MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE MELANIN BIOSYNTHETIC GENES FROM *Vibrio cholerae* 569B

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology, Faculty of Science, University of Cape Town, South Africa.

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ABSTRACT

V. cholerae 569B is a bacterium infamous for its role as the causative agent of the diarrhoeal disease cholera. Although the bacterium occurs naturally in brackish waters and estuaries, cholera outbreaks are closely linked to specific environmental conditions. For example, most outbreaks occur during the summer months when the bacterium experiences an increase in water temperature, UV-B radiation and salinity. Even though these conditions can play a role in activating virulence in *V. cholerae* 569B, the exact mechanism remains to be elucidated.

Previously it was observed that when *V. cholerae* is exposed to elevated temperature and salinity, the bacterium initiates the synthesis of a brown-black pigment known as melanin. The function of the pigment and the genes involved in its synthesis was unknown. We therefore set out to determine the function of pigmentation in *V. cholerae* 569B, since pigmentation could significantly enhance the survival of the bacterium during adverse conditions and therefore aid in the persistence of the organism in the environment.

Our first objective was to identify the genes responsible for pigmentation in the organism. For this, we constructed a *V. cholerae* genebank in *E. coli* HB101 using the suicide vector pEcoR251. This genebank was screened for *E. coli* clones that were able to pigment on Luria agar supplemented with L-tyrosine and copper. We isolated two phenotypically different pigmenting clones. Thus, *E. coli* clones harbouring pCM30 synthesized melanin which occurred extracellularly as a result of diffusion or export from the cell, whereas melanin remained intracellular when produced in *E. coli* transformed with pCM3. Southern hybridization studies revealed that the cloned *V. cholerae* DNA fragments were not linked on the chromosome, confirming that the genes were distinct. Restriction enzyme maps of both pCM30 and pCM3 were constructed and used to generate deletions within the cloned *V. cholerae* DNA in order to localise the regions responsible for pigmentation in the *E. coli* clones.

Subsequent sequencing of pCM30 revealed a 1.1 kb open reading frame (designated *ppdA)* encoding the enzyme 4-hydroxyphenylpyruvate dioxygenase. The enzyme catabolises tyrosine by initial deamination and conversion to hydroxyphenylpyruvate (pHpp). pHpp is then directly converted to homogentisic acid which then autopolymerises into pyomelanin. *In vitro*

transcription and translation of *ppdA* showed this locus to encode a 41 kDa protein and primer extension analysis identified the *ppdA* transcriptional start site and the putative promoter region.

In addition, the gene involved in pigment synthesis in pCM3 was sequenced. Sequence data suggested the presence of a 1.088 kb open reading frame encoding a protein of 39 kDa in size. *In vitro* transcription and translation confirmed the size of the synthesized protein, while homology searches of several databases showed that the protein displayed about 25% similarity to a hypothetical protein 54.9 minutes on the *E. coli* K12 chromosome. Despite this homology, we do not know how this enzyme synthesizes pigment. Primer extension analysis, however, allowed us to identify the transcriptional start site and to predict the putative promoter region.

Our second objective involved the characterization of the PpdA protein responsible for pyomelanin synthesis in *V. cholerae*. The protein was purified by means of the MalE protein fusion purification system. Antibodies against the purified protein were raised in rabbits and used in Elisa assays to monitor PpdA synthesis within the wild-type *V. cholerae*. These experiments showed that *ppdA* translation was initiated between 40 and 45 hours of growth and ended after 60 hours of growth. Contrary to this result, RT-PCR analysis revealed that *ppdA* transcripts were present in cells grown under both pigmenting-inducing and non-inducing culture conditions, suggesting either posttranscriptional or posttranslational control of *ppdA*. In addition, we showed that PpdA possessed alpha-haemolytic properties, a strategy usually employed by other pathogens to sequester valuable iron from their host.

Finally, we attempted to elucidate the function of pigmentation in *V. cholerae* 569B. Since melanins are pigments with excellent radical scavenging properties, we tested whether pigmentation could protect *V. cholerae* against severe oxidative stress. Initially we constructed a *ppdA*-minus mutant which was unable to pigment when exposed to elevated temperature and salinity, confirming the role of *ppdA* as mediator of melanogenesis in *V. cholerae*. The mutant displayed no growth defects during both exponential as well as stationary phase, suggesting that a defect in *ppdA* did not significantly affect this mutant. Both the mutant and wild-type strains were exposed to 5 mM H_2O_2 to determine whether pigmentation could protect the wild-type against oxidative stress. Indeed, after 2 days of growth under pigment inducing conditions, which corresponds to the time at which copious amounts of brown pigment are secreted into the culture media, the wild-type strain was found to survive oxidative stress better than the mutant. In addition, we showed that there was no overlap in protection against heat induced stress and oxidative stress, whereas increased salinity could confer resistance to various other stresses, including oxidative stress.

ABBREVIATIONS

A	adenine
Amp^r	ampicillin resistant
Arg	arginine
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
°C	degrees celsius
Ci	Curie
cpm	counts per minute
dATP	deoxy-adenine 5'-triphosphate
dCTP	deoxy-cytosine 5'-triphosphate
dGTP	deoxy-guanine 5'-triphosphate
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dTTP	deoxy-thymine 5'-triphosphate
DEPC	diethylpyrocarbonate
EDTA	ethylenediaminetetra-acetic acid
g	gram
G	guanine
Gly	glycine
Glu	glutamic acid
hr(s)	hour(s)
Kan^r	kanamycin resistant
kb	kilobase(s)
kDa	kilodalton(s)
kCal	kilocalories
L	Liter(s)
LB	Luria broth
M	molar
mA	milli-Amperes
mM	millimolar
ml	milliliter
min	minute(s)
mRNA	messenger RNA
mol	mole(s)
nm	nanometers
ng	nanograms

OD	optical density
IgG	Immunoglobulin G
Ile	Isoleucine
IPTG	Isopropyl-β-D-thiogalactopyranoside
ρ	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
%	percentage
Rif^r	rifampicin resistant
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
sec(s)	second(s)
SDS	sodium dodecyl sulphate
sp.	species
Str^r	streptomycin resistant
T	thymine
TAE	Tris-acetate-EDTA buffer
TB	Tryptone broth
TEMED	N,N,N',N'-tetramethylethylenediamine
U	unit of enzymatic activity
ug	microgram(s)
ul	microliter(s)
uM	micromolar
UV	ultraviolet
X-gal	5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside
α	alpha
β	beta
Δ	delta
γ	gamma
λ	lambda
σ	sigma

CHAPTER 1

GENERAL INTRODUCTION

CONTENTS

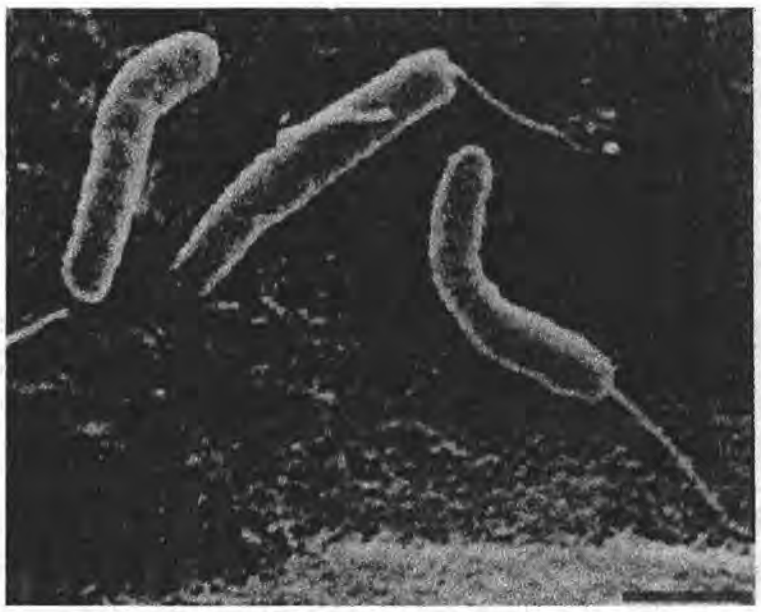


Figure 1. Electron micrograph showing the characteristic curved shape of *V. cholerae* cells. The single polar flagellum is clearly visible. Taken from Prescott *et al.*, (1996) In *Microbiology* (3ed), pp 769. WC Brown publishers, U.S.A.

and amount of cellular DNA (Baker *et al.*, 1983; Kjelleberg and Hermansson, 1984). Although these cells remain viable, they become non-culturable and demonstrate a predilection to associate with phyto- and zooplankton, as well as the mucilaginous sheath of algae (Tamplin and Colwell, 1986). It is now widely accepted that *V. cholerae* is in fact an autochtonous member of brackish water and estuarine environments (Singleton *et al.*, 1982).

1.3 Cholera

Cholera is a severe diarrhoeal disease that is caused by the enterotoxin produced by *V. cholerae*.

1.3.1 Symptoms

The most distinctive feature of cholera is the production of a voluminous watery stool that ultimately results in dehydration (Wachsmuth *et al.*, 1994). Because the bacterium does not invade the intestinal mucosa, it does not trigger a host inflammatory response, with the result that fever does not accompany cholera in patients. Diarrhoea is usually painless, although some intestinal cramping may occur as a result of fluid distension of the bowel. Vomiting is common, especially during the earlier stages of the disease.

1.3.2 Initial stages of infection

Once contaminated food or water has been ingested, the bacterium first needs to successfully transit the acid barrier of the stomach (Drasar and Forrest, 1996). Once in the small intestine, *V. cholerae* uses mucous dissolving enzymes to migrate through the mucous layers to ultimately reach the enterocytes. Motility plays a crucial role in these early stages of colonization. The vibrios adhere to the intestinal cells with the aid of pili. At this stage cholera toxin production is initiated.

1.3.3 Mode of action of the cholera enterotoxin

The cholera enterotoxin is a multimeric protein consisting of 5 non-covalently linked B-subunits and 1 A-subunit (Wachsmuth *et al.*, 1994). The A-subunit consists of two peptides, A_1 and A_2 that are linked via a disulphide bond. The B-subunit is responsible for binding the enterotoxin to ganglioside GM_1 of the intestinal cell. Binding involves at least two of the five subunits. Once bound, the disulphide bond between the A peptides becomes reduced and A_1 is released into the cell. The mechanism by which the A_1 peptide passes the B-subunit is not clear since simple insertion of the A_1 through the pore in the B pentamer, and subsequently, into the cell is not supported by crystallographic studies (Sixma *et al.*, 1991). The fate of A_2 is not known since there is little evidence that it actually enters the cell. The peptide may only serve to bind A_1 to the B-subunit (Gill, 1976).

Following translocation, the A_1 peptide catalyzes the ADP-ribosylation of the α subunit of the G_s protein (Fasano, 1998) (Fig. 2). Modification of $G_{s\alpha}$ results in the activation of adenylate cyclase. Adenylate cyclase mediates the conversion of ATP to cyclic AMP (cAMP). High cAMP levels activate the catalytic unit of a cAMP-dependent kinase, protein kinase A, which in turn phosphorylates membrane proteins involved in trans-epithelial ion transport. This then leads to an increase in Cl^- secretion by the intestinal crypt cells and decreased NaCl-coupled absorption by villus cells. The resultant osmotic driving force causes massive volumes of water to flow into the lumen which overwhelms the absorptive capacity of the intestine, resulting in diarrhoea.

1.3.4 Environmental conditions known to activate enterotoxin production

In vitro, environmental stimuli such as temperature, pH, osmolarity and the presence of amino acids have a dramatic effect on the activation of enterotoxin production in *V. cholerae* (Gardel and Mekalanos, 1994). For example, the classical strain, *V. cholerae* O1, produces much more toxin at 30°C than at 37°C. This temperature effect seems paradoxical since one would expect the amount of enterotoxin to increase as the bacterium enters the host. However, one might argue that during the infectious cycle, the pathogen encounters 37°C as soon as it enters the host, while toxin synthesis is only required once the invading pathogen colonizes the small intestine. Premature production of toxin might adversely affect early colonization with the result that the bacteria may be flushed out by the host's secretory processes before they have a chance to multiply. Thus, additional signals are required for activation of toxin production at 37°C (Gardel and Mekalanos, 1994).

Cholera toxin production is also influenced by the osmolarity of the medium (Gardel and Mekalanos, 1994). Optimal concentrations of NaCl for toxin production range from 66 mM to 86 mM. High levels of NaCl (250 mM) are inhibitory for toxin production. Culture media with a starting pH of 6.5 and the presence of the amino acids asparagine, arginine, aspartic acid, glutamic acid and serine also stimulates toxin production significantly. Interestingly, the conditions that favour optimal toxin expression in the classical biotype of *V. cholerae* O1 do not favour high levels of toxin expression in the El Tor biotypes and the mechanism underlying this difference is not presently understood.

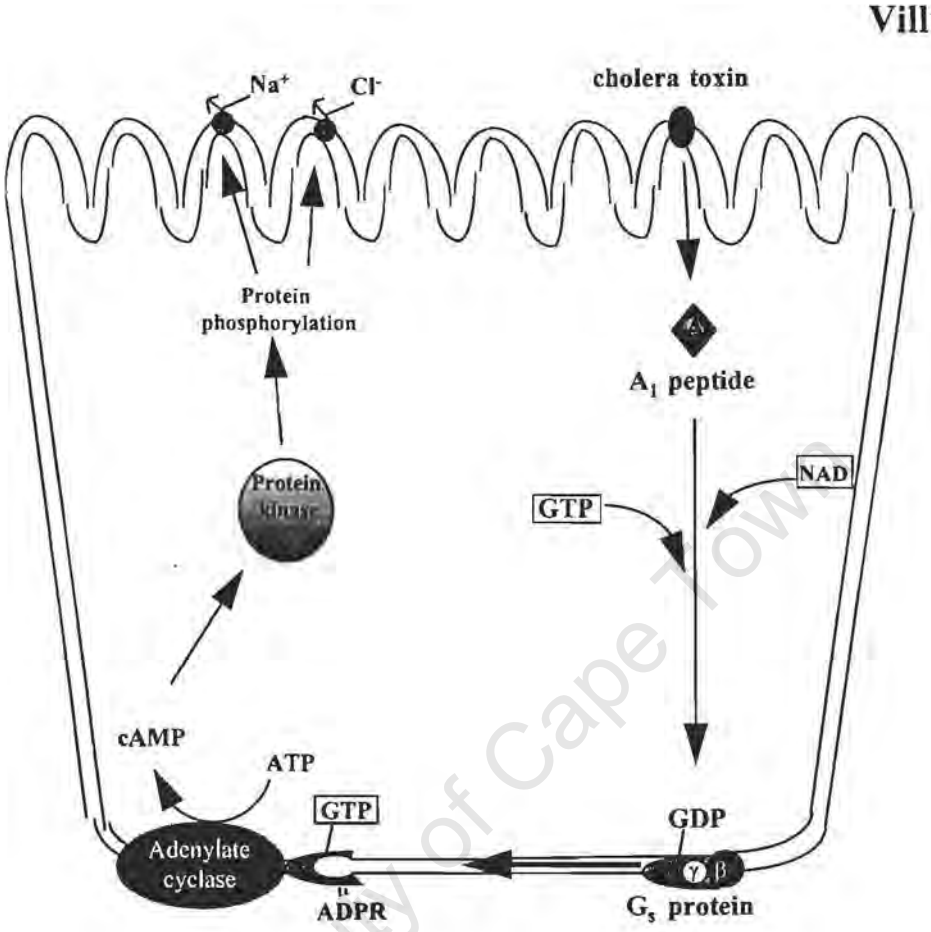


Figure 2. Cholera toxin binds the GM_1 ganglioside receptor via the B subunit. The A_1 subunit is transported into the cell and transfers NAD to the α subunit of the G_s protein. The ADP-ribosylated $G_{s\alpha}$ subunit dissociates from the other subunits and directly activates adenylate cyclase. This increases the cAMP levels which activates protein kinase A, leading to phosphorylation of membrane proteins. In the villus cells the result is the prevention of Na^+ and Cl^- coupled absorption. Taken from Fasano (1998) *Journal of Pediatric Gastroenterology and Nutrition* **26:** 520-535.

1.4 Regulation of the virulence cascade in *V. cholerae*

1.4.1 Components of the ToxR virulence regulon

Virulence in *V. cholerae* is under the control of the regulatory protein ToxR (Peterson and Mekalanos, 1988; Kovach *et al.*, 1996). ToxR is oriented in the inner membrane in such a way that its periplasmic domain is ideally situated for sensing environmental signals and it directly interacts with another transmembrane protein, ToxS (Miller *et al.*, 1989; DiRita and Mekalanos, 1991) (Fig. 3). ToxS plays an important role in stabilizing ToxR by driving the protein into a conformation that favours transcriptional activation. The cytoplasmic domain of ToxR can directly bind DNA in order to interact with the alpha subunit of RNA polymerase, and consequently, activate and coordinate gene expression. The genes under the control of ToxR are collectively known as the ToxR regulon which is comprised of genes which reside at different locations on the *V. cholerae* chromosome. Most important are the cholera toxin genes (*ctxAB*). These genes are located within the genome of a lysogenic filamentous bacteriophage, CTXΦ (Waldor and Mekalanos, 1996) (Fig. 3). Also very important components of the virulence response are the genes involved in colonization, *Tcp* and *Acf*, which comprise a separate pathogenicity island that may also have been a mobile genetic element at one time (Kovach *et al.*, 1996). *Tcp* (toxin-coregulated pilus), synthesized by eight to ten genes, is an essential intestinal colonization organelle (Taylor *et al.*, 1987; Peterson and Mekalanos, 1988). The *Acf* (accessory colonization factors) locus consists of a cluster of four genes whose products enhance colonization (Brown and Taylor, 1995). Separating the *Tcp* and *Acf* genes on the pathogenicity island is a gene encoding the positive regulatory protein ToxT (Fig. 3). ToxT is a member of the AraC family of transcriptional activators and is absolutely required for virulence (Higgins *et al.*, 1992).

Genes that are not associated with the two above mentioned elements, but which also form part of the ToxR virulence regulon, include the genes for the outer membrane proteins OmpU and OmpT, as well as genes involved in motility and chemotaxis (Miller and Mekalanos, 1988a; Sperandio *et al.*, 1995; Gardel and Mekalanos, 1996).

Only toxigenic strains of *V. cholerae* contain the lysogenic CTXΦ phage and the *Tcp-Acf* pathogenicity island (Kovach *et al.*, 1996), whereas ToxR seems to be present in all strains: pathogenic as well as non-pathogenic (Miller and Mekalanos, 1984). Recently, Hase and

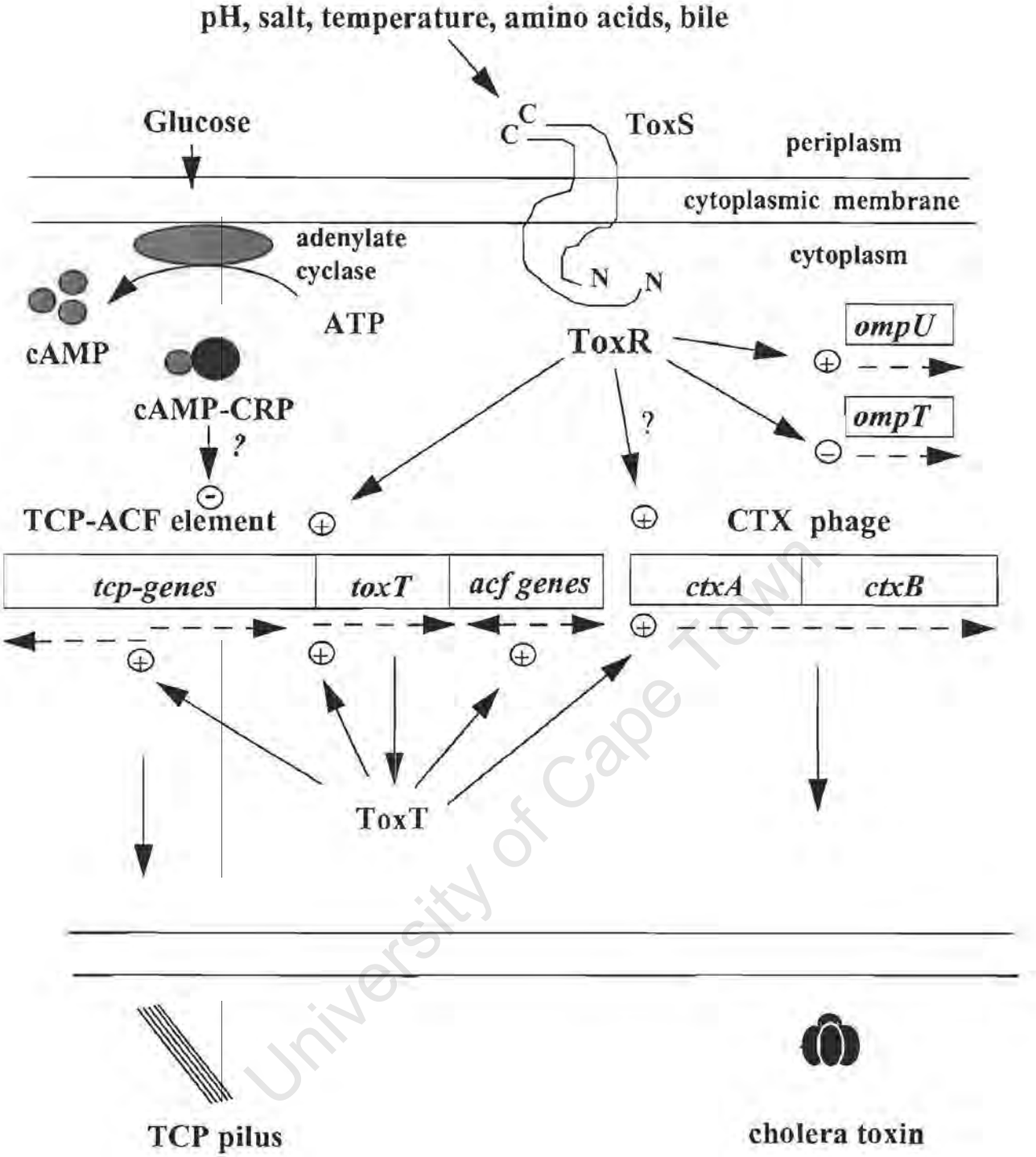


Figure 3. A model showing the influence of various regulatory pathways affecting the expression of the ToxR regulon. The plus or minus signs indicate either a positive or negative effect on the expression of a particular gene. Broken arrows show the relevant transcripts. It is not yet clear whether ToxR acts as an activator of *ctxAB* in *V. cholerae*. Taken from Skorupski and Taylor (1997a) *Molecular Microbiology* **25:** 1003-1009.

Mekalanos (1998) demonstrated that TcpP/TcpH constitute a pair of regulatory proteins functionally similar to ToxR/ToxS and activates transcription of the *toxT* gene. This suggests that ToxR most probably has other important regulatory functions in *V. cholerae* (Skorupski and Taylor, 1997a). For example, in the deep sea bacterium *Photobacterium* sp. strain SS9, ToxR was shown to directly mediate the expression of the outer membrane proteins OmpH and OmpL in response to pressure alterations in the environment (Welch and Bartlett, 1998).

1.4.2 Regulation of gene expression in the ToxR regulon

Activation of the genes in the ToxR regulon can be divided into two branches (Champion *et al.*, 1997). The first branch involves genes that are directly involved in virulence, and whose activation is dependent on both ToxR and ToxT. Activation of genes in the second branch only requires ToxR and is ToxT-independent.

ToxR controls virulence gene expression by activating the expression of ToxT (DiRita *et al.*, 1991) (Fig. 3). ToxT is then responsible for directly activating the *Tcp*, *Acf* and *ctxAB* genes. Expression of these genes is highly dependent on environmental stimuli and the same conditions that favour *ctxAB* expression, namely 30°C, pH 6.5 and 75 mM NaCl, are required for optimal expression of *Tcp* and *Acf* (Gardel and Mekalanos, 1994).

The expression of the genes encoding outer membrane proteins OmpU and OmpT is directly controlled by ToxR. (Champion *et al.*, 1997). ToxR positively regulates OmpU expression, but negatively regulates expression of OmpT (Miller and Mekalanos, 1988a; Sperandio *et al.*, 1995) (Fig. 3). The regulation and expression of ToxT-independent genes appears to be less influenced by environmental stimuli such as pH and temperature (Miller and Mekalanos, 1988a). This implies that specific environmental signals control and regulate the two branches of the ToxR regulon somewhat differently and this may be important in fine tuning gene expression throughout the infection process.

Although ToxR interacts directly with the upstream regions of *toxT*, *ctx* and *ompU*, no obvious consensus sequence exists among the promoters of these genes (Skorupski and Taylor, 1997a). Despite this, mutant ToxR proteins behave identically at both *ctx* and *toxT* promoters in gel shift experiments, suggesting a similar mechanism of DNA recognition at both these promoters (Higgins and DiRita, 1996). This raises the possibility that other factors may function together with ToxR to activate ToxT and possibly also provide important information regarding the status

of the environment. One such factor was recently identified as the cyclic AMP-CRP system (Skorupski and Taylor, 1997b).

1.4.3 Control of the ToxR regulon by cAMP-CRP

Cyclic AMP (cAMP) and its receptor protein CRP (cAMP receptor protein) functions as a global regulatory network to activate and repress the expression of many *E. coli* genes in response to carbon and energy sources in the environment (Kolb *et al.*, 1993). In the absence of cAMP, CRP is either free in solution or is bound to non-specific DNA (Fig. 4). Once adenylate cyclase is activated, the enzyme mediates the conversion of ATP to cAMP. cAMP then binds CRP and together the cAMP-CRP complex targets specific DNA where it interacts with the transcriptional machinery to either activate or repress gene expression. The amount of cAMP in the cell is dependent on the presence of the enzyme adenylate cyclase, which mediates the conversion of ATP to cAMP. In the absence of glucose, adenylate cyclase is activated with the result that the intracellular concentration of cAMP is higher than when glucose is available.

Classical biotypes, with a defective *crp* gene, displayed increased toxin and *Tcp* expression in Luria broth, pH 8.5, at 30°C compared to wild-type (Skorupski and Taylor, 1997b). This suggests that cAMP-CRP might inhibit expression of the *toxR* regulon under certain environmental conditions (Fig. 3). Furthermore, a putative cAMP-CRP binding site overlaps the -35 site of the most proximal promoter in the *tcp* operon, the *tcpA* promoter, which raises the possibility that cAMP-CRP negatively regulates toxin and *Tcp* expression by binding this site and thus not allowing preventing either RNA polymerase or other transciption factors from accessing the promoter (Thomas *et al.*, 1995; Ogierman *et al.*, 1996). If this is the case, the cAMP-CRP system will function through the ToxT-dependent branch of the regulon rather than the ToxT-independent branch. This is supported by the observation that, in *crp* mutants, no change in the expression of the ToxR regulated outer membrane proteins, OmpU and OmpT, is observed (Skorupski and Taylor, 1997a). This could explain why nutrient-rich environments, such as the intestine, favour expression of the regulon, whereas nutrient poor environments outside the host limit its expression. Although it has not been established whether carbon and energy sources in the environment serve as signals that influence expression of the ToxR regulon, their effects on intracellular cAMP levels suggest that this may be the case. Clearly, *V. cholerae* has evolved highly sophisticated systems for continuously monitoring the external

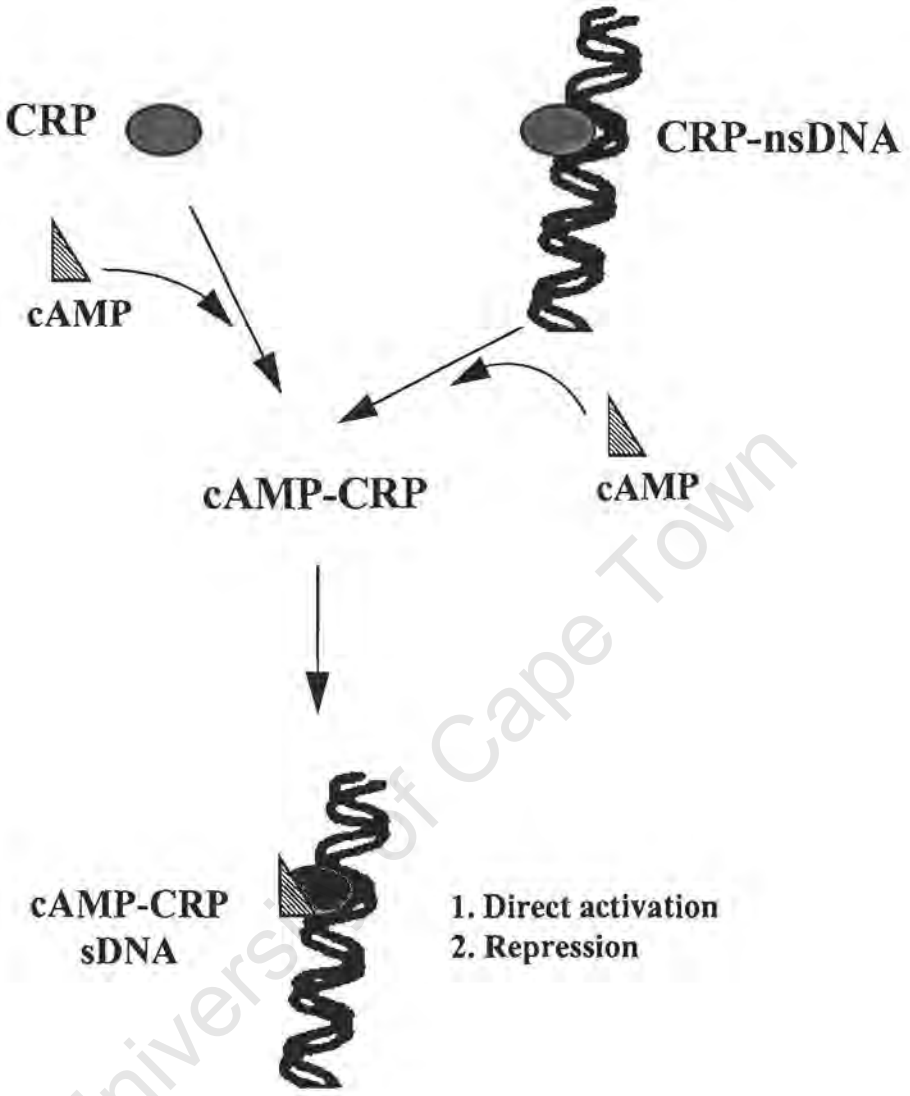


Figure 4. In the absence of cAMP, CRP is either free in solution or bound to non-specific DNA (nsDNA). In the presence of cAMP, CRP is bound to cAMP and the cAMP-CRP complex binds specific DNA (sDNA) to interact with the transcriptional machinery and so leads to activation or repression of gene expression. Taken from Kolb *et al.*, (1993) *Annual Review in Biochemistry* **62:** 749-795.

environment, both inside and outside the host, to ensure optimum gene expression so as to increase its chances of survival and multiplication.

1.5 The isolation of hypertoxigenic mutants of *V. cholerae*

In 1978, Mekalanos *et al.* reported the isolation of hypertoxigenic (Htx) mutants of *V. cholerae* through the use of N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis. Although the mutants produced 3-7 fold more enterotoxin compared to the wild-type, the elevated ratio of A to B chains remained constant, suggesting that the mutation was within a regulatory region rather than the toxin structural genes. Subsequent mapping of the *htx* locus indicated that it was situated between the *str* and *rif* markers on the *V. cholerae* chromosome (Mekalanos *et al.*, 1979). In addition to the hypertoxinogenic phenotype, Htx mutants displayed a slower growth rate compared to the parental strain and constitutively produced a brown diffusable pigment not previously observed in the wild-type strain. This was the first evidence that *V. cholerae* had the ability to produce pigment.

1.6 Characterization of the pigment produced by Htx mutants

The pigment produced by *V. cholerae* Htx mutants was characterized and shown to possess several properties common to microbial melanins (Ivins and Holmes, 1980). The amino acids L-phenylalanine, L-tyrosine and L-tyrosine plus L-cysteine, previously identified as precursors for pigmentation in other organisms, significantly stimulated pigmentation in Htx and other independently isolated mutants. Furthermore, the crude pigment purified from *V. cholerae* mutants was soluble under alkaline conditions, while the addition of H_2O_2 and glutathione resulted in profound bleaching of the pigment. Finally, the addition of $FeCl_3$ resulted in the formation of a black flocculant precipitate. All these observations provided strong evidence that the pigment produced by the *V. cholerae* mutants was indeed melanin.

1.7 Melanin pigments

Melanins are complex polyphenolic heteropolymers found in dark pigments produced by bacteria, fungi and higher organisms. Aside from plants, which make use of phenols and o-diphenols to form melanogenic pigments, the amino acid L-tyrosine is the main melanin precursor (Ruzafa *et al.*, 1995). The majority of melanins can be classified into four major classes depending on the substrate used for pigment synthesis.

1.7.1 Major classes of melanins

Eumelanins are black and are synthezised by the well-known Mason-Raper pathway in which L-

tyrosine is initially oxidized to dihydroxyphenylalanine (L-DOPA) and subsequently to L-dopaquinone by the enzyme tyrosinase (Raper, 1928; Mason, 1948). In the presence of O_2, L-dopaquinone auto-oxidizes and polymerizes to form eumelanin (Fig. 5).

Phaeomelanins are brown, red or yellow pigments that form when L-dopaquinone reacts with glutathione or cysteine prior to oxidation and polymerization (Coon *et al.*, 1994) (Fig. 5). Both eumelanins and phaeomelanins are mainly produced by higher organisms, but several micro-organisms have been shown to be capable of producing these pigments (Pomerantz and Murthy, 1974; Ivins and Holmes, 1980; Katz *et al.*, 1983; Kelley *et al.*, 1990; Hoti and Balaraman, 1993).

Allomelanins are mainly produced by plants, but are also produced by bacteria (Kubo *et al.*, 1996; Shivprasad and Page, 1989). The mechanism by which these melanins are formed has not been well characterized, although it is known that they are derived from compounds such as acetate, catechol and 1,8 dihydroxynaphthalene (Allport and Bu'lock, 1958; Wheeler, 1983).

Finally, pyomelanins are formed through tyrosine catabolism (Denoya *et al.*, 1994) (Fig. 5). L-tyrosine is first deaminated to 4-hydroxyphenylpyruvate, in a reaction catalyzed by transaminase. There are two possible pathways via which pyomelanin can then be formed. Firstly, the enzyme 4-hydroxyphenylpyruvate dioxygenase can convert 4-hydroxyphenylpyruvate directly into 2,5-dihydroxyphenylacetate (homogentisic acid), which then autopolymerizes into melanin. Alternatively, 4-hydroxyphenylpyruvate is metabolized to 4-hydroxyphenylacetate through 4-hydroxyphenylacetaldehyde which is then converted to homogentisic acid by the enzyme 4-hydroxyphenylacetate hydroxylase (Cooper and Skinner, 1980; Trias *et al.*, 1989; Coon *et al.*, 1994; Denoya *et al.*, 1994).

1.7.2 Properties of melanin

Although melanins are not considered essential for the growth and development of cells, they possess important properties which seem to play a crucial role in enhancing the ability of species to compete and survive under adverse conditions (Bell and Wheeler, 1986). These properties are discussed below.

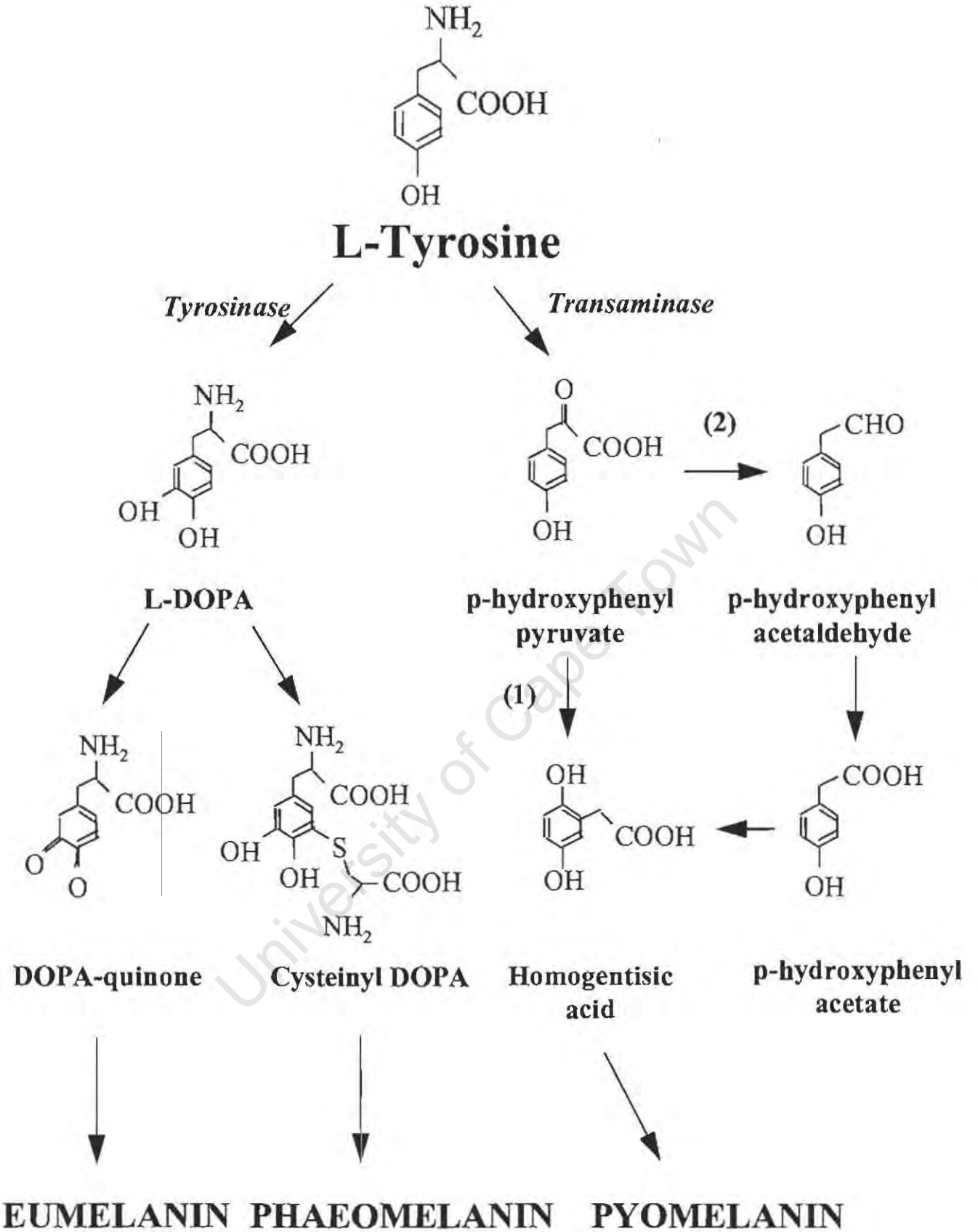


Figure 5. The melanin biosynthetic pathways for eumelanin, phaeomelanin and pyomelanin from L-tyrosine. (1) represents the enzyme 4-hydroxyphenylpyruvate dioxygenase whereas (2) represents the enzyme 4-hydroxyphenylacetate hydroxylase.

1.7.2.1 Protection against UV irradiation

In 1820, Sir Everard Home was the first to propose that the primary function of melanin was to act as a sunscreen. Since then, the sunscreen dogma has been widely accepted, with new evidence supporting this view. The logic behind this concept is simply based on the fact that people with dark skin are resistant to sunburn, while albinos and europeans living in the tropics and subtropics have a high incidence of skin cancer.

Sunlight is a mixture of ultraviolet (UV), visible and infrared wavelengths (Hill, 1992). The UV light can be divided into UV-A, UV-B and UV-C. UV-C has the shortest wavelength (254 nm), the highest energy and is the most harmful to cells. Fortunately, UV-C gets completely absorbed by the earth's atmosphere and consequently does not penetrate to the surface. UV-B has a longer wavelength of between 280 to 320 nm and is thought to play a dominant role in skin cancer. UV-A has the longest wavelength and the lowest energy, and together with UV-B penetrates the earth's atmosphere to reach the surface. All types of UV radiation are harmful and can damage DNA either directly or indirectly (Hill, 1992).

UV-C and UV-B directly damage DNA by causing the formation of pyrimidine dimers (Fig. 6). DNA damage by UV-A is mainly indirect. UV-A photons mediate damage through a process known as photosensitization, a mechanism whereby chromophores such as flavin or porphyrin absorb light and then transfer the energy to O_2 (Fig 6). Both types of DNA damage can be lethal.

Hill and Setlow (1982) investigated whether intracellular melanin could protect melanoma cellular DNA from the direct damage caused by UV-C and UV-B radiation. They determined the amount of enzyme sensitive sites present in the melanoma cellular DNA after treatment with each type of radiation. They found that intracellular melanin only protected DNA from direct damage caused by UV-C but not from the damage induced by UV-B. Similarly, Liu *et al.*, (1993) demonstrated that melanin protected the mosquito larvacidal activity of the *Bacillus thuringiensis* toxin, but only at a wavelength of 254 nm. These findings suggested that the sunscreening properties of melanin was most efficient at biologically irrelevant wavelengths. In contrast, Niggli (1990) compared the resistance of melanoma cells producing either large or small amounts of pigment to their ability to withstand direct DNA damage caused by UV-C and UV-B radiation. He found that regardless of the amount of pigment present, UV-C had an equally harmful effect on all the cells, whereas the damage induced by UV-B was less severe.

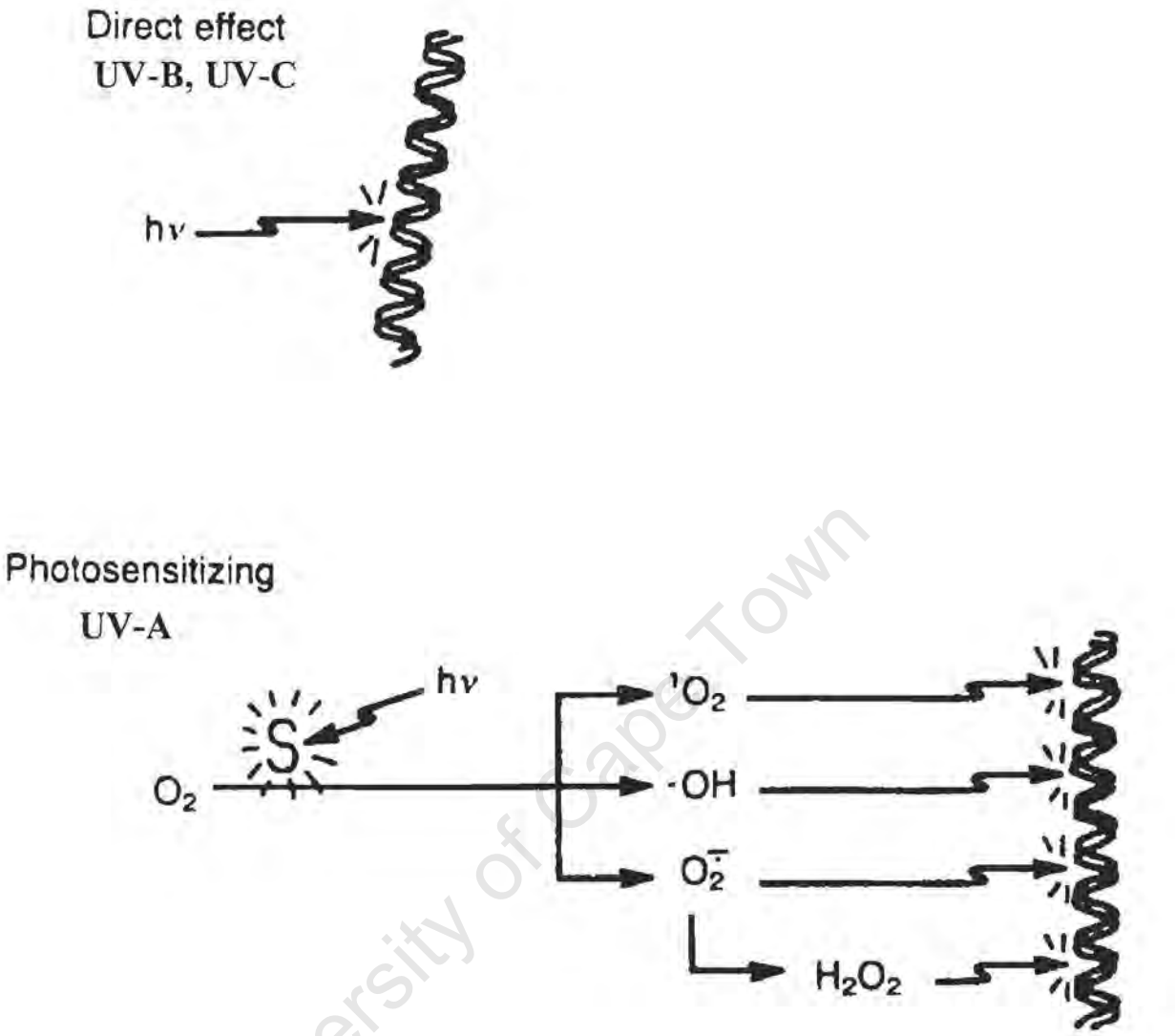


Figure 6. Radiation damage to DNA. Direct effect: photons from UV-B and UV-C interact directly with DNA to form pyrimidine dimers. Indirect effect: photons from UV-A damage DNA through photosensitization where flavin or porphyrin causes the formation of active oxygen species which damage DNA. Hill (1992) *Bioessays* **14**: 49-56.

Niggli measured DNA damage directly by degrading the radiated DNA to individual bases in order to determine the amount of pyrimidine dimers. These results, although contradictory to Hill and Setlow (1982), once again suggested that the sunscreening ability of melanin is rather poor.

1.7.2.2 Melanin as a free radical scavenger

Free radicals, or more specifically oxygen radicals, are extremely detrimental to living cells and have been implicated in human diseases such as arthritis, cancer and ageing (Imlay and Linn, 1988). Biologically, the two most important oxygen radicals are the hydroxyl radical and the superoxide anion.

Hydroxyl radicals ($OH^{\cdot}$) are extremely reactive and either react to or damage a nearby molecule as soon as the radical is generated (Storz *et al.*, 1990b). They can originate when cells are exposed to ionizing radiation from X -and gamma ray, cigarette smoke, asbestos or oxidation-reduction active drugs such as paraquat (Ahern, 1991). Another way in which hydroxyl radicals are produced is through the Fenton reaction, where intracellularly generated hydrogen peroxide (H_2O_2) reacts with metal ions such as Fe and Cu (Imlay and Linn, 1988; Storz *et al.*, 1990b).

Superoxide anions are by-products formed during cellular respiration and thus occur in all aerobic organisms. Although this anion is relatively stable, and only marginally reactive in aqueous environments, it is believed that much of its toxicity is due to its conversion to hydroxyl radicals.

The toxicity exerted by these radicals is mainly due to the fact that they attack cellular constituents, resulting in direct damage of macromolecules. They may attack DNA at either the sugar or the base (Imlay and Linn, 1988). Attacking a sugar results in sugar fragmentation, base loss and ultimately strand breakage. Attack at bases usually results in thymine fragments, adenine ring-opened products and hydroxymethyluracils.
Membrane damage from O_2 species is observable as an accumulation of lipid peroxides and/or the loss of the diffusion barrier to membrane-impermeable markers and cell lysis (Storz *et al.*, 1990b).

Fortunately, cells are well adapted and capable of defending themselves against oxidative stress. They employ defence enzymes such as superoxide dismutase (Carlioz and Touati, 1986) and catalase (Loewen *et al.*, 1985) to remove reactive radicals before they cause damage. In addition,

cells also have enzymes that function to repair damage as a result of free radicals. Examples of such enzymes include exonuclease III, endonuclease III and DNA polymerase I (Storz *et al.*, 1990b). However, when aerobic organisms lack one of these enzymes, they need to relieve oxidative stress via some alternative pathway. One such mechanism was proposed in *Azotobacter chroococcum* (Shivprassad and Page, 1989). This organism lacks catalase and has only low peroxidase activity. It produces melanin when grown at high aeration rates. Furthermore, melanogenesis was suppressed in the presence of charcoal (a free radical trap) and benzoic acid (a free radical scavenger), indicating a relationship between the presence of toxic oxygen species and pigmentation. Sarna *et al.*, (1986) examined the ability of synthetic melanins to scavenge radicals produced by ionizing radiation. Among the radicals studied, they found that hydroxyl radicals exhibited the strongest reactivity with melanin, and hence, were the most efficiently scavenged by the polymer. These observations suggest that melanin can scavenge radicals and can do so *in vivo*.

1.7.2.3 Melanin as a cation-exchanger

Melanins from both mammalian and invertebrate sources have the capacity to act as cation-exchange materials with considerable activity (White, 1958). As a result, melanins can bind many chemicals and drugs, and binding can be attributed to electrostatic forces due to the strong negative charges of the carboxyl groups in melanin (Hill, 1992).

In a study performed by Nair *et al.*, (1992), it was observed that pigmented marine bacteria exhibited increased tolerance to a variety of heavy metals and antibiotics compared to non-pigmented bacteria. This could be directly due to the binding of these compounds to melanin. Furthermore, stationary phase cultures of *Aspergillus nidulans* synthesize and deposit large amounts of melanin within their cell walls. Bull (1970) was able to demonstrate a direct correlation between the presence of melanin in the cell walls and *A. nidulans* resistance to lysis by a variety of polysacharases. He also showed that the melanin bound non-specifically to these polysacharases as was demonstrated by a decrease in the electro-negativity of melanin, most probably as a result of increased electrostatic interactions between the proteins and the polymer. It is clear that melanin can bind a vast array of different compounds, and thus, indirectly protect biological systems from their harmful effects.

1.7.2.4 Melanin as a semiconductor

Melanins act as amorphous semiconductors, meaning that they can participate in electron transfer

reactions (Crippa *et al.*, 1979). Thus, melanin can accept electrons from a molecule in an excited state, and leave the donor in the ground state. The energy in the excited state of melanin is transferred to internal degrees of freedom of the polymer; ie. converted to heat. This also explains why melanin has a black appearance: The energy from absorbed light is not re-radiated as visible or UV light, but converted to heat instead (McGinness and Proctor, 1973). This property of melanin could explain the presence of pigmentation in internal organs such as the brain and the inner ear (Proctor and McGinness, 1986).

1.7.2.5 Durability of melanin

In order for any substance to be effective as a protective agent, the agent itself should be either long-lived or it must be generated as rapidly as it is destroyed (Kuo and Alexander, 1967). Melanin is a polymer that is extremely resistant to hydrolysis and shown to be very durable (Kuo and Alexander, 1967). For instance, melanin appears to be highly resistant to microbial degradation as no organism capable of utilizing melanin as a sole carbon source has been isolated (Bloomfield and Alexander, 1967). This durability, in addition to the multiple characteristics of melanin, makes it understandable why so many organisms spend a significant amount of valuable energy in producing melanin pigments. It is clear that the role of melanins in nature is complex and requires more investigation.

1.8 Melanogenesis in *V. cholerae*

V. cholerae does not pigment under normal physiological conditions. However Coyne and Al-Harthi (1992) showed that *V. cholerae* 569B could be induced to produce pigment in response to a variety of stresses. They found that elevated temperatures of 30°C and above, in conjunction with increased salinity, induced the bacterium to pigment. Furthermore, pigmentation occurred at lower salinities when the bacterium was subjected to additional stress factors such as low organic nutrients or low pH. The observation that the osmoprotectants glycinebetaine and L-proline either delayed or prevented pigmentation respectively, confirmed the role of osmotic stress as the primary environmental factor which activates melanin formation in *V. cholerae*. No correlation between melanin synthesis and the production of cholera toxin was observed. In fact, physiological conditions that induced melanin synthesis in *V. cholerae* led to decreased toxin production. Furthermore, the ratio of OmpT and OmpU remained relatively constant under conditions at which pigment synthesis occurred. These results, however, do not exclude the possibility that melanin may function during the early stages of infection. The increased temperature and low pH of the stomach may cause invading bacteria to become susceptible to the

increased osmolarity of the mucosal lining of the upper small intestine (Coyne and Al-Harthi, 1992). The resulting hyperosmotic stress may then induce melanogenesis. Since leukocytes attack pathogens with a flux of secreted oxidants, any extracellular microbial product which neutralizes oxidants is likely to protect the pathogen and promote invasive disease. Indeed, melanin has been implicated as a virulence factor in a number of pathogenic fungi due to its ability to neutralize strong oxidants such as hypochlorite and permanganate (Jacobson and Tinnell, 1993; Jacobson *et al.*, 1995). Melanin deposited in the cell wall may also act as a shield, since no known enzyme hydrolyses melanin (Kwon-Chung *et al.*, 1982). Melanin may also prevent cellular dehydration of *V. cholerae* by sequestering compatible solutes from the intestine, as the efflux of Na^+ and the secretion of Cl^- from the intestinal cell could significantly affect the adhered bacterial cells.

Alternatively, the role of melanin could be to enhance the survival of *V. cholerae* in the estuarine environment. Again, melanin's free radical scavenging properties could turn out to be useful in alleviating oxidative stress, especially during the summer months where OH^- radical concentrations in the water column increase dramatically (Mopper and Zhou, 1990). Melanin can protect cells from cellular dehydration through binding solutes in the environment. Another important function may be that melanogenesis is a strategy employed by *V. cholerae* to convert potentially toxic, small molecular weight phenols or quinones occurring either inside the cell or in the extracellular environment into high molecular weight non-toxic pigments. Thus, whether melanogenesis has a specific role, or whether it assumes a more generalized role in terms of osmoregulation, remains to be elucidated.

1.9 Concluding remarks and aims of this study

Although characterization of the virulence response of *V. cholerae* is important from a clinical point of view, an understanding of how the bacterium persists in the environment for extended periods of time without triggering an epidemic is intriguing. In both the environments colonized by *V. cholerae*, namely the estuarine environment and the small intestine of humans, melanin has the potential to significantly enhance the fitness of the bacterium. Understanding the role of melanin and the signal(s) that induce the gene(s) involved in pigment synthesis can not only enhance our understanding of how pathogens interact with their host, but also provide valuable insight into how they persist in harsh environments. Clearly, environmental stimuli such as temperature, pH, salinity, availability of nutrients and many other environmental signals are key players in inducing the expression of specific genes and ultimately determining the physiological

state of the organism, and consequently its ability to compete and survive.

Since it is clear that a biosynthetic pathway for the production of melanin exists in *V. cholerae*, our first objective was to clone and identify the gene(s) responsible for encoding the proteins responsible for melanogenesis. This information provided an insight into the biochemical pathway employed by *V. cholerae* for pigment production, as well as the type of melanin that was produced. The second objective was to characterize the protein responsible for pigment synthesis in order to understand it in terms of the time at which it was expressed in the wild-type organism. This approach could possibly provide us with a valuable insight into the function of pigmentation in *V. cholerae* 569B. Purified protein was therefore used for raising polyclonal antibodies in rabbits which were employed in Elisa assays to monitor protein expression in wild-type cells over time. Our final objective was to determine the importance of the gene involved in melanogenesis in *V. cholerae* 569B by constructing a melanin-minus mutant. We then compared the overall fitness of this mutant to that of the wild-type strain with respect to its generation time and culturability under various culture conditions. In addition, we also tested whether melanin possibly played a role in alleviating oxidative stress by comparing the overall survival of wild-type and mutant strains following their exposure to H_2O_2. The implications of these findings are discussed in terms of the role played by melanin in the survival of *V. cholerae* 569B.

CHAPTER 2

THE CLONING AND SEQUENCING OF THE MELANIN BIOSYNTHETIC GENES FROM *V. cholerae* 569B

CONTENTS

Summary

A *V. cholerae* genebank was constructed in *E. coli* HB101 using the suicide vector pEcoR251. The genebank was screened for the melanin biosynthetic genes by assaying for *E. coli* clones capable of pigmentation on Luria agar containing L-tyrosine and copper. Two phenotypically different clones were identified. *E. coli* clones harbouring pCM30 synthesized melanin which occurred extracellularly as a result of diffusion or export from the cell, whereas melanin remained intracellular when produced in *E. coli* transformed with pCM3. Southern hybridization studies revealed that the cloned *V. cholerae* DNA fragments were not linked on the chromosome, confirming the genes to be different. Restriction enzyme maps of both pCM30 and pCM3 were constructed and used to generate deletions within the cloned *V. cholerae* DNA in order to localise the regions responsible for pigmentation in the *E. coli* clones. Subsequent sequencing of pCM30 revealed a 1.1 kb open reading frame (designated *ppdA)* which closely resembled a haemolysin from *V. vulnificus* as well as 4-hydroxyphenylpyruvate dioxygenase enzymes from various other organisms. In addition, the gene involved in pigment synthesis in pCM3 (designated *tyrM*) was sequenced. Sequence data suggested the presence of a 1.088 kb open reading frame encoding a protein of 39 kDa in size. Homology searches of several databases showed that this protein displayed about 25% similarity to a 33.9 kDa hypothetical protein 54.9 minutes on the *E. coli* K12 with unknown function. Primer extension analysis identified the *ppdA* and *tyrM* transcriptional start sites and allowed prediction of the upstream promoter regions.

2.1 Introduction

Melanin pigments are ubiquitous and are found in organisms from all the biological kingdoms (Hill, 1992). Melanins are classified according to the biosynthetic pathway by which they are formed and thus fall into four major categories: eumelanins, phaeomelanins, allomelanins and pyomelanins (Chapter 1 section 1.7.1). The genes and enzymes responsible for the synthesis of each of these melanin-types will be discussed.

2.1.1 Eumelanin biosynthesis

Eumelanins are black pigments and are responsible for the dark colour of hair, skin, feathers, insect cuticles, eggplants and over-ripe bananas. Eumelanin biosynthesis has been extensively studied in both mammalian and bacterial systems, and has been shown to be critically regulated by the enzyme tyrosinase (Aroca *et al.*, 1993). True tyrosinases are internal monooxygenases which require Cu^{2+} for activity (Chen *et al.*, 1993). They catalyze the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to dopaquinone (Fig. 1). In the absence of thiols, dopaquinone spontaneously forms dopachrome (Aroca *et al.*, 1993).

In most bacterial systems the metabolism of L-tyrosine into melanin occurs rapidly. The responsible enzyme, tyrosinase, is encoded for by a single gene and requires no additional catalytic intervention (Kelley *et al.*, 1990). One exception, however, is that of the Gram-positive, filamentous bacteria of the genus *Streptomyces*. In both *Streptomyces antibioticus* and *Streptomyces glaucescens*, the structural gene encoding tyrosinase, *melC2*, is located in a polycistronic operon (melC) (Lee *et al.*, 1988) (Fig. 2). Upstream from the *melC2* tyrosinase is a gene, *melC1*, whose product is essential for tyrosinase activity. MelC1 not only transfers copper to the MelC2 tyrosinase, but it also guides the tyrosinase into an active conformation. *In vitro* expression of *melC2*, in the absence of a functional *melC1*, results in inactive tyrosinase which can not be activated by the addition of copper ions (Chen *et al.*, 1993).

In contrast to bacterial systems, the enzymatic regulation of melanogenesis in mammals is complex and requires several genes. Tyrosinase is encoded for by the *albino* locus, and after catalyzing the formation of dopachrome which is spontaneously rearranged into 5,6-dihydroxyindole (DHI), tyrosinase also converts DHI into indole-5,6-quinone (Aroca *et al.*, 1993) (Fig. 1). This compound then polymerizes to form eumelanin. Apart from tyrosinase, two

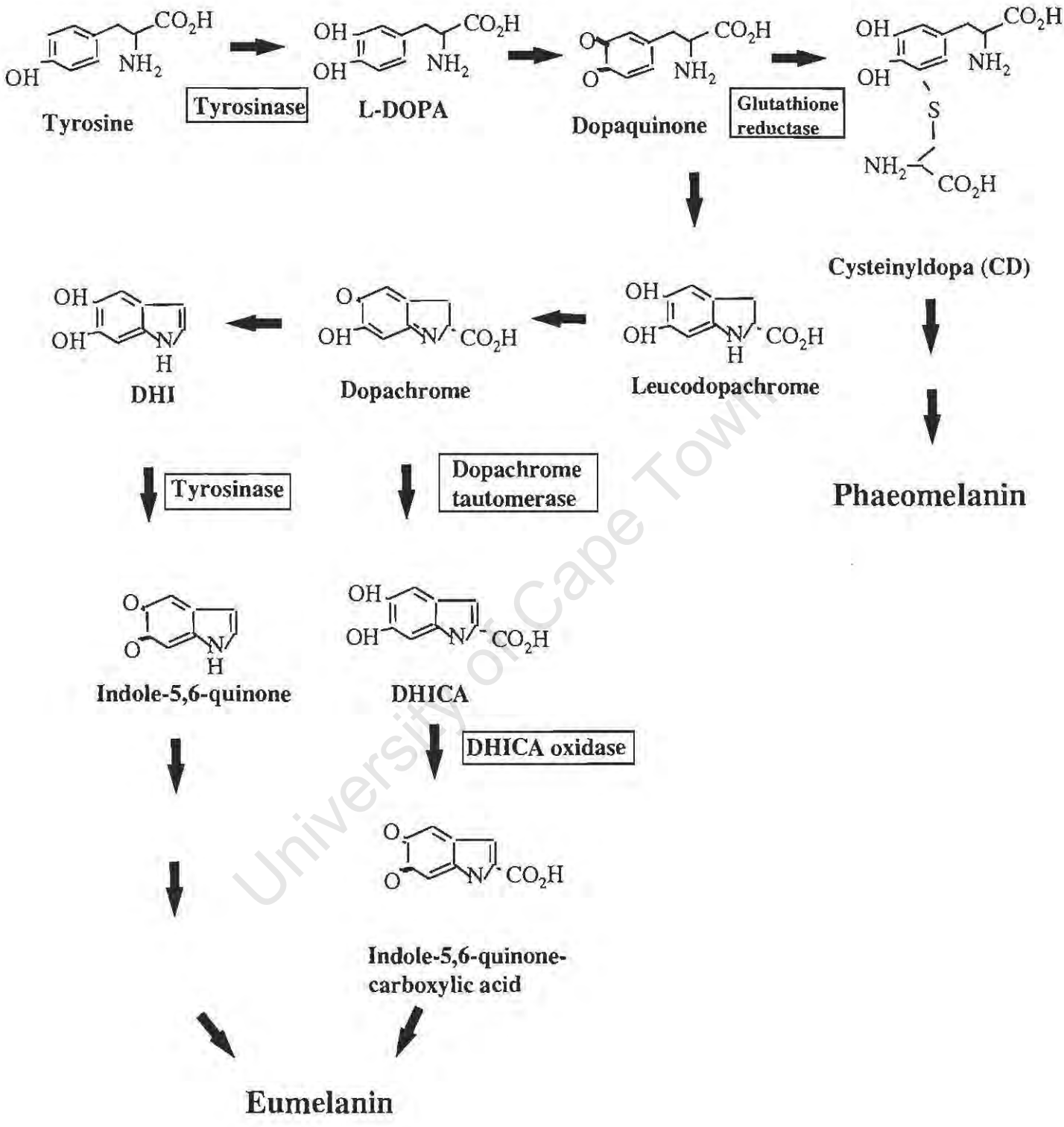


Figure 1. The melanogenic pathway for the synthesis of eu- and phaeomelanin in eukaryotes. Taken from Aroca *et al.*, (1993) *Journal of Biological Chemistry* **268:** 25650-25655.

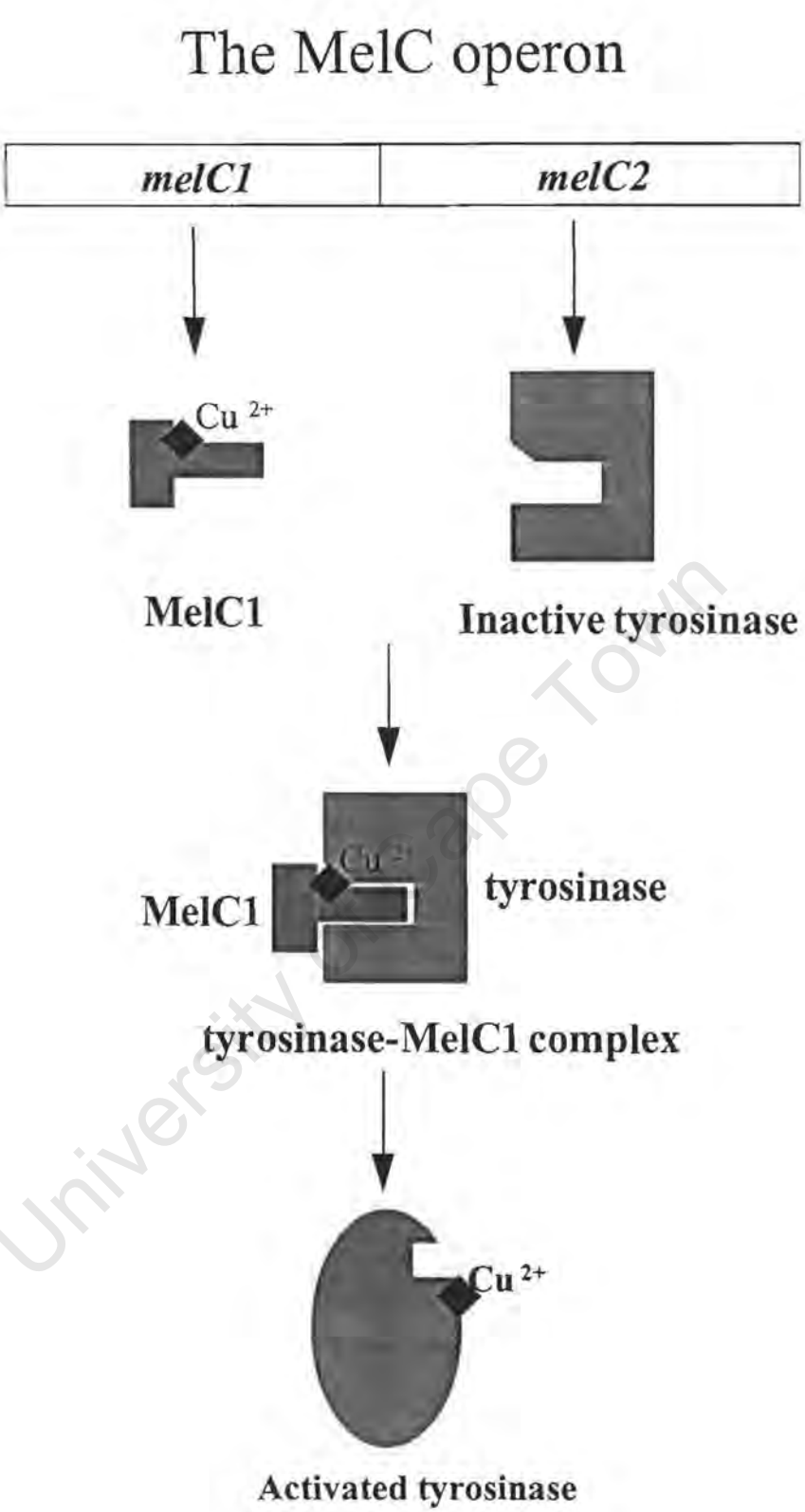


Figure 2. The MelC operon of the filamentous Gram-positive bacteria *S. antibioticus* and *S. glaucescens*. Eumelanin synthesis in these organisms is dependent on *melC2* gene product, namely tyrosinase. Tyrosinase activity, however, relies on the presence of another protein, MelC1. MelC1 activates tyrosinase by binding the inactive protein and transferring copper to the MelC2 tyrosinase. In addition, MelC1 also guides the tyrosinase to assume an active conformation.

additional enzymes, resembling tyrosinase in structure, but with distinct catalytic capacities, have also been identified. These have been shown to specifically affect the quantity and quality of the melanin that is produced. They are dopachrome tautomerase, encoded for by the *slaty* locus, and DHICA oxidase encoded for by the *brown* locus (Jackson *et al.*, 1992; Shibahara *et al.*, 1986). Dopachrome tautomerase diverts the spontaneous rearrangement of dopachrome to DHI to DHI-2-carboxylic acid (DHICA) (Aroca *et al.*, 1993) (Fig. 1). DHICA oxidase is then responsible for the oxidation and further metabolism of DHICA required for melanin polymerization (Kobayashi *et al.*, 1994) (Fig. 1). Dopachrome tautomerase and DHICA oxidase have only eumelanogenesis-specific functions whereas tyrosinase also plays a role in phaeomelanin biosynthesis (Ozeki *et al.*, 1997).

2.1.2 Phaeomelanin biosynthesis

Phaeomelanins are red and yellow pigments found in hair, feathers and freckles (Hill, 1992). Following the catalysis of the formation of dopaquinone by tyrosinase, the enzyme glutathione reductase is responsible for the addition of either glutathione or cysteine to form cysteinyldopa (CD) (Aroca *et al.*, 1993) (Fig. 1). The switch between eu- and phaeomelanin synthesis appears to be regulated by both the level of tyrosinase, as well as the availability of cysteine, in the cell (Ozeki *et al.*, 1997). As soon as the level of cysteine is higher than the level of dopaquinone being produced, CD is exclusively synthesized. Low concentrations of tyrosinase also seem to favour CD synthesis and therefore phaeomelanogenesis. Once produced, CD spontaneously polymerizes to form phaeomelanin.

2.1.3 Allomelanin biosynthesis

Allomelanins are brown-black pigments derived from phenols and catechols which lack nitrogen, and are mainly produced by plants and fungi (Kubo *et al.*, 1996; Polacheck *et al.*, 1982). Fungal allomelanins are produced by the polyketide biosynthetic pathway and the genes involved have been extensively characterized (Bell *et al.*, 1976; Perpetua *et al.*, 1996; Kimura and Tsuge, 1993; Vidal-Cross *et al.*, 1994). Three genes are required for melanin biosynthesis in *Colletotrichum lagenarium* (Fig. 3). The first is the *pks* gene which encodes a polyketide synthase responsible for converting acetate into scytalone (Takano *et al.*, 1995). Scytalone is converted to 1,3,8-trihydroxynaphthalene via the product of the *scd* gene encoding a scytalone dehydratase (Kubo *et al.*, 1996). Finally, the *thr* gene encodes a 1,3,8-trihydroxynaphthalene reductase which converts 1,3,8-trihydroxynaphthalene into vermelone (Perpetua *et al.*, 1996). Vermelone spontaneously

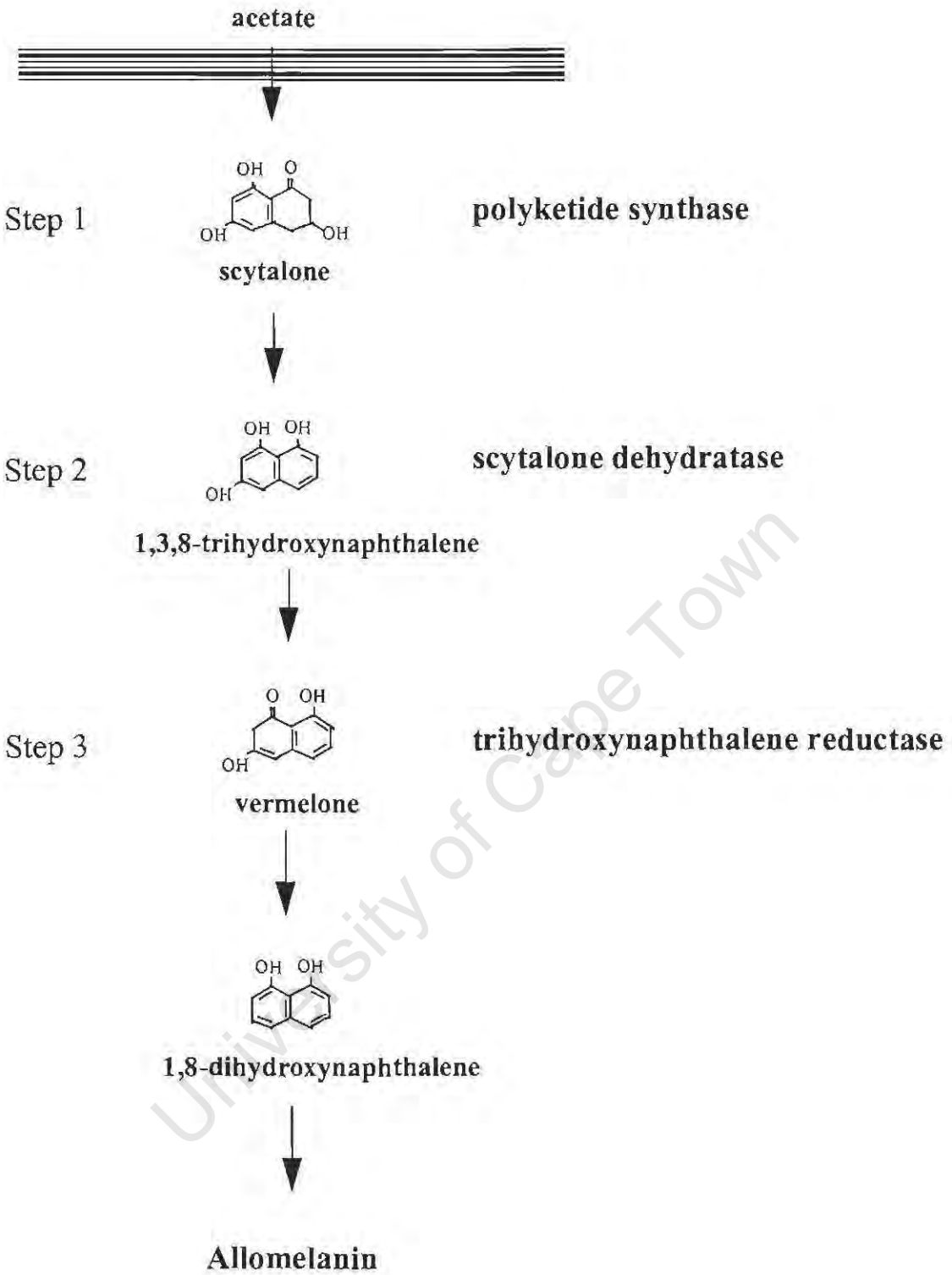


Figure 3. The polyketide biosynthetic pathway responsible for the production of fungal allomelanins. Step 1 involves the conversion of acetate to scytalone by the enzyme polyketide synthase. In step 2, scytalone dehydratase uses scytalone to produce 1,3,8-trihydroxynaphthalene. This compound is then converted to vermelone by trihydroxynaphthalene reductase (step 3). Vermelone spontaneously rearranges into 1,8-dihydroxynaphthalene and then to melanin. Taken from Kubo *et al.*, (1996) *Molecular Plant-Microbe Interactions* **9:** 323-329.

rearranges into 1,8-dihydroxynaphthalene and then to allomelanin. The allomelanin is deposited into the appressorial cell walls of the fungus where it mediates the build up of pressure required for the penetration of host tissues during infection (Kubo *et al.*, 1985).

2.1.4 Pyomelanin biosynthesis

Pyomelanins are brown-black pigments formed as a result of tyrosine catabolism (Yabuuchi and Ohyama, 1972). Tyrosine and phenylalanine are the only aromatic amino acids that can be degraded by mammals (Menon *et al.*, 1991). Aromatic transaminase converts tyrosine into 4-hydroxyphenylpyruvate by transamination with α-ketoglutarate. 4-Hydroxyphenylpyruvate is then oxidized to 2,5-dihydroxyphenylacetate (homogentisic acid (HGA)) by the enzyme 4-hydroxyphenylpyruvate dioxygenase (Hppd) (Lehninger, 1975) (Fig. 4). Hppd is an enzyme that is present in most organisms and the oxidation reaction catalyzed by this enzyme is complex (Denoya *et al.*, 1994). It involves hydroxylation of the phenyl ring as well as decarboxylation, oxidation and movement of the side chain. The gene encoding Hppd has been cloned and sequenced from a number of vertebrate livers, including human, pig and mouse (Ruetschi *et al.*, 1993; Endo *et al.*, 1992; Endo *et al.*, 1995). Once HGA is formed, it is further catabolized by the enzyme homogentisate 1,2-dioxygenase to fumarate and acetoacetate (Denoya *et al.*, 1994) (Fig. 4). These compounds can then be used in a number of metabolic pathways for the synthesis of various cell constituents. In humans, alkaptonuria is a rare hereditary metabolic disorder where the enzyme homogentisate 1,2 dioxygenase is deficient (Lehninger, 1975). Consequently, large amounts of HGA accumulates in the urine of these patients. On standing and exposure to oxygen, the urine turns dark due to the oxidation and polymerization of HGA into pyomelanin. Thus the role of Hppd in vertebrate metabolism seems to be mainly that of tyrosine catabolism.

Genes encoding Hppd have also been isolated from bacteria, fungi and protozoa (Fuqua *et al.*, 1991; Ruetschi *et al.*, 1992; Hammel *et al.*, 1992; Wintermeyer *et al.*, 1994; Denoya *et al.*, 1994; Wyckoff *et al.*, 1995). Expression of Hppd in these organisms can be directly correlated with pigment secretion, similar to that in alkaptonuria patients, into the culture media. This suggests a slightly different role for Hppd in these organisms, namely to produce HGA specifically for pyomelanin synthesis rather than for oxidation into fumarate and oxaloacetate. This would certainly seem paradoxical because large amounts of organic compounds in the environment are rich in aromatic amino acids. All these compounds become available when organisms die and organic matter is degraded by bacteria and fungi. An ability to use tyrosine as a carbon and

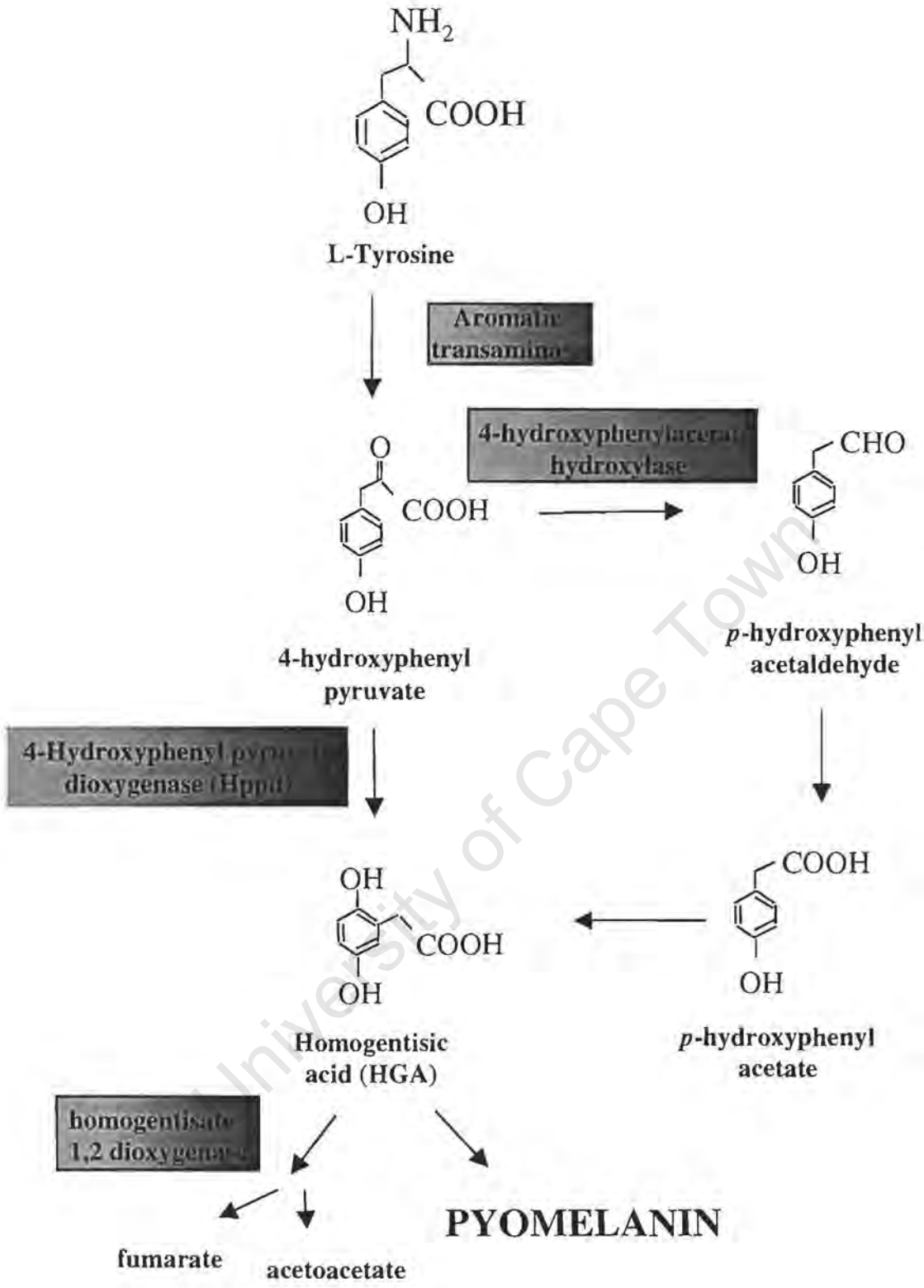


Figure 4. The melanin biosynthetic pathways for pyomelanin synthesis from L-tyrosine.

energy source would constitute a selective advantage over organisms that lack this ability. The utilization of a tyrosine catabolic pathway to produce pyomelanin seems wasteful, especially when faced with starvation. Nevertheless, in the majority of bacteria, pyomelanin synthesis occurs either during late log phase or during stationary phase. The intensity of the pigment is enhanced when the culture media is supplemented with L-tyrosine (Wintermeyer *et al.*, 1994; Coon *et al.*, 1994). This implies that the importance of synthesizing pyomelanin from tyrosine certainly exceeds its utilization as a nutrient source for survival.

In prokaryotes, pyomelanin synthesis can also occur via an alternative route involving 4-hydroxyphenylacetate hydroxylase rather than Hppd (Blakley, 1972; Hareland *et al.*, 1975) (Fig. 4). This enzyme produces HGA through 4-hydroxyphenylacetate, which then polymerizes into pyomelanin. As with Hppd, the expression of 4-hydroxyphenylacetate hydroxylase can be directly correlated with the synthesis of pigment in these organisms (Hareland *et al.*, 1975). Although pyomelanin has been implicated as a mechanism for protecting *Legionella pneumophila* against the harmful effects of light (Wintermeyer *et al.*, 1994), the role of this polymer in other organisms is still obscure and remains to be elucidated.

Melanogenesis is thus a result of one of several biosynthetic pathways and requires a wide variety of different genes to encode the proteins responsible for pigment production. The identification of these genes and their respective products has clearly increased our understanding of how the various types of melanin pigments are synthesized.

2.1.5 Aim of this chapter

Although melanogenesis has been extensively characterized in many organisms, the role of pigmentation in *V. cholerae* is not understood. Pigment synthesis occurs under stressful conditions such as high salt and temperature (Coyne and Al-Harthi, 1992). Pigmentation may therefore play an important role in protecting *V. cholerae* cells against adverse conditions which the pathogen will encounter in both the estuarine environment and the human host. In an attempt to determine the role of the melanogenic pathway whereby *V. cholerae* synthesizes its pigment, we first set out to clone the genes involved in pigment synthesis. This would provide information regarding the type of pigment *V. cholerae* produces and the biosynthetic pathway the organism uses for pigment synthesis.

In order to clone the genes involved in melanogenesis, a *V. cholerae* genebank was constructed in *E. coli* HB101 (Schroeder, 1993). *V. cholerae* chromosomal DNA was partially digested with the restriction enzyme *Sau*3A and the cleavage products were fractionated on a sucrose gradient to isolate fragments with an average size of 10 kb. These fragments were cloned into the *Bgl*II restriction enzyme site of the suicide vector pEcoR251 (Zabeau and Stanley, 1982). This strategy allowed us to directly select for only those transformants which harboured recombinant plasmids. The *V. cholerae* genebank was screened on media supplemented with L-tyrosine and copper in order to identify clones harbouring genes involved in melanin production. Out of a total of approximately 5800 clones, 50 *E. coli* clones were identified that were capable of synthesizing brown pigment. This chapter describes the sequencing and characterization of the cloned melanin biosynthetic genes from *V. cholerae* 569B.

2.2 Materials and methods

All media and solutions used in this study are listed in Appendix A.

2.2.1 Bacterial strains and plasmids.

The bacterial strains and plasmids that were used to clone and characterize the melanin biosynthetic genes from *V. cholerae* are listed in Table 1.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant features	Reference
Strains		
E. coli HB101	*hsdS20 recA13 leuB6 ara-14 roA2 lacY1 galK2 rpsL20 yl-5 mtl-1 sup E44*	Sambrook *et al.*, (1989)
V. cholerae 569B	Classical, Inaba	Mukherjee, (1978)
Plasmids		
pBluescript SK(+)	Amp^r, β-galactosidase	Short *et al.*, (1988)
pEcoR251	Amp^r, *Eco*RI restriction enzyme	Zabeau and Stanley, (1982)
pCM30	pEcoR251 containing 8.8 kb *V. cholerae* genomic DNA	This work
pCM3	pEcoR251 containing 8.1 kb *V. cholerae* genomic DNA	This work
pCM302	Derivative of pCM30 containing a 3.1 kb fragment from *V. cholerae* genomic DNA	This work
pCM32	Derivative of pCM3 containing a 2 kb fragment from *V. cholerae* genomic DNA	This work

2.2.2 Media and culture conditions

E. coli HB101 was grown in Luria broth (LB) at 37°C. *E. coli* transformants that harboured recombinant pEcoR251 and pBluescript SK plasmids were grown on Luria agar (LA) containing 100 ug/ml ampicillin. Pigmented *E. coli* clones were maintained on LA supplemented with 100 ug/ml ampicillin, tyrosine (5 mM) and $CuSO_4$ (5 uM). *V. cholerae* 569B was grown at 30°C in Tryptone broth (TB).

2.2.3 Phenotyping of pigmented *E. coli* clones

Fifty *E. coli* clones were isolated from a *V. cholerae* genomic library which were capable of pigmentation on media supplemented with tyrosine and copper. In order to confirm that the pigmented phenotype of these clones was plasmid linked, we extracted plasmid DNA from several pigmented clones and re-transformed the DNA into competent *E. coli* HB101 (Appendix B.2 and B.3). The resulting transformants were selected on LA containing ampicillin, tyrosine and copper in order to assess their ability to synthesize pigment.

2.2.4 Analysis of plasmid DNA isolated from pigmented clones

2.2.4.1 Southern hybridization analysis of recombinant plasmids

Once it was clear that pigmentation exhibited by *E. coli* transformants was plasmid linked, we had to establish whether the cloned DNA harboured on the recombinant plasmids was of *V. cholerae* origin. The two recombinant plasmids, pCM30 and pCM3, were isolated (Appendix B.1) from pigmented *E. coli* clones and used as probes against *V. cholerae* genomic DNA. The *V. cholerae* genomic DNA (extracted as described in Appendix B.4) was digested with restriction enzymes *Bam*HI and *Stu*I and the resulting fragments were separated on a 0.8% TAE agarose gel. pCM30 and pCM3 served as probes and were radio-labelled with [α-^{32}P] dCTP by nick translation using the Boehringer Manheim nick translation kit according to the manufacturer's instructions. The Southern hybridization procedure that was followed is described in Appendix B.11.

2.2.4.2. Restriction enzyme analysis of recombinant plasmids

The recombinant plasmids pCM30 and pCM3 were subjected to restriction enzyme mapping in order to determine whether the difference in phenotype displayed by the various clones harbouring these plasmids was a result of two completely different genes or due to a deletion within the same gene. The restriction enzymes that we initially used were those that cleaved within pEcoR251. These included *Pst*I, *Pvu*I, *Eco*RI, *Hind*III, *Cla*I and *Ava*I. Once the number of recognition sites for these enzymes were determined, additional enzyme sites could be identified

through double digestions with the above mentioned restriction enzymes. Restriction enzyme digestions were performed as outlined in Appendix B.5. The resulting restriction enzyme fragments were separated on 1% TAE agarose gels (Appendix B.6).

2.2.4.3 Deletion analysis of recombinant plasmid

Deletion analysis of pCM30 and pCM3 was employed in order to identify the regions within the cloned DNA that were responsible for pigment production in *E. coli*. Various constructs were obtained by cleaving plasmid DNA with either one or a combination of restriction enzymes and sub-cloning the resulting fragments into pBluescript SK.

Fragments subcloned from pCM30 included a 4.44 kb *Eco*RI (pCM301), a 3.14 kb *XbaI/Eco*RI (pCM302), a 4.12 kb *Hind*III/*Cla*I (pCM303) and finally a 5.45 kb *Cla*I (pCM304) fragment. Fragments that were sub-cloned from pCM3 were as follows: a 5.46 kb *Kpn*I (pCM31), a 2.66 kb *ClaI/Pst*I (pCM32), a 4.53 kb *Cla*I (pCM33) and finally a 3.75 kb *Bst*XI (pCM34) fragment. Restriction enzyme digestions, agarose gel electrophoresis and ligation procedures were performed as described in Appendix B.5, B.6 and B.9. The resulting constructs were transformed into *E. coli* (Appendix B.3) and subsequently assayed for their ability to produce pigment.

2.2.5 Henikoff shortening of pCM302 and pCM32

In order to sequence the *V. cholerae* genes responsible for pigmentation on the recombinant plasmids pCM302 (derivative of pCM30) and pCM32 (derivative of pCM3), the cloned regions were shortened with exonuclease III as follows:

pCM302 was cleaved with the restriction enzymes *Kpn*I and *Eco*RI to generate the 3'- and 5' overhangs required by exonuclease III for shortening in the forward direction. Reverse shortenings were obtained by cleaving pCM302 with *Sac*I (3' overhang) and *Xba*I (5' overhang). pCM32 was cleaved with *Apa*I (3' overhang) and *Xho*I (5' overhang) for isolating nested deletions in the forward direction. In order to isolate nested deletions in the reverse direction, pCM32 was digested with *Bst*XI (3' overhang) and *Bam*HI (5' overhang). Once cleaved, plasmid DNA was precipitated with 7.5 M ammonium acetate (Appendix B.10) and resuspended in exonuclease III buffer (Appendix A.2.10). To this, 150 U of exonuclease III was then added after which 4.5 ul aliquots of DNA were removed at 20 second intervals, transferred to tubes containing SI nuclease mix (prepared as described in Appendix A.2.10) and incubated for 30 min at room temperature. The reactions were stopped through the addition of 1.75 ul of S1 nuclease stop (Appendix A.2.10) and placed at –70°C for 10 min. In order to confirm the extent of the

shortening reactions, 2 ul from every 2nd time point was removed and run on a 1% agarose gel as described in Appendix B.6. In order to allow blunt-end ligations to proceed, overhangs generated by restriction enzymes were filled in with dNTPs using Klenow. This was achieved by the addition of 1.7 ul of klenow mix (Appendix A.2.10) to each tube as well as 1 ul of Klenow (1 U/ul). The reactions were left to proceed for 3 min at room temperature before 1 ul of dNTP's (0.5 mM) was added and the tubes incubated for 5 min at room temperature.

In order to re-circularize the shortened DNA fragments, ligations were performed at 15°C overnight as described in Appendix B.9. Half of each ligation mix was transformed into competent *E. coli* HB101 and the resulting transformants were selected on LA and ampicillin. Plasmid DNA isolated from transformants was cleaved with the restriction enzyme *Pvu*II in order to identify pBluescript SK containing the desired shortened inserts. We identified and isolated sequential deletions with 200 bp overlaps in both the forward and the reverse direction for both plasmids.

2.2.6 DNA sequencing of the pigmentation genes *ppdA* and *tyrM*

Double stranded sequencing of the shortened constructs (Chapter 2 section 2.2.5) was performed by the dideoxynucleotide chain termination method using the Sequenase sequencing kit (Amersham-Pharmacia) and [α-^{35}S]- dATP (Sanger *et al.*, 1977).

Ten ug of plasmid DNA was alkaline denatured in 2 M NaOH for 30 min at 37°C. The denatured templates were precipitated with 3 M sodium acetate (final concentration 0.3 M), and absolute ethanol at -70°C, washed in 70% ethanol to remove excess salt and air dried.

To the dried DNA pellets, annealing buffer (Appendix A.2.11), dH_2O and either a forward or reverse primer was added in a total volume of 10 ul. The forward universal pBluescript SK primer (5' AATACGACTCACTATAGGGCGAAT 3') and the reverse primer (5' GAGCGGATAACAATTTCACACAGG 3') were used for all sequencing reactions. Annealing of the primer to single stranded DNA templates was performed at 37°C for 30 min and subsequently, the tubes were slowly cooled to room temperature.

The following components were added to each of the primed DNA templates to allow for the labelling reaction: DMSO, lab mix (Appendix A.2.11), enzyme dilution buffer (Appendix A.2.11), T_7 DNA polymerase and labelled [α-^{35}S]- dATP. Labelling was performed at room

temperature for 10 min. Reactions were then terminated as follows: Four termination tubes (A, C, G, T) were prepared on ice. These contained extension mixes as well as the relevant ddNTP for termination. Aliquots of 4.5 ul were transferred from the labelling reaction tubes to each of the four termination tubes. The tubes were incubated for 5 min at 37°C before stop buffer (Appendix A.2.11) was added to each of the tubes. Labelled templates were separated on 6% PAGE / 7 M urea gels (Appendix A.2.11) in Taurine Tris buffer. Electrophoresis was performed at a constant current of 42 mA for either 2 or 4 hrs. After completion of each run, the gel was transferred to 3 MM Whatman filter paper and dried before overnight exposure to Curix X-ray film.

Sequence data was analyzed using the Genetics Computer Group software package. Homology searches with both DNA and protein sequences were carried out using the BLAST algorithm (Altschul *et al.*, 1990) provided by the internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

2.2.7 Recovery of RNA for primer extension analysis

Total cellular RNA was prepared from *E. coli* harbouring pCM302 and pCM32 using the method of Ausubel *et al.*, (1989). Ten ml of overnight cells were collected by centrifugation (10 000 rpm for 10 min), and resuspended in 10 ml protoplast buffer (Appendix A.2.12) to which 80 ul of lysozyme (80 mg/ml) was then added. The resulting protoplasts were collected by centrifugation (7 000 rpm for 5 min) and resuspended in lysis buffer (Appendix A.2.12) and 15 ul of DEPC. The tubes were incubated at 37°C for 5 min before salt-saturated NaCl (Appendix A.2.12) was added to precipitate most of the SDS contained within the lysis buffer. The white precipitate was removed by centrifugation at 14 000 rpm for 10 min. Supernatants were removed and the nucleic acid precipitated with absolute ethanol and centrifugation. Pellets were washed in 70% ethanol to remove any residual salt and resuspended in a final volume of 20 ul of DEPC treated water.

RNA samples were treated with DNAse I in order to remove any DNA contamination. Ten units of DNAse I was added to RNA in a final volume of 50 ul. The tubes were left at 37°C for 1 hr. The RNA was recovered by adding an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), centrifuging at 14 000 rpm for 10 min and transferring the resulting aqueous phase to a clean tube. In order to precipitate the RNA, 5 ul sodium acetate (3 M) and 150 ul absolute ethanol was added to the tube containing the RNA and centrifuged at 14 000 rpm for 10 min. Centrifugation resulted in the pelleting of the RNA which was subsequently resuspended in 20 ul

of DEPC treated dH_2O. RNA was quantified by reading the absorbance at 260 nm using a Beckman spectrophotometer.

2.2.8 Primer extension analysis of *ppdA* and *tyrM*

RNA isolated from *E. coli* harbouring pCM302 and pCM32 was used in primer extension analysis in order to identify the upstream transcriptional start sites as well as the putative promoter regions of each of the *V. cholerae* melanin biosynthetic genes harboured on these plasmids.

The oligonucleotide primers (100 ng) (5' GTGTTGAGTTGAAGTAACCATGG 3'and (5' GGCAAGATTGCGGTCTGTGACTTAAAC 3') were used to reverse transcribe RNA from pCM302 and pCM32, respectively. The primers were end-labelled with [γ-^{32}P] dATP using T_4 polynucleotide kinase (Appendix B.13). Labelled oligonucleotide was precipitated with 4 M ammonium acetate and absolute ethanol and finally resuspended in TEN 600 solution (Appendix A.2.13).

Radiolabelled oligonucleotide (5 x 10^4 cpm) was added to 50 ug of total cellular RNA. Hybridizations were performed at 65°C for 90 min. Reverse transciption of RNA to which the appropriate end-labelled primer was hybridized was carried out with AMV reverse transcriptase. The reaction was performed at 42°C for 1 hr since the enzyme stalls less frequently at this temperature. The reaction mixes were treated with 15 ul of RNase (10 mg/ml) to reduce the amount of total RNA in the sample and also to prevent aberrant electrophoresis of the primer extension products. Reverse transcribed products were extracted by adding 10 ul sodium acetate (3 M) and 100 ul phenol:chloroform:isoamyl alcohol (25:24:1). The tubes were spun at 10 000 rpm for 10 min before the acqueous phase was removed and the DNA precipitated by the addition of 100 ul of ice-cold absolute ethanol. The tubes were then spun at 10 000 rpm for 30 min and the DNA pellets were resuspended in 5 ul stop buffer (Appendix A.2.11). The DNA was denatured at 65°C for 5 min before separation on a 9% acrylamide / 7 M urea gel (Appendix A.2.13). The primer extension products were compared to a sequencing ladder, generated with the Promega sequencing kit and the primer used in the primer extension procedure.

2.3 Results

2.3.1. Phenotypic expression of pigment by *E. coli* clones harbouring *V. cholerae* genomic DNA

More than 50 *E. coli* clones capable of pigmentation were isolated after screening a *V. cholerae* genomic DNA library for melanin biosynthetic genes (Schroeder, 1993). Although all the clones produced pigment, they differed phenotypically with respect to the cellular localization of the pigment. The majority of the clones synthesized melanin which occurred extracellularly as a result of either diffusion or export of the pigment from the cell. The recombinant plasmid responsible for this phenotype was designated pCM30 (Fig. 5). The remaining clones, represented by the recombinant plasmid that was designated pCM3, produced melanin intracellularly (Fig. 5).

2.3.2. Southern hybridization studies

pCM30 and pCM3 were used as probes against wild-type *V. cholerae* chromosomal DNA to verify that the inserted DNA fragments carried by these recombinant plasmids were of *V. cholerae* origin, and that the pigmented phenotype in *E. coli* clones harbouring these plasmids was due to the presence of *V. cholerae* melanin biosynthetic genes (Fig. 6A and 6B). Indeed, pCM30 hybridized to a 20 kb *Bam*HI fragment and two *Stu*I fragments of 22 and 15 kb in size, whereas pCM3 was homologous to a 22 kb *Bam*HI fragment and *Stu*I genomic DNA fragments of 7.1 and 3.7 kb. There was no overlap between the *V. cholerae* genomic restriction enzyme fragments homologous to pCM30 and pCM3, suggesting that these plasmids encoded two distinct genes from *V. cholerae* which were responsible for pigmentation in *E. coli*.

2.3.3. Restriction enzyme mapping of recombinant plasmids, pCM30 and pCM3

In order to confirm that the recombinant plasmids pCM30 and pCM3 were different with respect to their insert DNA, the recognition sites of various restriction endonucleases were mapped within both plasmids (Fig. 7). The resulting restriction enzyme maps allowed the size of the cloned *V. cholerae* DNA fragments to be determined and clearly indicated a distinct restriction enzyme pattern for each of the recombinant plasmids. pCM30 harboured an insert of 8.8 kb whereas pCM3 had an insert of 8.1 kb in size. Both plasmids contained *Ava*I, *Bst*XI, *Stu*I and *Cla*I restriction enzyme sites. However, only pCM30 contained *Bam*HI, *Eco*RI, *Xba*I and *Hind*III restriction enzyme sites within the cloned *V. cholerae* DNA, while pCM3 contained *Eco*RV,

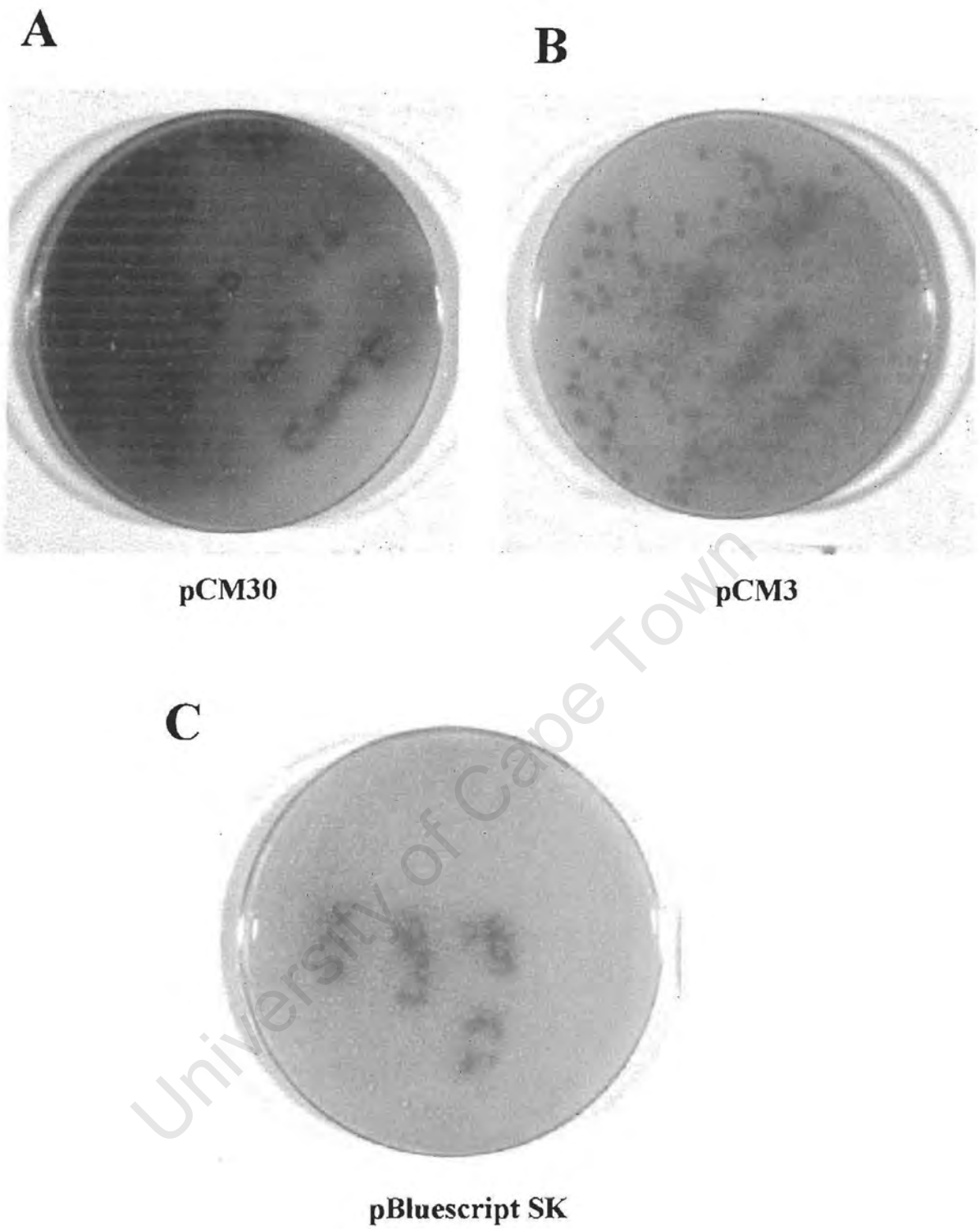


Figure 5. Phenotypic expression of cloned *V. cholerae* DNA responsible for pigmentation in *E. coli* HB101. A represents clones harbouring pCM30, B represents clones harbouring pCM3 and C represents clones that contain pBluescript SK.

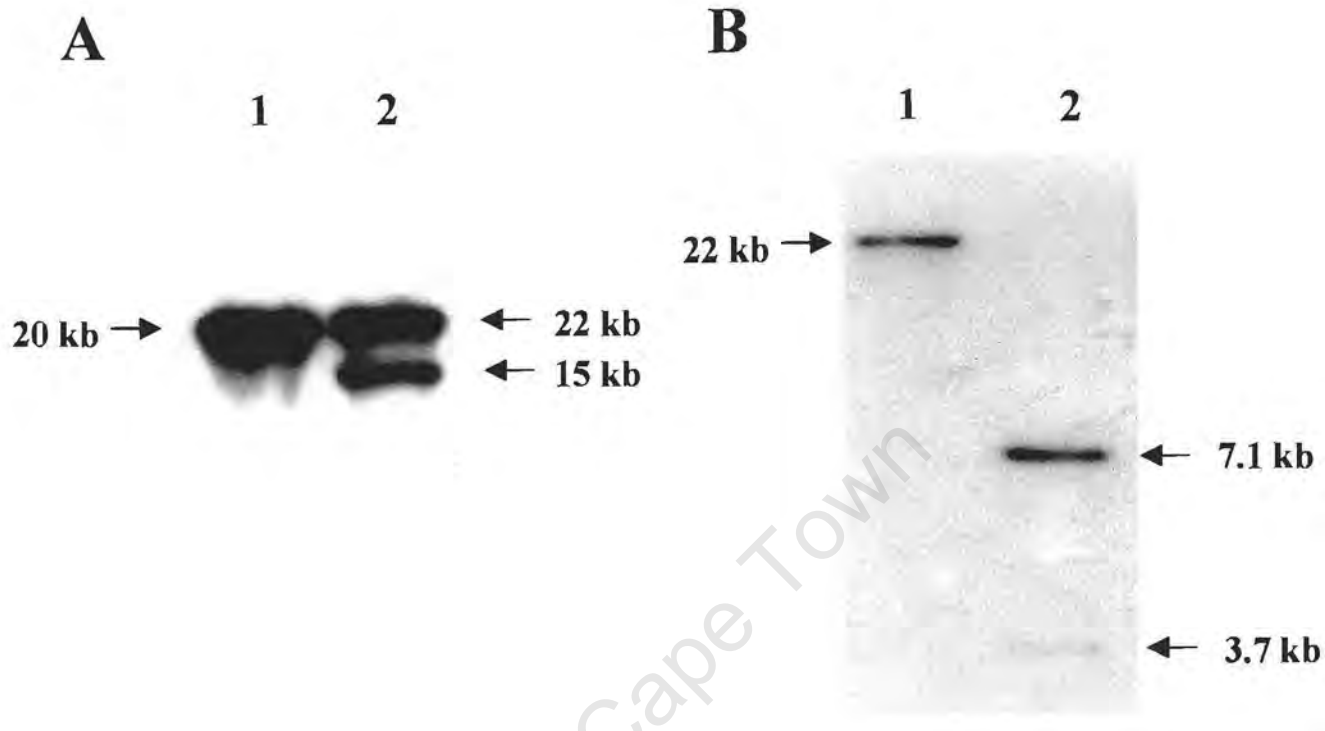


Figure 6. Southern hybridization of *V. cholerae* genomic DNA against (A) pCM30 and (B) pCM3. Genomic DNA was digested with *Bam*HI (lane 1) and *Stu*I (lane 2).

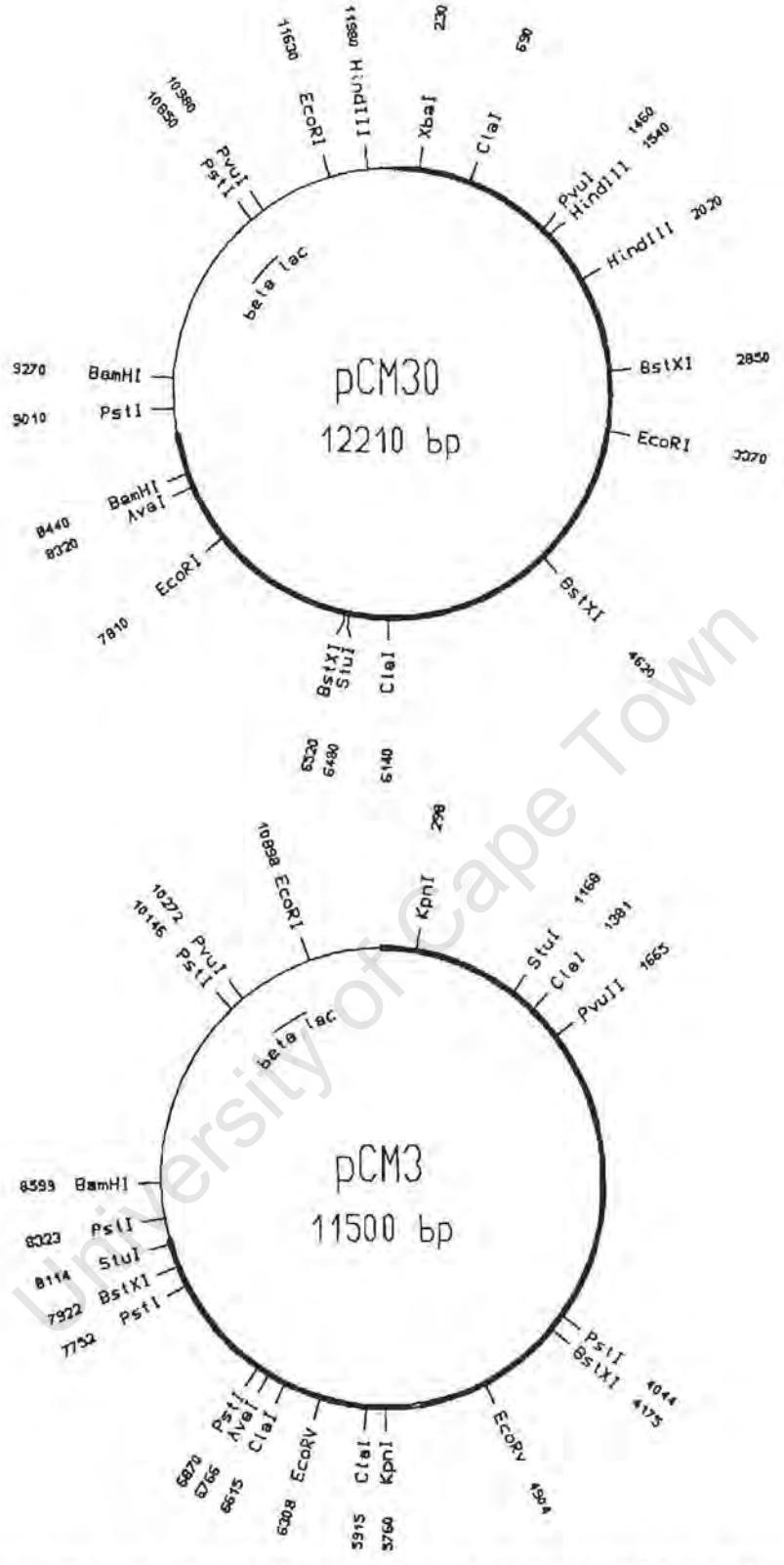


Figure 7. Restriction enzyme maps of the recombinant plasmids pCM30 and pCM3. The thick line represents cloned *V. cholerae* DNA whereas the thin line represents pEcoR251 DNA. pCM30 contains *Bam*HI, *Eco*RI, *Xba*I and *Hind*III restriction enzyme sites within the cloned *V. cholerae* DNA fragment. In contrast, pCM3 has *Eco*RV, *Pst*I, *Pvu*II and *Kpn*I restriction enzyme sites. The sizes of the recombinant plasmids are shown in base pairs (bp). The positions of the various restriction enzyme sites are indicated by the numbers. Finally, the position of the beta-lactamase gene (beta lac), encoding ampicillin resistance, is shown. The location of the beta lac was derived from sequence data.

*Pst*I, *Pvu*II and *Kpn*I restriction enzyme sites.

2.3.4. Deletion analysis of pCM30 and pCM3

Since both the recombinant plasmids harboured large inserts of *V. cholerae* genomic DNA (pCM30 has an insert of 8.8 kb and pCM3 an insert of 8.1 kb), it was necessary to identify which region of the *V. cholerae* DNA fragment was responsible for pigment production in the *E. coli* clones. This was achieved by subcloning various fragments from the two recombinant plasmids into pBluescript SK and visually scoring for pigmentation by comparing the ability of the resulting subclones to secrete pigment into the culture media. The deletion strategies that were followed are depicted in Figures 8 and 9.

Subcloning regions to the right from the *Eco*RI restriction enzyme site at position 3370 from pCM30, resulted in the elimination of the pigmented phenotype in subclone pCM301. PCM302 was the only subclone that maintained its ability to pigment. This plasmid harboured the *Xba*I (230)-*Eco*RI (3370) restriction enzyme fragment from pCM30. Subcloning DNA fragments downstream of the *Xba*I (230) restriction enzyme site resulted in either the loss of the ability to pigment (pCM303) or a decrease in the amount of pigment produced (pCM304).

Subclones harbouring regions to the left of the *Pst*I restriction enzyme site (4044) from pCM3 retained their ability to pigment in *E. coli* (pCM31, pCM32, pCM33). The smallest of these, pCM32, harboured a *Cla*I (1381)-*Pst*I (4044) restriction enzyme fragment. pCM34, carrying insert DNA from the region downstream of *Pst*I (4044), had lost its ability to synthesize pigment in *E. coli*. We concluded that the regions reponsible for pigmentation on recombinant plasmids pCM30 and pCM3 were localized within a 3 kb *Xba*I-*Eco*RI region (pCM302) and a 2.5 kb *Cla*I-*Pst*I region (pCM32).

2.3.5 DNA sequencing of the melanin biosynthetic genes from *V. cholerae*

In order to identify the genes responsible for pigment synthesis on pCM302 and pCM32, we sequenced various constructs containing nested deletions. Sequencing pCM302 revealed a single intact open reading frame (ORF) of 1.1 kb in size, designated *ppdA* (Fig. 10). The ORF, beginning at the ATG codon at position 352 and continuing to position 1462, thus codes for a protein of 370 amino acids. A putative Shine Dalgarno sequence at position 338-340 was present upstream from the translational start site. This sequence poorly resembled the AGGAGG normally regarded as the Shine Dalgarno sequence (Lewin, 1990). The 7 bp inverted repeat at positions

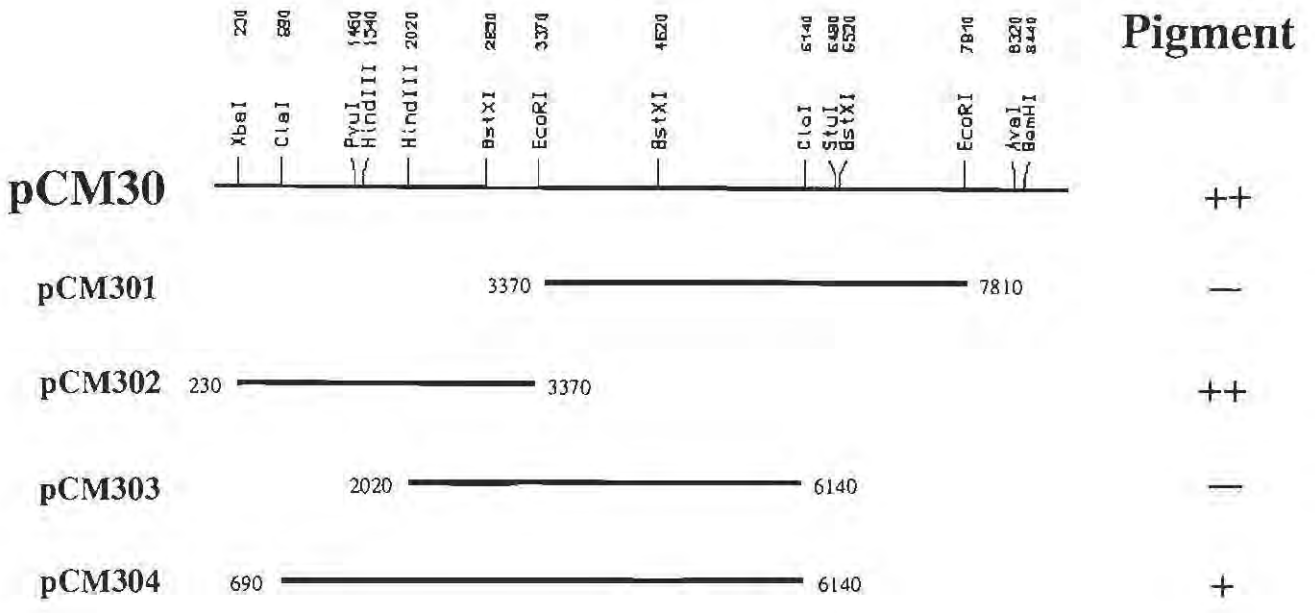


Figure 8. Subcloning of pCM30. Various regions of *V. cholerae* DNA from pCM30 were subcloned into pBluescript SK in order to localize the region containing the gene(s) responsible for pigmentation. pCM302, which harboured a *XbaI*/*Eco*RI fragment, was the smallest fragment which maintained the pigmented phenotype in *E. coli* clones. The plus signs indicate melanin production, whereas minus signs represent the absence of pigment production. The numbers indicate the relative positions of the various restriction enzyme sites. Only *V. cholerae* DNA is depicted.

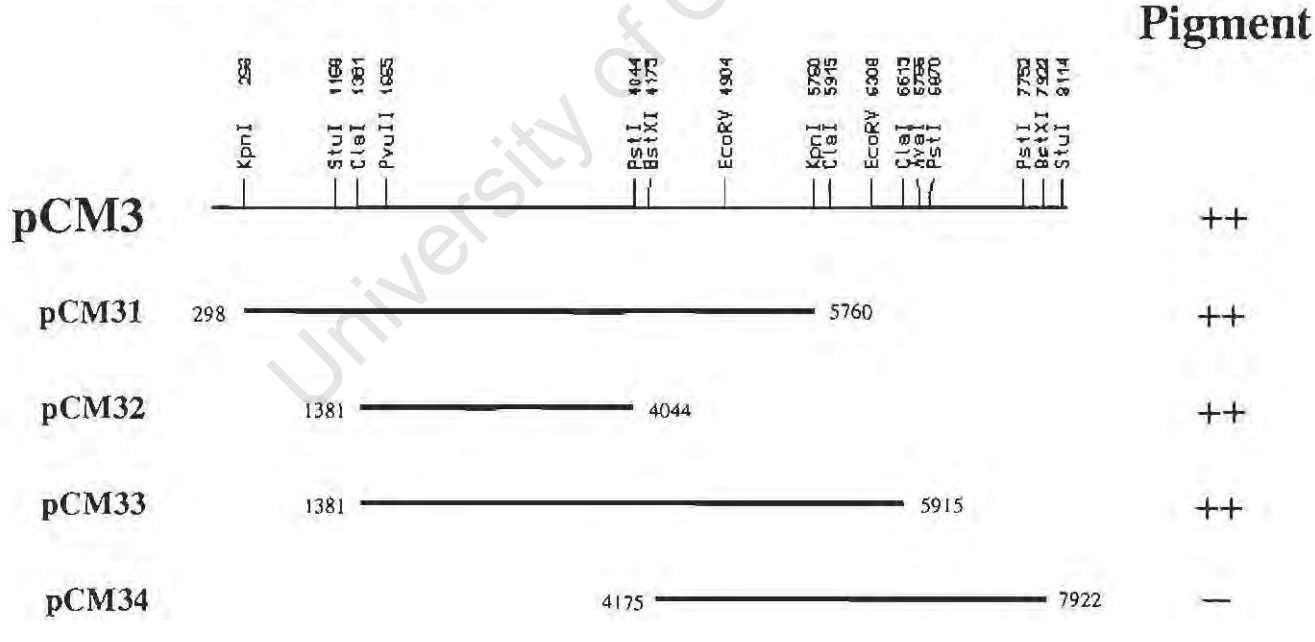


Figure 9. Subcloning of pCM3. Regions from within the cloned *V. cholerae* DNA harboured on pCM3 were subcloned into pBluescript SK. The resulting constructs were transformed into *E. coli* to analyze their ability to pigment. Clones harbouring pCM32 retained their pigmented phenotype on Luria agar supplemented with L-tyrosine. The plus signs represent clones that produced melanin, whereas the minus sign represents the absence of melanin production. The numbers indicate the relative positions of the various restriction enzyme sites. Only *V. cholerae* DNA is depicted.

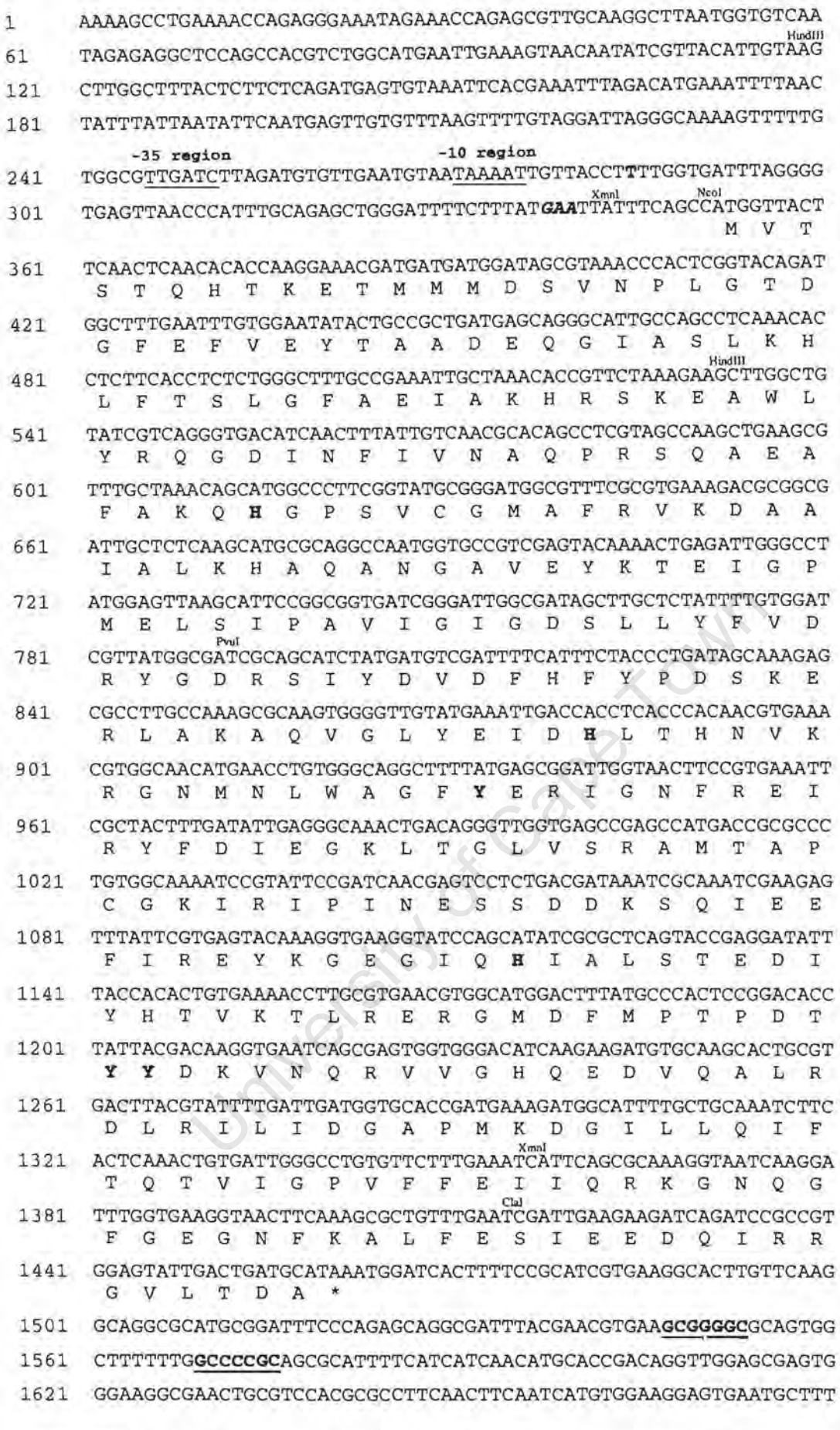


Figure 10. Nucleotide sequence and deduced amino acid residues of the cloned *ppdA* gene (accession number U31553/ GenBank). The putative -10 and -35 elements (underlined), transcriptional terminator (underlined and bold) and the conserved amino acids (bold) are shown. The predicted transciptional start site of *ppdA* is indicated by the bold nucleotide at position 285. The putative Shine Dalgarno sequence upstream from the translational start site is indicated (bold and italic).

1547-1553 and 1569-1575 has a calculated ΔG of -10.20 Kcal/mol (determined using DNAman for Windows version 2.2, Lynnon Biosoft), and thus, could serve as the transcriptional terminator of the *ppdA* gene.

The gene responsible for pigment synthesis in pCM32 consisted of a 1.088 kb ORF, designated *tyrM* due to the fact that gene expression of pCM32, in the presence of L-tyrosine, resulted in melanin synthesis. The *tyrM* ORF begins at nucleotide 217 and continues to position 1305 (Fig. 11) and encodes for a protein with a predicted molecular mass of 40 kDa. A putative Shine Dalgarno sequence could be identified at position 206-210. A transciptional termination sequence was not evident at the 3' end of the sequenced DNA.

2.3.6 Identification of the *ppdA* and *tyrM* transciptional start sites and putative promoters by primer extension analysis

A 23-mer oligonucleotide complementary to the mRNA transcribed from *ppdA* was used in primer extension analysis which placed the start site of the *ppdA* gene transcript at 67 nucleotides upstream of the putative translational start site (Fig. 12). Although a conserved promoter region could not be identified, the sequences TAAAAT at nucleotide positions -14 to -9 and TTGATC at -39 to -34 could possibly serve as the -10 and –35 regions of a putative promoter, respectively (Lewin, 1990). A 27-mer oligonucleotide complementary to the mRNA transcribed from *tyrM* was used to map the transcriptional start site of this gene (Fig. 13). The result shows that the start site of *tyrM* is 89 nucleotides upstream of the putative translational start site. The sequences GACAGT at positions -12 to -7 and TTGTGC at -35 to -30 could possibly serve as a -10 and -35 region of a *tyrM* gene promoter.

2.3.7 Homology searches

In order to identify the proteins encoded by *ppdA* and *tyrM*, we searched several databases for homologous protein sequences. The deduced amino acid sequence of *ppdA* most closely resembled the haemolysin from *Vibrio vulnificus* with an identity of 78% (Fig. 14), as well as proteins from various Gram-negative bacteria. These included the 4-hydroxyphenylpyruvate dioxygenase (Hppd) enzyme from a *Pseudomonas* sp strain P.J. 874 (51%), the legiolysin from *Legionella pneumophila* (49%), and the MelA from *Shewanella colwelliana* (37%). The Hppd enzymes from *Streptomyces avermitilis* and *Synechocystis* sp. shared 30% and 24% identity to the *ppdA* gene product, respectively (Fig. 14). The enzyme was less homologous (27-21%) to Hppd's from Vertebrates, Invertebrates and Protozoa (Fig. 14).

```
1       GCAAACGGCTTTTCACGTTTACTTGCAGCAGGGGGCTTTTCACTGGGCCAACGCATTGAC
                                                - 35 region      - 10 region
61      CCTACTCTTATTACTGTGGTTTGGCATTTACCTTGTGCAGAGTCGCCATGTACGCGACAG

121     TTTAAAAGCCCCCGCGTTGGATAAGGCATGATCATCATCAACGCGATGATTTTTCTTTTT

181     ATTACACTGAGCAAAACCGAAAAGTGAGGATTCGTTATGTTTAAGTCACAGACCGCAATC
                                 SD          M  F  K  S  Q  T  A  I

241     TTGCCAGAAGCTGGCCCGTTTGCCCTGTACACACTACTGAAAGTTCGTCAGAACCACGCC
         L  P  E  A  G  P  F  A  L  Y  T  L  L  K  V  R  Q  N  H  A

301     CATGTTTTGCAAGCGCTTAAGGCTTTGCCTGCGTTAGTTGAGGAAATCAATCAAAATCAG
         H  V  L  Q  A  L  K  A  L  P  A  L  V  E  E  I  N  Q  N  Q

361     CCGGGAGCCGAGCTGACGGTTTCTGTCGCGTTCAGCAAAGGTTTTTGGAGCCATTTTGAG
         P  G  A  E  L  T  V  S  V  A  F  S  K  G  F  W  S  H  F  E

421     ATGGCGTCGCCACCAGAACTGATTGATTTTCCTGAGCTGGGTGAGGGCGAAACTCACGCG
         M  A  S  P  P  E  L  I  D  F  P  E  L  G  E  G  E  T  H  A

481     CCAAGCACGGATGTGGATGTGTTGATCCATTGTCACGCCACGCGCCACGATTTGCTGTTT
         P  S  T  D  V  D  V  L  I  H  C  H  A  T  R  H  D  L  L  F

541     TATACTCTACGTAAAGGGATTAGCGACATAGCTCAAGATATTGAAATCGTGGATGAAACC
         Y  T  L  R  K  G  I  S  D  I  A  Q  D  I  E  I  V  D  E  T

601     TATGGTTTCCGTTATTTAGACGCACGTGACATGACGGGCTTTATTGATGGCACAGAAAAC
         Y  G  F  R  Y  L  D  A  R  D  M  T  G  F  I  D  G  T  E  N

661     CCGAAAGCTGAGAAACGTGCGGAAGTCGCGTTGGTGGCTGATGGCGACTTTGCGGGAGGC
         P  K  A  E  K  R  A  E  V  A  L  V  A  D  G  D  F  A  G  G

721     AGTTATGTGATGGTACAACGCTTTGTGCATAATTTGCCGGCGTGGAATCGTCTCAACTTA
         S  Y  V  M  V  Q  R  F  V  H  N  L  P  A  W  N  R  L  N  L

781     GCGGCGCAAGAAAAAGTGATTGGTCGCACTAAGCCTGATTCCGTCGAGCTAGAAAATGTT
         A  A  Q  E  K  V  I  G  R  T  K  P  D  S  V  E  L  E  N  V

841     CCTGCCGCTTCGCATGTAGGTCGAGTGGATATTAAAGAAGAAGGTAAAGGATTGAAGATT
         P  A  A  S  H  V  G  R  V  D  I  K  E  E  G  K  G  L  K  I

901     GTTCGCCACAGCTTGCCGTATGGCAGCGTGAGCGGCGATCACGGCCTACTGTTTATTGCG
         V  R  H  S  L  P  Y  G  S  V  S  G  D  H  G  L  L  F  I  A

961     TACTGTCACACGCTGCATAATTTCAAAACTATGCTGGAAAGCATGTACGGTGTCACTGAT
         Y  C  H  T  L  H  N  F  K  T  M  L  E  S  M  Y  G  V  T  D

1021    GGCAAAACAGACCAACTGCTACGCTTTACCAAAGACCGTGACCGGGGCTTATTTCTTTGC
         G  K  T  D  Q  L  L  R  F  T  K  D  R  D  R  G  L  F  L  C

1081    ACCGTCGCAAGTGATGTTGCAGGAACTGACACTCAAGAATCAATAATGCAACACTGTTGC
         T  V  A  S  D  V  A  G  T  D  T  Q  E  S  I  M  Q  H  C  C

1141    ATATGTTTCATGCCAAAGCCGAGCCTAGTGCTCGGTTTTTTGATTAAAGAAAACTCACTT
         I  C  F  M  P  K  P  S  L  V  L  G  F  L  I  K  E  N  S  L

1201    TACACCGATAAAAGAACAAGTAAGCAATCGTGTAAGGAGTGCAGCAGCCATGGCCATGAC
         Y  T  D  K  R  T  S  K  Q  S  C  K  E  C  S  S  H  G  H  D
                                                         M  A  M  T

1261    GGTAAATACCAATGTGTCTGCGCTGGTAGCACAGCGACATCTTAATTCTGCGTCCGAGAT
         G  K  Y  Q  C  V  C  A  G  S  T  A  T  S  *
          V  N  T  N  V  S  A  L  V  A  Q  R  H  L  N  S  A  S  E  M

1321    GCTCAATCAGTCTCTGGAGCGGCTCTCTTCTGGCAATCGAATCAACAGTGCCAAAGATGA
          L  N  Q  S  L  E  R  L  S  S  G  N  R  I  N  S  A  K  D  D

1381    TGCGGCAGGGCTGCAG
          A  A  G  L  Q
```

Figure 11. Nucleotide and deduced amino acid sequence of the cloned *tyrA* gene (accession number U62056/Genbank). The ORF extends from position 217 to 1305. The putative promoter region (-35 and -10 regions) are indicated (underlined) as well as the transciptional start site of *tyrA* (bold). A putative Shine Dalgarno sequence (bold, italic) occurs upstream from the translational start site. The *tyrA* gene overlaps *flaE* (bold nucleotide sequence), a gene encoding the *V. cholerae* flagellin (bold protein sequence).

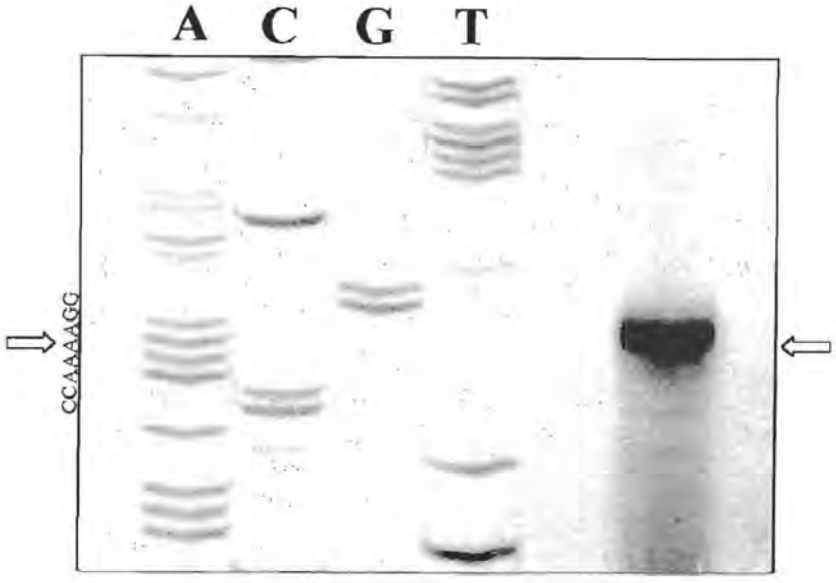


Figure 12. Mapping of the transcriptional start site of *ppdA* by primer extension analysis. The sequence ladder on the left is the reverse complement of the *ppdA* nucleotide sequence. The DNA sequence in the region of the transcriptional start site is indicated. The predicted position +1, which corresponds to a 67 nucleotide extension product, is marked by an arrow.

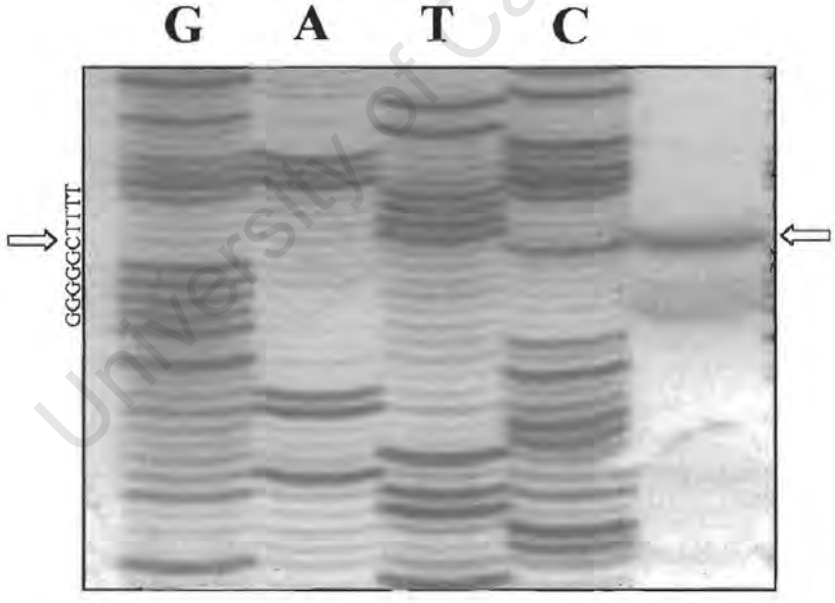


Figure 13. Mapping of the transcriptional start site of *tyrA* by primer extension analysis. The sequence ladder on the left is the reverse complement of the *tyrA* nucleotide sequence. The DNA sequence bracketing the *tyrA* transcriptional start site is indicated. The predicted transcriptional start site of *tyrA*, indicated by the arrow, corresponds to 89 nucleotides upstream from the translational start site.

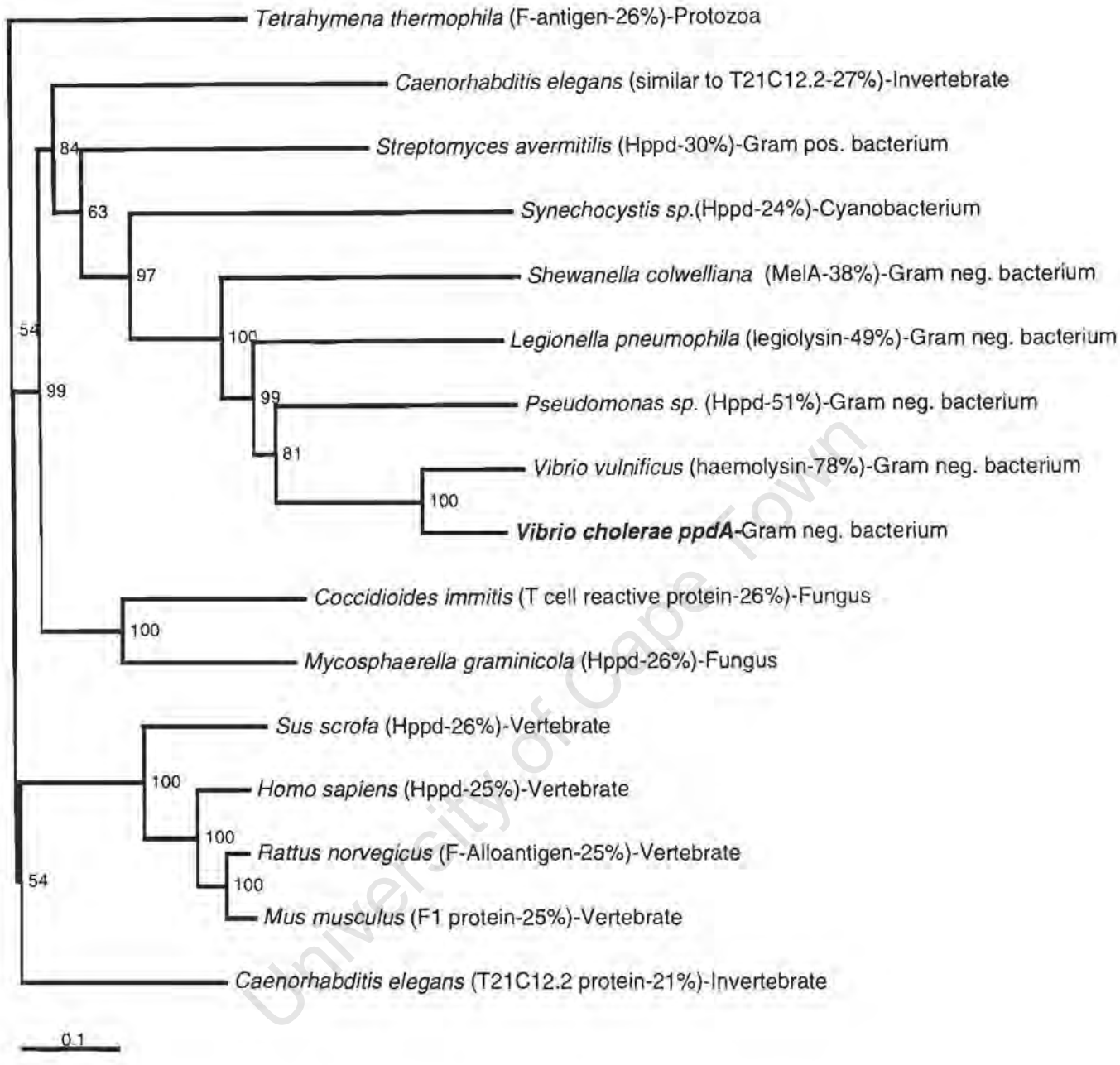


Figure 14. Phylogenetic relationship between the *V. cholerae ppdA* gene product and homologous proteins from various other organisms. The species name of each organism is followed by the name of the protein that shares homology to the *V. cholerae ppdA* protein in that particular organism (brackets), as well as the percentage identity that exists between the *V. cholerae ppdA* gene product and the respective protein. The group classification for each organism is given. Scale bar represents 0.1 nucleotide substitutions per site. Numbers at nodes indicate bootstrap values. The phylogenetic tree was constructed using Treeview (Page, 1996).

Multiple sequence alignment of the *ppdA* gene product and homologous proteins showed that sequence similarities occurred throughout the peptide chain. However, amino acid sequence conservation was most prominant at the C-terminal region of the peptide with several regions displaying between 60-100% homology (Fig. 15). Several histidine, tyrosine, and valine residues, as well as motifs such as GVQH and LLQIFT, were conserved in the PpdA protein (Fig. 10).

Homology searches of several databases showed that the deduced amino acid sequence of *tyrM* displayed 25% similarity to a 33.9 kDa hypothetical protein encoded by a gene at 54.9 minutes on the *E. coli* K12 chromosome. The function of this protein is currently unknown. Interestingly, the *tyrM* coding region overlaps the ORF of the *V. cholerae flaE* gene which encodes flagellin protein (Fig. 11) (GENBANK: accession number AF007122).

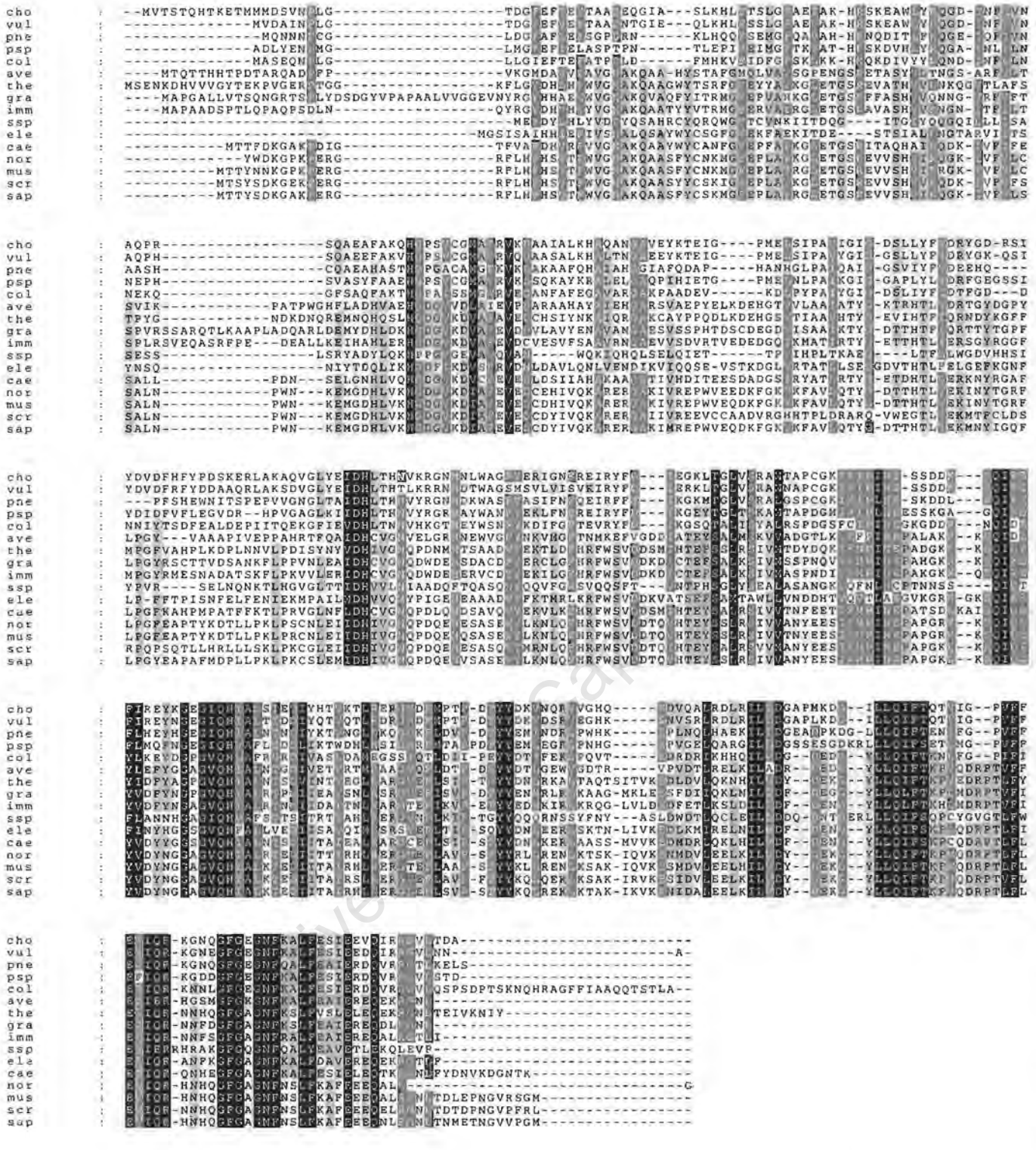


Figure 15. Multiple sequence alignment comparing the amino acid sequence of the *V. cholerae* Hppd (cho) with the corresponding enzymes form *V. vulnificus* (vul), *L. pneumophila* (pne), *Pseudomonas* sp. (psp), *S. colwelliana* (col), *S. avermitilis* (ave), *T. thermophila* (the), *M. graminicola* (gra), *C. immitis* (imm), *Synechocystis* sp. (ssp), *C. elegans* (ele), *C. elegans* (cae), *R. norvegicus* (nor), *M. musculus* (mus), *S. scrofa* (scr), and *H. sapiens* (sap). Black boxes represent regions with either 100% amino acid similarity or 100% functional amino acid similarity. The dark grey and light grey boxes indicate regions of 80% and 60% sequence similarity, respectively. The alignment was generated with Clustalw (Thompson *et al.*, 1994) and displayed with Genedoc (Nicholas and Nicholas, 1997).

2.4 Discussion

We identified two recombinant plasmids, namely pCM30 and pCM3, that were responsible for brown pigmentation in *E. coli* clones propagated on Luria agar supplemented with L-tyrosine and copper. Clones harbouring pCM30 secreted pigment into the extracellular media, whereas clones harbouring pCM3 maintained their pigment intracellularly. Southern hybridization studies and restriction enzyme mapping confirmed that genes harboured on pCM30 and pCM3 were not linked on the *V. cholerae* chromosome. This implies that *V. cholerae* possesses two different genes capable of synthesizing melanin when transformed into *E. coli* cells. Homology searches of several genetic databases revealed that the gene responsible for pigmentation within pCM30, designated *ppdA*, showed extensive homology to Hppd enzymes from a number of organisms. Multiple sequence alignment confirmed that PpdA, like other Hppd enzymes, contained a highly conserved C-terminal region that is thought to play a crucial role in the catalytic activity of this enzyme (Ruetschi *et al.*, 1993). In accordance with other Hppd's, the *ppdA* gene product contained a total of 14 tyrosine and 10 histidine residues. In fact, three histidine residues and three tyrosine residues in the PpdA protein occurred at positions which are conserved in all the other Hppd proteins (Fig. 15). These six amino acids are thought to function as ligands for iron which is required as a co-factor by Hppd (Bradley *et al.*, 1986; Ruetschi *et al.*, 1992). Furthermore, many of the previously characterized Hppd enzymes have been implicated in melanin biosynthesis. It is therefore reasonable to conclude that the protein encoded by *ppdA* is an Hppd enzyme. This is further supported by the fact that both transaminase activity and the release of copious amounts of homogentisate prior to pigmentation have been observed in *V. cholerae* (Ruzafa *et al.*, 1995). Since the *V. cholerae ppdA* gene encodes an Hppd enzyme, we propose that one of the melanins produced by this organism is a pyomelanin formed from L-tyrosine via homogentisate.

V. vulnificus is a halophilic Gram-negative bacterium which is notorious as the causative agent of wound infections and fatal septicaemia in humans (Chang *et al.*, 1997). One of the haemolysins produced by *V. vulnificus*, encoded for by the *vll*Y gene, has strong homology to other Hppd enzymes and exhibits haemolytic activity on blood agar plates (Chang *et al.*, 1997). Interestingly, the presence of *vll*Y in all clinical *V. vulnificus* isolates strongly implies that this protein plays a role in pathogenesis.

L. pneumophila resembles *V. vulnificus* in two ways. Firstly *L. pneumophila* has the ability to multiply within macrophages of different organisms, including humans, and is the causative agent of a severe pulmonary illness termed Legionaires disease (Hacker *et al.*, 1991). Thus, both *V. vulnificus* and *L. pneumophila* can be classified as human pathogens. Secondly, *L. pneumophila* also encodes a protein that exhibits strong homology to Hppd enzymes that is capable of haemolysing human erythrocytes (Wintermeyer *et al.*, 1994). This protein is known as legiolysin and is encoded for by the *lly* locus. However, Lly mutants displayed no defect in their ability to survive within macrophage-like cells (Wintermeyer *et al.*, 1994), whereas it has been shown to promote survival of *L. pneumophila* during periods of light stress (Steinert *et al.*, 1995). This suggests that legiolysin either plays a minor role, or has no role, in *L. pneumophila* pathogenesis.

The high degree of homology between the *V. cholerae* PpdA to both the *V. vulnificus* haemolysin and the *L. pneumophila* legiolysin, suggests that PpdA might be the haemolysin equivalent in *V. cholerae*. Haemolysins are exotoxins and are produced by many pathogenic bacteria. The toxin lyses erythrocytes through the formation of pores in the membrane through which haemoglobin and iron are then released (Krasilnikof *et al.*, 1992). The result is often anaemia and a consequent weakening of the host defences. In addition, valuable iron is made available to the pathogen in an environment where iron is extremely limiting (Sugawara *et al.*, 1997).

Contrary to most haemolysins which have been shown to be exported extracellularly to mediate pore formation (Sugawara *et al.*, 1997; Zitzer *et al.*, 1997), the *V. vulnificus* haemolysin has been localized to the periplasm (Chang *et al.*, 1997). Since we were unable to identify any signal sequences upstream from PpdA to aid in its extracellular secretion, PpdA might also be localized to the periplasm. This, however, needs to be tested.

Thus, PpdA could serve three possible functions in *V. cholerae*. Firstly, the protein could specifically be involved in tyrosine catabolism and thus allow *V. cholerae* to utilize tyrosine as a carbon and energy source. Secondly, the protein may only be involved in pyomelanin synthesis in order to promote survival of the pathogen against some specific stress encountered by the bacterium either in the human host or the estuarine environment, and finally, PpdA may aid in *V. cholerae* pathogenesis by acting as a haemolysin which scavenges iron from the human host. Since Ruzafa *et al.*, (1995) showed that *V. cholerae* was unable to grow on L-tyrosine as a sole

source of carbon and energy, it seems that PpdA in *V. cholerae* may either function as a haemolysin, a 4-hydroxyphenylpyruvate dioxygenase for the production of pyomelanin, or performs both functions in order to promote the survival of the bacterium.

The gene carried by pCM3 that was responsible for pigmentation in *E. coli*, designated *tyrM*, had revealed identity to only a single 33.9 kDa hypothetical protein from *E. coli* with unknown function. The exact mechanism whereby *tyrM* synthesizes melanin is unknown. The observation that the ORF of *tyrM* overlaps the *flaE* of *V. cholerae* is interesting. In *Serratia marcescens*, colour variation is a result of a 39 kDa protein that was shown to be closely associated with the flagella components of this organism (Parachuri and Harshey, 1987). Variation of this surface protein is proposed to provide *S. marcescens* with an alternate defence strategy for survival in the environment. Although the association of the *V. cholerae* TyrM with flagellin components could aid in deterring host defence systems, this possibility still needs to be tested.

The observation that *V. cholerae* has two different genes capable of producing melanin supports the theory that melanogenesis is an important trait in this organism. The exact roles of these pigments, and the respective proteins which synthesize them, is still unclear and requires further investigation.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF PpdA, A PROTEIN RESPONSIBLE FOR MELANOGENESIS IN *V. cholerae* 569B

CONTENTS

Summary

E. coli clones harbouring the recombinant plasmids pCM302 and pCM302-16 displayed α-haemolytic activity on Luria agar containing 5% human erythrocytes as opposed to β-haemolytic activity exhibited by the legiolysin of *L. pneumophila*. In addition, PpdA was unable to confer flourescent activity to the culture media as was shown to be the case for legiolysin. Thus, although the two proteins closely resembled each other with respect to their amino acid compositions, they displayed distinct catalytic properties. *In vitro* transcription and translation revealed that *ppdA* encoded for a protein with a molecular mass of 41 kDa which was in agreement with the DNA sequencing data we obtained in Chapter 2. In order to obtain pure PpdA, we used the MalE protein fusion purification system in *E. coli* TB1. Antibodies against purified protein were then used in Elisa assays in order to monitor PpdA synthesis in wild-type *V. cholerae* cells. These results showed that PpdA synthesis occurred at 44 hrs of growth and continued until 60 hrs. No PpdA protein could be detected in the cell extracts earlier than 43 hrs. Interestingly, RT-PCR analysis revealed the presence of *ppdA* transcripts in all cell extracts prepared from *V. cholerae* cells grown for 30-50 hrs, as well as in *V. cholerae* cells grown to exponential phase in Tryptone broth at 30°C. This suggested that *ppdA* expression occurred throughout the *V. cholerae* 569B growth cycle, even though the PpdA protein can only be detected after 44 hrs of growth.

3.1 Introduction

3.1.1 Properties of 4-hydroxypyruvate dioxygenases

4-Hydroxypyruvate dioxygenases (Hppd) are non-haem dioxygenases that catalyze a single reaction in the catabolism of tyrosine, namely the conversion of 4-hydroxyphenylpyruvate to homogentisic acid (Hamilton, 1974). These enzymes cannot utilize tyrosine as a substrate, and thus rely on aminotransferases for the transamination of tyrosine to 4-hydroxyphenylpyruvate before catalysis (Lehninger, 1975).

The oxidation reaction catalyzed by Hppd is complex and involves hydroxylation of the phenyl ring and decarboxylation, oxidation, and migration of the side chain (Hamilton, 1974). Consequently, both atoms of molecular oxygen are incorporated into 4-hydroxyphenylpyruvate to form homogentisic acid. Usually, this type of reaction requires the participation of a transitional metal (Ruetschi *et al.*, 1992). Not surprisingly therefore, the Hppd enzymes of human, chicken, pig and *Pseudomonas* have all been found to contain iron tightly bound to the enzyme. The iron centre has been shown to be most important for Hppd activity as it binds molecular oxygen which is subsequently incorporated into the substrate (Lindstedt and Rundgren, 1982). In addition to iron and oxygen, Hppd also requires DTT and ascorbate for activity *in vitro* (Lee *et al.*, 1996).

Hppd enzymes have been purified from porcine, human, avian and rat livers, as well as a *Pseudomonas* sp. (Buckthal *et al.*, 1987; Lindstedt and Odelhog, 1987a; Fellman, 1987; Lindstedt and Odelhog, 1987b). Mammalian Hppd enzymes are dimers, while the *Pseudomonas* enzyme exists as a tetramer consisting of four identical subunits. Further analysis of the *Pseudomonas* enzyme reveals that, although the protein has a unique structure, it consists mainly of mixtures of α helixes and β sheets which is a typical characteristic of globular proteins (Ruetschi *et al.*, 1992). All Hppds are rich in tyrosine and histidine residues which occur within the central domain of the protein and are absent at the C-terminal region (Fig. 15). These residues are important in the coordination of the iron co-factor molecule (Bradley *et al.*, 1986). The C-terminal end of Hppd is responsible for the catalytic activity and most conserved among both prokaryotic and eukaryotic enzymes (Lee *et al.*, 1996).

3.1.2 Relationship between Hppd and F-antigens

F-antigens are prominent liver proteins which exhibit a high degree of homology to Hppd enzymes, making it likely that these proteins are Hppd enzymes (Schofield *et al.*, 1991;

Gershwin *et al.*, 1987). The importance of the C-terminal domain in the catalytic activity of Hppd was illustrated when the human Hppd protein sequence was compared to that of the F-antigen that was originally cloned and sequenced from rat liver (Lee *et al.*, 1996). This F-antigen lacked 14 amino acids at its C-terminal end and *E. coli* clones harbouring this gene did not produce brown pigment on media supplemented with tyrosine. Thus, the enzyme was inactive, and consequently, was unable to convert 4-hydroxyphenylpyruvate into homogentisic acid. Hppd was later isolated from rat liver and found to be identical to F-antigen except for the additional amino acids that occur at its C-terminal end. The Hppd and F-antigen proteins in all species analyzed so far have apparent molecular masses of between 37 and 45 kDa.

3.1.3 The function of Hppd

The expression of Hppd genes from prokaryotic organisms can be directly correlated with the production of pyomelanin when their growth media is supplemented with L-tyrosine. This characteristic phenotype has led to the cloning and sequencing of Hppd genes from numerous organisms. The fact that these enzymes are widespread in nature and that their structure is evolutionary conserved in both prokaryotes and eukaryotes, suggests an important function for these enzymes. Although their role in vertebrates is that of tyrosine catabolism, a fact that is supported by their presence in mainly the liver and kidneys, their role in prokaryotes, protozoa and fungi remains obscure. Although both the *L. pneumophila* legiolysin and the *V. vulnificus* haemolysin are Hppd enzymes which have the ability to haemolyse erythrocytes (Wintermeyer *et al.*, 1994; Chang *et al.*, 1997), the significance of this trait and the mechanismof lysis is not yet understood.

3.1.4 The aim of this chapter

We cloned and sequenced the *ppdA* gene from *V. cholerae* and showed that this gene encoded a Hppd-like protein which also displayed significant homology to haemolysins from *V. vulnificus* and *L. pneumophila*. We therefore started the basic characterization of PpdA by assessing its haemolytic activity and its ability to confer fluorescence to the culture media. In order to gain insight into the importance of PpdA in *V. cholerae*, we employed Elisa assays to provide information regarding the time at which PpdA synthesis occured during the growth cycle of wild-type *V. cholerae*. In addition, this information allowed us to directly correlate the time of PpdA production with the appearance of brown pigment in the culture media. Finally, we examined *ppdA* gene expression using RT-PCR analysis so that we could compare the presence of PpdA in cellular extracts to the presence of *ppdA* transcripts in the same extracts. In this chapter we therefore outline the basic characterization of PpdA in terms of its haemolytic properties, the

time at which the protein is synthesized in *V. cholerae* 569B and gene expression under various culture conditions.

3.2 Materials and methods

All media and solutions used in this study are listed in Appendix A.

3.2.1 The bacterial strains and plasmids used in this study

The bacterial strains and plasmids that we used to characterize and purify the *V. cholerae* Hppd, namely PpdA, are listed in Table 1.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant features	Reference
Strains		
E. coli HB101	*hsdS20 recA13 leuB6 ara-14 roA2 lacY1 galK2 rpsL20 yl-5 mtl-1 sup E44*	Sambrook *et al.*, (1989)
TB1	*araΔ (lac pr AB) rpsL (Φ 80 lacZΔ M15) hsdR*	Johnston *et al.*, (1986)
V. cholerae 569B	Classical, Inaba	Mukherjee, (1978)
Plasmids		
pBluescript SK(+)	Amp^r, β-galactosidase	Short *et al.*, (1988)
pCM302	pBluescript SK with 3.1 kb insert, harbouring *ppdA*	Chapter 2
pCM302-16	pCM302 containing a 1.5 kb insert harbouring *ppdA*	This study
pMalC2	*malE* under control of P_{tac}, *lacZ*, *lacI*, Amp^r	Maina *et al.*, (1988)
pMalC2-PpdA	pMalC2 containing *ppdA* downstream from *malE*	This study

3.2.2 Fluorescent and haemolytic properties of *V. cholerae* PpdA

E. coli clones harbouring the gene encoding the *L. pneumophila* legiolysin, produce zones of lysis on Yeast Tryptone agar, containing canine, human and sheep erythrocytes (Wintermeyer *et al.*, 1994). In addition, these clones also exhibited yellow-green fluorescent activity under long UV light. Since *V. cholerae* PpdA shares significant homology to legiolysin, we tested whether *E. coli* clones harbouring *ppdA* exhibited fluorescent and haemolytic activities similar to that of legiolysin producing clones.

E. coli clones harbouring pBluescript SK, pCM302 and pCM302-16 were grown in Luria broth (LB) supplemented with L-tyrosine (2.5 mM) and ampicillin (100 ug/ml) (Appendix A.1 and A.2) at 37°C. These cultures were irradiated under a long wavelength UV lamp in order to detect fluorescence in the culture media.

In addition, we assessed the haemolytic activities of the above mentioned clones. Cells from each clone were harvested by centrifuging 4 ml aliquotes of each culture at 10 000 rpm for 10 min. The bacterial pellets were resuspended in 1 ml of phosphate buffered saline (PBS) (Appendix A.2.17). Cell suspensions were sonicated for 2x 30 second intervals with a 30 second interval in between to prevent overheating of cell extracts. The resulting cell extracts were diluted two fold in PBS and 50 ul of each dilution was loaded into wells made in Blood agar plates containing 5% human erythrocytes (Appendix A.1.4). The plates were incubated at 37°C overnight before scoring for haemolysis.

3.2.3 Size determination of the *V. cholerae* PpdA using *in vitro* transcription/translation

In order to determine the size of the protein encoded for by *ppdA*, the plasmids pCM302-16 and pBluescript SK were transcribed and translated *in vitro*, using the Promega *E. coli* S30 extract system for circular DNA in the presence of ^{35}S methionine. Reaction mixes were incubated for 1 hr at 30°C before the ^{35}S labelled proteins were precipitated with acetone and the pellets resuspended in 30 ul SDS-PAGE sample buffer (Appendix A.2.14). Samples were loaded and separated on a 12% SDS-PAGE gel as described in Appendix B.14. Gels were stained for 15 min in Coomassie blue R250 and destained in 7% glacial acetic acid before drying and exposure to X-ray film.

3.2.4 Purification of the *V. cholerae* PpdA

In order to purify PpdA, we used the New England Biolabs MalE protein and expression system, whereby *ppdA* was fused to *malE*. The *malE* gene codes for the maltose binding protein which

lacks the normal MalE signal sequence resulting in a cytoplasmically localized MalE protein. The resulting PpdA-MalE fusion protein was purified on an amylose affinity column and cleaved with factor Xa in order to liberate pure PpdA. The purification procedure is described below.

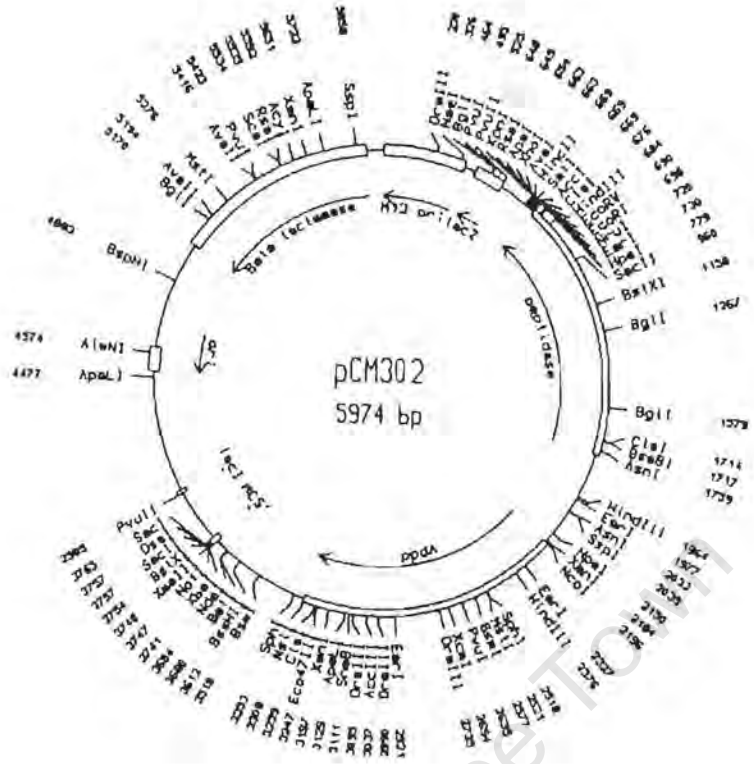
3.2.4.1 The cloning of *ppdA* downstream to *malE*

In order to create a fusion protein between the MalE and PpdA, we cloned *ppdA* downstream and in frame to *malE* on the pMalC2 vector.

pCM302-16 is a derivative of pCM302 that harbours only the *ppdA* gene from *V. cholerae* in pBluescript SK (Fig. 1). This recombinant plasmid contains three *Xmn*I restriction enzyme sites of which a partial 3.5 kb fragment (*Xmn*I sites at nucleotides 982 and 4 449) contains the entire *ppdA* gene (Fig. 1). In order to obtain partial *Xmn*I restriction enzyme fragments we cleaved 20 ug of pCM302-16 plasmid DNA with 10 units of *Xmn*I restriction enzyme and separated the partially digested fragments on a 1% agarose gel. (The restriction enzyme digestion and agarose gel electrophoresis procedures are described in Appendix B.5 and B.6). The 3.5 kb *Xmn*I restriction enzyme fragment was purified from the agarose gel by using electroelution as described in Appendix B.7. Once purified, the fragment was cleaved with the restriction enzyme *Xba*I (Fig. 2). This resulted in a 1.5 kb fragment which contained the entire *ppdA* gene. This fragment was ligated into the *Xmn*I/*Xba*I restriction enzyme sites of the pMalC2 polylinker using the standard ligation procedure outlined in Appendix B.9 (Fig. 2). This strategy ensured that *ppdA* was inserted directly downstream of *malE*, and that the protein sequence of PpdA was in frame to that of the MalE protein sequence (Fig. 3).

Ligation mixes were transformed into *E. coli* TB1 as described in Appendix B.3. Since TB1 encodes the α-subunit of β-galactosidase, insertion of *ppdA* between the *malE* and *lacZ* on pMalC2 would result in white colonies on LA containing X-gal (80 ug/ml) and ampicillin (100ug/ml) (Appendix A.2). We therefore only selected white colonies for further restriction enzyme analysis.

A



B

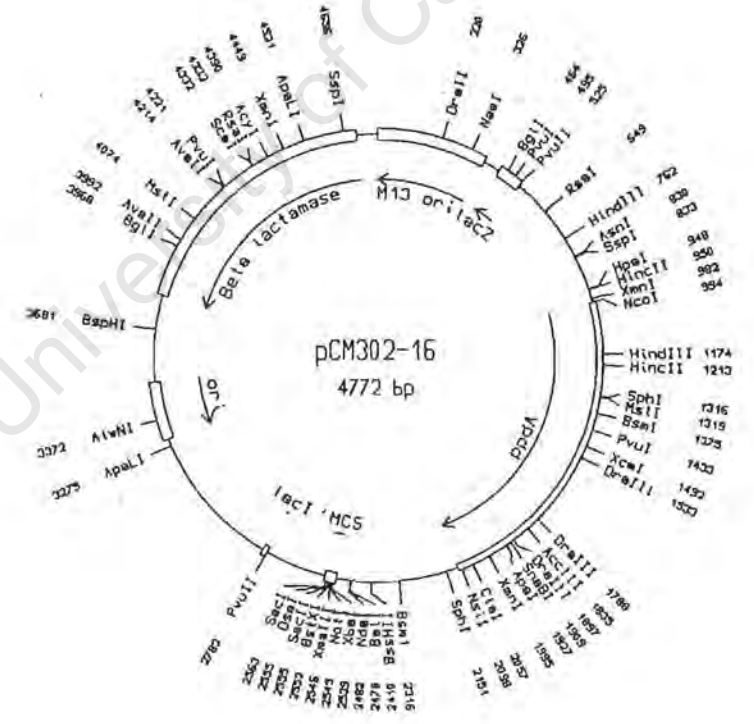


Figure 1. Restriction enzyme maps of pCM302 (A) and pCM302-16 (B). pCM302 harbours *ppdA* and a peptidase gene from *V. cholerae* 569B, whereas pCM302-16, a derivative of pCM302, only harbours the *V. cholerae ppdA*. The numbers indicate the respective positions of the restriction enzyme sites.

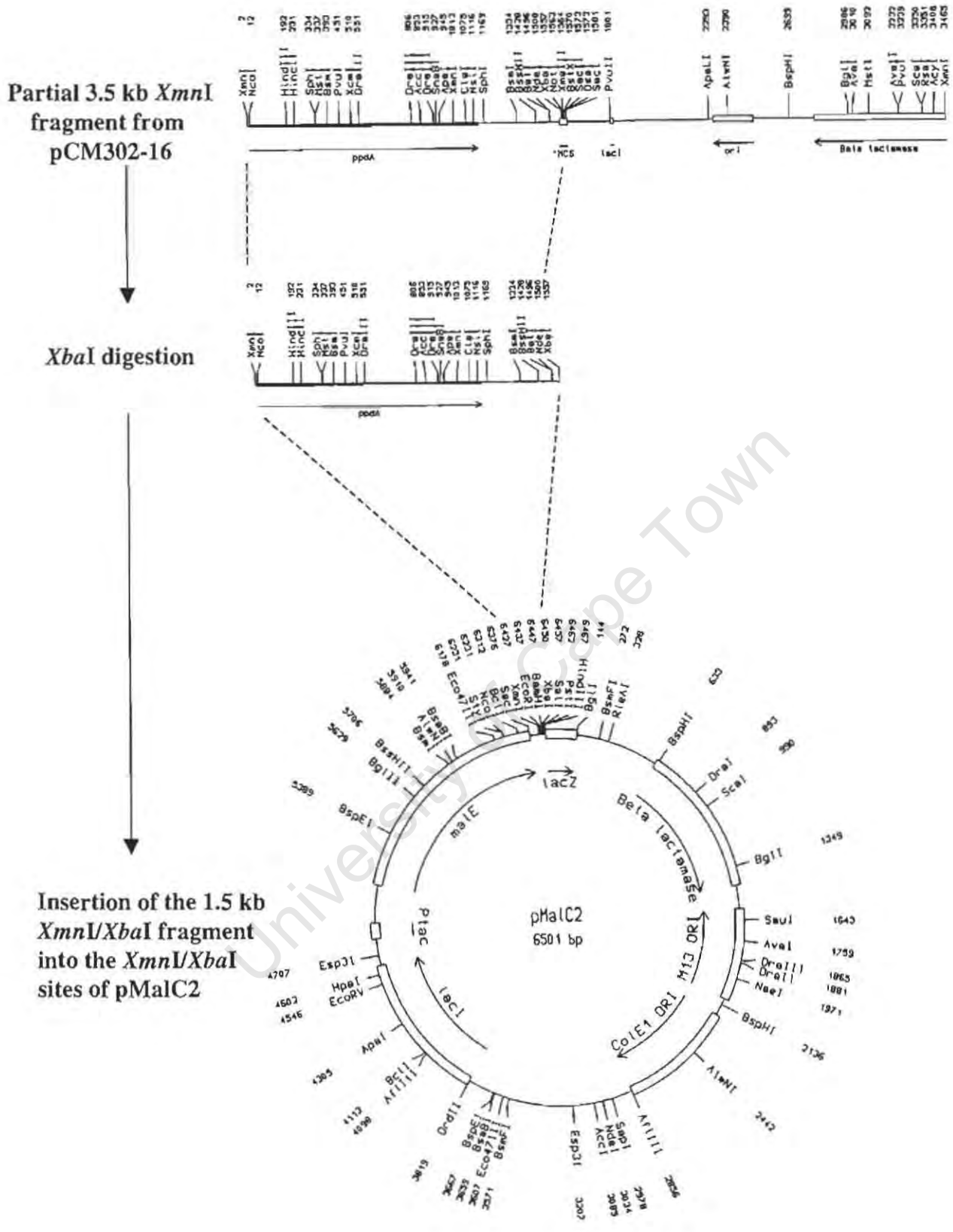
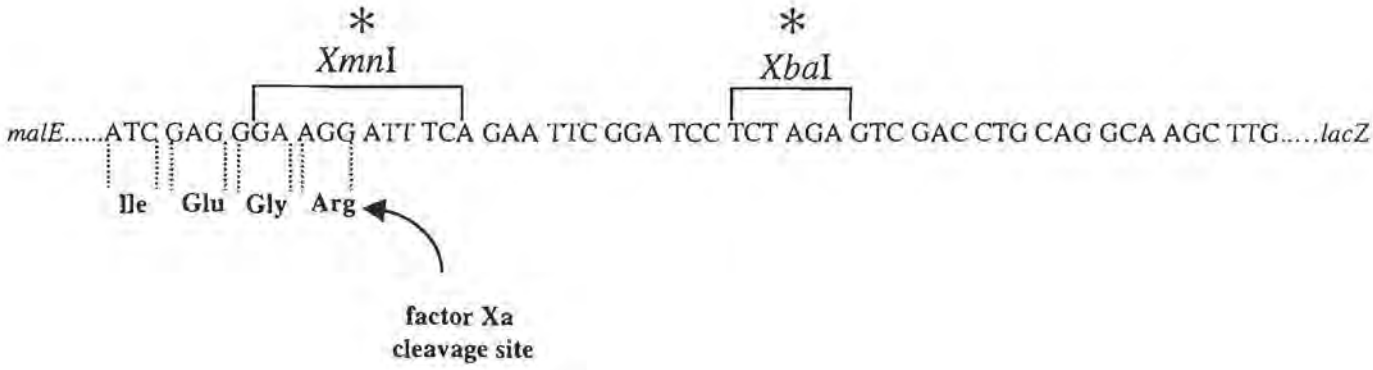


Figure 2. The subcloning of the partial *Xmn*I fragment (pCM302-16) into the *Xmn*I/*Xba*I restriction enzyme sites of the vector pMalC2. This strategy ensured that *ppd*A was inserted downstream and in frame to *mal*E. The position of the respective restriction enzyme sites are indicated by the numbers.

pMalC2 polylinker



pMalC2-PpdA

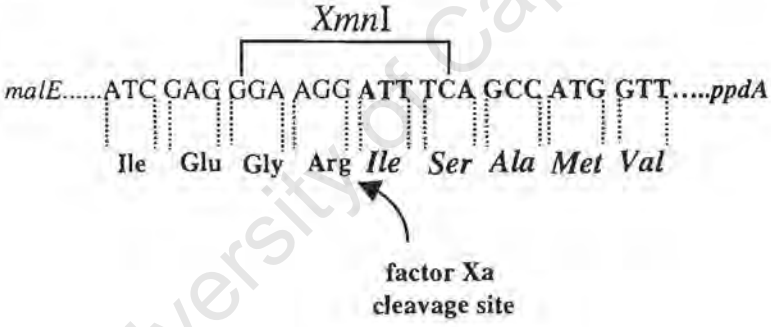


Figure 3. The pMalC2 polylinker provides several restriction enzyme nuclease sites for the insertion of a particular gene. We used the *XmnI*/*Xba*I (marked with asterix) sites to clone *ppdA* downstream and in frame to *malE*. This retained the factor Xa cleavage site between the two proteins shown in pMalC2-PpdA. The resulting fusion protein encoded for by pMalC2-PpdA is shown. The 3' amino acid sequence of MalE is indicated by the bold amino acids, and is directly followed by PpdA whose sequence of amino acids are shown in bold and italics.

3.2.4.2 Restriction enzyme analysis of pMalC2-PpdA

The DNA extracted from several white colonies was digested with various restriction enzymes to verify whether *ppdA* inserted into the correct position within pMalC (the DNA extraction and restriction enzyme digestion procedures are discussed in Appendix B.1 and B.5). Recombinant plasmids were cleaved with *Pst*I, *Sph*I, *Xba*I, *Cla*I, *Stu*I, *Bam*HI/*Cla*I, *Xba*I/*Nco*I, *Xba*I/*Sph*I, and the resulting fragments separated on 1% agarose gels as described in Appendix B.6.

3.2.4.3 Purification of the MalE-PpdA fusion protein

The *E. coli* clone harbouring the pMalC2-PpdA recombinant plasmid was grown at 37°C in 1 L of Luria broth (LB) containing glucose (0.2%) and ampicillin (100 ug/ml). The growth of the culture was monitored by reading the optical density (OD) at 600 nm. At an OD of 0.5, which corresponded to approximately 2 x 10^8 cells/ml, 3 ml of IPTG (1 M) was added to the culture media. This induced transcription from the P_{tac} promoter, and consequently, expression of the *malE-ppdA* fusion. The culture was incubated for an additional 2 hrs at 37°C before the cells were harvested (4 200 rpm for 20 min at 4°C) and the pellets resuspended in 50 ml of column buffer (Appendix A.2.18). To improve lysis, cells were incubated at -20°C overnight.

The cells were sonicated 3x 30 seconds with 45 second intervals on ice. In order to remove cell debris, 5 M NaCl to a final concentration of 0.5 M was added to the sonicated cells before centrifuging at 10 000 rpm for 30 min and discarding the pellet.

An amylose resin was prepared by adding 50 ml of column buffer to 15 g of resin. The resin was allowed to swell for 30 min before addition to a 2.5 x 10 cm column. After packing the column, the column was washed with eight volumes of column buffer containing 0.25% Tween 20. Diluted crude extract (1:5 in column buffer) was added to the column at a flow rate of 10x (diameter of column)^2 ml/hr. This corresponded to approximately 1 ml/min. The column was then washed with 12 volumes of column buffer containing 0.25% Tween 20. This was followed by a single wash with 5 volumes of column buffer. Fusion protein was eluted with column buffer containing 10 mM maltose. Fractions (1 ml) were collected which were then assayed for the presence of protein using the Bradford protein assay (Appendix B.16). Protein containing fractions were pooled and dialyzed against 400 volumes 10 mM Tris, 100 mM NaCl and 1 mM EDTA at 4°C overnight. This step removed excess maltose from the fusion protein. The dialyzed protein was concentrated with an Amicon Centricon filter system to a final concentration of 1 mg/ml.

3.2.4.4 Purification of PpdA

In order to separate PpdA from the MalE protein, purified fusion protein was cleaved with factor Xa protease. This protease cleaves after the arginine residue in the amino acid sequence Ile-Glu-Gly-Arg, thus cleaving MalE from PpdA (Fig. 3). We added 20 units of factor Xa to 20 ug of fusion protein and incubated the reaction mix at room temperature overnight. The cleaved products were diluted five fold in column buffer before addition to the amylose column. The eluant containing pure PpdA was collected in 1 ml fractions. The column was then washed with three column volumes of column buffer and the resulting eluant was collected as before. Bradford assays established which fractions contained the purified protein. These fractions were pooled and the purified protein concentrated by filtration through a PM10 Amicon filter to a final concentration of 160 ng/ul.

3.2.4.5 Western blot analysis of purified PpdA

To test whether the purified PpdA protein was indeed purified from MalE, we separated both PpdA and MalE proteins on 12% SDS-PAGE gels as described in Appendix B.14. The resulting proteins were transferred to a nitrocellulose membrane using the Hoeffer electroblotting apparatus as outlined in Appendix B.15. Western blot analysis was performed as follows: To prevent non-specific binding of antisera, the membrane was blocked by immersion in 100 ml blocking solution (1 g instant non-fat dried milk in PBS) for 2 hrs at room temperature. Anti-MalE antibodies were diluted 1:5000 in blocking solution and incubated with the membrane for 1 hr at room temperature. The membrane was washed 3x in 100 ml PBS for 15 min per cycle. Diluted alkaline phosphatase-goat anti rabbit IgG conjugate supplied by Sigma (diluted 1:5000 in blocking solution), was added to the membrane and incubated for 1 hr at room temperature. The membrane was washed 3x in 100 ml PBS as described previously. Freshly prepared substrate consisting of Nitroblue tetrazolium (75 mg/ml) and 5-Bromo-4-chloro-3-indolyl phosphate (50 mg/ml) in 100 mM Tris-HCl, 100mM NaCl and 5 mM $MgSO_4$, pH 9.2, was added to the membrane. Colour development was allowed to proceed for two to three minutes. The reaction was stopped by rinsing the membrane in water.

3.2.4.6 Antiserum production against purified PpdA

Antisera against purified PpdA protein was obtained by immunizing a rabbit with 100 ug of PpdA together with Freund's incomplete adjuvant. Three injections containing 100 ug PpdA each were given intravenously every 14 days over a period of six weeks. Seven days after the last injection the rabbit was bled to obtain serum containing polyclonal antibodies against PpdA.

3.2.5 PpdA synthesis in wild-type *V. cholerae* 569B

To monitor *ppdA* expression in *V. cholerae*, wild-type bacteria were grown at 37°C in Tryptone broth (TB) supplemented with 4% NaCl. A 5 ml overnight culture was prepared in TB at 30°C. This was used to inoculate a 250 ml flask containing 50 ml of media to a final OD_{600} of 0.05. Of this, 10 ml was used for RNA extractions and 10 ml for crude protein extraction at the various time points. These conditions were previously shown to result in pigment secretion into the culture media by *V. cholerae* (Coyne and Al-Harthi, 1992). Since Hppd production in other bacterial systems has been linked to the appearance of pigment in the culture media (Wintermeyer *et al.*, 1994; Chang *et al.*, 1997), we tested whether the time at which *ppdA* expression was induced could be correlated to pigment synthesis in cultures of *V. cholerae*. Thus, under these pigment-inducing culture conditions, we extracted RNA (Chapter 2 section 2.2.7) and obtained crude cellular extracts (section 3.2.5.1) from 20 ml aliquots sampled from the respective cultures over a 70 hr time space. In addition, we recorded the times at which pigmentation occurred in these cultures. Cellular extracts were used in Elisa assays to detect the presence of the PpdA protein, and purified RNA served as template for cDNA synthesis in RT-PCR.

3.2.5.1 Protein extraction for Elisa assays to determine the presence of PpdA

Total crude protein was isolated from *V. cholerae* cells as follows: Cells were collected by centrifugation (10 000 rpm for 10 min) and the pellets resuspended in 1.5 ml PBS (Appendix A.2.17). The cells were lysed by sonication for 3x 30 seconds with 45 second intervals on ice. Cell debris was removed by centrifugation at 10 000 rpm for 10 min. Cell extracts were serially diluted two fold in PBS and used in Elisa assays in order to detect the presence of PpdA.

3.2.5.2 Elisa assays

Indirect Elisa assays were performed to detect PpdA within the crude cellular extracts obtained from *V. cholerae* cells. Diluted extracts (100 ul) were added to microtitre plates and incubated at 37°C for 1 hr. The plates were washed 3x with PBS containing 0.05% Tween 20 for 5 min each. In order to minimize non-specific interaction between antibodies and cellular extracts, the plates were flooded with PBS containing 4% skim milk and incubated at room temperature for 15 min. The plates were beaten dry on paper towel before 100 ul of diluted anti-PpdA (1:100) was added to each well. The antibody was diluted in PBS containing 4% skim milk. The plates were then incubated at room temperature for 1 hr before they were washed in PBS containing 0.02% Tween 20 as described above. Alkaline phosphatase-goat anti rabbit IgG conjugate (diluted 1:1000 in PBS containing 4% skim milk) was added to the wells and the plates incubated at 4°C overnight.

The plates were washed 3x with PBS, before 150 ul 1 mg/ml 4-nitrophenyl-phosphate disodium salt in 10% diethanolamine was added to the wells. Colour reactions were allowed to proceed for no longer than 10 mins and the reactions were then stopped by the addition of 50 ul 3 M NaOH. The absorbance at 405 nm for each well in the microtitre plate was read using the Titertek Multiplus Elisa scanner. A standard curve (PpdA concentrations vs absorbance at 405 nm) was plotted and used to determine the concentration of PpdA that was present in each sample. As a control, we performed indirect Elisa assays on *V. cholerae* extracts using anti-MalE.

3.2.5.3 Reverse transcriptase PCR analysis (RT-PCR) of *ppdA* transcripts

Total RNA extracted from exponentially grown *V. cholerae* cells, and from cells grown for 39-50 hrs under pigment inducing conditions, was used to monitor *ppdA* transcription. The RNA extraction procedure that was used is outlined in Chapter 2 section 2.2.7. In order to detect the *ppdA* transcripts, 1 ug of purified RNA was analysed by RT-PCR using the Promega Access Reverse transcription kit. The primer 5' CCCTGACGATACAGCCAAGC 3' (247 bp downstream from the transcriptional start site of *ppdA* at position 404 in the *ppdA* sequence (Fig. 10)) was used for reverse transcription of the *ppdA* transcript. This primer, together with a second primer 5' CCCACTCGGTACAGATGGATTTG 3' (121 bp downstream from the transcriptional start site of *ppdA* at position 251 in the *ppdA* sequence (Fig. 10)), served as the forward and reverse primers for PCR amplification of the *ppdA* cDNA, respectively. The size of the PCR product was expected to be 150 bp in length. Reverse transcription was performed at 48°C for 45 min using AMV reverse transcriptase. The PCR conditions were as follows: denaturation was performed at 94°C for 45 secs, followed by an annealing step at 50°C for 1 min, followed by extension at 72°C for 1 min. The PCR was performed for 40 cycles using *Tfl* DNA polymerase. Negative controls were prepared for each sample which entailed the PCR of RNA without any prior reverse transcription. These controls were crucial for detection of DNA contamination within the RNA samples. A positive control, consisting of *V. cholerae* DNA, was included in the PCR reactions to verify the size of the amplification products that resulted from RT-PCR. In addition, we included a negative PCR control in which template was not added to the reaction tube in order to ensure that amplification products were not a result of contamination. Finally, we checked the validity of the PCR products by restriction enzyme digestion with the restriction enzyme *Hind*III since this enzyme should cleave the predicted amplification product once. PCR products, as well as the restriction enzyme digestion products, were separated on a 4.5% agarose gel as described in Appendix B.6.

3.3 Results

3.3.1 Fluorescent and haemolytic activities of PpdA

E. coli clones harbouring the recombinant plasmids pCM302 and pCM302-16 secreted copious amounts of brown pigment when grown in LB supplemented with L-tyrosine. Culture media from the pigmented clones were exposed to long wavelength UV light for 1 min to test for the presence of yellow-green fluorescent activity. Unfortunately, we were unable to detect any such fluorescence.

We tested the haemolytic activities of *E. coli* clones harbouring *ppdA* by loading crude cell extracts, prepared from the clones which had been grown under melanin-inducing conditions, into wells on Blood agar containing 5% human erythrocytes. *E. coli* clones harbouring pCM302 and pCM302-16, which both harboured *ppdA*, displayed haemolytic activity (Fig. 4A and 4B). Haemolytic zones appeared green in colour indicating the presence of α-haemolysis as opposed to β-haemolysis which results in complete hydrolysis of erythrocytes. In addition, the *E. coli* clone that harboured pCM302 displayed more haemolytic activity than *E. coli* harbouring pCM302-16, even though both plasmids harboured the full length *ppdA* gene. This was illustrated by the larger lysis zones produced by pCM302 cellular extracts, as well as the fact that the two-fold serial dilutions affected haemolytic activity to a much lesser extent in cell extracts prepared from *E. coli* containing pCM302 compared to that from clones harbouring pCM302-16 (Fig. 4A and 4B). Cellular extracts from *E. coli* harbouring pBluescript SK alone did not display haemolytic activity (Fig. 4C).

3.3.2 Determination of the size of PpdA

In vitro transcription/translation studies of pCM302-16 revealed the synthesis of a protein with a molecular mass of 41 kDa (Fig. 5). This is in agreement with the DNA sequence data of *ppdA* which predicts the synthesis of a 41 kDa protein from the 1.1 kb open reading frame. The 30 and 22 kDa proteins are the β-lactamase and β-galactosidase enzymes, respectively, which are encoded by genes carried on the pBluescript SK plasmid.

3.3.3 Purification of PpdA

In order to purify PpdA, we created a fusion protein between MalE and PpdA by cloning *ppdA* downstream of, and in frame to, the *malE* gene. The cloning of *ppdA* between *malE* and *lacZ* resulted in easily distinguishable white *E. coli* TB1 colonies when the transformants were

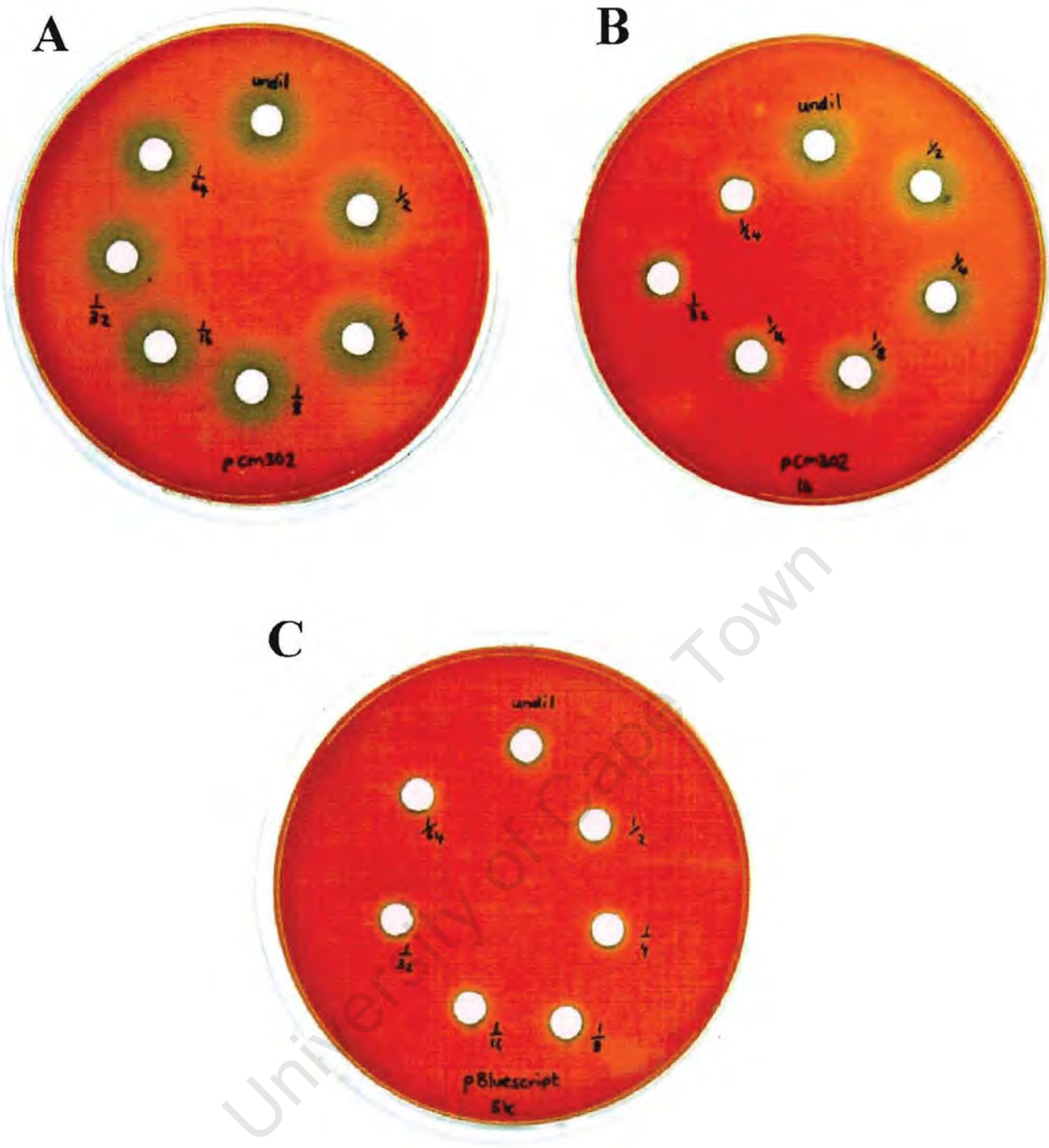


Figure 4. Haemolytic activity of extracts from *E. coli* expressing *ppdA*. Crude cell extracts of *E. coli* harbouring pCM302 (A) produced large haemolytic zones around the wells in the agar plate. The numbers seen next to the wells indicate the dilution factor for the various extracts. As can be seen in A, two fold dilutions of pCM302 cellular extracts did not significantly affect haemolytic activity. Total cellular extracts from *E. coli* harbouring pCM302-16 (B) produced smaller haemolytic zones and the activity was significantly reduced by the serial dilutions. Cellular extracts from *E. coli* harbouring pBluescript SK (C) did not exhibit haemolysis.

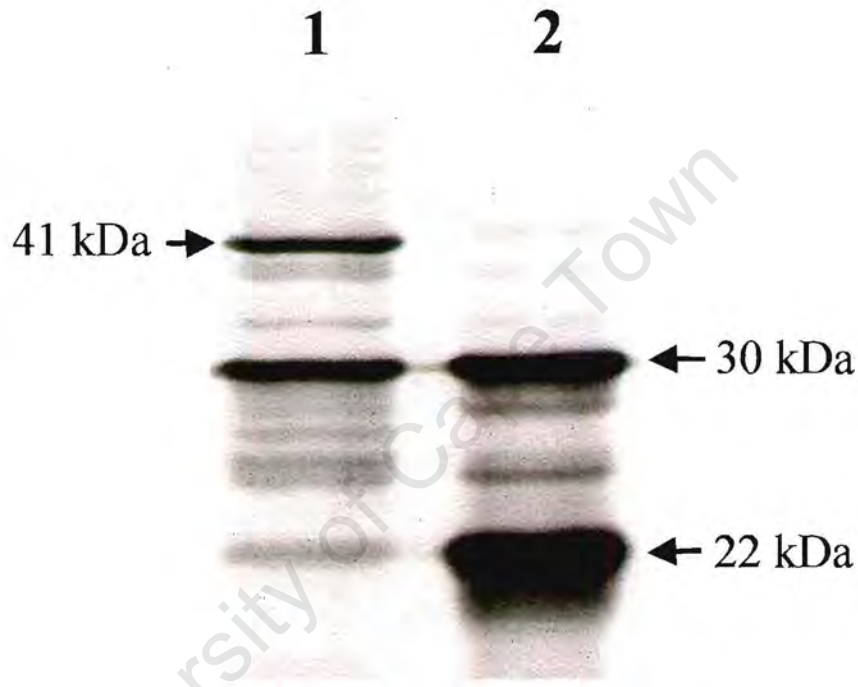


Figure 5. *In vitro* transcription / translation of pCM302-16
lane 1: protein product of *ppdA*, lane 2: pBluescript SK

selected on LB containing X-gal and ampicillin. Restriction enzyme analysis of the DNA isolated from several white clones confirmed the insertion of *ppdA* downstream of *malE* on pMalC2.

IPTG induction of *E. coli* TB1 containing pMalC2-PpdA resulted in the synthesis of large amounts of MalE-PpdA fusion protein which we purified on an amylose resin column. Purified fusion protein was cleaved with factor Xa and native PpdA was separated from MalE on a second amylose column. The size of the MalE and PpdA proteins were 40 kDa and 41 kDa, respectively. This made it difficult to distinguish the two proteins after separation on SDS-PAGE gels. Thus, in order ensure that PpdA was indeed purified from the MalE protein, we did Western blot analysis using anti-MalE antibodies. The results in figure 6B show that the anti-MalE antibodies cross-reacted very strongly to the MalE protein in lanes 3 and 4, and very weakly to the purified PpdA protein in lanes 1 and 2. This suggested that although PpdA was purified from MalE, a small amount of MalE was still present.

3.3.4 PpdA synthesis occurs in stationary phase

We analyzed PpdA synthesis in wild-type *V. cholerae* grown under pigment inducing conditions (TB supplemented with 4% NaCl at 37°C) using Elisa assays. Our results show that PpdA production occurred at 44 hrs of growth and corresponded directly to the appearance of brown pigment in the culture media (Fig. 7A). No PpdA protein could be detected in the cell extracts earlier than 43 hrs. High PpdA protein concentrations occurred between 48- and 58 hrs, after which the protein concentrations rapidly decreased (Fig. 7B). In addition, we were unable to detect any PpdA in exponential cultures of *V. cholerae* grown in TB at 30°C and TB supplemented with 4% NaCl at 37°C. In addition, we observed no cross reaction between anti-MalE and any of the *V. cholerae* cell extracts obtained from the various culture conditions.

3.3.5 Analysis of *ppdA* gene expression

RT-PCR was used to detect the presence of *ppdA* transcripts in *V. cholerae* grown under pigment inducing conditions. The reason why we employed RT-PCR rather than Northern blot analysis was due to the fact that the RNA isolated during late stationary phase was of very poor quality and as a result we were unable to detect any intact *ppdA* transcripts. The RNA we used for the RT-PCR was extracted from the same culture used to measure PpdA synthesis using Elisa (3.3.4). Interestingly, the expected 150 bp fragment was detected in all samples prepared from *V. cholerae* grown for 39- to 50 hrs, suggesting the presence of *ppdA* transcripts in *V. cholerae*

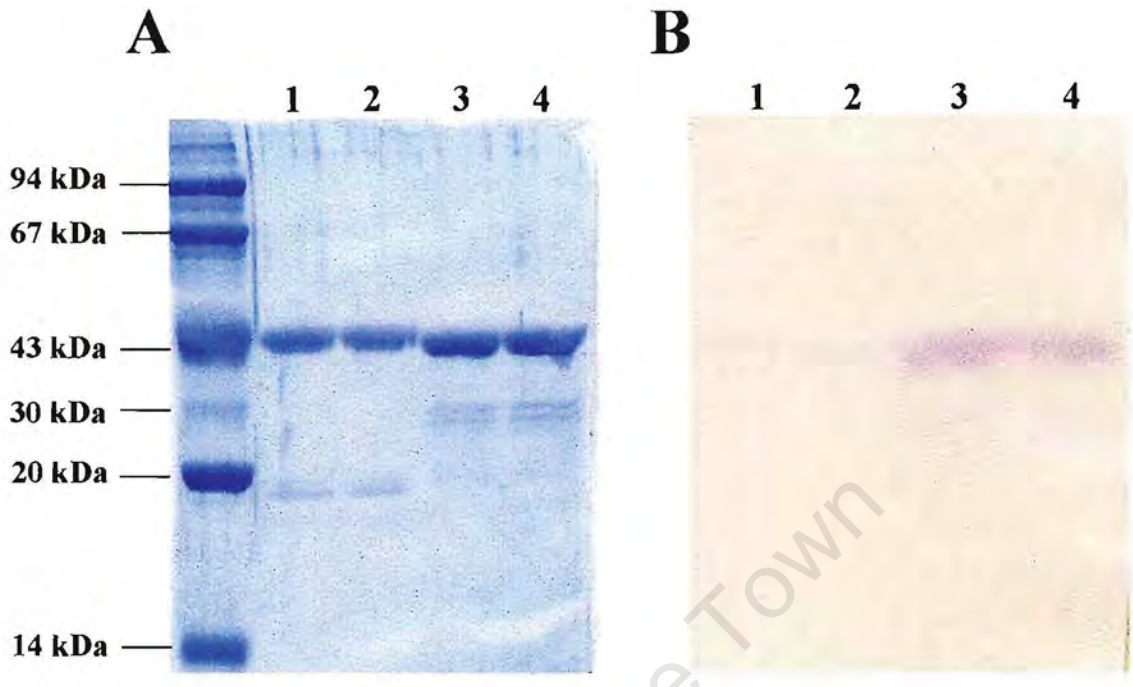


Figure 6. Purified PpdA and pure MalE were separated on a 12% SDS-PAGE gel (A). Lanes 1 and 2 contain the pure PpdA protein, whereas lanes 3 and 4 contain pure MalE. Panel B, shows theWestern blot of the PAGE gel depicted in (A) where MalE antibodies were used to assay the purity of PpdA. The antibody reacted strongly to the protein in lanes 3 and 4 and weakly to the PpdA protein in lanes 1 and 2.

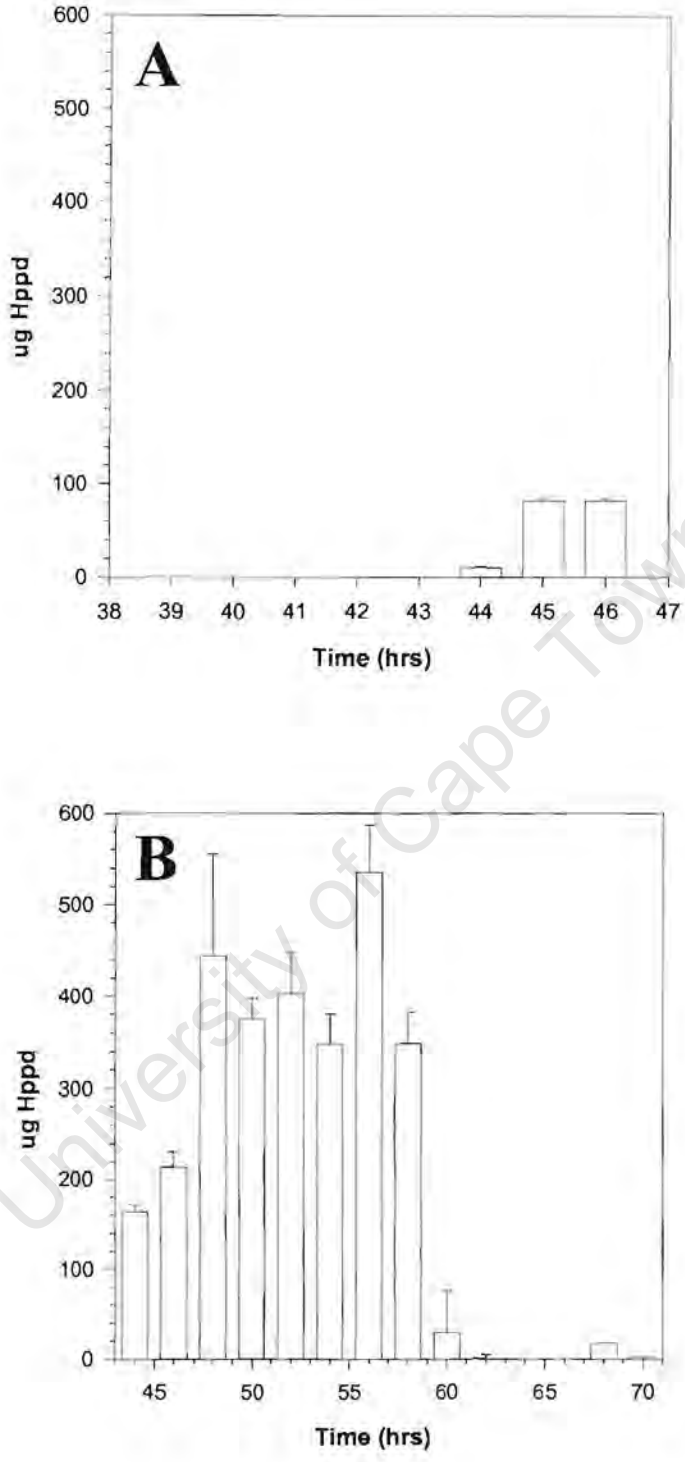


Figure 7. Elisa assays showing the time at which PpdA expression occurs in *V. cholerae* grown in TB containing tyrosine and 4% NaCl at 37°C. A, shows protein expression between 39-and 46 hrs, whereas B represents protein expression between 44- and 70 hrs. The results are averages between three experiments and the error bars depict the standard error between these results.

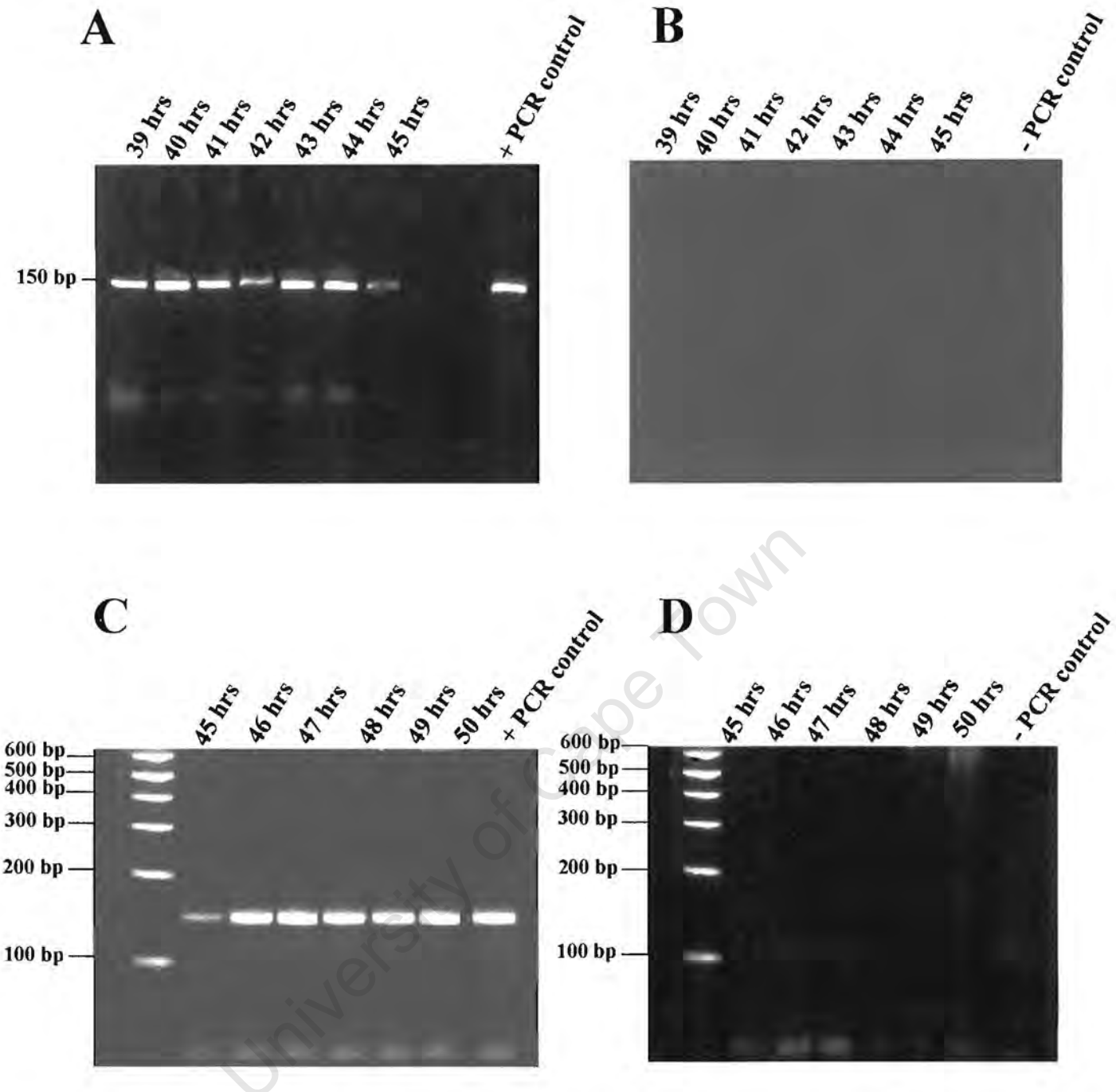


Figure 8. RT-PCR analysis of RNA extracted from *V. cholerae* grown for various times in Tryptone broth supplemented with 4% NaCl at 37^{0}C.
A. RT-PCR of RNA extracted from *V. cholerae* cells grown from 39 to 45 hr under pigment inducing conditions.
C. RT-PCR of RNA isolated from *V. cholerae* cells that were grown from 45to 50 hr under pigment inducing conditions.
B and D. PCR amplification of RNA samples shown in A and C (no reverse transcriptase) to ensure that the bands observed in A and C are not due to DNA contamination.
Positive PCR controls included *V. cholerae* genomic DNA as template, whereas the negative PCR controls lacked a RNA or DNA template.

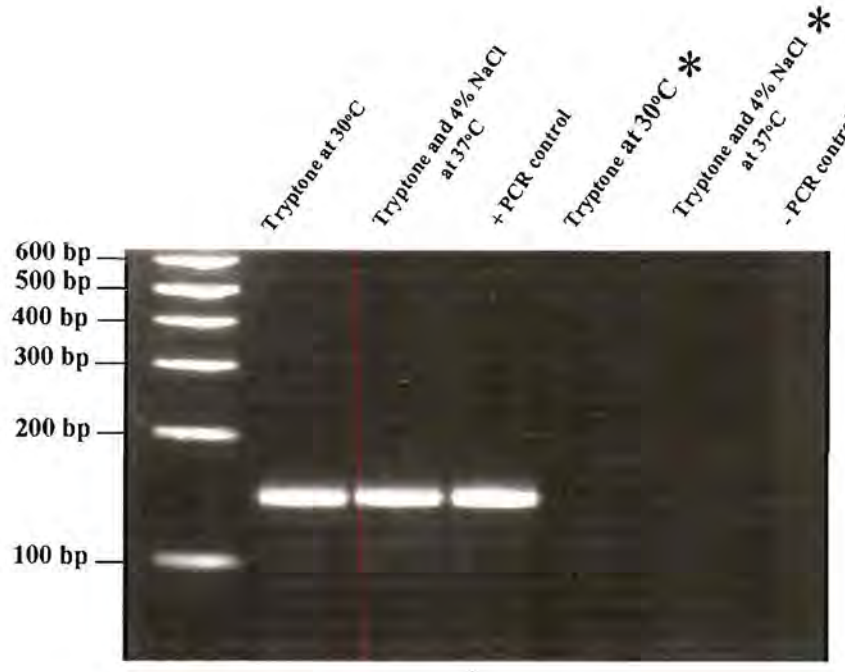


Figure 9. RT-PCR analysis of RNA extracted from *V. cholerae* during exponential growth (4 hrs after inoculation) from Tryptone at 30°C (non pigment-inducing) and Tryptone containing 4% NaCl at 37°C. The lanes marked with asterisks represent RT-PCR negative controls which are necessary to confirm the absence of DNA contamination. *V. cholerae* genomic DNA was used as the positive PCR control, so as to verify the size of the amplification product. A PCR negative control containing no template RNA or DNA was included to confirm the absence of any contamination.

Figure 10. *Hind*III restriction enzyme digestion of RT-PCR products obtained from the various RNA samples isolated at various times from a *V. cholerae* culture grown under pigment inducing conditions. The amplification product from *V. cholerae* genomic DNA (positive PCR control) was also *Hind*III digested (lane 9) while an undigested sample is in lane10.

sampled at all these time points (Fig. 8A and 8C). Furthermore, RT-PCR of RNA extracted from *V. cholerae* grown to exponential phase in TB at 30°C and TB supplemented with 4% NaCl at 37°C also detected the presence of *ppdA* transcripts (Fig. 9). All controls in which reverse transcriptase was omitted were negative (Figs. 8B, 8D and 9). Thus, it appears as if *ppdA* transcripts are present throughout the *V. cholerae* 569B growth cycle even though the PpdA protein can only be detected after 45 hrs of growth.

To confirm that the correct PCR fragment had been amplified, we tested the validity of the PCR products by restriction enzyme digestion using *Hind*III restriction enzyme. All PCR fragments, including the positive control, were cleaved by the restriction enzyme as seen in figure 10. The uncut amplification product is in the "+ PCR control" lane. Since we did not load similar amounts of DNA in these lanes, the intensity of the DNA bands cannot be compared.

3.4 Discussion

Characterization of the *V. cholerae* Hppd, namely PpdA, revealed that, although it is very similar in amino acid composition to legiolysin of *L. pneumophila,* the proteins differed in two respects. Firstly, PpdA was unable to confer fluorescence to the culture media as is the case with legiolysin. Secondly, PpdA displayed α-haemolytic activity which is characterized by greenish zones due to partial haemolysis of erythrocytes, compared to β-haemolysis characterized by a clear zone or complete lysis on the blood agar plate. The observation that the *E. coli* clones harbouring pCM302 and PCM302-16 displayed significantly different amounts of haemolytic activity is interesting. One reason could be due to the presence of additional DNA upstream of *ppdA* on pCM302. Thus, pCM302 may harbour a transcriptional enhancer sequence upstream from *ppdA* which can be recognized by the *E. coli* transcriptional machinery and therefore results in elevated *ppdA* expression in these clones compared to those harbouring pCM302-16. Alternatively, since pCM302 also harbours a gene with significant homology to peptidases, it is possible that this putative peptidase could affect the activity of PpdA *in trans*. Numerous peptidases have been implicated as activators of enzyme activity by cleavage of precursor molecules (Julius *et al.*, 1984; Francetic *et al.*, 1998). However, we do not yet know whether this is the case with PpdA, since no obvious signal sequence was observed at the N-terminal domain of the predicted protein sequence of PpdA that could serve as a cleavage site for this peptidase. Furthermore, *E.coli* clones harbouring pCM302-16 still produced haemolytically active PpdA even in the absence of the peptidase, suggesting an alternative reason for the observed difference in expression.

The significance of the haemolytic properties of PpdA is still unclear. Between 1989 and 1991, 31 patients infected with *V. cholerae* O1 strains that lacked the genes encoding enterotoxin, but still retained their haemolytic activity, were identified (Zitzer *et al.*, 1997). Furthermore, non-O1 strains that do not produce enterotoxin are haemolytic and have been recognized as important causes of diarrhoeal diseases world-wide (Blake *et al.*, 1980). Thus, the haemolytic phenotype is indeed an indicator of virulence in non-cholera toxin producing *V. cholerae*.

In both O1- and non-O1 strains of *V. cholerae*, the gene responsible for haemolysin production was identified as *hlyA* (Brown and Manning, 1985). In contrast to the watery fluid produced in response to the cholera toxin, the fluid produced in response to the HlyA haemolysin was invariably bloody (Ichinose *et al.*, 1987). HlyA is initially synthesized as an inactive 82 kDa

protein that is subsequently processed to a 65 kDa active cytolysin (Yamamoto *et al.*, 1990). The haemolysin binds to intestinal cells as monomers which assemble into detergent-stable tetra- or pentamers (Zitzer *et al.*, 1997; Ikigai *et al.*, 1997). Oligomer formation is accompanied by the generation of small pores in the membrane of the intestinal cell which allow rapid flux of K^+ but not an influx of Ca^{2+}. Pore formation results in irreversible ATP depletion and consequently death of the cell (Zitzer *et al.*, 1997). Although it has been proposed that the death of the intestinal cell could be the possible cause of the diarrhoea, a pathogenic role for HlyA has not yet been clearly established.

In the classical strain *V. cholerae* strain 569B, an 11 bp deletion has been identified in the *hlyA* gene that results in the synthesis of a truncated HlyA protein of 27 kDa (Alm *et al.*, 1988). This protein lacked the ability to lyse erythrocytes and therefore lacked haemolytic activity. The protein, however, retained enterotoxic activity which meant that it retained the ability to induce fluid accumulation in ileal loops. This then lead to the proposal that HlyA may be a bifunctional protein with the N-terminus carrying the enterotoxic activity and the C-terminus being the haemolysin (Alm *et al.*, 1991). PpdA could therefore constitute an additional haemolysin which could possibly complement the defect in HlyA in *V. cholerae* 569B.

In vitro transcription analysis confirmed the size of the PpdA protein as 41 kDa, which is in agreement with the DNA sequence data and falls into the molecular mass range reported for the Hppd enzymes that have been characterized to date. Whether PpdA exists in a dimeric or tetrameric state remains to be determined. A tetrameric configuration would strengthen the role of PpdA as a haemolysin, as haemolysins exhibit their effects on erythrocytes through their ability to form pores within the membrane of the cells (Sugawara *et al.*, 1997; Zitzer *et al.*, 1997).

Pigmentation in *V. cholerae* has been associated with certain culture conditions (Coyne and Al-Harthi, 1991). These conditions include elevated temperature of 30°C and above, in conjunction with increased salainity. Pigmentation occurs at lower salinities when the bacterium is subjected to additional stress factors such as low organic nutrients or low pH. Elisa assays confirmed that PpdA production directly correlated with the appearance of brown pigment in the culture media. PpdA protein synthesis increased to a maximal level around 50 hrs of growth. No protein could be detected before 43 hrs and when *V. cholerae* was grown to mid-exponential phase in either TB at 30°C or TB containing 4% NaCl at 37°C. This confirmed that PpdA production occurs

very late in stationary phase and that no protein is produced during the earlier stages of bacterial growth. The importance of PpdA in terms of its function in *V. cholerae* is therefore most probably linked to environmental conditions associated with late stationary phase. The putative promoter region identified for *ppdA,* however, displayed no homology to the tentative -35 and -10 consensus sequences identified for σ^s -regulated promoters (Lange and Hengge-Aronis, 1991).

Contrary to the Elisa assays, RT-PCR identified the presence of *ppdA* transcripts in all *V. cholerae* samples, even those grown under culture conditions where no PpdA protein could be detected, suggesting that *ppdA* is constitutively expressed. Since we were unable to detect any PpdA protein earlier than 45 hrs, our results suggest that the *ppdA* mRNA might be extremely unstable and is therefore degraded before it can be translated into functional PpdA protein. Alternatively, control of PpdA synthesis could occur at the posttranslational level, whereby PpdA synthesized during the earlier stages of growth is degraded rapidly and only once a certain set of conditions are met does effective stabilization of the protein occur in order for the protein to fulfil its function. This type of posttranslational control has been proposed to occur in *E. coli* in response to heat shock (Kanemori *et al.*, 1997). Under normal physiological conditions, ATP dependent proteases are responsible for the rapid degradation of both abnormal proteins as well as protein encoded for by the *rpo*H gene, namely σ^{32} (Chapter 4 section 4.1.1.2). Upon heat shock, the amount of misfolded proteins within the cell rapidly increases. This results in the indirect stabilization of the σ^{32} protein due to the fact that the proteases that are normally produced to degrade the sigma factor are diluted by an excess of misfolded proteins. The σ^{32} then becomes available to RNA polymerase for the transcription of the heat shock genes and thus allows induction of the heat shock response. Alternatively, unlike promoters which are uniformly accessible to RNA polymerase because they exist in the regular structure of duplex DNA, ribosomal binding sites may be trapped in the secondary structure into which most single stranded RNA molecules fall (Watson *et al.*, 1987). Thus, ribosomes will be unable to initiate protein synthesis from a *ppdA* transcript where the ribosomal binding site is inaccessible due to RNA folding. Only once the RNA structure is altered will translation be allowed to proceed. Such alteration could possibly occur once *V. cholerae* enters the late stationary phase of growth in order to allow synthesis of PpdA to occur. Indeed, the *ppdA* Shine Dalgarno sequence only weakly resemble the conventional AGGAGG Shine Dalgarno sequence, but it is difficult to conclude at this stage whether the folding of the RNA transcript is masking the sequence and

thus preventing access to ribosomes. Alternatively, increased ribosome stalling on the *ppdA* RNA could result in a decrease in translation from the downstream initiation codon and thus account for the absence of PpdA protein observed prior to 45 hr of growth. This type of translational attenuation has been demonstrated in several organisms including *Neurospora crassa* (Wang and Sachs, 1997) and *Bacillus subtilis* (Lee *et al.*, 1996), where the availability of tRNA molecules regulate translation of the mRNA by the ribosomes.

Finally, the fact that our results indicate similar levels of amplified product in cells grown under a variety of conditions, could be directly due to the sensitivity of the RT-PCR technique. In *S. colwelliana*, *melA* was indeed shown to be expressed constitutively even though melanin synthesis was dependent on the oxidation state of the culture medium (Fuqua and Weiner, 1992). Future work will therefore have to include quantitative RT-PCR to exclude the possibility of basal levels of *ppdA* transcription.

Whatever the role of PpdA is in *V. cholerae* 569B, its synthesis seems to be stationary phase specific under the culture conditions tested, and its appearance seems to be tightly linked to secretion of brown pigment into the culture media.

CHAPTER 4

THE EFFECT OF OXIDATIVE STRESS ON *V. cholerae* 569B

CONTENTS

Summary

In vitro mutagenesis of *V. cholerae* 569B and RM7 suggested that PpdA mediated melanogenesis in both these strains, since the resulting mutants were incapable of synthesizing pigment. Despite the lack of pigmentation, Mut2 and HX1 displayed no defect in their growth abilities compared to their parental strains. Analyzing the sensitivity of *V. cholerae* and Mut2 cells to 5 mM H_2O_2 showed that only after 2 days of growth under conditions of high salinity and temperature the wild-type strain was significantly more resistant to H_2O_2 compared to the mutant. This increased resistance correlated directly to the presence of copious amounts of pigment within the wild-type culture media, suggesting that the pigment was responsible for the difference in survival. In addition, we showed that prior exposure to heat did not make *V. cholerae* more resistant to subsequent oxidative stress, and that high salinity plays an important role in inducing cross protection mechanisms against various other stresses in *V. cholerae* 569B.

4.1 Introduction

Microorganisms have a limited capacity to control their environment, and consequently, respond to environmental changes by adjusting themselves both structurally and functionally. The ability to respond to changes in the environment is determined by the genome of the organism. Generally, microbes only express part of their genome to adjust to a certain set of conditions. The mechanisms whereby these responses enable the microorganism to cope with increased temperature, oxidative stress and salinity will be addressed.

4.1.1 The effect of elevated temperature on cells

Although bacteria can grow at a broader temperature range than most higher organisms, they remain sensitive to elevated temperature mainly due to the following reasons: During growth at elevated temperature, single and double stranded breaks occur in DNA (Pellon, 1983). The equivalent degree of DNA damage has not been observed at the same elevated temperatures *in vitro* (Woodcock and Grigg, 1972). This suggests that the *in vivo* process possibly leads to the activation of endonucleolytic activity which may be responsible for the damage. Furthermore, evidence also exists that DNA strand breakage occurs at growth permissive temperatures but that efficient repair processes possibly mask any outward signs of this damage (Woodcock and Grigg, 1972).

In addition to DNA damage, proteins and lipids are also affected by an increase in temperature (Neidhardt *et al.*, 1984). Denaturation of cellular proteins is induced, and the number of misfolded and unfolded proteins rapidly increases within the cell. The degree of saturation of the lipids that are incorporated into the outer and inner membranes of the cell envelope changes, with the result that membranes undergo a physical transition from a flexible fluidlike state to a more solid gel-like structure.

Thus, elevated temperature can be extremely harmful to bacterial cells and it is therefore not surprising that *E. coli* rapidly induces the expression of more than 20 genes upon exposure to elevated temperature to aid the cell in its survival (Bukau, 1993). This response is better known as the heat shock response.

4.1.1.1 Heat shock response

The induction of the *E. coli* heat shock response is mediated by the protein encoded by *rpoH*, namely σ^{32} (Bukau, 1993). Under normal physiological conditions, proteases such as HslVU

rapidly degrade any abnormal proteins within the cell as soon as they are synthesized (Kanemori *et al.*, 1997). In addition, HslVU is also responsible for the rapid turnover of the σ^{32} protein which results in extremely low (10-30 copies per cell) levels of σ^{32} (Kanemori *et al.*, 1997). However, when *E. coli* cells are exposed to high temperature (42°C), the amount of abnormal and misfolded protein rapidly increases within the cell (Bukau, 1993). The result is that the misfolded proteins rapidly bind and titrate HslVU and other proteases away from σ^{32}, resulting in its indirect stabilization (Kanemori *et al.*, 1997). Stabilized σ^{32} can then bind RNA polymerase and confers to the core RNA polymerase the specificity to transcribe the heat shock genes (Fig. 1).

Heat shock proteins (Hsps) fall into two main categories. The first class, which includes HtpG, DnaK, GroEL and GroES, are known as molecular chaperones (Hartl, 1996) (Fig. 1). These chaperones play a crucial role in protein folding and assembly during periods of elevated temperature. In the case of DnaK, two additional Hsps, DnaJ and GrpE are required to form functionally active chaperone machinery (Georgopoulos, 1992). Apart from having σ^{32} dependent promoters, *groEL*, *groES* and *grpE* have additional σ^{70}–dependent promoters that ensure a σ^{32} independent basal level expression (Zhou *et al.*, 1988).

The second class of heat shock proteins are the ATP-dependent proteases (Gottesman and Maurizi, 1992) (Fig. 1). Examples are Lon and ClpAP proteases which play major roles in proteolysis of any misfolded proteins that are synthesized during elevated temperature. Thus, by specifically inducing the genes involved in the heat shock response, the cell is able to survive by alleviating some of the harmful effects induced by elevated temperature.

4.1.2 The effect of oxidative stress on cells

Due to cellular respiration, active oxygen species occur naturally in aerobic cells. These radicals can cause a great deal of damage to macromolecules such as DNA, membranes, RNA and proteins (Farr and Kogoma, 1991). If this damage does not lead to radical induced death, at the very least, it wastes cell energy and resources since damaged molecules have to be repaired, degraded or replaced. It is therefore important that cells maintain a strong defence system against oxidative stress.

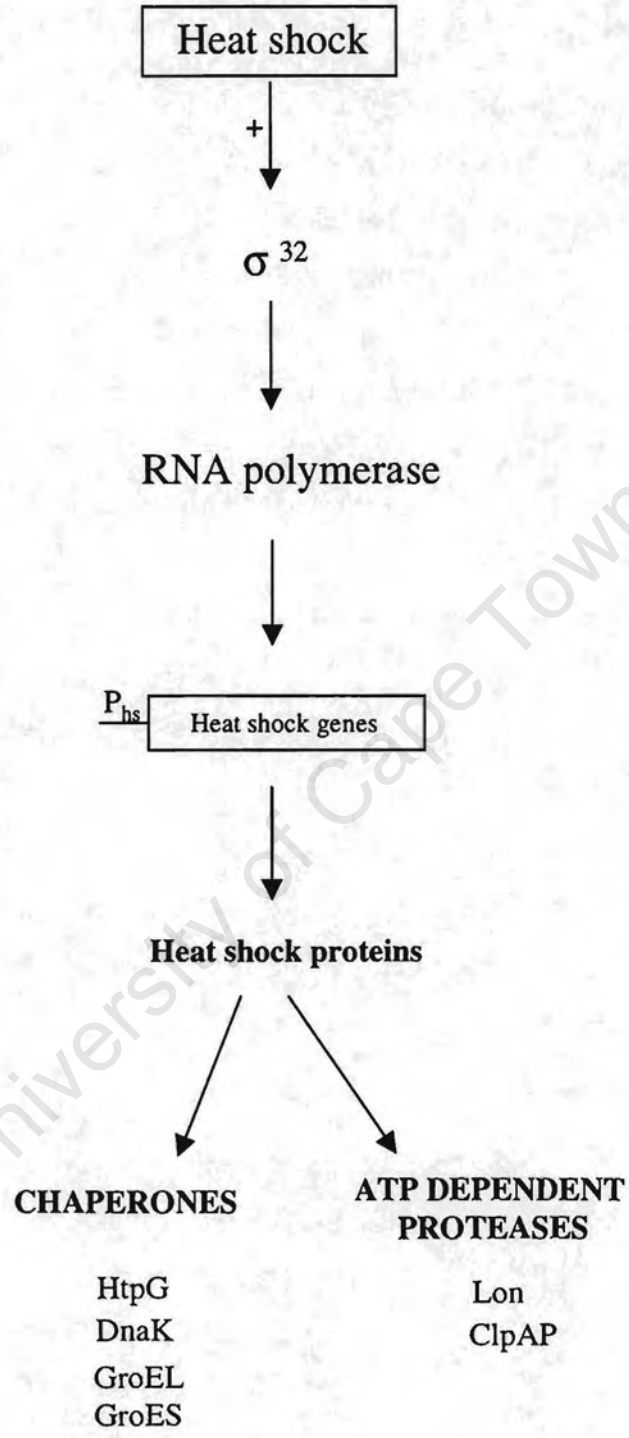


Figure 1. The heat shock response is mediated by σ^{32} which is directly involved in the activation of the heat shock proteins. The plus sign indicates the stabilization of σ^{32} due to tritration of HslVU.

In contrast to the heat shock response, bacteria respond to oxidative stress by invoking two distinct stress responses; the peroxide and the superoxide stress responses (Farr and Kogoma, 1991) (Fig. 2).

4.1.2.1. The peroxide stress response

The synthesis of 30 proteins are induced when *S. typhimurium* cells are exposed to H_2O_2 (Christman *et al.*, 1985). Nine of these proteins are positively regulated by OxyR, a protein encoded for by the *oxyR* gene. The genes encoding for these nine proteins includes *katG* and the *ahpCF* operon, and are collectively known as the OxyR regulon (Christman *et al.*, 1985) (Fig. 2). Upon oxidative stress, the increased flux of H_2O_2 converts OxyR protein to an oxidized form which interacts with RNA polymerase to activate transcription of the OxyR regulon (Storz *et al.*, 1990a). Thus, OxyR acts both as the sensor and transducer of the oxidative stress signal. The HP1 catalase encoded for by *katG* destroys H_2O_2 with remarkable rapidity, and the alkylhydroperoxide reductase (encoded for by *ahpC* and *ahpF*) provides additional defence by reducing various organic hydroperoxides (Farr and Kogoma, 1991).

In addition to catalase, the enzyme peroxidase is also able to destroy H_2O_2 (Fig. 2). Peroxidases, unlike catalases, require NADH or NADPH as an electron source (Farr and Kogoma, 1991). The electron source in the catalase reactions is from H_2O_2 itself, the reaction is exothermic and does not require ATP. Catalases therefore provide protection against H_2O_2 even in energy depleted cells, whereas the protective role of peroxidases, under conditions where reducing power is limited, is likely to be small (Farr and Kogoma, 1991).

4.1.2.2 The superoxide stress response

Aerobically growing *E. coli* cells are equipped with two superoxide dismutases (SODs); Mn-containing SOD (Mn-SOD, encoded by *sodA*) and Fe-containing SOD (Fe-SOD, encoded by *sodB*) (Farr and Kogoma, 1991) (Fig. 2). SODs are responsible for dismutating O_2^- to H_2O_2. In *E. coli*, more than 30 proteins are induced under O_2^- stress conditions. Most of these proteins form part of the SoxRS regulon which include Mn-SOD and Fe-SOD (Fig. 2). Two proteins, SoxR and SoxS are essential for the transcriptional activation of the SoxRS regulon in response to O_2^- stress (Tsavena and Weiss, 1990) (Fig. 2).

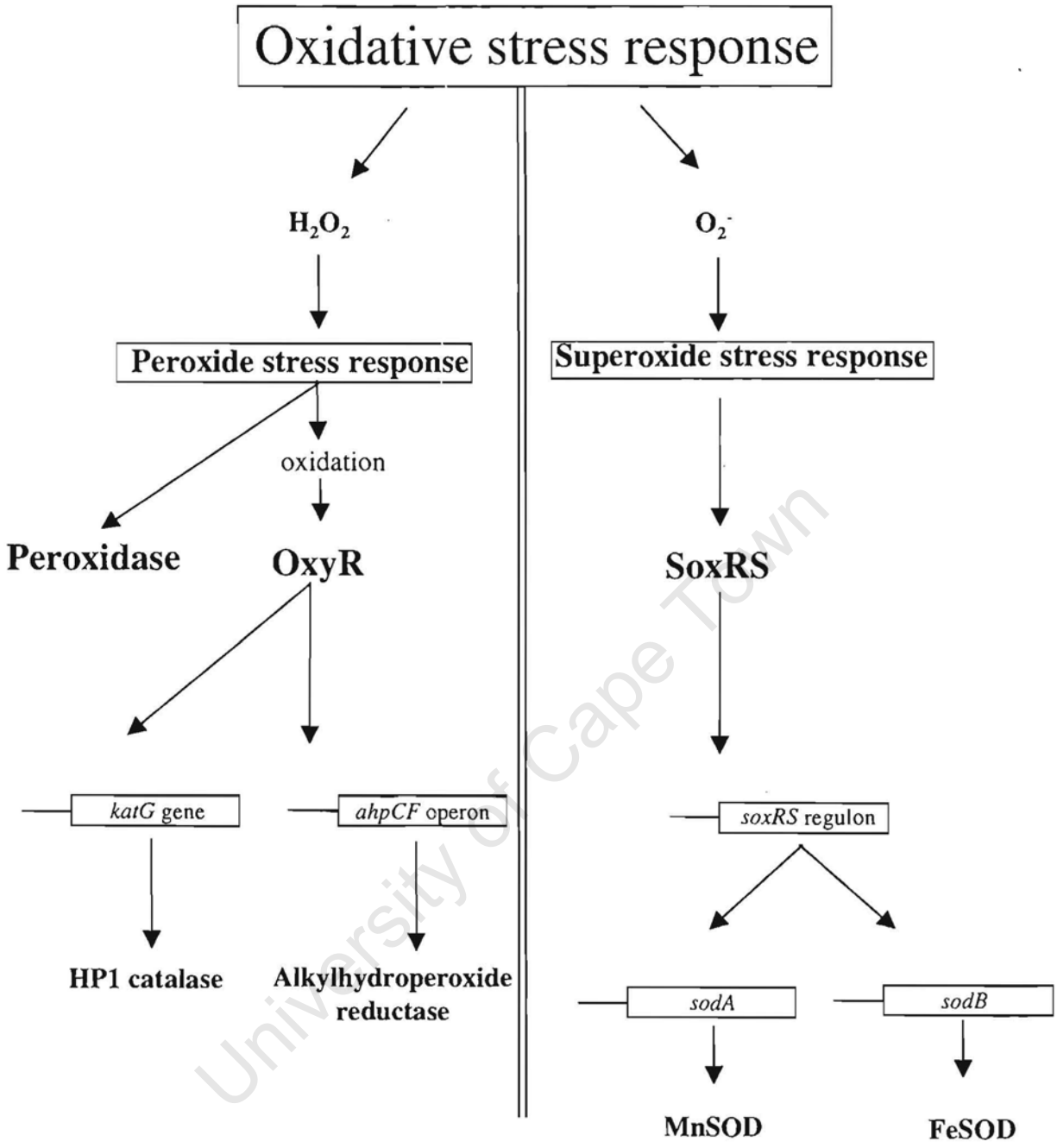


Figure 2. The two oxidative stress responses employed by aerobic microorganisms.

4.1.2.3 Role of heat shock proteins during oxidative stress

Interestingly, both peroxide and superoxide-mediated oxidative stresses have been shown to induce synthesis of the heat shock proteins GroES and GroEL, whereas DnaK is only induced by H_2O_2 (Morgan *et al.*, 1986; Walkup and Kogoma, 1989). This implies that these heat shock proteins also play a role in the oxidative stress response in *E. coli*. They might be necessary to handle the increased number of misfolded proteins resulting directly from damaged polypeptides or indirectly from mistranscribed or mistranslated genes (Farr and Kogoma, 1991). In addition, GroEL has been shown to facilitate export through the inner membrane, and thus increased levels of GroEL during oxidative stress may be required to compensate for damage sustained by the membrane export apparatus (Farr and Kogoma, 1991). Clearly, these observations indicate that several stress responses overlap.

4.1.3 The effect of salinity on cells

E. coli cells maintain a higher osmotic pressure in the cytoplasm compared to that of the surrounding media (Stock *et al.*, 1977). This results in an outward directed pressure known as turgor. Maintenance of turgor pressure is essential for cell division and growth (Meury, 1988). A sudden increase in the osmolarity of the growth media results in a rapid efflux of water from the cytoplasm and a concommitant loss of turgor. This can lead to plasmolysis which severely inhibits DNA replication, protein synthesis and finally, cell growth (Lucht and Bremer, 1994).

When *E. coli* cells experience a shift to high osmolarity, influx of potassium ions and the synthesis of glutamate are strongly stimulated (Csonga, 1989). This rapid response is followed by the accumulation of organic osmolytes. These compounds are polar, highly soluble and unlike most ions, they do not interfere with vital cellular functions. Furthermore, they protect the structure of proteins and other cellular components from denaturation in solutions of high ionic strength. Important examples of such compatible solutes are glycine betaine and the amino acid proline. With respect to regulatory mechanisms involved in osmoregulation, two systems have been studied in detail and are briefly discussed below.

4.1.3.1 The osmoregulatory mechanisms

The first osmoregulatory system involves the *proP* gene and the *proU* operon (Lucht and Bremer, 1994). These two transport systems mediate the uptake of organic osmoprotectants, including glycine betaine, in osmotically stressed cells. The *proP* encoded transporter consists of a single

polypeptide embedded in the cytoplasmic membrane (Culham *et al.*, 1993), whereas the *proU* operon (*proVWX*) encodes a multicomponent transport system (Gowrishankar, 1989).

The second defence against osmotic stress involves modulation of the OmpF/OmpC porins by the two component regulatory system OmpR-EnvZ (Mizuno and Mizushima, 1990). *E. coli* possesses two porin proteins, namely OmpF and OmpC. These proteins function as trimers to form pores in the outer membrane which allow passive diffusion of small hydrophilic molecules across the hydrophobic membrane barrier (Mizuno *et al.*, 1983). The pore formed by OmpF is slightly larger than the pore formed by OmpC and consequently, significantly faster rates of diffusion occur through an OmpF porin compared to an OmpC porin (Nikaido and Vaaro, 1987). Although the total amount of OmpF and OmpC remains constant in the outer membrane, their relative levels are regulated in a reciprocal manner by the two component regulatory system, OmpR and EnvZ (Pratt and Silhavy, 1995).

EnvZ is localized in the inner membrane in such a manner that its N-terminal domain extends into the periplasm and its C-terminal domain into the cytoplasm (Igo and Silhavy, 1988) (Fig. 3). It is the N-terminal region of the protein that functions directly to monitor the surrounding osmolarity. The nature of the periplasmic stimulus to which the N-teminal region of EnvZ responds during changes in osmolarity is unknown, ie. whether the stimulus is chemical (some small molecule) or mechanical (interaction with the cell wall) is still unclear (Pratt and Silhavy, 1995).

Under low osmolarity conditions, EnvZ exists in a phosphatase dominant state where the protein rapidly dephosphorylates the cytoplasmic regulatory protein, OmpR (Aiba *et al.*, 1989) (Fig. 3). It is the levels of phosphorylated OmpR which then dictates whether *ompF* or *ompR* expression is favoured. During periods of low osmolarity, transcriptional activator of *ompF* leads to OmpF-type porins dominating the outer membrane.

During elevated salinity, EnvZ is able to autophosphorylate itself at His-243 (Forst *et al.*, 1989). Autophosphorylation triggers the EnvZ kinase activity which overrides the protein's phosphatase activity. This results in a rapid transfer of the His-243 phosphate group to an Asp residue in OmpR (Aiba *et al.*, 1989). Phosphorylated OmpR then functions as a transcriptional repressor of *ompF* as well as a transcriptional activator of *ompC*. The net result is that OmpC type porins predominate within the outer membrane during high osmotic stress conditions. Thus, by

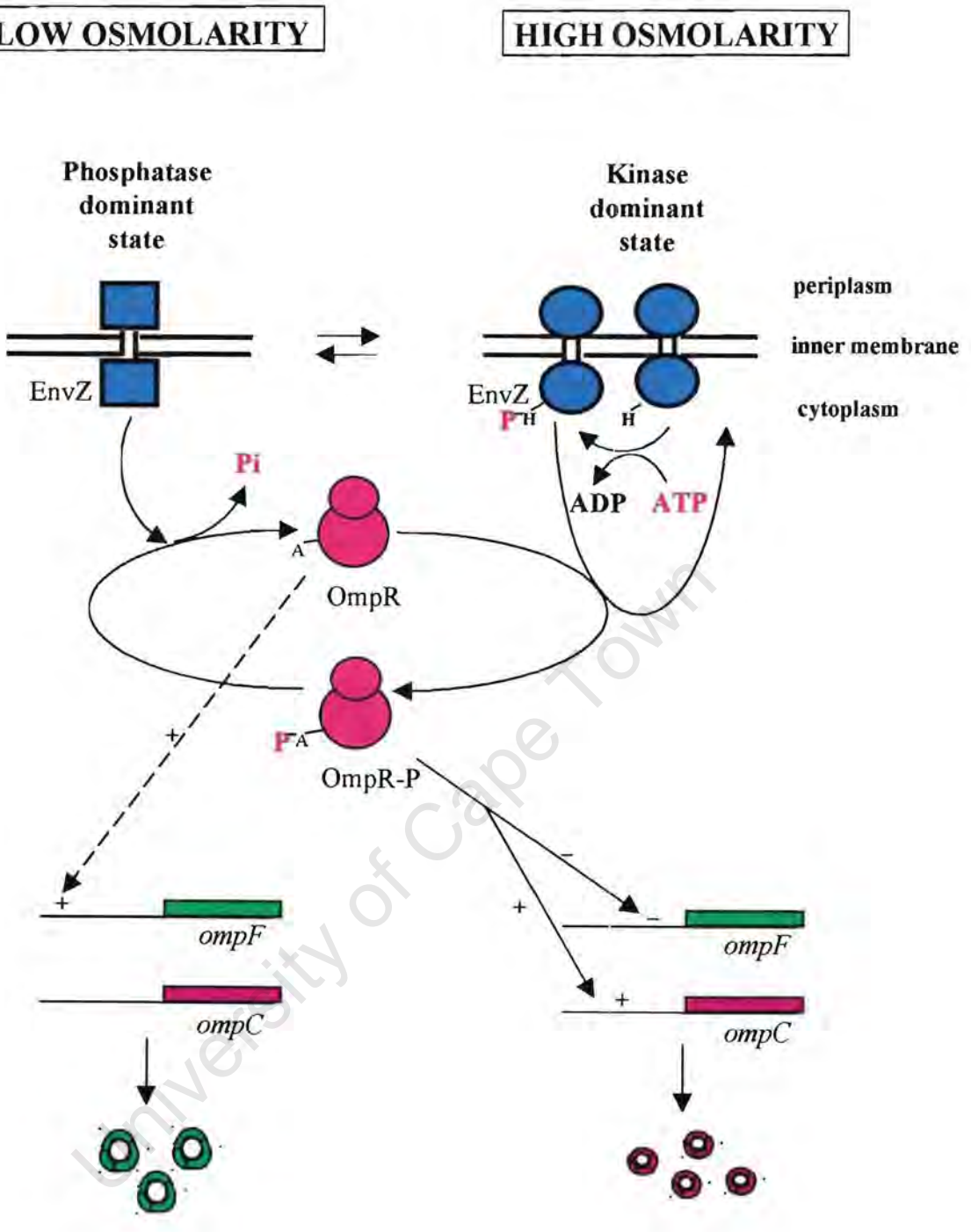


Figure 3. Regulation of porin expression in *E. coli* by the two component regulatory system, OmpR and EnvZ.

controlling the tendency of EnvZ to autophosphorylate, the cell regulates the two opposing enzymatic activities, namely phosphatase and kinase activity, of the EnvZ protein. Depending on the dominating enzyme activity of EnvZ, OmpR receives valuable information as to the osmotic state of the environment and can then respond appropriately. Although the above mentioned regulatory systems are effective in protecting the cells from osmotic stress, they do not seem to play a general role in the activation and regulation of other genes involved during osmotic stress. However, σ^S has recently been implicated as a mediator of several stress responses, including oxidative stress (Muffler *et al.*, 1996), and furthermore, might also serve as the primary inducer for osmoprotection. This, however, still needs to be tested.

4.1.4 σ^S, a mediator of the general stress response

During nutrient deprivation or stationary phase growth, *E. coli* cells induce the expression of an alternative sigma factor (σ^S) (Loewen and Triggs-Raine, 1984) (Fig. 4). This σ^S subunit of RNA polymerase controls the expression of more than 30 genes that are involved in starvation survival and multiple stress resistance during stationary phase (Lange *et al.*, 1995). σ^S is encoded for by the *rpoS* gene whose expression is induced during entry into stationary phase. Once induced, σ^S activates a large number of genes that are able to confer resistance to a number of different stresses. For example, σ^S has been shown to activate *otsBA*, *treA*, *osmB* and *osmY*. These genes are all involved in the protection of cells against osmotic stress during stationary phase. Furthermore, σ^S activates genes involved in the stationary phase oxidative stress response. These include *katE*, a HPII catalase which like KatG mediates the breakdown of H_2O_2 (Loewen *et al.*, 1985), *xthA*, an exonuclease III that is important in the repair of H_2O_2 mediated damage and *appA*, a gene encoding an acid phosphatase (Demple *et al.*, 1986; Touati and Danchin, 1987). In addition, three heat shock proteins (DnaK, GroEL and HtpG) have been shown to increase upon starvation, resulting in cells becoming resistant to elevated temperature (Jenkins *et al.*, 1991). Thus, stationary phase cells can develop tolerance to osmotic, oxidative, as well as, heat stress.

Recently, expression of *rpoS* was shown to occur in exponential phase cells in response to high osmolarity or heat shock (Muffler *et al.*, 1996; Muffler *et al.*, 1997) (Fig. 4). Whereas starvation stimulates *rpoS* expression at both the transcriptional and posttranscriptional levels, high osmolarity and temperature only influence the posttranscriptional stability of RpoS (Lange and Hengge-Aronis, 1991; McCann *et al.*, 1993; Loewen *et al.*, 1993). This increased stability of σ^S during the exponential growth phase under conditions of high osmolarity or elevated temperature

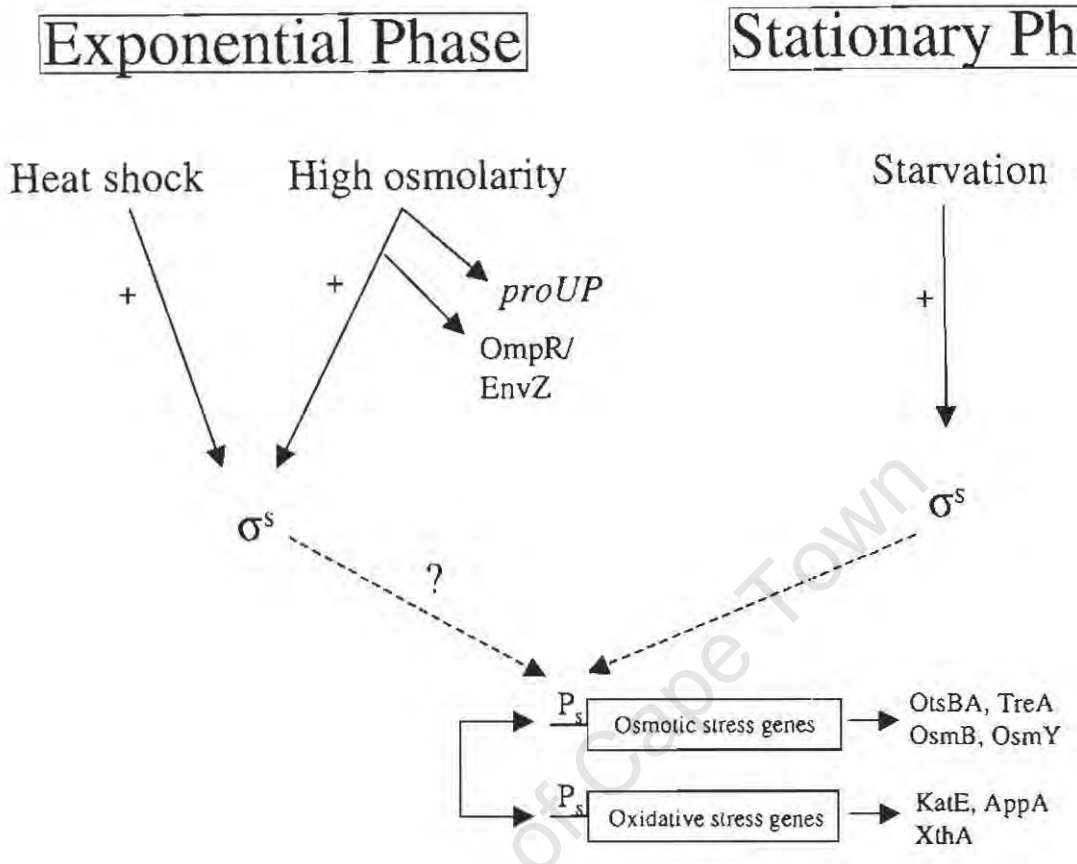


Figure 4. Activation of σ^s by various stresses. The plus signs indicate stabilization of σ^s. σ^s stabilized during stationary phase activates the expression of genes with σ^s-specific promoters. Whether σ^s activates the same set of genes during exponential phase is unclear.

is thought to be due to the DnaK chaperone which directly protects σ^S from degradation (Muffler *et al.*, 1997; Rockabrand *et al.*, 1998). One factor found to be crucial for *rpoS* expression is that of *oxyS* (Zhang *et al.*, 1998). *oxyS* is a small untranslated regulatory RNA whose expression is OxyR dependent. This small RNA represses *rpoS* at a posttranscriptional level. Thus, *rpoS* expression seems to be tightly regulated by a variety of signals. The σ^{32} mediated heat shock response and the *proP*, *proU* and OmpR/EnvZ osmoregulatory systems may therefore only be rapid emergency responses directed specifically against physical damage induced by high temperature and high osmolarity, respectively, whereas σ^S induction enhances cross protection against a variety of stresses.

4.1.5 Aim of this chapter

Since *V. cholerae* 569B produces pigment only under stressful conditions, pigmentation might play a role in protecting these bacteria against some form of stress. Thus, to determine the possible role of pigment synthesis in *V. cholerae*, and therefore the possible function of PpdA, we attempted mutating *ppdA* in wild-type *V. cholerae* 569B and the hypertoxigenic mutant RM7, using homologous recombination. We isolated two pigment-minus mutants designated Mut2 and HX1, respectively. The generation time of the resulting mutants, Mut2 and HX1, grown under different culture conditions was compared to their parental strains in order to assess whether these mutants displayed any defect in their culturability. Since Mut2 was defective in its ability to synthesize pigment, we measured its sensitivity to oxidative stress by challenging cultures grown under different conditions for various lengths of time with 5 mM H_2O_2. In comparison, we also assessed the ability of the wild-type strain to withstand oxidative stress following growth under different culture conditions. This chapter therefore provides an insight into the effects of various environmental stresses on the ability of *V. cholerae* to withstand oxidative stress, as well as the possible role of PpdA as a stress response mechanism in *V. cholerae* 569B.

4.2 Materials and methods

All media and solutions used in this study are listed in Appendix A.

4.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used to analyze the function of PpdA in *V. cholerae* are listed in Table 1.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant features	Reference
Strains		
E. coli SM10 λpir	*thi thr leu ton*A *lac*Y *sup*E *rec*A:: RP4-2T$_c$:: Mu (λpir R6K) Km^r	Miller and Mekalanos, (1988b)
V. cholerae 569B	Classical, Inaba	Mukherjee, (1978)
569B-Rif	569B, Rif^r	This study
Mut2	569B, *ppd*A::pCM704 Amp^r, Rif^r	This study
RM7	569B, *htx*	Mekalanos *et al.*, (1978)
RM7-Str	RM7, Str^r	This study
HX1	RM7, , *ppd*A::pCM704, Amp^r, Rif^r	This study
Plasmids		
pCM302-16	pCM302, *ppdA*	Chapter 2
pGP704	ori R6K, mob Rp4, MCS of M13tg131, Amp^r	Herrero *et al.*, (1990)
pCM704	pGP704 with 412 bp *Hind*III fragment of pCM302	This study

4.2.2 Media and standard culture conditions

E. coli SM10 (λ pir) was grown at 37°C in Luria broth (LB) containing 30 ug/ml kanamycin. Transformed *E. coli* SM10 (λ pir) harbouring pCM704 was grown on Luria agar (LA) containing 30 ug/ml kanamycin and 100 ug/ml ampicillin (Appendix A.2.1). *V. cholerae* 569B and RM7 were grown at 37°C in Tryptone Broth (TB). *V. cholerae* 569B-Rif was grown in TB containing 50 ug/ml rifampicin and *V. cholerae* RM7-Str in TB containing 100 ug/ml streptomycin. The *V. cholerae ppdA* mutant, Mut2, was grown in TB containing rifampicin (50 ug/ml) and ampicillin (100 ug/ml), and HX1 in TB containing streptomycin (100 ug/ml) and ampicillin (100 ug/ml).

4.2.3 *In vitro* mutagenesis

4.2.3.1 Isolation of a *V. cholerae* 569B rifampicin resistant mutant and a RM7 streptomycin resistant mutant.

In order to select for *V. cholerae* rifampicin resistant colonies, 100 ul aliquots from a 5 ml overnight TB culture of wild-type *V. cholerae* was spread onto rifampicin gradient plates. Gradients were prepared by pouring 14 ml of Tryptone agar (TA) containing 50 ug/ml rifampicin on top of 14 ml TA that were allowed to solidify at an angle. This created a rifampicin gradient from 0 to 50 ug/ml across the plate. The plates were incubated for 2 days at 30°C before rifampicin resistant colonies were streaked onto fresh TA plates containing 50 ug/ml rifampicin. Similarly, streptomycin resistant colonies of RM7 were obtained by preparing streptomycin gradient plates (0 to 100 ug/ml) and subsequently selecting for resistance on TA containing 100 ug/ml streptomycin.

4.2.3.2 Construction of pCM704 for *in vitro* mutagenesis

In order to determine the importance and function of *ppd*A in *V. cholerae* 569B, we attempted mutating the wild-type *ppdA* gene. A 412 bp *Hind*III fragment, incorporating the N-terminal region of *ppdA*, was obtained following restriction enzyme digestion of pCM302-16 with the restriction enzyme *Hind*III (Fig. 5). The resulting fragment was purified by electroelution (Appendix B.7), filled in with Klenow (Appendix B.17), and finally subcloned into the *Eco*RV restriction enzyme site of the suicide plasmid pGP704 (Fig. 5). The final construct, pCM704, was transformed into competent *E. coli* SM10 (λ pir), and introduced into rifampicin resistant *V. cholerae* 569B, as well as into streptomycin resistant RM7, by conjugation. Conjugal matings were performed overnight at 30°C on TA containing no antibiotic selection. Exconjugants were selected on TA

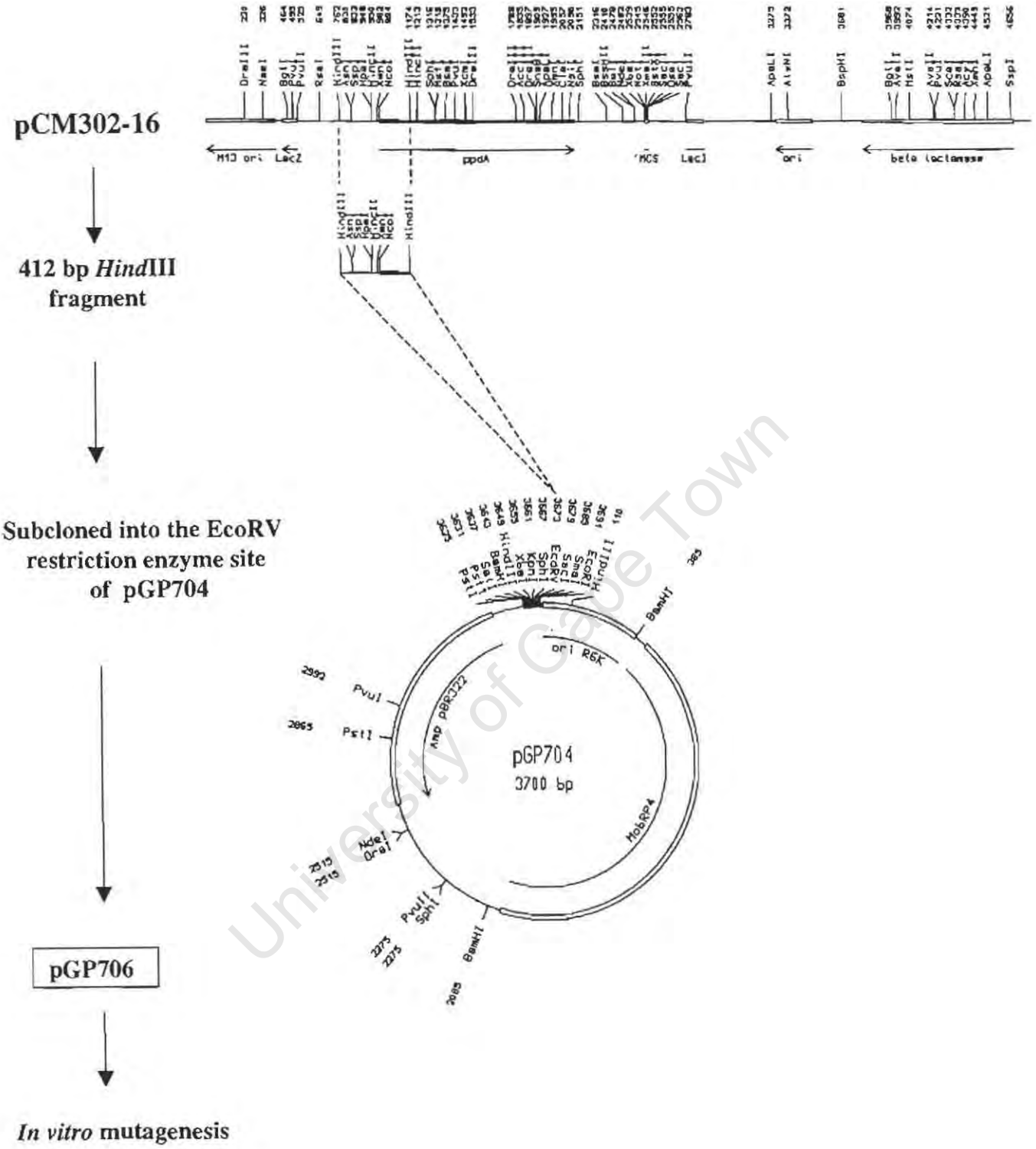


Figure 5. A 412 bp *Hind*III fragment from pCM302-16, which contained the N-terminal region of *ppdA*, was subcloned into the suicide vector pGP704. The resulting recombinant plasmid pGP706 was used for *in vitro* mutagenesis of *ppdA* on the chromosome of *V. cholerae* 569B and RM7.

containing ampicillin and rifampicin (569B) or TA containing ampicillin and streptomycin (RM7). Plates were subsequently incubated at 30°C for 24 hrs.

4.2.3.3 Analysis of the *ppdA* mutants, Mut2 and HX1

Two *ppdA* mutants, Mut2 (569B) and HX1 (RM7), were tested for their ability to pigment in response to elevated temperature and salinity by growing the cells in TB containing 4% NaCl at 37°C. Pigmentation was scored visually by comparing the ability of these mutants to secrete pigment into the culture media compared to their respective parental strains grown under the same set of conditions.

To confirm the integration of the recombinant suicide plasmid, pCM704, into *ppdA* on the *V. cholerae* 569B and RM7 chromosomes, we prepared chromosomal DNA from both mutants (Appendix B.4). Chromosomal DNA was restriction enzyme digested with the restriction enzymes *Cla*I, *Bam*HI and *Stu*I. The resulting products were then separated on a 0.8% agarose gel (Appendix B.6). Southern hybridization was performed as described in Appendix B.11, using an internal 1.3 kb *Xmn*I restriction enzyme fragment from *ppdA* as a probe.

In order to assess whether the inability to pigment altered the growth rate of the mutant strains, we grew the mutants under a variety of culture conditions and compared their generation times to that of their parental strains. The four conditions included 30°C in TB, 30°C in TB containing 4% NaCl, 37°C in TB and 37°C in TB containing 4% NaCl. A 5 ml overnight culture of each strain was prepared in TB at 30°C. This was used to inoculate a 250 ml flask containing 50 ml of fresh media to a final OD_{600} of 0.05. The flask was then incubated at the relevant temperature and the growth of the culture was monitored by reading the absorbance (OD_{600}) at various time intervals. The generation time for the different strains under the different conditions was calculated as follows: Generation time = $\ln 2/\mu$ (where μ represents the specific growth rate of the culture which is equal to the slope of the line corresponding to exponential growth).

4.2.4 Effect of oxidative stress on *V. cholerae* 569B and Mut2

Since Mut2 was defective in pigment synthesis, we assessed whether this would result in increased sensitivity to oxidative stress compared to the wild-type strain. To test this, we grew both *V. cholerae* 569B and Mut2 under a variety of culture conditions for various amounts of time, before subjecting both strains to 5 mM H_2O_2. Overnight *V. cholerae* 569B and Mut2 (5 ml TB at 30°C) cultures were used to inoculate the appropriate media (either TB containing 1%

NaCl, or TB containing 4% NaCl) to a final OD of 0.05. These cultures were incubated at either 30°C or 37°C. After exactly 24 hrs, 48 hrs and 72 hrs, 1 ml of cells was removed from the culture and diluted 10^{-1}, 10^{-3}, 10^{-5}, 10^{-6} and 10^{-7} in order to determine the number of viable cells that were present in the culture at the start of the experiment. Immediately after removal of the 1 ml of cells, H_2O_2 (1 M) was added to the culture media to a final concentration of 5 mM. At 1 hr intervals, 1 ml samples were removed from the cultures and diluted as described above in order to determine the number of surviving bacteria. The experiment was performed in triplicate using three independent cultures for each strain grown at a particular condition. TA plates were incubated at 30°C overnight before the number of colonies were scored.

4.3 Results

4.3.1. The *ppdA* mutants, Mut2 and HX1, were both defective in pigmentation

V. cholerae 569B synthesized melanin at both 30°C and 37°C when the cells were grown in TB amended with 4% NaCl (Table 2). *V. cholerae* 569B-Rif showed no defect in its ability to synthesize melanin under the conditions tested, indicating that rifampicin resistance did not affect melanogenesis in *V. cholerae* 569B (Table 2). In contrast to the wild-type strains, *V. cholerae* Mut2 was unable to produce any pigment under any of the above culture conditions (Table 2).

RM7 constitutively produced melanin at both 30°C and 37°C (Table 2). RM7-Str, like its parental strain, constitutively synthesized melanin which it secreted into the culture media under both the conditions tested, confirming that the streptomycin resistance mutation did not alter the pigmented phenotype of this strain. The *ppdA* mutant (HX1), however, completely lost the ability to constitutively synthesize pigment, and showed no pigmented phenotype when plated on Tryptone agar (Fig. 6).

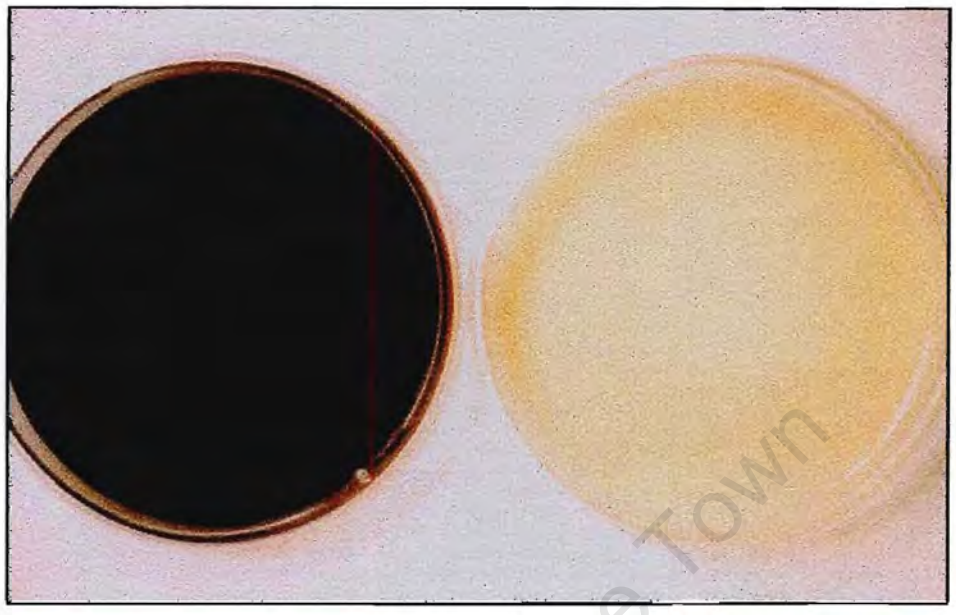
4.3.2 Southern hybridization confirmed the integration of pCM704 in the Mut2 and HX1 genomes

Figure 7 shows the homologous recombination strategy that we used in *V. cholerae* 569B and RM7. As a result of subcloning the 412 bp *Hind*III fragment into the *Eco*RV restriction enzyme site on pGP704, the resulting recombinant plasmid pCM704, lost one of the flanking *Hind*III restriction enzyme sites. The orientation of *ppdA* on pCM704 is indicated (Fig. 7). Homologous recombination between pCM704 and the N-terminal region of the wild-type *ppdA* gene led to integration of the vector resulting in a 3' truncated *ppdA* gene along with an intact full length *ppdA* gene. The vector position and the direction of β-lactamase expression responsible for ampicillin resistance in the mutants is shown (Fig. 7). We confirmed that pCM704 had integrated in the *V. cholerae* genome through Southern blot analysis (Fig. 8). Figure 9 shows the restriction enzyme map of *V. cholerae* 569B and RM7 *ppdA* loci, as well as the restriction enzyme pattern of the disrupted *ppdA* locus in Mut2 and HX1. The restriction enzymes *Cla*I and *Stu*I do not cleave within the vector sequence and therefore illustrate the integration of pCM704 into the *ppdA* locus on the chromosome. Integration resulted in an increase in the size of the wild-type *Cla*I restriction fragment from 5.4 kb to 8.7 kb and the *Stu*I fragment from 22 kb to 25 kb (Figs. 8 and 9). pCM704, however, contained two *Bam*HI restriction enzyme sites within its vector

Table 2. Melanin production in *V. cholerae* wild-type and mutant strains after 3 days of growth at 30°C and 37°C in TB supplemented with 1 and 4% NaCl.

Strain	1% 30°C	4% 30°C	1% 37°C	4% 30°C
V. cholerae 569B	–	+	–	+
V. cholerae 569B-Rif	–	+	–	+
V. cholerae Mut2	–	–	–	–
RM7	+	+	+	+
RM7-Str	+	+	+	+
HX1	–	–	–	–

+, indicates the presence of melanin; -, indicates the absence of melanin in the culture media.



RM7 HX1

Figure 6. Phenotypic difference between *V. cholerae* RM7 which constitutively synthesizes melanin and its *ppdA* mutant, HX1, which is unable to synthesize pigment.

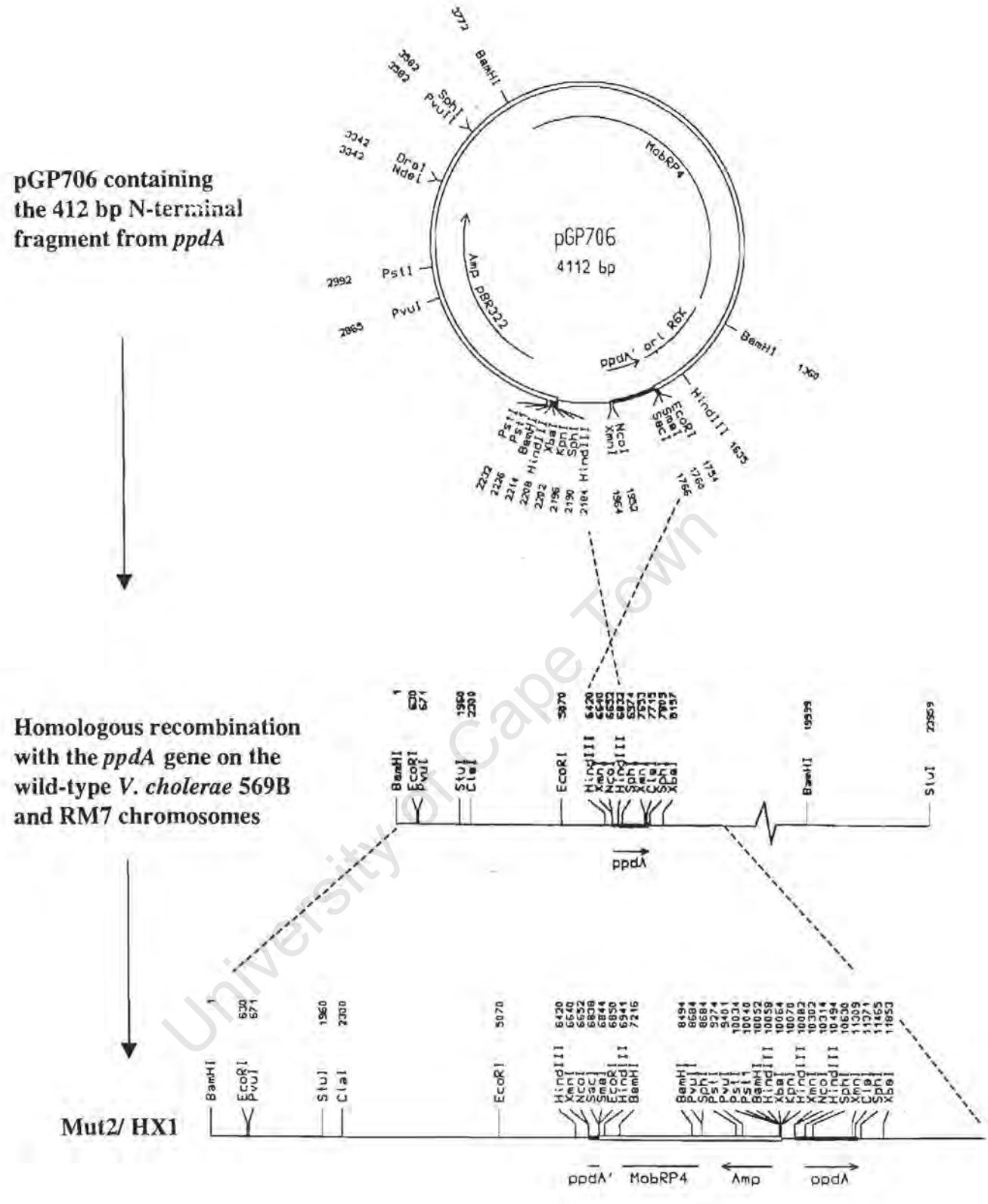


Figure 7. Homologous recombination between pCM704 and the N-terminal region of the *ppdA* gene resulted in the integration of pCM704 into the *ppdA* locus on the *V. cholerae* chromosome. The restriction enzyme maps for the wild-type *ppdA* locus and the resulting mutants are shown. The double lines represent pCM704 vector DNA, the thick black lines represent the *ppdA* gene and the thin black lines represent *V. cholerae* genomic DNA.

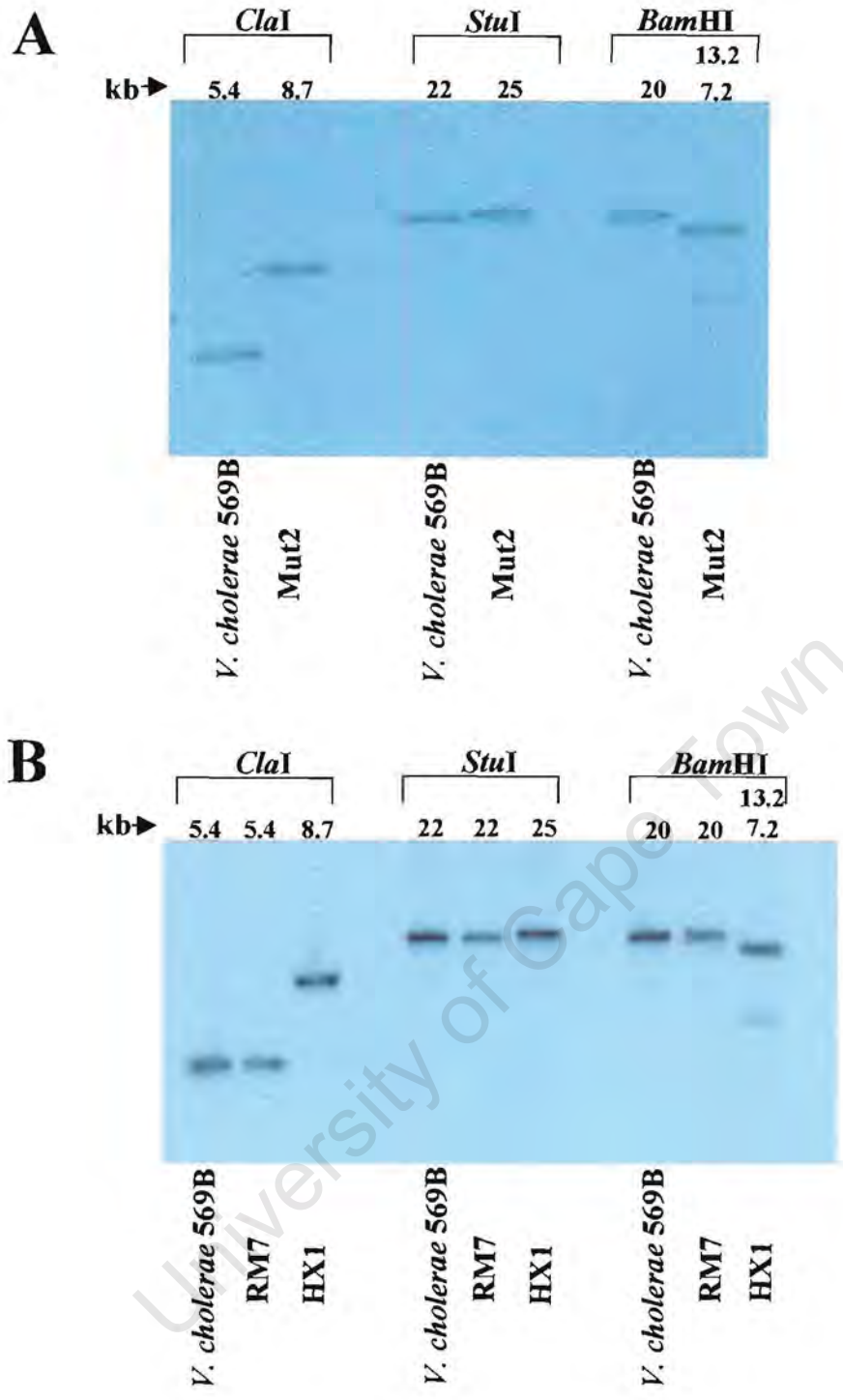


Figure 8. Southern hybridization analysis of Mut2 and HX1 *ppdA* mutants. Panel A represents Mut2 and *V. cholerae* 569B chromosomal DNA digested with various restriction enzyme nucleases and probed with the *XmnI* restriction enzyme fragment containing *ppdA*. Panel B represents *V. cholerae* 569B, RM7 and HX1 chromosomal DNA digested with various restriction enzymes and probed with the *XmnI* fragment containing *ppdA* (Fig.5). The figures above the lanes indicate the size of the hybridizing bands in kilobases.

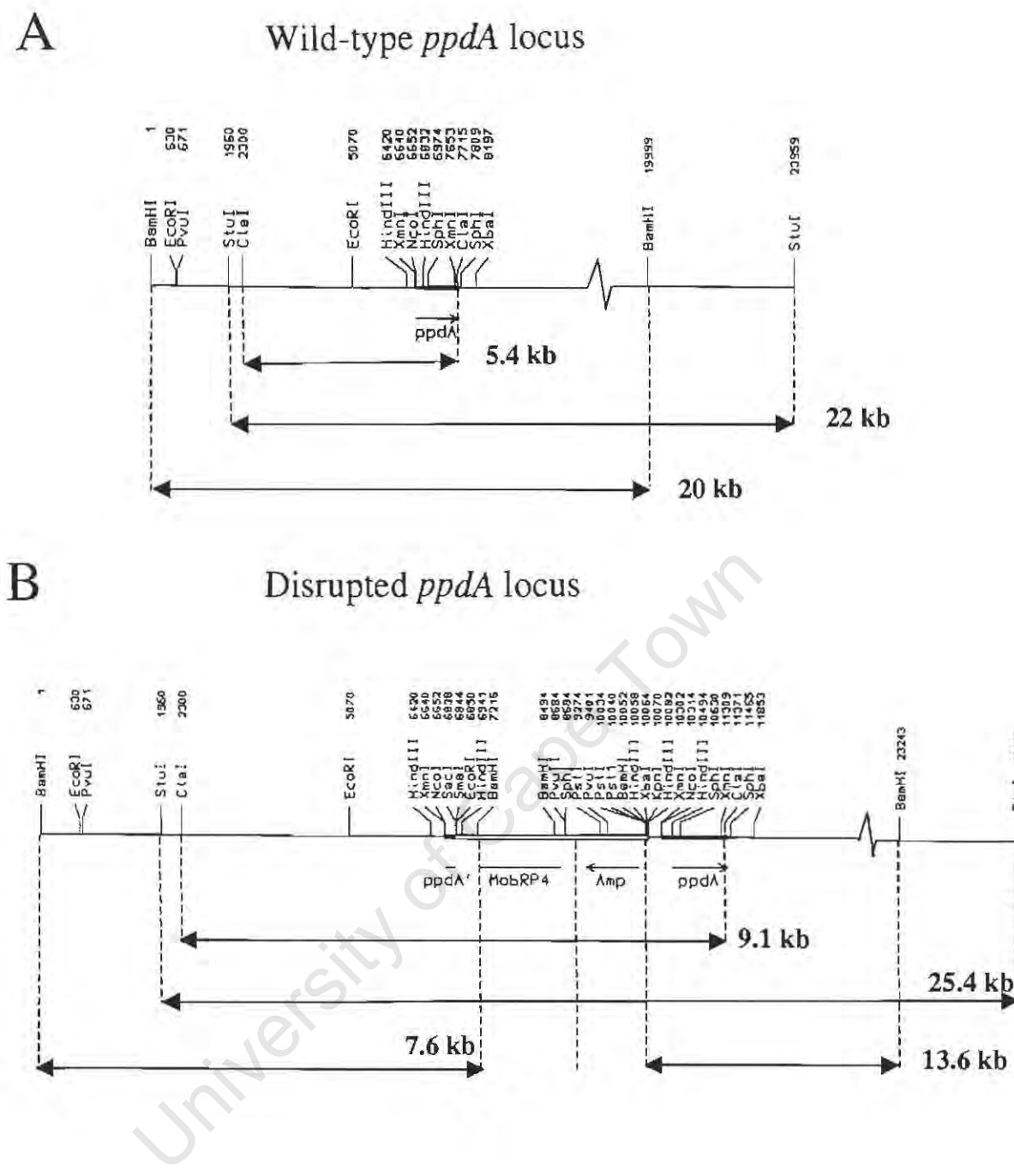


Figure 9. Restriction maps of the wild-type *ppdA* locus of 569B and RM7 (A) as well as the disrupted *ppdA* locus of the mutants Mut2 and HX1 (B). The sizes of the restriction enzyme fragments for the enzymes *Cla*I, *Stu*I and *Bam*HI are shown.

sequence which upon integration into the *ppdA* locus should result in the cleavage of the *ppdA* locus into several *Bam*HI fragments (Fig. 9). Indeed, in the wild-type strain, a single 20 kb *Bam*HI fragment is detected by the probe, whereas the integration of pCM704 into *ppdA* resulted in the detection of two restriction enzyme fragments of 13.2 kb and 7.2 kb. These are the only two fragments that contain *ppdA* sequence and therefore recognized by the probe. Since the larger 13.2 kb *Bam*HI fragment contains more of the probe sequence, this band appears much darker compared to the 7.2 kb fragment (Figs. 8 and 9). Furthermore, Figure 8B illustrates that despite the phenotypic dissimilarity with respect to pigmentation in *V. cholerae* 569B and RM7, there is no difference in the banding patterns that the probe generates upon hybridization to genomic DNA that had been isolated from these two strains and digested with the restriction enzymes *Cla*I, *Stu*I and *Bam*HI (Fig. 8).

4.3.3 The *ppdA* mutants, Mut2 and HX1, had no growth abnormalities

V. cholerae 569B cells grown at 30°C in TB containing 1% NaCl, displayed a generation time of 53 min (Table 3). In comparison, *V. cholerae* 569B-Rif and Mut2 had generation times of 49 and 52 min, respectively (Table 3). Increasing the temperature from 30 to 37°C resulted in a decrease in the generation time of all three strains with no significant difference in the resulting growth rate between the mutant (Mut2) and its parental strains. Under conditions of elevated salinity (TB containing 4% NaCl at 30°C), all three strains grew much slower with generation times of 72, 72 and 63 min, respectively. This indicated that the cells were severely affected by the increased salinity. Nevertheless, Mut2 displayed the fastest growth rate with a generation time of 63 min. Elevated salinity in conjunction with increased temperature dramatically lowered the generation time for all three strains, suggesting that the increased temperature allowed the cells to overcome the adverse effects induced by the presence of a high concentration of NaCl. No significant difference between the resulting growth rates existed between any of the strains.

The growth rate of RM7 under all four of the conditions tested, was always slower compared to *V. cholerae* 569B (Table 3). A temperature increase from 30°C to 37°C lowered the generation time in the RM7 strains grown in TB containing 1% NaCl. An increase in the salinity to 4% NaCl at both 30 and 37°C adversely affected the growth of these strains as could be seen from their increased generation times. The *ppdA* mutant HX1, like Mut2, did not display any disability with respect to its growth as can be seen from its generation times compared to its parental RM7 strains. In fact, the mutant grew better under certain conditions than its parental strains,

Table 3. Specific growth rates and generation times of the various strains grown under different conditions

Strain	Tryptone broth, 1% NaCl, 30°C		Tryptone broth, 1% NaCl, 37°C		Tryptone broth, 4% NaCl, 30°C		Tryptone broth, 4% NaCl, 37°C	
	Mean specific growth rate (μ)	Generation Time (min)	Mean specific growth rate (μ)	Generation Time (min)	Mean specific growth rate (μ)	Generation Time (min)	Mean specific growth rate (μ)	Generation Time (min)
V. cholerae 569B	0.79	53 (0.24)	1.03	41 (0.48)	0.58	72 (1.67)	1.02	41 (2.20)
V. cholerae 569B Rif	0.84	49 (2.17)	0.99	42 (0.29)	0.58	72 (1.84)	0.93	45 (0.91)
Mut2	0.80	52 (1.13)	0.97	43 (1.55)	0.67	63 (1.43)	0.95	44 (0.82)
RM7	0.71	59 (1.27)	0.77	54 (3.61)	0.44	94 (1.47)	0.63	67 (1.41)
RM7-Str	0.72	58 (0.26)	0.78	54 (2.71)	0.44	94 (3.49)	0.52	81 (3.53)
HX1	0.76	55 (2.61)	0.86	49 (0.53)	0.46	91 (3.91)	0.59	72 (4.77)

The numbers shown in parentheses represent the standard error calculated from the results of three experiments.

confirming that inactivation of *ppdA* did not alter the growth of the respective mutants during the exponential growth phase.

4.3.4 Stationary phase survival of wild-type and mutant strains

To investigate the survival of stationary phase cultures of both mutant and wild-type cells when exposed to various culture conditions, we determined the number of colony forming units (cfu) remaining in the culture following 24 hrs (1 day), 48 hrs (2 days) and 72 hrs (3 days) of incubation. Figure 10A represents the number of culturable wild-type and the mutant cells remaining after 1, 2 and 3 days of incubation in TB containing 1% NaCl at 30°C. Despite both strains having approximately the same generation time during exponential phase (Table 3), the wild-type survived much better than the mutant under these culture conditions. For example, after one day of incubation in TB containing 1% NaCl at 30°C, the number of culturable wild-type cells amounted to 5×10^9 cfu ml^{-1}, whereas the number of culturable mutant cells were approximately 9×10^8 cfu ml^{-1}. After three days of incubation, the number of culturable wild-type cells decreased to 5×10^5 cfu ml^{-1} compared to the 1.5×10^4 cfu ml^{-1} culturable mutants. Nevertheless, the rate at which the two strains lost their culturability over the three day period was similar (Fig. 10A).

Wild-type cells grown at 37°C in TB containing 1% NaCl experienced a rapid decline in culturability over the three day period (Fig. 10B). For example, after one day of incubation at 37°C in TB containing 1% NaCl, the number of culturable wild-type cells was 5×10^7 cfu ml^{-1}. However, after three days of incubation this number declined to less than 1×10^4 cfu ml^{-1}. This indicated that elevated temperature had an adverse effect on the culturability of the wild-type *V. cholerae* cells. The culturability of the mutant was less affected by the increased temperature compared to the wild-type strain. The number of culturable mutant cells was 3×10^7 cfu ml^{-1} after one day of incubation at 37°C in TB containing 1% NaCl. Of this, 1×10^6 cfu ml^{-1} mutant cells remained culturable after three days of incubation. This was considerably more than the 1×10^4 cfu ml^{-1} wild-type cells that remained culturable after three days of incubation. Thus, the rate at which the wild-type cells lost culturability was more rapid than the mutant strain (Fig. 10B).

Increasing the salinity of the growth media to 4% NaCl, in conjunction with an incubation temperature of 30°C, increased the culturability of both the mutant and wild-type strains dramatically (Fig. 11A). For example, after one day of incubation at 30°C in TB containing 4% NaCl, the number of culturable cells were 4×10^9 and 6×10^9 cfu ml^{-1} for the wild-type and

confirming that inactivation of *ppdA* did not alter the growth of the respective mutants during the exponential growth phase.

4.3.4 Stationary phase survival of wild-type and mutant strains

To investigate the survival of stationary phase cultures of both mutant and wild-type cells when exposed to various culture conditions, we determined the number of colony forming units (cfu) remaining in the culture following 24 hrs (1 day), 48 hrs (2 days) and 72 hrs (3 days) of incubation. Figure 10A represents the number of culturable wild-type and the mutant cells remaining after 1, 2 and 3 days of incubation in TB containing 1% NaCl at 30°C. Despite both strains having approximately the same generation time during exponential phase (Table 3), the wild-type survived much better than the mutant under these culture conditions. For example, after one day of incubation in TB containing 1% NaCl at 30°C, the number of culturable wild-type cells amounted to 5×10^9 cfu ml^{-1}, whereas the number of culturable mutant cells were approximately 9×10^8 cfu ml^{-1}. After three days of incubation, the number of culturable wild-type cells decreased to 5×10^5 cfu ml^{-1} compared to the 1.5×10^4 cfu ml^{-1} culturable mutants. Nevertheless, the rate at which the two strains lost their culturability over the three day period was similar (Fig. 10A).

Wild-type cells grown at 37°C in TB containing 1% NaCl experienced a rapid decline in culturability over the three day period (Fig. 10B). For example, after one day of incubation at 37°C in TB containing 1% NaCl, the number of culturable wild-type cells was 5×10^7 cfu ml^{-1}. However, after three days of incubation this number declined to less than 1×10^4 cfu ml^{-1}. This indicated that elevated temperature had an adverse effect on the culturability of the wild-type *V. cholerae* cells. The culturability of the mutant was less affected by the increased temperature compared to the wild-type strain. The number of culturable mutant cells was 3×10^7 cfu ml^{-1} after one day of incubation at 37°C in TB containing 1% NaCl. Of this, 1×10^6 cfu ml^{-1} mutant cells remained culturable after three days of incubation. This was considerably more than the 1×10^4 cfu ml^{-1} wild-type cells that remained culturable after three days of incubation. Thus, the rate at which the wild-type cells lost culturability was more rapid than the mutant strain (Fig. 10B).

Increasing the salinity of the growth media to 4% NaCl, in conjunction with an incubation temperature of 30°C, increased the culturability of both the mutant and wild-type strains dramatically (Fig. 11A). For example, after one day of incubation at 30°C in TB containing 4% NaCl, the number of culturable cells were 4×10^9 and 6×10^9 cfu ml^{-1} for the wild-type and

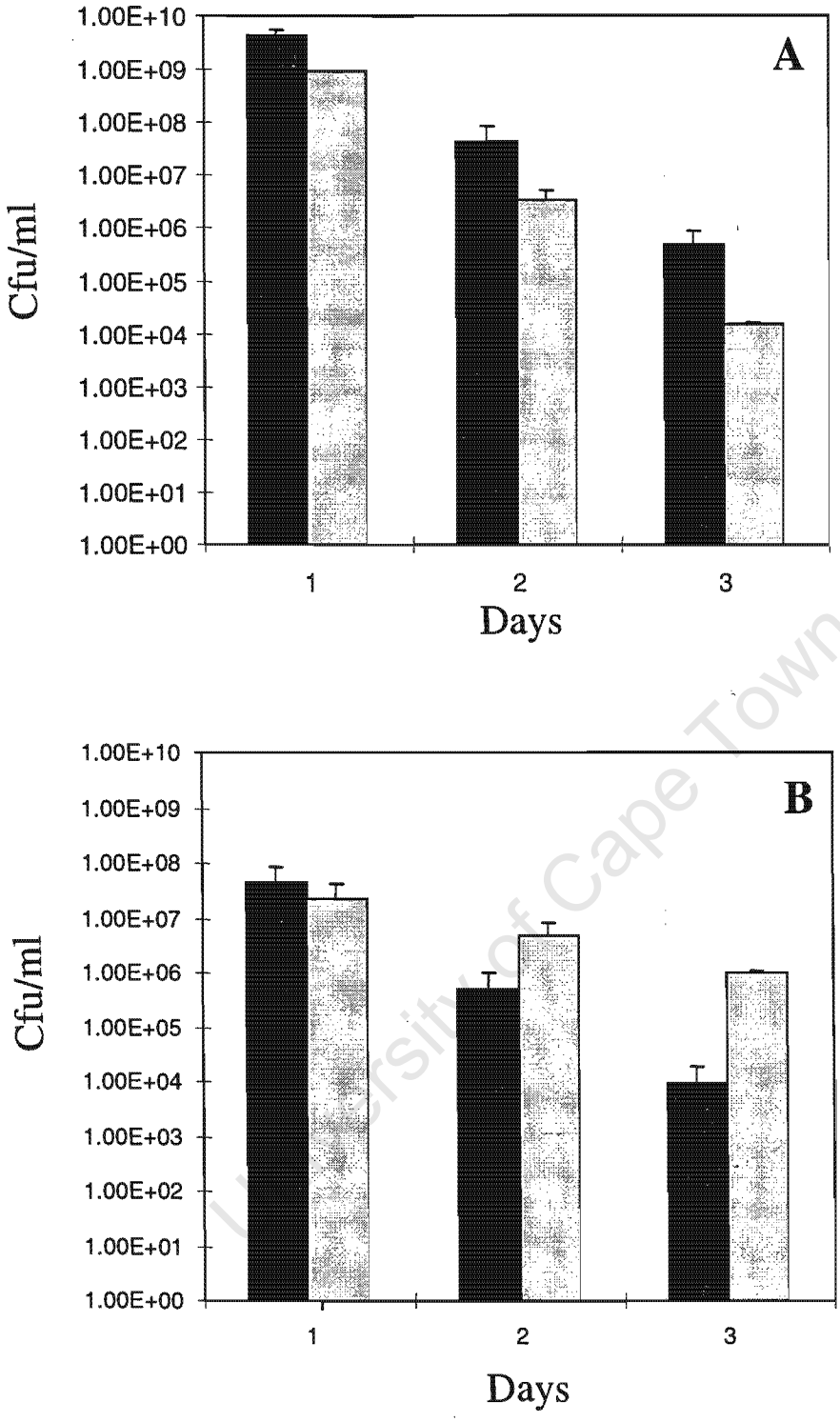


Figure 10. A represents the number of culturable cells remaining after 1, 2 and 3 days of growth in TB containing 1% NaCl at 30°C. B represents the number of culturable cells remaining after 1, 2 and 3 days of growth in TB containing 1% NaCl at 37°C. The black bars represent *V. cholerae* 569B, whereas the grey bars represent Mut2. Error bars represent the standard error calculated from three independent experiments.

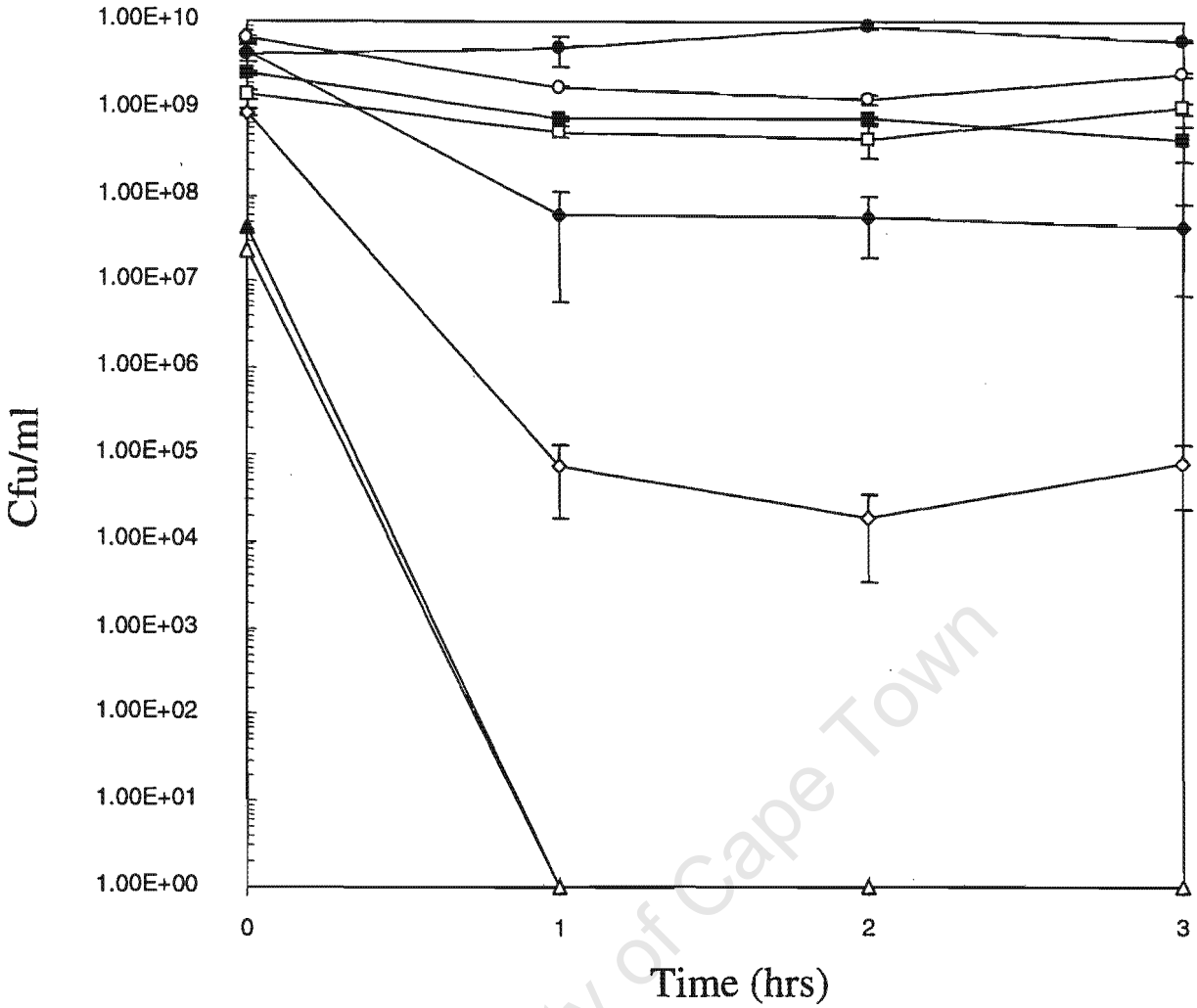


Figure 12. Survival of *V. cholerae* 569B and Mut2 after exposure to 5 mM H_2O_2. Both strains were first grown for 24 hrs under specific culture conditions before the addition of H_2O_2 to the culture media. Culture conditions were as follows: *V. cholerae* grown at 30°C in Tryptone broth (◆), Mut2 grown at 30°C in Tryptone broth (◇), *V. cholerae* grown at 37°C in Tryptone broth (▲), Mut2 grown at 37°C in Tryptone broth (△), *V. cholerae* grown at 30°C in Tryptone broth supplemented with 4% NaCl (●), Mut2 grown at 30°C in Tryptone broth supplemented with 4% NaCl (○), *V. cholerae* grown at 37°C in Tryptone broth supplemented with 4% NaCl (■), Mut2 grown at 37°C in Tryptone broth supplemented with 4% NaCl (□). Error bars represent the standard error calculated from the results of three independent experiments.

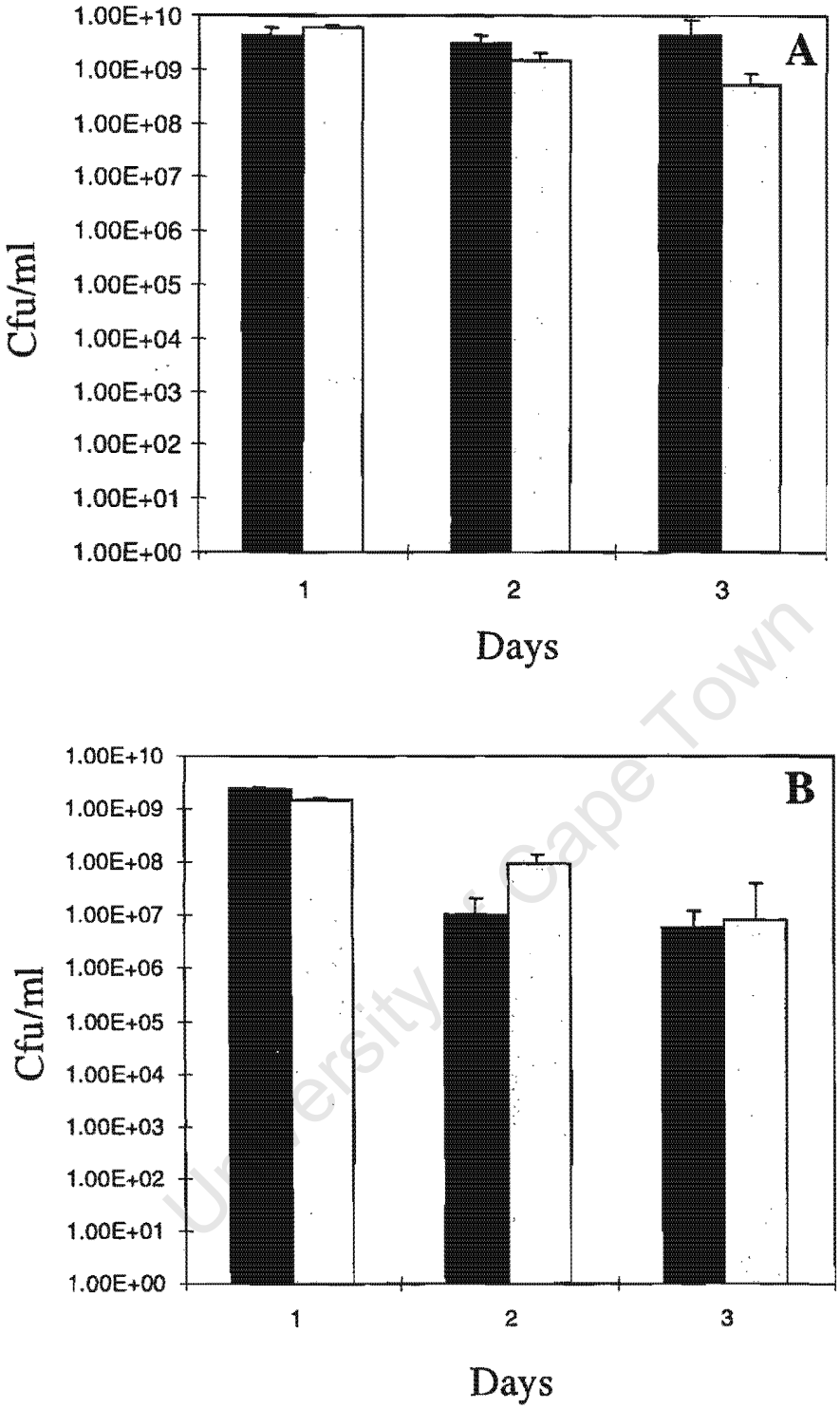


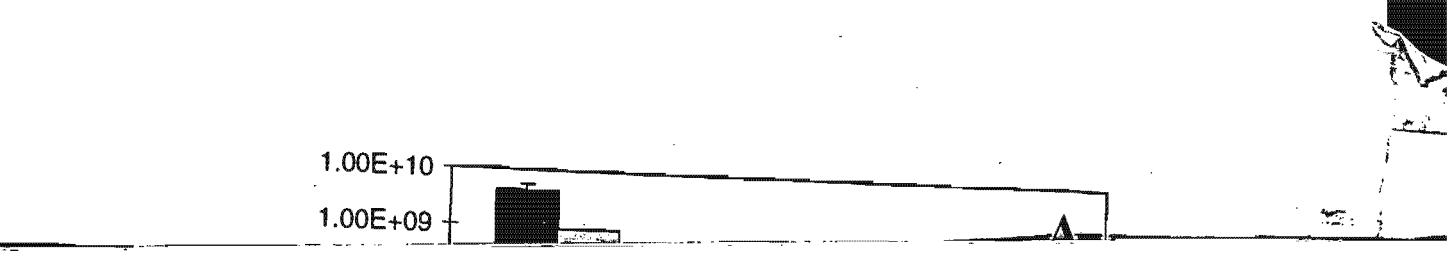
Figure 11. A represents the number of culturable cells remaining after 1, 2 and 3 days of growth in TB containing 4% NaCl at 30°C. B represents the number of culturable cells remaining after 1, 2 and 3 days of growth in TB containing 4% NaCl at 37°C. The black bars represent *V. cholerae* 569B, whereas the grey bars represent Mut2. Error bars represent the standard error calculated from three independent experiments.

mutant strains, respectively. After three days of incubation at the same culture conditions, the number of culturable cells was 5 x 10^9 cfu ml^{-1} for the wild-type and 5 x 10^8 cfu ml^{-1} for the [redacted] mutant (Fig. 11A). Thus, although growth at 30°C in TB containing 4% NaCl lowered the generation times of the both the wild-type and mutant strains during exponential phase (Table 3), these cells retained their culturability over the three day period tested. Furthermore, although the difference between the rate at which the two strains lost their culturability was small, the mutant cells did lose culturable cells slightly faster than the wild-type strain (Fig. 11A).

An increase in both the salinity and temperature of the growth media affected the culturability of both the wild-type and the mutant cells (Fig. 11B). After one day of incubation at 37°C in TB containing 4% NaCl, 2.5 x 10^9 cfu ml^{-1} and 1.5 x 10^9 cfu ml^{-1} of wild-type and mutant cells remained culturable, respectively. After two and three days of incubation, the culturability of wild-type cells decreased to 1 x 10^7 cfu ml^{-1} and then to 6 x 10^6 cfu ml^{-1}. The decrease in culturable mutant cells over the same period decreased to 1 x 10^8 cfu ml^{-1} and subsequently to 8 x 10^6 cfu ml^{-1}. Thus, the wild-type strain only lost 40% of its culturable cells between day two and day three, whereas 92% of the culturable mutant cells were lost over the same period. Therefore, the rate at which the mutant lost culturability after two days of incubation at 37°C in TB containing 4% NaCl was much faster than the wild-type strain (Fig. 11B). However, both strains had approximately 1 x 10^7 cfu ml^{-1} culturable cells remaining in the culture media after day three (Fig. 11B). Thus, although the number of wild-type cells drop by two logs between day1 and day2, the number of mutant cells dropped by only one log. However, wild-type cell numbers are stabilized between day2 and day3 compared to the amount of culturable mutant cells. This difference might be directly due to the accumulation of copious amounts of pigment which only accumulate after 2 days in the wild-type cultures and is absent in the mutant cultures.

4.3.5 Mut2 is more sensitive than the wild-type to oxidative stress when subjected to increased salinity and temperature

To assess whether pigmentation could in fact protect *V. cholerae* cells from the adverse effects of oxidative stress, we challenged both mutant and wild-type cells with 5 mM H_2O_2 and subsequently determined the total number of survivors. After 1 day of incubation at either 30 or 37°C in TB supplemented with 4% NaCl, both the mutant and the wild-type strains were resistant to exposure to 5 mM H_2O_2 (Fig. 12). Wild-type and mutant cells incubated at 30°C in TB containing 1% NaCl, however, were more sensitive to oxidative stress (Fig. 12). For example,



the number of wild-type cells decreased from 6 x 10^9 cfu ml^{-1} to 4 x 10^7 cfu ml^{-1} within only one hour of exposure to H_2O_2. A similar decrease in culturability was displayed by the mutant cells where the number of culturable cells decreased from 9 x 10^8 cfu ml^{-1} to 1 x 10^5 cfu ml^{-1} after one hour of H_2O_2 exposure. The decrease in the number of culturable cells was, however, much more rapid in mutant cells suggesting that these cells were more sensitive to the effects of H_2O_2. Both wild-type and mutant strains were extremely sensitive to oxidative stress after the cells were incubated at 37°C in TB containing 1% NaCl. This suggested that an increase in temperature was detrimental to the cells with respect to their long term survival (Fig. 10B) and when challenged with oxidative stress (Fig. 12).

Figure 13 shows the survival of 48 hr old mutant and wild-type cells following incubation under various culture conditions and subsequent exposure to H_2O_2. Wild-type and mutant cells incubated at 30°C in TB supplemented with 4% NaCl remained resistant to exposure to H_2O_2. Wild-type cells incubated at 37°C in TB containing 4% NaCl experienced a rapid decrease in the amount of culturable cells from 1 x 10^7 cfu ml^{-1} to 2 x 10^4 cfu ml^{-1} within one hr of exposure to H_2O_2. Mutant cells incubated at 37°C in TB containing 4% NaCl, however, did not show any capability to withstand oxidative stress, as no survivors remained after only one hour of exposure to H_2O_2 (Fig. 13). It should be pointed out that pigmentation occurred at this time in the culture media of the wild-type strain, and that the difference observed between the survival of the wild-type and mutant strains in this particular case could be the direct result of the lack of pigmentation in the mutant strain. Both strains were extremely sensitive to oxidative stress after incubation in TB containing 1% NaCl at both 30 and 37°C, with no survivors after only one hour exposure to 5 mM H_2O_2. After 3 days of incubation, none of the cells grown under any of the culture conditions tested could survive exposure to 5 mM H_2O_2 (Fig. 14).

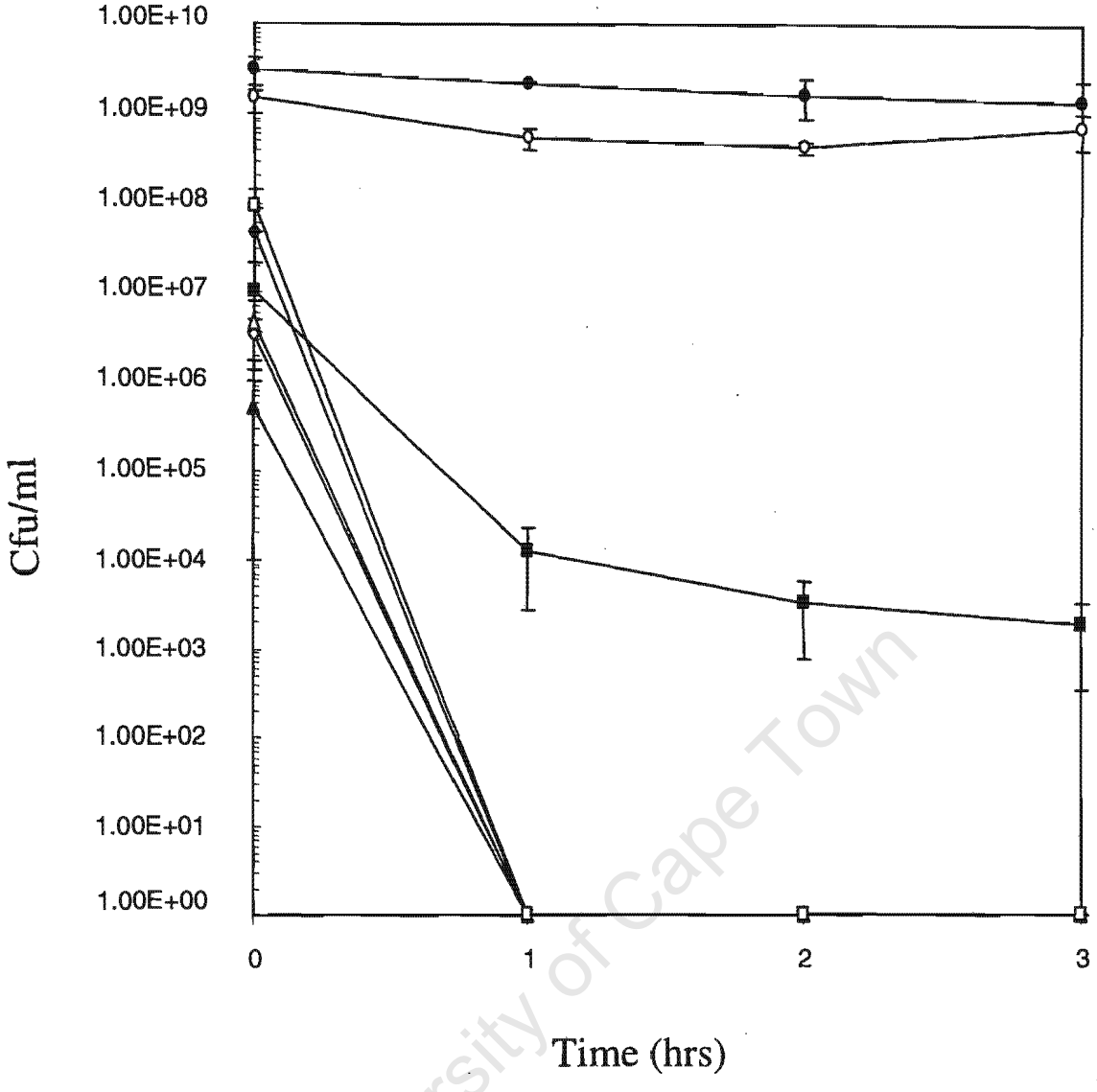


Figure 13. Survival of *V. cholerae* 569B and Mut2 after exposure to 5 mM H_2O_2.
Both strains were first grown for 48 hrs under specific culture conditions before the addition of H_2O_2 to the culture media. Culture conditions were as follows: *V. cholerae* grown at 30°C in Tryptone broth (◆), Mut2 grown at 30°C in Tryptone broth (◇), *V. cholerae* grown at 37°C in Tryptone broth (▲), Mut2 grown at 37°C in Tryptone broth (△), *V. cholerae* grown at 30°C in Tryptone broth supplemented with 4% NaCl (●), Mut2 grown at 30°C in Tryptone broth supplemented with 4% NaCl (○), *V. cholerae* grown at 37°C in Tryptone broth supplemented with 4% NaCl (■), Mut2 grown at 37°C in Tryptone broth supplemented with 4% NaCl (□). Error bars represent the standard error calculated from the results of three independent experiments.

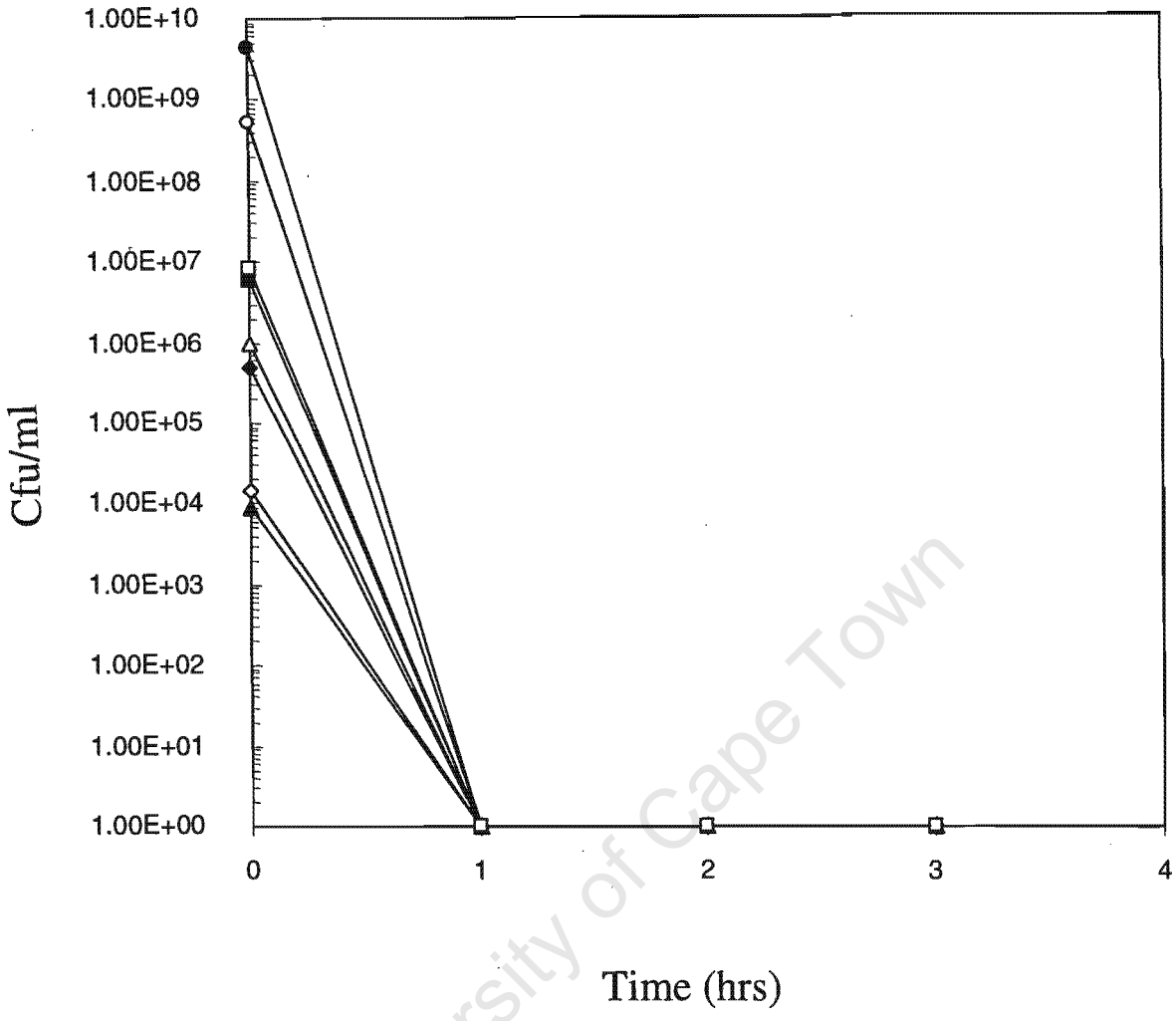


Figure 14. Survival of *V. cholerae* 569B and Mut2 after exposure to 5 mM H_2O_2. Both strains were first grown for 72 hrs under specific culture conditions before the addition of H_2O_2 to the culture media. Culture conditions were as follows: *V. cholerae* grown at 30°C in Tryptone broth (◆), Mut2 grown at 30°C in Tryptone broth (◇), *V. cholerae* grown at 37°C in Tryptone broth (▲), Mut2 grown at 37°C in Tryptone broth (△), *V. cholerae* grown at 30°C in Tryptone broth supplemented with 4% NaCl (●), Mut2 grown at 30°C in Tryptone broth supplemented with 4% NaCl (○), *V. cholerae* grown at 37°C in Tryptone broth supplemented with 4% NaCl (■), Mut2 grown at 37°C in Tryptone broth supplemented with 4% NaCl (□). Error bars represent the standard error calculated from the results of three independent experiments.

4.4 Discussion

Homologous recombination between pCM704 and *ppdA* in *V. cholerae* 569B and RM7 led to the inability of the resulting strains (Mut2 and HX1) to synthesize pigment. The reason for the lack of pigmentation in these mutants is still unclear. In addition to a truncated *ppdA* the mutants harbour an intact copy of the *ppdA* gene. Theoretically, this *ppdA* gene should remain functional regardless of the presence of the upstream integration event. One possible explanation for the pigment-minus phenotype in the mutant strains could be that the integration of pCM704 upstream from the *ppdA* gene, separated the intact copy of *ppdA* from either upstream activation sequences or additional promoter sequences that are required for transcriptional activation *ppdA* in *V. cholerae*. This, however, needs further investigation.

Southern hybridization confirmed the integration of pCM704 upstream from the *ppdA* locus of strains 569B and RM7. Furthermore, the *ppdA* locus in RM7 seemed unaltered compared to *V. cholerae* 569B, suggesting that the mutation in RM7 responsible for the constitutive production of pigment was most probably located in a regulatory region distinct from the *ppdA* structural gene. This result, however, does not exclude the possibility of a point mutation in the RM7 *ppdA* gene that could also result in the same phenotype.

Growth analysis of the *ppdA* mutant strains showed that the generation times of the mutants during the exponential growth phase, and their culturability over three days in stationary phase, compared well with their parental strains. Thus, disruption of the *ppdA* gene in 569B and RM7 did not alter their ability to grow and remain culturable.

Although there was no significant difference between the wild-type and mutant strains during these growth studies, the effect of the different culture conditions on *V. cholerae* was clear. Elevated temperature caused dramatic decreases in the generation times of all the strains. A similar increase in growth rate has been observed in *E. coli* cells during heat shock (Muffler *et al.*, 1997). Later it became evident that both *V. cholerae* wild-type and mutant cells that had been exposed to elevated temperature (37°C) in the presence of 1% NaCl, also displayed decreased culturability over a three day post-inoculation period. The rate at which the wild-type lost culturability, however, was significantly greater than for the mutant. The reason for this result is unclear, since pigmentation is not induced by 1% NaCl and an incubation temperature of 37°C. Nevertheless, cells exposed to 37°C and 1% NaCl were also very sensitive to the effects of

oxidative stress, with zero survival after only 1 hour of exposure to 5 mM H_2O_2. This implies that pre-exposing *V. cholerae* cells to elevated temperature did not confer resistance to subsequent exposure to oxidative stress. In *E. coli*, a deletion in *rpoH,* which prevented the induction of the heat shock response, resulted in sensitisation to both peroxide- and superoxide-mediated oxidative stress, suggesting that the heat shock proteins play some role in protection against oxidative stress (Kogoma and Yura, 1992). Since the *V. cholerae rpoH* gene has been shown to be 80% homologous to the *E. coli* σ^{32} (Sahu *et al.*, 1997), one could speculate that the heat shock response in *V. cholerae* is most probably mediated in a similar fashion to that of *E. coli*. The apparent lack of overlap between the heat and oxidative stress responses, however, suggests some difference in these two systems.

Increasing the salt concentration of the growth media to 4% adversely affected the exponential growth rate of all *V. cholerae* strains, as seen from the large increase in the generation times of these cultures. A similar decrease in the growth rate was also observed in *E. coli* cells that had been exposed to elevated salinity (Muffler *et al.*, 1997). Despite their long generation time, *V. cholerae* cells were better equipped to survive long term and to deal with oxidative stress. For example, wild-type and mutant cells incubated at 30°C in the presence of 4% NaCl maintained their culturability over all three days tested. These cells were also extremely resistant to subsequent exposure to H_2O_2 , indicating that exposure to salt stress could confer resistance to oxidative stress. Similarly, wild-type and mutant cells incubated at 37°C in TB containing 4% NaCl retained large numbers of culturable cells after one day of incubation. These cells also exhibited extreme resistance to subsequent H_2O_2 exposure. This confirms that increased salinity physiologically alters *V. cholerae* cells and allows them to overcome the detrimental effects associated with subsequent exposure to H_2O_2.

A likely explanation for the protective effect of elevated salinity on *V. cholerae* survival, could be that high osmolarity stabilizes the *V. cholerae* RpoS which then mediates resistance to various other stresses, including oxidative stress, by inducing catalase production which mediates the breakdown of H_2O_2. Indeed, the *V. cholerae* RpoS has been isolated and shown to play a crucial role in the survival of cells undergoing carbon starvation, hyperosmolarity and oxidative stress (Yildiz and Schoolnik, 1998). Furthermore, *V. fisheri* has been shown to contain a single catalase whose activity rapidly increases upon entering into stationary phase (Visick and Ruby, 1998). Although the *V. cholerae* catalase gene has not been cloned, catalase activity has been detected in

V. cholerae 569B during late exponential phase (Daily *et al.*, 1978) and it could play an important role in protecting *V. cholerae* cells from oxidative stress.

V. cholerae cells grown in 4% NaCl at 37°C displayed a generation time similar to that of *V. cholerae* cells grown at 37°C in 1% NaCl. This suggests that elevated temperature allowed the cells to overcome the adverse affects associated with high salt during exponential growth. This could be directly due to the induction of both σ^{32} and σ^{s}. Together, these sigma factors can induce a large set of genes that would allow the cells to cope with the stress associated with elevated temperature and salinity. Thus, both elevated temperature and increased salinity can play important roles in the induction of cross protective mechanisms that enhance the survival of *V. cholerae* in both its host and the natural environment.

A comparison of the differences in the culturability of the mutant and wild-type strains, and their sensitivity to oxidative stress, revealed interesting results. Firstly, the wild-type strain exhibited increased resistance relative to the mutant to oxidative stress after one day incubation at 30°C in TB containing 1% NaCl. The reason for this increased resistance is presently unclear. Secondly, wild-type cells incubated for two days at 37°C in TB containing 4% NaCl were much more resistant to oxidative stress compared to the mutant cells. The reason for this could be the presence of copious amounts of brown pigment that was synthesized by the active PpdA enzyme of *V. cholerae* 569B during this period. Thus, the presence of the pigment could be responsible for the difference in survival of the wild-type and mutant strains following exposure to H_2O_2. This result, together with the observation that wild-type cells grown at 37°C in TB containing 4% NaCl lost less culturable cells after two days of growth compared to the mutant, suggests that pigmentation plays a protective role in *V. cholerae*. Since melanin has excellent radical scavenging properties (Sarna *et al.*, 1986), this result would not be surprising. If this is the case, the role of *ppdA* would be to produce pyomelanin, which might to a certain extent can protect *V. cholerae* cells from oxidative stress, and therefore greatly enhance the chances of survival of the bacterium in both its host and the natural environment.

Both RpoH and RpoS can play key roles in protecting *V. cholerae* upon exposure to elevated temperature and salinity, by activating large numbers of genes that would assist the organism to not only cope with the harmful effects of these two stresses, but also with oxidative stress. In fact, resistance to H_2O_2 may be particularly important for aquatic organisms such as *V. cholerae*

because $OH^{\cdot}$ is generated by the action of UV radiation on water and is therefore expected to be produced within sun-illuminated aquatic habitats (Yildiz and Schoolnik, 1998). In addition, pathogens such as *V. cholerae* have to evade the human host defence system where macrophages produce active oxygen species that can severely limit the survival of the organism. However, induction of these protective mechanisms could prove energetically expensive and may only be effective for a short period of time. This would force the bacterium to employ some alternative protective mechanism. Since pigmentation occurs directly in response to elevated salinity and temperature, and considering the ability of pigmentation to increase the culturability of wild-type cells by directly protecting the cells from oxidative stress, this would indeed seem a likely possibility. Given the properties of melanin, protection against oxidative stress might only be one of the advantages conferred to pigmenting *V. cholerae* cells.

CHAPTER 5

GENERAL DISCUSSION

Melanins are not considered essential for the development and growth of cells, nevertheless, they possess important properties which may be crucial for the survival of organisms under adverse environmental conditions (Chapter 1). Although melanogenesis has been extensively characterized in many organisms, little was known about this process in *V. cholerae*. In this study, we investigated the significance of pigmentation in *V. cholerae* in order to generate new knowledge regarding this process.

Our first aim in understanding melanogenesis in *V. cholerae* was to identify the genes involved in pigmentation. Our study revealed that *V. cholerae* 569B harboured two distinct genes, *ppdA* and *tyrM*, both capable of synthesizing melanin in *E. coli* clones grown on LB supplemented with L-tyrosine. Further investigation revealed that *ppdA* displayed extensive homology to 4-hydroxyphenyl pyruvate dioxygenase (Hppd) enzymes from several organisms. Although the function of these Hppd enzymes has been well established in eukaryotic organisms, namely that of a mediator of tyrosine catabolism, their role in bacteria is still obscure. In fact, synthesis of Hppd in other bacteria can be directly correlated with pigment secretion into the culture media, whereas pigment accumulation in humans, as a result of Hppd synthesis, has only been associated with alkaptonuria, a rare hereditary disorder (Chapter 2 section 2.1.4). Since *V. cholerae* has been previously shown incapable of metabolizing tyrosine (Ruzafa *et al.*, 1995), the role of PpdA in *V. cholerae* may differ from the role Hppd enzymes play in humans.

Our second aim involved the basic characterization of the *V. cholerae* PpdA enzyme in terms of its haemolytic properties and the time at which protein synthesis and gene expression occurred in *V. cholerae*. We found that PpdA, although unable to confer fluorescence to the culture media, displayed α-haemolytic activity. This directly implicated PpdA as a virulence factor, since many pathogens employ haemolysis as a general strategy to weaken their host defence systems and to scavenge valuable iron for their growth (Sugawara *et al.*, 1997). Contrary to most haemolysins which have been shown to be exported extracellularly to mediate pore formation in the membranes of erythrocytes and consequently haemolysis (Sugawara *et al.*, 1997; Zitzer *et al.*, 1997), the *V. vulnificus* haemolysin has been localized to the periplasm (Chang *et al.*, 1997). Since the *V. cholerae* PpdA displays 78% sequence identity to that of the *V. vulnificus* haemolysin, and considering that the two proteins display identical haemolysin and pigmenting

properties, one could speculate that PpdA is probably also confined to the periplasm. This is strengthened by the fact that PpdA, similar to the *V. vulnificus* haemolysin, lacks a N-terminal signal sequence required for protein export. Certainly this then questions the role of these proteins as virulence factors in both *V. vulnificus* and *V. cholerae,* since their location would render the proteins inaccessible to host targets.

Furthermore, PpdA synthesis only occurs 45 hrs post-inoculation where *V. cholerae* cells had already been experiencing starvation for more than 24 hrs. Protein synthesis directly coincided with the appearance of brown-black pigment in the culture media, confirming the role of PpdA in *V. cholerae* melanogenesis. As was demonstrated by Elisa assays, PpdA synthesis was terminated around 60 hrs of growth which suggests that protein synthesis occurs only for a limited time in *V. cholerae* cultures. Contrary to the Elisa results, RT-PCR analysis clearly showed the presence of *ppdA* transcripts throughout the growth cycle of *V. cholerae*, regardless of the culture conditions. This result implies that *V. cholerae* either employs posttranscriptional or posttranslational control to regulate the synthesis of PpdA in the cell. At present we do not know which of the two mechanisms is of greater importance.

PpdA synthesis is thus closely associated with late stationary phase, and may therefore be of importance in viable but non-culturable *V. cholerae* cells whose physiological state most closely resembles that of stationary phase. Thus, the cells could be producing melanin when ingested by drinking contaminated water or eating contaminated shellfish. Furthermore, depending on the inoculum size and the susceptibility of the person who has been exposed, the incubation period for *V. cholerae* O1 varies from between 12 hr and 72 hr (Cash *et al.*, 1974). Since PpdA synthesis falls within this time period, the PpdA protein could play an important role in *V. cholerae* virulence by neutralizing oxidants and promoting invasive disease. In addition, the NaCl concentration in an adult cholera stool ranges from 90 - 130 mmol/L (Griffith *et al.*, 1967). Alternatively, PpdA might be important for preventing cellular dehydration of *V. cholerae* in cholera stools.

Our final objective was to determine whether pigmentation could render *V. cholerae* more resistant to oxidative stress. Our results suggested that pigmentation conferred protection against the adverse effects of oxidative stress and that this protection coincided directly with PpdA synthesis and secretion of pigment into the media. We therefore speculate that PpdA synthesis only continues until enough pigment has been accumulated within the culture media after which

it is terminated. Thus, we propose that the function of PpdA may be to specifically synthesize HGA for pyomelanin synthesis. Since HGA is a small, hydrophobic molecule, it can easily move across the outer membrane into the extracellular media. Spontaneous polymerization of homogentisic acid in the presence of oxygen then results in the formation of pyomelanin which surrounds the *V. cholerae* cells. The pyomelanin can then act as a free radical trap to alleviate some of the oxidative stress associated with stationary phase growth. Thus, melanin can clearly promote the survival of *V. cholerae* in the estuarine environment by scavenging free radicals that would otherwise damage and/or kill cells.

Although melanogenesis in *V. cholerae* thus appears to function to alleviate oxidative stress, this process is only confined to late stationary phase. This suggests that during exponential and early stationary phase, *V. cholerae* employs other mechanisms to cope with increased oxidative stress. Our results indicate that elevated salinity plays an important role in inducing these protection mechanisms as the cells were significantly resistant to subsequent oxidative stress and maintained culturability after growth in the presence of elevated salinity.

Finally, although this study has contributed to our understanding of melanogenesis in *V. cholerae,* many questions remain. Future work will include quantitative RT-PCR to determine the exact amount of *ppdA* transcripts present in the cell under non-pigment inducing conditions. This will confirm whether the data obtained with RT-PCR is due to basal levels of *ppdA* expression, which due to the sensitivity of the RT-PCR technique, results in the detection of similar levels of amplified product in cells grown under a variety of conditions. Furthermore, fusion of the *ppdA* promoter region to a reporter gene in *E. coli* and transforming this strain with a *V. cholerae* gene bank could allow us to isolate the regulatory genes involved in the melanin biosynthetic pathway. In addition, we can study the effects of global regulators such as RpoS and RpoH, in conjunction with various environmental signals, on reporter gene expression. These experiments would provide valuable insight into the regulation of melanogenesis in *V. cholerae* and whether this regulation occurs at a posttranscriptional or posttranslational level.

Although PpdA might be localized to the periplasm, as is the case with the *V. vulnificus* haemolysin, this does not exclude the possibility that the haemolytic capabilities of PpdA might contribute to *V. cholerae* virulence. Clearly the haemolytic characteristic of PpdA needs to be investigated further in order to explain why the *V. cholerae* PpdA protein and the legiolysin from *L. pneumophila* displayed different haemolysin and fluorescent activities. Furthermore,

understanding why *E. coli* clones harbouring pCM302 displayed increased haemolytic activity compared to clones harbouring pCM302-16 could only increase our understanding of PpdA.

In addition, we need to know what role *tyrM* plays in *V. cholerae* melanogenesis. Purification of the TyrM protein will allow us to obtain antibodies for use in *in situ* hybridization studies. This could determine whether *tyrM* is possibly associated with the flagella components of *V. cholerae*. Alternatively, these antibodies could be employed in Elisa assays in order to determine the culture conditions responsible for *tyrM* expression in wild-type *V. cholerae* 569B. Clearly much work remains to be done before we can fully understand the significance of pigmentation in *V. cholerae* 569B.

V. cholerae occupies two main niches, namely that of its human host and estuarine environments. The organism is therefore constantly challenged with changing conditions that threaten its survival. This danger can only be overcome if the organism can rapidly respond and adapt to these changing conditions. In order to do this, the organism employs regulatory proteins such as ToxR and many sigma factors to convey crucial signals to intracellular proteins as to the state of the environment and, in this manner, the bacterium is able to respond rapidly to particular environmental conditions. It is therefore also not surprising that *V. cholerae* can exploit the beneficial properties of melanin to aid it in its continuous battle for survival. Indeed, the fact that we were able to isolate two *V. cholerae* genes that allowed *E. coli* transformants to pigment, suggests that melanogenesis is an important trait in *V. cholerae* 569B.

Pigmentation is a trait found in most organisms. In mammals, birds and plants pigments play an important role in sexual reproduction and can even serve as warning signals to other organisms. In bacteria and fungi, however, the function of pigmentation is directly related to the chemical properties of the pigment rather than its visual properties. This is most probably due to the fact that these organisms employ pigments as direct armour against various stresses, especially that of oxidative stress (Shivprasad and Page, 1989). These pigments can also play an important role in virulence and ion scavenging processes (White, 1958; Kubo *et al.*, 1985). Whatever the role, pigmentation is an important component of the survival strategies employed by most organisms, and its ubiquity throughout the biological kingdom illustrates its importance.

APPENDIX A

MEDIA AND SOLUTIONS

CONTENTS

A.1 Media

A.1.1 Luria broth

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
H_2O to 1L	
Autoclave	

A.1.2 Luria agar

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
H_2O to 1L	
Autoclave	

A.1.3 Luria broth supplemented with tyrosine and $CuSO_4$

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
L-tyrosine (Sigma)	5 g
$CuSO_4$	5 mg
H_2O to 1L	
Autoclave	

For Luria agar supplemented with tyrosine and $CuSO_4$ add 15 g agar before autoclaving

A.1.4 Blood agar

Bacto blood base agar (Difco)	40 g
H_2O to 1L	
Autoclave	

Aseptically add 5% sterile defibrinated human erythrocytes

A.1.5 Tryptone broth supplemented with 1% NaCl

Tryptone	10 g
NaCl	5 g
L-tyrosine (Sigma)	5 g
$CuSO_4$	5 mg
H_2O to 1L	
Autoclave	

For Tryptone agar add 15 g of agar before autoclaving

A.1.6 Tryptone agar supplemented with 4% NaCl

Tryptone	10 g
NaCl	5 g
L-tyrosine (Sigma)	5 g
$CuSO_4$	5 mg
H_2O to 1L	

Autoclave
For Tryptone agar add 15 g of agar before autoclaving

A.2 Solutions

A.2.1 Antibiotic solutions

Ampicillin (100 mg/ml)
Dissolve 2 g in 20 ml water. Filter sterilize and store aliquotes at 4°C
Dilute 1:1000 into media for final concentration of 100 ug/ml

Kanamycin (30 mg/ml)
Dissolve 0.6 g in 20 ml water. Filter sterilize and store aliquotes at –20°C
Dilute 1:1000 into media for final concentration of 30 ug/ml

Rifampicin (50 mg/ml)
Dissolve 1 g in 20 ml methanol. Store aliquotes at –20°C
Dilute 1:1000 into media for final concentration of 50 ug/ml

Streptomycin (100 mg/ml)
Dissolve 2 g in 20 ml water. Filter sterilize and store aliquotes at 4°C
Dilute 1:1000 into media for final concentration of 100 ug/ml

A.2.2.General stock solutions

EDTA (0.5 M)
Dissolve 93.05 g EDTA in 400 ml dH_2O while adding 10 g NaOH pellets.
Adjust the pH to 8 and make up to a final volume of 500 ml
Autoclave

Tris base (1M)
Dissolve 12.1 g Tris in 100 ml dH_2O
Autoclave

Tris-HCl (1M)
Dissolve 12.1 g Tris in 80 ml dH_2O.
Adjust pH to required level with concentrated HCl.
Finally make up to a final volume of 100 ml

TE (Tris-EDTA)
Use Tris and EDTA stock solutions

Tris	10 mM, pH 8
EDTA	1 mM

Sterile dH_20

$MgCl_2$ (1 M)

Dissolve 20.3 g $MgCl_2.6H_2O$ in 100 ml H_2O.
Autoclave

$CaCl_2$ (1 M)

Dissolve 14.7 g ($CaCl_2.2H_2O$) in 100 ml H_2O
Autoclave

NaCl (5 M)

Dissolve 29.22 g in 100 ml dH_2O
Autoclave

EtOH (absolute)

Supplied by Merck
Store at –20°C

EtOH (70%)

Dissolve 70 ml of absolute EtOH in 100 ml H_2O
Store at –20°C

Isopropanol

Supplied by Merck
Store at room temperature

RNase (10 mg/ml)

Dissolve 0.1 g RNase A in 10 mM Tris-HCl, 15mM NaCl (pH 7.5)
Heat for 15 min at 100°C and allow to cool slowly to room temperature
Aliquot into eppendorf tubes and store at –20°C

Ammonium persulfate (10%)

Dissolve 1 g in 10 ml of dH_2O
Aliquot into eppendorf tubes and store at –20°C

TEMED (N, N, N', N'-Tetramethylethylenediamine)

Supplied by Sigma

DEPC (Diethylpyrocarbonate)

Supplied by Sigma

DEPC treated dH_20

Add 1 ml of DEPC to 1L of dH_2O
Autoclave

A.2.3 Solutions for Large scale preparation of plasmid DNA

Solution 1

0.25 M Tris-Cl pH 8
20% (w/v) glucose
0.1 M EDTA
Autoclave components separately, then mix

Solution 2

0.2 M NaOH
1% (w/v) SDS
Make fresh solution before use

Solution 3

3 M KOA (pH 4.8)
pH using glacial acetic acid
Autoclave

Salt saturated isopropanol

Add 300 ml 5 M NaCl in TE buffer to 600 ml isopropanol
Shake up and stand overnight

A.2.4 Solutions for Chromosomal DNA extractions

SDS 10% (Sodium dodecyl sulphate)

Dissolve 10 g in 100 ml dH_2O
Stir on warm plate and do not overheat

Proteinase K (20 mg/ml)

Dissolve 20 mg in 1 ml sterile dH_2O
Store at –20°C

CTAB/NaCl

Dissolve 4.1 g NaCl in 80 ml dH_2O
Slowly add 10 g CTAB (hexadecyltrimethyl ammonium bromide)
Heat while stirring slowly
If necessary, heat to 65°C to dissolve
Adjust to a final volume of 100 ml with dH_2O

Chloroform/isoamyl alcohol

Mix at ratio 24:1

A.2.5 Solutions for Southern hybridization analysis

0.25 M HCl

Dissolve 21.35 ml in 1L dH_2O

0.4 M NaOH

Dissolve 16 g in 1L dH_2O

0.4 M NaOH/1 M NaCl

NaOH	16 g in
NaCl	58.44 g

Dissolve and adjust the volume to 1L dH_2O

SSC 20x (Sodium chloride trisodium citrate)

NaCl	17.5 g
Tri-NaCitrate	8.82 g
dH_2O	80 ml

Adjust pH to 7.4 with NaOH
Make up to 100 ml with dH_2O
Autoclave

Reaction mixture
100 ul hexanucleotide mixture in 10x reaction buffer

STE (Sodium chloride-Tris EDTA)
TE buffer (pH 8) containing 0.1 M NaCl

Sephadex G-50
Slowly add 30 g Sephadex G-50 (medium) to 250 ml STE buffer
Autoclave
Store at 4°C

Tracking dye
Dissolve Blue dextran in 50 mM NaCl to a final concentration of 3%
Dissolve Orange G in the above solution to a final concentration of 1%

PB stock solution (1 M Na_2HPO_4, pH 7.2)

$Na_2 HPO_4. 7H_2O$	134 g
H_3PO_4 (85%)	4 ml

Make up to 1L with dH_2O
Autoclave

SDS 25% (Sodium dodecyl sulphate)
Dissolve 250 g in 1L dH_2O
Stir on warm plate and do not overheat

Church pre-hybridization buffer (pre CHB)

Non-fat dry milk	0.5 g
PB stock solution	50 ml
EDTA (0.5 M)	0.2 ml
SDS (25%)	28 ml

Make up to 100 ml with dH_2O

Church hybridization buffer (CHB)

PB stock solution	50 ml
EDTA (0.5 M)	0.2 ml
SDS (25%)	28 ml

Make up to 100 ml with dH_2O

Wash buffer A (WBA)

PB stock solution	20 ml
EDTA (0.5 M)	1 ml
SDS (25%)	100 ml

Make up to 500 ml with dH_2O

Wash buffer B (WBB)

PB stock solution	40 ml
EDTA(0.5 M)	2 ml
SDS (25%)	40 ml

Make up to 1L with dH_2O

A.2.6 Solution for Restriction enzyme digestions

Gel tracking dye

Bromophenol blue	62.5 g
Sucrose	10 g
EDTA (0.5 M)	1 ml

Make up to a final volume 25 ml in dH_2O
Autoclave

A.2.7 Solutions for Agarose gel electrophoresis

TAE 50x (Tris-acetate buffer)

Tris	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml

Make up to 1L with dH_2O
Autoclave

EtBr 10 mg/ml (Ethidium Bromide)
Dissolve 0.1 g in 10 ml dH_2O
Shake well to dissolve
Powerful mutagen-wear gloves and clean spills with isopropanol

A.2.8 Solution for Ligation reactions

Ligation buffer (10x)

Tris, pH 7.6	150mM
$MgCl_2$	50 mM
ATP	2.5mM
BSA	0.5 mg/ml

Make up to appropriate volume using sterile dH_2O.

A.2.9 Solution for Ammonium acetate precipitation

Ammonium acetate (7.5 M)
Dissolve 262.84 g in 400ml of dH_2O
pH to 7.5 using glacial acetic acid.
Make up to 500 ml with dH_2O
Autoclave

A.2.10 Solutions for Exonuclease III shortening

Exonuclease III buffer

Tris-HCl (pH 7.6)	66 mM
$MgCl_2$	660 uM
Autoclave	

S1 nuclease buffer (10x)

KOA (pH 4.6)	300 mM
NaCl	2 M
Glycerol	50%
$ZnSO_4$	9 mM
Autoclave	

S1 nuclease mix (enough for 15 tubes)

10x S1 nuclease buffer	24.6 ul
sterile dH_2O	155.4 ul
S1 nuclease	36 U
Autoclave	

S1 nuclease stop

Tris base	300 mM
EDTA (pH 8)	50 mM
Autoclave	

Klenow mix

Tris-HCl (pH 7.6)	20 mM
$MgCl_2$	7 mM
Autoclave	

Ligase mix (enough for 25 tubes)

10x Ligation buffer	50 ul
T_4 ligase (1 U/ul)	250 ul
sterile dH_20	150 ul
use 18 ul per tube	

A.2.11 Solutions for Sequencing

Silane

Silane	50 ul
Acetic acid	30 ul
dH_2O	300 ul
EtOH	9.62 ml

Gel mix for 6% PAGE/7 M Urea sequencing gel

Acrylamide (40%)	7.4 ml
Urea (Merck)	25.7 g
TTE (10x)	5 ml
Make up to 50 ml with dH_20	

Taurine Tris buffer (10x) (TTE)

Tris	108 g
Taurine	36 g
Na_2EDTA	3.72 g

Make up to 1L with dH_20

Annealing buffer (5x)

Tris-HCl, pH 7.5	200 mM
$MgCl_2$	100 mM
NaCl	250 mM

Labeling mix (Lab mix) (5x)

dGTP	7.5 uM
dCTP	7.5 uM
dTTP	7.5 uM

Enzyme dilution buffer

Tris-HCl, pH 7.5	10 mM
DTT (Dithiothreitol)	5 mM
BSA	0.5 mg/ml

Stop buffer

Formamide	95%
EDTA	20 mM
Bromophenol blue	0.05%
Xylene cyanol FF	0.05%

A.2.12 Solutions for RNA extractions

All solutions were either DEPC treated or made up in baked glass bottles using sterile DEPC treated dH_2O

Protoplast buffer

Tris-Cl, pH 8.0	15 mM
Sucrose	0.45 M
EDTA	8 mM

Made up to appropriate volume with DEPC treated dH_2O

Store at 4°C

Lysozyme (80 mg/ml)

Dissolve 0.8 g in 10 ml sterile dH_2O

Aliquot into eppendorf tubes

Store at –20°C

Lysis buffer

Tris-Cl, pH 8	10 mM
NaCl	10 mM
sodium citrate	1 mM
SDS	1.5%

Make up with DEPC treated dH_2O
Store at room temperature

Saturated NaCl

Dissolve 40 g NaCl in 100 ml in DEPC treated H_2O
Stir until solution reaches saturation
Autoclave

Phenol/chloroform/isoamylalcohol (25:24:1)

Mix at ratio 25:24:1
Store at 4°C

A.2.13 Solutions for Labelling oligonucleotide for primer extension

T_4 polynucleotide kinase buffer (10x)

1 M Tris, pH 7.6	1 ml
1 M $MgCl_2$	200 ul
500 mM DTT	200 ul
10 mM spermidine	200 ul
500mM EDTA	4 ul
dH_2O	396 ul

DTT (1 M) (Dithiothreitol)

Dissolve 0.3 g in 20 ml 10 mM sodium acetate (pH 5.2)
Filter sterilize and store aliquots at –20°C

Spermidine (1 M)

Dissolve 2.9 g in 20 ml dH_2O
Filter sterilize
Store aliquots at –20°C

Ammonium acetate (4 M)

Dissolve 30.8 g in 100 ml dH_2O
Autoclave

TEN 600

NaCl	600 mM

Make up in TE buffer, pH 7.5

Gel mix for 9% PAGE/7 M Urea sequencing gel

Acrylamide (40%)	11.25 ml
Urea (Merck)	25.7 g
TTE (10x)	5 ml

Make up to 50 ml with dH_2O

A.2.14 Solutions for SDS-PAGE gels

30% Acrylamide solution

Acrylamide	30 g
Bisacrylamide	0.8 g

Add dH_2O to a final volume of 100 ml
Store at 4°C

Separating gel buffer (4x)

Tris base	18.17 g
SDS (10%)	4 ml

Adjust pH to 8.8 with HCl and add dH_2O to a final volume of 100 ml
Store at room temperature

Stacking gel buffer (4x)

Tris base	6.06 g
SDS (10%)	4 ml

Adjust pH to 6.8 with HCl and add dH_2O to a final volume of 100 ml
Store at room temperature

SDS-PAGE Running buffer (10x)

Tris base	30 g
Glycine	144 g
SDS (10%)	100 ml

SDS-PAGE sample buffer (2x)

Glycerol	2 ml
SDS (10%)	2 ml
Bromophenol blue	0.25 mg
Stacking gel buffer (4x)	2.5 ml
β-mercaptoethanol	0.5 ml

Bring to a final volume of 10 ml with dH_2O
Store at room temperature

Coomassie blue dye staining solution

Isopropanol	250 ml
Glacial acetic acid	100 ml

Make up to 1L with dH_2O
Add 2.5 g Coomassie ^{R} brilliant blue R250
Store at room temperature

Destaining solution

Glacial acetic acid	70 ml

Bring final volume to 1L with dH_2O
Store at room temperature

A.2.15 Solution for Electroblotting proteins onto nitrocellulose

Blotting buffer

Tris	6 g
Glycine	28.8 g
Methanol	200 ml

Dissolve in 2L dH_2O

A.2.16 Solution for Determining protein concentration using Bradford assays

Bovine serum albumin (BSA) (1 mg/ml)

Dissolve 0.01 g in 10 ml sterile dH_2O

Aliquot and store at –20°C

NaCl (0.15 M)

Dissolve 0.88 g in 100 ml dH_2O

Autoclave

Coomassie brilliant blue solution

In a 1L volumetric flask dissolve 100 mg Coomassie brilliant blue G250 in 50 ml 95% EtOH.

Add 100 ml 85% phosphoric acid

Bring volume to 1L with dH_2O

Filter through Whatman no 1 filter paper

Store at 4°C

A.2.17 Solution for Western blot analysis

Phosphate buffered saline 10x (PBS)

NaCl	87 g
Na_2PO4	22.5 g
KH_2PO4	2 g

Dissolve in 800 ml with dH_2O

Adjust pH to 7.4

Autoclave

A.2.18 Solution for pMal protein purification system

Column buffer

Tris	20 mM
NaCl	0.2 M
EDTA	0.5 M

Adjust the pH to 7.4

Bring to appropriate volume with dH_2O

APPENDIX B

STANDARD METHODS

CONTENTS

B.1 Large scale preparation of plasmid DNA

(Taken from Ish-Horowicz and Burk, 1981)

1. Inoculate 5 ml Luria broth (LB) containing appropriate antibiotic selection (Appendix A.2.1) with a single colony of *E. coli* containing the desired plasmid.
2. Grow at 37°C with vigorous shaking overnight.
3. Inoculate 200 ml LB containing selection agent with 1 ml of overnight culture.
4. Grow at for 8-12 hrs at 37°C or until the culture is saturated.
5. Collect cells by centrifuging 10 min at 6 000 rpm, 4°C.
6. Resuspend the cells in 4 ml solution 1 (Appendix A.2.3) and leave at room temperature for 10 min.
7. Add 8 ml solution 2 (Appendix A.2.3) and mix well by shaking. Leave on ice for 10 min.
8. Add 6 ml solution 3 (Appendix A.2.3), shake well and leave on ice for 10 min.
9. Centrifuge at 10 000 rpm for 10 min and transfer the supernatant to a clean SS34 centrifuge tube.
10. Add 1 volume of isopropanol and incubate at room temperature for 10 min.
11. Pellet the DNA by centrifugation at 15 000 rpm for 15 min.
12. Wash the pellet with 10 ml 70% EtOH (Appendix A.2.2).
13. Resuspend DNA in 4.2 ml TE buffer (Appendix A.2.2).
14. Add 4.62 g CsCl and mix to dissolve.
15 Centrifuge for 10 min at 10 000 rpm.
16. Transfer the supernatant to a clean SS34 tube.
17. Add 200 ul of 10 mg/ml EtBr (Appendix A.2.7) and adjust the refractive index to 1.396.
18. Fill two Vti65 tubes and centrifuge at 55 000 rpm overnight at 20°C.
19. Visualize DNA bands under UV (310 nm) and remove the plasmid band using a 2 ml syringe to a clean eppendorf tube.
20. Add an equal volume of salt saturated isopropanol (Appendix A.2.3).
21. Discard the top phase.
22. Add two volumes of sterile dH_2O and one volume isopropanol.
23. Incubate on ice for 10 min.
24. Precipitate DNA by centrifuging at 14 000 rpm for 15 min.
25. Wash pellet with 500 ul 70% EtOH.
26. Resuspend pellet in 100 ul TE buffer and store at 4°C.

B.2 Preparation of competent *E. coli* cells

(Taken from Dagert and Ehrlich, 1979)

1. Inoculate a single colony of freshly streaked *E. coli* into a 5 ml LB and shake at 37°C for 2.5 hrs.
2. Inoculate this starter culture into 100 ml prewarmed LB and grow at 37°C until the OD_{600} reaches 0.35 (approximately 3.5-4.0 x 10^7 cells/ml).
3. Transfer the culture to a GSA tube and centrifuge for 5 min at 5 000 rpm, 4°C.
4. Decant the supernatant and resuspend the cells in 100 ml ice cold 0.1 M $MgCl_2$. Leave on ice for 1 min.
5. Collect the cells as before and resuspend in 50 ml 0.1 M $CaCl_2$. Incubate on ice for 2 hrs.
6. Collect the cells as before and resuspend them in 10 ml 0.1 M $CaCl_2$.
7. Aliquot 100 ul into 1.5 ml eppendorf tubes and store at –70°C.

B.3 Transformation of competent cells

(Taken from Dagert and Ehrlich, 1979)

1. Add 1 to 50 ng of plasmid DNA to 100 ul of competent cells.
2. Leave on ice for 10 min.
3. Heat shock cells at 42°C for 2 min or 37°C for 5 min.
4. Add 0.9 ml LB and allow expression at 37°C for 30-60 min.
5. Plate 100 ul of cells on LA containing antibiotic selection.
6. Incubate plates at 37°C overnight.

B.4 Large scale preparation of bacterial genomic DNA

(Ausubel *et al.*, 1989 unit 2.4)

1. Grow 100 ml culture of bacterial strain overnight.
2. Pellet the cells for 10 min at 4 000 rpm and discard the supernatant.
3. Resuspend the cells in 9.5 ml TE buffer (Appendix A.2.2).
4. Add 0.5 ml 10% SDS and 50 ul of 20 mg/ml proteinase K (Appendix A.2.4), mix and incubate 1 hr at 37°C.
5. Add 1.8 ml of 5 M NaCl (Appendix A.2.2) and mix thoroughly.
6. Add 1.5 ml CTAB/NaCl (Appendix A.2.4) solution and mix thoroughly.
7. Incubate for 20 min at 65°C.
8. Extract with an equal volume of chloroform/isoamyl alcohol (Appendix A.2.4).
9. Centrifuge at 6 000 rpm for 10 min, room temperature.
10. Transfer aqueous phase to a clean tube.

11. Precipitate DNA by adding 0.6 volumes isopropanol.
12. Centrifuge at 10 000 rpm for 10 min.
13. Wash pellet with 1 ml 70% EtOH (Appendix A.2.2).
14. Resuspend DNA in 1 ml TE buffer (Appendix A.2.2).
15 Measure the DNA concentration on a spectrophotometer.

B.5 Restriction endonuclease digestions

(Ausubel *et al.*, 1989 unit 3.1)

1. Pipette 0.1 to 4 ug of either plasmid or chromosomal DNA into a clean eppendorf tube.
2. Add 2 ul restriction enzyme buffer (The restriction enzyme buffers are supplied with their respective enzymes if obtained from Boehringer Manheim and Amersham).
4. Add 18 ul dH_2O.
5. Add restriction enzyme nuclease (1 to 5 U/ug DNA) to a final volume of 20 ul.
6. Incubate the reaction mixture for 1 to 2 hr at 37°C.
7. Stop the reaction by adding 5 ul gel tracking dye (Appendix A.2.6).
8. In order to perform multiple restriction enzyme digestions, first cleave with one of the restriction enzymes, precipitate the products using ammonium acetate precipitation (Appendix B.10) and finally cleave with the second restriction enzyme.
9. In order to perform partial restriction enzyme digestions, use 20 ug of DNA and only 10 U of restriction enzyme. Incubate for 1 hr at 37°C before stopping the reaction.

B.6 Agarose gel electrophoresis

(Ausubel *et al.*, 1989 unit 2.5)

1. Melt agarose in TAE (Appendix A.2.7) by heating in microwave and swirling to ensure even mixing.
2. Agarose concentrations can vary from 1% for separating plasmid DNA fragments to 0.8% for separating larger chromosomally restriction enzyme digested DNA fragments.
3. Add Ethidium bromide solution (10 mg/ml) (Appendix A.2.7) to a final concentration of 0.5 ug/ml.
4. Cool the melted agarose to 55°C before pouring onto the gel platform.
5. Seal the gel casting platform with masking tape if it is open at the ends.
6. Pour in the melted agarose and insert the gel comb, ensuring that no bubbles are trapped underneath the comb.
7. After the gel has hardened, remove the tape from the casting platform and withdraw the gel comb.

8. Place the gel casting platform containing the set gel in an electrophoresis tank.
9. Add sufficient TAE (Appendix A.2.7) to cover the gel.
10. Load DNA samples into the wells of the gel.
11. Attach leads so that DNA migrate into the gel toward the anode.
12. Run the gel at 1 to 10 V/cm until the dye in the loading buffer reach the end of the gel.

B.7 Electroelution of DNA restriction fragments from agarose gels

(Ausubel *et al.*, 1989 unit 2.6)

1. Digest sufficient DNA with the appropriate enzymes.
2. Separate the fragments on a TAE agarose gel (Appendix B.6).
3. Carefully cut out the target band under long UV light (310 nm) using a scalpel.
4. Rinse dialysis tubing (Spectra/por dialysis membrane tubing #4) with TAE buffer for 5 min.
5. Tie off the one end of the tubing using a dialysis clip and slide the gel into the tube.
6. Add 500 ul of TAE buffer (Appendix A.2.7) and seal the other end of the tube.
7. Place the sealed tube into a gel tank and fill the tank with TAE until the bag is covered by the solution.
8. Ensure that the bag is parallel to the electrodes, and that the gel slice is against the side of the tubing closest to the negative electrode.
9. Electroelute at a constant voltage of 2 V/cm between the two electrodes overnight.
10. Recover the DNA fragment using ammonium acetate precipitation (Appendix B.10).

B.8 Quantitation of DNA and RNA samples

(Coyne *et al.*, 1996)

B.8.1 Spectrophotometric quantitation of DNA and RNA

1. Perform a DNA or RNA scan of the DNA/RNA solution between 310-220 nm to determine the UV light absorbance of the sample.
2. The absorbance peak at 260 nm allows the calculation of the concentration of the DNA since 1 OD unit at 260 nm is equivalent to 50 ug/ml for double stranded DNA and 40 ug/ml for single stranded DNA or RNA.

B.8.2 Ethidium bromide fluorescent quantitation of DNA

1. Prepare three λ DNA standards with known concentrations: 5 ng/10ul, 10 ng/10ul and 20 ng/10ul.
2. Load 10 ul from each standard with 2.5 ul gel tracking dye (Appendix A.2.6) into the wells of a 1% TAE agarose gel (Appendix B.6).

3. Prepare several dilutions of DNA sample of unknown concentration in 10 ul.
4. Add 2.5 ul gel tracking dye (Appendix A.2.6) and load next to the standards on the agarose gel.
5. Electrophorese the samples at 100 V for 5 min.
6. Visualize the DNA bands using a 254 nm UV transilluminator.
7. Determine the concentration of the DNA sample by comparing the intensity of the DNA band to that of the standards. If you load 10 ul of a 1/10 dilution of the DNA sample, which corresponds to an intensity equivalent to that of the 10 ng standard, the DNA sample will have a concentration of 10 ng/ul.

B.9 Ligations

(Coyne *et al.*, 1996)

B.9.1 Intramolecular ligations

1. To recircularize plasmid DNA, use approximately 1 pmol of DNA.
2. Add 2 ul 10x ligation buffer (Appendix A.2.8).
3. Add 18 ul dH_2O.
4. Add 2 U of T_4 ligase to a final volume of 20 ul and incubate reaction mix at 15°C overnight.

B.9.2 Intermolecular ligations

1. In order to polymerize two distinct DNA fragments the total DNA concentration (vector plus insert) should not exceed 10 pmol.
2. Use ratios of vector to insert in the order of 1:1 to 1:4 pmol.
3. To an eppendorf add the vector and insert DNA.
4. Add 2 ul of 10x ligation buffer (Appendix A.2.8).
5. Add 2 U of T_4 ligase to a final volume of 20 ul.
6. When ligating DNA fragments with cohesive ends incubate reaction mixes at 15°C.
7. When joining blunt-ended DNA, use 10x more enzyme and incubate the reaction mixes at room temperature.

B.10 Ammonium acetate precipitation of DNA

(Coyne *et al.*, 1996)

1. Precipitation of DNA is carried out by adding half the volume of 7.5 M ammonium acetate, pH 7.5 (Appendix A.2.9) to the DNA suspension.
2. Incubate at room temperature for 15 min.
3. Centrifuge at 14 000 rpm for 15 min.

4. Transfer the supernatant to a clean eppendorf tube.
5. Add 2.5x volumes 100% EtOH and incubate at –20°C for 30 min.
6. Centrifuge at 14 000 rpm for 30 min at room temperature.
7. Wash the DNA pellet with 70% EtOH (Appendix A.2.2).
8. Resuspend DNA in 10 ul of TE (Appendix A.2.2).
9. Determine the DNA concentration via the Ethidium bromide fluorescent quantitation method (Appendix B.8.2).

B.11 Southern hybridization procedure

(Reed and Mann, 1985)

B.11.1 Southern transfer of DNA from agararose gel onto nitrocellulose membrane

1. Soak the agarose gel in 2x volumes 0.25 M HCl (Appendix A.2.5) for 5 min at room temperature.
2. Rinse the gel in 2x volumes of dH_2O.
3. Saturate 10 sheets (25 x 20 cm) Whatman 3MM paper with 0.4 M NaOH (Appendix A.2.5).
4. Place sheets on top of an inverted gel casting tray which has been placed in a tray covered with Saran wrap.
5. Add 0.4 M NaOH/1 M NaCl (Appendix A.2.5) to the tray so that the ends of the Whatman paper are submerged.
6. Invert the gel and place on top of the saturated Whatman paper. Ensure that no air bubbles remain trapped.
7. Cut Hybond N+ nylon membrane (15 x 20 cm).
8. Wet membrane in dH_2O and place on gel, ensuring that all air bubbles are removed.
9. Cover the edges with Saran wrap.
10. Place 3x sheets (20 x 15 cm) Whatman 3MM paper over the membrane, followed by a 10 cm stack of dry absorbant paper towel.
11. Place a glass plate on top of the towels, followed by a 0.2 to 0.4 kg weight.
12. Blot overnight.
13. Mark wells of the gel on the membrane with a pencil and rinse the membrane 2x SSC (Appendix A.2.5) for 5 min at room temperature.
14. Air dry the membrane on dry Whatman paper and store between 2 sheets of Whatman 3MM sheets at 4°C.

B 11.2 Labelling of DNA by nick translation

(Protocol used with Amersham labelling kit)

1. Place a 1.5 ml eppendorf on ice containing 10 ug of plasmid DNA and add the following reagents supplied as part of the Amersham Nick translation kit.
2. Add 10 ul of Nucleotide/buffer solution, 3 000 Ci/mmol [α-^{32}P] dCTP, and make up the reaction to 45 ul with sterile dH_2O.
3. Add 5 ul of DNA polymerase I/Dnase I solution.
4. Mix and incubate for 2 hr at 15°C.
5. Separate the labelled DNA from the unincorporated nucleotides using a spin column (Appendix B.11.4).

B.11.3 Labelling DNA by random prime labelling

(Protocol used with Boehringer Manheim labelling kit)

1. Denature 25 ng of DNA fragments by heating for 10 min at 95°C and subsequent cooling on ice.
2. Add 3 ul of dATP, dGTP, dTTP mixture supplied in the kit, and 2 ul of reaction mixture (Appendix A.2.5).
3. Add 5 ul 3000 Ci/mmol [α-^{32}P] dCTP and make up the reaction volume to 19 ul with sterile dH_2O.
4. Add 1 ul of Klenow enzyme and incubate the reaction mixture at 37°C for 30 min.
5. Stop the reaction by heating to 65°C for 10 min.
6. Separate the labelled DNA from the unincorporated nucleotides using a spin column (Appendix B.11.4).

B.11.4 Separation of radioisotope labelled DNA from unincorporated nucleotides using the spin column procedure

(Ausubel *et al.*, 1989 unit 3.4)

1. Plug the bottom of a 1 ml disposable syringe with a small amount of sterile glass wool.
2. Prepare a Sephadex G-50 (Appendix A.2.5) column with bed volume of 0.9 ml in the syringe.
3. Wash the column with 0.1 ml STE (Appendix A.2.5).
4. To the labelled DNA sample add 10 ul of tracking dye (Appendix A.2.5) and 40 ul STE buffer (Appendix A.2.5).

5. Place an eppendorf at the bottom of a bench top centrifuge and place the syringe containing the Sephadex column inside the eppendorf, so that the syringe will empty inside the tube.
6. Load the DNA onto the column and centrifuge for 4 min at 14 000 rpm.
7. The Blue dextran in the tracking dye will move with the labelled probe and will empty into the eppendorf tube, whereas the Orange G will remain with the unincorporated nucleotides on the column.
8. Determine the specific activity of the labelled DNA by counting 1 ul of probe in 2 ml scintillation fluid. Specific activity = counts per minute (cpm)/ug DNA.

B.11.5 Prehybridization, hybridization and washing of Southern blots

(Church and Gilbert, 1984)

1. Seal the Hybond N+ membrane containing transferred DNA in a plastic bag along with 0.2 ml of Church pre-hybridization buffer (pre CHB) (Appendix A.2.5) per cm^2 of membrane.
2. Incubate the sealed bag at 65°C for 1 hr with agitation.
3. Denature the labelled probe by heating at 100°C for 10 min and place on ice.
4. Remove pre CHB from the plastic bag and add fresh Church hybridization buffer (CHB) (Appendix A.2.5) (50 ul/cm^2 membrane) to the bag along with 1 x 10^6 cpm/ml of labelled probe.
5. Remove any bubbles and heat seal the bag.
6. Hybridize overnight at 65°C with agitation.
7. Wash membranes with Wash buffer A (WBA) and Wash buffer B (WBB) (Appendix A.2.5) at 65°C for 10 min and monitor the radioactivity between each wash on the membrane using a Geiger counter.
8. Once the radiation reach 200-500 cpm the membrane is sealed in a new plastic bag and placed in an X-ray cassette containing enhancer screens.
9. Expose the membrane to X-ray film and develop in an automatic X-ray film processor.

B.12 Sequencing

(Coyne *et al.*, 1996)

B.12.1 Preparing the sequencing gel

1. Mark the outer surface of two sequencing glass plates using tape, and wash the inner surfaces of both plates with detergent.

2. Add 1 ml silane (Appendix A.2.11) to the front plate and spread evenly over the entire inner surface.
3. Allow to dry for 5 min and wash gently with EtOH.
4. Arrange spacers on bottom plate and position the silanized top plate over the bottom plate.
5. Clamp the two plates together and place on gel pouring apparatus.
6. Prepare 50 ml gel mix (Appendix A.2.11) and filter through a 0.8 µm Millipore filter.
7. Add 200 ul 10% ammoniumpersulphate (Appendix A.2.2) and 45 ul TEMED (Appendix A.2.2).
8. Pour mix between glass plates.
9. Insert flat edge of spacer into the top of the gel at the top of the plate and leave the gel to polymerize.

B.12.2 Running of sequencing gels

1. Warm 2 liters of Tris Taurine buffer (TTE) (Appendix A.2.11) in microwave on high for 6 min.
2. Remove all clamps from glass plates as well as the bottom spacer.
3. Fill the bottom space of the gel with TTE (Appendix A.2.11).
4. Clamp the plates onto a sequencing gel tank and fill the bottom and top tanks with prewarmed TTE buffer.
5. Place shark tooth comb (points facing down) between the tanks until the tips of the teeth pierce the gel.
6. Load 4 ul of sequencing samples and run for 30 min to 6 hr at 42 mA.
7. On completion of the run, remove the buffer from the bottom tank.
8. Remove plates from gel tank and use a spatula to separate the two plates to expose the gel.
9. Place precut Whatman 3MM filter paper on gel.
10. Check that gel is adhering to filter paper before removing paper and gel from the sequencing plate.
11. Dry the gel at 75°C for 1 hr on dryer.
12. Expose to X-ray film to view sequences.

B.13 Labelling of oligonucleotide primer for primer extension

(Ausubel *et al.*, 1989 unit 4.8)

1. To an eppendorf tube, add the following reagents:

- 1 ul of dH_2O
- ul of T_4 10x polynucleotide kinase buffer (Appendix A.2.13)
- 1 ul of 0.1 M DTT (Appendix A.2.13)
- 1 ul of 1 mM spermidine (Appendix A.2.13)
- 100 ng/ul oligonucleotide primer
- 100uCi of [γ-^{32}P] ATP
- 20-30 U/ul T_4 polynucleotide kinase

Final volume 15 ul.

2. In order to avoid possible precipitation of the oligonucleotide primer, the spermidine and oligonucleotide should not be pre-mixed.
3. Incubate the reaction mix at 37°C for 1 hr.
4. Stop the reaction by adding 2 ul 0.5 M EDTA and TE buffer to a final volume of 25 ul.
5. Incubate for 5 min at 65°C to inactivate the kinase.
6. Precipitated the labelled oligonucleotide by adding 25 ul 4 M ammonium acetate (Appendix A.2.13) and 250 ul ice cold 100% ethanol. Leave overnight at -20°C.
7. Centrifuge at 14 000 rpm for 15 min and resuspended the pellet in 25 ul of DEPC treated dH_2O.
8. Repeat Steps 6 and 7 and resuspend the pellet in 100 ul of TEN 600 buffer (Appendix A.2.13).
9. Determine the specific activity of the labelled oligonucleotide by counting 1 ul in 2 ml scintillation fluid in a Beckman scintillation counter.

B.14 Preparing and running denaturing SDS-PAGE gels

(Ausubel *et al.*, 1989 unit 10.2)

1. Combine the following reagents for a 12% separating gel mix in a glass beaker.
2. The reagents are as follows:

- 30% acrylamide solution (Appendix A.2.14) 4 ml
- separating gel 4x buffer (Appendix A.2.14) 2.5 ml
- dH_2O 6.6 ml
- 10% ammonium persulfate (Appendix A.2.2) 50 ul
- TEMED (Appendix A.2.2) 15 ul

3. Pour the separating gel mix into the assembled gel plates, leaving sufficient space at the top for the stacking gel.
4. Gently overlay the gel mix with 0.1% SDS.

5. After polymerization, remove the overlay and rinse the surface of the separating gel to remove unpolymerized acrylamide.
6. Prepare the 5% stacking gel mix as follows:
    - 30% acrylamide solution (Appendix A.2.14) 625 ul
    - stacking gel 4x buffer (Appendix A.2.14) 1.25 ml
    - dH_2O 3.09 ml
    - 10% ammoniumpersulfate (Appendix A.2.2) 25 ul
    - TEMED (Appendix A.2.2) 15 ul
7. Pour the stacking gel mix and insert the comb immediately.
8. After the stacking gel has polymerized, remove the comb and rinse the wells to remove any unpolymerized acrylamide.
9. Place the assembled gel into the electrophoresis apparatus and fill the tank with SDS-PAGE running buffer (Appendix A.2.14).
10. Prepare protein samples by adding 5 ul of SDS-PAGE sample buffer (Appendix A.2.14) to 10 ul of protein sample. Denature protein samples by boiling for 3 min at 96°C.
11. Load samples into the bottom of the wells.
12. Run the gel at constant current of 15 mA in the stacking gel and 30 mA in the separating gel.
13. After electrophoresis, visualize the protein bands in the gel by staining with Coomassie blue dye (Appendix A.2.14) for 15 min at 37°C.
14. Destain the gel in destaining solution (Appendix A.2.14).
15. Dry the gel for 45 min at 70°C using a gel dryer.

B.15 Electroblotting of proteins onto a nitrocellulose membrane

(Towbin *et al.*, 1979)

1. Remove the SDS-PAGE gel from glass plate and soak the gel in blotting buffer (Appendix A.2.15) for 1 hr.
2. Pre-wet the nitrocellulase membrane in blotting buffer (Appendix A.2.15) and place gel on membrane.
3. Ensure that no air bubbles remain trapped.
4. Cut four sheets of Whatman 3MM filter paper (10 x 5 cm) and soak in blotting buffer (Appendix A.2.15).
5. Sandwhich the membrane and gel between the filter paper.
6. Clamp the entire sandwhich between two perforated sheets of perspex.

7. Load assembly into the transblot chamber filled with blotting buffer (Appendix A.2.15).
8. Ensure that the nitrocellulose is towards the anode (+).
9. Transfer at 15 V for 4 hrs.

B.16 Bradford protein assay for protein quantitation

(Ausubel *et al.*, 1989 unit 10.1)

1. Aliquot (in duplicate) the following amounts of 1 mg/ml BSA (Appendix A.2.16) and 0.15 M NaCl (Appendix A.2.16) into eppendorf tubes.

BSA	NaCl
2.5 ul (2.5 ug/ml)	97.5 ul
5 ul (5 ug/ml)	95 ul
10 ul (10 ug/ml)	90 ul
15 ul (15 ug/ml)	85 ul
20 ul (20 ug/ml)	80 ul

2. Add 100 ul of protein sample with unknown concentration (in duplicate) to an eppendorf tube.
3. Add 1 ml of Coomassie Brilliant Blue (Appendix A.2.16) to the standard and sample tubes.
4. Vortex for 5 seconds.
5. Allow the tubes to stand at room temperature for 5 min.
6. Determine the A_{595} of all the samples and plat a standard curve of A_{595} versus protein concentration, using the standards.
7. Use the curve to determine the protein concentration of the sample.

B.17 Repairing 3' or 5' overhanging ends to generate blunt ends

(Ausubel *et al.*, 1989 unit 3.5)

1. In a 20 ul reaction, digest 0.1 to 4 ug of DNA with restriction endonuclease (Appendix B.5).
2. Add 1ul of 0.5 mM dNTPs.
3. Add 1 ul of Klenow and incubate at 30°C.
4. Stop the reaction by heating to 75°C for 10 min of by adding 1 ul of 0.5 M EDTA (Appendix A.2.2).

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