

Responses of an Environmental Gram-negative Bacterium to Pollutant Stress

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By

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Declaration

I, *Buem-Seek Park* declare the results presented in this are solely my work, unless otherwise stated. The exceptions were PCR primers for presumptive GroEL gene identification were prepared by Dr. Maria D.E. Serafica and for 16S rRNA gene by Dr. Julian R. Marchesi and used with permission. Mammalian rRNA was prepared by Usula Manelpillai. DNA sequencing was completed at Department of Microbiology, Monash University using the VUT-Monash joint sequencing facility.

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I should, finally, also like to dedicate this thesis to every one of my family and wife's old home family, particularly, my lovely wife Sang-Woo Han and precious creature of our home, HwanHee and HwanSoul. All of my supporters are privileged to share in this thesis and with a constructive mind.

Summary

Several strains of pseudomonads which are normally found in natural water and soil environments, including *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and strains initially identified as *Pseudomonas paucimobilis* and *Pseudomonas maltophilia*, were used in this thesis and their stress responsive proteins were surveyed at the physiological level. Additionally, 16S rRNA gene comparison was employed to classify the pseudomonads and related species at the genetic level. In order to see to what extent the pseudomonads and related species responded to heat shock, cells were initially subjected to temperature upshift from 28°C to 37°C then disrupted by sonication or cells lysed by boiling in sample buffer, and the extracted total proteins were resolved on 1-D or 2-D SDS-PAGE. Their growth characteristics in different media (varying from rich to minimal) were determined and the physiological impacts on growth were examined at various concentrations of selected chemicals (metal ions, phenolics), to establish toxic and sub-lethal levels for use in determining stress responses. Production of novel proteins or elevated levels of normal proteins following exposure to different concentrations of toxicants was examined in detail in strain VUN10,077 (formerly *Ps. paucimobilis*) by sampling throughout the growth cycle and using ^{35}S -methionine incorporation into newly synthesized proteins.

Indigenous strain VUN10,077 presented abnormal rRNA pattern in standard RNA gels and 16S-like rRNA was analysed using Northern blot associated with 16S rRNA gene analysis which confirmed the identity of the known pseudomonads. An bands of MW 2.5 kb (faint), 1.38 kb, 1.2 kb and 0.1 kb were detected in RNA gels. The 1.38 kb band corresponded to 16S rRNA gene analysis, as shown by Northern blot analysis. 16S rRNA gene sequencing homology showed that strain VUN10,077 was a *Brevundimonas* species.

The pseudomonads and related species accumulated a GroEL-like protein under heat stress when cells were grown in LB broth and disrupted by sonication, and 2-D SDS-PAGE conditions were established using sonically disrupted cells of *Stenotrophomonas maltophilia* (formerly *Ps. maltophilia*). Cells lysed by boiling represented the total protein profile and included particulate or membrane-associated proteins, 70 kDa-like and 44-46 kDa proteins for strain VUN10,077. Selected pollutants impacted on the cell viability and sub-lethal levels of these were determined as follows; 2.0 gL⁻¹ for CdCl₂, 2.0 gL⁻¹ for CuCl₂.2H₂O, 2.0 gL⁻¹ for NiCl₂.6H₂O, 1.0 gL⁻¹ for CoCl₂.6H₂O, 0.025 gL⁻¹ for HgCl₂, 0.25 gL⁻¹ for SDS, 0.2 gL⁻¹ for NaAsO₂ and 0.5 gL⁻¹ for phenol, from A₆₀₀ readings and viable counts. Major HSPs were

present at relatively lower levels or were barely increased compared to controls, whereas low molecular weight proteins were significantly changed. A 20 kDa protein was commonly found in cadmium, copper, cobalt, nickel, arsenite and phenol stresses. However, a DnaK-like protein strongly reacted against commercially available DnaK antibodies on Western blot analysis and a visibly increased signal was seen for heat, cadmium, zinc, cobalt, phenol stresses and control, where represents in that order, whereas changes in the GroEL-like protein were less specific. Several minimal or defined minimal media were developed and used in heat stress and different protein profiles were seen. Medium (2) contained M9 salts medium (supplemented with glucose) plus casamino acids and medium (5) contained M9 salts medium (supplemented with glucose), vitamin solution, trace elements solution and casamino acids. Media (2) and (5) were found to be suitable for studying the physiological changes in strain VUN10,077, but growth rates in medium (2) were much less than seen in medium (5). Strain VUN10,077 presented major HSPs (70, 58 and 18 kDa proteins) in medium (5), similar to seen in Brain Heart Infusion (BHI) media. Pulse-chase ^{35}S -methionine labelling methods were established and known major HSPs were newly synthesised by strain VUN10,077 under heat and cadmium stresses, and these proteins gradually declined while 55 kDa, 50 kDa and 18 kDa proteins were detected under ongoing stress conditions. Presumptive *groEL* gene analysis was attempted and partial conserved *groEL* sequences were determined in the pseudomonads and related species, where similarity was 98-100%.

In parallel, a water-based *lux* system was established to examine physiological impacts of selected pollutants at the cellular level. *Lux* genes cloned from a marine bacterium were introduced into the background of the indigenous strain VUN10,077, providing a potentially sensitive tool for toxicity screening for terrestrial and fresh water samples. To show the use of bioluminescence in the indigenous (naturally non-bioluminescent) organism, research was performed on stable marking and optimisation of light output including marking of the organisms with *luxAB* or the whole *lux* cassette, which necessitated establishing gene transfer systems using available transposition vectors, optimisation of conditions for light output and stability. This approach would alleviate the requirement for salt or osmotic stabilizers in the test system to maintain bioluminescence or viability, which currently is a requirement of the MicrotoxTM system which employs *Photobacterium phosphoreum*.

Expression of bioluminescence genes in the indigenous strain employed the luminescence system of *Vibrio* species which is encoded by a gene cluster with a divergent transcriptional pattern. As strain VUN10,077 was a genetically uncharacterised natural isolate, this work was

achieved by establishing the parameters necessary for introducing genes after determining antibiotic sensitivity to determine which marker genes could be used in these backgrounds. The genes for two polypeptides (LuxA, LuxB) which form luciferase were transferred into strain VUN10,077. Introduction of the *luxAB* bioluminescence genes into a range of bacteria has been facilitated by using plasmid, pUT::Tn5-*luxAB* (de Lorenzo *et al.*, 1990), which carries a tetracycline resistance gene. The plasmid was introduced by bi- and tri-parental conjugation employing a helper plasmid pRK2013, if necessary, so that antibiotic resistance can occur in the transconjugants if transposition occurs. Alternatively, electroporation was used to introduce bioluminescence *lux* genes into strain VUN10,077 when cells were harvested at early-log and stationary phase. One of the engineered water-based *luxAB*-marked strain (VUN3,600) was used to detect the presence of toxic substances (determining selectivity and sensitivity). This bacterial luciferase system produces bioluminescence when supplied with an aliphatic aldehyde substrate, where the *luxAB* genes were expressed on the chromosome. The substrate delivery system and preparation of cells for bioluminescence toxicity test were achieved by diluting *n*-decyl aldehyde 10³-fold in Milli-Q water and resuspending cells in tap-water after mild centrifugation, when cells were harvested at A₆₀₀ 1.0-1.2. When a variety of common pollutants were tested in the water-based monitoring system, cells of VUN3,600 responded sensitively, rapidly and differentially to Cd, Pb, Hg, Zn, Co ions and SDS, and with less sensitivity to Ni, and As; responses were also dose-related in a short-term assay. DMF, a solvent used to dissolve or extract several organic compounds in environmental testing, stimulated light output but in a controlled fashion. Selected substituted heterocyclic compounds tested and phenol, δ-nitrophenol and strontium failed to cause dose-related inhibition, whereas di-, tri-, tetra- and penta-chlorophenol decreased light output in a long-term assay. DMF-dissolved PAH compounds were tested in the long-term assay and naphthalene, phenanthrene, fluorene, fluoranthene and benzo[α]pyrene presented dose-related responses while benz[α] anthracene and di-benzo[α,*h*]anthracene were not suitable for use in this type of assay. Although there is need to investigate extensive toxicity assays to gain broad acceptance, the water-based monitoring system showed repeatable and reliable toxicity assessment and has great possibility of usage in aquatic samples in either water-soluble or DMF-soluble forms.

This study allowed explanation of the stress responses of strain VUN10,077 to be examined at a variety of levels. Bioluminescence was the most sensitive approach to determining the lowest concentrations of pollutants which affected cell physiology, as this was obvious at concentrations lower than required to cause changes in viability or induce stress responses.

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Chapter 1

Introduction

1.1 Overview of the introduction

The most common approach to evaluate the impact of changed environmental conditions is to measure the change in species diversity and population numbers in an ecology. Although this suffices to maintain records or provide information on, for example, the ecological impact of pollutants, this approach often does not provide early warning of pending ecological degradation so that this can be avoided. Because bacteria respond rapidly to stimuli (including changes in the nutrient supply, presence of toxicants), it was postulated that the physiological or genetic changes of bacteria to the presence of pollutants might provide a system for detecting early warning signals of pending ecological perturbation. Most research on impacts of pollutants at the physiological or genetic level has been done on bacteria which were isolated from disturbed ecosystems (e.g. resistant to metal ions, from sites contaminated with petrochemicals) or from non-environmental sources, such as the gastrointestinal bacterium *Escherichia coli*. Moreover, the responses of bacteria from unpolluted environments (soil, river systems) are not well documented. To evaluate whether bacterial stress responses could be useful indicators of ecological perturbation, we must first understand how bacteria from nature react to chemicals which would be present only through pollution of natural environments. This thesis describes the investigation of the pollution stress responses of a bacterium originally isolated from an Australian river system and identified initially as a strain of *Pseudomonas paucimobilis* (personal communication from Dr. Paul Boon, CSIRO, Australia).

Several approaches were used to evaluate how this organism responds to pollutants, including: determining the impacts of selected chemicals on growth, determining whether stress proteins were synthesised in response to heat or pollution stresses, and developing variants which contained bioluminescence genes so that the impact of pollutants on the physiology of this strain could be measured. The three approaches were selected to gauge whether selected pollutants affected the bacteria at a genetic and/or physiological level and, indeed, if the pollutants were biologically available to this bacterium (i.e., reached a target site at the surface or intracellularly to alter traits). The literature review therefore covers several areas relevant to this theme, including a brief overview of cellular stress responses, the use of bioluminescence in biological monitoring and biosensor development.

1.2 Heat shock responses

1.2.1 A brief history of heat shock responses

Although the response of organisms to stressing environments, including sudden temperature change or heat shock, is now known to be an interesting genetic phenomenon, its history began as a molecular curiosity in fruit flies over 30 years ago (Ritossa, 1962). Detection of the heat shock responses occurred when Ritossa observed a novel puffing pattern in the polytene chromosomes of *Drosophila* larvae that demonstrated the rapid and selective expression of a group of proteins following exposure of fly larvae to temperatures above optimal for growth and development. Twelve years later, the identification of the proteins encoded in transcripts, indicated by these puffs, began in *Drosophila melanogaster* (Tissierse *et al.*, 1974). These proteins were referred to as heat shock proteins (HSPs) because of the nature of the stimulus that caused their induction. Subsequent studies later showed that similar changes in gene expression occurred when *Drosophila* tissues were exposed to uncouplers of oxidative phosphorylation, inhibitors of electron transport, or agents known to inhibit the activities of different enzymes, which also resulted in increased expression of one or more of the HSPs (reviewed by Ashburner and Bonner, 1979). The discovery that the exposure of cells to various amino acid analogues or the antibiotic puromycin also resulted in the increased expression of HSPs led to a hypothesis to explain how the heat shock response might be initiated (Kelly and Schlesinger, 1978; Hightower, 1980). Within a few years, HSPs of similar size to those seen in fly larvae were identified in the yeast *Saccharomyces cerevisiae*, and gene sequencing revealed surprisingly high homology (approximately 70%) between the corresponding insect and fungal proteins (Lindquist, 1986). These HSPs also had 40-50% homology to proteins of the bacterium *E. coli*, which was found to undergo a similar heat shock response (Yura *et al.*, 1993).

1.2.2 The heat shock family of proteins

To date, the most intensively studied stress response proteins are the HSPs, which are produced in response to sudden temperature shifts. This response is conserved in cellular systems and is a universal response to harsh conditions. The heat shock response is the molecular reaction to stressful, but sublethal, elevated temperature, and is characteristic of

Table 1.1. HSPs and related proteins in this family (based on review by Georgopoulos and Welch, 1993)

HSP type	Related proteins	Organism	Localization	References
Glyco-protein Hsp100	Hsp150	<i>S. cerevisiae</i>	NA	Lupashin <i>et al.</i> , 1992, Russo <i>et al.</i> , 1992
	Hsp104	<i>S. cerevisiae</i>	soluble	Parsell <i>et al.</i> , 1991
	Hsp98	<i>Neurospora crassa</i>		Vassilev <i>et al.</i> , 1992
	ClpB, ClpP	<i>E. coli</i>	soluble	Kroh and Simon, 1990, Squires <i>et al.</i> , 1991
Hsp90	Hsp83	<i>S. cerevisiae</i>	NA	Borkovich <i>et al.</i> , 1989
	HtpG	<i>E. coli</i>	NA	Lindquist and Craig, 1988
	NA	<i>Achlya ambisexualis</i>	NA	Brunt and Silver, 1991
	Hsp82	Fungus: <i>Histoplasma capsulatum</i>	NA	Minchiotti <i>et al.</i> , 1992
Hsp70	DnaK	eubacteria <i>Zymomonas mobilis</i>	soluble	Lindquist, 1986 Michel, 1993
	SSA1, SSA2, SSA1-4, SSB1, SSB2, SSC1, BiP	<i>S. cerevisiae</i>	mitochondria/ endoplasmic reticulum	Lindquist and Craig, 1988
	BiP	<i>Schizosaccharomyces pombe</i>	endoplasmic reticulum	Pidoux and Amstrong, 1992
	Hsp70-like	Fungus: <i>Ustilago maydis</i> <i>A. ambisexualis</i> <i>N. crassa</i> Protozoan: <i>Giardia lamblia</i> <i>Leishmania chagasi</i>	soluble	Holden <i>et al.</i> , 1989 Silver <i>et al.</i> , 1993 Fracella <i>et al.</i> , 1993
Hsp60	GroEL	eubacteria	soluble	Aggarwal <i>et al.</i> , 1988 Andersen <i>et al.</i> , 1996 Georgopoulos <i>et al.</i> , 1973, Hemmingsen <i>et al.</i> , 1988, Gupta, 1990
	Hsp60	<i>Mycobacterium leprae</i>	NA	Mehra <i>et al.</i> , 1986
	Hsp60 (Mif4)	<i>S. cerevisiae</i>	mitochondria	Cheng <i>et al.</i> , 1989
	Hsp60	mouse: <i>Mus musculus</i>	NA	Venner and Gupta, 1990
	NA	<i>Tetrahymena thermophila</i>	mitochondria	McMullin and Hallberg, 1987 and 1988

	NA	<i>N. crassa</i>	mitochondria	Hutchinson <i>et al.</i> , 1989
	RBP	plants: <i>Brassica napus</i>	chloroplast/ plastids	Hemmingsen <i>et al.</i> , 1988, Martel <i>et al.</i> , 1990
	TF55	archaeabacteria: <i>Sulfolobus solfataricus</i> <i>S. shibatae</i>	soluble	Knapp <i>et al.</i> , 1994 Trent <i>et al.</i> , 1991
	Thermosome	archaeabacteria: <i>P. occultum</i>	soluble	Phipps <i>et al.</i> , 1993
	CCT (TCP-1)	eukaryotes	cytosol, nucleus	Lewis <i>et al.</i> , 1992, Kubota <i>et al.</i> , 1994, Gao <i>et al.</i> , 1992.
DnaJ-related protein	DnaJ	<i>E. coli</i>	cytosol	Ueguchi <i>et al.</i> , 1994
		SCJ1, MDJ1, YDJ1/MAS5, SIS1, SEC63	<i>S. cerevisiae</i> mitochondria, cytoplasm, endoplasmic reticulum, nuclei	Blumberg and Silver, 1991, Caplan and Douglas, 1991, Rowley <i>et al.</i> , 1994, Luke <i>et al.</i> , 1991, Sadler <i>et al.</i> , 1989, Zhang <i>et al.</i> , 1992
Hsp10	GroES	<i>E. coli</i>	cytosol	Hemmingsen <i>et al.</i> , 1988
	Hsp10	rat pea and spinach	mitochondria chloroplast	Hartman <i>et al.</i> , 1992
α-crystallin family	Hsp30,26,12	<i>S. cerevisiae</i>	NA	Bertsch <i>et al.</i> , 1992 Petko and Lindquist, 1986, Horwich, 1992
	Hsp18	<i>Leuconostoc oenos</i> <i>Clostridium acetobutylicum</i>	membrane	Jobin <i>et al.</i> , 1997 Sauer and Dürre, 1993
	Hsp14	<i>Mycobacterium tuberculosis</i>	NA	Verbon <i>et al.</i> , 1992, Yuan <i>et al.</i> , 1996
	Hsp30	<i>N. crassa</i>	mitochondria	Plesofsky-Vig and Brambl, 1990
Ubiquitin	UB14	<i>S. cerevisiae</i>	NA	Seufert and Jentsch, 1990
	NA	mouse		Finley <i>et al.</i> , 1984

NA; not applicable

almost all organisms. When they are subjected to abnormal circumstances, cells rapidly redirect gene expression to maximize synthesis of this distinctive group of proteins which are essential components in a number of diverse biological processes. HSPs, and other relevant families of proteins are produced under stressing conditions, as well as being synthesized during cellular growth at all growth-related temperatures and occurring at significant levels in normal growth conditions: the heat shock family of proteins are highly conserved across genera (Morimoto *et al.*, 1990).

The spectrum of HSPs synthesized in different organisms displays significant similarities. Initially, four classes of HSPs were observed to be synthesized by eukaryotic organisms in response to high temperature: Hsp70, Hsp83/90, Hsp100, and the α -crystallin-related small HSPs. Several families of HSPs can be distinguished and are designated according to their average apparent molecular mass, including: Hsp100 (Hsp104 in *S. cerevisiae*), Hsp90 (Hsp83 in *S. cerevisiae*), Hsp70 (DnaK in *E. coli*), Hsp60 (the chaperonin or GroEL family), and small HSPs (Hsp30, Hsp26, and Hsp12 in *S. cerevisiae*) (Table 1.1). Small HSPs (low-molecular-weight HSP) are related to the α -crystallin family of HSPs which possess a chaperone-like function (see section 1.2.4) (Horwitz, 1992). The C-terminal region of the α -crystallin molecule is a conserved domain, often referred to as the α -crystallin domain, which constitutes the C-terminal halves of the proteins (de Jong *et al.*, 1993) and is important for the ability of this protein to protect other polypeptides against heat-induced aggregation (Takemoto *et al.*, 1993). The α -crystallin protein family is found in several prokaryotes, including *Clostridium acetylbutylicum*, *Leuconostoc oenos*, *Mycobacterium tuberculosis*, and *Stigmatella aurantiaca* (Verbon *et al.*, 1992; Heidelbach *et al.*, 1993; Sauer and Dürre, 1993; Yuan *et al.*, 1996; Jobin *et al.*, 1997). In addition, the rate of HSP synthesis was found to be related to several synthesis rates of other proteins in *S. cerevisiae* (e.g., ubiquitin, some glycolytic enzymes, and a plasma membrane protein) and was strongly enhanced upon exposure of cells to stress (Mager and de Kruijff, 1995; Plesofsky-Vig, 1996). Hsp60 and ubiquitin were found later to be notable additional HSPs in eukaryotes (Cheng *et al.*, 1989; Seufert and Jentsch, 1990).

In prokaryotes, HSPs were observed during genetic studies on bacterial host functions that were essential for bacteriophage growth. The genes coding for the constitutively expressed HSPs were found to map within specific operons and shown to take part in various aspects of bacteriophage DNA replication and morphogenesis (reviewed by Friedman *et al.*, 1984;

Georgopoulos *et al.*, 1990; Georgopoulos, 1992). For example, the DnaK protein (prokaryotic homologue of Hsp70) is required for bacteriophage DNA replication, while the GroEL-GroES proteins (prokaryotic homologues of Hsp60/Hsp10) were shown to be necessary for assembly of the bacteriophage head and tail components. DnaK, DnaJ, and GrpE are required for flagellum synthesis to maintain motility in *E. coli* (Shi *et al.*, 1992). HSPs such as DnaK (Hsp70) and GroEL (Hsp60) families are known to be conserved across genera and to play roles under non-stressed and stressed conditions, for example, participating in protein folding, protein translation, and perhaps highly ordered protein assembly (Lindquist and Craig, 1988; Morimoto *et al.*, 1990; Georgopoulos and Welch, 1993; Yura *et al.*, 1993). Synthesis of HSPs may also occur in response to natural environmental changes, which enables adaptation to the presence of pollutants at the cellular level (Selifonova *et al.*, 1993). In the following sections, some aspects of the regulation and function of HSPs are described, specifically in the context of relevance to this thesis.

1.2.3 HSP induction in transcription

Induction of stress inducible protein(s) occurs primarily at the level of transcription: transcription of heat shock genes is enhanced as a result of increased activity of transcription factors in prokaryotes and heat-shock factors (HSF) in eukaryotes such as yeast, flies, mice, chicken, and human cells (Allan *et al.*, 1988; Morimoto *et al.*, 1990; Hecker *et al.*, 1996). Expression of HSPs requires a change in the type of sigma factor associated with RNA polymerase and increased concentration of stress inducible protein(s) (Georgopoulos *et al.*, 1990; McCarty and Walker, 1994). Translocation of the sigma subunit is also one mechanism in response to environmental insult (Selifonova *et al.*, 1993).

1.2.3.1 Transcriptional induction in *E. coli*

In *E. coli*, transcription specificity occurs through the interaction between RNA polymerase (RNAP) holoenzyme (E) and the promoter site of genes and operons. The RNAP subunit which controls the specificity of this interaction has been shown to be the sigma factor, as the RNAP core itself does not recognise the promoter sites (Burgess *et al.*, 1969; Doi and Wang, 1986). The promoters of these heat shock genes are not recognized by the RNAP holoenzyme carrying the σ^{70} subunit, which carries out most transcription in the cell at

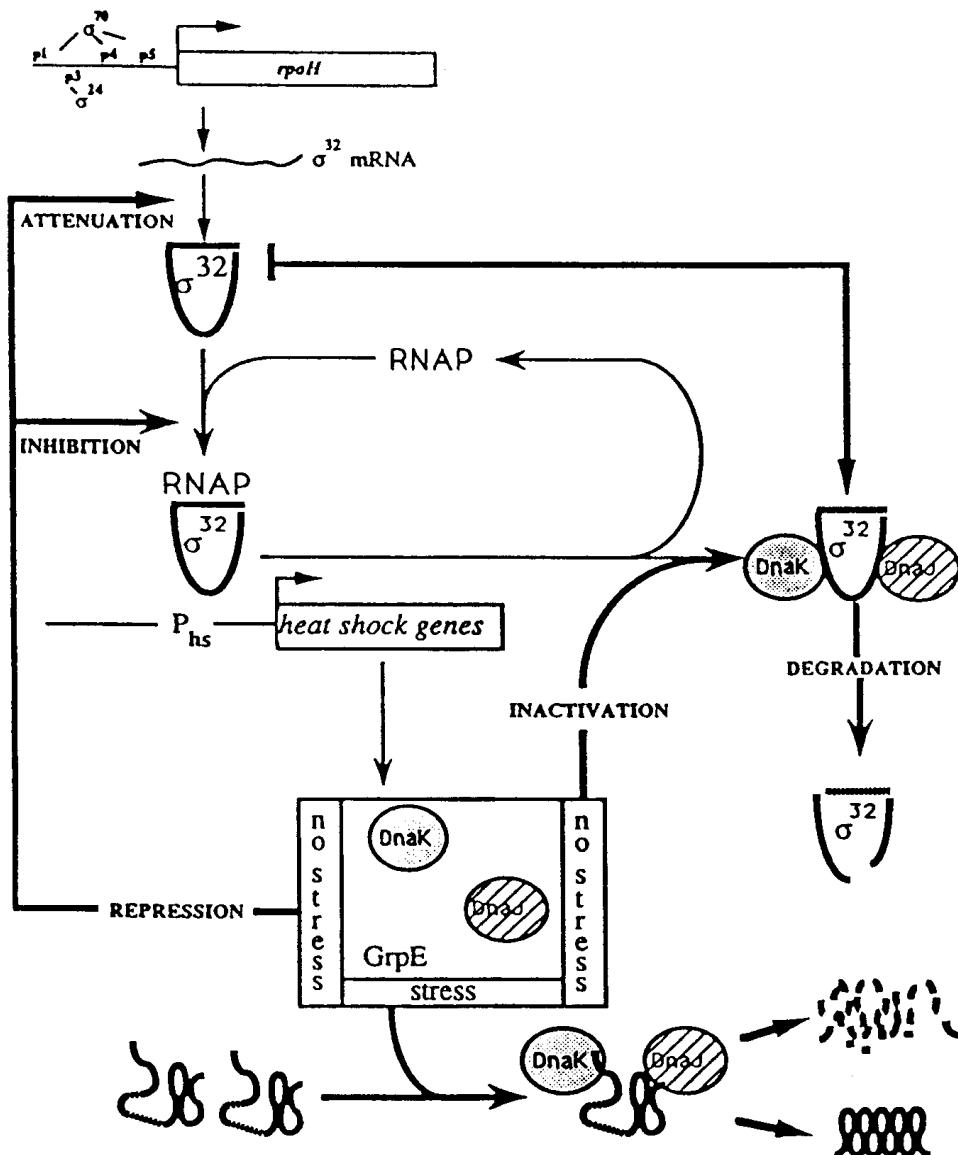


Figure 1.1. Regulation of heat shock-induced transcription in *E. coli*. Heat shock-induced transcription in *E. coli* is carried out by RNA polymerase (RNAP) associated with the heat shock-specific σ^{32} , the product of the *rpoH* gene. σ^{32} confers the specificity of the respective holoenzyme to bind to the promoters of the heat shock genes (P_{hs}). The level of heat shock gene transcription depends on the cellular concentration of σ^{32} . Under heat shock conditions, σ^{32} levels increase by enhanced synthesis, elevated stability, and increased activity of the factor. Among the proteins synthesized at high rate at the high temperature are DnaK, DnaJ, and GrpE, which play a central part in the stress response, by mediating refolding or degradation of heat-denatured polypeptides. Regulation of σ^{32} -mediated transcription activation occurs at various levels according to feedback mechanisms. Although transcription of the *rpoH* gene is rather complex (see the text), the main regulation of expression of *rpoH* is at the translational level: DnaK and DnaJ are implicated in attenuation of σ^{32} mRNA translation. In addition, these HSPs assist in the resumed rapid degradation of σ^{32} and in inhibiting the activity of the factor, probably by blocking association with RNAP (Mager and De Kruijff, 1995).

normal growth temperatures, but they are recognized by RNAP containing a heat shock promoter specific sigma subunit, σ^{32} , which is the product of the *rpoH* gene in *E. coli* (Doi and Wang, 1986; Bukau, 1993; Liberek and Georgopoulos, 1993; Mager and De Kruijff, 1995) (Figure 1.1).

1.2.3.1.1 Transcription regulation through induction of σ^{32}

Among prokaryotic organisms, the mechanisms underlying the induction of HSP synthesis have been studied most intensively in *E. coli* (Bukau, 1993; Yura *et al.*, 1993), although several mechanisms are switched on differently even within one given organism (Babst *et al.*, 1996; Hecker *et al.*, 1996). However, the so-called *rpoH* box is uniquely conserved among σ^{32} -like factors and this factor binds to DnaK with high affinity (Nakahigashi *et al.*, 1995; McCarty *et al.*, 1996). The heat shock regulon of *E. coli*, which consists of over 20 heat shock genes, was analysed by global transcriptional analysis with an overlapping lambda clone bank and 16 of these genes were activated *in vitro* by the E σ^{32} RNAP (Georgopoulos *et al.*, 1990; Chuang and Blattner, 1993). The factor was purified by Grossman *et al.* (1984) as a 32-kDa σ -factor that specifically recognized the heat inducible promoters, involving also the switching off of most genes located upstream of the heat shock genes (Taylor *et al.*, 1984).

It has been shown that σ^{32} is the limiting factor in the expression of HSPs. The level of heat shock gene transcription following temperature shift depends on the amount of σ^{32} produced (Yamamori and Yura, 1982), and increasing the rate of σ^{32} synthesis, without the temperature upshift, or addition of σ^{32} produced *in vitro* in a transcription- translation system also resulted in an elevated synthesis of HSPs (Bloom *et al.*, 1986). A lack of σ^{32} prevents *E. coli* growth at temperatures above 18°C, so σ^{32} plays an essential role at elevated temperatures (Zhou *et al.*, 1988; Tilly *et al.*, 1989). The low concentration of σ^{32} in the cell during steady-state growth conditions is limiting to heat shock gene expression (Craig and Gross, 1991), and there are two regulatory mechanisms, stability and synthesis rate, that keep the amount of σ^{32} at this level. The half-life of σ^{32} is approximately 4-5 minutes, so the synthesis of σ^{32} is repressed at some step after transcription initiation, perhaps at the level of translation or mRNA stability (Grossman *et al.*, 1987). In response to a temperature upshift, both the stability and synthesis of σ^{32} are transiently elevated, which causes an increase in the concentration of σ^{32} and rate of HSP synthesis (Yura *et al.*, 1993). The rate of synthesis of

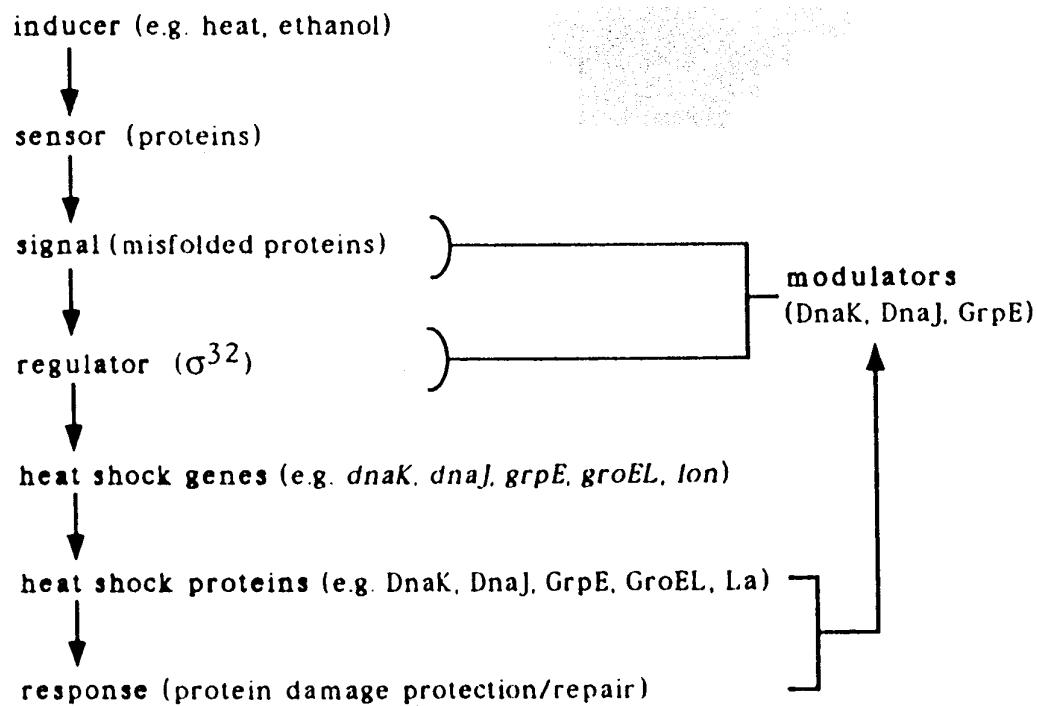


Figure 1.2. Feedback regulation on the signal-response transduction of the heat-shock regulon in *E. coli*. This model shows the elements of one major pathway that converts stress to a heat-shock response and is based on the general signal-response transduction scheme as given by Neidhardt (1987). DnaK, DnaJ and GrpE provide a negative feedback regulation. They repair proteins damaged by stress, and they are negative modulators of σ^{32} -dependent heat-shock gene expression. Damage repairs by DnaK, DnaJ, GrpE and other heat-shock proteins ameliorates the signal which then allow DnaK, DnaJ, GrpE to shut off the heat-shock response (Bukau, 1993).

HSPs is regulated by changes in the concentration of σ^{32} and synthesis of heat shock factors increases almost immediately, and this continues with increasing rates until it reaches a maximum (Tilly *et al.*, 1989; Bukau, 1993). The concentration of active σ^{32} limits the expression of heat shock genes and the stability of σ^{32} varies in conditions where heat shock gene expression is modulated. The limitation of the σ^{32} is likely to be essential for efficient adjustment of HSP levels in regulatory gene expression by the interaction between the concentration of active σ^{32} and the expression of heat shock genes (Grossman *et al.*, 1987; Straus *et al.*, 1987 and 1989; Tilly *et al.*, 1989; Bukau, 1993).

1.2.3.1.2 RNA polymerase modulation through σ^{32} protein binding to HSPs and the role of *rpoH*

Missense and null mutations of the *dnaK*, *dnaJ*, and *grpE* genes have been shown to lead to at least two- to five-fold elevated levels of heat shock gene expression at low temperature (30°C) and to prolonged synthesis of HSPs upon a shift to higher temperature (42°C) (Tilly *et al.*, 1983; Liberek and Georgopoulos, 1993). In all of these mutants, σ^{32} was markedly stabilized and showed a 10- to 30-fold slower decay rate compared with its behaviour in wild-type cells (Yura *et al.*, 1993). These observations strongly suggested that DnaK, DnaJ, and GrpE are key modulators of the heat shock regulon (Bukau, 1993). All three HSPs bind to free σ^{32} under non-heat shock conditions (Gamer *et al.*, 1992; Liberek *et al.*, 1992) and effectively sequester the majority of the σ^{32} molecules, hence repressing the expression of heat shock genes, including *dnaK* (Liberek *et al.*, 1992) (Figure 1.2).

Since the two major *dnaK* promoters are transcribed exclusively by σ^{32} (Cowing *et al.*, 1985; Liberek *et al.*, 1992), its rate of transcription could be regulated by at least both σ^{32} and DnaK. Although none of these HSPs themselves display proteolytic activity, HSPs such as DnaK, DnaJ, and GrpE form a complex with σ^{32} and deliver this to the proteolytic system under normal growth condition (Gamer *et al.*, 1996). In fact, it was demonstrated that DnaK, DnaJ, and GrpE specifically interact with the σ^{32} -dependent transcription machinery (Gamer *et al.*, 1992; Liberek *et al.*, 1992). Association of σ^{32} with DnaK and DnaJ occurs independently and each intermediate displays distinct biochemical properties (Gamer *et al.*, 1992). While ATP disrupts the association of DnaK and GrpE with σ^{32} , interaction of DnaJ with σ^{32} remains intact. These interactions affect the activity of σ^{32} . DnaJ may play a crucial role in establishing the cooperative repression of σ^{32} activity, since the presence of DnaJ alone

is sufficient to partially block transcription, whereas the presence of DnaK, even at higher concentrations, is less effective. The role of GrpE in repression is less clear, and experimental evidence suggested that it may be required to trigger the ATP-dependent release of DnaK (and DnaJ) from σ^{32} or E σ^{32} (Gamer *et al.*, 1992). All of this evidence supports the model that negative modulation of σ^{32} activity is based on the direct association of DnaK, DnaJ, and GrpE with the σ^{32} -dependent transcription machinery (Gamer *et al.*, 1996). According to the “DnaK/DnaJ titration model”, DnaK preferentially binds to denatured proteins accumulated under stress conditions and thus releases free σ^{32} , which in turn can associate with RNAP leading to the transcription of heat shock genes (Bukau, 1993).

The increased synthesis of σ^{32} upon heat shock is primarily due to elevated translation and stabilization of the protein (Nagai *et al.*, 1991; Yuzawa *et al.*, 1993). In regulation of *rpoH* mRNA translation, two proximal *cis*-acting elements of the *rpoH* mRNA presumably form a secondary structure that is thought to be responsible for translational repression under non-stressing conditions and for induction upon temperature upshift (Nagai *et al.*, 1991; Yura *et al.*, 1993). By extensive deletion analysis of an *rpoH-lacZ* gene fusion, two *cis*-acting mRNA regions within the *rpoH* coding sequence have been identified (Nagai *et al.*, 1991). One element, nucleotide 6 to 20, appeared to affect the rate of translation in a positive fashion, since deletions extending into this region from the 3' end exhibited an approximately 15-fold repression. The other control element region is located between nucleotides 15 and 247 and acts as a negative element in thermoregulation. Deletion of this region from both ends resulted in partially or fully constitutive expression. It has been proposed that the positive and negative *cis*-acting elements found in *rpoH* mRNA may form a secondary structure which plays a critical part in modulating the frequency of translation initiation in response to a heat (or other) stress environment (Nagai *et al.*, 1991; Yuzawa *et al.*, 1993).

1.2.3.2 Alternative sigma factors in prokaryotes

Induction of HSPs after heat shock or other stress conditions is a universal response common to all known organisms. Despite the universality of the heat shock response, the mechanisms of HSP induction varies in different organisms and heat shock gene induction by σ^{32} is just one of several mechanisms occurring in bacteria. However, the majority of stress genes is induced by an alternative sigma factor in different bacterial species (Babst *et al.*, 1996; Hecker *et al.*, 1996; Jishage *et al.*, 1996).

Besides σ^{70} (the *rpoD* gene product), six different molecular species of alternative σ subunits have been identified in *E. coli*; temperature upshift increases transcription of genes under the control of two heat shock σ subunits, σ^{32} (the *rpoH* gene product) (Erickson *et al.*, 1987; Straus *et al.*, 1987) and σ^{24} (the *rpoE* gene product). E σ^{32} transcribes the heat shock genes including those encoding chaperones and protease (Yura *et al.*, 1993), while the regulons under the control of σ^{24} are known to be involved in extracytoplasmic functions (Danese *et al.*, 1995; Raina *et al.*, 1995). σ^{53} (the *rpoN* gene product) is concerned with expression of a wide variety of genes including those involved in nitrogen metabolism (Magasanik, 1982; Merrick, 1993). σ^{38} (the *rpoS* gene product) is a key factor in the stress responses occurring during the transition from the exponential growth phase to the stationary phase (Loewen and Hengge-Aronis, 1994; Wise *et al.*, 1996) and response to various stress situations that include starvation, high osmolarity, and shift to acid pH (Muffler *et al.*, 1997).

Genes of the σ^{28} family (the *rpoF*, *sigD* and *fliA* gene product) are required for expression of motility and chemotaxis in several other organisms, including *Bacillus subtilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Legionella pneumophila* (Arnosti and Chamberlin, 1989; Mirel and Chamberlain, 1989; Ohnishi *et al.*, 1990; Starnbach and Lory, 1992; Heuner *et al.*, 1997). *FecI*, which is involved in the ferric citrate transport system, is identified as one of a number of new subfamily of σ subunits associated with extracytoplasmic functions in *E. coli* (Angerer *et al.*, 1995). In *Ps. aeruginosa*, two regions of conserved sequence which code for *algU*, *algD* and σ^E are believed to function as promoter recognition elements (Yu *et al.*, 1995; Schurr *et al.*, 1996), as well as *hrpL* (homolog to *algU*) in *Ps. syringae* (Xiao *et al.*, 1994).

The regulatory mechanisms for heat shock gene expression in Gram positive bacteria are based on repression at an inverted-repeat operator site located upstream of major heat shock genes and a highly conserved *cis*-acting regulatory element, a mechanism which has been found in *B. subtilis* and *Clostridium acetbutylicum* and some Gram negative bacteria (Narberhaus *et al.*, 1992; Hecker *et al.*, 1996; Segal and Ron, 1996). Because of its function as a regulatory element, this inverted repeat has been designated CIRCE (for controlling inverted repeat of chaperone expression) (Zuber and Schumann, 1994).

In *B. subtilis*, the general stress response is mediated by the alternative transcription sigma factor σ^B , whose activity is regulated in a complex and differential way by environmental signals including heat shock as well as starvation, on-set of stationary phase, and high osmolarity (Hecker *et al.*, 1996; Antelmann *et al.*, 1997). σ^B of *B. subtilis* is a putative repressor protein which binds to the CIRCE region and serves as an operator for a repressor encoded by *hrcA*, the first gene of the *dnaK* operon (Simon *et al.*, 1983; Yuan and Wong, 1995). σ^B is a stable protein and its effective concentration is controlled by the extent of its association with a specific anti- σ factor, RsbW (Redfield and Price, 1996). This sigma factor is different from σ^{32} and is regulated by a set of accessory factors (Hecker *et al.*, 1996). CIRCE- and σ^{32} -mediated heat shock regulation may occur within a single bacterial species, as is seen in *Agrobacterium tumefaciens* (Mantis and Winans, 1992), *Caulobacter crescentus* (Reisenauer *et al.*, 1996), and *Bradyrhizobium japonicum* (Babst *et al.*, 1996; Narberhaus *et al.*, 1996).

1.2.3.3 Transcriptional activators in eukaryotes

Transcriptional induction of most but not all eukaryotic heat shock genes in response to a temperature upshift and other forms of physiological stress is mediated by the binding of a transcriptional activator, HSF, to a short, highly conserved DNA sequence, the heat shock element (HSE). Initial proof for an activator of heat shock gene promoters was shown in *Drosophila* nuclei (Wu, 1984 and 1985) and from DNA-binding plus *in vitro* transcription studies with cell extracts (Parker and Topol, 1984; Topol *et al.*, 1985).

The gene encoding HSF was first isolated from *S. cerevisiae* (Sorger and Pelham, 1987; Wiederrecht *et al.*, 1988), when it was proven to be essential for viability at all temperatures (Sorger and Pelham, 1988). Later, HSF genes were also isolated from *Drosophila melanogaster* (Clos *et al.*, 1990), tomato cells (Scharf *et al.*, 1990), the yeast *Kluyveromyces lactis* (Jakobsen and Pelham, 1991), and mammalian cells such as human (Rabindran *et al.*, 1991; Scheutz *et al.*, 1991), mouse (Scheutz *et al.*, 1991), and chicken (Nagai and Morimoto, 1993). In higher eukaryotes, there is a family of HSFs containing at least three members, HSF1, HSF2, and HSF3 (Rabindran *et al.* 1991; Scheutz *et al.*, 1991; Nagai and Morimoto, 1993)

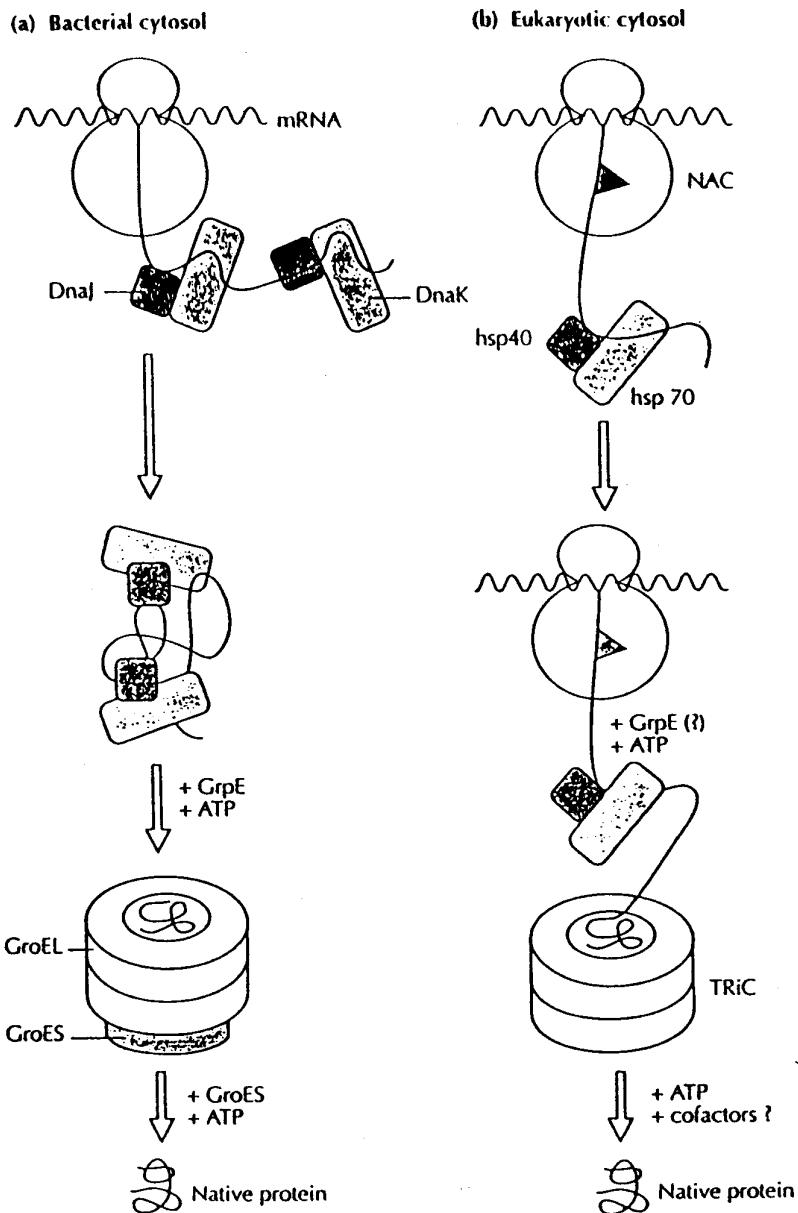


Figure 1.3. Pathways of chaperone-mediated protein folding in the cytosol of (a) bacterial cells and (b) eukaryotic cells as proposed in Langer *et al.* (1992) and Frydman *et al.* (1994). TRiC, TCP-1 ring complex; NAC, nascent polypeptide associated complex; Hsp40, a DnaJ homologue in the mammalian cytosol. TRiC has the ability to interact with ribosome-bound polypeptides, whereas binding of GroEL to nascent chains has not been observed. Alternative folding pathways involving different sets of chaperone components are also likely to exist (Hartl and Martin, 1995).

Expression of HSF in most species is constitutive and not responsive to stress, although some of the tomato HSF genes have been shown to be stress induced (Scharf *et al.*, 1990). The existence of a family of HSFs may be related to the finding that heat shock gene expression is induced during specific stages of development and differentiation. Despite the strong conservation of the HSE sequence, HSFs from different species show only limited sequence similarity. However, all different HSFs share several structural features: a DNA-binding domain at the amino terminus, an adjacent cluster of hydrophobic amino acids organized into heptad repeats (leucine zippers), and a distally located heptad repeat near the carboxyl terminus (reviewed by Mager and de Kruijff, 1995).

1.2.4 HSP mediated protein folding

Interaction of HSPs with other cellular proteins facilitates the appropriate folding of the target proteins, their assembly into protein complexes, and/or their translocation into organelles (Figure 1.3). Because of their effects on the conformation of other proteins, these HSPs have been termed molecular chaperonins and/or chaperones (Ellis, 1987 and 1992). Molecular chaperones occur in all cell types and compromise several protein families that are structurally unrelated. Many of them are classified as stress or HSPs, though they may also be required under normal growth conditions (Ellis, 1992; Hartl *et al.*, 1994). The first general function proposed for the HSPs was suggested for Hsp70 by Lewis and Pelham (1985), but this model applies to several other HSPs as well. They reported that exposure to amino acid analogues and the antibiotic puromycin, which affects protein conformation, caused abnormally folded proteins to accumulate, and this switched on a heat shock-like response. The increased expression and accumulation of the HSPs subsequently were believed to help cells cope with the increased burden of abnormally folded proteins (Hightower, 1980; Goff and Goldberg, 1985; Pelham, 1986). Chaperones can be defined broadly as proteins that bind to other unstable, non-native conformations of proteins, and facilitate correct folding by releasing the bound polypeptide in a controlled way (Hartl and Martin, 1995). Typically, the HSPs work together to mediate protein folding. The important folding process of chaperone action in protein folding have emerged, and this involves the several HSPs such as Hsp70 and Hsp60 families (Ellis, 1987 and 1992; Hartl *et al.*, 1994) (Figure 1.3). It is now generally considered that protein folding in the cell is mediated by molecular chaperone proteins (Ellis, 1987) and assembly of the protein folding machinery

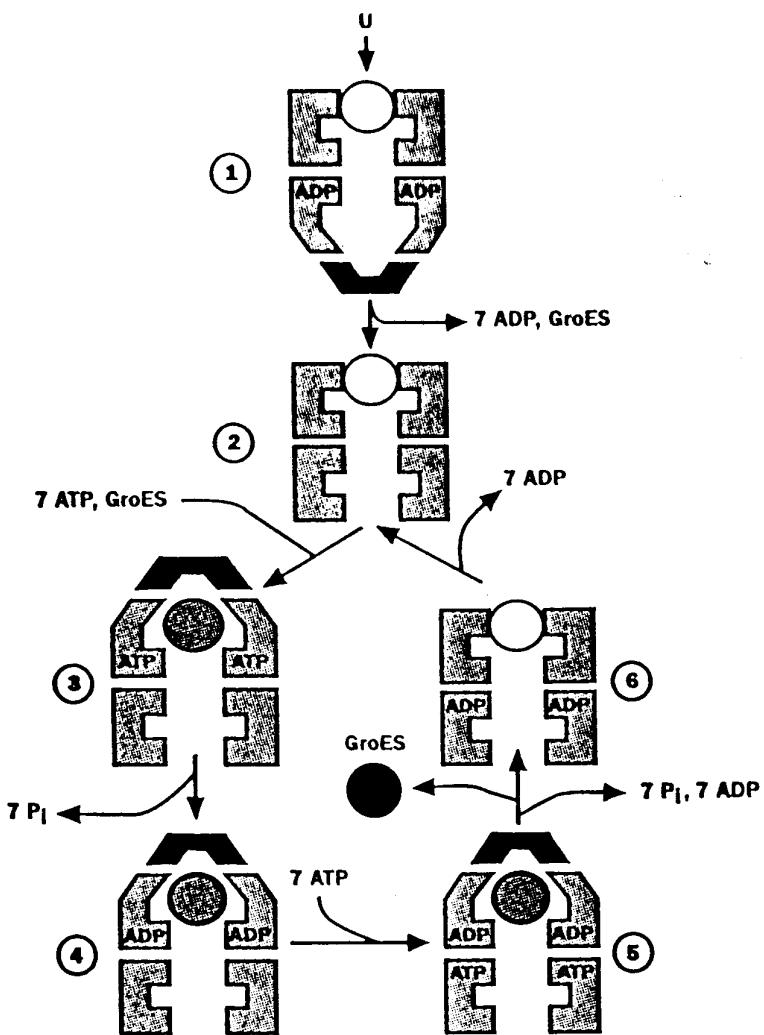


Figure 1.4. Model for the chaperonin-mediated folding of dihydrofolate reductase (DHFR) protein in *E. coli*. Steps 1 to 6 are hypothesised stages essential for operation of this model. GroEL is shown schematically as a vertical cut through the complex, reflecting the three-domain structure of the subunits and the conformational changes upon GroES binding (Chen *et al.*, 1994). ADP, the high-affinity ADP state in the seven subunits of GroEL that are bound to GroES; ATP, the subunits in a GroEL toroid in the ATP-bound state; U, unfolded polypeptide substrate as compact folding intermediate (open circle). The initial release of GroES from the *trans*-toroid of GroEL may be accompanied by a round of ATP hydrolysis in the polypeptide-containing toroid (not shown). Shaded spheres indicate that the population of GroEL molecules contain a mixture of folded and incompletely folded DHFR molecules. Folded DHFR is released (black circle) in between steps 5 and 6, whereas incompletely folded DHFR rebinds, resulting in structural rearrangement and unfolding (Mayhew *et al.*, 1996). See also Hartl and Martin (1995) and Burston *et al.* (1996).

chemical sequestration of these aggregation-prone species is a major function of GroEL (occurs in the bacterial cytosol, chloroplasts and mitochondria (Hartl *et al.*, 1994). Hsp60 in mitochondria plays an important role in mitochondrial protein transport across membranes (Cheng *et al.*, 1989) and Rubisco-subunit-binding protein (RBP) in chloroplasts assists in the assembly of Rubisco (ribulose-bisphosphate carboxylase) subunits (Hemmingsen *et al.*, 1988).

1.2.4.1 The role of Hsp60 protein folding in *E. coli*

The first discovered and most well studied chaperonin is GroEL of *E. coli* (Georgopoulos *et al.*, 1973), which is the ring-shaped ATPase that is capable of binding to a large spectrum of unfolded protein substrates. High resolution crystal structures of the GroEL tetradecamer (Braig *et al.*, 1994) showed its subunits had a relative molecular mass of 60,000 (Mr 60K). A functional mode of action of hetero-oligomeric TCP-1 (*t*-complex polypeptide 1) ring complex (CCT or TRiC) cytoplasmic chaperonin in eukaryotes is homologous to that of the Hsp60 chaperonins (Horwitz and Willison, 1993; Marco *et al.*, 1994), as both are large double-ring complexes that bind unfolded polypeptide within their central cavity and mediate folding by multiple rounds of ATP-dependent release and rebinding (Braig *et al.*, 1994; Ellis, 1994). An extensive mutational analysis of GroEL showed that different domains are important for the binding of GroES and that hydrophobic binding surfaces face the central channel of GroEL, where unfolded polypeptide interacts (Fenton *et al.*, 1994). Non-native substrate polypeptides bind to the end of the GroEL cylinder in the opening of its predominantly hollow core in the central channel: Chen *et al.*, 1994; Fenton *et al.*, 1994). In Figure 1.4, the GroES heptamer of 10K subunits is shown to provide a cavity within the dome which is continuous with the polypeptide binding chamber of GroEL in the chaperonin complex (Martin *et al.*, 1993; Hunt *et al.*, 1996) and this is positioned like a cap over the top of one of the two polypeptide binding chambers of GroEL in the stable GroEL₁₄-GroES₇-ADP₇ complex (Martin *et al.*, 1993). Cryo-electron microscopy and three-dimensional reconstruction were used to map out the domain movements in the chaperonin complex in the presence of nucleotides such as ADP, ATP, and the non-hydrolyzable ATP analog, AMP-PNP (Roseman *et al.*, 1996). It was concluded that critical steps of folding and rearrangement may occur in the shielded environment of the chaperonin cavity (Jaenicke, 1991; Hendrick and Hartl, 1993; Hartl *et al.*, 1994; Hartl and Martin, 1995).

Bound substrates are released from GroEL into solution, where they attempt to fold, then substrate proteins can leave GroEL as non-native intermediates. Folding is proposed to occur freely in solution while the protein substrate is trapped between GroEL molecules. Most of the non-native protein is bound to GroEL at any given time, however. It is also unclear how long the folding intermediates stay in solution before re-binding to GroEL (Gray and Fersht, 1993; Weissman *et al.*, 1994). The apical domain, particularly in the region of the substrate- and GroES-binding sites, shows a large repertoire of hinge rotations and distortions in the different functional states. Changes in the contacts between rings, which are likely to be fundamental to the mechanism by GroE system chaperonins protein folding, suggest a mechanism of allosteric switching *via* a direct connection to the ATP binding site and describe conformational changes (Roseman *et al.*, 1996).

Whether the productive protein folding reaction occurs with GroES and the substrate polypeptide on the same side (*cis*) or on opposite sides (*trans*) of the GroEL double cylinder remains obscure and evidence for either model is not conclusive. According to Burston *et al.* (1996), the substrate proteins are productively released from the *cis* ternary GroEL-GroES polypeptide complex in which both the native and non-native substrates are sequestered within the GroEL channel underneath GroES, in GroE-mediated protein folding. When GroES release is blocked by the use of either the non-hydrolyzable ATP analog or a single-ring GroEL mutant, the substrates complete their folding while remaining associated with the chaperonin (Burston *et al.*, 1996; Weissman *et al.*, 1996). Release of non-native forms gives the substrate proteins the opportunity to allow a kinetic partitioning among various chaperone systems. For example, the 60 kDa protein firefly luciferase forms a stable complex with GroEL *in vitro* but does not reach the native state even in the presence of GroES and ATP (Schroder *et al.*, 1993; Burston *et al.*, 1996). However, luciferase is efficiently refolded by the bacterial Hsp70 system, the triad of DnaK, DnaJ, and GrpE, even when initially bound at GroEL (Weissman *et al.*, 1996). On the other hand, dissociation of non-native intermediates reflects leakage that may occur from the GroEL-toroid in *trans* to GroES (Mayhew *et al.*, 1996), which was shown for malate dehydrogenase as a substrate for folding was observed to be predominantly in the *trans* position using cryo-electron microscopy analysis (Chen *et al.*, 1994). The premature release of non-native intermediates from GroEL is likely to occur physiologically to allow the degradation of damaged protein.

1.2.4.2 Chaperonin-mediated protein folding in eukaryotes

The eukaryotic cytosolic chaperonin called CCT (chaperonin containing TCP-1) (Gao *et al.*, 1992) or TRiC (Frydman *et al.*, 1992) plays an essential role in mediating ATP-dependent folding of two cytoskeletal components prone to aggregation, actin and tubulin. CCT has a function similar to GroEL in the prokaryotic cytoplasm, however CCT acts without the assistance of a co-chaperonin prokaryotic GroES analogue (Sternlicht *et al.*, 1993). CCT binds non-native forms of these proteins and, in the presence of ATP, discharges them in native form (Gao *et al.*, 1992). There is a large cavity at each end, which is the site of binding of unfolded substrate polypeptide (Marco *et al.*, 1994). In the case of GroEL, recent studies have shown that folding occurs in association with rounds of release from the chaperonin (Burston *et al.*, 1996; Mayhew *et al.*, 1996). Two different general mechanisms of action have been suggested in the case of CCT. In one, *in vitro* folding studies carried out with purified substrate and chaperonin proposed that the folding process, which is a cycle of binding and release, is linked to production of the native state (Gao *et al.*, 1994; Tian *et al.*, 1995). In the other, studies in a reticulocyte lysate system examining newly translated actin have shown that the native state is produced through a single round of association of the newly translated chain with a 70-kDa HSP and the chaperonin TCP-1 ring complex, followed by release of the native form (Frydman and Hartl, 1996; Farr *et al.*, 1997).

The eukaryotic cytosolic chaperone is composed of a complex with up to nine subunits which comprises two rings of a number of dissimilar subunits arranged back-to-back to form a cylinder. For example, CCT in mammalian testis was shown to be made up of nine different subunits using two-dimensional gel analysis, and six novel TCP-1-related polypeptides were isolated and sequenced in the mouse, where results suggested specific functions within the chaperone ring complex (Kubota *et al.*, 1994). Archaeabacteria and eukaryotic chaperonins (Hsp60 type) are related symmetrically to one another since they share up to 40% amino acid identity (reviewed by Ellis, 1992; Horwich and Willson, 1993; Willson and Kubota, 1994; Kubota *et al.*, 1995). The chaperonin, TF55, showed nine-fold symmetry when examined using the electron microscopic images of negatively stained TF55 (Trent *et al.*, 1991; Marco *et al.*, 1994), and another, thermosome, was composed of only two-subunits (Phipps *et al.*, 1993; Knapp *et al.*, 1994) in the archaeabacteria. This situation is reminiscent of the 20S proteosome which is a multi-toroidal, multi-subunit complex involved in proteolysis: the archabacterial proteosome is constructed from two subunits whereas the

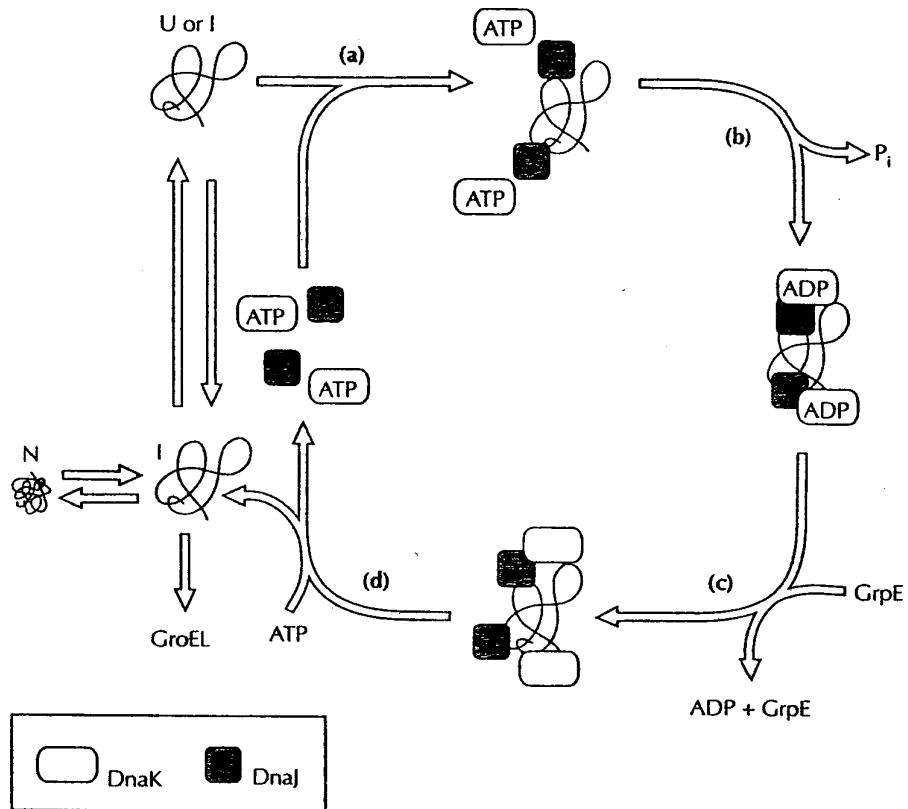


Figure 1.5. Model for the Hsp70 reaction cycle of DnaK, DnaJ and GrpE in protein folding in *E. coli*. (a) Unfolded (U) or partially folded (I) protein binds to DnaJ. (b) DnaK is recruited by the DnaJ-polypeptide complex and hydrolyzes bound ATP to ADP. (c) GrpE catalyzes the dissociation of ADP from DnaK. (d) Upon binding of ATP to DnaK, the ternary complex dissociates. The released protein may either fold to the native state (N), re-bind to DnaJ or be transferred to the chaperonin GroEL for folding. It is unclear whether *in vivo* the DnaK/DnaJ/GrpE system is sufficient to mediate protein folding or whether it functions predominantly as a holding cycle for unfolded polypeptide until GroEL is available (Hartl and Martin, 1995).

eukaryotic proteosome may have up to 14 different subunit types (Peters, 1994; Kubota *et al.*, 1995). Several eukaryotic chaperones are weakly, but significantly, related to the sequence of the eubacterial chaperonins, GroEL (Ellis, 1990; Gupta, 1990; Lewis *et al.*, 1992).

1.2.4.3 Hsp70 protein folding process

Another major class of molecular chaperones is the Hsp70 family, which compromises several closely related proteins of about 70 kDa (McCay *et al.*, 1994). Members of the Hsp70 family are found in the cytosol of prokaryotes and eukaryotes and in the luminal spaces of mitochondria, chloroplasts, and the endoplasmic reticulum. Both structure and function of the HSPs are well conserved. The DnaK protein of *E. coli* has 40-50% identity to the Hsp70 family of proteins from other species. Several eukaryotic homologs of DnaJ have been identified, and a homolog of GrpE has been shown in yeast mitochondria (Bolliger *et al.*, 1994; Laloraya *et al.*, 1994).

Current understanding of Hsp70 function follows from the initial suggestion by Pelham (Lewis and Pelham, 1985; Pelham, 1986) which proposed that these proteins bind to aggregation-prone, exposed hydrophobic surfaces that may be induced by stress. Rothman (1989) extended this by including the concept that both the response to stress and other functions, such as translocation and nascent chain folding involve the anti-folding activity of polypeptide binding to prevent aggregation. It is now generally accepted that Hsp70 chaperones promote protein folding, the assembly and disassembly of multimeric protein structures, protein synthesis and degradation, and the translocation of polypeptides across cellular membranes (Hendrick and Hartl, 1993; Glick, 1995).

In the case of DnaK, two additional HSPs, DnaJ, GrpE, are required to form a functionally active chaperone machinery (Georgopoulos, 1992; Hartl and Martin, 1995) (Figure 1.5). DnaK, DnaJ, and GrpE are involved in a large variety of cellular processes, including folding of nascent proteins (Grossman *et al.*, 1987; Gragerov *et al.*, 1992), DNA and RNA synthesis (Bukau and Walker, 1989; Hwang, *et al.*, 1990), ribosome assembly (Alix and Guerin, 1993), regulation of the heat shock responses (Straus *et al.*, 1990; Liberek *et al.*, 1992), proteolysis (Silhavy *et al.*, 1984; Keller and Simon, 1988; Straus *et al.*, 1988), λ , F, P1 replication (Kawasaki *et al.*, 1990; Hoffman *et al.*, 1992; Wickner *et al.*, 1992) and membrane translocation (Wild *et al.*, 1996). The molecular role of each of these proteins has been

investigated. DnaK binds to substrate molecules to influence the folding pathway, most generally by preventing off-pathway reactions, but also possibly by stabilizing certain folding intermediates. DnaK has a weak ATPase activity, which is important in chaperone function. Both DnaJ and GrpE accelerate the ATPase cycle of DnaK, the former by promoting nucleotide release. In addition, DnaJ binds independently to some substrates and may alter the substrate binding properties of DanK (Alfano and McMacken, 1989; Wickner, 1990; Gamer *et al.*, 1992).

1.2.5 Induction of stress proteins

1.2.5.1 Bacterial responses to stress

Induction of HSPs after heat shock or other stress conditions is a universal response common to all known organisms and these proteins are the most highly conserved group of proteins so far characterized (Morimoto *et al.*, 1990, Kubota *et al.*, 1994). Despite the universality of the heat shock response, the mechanism and production of HSPs varies greatly not only between prokaryotes and eukaryotes but also among different bacterial species and even differs within one species, depending on the nature of the stimulation (temperature, extreme pH, salinity, oxidative stress, hydrogen peroxide, detergent, macrophage infection and starvation conditions) (Lowe *et al.*, 1993, Babst *et al.*, 1996, Hecker *et al.*, 1996).

Stress responses have been noted to occur in *Pseudomonas* species and were initially reported in *Ps. aeruginosa* (Allan *et al.*, 1988). Allan *et al.* reported that a temperature shift from 30°C to 45°C caused elevated synthesis of 17 proteins. Two proteins (76 kDa and 61 kDa) were immunologically similar to the DnaK and GroEL of *E. coli* based on their cross-reactivity with antibodies raised against the *E. coli* heat-shock proteins. Ethanol stress produced seven new ethanol-induced proteins and three of them (91, 76 and 70 kDa) corresponded to HSPs in this strain.

The psychrophilic bacteria *Vibrio* species strain ANT-300 (Araki, 1991) and psychrotrophic *B. psychrophilus* (Whyte and Innis, 1992) have shown that cold acclimation proteins, also characterized as cold shock proteins, were identified after the temperature was shifted up or down and these were also seen during steady-state growth at the low (0°C) or supra-optimal

(13°C) temperatures. Similar proteins were also seen in the mesophilic bacterium *E. coli* when shifted from 13.5°C to 46°C (Herendeen *et al.*, 1979). Hebraud *et al.* (1994) reported that the relative synthesis levels of several proteins from the psychrotrophic bacterium *Ps. fragi* were different at temperature between 4°C and 30°C. One of the cold shock proteins (C8.0) cross-reacted immunologically with a major cold shock protein of *E. coli*, CspA. A temperature shift from 37°C to 15°C induced cold shock proteins in *B. subtilis* (Graumann *et al.*, 1996).

A subset of proteins induced at pH 4.3 were *rpoH* dependent in *E. coli* (Hyde and Portalier, 1990). A lactic acid stress response (pH 3.9) was examined in the lactic acid bacterium *Latococcus latics* subsp. *lactis* by pre-exposing the cells for 30 minutes to a mildly acid shock at pH 5.5. Among the proteins made were the major HSPs, DnaK and GroEL (Hartke *et al.*, 1996). Buchmeier and Heffron (1990) have reported that GroEL and DnaK are induced during *Salmonella* infection of macrophages. This is a condition that does not involve thermo-induction. Since the phagolysosome environment has been considered to be acidic, it may contribute vacuolar acid shock to the induction of these proteins (Foster, 1991). In *Le. oenos*, low acid shock induced the expression of a 18-kDa small HSP (Lo18), which was also produced in heat and ethanol stresses. This small HSP, related to α -crystallin family, was found to be peripherally associated with the membrane of *Le. oenos* (Jobin *et al.*, 1997).

The *groE* operon is induced in response to the formation of denatured or abnormal proteins. Oxidative stress may have a similar denaturing effect on cellular proteins. GroES, but not GroEL, is induced in *E. coli* by H₂O₂, though it is not an oxyR-regulated protein (VanBogelen *et al.*, 1987). Walkup and Kogoma (1989) reported that O₂⁻-generating conditions produced responses which were different to H₂O₂ stress, as no other HSPs were induced under O₂⁻-generating conditions, including DnaK and GroES, which were seen to be induced in both *S. typhimurium* and *E. coli* during H₂O₂ stress (VanBogelen *et al.*, 1987). It is also interesting that stress induced by the detergent sodium dodecyl sulfate appeared to be distinct rather than being a subset of the heat shock or oxidative stress responses of *E. coli* (Adamowicz *et al.*, 1991). Strain specific responses were observed in the anaerobic bacterium *Bacteroides fragilis* with oxidative stress. When cultures were shifted from anaerobic to aerobic conditions, or when up to 10 mM H₂O₂ was added to cultures, 24 to 28 new proteins were induced, as detected by 2-D PAGE. With a few exceptions, most of the proteins showed overlapping induction, unlike in *S. typhimurium* and *E. coli*, indicating that the synthesis of

these proteins may be due to a common response to oxidative stress. Surveys of stress inducible proteins indicated that overlapping responses appear to be a common strategy, although the extent of the overlap of proteins induced by a variety of stresses seen with *Ba. fragilis* is rare (Rocha *et al.*, 1996).

Carbon deprivation of *Ps. putida* KT2442 (Givskov *et al.*, 1994) caused synthesis of up to 72 new proteins, where similar responses are observed in multiple-nutrient starvation. Heat, NaCl, and H₂O₂ shock exposure of *Ps. putida* also caused production of clusters of new proteins, where some were similar to starvation-stress proteins and others were condition specific or similar to the HSPs. Regardless of whether the *E. coli* cells are starved for C, N or P, approximately 30% of the induced proteins were common to heat, oxidative, and osmotic shock proteins (Groat *et al.*, 1986; Lomovskaya *et al.*, 1994). Starvation induced Dps protein in *E. coli* was similar to the responses to glucose deprivation in *B. subtilis* (Antelmann *et al.*, 1997), indicating overlaps in responses across genera.

1.2.5.2 Application of HSPs

HSPs have a role in protecting cells against injuries and most types of stress. There has been interest in the role of HSPs, Hsp90, Hsp70, and Hsp60 as neo-antigens and/or immunodominant antigens in the immune system. Among the HSPs, immune responses (both antibody and T-cell responses) to Hsp60 are most frequently observed; before Hsp60 was recognised to be GroEL, it was called the ‘common antigen’ (see review by Jindal, 1996). During infections and certain autoimmune diseases, the rapidity of the stress response can be maintained and quickly restimulated by infection in the cellular immune responses, and thus, it may provide the basis for development of novel immunotherapeutics (Young, 1990; Jindal, 1996).

HSPs have been implicated as immunodominant antigens during parasite infection (Ardeshir *et al.*, 1987), as mediators of protective immunity against malaria (Renia *et al.*, 1990; Li *et al.*, 1993), or adjuvant-independent immunity (Barrios *et al.*, 1992), and as a potential mediator of immunopathology during infection (Fan and Davidson, 1996). In addition, Hsp70-like gene was specifically used to detect enteric protozoans of *Giardia* cysts which cause parasitic infections in humans and a lengthy diarrhea in infected individuals (Abbaszadegan *et al.*, 1997), and vaccination with heat shock protein-peptide complexes plays immune responses

against peptide-antigens bound with chaperone protein (Kaslow *et al.*, 1991; Craig *et al.*, 1994; Heike *et al.*, 1996; Stevens *et al.*, 1997). The immuno stimulation seen may not be against the HSP itself, but against the antigenic peptides ‘chaperoned’ by the HSP, based on the possible roles for gp96, Hsp70, and Hsp90 in antigen presentation. For example, antibody and lymphocyte proliferation responses to a recombinant 60 kDa *Brucella abortus* GroEL HSP caused resistance to infection by the virulent *Br. abortus* strain 2308. The results indicated that mice vaccinated had an immune response to GroEL during infection with the virulent strain 2308, although this response dose not appeared to have an essential role in vaccine-induced immunity to brucellosis (Stevens *et al.*, 1997).

1.3 Biosensors for detecting pollutants

1.3.1 A brief overview of biosensors

Intact organisms or enzymatic bioassays (see review Bitton and Koopman, 1992) can be used as specific and sensitive devices for sensing the bioavailability of particular pollutants or pollutant class. Living biosensors are based on the ability of pollutants to cause cytoprotective responses in organisms, including microorganisms. Increasing contamination in ecosystems by numerous toxic substances has prompted the development of living organism sensor systems, and this is an attractive approach to providing advanced warning of the presence of pollutants and toxicants. These include using green algae (predominantly *Selenastrum capricornutum* and *Scenedesmus subspicatus*), water fleas (*Daphnia magna*), fish (*Oncorhynchus mykiss*, *Cyprinus carpio*, *Lepomis macrochirus*, *Salmo gairdneri* and *Leuciscus idus melanotus*) and luminescent microbes (*Photobacterium phosphoreum*) (Ribo and Kaiser, 1983; Schmuck *et al.*, 1994).

The validity of living organism sensing methods rests on the premise that known correlations exist between their growth, or physiological state and their responses to toxins. For green algae, effects of toxicants on growth is measured over several generations (48 hours), and behavioural impairments and mortality to toxins are measured over 72 hours for water fleas. Lethality is the indicator of toxicity currently used in fish tests, which takes more than 96 hours to obtain results. For bioluminescent bacteria, the indicator is light output and

suppression of this in the presence of toxicants, where this occurs normally within an hour (Ribo and Kaiser, 1983). Water quality parameters (temperature, hardness, pH, suspended solids) are known to affect the toxicity of compounds (Doe *et al.*, 1988; Fisher, 1991). These parameters may affect toxicity testing over the testing period using algae, water flea and fish rather than when using bacteria.

Microbial biosensors have several advantages over alternative systems, providing rapid assays of superior sensitivity, specificity, quantitative response and results allow collection of real-time response data (Engebrect *et al.*, 1985; Meighen, 1991; Stewart and Williams, 1992; Wood and Gruber, 1996). Several microbially-based assays are commercially available: Microtox™ (bioluminescence), Polytox™ (respiration), ECHA Biocide Monitor (dehydrogenase activity), Toxi-Chromotest (enzyme biosynthesis), and MetPAD (enzyme activity). There is no single microbial bioassay that can detect all categories of environmental toxicants, including light emission systems (Bain, 1992; Bitton and Koopman, 1992; Myer *et al.*, 1993).

Because of the simplicity, cost effectiveness and time efficiency of using bacteria, there has been considerable effort put into developing microbially-based sensors based on natural or genetically-engineered, practically for use in testing water or soil samples. Naturally bioluminescent bacteria from marine environments have been used in toxicity testing previously, including strains of *Ph. phosphoreum* in the Microtox™ system (Bulich, 1982). Although this provides an efficient and easily used system, measurement of toxicants in aqueous samples (including aquatic soil samples) can pose problems due to the necessity of including NaCl in the assay system. Consequently, there is considerable scope to improve the use of bacterial bioluminescence as applied to aqueous systems. Genes from bacterial (*lux*) or eukaryotic (*luc*) sources can be expressed in a variety of organisms to produce bioluminescence in normally non-bioluminescent backgrounds (Meighen, 1993), producing attractive bioluminescent systems based on cloned *lux* or *luc* genes (Stewart and Williams, 1992; Van Dyk *et al.*, 1994; Britz *et al.*, 1997; Ben-Israel *et al.*, 1998).

Although production of bioluminescence is metabolically inexpensive, maintenance and expression of *lux* or *luc* genes can effect the physiology of the host organism. When employing the *lux* operon cloned in normally non-bioluminescent organisms, one important factor is the effect of the marker genes on host fitness and competitiveness subsequently if

destined for use *in vivo* in natural environments (Meighen, 1991; Prosser *et al.*, 1996). This phenomenon encourages using only part of the *lux* operon (*luxAB*) for marking hosts, as inclusion of full *lux* operon can reduce host cell activity, preventing detection of bioluminescence (de Weger *et al.*, 1991). Similarly, the metabolic load of the genes located on a plasmid may affect cellular physiology, as fitness may decrease with increased copy number (Hong *et al.*, 1991 and 1995). Moreover, the cost of substrates (FMN, aldehyde) is extremely low for the bacterial luminescence assay if used *in vitro*, and scintillation counter or photographic film exposure can be used for measurement, which are available in most laboratories (Meighen, 1991).

Chromosomal markers, introduced at low copy number, have less effect on host cell physiology when expressing luminescence. Levels of expression of marker genes will also determine fitness and stability over the entire testing period, where stabilization is best accomplished by chromosomal rather than plasmid marking (Meighen, 1991; Prosser *et al.*, 1996). Environmental strains are attractive for constructing bioluminescent strains using Tn5 disabled transposon systems, where insertion of *luxAB* into the chromosome is essentially random. The Tn5-*lux* transposon has been developed by Boivin *et al.* (1988) to introduce constitutive *luxAB* expression into a variety of Gram-negative bacteria including *Pseudomonas*, *Agrobacterium* and *Rhizobium* species. Expression from a promoter important for cell metabolism will reduce the fitness of the marked organism and a balance must therefore be obtained between maximisation of light output, through expression resulting from disruption of the expression of important genes (Prosser *et al.*, 1996).

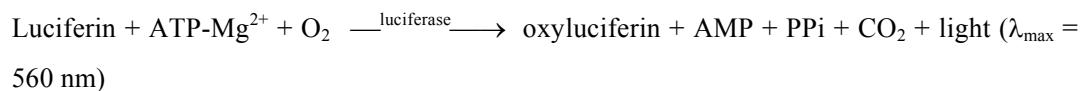
The monitoring of bioluminescent cells exposed to different stresses can be used in field applications including predictive microbiology. To predict how the organism is going to behave in certain environments is extended to provide more basic information, such capabilities of biosensor designs are well suited in fields such as biotechnology, medicine, food industry and environmental monitoring (Baker *et al.*, 1992; Stewart and Williams, 1992; Meighen, 1993; Prosser *et al.*, 1996). Living microbial sensors, particularly those based on *lux* genetic expression system, have the most potential for application in detection of environmental toxins or pollutants. The remainder of this section describes the biotechnology, genetic application of bacterial bioluminescence.

1.3.2 Bioluminescence

1.3.2.1 The bioluminescence reaction

Bioluminescence is the emission of the light resulting from the action of a luciferase enzyme on its substrate and this involves metabolism of oxygen or hydroxyl radicals resulting in the production of functionally important excited states of luciferase activity, plus the use of an alternative terminal oxidase. Bioluminescence is exhibited by both prokaryotes and eukaryotes (Meighen, 1988), including the luminescent system of marine bacteria (e.g., *Vibrio fischeri*, *Vibrio harveyi*, and *Ph. phosphoreum*), soil bacteria (*Photorhabdus luminescens* formerly *Xenorhabdus luminescens*), the firefly (*Photinus pyralis*), and the click beetle (*Pyrophorus plagiophthalmus*). Additionally, other luminescent bacteria are of interest including light-emitting *Vibrio cholera* strains found from brackish or freshwater (*Vibrio albensis*) and the aerobic *Shewanella (Alteromonas) hanedai* (Meighen, 1991 and 1993). Significant differences exist between the bioluminescence reaction of different organisms, including the structure and properties of the luciferase and substrates. The requirement for molecular oxygen is the one common feature of bioluminescence reactions.

The most common sources of *luc* genes are firefly and click beetle, and these luciferases catalyze the bioluminescent reaction most efficiently (McElory and Seigler, 1962; Ramanathan *et al.*, 1997). The substrate for the eukaryotic luciferase from the firefly is luciferin, a heterocyclic carboxylic acid, and its conversion is dependent on ATP rather than riboflavin 5'-phosphate (FMNH₂).



In the firefly, the enzyme catalyzes the ATP-dependent oxidative decarboxylation of luciferin. Eukaryotic luciferases from different sources have common substrates and emit light at different wavelengths. For instance, four different *luc* genes have been isolated from different species of click beetle and emit light at different wavelengths, in the range 547 to 593 nm (Wood *et al.*, 1989). The eukaryotic luciferases have provided the basis for a number of bioluminescent bacterial detection systems, where bacteria take up luciferin (Palomares *et al.*, 1989), but their use has been less widespread than systems employing the use of bacterial genes (Prosser *et al.*, 1996)

In the bacterial system, aldehydes are essential in the bioluminescence reaction, where the substrate is a long-chain aldehyde (tetradecanal) which is synthesized from a fatty acid precursor by a fatty acid reductase (Meighen, 1988; Tu and Mager, 1995). Light emission arises by the reaction of molecular oxygen with reduced (FMNH₂) by luciferase, to yield the corresponding fatty acid and FMN as shown below.



The primary source of energy for the light is supplied by the conversion of the aldehyde to the corresponding fatty acid. The reaction is highly specific for FMNH₂ which is formed by the reaction:



Bacterial luciferase is the enzyme catalyzing the bioluminescent reaction and it is linked to the respiratory pathway. The luciferase is a heteropolymeric protein with α and β subunits of approximately 40 to 44 kDa and 35 to 40 kDa, respectively, having arisen by gene duplication (Zeigler and Baldwin, 1981; Meighen, 1988). The active site is located primarily on the α subunit, but the β subunit is still essential for the light-emitting reaction. Neither the α or β subunit alone exhibits luciferase activity, but both preparations regain activity when combined with the second subunit, noting that the individual subunits do not possess an active site. The aldehyde binds at or near the interface of the luciferase α and β subunits (Hastings *et al.*, 1985; Baker *et al.*, 1992). In contrast, the firefly luciferase is active as a monomer with a molecular weight of approximately 62 kDa (deWet *et al.*, 1987).

It has been proposed that bacterial bioluminescent systems are a branch of the electron transport pathway in which electrons from reduced substrates are shunted to O₂ through two flavin enzymes, flavin mononucleotide reductase and luciferase (Hastings and Nealson, 1977). Luciferase may have evolved as a functional terminal oxidase alternative to the cytochrome system (Hastings, 1982), as the growth of cytochrome-deficient bacteria is dependent on luciferase induction and iron. Iron is required for cytochrome synthesis, but represses luciferase synthesis (Makemson and Hastings, 1982). Coupling between respiration and bioluminescence has been indicated by response to the respiratory inhibitors cyanide (Wada *et al.*, 1992) and carbonylcyanide-*m*-chlorophenylhydrazone (Grogan, 1983).

1.3.2.2 Bacterial *lux* genes

Bacterial bioluminescence has been characterized genetically as well as physiologically and biochemically (Hastings *et al.*, 1985; Meighen, 1988, 1991 and 1993; Stewart and Williams, 1992; Meighen and Dunlap, 1993). Engebrecht *et al.* (1985) first identified the enzymic and regulatory functions necessary for expression of the bioluminescent phenotype and determined the key aspects of genetic organization (Engebercht and Silverman, 1984). Genes coding for bacterial luciferase subunits (*luxAB*) and the fatty acid reductase polypeptides (*luxCDE*) responsible for biosynthesis of the aldehyde substrate for the luminescent reaction have been cloned and sequenced from *lux* operons of luminescent bacteria from at least four genera: *Photobacterium*, *Vibrio*, *Alteromonas* and *Photorhabdus*. The *luxCDE* genes flank the *luxAB* genes in the different luminescent bacterial species with transcription in the order *luxCDABE*, although an additional gene is located between *luxB* and *E* in *Ph. phosphoreum* (Meighen, 1991 and 1993; Meighen and Dunlap, 1993): the *lux* genes identified in specific luminescent strains are shown in Table 1.2. A multienzyme fatty acid reductase complex (r₄s₄t₂₋₄; 500 kDa) has been characterized from *Ph. phosphoreum* (Ferri and Meighen, 1991; Soly and Meighen, 1991).

The structural genes (*luxCDABE*) of *V. harveyi* and *V. fischeri* are highly conserved, indicating that the light emitting systems are very similar in the two bacteria. However, it was also found that the *lux* regulatory systems appear to have diverged. In *V. harveyi*, there was no open reading frame of greater than 40 codons within 600 bp of the start of *luxC*, which is where *luxI* is located in *V. fischeri*. The basic mechanism for the induction of luminescence or the location of the regulatory genes in relation to the structural genes differs in the two *Vibrio* systems (Miyamoto *et al.*, 1989).

1.3.2.3 Role of the HSPs in the regulation of the *V. fischeri lux* system

Regulation of light production by *V. fischeri* strains is controlled transcriptionally *via* a mechanism termed autoinduction. The autoinducer accumulates in the growth medium and, as the concentration of the inducer reaches a few molecules per cell, it acts as a specific

Table 1.2. The *lux* genes from bioluminescent bacteria (from Meighen and Dunlap, 1993).

Genes	Codons* (bp)	Protein functions and properties	Gene location
<i>luxA</i>	354-360	α subunit of luciferase ($\alpha\beta$), which catalyze the oxidation of reduced flavin mononucleotide (FMNH ₂) and a long-chain fatty aldehyde	Closely linked with upstream <i>luxB</i> flanked upstream by <i>luxCD</i> and downstream by <i>luxE</i>
<i>luxB</i>	324-328	β subunit of luciferase. Approximately 30% identical in amino acid residue sequence with <i>luxA</i>	Closely linked with downstream <i>luxA</i>
<i>luxC</i>	477-480	r (reductase). NADPH-dependent reduction of activated fatty acyl group to aldehyde and/or transferred back to the synthetase	Flanked upstream <i>luxAB</i> and closely linked with upstream <i>luxD</i>
<i>luxD</i>	305-314	t (transferase). Generation of fatty acids for the luminescence reaction	Flanked upstream <i>luxAB</i>
<i>luxE</i>	370-378	s (synthetase). ATP-dependent activation of fatty acyl group to form acyl-AMP	Flanked downstream <i>luxAB</i>
<i>luxF</i>	228-231	Non-fluorescent flavoprotein (23 kDa) with homology (40%) in sequence with the luciferase β subunits (Hastings <i>et al.</i> , 1985)	In most <i>Photobacterium</i> strains between <i>luxB</i> and <i>luxE</i> , excluded <i>Ph. leiognathi</i> species (Ruby and McFall-Ngai, 1992)
<i>luxG</i>	233-236	A 27 kDa protein related to that of a flavin reductase and other enzymes in electron transport, possibly implicating it in producing FMNH ₂ for luminescence reaction (Andrew <i>et al.</i> , 1992)	Found after <i>luxE</i> in marine but not terrestrial luminescent bacteria, <i>X. luminescence</i> (Meighen and Szittner, 1992)
<i>luxH</i>	230	A 27 kDa protein related in sequence (64% identity) to a new protein (HtrP) of <i>E. coli</i> which catalyzes the synthesis of a riboflavin precursor, implicating the <i>luxH</i> gene in riboflavin synthesis (Ritcher <i>et al.</i> , 1992)	Found in <i>V. harveyi</i> after <i>luxG</i> (Swartzman <i>et al.</i> , 1990)

<i>luxI</i>	193	Regulatory protein (22 kDa) required for synthesis of autoinducer, implicating LuxI protein as an autoinducer synthetase (Eberhard <i>et al.</i> , 1981; Salmond <i>et al.</i> , 1995)	Found in <i>V. fischeri</i> upstream of <i>luxC</i> (Engebrecht and Silverman, 1984)
<i>luxR</i>	250-252	Regulatory protein (29 kDa), a receptor for the autoinducer and activates the expression of the <i>lux</i> operon in <i>V. fischeri</i> (Sitnikov <i>et al.</i> , 1995)	Found in <i>V. fischeri</i> in a divergent operon immediately upstream of an operon containing the <i>lux</i> structural genes (Engebrecht and Silverman, 1984; Devine <i>et al.</i> , 1989)
<i>luxR</i>	205	Regulatory gene locus controlling expression of <i>lux</i> genes in <i>V. harveyi</i> . No homology in sequence to <i>luxR</i> in <i>V. fischeri</i> (Showalter <i>et al.</i> , 1990)	Gene locus in <i>V. harveyi</i> not linked to the <i>lux</i> structural genes (Showalter <i>et al.</i> , 1990)
<i>luxL</i>	189	LuxL protein tightly binds lumazine or riboflavin and can affect the wavelength, intensity, and decay of light produced by bacterial luciferase (Lee <i>et al.</i> , 1990)	Found 600 bp upstream of <i>luxC</i> in <i>Ph. phosphoreum</i> transcribed in the opposite direction (O'Kane <i>et al.</i> , 1991)
<i>luxY</i>	194	A yellow fluorescence protein (YFP, 22 kDa) that cause a shift in the luminescence from 490 nm to a higher wavelength resulting in the emission of a yellow rather than a blue-green light (Eckstein <i>et al.</i> , 1990)	Found in one strain of <i>V. fischeri</i> (Y1). Not linked to the <i>lux</i> structural genes (Baldwin <i>et al.</i> , 1990)

* The number does not include a stop codon. The range is indicated for different bacterial species. The nucleotide sequences of common *lux* genes (*luxCDABE*) were reported for three genera (i.e. *Vibrio*, *Photobacterium*, and *Photorhabdus*) and *lux* genes sequences of other species were identified in only specific luminescent strains (see reviewed by Meighen and Dunlap, 1993).

inducer of the *lux* operon (Ulitzur and Hastings, 1979). The autoinducer of *V. fischeri* produces a diffusible substance, identified as (N-[β -ketocaproyl] homoserine lactone) and analogues of this autoinducer could not stimulate luminescence in other species (Meighen, 1988). This compound is produced by the *luxI* gene product and acts as a positive regulator for the *lux* system in the presence of a functional *luxR* gene (Kaplan and Greenberg, 1985; Adar and Ulitzur, 1993). The chaperonins are involved in the regulation of *V. fischeri lux* system, *luxRICDABE*. Ulitzur and Kuhn (1988) have shown that the HSP encoded by *rpoH* (σ^{32}) supports high level of luminescence, as σ^{32} causes stimulation of expression of the GroES/L genes. LexA, another HSP, has also been implicated in the regulation of the *V. fischeri* luminescence system (Adar *et al.*, 1992). Shadel and Baldwin (1991) postulated that the overall effect of GroES/L proteins increases the rate of *luxR* transcription. The σ^{32} is involved in the folding and/or expression of the LuxR protein and not directly required for the transcription of *luxICDABE* in *E. coli* but is required for sufficient expression of *groE*, which in turn is required for the transcription of *luxICDABE* (Dolan and Greenberg, 1992). The role of GroES/L proteins in the regulation of the *lux* system is accomplished by stabilising the active form of the LuxR protein due to GroEL protein binding to the denatured form, where the stabilised protein is released from the complex through the aid of GroES protein and ATP. Stress signals, such as starvation or others, induce the formation of σ^{32} , which in turn induces the formation of the HSPs, including the GroES/L proteins. The GroES/L proteins assist the LuxR protein to fold into its active form without catabolite repression, which then forms a complex with the inducer. The full induction of the luminescence system seems to occur under certain stress conditions that activate σ^{32} genes and consequently lead to the formation of the heat shock proteins including GroES/L proteins.

Clearly, the *V. fischeri lux* system is under control of a multiple set of regulatory signals. Expression of luminescence is also subject to regulation by catabolite repression (reviewed by Meighen and Dunlap, 1993): glucose blocks *lux* gene expression in *V. fischeri* and *V. harveyi*. Furthermore iron represses luminescence in several species, oxygen tension causes different responses in different species, osmolarity effects both growth and bioluminescence as a function of osmotic strength, and luciferase mechanism involves several dark processes in dark or dim variants of bioluminescent strain (Lee *et al.*, 1990; Nealson and Hastings, 1992; Tu and Mager, 1995).

1.3.3 Bioluminescence in biomonitoring

When cells are exposed to toxins, there is a corresponding decrease in metabolic activity or the cells die, depending on the concentration of toxins present. Biosensors based on changes in metabolic activity can detect any soluble toxicant available to the test cells, but may not identify the type of toxin present (Ramanathan *et al.*, 1997). Considering this principle, simple microbial biosensors have been specifically developed for determining the biotoxicity of samples or the presence of specific nutrients and these have been commercialised for routine application (Lampinen *et al.*, 1990; Lee *et al.*, 1991). For example, a whole yeast cell biosensor has been developed and commercialized for monitoring biochemical oxygen demand (BOD), an indicator of the level of organic materials in polluted water (Karube, 1987; Tescione and Belfort, 1993). Other biosensors are based on natural bioluminescence or the response of bioluminescence genes in specific backgrounds. Some of these and their applications are described in the following sections.

1.3.3.1 Natural bioluminescent bacteria as biosensors

The commercially available Microtox® test (Beckman Instruments Inc., Carlsbad, California) is based on the inhibition of bioluminescence of the bacterium *Ph. phosphoreum* (Bulich, 1982) when it is exposed to toxic substances, including solvents and toxic metals (Bulich, 1986; Kamlet *et al.*, 1986). The freeze-dried cells are resuspended in sodium chloride (which is essential for bioluminescence) and exposed to aqueous samples. Changes in bioluminescence relative to a control used on the same day indicates the presence of toxicants, where the exact nature of the toxicant cannot be identified as this test indicates only the presence of some form of toxicant. However, the kinetics of the dose-related decline in bioluminescence can indicate the class of toxins present (Bulich, 1982 and 1986; Ribo and Kaiser, 1983; Kamlet *et al.*, 1986). The response time for this system ranges from 15 minutes to 1 hour (Tescione and Belfort, 1993). Furthermore, the intact freeze-dried cells have been used for testing toxicity in long-term based assays (overnight or longer incubation with toxic substances) in the Mutatox™ test (Arfstén *et al.*, 1994). The Mutatox™ test uses a dark variant of *V. fischeri* which produces bioluminescence after incubation at 27°C for 16-24 hours in the presence of genotoxic agents.

Although the Microtox™ system has broad acceptance as a laboratory test for monitoring

environmental samples, it could be improved significantly for more general application. For example, one problem in using *Ph. phosphoreum* is that it requires the use of high NaCl concentrations or other stabilisers which may lead to false negatives in samples containing metal ions. Additionally, an immobilized system has been studied for possible application in continuous water monitoring or for field applications. A strain of *Ph. phosphoreum* was immobilized in strontium alginate and alginate-glycerol suspensions stored at -80°C for up to 12 weeks which gave stable light output. This system showed a dose-related response to Pb(NO₃)₂ at concentration of 5, 10 and 50 ppm as well as for three other tested heavy metal ions (Chun *et al.*, 1996; Britz *et al.*, 1997).

1.3.3.2 Biosensors based on expression of *lux* genes in alternative hosts

The *lux* genes placed behind a suitable promoter and transferred into a naturally non-bioluminescent organism can be used as a sensor for a wide variety of processes or substances where the scope of application is limited only by the properties of the expression system and the organism (Meighen, 1993). Promoter probe vectors employing *lux* genes have been developed for both Gram positive and Gram negative bacteria (de Lorenzo *et al.*, 1990; Farinha and Kropinski, 1990; Park *et al.*, 1992; Van Dyk and Rosson, 1998). The promoter probe vectors have been constructed using *luxAB* or the entire *lux* pathway including the aldehyde synthesis genes using vectors such as pUCD615 (Rogowsky *et al.*, 1987). In the following sections, a number of examples have been selected to illustrate the variety of constructs that have been developed, including their sensitivity, specificity or general application.

1.3.3.2.1 Chromosomal marking using *lux* genes

The ubiquitous soil bacterium *Ps. fluorescens* was used to construct bioluminescent strains using either plasmid or chromosomally encoded *V. fischeri luxABE* genes. These strains were used to measure the bioluminescence in short response time periods (20 seconds) initiated by 1 µl of *n*-decyl aldehyde solution made up as 33% w/v in 100% ethanol. The bioluminescence declined sharply in the presence of sulfate salts of metals (Zn, Cu, Cd, Ni, and Cr) at concentrations up to 5 mgL⁻¹ at pH 5.5, but the kinetics showed no greater increased inhibition of subsequent concentrations of 5-25 mgL⁻¹ (Paton *et al.*, 1995). The order of sensitivity was Cu = Zn > Cd > Cr > Ni.

The *luxAB* genes were fused into the *E. coli* genome at a single location and screened for clones displaying transcriptional changes in the presence of metal ions. The Tn5-*luxAB* cassette was located in the metal-responsive gene, *fliC* (formerly called *hag*), which encodes flagellin, and *fliC*:Tn5-*luxAB* activation was detected by its increasing gene expression and light emission, with increasing concentrations of aluminium, copper, iron and nickel between 1 ppm and 10 ppm at pH 5.5 (Guzzo *et al.*, 1991).

Carmi *et al.* (1987) were the first to use luciferase as a marker for gene expression in *Bacillus* spp. as a non-disruptive indicator for developmental systems such as sporulation, where spore development is dependent on the architectural integrity of the mother cells. Spores produced from *lux* recombinant bacilli remain non-bioluminescent until triggered into biochemical activity through germination (Stewart and Williams, 1992). Cook *et al.* (1993) introduced the bioluminescence genes from *V. fischeri* and *V. harveyi* into *B. subtilis* using both plasmid and chromosomal integration. The chromosomally marked strains emitted less light but were found to be stable without a requirement for antibiotic selection, compared to the plasmid-bearing strains. This engineered *Bacillus* was designed for tracking bacteria in the environment and for use in enumerating viable cells following inoculation into soil (Cook *et al.*, 1993).

1.3.3.2.2 Use of stress promoters

Van Dyk *et al.* (1994) reported the development of a general pollution detection system which was based on *luxCDABE* (pUCD615) expression in *E. coli*, where the cassette was regulated by the *dnaK* and *grpE* promoters. When placed in suitable a host which enabled test substances to enter the cell (*tolC* mutant) for phenolic compounds, these workers were able to show that both promoters showed some reaction to the pollutants tested, such as ethanol, at concentrations 0.2-4% and 9-38 ppm of pentachlorophenol (dissolved in ethanol). The concentrations of different substances which stimulated luminescence were: 833 ppm of copper sulfate, 100 ppm of 2,4-dichlorophenoxyacetic acid (dissolved in ethanol), 40,000 ppm of methanol, 340 ppm of 2-nitrophenol (dissolved in methanol), 62 ppm of 4-nitrophenol (dissolved in methanol) and 1,390 ppm of phenol for *dnaKp::luxCDABE* and 1,000 ppm of copper sulfate, 133 ppm of 2,4-dichlorophenoxyacetic acid, 32,000 ppm of methanol, 113 ppm and 340 ppm of 2-nitrophenol, 37 ppm of 4-nitrophenol, 460 ppm of phenol and 1,250

ppm of xylene for *grpE'::luxCDABE*, while other concentration ranges showed less or no response when using either promoters. The induction of a *uspA'::lux* fusion by a variety of stresses was reminiscent of the heat shock responsive *grpE'::lux* fusion (Van Dyk *et al.*, 1995). The concentrations that produced maximum response were: 1% n-propanol, 1,040 ppm of phenol, 800 ppm of copper sulfate, 9,600 ppm of sodium propionate, 37.5 ppm of pentachlorophenol, 50 ppm of 4-nitrophenol, 12 ppm of CdCl₂, 800 ppm of CuSO₄ and no induction by HgCl₂. For both systems, alcohols tended to be the strongest inducers compare to other chemicals tested and UV irradiation. The strain containing the heat shock promoter fusion had elicited induction responses, better limits, and a broader range of detectable stresses by Van Dyk *et al.* (1995). Belkin *et al.* (1997) have further developed this system and Ben-Israel *et al.* (1998) described the quantification and quality assurance parameters required for use of this system for the identification of simple mixtures of toxic chemicals. The system provides a primary elucidation of the mode of action of different toxic agents the biological signatures of individual known compounds. *Mycobacterium tuberculosis* (contained plasmid pLUC10) was used to screen the efficacy of antimicrobial agents. Shuttle vector pMV261 was used to express a promoterless firefly luciferase gene, and luciferase was expressed under the control of a mycobacterial Hsp60 heat shock promoter. Bioluminescence was measured after 2, 7, and 14 days and the lowest concentration of antibiotics able to detected was 0.06 µg/ml for streptomycin, isoniazid, rifabutin, and rifampin (Cooksey *et al.*, 1993).

1.3.3.3.3 Metal detection biosensors

In order to understand the use of biosensors for monitoring metal concentrations, it is useful to understand the mechanisms that cells employ to regulate metal responsive operons and resistance to metals, which affects the sensitivity and selectivity of biosensors that utilise responsive cells (Ramanathan *et al.*, 1997). Microbial metal resistances are heterogeneous in both their genetic and biochemical bases and may be chromosomally-, plasmid-, or transposon-encoded with one or more genes being involved. At the biochemical level, microorganisms display a diversity of types of resistance mechanisms that have evolved, which includes six different fundamental types (see review by Rouch *et al.*, 1995). These different mechanisms may occur solely or in diverse combinations to produce resistance (Brown, 1985; Amyes and Gemmell, 1992; Silver and Ji, 1994; Rouch *et al.*, 1995; Ji and Silver, 1995). Specific metal responsive promoters in conjunction with bioluminescence genes have been studied to detect bio-hazardous materials.

Several *lux* operons originating from *V. fischeri* or other marine bacteria (Meighen, 1991) have been cloned so that the *lux* cassettes are under the control of a specific inducible promoter. Examples include the detection of mercurial compounds (Selifonova *et al.*, 1993; Tescione and Belfort, 1993) and, arsenic and cadmium (Corbisier *et al.*, 1993). In addition, biosensors have been used to detect specific responses to copper, nickel, zinc, chromate, and thallium ions by fusion of the *lux* genes to a number of metal ion-responsive promoters from *Alcaligenes eutrophus* (reviewed by Collard *et al.*, 1994). These whole-cell biosensors display rapid induction of bioluminescence in response to specific environmental pollutants and can complement analytical chemical methods for the detection of biologically-available metals in the environmental sample (Stewart and Williams, 1992; Ramanathan *et al.*, 1997).

For instance, a biosensor for the detection of Hg^{2+} in the environment was constructed in *E. coli* using fusions of a *Tn21* mercury resistance operon (*mer*) (which responds to Hg^{2+} but not to organo-mercurial compounds) with the promoterless *luxCDABE* genes from *V. fischeri* (Selifonova *et al.*, 1993). The use of *cadA-luxAB* and *arsB-luxAB* fusions as biosensors for detection of cadmium and arsenic ions was developed (Corbisier *et al.*, 1993), and a *celf::luxAB* transcriptional fusion displayed increased bioluminescence in the presence of 1 to 50 ppm of $NiSO_4$ in *E. coli* (Guzzo and DuBow, 1994).

Cyanobacteria, which efficiently utilize solar energy and carbon from the air, was used to express *lux* genes under the control of a methallothionein gene and this was reported to be a sensitive indicator of heavy metal cations in aqueous samples. A metal-responsive *smt* operator/promoter region of *Synechococcus* sp. was fused to the *luxCDABE* genes of *V. fischeri* (Erbe *et al.*, 1996). *Synechococcus* sp. PCC7942 (contained plasmid pJLE23) showed sensitivity to $ZnCl_2$ from 0.5 μM to 4 μM over a 5 hour time period; $CuSO_4$ was less effective than $ZnCl_2$ at these concentrations and the response to $CdCl_2$ was at 0.5 μM , 1 μM and 1.5 μM . Higher concentrations (more than 8-16 μM Zn^{2+} , 15 μM Cu^{2+} or 1.5 μM Cd^{2+}) caused less luminescence than the above concentrations tested which resulted in an initial increase in luminescence followed by a sharp decline toward baseline levels following 1 hour exposure time (Erbe *et al.*, 1996).

1.3.3.3 Use of other mechanisms

The use of *lux* genes systems has been extended to apply in the food industry for uses which include the detection of specific bacterial pathogens and indicator microorganisms, monitoring hygiene on-line, determining the effectiveness of spore destruction, monitoring starter culture integrity, biocide and virucide challenges, and studying recovery of sublethally injured cells (Ahmad and Stewart, 1991; Baker *et al.*, 1992; Stewart and Williams, 1992). For example, the introduction of the *lux* genes (*V. fisheri*) into the genome of a bacteriophage was developed by Ulitzur and Kuhn (1987), and similarly firefly luciferase genes used (Lee *et al.*, 1992). Recombinant *lux*⁺ bacteriophages could detect target bacteria without recovery or enrichment, provided that the bacteria were present in a food matrix at levels greater than 10⁴ per gram, and this phage assay could detect less than 10 enteric bacteria per gram of food matrix (Kodikara *et al.*, 1991).

In addition to using metal responsive promoters in conjunction with either *luxCDABE* or *luxAB*, promoters responding to naphthalene and salicylate (Burlage *et al.*, 1990; King *et al.*, 1990), toluene (Burlage *et al.*, 1994; Applegate *et al.*, 1997) or genotoxins (Ptitsyn *et al.*, 1997) have also been developed as possible specific biomonitoring tools.

A plasmid-borne transcriptional fusion between *narG* (*E. coli* nitrate reductase) promoter and *lux* (*Pho. luminescens*) operon provided an *E. coli* biosensor to detect nitrate in brewing water (Prest *et al.*, 1997). A bioluminescent bacterium, *Ps. fluorescens*, carries a reporter plasmid which contains a *nahG-luxCDABE* cassette, pUT21, encoding naphthalene degradation and the bioluminescence reporter function (King *et al.*, 1990). Exposure of *Ps. fluorescens* (containing plasmid pUT21) to both naphthalene and its degradation intermediate, salicylate, resulted in increased bioluminescence.

The SOS *lux* test was used for detection of environmental genotoxins (Quillardet *et al.*, 1982; Ptitsyn *et al.*, 1997). The bioassay was based on the recombinant plasmid pPLS-1 carrying the promoterless *luxCDABE* genes of *Ph. leiognathi* under the control of the SOS promoter that was downstream of a truncated *cda* gene from plasmid ColD. Responses to genotoxins could be detected within a second of exposure to growing cultures of *E. coli* C600 (containing plasmid pPLS-1), and assay results were obtained over periods up to 2 hours. The test showed a dose-response relationship for six genotoxins {mitomycin C, N-methyl-N'-nitro-N'-nitrosoguanidine (MNNG), nalidixic acid, dimethylsulfate, hydrogen peroxide, and formaldehyde}, UV and γ radiation. The minimum concentration of genotoxin are at which

the SOS response was induced ranged over two or more orders of magnitude, from 10 nM to 1 μ M for mitomycin C, from 0.5 to 50 μ M for MNNG, from 5 to 100 μ M for nalidixic acid, and 5 to 500 μ M for dimethylsulfate. Exceptions were H₂O₂ and CH₂O, which showed that SOS induction occurred over the range of 0.15 to 3 mM for H₂O₂ and from 0.3 to 0.75 mM for CH₂O, followed by significant cytotoxicity effects (Ptitsyn *et al.*, 1997).

A biosensor strain based on *E. coli* DH5 α , containing either plasmid pGEc74 or pJAMA7, carried a regulatory gene *alkS* from *Ps. oleovorans* and a transcriptional fusion of P_{*alkB*} from the same strain, with the promoterless luciferase *luxAB* genes from *V. harveyi*. These constructs were used to detect toxicants in groundwater samples contaminated with heating oil (Sticher, *et al.*, 1997). This biosensor responded to middle-chain-length alkanes, from pentane to hexadecane, branched alkanes (3-methylheptane) and petroleum ether but not to acrylic or aromatic compounds. Additionally, the *luxAB* genes derived from *V. harveyi* were expressed in *Nitrosomonas europaea* by transcriptional control of the promoter of the *hao* gene. When allylthiourea-treated intact cells were incubated with hydroxylamine or hydrazine, an increase in the intensity of light emission was observed. This biosensor strain showed that it was possible to evaluate overall ammonia-oxidizing activity and its inhibition using this approach (Iizumi *et al.*, 1998).

In developing biofilms, environmental stress factors were investigated by Rice *et al.* (1995). An alginic acid biosynthesis bioluminescent reporter plasmid, pUTK50, containing the regulatory region of the *algD* biosynthesis operon (alginic synthesis) fused with the promoterless *lux* gene cassette of pUCD615, was transconjugated into environmental strains of *Ps. putida*, *Ps. fluorescens*, and *St. maltophilia*. The culture was exposed to 100, 200 and 300 mM NaCl for testing medium osmolarity, and a maximum bioluminescence occurred in logarithmic phase growth. The induction gradually increased with increasing concentration of NaCl and this occurred only in the presence of 3.25 mM NH₄⁺ and 6.75 mM NO₃⁻ (Rice *et al.*, 1995). Although these results indicated that careful optimization for light output was required, one of the environmental isolates, *St. maltophilia*, showed a sustained ability for light production, under conditions necessary for biofilm growth and development.

For biosensor application, firefly luciferase or bacterial luciferase can utilize stable light-producing reagents and the concept of ‘continuous monitoring’ of biological reactions is applicable for both types (Lundin *et al.*, 1976; Lövgren *et al.*, 1982). Firefly luciferase

reagents have been used to detect ATP or metabolites or enzymes linked to ATP formation/utilization, and this luciferase and coupling enzymes were immobilized onto porous matrices (DeLuca, 1984; Ford *et al.*, 1996). In bacterial bioluminescence, the enzymes have been used to detect NAD(P)H or metabolites or enzyme linked to NAD(P)H formation/disruption and the soluble system was immobilised onto Sepharose for continuous-flow column NADH monitoring (Kurkijärvi *et al.*, 1982).

1.3.4 Challenges in using *lux*-cloned systems

Stabilization of light output following strain construction is a major problem, as the bioluminescence signal measured for whole-cell biosensors depends not only on the available concentration of the inducing substance but also on the stability of the luciferase in the particular strain (Table 1.3) and temperature stability (Mackey *et al.*, 1994), growth conditions as well as using suitable buffers (Korpela *et al.*, 1989), the strain's physiological state (Heitzer *et al.*, 1992), and the presence of other stimulating or inhibitory substances in the sample composition (Burlage *et al.*, 1994; Sticher *et al.*, 1997). For example, the substrate for bacterial luciferase reaction, *n*-decanal, easily penetrates across the cell membranes, however, at high concentrations this can inhibit the luciferase enzyme (Blouin *et al.*, 1996) and can be cytotoxic (Holzman and Baldwin, 1983). The *luxAB* system does not necessitate cell disruption thus the *in vivo* responses are estimated in real time. Accordingly, *E. coli* (Korpela *et al.*, 1989) and *B. subtilis* (Karp, 1989) were provided with cloned *lux* genes and certain strains capable of giving continuous reporting on bioluminescence detection. On the other hand, cells provided with cloned firefly luciferase genes (DeWet *et al.*, 1985) were not capable of doing this, owing to permeability difficulties of the luciferin cofactor for luciferase reaction.

The way in which cells are used is also important. Immobilized *Ps. fluorescens* containing the *nahG-luxCDABE* cassette was developed to detect bio-availability of naphthalene and salicylate in waste streams. The reporter culture was immobilised onto the surface of an optical light guide by using strontium alginate, which provided a stronger matrix than calcium alginate. This biosensor probe was then inserted into a measurement cell which

Table 1.3. Bacterial *lux* gene systems as sensors (Meighen, 1993).

Sources	<i>lux</i> genes	Applications
<i>Vh, Vf, Ph, Pp, Pl</i>	<i>luxAB</i>	Generates luminous prokaryotes on aldehyde addition. Sufficient FMNH ₂ in bacteria for high activity. <i>Ph</i> and <i>Vh luxAB</i> stable at 37°C.
<i>Vh, Vf, Ph</i>	<i>luxCDABE</i>	Luminous phenotype for prokaryotes without exogenous additions. Precursors (e.g., acyl-ACPs) are present for aldehyde biosynthesis catalyzed by <i>luxCDE</i> .
<i>Vh</i>	Fused <i>luxA-B</i>	Expression in eukaryote under a single promoter at less than 30°C. Most cases will require cell disruption to deliver FMNH ₂ and/or extraction to assay.

Ph, *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*); *Vh*, *Vibrio harveyi*; *Vf*, *Vibrio fischeri*; *Pl*, *Photobacterium leiognathi*; *Pp*, *Photobacterium phosphoreum*.

simultaneously received the waste stream solution and a maintenance medium. Exposure under defined conditions to both naphthalene and salicylate resulted in a rapid increase in bioluminescence (Burlage *et al.*, 1990; Heitzer *et al.*, 1994), and trichloroethylene and toluene were measured during on-line monitoring (Applegate *et al.* 1997).

Whole-cell systems exhibit reduced substrate specificities compared with enzymatic biosensors. However, the use of genetically-engineered bacteria with *lux* fused to the regulatory elements of inducible genes or operons largely overcomes the specificity problem and provides a general approach applicable to a large number of organic and inorganic pollutants for which degradation or detoxification pathways exist (King *et al.*, 1990; Silver and Walderhaug, 1992; Burlage *et al.*, 1994; Ji and Silver, 1995; Rouch *et al.*, 1995; Ramanathan *et al.*, 1997)

1.4 Aims of this thesis

This thesis was concerned with understanding how natural environmental bacteria responded to pollutants, such as metals and heat stress, in the context of developing bioindicators based on these stress responses for detecting toxic substances. Indigenous microbes are ubiquitous in soil and aquatic environments, and these environmental isolates were selected to provide a knowledge base for evaluating whether their responses would have utility in predictive models for pending environmental perturbation. The specific aims of this thesis were:

- To define responses to heat shock, at the protein level in natural Gram negative bacteria isolated locally.
- To discover what responses occurred to sub-lethal exposure to metal ions, in particular, and other common organic environmental pollutants using by 1-D/2-D SDS PAGE.
- To determine the level of physiological impacts of pollutants on cells using *lux*-marked strains to indicate these responses
- To evaluate the utility of the *lux*-marked strains in detecting the presence of pollutants.

This kind of work validates the approach which seeks to use pseudomonad species rather than *E. coli* for detection of specific chemicals and general toxicants. Indigenous species may have greater utility as bioindicators and biosensors because they are of aquatic, sediment or soil origin and may have response which closely resemble reactions which occur in their specific environments. The major aim of this thesis was to obtain knowledge of how aquatic microbes (pseudomonads related species) isolated from Australian waterways respond to pollution stress and the use of these responses for developing a luminescence-based reporter gene system, involving the induction of prokaryotic (*lux*) genes.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Bacterial strains and plasmids

The two aquatic indigenous strains, VUN10,077 (formerly identified as *Ps. paucimobilis*), and *Stenotrophomonas maltophilia*, VUN10,075 (formerly *Ps. maltophilia*) were natural isolates from northeastern waterway systems in Victoria, Australia, and were provided by Dr Paul Boon (when at CSIRO, personal communication). *Ps. aeruginosa*, VUN00030, and *Ps. fluorescens*, VUN00031, were obtained from the Victoria University of Technology (VUT) culture collection. Transconjugants of VUN10,077 containing chromosomally-located *luxAB* genes were designated as VUN3,600 and VUN3,601.

The plasmids and *E. coli* strains used are listed in Table 2.1. *E. coli* HB101 was used as the host for pRK2013 plasmid, strain DH5 α was used as the host for pUCD607, pUCD615 and pUCD623 plasmids. *E. coli* strain JM109 was used as the host for pGEMT plasmid (Promega) and strain CC118 and S17-1 were used as hosts for pUT::Tn5-*luxAB* plasmid. Tetracycline, ampicillin and kanamycin were used at 75, 100 and 100 μ g/ml, respectively, to retain plasmids.

2.1.2 Chemicals

All chemicals were analytical grade reagents obtained from Sigma (U.S.A.), BDH (UK) and other reputable supplies. Where they may have influenced the outcome of experiments, the specific source is designated.

For toxicity assessment, chemicals were used as described in the following: phenolic compounds (200 mg/ml) were dissolved in dimethyl formamide (DMF): phenol (BDH), 2,6-dichlorophenol (Sigma), 2,4,5-trichlorophenol (Sigma), 4-chlorophenol (Sigma), pentachlorophenol (Sigma) and δ -nitrophenol (Sigma). Heavy metal ions (50 mg/ml) were dissolved in Milli-Q water and filter sterilised (0.45 μ m, GS, Millipore): CdCl₂ (Sigma), CuCl₂.2H₂O (Sigma), CoCl₂.6H₂O (Sigma), HgCl₂ (Sigma), NiCl₂.6H₂O (Sigma), NaAsO₂ (Sigma), ZnCl₂ (Aldrich), FeCl₂.4H₂O (Sigma), MnCl₂.4H₂O (Sigma), SrCl₂.6H₂O (Sigma), and Pb(NO₃)₂ (Sigma). Sodium dodecyl sulphate (SDS) was dissolved as a 10% solution in sterilised water (Milli-Q) and used without sterilization

Table 2.1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description ^a	Reference of source
Plasmids		
pUT::Tn5-luxAB	Ap ^r , <i>tnp*</i> gene of Tn5-IS50R inserted in <i>SalI</i> site of pGP704. mini-Tn5luxAB reconstructed the promoterless luxAB unit from <i>SalI-BamHI</i> site of pFIT001 and pPANE001	de Lorenzo <i>et al.</i> , 1990
pRK2013	Cole1 <i>tra(RK2)</i> ⁺ Km ^r . Helper plasmid for conjugal transfer	Figurski and Helinski, 1979
pUCD607	<i>tet</i> promoter <i>luxCDABE</i> of <i>Vibrio fisheri</i> , <i>oriPE1</i> <i>oriPSa repA</i> Km ^r Ap ^r Sp ^r	Shaw and Kado, 1986
pUCD615	<i>oriPSa oriPB322 tra</i> Ap ^r Km ^r (Gm ^r), promoterless Tn5 <i>luxICDABE</i> of <i>V. fisheri</i> (<i>lux</i> genes from pJE347 inserted into pUCD5B)	Rogowsky <i>et al.</i> , 1987
pUCD623	<i>oriCole1 tra</i> , Tn4431 reconstructed the promoterless <i>luxCDABE</i> carried on pSa325, Tc ^r Cm ^r Ap ^r	Valentine and Kado, 1989
pUC19	ColE1 replicon, Ap ^r lacZα ⁺ , MCS ^a	Yanisch-Perron <i>et al.</i> , 1985
RP4	Ap ^r , Km ^r , Tc ^r	Datta <i>et al.</i> , 1971
<i>E. coli</i> strains		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac proAB) F'(traD36 proAB⁺ lacI^q ΔM15)</i>	Promega, Yanisch-Perron <i>et al.</i> , 1985
DH5α	F ⁻ <i>endA1 hsdR17(rK⁻mK⁺) supE44 thi-1 recA1 gyrA1 relA1 80dlacZΔM15 Δ(lacZYA-argF)U169</i>	Bethesda Research Laboratories, Gaithersburg, Md.
HB101	F ⁻ <i>lacY1 galK2 supE44λ ara-14 proA2 rpsL20 recA13 xyl-5 mtl-1 hsdS20(Sm^r) mcrB</i>	Boyer and Roulland-Dussoix, 1969
CC118(λpir)	Δ(<i>ara-leu</i>) <i>araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1</i> , lysogenized with λpir phage	Herrero <i>et al.</i> , 1990
S17-1(λpir)	<i>thi hsdR^r hsdM⁺ pro recA</i> , RP4-2-Tc::Mu km::Tn7Tp ^R Sm ^R , lysogenized with phage λpir	de Lorenzo <i>et al.</i> , 1993b

^a Km^r(Gm^r) designate Km^r genes that also encode resistance to gentamicin.

*tnp**, Tn5 transposase, The *tnp* product promotes the transposition of any DNA segment flanked by the 19-bp termini of Tn5 when the segment is located in *cis* with respect to *tnp**.

λpir is a λ phage carrying the *pir* gene, the product of which (the π protein) is essential for the replication of R6K plasmid and its derivatives.

2.1.3 Microbiological media

Luria-Bertani (LB) medium was used for growth of *E. coli* and contained 10 g of tryptone (Oxoid), 5 g of yeast extract (Oxoid), 5 g of sodium chloride per litre, pH 7.2 (Miller, 1972). Brain Heart Infusion media (BHI, Oxoid) was prepared according to the manufacturer's instruction. For plates, BHI and LB were solidified with 12 g/litre of agar (BHIA, LA). All antibiotics were added aseptically to the medium at 50°C, immediately prior to dispensing the medium.

The recovery medium used for transformants after electroporation was BHI supplemented with 10 mM CaCl₂, 10 mM MgCl₂.6H₂O and 0.5% glucose.

SOC medium (100 ml) contained 2 g of tryptone, 0.5 g of yeast extract, 1 ml of 1 M NaCl, 0.25 ml of 1 M KCl, 1 ml of 2 M Mg²⁺ stock (1 M MgCl₂.6H₂O/1 M MgSO₄.7H₂O) and 1 ml of 2 M glucose, pH 7.0 (Sambrook *et al.*, 1989). The medium was autoclaved at 121°C for 15 minutes except Mg²⁺ stock and glucose solutions were filter sterilised (0.22 µm, Millipore, GS) and added after autoclaving.

X-Gal medium prepared in LA containing ampicillin/IPTG/X-Gal. One hundred µl of 100 mM IPTG (isopropyl-β-D-thiogalactoside) and 20 µl of 50 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, dissolved in 1 ml of N,N'-dimethyl formamide) was spread over the surface of LA containing 100 µg/ml ampicillin and allowed to absorb for 30 minutes prior to use.

Various minimal media (Table 2.2) were prepared based on M9 salts solution (64 g of Na₂HPO₄.7H₂O, 15 g of KH₂PO₄, 2.5 g of NaCl and 5 g of NH₄Cl per litre) which was divided into 200 ml aliquots, sterilised and kept at -20°C (Sambrook *et al*, 1989). The components were prepared as follows and sterilised by autoclaving at 121°C, 20 minutes: 1 M CaCl₂, 1 M MgSO₄, trace elements solution (3 mg of MnCl₂.4H₂O, 20 mg of CoCl₂.6H₂O, 1 mg of NiCl₂.6H₂O, 200 mg of FeSO₄.7H₂O, 10 mg of ZnSO₄.7H₂O, 500 mg of Na₂MoO₄.2H₂O and 30 mg of H₃BO₃ per litre) (Bogardt and Hemimingen, 1992), and casamino acid (Difco) was dissolved as a 10% solution in Milli-Q water. The following were sterilised by Millipore filtration (0.22 µm, Millex®-GS): 20% glucose; vitamin solution (2 mg of biotin, 2 mg of folic acid, 5 mg of thiamine-HCL, 5 mg of D-calcium pantothenate, 5

Table 2.2. The composition of defined minimal media (per litre)

Medium ^a number	Glucose	CaCl ₂	MgCl ₂	Casamino acid	Sulphur-free amino acid mixture	Vitamin	Trace elements
1	20 ml	0.1 ml	2.0 ml	-	-	-	-
2	20 ml	0.1 ml	2.0 ml	10 ml	-	-	-
3	20 ml	0.1 ml	2.0 ml	-	50 ml	-	-
4	20 ml	0.1 ml	2.0 ml	10 ml	50 ml	-	-
5	20 ml	0.1 ml	2.0 ml	10 ml	-	0.1 ml	5.0 ml

^a All media contained 200 ml of M9 salts.

mg of vitamin B₁₂, 5 mg of riboflavin, 20 mg of nicotinic acid [niacin], 3 mg of pyridoxal-HCl and 2 mg of p-aminobenzoic acid per 100 ml) (Bogardt and Hemimingen, 1992); and sulphur-free 5% essential amino acids (each amino acids solution was prepared separately) included L-arginine, L-tryptophan, L-lysine, L-serine, L-phenylalanine, L-isoleucine, L-histidine, DL-threonine, L-proline, L-alanine, L-valine, L-glutamine, L-glutamic acid, L-leucine, L-aspartic acid, glycine, tyrosine and asparagine.

2.1.4 Buffers

All buffers were made up as described below, unless otherwise stated. The pH of each buffer was measured on a Orion model 410A pH meter and adjusted with solutions of salts, acids (10 M HCl) or bases (10 M NaOH) as needed before use. All buffers were prepared in glass with Milli-Q (Millipore) water and sterilised by autoclaving at 121°C, 20 minutes unless otherwise stated. Sugar components in buffers were added from sterile stock solutions of 50% (w/v) sucrose and 20% (w/v) glucose, autoclaved at 109°C for 25 minutes.

DEPC (diethyl pyrocarbonate, Sigma)-treated water and baked glassware were used for all RNA manipulation (1% DEPC dissolved completely in sterile Milli-Q water with stirring overnight, and autoclaved at 121°C, 20 minutes). All glassware was washed with detergent thoroughly and rinsed with sterile water and autoclaved. These glassware were baked in a dry oven at 150°C at least overnight. Electrophoresis gear was soaked into 0.5 M NaOH for 10 to 30 minutes and rinsed thoroughly with water, and plastic-ware was autoclaved at 121°C for 20 minutes.

TAE buffer (50×), pH 7.8, contained 200 mM tris(hydroxymethyl)aminomethane (Tris), 100 mM sodium acetate (NaOAc) and 10 mM ethylenediamine tetraacetic acid (EDTA, pH 8.0). The 50× concentrated stock was diluted in Milli-Q water to 1× TAE prior to use in agarose gel electrophoresis. When the composition of TAE was different from this formulation, this is noted in the sections where the buffer is used.

TBE buffer (5×), pH 8.2, contained 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA. The 5× concentrated stock solution was diluted into Milli-Q water to give the desired concentration and used for agarose gel electrophoresis.

TE buffer, pH 8.0, contained 10 mM Tris and 1 mM EDTA and sterilised Milli-Q water. This was used for dissolving, dilution and storing DNA.

DNA gel loading buffer was provided by Promega. RNA gel loading buffer contained 0.1% bromophenol blue, 20% Ficoll 400 (Pharmacia) and 10 mM disodium EDTA. Loading buffer was stored at -20°C in 1 ml aliquots. When the composition of gel loading buffer differed from this formulation, this is stated in the sections where the buffer is used.

MOPS (3-[N-morpholino]propanesulfonic acid, Sigma) buffer (10×), pH 7.0, contained 20 mM MOPS, 5 mM NaOAc, and 1 mM EDTA. The MOPS buffer was made as following: 41.2 g of MOPS was dissolved in 26.6 ml of 3 M NaOAc (sterilised and made in DEPC-treated water) and made up to 800 ml with DEPC-treated water, then pH was adjusted to pH 7.0 with 2 N NaOH or strong NaOH (10 M) solution then 20 ml of 0.5 M EDTA (made in DEPC-treated sterile water), pH 8.0, was added and the volume was adjusted to 1 litre with DEPC-treated water. After autoclaving, the buffer was stored in light-safe bottles and in the dark. The buffer was discarded if its color was not a pale yellow following storage.

Maleic acid buffer contained 0.1 M maleic acid (Sigma), 0.15 M NaCl and adjusted with NaOH (solid or 10 M stock solution) to pH 7.5 (20°C) and stored at room temperature. For RNA manipulation, DEPC-treated Milli-Q water was used.

Protein gel loading buffer (2×) contained 0.3 g of dithiothreitol (DTT, Sigma), 4.0 ml of 10% SDS, 1.6 ml of 1.25 M Tris-HCl (pH 6.8), 2.5 ml of 87% glycerol, 0.5 mg of bromophenol blue and made up to 20 ml with Milli-Q water, stored at -20°C in 1 ml aliquots.

Sample buffer for 2-D PAGE contained 27.0 g of urea, 1.0 ml of Triton X-100, 1.0 ml of 2-mercaptoethanol, 1.0 ml of one part of pH 4-7 and one part of pH 3-10 Biolyte™ (Bio-Rad), 70 mg of phenylmethylsulfonyl fluoride (PMSF, Sigma), and made up to 50 ml with Milli-Q water. This was stored at -20°C in 1 ml aliquots.

Electrode buffer (10×) for SDS-polyacryamide gel electrophoresis (SDS-PAGE) contained 30.3 g of Tris base, 144.2 g of glycine and 10.0 g of SDS and made up to 1 litre with Milli-Q water. The pH was not adjusted and the solution was stored at room temperature. The 10×

concentrated stock solution was diluted in Milli-Q water to 1× electrode buffer prior to use in SDS-PAGE.

Transfer buffer for Western blotting was low ionic strength (39 mM glycine, 48 mM Tris, 0.037% SDS) and contained 20% (v/v) methanol to minimise swelling of the gel as blotting progressed.

2.2 Microbiological methods

2.2.1 Maintenance and growth of the bacterial strains

Pseudomonad related strains (a term used throughout this thesis to describe *Pseudomonas* related species and the other Gram negative environmental isolates) were routinely cultured either in LB or BHI at 28°C. BHI medium was used for culture maintenance of pseudomonad related species, unless otherwise stated. *E. coli* strains were cultured in LB broth at 37°C overnight at 200 orbits per minute (o.p.m.) (model 013422, Paton Scientific Pty, Ltd.).

Operating cultures of pseudomonad related species were maintained by periodic subculturing from BHIA plates stored at refrigeration temperature (5-8°C). For longer-term storage, growth from plates was transferred into two-times BHI broth plus 50% glycerol as a cryoprotective agent and stored at -20°C and -70°C for 6 months to 3 years. Periodic viability tests were done to ascertain the viability of the cultures.

Mini-scale of culture (10 ml in MacCartney bottles, 25 ml capacity) were inoculated with a single colony from freshly prepared plates, whereas culture stocks stored at -20°C were transferred onto BHIA or LA plates with a sterilised loop and grown overnight at temperature 28°C for pseudomonad related species or 37°C for *E. coli*. This starter culture was used as inoculum for small (100 ml) and large (500 ml) cultures in Erlenmeyer flasks (250 ml and 3 litre capacity respectively). The inoculum was prepared depending on the size of culture required and the initial absorbance was adjusted to $A_{600} = 0.1$ or less before incubation was started. Growth rates were determined by measuring A_{600} , to take samples for analysis of

protein profiles following growth in stress or non-stressed conditions, and to isolate plasmids or genomic DNA. For preparing plasmids, selective pressure was provided by including 100 µg/ml ampicillin or 75 µg/ml tetracycline in LB, as appropriate.

Growth was monitored using an LKB Ultraspec spectrophotometer and absorbance measured at 600 nm against an appropriate medium blank; when A_{600} exceeded 0.6, cultures were diluted in the appropriate medium before measurement. Viable counts were performed using 10-fold serial dilutions of cultures in the growth medium used for that experiment. Dilution was made using 0.9 ml volumes of diluent in 1.5 ml Eppendorf tubes with 0.1 ml samples transferred. After vortexing tubes, samples of 200 µl were taken from tubes and spread onto appropriate pre-dried plates. Selective plates contained antibiotics (40 to 100 µg/ml nalidixic acid, 5 to 20 µg/ml tetracycline, alone or in combination) for screening transconjugants or transformants. All viable counts were performed in triplicate and plates were incubated at 28°C for up to 7 days.

2.2.2 Biochemical tests

Oxidase test was performed using a filter paper moistened with a few drops of Kovac's oxidase reagent and a small amount of bacterial growth was smeared onto the moist filter paper with a spatula or loop. In a positive test, a purple coloration developed immediately or within 10 seconds. Production of fluorescent pigment was examined by observing the plates under ultraviolet light. The inoculum was prepared from glycerol stock and the cells were incubated in LA and BHIA plates at 28°C and examined at room temperature. Bioluminescence was examined using the growing culture and a 0.5 ml sample was taken then examined using a scintillation counter (Pharmacia, Wallac1410). To carry out further biochemical tests, Microbact 24E (12E/12A+12B) identification kits were used and the manufacturer's instruction followed.

2.2.3 Induction of stress proteins

In order to evaluate the induction of heat stress proteins over the growth cycle, pseudomonad strains were grown in LB or BHI broth with a starting absorbance of ≤ 0.1 at

Table 2.3. Antibiotic stock solutions (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992).

Antibiotics*	Stock solution	Mode of resistance
Ampicillin	50 mg/ml in water	β -lactamase hydrolyzes ampicillin before it enters the cell
Carbenicillin	50 mg/ml in water	in place of ampicillin
Chloramphenicol	10 mg/ml in methanol	Chloramphenicol acetyltransferase inactivates chloramphenicol
Erythromycin	10 mg/ml in water	Macrolides inhibit protein synthesis by binding to 50S subunit of 70S ribosomes, mutation in modification of a protein in the 50S ribosomal unit.
Kanamycin	10 mg/ml in water	Aminoglycoside phosphotransferase (neomycin phosphotransferase) inactivates kanamycin
Lincomycin	10 mg/ml in water	MLS antibiotics which consisting of a substituted pyrrolidine linked to methyl- α -thiolincosamine. Inhibit protein synthesis
Nalidixic acid (pH to 11 with NaOH)	10 mg/ml in 1 N NaOH	Mutation in the host DNA gyrase prevent nalidixic acid from binding
Neomycin	10 mg/ml in water	Neomycin (aminoglycoside) phosphotransferase inactivates neomycin in place of ampicillin
Piperacillin	50 mg/ml in water	Mutation in the β subunit of RNA polymerase prevents rifampin from complexing
Rifampin	34 mg/ml in methanol	Mutation in the β subunit of RNA polymerase prevents rifampin from complexing
Streptomycin	50 mg/ml in water	Aminoglycoside phosphotransferase inactivates streptomycin; mutation in <i>rpsL</i> (<i>strA</i>) resulting in modified S12 protein, prevents streptomycin from binding
Spectinomycin	10 mg/ml in water	Mutation in <i>rpsE</i> , modifying ribosomal protein S5, prevent spectinomycin from binding
Tetracycline	5 mg/ml in 70% ethanol	Active efflux of drug from cell
Vancomycin	10 mg/ml in water	Actinomycetes inhibits peptidoglycan bio-synthesis by binding to pentapeptide, prevents transglycosylation step

*Stock solutions of antibiotics dissolved in Milli-Q water and sterilised by filtration through 0.22 μ m filter (Millipore, GS). Antibiotics dissolved in ethanol were not sterilised. All solutions were stored in light-safe containers at 4°C, except tetracycline, which was stored at -20°C and working solutions were freshly made prior to prescribed.

28°C. When A_{600} readings had reached 0.5-0.6, cultures were promptly transferred to the higher temperature and growth monitoring continued at appropriate intervals. Temperature upshift for indigenous strains and pseudomonads was from 28°C to 37°C which was achieved by placing flasks into a water-bath at 54°C for 1 minute, then growth continued at 37°C and 200 o.p.m. (Yamamori and Yura, 1980). Chemical stresses were achieved by adding toxicants when cell growth reached an A_{600} of 0.6 or less in BHI and optimised defined minimal media. Growth was monitored throughout the growth cycle and samples were withdrawn periodically up to 24 hours to detect induction of new proteins by SDS-PAGE. In heavy metal ion stresses, the cells were washed several times with 0.05 M Tris buffer (pH 8.0) and concentrated 10-fold in the same buffer. For all experiments, control cultures were incubated under identical growth condition to the tests but without temperature upshift or addition of chemicals.

2.2.4 Testing antibiotic sensitivity

Gradient plates (Sneath and Johnson, 1972; Phillips *et al.*, 1994) were prepared using BHIA for pseudomonads and related species, and LA for *E. coli* strains. Solidified agar plates containing selective antibiotics which prepared as follows: a 10 ml of LB or BHI medium containing 1.2% bacteriological agar was poured at an appropriate slant in a Petri dish. A further 10 ml of the same medium containing the prescribed antibiotics was poured on top of the base layer to give a flat surface. These plates were poured immediately prior to use. The following antibiotics were tested in antibiotic gradient agar medium at concentration ranges from 0 to 100 µg/ml initially: rifampin, ampicillin, chloramphenicol, tetracycline, streptomycin, spectinomycin, neomycin, kanamycin, lincomycin, erythromycin, vancomycin, carbenicillin, piperacillin and nalidixic acid (Table 2.3). Single colonies from freshly prepared LA or BHIA without selective pressure were streaked onto the selective medium using a spatular or loop. These tests were done in triplicate for each strain investigated and results were only recorded when control plates, containing no antibiotics, showed growth at 28°C for 24 hours. Furthermore, defined minimum inhibitory concentrations (MIC) were determined using solidified LA or BHIA plates at 28°C, where the MIC was defined as the lowest concentration of antibiotic that completely inhibited growth. Sensitivity patterns were recorded initially after 24 hours or up to 72 hours incubation for slowly growing cells.

2.2.5 Introduction of plasmid DNA into VUN10,077

2.2.5.1 Conjugation

Several systems were tested before transconjugants were obtained. The following method describes both the general approach used and the successful method developed for triparental matings.

Transfer of mobilizable plasmids from *E. coli* strains CC118 or S17-1, which contained the pUT::Tn5-luxAB plasmid, to recipient strain VUN10,077 was achieved by biparental and/or triparental bacterial conjugation with pRK2013 as the helper plasmid (de Feyter and Gabriel, 1991) in *E. coli* HB101. For spot-mating, mid-exponential phase cultures of recipient strain VUN10,077 were harvested by centrifugation (3,000 r.p.m., 15 minutes) at room temperature and resuspended in 0.05 volumes of BHI medium and aliquots of 10 µl spotted onto BHIA plates, then the spots allowed to dry on the surface. The *E. coli* donor and helper strains were grown to mid-exponential phase in LB broth containing 100 µg/ml ampicillin plus 75 µg/ml tetracycline for the donor strain and 25 µg/ml kanamycin for the helper strain. Prior to use, the donor and the helper strains were washed with fresh LB without antibiotics and concentrated 10-fold by centrifugation (5,000 r.p.m., 5 minutes). Then 10 µl of the cell suspensions of the *E. coli* donor and helper strains (mixed in a 1:1 ratio) was spotted onto the recipient strain spots. Plates were incubated at 28°C overnight. Cells from each spot were resuspended in 1.0 ml of sterile water and 200 µl of samples were plated onto selective BHIA containing nalidixic acid plus of tetracycline overnight. The plates were incubated at 28°C for up to 7 days or until exoconjugants were visible. The concentrations which successfully yielded transconjugants were 40 µg/ml of nalidixic acid plus 20 µg/ml of tetracycline. The parent strains were tested in parallel.

For filter mating, donor (*E. coli* S17-1) and recipient strains were prepared as described in the spot-mating method. The cell mixture (1:1 , 5:1 and 1:5 ratio of donor/recipient) was transferred using a 5 ml disposable syringe and filtered through a Millipore membrane (25 mm diameter, type HA, 0.45 µm) or Sartorius membrane filter (25 mm diameter, type SM, 0.45 µm) placed into a reusable filter case (Gelman Sci.). After filtering, the drained membranes were removed from the case with sterile tweezers, and placed onto the BHIA plates cell side up. The plates were incubated at 28°C overnight, to allow plasmid transfer. The membranes with the cell mixture were transferred into 5.0 ml of 10 mM MgSO₄. The cell suspensions

from control membrane filters (donor or recipient only) were directly plated onto BHIA for viable cell counting after 28°C for 2 days. The cell mixtures were either serially diluted then plated onto selective BHIA (40 µg/ml of nalidixic acid plus 20 µg/ml of tetracycline) or concentrated 10-fold before being plated similarly. Plates were incubated at 28°C for up to 7 days. Colonies were purified extensively on selective BHIA plates containing antibiotics and screened for luciferase reaction. They were then stored at -20°C and -70°C respectively for further application.

When transformation was performed using *E. coli* JM109, this is described in section 2.3.5.4 where the closely related methods were used.

2.2.5.2 Electroporation

The cells of strain VUN10,077 were cultured at 28°C and transformed using a Bio-Rad Gene Pulser according to the manufacturer instructions (Miller, 1994; Taghavi *et al.*, 1994), as described in the following paragraph.

To prepare cells of VUN10,077, a single colony was subcultured on BHIA, then a single colony was grown aerobically in 10 ml BHI broth. The next morning, five ml of culture was diluted into 100 ml of pre-warmed BHI medium and incubated at 28°C with shaking (200 o.p.m.) until the desired optical density (0.2, 0.4, 0.9, 1.1, 1.35, 1.8 or 3.2) at 600 nm was reached. Samples (10 ml) were collected and cells harvested by centrifugation at 5,000 r.p.m. for 5-10 minutes and washed once with the same volume of washing buffer (300 mM sucrose). After the washing step, the cells were concentrated 10-fold in 300 mM sucrose buffer. Aliquots of 40 µl of cells and 1 µg plasmid DNA of pUT::Tn5-*luxAB*, pUCD607, pUCD615 or pUCD623 (dissolved in 4 µl of sterile Milli-Q water) were prepared and transferred into pre-chilled 0.2 cm cuvette (Bio-Rad). Optimal results were obtained with the following settings: voltage 2.43 kV, capacity 2.5 µFD and external resistance 200 Ω. After electroporation, the cells (50 µl) were mixed with 950 µl of recovery medium (see section 2.1.3) and incubated at 28°C for 2-12 hours before being plated onto appropriate selective media (BHIA contained 5-10 µg/ml tetracycline) to select for transformants, and onto non-selective plates without antibiotics to determine the viable count. Transformants were examined for light emission using microtitre plate assays (Flemming *et al.*, 1994).

2.2.6 Measurement of bioluminescence

Many methods for measuring bioluminescence were tested and many ways of preparing the cells and substrate were tried (the approaches used are described in Chapter 5) but the methods described here gave the best results in terms of stabilization and reliability.

The following method was used for determining effects of potential toxicants on bioluminescent *luxAB*-marked VUN10,077 derivatives. Cultures were grown until the A₆₀₀ reached 1.0 to 1.2, at which point samples were promptly removed into 50 ml portions for centrifugation. The cells were centrifuged at room temperature for 15 minutes at 3,000 r.p.m.. After centrifugation, each portion was resuspended in tap-water without further concentration: 500 µl of resuspended cells was used as needed for measurements up to a 5 hour period, unless otherwise indicated. The resuspended cells remained at room temperature for up to 12 hours or until measurement started. The substrate solution, which was 10³-fold diluted decanal (*n*-decyl aldehyde, Sigma) in Milli-Q water, was freshly prepared by sonicating (Branson sonifier 450, microtip, 50% cycle, output 45 Watts) for 30 seconds (Schultz and Yarus, 1990). The luminescence reactions were initiated by the addition of 100 µl of substrate solution into 500 µl of the tap-water resuspended cells, and samples were analysed within the first 5-10 minutes after addition of toxicants, such as heavy metal ions, phenolic and PAH compounds, at room temperature. Bioluminescence intensity was measured using a liquid scintillation counter (LSC) (Wallac 1410, Pharmacia) and light output was expressed as RLU (relative light units). The RLU measurement was set in an out-of-coincidence mode and employed a user formula, left PM + right PM - 12,500, with single photon events counted in ³H mode. Light emissions were monitored over 10 second counting periods at room temperature for each vial during a cycle and recorded at intervals of 10 to 40 minutes. All experiments were performed in triplicate and mean values of the light intensity were the average of these measurements, where the usual standard deviation was less than 10%. Results were only deemed acceptable if the maximum standard deviation was less than 20%.

2.3 Preparation and analysis of nucleic acids

Standard recombinant DNA methods, including restriction enzyme digestion, agarose gel electrophoresis and plasmid purification, were performed using standard apparatus and methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992), unless otherwise stated, and these are

briefly described in the following.

2.3.1 Plasmid DNA purification

The following is a protocol for rapid isolation of plasmid DNA without column purification for small scale preparations or bands from CsCl gradients for large preparations (Sambrook *et al.*, 1989). Donor stains, *E. coli* cells (S17-1 and CC118 contained pUT::Tn5-luxAB plasmid), were grown overnight in 250 ml of LB broth contained 100 µg/ml ampicillin and 75 µg/ml tetracycline. Bacterial pellets were harvested at 5,000 r.p.m., 4°C, 15 minutes using a JH-2S refrigerated centrifuge (Beckman). The pellet was resuspended in 6 ml of freshly prepared lysis solution (25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme) by pipetting cells up and down thoroughly using a 10 ml pipette. The resuspended pellet was incubated in ice water for 20 minutes then 12 ml of a solution containing 0.2 M NaOH and 1% SDS was added. After mixing by inversion and incubating in ice water for 10 minutes, 7.5 ml of 3 M sodium acetate, pH 4.6, was added, mixed by inversion and incubation on ice water continued for 20 minutes. The sample was centrifuged (J2-HS, Beckman) at 15,000 r.p.m. for 15 minutes. The supernatant was collected and treated with 60 µl of RNaseA (1 mg/ml) for 30 minutes at 37°C. The sample was extracted twice with phenol:chloroform (1:1), then two volumes of ethanol added to precipitate the DNA at -20°C overnight. The sample was centrifuged at 10,000 r.p.m. for 10 minutes. The supernatant was removed and the pellet was washed with 70% ethanol. The DNA was dissolved in sterile Milli-Q water or TE buffer (pH 7.5) and stored at -20°C.

Alternatively, plasmid purification was achieved using a Quantum Prep® Plasmid Miniprep Kit (Bio-Rad) following the manufacturer instructions. Briefly, *E. coli* JM109 transformants, containing 16S rDNA PCR products ligated in the pGEM®T vector (Promega), were grown in LB (10 ml) containing 100 µg/ml ampicillin overnight at 37°C. Culture samples (2 ml) were centrifuged (Eppendorf) for 30 seconds and cells resuspended in the supplied resuspension (200 µl) and lysis solutions (250 µl), mixed by gentle inversion then the supplied neutralization solution (250 µl) was added and mixed by gentle inversion. The white precipitate was collected by centrifugation (Eppendorf, 14,000 r.p.m.) for 5 minutes and the supernatant collected and mixed with the supplied Quantum Prep matrix. This suspension was transferred to the supplied Spin filter and centrifuged for 30 seconds. After washing the

matrix in the supplied washing solution (500 µl) twice by centrifugation (30 seconds and 2 minutes), sterile Milli-Q water (100 µl) was added and the plasmid DNA was eluted by centrifugation for 30 seconds. The eluted plasmid DNA was analysed by gel electrophoresis then aliquoted and stored at -20°C.

For large preparations, caesium chloride-ethidium bromide (CsCl-EtBr) ultracentrifugation was performed as follows. Nine grams of CsCl was added to aqueous DNA mixtures (approximately 9 ml) then 0.92 ml ethidium bromide (EtBr, 10 mg/ml) added and mixed by inversion. The mixture was centrifuged (2,000 X g, 15 minutes, 4°C) and the clear red fluid decanted into a Quick-seal ultracentrifuge tube (16×76 mm, Beckman). The tubes were capped with light mineral oil, sealed and centrifuged (Beckman L-70 ultracentrifuge, Beckman 70 Ti/70.1 rotor) at 20°C, 60,000 r.p.m., for 16-20 hours. The lower band containing plasmid DNA was collected under long-wave light (302 nm, LKB 2011 Macrovue Transilluminator). EtBr was removed from the plasmid DNA solution by several extractions with equal volumes of isoamyl alcohol (Sigma) until the solution was clear. The plasmid DNA was precipitated in two volumes of cold ethanol at -70°C for 1 hour or -20°C overnight and collected by centrifugation (J2-HS, Beckman) then washed with 70% ethanol. The precipitate containing plasmid DNA was resuspended in sterile Milli-Q water (1 mg/ml), aliquoted, and then stored at -20°C.

2.3.2 Genomic DNA purification

The method used was modified from that described by Ausubel *et al.* (1992) and used to extract genomic DNA from pseudomonads and *E. coli* strains. Cultures (400 ml) were grown at 28°C overnight and cells collected by centrifugation for 10 minutes at 6,000 r.p.m. (J2-HS centrifuge, Beckman, JA-20 rotor) at 4°C. The pellet was washed with 50 ml of TE buffer (pH 8.0) and centrifuged as above. The pellet was resuspended in 9.5 ml of TE buffer (pH 8.0) then 1.0 ml of 10% SDS (pre-warmed at 37°C) and 50 µl of protease K (20 mg/ml in TE or 0.5 M EDTA, pH 9.0, previously autodigested at 37°C for 1 hour) was added and mixed. The sample was incubated for 1 hour at 37°C then 1.8 ml of 5 M NaCl and 1.5 ml of CTAB/NaCl solution (10% cetyl-trimethylammonium bromide/0.7 M NaCl) was added with continuous mixing. The sample was incubated for 20 minutes at 65°C in a water-bath. An equal volume (approximately 13.5 ml) of chloroform:isoamyl alcohol (24:1) was added and mixed. The

sample was centrifuged for 10 minutes at 7,000 r.p.m. using Beckman JA-20 rotor at room temperature or 4°C. The supernatant was transferred using a wide-bore pipette to fresh phenol/chloroform-resistant tubes (50 ml capacity polyallomer tubes). The chloroform:isoamyl alcohol step was repeated once, after adding one volume of TE buffer (pH 8.0) and mixing by inversion. One volume of phenol was added and the mixture shaken vigorously for 10 seconds then incubated at 28°C in an orbital shaker for 1 hour with gentle shaking. The sample was centrifuged at 15,000 r.p.m. in a Beckman JA-14 rotor, 4°C for 20 minutes, and then the sample allowed to stand at room temperature for 3 minutes. The top aqueous layer was removed without disturbing the protein precipitate. One volume of phenol and one volume of chloroform:isoamyl alcohol (24:1) were added. The sample was mixed well and centrifuged at 15,000 r.p.m. in a Beckman JA-14 rotor for 20 minutes at 4°C. The phenol and chloroform:isoamyl alcohol extraction step was repeated once, and the DNA solution was divided into pre-cooled Corex glass tubes in 2.5 ml aliquots. To each tube, a 0.1 volumes of sodium acetate, pH 4.8 was added then two volumes of ice-cold absolute ethanol were added and mixed by inversion. The sample tubes were incubated at -20°C overnight. The samples were centrifuged at 10,000 r.p.m. in a Beckman JA-14 rotor, at 4°C for 30 minutes. After collecting the pellet, 10 ml of ice-cold 70% ethanol was added and centrifuged at 10,000 r.p.m. in a Beckman JA-14 rotor, at 4°C for 25 minutes. The supernatant was drained off carefully. Tubes were inverted on Kim™ wipes or clean paper towels at room temperature. When dried, one ml of sterile TE buffer was added to dissolve the DNA and this was dialysed at 4°C in three changes of dialysis buffer (1 mM Tris, 1 mM EDTA, 1 mM NaCl [pH 8.0]) with two changes of dialysis buffer on the first day and another change the following day after overnight dialysis, if necessary.

The DNA concentration was determined using either the agarose plate or spectrophotometric methods (Sambrook *et al.*, 1989). For the agarose plate method, the DNA was spotted onto 1% agarose plates in 1× TAE buffer containing EtBr (0.5 µg/ml) and fluorescence compared with standardised amounts of salmon sperm DNA (0.05 to 4 µg/ml, Promega). For the spectrophotometric method, DNA was diluted 1:50 in Milli-Q water and absorbance readings taken at A₂₆₀ and A₂₈₀, and the A₂₆₀/A₂₈₀ ratio provided an estimation of the purity of samples. One A₂₆₀ unit (1 cm light path) is equal to 50 µg/ml DNA (Sambrook *et al.*, 1989).

2.3.3 Total RNA extraction

Cells were harvested from 200 ml cultures by centrifugation (J2-HS, Beckman), and washed once with DEPC-treated water. The cells were then lysed at 65°C for 10 minutes in a pre-warmed lysis solution containing 0.15 M sucrose, 10 mM sodium acetate (pH 5.2) and 1% SDS, followed by extraction with 4 ml of pre-warmed phenol (Wako chemicals) equilibrated with 0.1 M sodium acetate (pH 5.2). The bacterial DNA remains associated with the cell debris at the interface of the tube during phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) extraction. The upper phase was transferred to a fresh tube containing 0.1 volume of 3 M sodium acetate (pH 5.2; final concentration was 0.3 M), and 2.0-2.5 volumes of ethanol were added, then this stored at -20°C overnight to allow precipitation. After centrifuging the sample, the white pellets were washed with 80% ethanol to remove salts and trace amounts of organic solvents (Wilkinson, 1991). Total RNA was stored at -70°C in the presence of 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate, pH 4.8, or in DEPC-treated Milli-Q water. To determine the RNA concentration, spectrophotometric method was used (Ausubel *et al.*, 1992). The RNA sample was diluted 1:100 in Milli-Q water and absorbance readings taken at A₂₆₀ and A₂₈₀, and the A₂₆₀/A₂₈₀ ratio was considered for the purity of samples. One A₂₆₀ unit (1 cm light path) is equal to 40 µg/ml single-stranded RNA.

2.3.4 Gel electrophoresis of nucleic acids

2.3.4.1 DNA gel electrophoresis

Routine DNA gel electrophoresis was performed using horizontal 0.8% agarose (Promega, dissolved in running buffer) slab gels using a GNA-100, 10.5×8 cm apparatus (Pharmacia), unless otherwise stated. Electrophoresis was performed using 1× TAE or 0.5× TBE buffer at 65 to 100 V for 1 to 2 hours with 0.5 µg/ml EtBr in the gel. Samples contained 1-2 µg of genomic DNA or plasmid DNA or restriction enzyme digested genomic DNA fragments or PCR product was mixed with 6× gel loading buffer, then samples were briefly spun using an Eppendorf centrifuge before being loaded onto the gel. Large-scale DNA gels were performed using a GNA-200 apparatus (Pharmacia, approximately 20×30 cm, capacity 200 ml of agarose gel solution) for 12 to 24 hours (35 V, 1× TAE or 0.5× TBE) without EtBr. The run was usually stopped when the lowest molecular weight dye had reached the end of the gel. The gel was stained by EtBr (25 µl of 10 mg/ml EtBr in 500 ml water) for 15-30 minutes. The gel was

washed into Milli-Q water briefly, viewed and photographed on a UV transilluminator using a Polaroid MR-4 land camera, yellow filter with exposures of 1/4 to one seconds (Polaroid 667 film for positive) or 20-60 seconds (Polaroid 665 film for positive/negative). Appropriate molecular weight markers were run on gels in parallel with samples and approximately 0.5-1 µg of size markers was used: Lambda (*Hind*III digested) containing eight fragments (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 Kb), SPP1/*Eco*RI (Progen) containing 15 fragments (8,500, 7,350, 6,100, 4,840, 3,590, 2,810, 1,950, 1,860, 1,510, 1,390, 1,160, 980, 720, 480 and 360 bp) or 100bp DNA ladder (Promega) containing 11 fragments (1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp) and these were used for size determination (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992).

2.3.4.2 RNA gel electrophoresis

RNA gel electrophoresis was performed using either 1× MOPS buffer or 1× TAE buffer (10 times stock: 0.4 M Tris, 0.2 M NaOAc, and 20 mM disodium EDTA in DEPC-treated Milli-Q water, pH 7.4) in 0.4 M formaldehyde denaturing slab gels (Pharmacia, 10.5×8 cm). The agarose (0.4 g) was melted in sterile Milli-Q water (45 ml) then 5 ml of 10× MOPS or 10× TAE buffer and 2 ml of 37% formaldehyde (Sigma) were mixed in the fumehood. A denaturing mixture (10 µl of formamide, 2 µl of 10× MOPS buffer and 3.2 µl of formaldehyde) was added into each RNA sample (1 µg or less) and made up to 25 µl total volume with DEPC-treated water. The sample was incubated in a 65°C waterbath for 5 minutes then cooled on ice. Before electrophoresis, 5 µl of RNA gel loading buffer was added. After electrophoresis (1-3 hours or until the lowest molecular weight dye reached end of gel), the gel was stained using 5 µg/ml of EtBr solution in 1× TAE buffer before viewing on a transilluminator. RNA markers (G319, Promega) were run in gels in parallel with samples (3.5-5 µl) and contained nine fragments (6,583, 4,981, 3,683, 2,604, 1,908, 1,383, 955, 6233 and 281 bp).

Alternatively, denatured glyoxal/DMSO RNA gel electrophoresis was applied (McMaster and Carmichael, 1977; Ausubel *et al.*, 1992). Agarose gels were prepared as 0.8% in 10 mM sodium phosphate buffer (pH 7.0). Glyoxal (Sigma) was prepared freshly by treatment with 3 g of Amberlite IRN 150 resin per 5 ml of glyoxal, soaking for 1 hour with stirring to deionise, then filtering through Whatman No. 1 paper before use. The RNA samples were mixed in a denaturing mixture (1 µl of 100 mM sodium phosphate, 5 µl of DMSO, 1.5 µl of 6 M glyoxal

and made up to 9 μ l total volume with DEPC-treated Milli-Q water). The sample was mixed by vortexing, spun briefly (Eppendorf centrifuge), and incubated at 50°C for one hour in a waterbath. The sample was cooled on ice and 5 μ l of glyoxal gel loading buffer (10 mM sodium phosphate, pH 7.0, and 0.1% bromophenol blue) was added. The electrophoresis was performed at 80 V (4 V/cm) and paused every 20 minutes to remix the running buffer (10 mM sodium phosphate, pH 7.0). The gel was soaked in 0.5 M ammonium acetate for 20 minutes, twice, and stained with 0.05% toluidine blue in 10 mM sodium phosphate for 30 minutes, then destained with 20 mM sodium phosphate buffer (pH 7.0) overnight. After being photographed, the gel was dried using a GelAir dryer (Bio-Rad). The RNA markers (G319A, Promega) were treated the same as the RNA sample and run in gels in parallel with samples.

2.3.5 Manipulation of DNA

2.3.5.1 Restriction enzyme use

All restriction enzymes were obtained from various suppliers and used at concentrations and with buffers/incubation temperatures described by the suppliers.

2.3.5.2 Recovery of DNA fragments from agarose gels

Linear DNA fragments obtained from restriction enzyme digestion, such as *luxAB* genes or plasmid DNA, were recovered from 0.8% agarose gels and used in Southern hybridization experiments as probes to detect chromosomal integration of the *luxAB* genes. Ten μ g of pUT::Tn5-*luxAB* plasmid (*E. coli* CC118) was incubated in a total volume of 100 μ l of *Sa*I digestion reaction at 37°C overnight. One μ g of fragment was expected after 3 μ g of plasmid DNA (2.5 kb *luxAB*/8.2 kb whole plasmid) was digested with *Sa*I and the final concentration was assumed to decrease by 50% after the cleaning procedure. After electrophoresis (0.5× TBE, 100 V), the gel was stained and visualised under UV light. The *luxAB* fragment was excised and trimmed using sterile scalpel blade and isolated using BANDPURE™ (Progenius) as recommended by the manufacturer. The DNA concentration was determined using 1% agarose plates containing EtBr.

2.3.5.3 Polymerase chain reaction (PCR)

Oligonucleotides were synthesised by Pacific Oligos Pty. Ltd. and Fisher Biotech (Perth, Australia). PCR reactions were performed using a Peltier thermal cycler (PTC-200). 16S rDNA PCR amplification was performed according to the Expand™ Long Template PCR system (Boehringer Mannheim) protocol. The PCR mixtures (50 µl) contained 20 pmol of each appropriate primer, and approximately 500 ng of genomic DNA. Briefly, two mixtures (25 µl of master mix I [1.75 µl each 10 mM dNTP, downstream and upstream primer and template DNA] and 25 µl of master mix II [5 µl of 10 times PCR buffer with system 1 and 0.75 µl of amplify enzyme]) were prepared and mixed shortly before cycling. The cycle profile was as follows: denatured at 94°C for 2 minutes, 10 cycles at 94°C for 10 seconds, 65°C for 30 seconds, and 68°C for 90 seconds, 20 cycles at 94°C for 70 seconds, 65°C for 30 seconds, and 68°C for 90 seconds plus cycle elongation of 20 seconds for each cycle, prolonged elongation time was 2 minutes at 68°C. The PCR products were purified by Wizard™ PCR Preps DNA purification System (Promega) following the instruction manual of the supplier. The PCR products and 100sp bp ladder marker (Promega) were visualised by agarose gel electrophoresis (1.2% agarose gel using 1× TAE, 80 V for 1 hour).

The presumptive *groEL* gene was amplified using pseudomonad genomic DNAs and as follows. The PCR mixture (100 µl) contained 100 µM of primer, 10 mM dNTPs, 0.5 µl of Taq Amplify enzyme (Perkin-Elmer), 6 µl of MgCl₂ and 200 ng of template DNA. This was mixed shortly before cycling except Taq polymerase, which was added after the first cycle of denaturation of template DNA. The cycle profile was as follows: denatured at 97°C for 7 minutes, 35 cycles 94°C for 1 minute and 72°C for 2 minutes and elongated at 72°C for 5 minutes. The PCR products were purified using Wizard™ PCR preps DNA purification system (Promega) as recommended by manufacturer's instructions. After amplification of the presumptive *groEL* gene using the specific primers (*groEL* 9 and *groEL* 10, named and designed by Dr. M. Serafica, when at CBFT, VUT), the PCR products and 100sp bp ladder markers (Promega) were electrophoresed and visualised using a 1.2% agarose gel (1× TAE), run at 80 V for 1 hour.

2.3.5.4 Ligation and transformation for 16S rDNA gene analysis

Ligation was performed using the pGEM®-T vector system (Promega). The ligation and transformation was performed according to the instruction manual supplied, as follows. Briefly, the ligation reaction mixture contained 10× buffer (supplied), 50 ng of the supplied

pGEM-T vector, 65 ng of 16S rRNA PCR products, 1 μ l of the supplied T4 DNA ligase (3 Weiss units/ μ l) and sterile Milli-Q water made up to a final volume of 10 μ l. The ligation reaction was performed using a PCR machine overnight at 4°C. After ligation, transformation was achieved using *E. coli* JM109 competent cells (Promega). The ligation reaction (10 μ l) was mixed with 50 μ l of competent cells then incubated on ice for 20 minutes. The cells were heat treated for 50 seconds in a water-bath at 42°C then placed on ice for 2 minutes. A SOC medium (950 μ l) equilibrated at room temperature was added and incubated for 1.5 hours at 37°C with shaking on an orbital shaker incubator (150 o.p.m.). The cells were collected by centrifugation (Eppendorf) at 1,000 r.p.m. for 3 minutes, and resuspended in 200 μ l SOC medium. The cells were plated on LA plates containing ampicillin/IPTG/X-Gal (see section 2.1.3). The plates were incubated overnight or until colonies appeared at 37°C.

2.3.5.5 Sequencing

The ABI PRISM™ Big Dye terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) was used to directly sequence the PCR products and plasmid DNA by following the manufacturer's instructions. The reaction mixtures were sequenced automatically with the Applied Biosystems model 373A DNA sequencer at Monash University (Department of Microbiology) (joint facility with VUT).

2.3.6 Southern hybridization

2.3.6.1 Electrophoresis and transfer to membrane

The electrophoresis was performed using a large slab gel tank (20 cm \times 30 cm) in 0.5 \times TBE or 1 \times TAE buffers at 35 V overnight. The gel was cut to an appropriate size (approximately 5 cm \times 20 cm) and transferred into a tray containing 0.25 M HCl solution for 5 minutes and then rinsed with Milli-Q water several times. The gel was soaked in 100 ml denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30-45 minutes, shaking intermittently manually or on an orbital shaker (very low o.p.m.) at ambient temperature. The denaturing solution was discarded. The gel was rinsed in Milli-Q water. The gel was soaked in 150 ml neutralising solution (1.0 M NH₄OAc, 0.02 M NaOH) for 30 minutes, shaking intermittently or using an orbital shaker as above. The membrane (Hybond™-N⁺, Amersham) was aligned onto the gel.

The DNA was transferred to the membrane by the dry capillary method overnight (12-24 hours). The membrane was soaked in 2× SSC solution (diluted from 20× SSC; 3 M NaCl, 0.3 M Na-citrate.2H₂O, pH 7.0 adjusted with NaOH) for 5 minutes. The membrane was wrapped in plastic wrap and placed onto a UV transilluminator (2011 Micravue, LKB) to fix the DNA for 3 to 5 minutes. The membrane was kept at room temperature until used.

2.3.6.2. Use of radiolabelled probes

Hybridization buffer was prepared as follows: Denhardt's (5×) solution contained 2% Ficoll (type 400, Pharmacia), 2% polyvinylpyrrolidone (PVP), 2% BSA (Fraction V, Sigma), and was sterilised by filtration (0.22 µm, Millipore, GS) then stored at -20°C. Prehybridization solution (9.0 ml) was prepared by mixing 4 ml of 6× SSC, 4 ml of 5× Denhardt's solution, 0.5 ml of 10% SDS (mixed together and preheated at 55°C for 10 minutes before use), and 0.5 ml of denatured salmon sperm DNA (denatured at 95°C for 10 minutes using a water-bath, and placed on ice immediately). This solution (10-20 ml) was placed into a sealable plastic blender bag containing the membrane. The hybridization bag was sealed and placed on a glass tray or into a suitable container and prehybridized at 65°C for up to 8 hours.

Probes were prepared using GIGAPRIME DNA labelling kit (Bresatec). Two ng of probe (see details in section 2.3.5.2) was added to 6 µl of the supplied primer, 6 µl of dNTPs (without dATP), 5 µl of α -³²P dATP (3,000 Ci/mmol, aqueous solution), and 1 µl of the supplied Klenow enzyme, and made up to 30 µl with sterile Milli-Q water, with mixing by gentle tapping. The probe mixture was incubated at 37°C for 20 minutes and the enzyme was deactivated at 65°C for 10 minutes. The probe was then denatured at 100°C for 5 minutes, cooled on ice, and added into the hybridization bag containing the membrane in 10 to 20 ml of the hybridization buffer. The bag was sealed and the membrane was hybridized at 65°C overnight. Stock solution of SSPE (20×) contained 3.6 M NaCl, 0.2 M NaH₂PO₄ and 20 mM EDTA, pH 7.7. The membrane was washed twice with washing solution (2× SSPE plus 0.1% SDS) for 5 minutes, and soaked in the second washing solution (1× SSPE plus 0.1 % SDS) for 5 minutes then washed with 0.1× SSPE containing 0.1% SDS for 5 minutes. All of the above washing steps were performed at room temperature and the last washing step was repeated if required. The membrane was dried on filter paper and exposed to Kodak X-ray film at -70°C in an autoradiography cassette for up to 3 days. The film was developed as described in section 2.4.6.2.

2.3.6.3 Use of DIG-labelled probes

The membrane (Nylon, 0.45 µm, Boehringer Mannheim) was placed in a hybridization bag containing 10-20 ml prehybridization solution (DIG Easy HybTM, Boehringer Mannheim). The membrane was prehybridized at 40°C overnight.

In order to prepare the probe, 190-210 ng of template DNA was added to sterile water (Milli-Q) to a final volume of 40 µl in a 1.5 ml Eppendorf tube. The DNA was denatured by heating in a boiling water-bath for 10 minutes and chilled quickly on ice. DIG-High PrimeTM (4 µl, Boehringer Mannheim) was added to the aqueous DNA, mixed by centrifuging (Eppendorf) briefly. The probe was incubated overnight at 37°C. To achieve an accurate temperature control and high yield of DIG-labelled probe, a PCR machine was employed. The reaction was stopped by adding 5 µl of 0.2 M EDTA (pH 8.0) and the probe was stored at -20°C until used.

DNA probes (approximately 25-30 ng per 15 ml) were prepared by heating in boiling water for 10 minutes to denature the double-stranded DNA. The probe was chilled directly on ice. The probe was diluted in hybridization solution (5-20 µl in 15 ml) and filtered through a 0.45 µm filter (Millipore, GS) after pre-warming the hybridization solution (40°C). This solution was poured into the hybridization bag containing the membrane, and hybridized at 40°C overnight using the DIG Easy HybTM hybridization buffer. The membrane was placed into a hybridization wash solution (2× SSC plus 0.1% SDS) and washed twice with gentle shaking for 5-10 minutes at room temperature. The membrane was placed in the second washing solution (0.2× SSC plus 0.1% SDS) and incubated for 5 minutes at room temperature with gentle rocking. The membrane was semi-dried by placing it onto filter paper.

The following method was used to detect a signal using the manufacturer protocol (Detection Starter Kit II, Boehringer Mannheim). All incubations were performed at room temperature with agitation, unless otherwise stated. After hybridization, the membrane was washed briefly (1-5 minutes) in washing buffer (maleic acid buffer plus 0.3% TweenTM 20 [v/v]). The membrane was incubated for 25-30 minutes in 20-100 ml Blocking solution (10× supplied Blocking solution was diluted in maleic acid buffer). The membrane was incubated for 25-30 minutes in 20 ml buffer containing the supplied anti-DIG-AP conjugate (diluted to

75 mU/ml [1:10,000] in Blocking solution). The membrane was washed twice for 5 and 10 minutes (or twice for 15 minutes each) in 20 ml washing buffer each. The membrane was equilibrated for 2 minutes in 20 ml detection buffer (0.1 M Tris-HCl and 0.1 M NaCl, pH 9.5). The membrane was placed in a hybridization bag and about 20 drops (1 ml) of the supplied CSPD™ applied. The damp membrane was incubated for 5-15 minutes at 37°C. The membrane was exposed to X-ray film (Amersham, Hyperplus) for 2-30 minutes in an autoradiography cassette at room temperature and developed as described in section 2.4.6.2.

2.3.7 Northern hybridization

After electrophoresis in a standard formaldehyde gel, the gel was equilibrated in 20× SSC (DEPC-treated) for 15 minutes twice. The RNA was blotted onto a Nylon membrane (Boehringer Mannheim) by wet capillary transfer as follows. The perspex block of appropriate size was selected to support the gel within a baked glass tray holding a reservoir of 200 ml of 10× or 20× SSC. A wick was prepared by cutting filter paper to fit over the block, with the sides folded under the block so that wick touched the bottom of the baked tray. Six pieces of Whatman 3 mm filter paper and 1 piece of Nylon membrane were prepared to the size of the gel and soaked in 10× SSC or 20× SSC then 3 pieces of filter paper aligned on at the centre of the perspex block. The neutralised gel was placed, well-side up, on top of the 3 filters then the wet membrane placed on top of the gel without air bubbles trapped. The other 3 filter papers were placed on top of the membrane. A stack of paper towels (2-3 cm thickness) was cut to the same size as that of the filters then placed on top of the sandwich. About 500 g weight was placed on top of these. A capillary action was set up from the reservoir in the baked glass tray, through the bottom filters, gel, membrane, top filters, to the paper towels. Next day, the membrane was cross-linked without prior washing using a UV transilluminator for 5 minutes. After cross-linking, the membrane was rinsed briefly in DEPC-treated Milli-Q water, and the section of the membrane containing RNA markers (G319A, Promega) and duplicated wells of test RNA was removed using a scalpel blade and stained separately with 0.05% toluidine blue (Sigma) in 10 mM sodium phosphate buffer (pH 7.0) for 30 minutes at room temperature, then destained in the same buffer or DEPC-treated Milli-Q water. The membrane was placed in DIG-EASY™ prehybridization buffer at 55°C overnight. The method for making the double-strand DNA probe and detection of DIG-labelled nucleic acids were the same as for the DIG-labelled Southern hybridization (see section 2.3.6.3). The

amplified 16S rRNA gene PCR product was used as a probe which (40 µl) was diluted in hybridization solution (2 µl in 25 ml) and filtered through a 0.45 µm filter (Millipore, GS) after pre-warming the hybridization solution (55°C). The membrane was hybridized at 55°C overnight using the DIG Easy Hyb™ hybridization buffer. The membrane was washed twice in 2× SSC plus 0.1% SDS at room temperature for 15 minutes per wash and twice in 0.5× SSC plus 0.1% SDS at 68°C for 15 minutes per wash. After partially drying the membrane on the filter paper, the detection of a signal was achieved as described in the section 2.3.6.3 and the X-ray film (Amersham) was developed as described in section 2.4.6.2. All the above solutions were made in DEPC-treated Milli-Q water, except the supplied DIG-Easy Hyb™ solution.

2.4 Protein analysis

2.4.1 Preparation of cellular proteins

Samples (20 ml) were harvested during the growth cycle in 500 ml cultures by centrifugation (5 minutes, 10,000 r.p.m., 4°C, Beckman JH-20 rotor) and cells were resuspended in 50 mM Tris-HCl, pH 8.0 to concentrate these 10-fold. To extract the cellular proteins, the cells were disrupted by sonification (Branson sonifier 450, microtip, 50% cycle, output 45 Watts) for 2 minutes. The cells were kept on ice-water containing ethanol during sonification and the tip allowed to cool between 30 seconds bursts. Cell debris were removed by centrifugation (15 minutes, 12,000 r.p.m., Eppendorf centrifuge) and the supernatant was collected and kept at -20°C until use. The soluble protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) before performing gel electrophoresis.

Alternatively, bacterial cells were disrupted by boiling in protein gel loading buffer (2×). Samples (1.5 ml) were taken from the stressed and unstressed cultures during the growth cycle and centrifuged (Eppendorf) at room temperature for 5 minutes. The cells were washed in 50 mM Tris-HCl, pH 8.0, and resuspended in the same buffer to give a constant A₆₀₀ of 1.0. One volume of two-fold protein gel loading buffer was added to the sample then the mixture placed in a boiling water for 10 minutes then placed on ice. The samples were centrifuged for 2 minutes (Eppendorf) prior to loading onto the SDS-PAGE, to remove unlysed cells.

2.4.2 Estimation of protein concentration

The method modified from that described by Lowry *et al.* (1951) was used. Sample (10 µl in 50 mM Tris buffer, pH 8.0) was added to 0.5 ml of solution A (0.1 ml of 5% CuSO₄, 0.9 ml of Na₂CO₃ in 10 ml of 0.5 M NaOH). After incubation at 37°C for 10 minutes in a water-bath, 1.5 ml of solution B (1 ml Folin-Ciocalteu's, Sigma, reagent plus 10 ml Milli-Q water) was added and then vortexed immediately. The sample was incubated in a water-bath at 52°C for 20 minutes, and A₆₈₀ readings were recorded using an LKB spectrophotometer. Standards, ranging from 0 to 100 µg, were treated the same as samples and prepared from 1 mg/ml BSA solution (50 mM Tris buffer, pH 7.2). CuSO₄ solution (5%) was stored at 4°C and sodium carbonate solution was kept at room temperature. Reagents A and B were prepared immediately before use from stock solutions.

2.4.3 Pulse-chase radiolabelling

2.4.3.1 Radiolabelling of heat-stressed proteins

The slightly modified procedure described by Allan *et al.* (1988) was used as follows. The pseudomonads were grown at 28°C and *E. coli* HB101 at 37°C to an A₆₀₀ of 0.4 in minimal media used by Allan *et al.* (1988). Cells (0.5 ml) were concentrated 10-fold by centrifugation at room temperature and resuspended into fresh medium used. Cells were equilibrated at 28°C for 2 hours, then shifted from 28°C to 37°C for pseudomonads and from 37°C to 42°C for *E. coli* HB101, and shaken at 50 o.p.m.. At different times after temperature upshift, the cell suspensions were pulse-labelled using 10 µl of ³⁵S-methionine (ca. 10 mCi/ml, 1,100 Ci/mmol, Amersham) for 1 minute and chased for 1 minute by adding 0.5 ml of temperature-equilibrated medium supplemented with 400 µg/ml of L-methionine (Sigma). Protein synthesis was stopped by the addition of 0.1 ml of ice-cold chloroamphenicol (2.5 mg/ml in ethanol). The cells were harvested by centrifugation (Eppendorf) for 5 minutes and then resuspended in 0.1 volume of 50 mM Tris buffer (pH 8.0). They were boiled for 10 minutes after addition of two-fold protein gel loading buffer (1:1) and SDS-PAGE performed (see details in section 2.4.4).

2.4.3.2 Radiolabelling using optimised minimal or defined media

Alternatively, the optimised minimal or defined media developed for each pseudomonad strain was used for labelling without further concentration of the cell suspension before labelling. Pseudomonad strains were prepared in BHIA at 28°C and several colonies were inoculated into optimised minimal media (20 ml) at 28°C, 200 o.p.m. for up to 2 days. This starter culture was used to inoculate 100 ml of medium using 250 ml capacity of Erlenmeyer flasks and cultures were grown to an A_{600} of 0.6 then subjected to heat transfer (see section 2.2.3), phenol or heavy metal ion stresses. One ml of cell suspension was pulse-labelled with 15 μ l of ^{35}S -methionine (ca. 370 MBq/ml, 10 mCi/ml, Amersham) for 3 minutes and chased for 2 minutes with cold methionine (final concentration 20 $\mu\text{g}/\text{ml}$, Sigma). The labelling was stopped by adding 0.1 ml of cold chloramphenicol (2.5 mg/ml in ethanol). The labelled samples were treated the same as described in the section 2.4.3.1.

2.4.3.3 Measuring radioactivity incorporated into proteins

Samples (10 μl) of labelled proteins were precipitated by addition of 5 ml of 5% TCA (diluted from 100% stock solution, 500 g of trichloroacetic acid dissolved in 227 ml of Milli-Q water, Sigma) prior to SDS-PAGE analysis. After 20 minutes on ice, the precipitated proteins were collected by filtration onto glass fibre filters (Whatman GF/C, 2.5 cm) with mild suction. The filters were washed twice with cold absolute ethanol, dried in an oven at 120°C for 15 minutes and placed into 20 ml glass scintillation vials containing 4 ml of scintillation fluid (0.5% 2,5-diphenyloxazole [PPO, Sigma] in toluene fluid). The radioactivity incorporated into synthesized proteins was measured using the LS 50 scintillation counter (Wallac 1410, Pharmacia). The samples were analysed for two cycles with a minute counting period for each vial during a cycle. Incorporated ^{35}S activity was expressed as counts per minute (cpm).

2.4.4 Polyacrylamide gel electrophoresis

2.4.4.1 One-dimensional (1-D) gel electrophoresis

Proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), which was performed using pre-formed 12% homogenous and 10-15% or 10-12% gradient gels (Protean II unit, Bio-Rad) overlaid with a 4% stacking gel. Wells contained either 25 μg of proteins per sample or a constant amount of cellular material as determined

from A₆₀₀ of the original sample and later concentrating. Electrophoresis was carried out in 1× electrode buffer at 10°C, with temperature control achieved by a Grant cooling unit (Grant Instrument Ltd.). Samples were electrophoresed at 15 mA until the tracking dye entered the resolving gel, at which time the mA was increased to 20-30 mA for 6 to 8 hours or kept at 8 mA per gel for overnight runs. Low molecular weight (LMW) and pre-stained LMW standards (Bio-Rad) were used as markers. The polyacrylamide gel was visualised by either Coomassie blue or silver staining (see section 2.4.5), photographed when required, dried then exposed to Kodak X-ray film (or Amersham) as described in section 2.4.6, if appropriate.

2.4.4.2 Two-dimensional (2-D) gel electrophoresis

2-D gel electrophoresis was performed using 10-18% non-linear gradient PAGE. For the first dimension (isoelectric focusing), an Immobiline DryStrip™ Kit (Pharmacia) was employed. The strips had two different pH ranges, pH 4.0-7.0 and pH 3.0-10.5, and the narrow pH range was chosen for stress protein analysis. First-dimension gel electrophoresis was performed as described in the supplier's instruction manual. Briefly, the lysed protein samples prepared by sonication (1 mg) or boiling were mixed with sample buffer. The strips were in rehydration solution (24.0 g of urea, 0.25 ml of Triton X-100, 75 mg of DTT, 0.1 ml of 1.0 M/L acetic acid, and made up to 50 ml with Milli-Q water) for a minimum of 6 hours or overnight. The first-dimension electrophoresis was performed using a MultiphorII (Pharmacia-LKB) electrophoresis unit in a horizontal position at 15°C, the temperature controlled by the Grant cooling unit. A step-wise function of voltage was programmed into the computer controlled electrophoresis power supply (Model 3000Xi, Bio-Rad) as follows. The first step, 300 V, 1 mA, 1 W; second step, 2000 V, 1 mA, 1 W; and third step, 3000 V, 1 mA, 1 W at constant current (1 mA) for 30 to 48 hours. The isoelectrofocused strips were stored at -20°C until used.

The Immobiline DryStrips™ were equilibrated in the equilibration solution (20 ml 0.5 M Tris-HCl included PMSF, pH 6.8, 72 g of urea, 60 ml of glycerol, 2 g of SDS, and made up to 200 ml with sterile Milli-Q water). DTT (25 mg/ml) was added to the equilibration solution for the first equilibration step for 10 minutes and 0.45 g of iodoacetamide (Sigma) plus a few grains of bromophenol blue/10 ml solution added for the second step of equilibration for 10-30 minutes. The equilibrated strips were applied on a second-dimension gel electrophoresis (polyacrylamide slabs in the vertical position) using the Protean II unit (Bio-Rad) overlaid

with the 4% stacking gel in 1× electrode buffer. Electrophoresis was carried out at 8 to 25 mA/gel and 15°C until the tracking dye had reached the end of the gel. The gel was disassembled and visualised by silver staining or dried using a GelAir dryer (Bio-Rad) then exposed to the X-ray film (Amersham or Kodak) directly after drying. Autoradiography was performed as described in section 2.4.6.

2.4.5 Polyacrylamide gel staining

2.4.5.1 Coomassie brilliant blue staining

A 0.5 mg/ml solution of Coomassie brilliant blue R-250 (Sigma) was prepared by dissolving the dye in five parts methanol before addition of one part acetic acid and four parts Milli-Q water. Gels were stained for 30 minutes at room temperature with gentle shaking. The gels were rinsed in Milli-Q water and transferred into destaining solution (one part acetic acid, four parts ethanol and five parts Milli-Q water) then gently shaken at room temperature until blue bands and a clear background were obtained. Fresh destaining solution was added if required. The gels were kept in Milli-Q water overnight after destaining, then dried using a GelAir dryer (Bio-Rad).

2.4.5.2 Silver staining

Silver staining protocol was followed according to the instruction manual (Pharmacia) as follows. The gel was immersed in the fixing solution (one part acetic acid, four parts ethanol, and five parts Milli-Q water) for at least 30 minutes. The gel was placed in the incubation solution (60 ml of ethanol, 10.25 g of sodium acetate, anhydrous, 1.04 ml of glutaraldehyde, 0.4 g of sodium thiosulfate and made up to 200 ml with Milli-Q water) for more than 30 minutes. The gel was washed three times, each time for 15 minutes, in Milli-Q water. The gel was put into the silver solution (0.2 g of silver nitrate, 40 µl of formaldehyde and made up to 200 ml with Milli-Q water) for 40 minutes. The gel was placed into developing solution (5.0 g of sodium carbonate, 20 µl of formaldehyde, and made up to 200 ml with Milli-Q water) for 5-10 minutes. Extra formaldehyde was added if required. The gel was washed in Milli-Q water with one change for 5-10 minutes then preserved in a GelBond film (Pharmacia) as recommended by the manufacturer's instruction.

2.4.5.3 Gel photography

Gel photography was performed using a Polaroid MP-4 Land Camera if required. Typically, Type 667 black and white film was used for documentation at a shutter speed of 1/60 to 1/30 seconds with an aperture of F16. Type 665 positive/negative film used at a shutter speed 1/4 to 1/2 seconds and an aperture of F16. If the picture was too light, the shutter speed was increased or a higher F number was set or altered inversely when the picture was too dark (Ausubel *et al.*, 1992).

2.4.6 Autoradiography

2.4.6.1 Exposure to X-ray film

Gel fractionated ^{35}S -labelled proteins were visualised by autoradiography. The gel was not stained but placed in direct contact with an X-ray film (Kodak or Amersham) in a light-safe cassette for the appropriate length of time at room temperature or -70°C. Typically, 50,000 cpm of resolved total protein labelled with ^{35}S could be visualised by 4 days exposure and 15,000 cpm of supernatant protein labelled with ^{35}S needed 10 days exposure.

2.4.6.2 Developing of X-ray film

When the cassette was taken from -70°C, it was thawed at 37°C for about 15 minutes and/or placed at room temperature until thawed. The film was soaked in developing solution (Agfa) for a minimum of 3 to 5 minutes. Subsequently, the film was transferred in 3% acetic solution and soaked for 1 minute. The film was transferred into fixing solution (Agfa) for 4 minutes. The developed X-ray film was rinsed with tap water then dried and stored at room temperature.

2.4.7 Western blotting

Immunoblotting was performed using rabbit polyclonal anti-DnaK antibodies from two

different sources: commercially supplied from DAKO (used after diluting 1:1,500 in TBST solution) and antibodies from C. Georgopoulos (Zylicz and Georgopoulos, 1984) (used after diluting 1:3,000 in TBST solution). The TBST solution contained 1× TBS plus 0.02% Tween 20 (Sigma), or alternatively, saline was used in TBST solution instead of Tween 20. TBS (10×) stock solution was made of 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl and made up to 1 litre with Milli-Q water. Rabbit polyclonal anti-GroEL antibodies (Sigma, diluted 1:1,500 in TBST solution) were also used. Analytical SDS-PAGE was performed using a Mini Protean II electrophoretic unit (Bio-Rad), and 10 µg of proteins were loaded per well. The electrophoresis was carried out at 15 mA per gel at room temperature for 2 to 4 hours or until the tracking dye had reached the end of the resolving gel. Following SDS-PAGE, the gel was soaked in 50 ml transfer buffer for 15 minutes. A piece of nitrocellulose membrane (0.2 µm, Bio-Rad) and 10 pieces of Whatman No.1 paper were cut to the same size as the gel and submerged in transfer buffer for 15 minutes before blotting. The blotting apparatus (Bio-Rad) was wetted in transfer buffer and the Whatman No.1 paper, membrane, gel and Whatman paper placed in that order. The proteins in the gel were allowed to transfer electrophoretically overnight at a constant current of 0.2 mA. Pre-stained markers (Bio-Rad) were run on the gels to serve as internal markers for transfer and molecular weight measurement. To block the non-specific binding sites, the blotted membrane was incubated in 50 ml of blocking buffer (1% gelatine in TBST) for 2 hours at room temperature with shaking at 60 o.p.m. and washed twice for 10 minutes in TBST solution without gelatine. The membrane was soaked in the primary antibody solution with shaking at room temperature for 1 hour and washed twice for 10 minutes in TBST solution.

To detect bound antibodies, horseradish-conjugated goat anti-rabbit antibodies (Bio-Rad) were diluted 1:2,000 in TBST solution and used as the secondary antibody, with 4-chloro-1-naphthol (Sigma) as the substrate for detecting bound conjugated antibody. The membrane was soaked in the secondary antibody solution with gentle shaking for 1 hour and washed twice for 10 minutes in TBS solution at room temperature. The substrate was prepared by dissolving one pill of 4-chloro-1-naphthol (Sigma) in 10 ml of methanol then adding 50 ml of TBS and 35 µl of H₂O₂. The membrane was soaked in the developing solution for up to 20 minutes and washed with Milli-Q water for 10 minutes. The membrane was kept in Milli-Q water overnight if bands were not visible initially. The membrane was photographed using a Polaroid MP-4 Land Camera (Polaroid 667 or 665 film for negative), exposure of 1/125 seconds with an aperture of F16, on a light box.

Chapter 3

Characterization of the indigenous strain

3.1 Introduction

The indigenous strains were originally obtained from Dr. Paul Boon (when at CSIRO) and had been identified on the basis of their biochemical tests. However, speciation of Gram negative microbial bacteria is inexact if based only on such tests and identification is increasingly relying on genetic approaches, specifically based on 16S ribosomal RNA gene sequencing. It was therefore important to confirm the identity of these natural isolates to allow data obtained for them to be compared with previous literature reports on similar species and to provide a basis for ensuring that the same strains were being examined throughout this study. Smibert and Krieg (1981) noted the importance of stability of stock cultures in long-term studies.

The classification of prokaryotes has been extensively studied with regard to the molecular classification of bacterial species based on the small subunit rRNA, or 16S rRNA (Olsen *et al.*, 1994) although this may be misleading in constructing phylogenetic trees without G+C content data (Hasegawa and Hashimoto, 1993). These studies have led to the concept of dividing prokaryotes into two distinct monophyletic groups or domains, designated archaebacteria (or Archaea) which grow in unusual habitats (methanogens, extreme thermoacidophiles and extreme halophiles) and eubacteria (or Bacteria) (see Woese *et al.*, 1990). Within the eubacterial domain, phylogeny based on 16S rRNA has recognised several main divisions. These include numbers of the *Thermotogales*, green non-sulfur bacteria, deinococci, cyanobacteria, low G+C and high G+C Gram positive bacteria, flavobacteria, chlamydiae and planctomycetes, and purple bacteria to proteobacteria (Fox *et al.*, 1980; Woese, 1987; Olsen *et al.*, 1994; Gupta, 1998). Recently, a similar evolutionary relationship was examined between various representative taxa using the sequence data for the 70-kDa HSP (Hsp70) and GroEL (Hsp60) genes, which may assist in classifying the bacteria phylogenetically at the genus and species level (Viale *et al.*, 1994; Gupta *et al.*, 1997).

In this chapter, the characteristics of the main indigenous strain VUN10,077 used (originally identified as *Ps. paucimobilis*) are reported and compared with other representative α -proteobacteria. Analysis was also performed on another indigenous strain, *St. maltophilia* VUN10,075, which was originally classified as *Ps. maltophilia*, and on culture collection strains of *Ps. aeruginosa* and *Ps. fluorescens* to confirm their identity.

3.2 Morphological and physiological characteristics

Colonies of VUN10,077 had orange pigment and different elevation depending on the growth period. This strain showed convex elevation for less than two days and then gradually umbonate elevation appeared for colonies grown on BHIA plates when cultured at 28°C. Colonies also changed from circular to irregular according to the incubation period (Table 3.1).

For general characterization, Microbact 24E (12E/12A+12B) identification kits were used and results are shown in Table 3.2. Gram-negative rod-shaped indigenous strain VUN10,077 showed oxidase and lysine decarboxylase positive reaction. Indole was not fermented. Gelatin was not liquefied. Production of fluorescent pigment was examined by observing the plates under ultraviolet light. The test did not show a fluorescent zone in the agar surrounding the growth. Bioluminescence was examined because it is necessary to know whether all strains used were naturally non-bioluminescent organisms. Using overnight cultures, there was no bioluminescence detected when the cultures were tested either with or without *n*-decyl aldehyde using a scintillation counter (Wallac1410, Pharmacia).

Growth rates were monitored within the temperature range of 22°C to 42°C in LB medium (Table 3.3). The temperature of incubation dramatically affected the growth rate of the bacteria, since it affects the rates of all cellular reactions. Overnight starter cultures in LB were cultured at 28°C at 200 o.p.m. These cultures were then inoculated into 300 ml of broth in 1 litre conical flasks to give a starting A_{600} of approximately 0.2 or less, then incubation continued. Strain VUN10,077, *Ps. fluorescens* and *St. maltophilia* failed to grow at 42°C and A_{600} failed to change within 3-5 hours after inoculation, with viability remaining being tested by streaking onto LA plates. Unlike the other strains, *St. maltophilia* failed grow at 37°C when subcultured at this temperature. Although all strains could grow at 37°C, 28°C was chosen for subsequent studies as this was well within the range of temperatures that would be found in their natural environments and provided scope to perform heat shock experiments. BHI medium was used in subsequent experiments at it had been shown to give a 20% higher growth rate than LB medium for strain VUN10,077 (Yuriev *et al.*, 1997).

Table 3.1. Morphological features of the indigenous strain VUN10,077 on BHI medium.

Gram staining		negative
Cell shape		rod
Colony	form elevation	circular and irregular convex and umbonate
	margin	undulate
	pigmentation	orange
	opacity	opaque
	texture	butyrous
	surface	rough (bumpy)

Table 3.2. Physiological characteristics of Gram negative α -proteobacteria used in this study. The test organisms were incubated for up to seven days at 28°C before results were recorded.

strain VUN10,077		<i>St. maltophilia</i>	<i>Ps. aeruginosa</i>	<i>Ps. fluorescens</i>
Oxidase	+	+	+	+
Lysine	+	+	+	+
Ornithine	-	-	-	-
H ₂ S	-	-	-	-
Glucose	-	-	+	-
Mannitol	-	-	-	-
Xylose	-	-	+	+
ONPG	+	-	-	-
Indole	-	-	-	-
Urease	-	+	+	+
V.P.	-	-	N.A.	-
Citrate	+	+	+	+
TDA	-	N.A.	-	-
Gelatin	-	-	+	-
Malonate	-	+	+	+
Inositol	-	-	-	-
Sorbitol	-	-	-	-
Rhamnose	-	-	-	-
Sucrose	-	-	-	+
Lactose	-	-	-	-
Arabinose	-	-	-	-
Adonitol	-	-	-	-
Raffinose	-	-	-	-

Salicin	-	-	+	-
Arginine	+	+	+	+

+, positive reaction; -, negative reaction; and N.A., not applicable

Table 3.3. Cell specific growth rates for Gram-negative α -proteobacteria when cultured at temperatures between 22°C and 42°C in LB (the values are μ values, h^{-1}).

Temperature	strain VUN10,077	<i>Ps. fluorescens</i>	<i>Ps. aeruginosa</i>	<i>St. maltophilia</i>
22°C	0.316	0.429	0.444	0.462
28°C	0.444	0.600	0.667	0.600
30°C	0.400	0.353	0.545	0.480
37°C	0.600	0.273	0.500	0.500
42°C	-	-	0.500	-

-, A_{600} values did not increase

3.3 16S ribosomal RNA (rRNA) gene analysis

3.3.1 16S rRNA gene amplification

The analysis of 16S rRNA gene were aided by using PCR to amplify target sequences in the strain VUN10,077. Two PCR primers were used to amplify approximately 1,300 bp of a consensus 16S rRNA gene (Figure 3.1); forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') (Marchesi *et al.*, 1998) and reverse primer 1387r (5'-GGG CGG TGT GTA CAA GGC-3') (Pharmacia; Marchesi *et al.*, 1998) were prepared as described in the section 2.3.5.3. The PCR amplification was performed according to the Expand Long Template PCR system (Boehringer Mannheim) and 500 ng of genomic DNA was used as the template. The PCR product from strain VUN10,077 was smaller than similar fragments amplified from *E. coli* HB101, *Ps. aeruginosa*, *Ps. fluorescens* and *St. maltophilia* (Figure 3.1).

3.3.2 16S rDNA sequences

To obtain the sequences of the PCR fragments (Figure 3.1), TA cloning was employed using the pGEM[®]-T vector system (Promega). Ligation was performed at 4°C overnight directly after 16S rRNA gene amplification and transformation was carried out using *E. coli* JM109 competent cells. Plasmid DNA was purified using the Quantum Prep[®] Plasmid Miniprep Kit (Bio-Rad) from several blue or white colonies selected, which were plated on LA containing 100 µg/ml ampicillin then incubated at 37°C overnight. The plasmid DNA was analysed using restriction enzymes (*Apal* [*Bsp*1201] and *SacI* at 37°C for 1 hour), and this confirmed the presence of inserts of the correct size. Several amplification primers were used for sequencing the corresponding PCR products, including 63f and 1387r. The plasmid DNA (200 ng) was sequenced and two primers were used for both ends: T7 (5'TAA TAC GAC TCA CTA TAG GGC GA-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG AAT ACT-3'), and internal primers used for sequencing genes from all strains; 5'-TAC CGA CAG AAT AAG CAC C-3' (position 439-457) for *Ps. fluorescens*, 5'-ACC AAC AGA ATA AGC ACC-3' (position 440-457) for *Ps. aeruginosa*, 5'-ACC CAA AGA ATA AGC ACC-3' (position 448-415) for *St. maltophilia*, and two internal primers for strain VUN10,077, 5'-GAG TAT GAG AGA GGT GTG TGG-3' (position 555-575) and 5'-GTC GTA AGA TGT TGG GTT-3' (position 974-991).

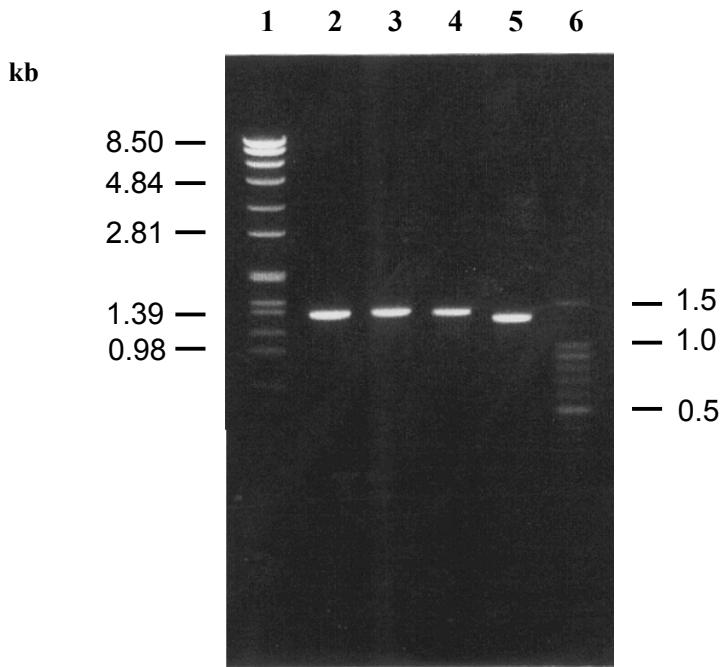


Figure 3.1. Agarose gel electrophoresis of 16S rRNA gene PCR amplification products.

Two different size markers were used, SPP1/*EcoRI* (Progen) for lane 1 (15 fragments contained with 360, 480, 720, 980, 1,160, 1,390, 1,510, 1,860, 1,950, 2,810, 3,590, 4,840, 6,100, 7,350, and 8,500 bp) and PCR markers for lane 6 (100bp DNA Ladder, Promega, consisted of 11 fragments with 1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp). Lane 2, *Ps. aeruginosa*; lane 3, *St. maltophilia*; lane 4, *Ps. fluorescens*; and lane 5, strain VUN10,077.

Table 3.4. Sequence similarity analysis of the strain VUN10,077 16S rRNA gene. The data was obtained from the GenBank database using FASTA program (search based on May, 1999 information).

Strain ^a	Source	Similarity(%)	Accession number
<i>Brevundimonas</i> sp.	?	100	BSJ22780
α -proteobacterium MBIC3965 ^b	Marine	100	AB19037
<i>Brevundimonas intermedia</i>	?	99.844	BIJ227786
<i>Caulobacter intermedius</i>	?	99.844	AB023784
Bacterial sp. isolate 4-8	Lake	99.766	BAJ832
Thin bent rods	Marine	99.688	TBJ00134
<i>Brevundimonas vesicularis</i> LMG 11141	Hemoculture	99.688	BVJ22778
<i>Brevundimonas vesicularis</i> LMG 2350	Leech	99.766	BVE7801
<i>Brevundimonas vesicularis</i> IAM 12105T	?	99.454	AB021414
<i>Caulobacter intermedius</i> ATCC15262	Pond	99.454	CIN7802
<i>Brevundimonas</i> sp. V4.BO.10	?	99.219	BSJ227801
<i>Brevundimonas aurantiaca</i>	?	99.063	BAJ22787
<i>Caulobacter henricii</i> strain: ATCC15266 ^b	Marine	99.063	AB016846

^a, the strain nomenclature is from the GenBank database.

^b, re-classified as *Brevundimonas* sp. (T. Hamada, personel communication)

?, source was not available.

Sequence similarity was checked against the 16S rDNA sequences of other bacteria in the GenBank database using the FASTA program (Pearson and Lipman, 1988), ANGIS. The sequences are shown in Figure 3.2, which were duplicated and aligned manually. *Ps. aeruginosa* and *Ps. fluorescens* were confirmed by comparing these in the GenBank data base sequences, and showed 99.853% and 99.263% similarities to known strains of these species (accession number PA38445 and PFIAM12), respectively. *St. maltophilia* showed 99.854% similarity to known species (accession number SMRR11087). Sequence data obtained for strain VUN10,077 showed that the size of the fragment was 1,281 bp compared to 1,351-1,367 for the other related species, which is consistent with the size of the PCR fragment seen on agarose gels. Strain VUN10,077 displayed a high level of DNA similarity more than 99% to the *Brevundimonas* type species and caulobacters (Table 3.4) and the isolate of freshwater *Caulobacter* strain FWC14 (97.982%) (Stahl *et al.*, 1992), but these strains are unknown species or from the marine environments or clinical settings. For example, marine isolate α -proteobacterium MBIC 3965 and *Caulobacter henricii* (ATCC15266) were re-classified as genus *Brevundimonas* by phylogenetic analysis (Tohru Hamada, personal communication). *Caulobacter intermedius* phylogenetically may belong to the genus *Brevundimonas* due to this organism having a stalk which may be distinctive for caulobacters (T. Hamada, personal communication). The pond water isolate *C. intermedius* type strain ATCC 15262 is the one most closely similar to strain VUN10,077 and is from a similar environment, however, this strain requires further classification. From these observations, indigenous strain VUN10,077 which was isolated from fresh waterways emerged as an unknown species which is most closely aligned to the *Brevundimonas* genus and most similar to species that are not fully classified. The sequences obtained have been deposited with GenBank (Figure 3.2). The GenBank accession numbers for the sequences described in this thesis are AF137356 (strain VUN10,077), AF137357 (*St. maltophilia*), AF137358 (*Ps. aeruginosa*) and AF137359 (*Ps. fluorescens*).

3.3.3 rRNA operon copy number

Based on partial rRNA sequence information, Southern blot analysis of genomic DNA digests was performed to provide some information on rRNA operon copy number and further analysis of the full 16S rRNA sequence. Southern blotting (section in 2.3.6.3) was performed using a PCR DIG-labelled probe constructed from strain VUN10,077 genomic DNA with two rRNA primers: 63f and 1387r. The probe was hybridized to restriction enzyme digested genomic

Figure 3.2. Sequences of 16S rRNA gene.

A, *Ps. aeruginosa*; B, *Ps. fluorescens*; C, *St. maltophilia*; D, strain VUN10,077.

A. 16s rDNA sequence of *Ps. aeruginosa*,

GenBank accession number AF137358

Length: 1356

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1 CAGGCCTAAC ACATGCAAGT CGAGCGGATG AAGGGAGCTT GCTCCTGGAT
51 TCAGCGGCGG ACGGGTGAGT AATGCCTAGG AATCTGCCTG GTAGTGGGG
101 ATAACGTCCG GAAACGGGCG CTAATACCGC ATACGTCCGT AGGGAGAAAG
151 TGGGGATCT TCGGACCTCA CGCTATCAGA TGAGCCTAGG TCGGATTAGC
201 TAGTTGGTGG GGTAAAGGCC TACCAAGGCG ACGATCCGTA ACTGGTCTGA
251 GAGGATGATC AGTCACACTG GAACTGAGAC ACGGTCCAGA CTCCTACGGG
301 AGGCAGCAGT GGGGAATATT GGACAATGGG CGAAAGCCTG ATCCAGCCAT
351 GCCGCGTGTG TGAAGAAGGT CTTCGGATTG TAAAGCACTT TAAGTTGGGA
401 GGAAGGGCAG TAAGTTATA CCTTGCTGTT TTGACGTTAC CAACAGAATA
451 AGCACCGGCT AACCTCGTGC CAGCAGCCGC GGTAATACGA AGGGTGCAAG
501 CGTTAACCGG AATTACTGGG CGTAAAGCGC GCGTAGGTGG TTCAGCAAGT
551 TGGATGTGAA ATCCCCGGGC TCAACCTGGG AACTGCATCC AAAACTACTG
601 AGCTAGAGTA CGGTAGAGGG TGGTGGATT TCCTGTGTAG CGGTGAAATG
651 CGTAGATATA GGAAGGAACA CCAGTGGCGA AGGCGACCAC CTGGACTGAT
701 ACTGACACTG AGGTGCAGAA GCGTGGGGAG CAAACAGGAT TAGATACCT
751 GGTAGTCCAC GCCGTAACCG ATGTCGACTA GCCGTTGGGA TCCTTGAGAT
801 CTTAGTGGCG CAGCTAACGC GATAAGTCGA CCCGCTGGGG AGTACGGCCG
851 CAAGGTTAAA ACTCAAATGA ATTGACGGGG GCCCCCACAA GCGGTGGAGC
901 ATGTGGTTA ATTCGAAGCA ACGCGAAGAA CCTTACCTGG CCTTGACATG
951 CTGAGAACTT TCCAGAGATG GATTGGTGCC TTCGGGAACT CAGACACAGG
1001 TGCTGCATGG CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC
1051 GTAACGAGCG CAACCCTTGT CCTTAGTTAC CAGCACCTCG GGTGGGCACT
1101 CTAAGGAGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA TGACGTCAAG
1151 TCATCATGGC CCTTACGGCC AGGGCTACAC ACGTGCTACA ATGGTCGGTA
1201 CAAAGGGTTG CCAAGCCGCG AGGTGGAGCT AATCCCATAA AACCGATCGT
1251 AGTCCGGATC GCAGTCTGCA ACTCGACTGC GTGAAGTCGG AATCGCTAGT
1301 AATCGTGAAT CAGAATGTCA CGGTGAATAC GTTCCCGGGC CTTGTACACA
1351 CCGCCC
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B. 16s rDNA sequence of *Ps. fluorescens*,
GenBank accession number AF137359

Length: 1357

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1 CAGGCCTAAC ACATGCAAGT CGAGCGGTAG AGAGAAGCTT GCTTCTCTTG
51 AGAGCGGCAG ACGGGTGAGT AAAGCCTAGG AATCTGCCTG GTAGTGGGGG
101 ATAACGTTCG GAAACGGACG CTAATACCGC ATACGTCCTA CGGGAGAAAG
151 CAGGGGACCT TCGGGCCTTG CGCTATCAGA TGAGCCCAGG TCGGATTAGC
201 TAGTTGGTGA GGTAGTGGCT CACCAAGGCG ACGATCCGTA ACTGGTCTGA
251 GAGGATGATC AGTCACACTG GAACTGAGAC ACGGTCCAGA CTCCTACGGG
301 AGGCAGCAGT GGGGAATATT GGACAATGGG CGAAAGCCTG ATCCAGCCAT
351 GCCCGTGTG TGAAGAAGGT CTTGGATTG TAAAGCACTT TAAGTTGGGA
401 GGAAGGGCAT TAACCTAATA CGTTAGTGTG TTGACGTTAC CGACAGAATA
451 AGCACCGGCT AACTCTGTGC CAGCAGCCGC GGTAAATACAG AGGGTGCAAG
501 CGTTAACCGG AATTACTGGG CGTAAAGCGC GCGTAGGTGG TTGTTAACGT
551 TGGATGTGAA ATCCCCGGGC TCAACCTGGG AACTGCATT AAAACTGACT
601 GACTAGAGTA TGGTAGAGGG TGGTGGATT TCCTGTGTAG CGGTGAAATG
651 CGTAGATATA GGAAGGAACA CCAGTGGCGA AGGCGACCAC CTGGACTAAT
701 ACTGACACTG AGGTGCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT
751 GGTAGTCCAC GCCGTAACAG ATGTCAACTA GCCGTTGGGA GCCTTGAGCT
801 CTTAGTGGCG CAGCTAACGC ATTAAGTTGA CCGCCTGGGG AGTACGGCCG
851 CAAGGTTAAA ACTCAAATGA ATTGACGGGG GCCCCCACAA GCGGTGGAGC
901 ATGTGGTTA ATTCGAAGCA ACGCGAAGAA CCTTACCAGG CCTTGACATC
951 CAATGAACCT TCTAGAGATA GATTGGTGCC TTCGGGAACA TTGAGACAGG
1001 TGCTGCATGG CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC
1051 GTAACGAGCG CAACCCTTGT CCTTAGTTAC CAGCACGTAA TGGTGGGCAC
1101 TCTAAGGAGA CTGCCGGTGA CAAACCGGAG GAAGGTGGGG ATGACGTCAA
1151 GTCATCATGG CCCTTACCGC CTGGGCTACA CACGTGCTAC AATGGTCGGT
1201 ACAGAGGGTT GCCAAACCGC GAGGTGGAGC TAATCCCACA AAACCGATCG
1251 TAGTCCGGAT CGCAGTCTGC AACTCGACTG CGTGAAGTCG GAATCGCTAG
1301 TAATCGCGAA TCAGAATGTC GCGGTGAATA CGTTCCCGGG CCTTGTACAC
1351 ACCGCC
```

C. 16S rDNA sequence of *St. maltophilia*,
GenBank accession number AF137357

Length: 1367

1 CAGGCCTAAC ACATGCAAGT CGAACGGCAG CACAGTAAGA GCTTGCTCTT
51 ATGGGTGGCG AGTGGCGGAC GGGTGAGGAA TACATCGGAA TCTACTTTT
101 CGTGGGGGAT AACGTAGGAA AACTTACGCT AATACCGCAT ACGACCTACG
151 GGTGAAAGCA GGGGATCTTC GGACCTTGC CGATTGAATG AGCCGATGTC
201 GGATTAGCTA GTTGGCGGGG TAAAGGCCA CCAAGGCAC GATCCGTAGC
251 TGGTCTGAGA GGATGATCAG CCACACTGGA ACTGAGACAC GGTCCAGACT
301 CCTACGGGAG GCAGCAGTGG GGAATATTGG ACAATGGCG CAAGCCTGAT
351 CCAGCCATAC CGCGTGGGTG AAGAAGGCCT TCAGGTTGTA AAGCCCTTTT
401 GTTGGGAAAG AAATCCAGCC GGCTAATACC TGGTTGGGAT GACGGTACCC
451 AAAGAATAAG CACCGGCTAA CTTCGTGCCA GCAGCCGCGG TAATACGAAG
501 GGTGCAAGCG TTACTCGGAA TTACTGGCG TAAAGCGTGC GTAGGTGGTT
551 GTTTAAGTCT GTTGTGAAAG CCCTGGGCTC AACCTGGGAA CTGCAGTGGA
601 AACTGGACAA CTAGAGTGTG GTAGAGGGTA GCGGAATTCC CGGTGTAGCA
651 GTGAAATGCG TAGAGATCGG GAGGAACATC CATGGCGAAG GCAGCTACCT
701 GGACCAAACAC TGACACTGAG GCACGAAAGC GTGGGGAGCA AACAGGATTA
751 GATAACCTGG TAGTCCACGC CCTAAACGAT GCGAACTGGA TGTGGGTGC
801 AATTGGCAC GCAGTATCGA AGCTAACGCG TTAAGTTCGC CGCCTGGGA
851 GTACGGTCGC AAGACTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG
901 CGGTGGAGTA TGTGGTTAA TTCGATGCAA CGCGAAGAAC CTTACCTGGC
951 CTTGACATGT CGAGAACTTT CCAGAGATGG ATTGGTGCCT TCGGGAACTC
1001 GAACACAGGT GCTGCATGGC TGTCGTAGC TCGTGTGCG AGATGTTGGG
1051 TTAAGTCCCG CAACGAGCGC AACCCCTTGT CTTAGTTGCC AGCACGTAAT
1101 GGTGGGAACCTAAGGAGAC CGCCGGTGAC AAACCGGAGG AAGGTGGGG
1151 TGACGTCAAG TCATCATGGC CCTTACGGCC AGGGCTACAC ACGTACTACA
1201 ATGGTAGGGA CAGAGGGCTG CAAGCCGGCG ACGGTAAGCC AATCCCAGAA
1251 ACCCTATCTC AGTCCGGATT GGAGTCTGCA ACTCGACTCC ATGAAGTCGG
1301 AATCGCTAGT AATCGCAGAT CAGCATTGCT GCGGTGAATA CGTTCCCGGG
1351 CCTTGTACAC ACCGCC

D. 16S rDNA sequence of the target indigenous strain VUN10,077,
GenBank accession number AF137356

Length: 1281

1 CAGGCCTAAC ACATGCAAGT CGAACGAAC TCTCGGAGTT AGTGGCGGAC
51 GGGTGAGTAA CACGTGGAA CGTCCTTTA GTTCGGAAT AACTCAGGGA
101 AACTTGTGCT AATACCGAAT GTGCCCTTCG GGGGAAAGAT TTATGCCCT
151 TAGAGCGGCC CGCGTCTGAT TAGCTAGTTG GTGAGGTAAA GGCTCACCAA
201 GGCGACGATC AGTAGCTGGT CTGAGAGGAT GATCAGCCAC ATTGGGACTG
251 AGACACGGCC CAAACTCCTA CGGGAGGCAG CAGTGGGAA TCTTGCACAA
301 TGGCGAAAG CCTGACGCAG CCATGCCCG TGAAATGATGA AGGTCTTAGG
351 ATTGTAATAT TCTTCACCG GGGACGATAA TGACGGTACC CGGAGAAGAA
401 GCCCCGGCTA ACTTCGTGCC AGCAGCCGCG GTAATACGAA GGGGGCTAGC
451 GTTGTCTCGGA ATTACTGGGC GTAAAGGGAG CGTAGGCGGA CATTAAAGTC
501 AGGGGTGAAA TCCCAGGGCT CAACCTCGGA ATTGCTTTG ATACTGGGTG
551 TCTTGAGTAT GAGAGAGGGT TGTGAACTC CGAGTGTAGA GGTGAAATT
601 GTAGATATTG GGAAGAACAC CAGTGGCGAA GGCGACACAC TGGCTCATTA
651 CTGACGCTGA GGCTCGAAAG CGTGGGGAGC AAACAGGATT AGATACCTG
701 GTAGTCCACG CCGTAAACGA TGATTGCTAG TTGTCGGGAT GCATGCATTT
751 CGGTGACGCA GCTAACGCAT TAAGCAATCC GCCTGGGGAG TACGGTCGCA
801 AGATTAACAC TCAAAGGAAT TGACGGGGC CCGCACAAGC GGTGGAGCAT
851 GTGGTTAAC TCGAAGCAAC CGCGAGAAC TTACCACCTT TTGACATGCC
901 TGGACCGCCA GAGAGATCTG GCTTCCCTT CGGGGACTAG GACACAGGTG
951 CTGCATGGCT GTCGTCAGCT CGTGTCTGA GATGTTGGGT TAAGTCCCGC
1001 AACGAGCGCA ACCCTGCCA TTAGTTGCCA TCATTTAGTT GGGAACTCTA
1051 ATGGGACTGC CGGTGCTAAG CCGGAGGAAG GTGGGGATGA CGTCAAGTCC
1101 TCATGGCCCT TACAGGGTGG GCTACACACG TGCTACAATG GCGACTACAG
1151 AGGGTTAAC TCTAAAAGTC GTCTCAGTTC GGATTGTCCT CTGCAACTCG
1201 AGGGCATGAA GTTGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
1251 AATACGTTCC CGGGCCTTGT ACACACCGCC C

DNA of the indigenous strain VUN10,077. The restriction sites in the 16S rRNA gene sequence (accession number AF137356) were used to determine restriction enzymes profile. The digests for strain VUN10,077 showed several bands depending on the restriction enzyme used. The blot results may propose a copy number for the rRNA operon in strain VUN10,077. The Southern blots shown in Figure 3.3 indicated one or two bands from *Hind*III, *Acc*I, *Sal*I, *Pst*I, *Bst*EII, *Clal* and *Eco*R321(*Eco*RV) digests, where these restriction enzyme sites were absent in the 16S rRNA gene sequence.

However, *Apal*, *Bsp*143I, *Bsr*BI, *Pvu*III and *Bam*HI digests showed several corresponding bands (Figure 3.3). *Apal*, *Bsp*143I and *Bsr*BI digests had recognition sequences within the PCR region amplified for probe synthesis, and from the observed results. *Pvu*III and *Bam*HI sites may probably exist in the complete 16S rDNA sequence, which would explain the multiple bands seen. From these observations, it is proposed that the copy number for the 16S rRNA gene of strain VUN10,077 is one to two. *E. coli* possesses seven rRNA operon copies (Kiss *et al.*, 1977) and *Sphingomonas* sp. strain RB2256 was suggested to have one by Fegatella *et al.* (1998) using similar analyses.

Full sequencing of the gene, which could be achieved using the *Hind*III fragment of the genomic DNA (lane 14, Figure 3.3), was not performed as it unlikely that this would provide any better diagnostic information than could be obtained by examining sequences from the normally conserved area.

3.4 Analysis of total cellular RNA

3.4.1 Comparison of total cellular RNA.

Total cellular RNA was extracted by the SDS-hot phenol extraction method (Wilkinson, 1991) for both *E. coli* HB101 and related species (section in 2.3.3). The RNA samples were loaded onto 0.4 M formaldehyde denaturing agarose gels as well as glyoxal/DMSO agarose gels for resolution by electrophoresis. For gels containing 0.4 M formaldehyde, both 1× MOPs

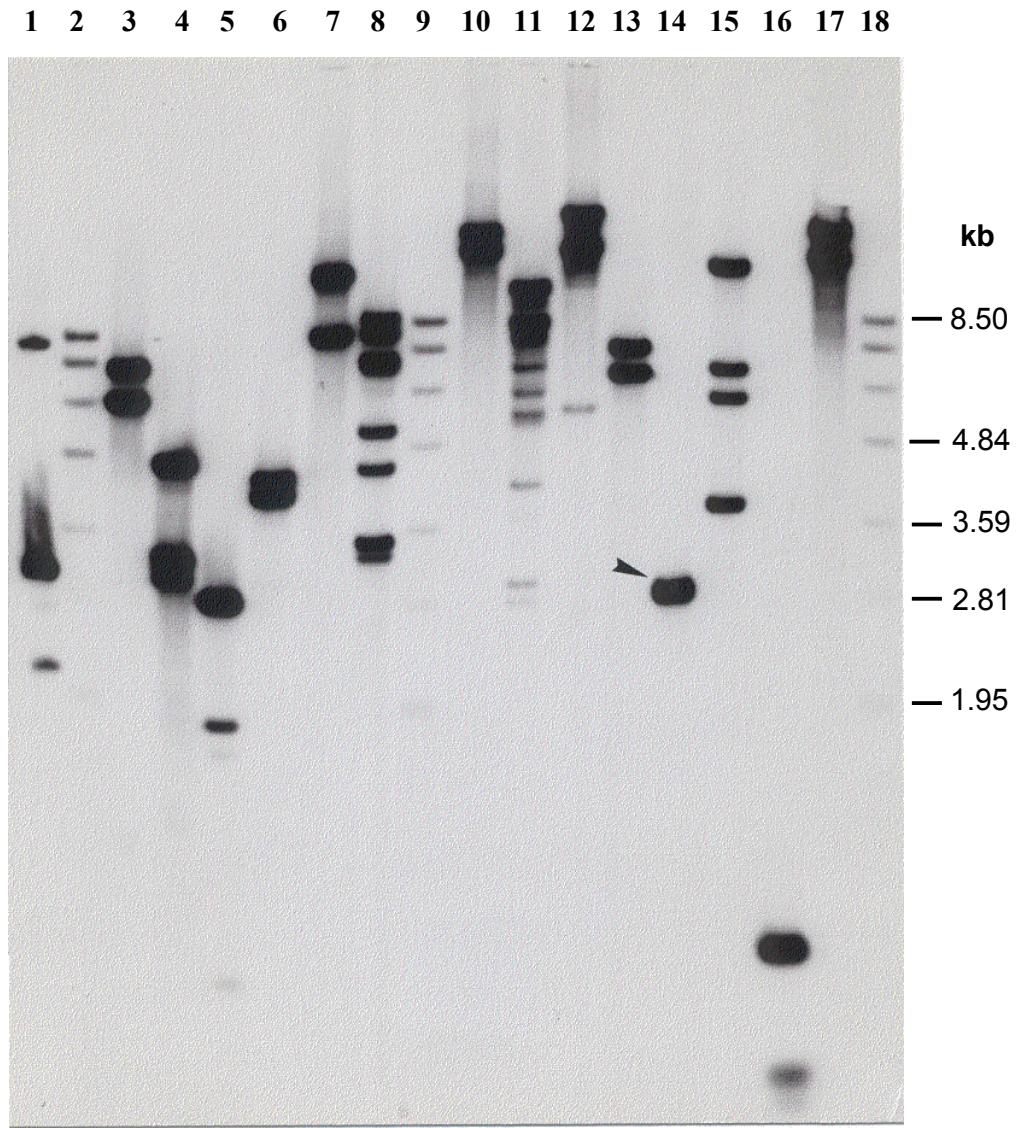


Figure 3.3. Analysis of the 16S rRNA gene of strain VUN10,077 by Southern hybridization of genomic DNA digests, probing with an amplified 16S rRNA PCR product.

The genomic DNA was digested with selected restriction enzymes at appropriate temperatures and 2 µg of DNA were resolved on large agarose slab gels by electrophoresis (section 2.3.4.1). Hybridization was performed by DIG™ labelling protocol as described in section 2.3.6.3. Lane 1, *BclI*; lane 3, *AccI*; lane 4, *AatIII*; lane 5, *BsrBI*; lane 6, *NcoI*; lane 7, *SalI*; lane 8, *PvuIII*; lane 10, *PstI*; lane 11, *BamHI*; lane 12, *BstEII*; lane 13, *ClaI*; lane 14, *HindIII*; lane 15, *Apal*; lane 16, *Bsp143I*, lane 17, *EcoR321* (*EcoRV*); and lanes 2, 9 and 18 were DNA markers (from top to bottom; SPPI/*EcoRI*, fractionated with 8,500, 7,350, 6,100, 4,840, 3,590, 2,810, 1,950, 1,860, 1,510, 1,390, 1,160, 980, 720, 480 and 360bp).

buffer and 1× TAE buffer were used at 80 V (5 minutes at 100 V at the beginning). When the gels were run in 1× TAE buffer, the gel was reversed every 10 minutes. Because preliminary results showed an unusual RNA profile for strain VUN10,077, different gel running conditions were tried in case these affected the running or denaturing conditions for this RNA. For example, glyoxal/DMSO gel loading buffer dose not contain Ficoll and this may affect the denaturation of the RNA sample. However, resolution of rRNAs was the same under all conditions tested and was as shown for the standard procedure used in Figure 3.4. *E. coli* HB101, *Ps. aeruginosa*, *Ps. fluorescens* and *St. maltophilia* contain two distinct bands of approximately 3.1 kb (23S rRNA species) and 1.6 kb (16S rRNA species) and one lower band of approximately 0.1 kb (5S rRNA species) which showed faintly in all tested strains (Figure 3.4). Strain VUN10,077 showed four distinct rRNA bands on the standard denaturing agarose gels: one pale band (approximately 2.5 kb), two thicker bands of approximately 1.38 kb and 1.2 kb, and 0.1 kb (lane 3 and 11). These bands did not correspond to mammalian rRNA (see lane 8, Figure 3.4), nor did the bands larger than 0.1 kb line up with any bands in *E. coli* and the other related species. The 1.38 kb rRNA is the most abundant of these rRNAs in this type of analysis. This phenomenon indicated a unique feature which was characteristic for this indigenous strain in denaturing conditions. The observation was extended to investigate which band from the target strain corresponded to the 16S rRNA gene.

3.4.2 Northern blot analysis

After electrophoresis on a 0.4 M formaldehyde gel, the RNA was transferred to Nylon membranes (Boehringer Mannheim) and these probed using the same conditions as described in the section 3.3.3. The hybridization temperature was 55°C and the signal was detected according to manufacturer's instruction (described in section 2.3.6.3).

The results of the hybridization showed that the 16S fragments from all strains tested hybridized to the probe (Figure 3.5), indicating that the strain VUN10,077-specific probe was homologous to the other tested strains, including *E. coli* HB101, *Ps. aeruginosa*, *Ps. fluorescens* and *St. maltophilia*. Preliminary data using the PCR amplification product from the 16S rRNA gene of VUN10,077 showed that the probe reacted in the 1.6 kb RNA band of *E. coli*, *Ps. aeruginosa*, *St. maltophilia* and *Ps. fluorescens*, as well as with the 1.38 kb band from strain VUN10,077. However, the amount of RNA loaded in this test (lanes 1-5, Figure 3.5) lead to a

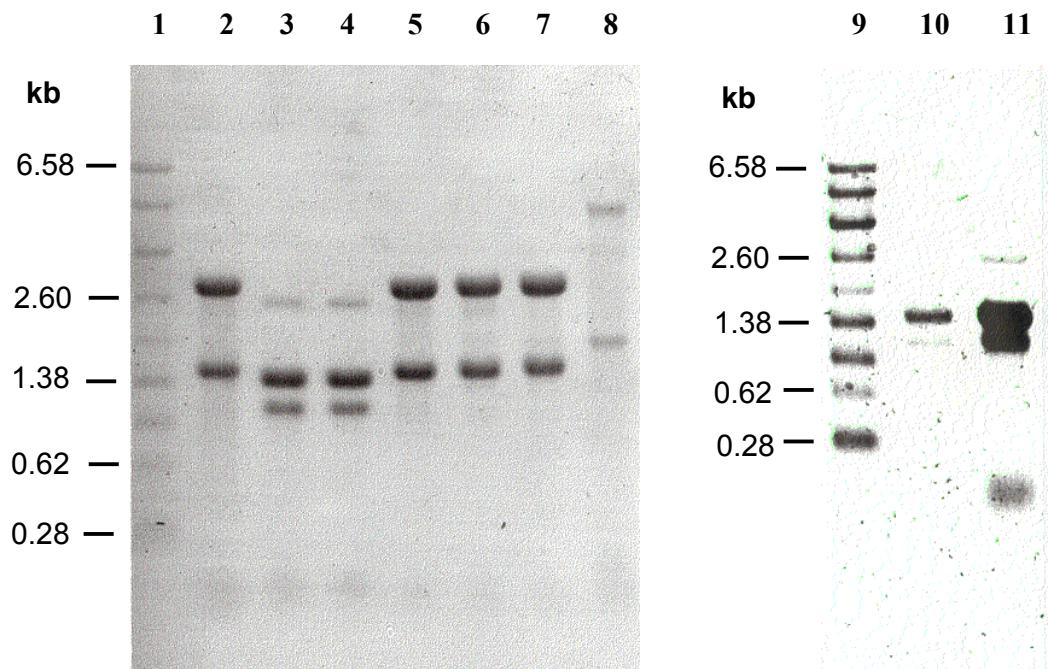


Figure 3.4. Total cellular RNA profiles on 0.4 M formaldehyde and glyoxal/DMSO denaturing agarose gel electrophoresis.

Electrophoresis was performed in 1× MOPs buffer at 80 V for the 0.4 M formaldehyde gel. Lanes 1 and 9 were RNA markers (G319, Promega, RNA fractionated according to 6,583, 4,981, 3,683, 2,604, 1,908, 1,383, 955, 623 and 281bp, from top to bottom); lane 2, *E. coli* HB101; lane 3, strain VUN10077; lane 4, transconjugant (VUN3,600); lane 5, *Ps. aeruginosa*; lane 6, *Ps. fluorescens*; lane 7, *St. maltophilia*; and lane 8, mammalian RNA sample (from human placenta and extracted by Ms. U. Manuelpillai). The total RNA extracted from VUN10,077 was run at concentrations of 1 µg (lane 10) and 3 µg (lane 11) with RNA markers G319 (lane 9) in glyoxal/DMSO denaturing agarose gel at 80 V in 10 mM sodium phosphate buffer (lanes 9,10 and 11), and the gel was stained with 0.05% toluidine blue in 10 mM sodium phosphate buffer pH (7.0).

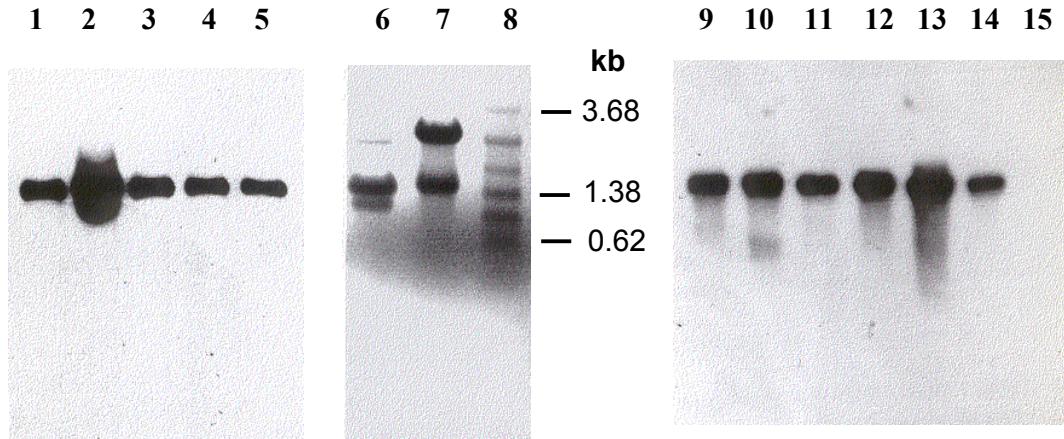


Figure 3.5. Northern blot analysis of 16S rRNA for several strains, probing with amplified 16S rRNA PCR product of VUN10,077.

A 0.4 M formaldehyde gel electrophoresis was performed in 1× MOPS buffer at 80 V before transfer (section 2.3.4.2). Lanes 1-7; approximately 3 µg of cellular RNA sample was loaded for each lane; and lanes 9-15, approximately 1 µg of cellular RNA sample was loaded for each lane except for lane 14 (100 ng) and lane 15 (10 ng). Lanes 1, 7 and 12, *E. coli* HB101; lanes 2, 6, 13, 14 and 15, strain VUN10,077; lanes 3 and 11, *Ps. fluorescens*; lanes 4 and 10, *St. maltophilia*; lanes 5 and 9, *Ps. aeruginosa*; and lane 8, RNA markers (G319, Promega). Lanes 6-8 were stained in toluidine blue (0.04% in sodium phosphate buffer) prior to hybridization using the DIG labelling protocol.

broad smeared band being detected despite similar amounts of rRNA loaded in parallel, so that it was hard to evaluate whether the probe also reacted with the 1.2 kb band (lane 6, Figure 3.5). To solve this, rRNA from VUN10,077 was run in parallel with the other strains but with lower amounts loaded (1 µg to 10 ng) and the Northern blot repeated (lanes 9-15, Figure 3.5), noted that 10 ng showed no band which indicated that this amount of rRNA was not suitable for blotting analysis. Under these conditions, only one band was detected using the PCR product from VUN10,077 as probe with 100 ng loaded suggested that the 1.2 kb fragment did not cross react with this probe and implying that this band derived from the equivalent of 23S rRNA. 23S rRNA was fragmented to two bands of mobility corresponding to 1.7 and 1.1 kb in *Salmonella typhimurium* (Winkler, 1979; Smith *et al.*, 1988). Similarly, Kordes *et al.* (1994) reported that a 23S rRNA is cleaved into 1.6 kb (16S) and 1.3 kb (14S) rRNA molecules in *Rhodobacter capsulatus* wild-type strains. These results confirmed that the 1.2 kb rRNA species of the strain VUN10,077 was not fragmented from the 16S-like species.

3.5 Discussion

The use of 16S rRNA in the classification of bacterial species has been well established, and its effects on biology have been profound. It was 16S rRNA data that provided convincing evidence that chloroplasts and mitochondria most likely arose from free-living bacteria (Fox *et al.*, 1980; Woese *et al.*, 1990). The general approach to determinative and environmental studies has been targeted at discrete regions of the rRNAs. Since rRNA gene sequences are conserved, the targeting of regions where greater or lesser conservation occurs offers exquisite control for classification purposes 23S molecules are around 3,000 nucleotides. When fully or almost fully analysed (at least >1,000 nucleotides should be determined), both molecules contain sufficient information for reliable phylogenetic analyses (Amann *et al.*, 1995). For the two indigenous strains used in this study 16S rRNA sequences were determined as 1,281 and 1,367 nucleotides respectively and the two *Pseudomonas* species were 1,356 and 1,357 nucleotides (Figure 3.2), using primers to amplify PCR products for conserved regions. 16S rRNA gene analysis of amplified 16S rRNA gene PCR products classified these indigenous strains as *St. maltophilia* and strain VUN10,077, formerly identified as *Ps. paucimobilis*, was an unidentified species but likely to be in the genus *Brevundimonas*. Strain VUN10,077 was a Gram-negative, rod-shaped, orange pigmented strain and could survive and replicate at the temperatures at least between

30°C and 37°C. These phenotypic observations are similar to *Brevundimonas* and to *Sphingomonas* and *Rhizomonas*. Unlike strain VUN10,077, *Sphingomonas paucimobilis* is normally oxidase negative and gelatin is liquefied (Yabuuchi *et al.*, 1990), and *Brevundimonas* gen. nov. species have negative reactions on β -galactosidase (ONPG) and in the lysine dehydrogenase test (Segers, *et al.*, 1994). It is quite difficult to translate rRNA similarity values into divergence and rRNA sequences from single isolates into nomenclature, at least at the level of a species or genus (Amann *et al.*, 1995; Clayton *et al.*, 1995). For example, Fox *et al.* (1992) reported 16S rRNA sequence identity between *Bacillus globisporus* and *Bacillus psychrophilus*, two species that are clearly justified by DNA-DNA similarity of less than 50%, and Woo *et al.* (1997) reported different sequencing outcomes between 23S and 16S rDNA sequences in *Leptospira interrogans*.

Ribosomes, the ribonucleoprotein organelles (approximately 25-30 nm diameter) which mediate protein synthesis and help determine function, may be either free or membrane-bound and are usually present in large numbers in cells. Ribosomal RNA constitutes the major proportion of RNA in the cell, and comprises about 66% of the bacterial ribosome and about 60% of the eukaryotic 80S ribosome: rRNA is currently believed to be primarily responsible for ribosome function. Different types of ribosomes are characterised particularly by their sedimentation coefficients on sucrose-gradient ultracentrifugation. Bacterial ribosomes in *E. coli* have a sedimentation coefficient of 70S, and each consists of one 30S and one 50S subunit, which is typical of bacteria. Ribosomes in chloroplasts and mitochondria may have sedimentation coefficients of 60S (in mammalian mitochondria), 70S (in chloroplasts in plants and algae and in mitochondria of lower eukaryotes, e.g. yeast), or 78S (in plant mitochondria). The eukaryotic 80S ribosome typically contains four rRNAs, commonly designated 28S, 5.8S and 5S (in the large subunit) and 18S (in the small subunit), although actual sizes vary somewhat with the source; the 28S and 18S rRNAs are more extensively modified by methylation (Kiss-László *et al.*, 1996) and/or pseudouridylation (Ganot *et al.*, 1997; Ni *et al.*, 1997) than are the 23S and 16S rRNAs in eubacteria. The bacterial (70S) ribosome normally contains one molecule each of three species of rRNA: 23S, 16S and 5S; the 23S and 16S rRNA contain modified nucleotides (Wrzesinski *et al.*, 1995), the 5S rRNA does not. The 16S rRNA occurs in the 30S subunit, the 23S and 5S rRNAs occur in the 50S subunit.

Ribosomal operons in bacteria and chloroplasts are typically arranged in the order of 16S-23S-5S. Archaea have the same organization for these rRNAs with some variation, such as a

tRNA^{ala} gene inserted between the 16S and 23S genes of methanogens and halophiles and a distal location of the 5S rRNA gene (Brown *et al.*, 1989; reviewed by Brown and Doolittle, 1997). Heterogeneity of the rRNA operons within a genome is a common feature and are this restriction fragment length polymorphisms (RFLPs) can be used to differentiate strains of bacteria (Hall, 1994). The RFLPs of rRNA genes were used for genotypic identification in *Streptococcus* (Rudney and Larson, 1994) and group A *Streptococcus* (GAS) isolates, which showed a rare lack of heterogeneity in all six rRNA operons as indicated by the same RFLP patterns (Sripakash and Gardiner, 1997). The number of rRNA operons varies in different bacterial species. For example, there are from one to four rRNA operons in various archaebacteria, seven in *E. coli*, and ten in *B. subtilis* (reviewed by Srivastava and Schlessinger, 1990). Some bacteria that have a low number of rRNA operons are involved in symbiosis or parasitism and this correlated with the low rates of growth in organisms, such as *Kryptophanaron alfredi* (Wolfe and Haygood, 1993), mycoplasmas (Suzuki *et al.*, 1987) and ultramicrobacterium (Fegatella *et al.*, 1998). Strain VUN10,077 may possess one or two rRNA operons (Figure 3.3) although the cells showed specific growth rates between 0.3 to 0.6 in the temperature range 22-37°C in BHI medium, which was similar to *Ps. aeruginosa* growth rates measured as between 0.4 to 0.67 at the same temperature range and medium (Table 3.3).

Although mammalian (28S, 18S, 5.8S, 5S) and *E. coli* (23S, 16S, 5S) rRNA sizes differ, many stem-loops and important parts of the structure are conserved; the 18S rRNA is homologous to the prokaryotic 16S rRNA, the 28S and 5.8S rRNA together are homologous to the prokaryotic 23S rRNA (Moore, 1997; Dube *et al.*, 1998). Abnormal total cellular RNA patterns were observed for strain VUN10,077 when the RNA was extracted by the hot-phenol method and analysed by 0.4 M formaldehyde or glyoxal/DMSO RNA gel electrophoresis. The pattern seen for strain VUN10,077 showed four rRNA components that were 2.5, 1.38, 1.2 and 0.1 kb in molecular weight in denaturing conditions (Figure 3.4). Interestingly, there was an absence of intact 23S rRNA, which was also seen previously in *S. typhimurium* (Winkler, 1979; Hsu *et al.*, 1994) and two strains of *Salmonella paratyphi* B (Smith *et al.*, 1988), *Brucella* and *Agrobacterium* (Hsu *et al.*, 1992), and *Rh. capsulatus* (Kordes *et al.*, 1994), resulting from fragmentation of the 23S rRNA. Similarly, *Leptospira interrogans* serovars hardjo and balcanica have been reported to contain four rRNA components (17S, 16S, 14S and 5S in size) and the abnormal rRNA pattern, 17S and 14S, were not degraded products of the 23S rRNA (Hsu *et al.*, 1990). The RNA extraction procedure for strain VUN10,077 was similar to the *Lep. interrogans* RNA extraction procedure that contained a high SDS concentration (1.7%) which

would be expected to inhibit any enzymatic reaction that might degrade the RNA molecules (Hsu *et al.*, 1990), and parallel tested proteobacterial strains did not exhibited degradation profiles using the same extraction and running procedures in parallel.

rRNA molecules can adopt complex secondary structures, extensive intramolecular base-pairing resulting in the formation of hairpins, stem-and-loop structures, *etc*. Certain regions of rRNA have been extremely highly conserved during evolution, and sequence homology studies in rRNAs are currently widely used to indicate evolutionary relationships between organisms. To obtain further information about the observed abnormal rRNA profile of strain VUN10,077, firstly, sucrose gradient sedimentation coefficient analysis needs to be carried out to determine the sedimentation properties of the ribosomal particles, which will provide information of the similarity, or otherwise, of the ribosomes to other Gram negative species, whatever the secondary structures may be. Although several running conditions were tried to see whether the observed patterns were affected by the denaturing agents or buffers, they had no affect on the patterns seen. However, the pattern may have arisen simply due to the lysis procedure used or the inherent nature of the RNA, which may be highly susceptible to RNase or may be fragile structurally, as found for *S. thypimurium* (Hsu *et al.*, 1994). It may also be necessary to investigate the secondary structure of the rRNA, which could be modified due to formation of hairpins, stem-and-loop structures, and secondary interactions due to base pairing, internal loops, unilaterally bulging residues or terminal extensions of helices (Gutell *et al.*, 1994).

Northern analysis (Figure 3.5) using a PCR product of the strain VUN10,077 16S rRNA gene interacted with the 16S rRNA band in all of the tested Gram negative strains as well as the corresponding band in strain VUN10,077. However, this probe did not interact with a second, smaller band (1.2 kb) which ran close to this (1.38 kb) 16S rRNA homologue when suitable loading conditions were used, indicating that the 1.2 kb gene most likely arose from a 23S ribosome subunit equivalent. The putative 16S rRNA of strain VUN10,077 was slightly smaller than in the other Gram negatives (1.38 kb compared with 1.6 kb), which was consistent with a smaller PCR product being amplified during strain speciation. These results indicated that strain VUN10,077 could be differentiated by 16S rRNA gene sequence and rRNA pattern. Conclusively, this indigenous strain VUN10,077 phenotypically and genetically closely resembled *Brevundimonas* sp., although some physiological characters are different and RFLP analysis is required. The indigenous strain is designated in this thesis as strain VUN10,077, to avoid misrepresenting its speciation.

Conclusion and Recommendation

The major conclusions of this chapter are summarised in dot points as follows:

- Strain VUN10,077 was classified as a *Brevundimonas* sp. using 16S rRNA gene analysis. The rRNA gene copy number was one and four rRNA bands were visualised in standard formaldehyde agarose gels when RNA was extracted by the hot-phenol method, which corresponded to a relatively weak 23S, 16S, 14S and 5S bands while the other pseudomonads and *E. coli* tested had known prokaryotic rRNA patterns in parallel. The MW of these bands showed that the strain VUN10,077 23S and 16S bands were smaller than their counterparts in other genera.

Areas for further basic- and applicable-level research are as follows:

- Abnormal rRNA patterns need to be explored using sucrose gradient ultracentrifugation to obtain exact coefficient values of each rRNA band seen in strain VUN10,077 to clarify the differences seen in MW. 16S rRNA gene analysis or secondary DNA structure should be explored to clarify the differences in size observed. The structure of the 23S-like band and its relationship to the 14S equivalent needs clarification. Morphological features using electron microscopy should be completed to allow comparison to be made for *Brevundimonas* and *Caulobacter* species, in addition to total rRNA patterns.

Chapter 4

Stress responses in strain VUN10,077 and related species

4.1 Introduction

When microbial species are subjected to abnormal environmental circumstances, the organisms rapidly redirect gene expression and produce a distinct range of newly synthesized proteins which assist their survival and adaptation to the new conditions. When organisms are confronted with sudden changes in temperature (heat shock), exposure to potentially toxic substances (ethanol, hydrogen peroxide, heavy metal ions, PAH compounds) or the onset of starvation, these stimulations induce the production of the so-called heat shock proteins or stress responsive proteins. The most thoroughly studied stress proteins include the heat shock proteins (HSP), the induction of which is the most highly conserved response across genera. This group of proteins interacts with other cellular proteins to facilitate appropriate folding, assembly into protein complexes, or their translocation into organelles (Morimoto *et al.*, 1990, Kubota *et al.*, 1994). Because of their effects on the conformation of other proteins, these HSPs, such as HSP60 (GroEL) and HSP70 (DnaK), have been designated as molecular chaperones (Ellis, 1987 and 1992).

Stress responses have been noted to occur in *Pseudomonas* species, as initially reported in *Ps. aeruginosa* (Allan *et al.*, 1988). Allan *et al.* (1988) reported that a rapid temperature shift from 30°C to 45°C caused elevated synthesis of 17 proteins soon after the temperature shock. Two proteins (76 kDa and 61 kDa) were immunologically similar to the DnaK and GroEL of *E. coli* based on their cross-reactivity with antibodies raised against the *E. coli* heat-shock proteins. Ethanol shock caused production of seven stress proteins, where three appeared to be heat-shock proteins. Nutrient (C, N, P) starvation of *Ps. putida* (Givskov *et al.*, 1994) caused synthesis of up to 72 new proteins, where approximately 20% of these proteins were common to all starvation conditions while the rest were condition specific. Heat, NaCl, and hydrogen peroxide shock exposure of *Ps. putida* also caused production of clusters of new proteins, where some were similar to starvation-stress proteins and others were condition specific or similar to the HSPs. Despite the universality of the heat shock response, the mechanism and production of HSPs varies greatly and also among different bacterial species, and even differs in one species depending on the stimulation (Babst *et al.*, 1996, Hecker *et al.*, 1996).

In this chapter, I report and compare the responses of indigenous strain VUN10,077 and related species (*Ps. aeruginosa*, *Ps. fluorescens* and *St. maltophilia*, formerly *Ps. maltophilia*) to heat shock, and responses seen following exposure to selected pollutants in the indigenous strain VUN10,077. These species were selected for study because they are commonly found in soil and aquatic environments: classification of the gene expression associated with pollutant stress responses may form the basis of biomonitoring systems for evaluating the ecological state of natural environments when these are disturbed by common pollutants.

4.2 Induction of stress-induced proteins

4.2.1 Analysis of heat shock-induced protein profiles

With the view of seeing to what extent the pseudomonads respond to heat treatment, four species of pseudomonad or related indigenous species (*Ps. aeruginosa*, *Ps. fluorescens*, *St. maltophilia*, and strain VUN10,077) were exposed to temperature upshift from 28°C to 37°C. Although the indigenous strain grew better at 37°C, 28°C was chosen as the growth temperature because bacteria would encounter this unnatural environments and this allowed scope to have a reasonable temperature shift in heat shock trials. Initially, the *St. maltophilia* strain was subjected to a temperature shift from 28°C to 42°C (similar to the conditions used by Allan *et al.* [1988] for *Ps. aeruginosa*), however, the cells did not replicate at this higher temperature, as measured from changes in absorbance readings at 600 nm and consistent with results reported in the Chapter 3. Subsequently, growth rates were checked within a temperature range of 22°C to 42°C (see Table 3.3), and Figure 4.1 shows the growth rates for heat-stressed and control cultures of the pseudomonads and related species. All tested strains showed an increased growth rate following temperature upshift to 37°C and entered stationary phase earlier than control cultures grown at 28°C in either LB or BHI broth, noting that *St. maltophilia* normally grows poorly at 37°C if growth is initiated at this temperature (see Table 3.3). Three strains, *Ps. aeruginosa*, *Ps. fluorescens*, and *St. maltophilia*, showed lower final absorbance readings compared to their control cultures in LB broth after entering the stationary phase (Figure 4.1). In contrast, strain VUN10,077 showed better absorbance readings throughout the growth period compared with control cultures when cultured in BHI

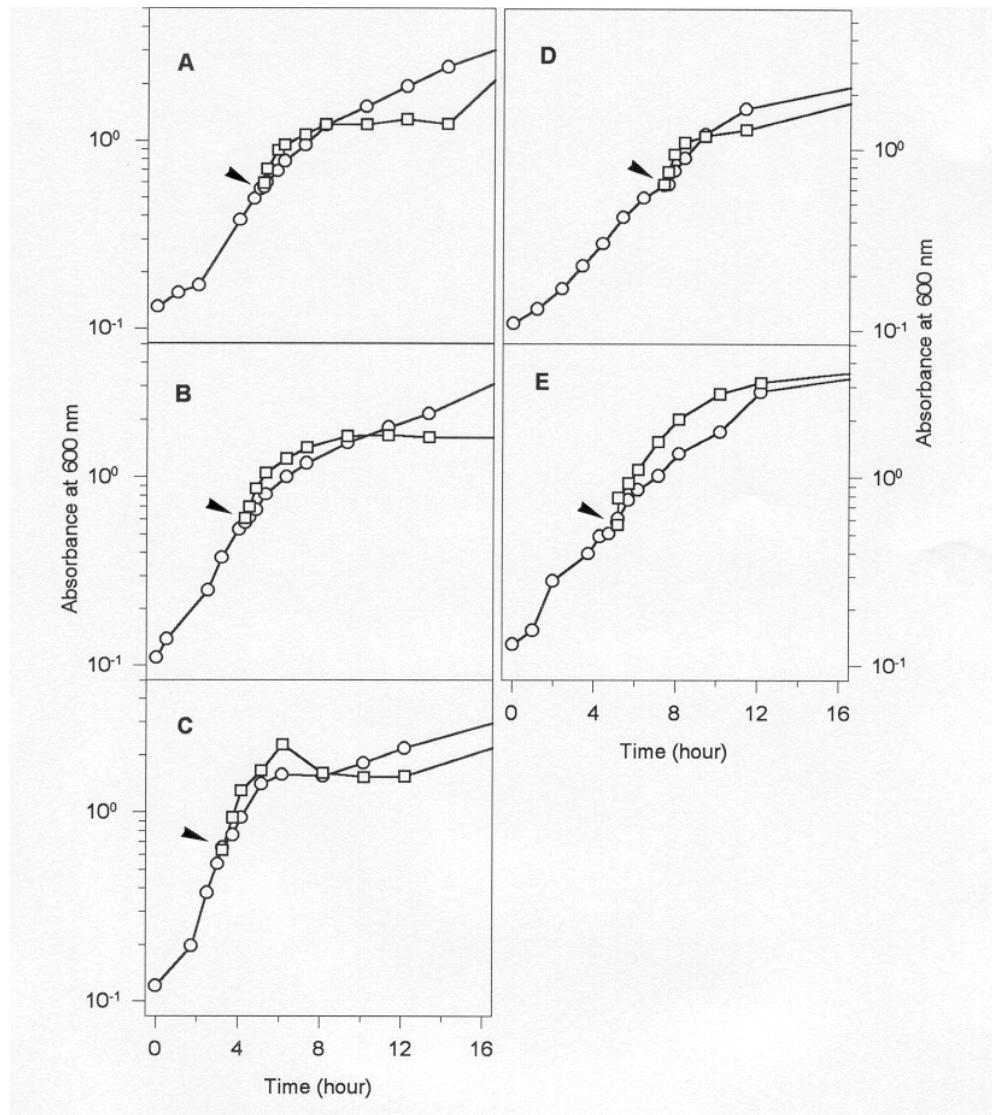


Figure 4.1. Effect of temperature upshift on growth rates of *St. maltophilia* (A), *Ps. fluorescens* (B), *Ps. aeruginosa* (C) and strain VUN10,077 strain (D and E). Strains were cultured in LB broth, except for strain VUN10,077 (E), which was grown in BHI broth, either at 28°C throughout the growth cycle (○) or heat shock (rapid transfer to 37°C) was applied at mid-exponential phase (arrow), then growth continued at 37°C (□).

broth (Figure 4.1 E), but a similar pattern of absorbance readings was observed in LB broth to other tested strains when cultured in LB broth (Figure 4.1 D) (Yuriev *et al.*, 1997). These results indicated that when cells were subjected to heat shock conditions (28°C to 37°C), the metabolic activity was accelerated in all tested strains and this depended on the given growth condition used for strain VUN10,077.

The kinetics of protein synthesis following heat stress was examined by SDS-PAGE analysis initially when cells were cultured in LB broth. The total soluble proteins were extracted by sonification from samples taken throughout the growth cycle and resolved using 1-D PAGE for all tested strains and 2-D PAGE for *St. maltophilia* (this strain was chosen at the beginning of this thesis for detailed study but emphasis later shifted to strain VUN10,077). Figure 4.2 shows protein profiles: the major heat-induced protein for the pseudomonads and related species had a molecular weight in the range of ~60 kDa, where elevated synthesis was first detected within 10 minutes of transferring to the higher temperature. This protein remained a major cellular component throughout the growth cycle in this type of analysis. The DnaK gene product equivalent was not prominent when the gels were visualized by Coomassie blue staining. These results indicated that the heat stress genes were strongly induced in these pseudomonads and related species, and that the extent of induction was strain dependent.

Each species showed synthesis of a range of other proteins in response to heat shock, where the nature of these proteins varied between species (Figure 4.2). For example, *Ps. aeruginosa* showed elevated synthesis of a 58 kDa protein, although Allan *et al.* (1988) reported induction of a protein of a slightly different size. Strain VUN10,077 showed elevated synthesis, relative to control cultures, for proteins with the following MW (kDa): 70, 58, 47, 29, 27, 20, 19 and 15. The 70 kDa, 58 kDa and 15 kDa proteins were increased relatively throughout sampling and remained as the major proteins when stressed by heat. The 29 kDa and 27 kDa proteins were induced at relatively high levels within 10 minutes exposure to high temperature and then declined upon ongoing stress, whereas the 20 kDa and 19 kDa proteins were induced after 2 hours stress.

The 58 kDa protein was likely to be a homologue to GroEL, Hsp60. The size of GroEL can differ between species, for example, *E. coli* synthesizes a heat-inducible 60 kDa protein, termed GroEL; *Ps. aeruginosa* showed a 61 kDa protein (GroEL) (Allan *et al.*,

Figure 4.2. The protein profiles form sonically disrupted cells of all tested strains grown in LB broth at 28°C throughout growth or heat-shocked at 37°C during mid-exponential growth and resolved using 10-15% gradient SDS-PAGE. The cultures were sampled at the times indicated and the protein markers (LMW, Bio-Rad) were 97.4, 66.6, 45, 31, 21.5 and 14.4 kDa (from top to bottom). The arrows show the position of protein of ~60kDa induced by heat shock or other major induced proteins.

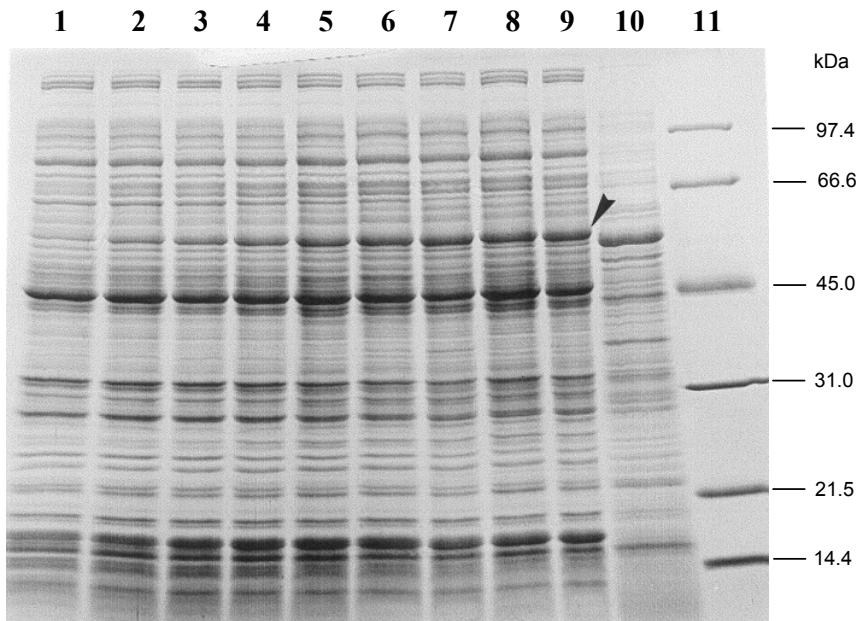
Panel A and B for *Ps. aeruginosa*.

Panel C and D for *Ps. fluorescens*.

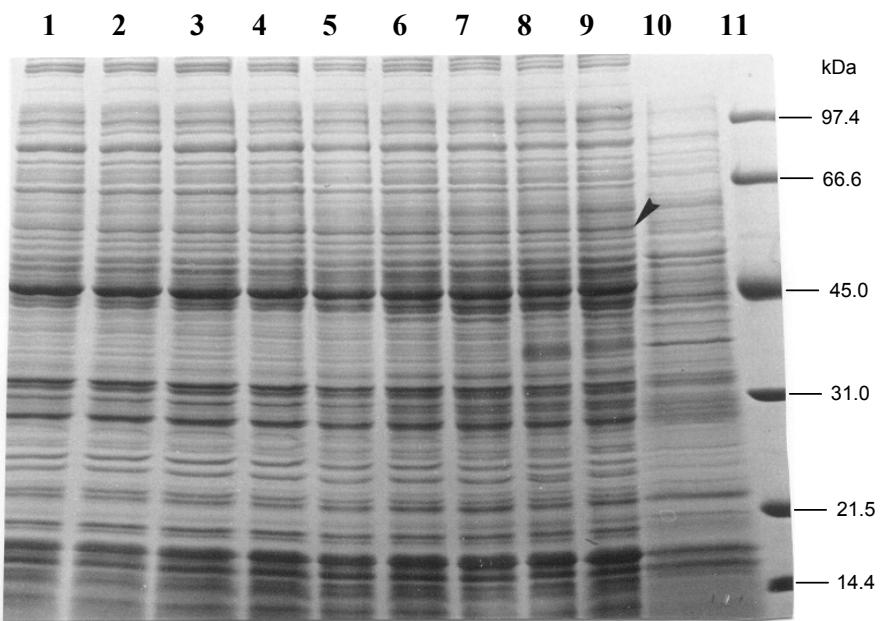
Panel E and F for *St. maltophilia*.

Panel G for strain VUN10,077.

A. Heat-stressed

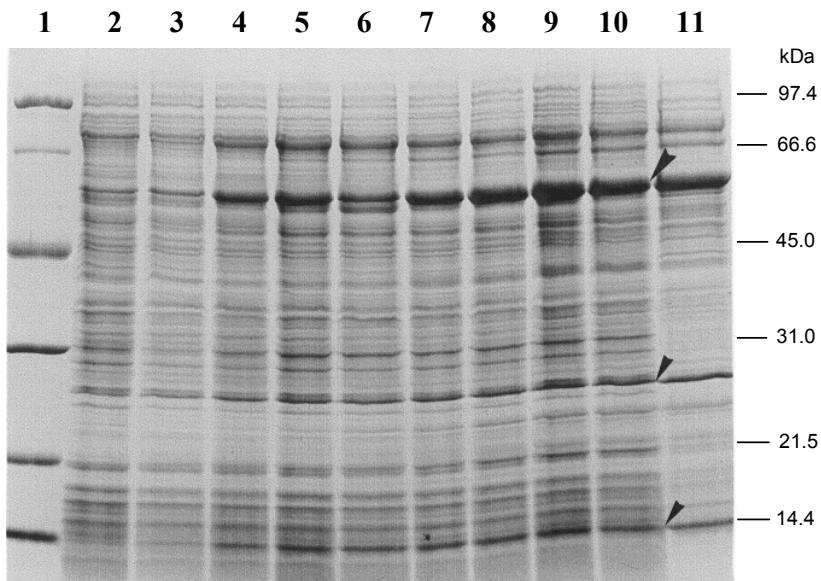


B. Control

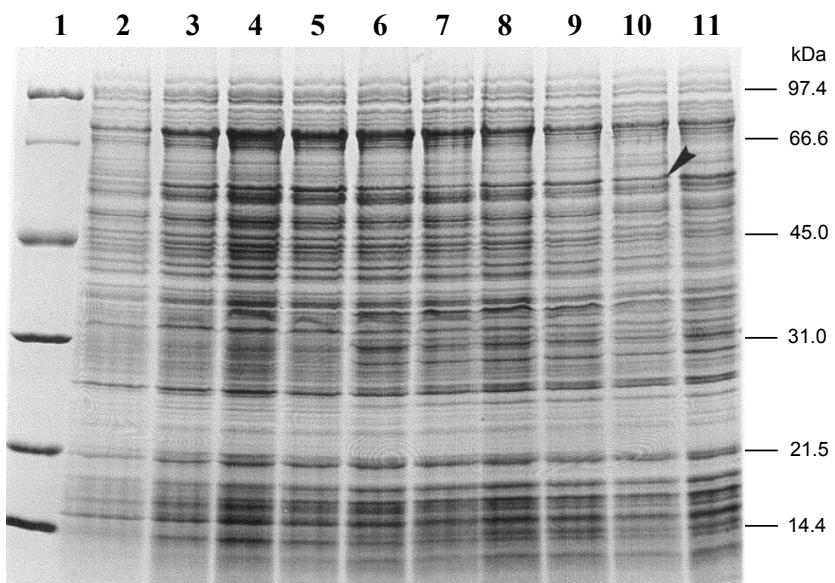


For panel A and B: Lane 1, time 0; lane 2, 10 minutes; lane 3, 30 minutes; lane 4, 1 hour; lane 5, 2 hours; lane 6, 3 hours; lane 7, 5 hours; lane 8, 7 hours; lane 9, 9 hours; lane 10, overnight and lane 11, markers.

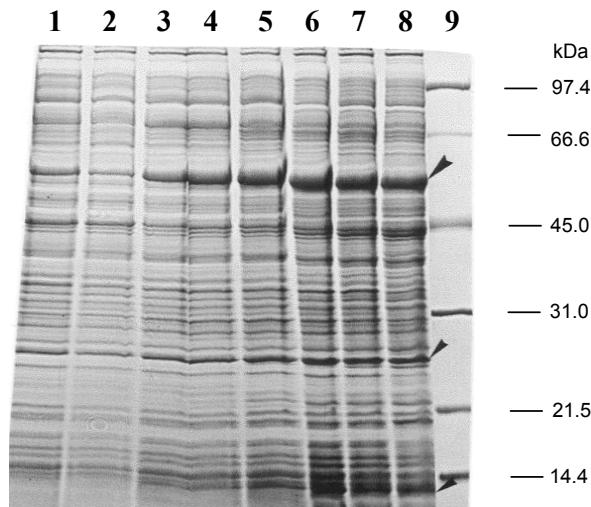
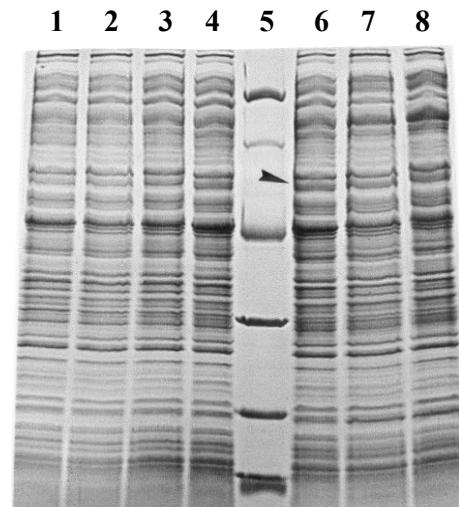
C. Heat-stressed



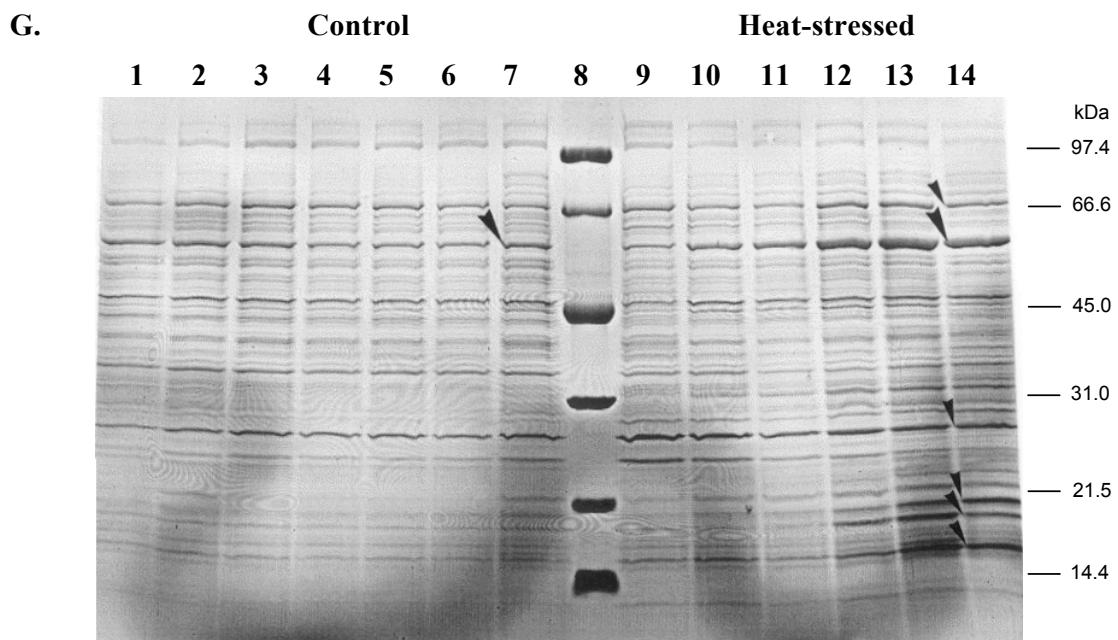
D. Control



For panel C and D: Lane 1, markers; lane 2, time 0; lane 3, 10 minutes; lane 4, 30 minutes; lane 5, 1 hour; lane 6, 2 hours; lane 7, 3 hours; lane 8, 5 hours; lane 9, 7 hours; lane 10, 9 hours and lane 11, overnight.

E. Heat-stressed**F. Control**

For panel D: Lane 1, time 0; lane 2, 10 minutes; lane 3, 30 minutes; lane 4, 1 hour; lane 5, 2 hours; lane 6, 4 hours; lane 7, 6 hours; lane 8, overnight and lane 9, markers. For panel F: Lane 1, time 0; lane 2, 10 minutes; lane 3, 30 minutes; lane 4, 1 hour; lane 5, markers; lane 6, 2 hours; lane 7, 4 hours and lane 8, 6 hours.

G.

For panel G: Lane 1, 10 minutes; lane 2, 30 minutes; lane 3, 1 hour; lane 4, 2 hours; lane 5, 4 hours; lane 6, 6 hours; lane 7, overnight for control samples (lanes 1-7), and lane 8, markers; lane 9, 10 minutes; lane 10, 30 minutes; lane 11, 1 hour; lane 12, 2 hours; lane 13, 4 hours; lane 14, 6 hours for heat-stressed samples (lanes 9-15).

1988), *Streptomyces albus* showed a 56- to 58-kDa (GroEL) protein following heat treatment (31 to 40°C) (Guglielmi *et al.*, 1991), and a 65 kDa protein from *Neisseria meningitidis* was similar to the *E. coli* GroEL (Arakere *et al.*, 1993). The 15 kDa protein may correspond to the equivalent of GroES in *E. coli*, where the synthesis level increased following temperature upshift of strain VUN10,077 and its kinetics paralleled synthesis of GroEL, Hsp60, as seen in *E. coli* (Chuang and Blattner, 1993). A similar response was noted for *Ps. aeruginosa* for a 16 kDa protein, as reported by Allan *et al.* (1988) previously.

For *Ps. fluorescens*, proteins of MW 75.5, 58, 27, 21 and 14 kDa showed elevated synthesis in addition to a 65 kDa protein. The 65 kDa protein was not observed until 1 hour after induction, and the 75.5 kDa and 21 kDa proteins were produced in relatively lesser amounts compared with the major heat-induced 58 kDa and 14 kDa proteins throughout the growth cycle, and the 27 kDa protein showed a similar response. The production of the 75 kDa protein peaked then declined during the course of growth for both the heat-shocked and untreated cultures.

For *St. maltophilia*, proteins of MW 56, 27, 21, 14.9, 14, and 13.5 kDa were induced. The 56 kDa, 27 kDa, 14 kDa and 13.5 kDa proteins were relatively increased upon ongoing heat treatment and the 14.9 kDa protein was produced during the later phases of growth. Two heat-induced proteins (MW 27 kDa and 14 kDa) were similar to ones seen for *Ps. fluorescens*.

Alternatively, BHIA plates were subjected to heat stress, which was achieved by transferring the plates from 28°C to 37°C for *St. maltophilia* and strain VUN10,077. The cells were spread onto the surface from liquid cultures at mid-exponential phase and plates were incubated for 8 hours before temperature upshift. Samples were taken at 8 and 12 hours after temperature upshift for tests and controls. The cells were collected using a scraper, adjusting the sample concentration to the equivalent of an absorbance reading (A_{600}) of 1.0 and cells were sonically disrupted. Proteins of MW 58 kDa and 90 kDa were observed for *St. maltophilia* and strain VUN10,077 from heat stressed plate cultures.

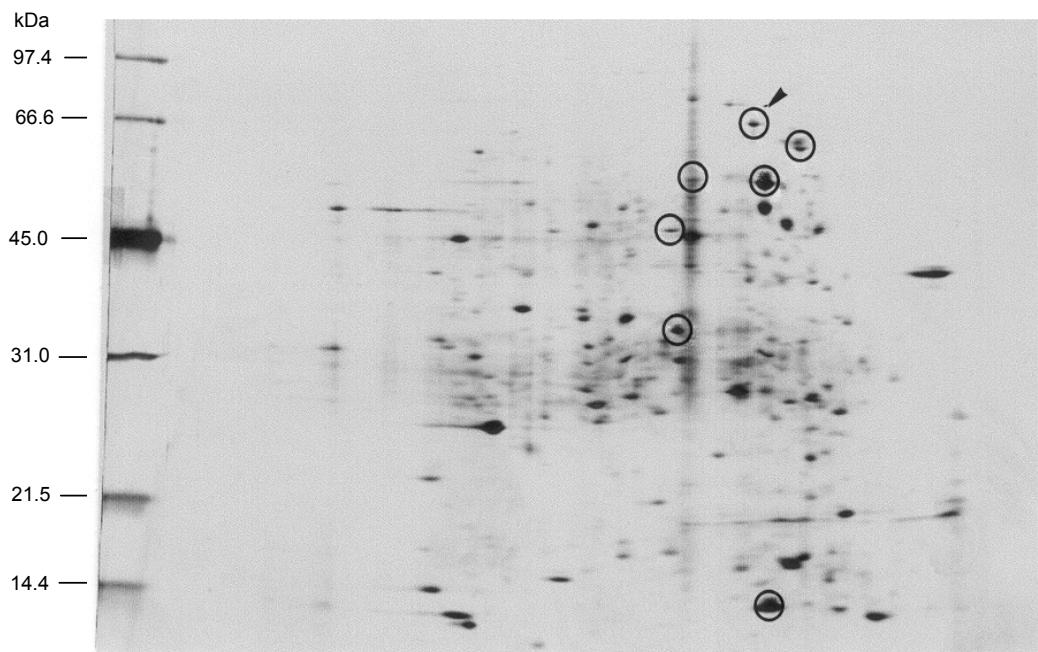
It was interesting that *Ps. aeruginosa* produced relatively high levels of the 58 kDa protein only, as detected using Coomassie stained gels, whereas Allan *et al.* (1988) reported synthesis of 5 major proteins (86.4, 76.0, 70.4, 60.6 and 15.7 kDa in molecular weight) and other pseudomonads were also reported to produce several new proteins (Guglielmi *et al.*, 1991;

Blom *et al.*, 1992; Lupi *et al.*, 1995). There is a difference in the experimented approaches used in that Allan *et al.* (1988) used a temperature upshift from 30°C to 45°C, whereas the *Ps. aeruginosa* used here was transferred from 28°C to 37°C. This may indicated that the protein profile difference depended on the degree of heat stress used for *Ps. aeruginosa* or that there was strain variation.

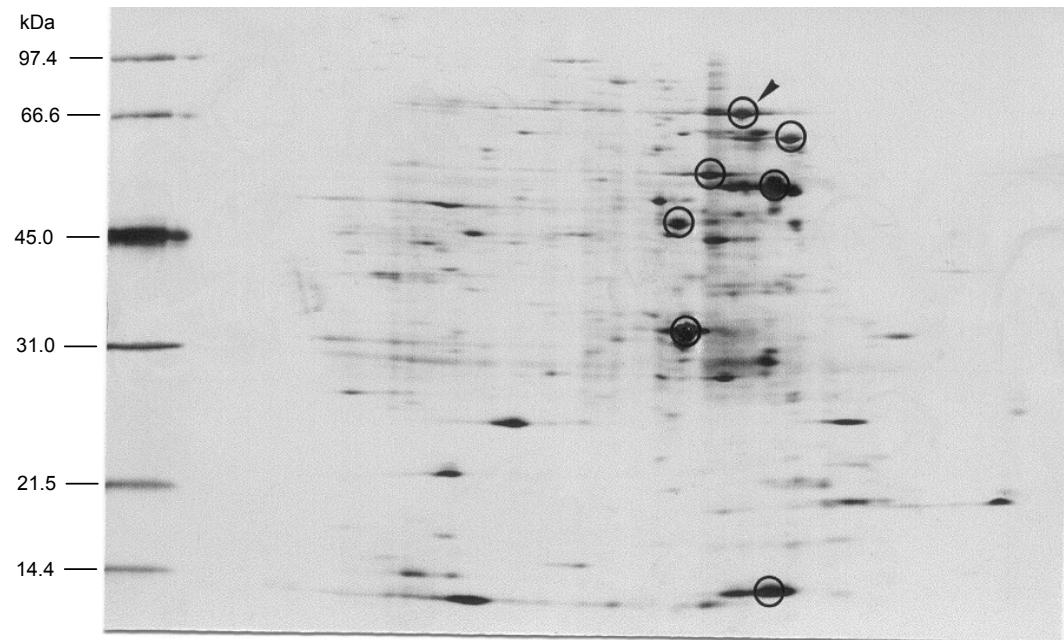
Samples taken throughout the growth cycle of *St. maltophilia* with and without heat stress were also examined on 2-D gels, using cell free extracts prepared by sonic disruption. Cells were taken at 37°C for *St. maltophilia* culture (LB) after 2 and 9 hours heat-stress and analysed on 2-D gels using pH 4 to 7 immobiline strips. Extracts from cells showed dramatic changes in protein profiles after 2 hours heat-stress, with the synthesis of more than approximately 35 discrete intracellular proteins detectable and, of these, seven major heat-induced proteins were circled (Figure 4.3). Spots corresponding to the GroEL proteins are indicated by arrow, and this was identified later using similar 2-D gel electrophoresis conditions used here for *St. maltophilia* by O. Yuriev (personal communication). During the 9 hours heat stress, the synthesis of a majority of cytoplasmic proteins decreased, whereas 13 proteins were expressed that mainly had a low molecular weight (below 21 kDa), as seen when comparing the 2 and 9 hour samples (Figure 4.3 B and C). Heat-induced protein profiles resolving on 2-D gels were performed independently using immobiline strips of pH 3 to 10.5: almost identical protein profiles were observed at corresponding pI regions and no distinct newly synthesized spots were detected below pH 4 and beyond pH 7 compare to strips of pH 4 to 7 (data not shown). Silver staining was sufficient to detect the synthesis of several new proteins following heat stress of *St. maltophilia*. Alternative approaches have used autoradiography to detect newly synthesized proteins. However, Graumann *et al.* (1996) showed that some proteins of *B. subtilis* were not visible using autoradiography as they did not contain methionine, whereas Coomassie blue stain was able to demonstrate newly synthesized proteins. Lupi *et al.* (1995) similarly reported that newly synthesized protein following chemical stress by 2-chlorophenol in *Ps. putida* KT2442 could be readily resolved by Silver staining, so that using labelled amino acid substrates was not necessary for these experimental purposes. As for the results described earlier above (see Figure 4.2), Coomassie stained 1-D SDS PAGE gels were able to discriminate the synthesis or accumulation of the major groups of new proteins synthesised upon heat stress, so that protein profiles were examined using 1-D. Coomassie blue staining following exposure to

Figure 4.3. Silver-stained 2-D PAGE from 10 to 15% gradient gels and protein extracts from sonically disrupted cells of *St. maltophilia* culture, LB grown at 28°C, and after heat-shock at mid-exponential phase. The samples were taken at 2 hours (B) and 9 hours (C) after transfer to 37°C and from controls kept at 28°C (A) in a parallel culture 2 hours after parallel cultures were heat stressed. All proteins are shown with isoelectric points in the range of pH 4 to 7 and with the same protein concentration, 40 µg/ml, loaded. The markers (LMW, Bio-Rad) were 97.4, 66.6, 45, 31, 21.5 and 14.4 kDa. Circled spots of selected proteins are shown on each gel and the presumptive GroEL equivalent is arrowed.

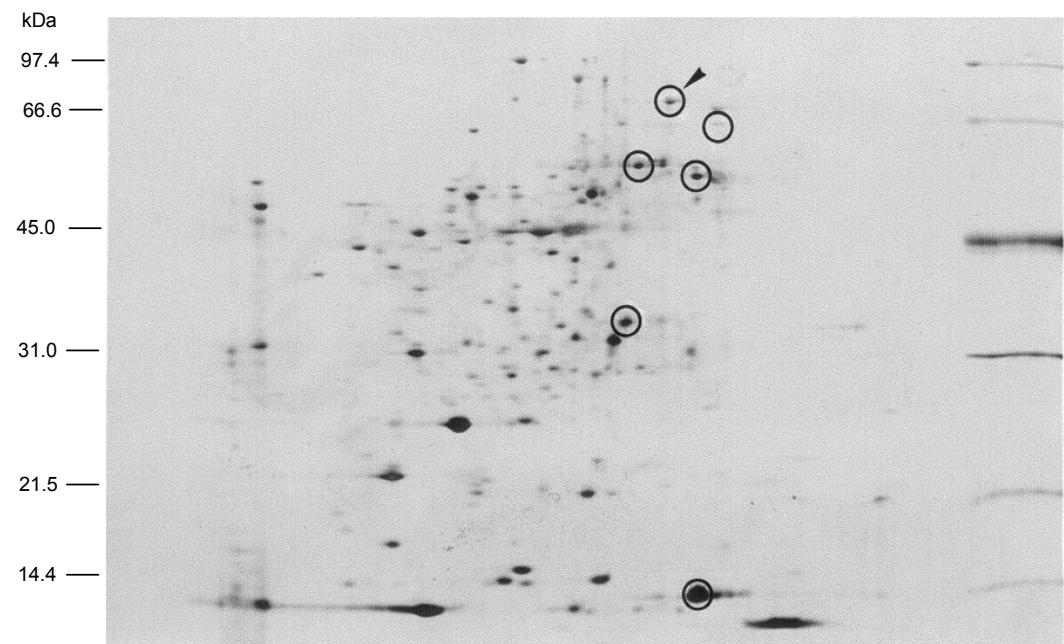
A. Control (2 hours)



B. Heat-stressed (2 hours)



C. Heat-stressed (9 hours)



various stress conditions, prior to using analysis by ^{35}S -methionine pulse labelling, in the following section

4.2.2 Induction of stress responses following exposure to chemicals

4.2.2.1 Protein profiles generated by different cell disruption methods

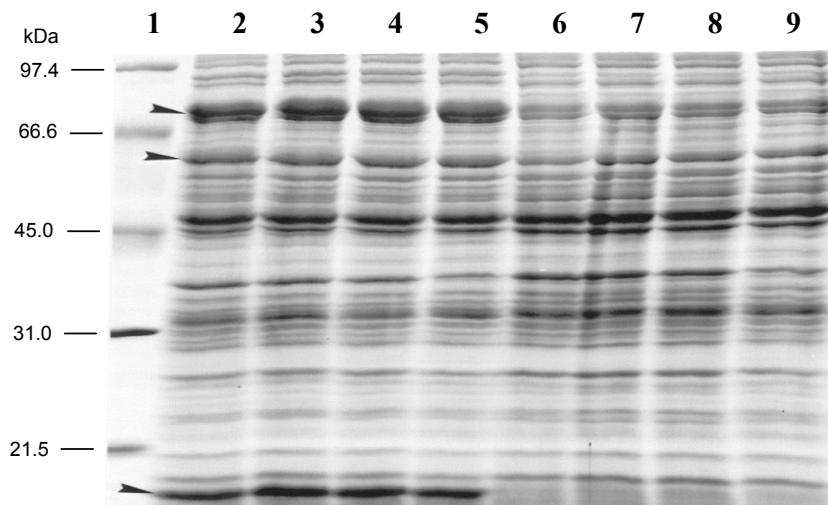
In the experiments reported in section 4.2.1, cells were disrupted by sonification and cell debris removed by centrifugation, giving the protein profile of the cytoplasmic rather than whole cellular proteins. Subsequently, strain VUN10,077 was subjected to heat stress or exposure to heavy metal ions to evaluate changes in the protein profiles. Growth inhibition tests were performed with chemicals added at early exponential growth phase (A_{600} between 0.2 and 0.3) for the following chemicals: heavy metal ions (cadmium chloride, cupric chloride, cobaltous chloride, ferrous chloride, manganese chloride, mercuric chloride, zinc chloride, strontium chloride, nickel chloride, sodium arsenite and lead nitrate), SDS, and chlorinated phenolic compounds dissolved in dimethyl formamide (DMF) (phenol, 2,6-dichlorophenol, 2,4,5-trichlorophenol, 4-chlorophenol, penta-chlorophenol and δ -nitrophenol). The above selected chemicals had impacts on the growth and inhibition was seen for absorbance readings, except for strontium and DMF. All of these selected chemicals were added to growing cells of strain VUN10,077, and protein profiles were investigated using 1-D SDS PAGE for samples prepared by boiling in SDS-PAGE loading buffer. Samples were taken periodically at 30 minutes and 2 hours after stress and gels were stained with Coomassie brilliant blue. However, this type of boiling lysis method was not efficient for strain VUN10,077 chemical stresses. Despite the broad range of chemicals tested, induction of the universal major HSPs was not visualized in this type of analysis. This may have been due to insufficient lysis of cells following boiling in a water-bath for the time used. The following experiments were therefore carried out to determine which cell disruption method was more effective for strain VUN10,077.

The boiling method and sonification were used by other workers to disrupt pseudomonad cells following exposure to stress conditions (Allan *et al.*, 1988; Jensen *et al.*, 1993). Also, these methods were assessed in context of dealing with numerous samples for analyzing protein profiles. Analysis of total proteins without centrifugation may also have identified

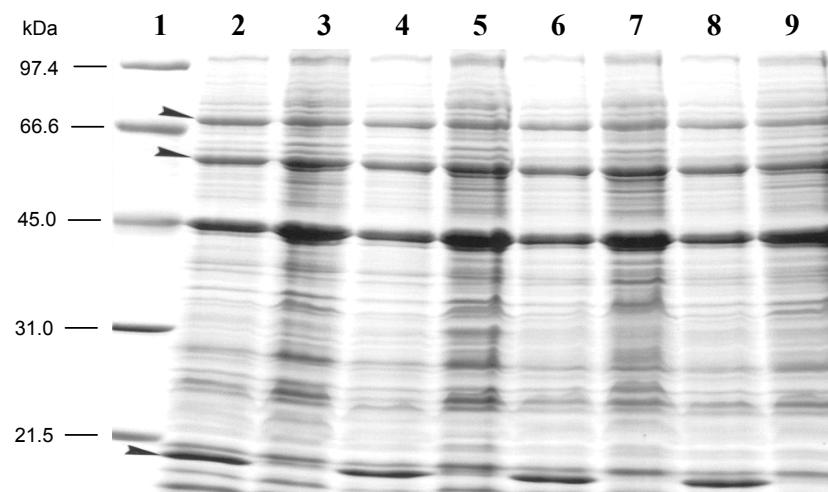
Figure 4.4. Evaluation of boiling in SDS-PAGE loading buffer to achieve cell lysis. Samples were taken from BHI cultures of *Ps. fluorescens* (A) or strain VUN10,077 (B) after 2 hours heat stress (28 to 37°C) at mid-exponential growth and control samples were taken at the same time. Cells were concentrated to a constant A_{600} equivalent of 1.0, resuspended in loading buffer and boiled for different periods up to 20 minutes, prior to analysis on 12% SDS-PAGE gels then visualised by Coomassie blue staining. The molecular weight markers (Bio-Rad) were 97.4, 66.6, 45, 31, 21.5 and 14.4 kDa (top to bottom).

Panel A: lane 1, markers; lanes 2-5, stressed and boiled for 5, 10, 15 and 20 minutes; lanes 6-9, unstressed and boiled for 5, 10, 15, and 20 minutes. Panel B: lane 1, markers; lane 2, 4, 6 and 8, stressed and boiled for 5, 10, 15 and 20 minutes; lane, 3, 5, 7 and 9, unstressed and boiled for 5, 10, 15 and 20 minutes.

A.



B.



the presence of membrane-associated HSPs as well as cytoplasmic HSPs. The efficiency of boiling cells in SDS-PAGE loading buffer to achieve cell lysis was evaluated using two strains: *Ps. fluorescens* and strain VUN10,077 (Figure 4.4). The cellular proteins seen differed from those seen for both strains when cells were disrupted by sonification followed by centrifugation, indicating that some membrane-associated proteins may have been lost using the former method. The protein profiles were similar for boiling times up to 20 minutes. In particular, it was interesting to note that heat stress caused relative increases in a 75.5 and 75 kDa protein in *Ps. fluorescens*, which was not apparent when using sonic disruption. Similar responses were noted for *Ps. aeruginosa*. These results indicated that this protein may be associated with membranes in this species. In parallel, GroEL/GroES-like proteins were also apparent here. A 72 kDa protein was also now visible in strain VUN10,077 but its relative levels did not increase during heat stress under the experimental conditions used (2 hours at 37°C). The GroES-like, 19 kDa protein, response was still apparent in strain VUN10,077, although the increase in GroEL-like was not as apparent as seen following sonification. Therefore, the altered method was used for the following sections unless otherwise stated. Although Allan *et al.* (1988) boiled *Ps. aeruginosa* cells for 5 minutes, 10 minutes was chosen for disrupting cells by boiling on a flame to prevent possible insufficient lysis for strain VUN10,077.

4.2.2.2 Kinetics of protein synthesis following addition of selected chemicals

Cadmium chloride, nickel chloride, mercuric chloride, cupric chloride, sodium arsenite and phenol were selected for detailed study and these were added to mid-exponential phase cultures (A_{600} 0.5-0.6), rather than early exponential phase, of strain VUN10,077. Following addition of different concentrations of the chemicals, samples were removed periodically from the BHI broth cultures for viable counts on BHIA and for analysis of protein by 1-D SDS-PAGE, preparing cells by boiling in gel loading buffer for 10 minutes and loading equal cell amounts in each lane. Control cultures (no addition of chemicals) were treated similarly.

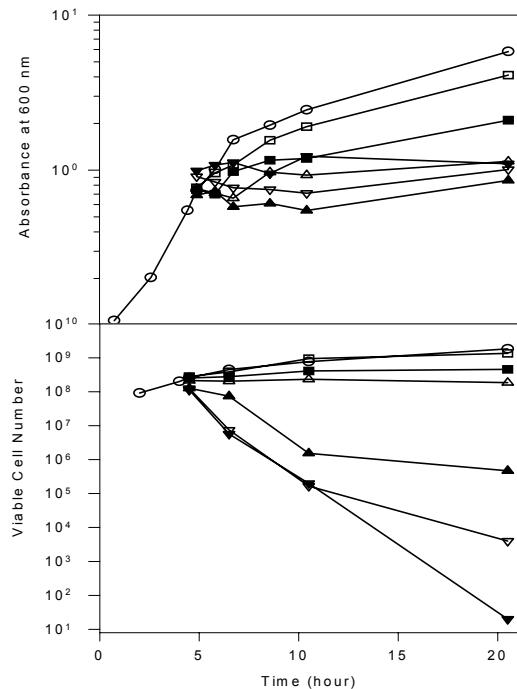
4.2.2.2.1 Cadmium and copper stresses

Cadmium and copper stresses showed significant impacts on the viability when strain VUN10,077 was exposed to specific concentrations of these. Cadmium chloride was added at concentrations of 0.5 to 5.0 gL⁻¹ (equal to 2.73 to 27.28 mM Cd²⁺) (Figure 4.5). Significant

growth inhibition was seen at concentrations above 1.0 gL^{-1} (5.46 mM) from absorbance readings and viable counts declined significantly after addition of 3.0 gL^{-1} (16.37 mM). The impact of addition of Cd^{2+} on protein profiles was determined after adding different concentrations of CdCl_2 to BHI cultures of strain VUN10,077 by analysing cellular proteins after 30 minutes and 2 hours stress, and cadmium induced proteins were of MW 58, 45, 38 and 20 kDa. A major stress-induced protein of 58 kDa was seen in control samples and was induced at the tested concentration range, and the relative portion of this increased following cadmium addition when samples were analysed after 30 minutes cadmium stress. A DnaK-like protein was not visible. Yuriev *et al.* (1997) had shown previously that a 60 kDa GroEL-like protein was also induced by cadmium in *St. maltophilia*, which also showed a dose-related response to increasing cadmium concentrations. Cadmium stress caused another significant protein response, a 45 kDa protein possibly a membrane-associated protein which appeared at concentrations over 1.0 gL^{-1} (5.46 mM) after 30 minutes exposure. The amount decreased after 2 hours exposure, through cells remained viable. At higher concentrations of cadmium, this protein was still seen in samples after 2 hours but cell viability had declined before this point, limiting cellular protein turnover. A minor stress-induced protein also detected that was the 20 kDa protein seen after 2 hours cadmium stress and similarly the 38 kDa protein was induced at concentrations of 3.0 gL^{-1} and 4.0 gL^{-1} cadmium, which was a dose-related response. *St. maltophilia* also showed a dose-related response for a 45 kDa protein when exposed to cadmium (Yuriev *et al.*, 1997).

Copper was added as $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ at concentrations of 0.5 to 2.6 gL^{-1} (1.09 to 5.69 mM Cu ion) (Figure 4.6). A_{600} was transiently increased at the tested concentrations, which was similar to the heat shock response after stress in terms of absorbance readings, and showed a constant level after entering stationary phase following copper stress. The cells were significantly inhibited in terms of viable cell number above 2.0 gL^{-1} copper concentrations, while A_{600} readings were not decreased. Copper stress produced proteins of MW 70 and 20 kDa. At 0.5 and 1.0 gL^{-1} $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ inhibited growth but did not cause decreases in cell number and showed relatively increased levels of a 70 kDa protein while the other proteins were not. The 70 kDa and 20 kDa proteins were produced at concentrations of 0.5 gL^{-1} and 1.5 gL^{-1} after 2 hours stress, and the 20 kDa protein was relatively increased at concentrations of up to 1.5 gL^{-1} but the 70 kDa protein was not seen at concentration of 1.5 gL^{-1} . However, at higher concentrations, cell viability declined significantly although analysis of protein at 30 minutes and 2 hours showed no significant change in protein profiles

Figure 4.5. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins from cells disrupted by boiling for strain VUN10,077 following addition of different concentrations of cadmium to mid-exponential BHI cultures at 28°C. Samples were taken periodically and absorbance at 600 nm was measured spectrophotometrically and viable counts performed on BHIA plates. Cadmium concentrations used were 0.5 gL⁻¹ (□), 1.0 gL⁻¹ (■), 2.0 gL⁻¹ (△), 3.0 gL⁻¹ (▲), 4.0 gL⁻¹ (▽), 5.0 gL⁻¹ (▼) and control (○).



Samples were taken at 30 minutes for lanes 1-7 and 2 hours for lanes 9-15 after addition of CdCl₂ at concentrations of 0.5 gL⁻¹ (lanes 1 and 9), 1.0 gL⁻¹ (lanes 2 and 10), 2.0 gL⁻¹ (lanes 3 and 11), 3.0 gL⁻¹ (lanes 4 and 12), 4.0 gL⁻¹ (lanes 5 and 13) and 5.0 gL⁻¹ (lanes 6 and 14) and control (lanes 7 and 15). Arrows mark induced proteins.

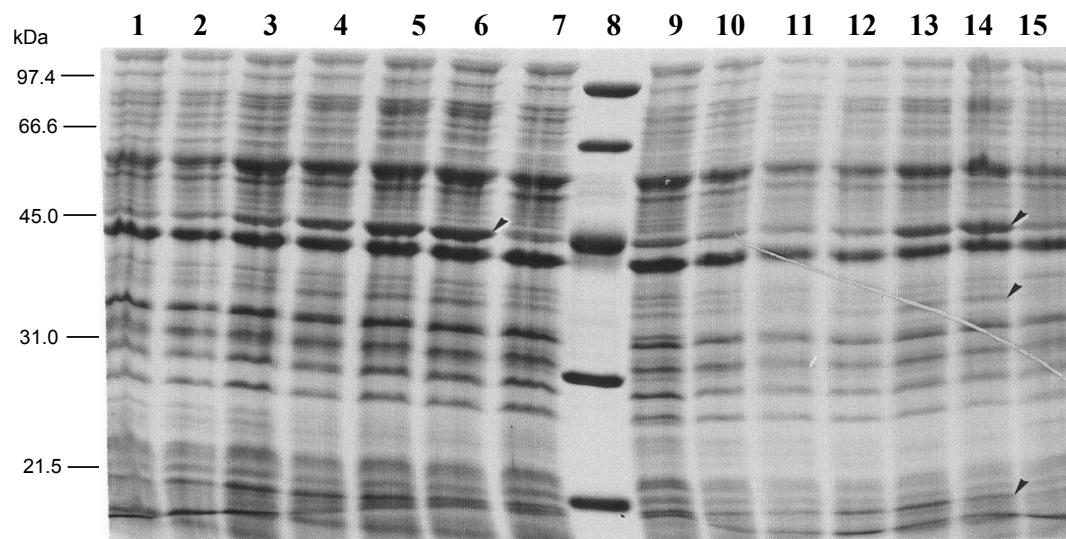
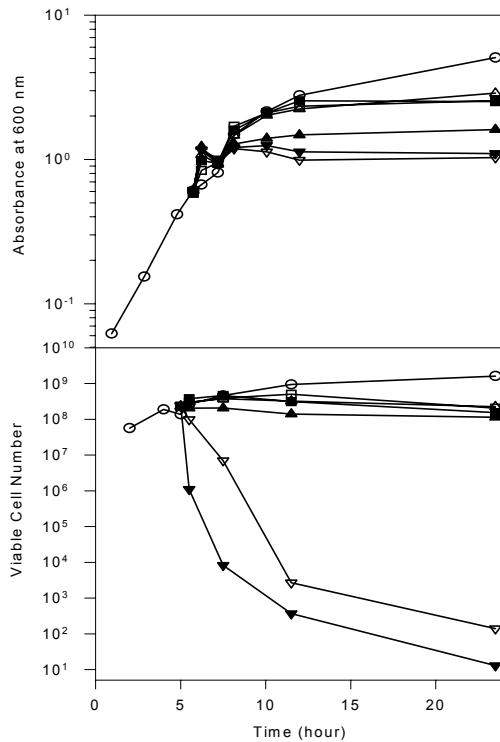
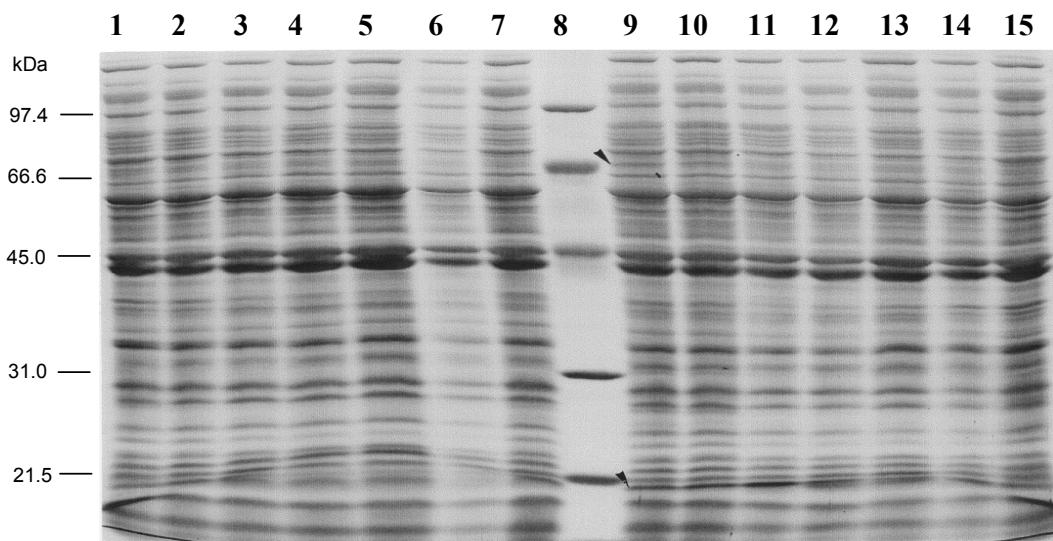


Figure 4.6. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins for strain of VUN10,077 following addition of cupric chloride, where conditions were the same as Figure 4.5. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ concentrations used were 0.5 gL^{-1} (\square), 1.0 gL^{-1} (\blacksquare), 1.5 gL^{-1} (\triangle), 2.0 gL^{-1} (\blacktriangle), 2.3 gL^{-1} (∇), 2.6 gL^{-1} (\blacktriangledown) and control (\circ).



Samples were taken at 30 minutes for lanes 1-7 and 2 hours for lanes 9-15 after addition of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ at concentrations of control (lanes 7 and 15), 0.5 gL^{-1} (lanes 1 and 9), 1.0 gL^{-1} (lanes 2 and 10), 1.5 gL^{-1} (lanes 3 and 11), 2.0 gL^{-1} (lanes 4 and 12), 2.3 gL^{-1} (lanes 5 and 13) and 2.6 gL^{-1} (lanes 6 and 14).



relative to the control samples grown similarly. These results indicated that when cells of VUN10,077 were exposed to sublethal concentrations of cadmium ion (≤ 10.9 mM), stress-induced proteins of 45 and 58 kDa were induced whereas sublethal concentrations of copper ion (≤ 4.4 mM) did not cause a similar response.

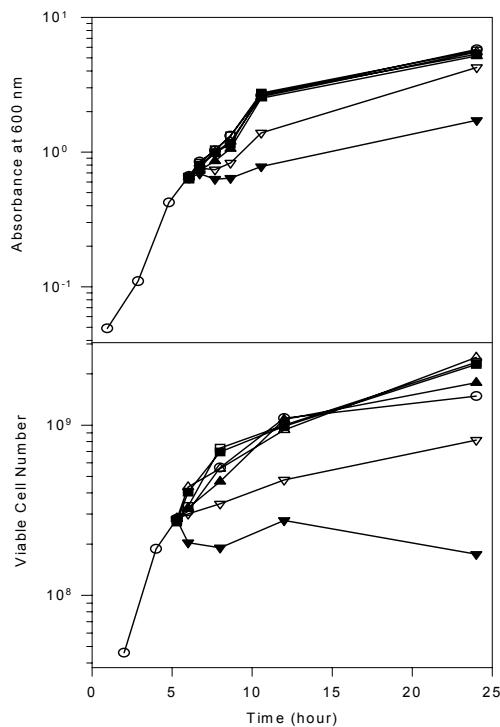
4.2.2.2.2 Nickel and cobalt stresses

Nickel stress showed a correlation between absorbance readings and cell viability in the tested concentration range from 0.1 gL^{-1} to 2.5 gL^{-1} (0.1 mM to 2.6 mM, nickel ion) as $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ (Figure 4.7). Nickel stress produced proteins of MW 75, 20, 16, 15, and 14 kDa. When nickel was added into growing BHI cultures at mid-exponential phase, A_{600} was similar to controls below nickel concentrations of 1.5 gL^{-1} , and viable cell number and A_{600} were inhibited at concentrations of 2.0 gL^{-1} and 2.5 gL^{-1} which was a dose related response. When viability declined at 2.5 gL^{-1} , protein profiles showed no changes, which was consistent with loss of viability at this concentration. The amount of 70 kDa protein declined following increasing concentrations and the low molecular weight proteins (in the size range below 20 kDa) increased to relatively similar levels for cells grown in nickel chloride at concentrations up to 2.0 gL^{-1} .

Cobalt stress showed a similar correlation between A_{600} and viable counts as seen for nickel stress for concentration of 0.5 gL^{-1} to 4.0 gL^{-1} (0.52 mM to 4.17 mM, cobaltous ion) as $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ (Figure 4.8). Viable number and A_{600} showed similar profiles as controls at concentrations of 0.5 gL^{-1} , while inhibition of growth was seen at higher concentrations. It was interesting that viable numbers declined immediately after addition of over 1.0 gL^{-1} cobaltous chloride, in parallel to changes in A_{600} . The gel shown in Figure 4.8 showed few changes in protein profile, however, a 20 kDa protein was increased relatively in the tested concentrations.

These compounds had little impact on the protein profile at the tested concentrations (Figure 4.7 and 4.8). Following addition of growth-inhibitory but sublethal concentrations, protein profiles of strain VUN10,077 did not change the major HSPs throughout the growth cycle. The concentration range used may not have been suitable for this strain or the changes may have been too subtle to detect using Coomassie stained 1-D PAGE analysis or the medium used may have complexed the ions. Similar patterns were observed in *St.*

Figure 4.7. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins for strain of VUN10,077 following addition of nickel chloride, where conditions were the same as Figure 4.5. $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ concentrations used were 0.1 gL^{-1} (●), 0.5 gL^{-1} (■), 1.0 gL^{-1} (Δ), 1.5 gL^{-1} (\blacktriangle), 2.0 gL^{-1} (∇), 2.5 gL^{-1} (\blacktriangledown) and control (○).



Sample was taken at 30 minutes for lanes 1-7 and 2 hours for lanes 8-15 after addition of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ at concentrations of control (lanes 7 and 15), 0.1 gL^{-1} (lanes 1 and 9), 0.5 gL^{-1} (lanes 2 and 10), 1.0 gL^{-1} (lanes 3 and 11), 1.5 gL^{-1} (lanes 4 and 12), 2.0 gL^{-1} (lanes 5 and 13) and 2.5 gL^{-1} (lanes 6 and 14).

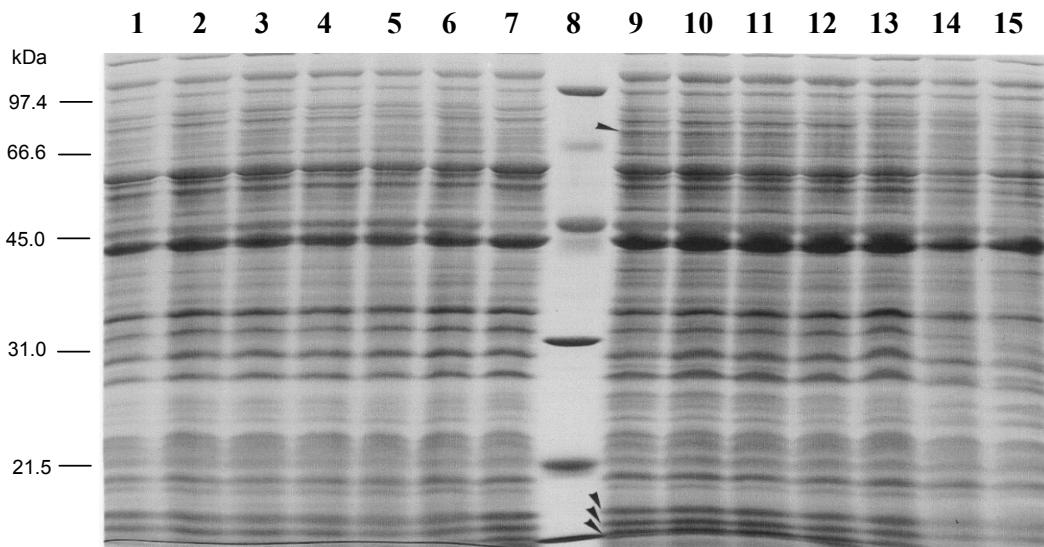
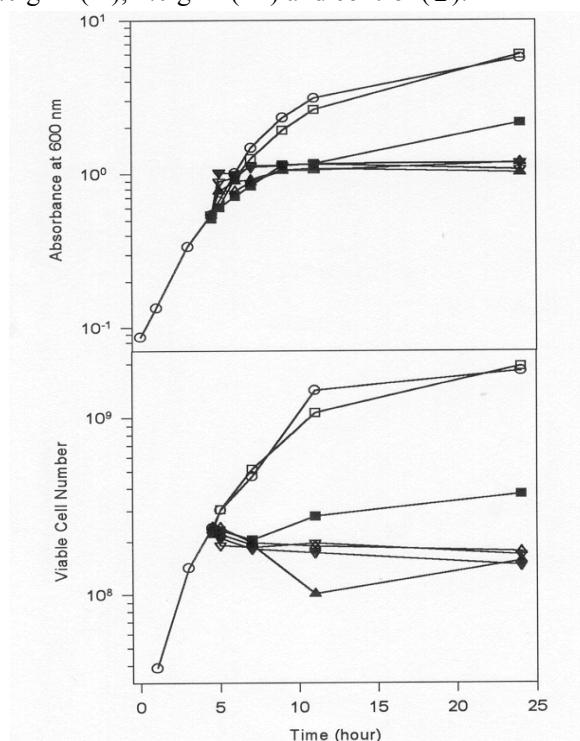
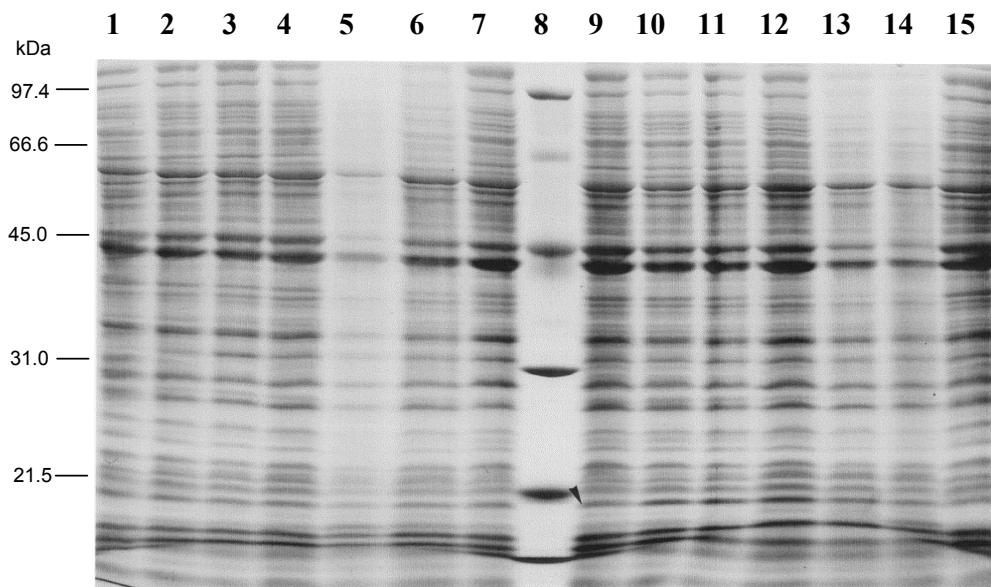


Figure 4.8. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins for strain of VUN10,077 following addition of cobaltous chloride, where conditions were the same as Figure 4.5. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ concentrations were used 0.5 gL^{-1} (\square), 1.0 gL^{-1} (\blacksquare), 1.5 gL^{-1} (\triangle), 2.0 gL^{-1} (\blacktriangle), 3.0 gL^{-1} (∇), 4.0 gL^{-1} (\blacktriangledown) and control (\circ).



Samples were taken at 30 minutes for lanes 1-7 and 2 hours for lanes 8-15 after addition of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ at concentrations of control (lanes 7 and 15), 0.5 gL^{-1} (lanes 1 and 9), 1.0 gL^{-1} (lanes 2 and 10), 1.5 gL^{-1} (lanes 3 and 11), 2.0 gL^{-1} (lanes 4 and 12), 3.0 gL^{-1} (lanes 5 and 13) and 4.0 gL^{-1} (lanes 6 and 14).

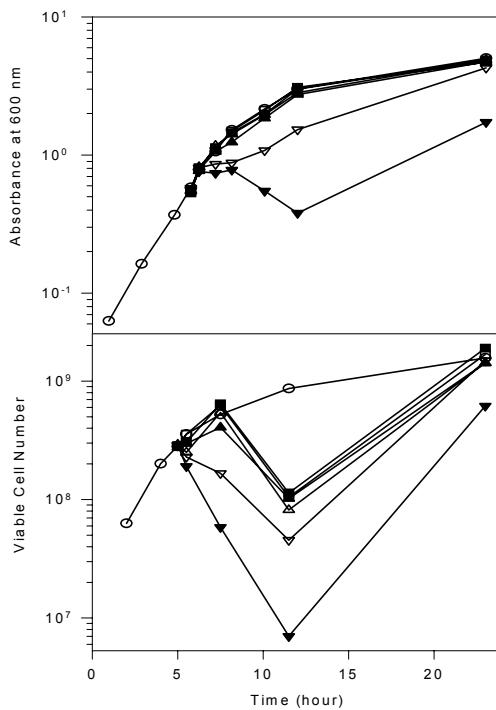


maltophilia previously (Yuriev *et al.*, 1997). These results indicated that induction of particular proteins presumed be case specific.

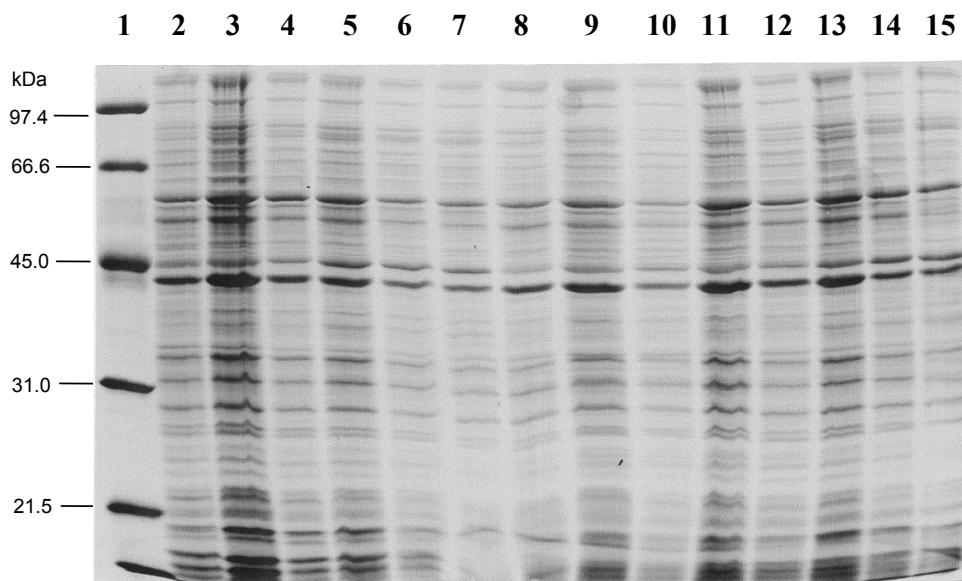
4.2.2.2.3 Mercury and SDS stresses

The production of stress-induced proteins was initially examined at concentrations of 0.05 to 3.0 gL⁻¹ HgCl₂ using early exponential phase. However, when strain VUN10,077 was cultured in concentrations above 0.5 gL⁻¹ HgCl₂, no increase in A₆₀₀ was seen and at higher concentrations caused significant decreases in A₆₀₀, presumably due to severe metabolic inhibition and cell death. It was also noted that at a concentration of 0.05 gL⁻¹ HgCl₂, cultures showed a sudden increase of absorbance readings in overnight samples which may have reflected a recovery of viability. Subsequently, HgCl₂ at concentrations of 0.001 gL⁻¹, 0.005 gL⁻¹, 0.01 gL⁻¹, 0.02 gL⁻¹, 0.05 gL⁻¹ and 0.1 gL⁻¹ (0.27 to 272 µM) were added to growing cultures of strain VUN10,077 at mid-exponential phase in BHI (Figure 4.9). Cell morphology was changed from the normal round rod-shape to a long-flat shape which coexisted with a flat rod-shape form, as observing under the light microscopy for cultures grown in the presence of 0.02 gL⁻¹ HgCl₂. The mercury ion apparently impacted on viable numbers during this stress, noting that the cells recovered viability on the culture period in concentrations of HgCl₂ up to 0.1 gL⁻¹, although A₆₀₀ readings did not follow this. Protein profiles were investigated using 1-D SDS-PAGE when cells were lysed by boiling on a flame. Unexpectedly, despite the changes in viability and later recovery seen during growth in the presence of HgCl₂, changes in protein profiles were not seen throughout the growth cycle (Figure 4.9). Also, in several trials the protein loading appeared to vary between lanes despite the calculated loadings being equal, which was not seen when the same method was used on cells exposed to other metals (see previous sections). Therefore, the lysis method was altered to the sand disruption method instead of boiling to lyse cells, to investigate if this approach would reveal different results. The concentration of HgCl₂ was selected at 0.005 gL⁻¹, 0.025 gL⁻¹ and 0.05 gL⁻¹ (13.6, 68.4 and 136.1 µM Hg ion), and a similar pattern of absorbance readings was observed for concentrations of 0.025 gL⁻¹ and 0.005 gL⁻¹ HgCl₂, with no significant changes in A₆₀₀ after stress. Subsequently, the concentrations of 0.005 gL⁻¹ and 0.025 gL⁻¹ mercury-stress cells were lysed using the sand disruption method and resolved on 1-D SDS-PAGE gels then stained with Coomassie blue staining. The level of stress-induced proteins after mercury exposure depended on the degree of the stress (Figure 4.10). An induction of proteins of 70, 60, 58, 46, 44, 33, 31, 29, 27, 22, 18 and 16 kDa was

Figure 4.9. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins for strain of VUN10,077 following addition of mercury chloride, where conditions were the same as Figure 4.5. HgCl_2 concentrations used were 0.001 gL^{-1} (□), 0.005 gL^{-1} (■), 0.01 gL^{-1} (Δ), 0.02 gL^{-1} (\blacktriangle), 0.05 gL^{-1} (∇), 0.1 gL^{-1} (\blacktriangledown) and control (○).



Samples were taken at 30 minutes for lanes 2-8 and 2 hours for lanes 9-15 after addition of HgCl_2 at concentration of control (lanes 2 and 9), 0.001 gL^{-1} (lanes 3 and 10), 0.005 gL^{-1} (lanes 4 and 11), 0.01 gL^{-1} (lanes 5 and 12), 0.02 gL^{-1} (lanes 6 and 13), 0.05 gL^{-1} (lanes 7 and 14) and 0.1 gL^{-1} (lanes 8 and 15).



seen in the presence of 0.025 gL^{-1} ($68.4 \mu\text{M}$). The 70 kDa protein (DnaK-like in size) was expressed at maximum levels after 2 hours exposure and this declined gradually as A_{600} , which was similar to changes seen in the 60 kDa and 16 kDa proteins. The 58 kDa protein (GroEL-like in size and induction amount) was induced in tests and controls, and became proportionally a major protein later in growth. A 46 kDa protein was almost not seen within 30 minutes after stress and the amount increased as viability recovered, whereas a 44 kDa protein was proportionally increased for up to 5 hours stress, similar to the 70 kDa protein. The 33 kDa, 29 kDa, 27 kDa, 22 kDa and 18 kDa proteins were relatively increased then declined, and the 31 kDa protein was induced, where the amount proportionally increased later in growth, similar to the 58 kDa protein. Strain VUN10,077 showed maximal stress response to mercury at 1 hour stress with $0.025 \text{ gL}^{-1} \text{ HgCl}_2$; most stress-responsive proteins had increased by this time and declined afterwards, and the 58 kDa and 31 kDa proteins were dominant after 5 hours stress as viability recovered, although the 46 kDa protein increased.

An induction of proteins of 70, 60, 58, 46, 44, 33, 31, 29, 27 and 16 kDa occurred in 0.005 gL^{-1} ($13.6 \mu\text{M}$) of HgCl_2 , and the higher concentration of mercury also caused dramatic changes in protein profiles as the viable number changed. After exposure for 30 minutes, the 70 kDa, 60 kDa, 58 kDa and 16 kDa proteins were relatively increased, and the 46 kDa protein was produced at levels similar to these seen for $0.025 \text{ gL}^{-1} \text{ HgCl}_2$. Interestingly, the 33 kDa and 27 kDa proteins were relatively increased then declined for up to 5 hours exposure. The higher concentration of HgCl_2 caused rapid induction after addition and the relative induction rate was high for up to 3 hours, and the response seen for the 18 kDa protein was similar to when the lower level of HgCl_2 was used. The 31 kDa and 44 kDa protein production was similar in both tested concentrations, and an relative increase of 22 kDa protein was not seen at the lower tested concentration. A similar induction pattern occurred for the 29 kDa protein at both of the tested concentrations as did the 16 kDa protein. It was interesting that the 70 kDa, 58 kDa and 16 kDa proteins increased immediately at the time of the growth recovery period, and the 70 kDa and 16 kDa proteins declined after recovery while the 58 kDa protein increased for up to overnight culture for both tested concentrations of HgCl_2 . Mercury stress caused a dose-related response which is seen by comparing the protein profiles in panel B and C: maximal stress responses occurred within 30 minutes and the responses seen at the higher concentration of HgCl_2 lasted longer, and

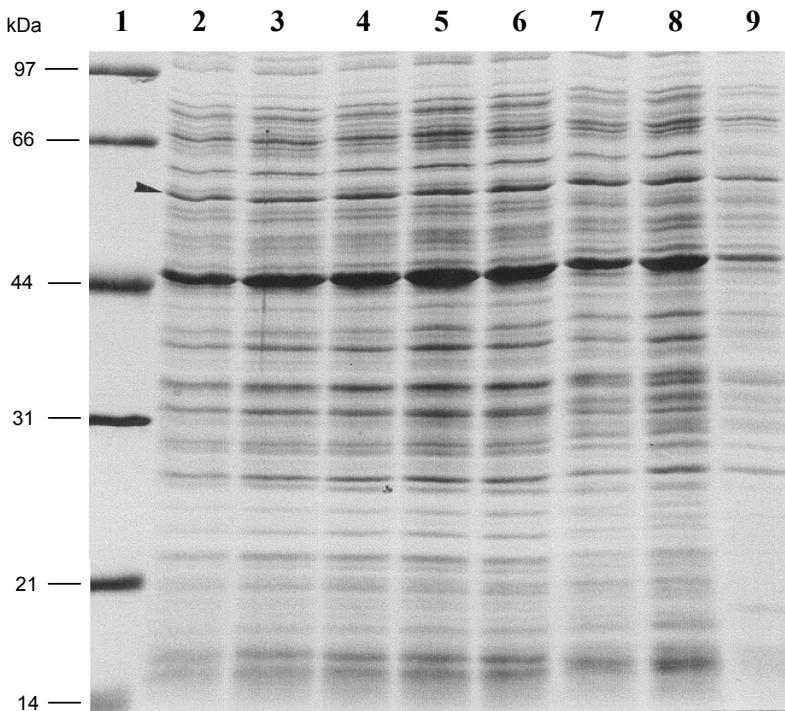
Figure 4.10. 1-D SDS-PAGE (10-15% gradient) analysis of proteins from cells disrupted by the sand lysis method for strain VUN10,077 following addition of HgCl_2 to mid-exponential BHI cultures at 28°C. Controls were treated similarly without addition of chemicals. Molecular markers (Bio-Rad) were 97, 66, 44, 31, 21 and 14 kDa proteins. Arrows mark proteins that change.

For panel A (control): markers (lane 1), time 0 (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 3 hours (lane 6), 5 hours (lane 7), 7 hours (lane 8) and overnight culture (lane 9).

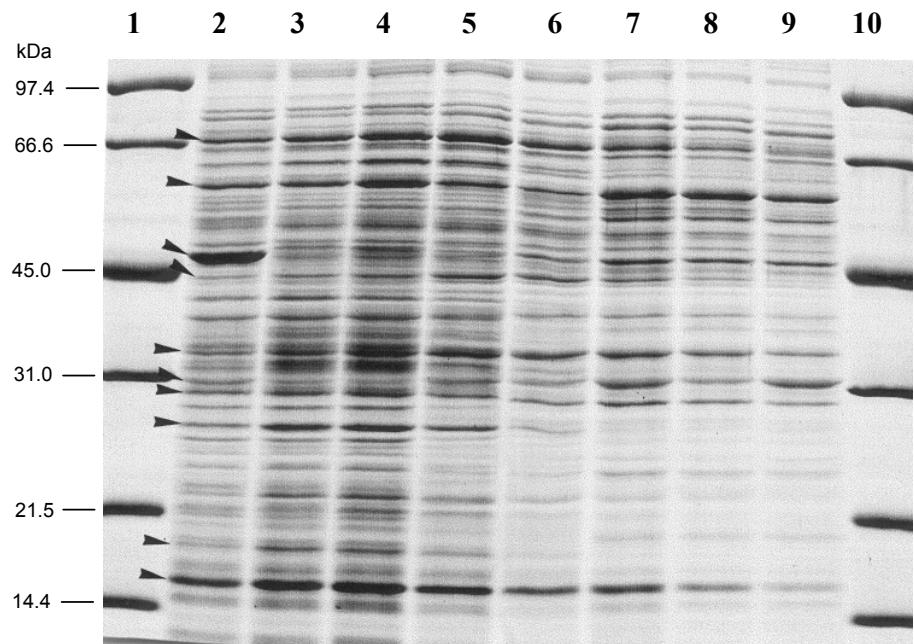
For panel B ($0.025 \text{ gL}^{-1} \text{ HgCl}_2$): markers (lanes 1 and 10), time 0 (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 3 hours (lane 6), 5 hours (lane 7), 7 hours (lane 8) and overnight culture (lane 9).

For panel C ($0.005 \text{ gL}^{-1} \text{ HgCl}_2$): markers (lanes 1 and 10), time 0 (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 3 hours (lane 6), 5 hours (lane 7), 7 hours (lane 8) and overnight culture (lane 9).

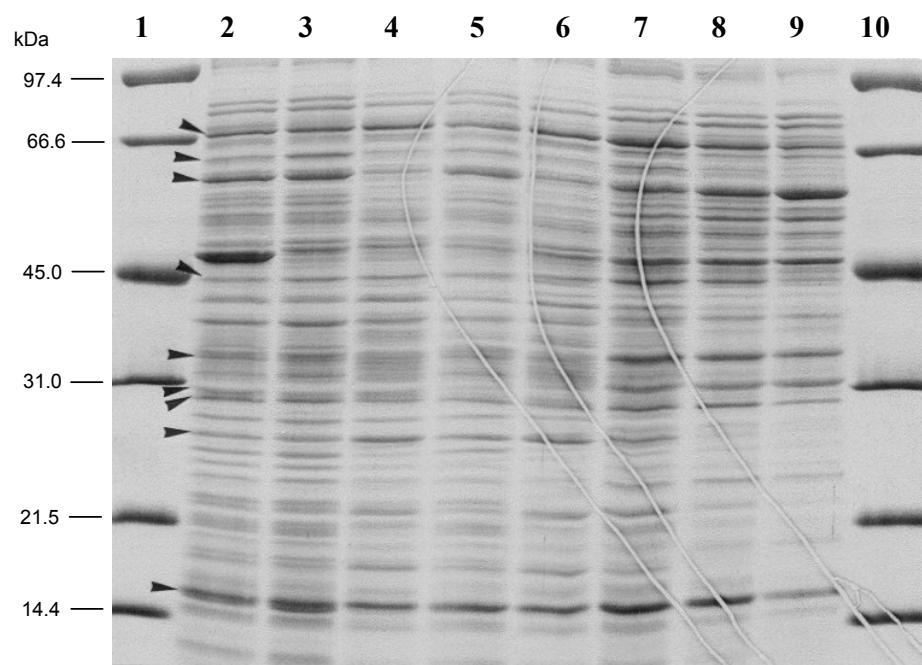
A. Control



B. Mercury-stressed (0.025 gL^{-1})

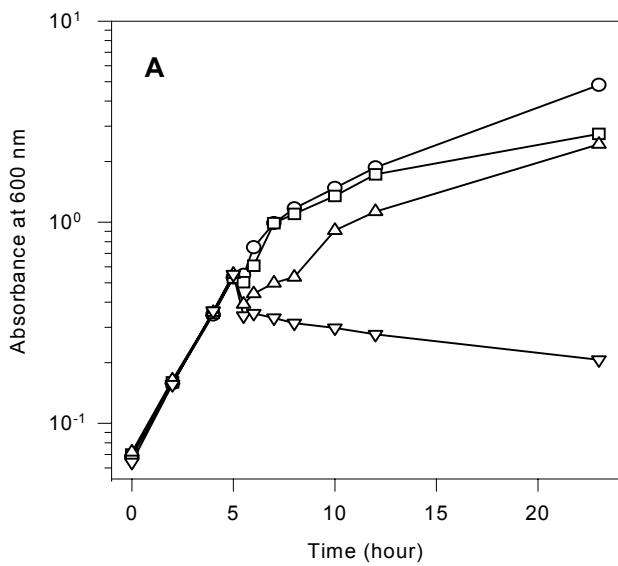


C. Mercury-stressed (0.005 gL^{-1})



there was a similar recovery pattern seen after 5 hours stress in both of the tested concentrations. The correlation between viability and protein profiles may indicate that strain VUN10,077 has a mercury detoxification systems, but this was not investigated further.

In preliminary trials, SDS stress showed similar patterns to HgCl_2 stress in terms of absorbance readings, when SDS was added at concentrations of 0.05 to 3.0 gL^{-1} into growing cultures of strain VUN10,077 in BHI. An A_{600} declined after addition of SDS and this was dose-related, except when 0.025 gL^{-1} SDS was added as this concentration caused little growth inhibition, similar to what aspect as 0.05 gL^{-1} HgCl_2 stress in terms of A_{600} related with viable counts, as judged from A_{600} of SDS stress. Viability tests were not performed due to apparent SDS lysis of cells for cultures showing decreased A_{600} following addition of high SDS concentrations. However, protein profiles were examined for cultures in the presence of SDS added to mid-exponential cultures at concentrations of 0.005 gL^{-1} , 0.025 gL^{-1} and 0.05 gL^{-1} , as the A_{600} readings at least for 0.005 and 0.025 gL^{-1} showed a recovery after initial inhibition (Figure 4.11). As expected from the preliminary trials, the low concentration of SDS did not show inhibition of growth in terms of absorbance readings, and the highest concentration (0.05 gL^{-1}) of SDS showed no recovery of A_{600} . The protein profiles did not change over the growth cycle when 0.005 gL^{-1} SDS added (data not shown). At the concentration of 0.025 gL^{-1} SDS, A_{600} increased after an initial decline, which was a similar pattern to that seen for addition of 0.05 gL^{-1} HgCl_2 (see Figure 4.9). Addition of 0.025 gL^{-1} SDS showed a recovery pattern over 3 hours later (Figure 4.11A), and SDS-induced proteins were: 75, 70, 58, 31, 29.5 and 16 kDa (Figure 4.11B). A maximal response occurred for up to 1 hour stress and the SDS-induced proteins declined relatively afterwards. Interestingly, the level of a 46 kDa protein was not immediately diminished, and levels of the 58 kDa and 31 kDa proteins were not seen to increase late in culture as seen in HgCl_2 stress. The 75 kDa, 70 kDa, 29.5 kDa and 16 kDa proteins declined relatively following ongoing stress. SDS-treated cells did not show a dramatic change in protein profiles compared with HgCl_2 stress, although the absorbance readings transiently declined and recovered after stress at a concentration of 0.025 gL^{-1} . It was interesting that a maximal response occurred for up to 1 hour stress and then relatively declined for both SDS and mercury stresses for strain VUN10,077. This may be implied that stress-specific mechanisms occurred in strain VUN10,077, seen in which the sand lysis method was able to show changes in protein profile



B. SDS-stressed (0.025 gL^{-1})

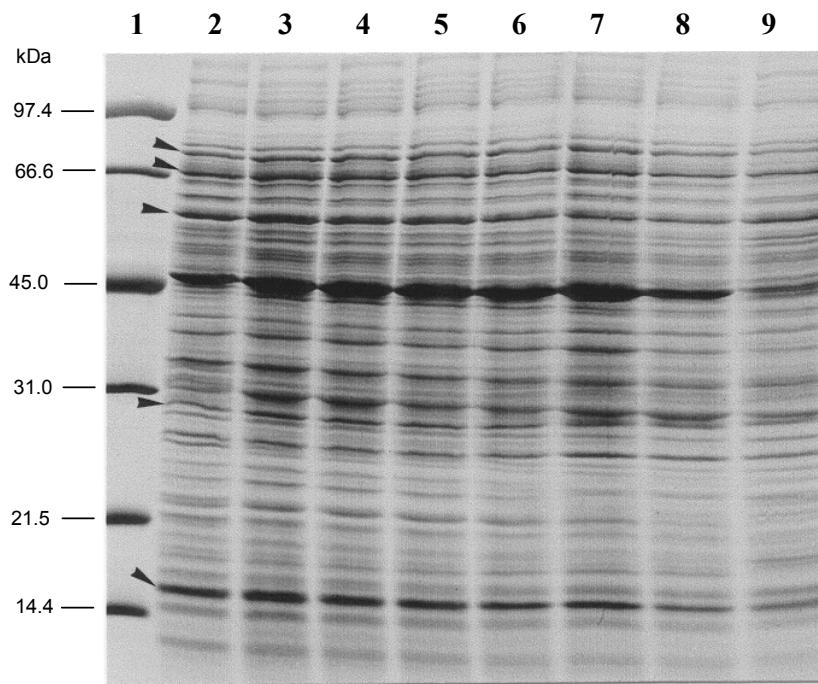


Figure 4.11. Effect on growth and 1-D SDS-PAGE (10-15% gradient) protein profiles of cells disrupted by the sand lysis method for strain VUN10,077 following addition of SDS at mid-exponential BHI cultures at 28°C . Control culture and markers were the same as Figure 4.10 (panel A) and the SDS concentrations used were 0.005 gL^{-1} (□), 0.025 gL^{-1} (Δ), 0.05 gL^{-1} (▽) and control (○). The gels for markers (Bio-Rad, lane 1), time 0 (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 3 hours (lane 6), 5 hours (lane 7), 7 hours (lane 8) and overnight culture (lane 9).

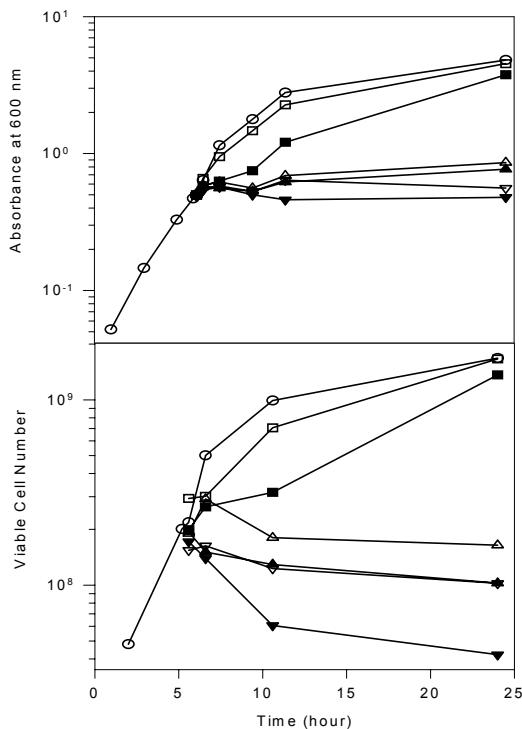
when cells recovered after initial inhibition by HgCl₂ or SDS. This method was also applied when testing impacts of 2,4,5-trichlorophenol and lead nitrate, which also showed no change on protein profiles when using cells lysed by boiling on a flame. However, no new protein patterns were seen using the sand lysis.

4.2.2.2.4 Arsenite and phenol stresses

Growth was significantly inhibited in concentrations of sodium arsenite $\geq 0.2 \text{ gL}^{-1}$ (0.81 mM), as reflected in detecting both changes in the rate of increase of A₆₀₀ and viable counts (Figure 4.12). Arsenite induced several proteins of MW around 20 kDa after 2 hours stress, which were 20, 16 and 15 kDa proteins. The low molecular weight proteins were seen within 30 minutes and ongoing after 2 hours stress.

In preliminary trials, phenol stress did not affect absorbance readings at concentrations between 0.005 gL⁻¹ and 1.0 gL⁻¹, but 3.0 gL⁻¹ phenol caused decline in A₆₀₀ when added at early exponential phase. Subsequently, phenol was added at concentrations between 1.0 gL⁻¹ and 3.0 gL⁻¹ at mid-exponential phase, and A₆₀₀ decreased at concentrations above 2.0 gL⁻¹ phenol. Therefore, cells were subsequently stressed at sublethal levels using various concentrations of phenol (0.5, 1.0, 1.2, 1.4, 1.6 and 1.8 gL⁻¹) and total proteins were resolved on 1-D SDS-PAGE after 30 minutes and 2 hours addition of different concentrations of phenol, when cells were lysed by boiling on a flame (Figure 4.13). When phenol was added at 0.5 gL⁻¹ (5.31 mM) and above it stopped further increases in viable cell numbers, despite continued small increases in A₆₀₀, and a concentration of 1.8 gL⁻¹ (19.1 mM) significantly decreased the viability of this strain. The relative levels of proteins of MW 62.5, 37, 20 and 16 kDa were significantly changed when compared to the controls for concentrations up to 1.6 gL⁻¹, including 75 and 58 kDa. When 1.8 gL⁻¹ of phenol was added, both the A₆₀₀ and viable counts decreased significantly, and the protein profile seen for 30 minutes and 2 hours samples were similar to the controls sampled at the same times (lanes 6 and 14 compared with 7 and 15 respectively). The exception was the relative decline in a 16 kDa protein. This protein was also seen to decline in both 30 minutes and 2 hours samples as the concentration of phenol increased, particularly at concentrations above 1.2 gL⁻¹. Similary, the 62.5 kDa and 37 kDa proteins also declined relative to the control when cells were exposed to lower concentrations (0.5 and 1.0 gL⁻¹) of phenol. Interestingly, a 20 kDa protein was induced with a steady increase in level up to 1.6 gL⁻¹ of phenol concentration at 2 hours

Figure 4.12. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins for strain of VUN10,077 following addition of sodium arsenite, where conditions were the same as Figure 4.5. NaAsO₂ concentrations used were 0.1 gL⁻¹ (□), 0.2 gL⁻¹ (■), 0.4 gL⁻¹ (△), 0.6 gL⁻¹ (▲), 0.8 gL⁻¹ (▽) and control (○).



Samples were taken at 30 minutes for lanes 1-7 and 2 hours for lanes 9-15 after addition of NaAsO₂ at concentrations of control (lanes 7 and 15), 0.1 gL⁻¹ (lanes 1 and 9), 0.2 gL⁻¹ (lanes 2 and 10), 0.4 gL⁻¹ (lanes 3 and 11), 0.6 gL⁻¹ (lanes 4 and 12), 0.8 gL⁻¹ (lanes 5 and 13) and 1.0 gL⁻¹ (lanes 6 and 14).

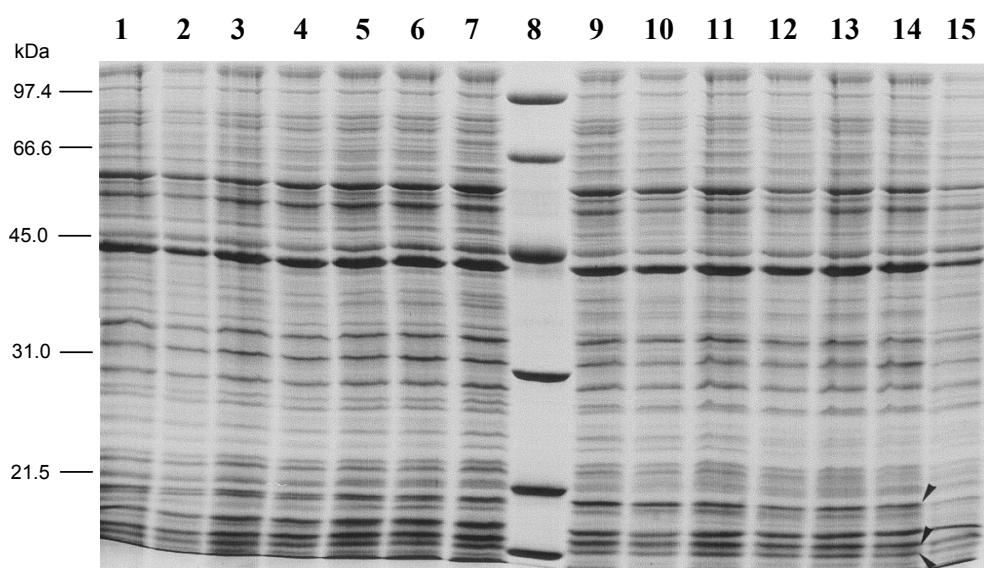
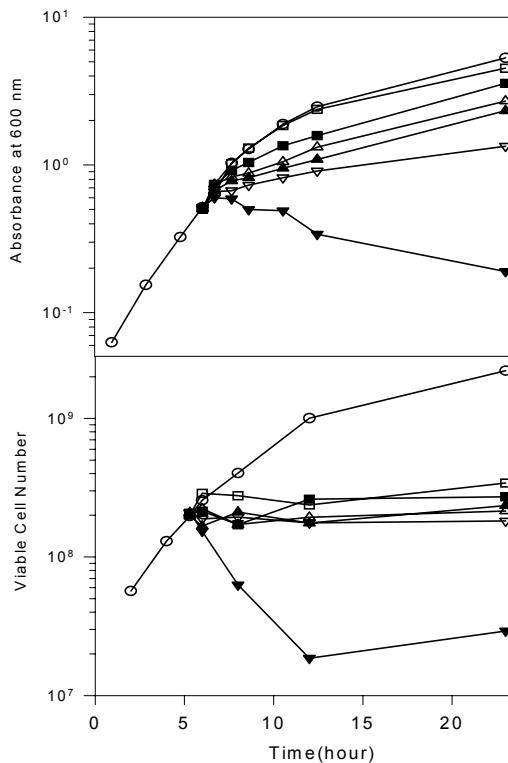
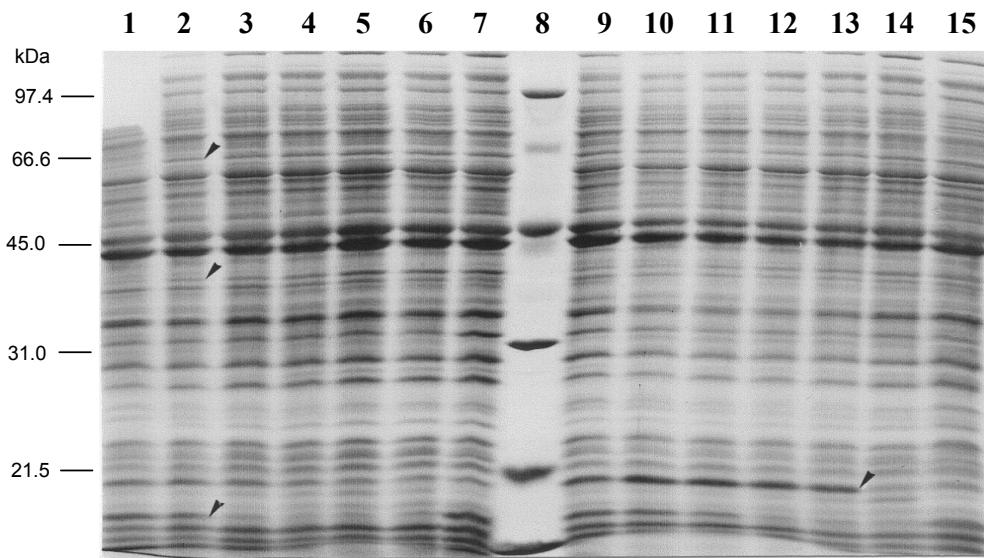


Figure 4.13. Viable cell count, absorbance readings and SDS-PAGE analysis of proteins for strain of VUN10,077 following addition of phenol, where conditions were the same as Figure 4.5. Phenol concentrations used were 0.5 gL^{-1} (□), 1.0 gL^{-1} (■), 1.2 gL^{-1} (Δ), 1.4 gL^{-1} (\blacktriangle), 1.6 gL^{-1} (∇), 1.8 gL^{-1} (\blacktriangledown) and control (○).



Samples were taken at 30 minutes for lanes 1-7 and 2 hours for lanes 9-15 after addition of phenol at concentrations of controls (lanes 7 and 15), 0.5 gL^{-1} (lanes 1 and 9), 1.0 gL^{-1} (lanes 2 and 10), 1.2 gL^{-1} (lanes 3 and 11), 1.4 gL^{-1} (lanes 4 and 12), 1.6 gL^{-1} (lanes 5 and 13) and 1.8 gL^{-1} (lanes 6 and 14).



stress.

The sodium arsenite and phenol stresses affected strain VUN10,077 less when compared with other tested chemicals, although viable numbers were affected. The exception was induction of a 20 kDa protein. In order to examine whether proteins were induced throughout the exposure period, a detailed time course was performed at phenol concentrations of 1.4, 1.6 and 1.8 gL⁻¹, with total proteins extracted by the boiling lysis method (Figure 4.14). Figure 4.14 is structured to show the relative level of induction in the region of MW 66 and 21 kDa for these concentrations, to allow comparison readily over time. In comparison to controls, the relative level of proteins of 75, 58 and 20 kDa increased and remained high when 1.4 or 1.6 gL⁻¹ of phenol was present. Again, when phenol was added at 1.8 gL⁻¹, growth ceased rapidly after addition and viable counts fell rapidly (see Figure 4.13). Although the amount of induction was not elicited, a protein of 75 kDa was increased in culture containing 1.4 and 1.6 gL⁻¹ phenol stress, but not 1.8 gL⁻¹ of phenol. Interestingly, 20 kDa protein was induced immediately and at a higher level at 1.6 gL⁻¹ compared to 1.4 gL⁻¹ phenol stress, but lesser response occurred in 1.8 gL⁻¹ phenol. These results indicated that the 20 kDa protein was a major phenol stress-responsive protein, although the 75 kDa protein was also seen to change in protein profiles, where the level of induction was a dose-related when analysed using Coomassie blue stained 1-D SDS PAGE gels.

4.2.2.3 Western blot analysis of stress-induced proteins

In the previous sections, it was repeated that heat and other chemical stresses caused relative increases in proteins of MW ~60 kDa and 70-75 kDa, which may have corresponded to the *E. coli* GroEL and DnaK proteins, respectively. Western blot analysis was therefore performed using antibodies known to detect these *E. coli* products and this was initially performed on the *St. maltophilia* strain, which showed an increase in a 60 kDa protein during heat stress but did not show obvious increases in a protein of 70 kDa under the conditions tested. Two sources of rabbit polyclonal DnaK antibodies were used: the commercially-available DAKO antibodies, used at a dilution of 1:1,500, and antibodies provided as a gift from Georgopoulos (Zylicz and Georgopoulos, 1984), used diluted 1:3,000. Another source of the anti-DnaK antibody was mouse monoclonal antibody (Sigma) and used diluted in 1:1,500. Cells were lysed by sonication and equal amounts of protein were applied to 12% SDS-PAGE gels for both *St. maltophilia* and *E. coli* tests and controls (Figure 4.15). The

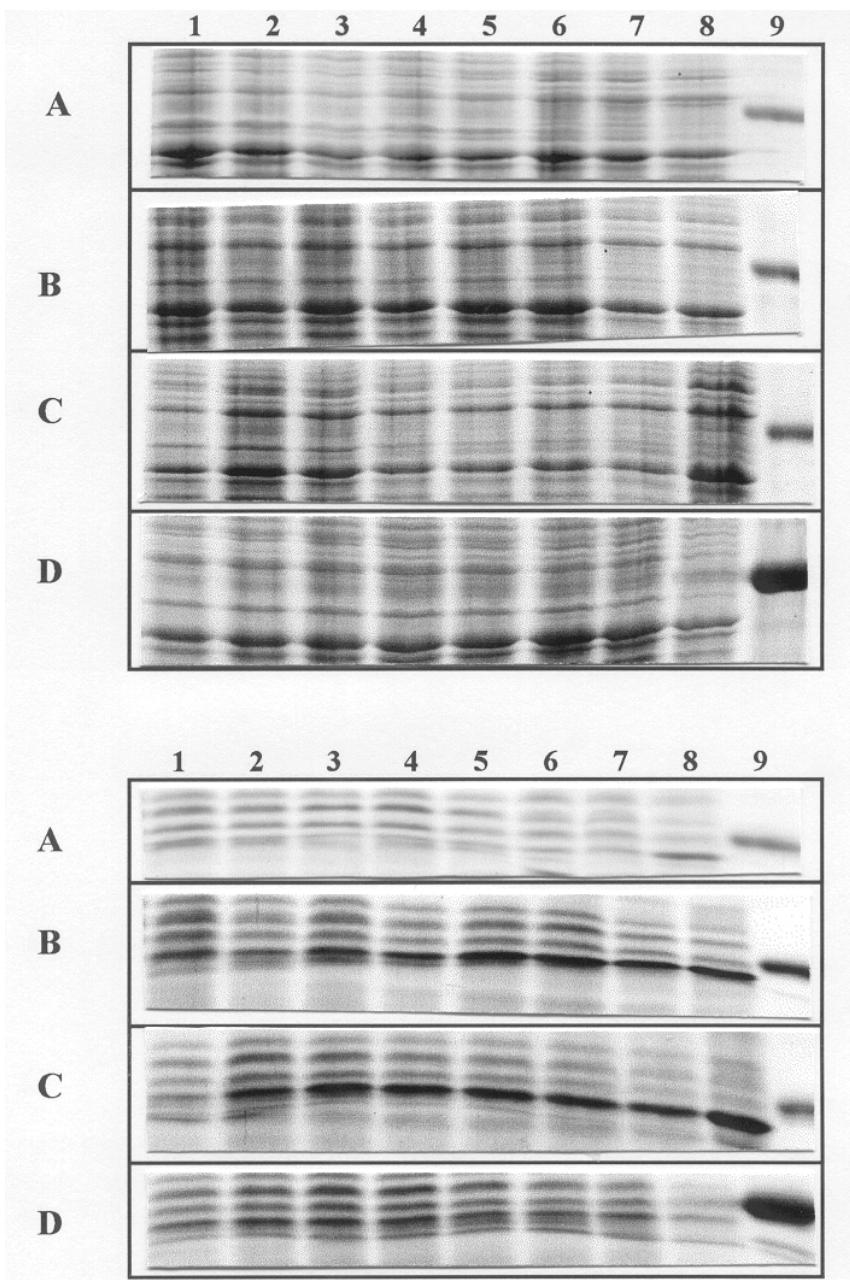


Figure 4.14. Time course of protein production during phenol stress for strain VUN10,077 and cells were lysed by boiling on a flame. In order to compare protein changes in relative amounts of protein produced, gel photography have been segmented in different MW ranges: upper group shows proteins produced in the region of 66 kDa MW marker and the lower group for protein in 21 kDa MW marker. A, unsupplemented (control); B, 1.4 g L^{-1} ; C, 1.6 g L^{-1} and D, 1.8 g L^{-1} . Samples were taken at 30 minutes (lane 1), 1 hour (lane 2), 2 hours (lane 3), 3 hours (lane 4), 5 hours (lane 5), 7 hours (lane 6), 9 hours (lane 7) and overnight (lane 8). Lane 9 indicated a molecular marker 66 kDa for top panel and 21 kDa marker for bottom panel.

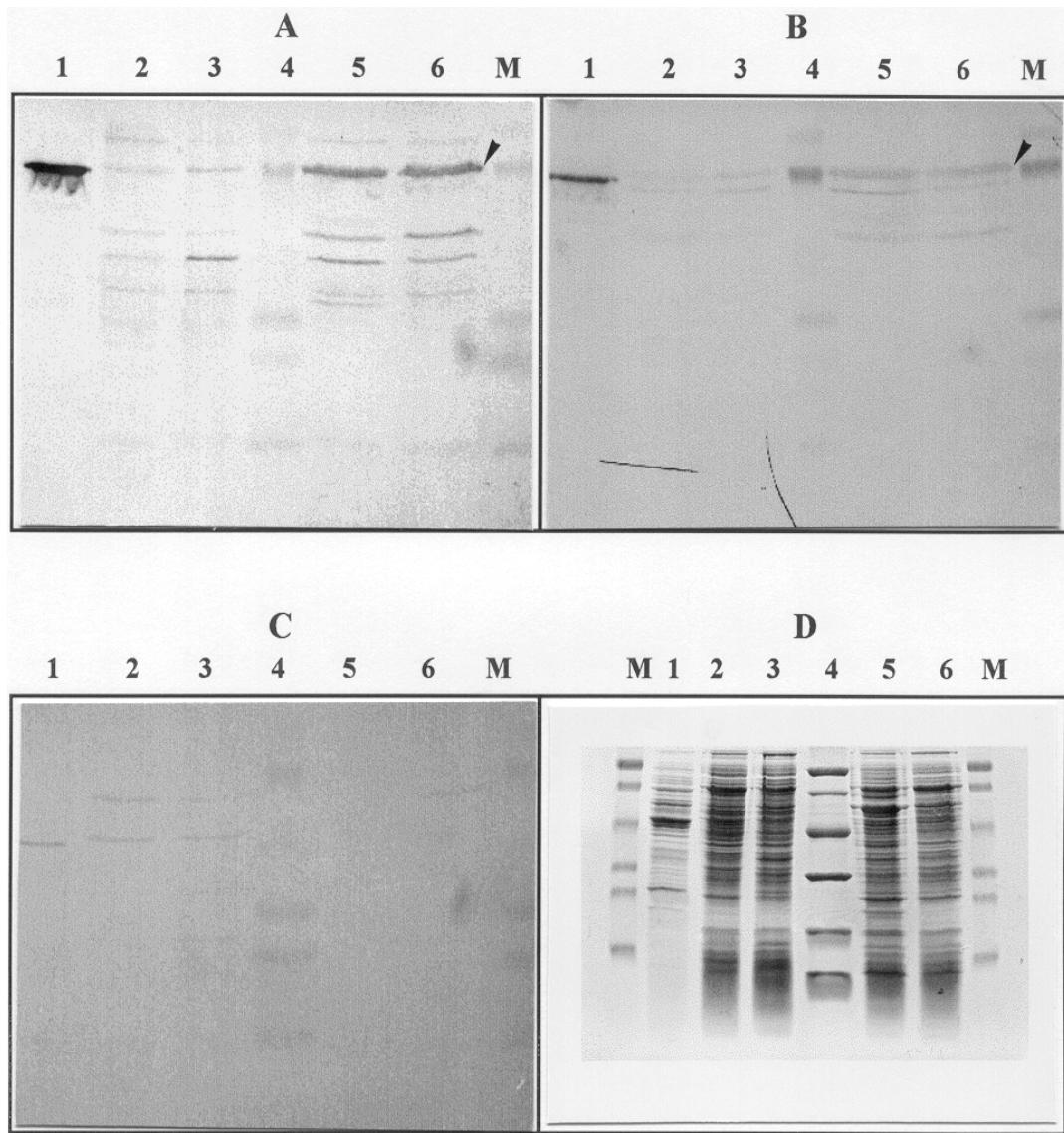


Figure 4.15. Evaluation of heat shock proteins of *St. maltophilia* using specific antibody. Samples were electrophoresed in 12% SDS-PAGE and electroeluted onto nitrocellulose at 15 V, 100 mA and 10°C overnight. The membrane was exposed to three types of Hsp70 antibody, DAKO (A, 1:1,500 dilution) and gift from Georgopoulos (B, 1:3,000 dilution) and monoclonal DnaK antibody, Sigma (C, 1:1,500 dilution). Panel D was a gel in parallel and stained with Coomassie blue. *St. maltophilia* cultures were grown in LB broth and heat transferred from 28°C to 37°C, and *E. coli* without stress was grown in LB broth at 37°C (lane 1). The cells were disrupted by sonication. Lanes for *St. maltophilia* are controls lane 2 (9 hours) and lane 3 (30 minutes); lane 5 (9 hours) and lane 6 (30 minutes) after heat shock; and lane 4 was LMW markers (Bio-Rad) and M designates the pre-stained MW markers (Bio-Rad).

polyclonal DnaK antibodies from DAKO and gift from Georgopoulos reacted strongly and specifically with the *E. coli* DnaK protein, which was easily detected in unstressed cells. When comparing *St. maltophilia* after heat shock, it was clear that a 70 kDa protein was increased but the reaction was not as intense as seen for *E. coli* (despite the obviously high amount of protein present) and many other bands were also seen in the *St. maltophilia* lanes under the conditions used to develop these Western blots. Polyclonal antibodies were reacted with unstressed *E. coli* sample, whereas these were not reacted well in the region of 70 kDa protein of *St. maltophilia*. Moreover, a similar result was seen for monoclonal anti-DnaK (Sigma) which was less specific in both tested strains. These results suggested that the *St. maltophilia* DnaK protein was different to the *E. coli* protein, and when monoclonal antibody used, the reaction may occur specifically, and considerable cross-reactivity occurred with other proteins in the tested antibodies used. Moreover, when the other pseudomonads were examined similarly, only faint reactions were observed in the region of 70 kDa MW similar to results seen for *St. maltophilia* reaction, and these bands were too pale to be recorded photographically. Use of sonicated cells may have influenced the results here, however, as Allan *et al.* (1988) reported specific reactions for a 75 kDa protein-anti DnaK polyclonal antibodies for *Ps. aeruginosa* (see below). The developing solution was altered so that TBS buffer containing saline instead of TBS buffer containing gelatin was used and the membrane was restored into Milli-Q water overnight, to improve the color reaction on the membranes in the following trials.

In the above, several heat shock antibodies were used for *St. maltophilia* and these antibodies showed strain and source specific reactions. Accordingly, whether strain VUN10,077 produced proteins which reacted with DnaK and GroEL antibody when stressed by several chemicals, was tested using cells disrupted by boiling on a flame and testing with polyclonal antibodies. Prior to performing Western blots, strain VUN10,077 was subjected to heat stress or exposure to heavy metal ions for a standard period of 2 hours and total proteins were prepared by the boiling lysis method (see Figure 4.17). Subsequently, Western blotting was performed with extracts of strain VUN10,077 following heat stress and other appropriate defined concentration of selected chemicals at sublethal level, using the same sources of anti-DnaK (DAKO, 1:1500 dilution) and polyclonal anti-GroEL (Sigma, 1:3000 dilution) (Figure 4.16). These antibodies reacted with stress induced protein(s) generated by heat shock and heavy metal ions (cobalt, zinc, cadmium and phenol). After reaction with the second antibody (goat anti-rabbit, Bio-Rad, 1:2,000 dilution), the membranes were washed

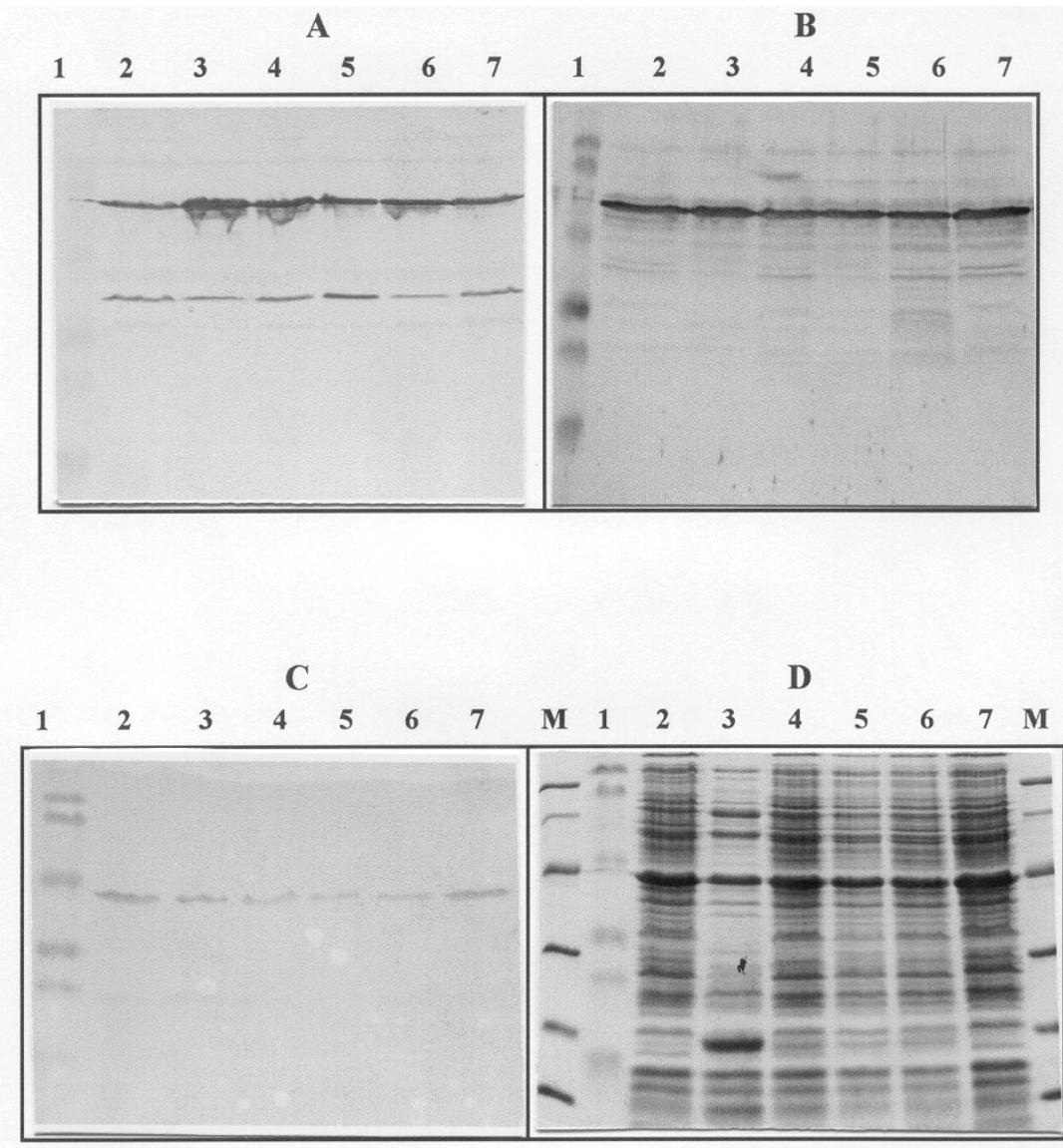


Figure 4.16. Western blotting analysis of proteins produced by strain VUN10,077 following growing in the presence of selected chemicals. A: Hsp70 (DAKO) antibody, B: Hsp60 (GroEL, Sigma) antibody, C: Hsp70, gift from Georgopolous and D: gel was run in parallel and stained with Coomassie blue. Samples were taken after 2 hours following stress and at the same time for the control grown in parallel. For panel A, B, C and D, lane 1, pre-stained markers (Bio-Rad); lane 2, control; lane 3, heat stress (28°C to 37°C); lane 4, cadmium (0.5 gL^{-1}); lane 5, cobalt (1.0 gL^{-1}); lane 6, zinc (1.0 gL^{-1}) and lane 7, phenol (1.0 gL^{-1}). M designates the LMW markers (Bio-Rad).

with TBS buffer containing saline. When the membrane was incubated in Milli-Q water overnight, the color of the signal was increasingly developed from a faint band and other cross-reactive bands were yielded. The DAKO Hsp70 antibody strongly reacted with all tests and also showed distinct reaction with a protein of approximately 40 kDa, which may form a truncated DnaK fragment (Krska *et al.* 1993). The dominant anti-DnaK reactive band was produced differentially following the treatment conditions. The visible signal strength may be in the order of heat stress > cadmium > zinc > cobaltous = phenol > control for detection using the DAKO Hsp70 antibody. Unlike the Hsp70 antibody, GroEL antibody showed a constitutive signal in the control and tested samples, and reacted with several other proteins in the fractions, mainly between 40 and 60 kDa (Figure 4.16 B) which was analogous to *Ps. aeruginosa* and *Pseudomonas cepacia* antigens prepared by sonication reacting with several other bands using both GroEL-monoclonal and -polyclonal antibodies (Jensen *et al.*, 1993). The signal intensity observed was consistent with 1-D SDS PAGE protein profiles obtained after stained with Coomassie brilliant blue, for cells disrupted by the boiling method (Figure 4.16). These results inferred that the method of cell disruption was important for determining the presence and location of stress inducible proteins. An outcome of this analysis was that GroEL and DnaK antibody detected sizable amounts of the 70 kDa and 60 kDa protein in stressed and non-stressed cells of strain VUN10,077, confirming the presence of homologs in this species.

4.2.2.4 Summary of chemical stress-responsive proteins produced in rich media

The strain VUN10,077 and related species responded to sudden temperature upshift and produced several different heat-induced proteins then chemical stress was pursued for strain VUN10,077. A detailed study of the proteins made in response to heat and chemicals showed stress-specific proteins, including Hsp70 and Hsp60. A Hsp70 and Hsp60 proteins were homologs to commercialised-DnaK and -GroEL antibodies respectively, when strain VUN10,077 was subjected to heat, cadmium, cobalt, zinc and phenol stresses (see section 4.2.2.3). When cells of VUN10,077 were heat stressed, apparently they produced a Hsp60 protein (measured size was 60-56 kDa protein in different experiments) but a Hsp70 (refer to ~70 kDa protein) was not obvious when cells were lysed by sonication. Although the relative level of Hsp70 was not increased by heat stress of strain VUN10,077, the Hsp60 protein was relatively increased when cells were lysed by sonication. However, the relative level of 70 kDa and 75 kDa proteins was seen to increase when cells were lysed by boiling on a flame

and the 60 kDa protein was not increased for strain VUN10,077 but the level was maintained. However, the relative level of a 20 kDa proteins were seen to increase when compared to controls whether cells were lysed by sonication or boiling. These observations emphasised the importance of the cell preparation methods in detecting stress responses and most experiments were then performed using the boiling methods.

To compare the impact of lysis methods on protein profiles, cells were prepared from cultures containing selected heavy metal ions at concentrations determined from previous growth inhibition tests: 1.0 gL⁻¹ for cobaltous chloride, zinc chloride and phenol, and 0.5 gL⁻¹ for cadmium chloride, or heat shock from 28°C to 37°C (Figure 4.17). The samples were taken at a standard period of 2 hours after stress and cells were lysed by sonication and boiling lysis method. After sonication, the protein concentration was measured by the Lowry method and sample volumes adjusted to load similar concentrations of protein in each well. For cells treated by boiling in SDS-PAGE loading buffer for 10 minutes, similar amounts of cells were loaded according to the A₆₀₀ when harvested. The resolution showed recognisable differences in protein profiles according to the disruption method (Figure 4.17). The protein profiles seen following boiling lysis showed more protein bands compared to sonification, especially in the high molecular region (over 45 kDa). A major membrane-associated protein was seen prominently in the range of 45 kDa following boiling lysis in addition to a 70 kDa protein. The 58 kDa (GroEL-like) protein was present in sonically disrupted cell extracts, but the 70 kDa (DnaK-like) protein was not prominent. It may be indicated that GroEL-like protein is located in the cytoplasm rather than binding with membranes in strain VUN10,077 when cells were stressed. Interestingly, the DnaK-like protein showed a different pattern. As illustrated in the above, this protein appeared to be removed by centrifugation, which inferred that this DnaK-like protein is closely related to membranes or particulate structures in strain VUN10,077. It also appeared to be present in amounts similar to the GroEL-like protein, which was not seen using sonification for sample preparation. The relative proportion of these two proteins were largely unchanged by chemical stresses but heat shock caused elevation of the proportion of both in comparison with other cellular proteins. When comparing the protein profiles of cells exposed to chemicals to control cells, little difference in the 60/70 kDa protein was seen. However, changes in protein profile were seen when cells were lysed by boiling following exposure of Zn²⁺ or Cd²⁺, in the size range 31-45 kDa. In particular, some bands were decreased in this range and relative proportions of others went up (MW 33 and 25 kDa). Relative increases in proteins were clearly seen when using

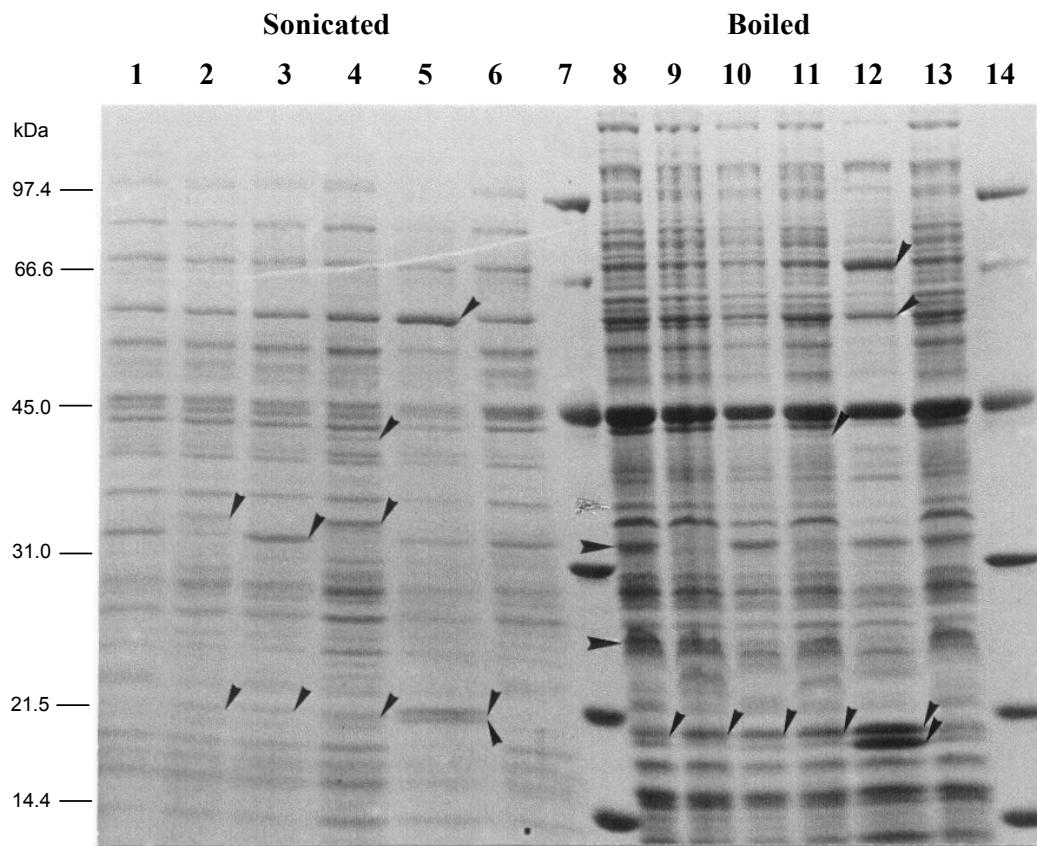


Figure 4.17. Comparison of total proteins detected using sonication (lanes 1-6) and boiling (lanes 8-13) lysis methods, using 1.5 mm thick 1-D SDS-PAGE and strain VUN10,077. Samples were taken after 2 hours stress and controls without stress were at the same time of point using BHI broth at 28°C. Chemicals were added at concentrations of 1.0 gL⁻¹ phenol (lanes 1 and 8); 1.0 gL⁻¹ zinc (lanes 2 and 9); 1.0 gL⁻¹ cobalt (lanes 3 and 10); 0.5 gL⁻¹ cadmium (lanes 4 and 11), and elevated heat from 28°C to 37°C (lanes 5 and 12). Control (lanes 6 and 13) and Bio-Rad LMW markers (lanes 7 and 14) were run parallel, which markers were from 97.4, 66.6, 45, 31, 21.5 and 14.4 kDa.

Table 4.1. Stress responsive proteins occurred upon heat shock in the strain VUN10,077 and related species when cells were lysed by sonication following growth in LB broth.

Strain	Response (kDa)	No response ^a (kDa)
<i>Ps. aeruginosa</i>	58	70, 75
<i>Ps. fluorescens</i>	75.5, 65, 58, 27, 21, 14	70, 75
<i>St. maltophilia</i>	56, 27, 21, 14.9, 14, 13.5	70, 75
VUN10,077	70 ^b , 58, 47, 29, 27, 20, 19, 15	75

a; Relative level of proteins was not increased.

b; detected on gels where cells were lysed by boiling on a flame.

Table 4.2. Stress responsive proteins were produced by chemical stresses for strain VUN10,077 when cells were lysed by boiling on a flame following growth in BHI broth.

Chemical stressor	Response (kDa)	No response ^a (kDa)
Cadmium	45, 38, 20	58, 70, 75
Copper	70 ^c , 20	58, 70, 75
Cobalt	20	58, 70, 75
Nickel	75 ^c , 20, 16, 15, 14	58, 70, 75
Arsenite	20, 16, 15	58, 70, 75
Phenol	75 ^c , 20, 16	58, 70, 75
Mercury ^b	-	58, 70, 75, sHSP ^d

a; Relative level of proteins was not increased and 58 kDa protein was produced as well in the control.

b; When lysed by the sand lysis method, protein profiles showed dramatic change.

c; Weak protein band produced at the specific concentration.

d; sHSP, small stress responsive protein in the range of 20 kDa.

sonically-disrupted extracts, where Zn^{2+} exposure caused induction of a 33 kDa protein, Cd^{2+} caused an increase in proteins of 38 kDa and 33 kDa, and phenol and cobalt stresses caused an increase in a 32 kDa protein. The gel shown in Figure 4.17 draws attention to proteins the range of 20 kDa: heat stress produced a doublet of about 21 and 21.5 kDa in size for sonically-disrupted samples, and 20 and 19 kDa in size for boiled lysis samples. These were deemed to be the same proteins as they were induced by heat shock conditions for strain VUN10,077 and small differences in molecular weight measured in the same gel may be from loading difference in each well or the lysis method. Phenol stress at 1.0 gL^{-1} produced relatively lesser amounts of a 20 kDa protein compared to 1.4 gL^{-1} and 1.6 gL^{-1} phenol stress (see Figure 4.14), and this response was not seen at this concentration when cells were lysed by sonication. When increases in the 70 kDa, 58 kDa and 20 kDa proteins occurred for any stress condition, these could be detected regardless of the lysis method used for strain VUN10,077. However, there appeared to be no uniform pattern of the stress responsive proteins under given conditions. Instead, qualitative as well as quantitative differences may be observed in the response following exposure to different stressors.

Table 4.1 shows a summary of the heat stress-responsive proteins for the pseudomonads tested when cells were lysed by sonication. The pseudomonads all produced Hsp60 protein, 56-58 kDa protein in size, in response to heat stress. Unlike the other pseudomonads, the major protein made by *Ps. aeruginosa* on heat stress was induction of a 58 kDa protein, as seen by sonic disruption. This may implied that the Hsp60 protein plays a major role in the cellular response following sudden temperature upshift from 28°C to 37°C . Although strain VUN10,077 showed increases in the relative level of ~ 70 kDa protein when cells were lysed by boiling on a flame following growth in BHI broth, a relative increase in a protein of ~ 58 kDa protein was not seen.

Chemical stress-responsive proteins for strain VUN10,077 when cells were lysed by boiling on a flame, are summarised in Table 4.2. Chemical stress-responsive proteins were induced as follows: 45 kDa, 75 kDa and sHSPs (small stress-responsive proteins), especially a 20 kDa protein. These proteins were condition-specific, for example, the 45kDa protein was produced by cadmium stress and showed a dose-related response, the 75 kDa protein was weakly induced by phenol stress and showed a dose-related response for concentrations up to 1.8 gL^{-1} , and sHSPs were induced as major stress-responsive proteins over the tested chemicals when they caused responses. Interestingly, copper stress decreased viability but no obvious change in

protein was observed. Mercury stress showed a correlation between changes in protein profiles and viability when cells were lysed by the sand disruption method. sHSPs were not induced immediately after stress except for the cadmium and mercury responses, which were relatively fast compared to copper, sodium arsenite, nickel and phenol, where proteins were induced 2 hours after being stressed. These results indicated that strain VUN10,077 responded to given conditions in a diverse way which was stressor specific and dose-related. In the next section 4.3, stress responses for cells cultured in more minimal or defined media are repeated.

4.3 Effects of medium composition on stress responses

Protein profiles of both prokaryotic and eukaryotic microorganisms depend on the environmental factors, including composition of the medium and growth temperature, and whether starvation conditions occur (Rossini *et al.*, 1993). Defined media may also affect cell growth rate compared to growth in rich medium or under stress conditions. The choice of medium is therefore important, as it must not only support growth to sufficiently high biomass but it must also be suitable for use in experiments to detect stress responses. Parameters important in the latter include suitability for use in ^{35}S -methionine uptake into newly synthesized proteins or use with heavy metal ions, where media components may interact with ions to form complexes or precipitates so that the effective concentration of metal available to cause stress responses is lowered. Effort therefore was put into developing media which were defined both in terms of the protein, hence amino acids, composition plus the presence of phosphate and other counter ions. Several basic and defined media were tested as follows (see Table 2.2); medium (1) contained M9 salts medium, medium (2) contained M9 salts medium plus casamino acids, medium (3) contained M9 salts medium plus defined amino acids mixture, medium (4) contained M9 salts medium, casamino acids and sulphur-free medium plus casamino acids, medium (5) contained M9 salts medium plus defined amino acids mixture, medium (6) contained M9 salts medium, casamino acids and sulphur-free defined amino acids mixture, medium (7) contained M9 salts medium, vitamin solution, trace elements solution and casamino acids (see Materials and Methods section 2.1.3).

4.3.1 Growth kinetics in various defined or minimal media

Pseudomonads and related species grown in commonly used minimal media which are based on M9 salts medium supplemented with other components for experimental needs (Allan *et al.*, 1988 ; Köhler *et al.*, 1989). Defined minimal media were reviewed by Guirard and Snell (1981) for *Ps. aeruginosa*, *Ps. fluorescens* and *St. maltophilia* which were composed of NH₄Cl, KH₂PO₄, NaCl, MgSO₄.7H₂O, and trace elements. *St. maltophilia* required methionine for growth in defined medium (Guirard and Snell, 1981). Growth in medium (1) by all strains was relatively poor. All of the pseudomonads and related species used in this thesis showed growth in medium (2) when starter cultures were in this medium (Figure 4.18), which was 0.05% (w/v) casamino acids in M9 salts medium and was previously used by Allan *et al.*, (1988) for *Ps. aeruginosa*. Initially, *St. maltophilia* was tested in medium (2) because *St. maltophilia* was used for investigation of stress responses in the beginning of this thesis (later the focus changed to strain VUN10,077), however, *St. maltophilia* grew poorly in medium (2) when starter cultures were prepared in this medium from pure colonies in BHIA or LA. *Ps. aeruginosa* and strain VUN10,077 reached A₆₀₀ 1.0 within 10 hours and *Ps. fluorescens* growth pattern was similar to *St. maltophilia* in terms of absorbance readings at 600 nm in medium (2) (Figure 4.18 A). When *St. maltophilia* was grown in medium (2), cells were not finely dispersed and coagulated yellow particles and precipitation occurred after taking samples. Medium (3) was also not suitable for growing of strains *St. maltophilia* and *Ps. fluorescens*, as inocula showed low turbidity when prepared in medium (3) at 28°C for up to 3 days. Similar results were seen for *St. maltophilia* in several trials which used media (1), (2) or (3) for preparing the starter cultures. Therefore, medium (4) was composed to examine the effect of the defined amino acids mixture on growth of the strain VUN10,077 and related species (Figure 4.18 B). *Ps. aeruginosa* and strain VUN10,077 showed similar growth rates, μ_{max} 0.490. *St. maltophilia* and *Ps. fluorescens* had an exponential phase within 10 hours of inoculation then growth was continued slowly. The results implied that amino acids are essential to support the growth of strain VUN10,077 and related species in these restricted nutrient conditions, furthermore, casamino acids are required for the growth of *St. maltophilia* and *Ps. fluorescens* rather than the defined amino acids mixture when compared to media (2), (3) and (4). Accordingly, casamino acids was chosen to prepare medium (5) to which trace elements and vitamin solution were added (Figure 4.18 C). Medium (5) had a significant affect on growth of the strain VUN10,077 and related species, where the growth curves showed had high μ_{max} and cultures entered

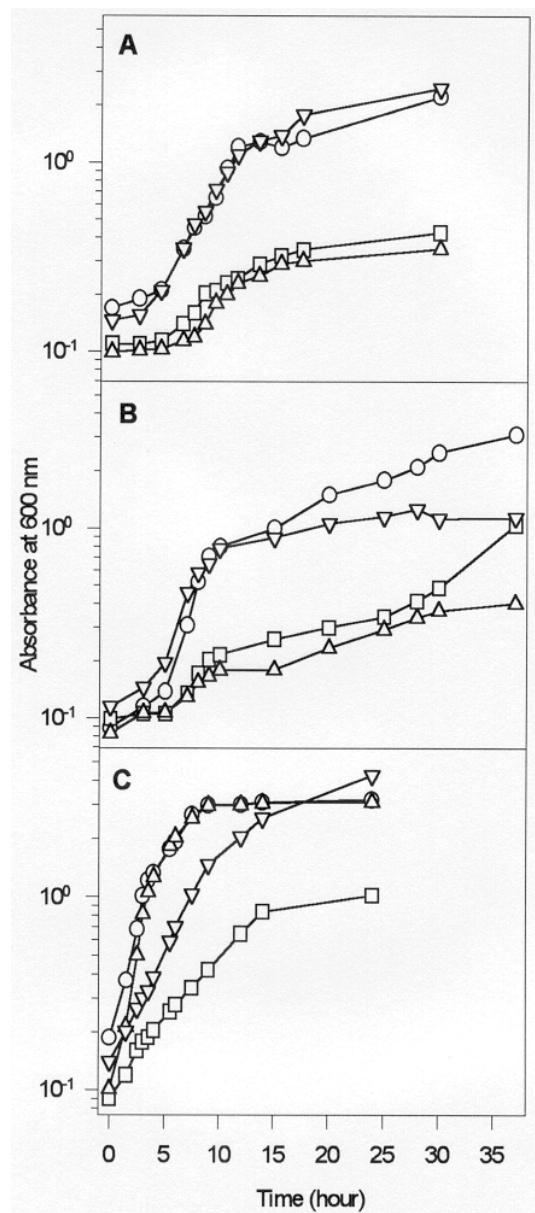


Figure 4.18. Growth curves in defined or minimal media. A, medium (2) (M9 salts and casamino acids); B, medium (4) (M9 salts, defined amino acids mixture and casamino acids); C, medium (5) (M9 salts, trace elements, vitamins and casamino acids). *Ps. aeruginosa* (○), *Ps. fluorescens* (△), *St. maltophilia* (□), and VUN10,077 (▽).

stationary phase earlier when compared to media (1), (2), (3) and (4). *Ps. aeruginosa* and *Ps. fluorescens* had similar growth rates in medium (5) and these were relatively high when compared to the locally isolated strains. To confirm that cell growth was not contaminated after overnight culture in the tested media, the culture broths were streaked on BHIA plates: cultures were always uncontaminated. When the strain VUN10,077 and related species grew in medium (5), *Ps. aeruginosa* showed a visible green color which may have been from alginic acid, *St. maltophilia* was a dark grey color, *Ps. fluorescens* had a yellowish color and strain VUN10,077 had a yellowish-orange color similar to seen when growing on BHIA. Conclusively, medium (5) was the optimal condition for the pseudomonads and related species compared to the other tested media.

Although it was found that medium (5) was appropriate for the locally isolated strains and *Pseudomonas* and related species (see Figure 4.18), the *St. maltophilia* strain only reached an A_{600} of 1.0 to 1.3 in this medium with a doubling time of approximately 5 hours, despite this being the optimum minimal medium thus far tested. Furthermore, *St. maltophilia* showed different growth rates in media (2) and (4) after overnight culture, which may have been due to the availability of the other added essential components. Therefore, the growth of this strain was further tested using medium (5) supplemented with methionine (40 mg per litre), defined amino acids mixture and uracil (Figure 4.19). Culture incubation was not extended to 35 hours because *St. maltophilia* had an exponential phase within 11 hours of inoculation in medium (5). Although *St. maltophilia* (formerly *Ps. maltophilia*) required methionine amino acid in defined medium (Guirard and Snell, 1981), medium (5) showed similar growth rates to medium (5) containing methionine for the environmental isolate strain *St. maltophilia*. In parallel, Amino acid assay medium (Difco) was used to examine the effect of methionine on growth (according to the manufacturer's instruction) but the environmental isolate strain *St. maltophilia* was not suitable for this type of experiment as it failed to grow on this medium. Medium (5) containing uracil did not affect the growth rate. Interestingly, this strain grew better when medium (5) was supplemented with defined amino acids either with or without methionine or uracil addition. This result indicated that the environmental isolate strain *St. maltophilia* required the defined amino acids mixture, rather than methionine alone for good growth in medium (5) at 28°C.

4.3.2 Induction of HSPs using defined minimal medium

4.3.2.1 Growth kinetics in various minimal media upon heat stress

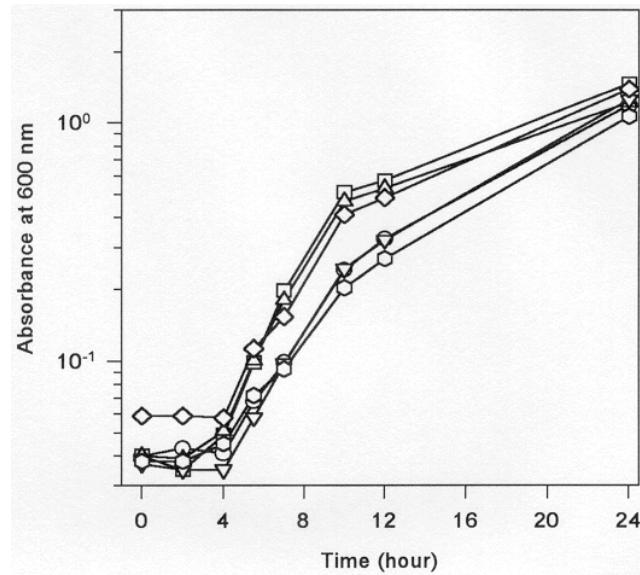


Figure 4.19. Effect of adding amino acids, methionine and uracil to medium (5) on growth of *St. maltophilia*. Medium (5) (O), plus defined amino acids mixture (□), plus methionine (▽), plus defined amino acids mixture including methionine (△), plus uracil (○), or plus defined amino acids mixture including uracil (◇).

Strain *St. maltophilia* showed slow adaptation to the harsh environments presented in the more minimal media tested, whereas strain VUN10,077 was capable of growing in several minimal or defined minimal media. Before attempting investigation of ^{35}S -methionine incorporation to stress-induced proteins, five different defined media which supported growth of strain VUN10,077 were evaluated in terms of detecting stress proteins using media (1), (2), (3), (4) and (5). In order to identifying major HSPs, protein profiles were generated by growing cells with and without temperature upshifts using Coomassie-stained 1-D SDS PAGE gels. Figure 4.20 shows the effect of temperature upshift on growth rates in the defined media, determined by measuring A_{600} . Although the starter cultures were prepared in each selected minimal or defined minimal media and different growth rate occurred in each, temperature stress was investigated using inocula when they had reached stationary phase, to set similar starting conditions regarding metabolic activity. The cultures were grown until mid-exponential phase then the temperature was elevated from 28°C to 37°C (Figure 4.20). Samples were taken periodically throughout the growth cycle for measurement of A_{600} and for protein analysis. Cells cultured in medium (1), showed early onset of stationary phase with no further increase in A_{600} and showed a transient increase in A_{600} following upshift. Temperature upshift here was at A_{600} 0.5 and growth trends were similar when transfer was at A_{600} 0.2, in terms of trends in absorbance readings. In media (3) and (5), growth was significantly impaired immediately after temperature upshift, although in late stationary cultures, the A_{600} had recovered, implying that growth continued but at a slow rate. In media (2) and (4), temperature upshift had little impact on either growth rate or final A_{600} . Growth in media (1) and (3) was significantly impaired after temperature upshift, although medium (3) contained defined amino acids mixture, whereas when M9 was supplemented with casamino acids (2) or casamino acids plus sulphur-free amino acids mixture (4), inhibition of growth was not apparent. In contrast, when medium (4) was further supplemented with trace elements and vitamins, temperature upshift again impaired growth, inferring that these further additions did not assist survival after upshift. This was not further investigated.

Media (2) and (4) already showed good growth rates and survival after temperature upshift to 37°C. Purines and pyrimidines were added to investigate whether these had any further impact on growth rates or final absorbance reached (Figure 4.21). Following heat elevation, growth rates in both of the supplemented media increased slightly relative to the unsupplemented control, but the effect was small. Purines and pyrimidine addition was not tested with the other media (1, 3 and 5) and the original media (1) to (5) were used in further experiments, as they all supported good growth.

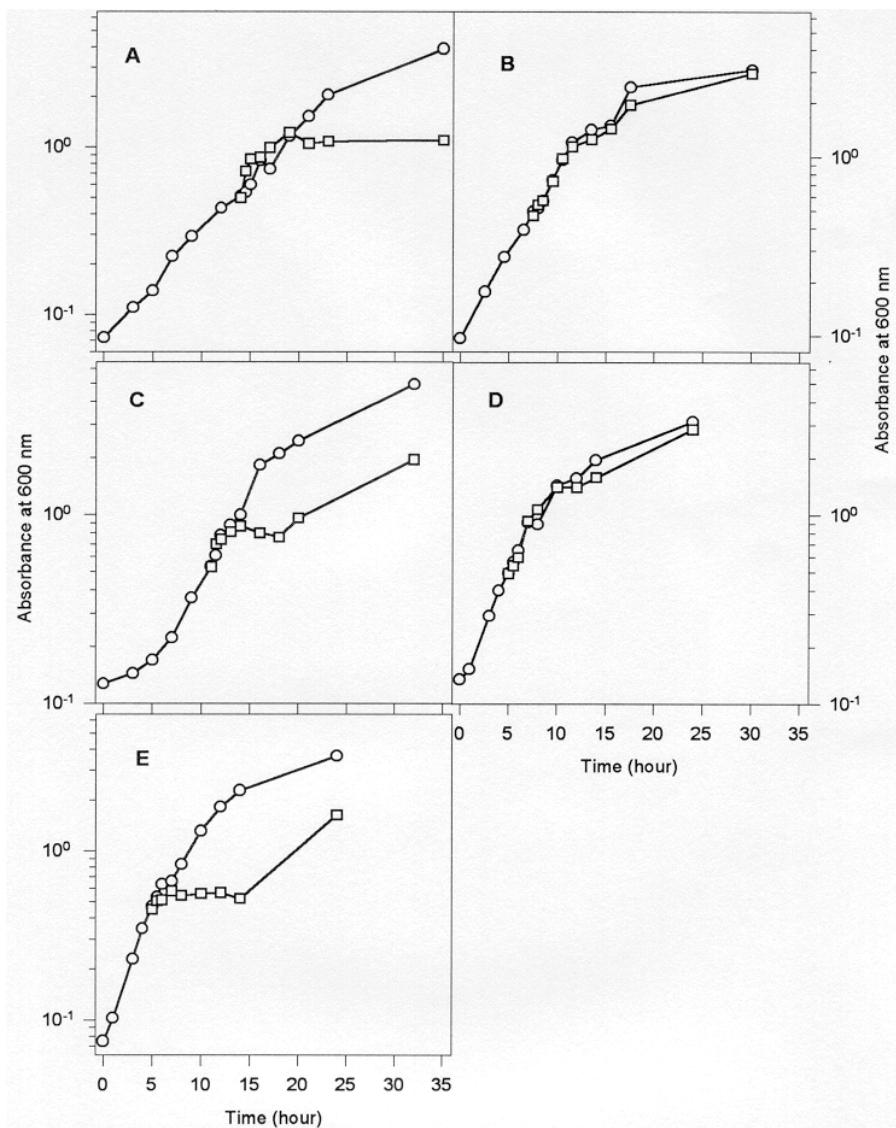


Figure 4.20. Effect on growth of strain VUN10,077 following transfer from 28°C to 37°C in various minimal or defined media: A, medium (1); B, medium (2); C, medium (3); D, medium (4) and E, medium (5). Cultures were initiated at 28°C (○) and growth was continued after heat elevation (□) respectively.

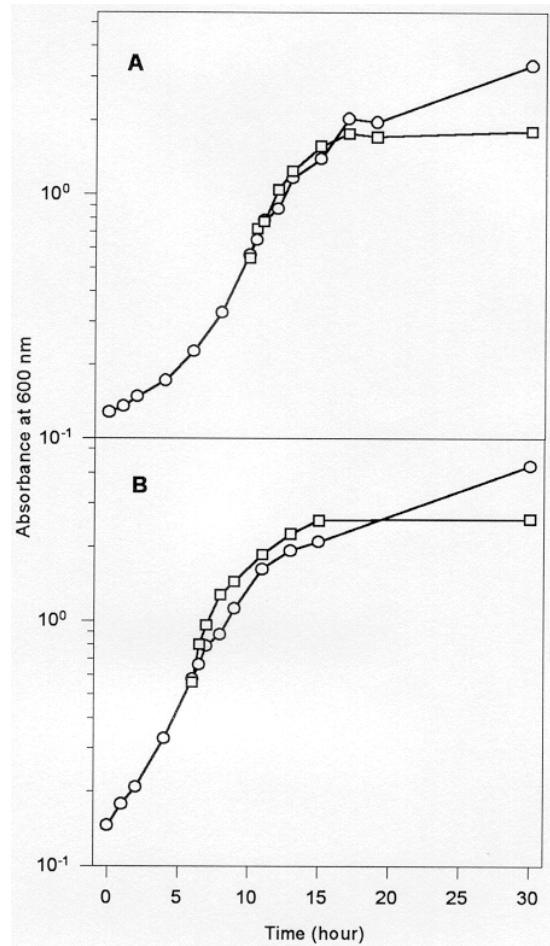


Figure 4.21. Effect of growth in medium (2) and medium (4) when purines and pyrimidines were added for strain VUN10,077 following transfer from 28°C to 37°C. A, medium (2) and B, medium (4). Cultures were initiated at 28°C (○) and growth was continued after heat elevation (□) respectively.

4.3.2.2 Protein profiles using various defined minimal media for VUN10,077

Heat shock trials were performed using defined media to examine whether stress proteins were made and whether nutrient deprivation (relative to BHI) caused changes in the protein profiles, generated by growing cells. Samples were subjected to analysis by 1-D SDS PAGE and cells were lysed by boiling on a flame, with gels visualised using Coomassie staining. When strain VUN10,077 was cultured in the various defined or minimal media (1) to (5), protein profiles changed depending on the medium used (Figure 4. 22). The medium (1) protein profile showed differences in the following proteins: 98, 26, and 17 kDa proteins increased, and a 58 kDa protein declined as a proportion of the total protein. The 26 kDa and 17 kDa proteins were induced late in the growth cycle and the 26 kDa protein increased relatively. For medium (2), the following proteins changed: 98, 47, 38, 17 and 15 kDa. The 47 kDa and 38 kDa proteins were increased proportionally. For medium (3), the following proteins changed: 98 and 38 kDa, and 75 kDa proteins were made at relatively high levels and a 70 kDa protein was relatively less produced. For medium (4), the following proteins changed: 98, 38, 26, 17 and 15 kDa. The 38 kDa, 26 kDa, and 17 kDa protein were relatively increased during growth. For medium (5), the following proteins changed: 26, 24.5 and 20 kDa, and 60 kDa proteins were produced at relatively high levels compared to the other tested media. The 26 kDa, 24.5 kDa, and 20 kDa proteins were relatively increased, while a 22 kDa protein declined during growth. Although the protein profiles differed slightly between the media used, the level of induction or decline in the relative proportions of particular proteins over the growth cycle was hardly noticeable and major protein changes were not seen when the gels were compared to heat stress conditions using the same media.

Heat shock increased 70 kDa, 58 kDa and sHSP (range of 20 kDa) proteins relatively and proportionally in all the tested media, including BHI, when temperature was shifted from 28°C to 37°C. Through medium (1) to (5) and BHI, significant differences were seen in expression of these HSPs compared to their controls. In medium (1), the following proteins were also relatively induced: 35, 20 and 19.6 kDa, and the major HSPs proteins declined during growth. Gel showed less intensity of protein bands when compare to the control, which may be because cells stop growing or entered death phase after heat shock, despite A₆₀₀ remaining constant. The 20 and 19.6 kDa proteins were expressed throughout the growth cycle after heat transfer, while a 36.5 kDa protein increased relatively late in growth and was induced less as compared to these smaller proteins. Most of synthesized major HSPs (70 kDa, 58 kDa and 55 kDa) declined during growth compared to the controls but the

Figure 4.22. Heat shock responses seen following growth of strain VUN10,077 in defined media or BHI broth. Heat stress occurred at mid-exponential phase as shown in Figure 4.20, transferring from 28°C to 37°C, and samples were taken periodically after stress for tests and controls (28°C throughout growth). Cells were lysed by boiling and equal cell numbers (judged from culture A₆₀₀ readings) were loaded. The molecular weight makers (Bio-Rad) were 97, 66, 44, 31, 21 and 14 kDa, except in panel J, where these were 116, 97, 66, 45, 31, 21 and 14 kDa (Bio-Rad).

A, medium (1) stressed; B, medium (1) control,

C, medium (2) stressed; D, medium (2) control,

E, medium (3) stressed; F; medium (3) control,

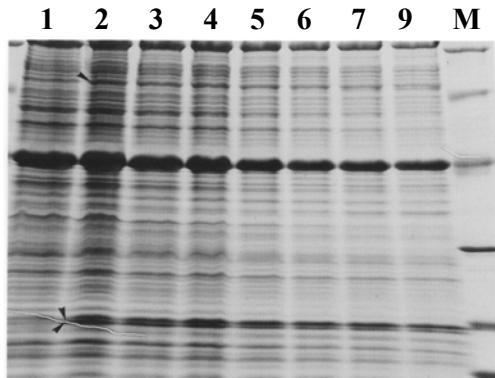
G, medium (4) stressed; H, medium (4) control,

I, medium (5) stressed; J, medium (5) control,

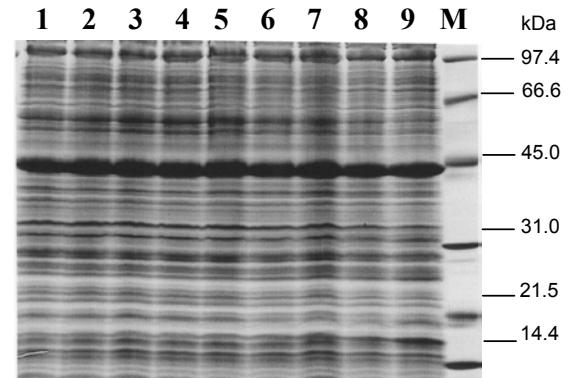
H, BHI stressed; I, BHI control.

Samples were taken at time 0 (lane 1), 30 minutes (lane 2), 1 hour (lane 3), 2 hours (lane 4), 3 hours (lane 5), 5 hours (lane 6), 7 hours (lane 7), 9 hours (lane 8), overnight culture (lane 9) and M designated the markers.

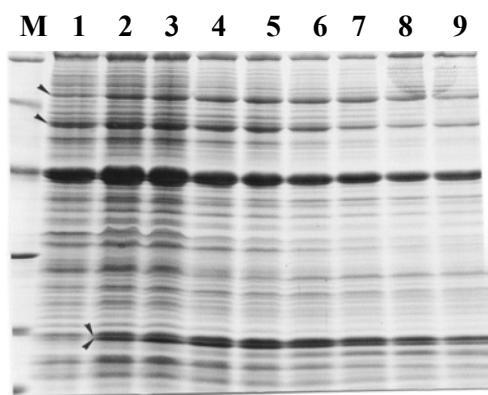
A. Medium (1), stressed



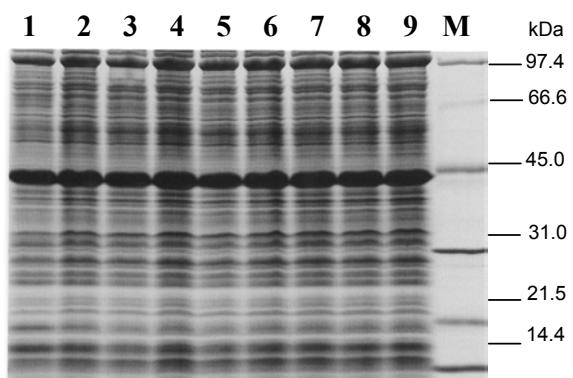
B. Medium (1), control



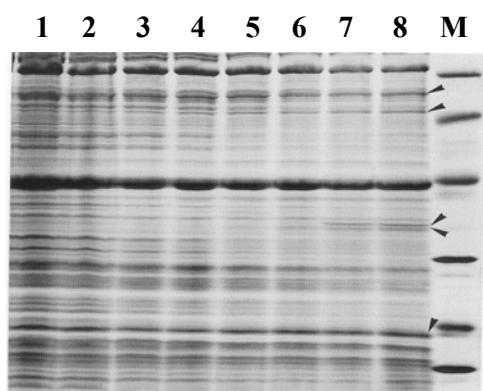
C. Medium (2), stressed



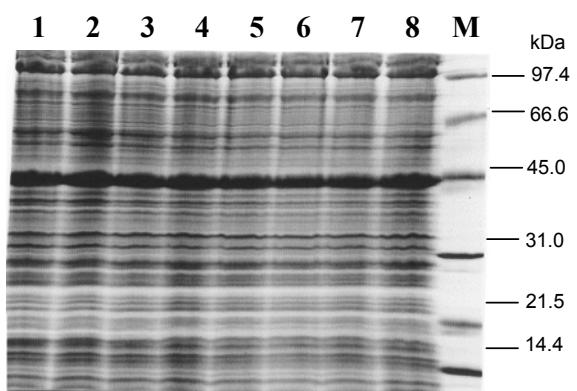
D. Medium (2), control



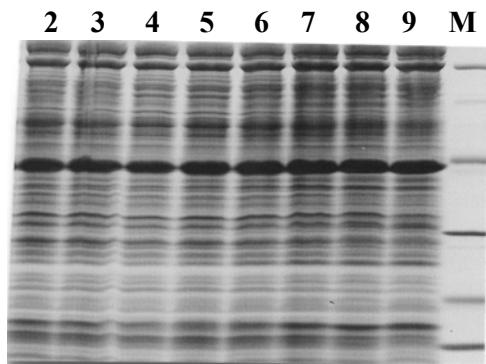
E. Medium (3), stressed



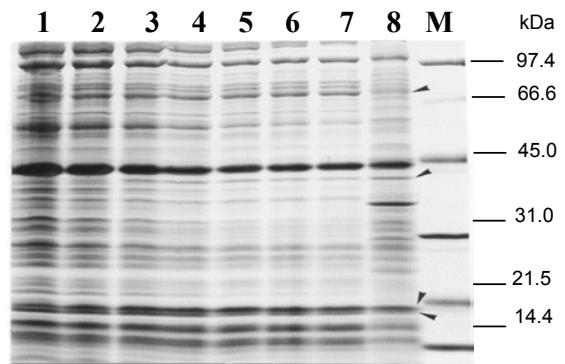
F. Medium (3), control



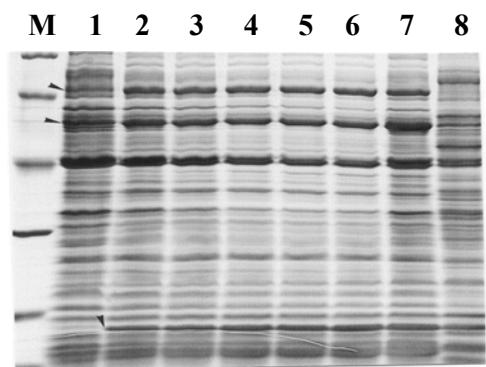
G. Medium (4), stressed



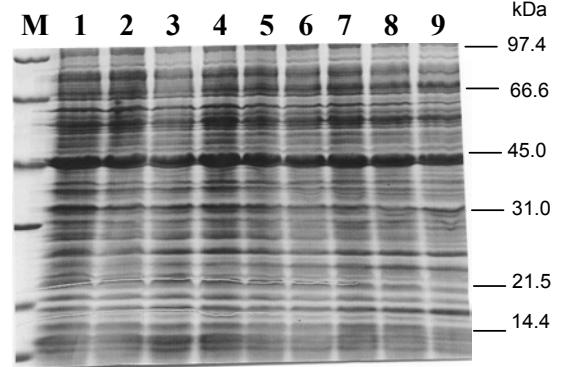
H. Medium (4), control



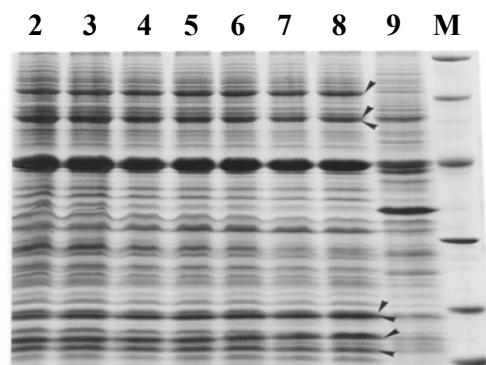
I. Medium (5), stressed



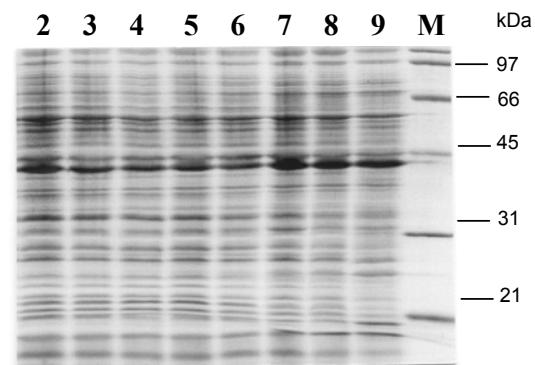
J. Medium (5), control



K. BHI, stressed



L. BHI, control



26 kDa and 17 kDa proteins remained dominant in all samples to late stationary phase. As the cells were no longer growing, this presumed be associated with the degradation of their proteins rather than continued induction of these proteins.

In medium (2), the following proteins were relatively induced: 70, 58, 28, 20, 19.6, 18, 17 and 15 kDa proteins. These proteins were induced within 30 minutes following heat stress and all declined except for the sHSPs in the range of 20 kDa. The 28, 20, 19.6 and 18 kDa proteins were relatively increased whereas the 17 kDa and 15 kDa proteins declined.

In medium (3), following proteins were relatively induced: 75, 70, 35, 34.5 and 20 kDa proteins, and 71, 58 and 55 kDa proteins declined gradually over the sampling period. The 35 kDa and 34.5 kDa proteins increased in the late stages of growth while the 20 kDa protein was synthesised at high levels throughout the growth cycle.

In medium (4), the following proteins were relatively induced: 70, 55, 38, 20 and 19.6 kDa proteins, and 37, 22, and 17 kDa proteins declined, which was similar to trends seen for the 55 kDa and 19.6 kDa proteins. The 70 kDa and 20 kDa proteins were synthesised and relatively increased by heat elevation. The 17 kDa protein was seen in both control and test cells, although a relative increase the 17 kDa protein was not seen for the test, which was as similar to profiles seen for medium (2). Several set of proteins were seen at overnight stressed culture as major proteins, for example, 55.5, 34, 31.5, 32, 29.5 and 17 kDa proteins. Some of these may due to contamination of the late stationary phase cultures and similar appearance of novel proteins late in sampling was seen for medium (5) and BHI overnight cultures.

In medium (5), the following proteins were strongly induced: 70, 58, and 18 kDa proteins. These proteins were synthesized and seen in relatively high proportions throughout the growth cycle. In BHI, the following proteins were synthesised: 70, 58, 20, 19.6, 17 and 15 kDa proteins, and a 17.5 kDa protein declined relatively while other synthesised proteins were relatively increased. When cells were stressed overnight, BHI protein profiles showed a set of proteins which were seen for medium (4). In Figure 4.21, the PAGE gels of boiled cells showed that there were detectable major HSPs produced following heat shock in media (2) and (5), similar to profiles seen for LB medium (see Figure 4.2). These results indicated that strain VUN10,077 showed diverse responses when heat shock was performed during growth in more minimal media. As the medium contained more supplements the strength of induction of heat shock proteins (GroEL/DnaK/GroES) increased, whereas under more

Table 4.3. Summary of proteins which showed different levels of synthesis in various define or minimal media when strain VUN10,077 was cultured at 28°C. Figures represent molecular weight (kDa).

Medium (1)	Medium (2)	Medium (3)	Medium (4)	Medium (5)
98	98	98	98	-
-	38 ↑	38	38 ↑	-
26*	-	-	26 ↑	26 ↑
-	-	-	-	24.5 ↑
-	-	-	-	22 ↓
-	-	-	-	20 ↑
17*	17	-	17 ↑	-
-	15	-	15	-

* Proteins synthesised in relatively high proportion late in the growth cycle.

↑ Protein synthesised at relatively increased or decreased (↓) levels during the growth cycle.

- No change during growth or protein not seen at all.

minimal conditions, these proteins were not elevated to these proportionally high levels.

Although strain VUN10,077 had a different protein profiles in various media without stress, the degree of induction level was minor compared with protein profiles arising after heat stress. Table 4.3 shows protein differences in terms of the visible relative increases or decreases in various minimal or defined media without heat shock, where the media differences observed occurred in proteins of < 40 kDa range. Unlike medium (5), media (1), (2), (3), and (4) produced a 98 kDa protein when cultured at 28°C and this higher molecular weight protein was also not seen for BHI control culture protein profiles. Medium (5) produced a 20 kDa protein and the synthesised level relatively increased; a similar 20 kDa protein was also seen for cells grown in BHI. 17 kDa and 26 kDa proteins appeared in overnight cultures of medium (1), which was the most minimal medium tested, suggesting that these proteins were made in responses to stress in these older cultures. The 17 kDa protein was also seen for cells grown in media (2) and (4) and the 26 kDa protein was seen in cells grown in media (4) and (5), with relative increases observed following culture aging. A 15 kDa protein was synthesised at relatively high levels in media (2) and (4) compared to media (1), (3) and (5), and was produced throughout the growth cycle for strain VUN10,077. Media (2), (3) and (4) produced a 38 kDa protein, although this protein was less obvious in medium (3), and was relatively increased in media (2) and (4). A 38 kDa protein was seen to be induced by cadmium stress in rich media for strain VUN10,077 but the relationship between these proteins remains unexplained.

Table 4.4 shows proteins synthesized upon heat elevation in the various defined or minimal media plus BHI for strain VUN10,077, compared to their respective controls. The DnaK-like, 70 kDa protein was visibly increased in Coomassie-stained gels for cells grown in media (2), (4), (5) and BHI, while induction of this protein was not prominent in media (1) and (3), which were the most minimal media tested. The GroEL-like, 58 kDa protein seen relatively increased in media (2), (5) and BHI, while cells from the other tested media showed decreased proportions of this protein. Interestingly, the GroEL-like protein was not relatively increased in media (1) and (3) although heat shock caused a definite impact on growth, which may indicate that cells were not responding to heat stress due to nutrient deprivation. In media (2) and (4), the growth rate was not affected by heat stress, but protein profiles were changed dramatically throughout the growth cycle. Temperature upshift using BHI medium was examined in parallel and samples were taken periodically then samples were treated the same as minimal media. The 70 kDa and 58 kDa proteins were the major HSPs seen. Interestingly, there also appeared to be sHSPs, in the range of 20 kDa,

Table 4.4. Comparison of proteins which showed proportionate increases or decreases in various media when strain VUN10,077 was treated with heat shock from 28°C to 37°C. Heat-induced proteins were compared with their controls sampled similarly. Figures represent molecular weight (kDa).

Medium (1)	Medium (2)	Medium (3)	Medium (4)	Medium (5)	BHI
-	-	75	-	-	-
70 ↓	70 ↑	70	70 ↑	70 ↑	70 ↑
-, 58 ↓	58 ↑	-, 58 ↓	58 ↓	58 ↑	58** ↑
38	38	-	38↑	-	-
36.5	36.5	-	-	-	-
35	-	35*	-	-	-
-	-	34.5*	-	-	-
-	28 ↑	-	-	-	-
20 ↑	20 ↑	20 ↑	20 ↑	-	20 ↑
19.6 ↑	19.6 ↑	-	19.6 ↓	-	19.6 ↑
-	18 ↑	-	-	18 ↑	-
17 ↓	17 ↓	-	17 ↓	-	17 ↑
-	15↓	-	-	-	15↑

* Proteins synthesised in relatively high proportion late in the growth cycle.

** This protein produced as doublet, 58 and 58.5 kDa.

↑ Protein synthesised at relatively increased or decreased (↓) levels during the growth cycle.

- No change during growth or protein not seen at all.

universally present in all tested media when stress occurred in strain VUN10,077. Two proteins of MW 20 kDa and 19.6 kDa proteins were co-synthesised in media (1), (2), (3) and (4), which was also seen for heat-stressed cells lysed by sonication or boiling methods for cells from BHI, although there was no visible doublet in medium (3). It was interesting that the doublet was not seen to be induced in medium (5) following heat stress, but a 18 kDa protein was induced, which was also induced in medium (2). A 17 kDa protein was induced initially after heat elevation in media (1), (2) and (4) then this declined proportionally. In BHI, increases in a 15 kDa protein were obvious but this protein was not prominent in any other media. A protein of MW 38 kDa was also seen to change in media (1), (2) and (4) and other proteins in a similar MW range (34.5-36.5) also increased after heat shock or late in the growth cycle. As the 38 kDa protein was also seen to vary in the control cultures depending on the medium type, this raise the question of whether these proteins were responding to heat stress or nutritional changes during the growth cycle, or a combination of the two effects.

It is possible that there were other stress-induced proteins produced which were not detected in this type of analysis, but 1-D gels were sufficient to provide a picture of the major heat-induced proteins, so 2-D gels with radiolabelling was not pursued. A size of protein synthesised may vary approximately \pm 2 kDa but all of these size determinations were estimated from the molecular weight markers on the 1-D gels which run in parallel. These results showed that media (2) and (5) could be used successfully to demonstrate heat-shock responses similar to those seen in complex media, such as BHI. Therefore, medium (2) was employed for ^{35}S -methionine radiolabelling initially as this medium was used by Allan *et al.* (1988) for *Ps. aeruginosa*, and strain VUN10,077 had similar growth rates although *St. maltophilia* grew poorly in this medium (see section 4.3.3.1). Chemical stresses were also performed in medium (5), as it provided relatively fast growth rates and heat shock responses were similar to rich media for strain VUN10,077 to evaluate ^{35}S -methionine incorporation into newly synthesized proteins following heat shock and other chemical stresses (see the following section).

4.3.3 Pulse-chase radiolabelling using ^{35}S -methionine

4.3.3.1 Heat shock responses detected using radiolabelling in strain VUN10,077 and related species

Heat shock experiments were performed in *Ps. aeruginosa*, *Ps. fluorescens*, *St. maltophilia*,

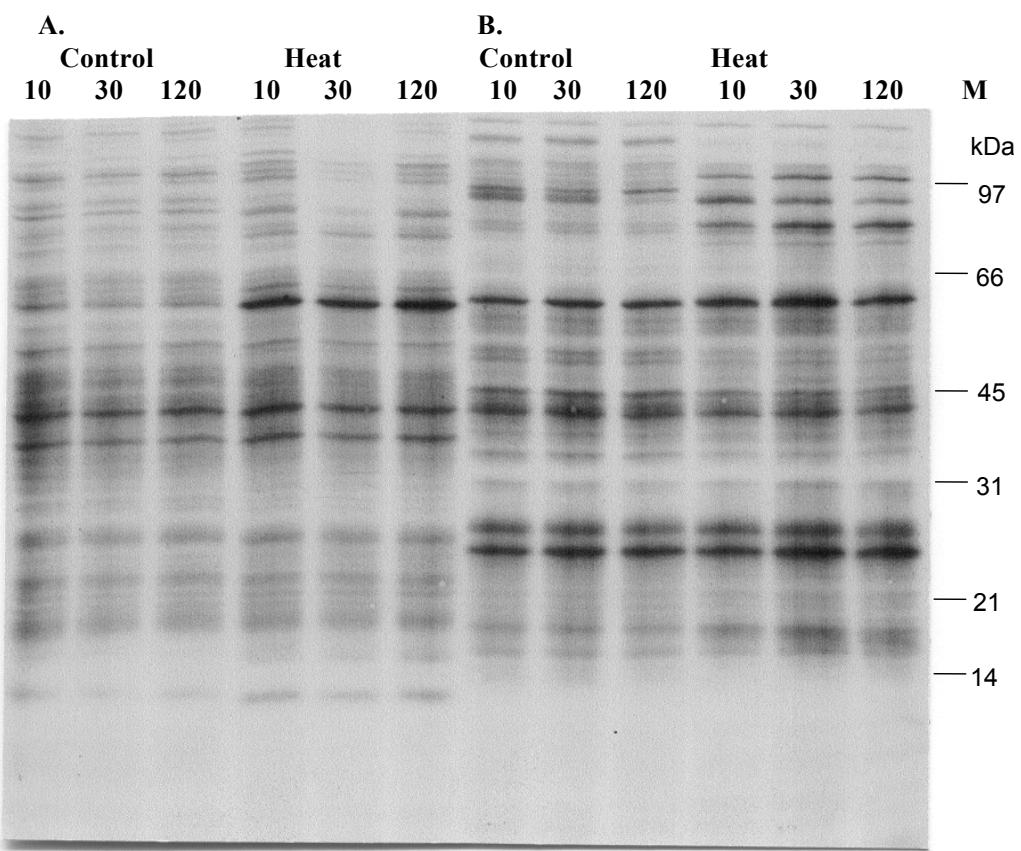
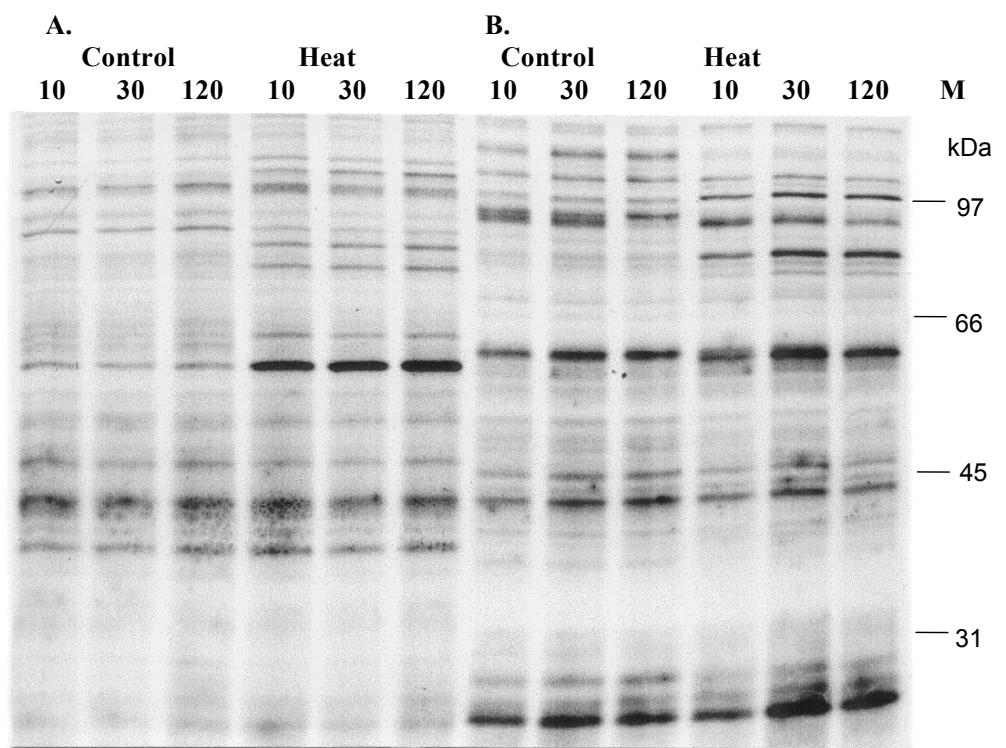
strain VUN10,077 and *E. coli* HB101 using experimental protocols described in materials and methods sections 2.2.3 and 2.4.3.1. Briefly, cultures were harvested at mid-exponential phase in medium (2) and concentrated 10-fold then cells were held for approximately 1 hour in fresh medium at 28°C. After heat shock, samples were taken periodically and labelled as described in section 2.4.3.1. Unlike the protocol used by Allan *et al.* (1988), cells were concentrated because *St. maltophilia* grew poorly in medium (2) while *Ps. aeruginosa* and *E. coli* HB101 grew well. Similar methods have been employed recently to detect induction of the stress proteins. A starvation conditions were achieved resuspended in pre-heated AB minimal medium (plus 0.02% glucose) after harvested from growing cultures in LB (Givskov *et al.*, 1994), similar to acid adapted conditions (Hartke *et al.*, 1996). DnaK heat shock protein was induced using *E. coli* quiescent or resting cells (Cha *et al.*, 1999). In Cha *et al.* (1999) experiments, cells were grown to early log phase at 30°C in LB broth then washed with phosphate saline buffer after harvesting and resuspended in phosphate saline buffer before heat stress tests. Similar approaches have also been used to induce HSPs in yeast (Yapping Chen, CBFT, personal communication).

Medium (2) was similar to the minimal medium used by Allan *et al.* (1988) for *Ps. aeruginosa* and this consisted of M9 basal salts medium plus 0.05% (w/v) casamino acids. In the previous section 4.2.1, a 58 kDa protein was seen to be induced across the tested strain VUN10,077 and related species in Coomassie stained gels while a 70 kDa protein was not seen when cells were lysed by sonication. However, these proteins were easily seen when cells were lysed by boiling on a flame (see section 4.2.2.1), which was the method used by Allan *et al.* (1988), Walkup and Kogoma (1989), and Jensen *et al.* (1993) for *Ps. aeruginosa* and *E. coli*. Therefore, boiling lysis method was employed and total soluble protein was analysed using ³⁵S-methionine labelling for *Ps. aeruginosa* and *E. coli* HB101 prior to trials in the locally isolated strains (Figure 4.23). The commonly found HSPs of sizes corresponding to GroEL and DnaK, were newly synthesized within 10 minutes when whole boiled cell lysates were analysed. *Ps. aeruginosa* synthesised the following proteins: 100.5, 99.5, 98, 97, 85, 75, 71, 61, 59, 20 and 19 kDa proteins. Allan *et al.* (1988) reported heat induced proteins of 86.4, 76, 70.4, 60.6 and 15.7 kDa in molecular weight, so that these proteins of MW 76, 70.4 and 60.6 kDa most likely corresponded to the 75 kDa, 71 kDa and 59 kDa proteins detected here, although the protocols used were modified. The 59 kDa protein was synthesized as a major protein, as seen for Coomassie stained gels which detected a 58 kDa protein (see Figure 4.2) and corresponding to the 60.6 kDa protein reported by Allan *et al.* (1988) for *Ps. aeruginosa*. In contrast, the 100.5 kDa, 99.5 kDa, 98 kDa and 95 kDa proteins were seen clearly in this type of analysis while Allan *et al.* (1988)

Figure 4.23. 1-D SDS PAGE of proteins synthesized during constant temperature growth (28°C) or upon temperature elevation (37°C). At each time point during growth in medium (2), 0.1 ml portions of cells were taken and labelled with ^{35}S -methionine for 1 minute and chased with cold methionine for 1 minute. Cells were disrupted by boiling in loading buffer. Total proteins were analysed using 10% homogenised (top) and 10-12% gradient (bottom) gels. Each lane received 100,000 cpm (top) and 200,000 cpm (bottom) of labelled protein, and the films were exposed for up to 5 days at -80°C. The number indicated the time of sampling (minutes). Molecular weights of markers (M) are given in kDa (top to bottom; 97, 66, 44, 31, 21 and 14) and bands corresponds from top to bottom which was Commassie stained separately.

A, *Ps. aeruginosa*.

B, *E. coli* HB101.



did not report detecting these. It may be possible that the difference in protocols caused induction of these high molecular weight proteins, as the temperature change used was from 28°C to 42°C (Figure 4.23), whereas Allan *et al.* (1988) used from 30°C to 45°C for *Ps. aeruginosa*. The 75 kDa and 60 kDa proteins were deemed by Allan *et al.* (1988) DnaK and GroEL respectively from Western blot analysis when using polyclonal antibodies to *E. coli* DnaK and GroEL. These observations indicated that the major heat-induced protein is GroEL for *Ps. aeruginosa*, which was seen for both ^{35}S -labelling and Coomassie stained gels, and similar major HSPs were seen using different labelling conditions. For *E. coli* HB101, the following proteins were synthesised following heat shock for up to 120 minutes: 99, 92, 85, 75, 60, 20 and 19 kDa proteins in this type of analysis.

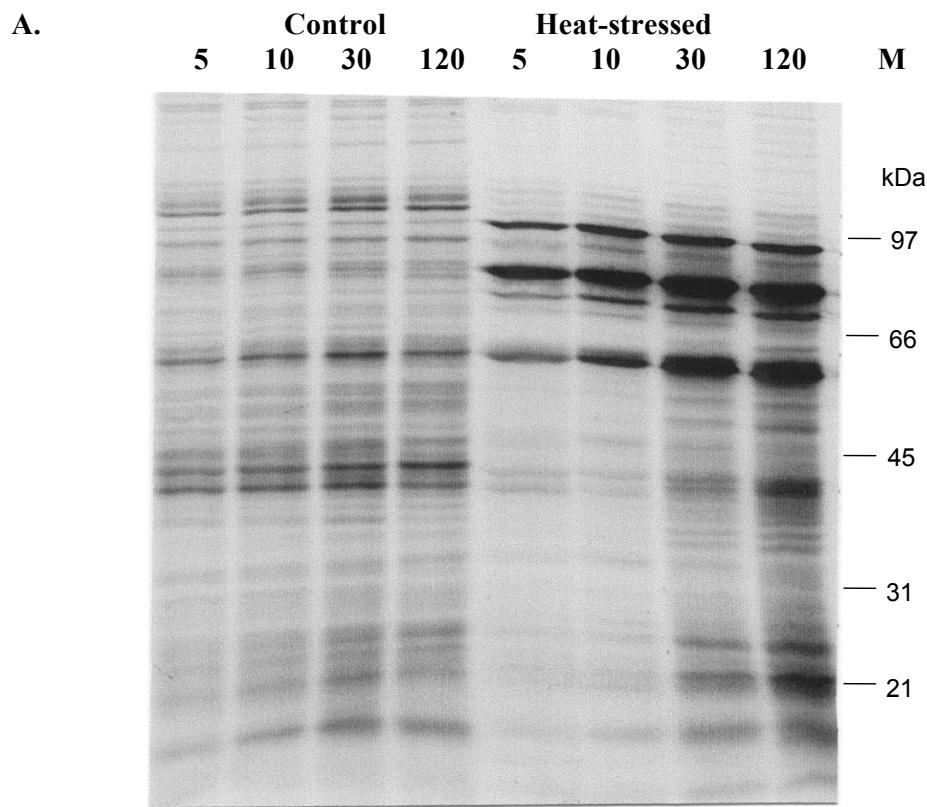
The labelling method used was validated by comparisons between the control and heat-stressed cultures, as described in the above. Therefore, ^{35}S -methionine labelling experiments were performed in the locally isolated strains and *Ps. fluorescens* (Figure 4.24). Major HSPs were synthesized within 5 minutes of heat shock in the strain VUN10,077 and related species. Unlike *Ps. aeruginosa*, *Ps. fluorescens* synthesized less heat-induced proteins within 10 minutes as follows: 98, 84, 76, and 63 kDa proteins, and 66, 58, 55, 33, 30, 27 and 25 kDa proteins were induced after 30 minutes heat-stress. For *St. maltophilia* the following proteins were synthesized: 98, 93, 75, 62 and 54 kDa proteins. The heat-induced proteins were recognised within 1 minute of heat elevation from 28°C to 37°C relative to controls, and relatively high levels of induction were visualised within 5 minutes, with synthesized levels increasing over the sampling period for *St. maltophilia*. The 54 kDa protein was synthesized as the major heat-induced protein, which was similar to the 56 kDa protein seen on Coomassie stained gel (see Figure 4.2) and also similar to the 59 kDa protein in *Ps. aeruginosa* in terms of level of induction. For strain VUN10,077, the following proteins were synthesized: 98, 94, 75, 62.5 and 62 kDa proteins, and the control showed a doublet in the MW range of 44 kDa, which was similar to Coomassie stained controls (see Figure 4.22). Proteins equivalent to DnaK and GroEL (based on MW) proteins were seen, and another doublet occurred in the range of 62 kDa for strain VUN10,077 which may correspond with a 58 kDa protein seen for cells which were heat shocked following growth in BHI. Proteins of MW corresponding to GroES were not seen for the tested strain VUN10,077 and related species, except for *Ps. aeruginosa*. Unexpectedly, low molecular weight proteins which were observed as major heat-induced proteins on Coomassie stained gels were not seen in this type of analysis. It was an interesting observation that *Ps. fluorescens*, *St. maltophilia* and strain VUN10,077 synthesised less heat-induced proteins (4 to 5 proteins) within 10 minutes compared to proteins synthesized by *E. coli* and *Ps. aeruginosa* (7 to 11 proteins),

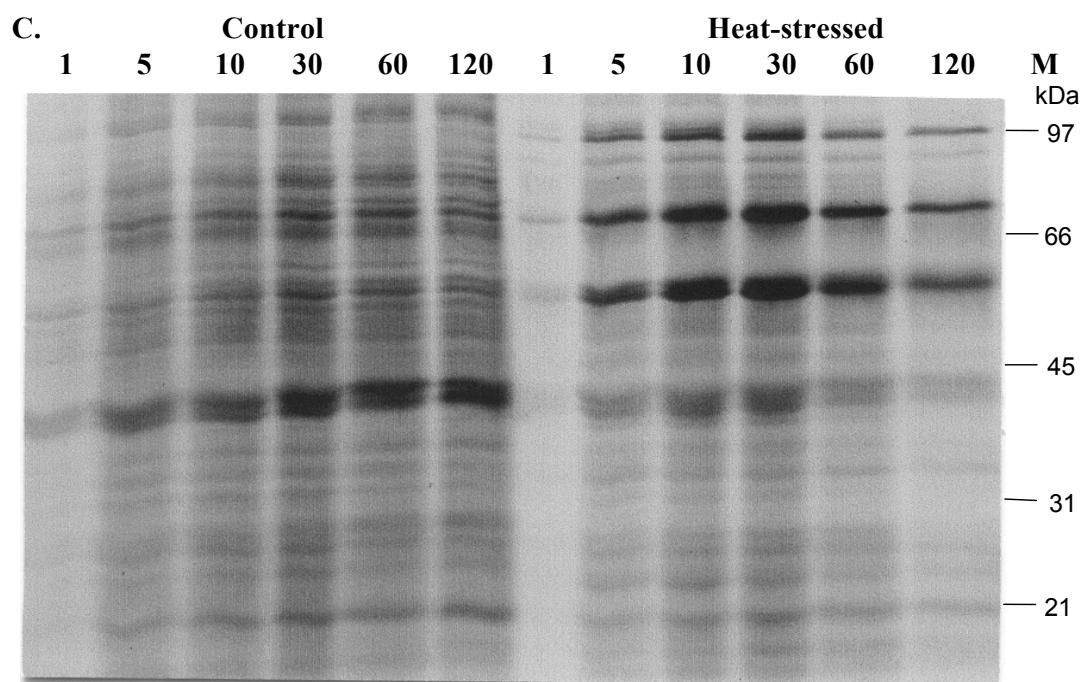
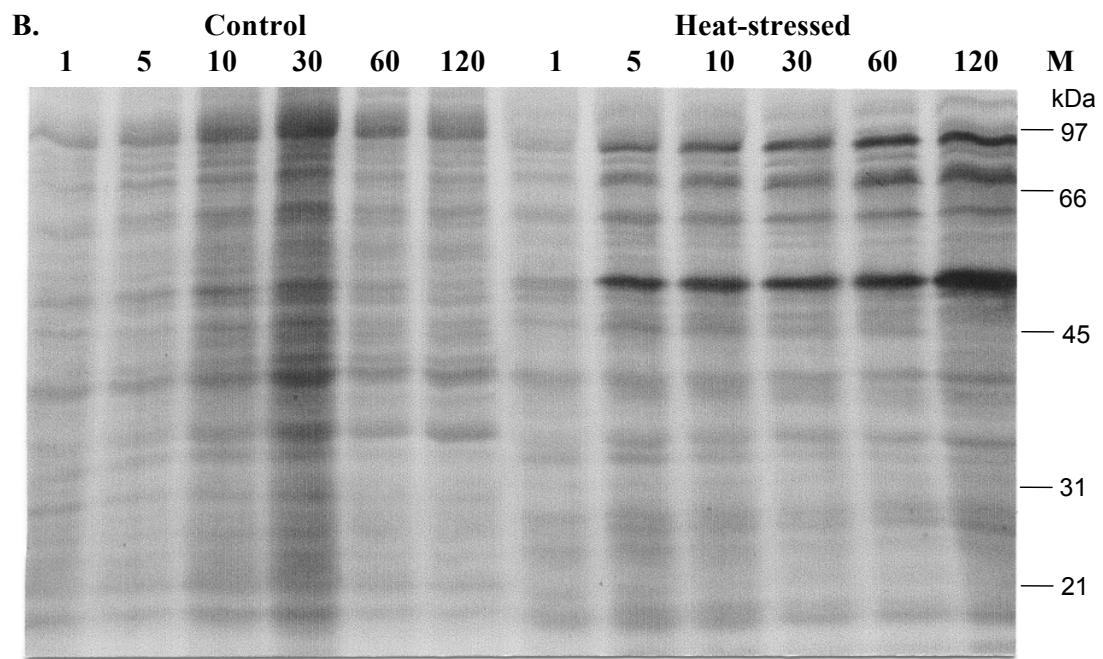
Figure 4.24. 1-D SDS PAGE of proteins synthesized during constant temperature growth or upon temperature elevation using 12% homogeneous gel. The experimental protocol was the same as in Figure 4.19. The number indicated the time of sampling (minutes). Molecular weights of markers (M) are given in kDa (top to bottom; 97, 66, 44, 31, 21 and 14) and bands correspond from top to bottom which was Coomassie stained separately. Each lane received 100,000 cpm of labelled proteins, and the films were exposed up to 5 days at -80°C.

A, *Ps. fluorescens*.

B, *St. maltophilia*.

C, strain VUN10,077.





which may be related to their environmental habitats or temperature tolerance: the strains of *Ps. fluorescens*, *St. maltophilia* and VUN10,077 were not capable of growing above 37°C and their optimal temperature was 28°C, although strain VUN10,077 could survive at 37°C (see Table 3.3). It was interesting to note that ^{35}S -labelling detected production of a 98 kDa protein following heat shock of strain VUN10,077 in medium (2), although this was not detected in Coomassie stained gels (Table 4.2). These results indicated that cells of all the strains tested apparently responded to heat stress conditions but that their reaction mechanisms and behavior differed under the given conditions.

4.3.3.2 Detection of proteins induced by chemical stressors using pulse-chase radiolabelling

Although the above method worked, the step involving cell concentration made it difficult to evaluate the kinetics of response during growth. Methods used by Allan *et al.* (1988) were not explicit but involved 0.5 ml samples of culture being shifted to 45°C and shaken at 50 r.p.m.. The samples were labelled for 1 minute and chased for 1 minute with 0.5 ml of temperature-equilibrated medium supplemented with cold methionine and stopped by addition of ice-cold chloramphenicol. Several attempts were made to replicate this approach using strain VUN10,077 in medium (5), where ^{35}S -methionine was added to cells in 0.5 ml aliquots, heat shocked by transfer to 37°C but poor incorporation was observed when labelling for 1 minute and labelled proteins were not resolved on 1-D SDS-PAGE, in contrast to results reported by Allan *et al.* (1988). This problem was addressed as described in the following: cells were cultured in 100 ml volumes in medium (5) at 28°C. At mid-exponential phase, cultures were either kept at 28°C or transferred rapidly to 54°C for 1 minute to elevate the temperature rapidly to 37°C. For both tests and controls, several 0.5 ml samples were removed then 10 µl of ^{35}S -methionine added to each and labelled at 37°C. Cold methionine was added to chase and labelling was stopped by addition of ice-cold chloramphenicol then ^{35}S -methionine incorporation examined. The labelling time was examined similarly for controls. High ^{35}S incorporation occurred in 3 minutes in both test and control samples. Strain VUN10077 was cultured using its optimal defined medium (5) composed of M9 basal salts, trace elements, vitamins and casamino acids. When the culture was heat-stressed, the amount of ^{35}S -methionine incorporated during pulse labelling declined when the time period for labelling increased to 3 minutes (Figure 4.25). In contrast, when cells were not heat stressed, the amount of label incorporated continued to increase with prolonged incubation. A pulse-labelling period of 3 minutes was therefore selected for

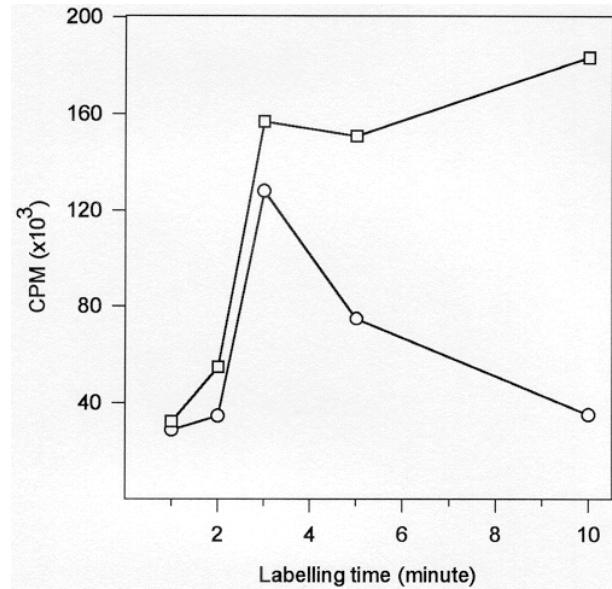


Figure 4.25. Optimization of ^{35}S -methionine labelling time in strain VUN10,077 using medium (5). Cultures were grown at 28°C and temperature elevated to 37°C at mid-exponential phase, and samples were taken after 1 hour stress (○) and control samples were taken at the same time (□). Several 0.5 ml samples were removed and each supplied with 10 μl of ^{35}S -methionine. After time periods of 1, 2, 3, 5 or 10 minutes at ambient temperature, cold methionine was added and cells stored on ice before the level of incorporated was measured (see section 2.4.3.3).

analysing cultures stressed by heat or exposure to chemicals in medium (5). However, when several trials were conducted on heat-stressed cells labelled at 37°C, labelled proteins could hardly be detected on SDS-PAGE gels, even when the labelling period was extended. Consequently, the temperature used for pulse-labelling was dropped to $\leq 28^{\circ}\text{C}$, even after heat shock. Under these conditions, label was incorporated and ^{35}S -proteins were detected on gels. The labelling temperature was not specified by Allan *et al.* (1988).

Strain VUN10,077 showed a relatively fast growth rate in medium (5) compared to the other tested defined or minimal media. When compared to the growth rate in BHI, μ_{max} 0.625, medium (5) had a μ_{max} of 0.476, which was similar to growth in LB (μ_{max} 0.455) at 28°C. Although medium (5) gave a similar growth rate to LB, lag phase was significantly longer than seen for LB. A similar pattern occurred when comparing LB and BHI, as LB had a 50% longer lag phase compared to BHI broth. It was interesting that VUN10,077 cells reflected different heat shock kinetics for the different media used when they were subjected to sudden temperature upshift, as A_{600} readings were higher in the rich media in the order of BHI, LB and medium (5) (see Figure 4.1 and 4.19). However, the heat induced proteins seen were similar for BHI and medium (5) (see Table 4.2 and 4.4). Accordingly, the chemical stress conditions selected to study the kinetics of induction of HSPs in medium (5) were based on the results obtained for chemical stresses in BHI (see section 4.2.2). Strain VUN10,077 was cultured in medium (5) at 28°C and chemical stresses performed when cultures had reached mid-exponential phase (Figure 4.26). Cultures were exposed to cadmium chloride concentrations of 0.005 to 3.0 g L^{-1} . Absorbance readings obtained were relatively high at concentrations below 0.5 g L^{-1} CdCl₂. Interestingly, pigmentation of colonies on BHIA was discoloured at higher concentrations when viability was confirmed by streaking. The selected test concentration of 0.5 g L^{-1} had an impact on growth and A_{600} increased immediately after addition, which was similar to BHI stressed cultures. NaAsO₂ was added at a concentration of 0.1 g L^{-1} to 1.0 g L^{-1} . All the tested concentrations in this range showed similar growth inhibition trends in medium (5) (Figure 4.26 B), and similar changes in A_{600} were observed for 0.1 g L^{-1} NaAsO₂ in BHI. The lowest tested concentration was chosen to examine pulse labelling for arsenite stress, which was shown to leave cells viable and growing in BHI. Phenol was added at concentrations of 0.005 g L^{-1} to 1.0 g L^{-1} . These concentrations had no significant effect in terms of absorbance readings and A_{600} readings were relatively higher compared to control cultures over for up to 5 hours after stress. Addition of 1.0 g L^{-1} showed A_{600} increases and growth was similar to control culture after stress. Higher concentrations of phenol were not pursued because viable cell numbers

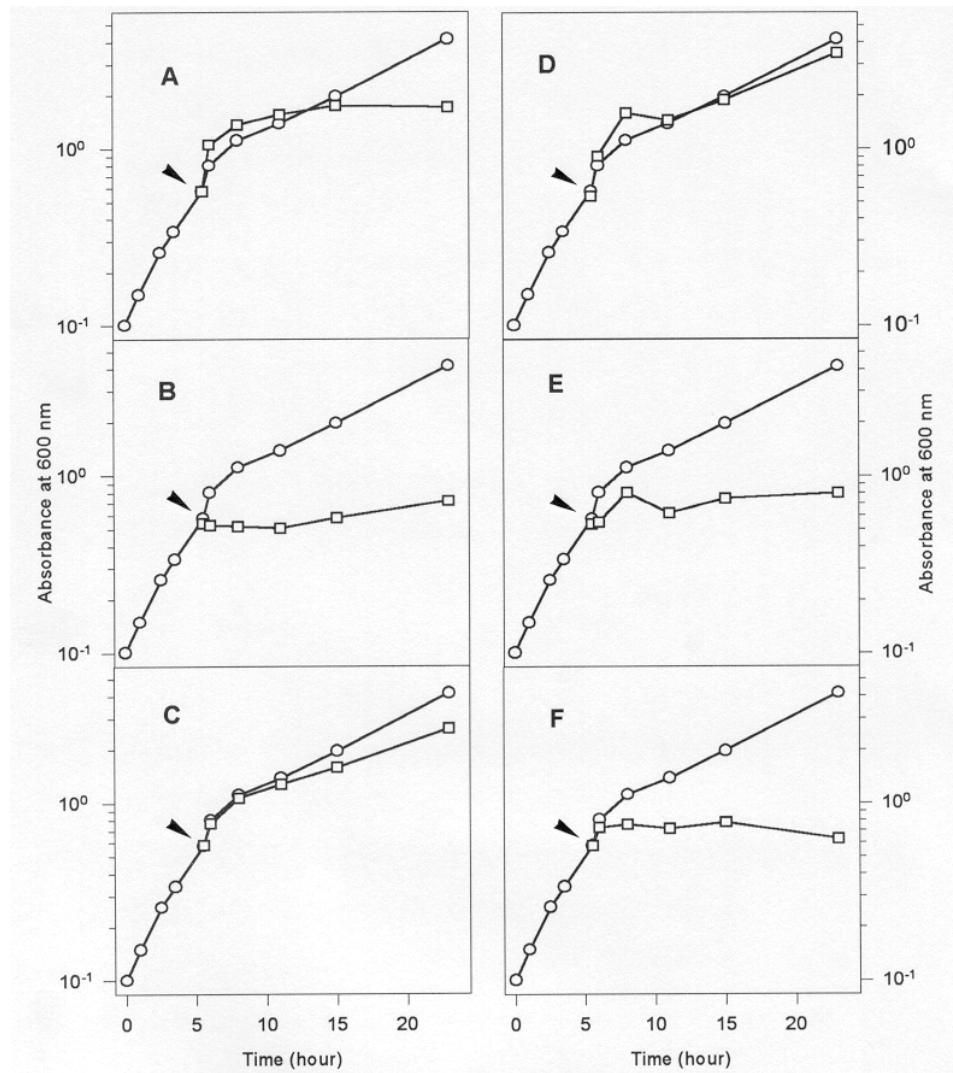


Figure 4.26. Effect on growth of strain VUN10,077 following chemical stresses in medium (5). Cultures were grown to mid-log phase then subjected to each stress (arrow) and growth was continued after chemical stresses (□) and without chemicals (○) at 28°C. A, 0.5 gL⁻¹ of CdCl₂; B, 0.1 gL⁻¹ of NaAsO₂; C, 1.0 gL⁻¹ of phenol; D, 1.0 gL⁻¹ of CuCl₂.2H₂O; E, 0.5 gL⁻¹ of NiCl₂.6H₂O and F, 0.005 gL⁻¹ of HgCl₂.

did not increase at 1.0 gL^{-1} to 1.6 gL^{-1} when strain VUN10,077 was stressed in BHI. Cupric chloride was added at concentrations of 0.5 to 2.6 gL^{-1} , similar to cultures stressed in BHI. Unlike cultures stressed in BHI, absorbance readings were higher at concentrations above 0.5 gL^{-1} compared to controls over the sampling time, which may be because added $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ caused precipitation with the medium (5) components. The lowest tested concentration was chosen for pulse labelling in medium (5). Nickel chloride was added at concentrations of 0.1 to 2.5 gL^{-1} . The concentration of 0.5 gL^{-1} was chosen to examine $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ stress in medium (5) because strain VUN10,077 showed no impacts at concentrations of up to 1.5 gL^{-1} when cells were stressed in BHI. Interestingly, when mercury chloride was added into BHI and medium (5) during mid-exponential phase, there was no significant change in A_{600} at concentrations from 0.1 mgL^{-1} to 0.005 gL^{-1} . Concentrations of 0.005 and 0.001 gL^{-1} HgCl_2 were chosen for use in medium (5) because viable cell numbers declined immediately then recovered in parallel with the control in BHI. Figure 4.26 shows the impact on A_{600} when the selected concentrations of metals were added to medium (5). Cadmium and copper stresses immediately increased A_{600} after stress, and arsenite, nickel and mercury stresses showed similar trends (all decreasing growth rates immediately), whereas phenol and copper stresses had similar absorbance readings compared to controls (Figure 4.26).

When the cultures had reached mid-exponential phase at 28°C , the cells were subjected to each stressor in medium (5) for strain VUN10,077, and selected stressors were as follows: temperature upshift from 28°C to 37°C , 0.5 gL^{-1} of CdCl_2 , 1.0 gL^{-1} of phenol, 1.0 gL^{-1} of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 gL^{-1} of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.005 and 0.001 gL^{-1} of HgCl_2 , and 0.1 gL^{-1} of NaAsO_2 (Figure 4.27). Samples were taken at 5 , 30 , 60 and 120 minutes after stress, noting that total ^{35}S -methionine incorporated was significantly decreased with ongoing stress while control cultures could readily take up the ^{35}S -methionine throughout. This may be because the metabolic activity of cells had declined and they had entered stationary phase and ceased metabolism under these stress conditions. This is consistent with the observed decline in growth rate and drop in viable cell counts seen previously (see section 4.2.2.2). Mercury and nickel stresses were carried out but either the cells did not produce newly synthesized labelled proteins or changes were too subtle to detect using this approach. Initially, 0.005 gL^{-1} HgCl_2 was added into growing cultures at mid-exponential phase and labelled proteins were resolved on 1-D SDS PAGE but gel autoradiography showed no labelled protein, and similar results occurred when 0.001 gL^{-1} HgCl_2 was added. Heat shock of strain VUN10,077 showed more than 11 newly-synthesized proteins in parallel. Cadmium, sodium arsenite and phenol stresses produced the common major HSP and over 6 to 8 new proteins were also produced by the specific stressors (Figure 4.27).

Figure 4.27. Autoradiograms of the polypeptide composition and kinetics of HSP-induction in strain VUN10,077 by heat shock, cadmium, mercury, nickel, phenol, copper and arsenite. A one ml portion of cells was removed from 100 ml of culture after stresses and labelled with ^{35}S -methionine for 3 minutes and chased with cold methionine for 2 minutes. The cells were disrupted by boiling on a flame in gel loading buffer, and labelled proteins were analysed by 12% SDS-PAGE. For gel-electrophoresis, 200,000 cpm of labelled proteins were loaded for each lane and the film was exposed for up to 4 days at room temperature. The number indicated the time of sampling (minutes). Molecular weights of markers (M) are given in kDa (97, 66, 44, 31, 21 and 14) and bands correspond from top to bottom which was Coomassie stained separately.

Control, without stress.

Heat, temperature upshift from 28°C to 37°C.

Cd, 0.5 gL⁻¹ of CdCl₂.

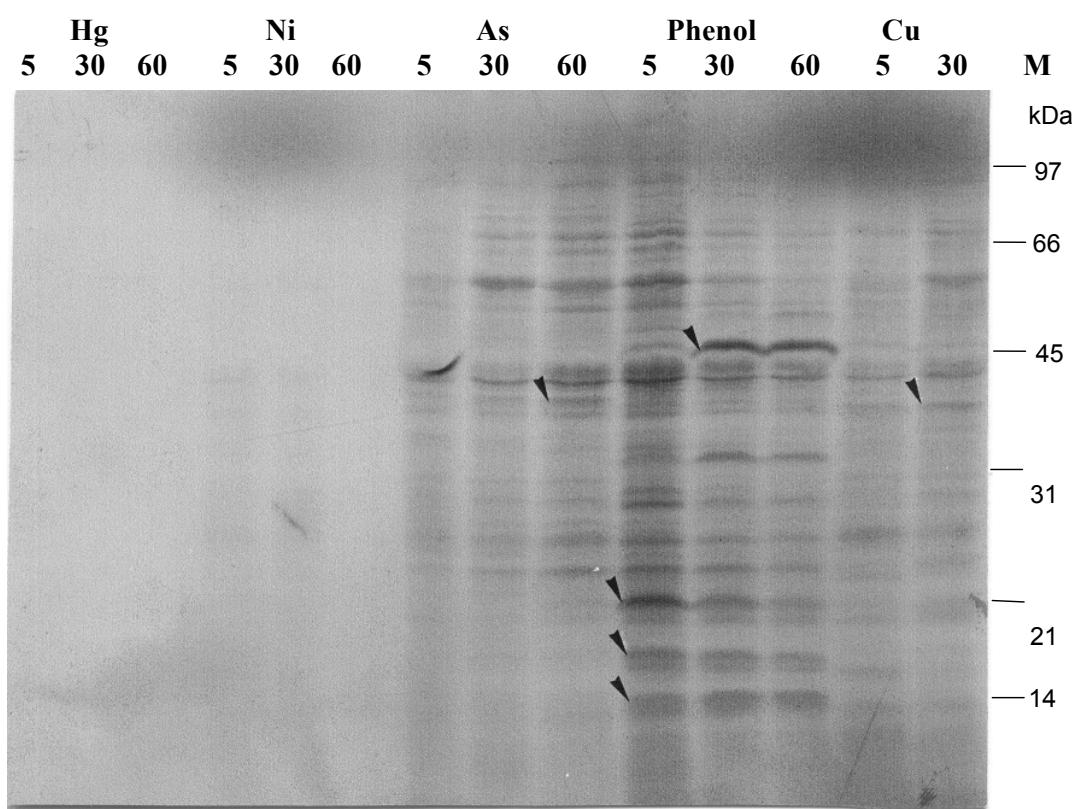
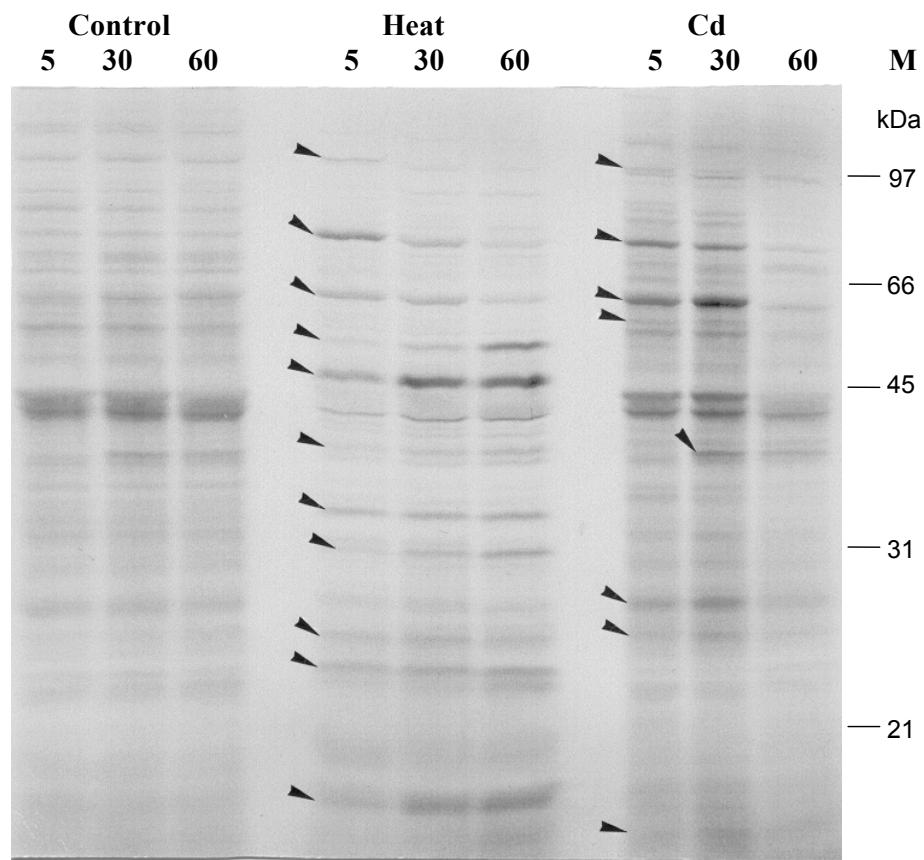
Hg, 0.001 gL⁻¹ of HgCl₂.

Ni, 0.5 gL⁻¹ of NiCl₂.6H₂O.

As, 0.1 gL⁻¹ of NaAsO₂.

Phenol, 1.0 gL⁻¹ of phenol.

Cu, 1.0 gL⁻¹ of CuCl₂.2H₂O.



Heat shock induced proteins were as follows: 98, 72, 62, 55, 50, 34, 32, 27, 25, and 18 kDa proteins. The 98 kDa protein was produced within 5 minutes but declined with prolonged incubation at 37°C. The 72 kDa and 62 kDa proteins were synthesized at relatively high levels within 5 minutes and the relative level of synthesis declined while other proteins became dominant after 1 hour stress, such as the 55 kDa, 50 kDa, 32 kDa and 18 kDa proteins. The 34 kDa, 27 kDa and 25 kDa proteins were induced at a relatively steady level. Doublet proteins in the range of 44 kDa were not seen in medium (5) while it was seen in medium (2) when heat stress was applied. The 55 kDa, 50 kDa and 18 kDa proteins were detected as an acute response to sudden temperature upshift, followed by production of new proteins during prolonged exposure to the elevated, growth permissive temperature. The role of these proteins as chaperonin or otherwise is not known but warrants investigation.

Cadmium stress caused synthesis of proteins as follows: 98, 87, 72, 62, 60, 28, 38, 27 and 17 kDa proteins. The 38 kDa protein was synthesized after 30 minutes exposure time to cadmium, and the 72 kDa and 28 kDa proteins were increased relatively compared to 5 minutes exposure time. A doublet, 44 kDa and 45 kDa, was synthesized when 0.5 gL⁻¹ of cadmium was used which was not seen heat stress. These bands were seen before on Coomassie-stained gels. When prolonged exposure to cadmium occurred for up to 1 hour, the rate of newly synthesized proteins declined, which is consistent with viability and A₆₀₀ decreases seen previously. The 38 kDa protein is unique to cadmium stress compared to the 72 kDa and 62 kDa proteins which overlapped in heat and cadmium stresses.

Sodium arsenite stress induced synthesis of proteins as follows: 87, 72, 62, 40, 38, 29 and 27 kDa proteins, which were seen at exposure time of 1 hour. Arsenite stress did not show a strong induction, which was consistent with the A₆₀₀ readings in medium (5) (see Figure 4.25), although the 72 kDa and 62 kDa proteins were relatively increased when the exposure time was prolonged for up to 1 hour. Phenol stress synthesized proteins as follows: 87, 72, 62, 60, 50, 38, 35.5, 33, 32, 29, 27, 25, 20, 19.5, 16.5 and 16 kDa proteins. Interestingly, phenol stress caused the most diverse response of the thus far tested chemicals and heat treatment and this occurred within 5 minutes. The diverse response may be possibly incurred by adaptation and induction due to utilization of phenol. The 87 kDa, 72 kDa, 38 kDa, 33 kDa, 32 kDa, 29 kDa, 27 kDa and 25 kDa proteins were relatively decreased, and the 50 kDa protein was increased upon increased exposure time, similar to newly-synthesized heat shock proteins, whereas Coomassie-stained gels did not detect this. The exception was 20 kDa and 16 kDa proteins, as seen in Coomassie-stained gels, although these were induced

Table 4.5. Heat shock induced proteins generated using ^{35}S -methionine labelling in medium (2) for the strain VUN10,077 and related species.

Strain	Synthesized proteins
<i>E. coli</i> HB101	99, 92, 85, 75, 60, 20, 19
<i>Ps. aeruginosa</i>	100.5, 99.5, 98, 97, 85, 75, 71, 61, 59, 20, 19
<i>Ps. fluorescens</i>	98, 84, 76, 63
<i>St. maltophilia</i>	98, 93, 75, 62, 54
VUN10,077	98, 94, 75, 62.5, 62

Table 4.6. Stress responsive proteins generated using ^{35}S -methionine labelling in medium (5) for strain VUN10,077.

Stressor	Synthesized proteins
Heat (27°C to 37°C)	98, 72, 62, 55, 50, 34, 32, 27, 25, 18
Cadmium	98, 87, 72, 62, 60, 38, 28, 27, 17
Arsenite	87, 72, 62, 40, 38, 29, 27
Phenol	87, 72, 62, 60, 50, 38, 35.5, 33, 32, 29, 27, 25, 20, 19.5, 16.5, 16
Copper	72, 62, 50

independently. The 60 kDa protein was induced after 1 hour exposure in medium (5). In contrast, copper stress hardly caused production of newly-synthesized proteins for up to 30 minutes exposure compared to other stresses, although a weak response was seen for 72 kDa, 62 kDa and 50 kDa proteins after 30 minutes exposure. When copper was added into medium (5), copper caused precipitation with medium (5) components and the A_{600} increased in all tested concentrations similar to controls so that the effective concentration was probably low. Unlike the control, heat and cadmium stresses, ^{35}S -methionine incorporation during arsenite, phenol and copper stresses was poor and for mercury and nickel stress this was not incorporated. This may imply that the metabolic activity of strain VUN10,077 was low under some chemical stresses or these compounds inhibited ^{35}S -methionine uptake or medium (5) was not suitable due to chemical interaction.

Table 4.5 and 4.6 show a summary of stress responsive proteins under the given conditions, indicating newly-synthesized proteins detected within the given time course. Medium (2) was used for Table 4.5 and medium (5) was used for Table 4.6 and detailed methods were described in section 2.4.3. The ~98 kDa, ~75 kDa and ~60 kDa proteins were universally synthesized for the pseudomonad and related species medium (2) in addition to medium (5) for strain VUN10,077. When cells were under extended heat shock conditions, synthesis of GroEL-like (~62 kDa) and DnaK-like (72-75 kDa) proteins gradually declined while other newly-synthesized proteins were maintained or increased upon ongoing growth. For example, 55 kDa, 50 kDa, 32 kDa and 18 kDa proteins increased for up to 1 hour following heat stress in medium (5). It was found that the 55 kDa, 50 kDa and 18 kDa proteins were overproduced after the decline seen for the known major HSPs, where these proteins may play major chaperonin roles whilst strain VUN10,077 is being heat stressed, although these proteins were not seen when cells were grown in medium (2) and labelled.

Two proteins were induced by heat as well as by phenol stress, 50 kDa and 34 kDa proteins, while two separate sHSPs appeared to be unique to phenol-stressed (MW 20 and 19.5 kDa, 16 and 16.5 kDa). Strong induction of the major HSPs was not seen for arsenite, copper, nickel and mercury stresses on stained gels or for labelled proteins but this may have been due to the experimental design. The concentrations of metals added were selected on the basis of the impacts on A_{600} and viable counts observed when these were added in BHI. When strain VUN10,077 was stressed at mid-exponential phase, A_{600} readings did not increase or addition of the metal ions caused precipitation, which was consistent with failure to incorporate ^{35}S -methionine.

4.3.4 Preliminary genetic analysis of the presumptive *groEL* genes in the strain VUN10,077 and related species used in this study

The primer sets used (*groEL* 9 and *groEL* 10) were designed by Dr M. Serafica (when at CBFT, VUT) using known sequences of conserved regions of the *groEL* gene from several Gram-negative species. The predicted amplification product was ~200 bp. The conditions used for preparation of genomic DNA from *Ps. aeruginosa*, *Ps. fluorescens*, *St. maltophilia* and strain VUN10,077 are described in section 2.3.2 and the PCR reaction conditions in section 2.3.5.3. PCR products were electrophoresed and visualised using 1.2% agarose gel (1× TAE), 80 V for 1 hour. The synthesized products were approximately ~200 bp. These fragments were sequenced directly from PCR products following the kit manufacturer's instructions and autosequenced using the Applied Biosystems model 373A DNA sequencer at Monash University. Comparison of the sequences using GenBank showed 98-100% homology with each other (Figure 4.28) using BLAST program in ANGIS (Altschul *et al.*, 1990). The *St. maltophilia* presumptive *groEL* sequences showed 100% homology with known *St. maltophilia* *groEL* and *groES* cassettes, and to the *E. coli* *groE* operon. Multigene sequence alignment showed 100% homology in the pseudomonads and related species from positions 25 to 221.

Restriction enzymes were used to digest genomic DNA of strain VUN10,077 as follows: *EcoRI*, *KpnI*, *BamHI*, *SalI*, *HindIII*, *SphI* (*BsrBI*), *Clal*, *BstEII*, *SacI*, *SmaI*, *BclI*, *AatII*, *NcoI*, *PvuIII*, *ApaI*, *Bsp143I*, *EcoRV* and *AccI*. The *AccI*, *PstI*, *BsrBI*, *SalI*, *Clal*, *BstEII*, *BamHI*, *BclI*, *AatII*, *NcoI*, *PvuIII*, *ApaI*, *Bsp143I* and *EcoRV* enzymes digested strain VUN10,077 genomic DNA, and the *SacI*, *SmaI* and *HindIII* showed a partially digested band, and the *KpnI* and *EcoRI* did not digest genomic DNA of strain VUN10,077 despite the digestion reaction being prolonged for up to 24 hours at 37°C. The *BsrBI* (*SphI*) enzyme cuts in the polycloning sites in pUC18 or pUC19 vectors, and the *Clal*, *ApaI* and *EcoR321* (*EcoRV*) enzymes cuts in the polycloning site in pBluscript II SK (+/-) phagemid vector, and the *SalI*, *AccI*, *PstI* and *BamHI* enzymes cut both plasmid vectors. Probing VUN10,077 genomic DNA digested with *BsrBI* (*SphI*), *PvuII*, *PstI*, *BamHI*, *BstEII*, *Clal*, *HindIII*, *ApaI* or *Eco321* (*EcoRV*) enzymes using the PCR product from VUN10,077 resulted in one band on Southern blot analysis (Figure 4.29). Obtaining one band in this blot indicated that there may be a single copy of the presumptive *groEL* gene in the genome of strain VUN10,077, because these enzymes did not cut within the PCR product and one band only was seen. Unlike ³²P-labelling, DIG-labelling showed several pale, smeared bands in the *BamHI*, *Clal*, *HindIII*, *ApaI* and *EcoRV* digests. The probe reacted uniquely with a *PstI* fragment of approximately

Figure 4.28. Partial *groEL* sequence results from strain VUN10,077 and related species. The sequences were aligned using MultAlign program in ANGIS.

A, *Ps. aeruginosa*; B, strain VUN10,077; C, *Ps. fluorescens*; D, *St. maltophilia*; E, consensus sequence

	10	20	30	40	50	60
A. 1	GGTTGCTTTTCCACTCTT-TC-GCTCCATACCGATCTTCAGAGATCACGGTACCGC					
B. 1	----- GGTTGCTTTT-CCAGCTCCATACCGATCTTCAGAGATCACGGTACCGC					
C. 1	----- TTGCTTTT-CCAGGCCATACCGATCTTCAGAGATCACGGTACCGC					
D. 1	----- GGTTGCTTTTCCAGCTCCATACCGATCTTCAGAGATCACGGTACCGC	***	***	*	*****	*****
E.	RMWWWKTTGCTTTTWCCAGCTCCATACCGATCTTCAGAGATCACGGTACCGC
	59 CAGTCAGGGTTGCATATCCTGCAGCATAGCTTACGACGATCGCCGAAGCCGGTGCTT					
	51 CAGTCAGGGTTGCATATCCTGCAGCATAGCTTACGACGATCGCCGAAGCCGGTGCTT					
	49 CAGTCAGGGTTGCATATCCTGCAGCATAGCTTACGACGATCGCCGAAGCCGGTGCTT					
	52 CAGTCAGGGTTGCATATCCTGCAGCATAGCTTACGACGATCGCCGAAGCCGGTGCTT	*****	*****	*****	*****	*****
	CAGTCAGGGTTGCATATCCTGCAGCATAGCTTACGACGATCGCCGAAGCCGGTGCTT
	119 TAACCGCAGCGACTTCACGATGCCACGCATGGTGTAAACAACCAGAGTTGCCAGCGCTT					
	111 TAACCGCAGCGACTTCACGATGCCACGCATGGTGTAAACAACCAGAGTTGCCAGCGCTT					
	109 TAACCGCAGCGACTTCACGATGCCACGCATGGTGTAAACAACCAGAGTTGCCAGCGCTT					
	112 TAACCGCAGCGACTTCACGATGCCACGCATGGTGTAAACAACCAGAGTTGCCAGCGCTT	*****	*****	*****	*****	*****
	TAACCGCAGCGACTTCACGATGCCACGCATGGTGTAAACAACCAGAGTTGCCAGCGCTT
	179 CGCCTTCTACATCTCAGCGATGATCAGCAGCGGTTGCCTGCTTGGCAA- CAGCT- CC					
	171 CGCCTTCTACATCTCAGCGATGATCAGCAGCGGTTGCCTGCTTGGCAA- CAGCTTCC					
	169 CGCCTTCTACATCTCAGCGATGATCAGCAGCGGTTGCCTGCTTGGCAAACAGCTTCC					
	172 CGCCTTCTACATCTCAGCGATGATCAGCAGCGGTTGCCTGCTTGGCAA- CAGCTTCC	*****	*****	*****	*****	*****
	CGCCTTCTACATCTCAGCGATGATCAGCAGCGGTTGCCTGCTTGGCAAAMCAGCTTCC
237 AGAACCGGCAGCATTGCGGGAT.	A, <i>Ps. aeruginosa</i>					
230 AGAA-----	B, strain VUN10,077					
229 AGAACCG-----	C, <i>Ps. fluorescens</i>					
231 A-----	D, <i>St. maltophilia</i>					
*...						
AGAACCGGCAGCATTGCGGGAT	E, CONSENSUS SEQUENCE					

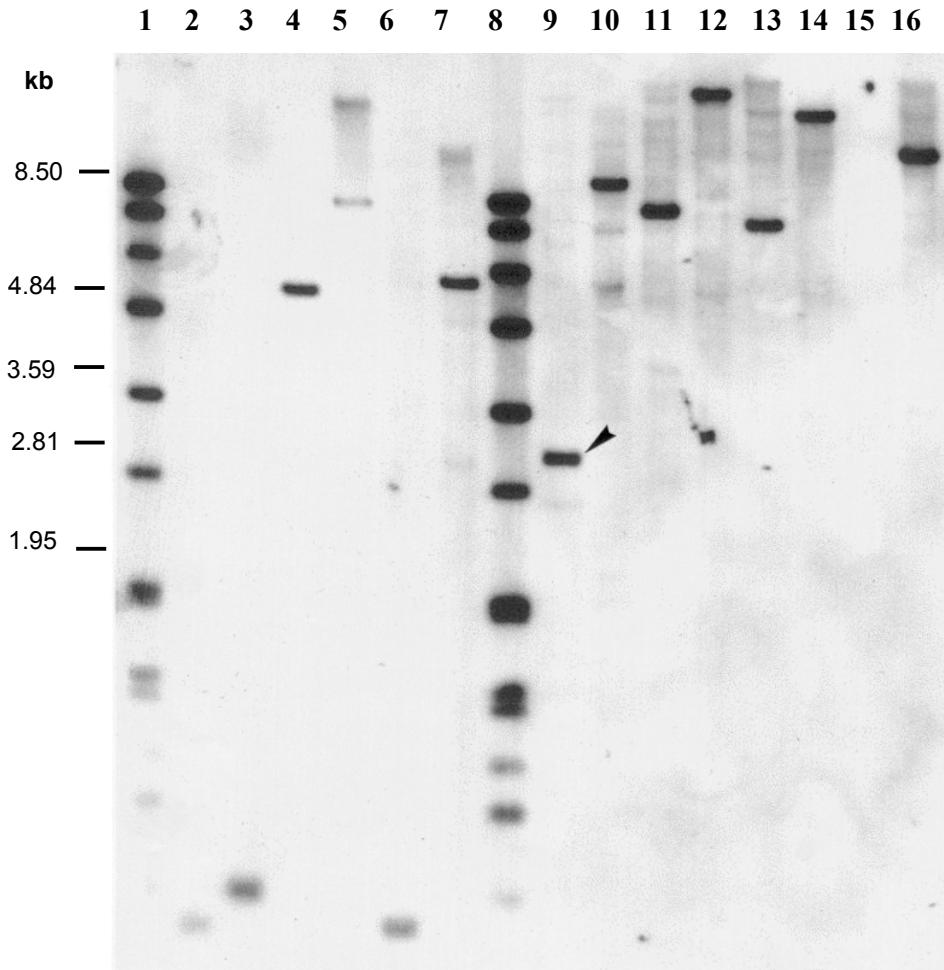


Figure 4.29. Southern blot analysis of strain VUN10,077 using a non-radioactive detection method (DIG™-labelling). The genomic DNA was digested with a range of selected restriction enzymes at appropriate temperatures and 2 µg of genomic DNA was resolved using a large slab gel apparatus. Hybridization was performed using the *groEL* PCR product for strain VUN10,077, as described in section 2.3.6.3.

The lanes were as follows: Lanes 1 and 8, DNA markers (SPPI/*Eco*RI, fractionated with 8,500, 7,350, 6,100, 4,840, 3,590, 2,810, 1,950, 1,510, 1,390, 1,860, 1,160, 980, 720, 480, and 360 bp, top to bottom); lane 2, *Acc*I; lane 3, *Aat*II; lane 4, *Bsr*BI; lane 5, *Nco*I; lane 6, *Sall*; lane 7, *Pvu*II; lane 9, *Pst*I; lane 10, *Bam*HI; lane 11, *Bst*EII; lane 12, *Cla*I; lane 13, *Hind*III; lane 14, *Apal*; lane 15, *Bsp*143I and lane 16, *Eco*R321 (*Eco*RV).

3.3 kb and *Pst*I also cut in the polylinker site of both pUC19 and pBluescript plasmids, which would be an expedient approach to chasing and analysing the complete *groE* operon gene in this strain.

4.4 Discussion

When organisms adapt to environmental circumstances, they are able to cope with sub-lethal but harsh conditions through responses involving the activation of HSPs, which act to protect existing non-heat-shock proteins and repair other cellular damages. Genes for HSP expression are conserved across the genera, but earlier studies of HSPs lean on data for enteric bacteria rather than on data collected on environmental strains (Morimoto *et al.*, 1990). Models for regulation of expression of HSP synthesis have shown differences and commonalities between *E. coli* and environmental species such as *B. subtilis*: information on a variety of environmental species is still emerging. Environmental bacterial strains can use and induce prompt responses when cells are confronted with abnormal circumstances. Induction of stress proteins in response to a diverse range of stresses or starvation conditions provides nonspecific protection, in contrast to stress-specific proteins, that are referred to as universal or general stress responses (Nyström and Neidhardt, 1992; Bernhardt *et al.*, 1997). However, the general stress response seen across genera is analogous to the σ^S -dependent general stress and starvation responses of *E. coli* (Hecker *et al.*, 1996) and *Ps. putida* (Givskov *et al.*, 1994). For the environmental isolate strain VUN10,077, it was useful to investigate induction of this kind of universal or general stress response associated with induction of HSPs because the earliest responses would be genuine stress responses which may not produce modified or adapted signal production in the cell. The response of a microorganism to heavy metal is influenced by biotic and abiotic factors. The biotic factors of importance include the physiological state of the organism in question (nutritional value, acclimation/adaptation to the toxic substances, genetic adaptations such as plasmid-conferred resistance) while the abiotic factors include the physicochemical characteristics of the environment (precipitation and complexation of the metals, pH, temperature, and redox potential). Moreover, the inhibitory concentrations are varied between the bacterial species. Therefore, this chapter are necessary to limited that a means to better understand the sensitivity of the strain VUN10,077 towards selected toxic substances by either viability and

protein profies. Firstly, to examine the genuine stress responses using indigenous strains, the habitat status of any particular strain should be considered. For example, strain VUN10,077 is adapted to aquatic or soil environments, and *B. subtilis* status spend most of their life cycle in a naturally stressful environmental ecosystem causing a starvation or non-growing state in the soil (for a review, see Hecker *et al.*, 1996). The origin of the strain may as well influence the design of experiments to evaluate responses to stressful conditions.

As a basic starting point for experiments, the strain VUN10,077 and related species were grown in LB or BHI then heat stressed from 28°C to 37°C. The growth patterns following heat stress were seen to be different in the different media (Figure 4.1). Strain VUN10,077 showed a similar pattern of growth rate until entering stationary phase when grown in either LB or BHI broth, but heat-stressed cultures were sustained better in BHI than LB broth after stationary phase in terms of absorbance readings (Yuriev *et al.*, 1997), suggesting that BHI broth was nutritionally less limiting than of LB. Cells growing at a higher growth rate before being subjected to temperature upshift were better equipped to adapt to stressful environments. It has been reported that *E. coli* responds to stressful temperature upshift by making a number of HSPs, which are involved in various cellular activities such as DNA replication, RNA synthesis, and production, processing, repair, and degradation of proteins (Neidhardt *et al.*, 1984; Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993; Ryan *et al.*, 1996). Based on these observation, it was proposed that the differences in the post-induction cell growth behaviour were directly influenced by the heat shock response, which can significantly impact on both carbon metabolism and protein synthesis in general (Ryan *et al.*, 1996).

The HSPs were strongly expressed in the strains tested in this thesis and different kinetics were seen for each (Figure 4.2). A GroEL-like protein increased gradually by 50% over 24 hours following heat shock in *Ps. aeruginosa*, starting within 10 minutes and with increasing accumulation throughout the heat stress period. The *Ps. fluorescens* strain did not show the same gradual increase of the 60 kDa and 58 kDa proteins (sometimes resolved as two bands) compared with similar ones in *St. maltophilia* and strain VUN10,077, which only showed small increases in this major cellular protein compared to initial gene expression of this protein. This phenomenon indicated that the cells from different species responded and behaved differently and this may depend upon the level of stimulation of cells and/or implies a strain selectivity for biomonitoring implications (Stewart and Williams, 1992; Ramanathan *et al.*, 1997). The analytical methods used initially involved 1-D SDS-PAGE, which detected total Coomassie blue stained proteins released by sonication. 2-D analysis was trialed using

St. maltophilia and 2-D silver staining. At least 35 distinct spots were displayed in silver-stained 2-D analysis of heat-stressed *St. maltophilia*, including the major HSPs, implying that these proteins are probably essential for continued metabolism under heat stress. It is interesting that these proportionally increased proteins were decreased in 2-D analysis, with less total synthesis proteins synthesized when cells were stressed for a long period (9 hours) compared to short-term (2 hours) stress, although these proteins were visualised and seen to accumulate in 1-D analysis. The rates of synthesis of these proteins quickly stabilized at new basal levels specific for the new growth temperature. In other systems, returning cells to the original growth temperature caused the levels of heat stress proteins to return to their previous level (Lindquist and Craig, 1988) and it was noted that *St. maltophilia* synthesized the major HSPs at a higher level after adapting at this temperature (Figure 4.3). In initial trials using heat shock or exposure to chemicals, either some HSPs did not increase or few definite responses were detected using sonicated, cleared cell lysates when analysed by 1-D SDS-PAGE with Coomassie brilliant blue staining. The ability to detect new proteins depended on the methods used to disrupt cells but the Hsp60 and Hsp70 proteins were detected by pulse-chase radiolabelling as were other major new proteins made following altered growth and metabolic activity, as discussed in the following.

Major HSPs were accumulated in cells when growth continued at elevated temperatures when Coomassie stained gels were used with extracts of strain VUN10,077 and related species (Figure 4.2) and others (Morimoto *et al.*, 1990). These kind of HSPs have been investigated over decades using several different approaches to detect either transient or prolonged induction, employing ^{35}S -methionine, mixture of ^{35}S -methionine and ^{35}S -cysteine, ^{14}C -leucine or $\text{Na}_2^{35}\text{SO}_4$ labelling, or green fluorescent protein expression in Gram negative and Gram positive bacteria (Allan *et al.*, 1988; Walkup and Kogoma, 1989; Givkov *et al.*, 1994; Graumann *et al.*, 1996; Hartke *et al.*, 1996; Cha *et al.*, 1999). These labelling methods or 1-D or 2-D gels with staining were used to detect both general and condition-specific responses and led to similar observations in terms of major HSPs or specifically induced proteins (e.g. see Lupi *et al.*, 1995). After labelling, cells have been disrupted *via* physical extraction, boiling lysis and alkali lysis or combinations of these (Lutkenhaus, 1977; Allan *et al.*, 1988; Walkup and Kogoma, 1989; Graumann *et al.*, 1996; Hartke *et al.*, 1996). When strain VUN10,077 cells were disrupted using sonication followed by centrifugation, a 72 kDa protein (DnaK-like) was not visible following heat shock suggesting that this protein was removed by centrifugation. When cells were lysed by boiling in loading buffer, the relative levels of membrane-associated proteins were increased, specifically a 44-45 kDa protein, and changes in levels of a DnaK-like protein were then detectable (Figure 4.4).

The comparison of lysis methods used was extended to chemical stresses after detecting production of stress responsive proteins, in context of the impact of the addition on viable counts. The absorbance readings did not necessarily correlate well with viable counts when strain VUN10,077 was subjected to selected chemical stresses such as cadmium, copper, mercury, SDS, arsenite and phenol, with nickel and cobalt exposure being exceptions. Comparably nickel and cobalt caused less effect on protein profiles at the sub-lethal levels used. The concentrations used may not have been sufficient to stimulate stress responses at a translational level due to the inherent resistance of this strain to selected chemicals or the amounts of newly synthesized protein were too low to be visualised by Coomassie stained-gels or co-precipitated with nutrient components in the media. Condition specific or chemical specific proteins were induced as a result of exposure to certain chemicals (see section 4.2.2.2), which has also been seen for other bacterial systems for different chemicals. Ethanol is probably the most tested chemical stress used in parallel with early work on heat stress using *E. coli* and *Ps. aeruginosa* (VanBogelen *et al.*, 1987; Allan *et al.* 1988), where ethanol caused enhanced protein synthesis and these were compared in terms of similarity or homology to HSPs. Later on, the focus was also on chemical-specific induced proteins as new or having overlaps with all of the known HSPs (Adamowicz *et al.*, 1991; Blom *et al.*, 1992; Lupi *et al.*, 1995). These specific proteins have been identified as enhanced spots on autoradiograms of two-dimensional gels and individual proteins with discrete function were identified using either N-terminal sequencing of the proteins or disruption of the gene by insertional inactivation or deletion (Bernhardt *et al.*, 1997). The genetically uncharacterised strain VUN10,077 produced chemical specific proteins and showed relative increased or accumulated levels of proteins or below a MW of 20 kDa (using 1-D SDS-PAGE), while the major HSPs were not significantly elevated (Table 4.2). It was found that induction of the \leq 20 kDa proteins occurred across the tested chemicals, with exception of mercury, when cells were lysed by boiling. Both general and condition specific responses were detected when cells were lysed by sonication or boiling, when using 1-D SDS-PAGE analysis, although the profiles seen were different (Figure 4.17).

The addition of mercury and SDS at the concentrations used caused a transient decline in A_{600} then growth continued slowly under stress. Interestingly, viable counts had recovered in overnight samples of mercury-stress cultures after the initial dose-related drop in viability caused by addition of mercury in BHI (Figure 4.9). Mercury ion (Hg^{2+}) is purely toxic and has no metabolic function in bacteria, which is similar to Cd^{2+} , AsO_2^- , CrO_4^{2-} , Sb^{3+} and TeO_3^{2-} , whereas Co^{2+} , Ni^{2+} and Zn^{2+} are toxic in excess but are also essential nutrients

(Brown *et. al.*, 1995; Ji and Silver, 1995; Lovley, 1995). The recovery in viability may have inferred that strain VUN10,077 was resistant to Hg²⁺ due to the presence of a detoxification pathway. Moreover, the amount of protein synthesis is relatively decreased after mercury stress (indicated by a decrease in total Coomassie stained proteins being seen despite constant cell loadings) and this gradually recovered, along with increased viable numbers, at concentrations of mercury of 0.025 gL⁻¹. A lesser degree of protein profile change occurred at lower concentrations of mercury such as 0.005 gL⁻¹ as detected using sand-lysed cells (Figure 4.10). Surface layer associated ~70 kDa and 44-45 kDa proteins were induced at maximum levels after 2 hours exposure time to mercury, while viable cell counts were still in decline which may have been related to the role that mercury plays in the breakdown of the cell membrane as an essential barrier in viable cells (Passow and Rothstein, 1960). The cell membrane may be a major barrier to entry of hydrophilic metal ions into the interior of the cell when exposed to metal ions when the first sites of interaction (Passow and Rothstein, 1960; Nikaido and Vaara, 1985; Rouch *et al.*, 1995), and also the outer envelop may act as a limited trap for heavy metals by non-specifically binding them, providing natural metal tolerance to cells (Bitton and Freihofer, 1978; Rouch *et al.*, 1995). Thus far, mercury resistance mechanism which occurs at the stage before Hg enters cells is presumptively associated with surface layer-linked proteins. The role of the ~70 and 44-45 kDa surface proteins that were observed by change during exposure to Hg in survival or handling is not known, nor is how induction of intracellular proteins relates to subsequent observed recovery of viability. However, it is not likely that changes in intracellular proteins in response to Hg stress, which paralleled a dose-related decline in viable counts, is sufficient to account for the subsequent recovery in cell viability of strain VUN10,077 cells. This is because S-H related metabolic processes are strongly inhibited after mercury is taken into bacterial cells (Wakatsuki, 1995). Hence, it is proposed that strain VUN10,077 may contain a mercury detoxification system. Mercury oxidoreduction reactions primarily occur due to electron transport systems in the plasma membrane in the prokaryotic bacteria, while eukaryotes mainly supply electron donors in mitochondria and this may be involved in transplasma-membrane redox systems in the cell surface layers (Crane *et al.*, 1985; Wakatsuki, 1995). Fox and Walsh (1982) proposed the equation of the reductase reaction to be: NADPH + RS-Hg-RS + H⁺ → NADP⁺ + Hg⁰ + 2RSH. Strain VUN10,077 had an effective mercury-responding stress system for concentrations up to 0.025 gL⁻¹ of HgCl₂, associated with the major HSPs. Further research is required to show whether a specific resistance mechanism or/and detoxification pathway is present in strain VUN10,077, which may be associated with a switching system, the acute stress-mediated response, or the physicochemical properties of the cell envelop.

It is well known that the major HSPs are produced in unstressed bacterial cells but at lesser amounts and these can be detected using antibodies and also when cells were subjected to harsh conditions, as the cellular concentration of proteins increase or decrease (Morimoto *et al.*, 1990). In Western blot analyses, the levels of signal intensity detected (Figure 4.16) indicated that the severity of the environmental stress applied had an impact on the extent of HSPs accumulation. However, the antibodies reacted with several other bands on the gels, which may have been due to the way in which the antibodies were used to obtain positive reactions or non-specific reactions with other cellular components (e.g. phospholipids or lipopolysaccharides) present on the gels (Jensen *et al.*, 1993; Richarme and Kohiyama, 1993). The signal detected using antibody against *E. coli* DnaK intensified in order of heat stress = cadmium > zinc > cobaltous = phenol > control, whereas, antibody to GroEL reactivity did not show a dramatic change in this type of analysis (Figure 4.16). This kind of differential response to various conditions, including heat shock, has been noted for the major HSPs of *D. melanogaster* (Lindquist, 1980) and *Tetrahymena thermophila* (McMullin and Hallberg, 1987 and 1988). Additionally, antibodies to DnaK have been used to demonstrate the distribution of the epitope among a variety of species, although differential reactions have been reported. The 2G5 DnaK epitope was found in Gram-negative bacteria such as *Ps. fluorescens* and *S. typhimurium* with an apparent MW of 70 kDa, whereas *Ps. aeruginosa* and *Ps. syringae* showed a slightly different MW while negative reactions occurred with the tested Gram-positive bacteria (Krska *et al.*, 1993).

The newly synthesized protein(s) are commonly detected by pulse-chase radiolabelling during heat stress conditions. However, there have been few similar studies on proteins produced following exposure to pollutants, with the exception of the work by Blom *et al.* (1992). This group showed that when *E. coli* was exposed to selected pollutants unique and condition-specific proteins were induced, where some of these were in common with HSPs or starvation-induced proteins. To undertake more detailed studies in strain VUN10,077, it was necessarily to determine a suitable minimal or defined medium for pulse-chase labelling experiments and validate its use by detecting heat stress responses, in context of this strain being genetically and physiologically uncharacterised. Different heat induced proteins were detected in strain VUN10,077 where the kinetics of induction and the number of proteins and sizes seen varied with the media used. The labelling method and media used may have caused these differences, which inferred that the choice of medium was important rather than labelling method because a similar observation was seen when strain VUN10,077 was heat shocked using two rich media, LB and BHI (Figure 4.2 and 4.17). The choice of minimal

medium is important in that it makes pulse-labelling easier and more sensitive than experiments performed in complete medium, because the uptake level of labelled amino acids is greater in the minimal media background, although HSPs were detected in *St. maltophilia* when labelled with ^{35}S -methionine using complex medium under similar heat stress conditions (O. Yuriev, personal communication). When bacteria growing are subjected to nutrient deprivation, the cells enter stationary phase and cease growth, often retaining viability for a considerable period. The adaptation to this stage demands many physiological changes, including reduction of global gene expression, accompanied by induction of specific genes (Siegele and Kolter, 1992; Miksch and Dobrowolski, 1995). Recently, different induction levels of stress responsive proteins were reported in Gram negative and Gram-positive bacteria (Free and Dorman, 1997; Graumann *et al.*, 1996). Heat shock (30°C to 37°C) and osmotic shock induced a 13.5 kDa protein (*stpA* gene), a different level of induction between rich medium and minimal medium at the transcriptional level in *E. coli* being seen (Free and Dorman, 1997). Moreover, detection of cold shock responses depends on the presence of amino acids in a labelling minimal medium, which was M9 medium supplemented with tryptophan, phenylalanine and yeast extract in *B. subtilis* (Graumann *et al.*, 1996). In order to select the optimal medium, the heat shock response was examined upon elevating the temperature from 28°C to 37°C in strain VUN10,077 (Figure 4.20 and 4.22).

Strain VUN10,077 showed diverse responses compared to the environmental isolate *St. maltophilia* in terms of absorbance readings when the cells were subjected to heat stress in media (1) to (5) (Figure 4.20), and different protein profiles were seen in minimal or defined media (Figure 4.22) and for *E. coli* (Herendeen *et al.* 1979). When cells of VUN10,077 were subjected to heat stress, the heat stressed cells showed slower growth rates which may also have been affected by the initial growth temperature, as previously seen in Table 3.3 and for *Ps. putida* (Givskov *et al.*, 1994). Levels of major HSPs, the GroEL-like (~58 kDa) and DnaK-like (~70 kDa) proteins, were changed following growth in the minimal media, which was seen earlier in LB (see Figure 4.2) as well as BHI broth (Figure 4.22) under heat stress from 28°C to 37°C. It was also revealed that LMW HSPs were expressed differently and independently in either the minimal or defined minimal media (1) to (5). The major HSPs declined or were weakly induced in medium (1) throughout the sampling period, which was similar to results for medium (3), but these HSPs were increased in media (2) and (5), as seen in BHI medium. An exception was medium (4), where the 70 kDa protein was relatively increased while the 58 kDa protein decreased. Unlike the major HSPs, a 20 kDa protein was relatively increased in the defined media, except for medium (5), while other LMW proteins

varied (Table 4.4). This protein was co-expressed with a 19.6 kDa protein as a doublet, except in medium (3), whereas an 18 kDa protein increased as the 58 kDa protein increased in the optimum defined medium (5), similar to results reported in *Ps. aeruginosa* and *E. coli* (Allan *et al.*, 1988; Chuang and Blattner, 1993). However, it was found that the 20 kDa protein was relatively increased without co-expression of the 58 kDa protein when growth continued at 37°C after heat stress in media (1), (3) and (4), which was similar to the results seen for selected heavy metal stresses in rich media (Table 4.2). Also, this protein was present at high levels in medium (2) and BHI without stress and a protein of slightly lower MW was also seen at similar high levels in cells grown in medium (5). The GroEL-like and DnaK-like proteins were constitutively expressed at high levels in defined media (2), (5) and BHI medium, which was similar to the GroEL-like protein synthesised in both *Streptomyces albus* and *Caulobacter crescentus* (Gomes *et al.*, 1986; Guglielmi *et al.*, 1991), whereas this did not occur in other tested media.

Chuang and Blattner (1993) studied the *E. coli* heat shock gene expression by global transcription analysis. While GroES (MopB) synthesis was about equally stimulated in both *in vivo* and *vitro* conditions, GroEL (MopA) synthesis was much more stimulated *in vivo*, and synthesis of the uncharacterized 22 kDa protein showed higher stimulation *in vitro*. Synthesis of Lon from clone 148 was also stimulated much more *in vivo* whereas a 49 kDa protein of clone 334 was synthesized much more *in vitro*. The above results indicated that *in vitro* study does not always reflect the situation *in vivo*, where among the σ^{32} -induced heat shock genes, some were drastically activated *in vivo* while some were more stimulated *in vitro*. This phenomenon implied that other factors are involved in fine-tuning the response to heat shock. *S. albus* and *Streptomyces lividans* have two proteins, Hsp56 and Hsp58, whose size and N-terminal sequences showed that they were similar to GroEL-like proteins of other organisms (Guglielmi *et al.*, 1991). These proteins are similar to those induced in *Ps. fluorescens* following heat shock, where the response induced a doublet of 56/58 kDa (see Figure 4.2). A Hsp18 protein was synthesized throughout the sampling period and represented a third *S. albus* GroEL-like protein with an N-terminal amino acid sequence that was similar to that of Hsp58 (Guglielmi *et al.*, 1991). The role of this heat-induced protein was further investigated by Servant and Mazodier (1995 and 1996), who showed transcription of *hsp18* is induced by heat shock, but no *hsp18* mRNA could be detected at 30°C in wild type of *S. albus* G. Thus far, observations reported that the LMW protein was co-synthesized or weakly expressed when the GroEL-like protein is expressed together. However, the 20 kDa protein of strain VUN10,077 was increased whether the GroEL-like protein was increased or not, and synthesis of several other LMW also varied: the LMW

stress responsive proteins were observed and migrated in the size range of 14 kDa to 20 kDa, and these were significantly overproduced through various types of stresses, such as nutrient deprivation, heat elevation and heavy metal stresses in strain VUN10,077 (Table 4.1, 4.2 and 4.4), even when the major HSP-like proteins were not strongly induced which was seen in Coomassie-stained gel following stress in minimal nutrient conditions and heavy metal stress. Under some of these stress conditions, the cells had ceased growing when the LMW proteins increased, which may be similar to the *in vitro* expression of the 22 kDa protein of *E. coli* (Chuang and Blattner, 1993).

Pulse-chase ^{35}S -methionine experiments showed the newly-synthesized proteins to either heat shock or chemical stressors. Temperature is a major parameter governing growth and metabolism of organisms. Increased synthesis of a small specific subset of proteins by exposure of cells to temperature upshift is universal with all organisms so far examined, although strain specific HSPs are induced. These strain-specific HSPs were stimulated by heat elevation in the strain VUN10,077 and related species, and the levels of induction of the subset of HSPs produced steadily increased with increasing temperature in *E. coli* and *Ps. aeruginosa*, comparable to that of strains grown continuously at 28°C (Figure 4.23). By transient induction of HSPs, maximum rates of protein synthesis in most bacteria studied so far occurs 5-60 minutes after heat elevation and then shows a rapid decline with the onset of normal protein synthesis. This recovery period is variable but generally occurs 60-90 minutes after incubation at the heat-treatment temperature or at the normal growth temperature (Watson, 1990) and this pattern of protein synthesis is also analogous to cold shock response in *B. subtilis* (Graumann *et al.*, 1996). Recently, studies on prolonged chemical stresses have progressed in environmental organisms, showing that new proteins are synthesized after 3 hours of chemical stress in *E. coli* (Blom *et al.*, 1992) and 1.5 hours for 2-chloropheol stress in *Ps. putida* (Lupi *et al.*, 1995). Hsp28 was synthesized while Hsp70 and Hsp60 were relatively less produced for up to 4 hours exposure to heat stress, 100 μM cadmium chloride and arsenite in mammalian cells (Wiegant *et al.*, 1995). Similar phenomena were observed in cadmium-stress for growing VUN10,077 cells, where major stress-induced proteins were expressed by 1 hour exposure and normal cell growth metabolism and recovered relative to un-stressed cells (Figure 4.27). In contrast, heat-stressed cells synthesized another subset of proteins while major HSPs, GroEL-like and DnaK-like, declined. It was revealed that the second subset of heat-induced proteins, 54, 48, 34 and 20 kDa, were steadily synthesized or increased after heat-stress for 1 hour at 37°C using ^{35}S -methionine incorporation (Figure 4.27). The five stressor conditions lead to the clear induction of major stress induced proteins within 5 minutes, including DnaK-like and

GroEL-like proteins. However, radiolabelled uptake following mercury and nickel stresses did not occur, possibly because these chemicals were not compatible with methionine in the optimised defined media (5) or the cells had lost their ability to express new genes due to their low viability or lack of incorporated ^{35}S -labelled methionine into mercury stress induced-proteins.

Models for chaperonin function propose that GroEL is generally involved in protein folding and is assembled in the bacterial cytosol with assistance of the GroES (Weissman *et al.*, 1996), whereas eukaryotic CCT (which has a similar function to GroEL) does not require a co-chaperonin analogue (Sternlicht *et al.*, 1993). However, when the known major HSPs were expressed in strain VUN10,077, a 20 kDa protein was induced during cell growth under elevated temperature conditions while another three heat-induced proteins gradually increased (Figure 4.27). This result may indicate that the 20 kDa heat-induced protein does not belong to the GroES family although this protein's regulatory pattern did not appear during chemical stresses when analysed by ^{35}S -methionine incorporation. Upon heat shock treatment, expression of most genes other than the heat shock ones decreased. In contrast to the extensive studies on the mechanism of heat shock activation, relatively less is known about repression of the major HSPs (Chuang and Blattner, 1993). Recently, expression of the *E. coli* *stpA* gene, which encodes a 15.3 kDa protein and might act as a molecular backup of H-NS, had its expression strongly down-regulated by a product of a related *hns* gene, with the result that *stpA* transcription is undetectable in a wild-type strain producing H-NS protein (Zhang *et al.*, 1996; Free and Dorman, 1997).

Low molecular weight (LMW) HSPs belong to the family of bacterial small HSPs for which the function remains unclear (Yuan *et al.*, 1996; Jobin *et al.*, 1997). However, a chaperone-like activity was recently revealed for a 13.5 kDa (UspA) cytoplasmic protein in *E. coli* (Nyström and Neidhardt, 1992), a 21 kDa (*hspA*) protein response to stress situations such as heat shock, anoxia, or starvation in the Gram-negative myxobacterium *Stigmatella aurantiaca* (Heidelbach *et al.*, 1993), a 16 kDa alpha-crystallin-like small heat shock protein (smHSP) from *M. tuberculosis* (Yuan *et al.*, 1996), an 18 kDa smHSP associated with membranes of *Leuconostoc oenos* which can respond to different stress factors independently of an alternative σ^{32} factor or CIRCE element (Jobin *et al.*, 1997) similar to genes encoding smHSP of *S. albus* (Servant and Mazodier, 1995) and for a few genes of *B. subtilis* including *lon* and *clp* (Hecker *et al.*, 1996). The eukaryotic LMW HSPs are known to form high-molecular-weight complexes, the so called heat shock granules, during heat shock response, whereas for mammalian LMW HSPs a role in the acquisition of thermoresistance has not

been clear but *in vitro* study showed a chaperone-like function (Jakob *et al.*, 1993). The LMW HSPs of plants are involved in the regulation of translation during the heat shock response, for example, heat shock granules of tomato cells contain a particular subset of mRNAs which may be used after heat shock (Nover *et al.*, 1989). The LMW HSPs made by strain VUN10,077 detected following heat shock using ^{35}S pulse-chase labelling (Figure 4.27), however, the synthesized levels were not overproduced compared to controls over the 2 hours incorporation period and in contrast to results seen in Coomassie-stained gels. A similar small cytoplasmic protein, 13.5 kDa, was increased during growth inhibition caused by nutrient limitation or by toxic agents including cadmium chloride, osmotic stress, and heat shock in *E. coli* (Nyström and Neidhardt, 1992). In *S. albus*, similar small HSPs were produced in parallel to Hsp56/58 proteins (Guglielmi *et al.*, 1991; Servant and Mazodier, 1995). The synthesis rate of a protein around 20 kDa was increased while major HSPs were repressed by heat stress but phenol stress showed a different pattern (Figure 4.27), which may imply that production of LMW HSPs was condition-specific when cellular proteins were analysed using ^{35}S incorporation.

Many environmental stresses are known to induce the production of HSPs that can help protect an organism from damage until stress is removed. Temperature change is regarded as one of the environmental stresses that activates a specific set of genes called the heat shock genes, which are associated with newly synthesized mRNA (Lindquist, 1986; Servant and Mazodier, 1996). Presumably mRNA synthesis also occurs in strain VUN10,077 when this organism responds to pollutant stress. Heat shock mRNA appears in the cytoplasm of a viable cell with in a few minutes of temperature elevation and is immediately translated with very high efficiency into a number of highly conserved proteins. The cell responses to stress conditions can be used as unique and sensitive tools for detecting the bioavailability of specific toxicants or potentially toxic elements. Accordingly, there has been increasing interest in investigating the heat shock-like genes, their promoter regions and other stress-related genes, that presumed be specific to certain stresses or general stresses. Van Dyk *et al.* (1994 and 1995) and Belkin *et al.* (1996 and 1997) have used various promoter genes under the control of several global regulatory elements fused in *luxCDABE*, including *rpoH* (*rpoH*-controlling protease), *soxRS* (responsive to superoxide), *oxyR* (controlling catalase), *fadR* (controlling a β -hydroxydecanoyl-thioester dehydrase), *uspA* (universal stress), and *grpE*. The above fused stress promoters were examined for toxicity assessment (Ben-Israel *et al.*, 1998). General characterisation of the stress responses seen in the selected aquatic indigenous organisms and related species were reported in this chapter. Especially, nutrient, temperature and heavy metal ions affected the growth rate, metabolic pattern and protein

profiles of strain VUN10,077. Some biophysical factors affecting bacterial growth were controlled primarily by the constituents of the culture medium (nutrient, pH, water activity and osmotic pressure) and others, especially, by the external environmental (temperature, oxygen and applied stresses such as heavy metal ions) (Costilow, 1981). Future research on the stress response of strain VUN10,077 should concentrate on characterising the differential expression of the observed stress proteins at the transcriptional level. This would provide a fundamental understanding of the regulatory mechanisms involved and the relationship between the classical HSPs and the various low MW stress-induced proteins. In order to further characterise the physiological response of strain VUN10,077, the next chapter reports the results obtained using *lux*-marked variants of this strain.

Conclusion and Recommendation

The major conclusions of this chapter are summarised in dot points as follows:

- The strain VUN10,077 and related species showed different heat-shock protein profiles and a GroEL-like protein was common to all but a DnaK-like protein was barely seen to change when cells were disrupted by sonication. The DnaK-like and 44-46 kDa proteins were clearly seen when cells were lysed by boiling. This suggested that these proteins were associated with membranes or particulates.
- Low molecular weight proteins were accumulated during ongoing chemical stresses rather than the major HSPs, when proteins were extracted by the boiling method and analysed using 1-D SDS PAGE. A common chemical stress responsive protein was found to be a 20 kDa protein in strain VUN10,077 and several LMW stress responsive proteins accompanied this, which was dependent on the degree of stimulus, the period of stress and the nature of the chemical (cadmium, copper, cobalt, nickel, arsenite and phenol stresses).
- HgCl_2 stress caused significant and dramatic protein profile changes to strain VUN10,077 and dose-related responses were seen following physical disruption of cells using sand. More severe protein changes occurred at concentrations of 0.025 g L^{-1} compared to 0.005 g L^{-1} HgCl_2 at the sublethal level: 70 kDa and 58 kDa proteins were strongly involved as up- and down-regulated proteins over ongoing stress, along with the surface layer associated proteins of 44-46 kDa. Because cells recovered viability on prolonged exposure to Hg^{2+} , a detoxification mechanism may have been present.
- Defined or minimal media were determined for the indigenous strains and minimal media dependent heat-shock proteins were differentiated. Media (2) and (5) produced major HSPs consistent with BHI under given heat stress conditions and cell growth rates were relatively less impaired in medium (5) and starvation induced proteins levels were lower.
- Growth dependent stress responsive proteins were seen at the molecular level using pulse-chase ^{35}S -methionine labelling. *E. coli* and *Ps. aeruginosa* synthesised HSPs in controlled ways when cells were stressed in medium (2) compared with *St. maltophilia*, *Ps. fluorescens* and VUN10,077, which grew poorly in medium (2).

- Strain VUN10,077 synthesized new proteins under given heat-shock and cadmium stress conditions in the optimised defined minimal medium, and the major HSPs varied. Proteins of MW 72 kDa and 62 kDa were common to the two stresses while a 38 kDa protein was specific for cadmium stress. These overlapped proteins declined after 1 hour stress and 55 kDa, 50 kDa, 32 kDa and 18 kDa proteins emerged upon prolonged heat stress, and membrane associated proteins were increased in prolonged cadmium stress compared to heat stress.

Areas for further basic- and applicable-level research are as follows:

- To compare the stress responsive proteins, optimised ^{35}S -methionine labelling method should be applied to the other aquatic pseudomonads and related species, and broad chemical responses, including starvation conditions, determined using 2-D electrophoresis. The physiological response mechanisms, molecular make-up of major known HSPs or strain specific cellular systems, should be explored at a transcriptional level using established RNA extraction methods and Northern analysis.

Chapter 5

**Expression of bioluminescence genes in strain
VUN10,077**

5.1 Introduction

The development of techniques for environmental monitoring in natural environments has been greatly simplified by using microbes. Microbial biosensors are designed to employ the physiological responses of whole living cells as the sensing components which are able to detect a variety of stimuli such as oxygen consumption, surface chemical potential, genetic activity or induction of HSPs (Karube and Masayasu, 1990). The increases in the production of HSPs are common under ‘stress’ situations. The synthesis of HSPs as a response to heat elevation is conserved throughout the cellular system and is apparently a universal response to harsh conditions. HSPs are also induced when cells are exposed to a variety of other potentially deleterious environmental situations, as demonstrated for strain VUN10,077 in the previous chapter. Synthesis of HSPs is a natural response that enables survival in the presence of pollutants, at the cellular level. Selected pseudomonad related strains have been used in this thesis and the stress responses surveyed in these isolates from natural environments, at the level of protein synthesis and transcription resulting in new protein synthesis. In this chapter, these bacteria were used for monitoring environmental stress responses using the expression of bacterial luciferase to detect early responses to sublethal levels of pollutants and changed environmental conditions.

The objective of this study was to develop bioluminescent bio-indicators for environmental pollution monitoring in aqueous samples, where the system would be based on aquatic rather than marine bacteria. This approach would alleviate the requirement for salt or osmotic stabilisers in the test system to maintain bioluminescence or viability, which is currently a requirement of a commercialised system which employs *Ph. phosphoreum*. There are two stages in this process: firstly, introducing bioluminescence genes into the indigenous strain VUN10,077, and obtaining detectable, stable light output and then, secondly, evaluating this system for detecting pollutants such as heavy metal ions, phenolic compounds and PAH compounds. Furthermore, an important question was whether the cloned system in strain VUN10,077 could detect general responses to stressors or whether particular compounds changed the bioluminescent output. In the previous chapter, it was shown that both HSPs and compound-specific proteins were induced under different circumstances and that loss in viability occurred at particular concentrations of the compounds tested. As well as being potentially useful as a biomonitoring tool, the *lux*-marked VUN10,077 strains would also provide a tool for evaluating the physiological impact of sublethal concentrations of

pollutants, which would then allow some prediction to be made about the potential impact of these in nature. Once bioluminescent transconjugants were obtained, one of these, strain VUN3,600 was used to detect the presence of toxic substances as shown by a decrease in light emission. This, however, firstly necessitated developing systems for stabilizing light output. Results of this work are reported in this chapter.

5.2 Establishing methods for introducing *lux* genes into natural isolates

Indigenous strain *St. maltophilia*, VUN10,075, was initially selected to develop a microbial indicator of environmental stress. One difficulty was that this locally isolated strain displayed naturally high resistance to the antibiotic tetracycline that was carried by the mini-Tn5 *luxAB* PUT-derivative plasmid construct when compared to concentrations recommended by de Lorenzo *et al.* (1990), as well as penicillins and kanamycin, so that vectors using these selectable markers were not suitable for use. Thus another aquatic pseudomonad was chosen as the recipient strain for introducing the bioluminescence genes, strain VUN10,077. As strain VUN10,077 was a genetically uncharacterised natural isolate, the first part of this work was establishing the parameters necessary for introducing genes: determining antibiotic sensitivity (to determine which marker genes could be used in this background) and establishing gene transfer systems (conjugation/electroporation) using the transposition system.

5.2.1 Antibiotic sensitivity testing

The MICs of the antibiotics were determined prior to introduction of plasmids into VUN10,077 strain. Then, genetic exchange methods for bioluminescence encoding genes were established for the VUN10,077 strain, employing bi- and tri-parental matings and electroporation was performed in the following sections. The MICs of antibiotics for counter selection of transconjugants or transformants were examined using antibiotic gradient agar plates at concentration ranges from 0 to 100 µg/ml initially. The antibiotics tested were rifampin, ampicillin, chloramphenicol, tetracycline, streptomycin, spectinomycin, neomycin, kanamycin, lincomycin, vancomycin, carbenicillin, piperacillin and nalidixic acid. In

Table 5.1. Determination of MICs of selected antibiotics. The antibiotics were tested at concentrations of 5, 10, 20, 50, 100, 150 and 200 µg/ml in BHIA and recorded after incubation at 28°C for up to 3 days and the number shown are MICs within the tested range.

Antibiotics	<i>Ps. erugi-nosa</i>	<i>St. maltophilia</i>	<i>Ps. fluorescens</i>	Strain VUN10,077	<i>E. coli</i> CC118	<i>E. coli</i> S17-1
Tetracycline	100	100	10	<	> 200	> 200
Kanamycin	> 200	> 200	100	<	50	50
Rifampin	50	10	50	<	> 200	> 200
Streptomycin	> 200	> 200	> 200	20	20	50
Ampicillin	> 200	> 200	> 200	50	> 200	> 200
Piperacillin	> 200	> 200	150	> 200	> 200	> 200
Nalidixic acid	> 200	50	> 200	> 200	10	10

>, the tested strains grew up to this tested concentration.

<, the tested strains failed to grow at the lowest concentration tested.

Table 5.2. Selection of antibiotic markers to introduce bioluminescence genes to strain VUN10,077, basis on results in Table 5.1. The symbols indicate (+) growing and (-) non-growing status. Antibiotics concentrations are described in the text.

	Tetracycline	Nalidixic acid	Ampicillin	Tetracycline plus nalidixic acid
Donor strain: <i>E. coli</i> S17-1 and CC118	+	-	+	-
Recipient strain: VUN10,077	-	+	-	-

Transformants	+	+	-	+

gradient agar plates, *St. maltophilia* was resistant to ampicillin, streptomycin, neomycin, kanamycin, lincomycin, vancomycin and spectinomycin. Interestingly, strain VUN10,077 was relatively sensitive to the tested antibiotics, except for lincomycin, when compared to the other pseudomonads. *Ps. aeruginosa* and *Ps. fluorescens* were resistant to ampicillin, lincomycin and vancomycin, and *Ps. aeruginosa* and *Ps. fluorescens* showed a few resistant colonies on gradient agar plates containing streptomycin while *St. maltophilia* was resistance to streptomycin. Donor strains were resistant to ampicillin, tetracycline, lincomycin, vancomycin, carbenicillin and rifampin. The shape of the streaks represented different visual patterns which indicated antibiotic sensitivity or patterns of inhibition. When they showed no inhibition pattern in gradient agar plates, the pseudomonads were examined at higher antibiotic concentrations of 100, 150 and 200 µg/ml in LA. Several colonies appeared at a concentration of 100 µg/ml tetracycline for *Ps. aeruginosa* and *St. maltophilia* but not at 200 µg/ml in LA. When the tested strains showed abrupt inhibition on the gradient plates, defined MICs were carried out using LA and BHIA at 28°C using a range of concentrations of selected antibiotics. BHIA agar was selected as it gave better growth for all strains relative to LA. Furthermore, when the defined MICs were performed in LA and BHIA containing kanamycin, streptomycin, piperacillin, nalidixic acid and rifampin at concentration ranges of 5 to 200 µg/ml, the pseudomonads related species displayed better resistance or inhibition patterns or higher MICs in BHIA compared to LA (Table 5.1), and selection marker concentrations were chosen on the basis of these antibiotic sensitivity patterns (Table 5.2). The pseudomonad strains showed sensitivity at concentration of up to 10 µg/ml in LA containing piperacillin at 28°C, noting that a few colonies appeared at concentrations of up to 200 µg/ml for these strains in BHIA, whilst *E. coli* donor strains showed strong resistance to piperacillin in both media in addition to resistance to tetracycline and ampicillin, as expected from their reported phenotypes and plasmids present. The *E. coli* strains showed similar results when they were incubated at 37°C in LA. Strain VUN10,077 was relatively highly resistant to nalidixic acid compared to inhibition patterns of *Ps. aeruginosa* and *Ps. fluorescens*, although they showed resistance at the highest concentration tested. *Ps. fluorescens* and *St. maltophilia* showed normal growth on plates containing kanamycin while strain VUN10,077 showed no growth at the lowest concentration tested. Donor strains (*E. coli* S17-1 and CC118) showed sensitivity to kanamycin and streptomycin with a few colonies appearing at concentrations of 20 µg/ml but not at 50 µg/ml, and nalidixic acid at a concentration of 10 µg/ml in this type of analysis. It was interesting that *St. maltophilia* showed relatively high resistance to the tested antibiotics except rifampin, whereas strain

VUN10,077 showed sensitivity to the antibiotics except nalidixic acid. The experimental results indicated that 50 µg/ml of tetracycline plus 100 µg/ml of nalidixic acid could be used to select transconjugants in BHIA and suppress the growth of parental strains after conjugation or electroporation for the pseudomonads, except for *St. maltophilia*.

5.2.2 Introduction of bioluminescence *luxAB* genes

A mini-Tn5 *luxAB* pUT derivative-plasmid construct (pUT::Tn5 *luxAB*) in *E. coli* S17-1 or *E. coli* CC118 has been used to express bioluminescence in *Pseudomonas* species (de Lorenzo *et al.*, 1993b), with mini-Tn5 described by Herrero *et al.* (1990) and de Lorenzo *et al.* (1990 and 1993b). This transposon system produces random insertions into the chromosome of a variety of Gram negative bacteria which utilize the same Tn suicide delivery system and *luxAB* inserts are stable due to the loss of the transposase gene during transposition (de Lorenzo *et al.*, 1993b). Initially, attempts were made to transfer the pUT::Tn5 *luxAB* into strain VUN10,077 by conjugal transfer.

5.2.2.1 Bi-parental matings

Recipient cells of strain VUN10,077 were cultured till mid-exponential phase at 28°C in BHI broth and donor strains, *E. coli* S17-1 and *E. coli* CC118, were cultured in LB broth plus 50 µg/ml tetracycline and 100 µg/ml ampicillin at 37°C. For filter bi-parental matings, 10 to 50 µl of concentrated cell suspensions were mixed in a ratio (donor versus recipient) of 1:0, 1:1, 1:5 and 0:1 in 5 ml of 10 mM MgSO₄, and filtered then incubated for 8, 18, 24, or 48 hours at 28°C. To allow the transfer of plasmid DNA, the overnight incubation period was considered to be optimum because the recipient strain showed its typical visible phenotype of orange colour which indicated the growing of cells. The cells on filters were resuspended into 10 mM MgSO₄ solution, and the suspensions were transferred onto selective medium containing 100 µg/ml nalidixic acid and 50 µg/ml tetracycline which was similar to beetle luciferase mini-Tn5-*lucOR* insertion into *Ps. putida* (Vázquez *et al.*, 1994). Viable cell count was performed for control filters only after overnight incubation and results were 2.45×10⁸ for the recipient, 3.2×10⁸ for the *E. coli* CC118 and 1.5×10⁸ for the *E. coli* S17-1. The parental strains did not appear on the selective medium, but also no transconjugants were isolated.

5.2.2.2 Tri-parental matings

The recipient and donor strains were cultured as in the previous section 5.2.2.1, and *E. coli* HB101 containing pRK2013 helper plasmid was cultured in LB broth containing 20 µg/ml kanamycin at 37°C. Spot mating was performed using tri-parental matings (de Feyter and Gabrielle, 1991). For spot matings, 10-fold concentrated cells of recipient, donor and helper strains were spotted in the same ratio for up to 9 to 44 spots on BHIA then incubated at 28°C without selection overnight or until typical cell colour appeared on the BHIA plates, which was considered to correspond to growth. Several sets of experiments were tried using selective medium containing 50 µg/ml tetracycline and 100 µg/ml nalidixic acid, but these were not successful, similar to filter matings above. It was possible that the concentration of antibiotics used was too high for screening of transconjugants. In order to select the lowest MICs of tetracycline for strain VUN10,077, the cells were concentrated 10-fold when harvested at mid-exponential phase then spread onto BHIA contained 2, 5, 10, 20, 30, 40, or 50 µg/ml of tetracycline. Several colonies appeared at concentrations up to 5 µg/ml of tetracycline, but not at 10 µg/ml, and similar trials were made to determine an appropriate nalidixic acid concentration for donor and helper strains. The helper strain containing pRK2013 plasmid did not grow at this concentration of tetracycline (10 µg/ml) and nalidixic acid (40 µg/ml). The spot matings were carried out using BHIA containing 40 µg/ml nalidixic acid and 10 µg/ml tetracycline, but transconjugants were not isolated even in these trials. Accordingly, the selection procedure was modified as follows. The spot was resuspended in BHI containing 40 µg/ml nalidixic acid then incubated overnight and plated for single colonies. The single colonies were transferred onto fresh BHIA plates containing 10 µg/ml tetracycline and incubated until transconjugants grew. Using this approach, only two bioluminescent transconjugants out of several hundred colonies screened were isolated. These transconjugants showed bioluminescence reactions when supplied with external aldehyde and they were Gram negative and oxidase positive. These transconjugants were named VUN3,600 and VUN3,601, and stored as glycerol stocks in BHI at -80°C.

5.2.2.3 Preliminary characterization of transconjugants

5.2.2.3.1 Tetracycline and heat shock effects on *luxAB*-marked strain

The transconjugants were examined in terms of light output in the presence of tetracycline using a LSC (Wallac 1410, Pharmacia) and light output was affected by the tetracycline

concentration used (Figure 5.1). Antibiotic sensitivity tests were repeated for transconjugants, parent strain, donor strain and helper strain at concentrations of 10, 20, 30, 40 and 50 µg/ml of tetracycline or nalidixic acid in BHIA at 28°C: the transconjugants and parent strain grew at up to 50 µg/ml nalidixic acid, while the donor strains grew at this concentration of tetracycline, and the helper strain did not grow on either antibiotics tested. It was noted that the transconjugants did not grow when the tetracycline concentrations exceeded 10 µg/ml in BHIA. BHI broth was used for growing the transconjugants at concentrations of 1 and 10 µg/ml tetracycline initially and, as expected, the cells did not grow in the presence of 10 µg/ml tetracycline. To test whether tetracycline affected the light emission for transconjugants, samples were taken during the growth cycle and bioluminescence was measured using LSC after delivering the external substrate, and transconjugants showed different induction level of bioluminescence depending on the tetracycline concentration used. To examine the light output throughout the growth cycle, the transconjugants were cultured in BHI broth containing 1 µg/ml and 5 µg/ml of tetracycline and these were compared with non-*luxAB*-marked parent strain VUN10,077 grown without antibiotics. The external substrate was applied by evaporation from 1 µl of decanal placed onto lid of the scintillation vial in tests, and bioluminescence was measured over 1 minute and initial values were recorded as CPM. The transconjugants showed similar growth rate compared to the parent strain VUN10,077, even in the presence of the tested tetracycline concentrations, in terms of absorbance readings at 600 nm. Transconjugant VUN3,600 gave better luciferase reaction at 1 µg/ml tetracycline compared to 5 µg/ml tetracycline, whereas transconjugant VUN3,601 showed higher bioluminescence reaction at 5 µg/ml tetracycline in this type of analysis. When bioluminescence was examined after several subcultures on BHIA, VUN3,600 had a similar profile to previous trials but VUN3,601 had relatively lower bioluminescence at 5 µg/ml tetracycline, which was similar to VUN3,600. These results implied that VUN3,600 was stably marked with the *luxAB* genes on the chromosome compared with VUN3,601 in this analysis, or the 1 minute testing period may have affected the bioluminescence outcome or initial measurements of light output value were not suitable for the *luxAB* engineered strains. Transconjugants were further examined using semi-continuous measurement instead of recording initial light output only. Overnight cultures were tested using LSC for up to 50 minutes and measured for 10 seconds intervals: similar values were observed within 10 minutes, although VUN3,600 showed higher bioluminescence later than VUN3,601, which may have inferred that measurement of

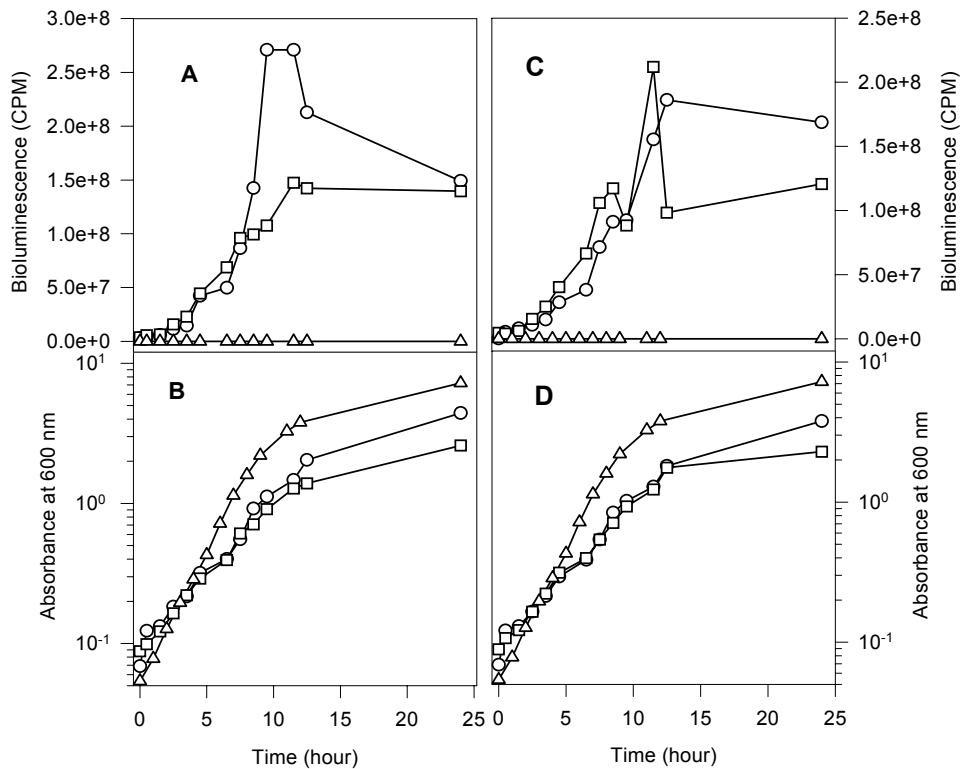


Figure 5.1. Kinetics of bioluminescence reaction and growth curve of the *luxAB*-marked VUN10,077. Bioluminescence reaction (A and C) and growth curve (B and D) samples were taken at the same time. Strains were cultured in BHI broth at 28°C for wild type VUN10,077 (Δ), VUN3,600 (\circ) and VUN3,601 (\square) throughout the growth cycle. Bioluminescence was measured for 60 seconds (CPM) using LSC, and VUN3,600 (A and C) and VUN3,601 (C and D) were grown in the presence of tetracycline 1 μ g/ml (A and B) and 5 μ g/ml (C and D), and wild type grown without antibiotics. One μ l of *n*-decyl aldehyde was applied on the top of the lid of the scintillation vial and 100 μ l testing volume of culture was used.

bioluminescence was not affected by the exposure period to external aldehyde in this type of analysis. However, when transconjugants were measured for a 10 seconds testing period as CPS, during the growth phase with 0, 1 or 5 µg/ml tetracycline, bioluminescence reactions were affected by tetracycline concentration. When 1 µg/ml tetracycline was added into BHI broth, both transconjugants produced higher levels of induction compared to 5 µg/ml tetracycline and no addition of tetracycline gave similar results for VUN3,600 and VUN3,601 (Figure 5.2), with light emission occurring from A_{600} 0.05 and this was maintained over the culture period and overnight cultures retained their luminescence ability. Relatively higher light output occurred when the test culture samples were taken from growing cultures at A_{600} between 0.8 and 1.2 for the given measurement conditions (see Figure 5.1). It was interesting that transconjugants again showed better bioluminescence reaction in the presence of 1 µg/ml tetracycline compared to 5 µg/ml tetracycline in BHI broth throughout the growth cycle: A_{600} readings were similar when grown at 28°C (Figure 5.2 and 5.3) and viable counts did not show significant differences for up to 9 hours of culture for the tested concentrations of tetracycline and parallel control. VUN3,600 had relatively high levels of induction of bioluminescence even in the presence of various concentrations of tetracycline on the growth compared with VUN3,601 and results were similar to those seen in Figure 5.1. These results indicated that the transconjugants were affected by tetracycline concentrations even when using different measurements modes where growth in the presence of 1 µg/ml tetracycline gave higher induction levels of light output and VUN3,600 had stable bioluminescence reactions compared to VUN3,601. However, the bioluminescence reaction could also occur without tetracycline even after several subcultures in BHIA at 28°C, and the transconjugants had similar growth rates compared to the parent strain VUN10,077.

Bioluminescence was significantly decreased when the cells were subjected to heat shock (Figure 5.2). Although the bioluminescence reactions were different for tetracycline concentrations of 0, 1 µg/ml, 5 µg/ml, the light output was significantly affected by rapid heat transfer to 37°C followed by continued growth at this temperature, for both transconjugants VUN3,600 and VUN3,601. In contrast, A_{600} values continued to increase for up to 4 hours after heat shock although the A_{600} had not increased significantly in overnight cultures. Final A_{600} readings were affected by different concentrations of tetracycline following heat shock while the control culture showed similar final A_{600} readings with or without heat shock (Figure 5.3). Transconjugant VUN3,600 had final A_{600} readings of 1.23, 1.14 and 1.91 in the presence of tetracycline 1 µg/ml, 5 µg/ml and none respectively.

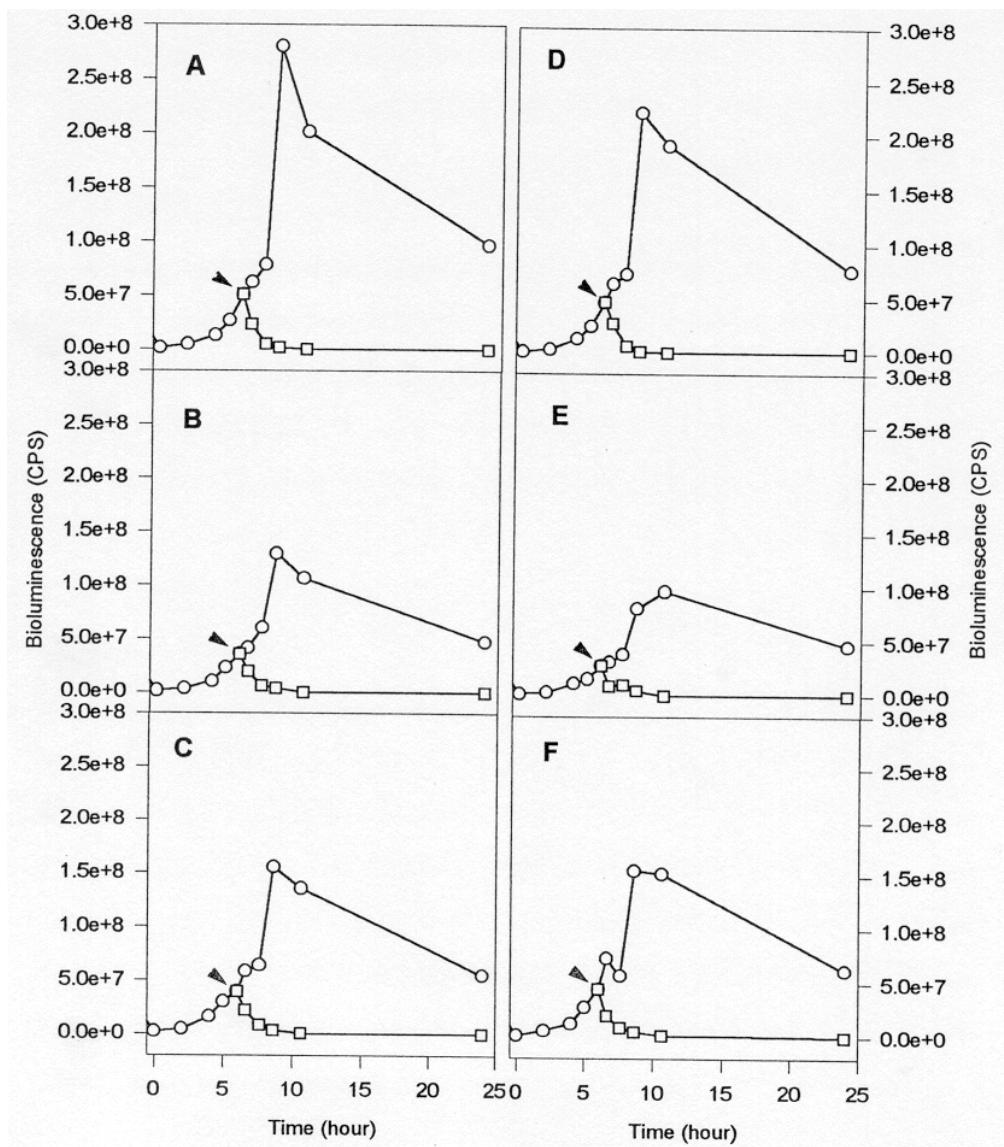


Figure 5.2. Kinetics of bioluminescence reaction context with heat shock from 28°C to 37°C in BHI broth. Strains were cultured in BHI broth either at 28°C throughout the growth cycle (○) or heat shock (rapid transfer to 37°C) was applied at mid-exponential phase (arrow), then

growth continued at 37°C (□). Bioluminescence was measured 10 seconds (CPS) using LSC, and VUN3,600 (A, B and C) and VUN3,601 (D, E and F) grown in the presence of tetracycline 1 µg/ml (A and D), 5 µg/ml (B and E) and without (C and F). One µl of *n*-decyl aldehyde applied on the top of lid and 100 µl testing volume was used.

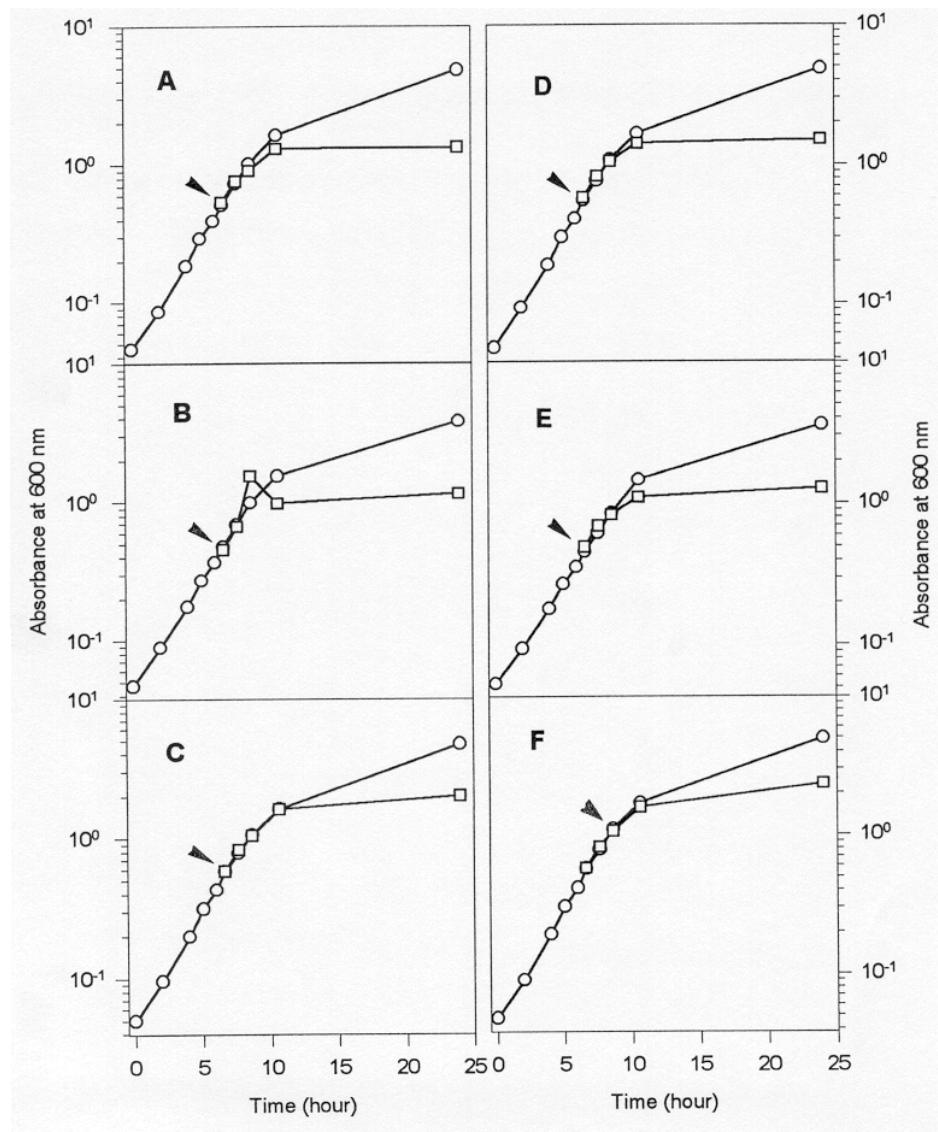


Figure 5.3. Effect of temperature upshift on growth rates of transconjugants. Strains were cultured in BHI broth either at 28°C throughout the growth cycle (○) or heat shock (rapid transfer to 37°C) was performed at mid-exponential phase (arrow) then growth was continued at 37°C (□) when VUN3,600 (A, B and C) and VUN3,601 (D, E and F) grew in the presence of tetracycline 1 µg/ml (A and D), 5 µg/ml (B and E) and without tetracycline (C and F).

Similarly, transconjugant VUN3,601 had A_{600} readings of 1.39, 1.14 and 2.02 at similar concentrations of tetracycline respectively. When the transconjugants grew without antibiotics, A_{600} values were relatively high compared to tetracycline supplemented cultures throughout the growth cycle and also higher final A_{600} readings were obtained at a concentration of 1 $\mu\text{g}/\text{ml}$ tetracycline compared to 5 $\mu\text{g}/\text{ml}$ tetracycline. Similar growth kinetics were not observed when the parent strain was heat shocked from 28°C to 37°C (see Figure 4.1), where growth was stimulated in BHI immediately following temperature upshift although there was an early onset of stationary phase. These results indicated that transconjugants had better tolerance to heat stress without tetracycline, which may indicate that tetracycline affects the cell metabolism so that the heat shock response is impaired or that the *luxAB*-marked strains have altered heat stress responses. This was not further investigated.

5.2.2.3.2 Southern blot

The above differences in the kinetics of growth and bioluminescence may have arisen in transconjugants VUN3,600 and 3,601 due to different *luxAB* marking on the chromosome. To confirm the presence and relative location of *luxAB* integration onto the chromosome, Southern hybridization (Figure 5.4), and several biochemical tests were performed. Transconjugants VUN3,600 had similar biochemical characteristics to the parent strain, such as Gram negative and oxidase positive, and also morphological features (see Tables 3.1 and 3.2). These transconjugants showed constant luciferase reaction when the external substrate was delivered into cultures, which normally occurs because the light emitting genes, *luxAB*, are located on the chromosome within genes which are constitutively expressed (de Lorenzo *et al.*, 1990). The genomic DNAs for Southern hybridization were purified using the alkaline lysis method as described by Sambrook *et al.* (1989), and α -³²P dATP (3,000 Ci/mmol, Amersham, aqueous solution) labelled as described in section 2.3.6.2. The *luxAB* probe was prepared as described in section 2.3.5.2. The nitrocellulose membrane was washed with SSPE buffer containing 0.1% SDS at room temperature and exposed to Kodak X-ray film at -80°C. The film was developed as described in section 2.4.6.2. The two transconjugants VUN3,600 and VUN3,601 showed different genotypes as shown by Southern hybridization blot analysis. *EcoRI* digests did not react with the *luxAB* probe (due to small fragment generated) but, as expected, *Sall* digests produced one band for VUN3,600 and 3,601 as well as for the control *E. coli* DNAs, noting that *Sall* cuts either side of the *luxAB* in the vectors

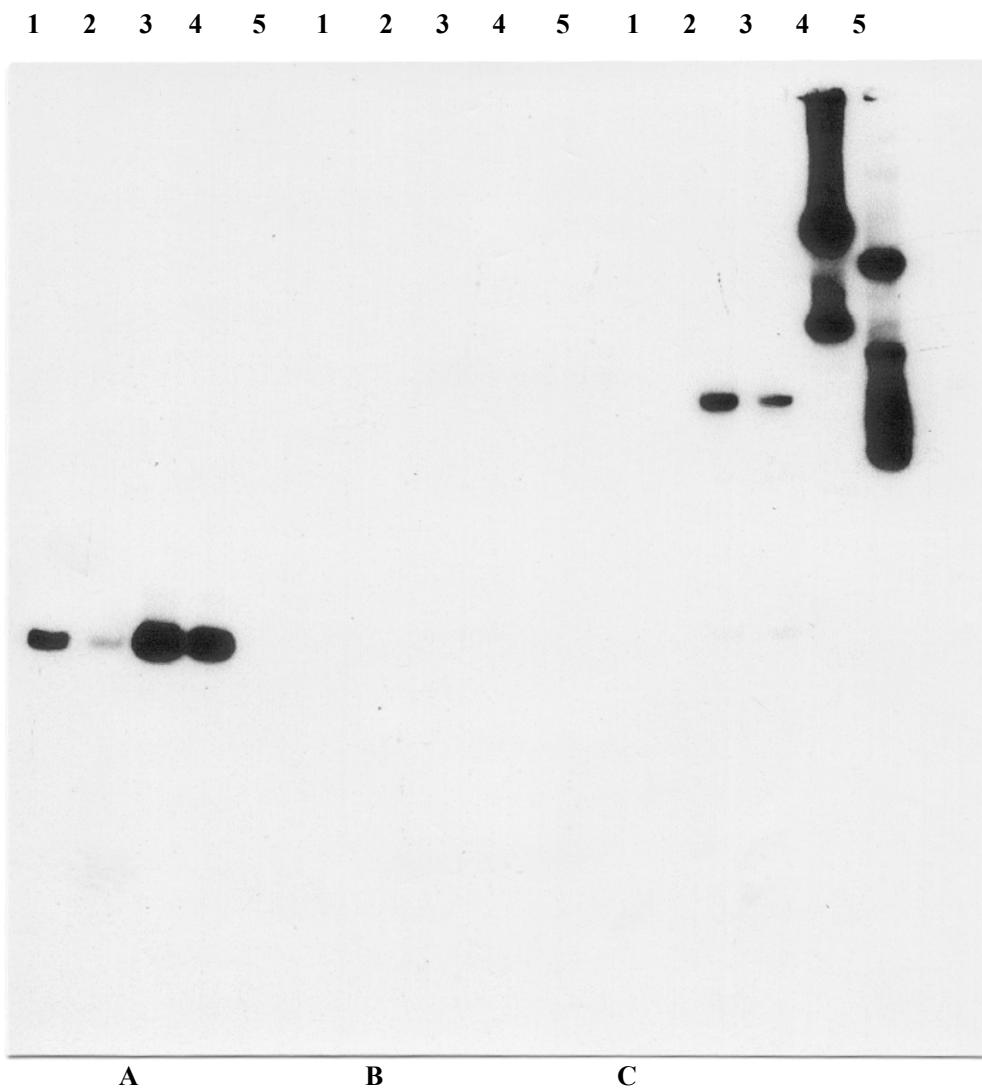


Figure 5.4. Southern hybridization of transconjugants using a *luxAB* probe. The probe was recovered from 0.8% agarose gel after *SalI* digestion of pUT::Tn5 *luxAB* and labelled with α -³²P dATP using Klenow enzyme. The membrane was hybridized at 65°C overnight and washed with SSPE buffer contained 0.1% SDS at room temperature. The genomic DNA was extracted from *E. coli* S17-1 (lane 1), *E. coli* CC118 (lane 2), VUN3,601 (lane 3), VUN3,600 (lane 4) and parent strain VUN10,077 (lane 5) and digested with *SalI* (A), *EcoRI* (B) and *BstEII* (C). The X-ray film was developed after 3 days at -80°C.

used. Transconjugant VUN3,600 and VUN3,601 showed different patterns of hybridization with the *luxAB* probe in *Bst*EII digests, which had several strong bands which did not line up between digests of VUN3,600 and VUN3,601. The parent strain VUN10,077 did not show bands on the blots when the genomic DNA was digested with *Sa*II, *Eco*RI and *Bst*EII. This result indicated that two transconjugants had the different genotypes and this may have given rise to the different induction levels of bioluminescence seen.

5.2.3 Attempts at isolation of a positively regulated *lux* gene system

Attempts to obtain transconjugants of VUN10,077 showed limited success, as only a few transconjugants were produced and very poor efficiency was seen in the previous section 5.2.2. This prompted an examination of using electroporation and the first experiments examined the effect of growth phase on electrotransformation of strain VUN10,077. The cells were grown at 28°C in BHI broth to the desired A₆₀₀ and harvested by centrifugation. One µg of plasmid DNAs were mixed with a 10-fold concentrated cell suspension and electroporation was performed as described in section 2.2.5.2. The recipient cells were used directly after cell harvesting. Electroporation buffer containing 300 mM sucrose instead of 15% glycerol was used to improve the transformation efficiency in pseudomonads (Miller, 1994; Taghavi *et al.*, 1994). The cells were transferred to BHI recovery medium contained 0.5% glucose, 10 mM MgCl₂ and 10 mM CaCl₂ and incubated for an appropriate period then plated onto selective medium. Bioluminescence genes carried by plasmids pUT::Tn5 *luxAB*, pUCD607, pUCD615 and pUCD623, were transformed into strain VUN10,077 and transformants were isolated from appropriate selective medium then these isolates were examined for light output under heat stress conditions. The phenotype being sought was transconjugants where the *luxAB* genes had been integrated into heat-inducible chromosomal genes.

5.2.3.1 *LuxAB*-marking system

Electroporation buffers, 15% glycerol and 300 mM sucrose, were used for pUT::Tn5 *luxAB* transformation initially. The recipient cells were harvested at A₆₀₀ 0.488 (1.69×10^8). After electroporation using pUT::Tn5 *luxAB*, aliquots of 200 µl were plated onto BHIA containing 50 µg/ml tetracycline and 50 µg/ml ampicillin, and incubated for 3 days at 28°C. Two

Table 5.3. Transformation strain VUN10,077 using *luxAB* and electroporation. One µg of PUT::Tn5 *luxAB* plasmid DNA and 10 µg/ml tetracycline selective medium were used. Cells were incubated for 2 or 12 hours in BHI recovery medium containing 0.5% glucose, 10 mM MgCl₂ and 10 mM CaCl₂. The time constant was between 4.1 to 4.8.

Viable cell number ($\times 10^6$) (A ₆₀₀)	Recovery time			
	2 hours		12 hours	
52.7 (0.2)	2.67	1	17.9	750
134 (0.4)	25.68	0	15	0
190 (0.55)	41.0	0	24	2
253 (0.9)	43.7	0	8	0
293 (1.1)	310.0	0	17	0
226 (1.35)	900.0	25	15.6	30
350 (1.8)	135.0	28	-	-
653 (3.2)	-	-	27	3

a, transformants/ml

transformants were isolated from each buffer used and bioluminescence reactions did not occur when these transformants were examined using LSC for 1 minute for samples taken throughout the growth cycle, as readings were 25-53 CPM while *E. coli* S17-1 gave highest counts of 3,502,589 CPM. Moreover, further transformants were not isolated after several sets of trials using this approach, which may have been due to the selection pressure being high (note that these experiments were done before the low tetracycline resistance of the transconjugants was known). The selective medium was then modified as follows: BHIA plates contained 10 µg/ml tetracycline and recipient cells were harvested using 300 mM sucrose buffer at various growth stages which were A_{600} 0.225, 0.499 and 0.785. One transformant was screened at A_{600} 0.785, although the level of induction of light was 104.5 RLU after delivering the substrate when compared to the control of -0.2 RLU and VUN3,600 of 7,500 RLU. These results indicated that the growth stage may be important for successful transformation of the pUT::Tn5 *luxAB* into strain VUN10,077. Results of the effect of growth phase and recovery time on transformation efficiency are shown in Table 5.3: several transformants were isolated when cells from early-log or stationary phase were used in this systematic approach. This locally isolated strain VUN10,077 was not capable of being transformed at mid-exponential phase for *luxAB* gene insertion onto the chromosome. The number of transformants per 1.0 ml sample plated was 1, 25 and 28 when cells were harvested at A_{600} 0.2, 1.35 and 1.8 respectively after 2 hours incubation in BHI recovery medium, and no transformants were isolated when cells were harvested at the rest of the A_{600} readings. Similar observations were made when recipient cells were kept on ice for up to 4 hours after 10-fold concentration then incubated for 2 hours in recovery medium after electroporation: the number of transformants was 1, 9 and 29 when cells were harvested at A_{600} 0.2, 1.35, and 1.8 respectively. Interestingly, when the pUT::Tn5 *luxAB* plasmid DNA concentration was increased 2-fold, the efficiency seen was not affected and the numbers of transformants remained low (14 obtained when cells were harvested at A_{600} 1.8). The growth phase and recovery time were both important for obtaining transformants, and this was not affected by the recipient cell storage time on ice or 4°C after cells were concentrated 10-fold at the different growth stages. When cells were incubated for 12 hours in BHI recovery medium, the transformation efficiency was significantly increased when cells were harvested and concentrated at A_{600} 0.2, and similar numbers of transformants were isolated at A_{600} 1.35; no transformants were isolated at A_{600} 0.4, 0.55, 0.9 or 1.1, which was a similar result as seen for 2 hours incubation after electroporation.

It was found that the resulting transformants did not require a high salt concentration to maintain the light output and viability, however, the light output was less than seen for transconjugants VUN3,600 and VUN3,601: when randomly selected transformants were screened using the same methods applied to the transconjugants, light output using LSC was around 100 RLU or lower. Therefore, all obtained transformants were subjected to heat stress using a microtitre plate assay to screen large numbers (Flemming *et al.*, 1994) to see whether these transformants were positively regulated by heat-inducible promoters in terms of bioluminescence reaction.

Cells were harvested at A_{600} 0.2 and 1.2, concentrated, electroporated and incubated for 12 hours in BHI recovery medium before selection on BHIA containing 5 μ g/ml tetracycline. More than two thousands transformants were screened using selective medium containing 5 μ g/ml tetracycline in microtitre plates to analyse the bioluminescence reaction and each single colony was grown in BHI broth at 28°C. The 21 microtitre plates were exposed on X-ray film (Kodak) for 10 minutes, 30 minutes, 1 hour and 2 hours, but these exposure times did not detect clear induction of bioluminescence (as distinguished by the difference of intensity of each well), which was similar to the results obtained using LSC for randomly selected transformants. Similar observations occurred after heat stress from 28°C to 37°C for 2 hours using a heating block and external substrate delivered by evaporation (section 2.2.6). Conditions of X-ray film development were standardized to obtain relative signal intensity, which was develop for 5 minutes, 37% acetic acid for 1 minute, fixing step for 5 minutes and washing with tap water. Several hundred single colonies were screened by X-ray film development and the signals were verified using laser densitometry scanning when the intensity of each well was discerned. In parallel, "luxdot" assay was tried on nitrocellulose transfer membranes (Bartelt Instrument Pty, Ltd.) using *luxAB* engineered strains (Legocki *et al.*, 1986), but the results were not reliable and repeatable because signal strength depended on dryness of the samples which may cause false-positive interpretation. Although several trials were made using this approach, *luxAB*-marked transformants which gave a positive bioluminescence reaction under heat shock conditions were not isolated, noting that strain VUN10,077 showed major HSP induction during heat-stress under these conditions (described in Chapter 4). This failure may have been due to failure to mark the chromosome with *luxAB* genes in heat inducible operons or the method of screening being unable to detect induction. For example, transconjugants may have grown on the bottom of well in the absence of good mixing, so that external aldehyde did not penetrate sufficiently to cells to

Table 5.4. Transformation efficiency of the whole *lux* operon by electroporation (transformants per μg DNA). The recipient cells were grown in BHI broth at 28°C and the cells were subjected to 12 hours recovery time in BHI recovery medium used the same as Table 5.3. The time constant was between 4.2 and 4.7.

Plasmid	A_{600}						
	0.21	0.41	0.55	0.93	1.1	1.3	3.17
<hr/>							
pUCD607							
transformants ($\times 10^2$)	12 ^a	5	1	2	2.5	4	7.5
survival cells ($\times 10^6$)	1.93	1.51	1.85	2.07	1.66	1.87	2.72
pUCD615							
transformants ($\times 10^2$)	16.5	16	0.12	0.65	3	3	15
survival cells ($\times 10^6$)	1.86	1.33	1.70	2.56	1.80	1.05	2.76
pUCD623							
transformants ($\times 10^2$)	0	0	0	0	0	0	0
survival cells ($\times 10^6$)	2.02	2.48	1.68	1.86	1.45	2.07	4.55

a, transformants per μg of DNA on appropriate selective media (see text).

induce the light output, noting that overnight flask cultures of strain VUN10,077 settled when left on the bench without shaking. It was determined to use the whole *lux* operon for marking strain VUN10,077, since the *lux* system would not require exposure to the external substrate.

5.2.3.2 Whole *lux* operon-marking system

As shown by others and later in this thesis, when *luxAB* is located in naturally non-bioluminescent organisms, difficulties in quantification of bioluminescence arise using extracellular substrate because the added aldehyde is injurious and can vary in intracellular concentration as a function of cell age. Light decaying rates are often so fast, for example the half-life of initial light output is within a minute in transconjugants, so that screening methods may fail to detect these. These limitations needed to be investigated using the whole promoterless *lux* operon, *luxCDABE*, using plasmids pUCD615, pUCD623 and pUCD607. These systems can provide an endogenous aldehyde from the cellular fatty acid and directly eliminate the problem of substrate delivery. The pUCD623 Tn4431 plasmid contains a promoterless *lux* operon, a constitutive tetracycline gene, a resolvase and a transposonase derived from Tn1721 (Shaw *et al.*, 1988). The vector can be transferred to the desired organism by electroporation, in which it is unable to replicate so that expression requires that the transposon is mobilised onto the chromosome. The broad-host-range vector pUCD615 plasmid was used to screen for promoter activities through the formation of gene fusions with the promoterless *lux* operon in this vector once transposition had occurred. The vector was designed to allow fusion of *lux* to promoters of interest so that promoter activity could be measured (Rogowsky *et al.*, 1987), in addition to pUCD607 plasmid, which carries a *tet-lux* fusion (Shaw and Kado, 1986) (see Table 2.1).

In preliminary experiments, selective media were tested using 10-fold concentrated VUN10,077 cells harvested at A₆₀₀ 0.2 or 1.0. Under these conditions, strain VUN10,077 could grow in the presence of 50 µg/ml ampicillin and several colonies appeared on the BHIA plates containing 10 µg/ml and 25 µg/ml kanamycin after 3 days and 4 days incubation respectively. Consequently, the selective medium used for experiments using pUCD615 was BHIA containing 20 µg/ml kanamycin, 15 µg kanamycin plus 150 µg/ml ampicillin for pUCD607, and 10 µg/ml tetracycline plus 40 µg/ml nalidixic acid for pUCD623 to select transformants of VUN10,077. Transformation efficiency was then examined throughout the growth cycle (Table 5.4). Unlike results for pUT::Tn5 *luxAB*, the number of transformants

obtained for pUCD607 and pUCD615 was high across the growth phases but the efficiency was still better if cells were harvested at either early exponential or stationary phases. The pUCD615 plasmid that contains the promoterless whole *lux* operon transformed at high efficiency at A_{600} 0.2 but this declined at A_{600} 0.55 and 0.93 and the number of transformants increased at stationary phase, when transformants were screened on BHIA containing 20 µg/ml kanamycin after 3 days incubation (Table 5.4). The pUCD607 plasmid transformation efficiency showed a similar pattern to the pUCD615 plasmid transformation throughout the growth phase. However, transformants were not isolated when using pUCD623, despite its successful use previously with *X. campestris* (Shaw *et al.*, 1988) and *Ps. fluorescens* 5R (King *et al.*, 1990). This may have occurred due to lack of successful electroporation or failure of the Tn443I to transpose.

The electroporation frequencies were sufficient to screen numerous single colonies from selective medium, however, the light emission level of transformants was very low in cultures using LSC, with levels of induction similar to controls: one ml of growing cultures of transformants showed less than 1.0 RLU. When CdCl₂ was added at concentrations of 0.001 gL⁻¹ and 0.0005 gL⁻¹ to apply stress, transformants from pUCD615 still showed very low light output (0.2-0.7 RLU). In parallel, the isolated transformants were examined using microtitre plate assays to screen for light emitting transformants under heat stress and several intensified spots were verified after heat shock from 28°C to 37°C (Figure 5.5). Transformants obtained from pUCD615 plasmid transformation were transferred into microtitre plates and incubated for up to 3 days, when discrete cell turbidity or typical orange color appeared as indicators of growth, and the standardised conditions used to develop X-ray films after exposure to microtitre plates for 2 hours, as described in the section 5.2.3.1. This assay provided sufficient information about different levels of light emission and relatively strong induction occurred in 4-5% of the 2000 tested transformants. Relative light output was confirmed using laser densitometry, which showed that the level of bioluminescence induction was 3.5 times higher compared to the lowest induction observed. This assay could be improved significantly, as the configuration used did not account for cell numbers in inocula so that qualitative information only was obtained. Presumptive positive clones were selected and examined further using larger scale culture and cells were subjected to rapid transfer to 37°C then bioluminescence measured using LSC: results were largely similar to the untransformed, parent controls. Great difficulty was encountered in measuring the positive regulation of light output using LSC even for the control cultures, which may demand

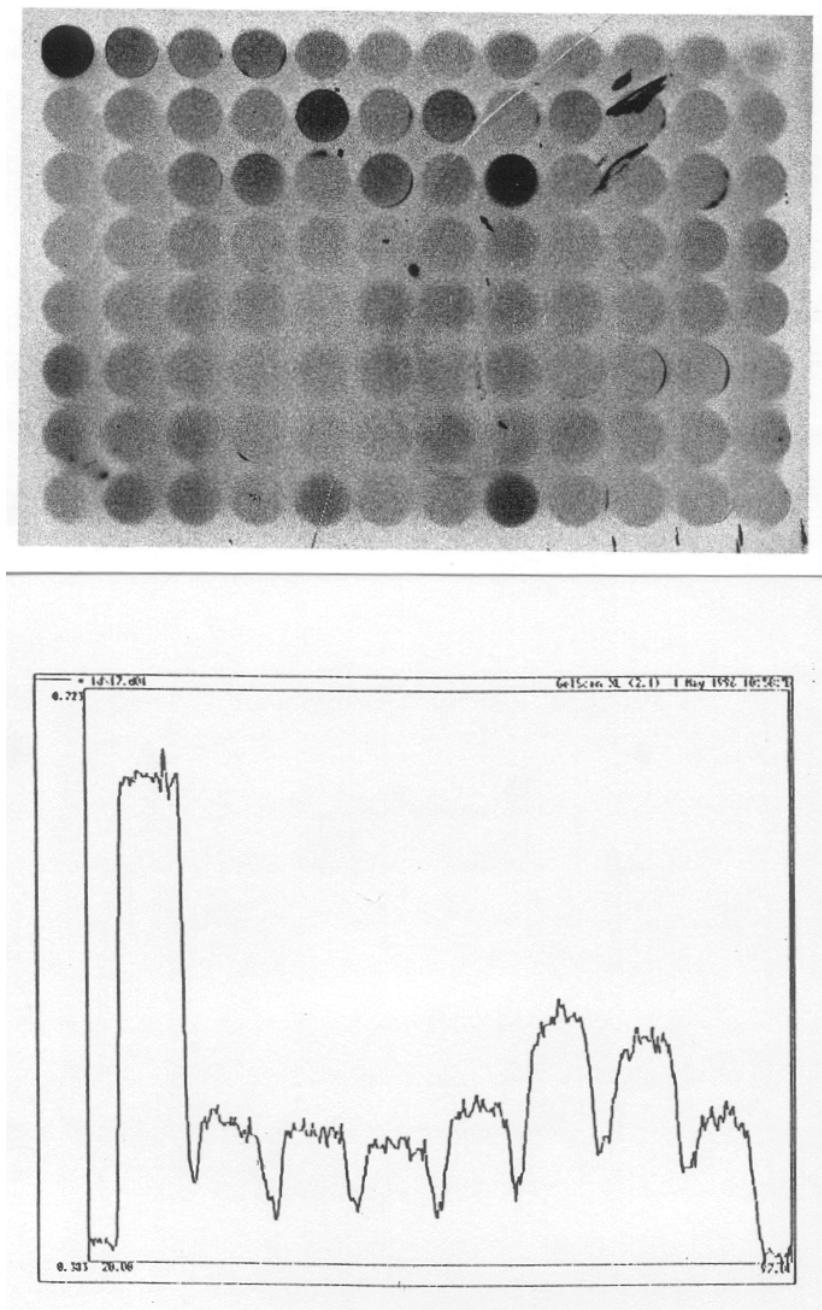


Figure 5.5. Laser densitometry analysis of X-ray film exposed to whole *luxCDABE*-marked transformants of strain VUN10,077. The individual single colonies were transferred to microtitre plate wells containing BHI broth and incubated for 3 days at 28°C. The X-ray film (top) was exposed for 2 hours after heat shock at 37°C and scanned with a laser densitometer (bottom) from one extreme left lane of the above microtitre plate.

thermocontrol during the testing period or light emission levels were too subtle to detect in this type of analysis. Despite extensive efforts to isolate strains of VUN10,077 with the whole *lux* operon on the chromosome under control of either a constitutively expressed or inducible promoter, candidate transformants were not obtained due to failure to integrate/transform the vector or the resulting presumptive marked transformants had low light output. Efforts were therefore concentrated on the previously isolated transconjugants.

5.3 Optimisation and stabilisation of light output by *luxAB*-marked transconjugant VUN3,600

Stabilisation of light output by transconjugant VUN3,600 was accomplished by finding the optimal growth conditions and methods of supplying the external substrate, as well as defining suitable cell preparation and measuring buffers. In early experiments, light emission was seen at all growth phases in the transconjugants when the external substrate was delivered, while this type of reaction was not observed in an engineered *E. coli* strain (Ulitzer and Kuhn, 1988). Despite the earlier described practical problems using decanal as an *in vivo* assay reagent, this appears to be the optimal substrate in terms of the biochemistry of luciferase, although other shorter-chain aldehydes and other proposed substitutes have been studied (Hastings and Gibson, 1963; Meighen, 1988; Ismailov *et al.*, 1990). Also, facilitated bioluminescence has been obtained using decanal with *luxAB* gene constructs only out of the up to 13 genes of the *lux* operons in several natural bioluminescent organisms (Meighen and Dunlap, 1993; Blouin *et al.*, 1996). The transconjugant VUN3,600 produced stable and higher light output compared with VUN3,601 under similar assay conditions, which was described in section 5.2.2.3.1. Moreover, transformants obtained using the same vector (pUT::Tn5 *luxAB*) by electroporation were similar but no better than the transconjugants and transformants obtained using the whole *lux* operon gave poor light output under the given conditions. On these grounds, the *luxAB*-marked transconjugant VUN3,600 was selected for further examination as reported in this section. The *luxAB*-marked VUN3,600 had a typical luminescence reaction pattern, which caused a problem in measuring light output in assays where the substrate is added externally. Substrate delivery systems and changes in light emitted at different growth phases (Korpela *et al.*, 1989; Eaton *et al.*, 1993; Iizumi *et al.*, 1998) were areas to eliminate the intrinsic problems of *luxAB*-marked strains (Blouin *et al.*,

1996) using continuous and/or semi-continuous measuring systems, as reviewed by Burlage and Kuo (1994). When transconjugant VUN3,600 was grown at 28°C in uninduced conditions, cells retained their capability to produce relatively stable light output compared to output seen under heat stress conditions (see section 5.2.2.3.1 and Korpela *et al.*, 1989). A similar observation reported in engineered yeast by Y. Chen (CBFT, personal communication). The bioluminescence reaction was measured using LSC, when samples were prepared by centrifugation and stored at room temperature (Korpela *et al.*, 1989; Tescione and Belfort, 1993) after determining a substrate concentration to adjust the luciferase activity. The *in vivo* activity of luciferase was expressed as RLU using LSC as described in the methods and material section 2.2.6.

5.3.1 Preliminary optimization of light output

The substrate delivery system had emerged as a problem in earlier assays because evaporation resulting in weak or no response to cadmium (CdCl_2) or mercury (HgCl_2). In preliminary experiments, cells were grown in BHI to A_{600} 1.1-1.4 and tested in the presence of aqueous solutions of cadmium and mercury in concentration ranges of 0.001 gL^{-1} to 1 gL^{-1} and water was added alone as a control, and bioluminescence was measured using LSC after delivering the $1 \mu\text{l}$ of *n*-decyl aldehyde by evaporation. Bioluminescence values declined and dose-related responses were observed when CdCl_2 was added into VUN3,600 cultures after harvest, while when HgCl_2 was added this did not cause a dose-related response and relative bioluminescence values were even higher than for water added alone. Moreover, HgCl_2 at 1 gL^{-1} did not affect bioluminescence compared to other tests, when light output was measured semi-continuously for 12 minutes. This observation was inconsistent with viable counts data which were closely related to protein profile changes when HgCl_2 was added into growing cultures of strain VUN10,077 (see Chapter 4): viable cell counts immediately declined when 0.005 gL^{-1} HgCl_2 was added and 1 gL^{-1} HgCl_2 dropped the A_{600} readings 3-fold immediately and viability did not recover at all during the test period, which implied that cells lost viability or completely shut down their metabolism. Furthermore, transconjugant VUN3,600 had less tolerance to heat stress compared with strain VUN10,077 under similar conditions (see section 5.2.2.3.1). On the basis of these observations, transconjugant VUN3,600 was obviously impaired when compared with strain VUN10,077, which may have been related to random insertion of *luxAB* genes on chromosome of VUN3,600. Therefore, relative bioluminescence

values were expected to decline at 1 gL⁻¹ HgCl₂ compared to lower concentrations of added HgCl₂. These results implied that mercury did not affect bioluminescence in a controlled way in this type of analysis or that substrate delivery method was not effective, and the solvent itself could affect the bioluminescence. Therefore, the substrate delivery system was investigated initially before investigating the effects of other parameters, such as growth phase, on light output.

5.3.1.1 Selection of substrate delivery system

Bioluminescence reactions were measured continuously to see the kinetics of total reactions using LS50 luminometer (LS50 luminescence spectrometer, Perkin Elmer) in phosphorescence mode. Several different solvent systems were selected to determine the substrate delivery system such as 1% substrate in ethanol, 10³-fold diluted into water, water plus ethanol mixture and 2×10³-fold diluted in 0.9 M NaCl. The application of the substrate delivery systems was as follows: one μ l of aldehyde applied on the top of the lid of a scintillation vial to allow evaporation, 10 μ l of 10³-fold aldehyde diluted into Milli-Q water (10 μ l of decanal in 10 ml of Milli-Q water) was added after sonically dispersing (Schultz and Yarus, 1990), 20 μ l of 2×10³-fold diluted aldehyde in 0.9 M NaCl was injected after sonically dispersing (Legocki *et al.*, 1986), 1 μ l of a mixture of one part aldehyde in 9 parts ethanol plus 90 parts water (Sizemore *et al.*, 1993), and 1 μ l of 1% aldehyde in ethanol was added into 100 μ l of test sample (Gordon *et al.*, 1994). Initially, these external substrate concentrations were examined using LSC but results were varied making it difficult to compare between delivery systems, which may have been due to the short periods used for measurement and initial reaction only being detected (see section 5.2.2.3.1). Subsequently, all the substrate solutions were freshly prepared and the same external substrate ratio was applied into 3 ml volumes of tests. Assays *in vivo* were done by quickly mixing various substrate solutions plus tests as culture suspension and measuring using the LS50 luminometer (Figure 5.6). Although the evaporation delivery system was not carried out at the same time due to lack of aeration and equipment, all tested delivery systems showed a rapid decline following an initial burst output phase within a minute, except for aldehyde diluted in 0.9 M NaCl. Highest intensity was 448.5 for 1% aldehyde in ethanol, 227.1 for 10³-fold diluted aldehyde into water, 337.3 for water and ethanol mixture and 30 for aldehyde in 0.9 M NaCl. A secondary burst phase occurred for aldehyde diluted in water and the water/ethanol mixture, but 1% aldehyde in ethanol kept the intensity of light output at a level

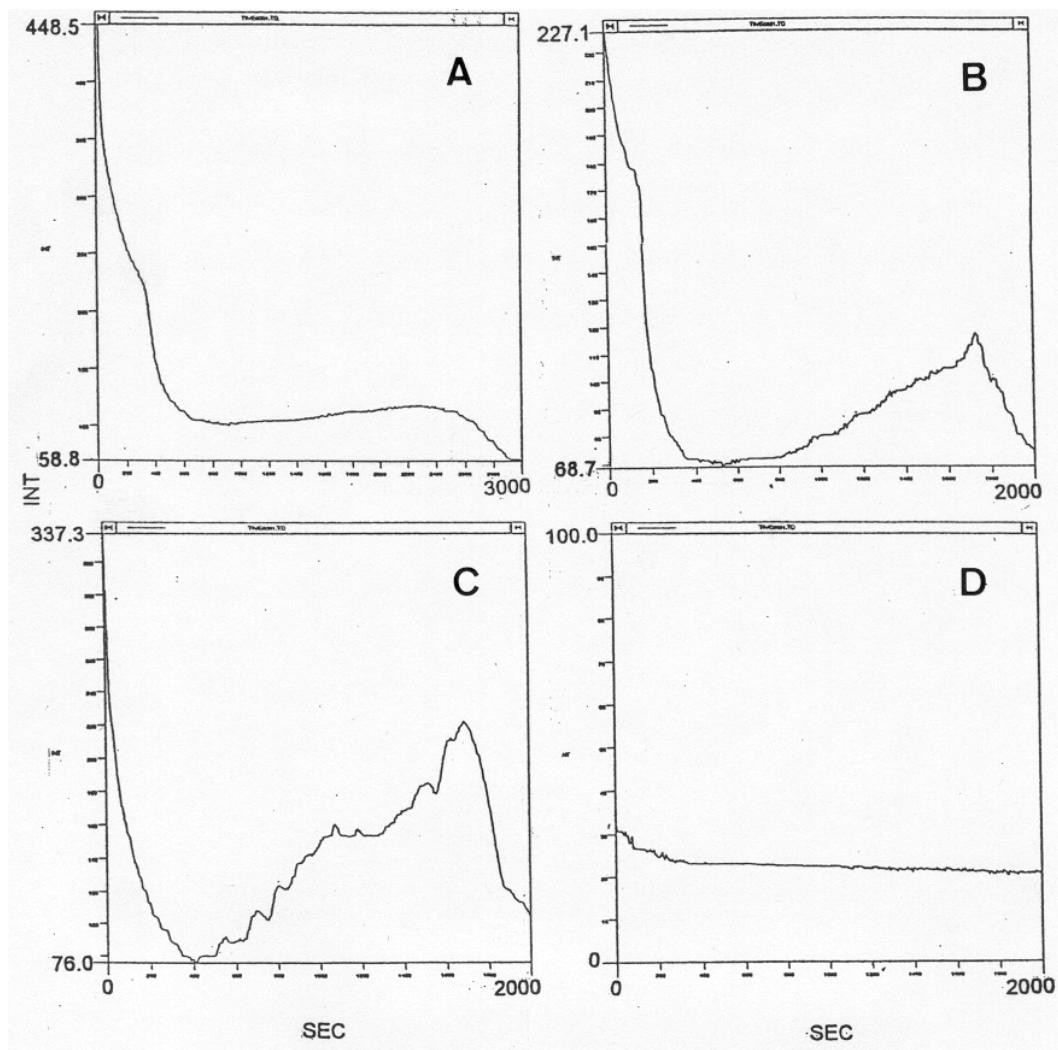


Figure 5.6. Time-course of bioluminescence in different substrate delivery systems using LS50 luminometer (Perkin Elmer). Four systems were examined using 3 ml cultures and substrates supplied as follows: 30 μ l of 1% aldehyde in ethanol (A), 300 μ l of 10³-fold diluted aldehyde (10 μ l of decanal in 10 ml of Milli-Q water) into Milli-Q water was added after sonication (B), 30 μ l of one part substrate in 9 parts ethanol plus 90 parts water mixture (C) and 600 μ l of 2 \times 10³-fold diluted aldehyde in 0.9 M NaCl was added after sonication (D) into 3 ml of culture suspension.

of approximately 100 after 7 minutes and this was longer than seen for other tests, then this declined at the end of the test period. The delivery system of 1% aldehyde in ethanol produced a relatively stable light emission stage compared with the other tests, noting that 10^3 -fold diluted aldehyde into water produced a similar bioluminescence reaction. The delivery system of 1% aldehyde in ethanol was optimal of the thus far tested conditions when external aldehyde was added into culture suspensions.

5.3.1.2 Light output with growth phase

Transconjugant VUN3,600 had shown various levels of bioluminescence at different growth phases, as seen in section 5.2.2.3.1. Bioluminescence reactions were measured using LSC and the LS50 luminometer at the same time and maximum light output occurred at an A_{600} 0.8-1.2 (Figure 5.7 and 5.8). Ten μl of substrate solution (1% in ethanol) was added to deliver the external substrate into 1 ml of test samples taken from BHI culture broth and the reaction was recorded by RLU using LSC and 30 μl of substrate solution delivered to 3 ml culture suspension when measuring using LS50 luminometer. The light emissions occurred from A_{600} reading of 0.1 and were maintained during the growth cycle, noting that overnight cultures retained bioluminescence with small standard deviations although the intensity was lower than maximum light output at A_{600} 0.8. When samples were taken at/or below A_{600} 0.4, relatively less bioluminescence was observed compared to other tests. The samples taken from growing cultures at A_{600} 0.8 produced maximum light output as measured using LSC (Figure 5.7). Although higher levels of induction occurred at mid-exponential phase just before cells entered stationary phase, at A_{600} 0.8 the standard deviations were greater than for sample taken at early-stationary phase, A_{600} 1.1-1.2, and a similar pattern was observed with 100 μl of test volume. When measured using LS50 luminometer, samples at A_{600} 1.6 produced maximum light output over the test period (Figure 5.8). Lower light intensity was observed at A_{600} 0.4, similar to when measured using LSC. Overnight culture samples produced relatively less light output and this was retained for up to 30 minutes before light output ceased, whereas different kinetics were seen when using LSC for measurement. Maximum light output occurred from A_{600} 0.8 to 1.6 and relatively stable light output was produced at A_{600} 1.1-1.2. Interestingly, the kinetics of induction and relative level of light intensity differed between the measurement systems used, which may have been due to different culture volumes, although similar ratios were used between substrate solution and sample volume, or cells settled at different rates during measurement (as described in

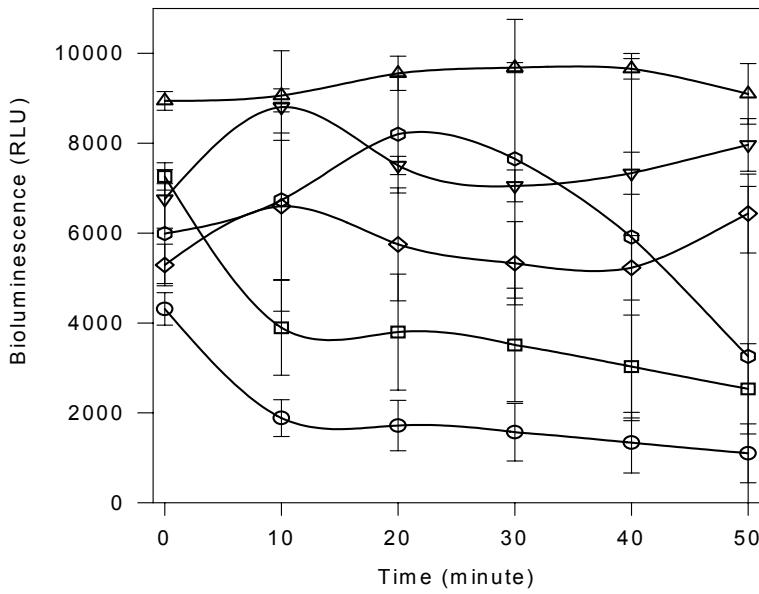


Figure 5.7. Light emission with growth phase of VUN3,600. Samples were taken from growing cultures of VUN3,600 and growth continued at 28°C in BHI. The luminescence reaction was initiated by adding of 10 μ l substrate solution, 1% aldehyde in ethanol, to the vial containing 1 ml test culture suspension. RLU values were measured using LSC and recorded semi-continuously at A_{600} 0.2 (\circlearrowleft), 0.4 (\square), 0.8 (\triangle), 1.1 (∇), 1.6 (\diamondsuit) and overnight culture (\circ). Standard deviations were based on triplicate analyses.

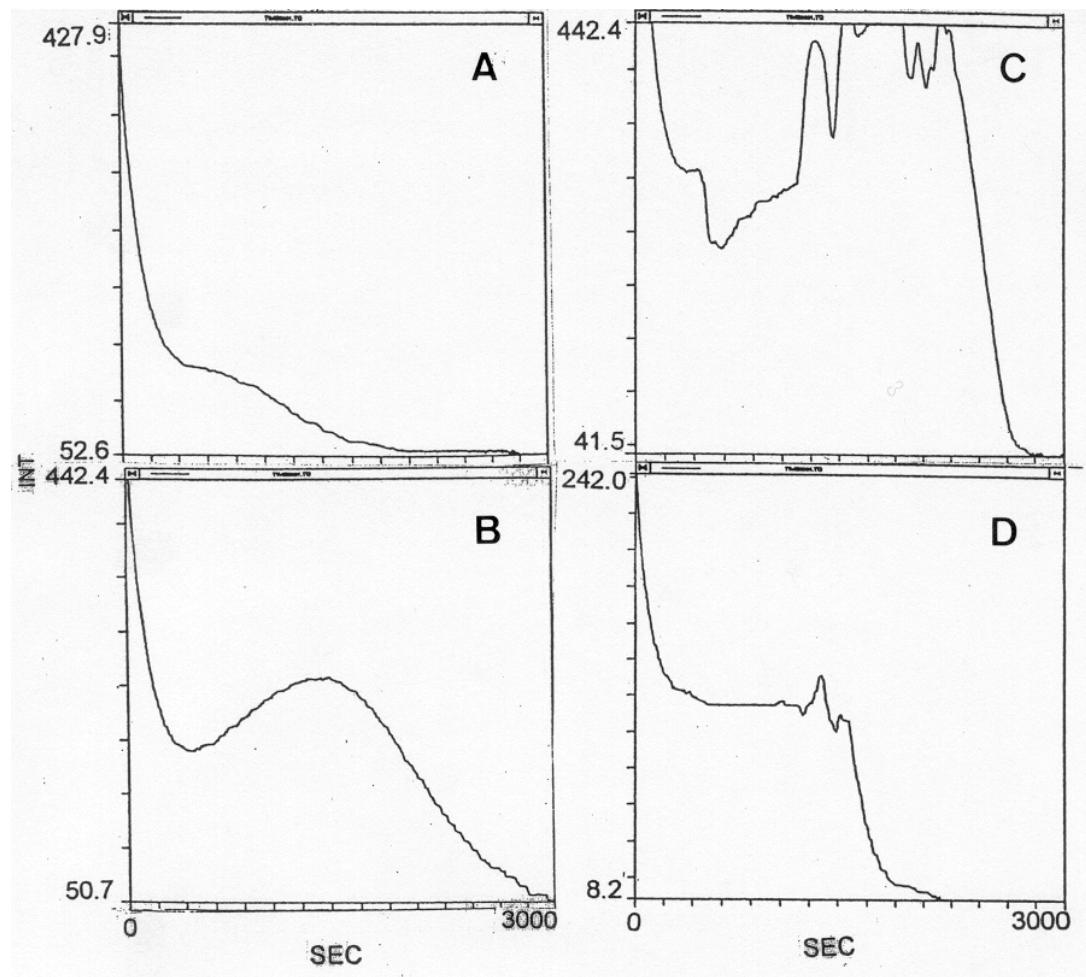


Figure 5.8. Light emission with growth phase at A_{600} 0.8 (A), 1.1 (B), 1.6 (C) and overnight culture (D) using the LS50 luminometer. Cultures and initiation of bioluminescence reactions were similar to those in Figure 5.7, except 30 μ l of external aldehyde was used with 3 ml of culture suspension.

microtitre assays) (see section 5.2.3.1). These results indicated that transconjugant VUN3,600 produced different levels of light output with growth phase and stable light output occurred at the A_{600} 1.1-1.2 in both measuring systems. Similarly maximum light output occurred at this region of growth phase when the external substrate was delivered by evaporation (see section 5.2.2.3.1).

5.3.1.3 Toxicity test using the preliminary optimised delivery system

Toxicity tests were performed using the thus far optimum delivery system, 1% aldehyde in ethanol, for representative heavy metal ions and water was added alone as the control. Transconjugant 3,600 was grown in BHI broth and culture suspensions were exposed to 1% aldehyde in ethanol then 0.334 gL^{-1} of CdCl_2 was added into 3 ml sample volumes or the same volume of water was added alone (Figure 5.9). Control samples without addition of cadmium or water alone showed similar luminescence reaction to these seen previously (see Figure 5.6), although slightly less intensity of bioluminescence occurred. Cadmium affected the bioluminescence reaction clearly in a positive way when 0.334 gL^{-1} of CdCl_2 was added. Bioluminescence rapidly declined initially and then this started to increase over 13 minutes exposure, with the highest value of light intensity exceeding the limit of detection before declining immediately at the end of test period. Similar increased level of induction was observed at concentrations of CdCl_2 of 0.5, 0.05 and 0.005 μg in 3 ml test volumes of culture, but these observations were not suitable to compare between tests as different volumes of cadmium solution were added. However, transconjugant VUN3,600 responded to the presence of cadmium ions. It was most interesting to see that the cells also responded to water added alone using this approach: adding 200 μl of water, which was the same volume of cadmium solution added, transconjugant VUN3,600 showed a similar response to cadmium addition, when culture suspensions were tested after delivering the 30 μl of 1% aldehyde in ethanol. Although the level of intensities was different, the mode of bioluminescence reaction was similar to when 10^3 -fold aldehyde diluted in Milli-Q water was used (see Figure 5.6). Maximum light intensity occurred as 445 over 35 minutes exposed to water added alone while cadmium induced bioluminescence was over 448.5, and the control (no additions) showed a light output of 80. The culture suspension was tested after 2 days standing on room temperature and similar kinetics of bioluminescence output to shown in Figure 5.9 was observed: light output was steady over 9 minutes using 30 μl of 1% aldehyde in ethanol to 3 ml of culture, although maximum intensity was 228.1 for the stored cells

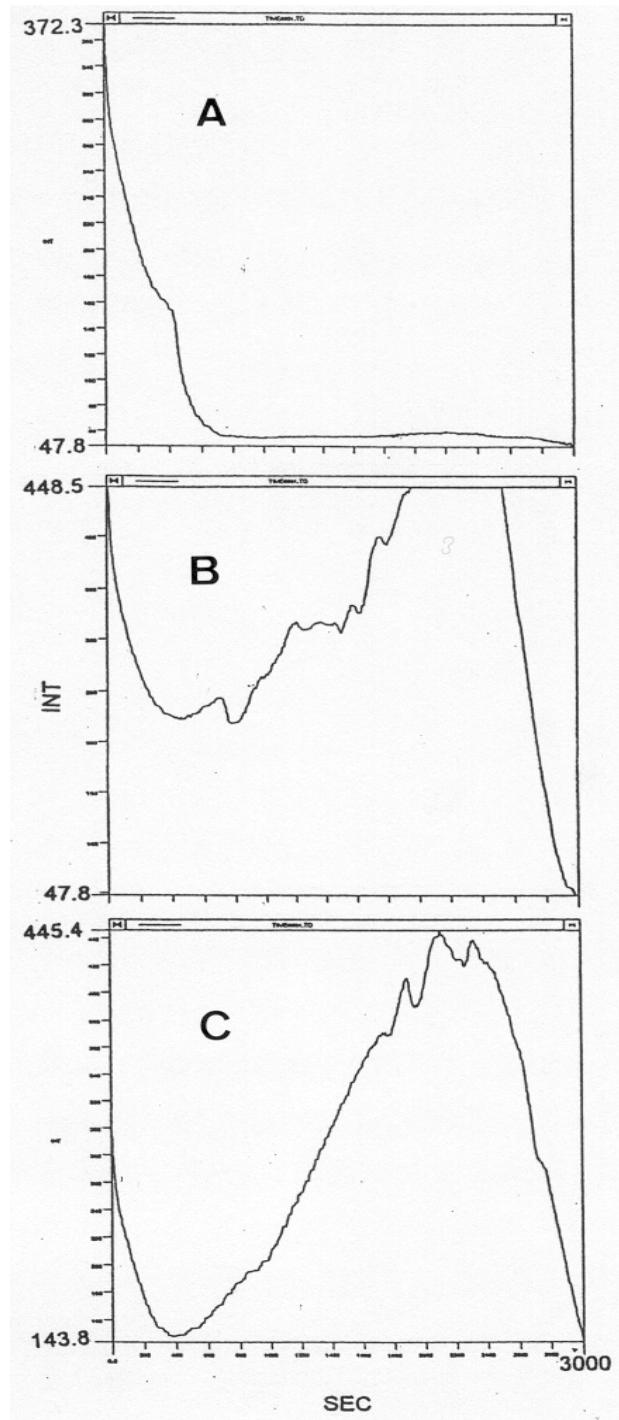


Figure 5.9. Time course of bioluminescence with CdCl_2 and water that added alone. Culture suspension and initiation of bioluminescence reaction was similar to that described in Figure 5.7, except 30 μl of external aldehyde was added to 3 ml of culture suspension, which was taken from overnight cultures. Control (A), 0.334 g L^{-1} of CdCl_2 (B) and same volume of solvent added without CdCl_2 (C).

compared to 448.5 for the directly tested growing culture. When retested with similar cadmium concentrations, maximum light intensity was 250, which was similar to the light output seen when the same volume of water was added alone. These results implied that adding water alone caused positive false or synergistic effects on bioluminescence reactions in transconjugant VUN3,600. This result prompted the examination of the preparation of cells or use of buffer system which may alleviate this effect.

5.3.2 Preparation of cells for toxicity testing

It is hard to approach or understand the phenomenon observed in the above section 5.3.1.3 which used 1% aldehyde in ethanol to initiate the bioluminescence reaction. However, the indigenous strain VUN10,077 exists in aquatic or water/soil environments naturally and showed tolerate harsh conditions or survive in water environments. The original habitat and hence the physiology of this strain may influence the way in which this species responds to water added to culture fluids. Transconjugant VUN3,600 was grown in BHI at 28°C and harvested at A_{600} 1.1-1.2, then cells were pelleted and resuspended in the same volume of tap water and light output measured as RLU using LSC. To avoid artefacts that may arise due to adding ethanol in addition to water, the substrate was used as 103-fold aldehyde diluted in Milli-Q water, which had produced similar kinetics to 1% aldehyde in ethanol (see section 5.3.1.1). Initially, a ratio of 10:1 of cells to substrate was used in controls and tests with cadmium, nickel and mercury at concentrations of 0.01 μg and 1 μg into 100 μl test cell volumes. The lowest concentrations of cadmium and nickel tested increased light output for up to 8 hours, although the tested concentrations of mercury had less light output compared to the control. Moreover, the same volume of solvent without heavy metal ions showed similar bioluminescence to the control, and the highest level of induction seen was similar to the lowest concentration of nickel added. To prepare the cells in toxicity testing, the ratio of cells to substrate was investigated to obtain the best light output conditions, and this was achieved at a ratio of 5:1 of cells to substrate and addition of water instead of the same volumes of toxicants showed no influence when using this ratio. Data are summarised in the following sections.

5.3.2.1 Substrate concentration and ratio of cells to substrate

Diluted *n*-decyl aldehyde (10^3 -fold in Milli-Q water) was used to determine conditions for stable light output. When strain VUN3,600 was exposed to the external substrate, bioluminescence was affected by the concentration of aldehyde in context of the ratio of cells to substrate. It was interesting that different cell volumes resulted in different bioluminescence reactions when different volumes of substrate solution were added (Figure 5.10). Bioluminescence reactions with similar kinetics occurred when using a ratio of 10:1 for 100 μ l of cell suspension, 4:1 for 200 μ l cells, 5:1 for 500 μ l cells and 20:1 for 1,000 μ l cells, where there was an initiation of reaction phase follow by a decline period. When the tested volume of cells was increased for up to 500 μ l, bioluminescence reactions were prolonged relative to other tests. The lowest tested volume of cells did not produce detectable light output except at a cell:substrate ratio of 10:1, and similar observations were made for the highest concentration of aldehyde solution added into tests, with 200, 500 and 1,000 μ l of cell suspension. These results indicated that excess amounts of aldehyde apparently inhibited strain VUN3,600 so that the bioluminescence reaction did not occur, as previously reported by Meighen (1988). When the bioluminescence was measured using 500 μ l of cells, high bioluminescence was seen and the standard deviations between replicates were lower than seen for the other tests which showed higher induction of bioluminescence. Although a clear relationship between cell volume and substrate added was not seen, the best outcome of light output was found when 100 μ l of freshly prepared substrate solution was added into 500 μ l of aqueous cell suspension to provide a water-based *luxAB* system for use in pollutant testing at ambient temperature.

5.3.2.2 Preparation of cells using a water-based system

Initially attempts at stabilizing the light output of cells from BHI cultures used water or buffers (sodium phosphate or Tris, pH 8.0) to dilute the cultures in ratios of 1:1 to 1:20 in these. When tested with 10^3 -fold water diluted aldehyde, light output was unstable still. Accordingly, it was decided to test water suspended cells, which were prepared by resuspending cells into water after mild centrifugation (Tescione and Belfort, 1993; Cha *et al.*, 1999), as most of the toxic substances to be tested were dissolved in water. The test system consisted of 500 μ l of cells (either BHI culture or centrifuged and resuspended in tap water) harvested at A_{600} 1.1-1.2 plus 100 μ l of substrate (aldehyde diluted 10^3 -fold in Milli-Q water) (see Figure 5.7). A further 2 μ l of water from different sources was then added to test the impact on bioluminescence. For cells in culture medium, addition of water significantly

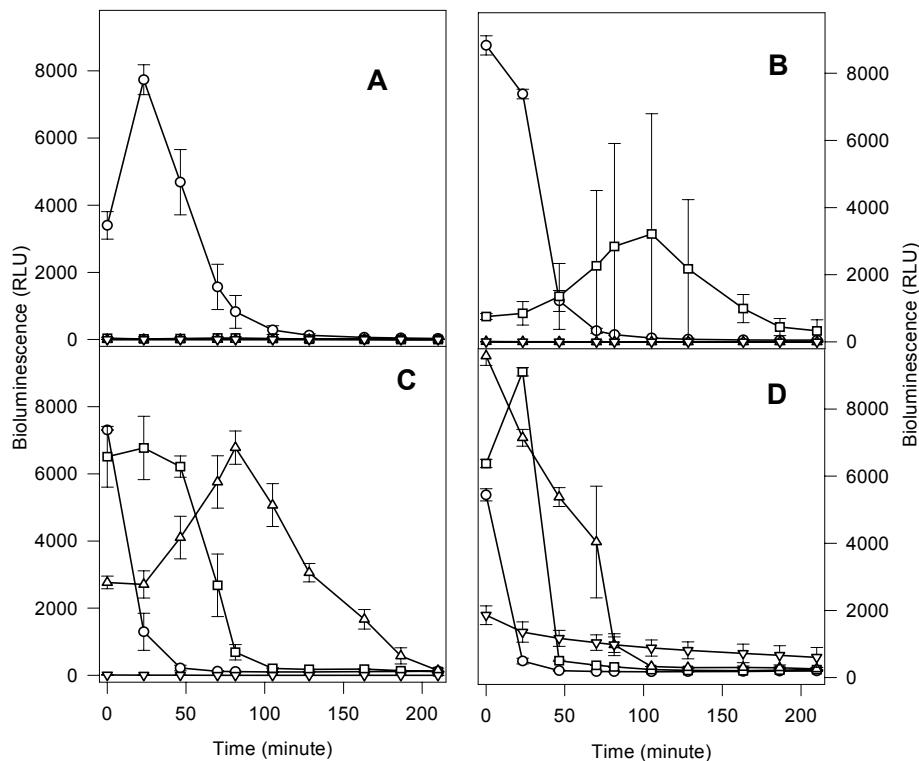


Figure 5.10. Effect of ratio of bacterial cells suspended in water and substrate and amount of substrate supplied, on light output of VUN3,600. Cells were taken from a BHI culture at A_{600} 1.0-1.2, pelleted and resuspended in tap water and external substrate was added to measure RLU using LSC. The cells were tested using a sample volume of 100 μ l (A), 200 μ l (B), 500 μ l (C) and 1,000 μ l (D) and 10^3 -fold diluted substrate in Milli-Q water was used and at different volumes: 10 μ l (\circ), 50 μ l (\square), 100 μ l (Δ), and 200 μ l (∇).

impacted on the kinetics of light output. In contrast, the control had stable light output with relatively small standard deviations, which was similar to results seen previously in Figures 5.7 and 5.8, although a different delivery system was used. It was interesting that the two sources of water affected bioluminescence differently over the 4 hours testing period in several repeated sets of experiment. When 2 µl of Milli-Q water was added into tests, the highest standard deviations occurred and the level of light induction was lower, whereas pure water (supplied by Promega) showed relatively higher bioluminescence and small standard deviations compared to Milli-Q water or the control. This result may have been caused by pH changes (Cronin and Dearden, 1993; Paton *et al.*, 1995) because the Milli-Q water available at VUT had a pH 5.5-6.5 and pure water was neutral pH. Alternatively, VUN3,600 cells were resuspended in tap-water after mild centrifugation. These tap-water resuspended cells were similarly tested with Milli-Q or pure water and bioluminescence was not significantly affected relative to the control (Figure 5.11 B) when compared with tests using cells in culture fluid. It was concluded that tap-water resuspended cell with sonically dispersed, water-diluted aldehyde was the best approach in this type of analysis.

5.3.3 Stabilization of light output produced by VUN3,600

To this point, the potential water-based monitoring system was relatively optimised. Conditions involved 100 µl of aldehyde (10^3 -fold diluted in Milli-Q water) using sonication for up to 30 seconds to disperse and 500 µl of cells were taken at A_{600} 1.1-1.2 in BHI at 28°C, then pelleted and resuspended into the same volume of tap water. Moreover, addition of water caused no impact on bioluminescence so that this system was suitable for testing pollutants dissolved into Milli-Q water. However, transconjugant VUN3,600 had a typical bioluminescence reaction pattern which included a prolonged reaction phase, and toxicity tests would probably need to rely on changes in the secondary burst output phase (see Figures 5.10 and 5.11). This system was further developed to obtain reliable, repeatable and easily visualised toxicity assay patterns using this optimised water-based system which was used at room temperature. This was achieved by incubating the tap-water resuspended cell suspension at room temperature for up to 8-10 hours before assay. Cells stored at room temperature showed steady light output. Supporting data are presented in the following sections.

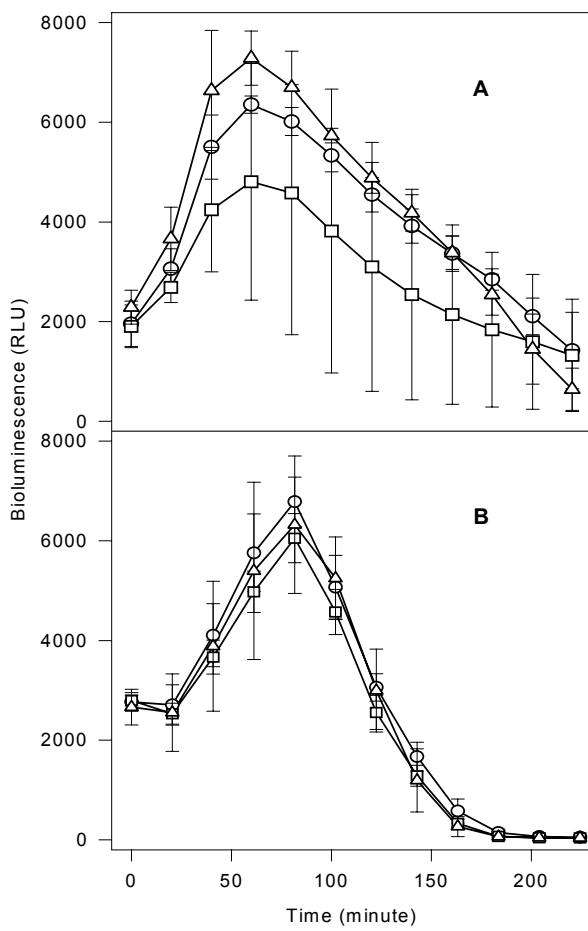


Figure 5.11. Comparison of the kinetics of bioluminescence output for cells in culture medium (A) or resuspended in tap water (B). Cells were grown at 28°C in BHI broth and samples were taken at A_{600} 1.0-1.2 then bioluminescence measured directly (A) or when cells were resuspended tap water (B). Reaction mixtures contained 500 μ l of cells plus 100 μ l of 10³-fold water-diluted aldehyde to initiate bioluminescence and 2 μ l of Milli-Q water (□), molecular biology grade water added which supplied by Promega (△), or no further addition of water (○).

5.3.3.1 Typical bioluminescence reaction pattern

The selected water-based monitoring system was investigated to see whether this system behaved in a manner similar to that seen for cells in BHI (see section 5.3.2.2). The sample was taken from a growing culture of VUN3,600 in BHI at A_{600} 1.0-1.2 and 500 μl of culture directly supplied with 100 μl of 10^3 -fold aldehyde diluted in Milli-Q water after which the bioluminescence reaction was measured using LSC. A typical burst-out phase seen in *luxAB*-marked strain was seen in the transconjugant VUN3,600 in several trials (Figure 5.12), where the initial burst-output phase occurred within less than one minute. This representative phase occurred immediately after supplying the external aldehyde as seen in Figure 5.12 and RLU values could be higher than recorded because readings could only be started after samples were mixed and placed in the LSC. Three burst-output phases were seen in VUN3,600: the initial phase was followed by a small increase phase which occurred at 100 minutes exposure time and a significantly increased phase occurred at 160 minutes then a gradual decline. The kinetics of bioluminescence output by the engineered bacterium VUN3,600 had similar characteristics using this approach in terms of biochemistry of luciferase reaction as other *luxAB*-marked strains (Blouin *et al.*, 1996). The effects on the incubation time in toxicity testing which were partly described in previous sections were then investigated to determine whether light output could be stabilised further.

5.3.3.2 Incubation period of cells affects stabilization of light output

Transconjugant VUN3,600 responded to cadmium at a concentration of 0.334 gL^{-1} and different responses were observed when using overnight and 2 day old cultures incubated at room temperature (see section 5.3.1.3). This implied that cell suspensions could be stored at room temperature for up to 48 hours and retain light output. Similarly, cell suspensions were incubated at room temperature after pelleting and resuspending in the same volume of tap water when cells were harvested at A_{600} 1.0-1.2. Bioluminescence was measured using incubation periods of 0.5, 4, 8, 12, 24, 72 hours and 5 days after addition of 100 μl of substrate (10^3 -fold aldehyde diluted in Milli-Q water) into tests using 500 μl of cells. Interestingly, relatively high levels of light output occurred for cells stored for 4 hours to 24 hours (Figure 5.13). Bioluminescence gradually declined at 0.5 hours incubation after supplying the external aldehyde, which was different to results seen in Figure 5.11, where the 0.5 hours incubated cell suspension did not show a secondary burst-output phase. However,

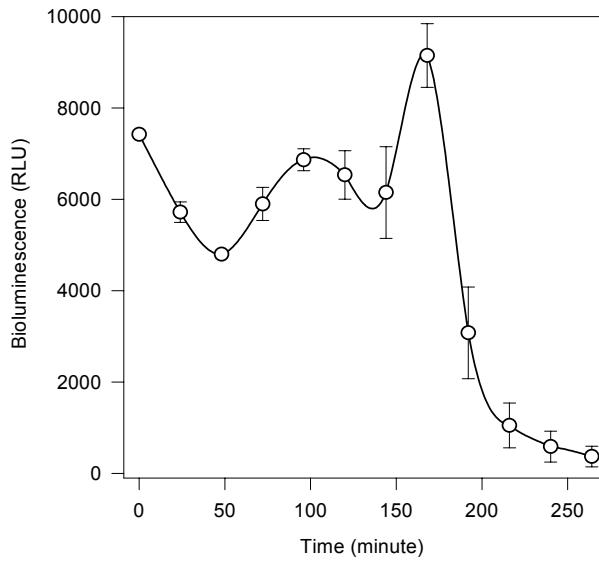


Figure 5.12. Typical bioluminescence reaction in *luxAB*-marked transconjugant VUN3,600. The sample was taken from growing culture in BHI broth at A_{600} 1.0-1.2 and supplied with 100 μ l of external substrate (10^3 -fold diluted into Milli-Q water) into 500 μ l of culture suspension, and bioluminescence was measured using LSC.

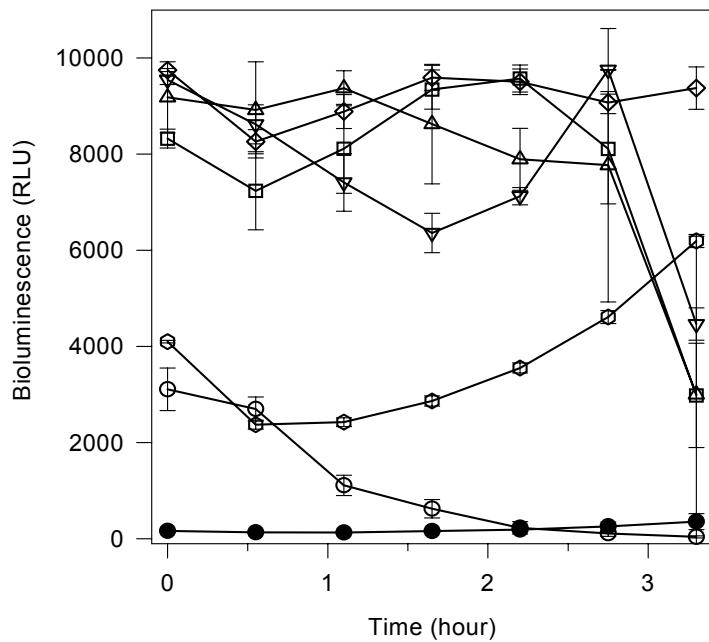


Figure 5.13. Effects of incubation period of cell suspensions on light output. Samples were stood at ambient temperature after pelleting and resuspending in tap-water in the same volume when cells were harvested at A_{600} 1.0-1.2 and bioluminescence measured at 0.5 hour (○), 4 hours (□), 8 hours (△), 12 hours (▽), 24 hours (◇), 72 hours (○) and 5 days (●) using 500 μl of cell suspension supplied with 100 μl of external aldehyde (10^3 -fold diluted in water).

bioluminescence was significantly increased by approximately 2-fold for cells started for up to 4 hours at room temperature and the mode of bioluminescence reaction was similar to that seen in Figure 5.11. This reaction mode was also seen at 12 hours storage. When the cell suspension was incubated for 8 and 24 hours at ambient temperature, relatively plateau-like bioluminescence output was seen then this declined rapidly after 2.5 hours which was similar to the decline seen for fresh cells in Figure 5.12. The 24 hours incubated cell suspension kept up a level output with less standard deviations throughout the testing period for up to 3.3 hours. Light output after 72 hours storage was much lower relative to other earlier tests and the bioluminescence reaction slowly increased over the assay period and this lasted longer than seen for samples stored for shorter periods and showing high light output.

It was found that the 8 hours incubated cell suspension gave a relatively stable and high level of light output with no significant decline or increase in light output for up to 2.7 hours following addition of the external aldehyde. Bioluminescence declined after 3 hours reaction, as seen in the typical bioluminescence reaction for samples from growing cultures of transconjugant VUN3,600. These results indicated that VUN3,600 retained its metabolic activity for up to 72 hours when suspended in tap water, as indicated by its continued ability to support bioluminescence when the external substrate was provided for the *luxAB* system. Long term incubation at ambient temperature improved the stability of light output and, although posing problems for routine analysis, this system was used to test the suitability of VUN3,600 for detecting the presence of water-soluble pollutants.

5.4 Evaluation the toxicity testing using VUN3,600

The potential of using the *luxAB*-marked pseudomonad related species VUN3,600 for monitoring environmental toxicants was evaluated using the thus far optimised conditions, using LSC for detection. The engineered bacterium was cultured at 28°C, in BHI broth until the optical density reached A_{600} 1.0-1.2. The cells were harvested using centrifugation at 3,000 r.p.m. for 15 minutes and cells resuspended in the same volume of tap-water. The resuspended cell suspension was kept at room temperature for up to 10-12 hours before commencing the toxicity testing (see section 5.3.3.2). For bioluminescence measurement, *n*-decyl aldehyde was

Table 5.5. Impact of chemicals on light output of *luxAB*-marked VUN3,600. The impact of the presence of 0.02 mgL^{-1} to 0.2 gL^{-1} of the chemicals was recorded after adding external aldehyde and the chemicals in that order then incubating ~ 10 minutes before measuring light output for 10 seconds using LSC. Cells were harvested at A_{600} 1.0-1.2 and resuspended in tap-water for 10-12 hours. All figures are RLU (average of triplicate assays), where the control (water replacing the test compounds) was 9,400 RLU over the same period.

Compound	0.02 mgL^{-1}	0.002 gL^{-1}	0.01 gL^{-1}	0.02 gL^{-1}	0.1 gL^{-1}	0.2 gL^{-1}
CdCl ₂	7,720	4,031	2,279	1,112	479.7	443.3
Pb(NO ₃) ₂	7,739	5,812	4,789	2,954	791*	382
ZnCl ₂	6,958	1,578	515	703.7	340.3	311.2
NiCl ₂ .6H ₂ O	6,499	6,664	7,533	7,299	4,107	3,309
NaAsO ₂	8,378	7,975	6,038	5,395	4,634	4,791
CoCl ₂ .6H ₂ O	8,274	175.5	111.2	78.3*	46*	139.0*
HgCl ₂	7,625	275	84*	11.1	13.2*	2.7*
CuCl ₂ .2H ₂ O	9,763	9,499	1,747*	448*	27.1	33.7*
SDS	9,766	9,610	5,037*	834*	150.7	49.7

*, more than 20% standard deviation error occurred in this assay

diluted 10³-fold in Milli-Q water and this was freshly prepared by sonification in every set of experiments. The substrate solution was allowed to stand for 5 minutes to evaporate excess volatile components, if necessary. Measurement was commenced by the addition of 100 µl of substrate solution followed by addition of selected chemicals to 500 µl of tap-water resuspended cells. All the heavy metal salts were made up as stock solutions of 50 mg/ml in Milli-Q water and sterilized by filtration (0.45 µ/ml, GS, Millipore). Chlorinated phenol compounds were dissolved in DMF at 200 mg/ml without sterilization and stored at room temperature. In all the experiments, results were related to controls lacking test chemicals and the kinetics of inhibition were recorded in triplicate. These trials showed a range of responses when using heavy metals and chlorinated phenolic compounds, whereas there was no impact of adding water alone or 0.4% DMF to 500 µl tap-water resuspended cells.

5.4.1 Heavy metal ions and SDS assessment

The optimised light output conditions were used to assess heavy metal ions and SDS and the individual chemicals generally caused certain degrees of bioluminescence inhibition at different concentrations. The 100 µl of aldehyde was normally added to the cell suspension to initiate the luciferase reaction then the chemicals were added. Interestingly, when the order was reversed, the level of light output was lower and dose-related responses were barely observed. When the freshly prepared external aldehyde was evaporated at room temperature for approximately 5 minutes after sonication, dose- related responses were better than when the substrate was immediate used as measurements gave the smaller standard deviations. As expected, the initial burst output phase was affected in toxicity assays when measurements commenced immediately after the addition of external aldehyde and chemicals, but this was avoided when the substrate was incubated for 10 minutes at room temperature after the addition of external aldehyde and tested chemicals in that order. The optimised toxicity test conditions for VUN3,600 were as follows; Chemicals were added after the luciferase reaction was initiated by the addition of external aldehyde, and incubated for approximately 10 minutes before RLU values were recorded using LSC for 10 seconds in triplicate assays at room temperature. Representative heavy metal ions and SDS were added at a range of concentration of 0.02 mgL⁻¹ to 0.2 gL⁻¹ (Table 5.5). Transconjugant VUN3,600 had ‘acute’ bioluminescence responses to CdCl₂, Pb(NO₃)₂, ZnCl₂, CoCl₂.6H₂O, NaAsO₂ and HgCl₂ at up to concentration of 0.02 gL⁻¹ with less than 10% standard deviations between triplicates although zinc had higher RLU values at concentration of 0.01 gL⁻¹ than 0.02 gL⁻¹.

CdCl_2 caused relatively consistent response pattern at the tested concentration range, where bioluminescence gradually declined at concentrations of up to 0.1 gL^{-1} , while lead showed a similar response pattern over the tested concentration range. NaAsO_2 showed relatively high levels of bioluminescence at up to 0.2 gL^{-1} with sensitivity which was similar to $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ tests. When $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ was tested over a concentration range of 0.02 to 0.2 gL^{-1} , inhibition of bioluminescence was less across the concentration range, despite relatively more inhibition at the lower concentrations. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and SDS affected luminescence at concentrations from 0.002 gL^{-1} to 0.02 gL^{-1} although the higher concentrations had more than 20% standard deviations in assays, and luminescence was slightly increased compared to the control at the lowest concentration of SDS. The toxicity tests indicated that transconjugant VUN3,600 can discriminate the tested chemicals using this concentration range despite relatively higher standard deviations occurring when toxicant concentrations increased.

To validate the data reported in Table 5.5, the bioluminescence reaction was prolonged to obtain information about the kinetics of the dose-related responses. The time course of changes in luminescence intensity by CdCl_2 , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, HgCl_2 and $\text{Pb}(\text{NO}_3)_2$ was investigated and Figure 5.14 presents the typical kinetics of light output. A clear dose-related response was seen over the 2 hours test time after the addition of chemicals to the reaction mixture for the cadmium and mercury in the long-term assay. RLU values were recorded at 20 minutes intervals and impacts could be seen for all chemicals in this period for the lowest concentrations that caused an impact: 0.2 - 1.0 mgL^{-1} of CdCl_2 ; 0.1 - 0.2 mgL^{-1} of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.02 - 0.4 mgL^{-1} of HgCl_2 ; 1.0 mgL^{-1} of $\text{Pb}(\text{NO}_3)_2$. When HgCl_2 was added at concentrations of 0.1 mgL^{-1} and 0.2 mgL^{-1} , the kinetics were similar or a little lower than presented in Figure 5.14 for 0.02 mgL^{-1} HgCl_2 . The maximum luciferase reaction occurred at the beginning and afterwards it decreased gradually then showed dose-related kinetics, and a similar induction pattern was displayed for CdCl_2 and HgCl_2 , with impacts seen in two areas; the initial rate of inhibition and a dose-related response to CdCl_2 and HgCl_2 for up to 40 minutes, followed by asymptoting to a constant, lower level output. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ showed a different pattern of inhibition, as Co tests already showed suppressed starting values which were seen within 10 minutes of exposure for concentrations of 0.02 mgL^{-1} to 0.002 gL^{-1} (see Table 5.5). The Pb stress showed less sensitivity in long-term assays compared to the other tested metal ions, although a dose-related response was seen in the test period over a concentration range of 0.001 gL^{-1} to 0.1 gL^{-1} , although some precipitation occurred in the assay. Using this water-based monitoring system, the selected heavy metal stresses could be detected with less than 10% standard deviations in triplicate assays for levels of $\text{CdCl}_2 < 0.01 \text{ gL}^{-1}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O} < 0.002 \text{ gL}^{-1}$, $\text{HgCl}_2 < 0.002 \text{ gL}^{-1}$ and concentrations up to 0.1 gL^{-1} $\text{Pb}(\text{NO}_3)_2$ in tap-water resuspended cell suspensions. The test system used was successful

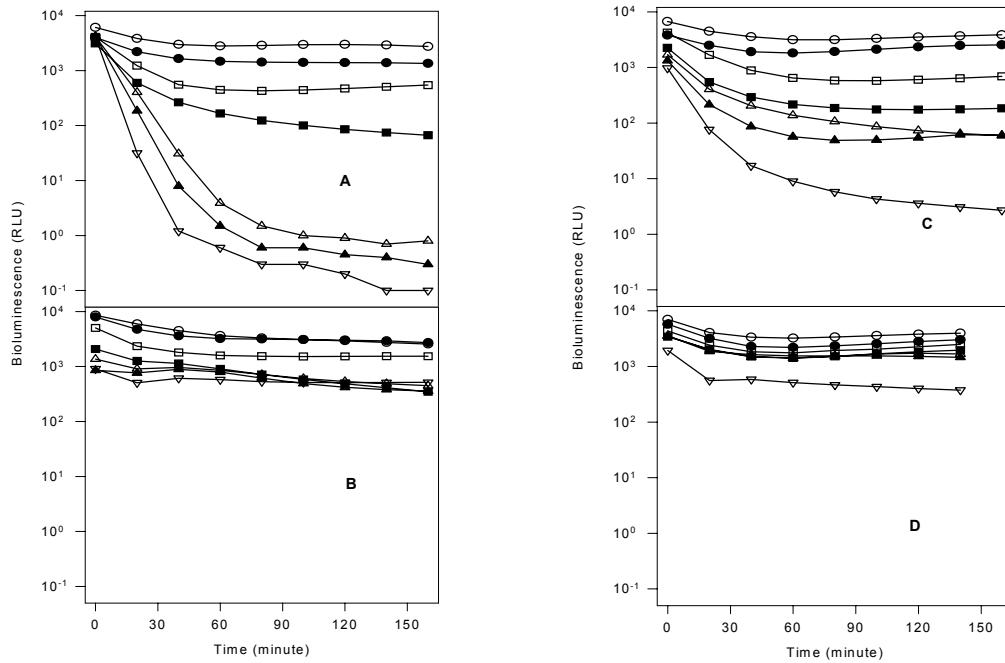


Figure 5.14. Kinetics of dose-related response to selected heavy metal ions. The CdCl₂ (A), CoCl₂.6H₂O (B), HgCl₂ (C), and Pb(NO₃)₂ (D) were added to 0.5 ml tap-water resuspended cell suspensions where the conditions were similar to Table 5.5 and measured at 20 minute intervals using LSC for 10 seconds. The values are by logarithm and mean of triplicate assays.

The legend designates for A: control (○), 0.2 mgL⁻¹ (●), 0.001 gL⁻¹ (□), 0.002 gL⁻¹ (■), 0.004 gL⁻¹ (△), 0.006 gL⁻¹ (▲) and 0.01 gL⁻¹ (▽);

B: control (○), 0.1 mgL⁻¹ (●), 0.2 mgL⁻¹ (□), 0.4 mgL⁻¹ (■), 0.8 mgL⁻¹ (△), 0.001 gL⁻¹ (▲) and 0.0016 gL⁻¹ (▽);

C: control (○), 0.02 mgL⁻¹ (●), 0.4 mgL⁻¹ (□), 0.6 mgL⁻¹ (■), 0.8 mgL⁻¹ (△), 0.001 gL⁻¹ (▲) and 0.0016 gL⁻¹ (▽);

and D: control (○), 0.001 gL⁻¹ (●), 0.01 gL⁻¹ (□), 0.02 gL⁻¹ (■), 0.04 gL⁻¹ (△), 0.08 gL⁻¹ (▲) and 0.1 gL⁻¹ (▽).

in long-term assays which detected impacts on the kinetics of bioluminescence inhibition or in short-term tests which provided a single time point assay, where both generally detected similar concentration ranges of metal ions. In particular, this system could clearly detect and respond to CdCl₂ and HgCl₂ in the long-term assay as well as the combined assay between long-term and single point (short-term) assays may provides a qualification of metal ions in water environments.

5.4.2 Effect of storing cells at 4°C or room temperature

: bioluminescence and detection of toxicants

Cells of transconjugant VUN3,600 were harvested at A₆₀₀ 1.0-1.2 and resuspended in tap-water after mild centrifugation. The kinetics of bioluminescence output was examined immediately in triplicate assays, where the results showed < 5% differences between the replicates. The cell suspension was then stored at either 4°C or room temperature and assays for detecting bioluminescence and the effect of toxicants were performed periodically for up to 2 weeks (Figure 5.15). However, bioluminescence was retained at 4°C after one day but with large differences between replicates, whereas room temperature stored cell suspensions were relatively stable (see Figure 5.13). These 4°C stored cells remained sensitive to CdCl₂ and HgCl₂ at 0.001 gL⁻¹ and 0.01 gL⁻¹, but standard deviations were more than 20%. When the storage time was extended up to 2 weeks at 4°C, bioluminescence could still be detected and this was maintained for up to 3 hours but with 10-fold less intensity (Figure 5.15 A). In contrast, cells of transconjugant VUN3,600 stored at room temperature could not maintain bioluminescence which declined by 5 days (see Figure 5.13). Cells of VUN3,600 stored at 4°C for up to 2 weeks could be used to detect responses to toxicants after they were warmed to room temperature for 20-30 minutes. However, when water was added as a control for heavy metal tests, bioluminescence was slightly stimulated but maximum levels of intensity were similar to the control without additions (Figure 5.15 A), whereas cells assayed directly from 4°C storage showed low bioluminescence.

Despite the slight stimulation by water alone, it was possible to detect the addition of metal ions by suppression of bioluminescence (Figure 5.15 B) for 0.02 mgL⁻¹ and 0.002 gL⁻¹ of CdCl₂ or NiCl₂.6H₂O and HgCl₂ respectively. Incubation time and temperature of storage following cell harvesting caused different sensitivity to the test compounds. It was found that the sensitivity to the test compounds varied with the length of the storage period when the cells were suspended in tap-water following growth and harvesting. An incubation period of 10-12

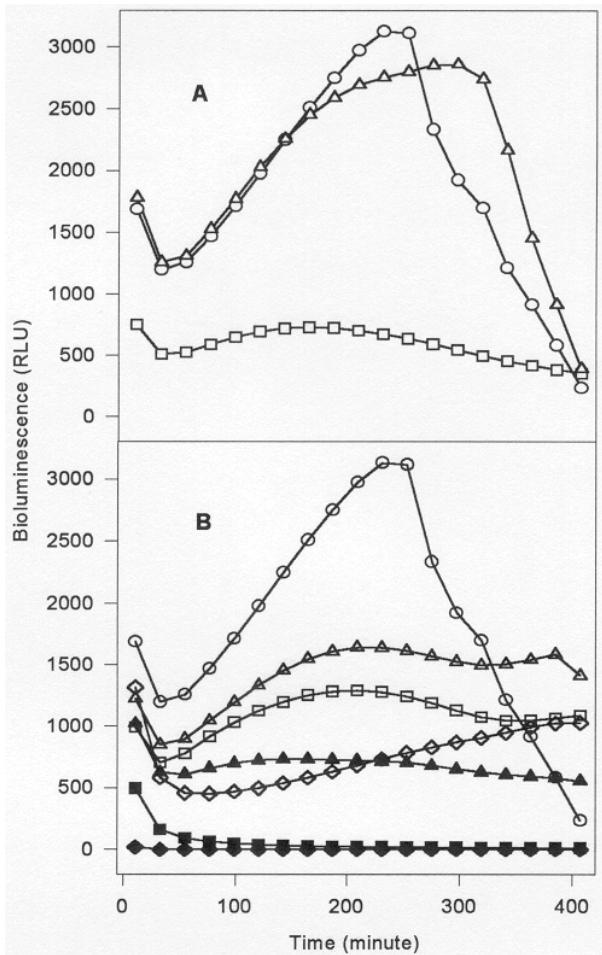


Figure 5.15. Estimation of luminescence in the presence and absence of metals following storage of cells at 4°C. Cells were kept at 4°C for 2 weeks following harvesting and resuspending in tap water. After storage, they were warmed to room temperature for 30 minutes (panels A and B) except for direct measurement of bioluminescence from 4°C in panel A (\square) only. The bioluminescence reaction was initiated by adding 100 μ l of aldehyde (10³-fold diluted in Milli-Q water) to 500 μ l of cell suspension, and RLU was measured using LSC. The legends are A: no additions (\circ) and (\square), and Milli-Q water was added to warmed cells (Δ); and B: no additions (\circ), 0.02 mgL⁻¹ of CdCl₂ (\square), NiCl₂.6H₂O (Δ), HgCl₂ (\diamond) and 0.002 gL⁻¹ of CdCl₂ (\blacksquare), NiCl₂.6H₂O (\blacktriangle), HgCl₂ (\blacklozenge).

hours at room temperature was shown earlier to be effective for toxicity measurements. The shorter incubation period resulted in a non-dose-related quick response, and longer period of storage showed relatively slow response to the chemicals. Although the response time was extended over 2-fold for CdCl₂, NiCl₂.6H₂O and HgCl₂, these results indicated that it was possible to store cells at 4°C in tap-water suspension for up to 2 weeks and retain activity in toxicity assessment.

5.4.3 DMF-dissolved chemicals toxicity assessment

5.4.3.1 DMF effects on bioluminescence

DMF prolonged light emission of strain VUN3,600 and slightly stimulated this (Figure 5.16). The naturally bioluminescent bacterium, *Ph. phosphoreum*, and a bioluminescent yeast constructed in CBFT were sensitive to DMF by inhibition (Y. Chen, personnel communication). Interestingly, this solvent was used to dissolve chlorinated phenolic and other water insoluble compounds, such as PAHs, so that these systems were not suitable for use in assays for these compounds. In contrast, bioluminescent VUN3,600 was not inhibited by DMF, and DMF did not induce the major stress inducible proteins in the parent strain VUN10,077 at concentrations of up to 3.0 gL⁻¹, as determined by using 1-D PAGE. All the tested phenolic and PAH (polycyclic aromatic hydrocarbon) compounds were dissolved in 0.4% (54.5 µM) DMF and added to 500 µl tap-water resuspended cell suspensions at volumes similar to heavy metal ions dissolved in water. Stimulation of light output relative to the control is shown in the Figure 5.16, possibly allowing higher concentrations of decanal to enter cells, but the mechanisms were not further investigated here. However, it may be possible to use the *luxAB*-marked strain VUN3,600 to measure the phenolic and PAH compounds which can dissolve in DMF or exchanged into DMF.

5.4.3.2 Detection of responses to substituted heterocyclic compounds in long-term assay

The transconjugant VUN3,600 exhibited a response to chlorinated phenolic compounds dissolved in DMF when these were tested in assays incubated for extended periods (Figure 5.17). Initially, the phenolic compounds were tested in short-term assays, but most DMF-dissolved phenolic compounds were not suitable for this type of assay which showed non-dose-related responses and initial light output declined irregularly over 30 minutes while the initial decline pattern was similar to controls when 0.4% DMF was added to 500 µl of tap-water

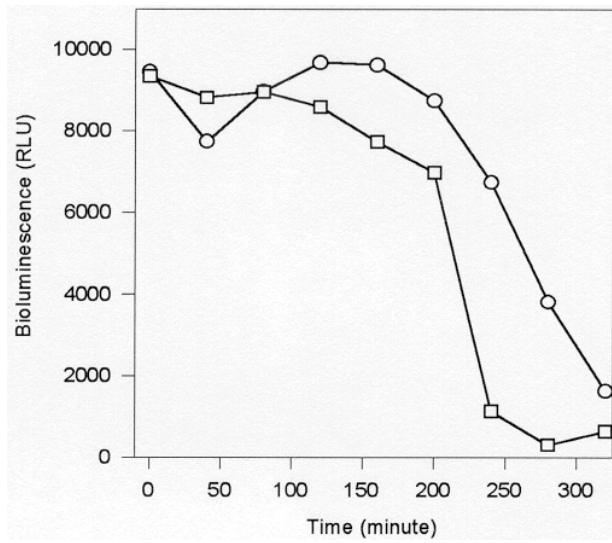


Figure 5.16. Effect of DMF on light output by *luxAB*-marked VUN3,600. The assay contained 500 μ l of cell suspension harvested at A_{600} 1.0-1.2 and resuspended in tap water then incubated at room temperature for 10-12 hours. Legend: control (\square) and 0.4% (54.5 μ M) DMF added (\circ).

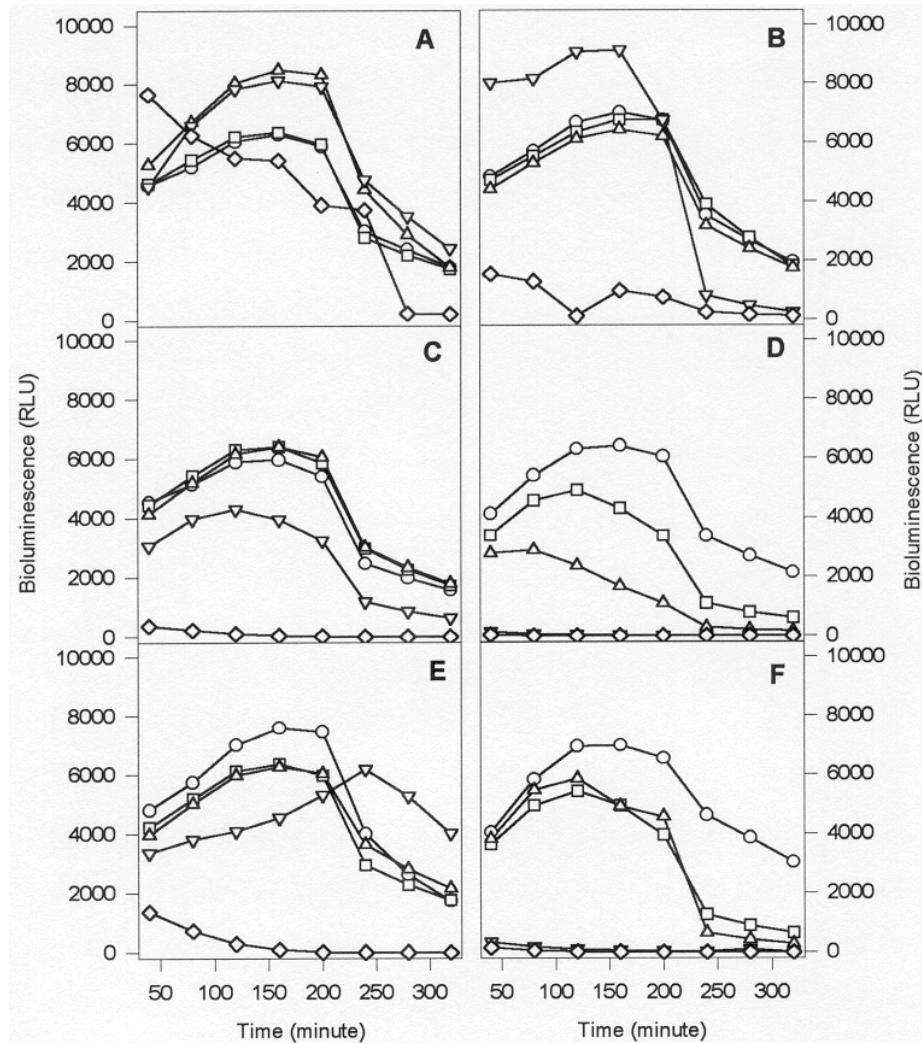


Figure 5.17. Response of VUN3,600 cells to DMF-dissolved heterocyclic compounds. Phenol (A), δ-nitrophenol (B), di-chlorophenol (C), tri-chlorophenol (D), tetra-chlorophenol (E) and penta-chlorophenol (F) were dissolved in DMF and added to 0.5 ml tap-water resuspended cells, with assay similar to Table 5.5 and measured at 40 minute intervals using LSC for 10 seconds. The test concentration range was 0.002 gL⁻¹ (□), 0.01 gL⁻¹ (△), 0.02 gL⁻¹ (▽), 0.1 gL⁻¹ (◇) and 0.2 gL⁻¹ (○) and all tests contained 54.7 μM DMF. The control was the same as in Figure 5.16, which contained 54.7 μM DMF.

resuspended cells. In contrast, penta-chlorophenol caused a rapid response at concentrations from 0.002 gL⁻¹ to 0.1 gL⁻¹. The concentration of 0.002 gL⁻¹ to 5,584 RLU, 0.01 gL⁻¹ to 3,967 RLU, 0.02 gL⁻¹ to 3,117 RLU, and 0.1 gL⁻¹ to 706 RLU were seen, whereas DMF added in the control had a light output of 8,000-10,000 over the 30 minutes test. This implied that penta-chlorophenol could be detected in this short-term assay when dissolved in DMF. Therefore, the tests were performed for longer than 40 minutes incubation at room temperature after adding the external aldehyde and the chemicals, with readings commencing after 10 minutes incubation (conditions similar to those used for heavy metal ions in short-term assays). In these tests, phenolic compounds were dissolved in 54.7 µM DMF at concentration of 0.002 gL⁻¹, 0.01 gL⁻¹, 0.02 gL⁻¹, 0.1 gL⁻¹ and 0.2 gL⁻¹. The intensity of the bioluminescence responses varied with the concentrations of chlorinated phenolic compounds used and the period of incubation required to detect these compounds was 100-200 minutes after the addition of external aldehyde and chemicals (Figure 5.17). This was in contrast to the rapid or ‘acute’ response noted when testing heavy metal ions. Phenol and δ-nitrophenol, which can dissolve in either water or DMF, did not show a dose-related response. Different concentration of these chemicals showed similar levels of bioluminescence over the test period for 0.002 gL⁻¹ and 0.01 gL⁻¹ of phenol, and 0.002 gL⁻¹, 0.01 gL⁻¹ and 0.02 gL⁻¹ of δ-nitrophenol. Moreover, certain concentrations of these chemicals increased the bioluminescence (0.1 gL⁻¹ and 0.2 gL⁻¹ to phenol, and 0.1 gL⁻¹ to δ-nitrophenol) as was seen for nickel in a short-term assays (see Table 5.5), but higher concentrations inhibited light output.

The cells showed a dose-related response to the following concentration ranges, relative to a DMF-treated control (see Figure 5.16): 0.01 gL⁻¹ to 0.1 gL⁻¹ for di-chlorophenol, 0.002 gL⁻¹ to 0.02 gL⁻¹ for tri-chlorophenol, 0.002 gL⁻¹ to 0.1 gL⁻¹ for tetra-chlorophenol after 2 hours assay. These chemicals increased light output for up to 3.3 hours exposure time then declined, but the higher concentration significantly inhibited the bioluminescence in the assays. Above 0.1 gL⁻¹ of di-chlorophenol, a similar level of light output was seen throughout the test period, whereas in assays for tetra-chlorophenol a clear dose-related response was seen. Similar overall patterns of light output were observed in the penta-chlorophenol assays, although a rapid response was also observed in the beginning over the tested concentration range, which was similar to the kinetics seen for NaAsO₂: the inhibitory effects of NaAsO₂ were dose-related for up to 1 hour assay over the concentration range from 0.002 gL⁻¹ to 0.1 gL⁻¹. However, when the assay was further extended for up to 4 hours, bioluminescence was increased at concentrations of 0.02 gL⁻¹ and 0.1 gL⁻¹ of NaAsO₂, while other tested concentrations caused further decreases in the bioluminescence linearly (rapid initial response, followed by a slower decline later in the assay

Table 5.7. Structure and physical-chemical properties of some PAH compounds (adapted from Sims and Overcash, 1983).

*PAHs	N° of Rings	mp ^a (°C)	bp ^b (°C)	Sol ^c (mg/ml)	log K _p ^d	Vapour pressure ^e
Naphthalene (C ₁₀ H ₈)	2	80	218	30	3.37	4.92×10 ⁻²
Phenanthrene (C ₁₄ H ₁₀)	3	101	340	1.29	4.46	6.8×10 ⁻⁴
Fluorene (C ₁₃ H ₁₀)	3	116	293	1.98	4.18	1.3×10 ⁻²
Fluoranthene (C ₁₆ H ₁₀)	4	111	250	0.26	5.33	6.0×10 ⁻⁶
Benz[α]anthracene (C ₁₈ H ₁₂)	4	158	400	0.014	5.61	5.0×10 ⁻⁷
Benzo[α]pyrene (C ₂₀ H ₁₂)	5	179	496	0.0038	6.04	5.0×10 ⁻⁷
di-benzo[α,h]anthracence (C ₂₂ H ₁₂)	5	262	524	0.0005	5.97	1.0×10 ⁻¹⁰

^amp; melting point, ^bbp; boiling point, ^cSol; aqueous solubility, ^dLog K_p; logarithm of the octanol:water partition coefficient, ^eVapour pressure; torr at 20°C.

*EPA (Environmental Protection Agency) priority pollutants

period). This phenomenon was also seen at 0.1 gL^{-1} of tetra-chlorophenol within 2 hours assays.

While some of the heavy metals and chlorinated phenolic compounds tested elicited a readily detectable and sensitive response from the transconjugant VUN3,600, other compounds such as strontium salt caused no response up to 0.2 gL^{-1} . The phenol and δ -nitrophenol toxicity kinetics behaved differently and particular concentration ranges caused increases in light output compared to lower concentrations tested. These results indicated that impacts on bioluminescence were different depending on the challenge used. Consequently the intensity of inhibition seen for particular concentration ranges varied with the toxicants tested.

5.4.4.3 Effect of PAHs on bioluminescence of VUN3,600

A range of higher MW PAHs dissolved in DMF were tested using VUN3,600. These highly cytotoxic, carcinogenic and mutagenic compounds are often extracted from soils using DMF or DMSO solvents. The properties of selected PAHs are shown in Table 5.7 and all the compounds were dissolved in DMF not in DMSO. The tap-water resuspended cells of VUN3,600 were used for PAH monitoring after harvesting at A_{600} 1.0-1.2 and tests were performed after incubating these at room temperature for 10-12 hours (Figure 5.18). When the incubation period was 8 hours, the sensitivity detected was less (data not shown). A distinctive kinetic pattern and a clear dose-related response over a broad concentration range (1.0 and 10.0 gL^{-1}) was seen where bioluminescence declined after initiating the reaction. A lower concentration range (0.01 and 0.1 gL^{-1}) showed an up-regulation of the light emission after an initial decline, and this was retained or slightly increased for four PAHs (naphthalene, phenanthrene, fluorene and flouranthene) tested. Interestingly, benzo[α]pyrene has a lower aqueous solubility (0.0038 mg/ml) compared to benz[α]anthracene (0.014 mg/ml), but bioluminescence kinetics were similar to the above four PAHs which are more soluble. More higher MW of PAHs were tested over the same assay period, and benz[α]anthracene and di-benzo[α,h]anthracene showed no effect up to concentrations of 10.0 gL^{-1} , although initial light output was deemed to be inhibited for benz[α]anthracene, which may be due to their lower aqueous solubility (Sims and Overcash, 1983) and/or chemical reactions with *n*-decyl aldehyde. These three compounds caused prompt precipitation as soon as the chemicals were added to the tap-water resuspended cell suspension. Attempts to improve the solubility of the samples were made by dissolving the PAHs in various concentrations of DMF in water (10, 20, 30, 40, 50, 60,

70, 80, 90, 100%) but these higher MW PAHs precipitated below 40% solvent. The test system using VUN3,600 could not be used to test these compounds.

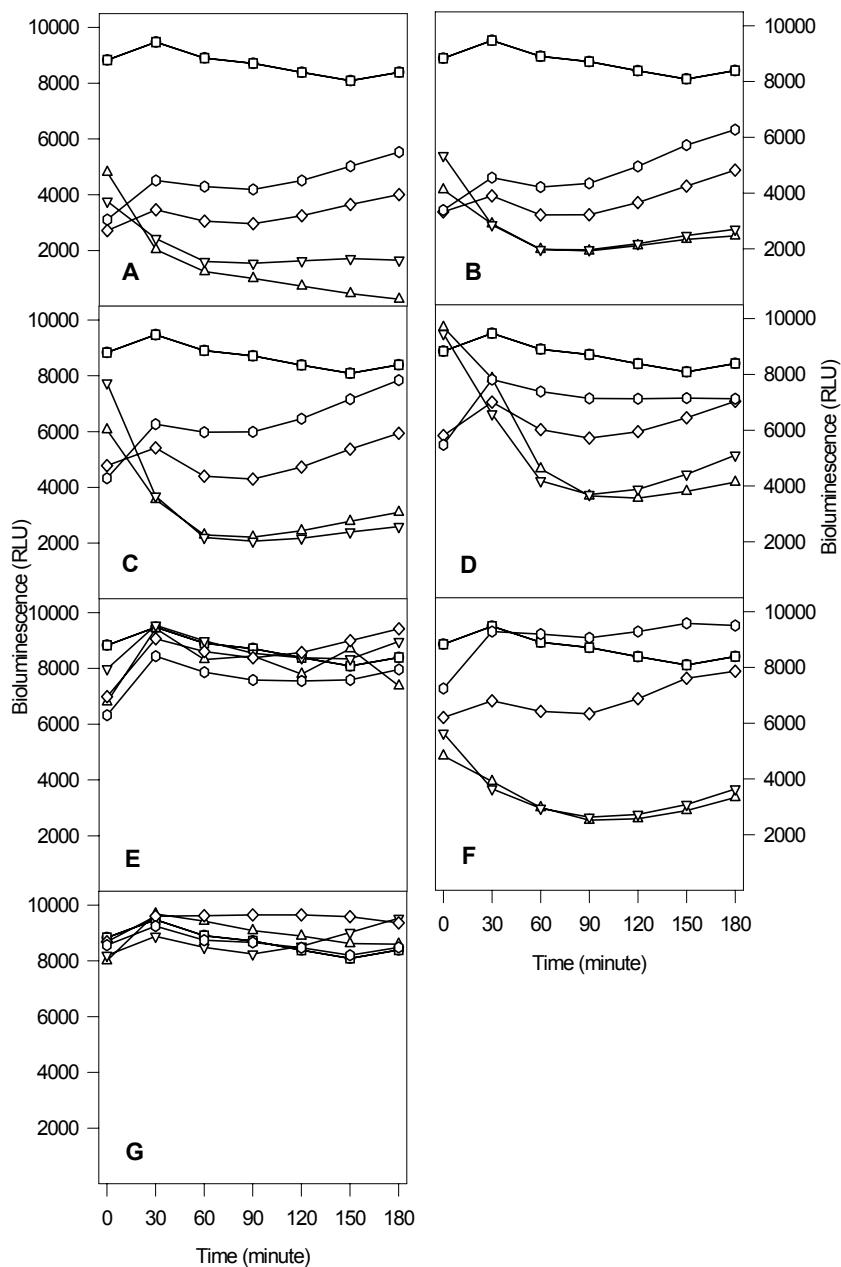


Figure 5.18. Bioluminescence monitoring of PAH compounds in VUN3,600 at different concentrations of PAHs: 0.01 gL^{-1} (\circ), 0.1 gL^{-1} (\diamond), 1.0 gL^{-1} (∇) and 10.0 gL^{-1} (Δ), and the control (\blacksquare), which contained $54.7 \mu\text{M}$ DMF added at the same time as the tests. Tap-water resuspended cells were used and assay conditions were as described in Figure 5.17, with measurements at 30 minute intervals using LSC for 10 seconds. The selected PAHs were naphthalene (A), fluorene (B), phenanthrene (C) and fluoranthene (D), benz[α]anthracene (E), benzo[α]pyrene (F) and di-benzo[α,h]anthracene (G).

Although there is a requirement for this to be investigated further, these results showed that this system had some potential for environmental monitoring to detect selected high molecular weight PAHs with limited aqueous solubility.

5.5 Discussion

Natural living microbial sensors are potentially powerful tools and provide the opportunity for directly revealing toxic conditions at the cellular level. Detection of environmental toxins has become the most widely sought application of genetically-marked microbial sensors (Stewart and Williams, 1992; Meighen, 1993; Prosser *et al.*, 1996; Ramanathan *et al.*, 1997). Moreover, genetically modified strains may be released into the field then used as sensors directly (Prosser *et al.*, 1996), but this approach would inevitably cause perturbation in the ecosystem. A wide variety of reporter gene systems for the detection of environmental pollutants has been described detecting substances such as naphthalene (Burlage *et al.*, 1990; Heitzer *et al.*, 1995) or toluene (Applegate *et al.*, 1997) or middle-chain alkanes (Sticher *et al.*, 1997) or metal ions (Herrero *et al.*, 1990; Guzzo *et al.*, 1991; Corbisier *et al.*, 1993; Selifonova *et al.*, 1993; Paton *et al.*, 1995; Rouch *et al.*, 1995; Erbe *et al.*, 1996), while others are not directly dependent on stimulation of a particular promoter to switch on luminescence, such as *lux* expression in *Bradyrhizobium* (O’Kane *et al.*, 1988) or *Lactobacillus* (Ahmad and Stewart, 1991). Non-specific whole cell biosensors for the detection of toxins include the commercially available Microtox® system (Hermens *et al.*, 1985; Kamlet *et al.*, 1988), or natural bioluminescent *V. harveyi* (Thomulka and Lange, 1995; Lange and Thomulka, 1997), whereas cloned systems which perform a similar function use stress responsive promoters (Van Dyk *et al.*, 1994; Belkin *et al.*, 1996; Ben-Israel *et al.*, 1998).

Unlike the above systems which are largely based on well-established bacterial systems as the basis for developing biosensors, the approach used in this thesis was to use a natural isolate from an aquatic environment to develop a biosensor for maintaining pollutants in water samples. Indigenous strain VUN10,077 was characterised phenotypically and biochemically in Chapter 3 and results for 16S rRNA gene analysis were also reported. However, this strain was largely genetically and physiologically uncharacterised although this thesis described the abnormal rRNA pattern, the optimal defined medium and differentiation of HSPs under various stress

conditions. Moreover, strain VUN10,077 showed dose-related responses to selected heavy metal ions during growth and this was described in Chapter 4. Analysis of the physiological response to stress environments such as pollutants was extended to expression of bioluminescence, where the level of reactions provided fundamental early warning of toxins both selectively and specifically using a water-based detection system. Environmental stress responses take place in the intact organisms and mostly indigenous organisms are confronted by these unnatural conditions in natural environments. In taking this approach, the construction of an indigenous strain with stable light output enabled both its testing as a biosensor for detecting pollutants and a comparison to be made between the impacts of pollutants on the physiology and genetics (induction of stress responses) in this strain. The latter is useful as it provides a tool for determining likely impacts on this type of organism in natural settings, whereas other laboratory tests may give false impression of this because of the crude measurements used. For example, changes in viability or growth rate occurred at much higher concentrations of heavy metal ions when compared with the concentrations which affected cell physiology by decreasing bioluminescence.

The *luxAB* gene insertion may affect cell metabolism less compared to expressing the whole *lux*-operon because bioluminescence gene expression does not only need luciferase stability but it also interacts with the cell metabolism once the genes are integrated so that light expression depends on the biochemical and physiological state of the strain (Korpela *et al.*, 1989; Heitzer *et al.*, 1992; Tu and Mager, 1995; Sticher *et al.*, 1997). This bacterial reporter-gene system produced bioluminescence in the presence of the aliphatic aldehyde substrates, where the substrate can be made by genes expressed on the chromosome or by supplying the substrate exogenously. The first step in introducing the *lux* genes was to determine the MICs of antibiotics for all of the pseudomonad related species. Preliminary drug tests were done to determine the MICs in antibiotic gradient agar plates with concentration ranges of 0 to 100 µg per ml. Strain VUN10,077 was sensitive to the tested antibiotics except for nalidixic acid, whereas *St. maltophilia* was resistant to tetracycline at up to 150 µg/ml, kanamycin up to 200 µg/ml, and ampicillin up to 200 µg/ml (Table 5.1) making this strain unsuitable for use with the available plasmids. Based on antibiotic sensitivity, tetracycline and nalidixic acid were chosen to suppress the parental strain for conjugal transformation of strain VUN10,077 (Table 5.2). The conjugation system for introducing bioluminescence genes into the strain VUN10,077 was established. Mini-Tn5 pUT derivative plasmid carrying a standard tetracycline resistance marker gene (de Lorenzo *et al.*, 1990) was integrated into strain VUN10,077 by random insertion onto the chromosomal DNA using bi- and tri-parental matings (de Lorenzo *et al.*, 1993b; Waterhouse and Glover, 1994; Vázquez *et al.*, 1994). Unexpectedly, a low frequency of

light emitting mutants was seen despite extensive screening, and two transconjugants, VUN3,600 and VUN3,601, which constantly emitted light output throughout the growth cycle (Figure 5.1 and 5.2) were isolated. This may have been because the light emitting genes were located on the chromosome in a constitutively expressed gene and/or a specific gene promoter which can be activated by *luxAB*, and related to a host fitness as the *lux*-marked strains were not impaired greatly in terms of growth rate (Figure 5.1 and 5.3) (Stewart and Williams, 1992). This may also have indicated the lack of *luxI* homologues cause cell density-dependent induction of bioluminescence in *V. fischeri* (Swift *et al.*, 1993). The simplest test for impairment of host fitness is comparison of specific growth rates of marked and parental strains in liquid batch culture, because bacterial luciferase may generate superoxide ions when strains are expressing *luxAB*, which could be toxic to the cells (González-Flecha and Demple, 1994; Liu *et al.*, 1995). Rattray *et al.* (1993) and Cebolla *et al.* (1993) reported that *lux* marked strain of *Ps. fluorescens* 10568 and *luc*-marked *Rhizobium meliloti* showed no impairment of specific growth rate or survival relative to parent strains. The relatively stable transconjugant VUN3,600 was maintained using BHI without supplement of antibiotics and showed a growth rate similar to the parent as well as light output throughout the growth cycle. Light emission of VUN3,600 reached a peak before the culture entered stationary phase and thereafter this declined rapidly (Figure 5.1 and 5.3), similar to other Gram negative and positive *luxAB*-marked strains (Eaton *et al.*, 1993; Iizumi *et al.*, 1998).

Several locations of the *luxAB* genes on the chromosome were revealed by Southern blot analysis (Figure 5.4), and these different locations may have affected stability of light output and expression of light emission, as differences were seen between two transconjugants, VUN3,600 and VUN3,601 (Figure 5.1 and 5.2), although the growth rates were less affected (Figure 5.3). The *luxAB* genes can influence the function of constitutive and/or specific promoters to express light, and this may be due to the first 200 nucleotides of the *lux* coding sequence which contains tracts of A residues which can form a curve in the DNA (Owen-Hughes *et al.*, 1992) or stem-loop structure possibly involved in stability of the upstream mRNA coding for *luxA* and *luxB* genes (reviewed by Meighen and Dunlap, 1993). It is well known that curved DNA can influence promoter activity (see reviewed by Travers, 1991). For example, a downstream curved DNA element was required for normal repression of the *proU* promoter (Owen-Hughes *et al.*, 1992) and a chromatin-associated protein H-NS interacts at curved sequence elements in the *luxAB* genes and influences DNA topology (Tupper *et al.*, 1994). The commonly used *luxAB* reporter genes influence transcription from a subset of promoters, and this may account for the observation that some promoters are active (*leu-500*) or inactive (*proU*) while some are unaffected (*gyrB*) by the insertion of the *luxAB* genes from *V.*

harveyi (Forsberg *et al.*, 1994). These effects may also be related to an intrinsically curved DNA segment in the 5' coding sequence of the *luxA* gene. Forsberg *et al.* (1994) proposed that if the advantages of the *lux* reporter system are to be retained, then it may be pertinent to remove the intrinsically curved DNA element by introducing multiple mutations without disrupting the coding sequence or by reconstructing a synthetic gene with altered codon usage. This may be one of the factors important for using the *lux* reporter gene system in data analysis and in field applications. However, *luxAB* expression by the transconjugants of VUN10,077 was adequate.

Conjugal transformation of strain VUN10,077 achieved, however, only a limited number of mutants of the clones screened. Electroporation of *lux* genes into strain VUN10,077 succeeded and better results were found when cells were harvested at early-exponential or stationary phases then recovered for 12 hours at 28°C following electroporation (Table 5.3 and 5.4) (Miller, 1994). The volatility of *n*-decyl aldehyde allowed it to disperse and penetrate into cell suspensions to initiate luciferase induction when using enough top space in the measuring container and healthy cells. However, there was great difficulty in delivering the external substrate to clones being screed by growth in microtitre trays and testing expression by exposure to X-ray film (Flemming *et al.* 1994; Virta *et al.*, 1995), similarly for agar plates exposed to X-ray film (Miyamoto *et al.*, 1985; Guzzo *et al.*, 1991). These limitations prompted using the whole promoterless *lux* operon, which can provide an endogenous aldehyde from the cellular fatty acid and therefore decrease the problems of substrate delivery. The pUCD615 plasmid containing *luxICDABE* (Rogowsky *et al.*, 1987) was useful for analysis of heat shock protein promoter activity (Van Dyk *et al.*, 1994; Van Dyk *et al.*, 1995; Belkin *et al.*, 1996; Ben-Israel *et al.*, 1998), where DNA sequencing of the *luxR-luxI* region revealed the presence of a promoter region of a kind typical of σ^{32} sequences at the beginning of the *luxI* gene. Moreover, starvation and heat shock conditions did not induce luminescence in *luxI* deleted engineered *E. coli* in the presence of σ^{32} (Ulitzur and Kuhn, 1988). It was possible to screen for positively regulated responding transformants by heat treatment at 37°C, however, the light emission levels were so low that great difficulty was encountered measuring a dose-related toxic substance response (Figure 5.5). In a study of temperature responsive promoters in luminescent organisms, the luciferase of *V. harveyi* and *Xenorhabdus luminescens* were found to be stable at 37°C and 42°C, respectively (Szittner and Meighen, 1990), in contrast *V. fischeri* luciferase was rapidly inactivated at 37°C but was stable at 30°C (Ahmad and Stewart, 1991). *LuxAB* engineered *E. coli* grew at 37°C and bioluminescence was not impaired, although this may be less than optimal (Phillipes-Jones, 1993). The light output was differentiated by whole *lux-*

marked strains of VUN10,077, mostly from pUCD615 plasmid, on X-ray film then bright clones analysed by densitometry, but the signal strength was not enough to carry out further toxicity tests using either LSC or LS50 luminescence spectrometry. The other disadvantages of using this method were: the wild type strain of VUN10,077 had a naturally adhesive character and precipitated to the bottom of wells or flasks when standing without shaking, which may cause problems with the even distribution of cells when measuring bioluminescence or turbidity at late stages of growth; less reproducibility may be due to temperature dependent instability of luciferase induction after transferring the bioluminescence genes or individual transformants have different growth rates or introduction of the *lux* operon could cause additional energy constraints for the transformed cells (Brennerova and Crowley, 1994).

To be effective, the genetic-based microbial responses must be translated into readily observable outcomes, that is, which convert the state of the sensing system within the microbes into an external signal. The stability of signal and the physiological state of the biosensor cells can be reasonably controlled by optimised and standardized assay conditions as described in this chapter (substrate delivery system, solvent system and incubation period). Although practical problems arise in using decanal as an *in vivo* assay reagent, because it is volatile, lipophilic, poorly soluble in aqueous system and cytotoxic, this appear to be the optimal substrate in terms of the biochemistry of the luciferase reaction (Hastings and Nealson, 1977; Hastings *et al.*, 1985; Blouin *et al.*, 1996). Thus several different solvent systems (ethanol, water, ethanol plus water mixture and 0.9 M NaCl) were tried as substrate delivery systems (Figure 5.6). Diluting in water was chosen after evenly dispersing the substrate in the Milli-Q water using sonication, although dissolving in ethanol was also to be applied to VUN3,600. However, ethanol may have caused synergistic induction by the tested chemicals in the chromosomally marked strain (Figure 5.9) (Van Dyk *et al.*, 1995a; Belkin *et al.*, 1996). Additionally, the whole *lux* gene biosensors with stress-inducible promoters showed increased bioluminescence in ethanol and penta-chlorophenol treatment assays in *E. coli* (Van Dyk *et al.*, 1994). To determine the best light output of VUN3,600, samples were taken during the growth cycle and measured using LSC and the LS50 luminometer. Relatively high and reproducible light output occurred at an A_{600} 1.0-1.2 when transconjugant VUN3,600 grew in BHI at 28°C (Figure 5.7 and 5.8). Similary, *luxAB* genes of *V. fischeri* in *L. lactis* subsp. *lactis* showed that the expression reached a peak in the late exponential phase and rapidly decreased as cells entered stationary phase, when they were induced during growth on lactose (Eaton *et al.*, 1993). The decanal concentrations affect the luciferase reaction in that high decanal concentrations can inhibit the luciferase enzyme (Holzman and Baldwin, 1983; Blouin *et al.*, 1996; Sticher *et al.*, 1997). Schultz and Yarus (1990) used 20 µl of *n*-decyl aldehyde in water (1:1,000) after sonication and

addition to 200 µl of sample to initiate light output in *E. coli*, and this system was applied to transconjugant VUN3,600 in the same ratio: the luciferase reaction occurred promptly then dropped dramatically. Different ratios of substrate to cells or decanal concentrations caused various patterns of induction in VUN3,600 and the most reproducible luminescence reaction was obtained when substrate:cell suspension was 100 µl:500 µl (Figure 5.10), which presented a controlled secondary phase-like reaction for biosensor application (Blouin *et al.*, 1996). This substrate delivery system incurred a higher level of light emission by VUN3,600, so the system may be sufficient for *in vivo* luminescence assay.

The *luxAB*-marked VUN10,077 is a naturally dark organism until the substrate is delivered and difficulty was encountered in quantifying the bioluminescence which had several peaks after initial induction, even when using the optimized external substrate delivery system (Figure 5.12), and tap-water resuspended cells appeared also to have a peak or burst phase (Figure 5.11). When bioluminescence was measured using growing cultures of VUN3,600 in BHI, the light decaying rates varied quickly and showed a typical pattern of induction seen for other *luxAB*-marked strains (Blouin *et al.*, 1996), and this may have been due to the half-life of initial light output being within a minute. Phillips-Jones (1993) reported a initial burst output phase where bioluminescence increased immediately then decayed exponentially in the anaerobe *Clostridium perfringens*. A steady increase then slow decay was reported for *E. coli*, *luxAB* harbouring plasmid pPS14 which occurred within 20 minutes when samples were taken at exponential phase and using 1 µl of 10% aldehyde in ethanol to 100 µl samples. Recently, *Caulobacter crescentus* CB2A and *E. coli* harbouring plasmid RTB7 and pSW23 (*luxAB*) produced prototypic kinetics of *in vivo* bioluminescence which had two major phases and the light emission ceased almost within 25 minutes, when decanal was dissolved in a water and methanol mixture (1:1) (Blouin *et al.*, 1996). Healthy growing cells of transconjugant VUN3,600 emitted light over the 4 hours test period and three burst phases were represented (Figure 5.17). This differentiation of induction could have been due to different sensitivity of these cells to inducer and/or different intracellular concentrations of substrate and/or the solvent system used. Interestingly, typical kinetics seen for decanal induced light emission by biosensors developed in several studies are dependent on the assay system used. Sticher *et al.* (1997) also reported that light emission of *E. coli* DH5 α containing pGEc75 and pJAMA7 plasmids was dependent on the concentration of decanal in the assay when using a 1:1 mixture of water and ethanol in the decanal solution.

The water-based *luxAB*-marked system (cells suspended in tap water) showed stability and reproducibility of light output using LSC and the output showed a relatively plateau-like

induction compared to that seen for growing cells. This system was successfully used for water-soluble heavy metal ions and SDS or DMF-dissolved chemicals assays, where the solvent used alone, water or DMF, did not cause false-positive or -negative results in the assay (Figure 5.11 and 5.16). Recently, Iizumi *et al.* (1998) used water as the solvent for testing bioluminescence of *luxAB*-marked *Nitrosomonas europaea*, with light output initially using 2.5 µl of 10% aldehyde in ethanol and a similar approach was used by Ptitsyn *et al.* (1997). It is well known that a number of solvents can reduce the bioluminescence response in *Ph. phosphoreum* in the Microtox™ assay (Bulich *et al.*, 1986), including DMF, and water alone causes similar patterns due to the osmotic instability of this marine bacterium. Additionally, Heitzer *et al.* (1995) reported that water alone did not cause a bioluminescence response in their continuous monitoring system using naphthalene-*luxCDABE* construct in *Ps. fluorescence* HK44.

The bioluminescent transconjugant VUN3,600 was employed for toxicity assessment using the optimized conditions of light output. The concentration of tetracycline used to culture cells affected bioluminescence (Figure 5.1) and the ethanol substrate delivery system caused false-positive results when overnight culture suspensions were used (Figure 5.9). Optimised conditions included using cells harvested at A_{600} 1.0-1.2 from BHI cultures grown at 28°C, then resuspending cells in the same volume of tap-water after mild centrifugation and storing the tap-water suspension for 10-12 hours at room temperature before use to stabilise the light output. The tap-water resuspended bioluminescent cells could also be used after long-term storage at 4°C if they were incubated for 30 minutes at ambient temperature before use and toxicity responses were still retained (Figure 5.15). Bioluminescence was initiated by supplying freshly prepared 100 µl of *n*-decyl aldehyde diluted 10³-fold in Milli-Q water, sonicating to disperse it, to 500 µl of tap-water resuspended cells. Toxicity measurements were recorded approximately 10 minutes later in triplicate assays using LSC for 10 seconds. The external aldehyde and 0.4% of the assay volume toxicants were added in that order.

Heavy metals could be detected both qualitatively and quantitatively over different concentration ranges in either short-term or long-term assays (Table 5.5 and Figure 5.14). Whole cell *luxAB* constructs have been rarely challenged in toxicity assays (Paton *et al.*, 1995), whereas specific gene promoters have been popularly used to detect specific compounds (Guzzo *et al.*, 1991; Corbisier *et al.*, 1993; Eaton *et al.*, 1993; Guzzo and DuBow, 1994; Virta *et al.*, 1995; Iizumi *et al.*, 1998). However, whole *lux* operon mutants have been applied for toxicity assays when associated with stress or specific metal responsive promoters (Selifonova and Barkay, 1994; Van Dyk *et al.*, 1994; Rouch *et al.*, 1995; Belkin *et al.*, 1996; Ptitsyn *et al.*, 1997;

Ben-Israel *et al.*, 1998). Interestingly, heat shock-bioluminescence gene constructs were tested in LB medium: Van Dyk *et al.* (1994 and 1995) used 50 µl of LB-kanamycin medium directly and 50 µl of toxin was added, and similarly Belkin *et al.* (1996) used 50 µl of cells resuspended in LB without kanamycin after 2-3 hours. Ben-Israel *et al.* (1998) used 10 µl of cells chilled on ice where the cells were a culture suspension in LB-ampicillin medium resuspended in the same volume of cold LB broth then a final volume of 150 µl was used for assay, moreover, they used the same or similar stress promoter-*lux* constructs in several *E. coli* host systems. In comparison, VUN3,600 emitted relatively low bioluminescence when growing cells were diluted in the same volume of fresh rich medium compared to growing culture suspensions. The commercially available Microtox™ system may avoid to compare due to use a high salt concentration in tests, however, these were reported to compare favourably in terms of detecting a range of pollutants as well as in *luxAB* whole cell microbial sensors (Paton *et al.*, 1995; Iizumi *et al.*, 1998). In this study, the water-based system could detect heavy metal ions and SDS when they were added at a range of concentration of 0.02 mgL⁻¹ to 0.2 gL⁻¹ in short-term assays (Table 5.5), in addition to long-term-assays for CdCl₂ and HgCl₂ (Figure 5.14). Transconjugant VUN3,600 presented sensitivity to CdCl₂, Pb(NO₃)₂, ZnCl₂, CoCl₂.6H₂O, NaAsO₂ and HgCl₂ up to a concentration of 0.02 gL⁻¹ with less than 10% standard deviations between triplicate assays. The use of VUN3,600 provided a sensitive assessment to toxicity as follows: cadmium, lead, zinc, nickel, arsenite, cobalt and mercury had equivalent EC₅₀ values of 2.0 mgL⁻¹, 0.01 gL⁻¹, >0.02 mgL⁻¹, 0.1 gL⁻¹, 0.1 gL⁻¹, 0.4 mgL⁻¹ and 0.6 mgL⁻¹, respectively. When comparing results for cadmium, zinc and nickel tests, the Microtox™ EC₅₀ values were 2.35 mgL⁻¹, 5.5 mgL⁻¹ and 4.35 mgL⁻¹, respectively, which differed from those reported by Xu and Dutka (1987) and Paton *et al.* (1995). Cadmium and zinc tests were sensitive and nickel was less sensitive in the water-based monitoring system developed here, whereas *Ps. fluorescens* 10568s was reported to have more sensitive EC₅₀ values by Paton *et al.* (1995); 0.98 mgL⁻¹ to Cd, 0.89 mgL⁻¹ to Zn and 2.49 mgL⁻¹ to Ni.

Unlike heavy metal ion toxicity assays, few researchers have studied the detection of bioavailable hydrophobic carbon compounds, which may be due to their lower water solubility (Menn *et al.*, 1993; Selifanova and Eaton, 1996; Iizumi *et al.*, 1998). Selected PAH compounds have low aqueous solubility (Table 5.7), while they dissolved readily in DMF or DMSO, which are commonly used in media for degradation of PAHs using fungal and *Pseudomonas* species (Monticello *et al.*, 1985, Cerniglia *et al.*, 1989, Pothuluri *et al.*, 1990). The selected PAHs were dissolved in DMF and biological impacts were examined using the water-based monitoring system developed here. The system showed potential to measure either partly water-dissolved

or DMF-dissolved PAHs such as naphthalene, phenanthrene, fluorene, flouranthene and benzo[α]pyrene, which presented distinctive patterns and dose-related responses in this approach, but benz[α]anthracene and di-benzo[α,h]anthracence could not be assayed (Figure 5.18). Detection of benz[α]anthracene and di-benzo[α,h]anthracence caused great difficulty in assays despite using high concentrations of up to 10.0 gL⁻¹; this may have been due to low aqueous solubility and/or chemical reaction with *n*-decyl aldehyde. It should be noted, however, that benzo[α]pyrene could be detected despite having lower water solubility than benz[α]anthracene. In nature, pollutants would be present as mixtures, and this may be more difficult to control and possibly lead to false-positive and false-negative measurements (Burlage *et al.*, 1994; Heitzer *et al.*, 1994). The difficulty of measuring the light emission in certain chemicals may be removed by taking several precautions, including choice of solvent system, substrate delivery system and/or data interpretation. Misinterpretation of false-positive results (i.e., partial or full induction of the reporter without its cognate inducer) can be avoided by a clear understanding of the biosensor specificity and knowledge of other potential inducers in typical pollutant mixtures. False-negative results (i.e., partial or full inhibition of the signal expected from a particular concentration) are mainly due to toxic compounds in the sample like some PAH compounds: benz[α]anthracene and di-benzo[α,h]anthracence caused prompt precipitation as soon as the chemicals were applied in the tap-water resuspended cells making them unsuitable for assay. Using suitable controls or applying an additional, constitutively expressed reporter system to account for the observed biosensor activity can help overcome these constraints (Burlage *et al.*, 1994; Heitzer *et al.*, 1992; Wood and Gruber, 1996).

The water-based monitoring system described in this chapter could be used for water-soluble or DMF-dissolved chemicals, and was successfully applied in assaying several representative chemicals; however, it may be unattractive for either continuous monitoring or routine analysis of water samples because detection of pollutants required careful optimisation and it was necessary to deliver the luciferase substrate, deanol, in a controlled fashion to stimulate light output. The quality assurance parameters for routine use of the constructed microbes have not been defined fully yet but this aspect of the work is worthy of pursuit because of the potential advantages of the water-based testing systems, which include that they are not restricted to aseptic conditions, they are not influenced by non-luminescent bacteria during the incubation or test period, and they are sensitive to common pollutants. The water-based monitoring system shows potential as an environmental microbial sensor because most toxicants are water soluble and sampling places are fresh waterways and ground water, not in the sea or over 3% salt containing streams.

Conclusion and Recommendation

The major conclusions of this chapter are summarised in dot points as follows:

- Antibiotic susceptibility profiles were determined and tetracycline and nalidixic acid were chosen to select strain VUN10,077 *lux*-marked mutants for bioluminescence genes transferring in by either conjugation or electroporation. Although conjugal transformation gave limited success, early-exponential and stationary phase cells were suitable for electroporation when cells were recovered by incubating in BHI recovery medium for 12 hours at room temperature.
- Relatively plateau-like bioluminescence was observed in a water-based monitoring system for *luxAB*-marked mutant VUN3,600. This was achieved using tap-water resuspended cells supplied with external substrate prepared by diluting 10³-fold in Milli-Q water. Cell suspensions were incubated at room temperature for 10-12 hours to give assays with less than 5% standard deviations between triplicates.
- The tap-water resuspended cells were not affected by the addition of either water alone or DMF, which was used as the solvent for dissolving organic toxicants. Clear dose-related responses were obtained for CdCl₂, Pb(NO₃)₂, ZnCl₂, CoCl₂.6H₂O, HgCl₂ and SDS while NiCl₂.6H₂O and NaAsO₂ caused chemical specific responses in short-term assays. CdCl₂ and HgCl₂ exhibited clearly dose-related response in long-term assay. DMF-aided toxicity assays were subjected to long-term assays, and chlorinated phenolic and PAH compounds were tested. Dose-related responses occurred in di-chlorophenol, tri-chlorophenol, tetra-chlorophenol and penta-chlorophenol but not for phenol and δ-nitrophenol; naphthalene, phenanthrene, fluorene, fluoranthene and benzo[α]pyrene were also detectable but benz[α]anthracene and di-benzo[α, h]anthracence were not.

Areas for further basic- and applicable-level research are as follows:

- Once the genes for the proteins that respond to heat, Cd²⁺ and Hg²⁺ have been identified, it would be possible to explore the switching mechanisms for these and determine the nature of

the stimuli which can up-regulate these promoters (including overlaps in switching). These promoters may well provide an alternative to the existing systems (heat-stress, compound-specific) available for toxicity testing.

Conclusion Remarks

Throughout this thesis, the indigenous strain VUN10,077 remarked affection at the physiological level when cells were subjected to different pollutant classes and chemical assaults. Specific concentrations of the chemicals which affected viable counts and intracellular proteins were: above 2.0 gL⁻¹ of CdCl₂, 2.5 gL⁻¹ of NiCl₂.6H₂O, 4.0 gL⁻¹ of CoCl₂.6H₂O, 0.5 gL⁻¹ of HgCl₂, 0.5 gL⁻¹ of SDS, 1.0 gL⁻¹ of NaAsO₂ and 1.8 gL⁻¹ of phenol significantly decreased the viable number of cells. Dose-related viable counts and bioluminescence toxicity assays overlapped in the case of cadmium stress in addition to protein profile changes in prolonged stress proteins when analysing profiles by ³⁵S-methionine labelling.

The major conclusion remarks of this thesis are summarised in dot points as follows:

- In toxicity assays, CoCl₂.6H₂O was relatively less effective at concentrations of up to 2.0 gL⁻¹ but significantly decreased viable counts at concentrations of 2.3 gL⁻¹ similar to bioluminescence toxicity assays which did not respond at concentrations of up to 0.002 gL⁻¹ then abruptly decreased bioluminescence at concentrations of 0.01 gL⁻¹ in single point assay.
- Viable counts were slightly increased below 1.0 gL⁻¹ of NiCl₂.6H₂O stress and decreases in the viability started above 1.5 gL⁻¹ of NiCl₂.6H₂O, similar to bioluminescence toxicity assays which showed relatively less sensitivity at concentrations of up to 0.02 gL⁻¹ of NiCl₂.6H₂O then showed sensitivity above this concentration.
- CoCl₂.6H₂O at the concentrations used did not affect viable counts as much when compared with other tests, whereas bioluminescence toxicity assay showed a sensitive response at the lower concentrations between 0.02 mgL⁻¹ and 0.002 gL⁻¹ in single point assay not long-term assay.

- At concentrations of up to 1.0 gL^{-1} of NaAsO_2 , viable counts were differentiated in detailed fashion similar to bioluminescence toxicity assays at the tested concentration range from 0.02 mgL^{-1} to 0.2 gL^{-1} .
- Although HgCl_2 stress affected viable cell changes dramatically in the concentration range from 0.001 gL^{-1} to 0.025 gL^{-1} , intracellular protein changes were dose-related at concentrations up to 0.025 gL^{-1} of HgCl_2 , whereas bioluminescence toxicity assays showed sensitivity at concentrations between 0.02 mgL^{-1} and 2.0 mgL^{-1} .
- Response to SDS was not sensitive as mercury stress in terms of toxicity assays and protein profile changes.
- Response to the thus far tested important classes of environmental pollutants showed good correspondence between physiological changes and bioluminescence toxicity, although bioluminescence changes often occurred at much lower concentrations.

Conclusively, it is possible to detect early warning of changed environments using the physiological state of cells from their responses in bioluminescence toxicity assays, which may discriminate toxic compounds using this approach. However, this analytical application needs more careful examination and further optimization.

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