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**THE ROLE OF MELANIN PRODUCTION IN THE SURVIVAL OF
*VIBRIO CHOLERA*E IN THE MARINE ENVIRONMENT**

BY

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Abstract

The marine bacterium *Vibrio cholerae* produces pyomelanin through the catabolism of L-tyrosine to homogentisic acid. Various types of melanins are used by microbes as defense mechanisms against a variety of environmental stresses. This thesis investigated the defensive role of *V. cholerae* pyomelanin against exogenous stresses, particularly hydrogen-peroxide (H_2O_2).

A *V. cholerae* 569B melanin-deficient mutant was created by insertional inactivation of *ppdA*, which encodes for *p*-hydroxyphenyl pyruvate dioxygenase, with the aid of suicide vector pGP704 that contained an internal *ppdA* fragment. The melanin mutant was more sensitive than the wild type to the presence of 5 mM H_2O_2 . Pyomelanin also protected melanised *Escherichia coli* expressing *ppdA* from the low copy number plasmid pACMel against H_2O_2 . Although both microorganisms showed greater resistance to H_2O_2 , the melanised bacteria always resulted in lower bacterial numbers (in the absence of H_2O_2) in comparison to non-melanised *V. cholerae* and *E. coli* cultures.

Complementation of the *ppdA* mutation with *ppdA* expressed from the high copy number plasmid pCM302-16 did not restore resistance to H_2O_2 , but instead resulted in enhanced sensitivity to H_2O_2 . Increased sensitivity also occurred in *V. cholerae* wild type and *E. coli* cells when melanin was produced in these strains from plasmid pCM302-16. The decline in the cell number of melanised cultures, together with the enhanced bactericidal effect of H_2O_2 on bacterial strains overproducing melanin, suggested that a by-product of melanin production was detrimental to cellular fitness and enhanced killing by exogenous H_2O . It is known from published literature that H_2O_2 is formed during the autopolymerization of homogentisic acid, and that it causes oxidative DNA damage. However, we were unable to demonstrate DNA damage in melanised cultures using alkaline agarose gel electrophoresis.

The *V. cholerae ppdA* mutant was more sensitive than the wild type strain to copper chloride, but not ferrisulphate. In contrast, *V. cholerae* 569B was more sensitive to silver nitrate and sodium hypochlorite than the *ppdA* mutant.

The role of melanin as an antioxidant metabolite was further investigated in a *V. cholerae* strain that lacked the catalase-peroxidase PerA. PerA was found to be a heat-labile protein, 724 amino acids in length, and the sole catalase produced by *V. cholerae* 569B during the stationary phase of growth. The *perA* gene was isolated from a *V. cholerae* 569B genebank and used to insertionaly inactivate *perA*. The *perA V. cholerae* mutant was more sensitive to 1 mM H_2O_2 compared to the wild type strain when the cells were either in log phase or were twenty-four hours old. In contrast, melanised *V. cholerae perA* mutant cultures, i.e. two day old cultures grown under melanin-inducing conditions, exhibited increased sensitivity to 1 mM H_2O_2 in comparison to the non-

melanised *perA* mutant cultures. In addition, we observed a severe decline in melanised *perA* mutant cell numbers, even in the absence of exogenous H₂O₂, suggesting that H₂O₂ is indeed a by-product of melanization in *V. cholerae*.

We conclude that melanin protects *V. cholerae* against H₂O₂ only when homogentisic acid is produced at physiological levels, that H₂O₂ is formed during melanogenesis and that PerA is required for the removal of the H₂O₂ formed during melanogenesis. Finally, melanin protects *V. cholerae* against some, but not all, exogenous stresses.

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CHAPTER 1
LITERATURE REVIEW

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1.1 Cholera: A Short history, aetiology and regions affected

Cholera is a water-borne bacterial disease that presents clinically in humans as a watery diarrhea. Death is the result of severe dehydration (Jawetz et al., 1995). The recorded history of the last few hundred years provide useful information on the epidemiology and etiology of the disease (Faruque et al., 1998; Shah et al., 1998; Codeco, 2001; Colwell, 2002; Pascual et al., 2002). Seven pandemics are recorded to date. The seventh pandemic has spread from Sulawesi, Indonesia to Asia, Africa and South America (Faruque et al., 1998; Codeco, 2001; Colwell, 2002; Pascual et al., 2002). Modern microbiological techniques, as well as the use of molecular biology tools helped to identify and characterise bacterial isolates responsible for epidemics both past and current (Karaolis et al., 1994; O'Shea et al., 2004; Smirnova et al., 2004). These tools also help with the identification of epidemiological patterns as well as the identification of environmental reservoirs for the offending bacteria (Karaolis et al., 1995; Karaolis et al., 2001; Faruque and Mekalanos, 1998; Faruque et al., 2003; Binsztein et al., 2004; Faruque et al., 2005).

Different strains of *V. cholerae*, the causative agent of cholera, predominated with each pandemic. Strain evolution, as well as replacement or alternation of strains is clearly seen on the Indian subcontinent, where strain 569B was isolated in the early 1900s (caused the 6th pandemic), while the El Tor strain predominated in the 7th pandemic, followed by the emergence of the O139 (or Bengal) strain in the early 1990s (Faruque et al., 1997; Shah et al., 1998). This Bengal strain is closely related to the El Tor strain (Berche et al., 1994; Mooi and Bik, 1997), and seems to have evolved rapidly into different variants, exhibiting different antibiotic resistance profiles (Mitra et al., 1996; Faruque et al., 1997; Faruque, 1998; Faruque et al., 2000; Faruque et al., 2003). Evolution into different variants most probably contributes to the success of the causative bacterium as a pathogen.

Cholera is endemic to the Indian sub-continent (Fig.1.1) (Pascual et al., 2002), while recurring epidemics also occur in South America (Kumate et al., 1998), Africa (Dalsgaard et al., 2001; Naidoo and Patric, 2002), and South-Eastern Europe, e.g. Romania (Damian et al., 1998; Israil et al., 1998). In the case of the South American epidemics, the source of the initial contamination was traced to contaminated ballast water from an Asian freighter (Kumate et al., 1998). The introduction of cholera, caused by an El Tor strain, to South Africa was partly attributed to the migration of mineworkers from Mozambique, the latter being a country with frequent cholera epidemics (Dalsgaard et al., 2001). Sporadic incidents of cholera occur in North America (Steinberg et al., 2001) and parts of Europe (Schurmann et al., 2002), mainly due to ingestion of contaminated seafood.

The presence of cholera is associated with water masses, for example the South American west coast (Kumate et al., 1998), the Indian Sub-continent (Fig.1.1) (Pascual et al, 2002), as well the Danube River Delta (Damian et al., 1998; Lobitz et al., 2000). The disease occurs annually, coinciding with specific climatic events. On the Indian subcontinent, for example, the incidence of cholera coincides with an increase in sea surface temperatures as well as an increase in the numbers of phyto- and zooplankton (Lobitz et al., 2000). Outbreaks correlate positively with the warmer months of the year, particularly the monsoon season. A similar pattern is observed in South Africa, with cholera infections appearing mainly in the summer months (Kustner and Du Plessis, 1991). The relationship between elevated sea surface temperatures, plankton and *V. cholerae* was also observed along the Peruvian coast on the South American continent (Gil et al., 2004).

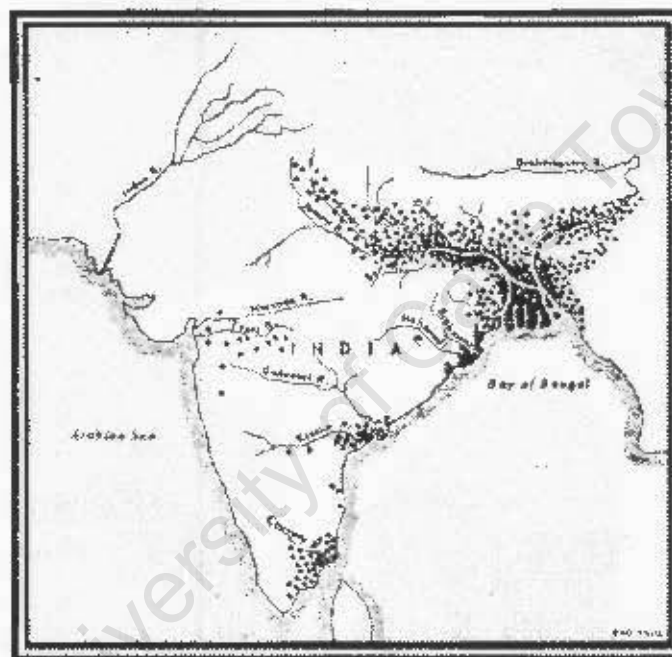


Fig. 1.1 The distribution of cholera on the South-East Asian continent. A rise in sea-surface and water temperature in the Bay of Bengal leads to increased influx of seawater into the river delta, thereby aiding the influx of contaminated plankton into river basins. Plankton contaminated with *Vibrio cholerae* aid the spread of the disease along the river deltas (Codeco, 2001, Lobitz et al., 2000, Pascual et al., 2002). Picture from Pascual et al., 2002. The black dots indicate areas affected by cholera.

1.2 *Vibrio cholerae*, the cause of cholera, a waterborne bacterial disease

Cholera was first associated with contaminated water by Lord Snow (Codeco, 2001; Colwell, 2002). A Gram-negative bacterium, named *Vibrio cholerae*, was identified as the causative agent of the disease.

1.2.1 The genus *Vibrio*

The family *Vibrionaceae* includes a group of Gram-negative comma-shaped bacilli, commonly referred to as vibrios. Vibrios are oxidase positive, catalase positive, non-spore-forming, comma-shaped and motile (Jawetz et al., 1995). These bacteria are most frequently isolated from waters with an intermediate salt-content, such as estuaries, but are also cultivated from fresh water sources as well as seawater (Singleton et al., 1982). *V. cholerae* is differentiated from Gram-negative enteric bacilli through its ability to grow on thiosulphate-citrate-bile-sucrose (TCBS) agar. It grows well at an alkaline pH, but in general is rapidly killed by acid. *Vibrio cholerae* is able to grow in media with up to 6% NaCl (Jawetz et al., 1995).

The genus *Vibrio* includes both pathogenic and non-pathogenic species. A number of diseases are caused by vibrios. *Vibrio parahaemolyticus* (Hornstrup and Garhm-Hansen, 1993), *Vibrio mimicus* (Shandera et al., 1983), *Vibrio vulnificus* (Patel et al., 2002) and *Vibrio alginolyticus* (Hornstrup and Garhm-Hansen, 1993) are indicated in gastroenteritis and extra-intestinal infections. *Vibrio cholerae* causes profuse diarrhoea, but is rarely responsible for infections outside of the gastrointestinal system (Jawetz et al., 1995).

Three serogroups have been described for *Vibrio cholerae*, namely O1, O139 and non-O1 serogroups. The cholera disease is associated mainly with toxinogenic (strains producing cholera toxin) *V. cholerae* belonging to the O1 serogroup, although sporadic cases of cholera-like diarrhoea by non-O1 *V. cholerae* have been reported (Jawetz et al., 1995; Chan et al., 1994). Two major serotypes, Ogawa and Inaba, were identified in *V. cholerae* strains belonging to the O1 serogroups. Vibrios from the O1 serogroup are able to seroconvert between the Ogawa and Inaba serotypes (Stroeher et al., 1992).

The virulence of toxinogenic strains (compared to those strains not capable of causing disease), is contributed to the presence of essential colonization factors and, most importantly, the production of cholera toxin (CT) (Champion et al., 1997). The genes encoding for CT, that is *ctxAB*, are not a part of the ancestral *V. cholerae* genome, but actually originates from the genome of a filamentous phage. This phage, CTX ϕ is able to infect *V. cholerae* and integrate into the bacterial genome (Waldor and Mekalanos, 1996). Other genes in this phage element include genes responsible for phage packaging and secretion. Another set of virulence factors, including the toxin co-regulated pilus (TCP), is encoded by genes found on the *Vibrio* pathogenicity island (VPI). There are some indications that VPI might be a filamentous phage, but other evidence contests this hypothesis (Karaolis et al., 1999; Davis et al., 2000; Davis and Waldor, 2003; Faruque et al., 2003).

1.2.2 The habitat of *V. cholerae*

Vibrio cholerae is a native inhabitant of aquatic environments and is associated with a wide variety of aquatic fauna and flora. The organism has been isolated from fresh and salty waters, although greater numbers were obtained from aquatic environments of intermediate salt-content, such as estuaries (Hood and Ness 1982; Singleton et al. 1982; Shukla et al. 1995).

The growth of the organism is influenced by water temperature and salinity, as well as the availability of nutrients. Nutrient deprivation, as well as unsuitable salinity and temperature cause the induction of the viable, but non-culturable (VBNC) state (Baker et al., 1983, Colwell et al., 1985). The bacterium exists as small cocci, exhibits a reduction in the nucleic acid content and changes in the membrane lipid composition when in this non-culturable state (Wai et al., 1999; Reidle and Klose., 2002).

The bacterium is able to fully persist in a viable form as a free-living organism in water outside of a host, and the number of vibrios in seawater increases during phytoplankton blooms (Worden et al., 2006). It was also found to survive on coral reefs and grassy marine areas and marine sediments (Perez-Rosas and Kazen, 1988). The organism was also isolated from a variety of freshwater and marine algae and plants. It adhered to freshwater plants while persisting in the VBNC state, but converted back to the culturable state and induced cholera symptoms when introduced to a suitable host (Shukla et al., 1995). The organism thus retains its pathogenicity in the environment.

The bacterium was found in association with phytoplankton and zooplankton (Huq et al., 1983; Huq et al., 1984; Tamplin et al., 1990; Binsztein et al., 2004), arthropods (Shukla et al., 1995), crabs (Huq et al., 1986), eggs of chironomids (Halpern et al., 2004, Broza, 2001; Broza et al. 2005; Halpern et al., 2006) and shellfish (Murphree and Tamplin, 1995). Most importantly, chironomids, or midges, carried *V. cholerae* 0139 from an infected water source to an uninfected source in *in vitro* studies (Broza et al., 2005). This implies that this fresh water insect might play an important role in the dissemination of *V. cholerae* amongst aquatic sources in different geographical locations. The bacterium was also isolated from houseflies in an endemic cholera region (Echeverria et al., 1983; Echeverria et al., 1995). Humans are, without a doubt, a very suitable host and vector for the organism, as evidenced by the rapid spread of the disease during an epidemic. The bacterium was found to be more virulent after isolation from humans, indicating that *V. cholerae* converts to a highly infectious state inside the host (Merrell et al., 2002). The persistence of the bacterium in aquatic environments is aided by various ligands on the cell surface, such as the mannose-sensitive haem agglutinin (Tarsi and Pruzzo, 1999; Chiavelli et al., 2001; Zampini et al., 2005).

Lastly, *V. cholerae* forms biofilms (Watnick and Kolter., 1999), and forms a rugose type colony when exposed to starvation conditions (Yildiz and Schoolnik, 1999). Both these phenomena are

known to be advantageous for microorganisms in terms of protection and survival against unfavourable conditions such as oxidative stress (Stickler, 1999; Yildiz and Schoolnik, 1999). Recent work indicates that biofilms play a valuable role in the persistence and dispersal of pathogens in the environment (Hall-Stoodley and Stoodley, 2005).

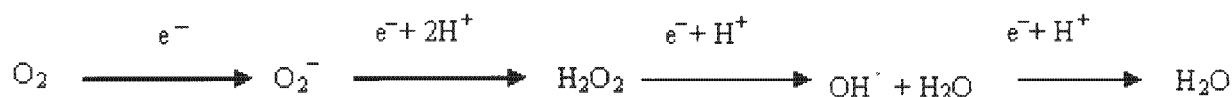
From the wide variety of hosts for *V. cholerae*, its ability to persist as free-living organisms and the recurrence of epidemics in particular areas, it appears that *V. cholerae* is well adapted to survive in a wide variety of hosts and environments. This bacterium thus should possess a number of traits that allows it to (1) obtain food in a nutrient poor environment, (2) to adapt to unfavourable temperature and osmotic shifts, and (3) to defend against oxidative radicals. The latter are present in marine waters and, in addition, are produced by the invertebrate hosts of *V. cholerae*. As a successful colonizer, the bacterium therefore should contain anti-oxidant molecules and proteins. In line with the theme of this thesis, the next section of this review will focus on oxidative stress and defence in bacteria, particularly H₂O₂ and catalases.

1.3. Oxidative stress and bacteria

Oxidative stress occurs in an organism when reactive oxygen intermediates (ROIs) are present in higher concentrations than what can be detoxified efficiently by the cellular defence mechanisms (Farr and Kogoma, 1991). This leads to a decline in bacterial fitness or even death. The most prevalent of these radicals are the superoxide radical (O₂⁻), hydrogen-peroxide (H₂O₂) and the hydroxyl radical (OH[·]).

1.3.1. The origin of oxidative radicals

Superoxide (O₂⁻) is a by-product of aerobic respiration. It is formed at specific points in electron transport chains as a result of the autoxidation of electron (e⁻) carriers (Storz et al., 1990; Messner and Imlay, 1999). NADH dehydrogenase I, ubiquinone and cytochrome C were identified as the specific enzymes in electron-transport chains where production of mainly O₂⁻ occurs (Gonzalez-Flecha and Demple, 1995; Messner and Imlay, 1999). With the addition of more e⁻ and protons (H⁺) to O₂⁻, other radicals such as H₂O₂, and OH[·] are formed (See Reaction 1).



Reaction 1

The superoxide radical (O_2^-) represents a substantial threat to biological molecules, and consequently aerobic respiring organisms produce superoxide dismutase (SOD) to break down this radical. It is known that bacterial fitness declines with a loss of SOD (Carlioz and Touati, 1986; Sadosky et al., 1994; Inaoka et al., 1999; Strohmeier Gort and Imlay, 1998; Yesilkaya et al., 2000). A number of bacteria struggled to grow in the absence of SOD (Carlioz and Touati, 1986; Inaoka et al., 1999), while SOD mutants of *E. coli* exhibited increased DNA damage (Strohmeier Gort and Imlay, 1998). These observations indicate that SOD is essential for detoxification of internally and externally produced O_2^- .

The end-product of O_2^- dismutation is H_2O_2 , and the majority of intracellularly produced H_2O_2 is therefore the result of SOD activity (Messner and Imlay, 1999). The internal amount of O_2^- and H_2O_2 produced in *E. coli* was shown to peak during the exponential phase of growth (optimal phase of growth) and ceased with a decline in cell growth. Since H_2O_2 also poses a threat to biological molecules, bacteria produce scavenger enzymes to detoxify this oxidant swiftly. In a bacterium such as *E. coli* the intracellular concentration of H_2O_2 is maintained at a steady state (less than 10^{-7} M) through the action of enzymes such as catalases and other hydroperoxidases (Seaver and Imlay, 2001). Lastly, the hydroxyl radical (the most damaging of the three mentioned radicals) is formed inside the cell through the interaction of O_2^- and H_2O_2 with each other and metal ions (Imlay et al., 1988). When these reactions involve iron, they are called Fenton reactions.

In the marine environment, all three radicals form through the action of heat and ultraviolet (UV) light (Alam and Ohgaki, 2002). Nanomolar amounts of the hydroxyl radical (OH^\cdot) were detected in seawater after irradiation with sunlight and the amounts decreased from coastal waters to deep-sea surface waters (Mopper and Zhou, 1990). Larger quantities of OH^\cdot were measured in waters rich in dissolved organic material (DOM) (Mopper and Zhou, 1990). As DOM is the major source of nutrients for marine microorganisms, it can be inferred that bacteria in this environment are constantly at risk of exposure to the radical. A study done on the enteric bacterium *E. coli* showed that sunlight irradiation of the bacterium, when resuspended in seawater, caused the bacterium to exhibit a loss in culturability (Gourmelon et al., 1994). This effect was inhibited in the presence of iron chelators, catalase enzyme, a hydroxyl radical scavenger or anaerobic conditions. These results suggested that oxidative radicals such as H_2O_2 and hydroxyl radicals are formed in irradiated water. Marine bacteria exhibited similar inactivation in water irradiated by ultraviolet (UV) light (Liltved and Landfald, 2000), therefore one expects that antioxidant mechanisms of protection expressed by aquatic bacteria are essential for survival against UV-induced phototoxicity.

Another source of oxidative radicals is the autoxidation of transition metals. Autoxidation of metals such as Fe (II) and Cu (I), results in oxidative stress from ROIs such as O_2^- , H_2O_2 and OH^\cdot (Halliwell and Gutteridge, 1992; Imlay et al., 1988). Inside the bacterial cell, haem-containing

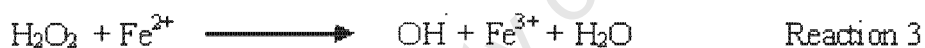
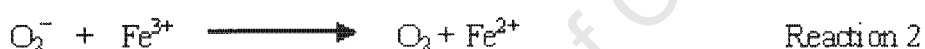
enzymes and proteins with iron-sulphur (Fe-S) clusters are another source of radicals as these metals can be oxidised or removed from the protein (Kono and Fridovich, 1982; Djaman et al., 2004). Free pools of Fe in the cytoplasm can then react in Fenton reactions to generate harmful radicals such as the hydroxyl radical (McCormick et al., 1998).

1.3.2. Damage caused to biological molecules by O_2^- , H_2O_2 and OH^\cdot Radicals.

1.3.2.1. DNA oxidation

Oxidative radicals attack the sugars and bases of DNA. This leads to the formation of 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, adenine ring-opened and ring-saturated products (Halliwell and Aruoma et al., 1991; Marnett, 2000; Marnett, 2002).

Damage to DNA mediated by O_2^- and H_2O_2 is thought to occur indirectly through the production of OH^\cdot (Imlay et al., 1988). The model for O_2^- -mediated damage to DNA involves oxidative radicals that are products of the Fenton reaction (Reaction 2 and 3). This damage is the result of the interaction of O_2^- , ferric iron (Fe^{3+}) and H_2O_2 to produce the deleterious OH^\cdot . The latter is a known culprit of mutational lesions in DNA.



Initially it was thought that reactions (2) and (3) occur with ferric iron (Fe^{3+}) bound to DNA (Imlay, 1988). However, further investigation showed that DNA-damage might be mediated through interaction with free iron inside bacterial cells (Keyer et al., 1995; Keyer and Imlay, 1996). It was subsequently shown that the presence of superoxide leads to an increase in the levels of free iron (Keyer et al., 1995). This pool of free iron is obtained through the loss of iron from proteins containing [Fe-S] clusters. Such proteins, such as aconitases and dehydratases, are inactivated by H_2O_2 and O_2^- , and the mechanism of inactivation is due to the loss of iron from catalytic centers (Flint et al., 1993; Gardner and Fridovich, 1992; Keyer et al., 1995; Keyer and Imlay, 1996; Strohmeier Gort and Imlay, 1998). This generation of free pools of Fe in the cytoplasm contribute to oxidative damage of biological molecules such as DNA (McCormick et al., 1998).

1.3.2.2. Damage to proteins

As mentioned above, oxidative stress leads to the loss of iron in proteins containing [Fe-S] clusters (Gardner, 1991; Djaman et al., 2004). Proteins can also be inactivated by oxygen radicals such as H_2O_2 and superoxide, when specific amino acid residues or catalytic sites become irreversibly damaged (Kono and Fridovich, 1982; Farr and Kogoma, 1991). An example of such oxidative damage is found in haem-containing catalases, the latter being inactivated by high concentrations of H_2O_2 (Switala and Loewen, 2002). This inactivation is due to the irreversible oxidation of the Fe^{3+} in the haem centre by the oxidant.

1.3.2.3 Damage to membranes

Oxidative radicals cause damage to membranes through a process known as lipid peroxidation. In the process of lipid peroxidation hydrogen is extracted from polyunsaturated fatty acid residues in the membrane. A sequence of oxidation events ensues, leading to the formation of a fatty acid hydroperoxide, carbonyl products (such as malondialdehyde (MDA)) and other lipid radicals (Farr, and Kogoma, 1991; Marnett, 2002). The formation of lipid radicals, shorter lipids and other products cause the membrane to become distorted and more fluid. Subsequently, there is a loss of membrane transport and proton gradient across the membrane, more O_2^- is then formed, resulting in even more damage. In addition, the products of lipid peroxidation (eg. MDA) have also been implicated in DNA damage in *E. coli* (Marnett, 2002; Yoon et al., 2002). H_2O_2 , and other organic peroxides are known inducers of lipid peroxidation (Park et al., 2002).

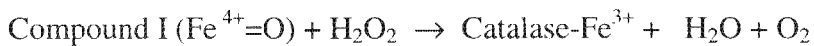
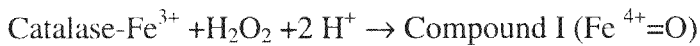
1.4 Bacterial defence against oxidative stress.

Bacteria combat oxidative stress by preventative as well as repair mechanisms. In addition, bacteria are able to adapt swiftly to oxidative insult from the external environment by the up-regulation of specific genes (Storz et al., 1990).

Specific scavenger enzymes and metabolites are produced to detoxify oxidative radicals. For example, aerobic respiring bacteria produce SOD to break down O_2^- , while the catalase enzyme is the main bacterial scavenger of H_2O_2 , though enzymes such as alkyl hydroperoxidase also remove H_2O_2 . Bacteria also produce DNA-binding proteins, such as Dps, that prevent damage by literally excluding radicals from the DNA strand. Oxidised proteins are removed by specific proteinases. Lastly, specific DNA-repair enzymes are responsible for keeping the genetic code intact by removing damaged bases or regions of damaged bases (Farr and Kogoma, 1991).

1.4.1 Catalases

Catalases are the main scavenger enzymes of H₂O₂ and are found throughout the prokaryotic and eukaryotic kingdoms. This enzyme breaks down H₂O₂ to form H₂O and O₂ through the following reactions:



1.4.1.1. Classification of catalases. Catalases are grouped according to phylogenetic traits as well as their protein characteristics.

1.4.1.1.1 Typical or “true” catalases. This group consists of those catalases containing a haem group as the active centre. The enzymes in this group are known as monofunctional catalases because they only decompose H₂O₂ through the typical catalase reaction, i.e. 2 molecules of H₂O₂ are converted to H₂O and O₂ (Zamocky and Koller, 1999). Monofunctional catalases are found in a number of microorganisms including *Escherichia coli*, *Xanthomonas campestris*, *Bordetella pertussis*, *Pseudomonas*, *Brucella abortus*, *Saccharomyces cerevisiae*, *Aspergillus niger*, and many more microorganisms (Switala and Loewen, 2002). Amongst the genus *Vibrio*, monofunctional catalases were identified in *V. fischerii* (Visick and Ruby, 1998), and *V. cholerae* (KatB, locus VC1585, <http://www.tigr.org>). Monofunctional catalases consist of homotetramers (Zamocky and Koller, 1999), differ in size, their sensitivity to heat and inactivation by inhibitors (Switala and Loewen, 2002). Monofunctional catalases with bigger subunits (such as is found in *E. coli* and *A. niger*) are more heat stable. The larger size of these subunits is due to addition of 70 amino acids on the N-terminus and 150 amino acids on the C-terminus on either side of the conserved central catalase core (Klotz et al., 1997).

Phylogenetic analysis of 70 monofunctional catalases (Klotz et al., 1997) revealed that these catalases group into separate plant, animal, fungal and bacterial catalases. The latter two groups were further split into 2 and 3 separate groups respectively. Amongst the bacterial catalases, Group I bacterial catalases are closely related to plant catalases, Group II consists of bacterial catalases with large sub-units, while Group III bacterial catalases resemble the fungal catalases.

1.4.1.1.2 “Atypical” catalases, or catalase-peroxidases. These catalases are bi-functional, because they decompose H₂O₂ through the “typical” catalase reaction and also through a peroxidase reaction (Zamocky et al., 2000; Zamocky et al., 2001). In the latter reaction, one molecule of H₂O₂

serves as the electron donor for the formation of Compound I, while another donor molecule (not H₂O₂) serves as the second electron donor. Normally the peroxidatic activity of the catalase-peroxidases is much smaller than the catalase activity, such as was found in KatB, the catalase-peroxidase of *Legionella pneumophila* (Bandyopadhyay and Steinman, 1998).

The second hydrogen donor can be molecules such as methanol, ethanol, propanol, formate and phenols, NADPH, guaiacol, pyrogallol and *o*-dianisidine (Nagy et al., 1997, Regelsberger et al., 1999). A catalase-peroxidase isolated from *Synechocystis* PCC 6803 also interacted with a number of aromatic substrates and received one e⁻ from donors such as *p*-hydroxyaniline, *p*-toluidine, aniline, *p*-hydroxyanisole, phenol and *p*-chlorophenol (Regelsberger et al., 1999).

Phylogenetic analysis of the catalase-peroxidases revealed a close relationship between bacterial catalase-peroxidases and eukaryotic peroxidases belonging to the Class I peroxidase superfamily (Welinder, 1991, Zamocky et al., 2000). These eukaryotic peroxidases seem to have evolved from a prokaryotic origin and include ascorbate peroxidases from higher plants and cytochrome C peroxidase from *Saccharomyces cerevisiae*. A characteristic feature of the catalase-peroxidases is the duplication of specific ligands in the C and N termini of the protein (Zamocky et al., 2000). The duplicated domains show strong similarity with the cytochrome c peroxidase from yeast (Zamocky et al., 2000; Zamocky et al., 2001). Striking sequence similarities that are centred around specific amino acid (histidine) residues, exist in the distal and proximal haem ligands of sequenced bacterial catalase-peroxidases (Zamocky et al., 2000). The catalytic activity of KatG reside in the N-termini of the protein (Carpena et al., 2004). Recent work also indicates that the C-terminus of KatG proteins might play a role in formation of the dimeric protein, as well as folding of the protein (Carpena et al., 2004).

The catalase-peroxidases generally consist of more than 700 amino acids (Zamocky et al., 2000, Zamocky et al., 2001). Another feature of catalase-peroxidases is the fact that they are inactivated by high temperatures. For example, Hydroperoxidase I (or HPI), the catalase-peroxidase of *E. coli*, is inactivated by exposure to 55°C (Visick and Clarke, 1997), whereas the monofunctional Hydroperoxidase II (HPII) is not affected by exposure to this temperature.

Bacteria that possess catalase-peroxidases include *Mycobacterium tuberculosis* (Nagy et al., 1997; Pym et al., 2001), *E. coli* (Loewen et al. 1985; Hillar et al, 1999), *Streptomyces reticuli* (Zou et al., 1999; Zou and Scrempf, 2000), *Yersinia pestis* (Garcia et al., 1999) and *Synechocystis spp.* (Tichy and Vermaas, 1999). A gene encoding for a catalase-peroxidase, *perA*, was identified in the genome of *V. cholerae* El Tor N16961 (locus VC1560, <http://www.tigr.org>).

1.4.1.1.3 Pseudo-catalases are monofunctional catalases that contain manganese instead of iron at the active centre. Manganese-containing catalases have been described in *Lactobacillus*

plantarum (Igarashi et al., 1996; Barynin et al., 2001), *Salmonella enterica* (Robbe-Saule et al., 2001), as well as a thermophilic bacterium, *Pyrobaculum calidifontis* VA1 (Amo et al., 2002). Catalases belonging to this group form homopentamers or homohexamers (Beyer and Fridovich, 1985), with subunits of between 28- 37 kDa (Robbe-Saule et al., 2001). The identity between the sequenced Mn-catalases is very low (34 - 52%) (Robbe-Saule et al., 2001). Another group of enzymes, namely bromoperoxidases, might also be part of the monofunctional atypical catalase family. Haem-containing bromoperoxidases contain ferriprotoporphyrin IX and have catalase activity. Such a bromoperoxidase was isolated from *Streptomyces venezuela*. This enzyme shows sequence homology to bacterial monofunctional catalases of the Group I bacterial catalases as described by Klotz et al. (Facey et al., 1996; Klotz et al., 1997).

A single species or strain of bacteria might contain one or more of the above catalases. *E. coli*, for example, encodes for HPI and HPII (Loewen et al., 1985; Loewen et al., 1987; Visick and Clarke, 1997). Each of these enzymes fulfills specific roles, namely regulation of internally produced H₂O₂, dismutation of externally added H₂O₂, and protection during the different bacterial growth stages. HPI is located in the periplasmic space (Hillar et al., 1997) and is more important in the logarithmic phase of growth, while HPII is found in the cytoplasm and is more relevant as a stationary phase protein. The two catalases are furthermore divergently regulated by the catalase regulators OxyR and RpoS (Loewen et al., 1985; Tanaka et al., 1997; Visick and Clarke, 1997; Hillar et al., 1999). Divergent regulation of catalases was also described in *Aspergillus nidulans* (Kawasaki and Aguirre, 2001).

The catalase and peroxidase activity of a bacterium can be modulated between different growth stages. The activity of the 2 catalase-peroxidases (KatA and KatB) from *L. pneumophila*, for example, differed between log and stationary phase cells (Bandyopadhyay and Steinman, 1998). KatB was induced in log phase, whereas KatA was the predominant enzyme during stationary phase. The catalase activity during the log phase was found to be high, with little peroxidase activity, whereas the peroxidase activity was higher than the catalase activity during stationary phase. The latter peroxidase activity was due to KatA induction and not KatB. KatB mutants were further attenuated in their ability to infect host immune cells (Bandyopadhyay and Steinman, 1998), indicating a role for KatB as protection against host-mediated oxidative radicals.

The amount of catalase produced by a bacterium depends on its environmental niche. For example, *Vibrio rumoiensis*, a bacterium isolated from fish tanks cleaned with H₂O₂, showed very high catalase activity, with the catalase enzyme comprising 2% of total cellular protein (Ichise et al., 1999; Yumoto et al., 1999; Yumoto et al., 2000). This bacterium produced approximately 25 000 U/mg protein after 25 hours of growth, without induction by externally added H₂O₂. In addition, it produced 1500 U of catalase per ml of culture after 10 minutes of growth in the presence of 5 mM

of the oxidant. It is known that bacteria can adapt to produce higher amounts of catalase when exposed to small doses of H_2O_2 (Mongkolsuk and Hellman, 2002). This mechanism enables bacteria to survive subsequent lethal doses. In the case of *V. rumoiensis*, the H_2O_2 used to decontaminate the fish-processing plant clearly lead to the adaptation of the bacterium (Yumoto et al., 1999; Ichise et al., 1999).

1.4.1.2 The structure of catalases.

Catalases (hydrogenperoxide: hydrogenperoxide oxidoreductase, EC 1.11.1.6) belong to the haem hydroperoxidases. This group of enzymes includes catalase, cytochrome oxidase, ascorbate peroxidase and alkylhydroperoxidase (ahp). The catalytic centres of these enzymes contain protoporphyrin complexed with Fe^{3+} as the haem prosthetic group (Zamocky and Koller, 1999; Zamocky et al., 2002).

The haem site of the catalase is buried deep inside the protein. A funnel-shaped substrate channel has been shown to provide access to the active centre of monofunctional catalases (Reid et al, 1981; Fita and Rossmann, 1985). The small diameter of the entrance to the haem site, as well as the buried haem site, limits the substrates to very small molecules, such as H_2O_2 . The nature and size of the channel plays a part, not only in substrate specificity, but also directs the orientation of the substrate when it enters the active site (Switala and Loewen, 2002).

1.4.1.3. The catalase reaction.

A typical catalase reaction involves the oxidation of Fe^{3+} . This divalent metal is complexed to protoporphyrin IX in the haem centre. In a typical catalase reaction, 2 molecules of H_2O_2 are broken down to form 2 molecules of H_2O and 1 molecule of O_2 (Zamocky and Koller, 1999). Oxidation of Fe^{3+} by this molecule of H_2O_2 produces the oxo-ferryl complex, or Compound I (containing Fe^{4+}). The second molecule of H_2O_2 serves as an electron donor for Compound I, thereby causing reversion of the active site to the resting state of catalase.

The reactions as discussed above are widely accepted, but the precise reaction mechanism is still unclear. For example, the precise mechanism of reversion back to the native state, the role of NADPH in the reversion process, the intermediate compounds formed, as well as the specific channel used for substrate entry and exit, all requires further investigation. It was found, for example, that another intermediate compound, compound II, might also form during the breakdown of H_2O_2 (Kirkman and Gaetani, 1984; Kirkman et al., 1987; Kirkman et al, 1999; Zamocky and Koller, 1999). In order to convert Compound II back to the native state, more electrons have to be added to the formed Fe^{4+} complex. Various electron donors potentially might serve this purpose. A potential electron donor is NADPH. The potential of NADPH as electron donor was indicated

when it was observed that bovine liver catalase (Fita and Rossmann, 1985) and human erythrocyte-derived catalase (Kirkman and Gaetani, 1984) bound approximately 4 molecules per homotetramer. It was observed that the native state of the enzyme was restored more readily in the presence of NADPH (Kirkman et al., 1987; Kirkman et al., 1989), whereas high doses of H₂O₂ normally inactivate catalases (Switala and Loewen, 2002). As NADPH is a strong electron donor, it thus could serve in the catalase as a reducer of Fe⁴⁺ to Fe³⁺ (Zamocky and Koller, 1999).

1.4.2. Other mechanisms of protection against oxidative stress

Alkyl hydroperoxidase (Ahp), a flavoprotein and a member of the thioredoxin reductase family, is able to use a number of organic peroxides (such as tert-butyl peroxide; cumene peroxide), as well as H₂O₂ as substrates. These substrates are converted to alcohols. The active Ahp enzyme consists of 2 units, namely AhpC (57 kDa) and AhpF (21 kDa) (Storz et al., 1989; Tartaglia et al., 1990). Both these units are needed for activity of the protein. For activity, the protein needs NADPH or NADH as co-factor (Poole and Ellis, 1996). Although the H₂O₂ scavenging ability of Ahp is not as efficient as catalases, this enzyme was found to be the primary scavenger of intracellular generated H₂O₂ (Seaver and Imlay, 2001) in *E. coli*. The protein was identified in a number of bacteria, including *Campylobacter jejuni* (Baillon et al., 1999), *Xanthomonas* (Loprasert et al., 1997; Mongkolsuk et al., 2000), *Mycobacterium tuberculosis* (Master et al., 2002) and *S. typhimurium* (Poole and Ellis, 1996). In *V. cholerae* El Tor N16961, a putative Ahp homologue is encoded by the VC0731 locus.

The **DNA protection protein, Dps** binds DNA, thereby mediating protection against oxidative damage (Almiron et al., 1992). This protein binds DNA in a non-specific manner, thereby physically excluding radicals from the DNA strand. In the presence of Dps, the number of strand breaks and mutational damage is significantly reduced (Martinez and Kolter, 1997). Dps has been described in a number of bacteria, including *E. coli* (Almiron et al., 1992), *Bacillus subtilis* (Chen et al., 1995), and *Synechocystis spp.* (Dwivedi et al., 1997). The putative homologue for Dps in *V. cholerae* is VC0139 (tigr.org).

Specific repair mechanisms are used by bacteria to repair damage to biomolecules. DNA damage by oxidative radicals includes purine and pyrimidine base modifications, oligonucleotide strand breaks, DNA-protein cross-links and the creation of abasic sites. Repair of DNA lesions occurs by either using the base-excision repair (BER) or nucleotide excision repair (NER) systems. In BER, the *N*-glycosidic bond of oxidised bases is cleaved, followed by removal of damaged bases by excision repair enzymes. In NER, the mutated region is marked by a protein before other factors bind that will remove the damaged nucleotide (Lee and Chung, 2003; Lage et al.2003).

Bacteria also possess **non-enzymatic strategies** to detoxify oxidative radicals. In the case of *V. cholerae*, it was observed that the organism presents a rugose colony form upon starvation (Mizunoe et al., 1999). Rugose colonies are more resistant to oxidative stress (Rice et al., 1993). Cellular metabolites, such as melanin and ubiquinone, are also used by microorganisms against oxidative stress. Melanin possesses antioxidative properties and is known to interact with oxygen and oxygen radicals (Goodchild et al., 1981; Zughaier et al., 1999; Jacobson et al., 1995; Korytowski and Sarna, 1990). Adding to the theory that melanin can serve as an antioxidant, black melanin from a number of fungi conferred protection against oxidants (Jacobson et al., 1995; Zughaier et al., 1999).

V. cholerae produces melanin in response to nutritional and osmotic stress (Coyne and Al-Harhi, 1992; Kotob et al., 1995; Sanchez-Amat et al., 1998). Melanin production in *V. cholerae* therefore poses a question about its role in this bacterium, especially as oxidative radicals are present in its natural habitat (Mopper and Zhou, 1990; Alam, 2002). The next section of this review will focus on melanin, its characteristics and functions.

1.5. Melanin

The melanin pigment has been described in a variety of living organisms, including microorganisms, mammals and birds (Agodi et al., 1996; Price and Bontrager, 2001; Novellino et al., 2000). Although many characteristics of the pigment have been elucidated, melanin thus far remains difficult to completely characterise chemically. The reason for this is that melanin is found in nature complexed to proteins, metals and other substances (Novellino et al., 2000; Fogarty and Tobin, 1996). Research by Hong et al. (2004) showed that various metal ions bind to different reactive groups in melanin, e.g. Cu (II) binds to hydroxyl groups, whereas Fe (III) binds to amine groups.

A true melanin is soluble in an alkali, precipitates with HCl, flocculates in the presence of FeCl₃ and is bleached by H₂O₂ (Korytowski et al., 1990). The chemical reactivity of the pigment is attributed to various chemically reactive groups (e.g. quinonic groups, carboxyl groups) available in the heteropolymer (Korytowski et al., 1990).

1.5.1. Types of melanin

Pure black to dark brown pigment, such as that from natural black hair, belongs to the eumelanins (Nofsinger et al., 2002). This group of dark brown or black melanins is derived from L-dihydroxyphenylalanine (L-DOPA, Fig. 1.2). A second type of melanin is also derived from L-DOPA, namely yellow and red phaeomelanins (found in hair and feathers). Phaeomelanin contains nitrogen and sulphur due to the presence of cysteine. A third type of melanin is derived from

homogentisic acid (HGA) (Fig. 1.5). Bacteria produce either pyromelanin (derived from homogentisic acid) or melanin derived from L-DOPA (Table 1.1). The abovementioned melanins are produced in pathways with L-tyrosine as the precursor. Fungi produces catechol melanin (L-DOPA or dopamine as precursors) or melanin derived from 1,8 dihydroxynaphtalene (1,8 DHN, Fig 1.6) (Langfelder et al., 2003; Polacheck et al., 1982; Takana et al., 2000; Kubo et al., 1996).

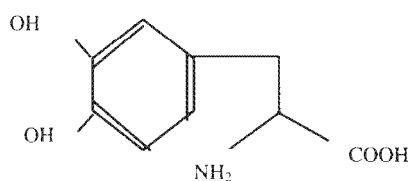


Fig. 1.2. Dihydroxyphenyl alanine (L-DOPA). This substance is the precursor of both eumelanin and phaeomelanins. Phaeomelanin contains additional sulphur groups.

1.5.2. Melanin formation Pathways

1.5.2.1 Melanin produced through the action of polyphenol oxidases.

Eumelanin and phaeomelanin are formed through the catabolism of L-tyrosine. In the classic Mason-Raper pathway, the tyrosinase enzyme (a polyphenol oxidase) is essential in the breakdown of L-tyrosine to form eumelanin. Polyphenol oxidases (PPO's) are a group of copper-containing monooxygenases (oxidoreductases).

The basic reaction for these monooxygenases is: $R-H + O_2 \rightarrow R-OH + H_2O$ (R-H represents a substrate). PPOs catalyse the oxidation of mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate (Paul and Ladd, 1981). PPO include laccases, tyrosinases and peroxidases, with the former two enzymes involved in melanization in organisms.

Tyrosinases are wide-spread amongst bacteria (Pomeranz et al., 1974; Serre et al., 1999; Wang et al., 2000), fungi (Van Gelder et al., 1997), plants (Gowda and Paul, 2002), invertebrates and vertebrate animals (Sánchez-Ferrera et al., 1995). This enzyme catalyzes more than one reaction in the eumelanin pathway. The *cresolase* activity (monophenol monooxygenase) of tyrosinases refers to the hydroxylation of a monophenol, such as tyrosine, to a catechol or diphenol (*o*-diphenol:oxygen-oxidoreductase), while the *catecholase* (or diphenolase) activity describes the dehydrogenation (removal of H₂O) of diphenols to form quinones. Hydroxylation of tyrosine (*cresolase* activity) by tyrosinase therefore leads to the formation of L-DOPA (the diphenol, Fig. 1.3), with subsequent formation (*catecholase* activity) of DOPA-quinone.

DOPA-quinone is further metabolised via 2 different pathways, eventually leading to the formation of cysteine- containing phaeomelanin or dihydroxyindole containing eumelanins (Fig. 1.3). The pathway diverges to form either eumelanin or phaeomelanin. With the addition of cysteine or glutathione to dopaquinone, the pathway proceeds to form yellow phaeomelanins. Spontaneous decarboxylation of dopaquinone leads to the formation of 5,6-dihydroxyindole (DHI, Fig. 1.4), whereas 5,6-dihydroxyindole-2-carbolic acid (DHICA, Fig. 1.4) forms through the action of DOPACHrome tautomerase. DHI and DHICA form eumelanin spontaneously by consuming oxygen, but also form eumelanin through the enzymatic action of tyrosinase, peroxidase and /or DHICA oxidase (Jimenez-Cervantes et al., 1994; Tsukamoto et al., 1992; Nappi and Vaas, 1996). H₂O₂ is produced when eumelanin formation proceeds enzymatically via the action of tyrosinases and peroxidase (Nappi and Vaas, 1996).

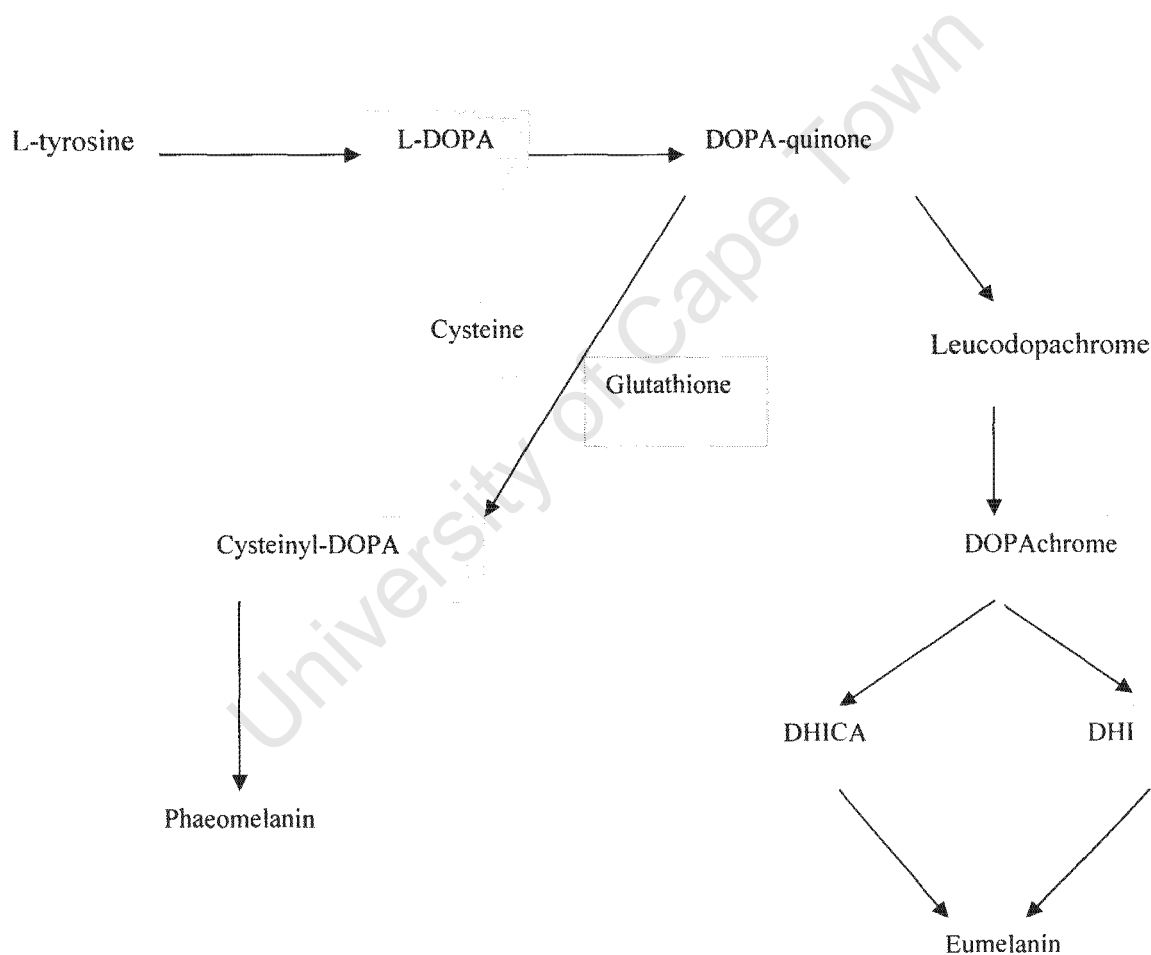


Fig 1.3. Phaeomelanin and Eumelanin formation. L-tyrosine is catabolyzed to form DOPA-quinone. At this point two different types of melanins might form: In the one pathway, the amino acid L-cysteine is added to DOPA-quinone to form cysteinyl-DOPA. Yellow to brown phaeomelanin forms in this pathway. In the other pathway, DOPA-quinone is metabolized to form either dihydroxy-indole (DHI) or dihydroxy-indole carbolic acid (DHICA). Black or dark brown eumelanin forms from the oxidation of these precursors.

Table 1.1. Melanin formation by bacteria.

Bacteria	Enzyme	Precursor	Reference
<i>Aeromonas liquefaciens</i>	PPO	L-Dopa, catechol	Aurstad and Dahle , 1972
<i>Azotobacter chroococcum</i>	ND	Catechol	Shivprasad and Page, 1989
<i>Azotobacter salinestris</i>	ND	Catechol	Shivprasad and Page, 1989
<i>Bacillus thuringiensis</i>	Tyrosinase?	L-Dopa	Hoti and Balaraman, 1993
<i>Hyphomonas</i>	HPPD	HGA	Kotob et al., 1995
<i>Legionella pneumophila</i>	HPPD	HGA	Steinert et al., 2 001
<i>Marinomonas mediterranea</i>	PPO,tyrosinase	ND	Solano et al., 1997
<i>Proteus mirabilis</i>	Tyrosinase?	ND	Agodi et al., 1998
<i>Pseudomonas fluorescens</i>	ND	Chlorinated catechol	Fava et al., 1993
<i>Pseudomonas maltophilia</i>	Tyrosinase	L-Dopa	Wang et al., 2 000
<i>Rhizobium meliloti</i>	Tyrosinase	ND	Mercado-Blanco et al., 1983
<i>Shewanella algae</i>	ND	HGA	Turick et al., 2002
<i>Shewanella colwelliana</i>	HPPD	HGA	Coon et al., 1994; Kotob et al., 1995; Fuqua et al., 1991; Fuqua and Weiner, 1993; Eylem et al., 1996
<i>Streptomyces antibioticus</i>	Tyrosinase	ND	Katz et al., 1983
<i>Streptomyces avermitilis</i>	HPPD	HGA	Denoya et al., 1994
<i>Streptomyces spp.</i>	Tyrosinase	ND	Lee et al., 1988
<i>Thermomicrobium roseum</i>	Tyrosinase	L-Dopa, catechol	Kong et al., 2000
<i>Vibrio cholerae</i>	HPPD	HGA	Sanchez-Amat et al., 1998;
<i>Vibrio tyrosinaticus</i>	Tyrosinase	ND	Pomerantz and Murthy, 1974
<i>Yarrowia lypolytica</i>	ND	HGA	Carreira et al., 2001

PPO, polyphenol oxidase - used where type of PPO not identified (i.e a tyrosinase or a laccase). ND, not determined; HPPD, hydroxyphenyl pyruvate dioxygenase; HGA, homogentisic acid; L-DOPA, dihydroxyphenylalanine.

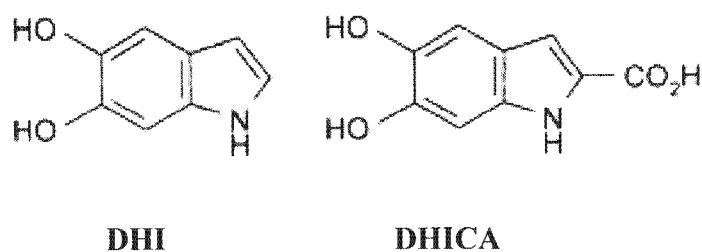


Fig. 1.4. The indolic precursors of eumelanin. DHI, dihydroxy-indole; DHICA, dihydroxy-indole carboxylic acid

Laccases are a part of the family of copper-containing monophenol oxygenases. They have been described in a variety of bacteria, including *Sinorhizobium meliloti* (Castro-Sowinsky et al., 2002) and *Marinomonas mediterranea* (Lucas-Elio et al., 2002), as well as fungi such as *Cryptococcus neoformans* (Williamson, 1997). The enzyme has *p*-diphenol:oxygen-oxidoreductase activity (Williamson, 1997), which results in the oxidation of dihydroxydiphenols to form quinones.

1.5.2.2. Melanin formed via the action of hydroxyphenyl pyruvate dioxygenase

Homogentisic acid forms when L-tyrosine is converted to hydroxyphenyl pyruvate (HPP) by tyrosine amino transferase (Fig.1.5). HPP then serves as the substrate for hydroxyphenyl pyruvate dioxygenase (HPPD), where it is converted into homogentisic acid (HGA). The formation of HGA, through the action of HPPD, includes (1) the decarboxylation of the pyruvate side-chain, (2) incorporation of molecular oxygen, and (3) rearrangement of the formed side-chain (Lindstedt and Rundgren, 1982). The final step in the pathway is the spontaneous autoxidation of HGA to form melanin. This step occurs without enzymatic intervention through the consumption of molecular oxygen by HGA.

HPPD had been identified in mammals (Bradley et al., 1986; Ruetchi et al., 1993), plants (Garcia et al., 1999), fungi (Keon and Hargreaves, 1998; Wyckoff et al., 1995) and bacteria (Table 1.1). The crystal structure of HPPD from *Pseudomonas fluorescens* revealed two barrel-shaped domains, and one non-haem iron atom per monomer. The protein consists of homotetramers in bacteria or homodimers in eukaryotes (Lindstedt et al., 1982; Serre et al., 1999) and needs iron for activity (Bradley et al., 1986).

1.5.2.3. Melanin formed via the Polyketide pathway

The polyketide pathway has been described in a number of pathogenic melanised fungi such as *Aspergillus fumigatus* (Tsai et al., 1999), *Magnaporthe grisea* (Vidal-Cros et al., 1994), *Alternaria alternata* (Kimura et al., 1993) and *Colletotrichum lagenarium* (Kubo et al., 1996). These fungi all form brown or black melanin. Acetate is one of the precursors for dihydroxynaphtalene (DHN) melanin. A series of enzyme-catalysed reductions (Fig. 1.6) and dehydrations leads to the formation of 1, 8 dihydroxynaphtalene (1, 8-DHN). Melanin is then formed from 1, 8 DHN, through the action of a laccase. Fungal melanin is an important virulence, pathogenicity and growth development factor (Table 1.2).

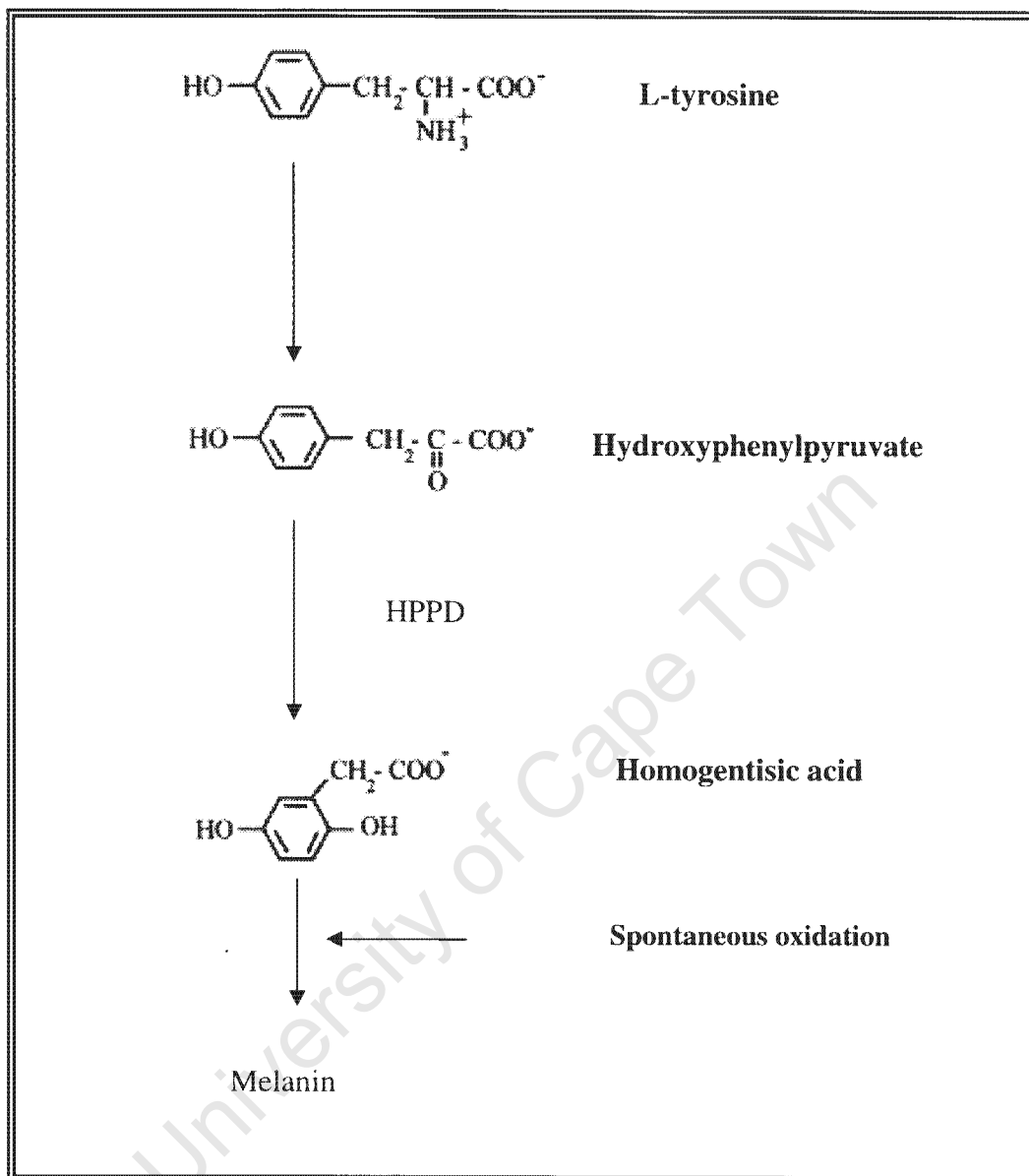


Fig 1.5. Melanin formation via homogentisic acid. Melanin formation from homogentisic acid (HGA) proceeds spontaneously, through the interaction of HGA and molecular oxygen. HPPD, hydroxyphenyl pyruvate dioxygenase.

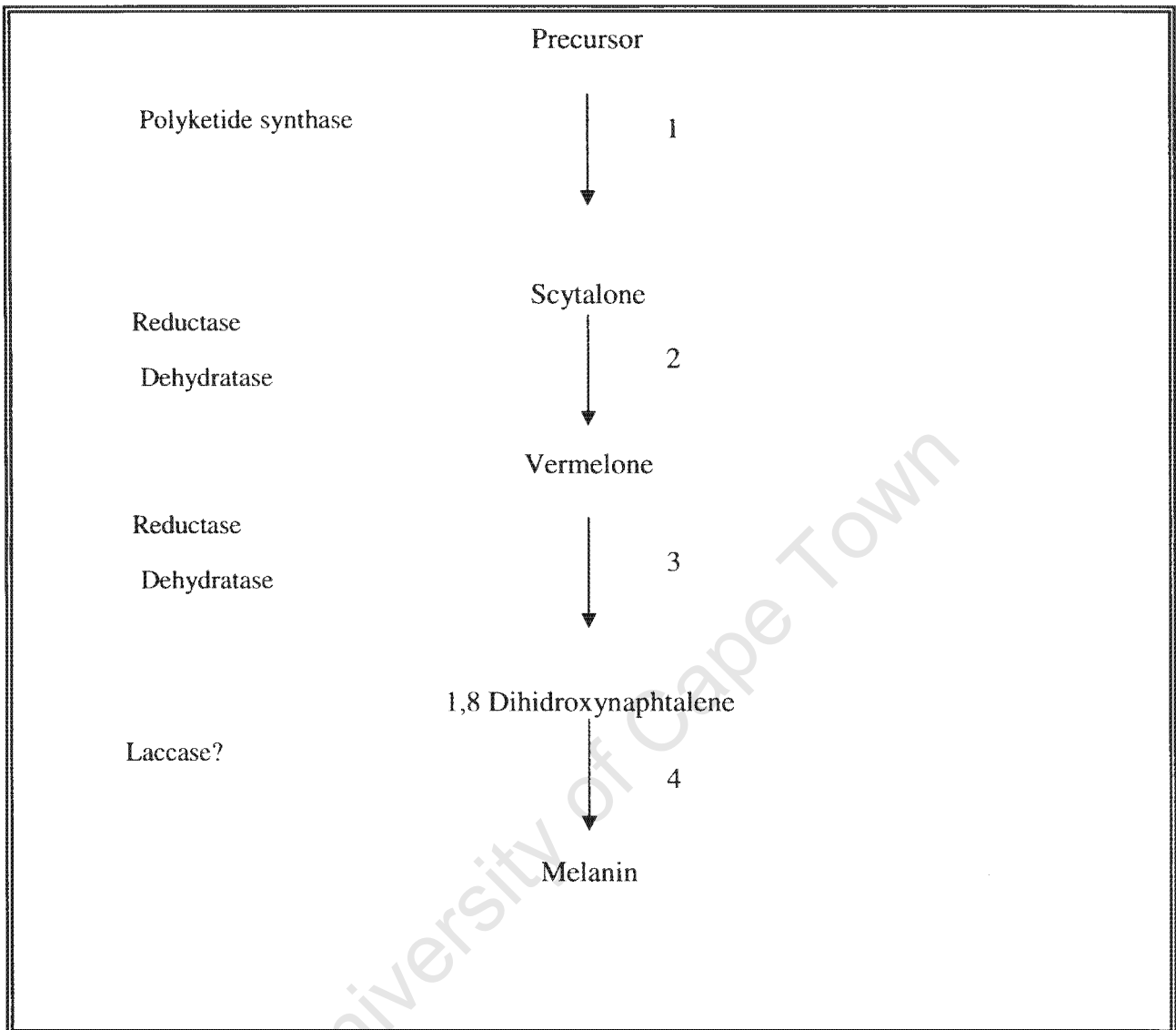


Fig. 1.6. The formation of fungal melanin via the polyketide pathway. The formation of scytalone from a precursor (eg. acetate), is catalysed by polyketide synthase. Steps 2 and 3 consists of a set of reduction reactions (catalysed by a number of reductases) and dehydrogenations (catalysed by dehydratases) to form 1,8 Dihydroxynaphtalene. The final formation of melanin from DHN is thought to be catalysed by a laccase (Adapted from Kimura et al., 1993; Tsai et al., 1999).

Table 1.2. The role of melanin in fungal pathogenesis and virulence.

Fungus	Melanin	Function /Role	Reference
<i>Alternaria alternata</i>	DHN	Protect against hypochloride; permanganate	Jacobson et al., 1995
<i>Alternaria alternata</i>	DHN	Affect conidial development	Kawamura et al., 1999
<i>Aspergillus nidulans</i>	DHN	Inhibits mycolysis	Bull, 1970 Kuo and Alexander, 1967
<i>Aspergillus niger</i>	L-DOPA	Activates alternative complement pathway	Rosas et al., 2002
<i>Burkholderia cepacia</i>	DHN	Scavenge O ₂ ⁻	Zughaier et al., 1999
<i>Colletotrichium lagenarium</i>	DHN	Aids penetration by appresoria	Kubo et al., 1996 Perpetua et al., 1996
<i>Cryptococcus neoformans</i>	L-DOPA	Activates alternative complement pathway	Rosas et al., 2002
<i>Cryptococcus neoformans</i>	L-DOPA	Potentiates virulence	Kwon-Chung et al., 1982
<i>Cryptococcus neoformans</i>	L-DOPA	Protects against UV-light	Wang and Casadevall, 1994
<i>Cryptococcus neoformans</i>	DHN	Redox buffer by interaction with Fe	Jacobson and Hong, 1997
<i>Exophiala dermatitidis</i>	DHN	Block oxidative burst and killing by neutrophils	Schnitzler et al., 1999
<i>Exophiala dermatitidis</i>	DHN	Protect against hypochloride; permanganate	Jacobson et al., 1995
<i>Pyricularia oryzae</i>	DHN	Aids penetration by appresoria	Vidal-Cross et al., 1995
<i>Sporothrix schenckii</i>	DHN	Protects against UV light; Protects against H ₂ O ₂ , NaNO ₂	Romero-Martinez et al., 2000

DHN, Dihydroxynaphtalene; L-DOPA, dihydroxyphenylalanine

1.5.3 The role of melanin: A pigment that both protects and destroys

Many wonderful functions have been ascribed to the pigment, but a thorough investigation of available literature showed that melanin plays both a positive (protective) role, as well as a negative (damaging) role in biological systems.

According to Hill (1992), many of the assumed characteristics of melanin are the result of speculation based on the known chemical nature of the pigment. The interactions of melanin with various metals and oxidants are dependent on the chemical composition of the melanin, the chemically reactive groups available as well as the type of interaction with other chemically active molecules. This section of the literature review will show that melanin protects cells, but it also contributes to cell damage or even death.

1.5.3.1 Melanin acts as a photoprotector.

Melanin's claim to fame is most probably the highly disseminated fact that it functions as a sunscreen. DOPA-derived melanins were indeed shown to be absorbing in the UV range (Ortonne, 2002). Consequently, people with higher percentages of melanin in their skins absorb more UV-light and are at a reduced risk of developing skin cancer (Kolias et al., 1991). A further correlation was made between the incidence of skin cancer and the ratio of DOPA-derived eumelanin:phaeomelanin in the skin. Individuals with more phaeomelanin (yellow or red) than eumelanin (black melanin) are at a greater risk of developing melanoma (Vincensi et al., 1998). Thus, melanin confers photoprotection depending on the type of melanin present and degree of melanization (basically the skin type).

Fungal melanin protected *Wangiella dermatitidis* (Schnitzler et al., 1999), *Sporothrix schenckii* (Romero-Martinez et al., 2000), and *Cryptococcus neoformans* (Wang and Casadevall 1994) against ultraviolet light. In bacteria, melanin derived from *Streptomyces* protected *Bacillus thuringiensis* toxin from inactivation by sunlight (Liu et al., 1993). This protection is useful in the environment as this toxin is rapidly inactivated by sunlight, thereby its effectiveness is reduced in the environment.

Although melanin protects certain microorganisms against damage caused by UV light, it was observed that radicals such as H_2O_2 and OH^\cdot are formed when melanin is irradiated with UV-light (Korytowski and Sarna, 1990). In a later study, it was found that the aggregation and disaggregation of eumelanin directs the production of H_2O_2 and O_2^\cdot . The amounts of these radicals produced depend on the degree of aggregation of the polymer subunits (Nofsinger et al., 2002). Melanin polymer subunits with a "molecular weight" of less than 1000 are actively involved in the consumption and photo-activation of O_2 , leading to the production of O_2^\cdot . Therefore, more H_2O_2

was formed by the smaller “subunits” than aggregated eumelanin. This phenomenon was ascribed to the fact that less surface hydroquinonic groups are available in bulk eumelanin. In earlier work, it has been postulated that these quinonic groups react with O_2^- to yield H_2O_2 (Nappi and Vass, 1996). As such, it was hypothesized that the disruption or breakdown of bulk eumelanin could potentially lead to increased oxidative stress in the area where it is deposited e.g. keratinocytes of the skin (Nofsinger et al., 2002).

1.5.3.2 Melanin acts as an anti-oxidant molecule.

Melanin protects microorganisms against oxidants. The ability of melanin to scavenge O_2^- *in vitro* was first reported by Goodchild (1981). This ability of melanin to scavenge O_2^- was thus implicated in the resistance of melanomas to radiotherapy, a known generator of this radical. Similarly, various types of melanins produced by microorganisms were shown to be able to scavenge oxidizing radicals. Examples of these are melanin from *Proteus mirabilis* that scavenges the superoxide radical (Agodi et al., 1996), and the melanin-like pigment from a highly infectious strain of *Burkholderia cepacia* that scavenges O_2^- produced by human monocytes (Zughaier et al., 1999). It was suggested that this intracellular pathogen should be able to overcome the oxidative burst of monocytes *in vivo*, because of the antioxidant properties of the melanin it produces. In this case, production of the radical actually might be beneficial to the pathogen. It was postulated that the respiratory burst by monocytes would be ineffective, and since LPS from this bacterium is primed by monocytes, accumulation of recruited inflammatory leukocytes would increase radical-induced tissue damage in the infected area, thereby indirectly aiding the spread of the bacterium. In addition, catechol-melanin from *Azotobacter chroococcum* was postulated to act as a free radical trap, thereby providing protection against damaging oxidants (Shivprasad and Page., 1989).

The interaction of melanin or melanin precursors with H_2O_2 seems complex. In some instances, the presence of melanin leads to neutralization of H_2O_2 , while under different conditions, it can lead to the production of more toxic radicals *in vitro*. This behaviour is dependent on the presence or absence of metals, the pH of solutions, the presence or absence of light, and the availability of reactive groups on the melanin (Korytowksi and Sarna, 1990; Nappi and Vass, 1996; Pilas et al., 1988). Thus DOPA-melanin is bleached by H_2O_2 prepared in an alkaline solution, but resistant to degradation by H_2O_2 when incubated in a neutral solution in the dark (Korytowski and Sarna, 1990). Bleaching (the result of the decomposition of melanin) by H_2O_2 is enhanced if the melanin is irradiated by UV-A light (causing photo-activation), or exposed to H_2O_2 in the presence of copper ions. The formation of the hydroxyl radical when melanin or its precursors are incubated in the presence of copper suggests the involvement of a Fenton-like reaction. The autoxidation of the DOPA melanin precursors DHI and DHICA might also lead to the generation of H_2O_2 (Nappi and

Vass, 1996). This event occurs if quinone intermediates are formed enzymatically from either DHI or DHICA through the action of tyrosinase or peroxidase or DHICA oxidase. The production of peroxide is suggested by the fact that addition of catalase inhibited the formation of H_2O_2 . When eumelanin was produced *in vitro* through this process, it lead to the generation of hydroxyl ions, once again via the Fenton reaction.

As radicals generated by the Fenton reaction are implicated in cytotoxic damage, it would seem that the interaction of copper, H_2O_2 and melanin would have negative implications for biological systems. The observed H_2O_2 and subsequent hydroxyl radical formation might be clinically relevant. For example, a decrease in catalase activity was shown to be a characteristic of the affected skin areas of vitiligo patients (Courtney et al., 2002). This pigmentary disorder, amongst other traits, is characterized by a loss of melanocytes and increased H_2O_2 in depigmented areas. Treatment of patients with substances with pseudocatalase activity restores pigmentation to vitiligo patients. Thus, in at least one clinical condition, the lack of removal of H_2O_2 is linked to depigmentation and destruction of keratinocytes.

1.5.3.3 Melanin binds to heavy metals.

Melanin is an effective chelator of metals. Fungal melanin, in particular, was shown to bind to a large number of metals, including copper, iron, silver, nickel, lead and zinc (Fogarty and Tobin, 1996; Gadd, 2 000).

1.5.3.3.1 Melanin and iron.

Melanin produced by a number of microorganisms interacts with iron. Firstly, membrane-bound catechol-melanin from *Azotobacter salinestris* was found to bind iron in a non-specific manner (Page and Shivprasad, 1995). Secondly, the dihydroxy-indole (DHI) derived-melanin produced by the fungus *Cryptococcus neoformans* was reduced, *in vitro*, by ferrous Fe (II) iron and oxidised by ferric Fe (III) iron (Jacobson et al., 1997). This interaction with iron was also observed with synthetic DOPA-melanin that chelated Fe (II), thereby preventing OH[•] radical formation through a Fenton-type reaction in the presence of H_2O_2 (Pilas et al., 1988; Korytowski and Sarna, 1990). This observed inhibition or scavenging of hydroxyl radicals happened in the presence of a weak competitive chelator, but not a strong chelator such as EDTA. Thus it was postulated that melanin can act as a weak chelating agent for Fe (II) *in vivo*. *In vivo* therefore, melanin should be able to remove Fe (II) introduced into the environment of a melanised microorganism. In contrast, interaction of melanin with Fe (III) generates hydroxyl radicals in the presence of H_2O_2 . This behaviour is due to the reduction of Fe (III) to Fe (II). The generation of Fe (II) thus seems to promote the Fenton reaction in the presence of H_2O_2 .

Pyomelanin produced by *Shewanella algae* (Turick et al., 2002, Turick et al., 2003) was also found to be a chelator of iron. Similar to humic acid, the HGA melanin produced by this bacterium was found to serve as a tool for electron transfer. In the case of *Cryptococcus neoformans*, melanin potentially serves as a redox buffer when bound to iron (Jacobson et al., 1997).

1.5.3.3.2. Melanin and copper. The structure of chemically synthesized melanin was found to change from a sheet-like particle to long, rod-like structures when copper ions were added in increasing concentrations. Hypothetically, this would affect the availability of reactive groups in melanin, and therefore the kind of interactions it participates in (Gallas et al., 1999).

The interaction of melanin or melanin precursors with copper seems to be detrimental for biological systems. The highly reactive hydroxyl radical was shown to be formed in the presence of DOPA-melanin and copper (Korytowski and Sarna, 1990), via a mechanism thought to be similar to the Fenton reaction. The interaction of copper with DOPA-melanin or melanin precursors has also been implicated in DNA damage (Husain et al., 1995; Spencer et al., 1994) and cytotoxicity (Wick, 1977; Snyder and Friedman, 1998).

A number of debilitating conditions involves melanin or melanin precursors (Hegedus, 2000). In the case of Parkinson's disease, the damage or death of neurons is postulated to be a result of the autoxidation of dopamine, with the formation of quinones, semi-quinones and reactive oxygen species (Enochs et al., 1994; Snyder, 1998; Hegedus, 2000). In addition, elevated copper concentrations are generally observed in persons with Parkinson's disease. Due to a lack of dopamine, these patients are treated with the neurotransmitters L-DOPA and dopamine. However, it is becoming more evident that the combination of these neurotransmitters and the observed free copper, is possibly responsible for the cytotoxic and clastogenic effects seen in PD patients. In fact, it would seem that the damage to neurons is enhanced in patients treated with this combination. In addition, Snyder and Friedman (1998) showed that a combination of L-DOPA or dopamine and copper or manganese inhibited the proliferation of hamster lung cells and caused increased formation of micronuclei. The latter is associated with clastogenic events, fragmented chromatin and chromosomal aberrations. In an earlier study (Spencer et al., 1994) it was demonstrated that L-DOPA and dopamine caused extensive DNA damage in the presence of H₂O₂ and traces of copper. Copper further caused DNA strand scission and breakage and this process was enhanced in the presence of H₂O₂ (Husain et al., 1995; Husain et al., 1998). This damage once again is thought to be as a result of the generation of the hydroxyl radical.

1.5.4. Melanin is both protecting and cytotoxic to microbes.

DHN-derived melanin from the pathogenic fungi *Wangiella dermatitidis* and *Alternaria alternata* was shown to scavenge oxidants such as permanganate (MnO_4^-) and hypochloride (ClO^-) (Jacobson et al. 1995). Indeed the minimum fungicidal concentration of MnO_4^- and ClO^- were found to be much higher for melanised fungi compared to their albino counterparts, indicating that melanin offered protection against these oxidants. The same protection was not afforded against H_2O_2 , though the authors ascribed this to the fact that this oxidant requires alkaline conditions for effectiveness, or alternatively, to the fact that H_2O_2 readily diffuses through membranes. However, in another report it was demonstrated that melanin protected against H_2O_2 , and has lengthened the killing time of H_2O_2 and NaNO_2 against melanised *Sporothrix schenckii* cells, compared to albino cells (Romero-Martinez et al., 2000).

A number of reports indicated that the presence of melanin might be detrimental to microorganisms. Production of melanin by *Pseudomonas fluorescens* lead to a cell phenotype that presented with damage to the cell wall (Fava et al., 1993). This lead to the hypothesis that the melanin might probably cause premature cell death in this bacterium. A mutant of *Bacillus thuringiensis* that produced melanin (as a result of a mutation) was found to be more lethal to mosquito larvae (Liu et al., 1993). Hyphal growth of *Aphanomyces spp.* previously incubated in the presence of DHI and DHICA lead to a decline in the culturability of the hyphae (Söderhäll and Ajaxon, 1982). A similar decline in culturability in the presence of a non-melanin pigment was described in *Phaffia rhodozyma* (Schroeder and Johnson, 1993). This pigment, although not a melanin, also contained quinonic groups, just like melanin. The reason for the growth inhibition could not be explained by the authors.

A study by Kruk et al. (1999) showed that the generation of ROIs by oxidation of L-DOPA was inhibited by mannitol (hydroxyl anion scavenger), superoxide dismutase (superoxide scavengers) and catalase (peroxide scavenger). In a different study it was demonstrated that the superoxide anions were produced when melanin and ferric ions were incubated together (Sotomatsu et al., 1994). In this study, the presence of SOD inhibited the formation of the radical. Therefore, it is quite clear that damaging oxidative radicals form during the process of melanization. Accordingly, DNA damage occurs when homogentisic acid or L-DOPA, both melanin precursors, are incubated with DNA, but damage is inhibited in the presence of scavengers of oxidative radicals (Hiraku et al., 1998; Husain et al., 1998).

1.5.5 Other functions of melanin

Other functions for melanin described in the literature includes protection of fungi against lytic enzymes (Kuo and Alexander, 1967; Bull, 1970) as well as a disease-resistance factor in developing

insect larvae (Wilson et al., 2001). Melanin from various pathogenic plant fungi was also shown to be an important virulence factor (Table 1.2). In these cases, melanin was usually found to be present in penetrating tips of hyphae. Loss of the melanin resulted in a number of plant pathogens losing the ability to penetrate the cell walls of host plants. The absence of melanin in hyphal tips is correlated with a decrease in turgor pressure. The latter is thought to be responsible for the mechanism of penetration in invasive plant fungi (Howard and Valent, 1996).

1.5.6 Melanin production in *V. cholerae*

Melanin production was first observed in a toxinogenic strain of *V. cholerae* after chemical mutagenesis of the bacterium (Ivins and Holmes, 1980). Later work identified the precursor of the pigment as homogentisic acid (Kotob et al., 1995; Ruzafa et al., 1995). It was further discovered that the wild type strain can produce melanin *in vitro* under specific conditions. These conditions are increased osmotic stress and starvation culture conditions (Ivins and Holmes, 1981; Coyne and Al-Harthi, 1992; Ruzafa et al., 1995; Sanchez-Amat et al., 1998). The inclusion of copper in the culture media enhanced the formation of pigment (Coyne and Al-Harthi, 1992). Melanogenesis begins at 44 hours of growth under starvation conditions (Ruzafa et al., 1995; Sanchez-Amat et al., 1998), and osmotic stress (Schroeder, 1998). This coincides with the detection of HPPD in cell extracts (Schroeder, 1998). The gene encoding for hydroxyphenyl pyruvate dioxygenase (HPPD) was identified and sequenced in both the classical strain 569B (Schroeder, 1998), and the El Tor strain N16961 (www.tigr.org).

1.6 The aim of this thesis

In its natural habitat, as well as inside its hosts, *V. cholerae* is exposed to oxidative and other abiotic stresses. As discussed earlier, oxidative radicals form in seawater due to the interaction of ultraviolet light, seawater and substances such as metals and dissolved organic matter in water (Mopper and Zhou, 1990). Marine bacteria are exposed to these oxidative radicals produced in water, as well as by that produced by the immune responses of their various hosts. Aquatic environments also show increasing levels of pollution due to human activity (Fayer et al., 2004). Pollution from human activity includes fecal material, agricultural run-off and industrial waste such as various metals. Heavy metals accumulate in marine organisms, causing a number of unhealthy effects (Samanta et al., 2005). Ultimately, bacteria in water and inside marine animals are exposed to heavy metal pollutants. Exposure to heavy metals has already resulted in increased isolation of aquatic bacteria exhibiting resistance to various heavy metals, such as mercury (De et al., 2003).

Since *V. cholerae* is highly successful both as a pathogen and in its ability to persist in the environment, it should have mechanisms that protect against threats such as oxidative radicals and

heavy metals. A variety of studies showed that the properties of melanin provided protection against a number of abiotic stresses, including oxidative stress and heavy metals. A number of microorganisms, such as *Shewanella spp.*, use melanin to accumulate metals. The aim of this thesis therefore was to evaluate the functional role of melanin in *V. cholerae*, particularly as a possible means of protection against H₂O₂ which is one of the damaging radicals encountered by the bacterium in the environment. We further investigated the ability of melanin to protect *V. cholerae* against a number of metals.

University of Cape Town

CHAPTER 2.

CONSTRUCTION OF A MELANIN-DEFICIENT STRAIN DERIVED FROM *V. CHOLERAE* 569B.

University of Cape Town

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SUMMARY

Melanin production occurs in *V. cholerae* upon exposure to conditions of starvation or osmotic stress. The pigment is formed from L-tyrosine and involves the spontaneous oxidation of homogentisic acid to form pyomelanin. Since the production of homogentisic acid is facilitated by the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD), a mutation that prevents the production of active HPPD enzyme should lead to melanin-deficiency. To achieve this, a gene encoding for HPPD was disrupted through insertional inactivation. A suicide construct containing a fragment from the target gene was delivered into *V. cholerae* 569B through mating with an appropriate *E. coli* strain. No melanin formation was observed in the melanin-deficient mutant. The insertion of the suicide construct into the *ppdA* gene was confirmed by Southern blot analysis. Mutation of *ppdA*, the gene encoding for HPPD, did not affect the growth of *V. cholerae*. The lack of pigmentation affected the survival of *V. cholerae* when exposed to H₂O₂. Contrary to expectations, it was observed that complementation of the mutated gene by a wild-type *ppdA* gene, harboured on plasmid pBluescript SK (+), negatively affected survival and growth of *V. cholerae*. This result was duplicated in *E. coli* JM109 containing *ppdA* on plasmids of differing copy number, i.e. intermediate production of melanin provided protection against oxidative stress, but overproduction negatively affected survival. These results indicate that *V. cholerae* melanin would be advantageous to the microorganism only at the amounts produced by the wild type, but that overproduction of melanin was harmful to the bacterium.

2.1 Introduction

V. cholerae produces melanin upon exposure to starvation (Ruzafa et al., 1995) or osmotic stress (Coyne and Al Harthi, 1992). The process of melanization appears to be independent of temperature, since pigmentation occurs at both 30 °C and 37 °C, but only in the presence of osmotic stress or starvation (Coyne and Al-Harthi, 1992; Ruzafa et al., 1995; Schroeder, 1998). The melanin synthesized by this organism is a pyomelanin, formed through autopolymerisation of homogentisic acid (HGA), with L-tyrosine acting as the precursor molecule (Kotob et al., 1995; Sanchez-Amat et al., 1998). L-tyrosine is converted to p-hydroxyphenylpyruvate (HPP) through transamination, a reaction catalysed by tyrosine aminotransferase. HPP, in turn, is converted to homogentisic acid through the action of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). This reaction involves the oxygenation of HPP, followed by decarboxylation and rearrangement of the side-chain, to form HGA (Pascal et al., 1985; Ruetchi et al., 1993). The enzyme activity depends on the presence of ferrous ion (Fe^{2+}), O_2 , and ascorbate (Lindstedt, 1982). The latter is needed to keep the iron in the ferrous form.

The *ppdA* gene encoding for HPPD (Genbank accession number of U31553) in *V. cholerae* 569B was identified and sequenced (Schroeder, 1998). The same gene was identified in the El Tor strain N16961, where it was named *vllY* (<http://www.tigr.org/tbd/mdb>). HPPD, the product of *ppdA*, has a size of 41kDa (Schroeder, 1998).

Melanin production by *V. cholerae* seems to be dependent on that portion of the L-tyrosine catabolic pathway that follows after homogentisic acid production. This part of the pathway contains the enzymes homogentisic acid oxygenase, maleylacetoacetate isomerase and fumarylacetoacetate hydrolase. Homogentisic acid oxygenase converts homogentisic acid into maleylacetoacetate. This substance is then broken down to acetoacetate and fumarate, through the actions of the remaining two enzymes (Sanchez-Amat et al., 1998). In a study with two *V. cholerae* strains, one melanogenic and the other non-melanogenic, it was observed that the melanogenic strain produced lesser amounts of homogentisic acid oxygenase, maleylacetoacetate isomerase and fumarylacetoacetate hydrolase (Sanchez-Amat et al., 1998). No melanin was formed in the strain where greater quantities of these enzymes were measured. This implies that melanization in *V. cholerae* is a result of insufficient removal of homogentisic acid, since the low levels of homogentisic acid oxidase are insufficient to convert all the produced homogentisic acid to maleylacetoacetate. This would cause spontaneous oxidation of HGA to form pyomelanin, a process that occurs spontaneously without enzymatic intervention.

2.1.1 The aim of this chapter

The primary aim of this thesis was to evaluate the role of melanin in *V. cholerae*. In an earlier study (Schroeder, 1998), it was observed that the absence of melanin in *V. cholerae* affected the survival of the organism following exposure to H₂O₂. It is known that melanin production in *V. cholerae* is induced by stress, namely starvation or osmotic stress (Coyne and Al-Harthi, 1992). As an autochthonous member of the marine environment the bacterium is exposed to starvation, osmotic stress and H₂O₂ (and other oxygen radicals) in its natural habitat. It is plausible that melanization is a mechanism of protection against these adverse conditions.

This chapter describes the creation of a melanin-deficient *V. cholerae* mutant. Inactivation of *ppdA*, the gene encoding for HPPD, was achieved through insertional inactivation, a method through which the coding region of a specific gene is disrupted with the aid of a suicide plasmid (Reyrat et al., 1998). Suicide vectors are designed so that they only replicate in specific bacterial hosts. Once introduced into a target bacterium, homologous recombination between a fragment contained on the suicide plasmid and its homologous equivalent on the bacterial genome occurs. In the process, the suicide plasmid integrates into the genome, interrupting the target gene on the genome. Strains containing the suicide vector become resistant to an antibiotic marker on the plasmid, enabling specific selection of mutants.

In order to mutate *ppdA*, an internal fragment derived from *ppdA* was cloned into the suicide vector pGP704 (Herrero et al., 1990). This suicide plasmid carries elements that allow replication of the plasmid only in *E. coli* strains that carry the lambda pir prophage (e.g. *E. coli* SM10λpir). The R6K origin of replication (*oriR6K*), in the presence of the Pi protein (encoded by the *pir* gene), allows plasmid replication in suitable hosts. The pGP704 vector also contains a fragment encoding the *oriT* region from plasmid RP4. This *oriT* is the origin of transfer of the plasmid into a recipient bacterium (Simon, et al., 1983; Kolter et al., 1978; Miller and Mekalanos, 1988). The *E. coli* host SM10λpir was used to transfer the plasmid from the host bacterium to *V. cholerae* 569B.

Homologous recombination between the *ppdA* fragment cloned into pGP704 and its homologous equivalent in the *V. cholerae* genome lead to the insertion of the suicide vector into the chromosomal copy of *ppdA*. The interruption of *ppdA* was confirmed by restriction enzyme analysis and Southern hybridization. Finally, the effect of the mutation on survival of *V. cholerae* was assessed.

2.2 Materials and methods

2.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2.1.

2.2.2. Media and culture conditions

Escherichia coli JM109 and SM10 λ pir were grown in Luria broth (LB), containing tryptone, sodium chloride and yeast extract (Appendix A1). *V. cholerae* 569B was grown at 30°C in tryptone broth (TB) containing 1% tryptone and 1% NaCl (Appendix A1). For induction of melanogenesis, the bacterium was grown in melanin-inducing media, i.e. tryptone broth amended with 4% NaCl, 5 mM L-tyrosine and 5 μ M CuSO₄ (Appendix A1).

Where applicable, media was supplemented with ampicillin at a concentration of 100 μ g / ml. In addition, *V. cholerae* 569B^{Rif}, a rifampicin-resistant strain, was grown in TB amended with rifampicin at a concentration of 50 μ g / ml.

2.2.3. Construction of suicide construct pGP259

Plasmid pCM302-16, harboring *ppdA*, was digested with *Hind*III and *Cla*I. Resultant fragments were separated by gel electrophoresis (Appendix B6). A *Hind*III - *Cla*I (883 base pairs in length) fragment was excised from the gel under long-wavelength (320nm) UV light, purified with Gene-Clean (Appendix B14), the purified fragment blunted by a polymerase filling-in reaction (Appendix B11) and ligated (Appendix B11) into *Eco*RV-linearised pGP704. The resultant plasmid (pGP883) thus contained an internal *ppdA* fragment (Fig 2.1). Partial digestion of pGP883 with *Pvu*I and subsequent religation (Appendix B11) of the shortened fragment produced plasmid pGP259 (Fig 2.1).

2.2.4. Insertional inactivation of *ppdA*

V. cholerae 569B^{Rif} was grown overnight in TB amended with rifampicin (50 μ g / ml). *E. coli* SMG883 (Table 2.1) was grown at 37°C for 18 hours in Luria Broth (LB, Appendix A.1) amended with ampicillin (100 μ g/ml). The next day, 100 μ l from each culture was mixed on a 0.22 μ filter (Millipore) placed on the surface of a TB agar plate. The plate was incubated overnight at 30°C to allow mating to occur. After overnight incubation, the bacteria on the filter were rinsed off with sterile TB. Dilutions of the bacterial suspensions were plated on TB agar containing rifampicin (50 μ g/ml) and ampicillin (100 μ g/ml). This ensured selection against *E. coli* and wild type *V. cholerae* cells respectively. However, resultant ampicillin-resistant isolates were still pigmenting. Therefore, a second construct, pGP259, was created as described in section 2.2.3. This construct, containing a

Table 2.1. Bacterial strains and plasmids

Strain or plasmid	Genotype / relevant features	Reference
<i>V. cholerae</i> 569B	Classical biotype, O1 serogroup, Inaba serotype	Mukherjee (1998)
<i>V. cholerae</i> 569B ^{Rif}	569B, Rifampicin resistant	Schroeder (1998)
<i>V. cholerae</i> 569BSK	<i>V. cholerae</i> 569B containing pBluescript SK(+)	This study
<i>V. cholerae</i> Mut32	<i>V. cholerae</i> 569B <i>ppdA</i> , amp ^r , Rif ^r	This study
<i>V. cholerae</i> Mut3216	Mut32, containing pCM302-16	This study
<i>V. cholerae</i> 5616	<i>V. cholerae</i> 569B, containing pCM302-16	This study
<i>E. coli</i> JM109	<i>F'</i> <i>traD36 proA⁺ proB lacI lacZΔM15/end A1 gyrA96 (Nal) thi hsd17 supE44 relA1 Δ(lac-proAB) mcrA</i>	Ausubel et al., 1989
<i>E. coli</i> JM177	JM109, containing pACYC177	This study
<i>E. coli</i> JMAC	JM109, containing pACMel	This study
<i>E. coli</i> JM16	JM109, containing pCM302-16	This study
<i>E. coli</i> SM10λpir	<i>thi thr leu tonA LacY supE recA:: RP4-2T::Mu</i>	Miller and Mekalanos (1998)
<i>E. coli</i> SMG883	<i>E. coli</i> SM10 harboring pGP883	This study
<i>E. coli</i> SMG259	<i>E. coli</i> SM10 harboring pGP259	This study
Plasmids		
pBluescript SK(+)	Amp ^r , β - galactosidase	Short et al. (1988)
pCM302-16	pBluescript SK(+) carrying <i>ppdA</i>	Schroeder (1998)
pGP704	ori R6K, mob Rp4, MCS of M13tg131, Amp ^r	Herrero et al. (1990)
pGP883	pPGP704 carrying HindIII-ClaI (883 bp) internal fragment from <i>ppdA</i>	This study
pGP259	Shortened fragment of pGP883 carrying a HindIII-PvuI internal fragment from <i>ppdA</i>	This study
pACYC177	Low copy number plasmid, Amp ^r	Rose (1988)
pACMel	pACYC177, carrying <i>ppdA</i>	This study

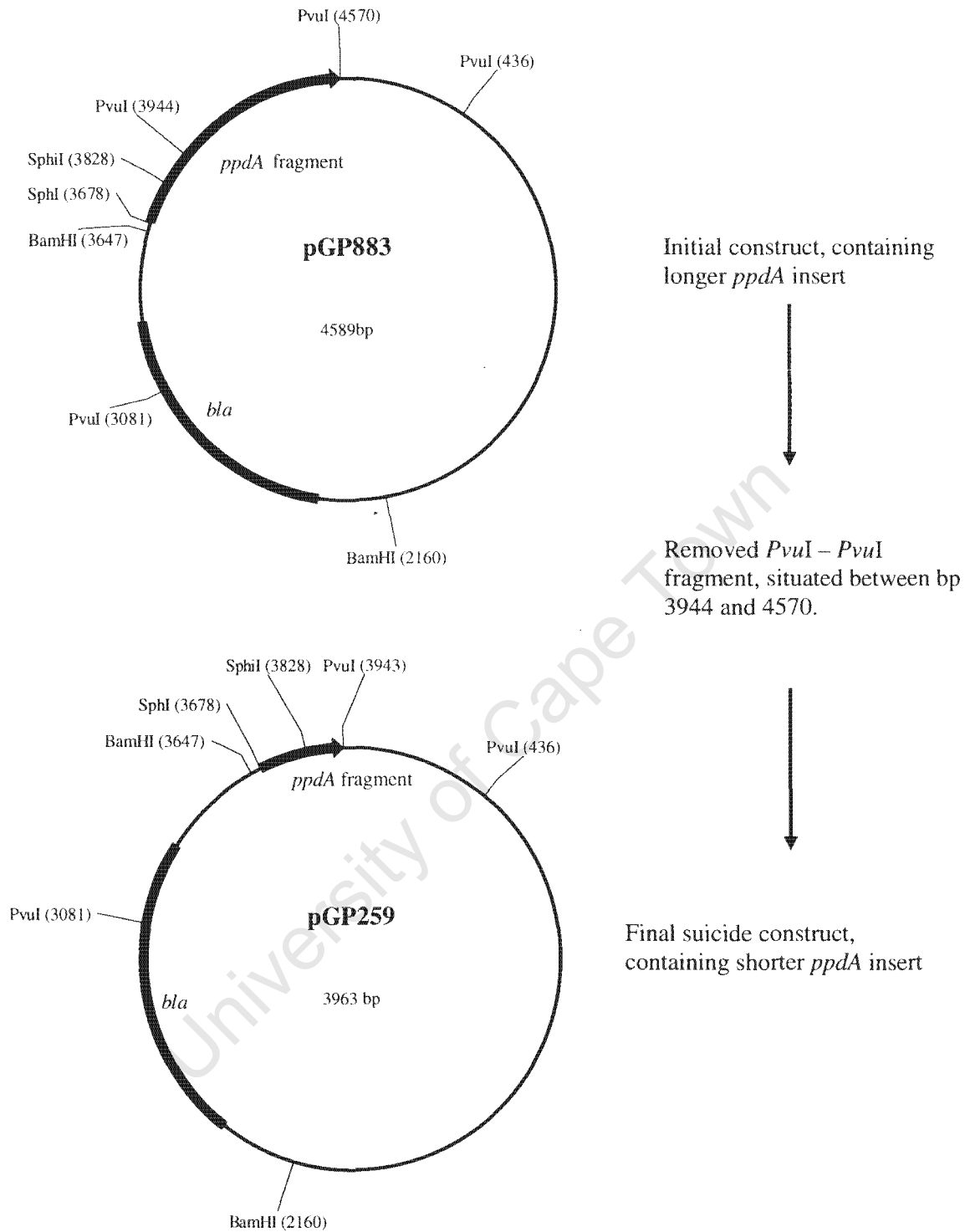


Fig. 2.1. Suicide constructs used to generate mutants of *V. cholerae*. A *Hind*III – *Cla*I fragment, derived from *ppdA*, was cloned into *Eco*RV-linearised pGP704 to create pGP883. A shorter *ppdA* fragment remained in pGP259 construct after deletion of the *Pvu*I fragment of the *ppdA* insert. *bla*, gene encoding for *b*-lactamase.

shorter *ppdA* fragment, was transformed into *E. coli* SM10 λ pir, and delivered into *V. cholerae* through mating as described above.

Prospective mutants were tested for pigmentation by growth in melanin-inducing media. After 2 days of growth on a shaker at 37°C, the cultures were visually inspected for the presence of melanin. One putative mutant, named *V. cholerae* Mut32, was selected for further analysis.

2.2.5 Characterisation of *V. cholerae* Mut32, a melanin-deficient *V. cholerae* mutant

2.2.5.1. Confirmation of the interruption of *ppdA* by pGP259

Genomic DNA was prepared (Appendix B3.1) from *V. cholerae* 569B and the melanin-deficient mutant *V. cholerae* Mut32. DNA was digested with *Cla*I, *Bam*HI, or both restriction enzymes and the resultant fragments separated on a 0.8% agarose gel (Appendix B6). Southern hybridisation (Appendix B12) was performed using an α -³²P [dCTP] radiolabelled *Hind*III - *Cla*I fragment (derived from *ppdA*) as the probe. Probes were labelled with a random priming kit according to the manufacturer's instructions (Appendix B12).

2.2.5.2. The effect of the *ppdA* mutation on the growth of *V. cholerae*.

V. cholerae strains 569B and Mut32 were grown either under conditions representing no stress (TB containing 1% tryptone, 1% NaCl, incubation at 30°C), or a combination of osmotic and temperature stress (TB broth containing 1% tryptone, 4% NaCl, incubation at 37°C). Growth was monitored by determination of the optical density (OD at 600 nm) every hour for 7 hours.

2.2.5.3. The effect of the *ppdA* mutation on the survival of *V. cholerae* upon exposure to oxidative stress.

Vibrio cholerae strains 569BSK and Mut3216 were created by electroporation of plasmids pBluescript SK(+) and pCM302-16 into *V. cholerae* 569B and the melanin-deficient strain Mut32 respectively. Electroporation was done as described before (Hamashima et al., 1995). These strains were grown in melanin-inducing media at 37°C. After two days of growth, 1 ml was removed from each culture before H₂O₂ was added to the cultures to a final concentration of 5 mM. After an incubation period of 30 min, another 1ml aliquot was removed from each culture. Serial dilutions of the culture samples were subsequently plated on tryptone agar. Colonies were enumerated after overnight incubation at 30°C.

2.2.5.4. The effect of melanin on bacterial cell numbers

V. cholerae strains 569BSK, Mut32, and Mut3216 were grown in melanin-inducing media at 37°C with increased aeration, thereby increasing melanin production levels. Aliquots were removed from these cultures after one and two days of growth at 37°C. Serial dilutions of the culture samples were plated on tryptone agar. Colonies were enumerated after plates were incubated overnight at 30°C.

In order to assess whether the detrimental effect of melanin, as observed in *V. cholerae*, could be duplicated in *E. coli*, various plasmids containing a wild type copy of *ppdA* were introduced into *E. coli* JM109. One of the plasmids, pACMel, was created by cloning a 2 kb *XbaI-PvuII* fragment derived from pCM302-16 into the low copy number plasmid pACYC177. The second plasmid, pCM302-16 (Schroeder, 1998), contains *ppdA* cloned into pBluescript SK(+), a plasmid with a much higher copy number than pACYC177. The resultant strains were grown overnight at 37°C in Luria broth amended with 5 mM L-tyrosine and 5 µM CuSO₄. After overnight growth at 37°C with shaking, aliquots were removed from each culture, dilutions were made and plated on Luria agar. H₂O₂ was added to the remainder of the cultures to a final concentration of 100 mM. After 30 minutes of exposure at 37°C, additional aliquots were removed, serial dilutions made and plated in triplicate as above.

2.2.5.5. The effect of melanin on the integrity of genomic DNA

Previous research showed that oxidative DNA damage occurs in the presence of homogentisic acid and copper (Hiraku, 1998). To investigate whether DNA damage was the cause of the lower cell numbers observed in the presence of melanin, we isolated genomic DNA from *V. cholerae* strains 569B, Mut32 and Mut3216, as well as from *E. coli* strains JMSK, JMAC and JM16. These strains were grown under pigment-inducing conditions to promote melanin formation. Genomic DNA was isolated (Appendix B3.) and separated on an alkaline agarose gel (Appendix B14) to assess whether oxidative damage occurred in melanin-producing bacteria. Electrophoresis was performed overnight at 10 volts.

2.3. Results

2.3.1. Isolation and characterisation of Mut32, a melanin-deficient mutant derived from *Vibrio cholerae* 569B.

Mating of *V. cholerae* with *E. coli* SMG259 resulted in the creation of a melanin-deficient *V. cholerae* mutant. This mutant, *V. cholerae* Mut32, did not produce melanin when grown under pigment-inducing conditions (Fig 2.2).

Interruption of *ppdA* was confirmed by Southern hybridization which showed that *ppdA* was inactivated due to the integration of pGP259 into the *V. cholerae* genome. Integration of pGP259 led to the introduction of *Bam*III sites, originating from the suicide vector, into the *ppdA* genomic area. Digestion of Mut32 genomic DNA with *Bam*III therefore showed that a 20 kb *Bam*HI fragment, as seen in genomic DNA isolated from the wild type *V. cholerae* (Fig 2.3, lane 1), was split into 2 fragments of approximately 13 kb and 7 kb (Fig 2.3, lane 2) in genomic DNA from Mut32. The integration of the suicide vector into *ppdA* also lead to an increase in the size of the *Clal* band observed in wild type *V. cholerae* (Fig 2.4: lanes 3, 4). Genomic DNA isolated from wild type *V. cholerae* yielded a *Clal* fragment of approximately 5 kb in size, whereas this fragment increased in size to 9 kb due to the integration of the suicide vector.

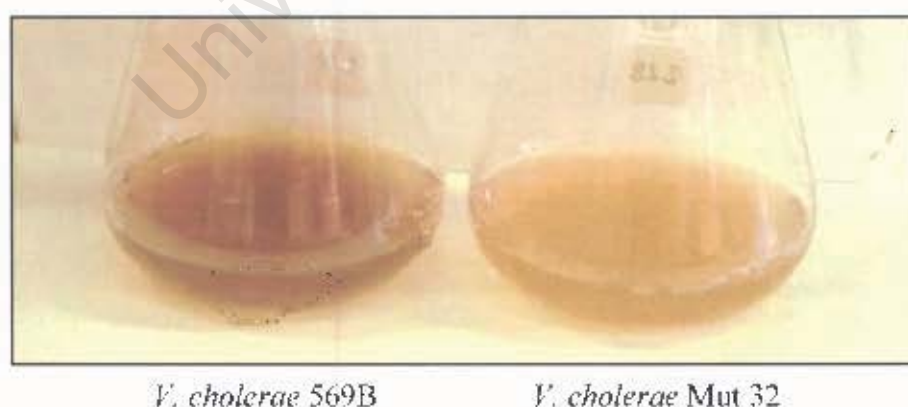


Fig 2.2. Pigmentation in *V. cholerae* 569B and Mut32, a melanin-deficient mutant. Cells were grown for 2 days under pigment-inducing conditions.

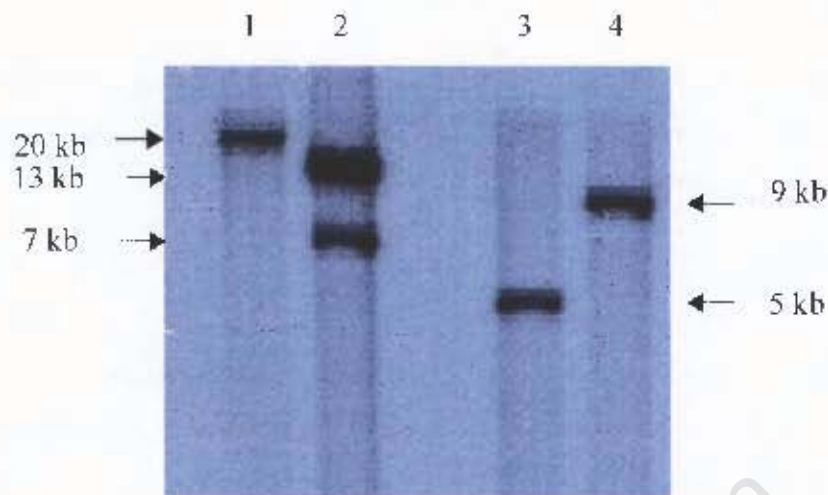


Fig 2.3. Southern blot analysis of *V. cholerae* Mut32, indicating interruption of *ppdA*.

Autoradiograph of genomic DNA from *V. cholerae* 569B (Lanes 1 and 3) and Mut32 (Lanes 2 and 4), probed with an α - ^{32}P [dCTP] labelled internal *ppdA* fragment. Lanes 1, 2: *Bam*HI digestions; Lanes 3, 4: *Cla*I digestions.

2.3.2. The effect of the *ppdA* mutation on the growth and survival of *V. cholerae*.

There was no difference in growth between *V. cholerae* strains 569BSK and the melanin-deficient strain Mut32 when grown over a period of 7 hours (Fig. 2.4). This was true when strains were grown at 30 °C or 37 °C, as well as in the presence of 1% or 4% NaCl.

When survival of *V. cholerae* strains exposed to H_2O_2 was compared, it was clear that melanin protected against H_2O_2 when produced at levels similar to that of the wild type (Table 2.2). In the absence of melanin, only 54% of the bacteria survived. Contrary to expectations, complementation of the *ppdA* mutation by a wild type copy of the *ppdA* gene carried on a pBluescript (SK)+ plasmid did not restore the protective effect of melanin. Instead, killing by H_2O_2 increased in the presence of the elevated levels of melanin generated by expression of *ppdA* from a high copy number plasmid. Even before H_2O_2 was added, it was clear that the excess melanin had a negative effect on the culturability of the cells, as seen in the decline in cell numbers (0 mM H_2O_2 Table 2.2). Melanin, therefore, protected *V. cholerae* against exposure to H_2O_2 , but had a negative effect on cell growth and survival when overproduced.

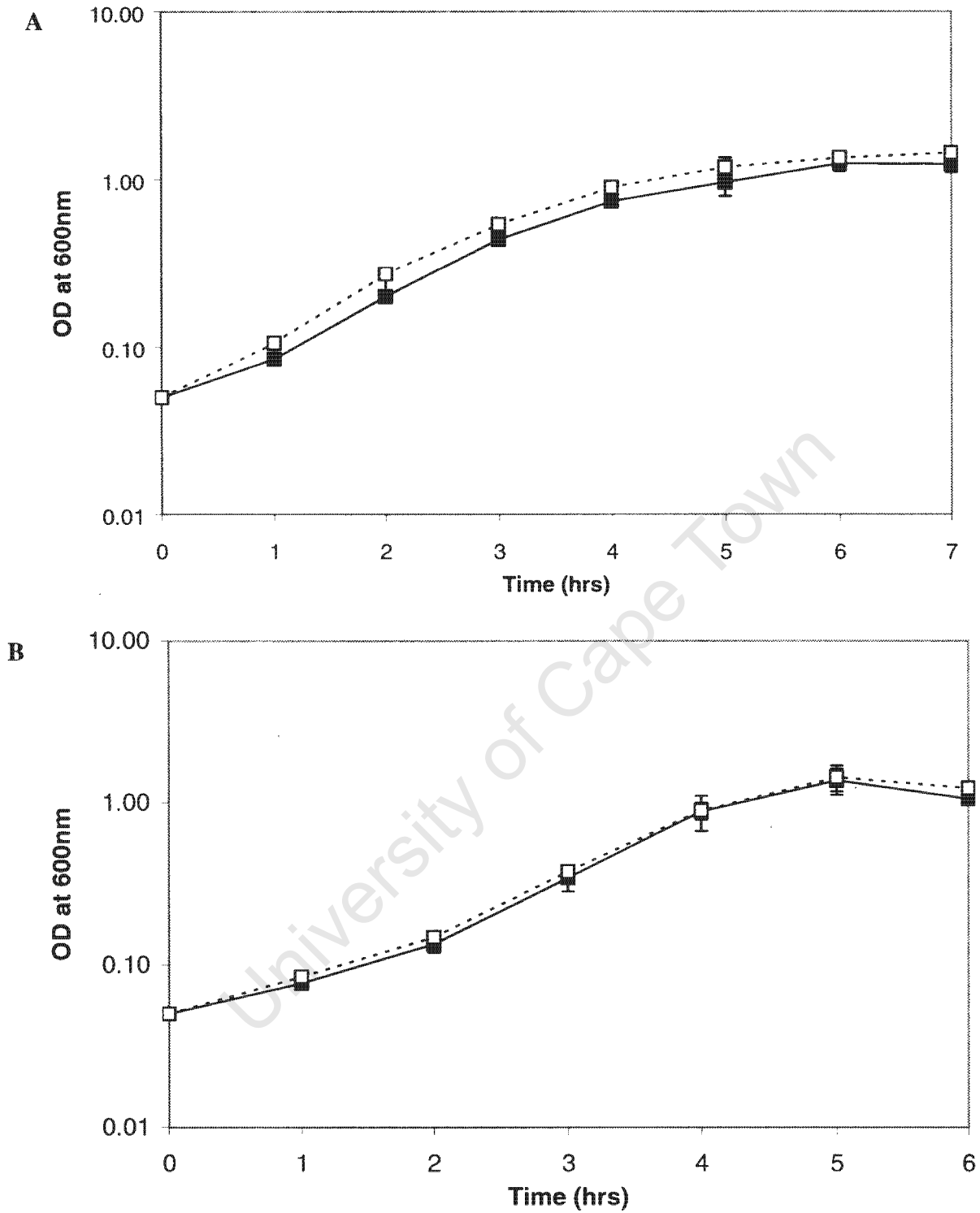


Fig 2.4. Growth of *V. cholerae* 569B and the melanin-deficient strain Mut 32. Open symbols, melanin mutant, Mut 32; Closed symbols, wild type. A, 30 °C, growth in tryptone broth containing 1% NaCl; B, 37°C, growth in tryptone broth containing 4% NaCl. Data represent the mean of 3 experiments.

Table 2.2. Survival of *V. cholerae* exposed to 5 mM H₂O₂.

Strain	Mean CFU ml ⁻¹ (SE)*		% Survival
	0 mM H ₂ O ₂	5 mM H ₂ O ₂	
569BSK	1.48 x 10 ⁸ (±3.51 x 10 ⁷)	1.85 x 10 ⁸ (±7.21 x 10 ⁷)	>100
Mut32	1.42 x 10 ⁸ (±5.61 x 10 ⁷)	7.70 x 10 ⁷ (±4.68 x 10 ⁷)	54
Mut3216	3.75 x 10 ⁶ (±1.31 x 10 ⁶)	3.78 x 10 ⁴ (±2.16 x 10 ⁴)	1

Cells were grown for 2 days under pigment-inducing conditions, i.e. at 37 °C in broth containing 1% tryptone, 5 mM L- tyrosine, 5 µM CuSO₄ and 4% NaCl. Values are the means of two independent experiments, each plated in duplicate. * SE, standard error

2.3.3. The effect of the *ppdA* mutation on the culturability of *V. cholerae*.

The detrimental effect that an excess of melanin had on the culturability of *V. cholerae* Mut3216 was investigated further. *V. cholerae* strains 569BSK, 5616, Mut32 and Mut3216 were compared in terms of culturability when grown under pigment-inducing conditions and increased aeration. More melanin was produced when the aeration of the culture was increased. After one day of growth under pigment-inducing conditions, all four strains reached a cell density of 10⁸ CFU/ml of culture (Table 2.3). However, the cell numbers declined, both in the presence and absence of melanin, after 2 days of growth. The culturability of Mut32 (no melanin production) only declined by 44.7 %, whereas the CFU/ml of the wild type declined by 94.9 %, and the two melanin-overproducing strains by 99 % (wild type, carrying pCM302-16) and more than 99.9 % (Mut32 carrying pCM302-16), respectively.

2.3.4. The effect of melanin on the culturability and survival of *E. coli* JM109.

E. coli JM109 strains that produced either no melanin, intermediate or excess amounts of melanin were created by transforming either pBluescript SK(+), a low copy number plasmid (pACMel) containing *ppdA*, or a high copy number plasmid (pCM302-16) into *E. coli* JM109 (Fig 2.5). After one day of growth the melanin-deficient control strain yielded 7.7 x 10⁸ cfu/ml (Table 2.4). Similar to *V. cholerae*, the culturability of *E. coli* declined with increasing amounts of melanin: *E. coli* synthesizing intermediate amounts of melanin (strain JMAC, Fig. 2.5) yielded 4 x 10⁸ colonies,

compared to 6×10^7 colonies of the overproducing strain (JM16, Fig. 2.5). When these strains were exposed to H_2O_2 , *E. coli* JMAC yielded the highest number of culturable cells (31% survival), whereas *E. coli* JM16 did not survive. Thus, similar to *V. cholerae*, melanin protected against H_2O_2 , whereas an excess or a complete absence of melanin was found to be detrimental to the bacterium.

Table 2.3. The effect of melanin on the culturability of *V. cholerae*

STRAIN ¹	CFU/ML OF CULTURE (SE) ²		MELANIN ³	% SURVIVAL
	Day 1 post inoculation	Day 2 post inoculation		
569BSK	$1.49 \times 10^8 (\pm 1.63 \times 10^7)$	$7.66 \times 10^6 (\pm 3.32 \times 10^6)$	+	5.1
Mut32	$4.67 \times 10^8 (\pm 1.62 \times 10^8)$	$2.54 \times 10^8 (\pm 8.40 \times 10^7)$	-	55.3
5616	$6.25 \times 10^8 (\pm 1.84 \times 10^8)$	$6.83 \times 10^6 (\pm 3.19 \times 10^6)$	+-	1.1
Mut3216	$3.88 \times 10^8 (\pm 7.80 \times 10^7)$	$1.77 \times 10^4 (\pm 8.88 \times 10^3)$	++	0.01

¹569BSK, *V. cholerae* containing pBluescript SK(+); Mut32, melanin-deficient mutant of *V. cholerae*; 5616, *V. cholerae* carrying pCM302-16; Mut3216, Mut32 carrying pCM302-16. ² Cells were grown for two days under pigment-inducing conditions; i.e. at 37°C, in tryptone containing 4% NaCl, 5 mM L-tyrosine and 5 μ M $CuSO_4$. Values represent the means of 2 independent experiments. Dilutions from each experiment were plated in triplicate. ³ Melanin production as evaluated by visual inspection of cultures: +, melanin as produced by wild type bacterium; ++ overproduction of melanin as a result of overexpression of *ppdA* carried on a plasmid; -, no melanin

Table 2.4. Survival of *E. coli* exposed to 100 mM H_2O_2 in the presence or absence of melanin.

STRAIN	0 mM H_2O_2	100 mM H_2O_2	% SURVIVAL
JM177	$7.70 \times 10^8 (\pm 3.46 \times 10^8)$	$9.07 \times 10^5 (\pm 18.57 \times 10^5)$	11.8
JMAC	$4.00 \times 10^8 (\pm 2.15 \times 10^8)$	$1.27 \times 10^8 (\pm 6.96 \times 10^7)$	31.8
JM16	$5.97 \times 10^7 (\pm 9.84 \times 10^6)$	0	0

Cells were grown for 24 hours in Luria broth containing 5 mM L-tyrosine and 5 μ M $CuSO_4$. Cultures (24 h) were exposed for 30 min to oxidative stress (100 mM H_2O_2). Values represent the mean CFU/ml obtained from 3 independent experiments. JM177, *E. coli* containing pACYC177; JMAC, *E. coli* containing pACMel; JM16, *E. coli* containing pCM302-16.

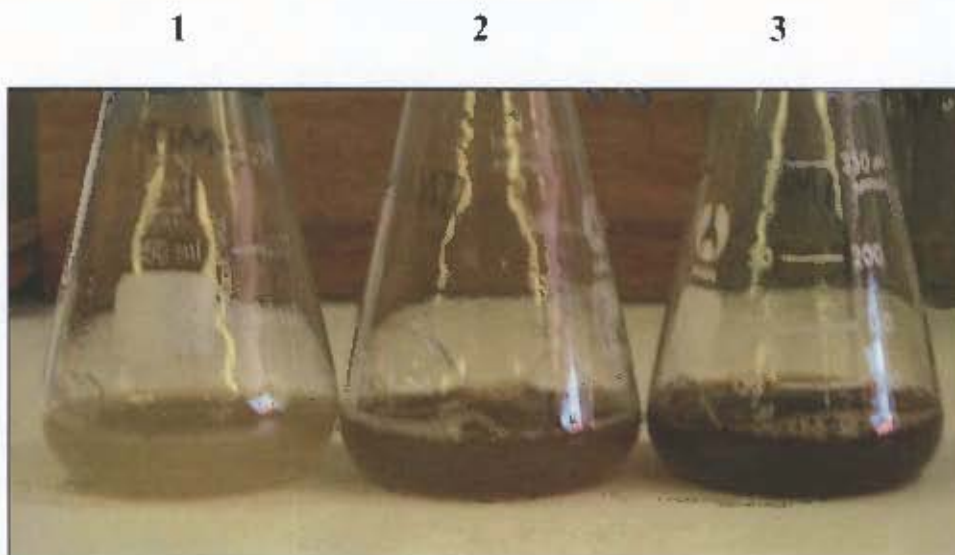


Fig. 2.5 Melanin production in *E. coli* JM109. *ppdA* was expressed from plasmids with either a low or a high copy number. 1 - JMSK, *E. coli* containing pBluescript SK. 2 - JMAC, *E. coli* containing pACMel. This plasmid contains a wild-type copy of *ppdA*, cloned into the low copy number plasmid pACYC177. 3 - JM16, *E. coli* containing pCM302-16. This plasmid contains a wild-type copy of *ppdA*, cloned into pBluescript SK (a high copy-number plasmid).

2.3.5. The effect of melanization on the quality of genomic DNA isolated from melanin-producing strains.

Melanin and melanin pre-cursor molecules induces oxidative DNA damage (Husain, 1995; Snyder, 1998; Hiraku, 1998; Urios et al., 2003). To investigate if the decline in cell numbers in melanised *V. cholerae* cultures was the consequence of melanin-induced DNA damage, we isolated DNA from melanised and non-melanised cultures of the bacterium, as well as from *E. coli* cells that carried the *ppdA* gene on plasmids. There was no obvious difference in the quality of DNA isolated from either *V. cholerae* or *E. coli* strains (Fig. 2.6). Alkaline gel electrophoreses did not reveal any difference in the degree of DNA cleavage (indicative of oxidative damage) of DNA in the presence or absence of melanin.

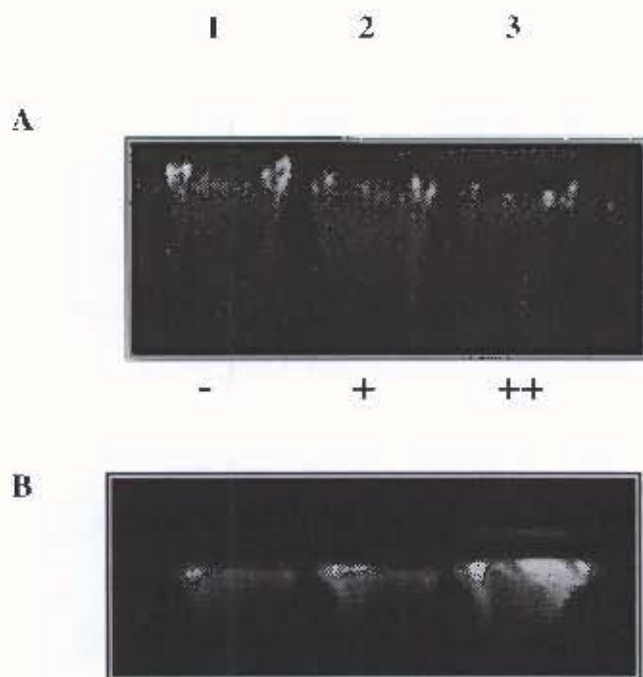


Fig. 2.6. The effect of melanin on the integrity of genomic DNA isolated from melanin-producing bacteria. A. Genomic DNA isolated from *V. cholerae* 569BSK (Lane 1), Mut32 (Lane 2) and Mut3216 (Lane 3). B. Genomic DNA isolated from *E. coli* JM177 (Lane 1), JMAC (Lane 2) and JM16 (Lane 3). -, no melanin; +, melanin as produced by wild type *V. cholerae* or as produced in *E. coli* with *ppdA* expressed from a low copy number plasmid; ++, melanin levels as produced through *ppdA* expressed from a high copy number plasmid

2.4. Discussion

Inactivation of *ppdA* led to the production of a melanin deficient strain of *V. cholerae* 569B. There was no difference in growth between the wild type strain and the mutant, even under conditions of elevated salinity and temperature. These results indicate that the gene might not be essential for growth of the organism before the onset of L tyrosine metabolism. Earlier work showed that L-tyrosine is only utilized by *V. cholerae* after more than 12 hours of growth (Ruzafa et al., 1995; Sanchez-Amat et al., 1998). HPPD is only detected after approximately 44 hours of growth (Schroeder thesis, 1998). The appearance of the protein coincides with the disappearance of L-tyrosine, as well as the appearance of homogentisic acid and melanin in the media (Ruzafa et al., 1995; Sanchez-Amat et al., 1998, Schroeder thesis, 1998). The absence of this gene would therefore only affect the bacterium once the L-tyrosine catabolic pathway is active.

However, the absence of melanin affected the survival of the bacterium upon exposure to oxidative stress, since the *ppdA* mutant did not survive to the same extent as the melanised wild type cells when exposed to H_2O_2 . Melanin thus has an antioxidant function in *V. cholerae*. Similar antioxidant functions were exhibited by melanin derived from *Exophiala dermatitidis* (Schnitzler et al., 1999), *Alternaria alternata* (Jacobson et al, 1995) and *Cryptococcus neoformans* (Casadevall et al., 2000).

Contrary to expectations, complementation of the mutation by a wild type copy of *ppdA* carried on pCM302-16 did not restore protection against oxidative stress. Instead, overproduction of melanin occurred when a copy of *ppdA* was expressed on a multi-copy number plasmid and these cultures were much darker than those of the wild type bacterium. Although this caused a decline in bacterial numbers, a higher degree of death was observed when these cultures were exposed to H_2O_2 (only 1% or less of the cells remained in cultures that overproduced melanin). In comparison, only half of the melanin-deficient cells were killed and none of the wild type. A similar result was obtained with *E. coli*, i.e. melanin protected against H_2O_2 , but only if it was produced in "intermediate" amounts. *E. coli* that produced excess amounts of melanin did not survive exposure to the oxidant. These results therefore indicate that melanin would protect against H_2O_2 exposure, but that an excess of the pigment is potentially lethal to the bacterium.

The reason for the cytotoxic effect warrants further investigation. Various types of pigments, including melanin and melanin precursors have been identified as the source of negative growth effects in a number of microorganisms. In the case of *Aphanomyces*, a fungal pathogen of crayfish, it was found that inhibition of hyphal growth occurred when hyphae were incubated beforehand in the presence of the DOPA-melanin precursors DHI and DHICA (Söderhäll and Ajaxon, 1982). Another fungus, *Phaffia rhodozyma*, showed increased sensitivity to H_2O_2 when its pigment (not a

melanin, but a carotenoid) was overexpressed (Schroeder and Johnson, 1993). The common denominator in the abovementioned cases is the fact that the pigments and/or the pigment precursors contain quinonic groups. Quinones are chemically active groups that interact with a number of substances to generate oxidative radicals. These radicals damage biomolecules such as proteins, lipids and nucleic acids. An indication of the damage caused to microbes by melanin was shown in a study on *Pseudomonas fluorescens* (Fava et al., 1993). This organism produces melanin-containing granules in the cytoplasm. The cytoplasm and cell wall of *Ps. fluorescens* cells exhibited extensive damage, as well as leakage of the cytoplasmic content into the environment, in the presence of these melanin-containing granules. A similar damaging effect was demonstrated in *Sclerotium rolfii*, a fungal pathogen that forms sclerotia in response to unfavourable environmental conditions (Abo Ellil, 1999). In this instance, it was found that the degree of melanization increased towards the centre of the sclerotia. Concomitantly, the degree of lipid peroxidation also increased in a similar manner. It was further observed that melanin production correlated positively with oxidative stress. When antioxidant substances such as ascorbate were added to cultures, the number of formed sclerotia decreased, as well as the degree of pigmentation. This was a clear demonstration of the relationship between the formation of sclerotia, oxidative stress, melanin and oxidative damage (in the form of lipid peroxidation) in this fungus.

The cytotoxicity of homogentisic acid (and other phenolic compounds) towards *E. coli* was displayed in an OxyR mutant of *E. coli*. OxyR is a protein sensitive to the redox status of bacterial cells. This DNA-bound protein undergoes a conformational change in the presence of H₂O₂, thereby causing the induction of genes encoding for antioxidant molecules such as catalases (Storz et al, 1990). The OxyR mutant of *E. coli* displayed a reduction in cell numbers when incubated in the presence of homogentisic acid. This indicates that the bacterial cell could not cope with reactive oxygen species, such as H₂O₂. Any H₂O₂ produced by the mutant bacterium would be detrimental, since the inducing sensor molecule OxyR was inactivated in this particular mutant. Therefore catalase production would be defective as a consequence of the OxyR mutation (Urios et al., 2003). This cytotoxic effect of homogentisic acid was not seen in *E. coli* cells that produced OxyR.

In vitro studies with purified melanin precursors confirms that the toxicity of melanin involves oxidative radicals. Homogentisic acid, as well as other melanin precursors (L-DOPA, DHI, DHICA), was shown to generate reactive oxygen species during the process of autopolymerisation to form melanin. These species are known to damage DNA, lipids and proteins (Marnett, 2002). Lipid peroxidation, due to the presence of a melanin precursor, was also demonstrated in eukaryotic cells (Sotomatsu et al., 1994; Park et al., 2002).

In vitro assays with DNA (plasmid and genomic) provided further evidence of DNA damage caused by melanin precursors. Incubation of DNA fragments or plasmids in the presence of melanin precursors and copper resulted in fragmentation of genomic DNA and nicking of plasmid DNA (Husain, 1995; Snyder, 1998; Wick, 1977; Hiraku, 1998; Urios et al. 2003). As a result of nicking, supercoiled plasmid DNA disappears while the less compact forms become more evident. DNA damage was enhanced in the presence of a metal such as copper, but was limited in the presence of ROI scavengers (in particular catalase and mannitol) and copper scavengers (Hiraku, 1998). This indicates that precursors can interact with metals, H_2O_2 is formed during melanin formation, and that hydroxyl radicals are the actual culprits causing damage to DNA.

Thus a number of mechanisms exist that might explain the negative effect of melanin overproduction in *V. cholerae* and *E. coli*. We investigated whether the presence of melanin would cause oxidative damage to DNA in both *E. coli* and *V. cholerae*. No difference in the quality of the DNA isolated from melanin-deficient/proficient strains could be demonstrated. This indicated that the cytotoxic effect of excess melanin might be due to causes other than DNA damage. Damage to other cellular components and biomolecules, such as proteins and membranes, might offer an alternative explanation for the negative effect of increase melanin production observed in this study.

In conclusion, we demonstrated that melanin protected *V. cholerae* and *E. coli* against H_2O_2 , but conversely, that overproduction of the pigment was detrimental to these bacteria: The reason for the observed decline in cell numbers in the presence of excess amounts of melanin remains unclear, as we could not demonstrate that the excess melanin caused oxidative DNA damage. Future investigation of the effect of excess melanin on other cellular components might provide an answer to this question.

CHAPTER 3
ISOLATION AND CHARACTERISATION OF A
CATALASE GENE FROM *V. CHOLERA* 569B

University of Cape Town

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University of Cape Town

SUMMARY

The relevance of melanin as a H₂O₂ scavenger in *Vibrio cholerae* 569B should ideally be assessed in a strain that has diminished or no capacity to scavenge H₂O₂ through enzymes such as catalases. In order to achieve this, a plasmid containing a catalase gene was isolated from a *V. cholerae* genebank. This plasmid, with a *V. cholerae* genomic DNA insert of approximately 7.3 kb, restored the ability of an *E. coli* catalase mutant to dismutate H₂O₂. The catalase gene was identified as *perA*, encoding for a catalase-peroxidase previously identified in the El Tor strain *V. cholerae* N16961. The sequence of *perA* from the classical strain 569B was found to be 99% identical to *perA* of the El Tor strain. Zymogram analysis of cell extracts showed that this catalase-peroxidase appears mainly after six hours of growth in tryptone broth, suggesting its possible importance for protection against H₂O₂ when bacteria are in the stationary phase of growth. The catalase activity of *V. cholerae* 569B was increased upon exposure to sub-lethal doses of H₂O₂. This induction led to the protection of *V. cholerae* cells against subsequent lethal doses of the oxidant. However, zymogram analysis showed that it is not the *perA* gene product that was induced, but instead another catalase that migrated slower than PerA. PerA, like other catalase-peroxidases, was inactivated by high temperature (55°C).

3.1 Introduction

According to Farr and Kogoma (1991) “oxidative stress can be functionally defined as an excess of prooxidants in the cell”. Oxidative stress thus refers to the state of an aerobic respiring cell when antioxidant enzymes and metabolites are insufficient to detoxify reactive oxygen species (ROS) produced intracellularly or in the environment. ROS damage cellular proteins (Gardner, 1992), lipids (Abo Ellil, 1991) and nucleic acids (Keyer, 1995). The most damaging oxygen radicals formed in aerobic respiring organisms are the hydroxyl radical (OH^\cdot), superoxide (O_2^-), and hydrogen peroxide (H_2O_2). The OH^\cdot and O_2^- radicals cause the most damage to cellular components due to their highly reactive nature, while H_2O_2 , due to its more chemically stable nature, is the least reactive radical of the three oxygen radicals formed (Farr, 1991; Touati et al., 1995, Touati, 2000).

In response to oxidative stress, bacteria produce enzymes (such as superoxide dismutase and catalases), DNA-binding proteins and antioxidant metabolites (Farr, 1991). Amongst the genus *Vibrio*, catalase and or catalase-peroxidase genes have been identified in *V. rumoiensis* (Genbank accession nr. BAB12412), *V. fischeri* (locus VFA0009), *V. vulnificus* CMCP6 (locus VV12755) *V. cholerae* (locus VC1560 and VC1585), *V. parahaemolyticus* (locus VPA0453 and VPA0768)*. Both monofunctional and/ or bi-functional catalases have been identified in bacteria belonging to this genus.

3.1.1. The aim of the chapter

V. cholerae and other marine organisms are constantly exposed to oxidative radicals such as H_2O_2 . These radicals form through the action of sunlight penetrating into water (Alam, 2002). The persistence of *V. cholerae* in aquatic environments (Codeco, 2001) suggests that this bacterium possesses the necessary mechanisms, such as scavenger enzymes, to combat the oxidative stress it might encounter in its natural environment. *V. cholerae* also produces melanin, a pigment known to protect other microbes against oxidants such as H_2O_2 (Jacobson et al., 1995; Jacobson, 2000; Zughailer et al., 1999). However, as bacteria mainly combat H_2O_2 via the action of catalases, the true importance of melanin as an antioxidant should be determined in a genetic background either devoid of a functional catalase or with diminished catalase activity.

To obtain a catalase mutant, the gene(s) encoding for catalase in *V. cholerae* 569B would have to be identified and subsequently mutated (Chapter 4). This chapter describes the isolation and characterisation of a catalase gene from *V. cholerae* 569B. A plasmid carrying a *V. cholerae* gene that conferred catalase activity to an *E. coli* catalase mutant was isolated. Sequencing of the gene

* Numbers in brackets, except for *V. rumoiensis*, denotes the locus name in published genomes. Sequences available at www.ncbi.nih.gov/.

on the plasmid identified it as *perA*, which encodes for a catalase-peroxidase consisting of 724 amino acids. The sequence of *perA* was compared, at the nucleotide and amino acid levels, with the catalase-peroxidases from other bacteria.

University of Cape Town

3.2 Materials and methods

3.2.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 3.1. The catalase-deficient *E. coli* UM2 mutant was a kind gift from P. Loewen. This mutant *E. coli* strain does not produce any functional catalases (Loewen, 1985).

3.2.2 Media and culture conditions

Escherichia coli UM2 and *V. cholerae* were maintained on Luria agar or tryptone agar respectively (Appendix A). Bacteria harboring plasmids were cultured on media amended with ampicillin (at a concentration of 100 µg/ml of media). *E. coli* was incubated at 37°C, whilst *V. cholerae* was incubated at 30°C, unless stated differently.

3.2.3 Screening of a DNA genebank from *V. cholerae* 569B

Competent *E. coli* UM2 cells were obtained with the CaCl₂ method (Appendix B1) and transformed (Appendix B2) with plasmids from a *V. cholerae* genebank. This genebank consists of *V. cholerae* genomic DNA fragments (10- 14kb in size) that were ligated into plasmid pEcoR251 (Schroeder, 1998). In order to identify a plasmid containing a catalase gene, *E. coli* transformants were tested in the following manner: H₂O₂ (3%, w/v) was applied to the edge of individual colonies to check for the generation of oxygen bubbles (the presence of oxygen bubbles indicates that H₂O₂ is being broken down into water and O₂). Colonies exhibiting oxygen bubbles were inoculated into 5 ml Luria broths, grown overnight at 37°C in the presence of 100 µg/ml of ampicillin, before plasmids were isolated and analysed.

3.2.4 Analysis of plasmid pKat18

3.2.4.1 Confirmation of the identity of the DNA fragment contained on pKAT18.

Genomic DNA was extracted from cultures of *V. cholerae* 569B and *E. coli* as previously described (Appendix B3). Five µg of genomic DNA from *V. cholerae* and *E. coli*, as well as 100 ng of plasmid pKat18, were digested with either *Cla*I, *Sal*I, *Sph*I or *Hpa*I. Fragments were separated by agarose gel electrophoresis (Appendix B6), before being transferred to a nitrocellulose membrane (Hybond N+, Amersham) by capillary transfer (Appendix B12). A 2 kb fragment obtained by restriction digestion of pKat18 with *Cla*I and *Sal*I, was purified and radiolabelled with [α -³²P] dCTP by random prime labeling (Appendix B12). Southern hybridisation and visualisation of the bands by autoradiography was as previously described (Appendix B12).

Table 3.1. Bacterial strains and plasmids

STRAIN OR PLASMID	GENOTYPE / RELEVANT FEATURES	REFERENCE
<i>E. coli</i> UM2	λ^- e14 ⁻ araD139 leu B6 azi-6 fhu A23 lacYI proC83 tsx-67 purE42 glnV44 galK2 trpE38 xthA15 his208 rfbD1 mgl-51 argG77 rpsL109 glpR201 xylA5 mtl-2 ilvA681 thi-1 met160 katE32 katG15	Loewen et al. (1985)
<i>E. coli</i> UM2BV	<i>E. coli</i> UM2 carrying pSKBV	This study
<i>E. coli</i> JM110	rpsL (Strr) thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ (lac-proAB) [F' traD36 proAB] lacIqZAM15	
<i>V. cholerae</i> 569B	Classical biotype, O1 serogroup, Inaba serotype	Mukherjee, (1998)
Plasmids		
pBluescript SK(+)	Amp ^r , β - galactosidase	Short et al. (1988)
pEcoR251	Amp ^r , <i>EcoRI</i> endonuclease gene	Zab�eau and Stanley (1982)
pKat18	pEcoR251 derivative containing genomic DNA fragment derived from <i>V. cholerae</i>	Schroeder (1998) ^a
pSKBV	pSK derivative containing <i>SalI</i> - <i>EcoRV</i> fragment from pKat18	This work

Amp^r, ampicillin resistance. ^a, Plasmid obtained from a genebank constructed by Schroeder, IC (PhD thesis, 1998)

3.2.4.2 Analysis of pKat18 and sub-cloning into pBluescript SK (+)

Plasmid DNA was extracted with the Nucleobond Plasmid MaxiKit (Clontech). Restriction enzyme analysis was performed according to standard methods. In order to assess the region that contained the catalase gene within the genomic DNA insert on the plasmid, various fragments were deleted from pKat18. This was achieved by restriction enzyme digestion, blunting of the fragments and religation of blunt ends. Blunt ends were obtained by treatment of digests with the Klenow

fragment of DNA polymerase as previously described (Appendix B). Ligations were transformed into *E. coli* UM2. After overnight incubation on Luria agar containing ampicillin, colonies were tested for catalase activity by applying H₂O₂ to the edge of colonies. A *Bam*HI – *Eco*RV DNA fragment, derived from pKAT18, was ligated into pBluescript SK (+) to create plasmid pSKBV. This plasmid was used in all further experiments.

3.3 Sequencing of the gene

3.3.1 Creation of constructs for sequencing

Small, overlapping DNA fragments of approximately 500 to 1000 bp were obtained by digesting plasmid pSKBV with restriction enzymes, or by PCR amplification, as indicated in Figure 3.1. These DNA fragments were cloned into pBluescript SK (+). The primers to amplify Fragment 6 were designed with the aid of the nucleotide sequence obtained from *perA* of *V. cholerae* El Tor N16961 (VC1560). The sequences of the forward and reverse primers for Fragment 6 were 5' ATTGTTCTGGCTGGTAACGTAGG 3' and 5'GGTGAGGTTAACGAAGAAATCGTT 3' respectively.

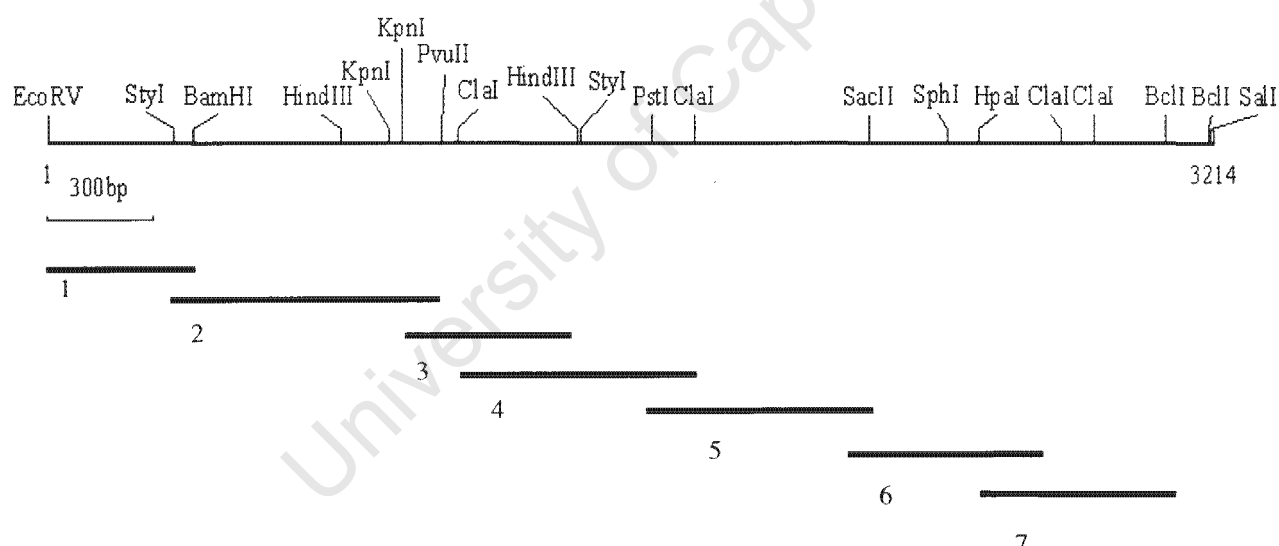


Figure 3.1. Inserts cloned into pSK to sequence the entire catalase gene.

Restriction map of the fragment containing the catalase gene on plasmid pSKBV. All fragments, except Fragment six, were obtained with restriction enzyme digestion of pSKBV. Fragment 6 was obtained by PCR. Fragment 1, *Eco*RV – *Bam* HI; Fragment 2, *Sty*I – *Pvu*II; Fragment 3, *Kpn*I – *Hind*III; Fragment 4, *Cla*I – *Cla*I; Fragment 5, *Pst*I – *Sac*II; Fragment 6, obtained with PCR; Fragment 7, *Hpa*I – *Bcl*I

Plasmids were prepared with the High Pure Plasmid Kit (Roche Biochemicals) and inserts were sequenced, in both directions, with the automated Megabase (MegaBACE 500; Amersham Biosciences). Sequencing reactions were performed according to the instructions of the DYEnamic ET Dye terminator Cycle sequencing Kit. The sequences of the M13 primers used were 5' CGC

CAG GGT TTT CCC AGT CAC GAC 3' (Forward primer) and 5' GAG CGG ATA ACA ATT TCA CAC AGG 3' (Reverse primer).

3.3.2. Analysis of sequencing results

Sequence analysis and database searches were performed using the Chromas (Version 2.01, Technelysium Pty Ltd) and DNAMAN (Version 4.13, Lynnon BioSoft) software packages, the National Center for Biotechnology Information BLAST server (<http://www.ncbi.nlm.nih.gov/>), and TIGR-CMR BLAST (<http://www.tigr.org/cmr-blast/>). The protein secondary structure prediction was performed with PSIPRED (<http://bioinf.cs.ucl.ac.uk/cgi-bin/psipred/>).

3.4. Induction assay and zymogram analysis of *V. cholerae* catalase activity

Vibrio cholerae 569B was inoculated into 5 ml of tryptone broth (1% tryptone, 1% NaCl) and grown overnight at 30°C. The next day, 100 ml sterile tryptone broths were inoculated, with the overnight cultures, to an optical density of 0.05 and incubated for three hours, i.e. until cells reached the log phase of growth and an optical density of approximately 0.4 at 600 nm. Log phase cultures were split into two fractions in sterile flasks. One of these fractions was exposed for 30 minutes to H₂O₂, at sub-lethal dosages (i.e. 50 µM of the oxidant), before cells were harvested by centrifugation. The other half of the culture was incubated for the 30 minute period without any exposure to the oxidant, and therefore served as the control (uninduced) culture. These cells were also harvested by centrifugation. To test whether osmotic and temperature stress would induce catalase activity, *V. cholerae* 569B was also inoculated into tryptone broth amended with 4% NaCl and grown at 37 °C for three hours. As a positive control, *E. coli* UM2BV (*E. coli* catalase mutant carrying pSKBV) was inoculated into 5 ml Luria broth containing ampicillin at 100 µg/ml and grown for 16 hours at 37°C.

Cells were harvested by centrifugation (10000 rpm for 10 minutes), washed once in phosphate buffer (0.05 M, pH 7) and resuspended in 1 ml phosphate buffer (Appendix B). Crude cell extracts were obtained through sonication (4 pulses of 20 seconds each, with 20 seconds pauses in-between, Virsonic Sonicator, Appendix B) in an alcohol ice-water bath. Cell debris was removed from cell extracts by centrifugation. The protein content of the crude cell extracts was determined by the Bradford assay (Ausubel et al., 1989).

Equal amounts of protein (100 µg) from each extract (i.e. *E. coli* UM2BV and *V. cholerae* cultures) were separated by non-denaturing PAGE (10% acrylamide, Appendix B13). The acrylamide gel contained only a separating gel, but no stacking gel. The acrylamide gel and buffers used for PAGE contained no SDS in order to prevent denaturation of proteins. Electrophoresis was performed for 2.5 hours at 140 V in a Mighty Small II SE250 (Hoefer Scientific Instruments). After

electrophoresis, the gel was rinsed in distilled water, followed by a 15 minute incubation step in the presence of 0.003% H₂O₂. Peroxide was removed, the gel rinsed with distilled water and catalase activity in the gel was visualised by staining (Wayne and Diaz, 1986; Appendix B) using a solution containing ferricyanide (1%) and ferrichloride (1%) until the acrylamide gel turned green, except for regions containing the active catalase protein. The latter turns yellow in colour.

3.5 Investigation of other properties of the catalase-peroxidase

3.5.1 Sensitivity of the catalase-peroxidase to heat

V. cholerae 569B was inoculated into 5 ml LB broths and grown overnight at 30°C. The next day, the cells were harvested and cell-free extracts obtained as in Section 3.4. A fraction of each cell extract, containing 100 µg of crude protein, was heated at 55°C for 15 minutes (Visick and Clarke, 1997), in order to determine the sensitivity of the catalase to heat. Heat-inactivation of catalase activity was determined by analysis with non-denaturing PAGE as described in Section 3.4.

3.5.2 Quantitative analysis of the induction of catalase activity by H₂O₂

V. cholerae 569B was inoculated into 5 ml tryptone broth and grown for 16 hours at 30°C. Sterile tryptone broths (400 ml) were inoculated with aliquots from these cultures to achieve an optical density of 0.05 at 600 nm. Each 400 ml culture was grown at 30°C for 3 hours, before it was split into four 100 ml aliquots, each in a sterile 500 ml flask. Two of the cultures served as control cultures, while H₂O₂ was added to the remaining two cultures to a final concentration of 50 µM. All cultures were incubated for a further 90 minutes. At this time, 50 ml aliquots were removed from all the cultures. These aliquots were centrifuged (10000 rpm for 10 minutes, at 4°C), the cell pellets were washed in phosphate buffer (0.05 M, pH 7), resuspended in 2 ml of the same buffer and cell-free extracts prepared as described in Section 3.4. The catalase activity was determined quantitatively as described before (Appendix B).

The remainder of each culture (50 ml) was treated as follows: An aliquot of 100 µl was removed from each culture, dilutions made and plated in duplicate on TB agar. The rest of the culture was exposed to a lethal dose of H₂O₂, namely 250 µM, and incubated for a further 45 minutes. After this incubation time, another aliquot of 100 µl was removed from each culture. Dilutions of these aliquots were plated in duplicate on tryptone agar. Plates were incubated overnight at 30°C. Colonies were counted and the mean CFU/ml was determined.

3.6 Results

3.6.1 Analysis of pKat18, a plasmid containing a catalase gene from *V. cholerae* 569B

A catalase-deficient *E. coli* strain was used to screen a *V. cholerae* genebank for the presence of a plasmid that contains a catalase gene. Two colonies exhibiting catalase activity were obtained after transformation of *E. coli* UM2 with plasmids from this genebank. The catalase-positive colonies exhibited an identical restriction enzyme profile, thus only one was used for further analysis. This plasmid was named pKat18.

3.6.2 Confirmation of the origin of the insert on pKat 18

Southern hybridization analysis revealed that a 2 kb *SalI* - *ClaI* fragment obtained from pKAT18 hybridized to *V. cholerae* genomic DNA (Fig. 3.2, lanes 2, 4, 6). No hybridization was observed to genomic DNA extracted from *E. coli* (lanes 3, 5, 7). The probe hybridized to a fragment of approximately 2 kb in size in lanes containing *ClaI* and *SalI* double digests of pKat18 (lane 1) and *V. cholerae* genomic DNA (lane 2, Fig 3.2). Single restriction enzyme digests of genomic DNA with these enzymes yielded bands of approximately 5.5 kb (*ClaI* restriction enzyme digest) and 11 kb (*SalI* restriction enzyme digest), respectively.

3.6.3 Construction of a restriction enzyme map of pKat18

Restriction enzyme analysis revealed that plasmid pKAT18 is approximately 11 kb in size. The size of plasmid pEcoR251 is 3349 bp, thus the genomic DNA insert contained on pKAT18 is calculated to be approximately 7 700 bp in size. Various restriction enzymes were used to construct a restriction enzyme map (Fig. 3.3) of plasmid pKAT18. The restriction fragment profile of pKAT18 was found to be similar to the restriction enzyme map constructed of the region containing *perA* of *V. cholerae* N16961, that is the region that contains locus VC1560. However, the sequence of *perA* from *V. cholerae* N16961 contains 3 more *ClaI* restriction sites than what is seen with plasmid pKAT18 isolated from *E. coli* UM2. In order to verify the presence of the 3 additional sites, plasmid pSK18 was transformed into *E. coli* JM110, a methylase-deficient strain of *E. coli*. Plasmid DNA isolated from *E. coli* JM110 revealed that the 3 *ClaI* restriction sites, as seen in the *perA* map of the El Tor strain, were indeed present. The presence of these sites was further confirmed when the gene was sequenced.

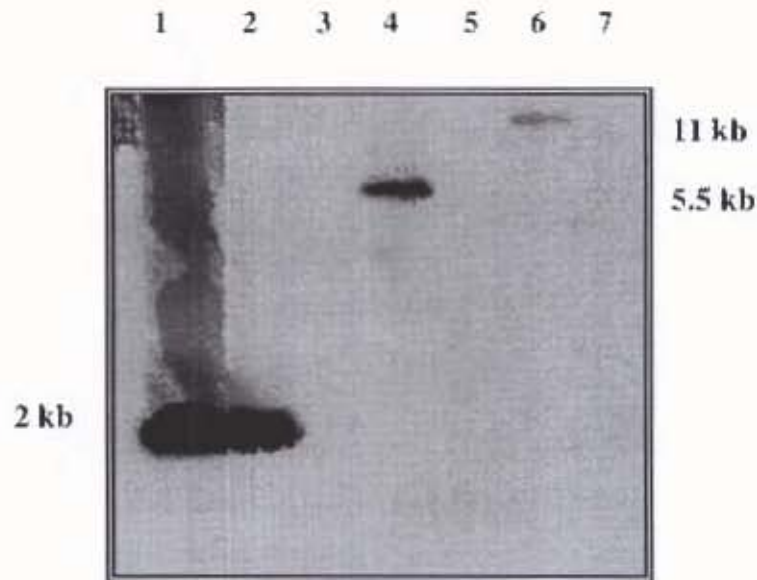


Fig. 3.2. Southern hybridisation to confirm the source of the pKat18 DNA insert. Lane 1, pKAT18, digested with *ClaI* and *SalI*; Lanes 2, 4, 6, genomic DNA extracted from *V. cholerae*; Lanes 3, 5, 7, genomic DNA extracted from *E. coli* UM2. Restriction enzyme digests of DNA: Lanes 1 - 3, digested with *ClaI* and *SalI*; Lanes 4 and 5, DNA digested with *ClaI*; Lanes 6 and 7, DNA digested with *SalI*. Five micrograms of genomic DNA was loaded and 1 μ g of plasmid.

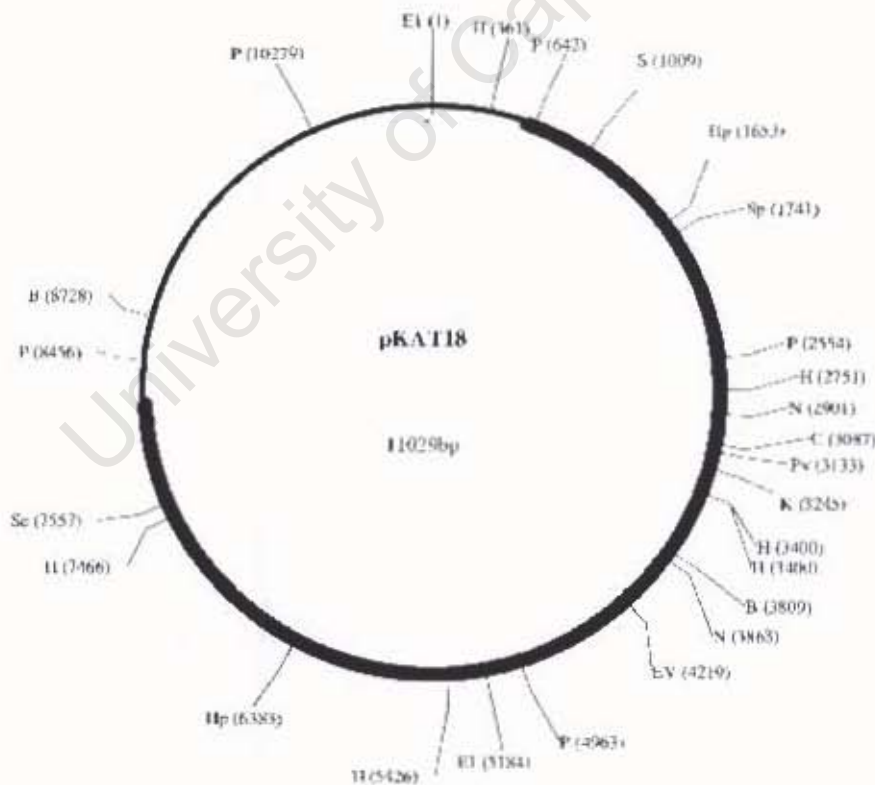


Fig 3. 3. Plasmid pKAT18, isolated from a *V. cholerae* 569B genebank. Abbr. for restriction enzymes: B, *Bam*HI; C, *Cla*I; EL, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sc, *Spe*I; Sp, *Sph*I. The insert is indicated by the thick black line, while the thin line represents the vector. Numbers in brackets indicate the position of the restriction site in base pairs.

3.6.4 Deletion analysis of pKat18

In order to obtain the smallest fragment able to confer a catalase-positive phenotype to *E. coli* UM2, various DNA fragments were deleted from pKAT18 (Fig 3.4). A *SalI* - *Bam*HI fragment was the smallest fragment to confer a catalase positive test. Deletions made with enzyme sites between *Bam*HI and *SalI* generated colonies that were catalase negative (Fig 3.4).

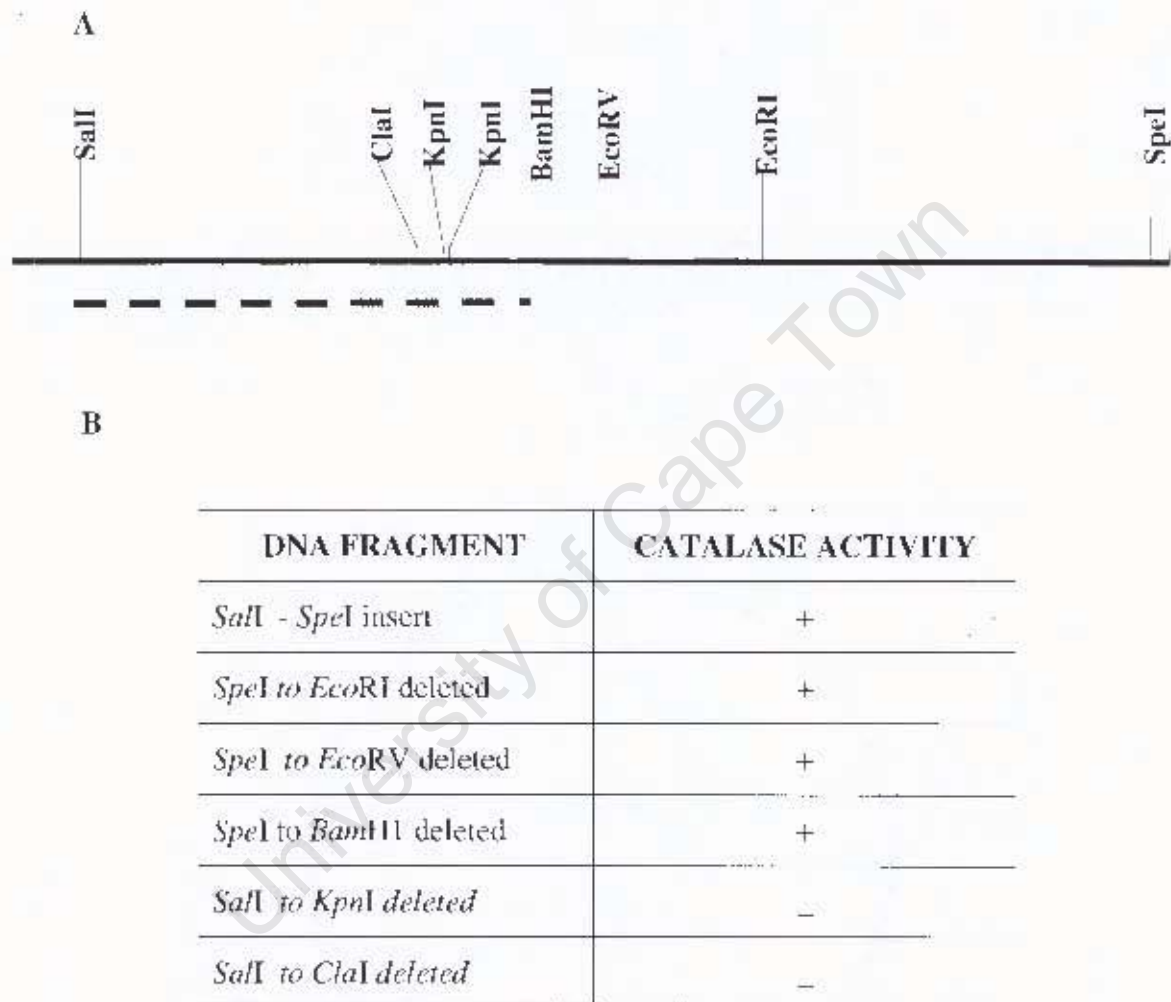


Fig. 3.4 **A.** The restriction map of the *V. cholerae* DNA insert on pKAT18. The dashed line indicates the region that confers a catalase positive phenotype to *E. coli* UM2. **B.** Ability of pEcoR251-derived plasmids to confer a catalase positive phenotype to *E. coli* UM2. Fragments were deleted from the original plasmid. + sign indicates a positive test, i.e. O₂ bubbles are generated in the presence of H₂O₂; - sign indicates a negative catalase test.

3.6.5. Induction of the catalase activity of *V. cholerae*

Zymogram analysis of cell-free extracts of *V. cholerae* cells and *E. coli* UM2BV (catalase mutant carrying plasmid pSKBV) revealed that the catalase encoded on pKAT18 is either not produced in log phase *V. cholerae* (lane 2), or alternatively, is not produced in amounts detectable by the zymogram (Fig. 3.5). The catalase encoded on pSKBV in *E. coli* migrated the same distance to a band seen in a stationary phase cell extract from *V. cholerae* (Lanes 1 and 6). The zymogram also revealed that catalase activity is induced in log phase in *V. cholerae* upon exposure to H_2O_2 . However, the increased amounts of catalase activity are not due to the catalase encoded by the cloned *V. cholerae* genomic DNA on the plasmid, but instead by a catalase that migrated at a slower rate than the catalase expressed from the plasmid (Lane 3). The cloned catalase was also not visible in extracts from log phase *V. cholerae* cells, even when grown under elevated osmotic and temperature conditions (Lane 5).

Quantitative catalase assays revealed that log phase *V. cholerae* cells (3 hours old), as cultured under our conditions (i.e. in broth containing 1% tryptone and 1% NaCl, incubated at 30 °C), produced barely detectable amounts of catalase (less than 2 U/mg protein). However, when cells were pre-exposed to a non-lethal dose of H_2O_2 , the catalase activity increased to 12.4 U/mg of protein. This induction resulted in the protection of induced cells when challenged later with an elevated dose of H_2O_2 (Fig 3.6). Thus, when uninduced and pre-exposed cells were subsequently exposed to 250 μ M of the oxidant, only 38% of the uninduced cells survived, whereas the induced cells were not affected by the elevated dose of the oxidant (Fig 3.6).

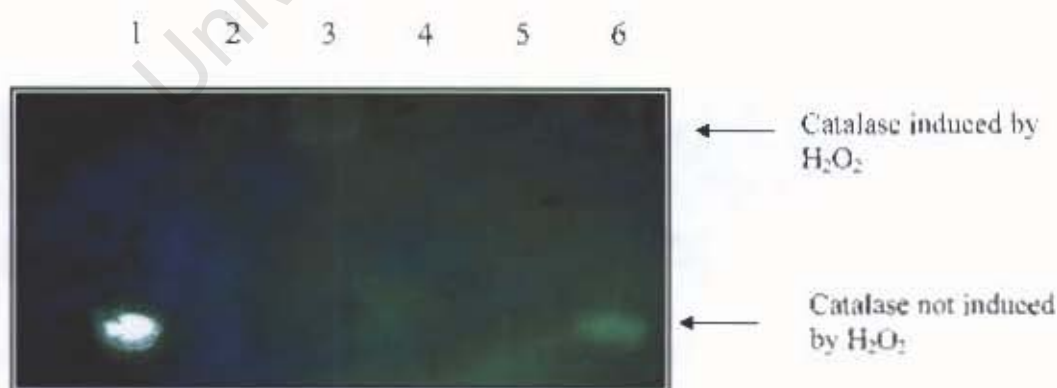


Fig. 3.5. Zymogram of cell-free extracts derived from *E. coli* and *V. cholerae*.

Lane 1, *E. coli* UM2BV; Lane 2, log phase cells of *V. cholerae*, grown at 30°C in the presence of 1% NaCl; Lane 3, log phase cells of *V. cholerae*, grown at 30°C in the presence of 1% NaCl, and induced with H_2O_2 ; Lane 4, empty lane; Lane 5, log phase cells of *V. cholerae*, grown at 37°C in the presence of 4% NaCl; Lane 6, Stationary phase cells of *V. cholerae* 569B, grown at 30°C in the presence of 1% NaCl. Cell-free extracts were obtained by sonication

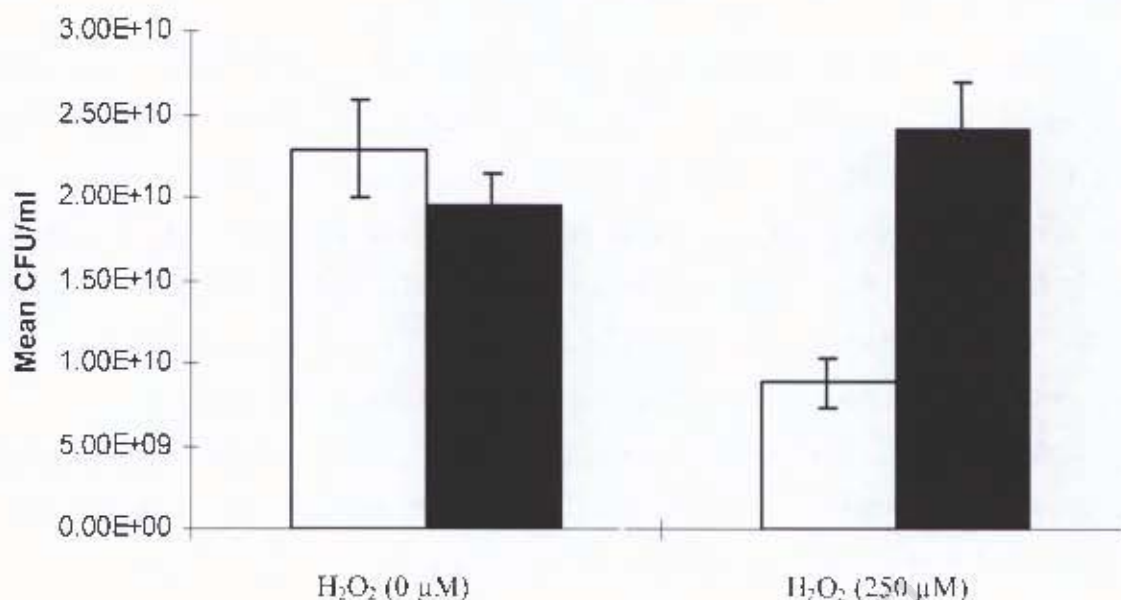


Fig 3.6 Adaptation of *V. cholerae* 569B log phase cells induced by 50 μM H₂O₂ before exposure to an elevated dose of H₂O₂ (250 μM). White bars, uninduced cells; black bars, induced cells. The mean CFU/ml was calculated from 3 independent experiments. Each experiment was done in duplicate. Error bars depict the standard error. H₂O₂ (0 μM), aliquot of culture plated before addition of the lethal dose of H₂O₂ (250 μM). H₂O₂ (250 μM), aliquot of culture plated after addition of the lethal dose of H₂O₂ (250 μM).

3.6.6. Analysis of the sensitivity of the *V. cholerae* catalase to heat.

When cell-free extracts of *V. cholerae* were heated to 55 °C, the catalase was inactivated, as evidenced by the disappearance of the catalase band (Fig. 3.7). In a similar manner, *E. coli* HPI was inactivated when heated to 55 °C (Lane 2). Incubation of a cell-free extract from *V. cholerae* at 42 °C had no effect on the catalase activity of *V. cholerae* (Lane 5).

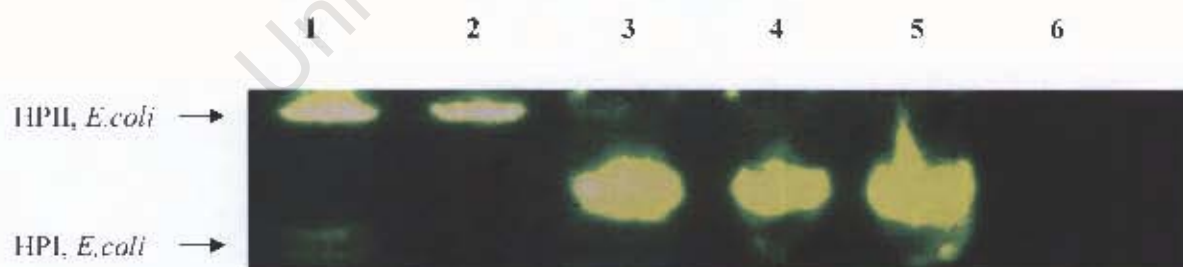


Fig 3.7. Zymogram to illustrate the heat-lability of the *V. cholerae* 569B catalase.

Cell-free extracts were prepared by sonication. To test sensitivity to heat, the extracts were treated as follows: Lanes 1 and 3, kept on ice until electrophoresis; Lane 4, incubated at room temperature; Lane 5, incubated at 42 °C; Lanes 2 and 6, incubated for 15 minutes at 55 °C. Lanes 1, 2: Extracts from *E. coli* JM109; lanes 3- 6, extracts from *V. cholerae*. HPI, catalase-peroxidase from *E. coli*; HPII, catalase from *E. coli*.

3.6.7 Sequencing of the *V. cholerae* 569B catalase gene

Sequence analysis identified the catalase gene on pKat18 as *perA*, with 99% identity to the *perA* catalase-peroxidase from *V. cholerae* El Tor N16961 (Genbank accession number: AAF94714.1) (Table 3.2). *perA* cloned from the classical strain consists of 2172 bp, encoding for a protein of 724 amino acids (Fig. 3.8). The molecular weight of PerA from *V. cholerae* 569B is calculated to be 80.572kDa and the protein has a predicted pI value of 5.72. The *perA* gene from strain 569B differs by only 1 nucleotide base from the sequenced *perA* of the El Tor strain, i.e. the gene from the classical strain contains an adenine residue at position 1293, whereas the El Tor strain contains a guanine residue (Fig 3.8). This resulted in the substitution of an alanine residue in the El Tor protein with a threonine residue in the protein from the classical strain.

The catalase-peroxidase from *V. cholerae* 569B exhibited high homology and identity to other bacterial catalase-peroxidases (Table 3.2, Fig 3.9). Of the published catalase-peroxidase genes, PerA was found to be most similar to bi-functional catalases from other members of the genus *Vibrio*. PerA has 93% and 91% similarity to the catalase-peroxidases of *V. vulnificus* protein and *V. parahaemolyticus* respectively. PerA is approximately the same length as other bacterial catalase-peroxidases (Table 3.2). The protein contains two peroxidase domains, where one of the domains is a truncated duplicate of the first domain (Fig. 3.10). The predicted secondary structure of PerA is characteristic of catalase-peroxidases as reflected by the typical helix-turn-helix structure which is present in KatGs (Fig. 3.11).

Table 3.2. Homology analysis of the catalase-peroxidase of *V. cholerae* 569B.

BACTERIA	NAME	LOCUS/ ACC*	AA NUMBER	% IDENTITY	% SIMILARITY
<i>V. cholerae</i> N16961	PerA	VC1560	724	99	99
<i>V. vulnificus</i>	HPI	VV12755	723	86	93
<i>V. parahaemolyticus</i>	N/A	VPA0768	723	82	91
<i>Edwardsiella tarda</i>	OrfA	AY643478.1:33997..36171	724	82	90
<i>Synechocystis</i> PCC 6301	N/A	AF197161.1:241..2403	714	80	89
<i>E. coli</i> K12	KatG or HPI	AAC76924	725	59	72

* Locus number as annotated in genome of the relevant bacterium. Acc., accession number in Genbank. N/A, no name assigned, simply identified as "catalase-peroxidase".

1 ATGGAGCACAATAAAGCGGGTTCAAGCGGTCAATGCCCTGTCATGCACGGTGGCTTGAAT
1 M V E N K A G S S G Q C P V M H G S L T
61 TCGGGCAGCAATTTCCAAACATGGACTGGTGGCCAAAGCGCTAAACTTAGACATCCTTCAT
21 S A S M S N M E W W P K A L N L D I I F
121 CAGCATGACAGCAAAAACCAATCCACTTGGTGTCTGATTTCAACTATCGCGAAGAGCTGAAA
41 Q H D S K T N P L G A D F N Y R K F I K
181 AAGCTTGTATGTAGAAGCGCTAAAACCGGATCTGAAAGCGTAAATGACCAATAGCCAAAG
61 K L D V F A L K R D D K A L K T N S Q E
241 TGGTGGCCGCGGACTGEBBACATTACGGCGEHTTTGATGATTGCTACGSCATGSCACTCT
81 W W P A D W S H Y S G L M I R M A W H S
301 GCGGGTACCTACCGTATTTCCGATGGTCTTGGCGGCGCGGCTACCGCTAACCAACGTTTT
101 A S T Y R I A D G R G G S G T G N Q R F
361 GCCCCACTGAACTCATGECCTGATAAAGCCCAACTTACATAAAGCAGCCCTTTTGGCTGG
121 A P D K S W P D N A N I D K A R R L D W
421 CCTATCAAAACAAAAGTACGGCTACAAAATCAGCTGGCGGATTTGATGATCCCTGGCGGG
141 P I K Q K Y G N K I S W A D L M T L A G
481 AACATGGCTTADBAATCGATGGGCCTCAAAAACCTTTGCTTTTGGCTTTGGCGCGGAGAC
161 N M A Y E S M G L K T K G P A F G R E D
541 ATCTGGCAGCCAGAAAAGACATTTACTGGGGTTCTGAAAAGAGTGGCTTGGCAAACTT
181 T W H P E K E I Y W S S E K R W L A K S
601 GCGCGTGAATAATAGCCCTTACTGGCTCAGCGCGATCTGAAAACCCACTTGGCAGCGTC
201 G G N N S R Y S G Q R D L N N P L A T V
661 ATGATCGCGCTTATCTACGTCGAAACCCAGAGGTGTGGATGCAAAACCCAGATCCACTGAAA
221 M M G L I Y V N P R S V E G N P D P I K
721 ACGGCGCAAGACATGCGTGTCACTTTTGGCTGCGCATGGCAATGAATGACGAAGAGACCGTC
241 T A Q D M R V T F A R M A M N D E E T V
781 GCTCTGACGGCTGCGCGCCATACGGTGGGTAAGCGGACGGTAATGGCAAAGCTTCTAAC
261 A L T A G S E T V S K A E S N G K A S N
841 CTTGCGCCTGATCCTGAAGGCGCTGAACTGCACGAAACAAGGCTTAGGTGGBAACAAACCAC
281 D G P D P E G A N D H R Q G D G W N N H
901 ACCAGCGCTGGGATTCGTCGAAAACCGCTCACCAGCGTATTGAAAGCTGCATGACACCAC
301 V S R G I G R N T V T S G I E G A W T T
361 CACCCAACCCGTTGGGATAACGATATTTCTACTTACTGCTCAGCTATGAATGGCAGCTC
421 H P T R W D N E Y F Y L D L S Y E W O L
1021 ACTAAGAGCCCTGCGAGGTGCGTGGCAATCGGAACCTGTCAACATCAAAAGAGGAAGATAAA
341 T K S P A G A W Q W E P V N I K E E D K
1081 CCGGTGAGCTGGAAGACCCAAAGTATTCGCTACAACCCCAATGACGACCGGATGCGGATATG
361 P V D V E D P S I R Y N P M M T D A D M
1141 GCTCTGAAAATGATCCGGAACCTCGCAACATTTTCAGAGCGCTTCTACAAAAGATCCCGCT
381 A L K I D P E Y R K I S E R F Y K D P A

1201 TACTTCTCAGAAAGCGTTTGGCGGACCTTGGTTTAAGCTCACTCACCGGATATGGGACCA
 401 Y F S E V F A R A W F K L T H R D M G P

 1261 AAAGCGCGCTACCTTGCOCOCAGATGTTCCGGCAGAAGACTTGATCTGGCAAGACCCCTGT
 421 K A R Y F G P L V F A E E L I W Q D P V

 1321 CCAGCAGGTGGTAAAGATTATGATGTC AATGCGGTCAAAGCAAAAATCGCGGCAAGCGGT
 441 F A G R K D Y E V N A V K A K I A A S G

 1381 STAAGCATCAGTCAAATGGTCAGCACCGCTTGGGATAGCGCTCGTACTTTCGGTGGTTCCT
 461 L S I S E M V S T A W D S A R T F R G S

 1441 GATAAACGTGGGGGAGCCAAACGGGGCGGTATTGCGCTCGCTCCACAAAAAGATTGGCGAA
 481 D K R G C A N G A R I R L A P Q K D W E

 1501 GGCAACGAACCTGCGCGTCTTGGTAAAGTTCGGCGGTACTAGAGAAAAATGCGCGCTCAA
 501 G N E P A R L G K V L A V L E K I A A E

 1561 TCTGGCATCAGTATCGCCGATACGATTGTTCTGGCTGGTAAAGTAGGTATCGAACAAAGCG
 521 S G I S I A D T I V L A G N V G I E Q A

 1621 GCTAAAGCCGCGGGCGTGAATGTGACCGTACCGTTGCGACCCGGGACGCGCGGATGGGACA
 541 A K A A G V N V T V P F A D G R G D A T

 1681 ATCGAACAAACCGATGTTGAGTCCTTTGAAGTGCTTGAGCCCTCTCGCGGATGGCTTCGGT
 561 I E Q T D V E S F E V L E P L A D G F R

 1741 AACTGGCAGAAAAAGCAATTATGTGGTCACACCGGAAGAAATGTTGCTCGACAAAAGCGCAA
 581 N W Q K K H Y V V T F E E M L L D K A Q

 1801 CTGCTGCGCCTCACCGCTCCAGAAATGACGGTGTAAATTGGTGGCATGCGTGTGCTTGGC
 601 L I R L T A F E M T V L I G G M R V L G

 1861 ACTAACTACGGTGGCAGCCAAACACGGCGTGTTTACCGACCGCGTGGGTGCGTGTGACCAAC
 621 T N Y G G S Q H G V F T D R V G A L T N

 1921 GATTTCCTCGTAAACCTCACCGACATGAGTTACACCTGGAAACCAACTGGTCCCAACTCT
 641 D F F V N L T D M S Y T W K P T G R N S

 1981 TACGAGATTGTTGAACTAAGAGCGGCCAAAGTGAAGTGGACAGCCACTCGTGTTCGATCTG
 661 Y E I V E R K S G K V K W T A T R V D L

 2041 CTGTTTGGTTCCTAACTCCATCCTACGTGCTTATGCGGAAGTGTACGCCACAGGACGACAAC
 681 V F G S N S I L R A Y A E V Y A Q D D K

 2101 AAAGAAAAATTCCTCAAAGACTTTGTTGCCGCATGGACCAAAAGTAAATGAATGCGGATCGA
 701 K E K F V K D F V A A W T K V M N A D R

 2161 TTTGATCTGGTG
 721 F D L V

Fig 3.8. Sequence of the *perA* gene and the protein sequence of the catalase-peroxidase encoded by *perA* from *V. cholerae* 569B. The top strand represents the genic code, whereas the bottom strand represents the deduced amino acid sequence of the catalase-peroxidase encoded by *perA*.

569B	MEHNKAGSSGQCQVMEGGDITSAASYNMDWWPKALNIDT.EQHDSKTNP.LADPNYREELK	50
VC16961	MEHNKAGRSQCQVMEGGDITSAASYNMDWWPKALNIDTILHQHDSKTNP.LADPNYREELK	50
VV12755	MEHNKPTETSQCQVMEGGDITSAASYNMDWWPKALNIDT.EQHDSKSNPMDGADPSPREELK	50
VPA0453	MIGQNAHRSRQKQVMEGGDITNNRIEKNWVPEKLNLDLILHQDAKTNPMPSPDFVYEEVVK	50
VPA0768	MSITNGGVSQKQVMEGGDITSDKSVMDWVWVWALNLDLILHQHDSKTNP.LADPNYREELK	50
Consensus	r g qvvdg d wwp laldilqhg k r p d f y ee k	
569B	KIDVEAIKRDIKALMTNSQDWVADWGHYGGLMIRMAWHSAGTYRIADGRGGGGTGNQRF	120
VC16961	KIDVEALKRDLKALMTNSQDWVADWGHYGGLMIRMAWHSAGTYRIADGRGGGGTGNQRF	120
VV12755	KIDVEAIKRDILKALMTNSQDWVADWGHYGGLMIRMAWHSAGTYRIADGRGGGGTGNQRF	120
VPA0453	KIDVSAALKQDITALMTNSQDWVADWGHYGGLMIRMSWHAAGTYRIADGRGGGGTGNLRF	120
VPA0768	KIDVDALKQDLKALMTNSQVWVWVADWGHYGGLMIRMAWHSAGTYRIADGRGGGGTGNQRF	120
Consensus	kld alk dl r g wwpdwhhygglmirm wh ag yr dgrg tgn r f	
569B	APLNSWPDNANLDKARRILWPIKQKYGKKSIAWADLMIAGNMAYBSMG.KTFGPAFGRED	150
VC16961	APLNSWPDNANLDKARRILWPIKQKYGKKSIAWADLMIAGNMAYBSMG.KTFGPAFGRED	150
VV12755	APLNSWPDNANLDKARRILWPIKQKYGKKSIAWADLMIAGNMAYBSMG.KTFGPAFGRED	150
VPA0453	APLNSWPDNANLDKARRILWPIKQKYGKKSIAWADLMIAGNMAYBSMG.KTFGPAFGRED	150
VPA0768	APLNSWPDNANLDKARRILWPIKQKYGKKSIAWADLMIAGNMAYBSMG.KTFGPAFGRED	150
Consensus	apl nswpdnanldkarrilwpi k q k yg k k s i w a d l m i a g n m a y b s m g . k t f g p a f g r e d	
569B	IWHPKDIYWGSEKEW.AKSGGNSRYSGQRDLKPLA.VMMGLIYVNPBGVGGCPDLK	240
VC16961	IWHPKDIYWGSEKEW.AKSGGNSRYSGQRDLKPLA.VMMGLIYVNPBGVGGCPDLK	240
VV12755	IWHPEKLIYWGAELEWDAFSGAENSRYSGERDLKPLA.VMMGLIYVNPBGVGGCPDLK	240
VPA0453	IWHPKDIYWGSEKEW.APTNPNPSRYSGERTLNP.AAVMMGLIYVNPBGVGGCPDLK	240
VPA0768	IWHPKDIYWGSEKEW.AKSGGNSRYSGERDLKPLA.VMMGLIYVNPBGVGGCPDLK	240
Consensus	iwhpkd yw e kw l a k s g g n s r y s g q r d l k p l a . v m m g l i y v n p b g v g g c p d l k	
569B	TAQDMAVTFARMAMNDEETVALTAGGHTVGAHQNGAANI.GPDPGADVHROG.GWMMH	300
VC16961	TAQDMRVTFARMAMNDEETVALTAGGHTVGAHQNGAANI.GPDPGADVHROG.GWMMH	300
VV12755	TAQDMAVTFARMAMNDEETVALTAGGHTVGAHQNGAANI.GADPESADLHROGLWNH	300
VPA0453	TAQDMRVTFARMAMNDEETVALTAGGHTVGAHQNGAANI.GPEPEGADHROGLWLNK	300
VPA0768	TAQDMRVTFARMAMNDEETVALTAGGHTVGAHQNGAANI.GPDPGADVHROG.GWMMH	300
Consensus	t a q d m a v t f a r m a m n d e e t v a l t a g g h t v g a h q n g a a n i . g p d p g a d v h r o g . g w m m h	
569B	TSRGIQRTIVTSGLEGAWTIHPIDNDNEYFYLLESYEWOLTXSPAGAWQWEPVNIKEEDK	360
VC16961	TSRGIQRTIVTSGLEGAWTIHPIDNDNEYFYLLESYEWOLTXSPAGAWQWEPVNIKEEDK	360
VV12755	TSRGIQRDIIVTSGLEGAWTIHPIDNDNEYFYLLESYEWOLTXSPAGAWQWEPVNIKEEDK	360
VPA0453	ITRQVGNNAVTSGLGGAWTSQPTQWNGYFHLIDYDWELKXSPAGAWQWEPDIKAFEDK	360
VPA0768	KTRGIQRDIIVTSGLEGAWTIHPIDNDNGYFYLLESYDWELKXSPAGAWQWEPDIKEEDK	360
Consensus	t s r g i q r t i v t s g l e g a w t i h p i d n d n e y f y l l e s y e w o l t x s p a g a w q w e p v n i k e e d k	
569B	FVDVDETSIRYKPMIADAMALKIDPEYRKISERFYKDPAYFSEVFARAWFKLTHRDMGP	420
VC16961	FVDVDETSIRYKPMIADAMALKIDPEYRKISERFYKDPAYFSEVFARAWFKLTHRDMGP	420
VV12755	FVDVDEPTIRNFIMTDADMALKIDPEYRKISERFYKDPAYFSEVFARAWFKLTHRDMGP	420
VPA0453	FVDVDETSVRRNPIIMTDADMAMKIDPEYRKISERFHSOPAYFADTFARAWFKLTHRDMGP	420
VPA0768	FVDVDETSKRYNPIIMTDADMAMKIDPEYRKISERFQDPAYFSEVFARAWFKLTHRDMGP	420
Consensus	f v d v d e t s i r y k p m i a d a m a l k i d p e y r k i s e r f y k d p a y f s e v f a r a w f k l t h r d m g p	
569B	KARYEGPDVPAEDLIWQDPVFAGRKDYDNAVKAKTAAAGSISEMVSSTAWDSARTFRGS	480
VC16961	KARYEGPDVPAEDLIWQDPVFAGRKDYDNAVKAKTAAAGSISEMVSSTAWDSARTFRGS	480
VV12755	KARYEGPDVPAEDLIWQDPVFAGRKDYDNAVKAKTAAAGSISEMVSSTAWDSARTFRGS	480
VPA0453	KARYEGPDVPELELIWQDPVFNAGANYDIDAVKAKTAAAGSISVDMVSTAWDSARTFRGS	480
VPA0768	KSCDLPQVPSRDLWQDPTFAGK.DYNVDIYKGLASGLIADI.VATAWDSARTFRGS	480
Consensus	k a r y e g p d v p a e d l i w q d p v f a g r k d y d n a v k a k t a a a g s i s e m v s s t a w d s a r t f r g s	
569B	DKRGGANGARIRLAPQKDWLGNPEFKLAKVAVLERTAAESGISIACTIVLAGNVGIEQA	540
VC16961	DKRGGANGARIRLAPQKDWLGNPEFKLAKVAVLERTAAESGISIACTIVLAGNVGIEQA	540
VV12755	DKRGGANGARIRLAPQKDWLGNPEFKLAKVAVLERTAAESGISIACTIVLAGNVGIEQA	540
VPA0453	DKRGGANGARVRLADQKDWQGNFPERLARVLEVLSSIAKDTGASVAVVVLACNVGIEQA	540
VPA0768	DKRGGANGARIRLAPQKDWQKPEPERIRVLAVERLSSIAAREGCSVADATVLAGNVGIEQA	540
Consensus	d k r g g a n g a r i r l a p q k d w l g n p e f k l a k v a v l e r t a a e s g i s i a c t i v l a g n v g i e q a	
569B	AKAAGVNVTVVFPAGRGDATTIEQIDVESFVLEPLADGFRNWQKHHVVTPEENLLOKAO	600
VC16961	AKAAGVNVTVVFPAGRGDATTIEQIDVESFVLEPLADGFRNWQKHHVVTPEENLLOKAO	600
VV12755	AKAAGVNVTVVFPAGRGDATTIEQIDVESFVLEPLADGFRNWQKHHVVTPEENLLOKAO	600
VPA0453	AKAAGVNVTVVFPAGRGDATTIEQIDVESFVLEPLADGFRNWQKHHVVTPEENLLOKAO	600
VPA0768	AKAAGVNVTVVFPAGRGDATTIEQIDVESFVLEPLADGFRNWQKHHVVTPEENLLOKAO	600
Consensus	a k a a g v n v t v v f p a g r g d a t t i e q i d v e s f v l e p l a d g f r n w q k h h v v t p e e n l l o k a o	
569B	ILGLTAFEMTVLIGCMRVLCINYGGS.LHCVPTDRVQALTNDFVNLDMYETWXPTRGNS	660
VC16961	ILGLTAFEMTVLIGCMRVLCINYGGS.LHCVPTDRVQALTNDFVNLDMYETWXPTRGNS	660
VV12755	ILGLTAFEMTVLIGGLVAVLEINEGYKHGVFTDNVQALNDFVNLDMYETWXPTRGNS	660
VPA0453	LMGLTAFEMTVVQCMRVLCINLGSRECVPTDRVQALTNDFVNLDMYETWXPTRGNS	660
VPA0768	LMGLTAFEMTVLIGSLRVVLSNYGGS.KGVFTDRVGTLENDFFVNLDMYETWXPTRGNS	660
Consensus	l i g l t a f e m t v l i g c m r v l c i n y g g s . l h c v p t d r v q a l t n d f v n l d m y e t w x p t r g n s	
569B	YEIVREKSKVKKWATRVVIVFGNSILRAYAVVAQDNRKXVVFVAANTKVMNADR	720
VC16961	YEIVREKSKVKKWATRVVIVFGNSILRAYAVVAQDNRKXVVFVAANTKVMNADR	720
VV12755	YEIVREKSKVKKWATRVVIVFGNSILRAYAVVAQDNRKXVVFVAANTKVMNADR	720
VPA0453	YEIVREKSKVKKWATRVVIVFGNSILRAYAVVAQDNRKXVVFVAANTKVMNADR	720
VPA0768	YEIVREKSDVKKWATRVVIVFGNSILRAYAVVAQDNRKXVVFVAANTKVMNADR	720
Consensus	y e i v r e k s k v k k w a t r v v i v f g n s i l r a y a v v a q d n r k x v v f v a a n t k v m n a d r	
569B	FDL	723
VC16961	FDL	724
VV12755	FDL	723
VPA0453	F	721
VPA0768	FDL	724
Consensus	f	

Fig 3.9. Amino acid sequence alignment of PerA from *V. cholerae* 569B and sequenced catalase-peroxidases from other vibrios. 569B, *V. cholerae* 569B; VC1569, *V. cholerae* N16961; VV12755, *V. vulnificus*; VPA0453 and VP0768, *V. parahaemolyticus*. Sequences were obtained from the sequenced genomes, available at www.ncbi.nih.gov. Light blue shading depicts 100% homology; orange shading, 80% homology, light purple, less than 75% homology.

3.7 Discussion

The nucleotide sequence of the *V. cholerae* N16961 genome revealed the presence of two catalases, namely, a monofunctional catalase and a bifunctional catalase-peroxidase (www.tigr.org). In this study, we isolated a *V. cholerae* 569B gene encoding for a bifunctional catalase-peroxidase identical to PerA from *V. cholerae* N16961. Complete sequencing of the gene revealed a single base-pair difference to *perA* from the El Tor strain. This base change resulted in the replacement of an alanine residue in PerA from the El Tor strain with a threonine residue in the classical strain.

PerA is a typical catalase-peroxidase; it consists of 724 amino acids, with a monomeric molecular weight of 80.59kDa. The size of 724 amino acids is characteristic of bacterial catalase-peroxidases (Table 3.2). Similar to other catalase-peroxidases (e.g. the HPI catalase-peroxidase from *E. coli*) PerA was found to be heat-labile (Visick and Clarke, 1997; Klotz et al., 1997).

Like other bacterial catalase-peroxidases, PerA contains certain conserved histidine residues typically found in peroxidases belonging to the Class I plant peroxidases. Bacterial catalase-peroxidases are reportedly more closely related to these plant peroxidases than they are to bacterial monofunctional catalases (Welinder, 1991; Zamocky et al., 2000; Zamocky et al., 2001). A duplicated peroxidase domain, typical in all catalase-peroxidases, was present in PerA, with the peroxidase moiety on the C-terminal portion of the protein shorter than the peroxidase moiety on the N-terminal end. This duplication of the peroxidase moiety is known to be centered around highly conserved histidine residues (Welinder, 1991; Zamocky et al., 2000; Zamocky, 2001), and in the *V. cholerae* PerA sequence these conserved residues are represented by the distal His 99 and the proximal His 267 (www.ncbi.nlm.nih.gov). In addition, the amino acids adjacent to these conserved His residues were identical and found in the same arrangement, namely RMAWH (for His 99) and GGHT (for His 267), as in other catalase-peroxidases. These histidine residues are important haem ligands in the enzyme (Zamocky et al., 1999).

An essential tryptophan residue, found at position 318 of the aa sequence, is necessary for catalytic activity and forms a part of a catalytic triad of amino acids, namely His-Asp-Trp. These 3 amino acids are a part of a region in plant peroxidases that forms the frontal edge of the catalytic haem centre and thus control access to the catalytic centre (Zamocky et al., 2001). In PerA this conserved region starts at Gln293 and comprises QGLGWNNHTSRGIGRNTVTSGIEGAW. The last residue in this sub-sequence is the highly conserved Trp318.

PerA has the typical helix-turn-helix (HTH) structure found in KatGs (Zamocky et al., 2001). It was reported recently that a short sequence (35aa) of amino acids between helices F and G is

important for the interaction of the enzyme with its substrate, H₂O₂ (Li and Goodwin, 2004). It seems also that this region lends structural support to the enzyme. A mutant catalase-peroxidase (in which these 35 amino acids were deleted) exhibited severely diminished (only 0.2% remaining) catalase activity, although access of other electron donors to the active site of the enzyme were not as restricted compared to the native protein (Li and Goodwin, 2004).

Although the genome of *V. cholerae* encodes for two putative catalases, zymogram analysis of cell free extracts from the bacterium revealed that only one catalase is expressed in log phase and stationary phase cells of the bacterium. This band of catalase migrated the same distance than to the *V. cholerae* catalase expressed in *E. coli* transformed with a recombinant plasmid carrying *perA*. A second band of catalase activity appeared on zymograms only when *V. cholerae* cells were pre-induced by a sub-lethal dosage of H₂O₂.

The pattern of catalase production in *V. cholerae* is similar to that described in *V. vulnificus* ATCC293077 (Park et al., 2004). Firstly, although the genomes of both bacteria contain genes for a monofunctional and a bifunctional catalase, zymogram analysis revealed the presence of only the bi-functional PerA of *V. cholerae* (this study) and HPI (the catalase-peroxidase) of *V. vulnificus*. This was, in both cases, true for both the exponential and stationary phases of growth. Exposure of exponential phase cells of *V. vulnificus* to sub-lethal doses of H₂O₂ caused induction of the bi-functional catalase. This induction of the *V. vulnificus* catalase-peroxidase is contrary to what was observed in this study, since we observed induction of a band of catalase other than PerA. Induction of HPI by H₂O₂ suggests that a redox-type regulator, such as OxyR, might be involved in the transcriptional regulation of *V. vulnificus* HPI. Since the *V. cholerae perA* gene product was not induced upon exposure to H₂O₂, but instead increased as cells entered stationary phase of growth, it suggests regulation of *perA* by a protein other than the redox-sensitive OxyR regulator. It is possible that PerA might be regulated by RpoS, the regulator of genes essential for bacterial survival in stationary phase (O'Neal et al., 1994). An earlier *V. cholerae* study showed that inactivation of *rpoS* sensitized *V. cholerae* cells to H₂O₂ (Yildiz and Schoolnik, 1998). Although Yildiz and co-workers did not quantitate or visualize (on zymograms) the actual catalase activity, one can assume that one of the genes to be down-regulated in the *rpoS* mutant is a catalase gene, since catalase is one of the main bacterial defences against H₂O₂. In the study with *V. vulnificus*, a *rpoS* mutation caused decreased HPI activity in the bacterium, indicating that this regulator is important for the expression of the catalase-peroxidase during the stationary phase of growth (Park et al., 2004). It therefore seems that both OxyR and RpoS is involved in the regulation of HPI of *V. vulnificus*.

As mentioned above, we found that the catalase activity of log phase cells of *V. cholerae* 569B was found to be induced six-fold by sub-lethal doses of H₂O₂. This increased activity led to protection of cells against subsequent lethal doses of the oxidant. Similar up-regulation in response to a sub-lethal threat of an oxidant has been described for monofunctional catalases from *V. harveyi* (Vatanaviboon et al., 2001) and *V. fischeri* (Visick and Ruby, 1998). Zymogram analysis of *V. cholerae* 569B extracts confirmed that the increased catalase activity of *V. cholerae* was due to induced expression of a catalase other than PerA. This other catalase is most probably KatB, the other catalase identified in the genome of *V. cholerae* N16961.

PerA from *V. cholerae* was found to be heat-labile, being inactivated by exposure to 55°C. Monofunctional catalases, particularly the larger ones, are more resistant to heat denaturation, than bi-functional catalase-peroxidases. Comparisons between HPI (catalase-peroxidase) and HPII (catalase) from *E. coli* showed that HPI is denatured, like PerA, at 55°C, whereas HPII can withstand temperatures as high as 70°C (Loewen and Switala, 1986, Visick and Clarke, 1997, Switala and Loewen 2000). Monofunctional catalases containing large sub-units (such as *E. coli* HPII) are generally more heat-stable than small sub-unit monofunctional catalases (like bovine liver catalase) and bi-functional catalase-peroxidases. This is attributed to stronger association of subunits and greater stability in folding and structure, apparently being conferred by the extra amino acids present in large sub-unit catalases (Switala et al., 1999).

In conclusion, we isolated a gene encoding for a heat-labile catalase-peroxidase from *V. cholerae* 569B. This gene differs by only one base from the sequence of the El Tor strain N16961. We showed that the gene product is not induced when the organism is exposed to H₂O₂, but is expressed in response to an increase in the age of the bacterium. The protein showed the typical characteristics exhibited by bacterial catalase-peroxidases and Class I plant peroxidases.

CHAPTER 4

THE ROLE OF *V. CHOLERAE* 569B PerA AS DEFENCE AGAINST OXIDATIVE STRESS.

University of Cape Town

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Summary

Catalases are antioxidant enzymes that detoxify H_2O_2 . This chapter describes the creation and characterisation of a *perA* catalase mutant derived from *V. cholerae* 569B. Insertional inactivation of the *perA* gene was achieved with the aid of a suicide plasmid pGP1.5. This plasmid contained an internal 1.5 kb fragment derived from *perA*, resulting in recombination between the fragment on the plasmid and its homologous equivalent on the *V. cholerae* genome. Zymogram analysis of *V. cholerae* cell extracts revealed that the interruption of the *perA* gene led to a loss in the production of active PerA protein. The *perA* mutation did not affect the growth of the mutant in tryptone broth, although growth under osmotic and temperature stress was slightly retarded. The mutant was more sensitive to H_2O_2 than wild type *V. cholerae* 569B. The catalase-peroxidase from *V. cholerae* 569B therefore seems to be an important mechanism of protection of *V. cholerae* against externally added H_2O_2 . In addition to the loss of protection against H_2O_2 , the absence of the PerA protein also led to a severe decline in cell numbers when bacterial cells were cultured under melanin-producing conditions. This suggests that H_2O_2 forms during the autopolymerization of HGA and that PerA is essential for protecting the bacterium against the oxidant. It was concluded that PerA is an important defence for *V. cholerae* against H_2O_2 .

4.1 Introduction

The catalase enzyme is an important bacterial defence against H_2O_2 . Loss of catalase activity affects bacterial growth and survival. Catalase mutants are susceptible to H_2O_2 (Vattanaviboon et al., 2000), which leads to a reduction in their ability to colonise a host (Visick and Ruby, 1998) or to persist in a host after colonization (Harris et al. 2003). It also affects bacterial survival upon the onset of the stationary phase of growth (Shiba et al., 1997). The loss of catalase activity is not always a disadvantage to bacteria. Loss of KatN, the manganese-containing catalase from *Salmonella enterica* serotype *Typhimurium*, had no significant effect on its survival upon exposure to oxidative stress (Robbe-Saule et al., 2001). In cases like these, more than one catalase is usually present in the bacterium. The loss of one of the catalases is compensated for by the other catalases produced by the bacterium. *Salmonella* indeed contains two catalases other than KatN (Robbe-Saule et al., 2001). In *Mycobacterium tuberculosis*, the loss of the catalase-peroxidase (KatG) is advantageous to the bacterium, because it results in isoniazide (INH) resistance. Point mutations in *katG* prevent KatG-mediated oxidative activation of the INH pro-drug, causing resistance to INH (Slayden and Barry, 2000).

4.2. The aim of this chapter

Aerobic respiring bacteria such as *V. cholerae* possess well-defined defence mechanisms, such as the catalases, to deal with H_2O_2 . Two catalase genes were identified in the *V. cholerae* N16961 genome project (www.tigr.org). The *katB* gene putatively encodes for a monofunctional catalase, whilst *perA* encodes for a bi-functional catalase-peroxidase. In this study, a catalase peroxidase gene, identical to *perA* from the El Tor strain, was isolated from the classical strain *V. cholerae* 569B.

In order to establish the importance of melanin as a scavenger of H_2O_2 , the efficiency of melanin as a potential defence mechanism was evaluated in the absence of the PerA catalase. This catalase is mainly produced as the bacterium enters the stationary phase of growth. KatB, the other catalase from *V. cholerae*, was not detected in zymograms of *V. cholerae* cell extracts, unless the bacterial culture was induced by sub-lethal amounts of H_2O_2 (Chapter 3). PerA was also the only catalase observed in cell free extracts obtained from stationary phase *V. cholerae* cells. The *perA* gene was therefore targeted for mutagenesis. We used insertional inactivation to obtain the *perA* mutant. The *perA* mutant, named *V. cholerae* PERM1.5, was characterised phenotypically and genotypically. This was achieved by firstly analyzing the genetic rearrangement of the *perA* region through Southern hybridization and, secondly, by comparing growth patterns of the wild type and the mutant. Finally, the ability of the *perA* mutant to defend itself against H_2O_2 in the presence and absence of melanin was also investigated.

4.3 Materials and methods

4.3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 4.1.

Table 4.1. Bacterial strains and plasmids

Strain or plasmid	Genotype / relevant features	Reference
<i>E. coli</i> SM10λpir	<i>thi thr leu tonA Lac Y supE recA:: RP4-2T::Mu</i>	Miller and Mekalanos (1998)
<i>E. coli</i> SM1015	<i>E. coli</i> SM10λpir, containing suicide plasmid pGP1.5	This study
<i>V. cholerae</i> 569B	Classical biotype, O1 serogroup, Inaba serotype	Mukherjee, (1998)
<i>V. cholerae</i> 569B ^{Rif}	<i>V. cholerae</i> 569B, Rifampicin resistant	Schroeder (1998)
<i>V. cholerae</i> 569BSK	<i>V. cholerae</i> 569B containing pBluescript SK(+)	This study
<i>V. cholerae</i> PERM1.5	<i>V. cholerae</i> 569B <i>perA</i> , <i>amp</i> ^r , <i>Rif</i> ^r	This study
Plasmids		
pBluescript SK(+)	<i>Amp</i> ^r , β - galactosidase	Short et al. (1988)
pSK18	pBluescript SK(+) derivative containing a <i>SalI</i> - <i>SpeI</i> fragment from pKat18	This study
pSKBV	pBluescript SK(+) derivative containing a <i>BamHI</i> - <i>EcoRV</i> fragment derived from pSK18	This study, Chapter 3
pGP704	<i>ori</i> R6K, <i>mob</i> Rp4, MCS of M13tg131, <i>Amp</i> ^r	Herrero et al. (1990)
pGP1.5	pPGP704 derivative, containing a 1.5 kb internal fragment from <i>perA</i>	This study

4.3.2 Media and culture conditions

Escherichia coli SM10λpir was cultured in Luria broth (LB) (Appendix A1) at 37°C and maintained on Luria agar (Appendix A1). *E. coli* strains harboring suicide plasmids pGP704 or pGP1.5 were grown in Luria broth containing ampicillin at a concentration of 100 µg/ml. *V. cholerae* 569B^{Rif} was grown in tryptone broth (Appendix A1) containing rifampicin at a concentration of 50 µg/ml. Unless stated otherwise, *V. cholerae* 569B strains were cultured at 30°C. Bacterial strains harbouring plasmids were maintained on solid media containing ampicillin at a concentration of 100 µg/ml. For growth curves, *V. cholerae* strains 569BSK and PERM1.5 were cultured in broth containing 1% tryptone and either 1% or 4% NaCl.

4.3.3 Construction and identification of a suicide construct, pGP1.5, for mutation of *perA*

Plasmids pSK18 and the suicide vector pGP704 were digested with the restriction enzymes *KpnI* and *SphI* (Fig. 4.1, Appendix B.5). The generated fragments were separated by agarose gel electrophoresis (Appendix B.6), an internal fragment of approximately 1.5 kb was cut out from low-melting point agarose and ligated into pGP704 (previously linearised by digestion with *KpnI* and *SphI*) (Appendix B.11). Plasmid DNA was extracted from transformants (Appendix B.4) and its identity confirmed by restriction enzyme analysis.

4.3.4 Insertional inactivation of *perA*

A single colony of *V. cholerae*^{Rif} was inoculated into 5 ml of tryptone broth and grown overnight at 30 °C in the presence of rifampicin. Suicide construct pGP1.5 was transformed into competent *E. coli* SM10λpir and grown overnight, at 37 °C, in 5 ml LB (amended with ampicillin at 100µg/ml). To achieve mating of *V. cholerae*^{Rif} and *E. coli* SM1015, 100 µl from each overnight culture were mixed and pipetted onto a membrane filter (placed beforehand on TB agar containing no antibiotic). The plate was incubated overnight at 30°C in an upright position. The next day, growth on the filter was washed off with 5 ml sterile TB. An aliquot of 100 µl was used to make dilutions in tryptone broth. Aliquots of 100µl from these dilutions were plated on tryptone agar containing ampicillin (100 µg/ml) and rifampicin (50µg/ml). Plates were incubated at 30°C. Plating on media containing rifampicin and ampicillin enabled selection against *E. coli* SM1015 (sensitive to rifampicin) and wild type *V. cholerae* 569B^{Rif} (sensitive to ampicillin). A visual inspection for growth was performed after 1, 2 and 3 days. Colonies that grew were streaked onto TCBS agar to confirm that they were *V. cholerae* and not *E. coli*.

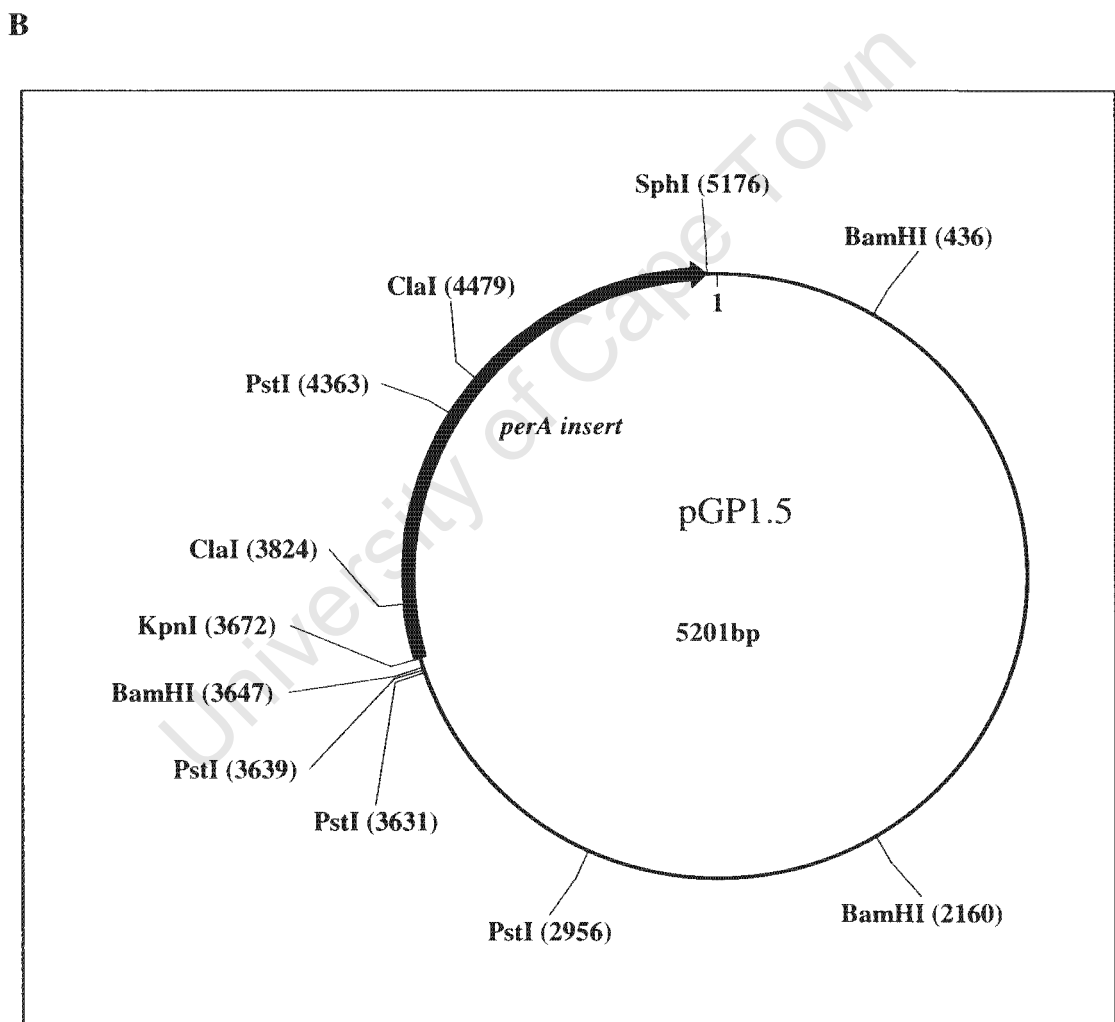
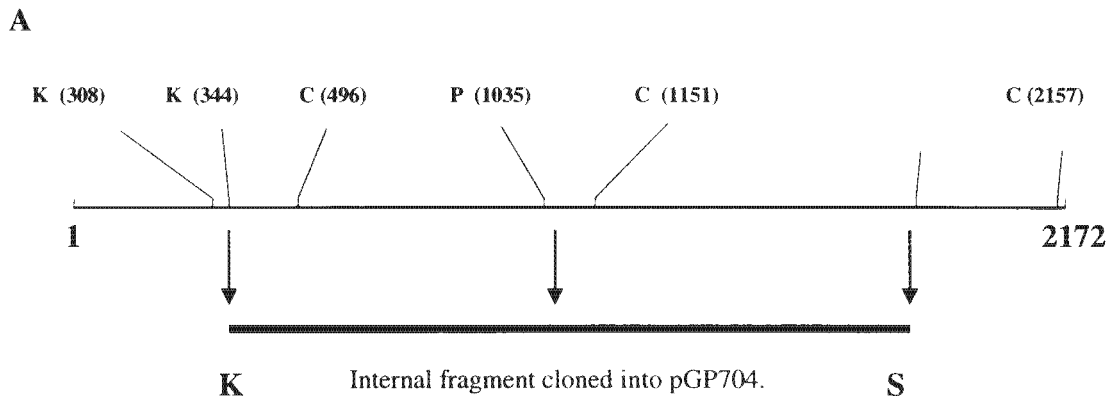


Fig. 4.1. Suicide construct pGP1.5. An internal *Kpn* I – *Sph* I fragment (A) from *perA* was cloned into pGP704 to create pGP1.5 (B). B: Restriction map of suicide construct pGP1.5. Abbreviations: K, *Kpn*I; C, *Cla*I, P, *Pst*I; S, *Sph*I.

4.4. Characterisation of *V. cholerae* PERM1.5

Prospective mutants were selected based on their ability to grow on TB agar containing rifampicin and ampicillin, as well as on TCBS agar. One of these mutants, named PERM1.5, was selected for further analysis.

4.4.1 Confirmation of the interruption of *perA* by pGP1.5

Genomic DNA was extracted (Appendix B.3.2) from *V. cholerae* 569B and PERM1.5. The genomic DNA from *V. cholerae* 569B and *V. cholerae* PERM1.5 was digested with *Cla*I and *Pst*I before the restriction fragments were separated by agarose gel electrophoresis. DNA fragments were transferred to a nylon membrane through capillary transfer (Appendix B). An internal *Cla*I-*Sph*I fragment from *perA* was radiolabelled by random priming (Appendix B) with [α - 32 P]dCTP and used to probe the genomic DNA extracted from wild type and putative *perA* mutant cells.

4.4.2 Zymogram analysis of *V. cholerae* catalase-peroxidase mutant, PERM1.5

V. cholerae strains 569BSK and PERM1.5 were inoculated into 50 ml of tryptone broth (amended with ampicillin, 100 μ g/ml) and incubated at 30 °C. *E. coli* UM2 harboring pSKBV (contains a copy of *perA* on the plasmid) was grown at 37°C in 5 ml of LB containing ampicillin at 100 μ g/ml. After 24 hours of growth, 20 ml of the cells were pelleted by centrifugation at 10 000 rpm for 10 minutes. Cells were washed in 10 ml of phosphate buffer before being centrifuged again. Finally, the pellets were resuspended in 2 ml of phosphate buffer and the cells lysed by sonication (4 rounds of 30 second bursts at 30% output using a Virsonic sonicator). Cell debris was removed by centrifugation at 15 000 rpm for 10 minutes at 4°C. The supernatant fraction was stored frozen until assayed.

Aliquots of the extracts, each containing 100 μ g of crude protein, were separated through native PAGE (Appendix B13). Electrophoresis was performed at 140V for 3 hours in the Mighty Small II SE250 (Hoefer Scientific Instruments). The gel was stained with ferricyanide as described by Wayne and Diaz (1986) (Appendix B13).

4.4.3 The effect of the *perA* mutation on growth

V. cholerae 569BSK and the *perA* mutant, PERM1.5, were inoculated from overnight cultures into 50 ml of tryptone broth containing 1% tryptone, amended with either 1% or 4% NaCl. The broths were inoculated to the same starting optical density (OD) at 600 nm, namely 0.05. Broths were incubated at either 30 °C or 37 °C, whilst continuously shaking. The optical densities at 600 nm for each culture were determined at hourly intervals. Experiments were repeated twice for each strain.

4.4.4 The effect of the *perA* mutation on the sensitivity of *V. cholerae* to H₂O₂

4.4.4.1 The sensitivity of *V. cholerae* to H₂O₂ in the presence and absence of *perA*.

The effect of the *perA* mutation on *V. cholerae* 569B was tested by exposing the bacterium to H₂O₂. Firstly, a semi-quantitative disk-diffusion method was used to determine the sensitivity of the wild type and the mutant against H₂O₂. *V. cholerae* 569B and PERM1.5 were grown overnight in 5 ml of tryptone broth. The next day, fresh broths were inoculated from the overnight cultures and grown until the optical density at 600nm reached approximately 0.4. One hundred microliters from each culture were spread-plated, in triplicate, onto tryptone agar plates. Plates were allowed to dry, before a Whatman filter paper disk that contained 5 µl of H₂O₂ (30% stock) was placed in the centre of each plate. Plates were incubated overnight at 30°C. The next day, zones of inhibition of growth (measured in mm) around the disks were measured. This experiment was repeated once.

In the second experiment, the wild type (*V. cholerae* 569BSK) and the catalase mutant were grown at 30°C or 37 °C in 50 ml of tryptone broth containing 1% or 4% NaCl, and amended with ampicillin at a concentration of 100 µg/ml of culture. Cells were grown to either the log phase (when cells reached an optical density of approximately 0.4) or for 24 hours before exposure to H₂O₂. The cells grown for 24 hours were adjusted to an OD of 0.4 (that is, approximately 10⁸ -10⁹ cells). Five millilitres of the log phase and 24 hour cultures were dispensed into sterile universals, before H₂O₂ was added at increasing concentrations (500 µM, 1000 µM, 2000 µM and 4 000 µM final concentration). After 30 minutes in the presence of the oxidant, dilutions of the cultures were made and 100 µl aliquots were plated in triplicate on tryptone agar (containing no antibiotics). *V. cholerae* colonies were counted after overnight growth at 30°C. The experiment was repeated once.

4.4.4.2 The sensitivity of *V. cholerae* PERM1.5 to H₂O₂ in the presence and absence of melanin.

V. cholerae PERM1.5 was grown overnight in 5ml of tryptone broth. The next day, 2 tryptone broths (50 ml each), containing 4% NaCl were inoculated from overnight cultures to a starting optical density of 0.05. One of these broths contained 20 mM L-proline, in order to inhibit melanogenesis. All cultures were incubated at 37°C for two days, prior to exposure to 1 mM H₂O₂. One hundred microliter aliquots from each culture were removed from cultures before and 30 minutes after exposure to the oxidant. Dilutions were made and plated, in triplicate, on tryptone agar. Colonies were counted after overnight growth at 30°C. The experiment was repeated once.

4.5 Results

4.5.1 Isolation of *perA*-deficient mutants

Eight putative mutants grew on TCBS agar, confirming their identity as *V. cholerae*. They also grew on TB agar containing ampicillin and rifampicin. These selected ampicillin-resistant *V. cholerae* colonies therefore contain possible insertions of pGP1.5 in the *perA* gene.

4.5.2 Mapping of the interruption of *perA* by suicide plasmid pGP1.5

One of the putative *perA* mutants was selected for further analysis. In order to confirm the interruption of the *perA* gene, genomic DNA from the wild type and selected mutant, PERM1.5, was digested with *Cla*I, *Pst*I, or both restriction enzymes. Southern hybridization confirmed that integration of the suicide plasmid into the genome interrupted *perA* and introduced extra restriction sites (Fig 4.2 and 4.3). The additional *Cla*I and *Pst*I sites (as depicted in Fig. 4.3B) led to the generation of extra bands in the mutant, as seen in Fig. 4.2 (Lanes 2, 4 and 6).

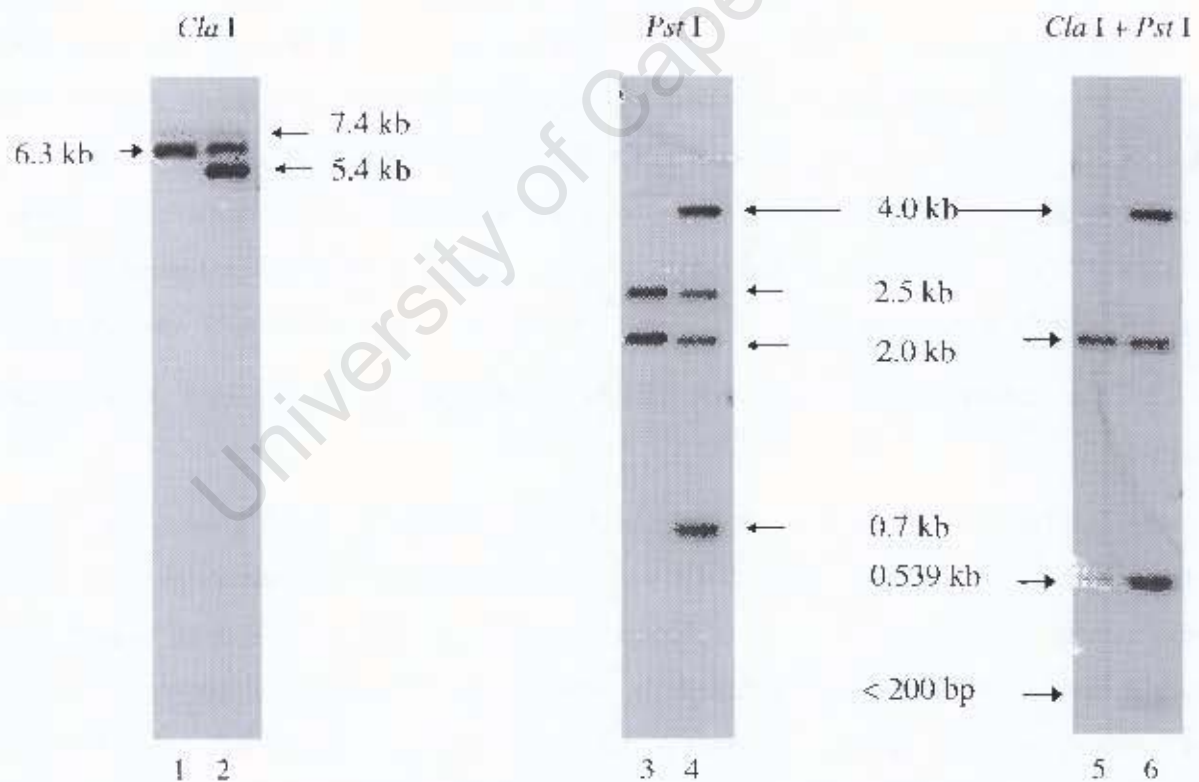


Fig 4.2 Southern blot to confirm the genomic rearrangement of *perA*.

Genomic DNA from Wild type, lanes 1, 3, 5. Genomic DNA from Mutant, lanes 2, 4, 6. Restriction enzyme digests: Lanes 1, 2: *Cla*I; Lanes 3, 4: *Pst*I; Lanes 5, 6: *Pst*I and *Cla*I.

An equal amount of DNA was loaded in each lane. An internal fragment from *perA* was radiolabelled and used as probe.

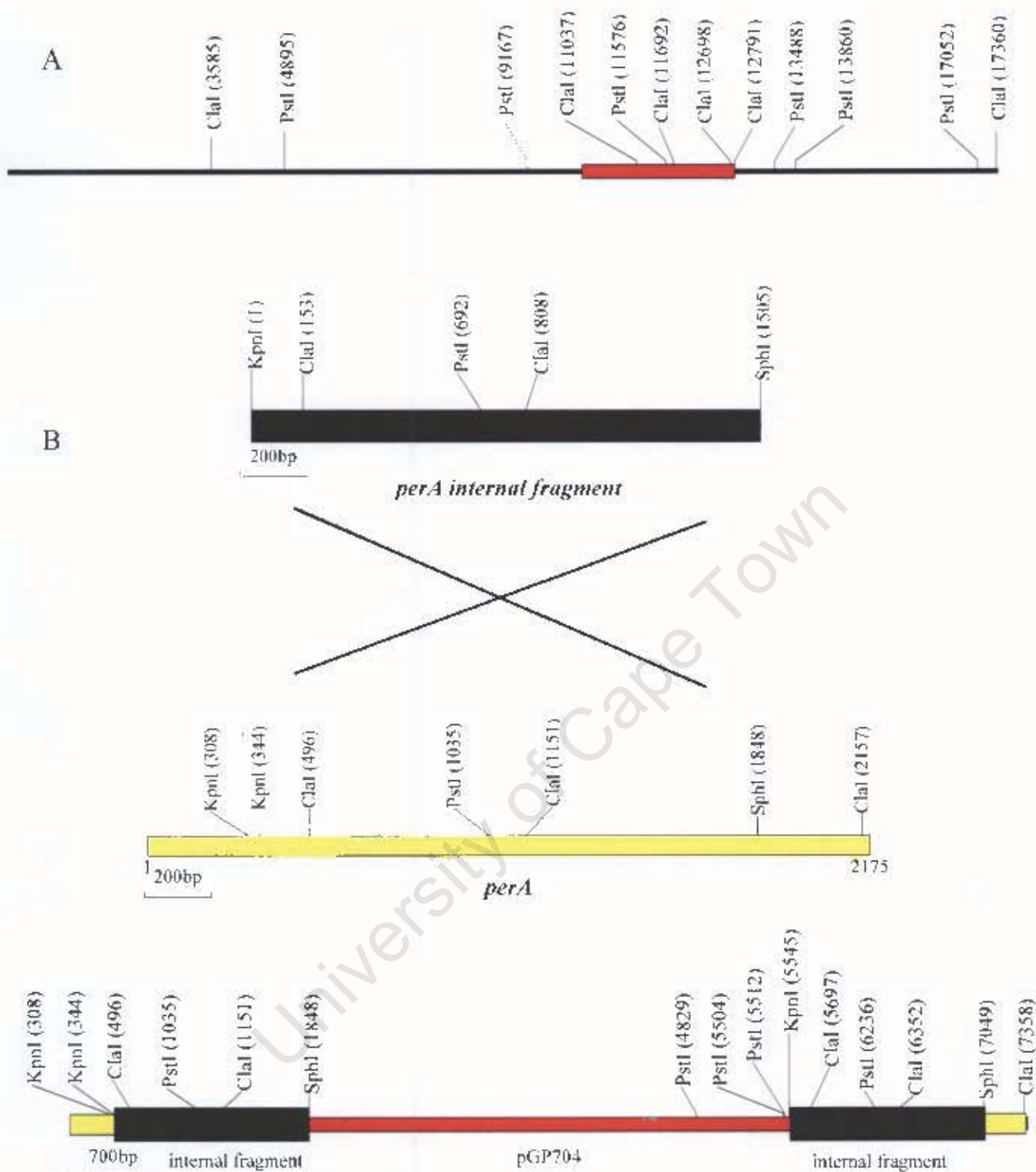


Figure 4.3 Mutagenesis of *V. cholerae* 569B *perA*.

A. Restriction map of the region of *V. cholerae* N16961 genomic DNA, containing the *perA* gene. The gene is represented by the red bar. The *Clal* sites in this region are sensitive to methylation, and sites at positions 11692, 12698 and 12791 do not cut with this enzyme (See Chapter 3). Restriction enzyme digestion of *V. cholerae* 569B genomic DNA from this region with *Clal* therefore yields bands of 6.3 and 7.4 kb respectively (see Figure 4.2). The sequence of *perA* from the wild type bacterium yielded an identical restriction map as strain N16961. The sequence of the latter strain was obtained from www.tigr.org

B. Mutation of *perA* with the aid of a suicide vector pGP1.5. Thick black bar on pGP1.5 – represents an internal *perA* fragment ligated into the suicide vector; Yellow bar – represents the genomic copy of *perA*; Red line – represents pGP704 DNA derived from the suicide plasmid.

When genomic DNA from *V. cholerae* 569B was digested with *Cla*I, the *perA* probe hybridised to two restriction fragments of 7.4 kb and 6.3 kb (Fig.4.2, lane 1). This size is comparable to that calculated for the same region in *V. cholerae* N16961 (Fig. 4.3). According to Fig. 4.3, three restriction fragments should be obtained when *perA* is digested with *Cla*I. Instead, the *perA* probe only hybridised to two bands of 7.4 kb and 6.3 kb, indicating that *Cla*I sites situated in *perA* were not cut with the enzyme. An extra *Cla*I restriction fragment of approximately 5.4 kb, absent in genomic DNA from the wild type, was observed in genomic DNA isolated from the *perA* mutant (Fig 4.2, lane 2). When the genomic DNA from both the wild type and mutant were digested with *Pst*I, the *perA* probe hybridised to restriction fragments of approximately 2 kb and 2.5 kb respectively (Fig. 4.2). The actual calculated band sizes (as determined with the aid of sequence from *V. cholerae* N16961) are 1.912 and 2.409 kb (Fig. 4.3). In lanes containing DNA isolated from the mutant, the probe also hybridised to genomic DNA restriction fragments of approximately 4 kb and 0.7 kb (Fig. 4.2, Lane 4).

When genomic DNA was digested with *Cla*I and *Pst*I, the probe hybridized to 2 restriction fragments from the wild type, compared to 4 bands from the mutant (Lanes 5 and 6). Firstly, DNA from both strains exhibited a restriction fragment of 2 kb when digested with both enzymes, similar to DNA cut with *Pst*I alone. The 2.5 kb restriction fragment in *Pst* I genomic DNA digests, was absent when DNA was subjected to a double digest with *Cla*I and *Pst*I, while a smaller fragment of approximately 539 bp appeared in lanes containing genomic DNA from both strains. This indicates that the original 2.5 kb fragment was split into two restriction fragments (2kb and 0.5 kb). This pattern was confirmed by the *perA* restriction map (Fig 4.3). However, the 539bp signal is stronger in the mutant, indicating the presence of a possible doublet (Lane 6). Lastly, *Cla*I – *Pst*I digestion of mutant DNA also exhibited a restriction fragment of approximately 200 bp that was not present in mutant DNA digested with *Pst*I only.

4.5.3. Confirmation of the absence of PerA

Zymogram analysis of cell-free extracts from *E. coli* UM2 (pSKBV) and *V. cholerae* 569B showed catalase activity, with a band that migrated the same distance through the acrylamide gel (Fig.4.4).



Fig 4.4 Zymogram to test for catalase activity from *V. cholerae* PERM1.5. Each lane contains 100 µg of crude extract from sonicated bacterial cells. Lane 1, *V.cholerae* 569B; lane 3, *E. coli* cell extract containing the *perA* gene expressed from plasmid pSKBV; Lane 5, *V. cholerae* PERM1.5. The larger band in lane 3 is due to the fact that the catalase is expressed from a plasmid in *E. coli* and therefore contains increased amounts of the catalase protein per 100 µg of crude protein. Lanes 2 and 4 were empty.

E. coli UM2 is a catalase-deficient mutant, therefore the catalase activity seen is from the catalase produced from the gene on plasmid pSKBV (Chapter 3, Table 3.1). This band of catalase activity is absent in cell extracts from *V. cholerae* PERM1.5, confirming that the *perA* mutant lacked PerA activity.

4.5.4 The growth of *V. cholerae* in the presence and absence of PerA

In order to determine whether the *perA* mutation affected the growth of *V. cholerae*, the wild type and the *perA* mutant were grown over a period of 7 hours to determine the growth pattern. The *perA* mutation did not alter the growth pattern of *V. cholerae* since the wild type and the mutant grew at a comparable rate. This was true when cells were grown at 30 °C in the presence of 1% NaCl and 1% tryptone (Fig 4.5). However, the mutant was slightly impaired in growth when cells were cultured under pigment-inducing conditions (i.e. cells were cultured at 37°C in the presence of 4% NaCl and 1% tryptone), in comparison to the wild type strain (Fig 4.5).

4.5.5 The effect of the *perA* mutation on the survival of *V. cholerae*

4.5.5.1 Semi-quantitative determination of the sensitivity of *V. cholerae* upon exposure to H₂O₂.

The disk-diffusion method is a quick, albeit semi-quantitative method, to determine sensitivity of an organism to H₂O₂. A filter disk, saturated with 5 µl H₂O₂ (30% v/v) is placed on an agar plate containing an inoculum of a particular bacterium. Zones of growth inhibition surrounding the disk would indicate sensitivity to H₂O₂. When *V. cholerae* cells were plated on tryptone agar containing a paper disk saturated with H₂O₂, the mean zone of inhibition surrounding the saturated disks was 23.00 mm (± 0.45 mm) in the case of *V. cholerae* PERM1.5, in comparison to a 17.50 mm (± 0.50 mm) zone of inhibition for the wild type bacterium.

4.5.5.2 Quantitative determination of the sensitivity of *V. cholerae* to H₂O₂

Although the disk-diffusion method revealed differences in the sensitivity of *V. cholerae* 569B and the *perA* mutant to H₂O₂, this method is not quantitative. A more accurate measure of survival was obtained by exposing liquid cultures of the two strains to H₂O₂, followed by enumeration of the surviving cells by plating aliquots of cultures before and after exposure to H₂O₂. The *perA* mutation rendered *V. cholerae* PERM1.5 more sensitive to H₂O₂ than the wild type (Fig 4.6 and Fig. 4.7) when exposed to increasing amounts of H₂O₂. This was true for both log phase (Fig. 4.6) and the 24 hour old cells (Fig. 4.7).

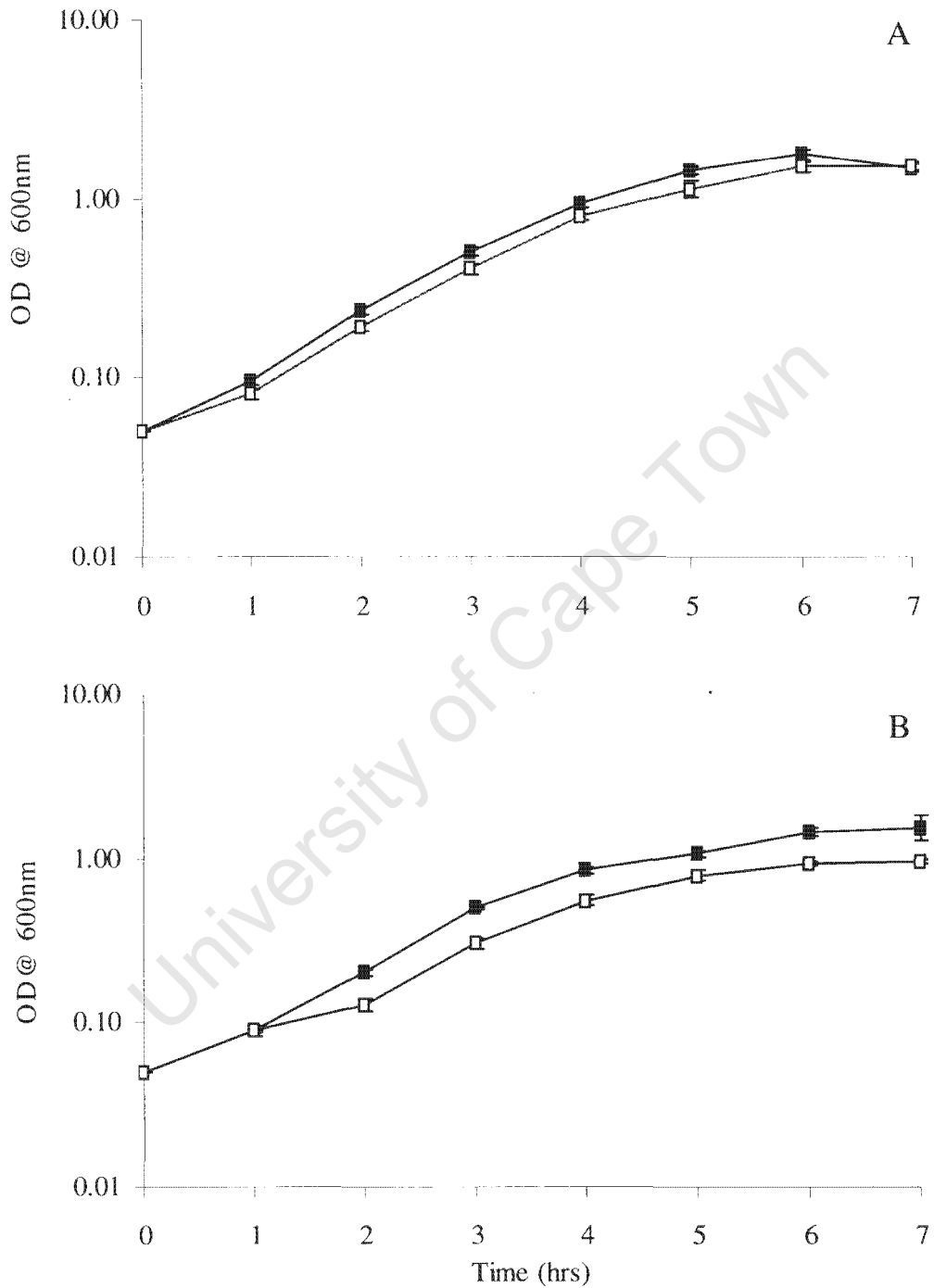


Fig. 4.5. The growth of *V. cholerae* 569B and the catalase mutant PERM1.5.

A, Growth at 30 °C, in tryptone broth amended with 1% NaCl. B, Growth at 37 °C, in tryptone broth amended with 4% NaCl. Mean OD values calculated from 3 independent experiments. Closed squares, wild type; Open squares, mutant.

Wild type cells grown for three hours at 30 °C in TB containing 1% NaCl, were not affected by H₂O₂ when exposed to the lowest dose of H₂O₂ (250 µM), whereas the *perA* mutant declined in cell numbers from 10⁹ to 10⁷ cfu/ml (Fig. 4.6A). When cultures were exposed to higher doses (500 to 1 000 µM) of H₂O₂, the wild type was also affected by H₂O₂, but higher numbers of cells were always recovered from the wild type compared to the mutant. After exposure to 1 000 µM of H₂O₂, less than 10⁴ cfu/ml of the mutant remained, compared to 10⁵ cfu/ml of the wild type.

When *V. cholerae* was grown for three hours at 30 °C in broth containing 4% NaCl, or at 37 °C in TB containing 1% NaCl, the wild type cells were more resistant to H₂O₂ in comparison to cells grown at 30 °C in the absence of osmotic stress (Fig 4.6 A, B and C). When the bacterium was incubated in the presence of elevated salt concentrations before exposure to H₂O₂, wild type cell numbers declined from approximately 10⁹ cfu/ml to 10⁸ cfu/ml, even in the presence of H₂O₂ as high as 1000 µM H₂O₂ (Fig. 4.6B). In contrast, the mutant declined from 10⁹ cfu/ml (0 µM) to 10⁴ cfu/ml (250 and 500 µM H₂O₂). Finally, no cells remained when the mutant was exposed to 1000 µM of H₂O₂ (Fig 4.6B).

When the *perA* mutant strain was exposed to high temperature and osmotic stress prior to H₂O₂ exposure, it was resistant to killing by 500 µM of H₂O₂ (Fig. 4.6 and Fig. 4.7). However, when it was exposed to a higher concentration of the oxidant, 100% killing of the mutant was observed, whereas more than 10⁸ cfu/ml of the wild type cells still remained.

V. cholerae 569B cultures grown for 24 hours at 30 °C in TB (containing 1% tryptone and 1% NaCl) prior to H₂O₂ exposure, exhibited increased resistance to H₂O₂, compared to log phase cultures. Cells survived exposure to H₂O₂ at a concentration of 2 mM (Fig 4.7A). The *perA* mutant cell numbers declined upon exposure to increasing amounts of the oxidant. The number of surviving cells declined from 10⁹ cfu/ml before exposure to 10⁶ cfu/ml upon exposure to 2 mM of H₂O₂. Lastly, 100% killing of the mutant was observed after exposure to 4 mM H₂O₂, whereas 10⁷ cfu/ml of the wild type cells still remained. When cells were grown for 24 hours at 37 °C in TB containing 4% NaCl, prior to H₂O₂ exposure, both the wild type and mutant cells were resistant to 500 µM H₂O₂ (Fig. 4.7B). However, exposure to a concentration of 2 mM H₂O₂ led to a decline in cell numbers from 10⁹ cfu/ml to 10⁷ and 10³ cfu/ml for the wild type and mutant respectively. When the cells were exposed to 4 mM of H₂O₂, none of the mutant cells survived, whereas 10⁴ wild type cells still remained. Lastly, cells grown under osmotic stress and high temperature (Fig 4.7B) were more susceptible to killing by exposure to 2 and 4 mM H₂O₂, than cells grown for 24 hours at 30°C in broth containing 1% NaCl (Fig. 4.7A). This is reflected by the fact that over 10⁷ cfu/ml of the wild type remained after exposure to 4 mM H₂O₂, whereas approximately 10⁴ cfu/ml remained when the wild type was grown at 37 °C in the presence of osmotic stress.

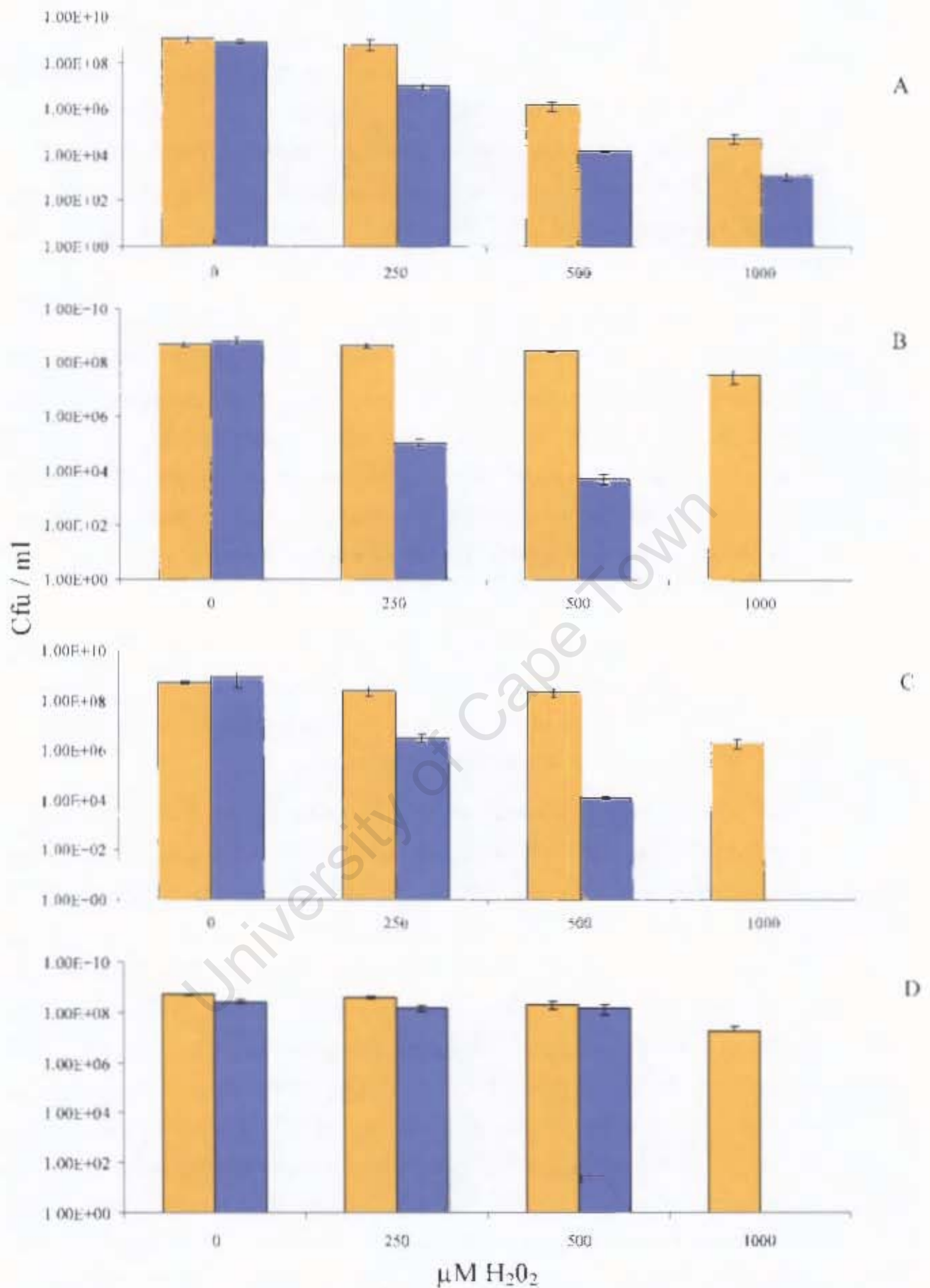


Fig 4.6. The effect of the *perA* mutation on survival of *V. cholerae*. Cells were grown to log phase, in tryptone broth containing 1% tryptone and either 1% or 4% NaCl. Yellow bars, wild type; blue bars, *perA* mutant. Culture conditions: (A) Cells were grown at 30 °C, 1% NaCl (B) Cells were grown at 30 °C, 4% NaCl (C) Cells were grown at 37 °C, 1% NaCl (D) Cells were grown at 37 °C, 4% NaCl. The mean was calculated from two independent experiments, each done in triplicate.

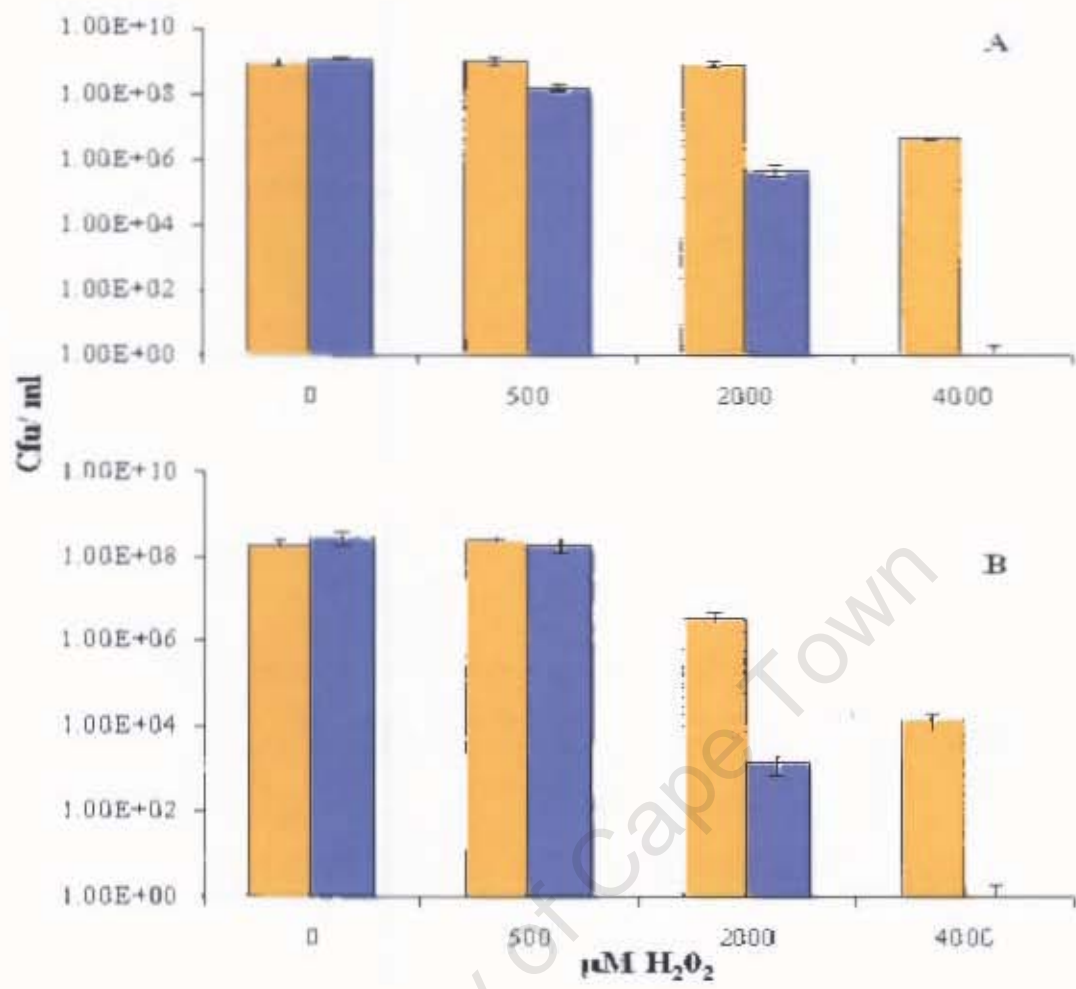


Fig 4.7. The effect of the *perA* mutation on survival of *V. cholerae*. Cells were grown for 24 hours in tryptone broth containing 1% tryptone and either 1% or 4% NaCl. Yellow bars, wild type; blue bars, *perA* mutant. Culture conditions: (A) Cells were grown at 30 °C, 1% NaCl. (B) Cells were grown at 37 °C, 4% NaCl. The mean was calculated from two independent experiments, each done in triplicate.

4.5.6. The effect of melanin on the *V. cholerae perA* mutant.

The PerA mutant was cultured under pigment-inducing conditions (i.e. tryptone broth amended with 4% NaCl, 5 mM L-tyrosine and 5 μ M CuSO₄ (Appendix A1) to assess whether melanin protects *V. cholerae* against H₂O₂ in the absence of the catalase. L-proline was added to one set of cultures in order to inhibit melanogenesis (Coyne and Al-Harhi, 1992). It was observed that, after two days of growth, without exposure to externally added H₂O₂, only 10⁴ cells of the melanized *perA* mutant remained (Table 4.2). In contrast, 10⁹ cfu/ml of the non-melanized *perA* mutant were still culturable after two days of growth. After exposure to 1mM H₂O₂, none of the melanized cells survived, whereas the non-melanized mutant cell numbers (cultures where proline was added) only declined from 1.39x 10⁹ ($\pm 7 \times 10^7$) to 1.48x 10⁸ ($\pm 1.7 \times 10^7$) cfu/ml (that is, 10% of the mutant cells still remained viable).

Table 4.2. The effect of melanin on the survival of *V. cholerae* PERM1.5.

Condition	Mean cfu/ml (SE)	Mean cfu/ml (SE)	Melanin
	Before exposure*	After exposure*	
Culture minus proline	2.83 x 10 ⁴ (1.1 x 10 ⁴)	0	++
Culture plus proline	1.39 x 10 ⁹ (7.09 x 10 ⁷)	1.48x 10 ⁸ (1.7 x 10 ⁷)	-

SE, standard error; Proline was added at a concentration of 20 mM to prevent melanogenesis. A plus sign indicates the presence of melanin, a minus symbol indicates the absence of melanin. The experiment was repeated once. The mean value was calculated from six determinations. *, exposure to 1mM H₂O₂.

The severe decline in the melanized catalase mutant cell numbers, even before exposure to H₂O₂, prompted further experiments to explore the reason for the low number of culturable cells. In order to assess whether the presence of melanin was the cause of the low cell numbers, the PerA mutant was cultured with different degrees of aeration (causing different degrees of pigmentation in *V. cholerae*) in 250 ml flasks containing either 30 ml (broth:total flask volume approximately 1:8) or 60 ml (broth:total flask volume approximately 1:4) broths. L-proline was added to control flasks in order to prevent pigmentation, regardless of the degree of aeration.

When the mutant was grown with higher aeration (30 ml cultures, no added L-proline, therefore melanin produced by bacterium) the cell numbers declined to 10⁴ cells, whereas non-melanized cells (30 ml cultures, added L-proline) yielded 10⁸ cells (Table 4.3). When the cells were grown in

60 ml broths (less aeration, degree of pigmentation less), 10^8 cells were obtained in melanized cultures, compared to 10^9 cells in cultures with no melanin.

Table 4.3. The effect of increased amounts of melanin on cell numbers of *V. cholerae* PERM1.5.

Condition	Broth volume/ 250 ml flask	Mean CFU/ml (se)	Melanin
Culture minus proline	30 ml	3.2×10^4 (4.24×10^3)	++
Culture plus proline	30 ml	6.45×10^8 (2.98×10^7)	-
Culture minus proline	60 ml	6.67×10^8 (5.98×10^7)	+
Culture plus proline	60 ml	1.66×10^9 (1.6×10^8)	-

SE, standard error; proline was added to a final concentration of 20 mM to prevent melanogenesis. A plus sign indicates the presence of melanin, a minus symbol indicates the absence of melanin. The experiment was repeated once. Mean values were calculated from six determinations. Broth volumes in flasks were varied, causing different degrees of aeration, and consequently different degrees of melanization

4.6 Discussion

Mutation of the gene encoding for the *V. cholerae* catalase-peroxidase was achieved by insertional inactivation. Insertion of a suicide plasmid, pGP1.5, into the coding sequence of *perA* led to the absence of an active PerA protein in *V. cholerae* 569B. The absence of a functional protein was confirmed with zymogram analysis.

When grown at 30 °C, in the presence of 1% tryptone and 1% NaCl, the *perA* mutation did not affect growth of *V. cholerae*, since the mutant grew in a similar fashion to the wild type. This indicated that the bacterium would be able to survive, under similar conditions, as a free-living organism in the absence of PerA. However, the mutant did not reach quite the same optical density in comparison to the wild type, when exposed to increased temperature (37 °C) and salinity (4% NaCl), indicating that *V. cholerae* might be slightly impaired in growth in its natural environment (such as estuaries) if, for whatever reason, it is defective in this catalase-peroxidase. The natural environment of *V. cholerae* is aquatic environments with moderate to high salinity (2% and higher), such as estuaries and seawater. The salinity of these habitats increases during summer months, due to increased evaporation of water. It is known that ROS such as superoxide and H₂O₂ are produced in seawater and some marine animals when water temperature and salinity increases, and that H₂O₂ and other oxidative radicals are naturally formed in water due to photo-activation (Mopper and Zhou, 1991, Joux et al., 1999, Filho et al., 2001). It is further known that H₂O₂ formed in this manner inactivates bacteria in water (Arana et al., 1992, Sinton et al., 2002). The absence of the catalase-peroxidase would therefore be disadvantageous to *V. cholerae*, especially in the warmer months of the year.

The wild type strain showed increased resistance to H₂O₂ when grown in the presence of elevated temperature and salinity (Fig. 4.7). This increased resistance is absent in the *perA* mutant, indicating modulation of catalase production by environmental temperature and salinity. A study by Kaku et al. (2000) demonstrated that *Synechococcus* sp. PCC 7942, overexpressing the *E. coli* catalase gene *katE*, grew better in a high saline environment, compared to the same strain without excess catalase. This indicates that osmotic stress induces oxidative stress in aquatic environments and that H₂O₂ is the causative agent of this stress. It is deduced therefore that a catalase-negative strain of *V. cholerae* would struggle to survive in estuaries or open ocean, especially in summer. It should also struggle in the small intestine of humans, where the bacterium encounters bile salts at a concentration equivalent to 0.2 -2% (Gupta and Chowdury, 1997), as well as the human body temperature of 37 °C.

Both log phase and stationary phase *V. cholerae* cells were affected by the absence of PerA. In Chapter 3 of this thesis it was demonstrated that PerA was observed mainly upon the onset of the

stationary phase of growth in *V. cholerae*. One would therefore expect that H₂O₂ would not affect log phase cells of the bacterium. However, the mutant was negatively affected by the *perA* mutation upon exposure to H₂O₂, irrespective of the phase of growth. This indicates that there might be either small quantities of the PerA protein produced during the log phase of growth of the bacterium or, alternatively, that other antioxidant strategies are utilised by the bacterium to protect against the oxidative stress imposed by H₂O₂. The absence of catalase activity observed in non-induced log phase cell-free extracts might be due to low levels of catalase, too little for detection with this particular zymogram method. A more sensitive method of staining, or alternatively, detection of *perA* RNA transcripts or the protein with antibodies would provide the answer to this question.

Exposure of stationary phase *V. cholerae* 569B cells to H₂O₂ at concentrations of more than 500 µM severely affected the survival of the mutant, whilst a lesser degree of killing was observed with regard to the wild type. When the mutant was in stationary phase, more than 99% of the cells were killed when exposed to 2 mM of the oxidant, while none remained following exposure to 4 mM H₂O₂. It is therefore obvious that the catalase-peroxidase of *V. cholerae* is essential for the organism to survive exposure to high concentrations of exogenously added H₂O₂. From these results, we concluded that *V. cholerae* PerA is essential as an antioxidant enzyme when cells are in the stationary phase of growth.

Interestingly, *V. vulnificus*, a human pathogen associated with shellfish, exhibited increased sensitivity to H₂O₂ when *rpoS*, a regulator of stationary phase proteins, was mutated (Park et al., 2004). The increased sensitivity was observed in log phase cell-free extracts. Upon more detailed investigation, it was found that the *rpoS* mutation resulted in a lack of synthesis of the catalase-peroxidase of *V. vulnificus*. Similar to *V. cholerae*, *V. vulnificus* also contains two genes encoding for catalases, a monofunctional catalase and a bi-functional catalase-peroxidase. However, as was found in this study, only the catalase-peroxidase was observed in zymograms (See Chapter 3). In the case of *V. vulnificus*, the catalase-peroxidase was present in log phase cells, whereas we did not detect PerA in cell free extracts from uninduced *V. cholerae* log phase cultures. However, in this study, the *perA* mutation clearly affected cells in the log and stationary phase of growth. This indicates that the protein is produced in the log phase, but in amounts too low for detection on a zymogram.

Similar to *V. vulnificus*, it was demonstrated that a *V. cholerae* *rpoS* mutant exhibited increased sensitivity to H₂O₂ (Yildiz and Schoolnik, 1998). Although the study by Yildiz and Schoolnik (1998) did not investigate which specific catalase might have been affected by the *rpoS* mutation, it is plausible that *perA* was affected since RpoS affects stationary phase proteins and we observed PerA only once the stationary phase of growth had commenced.

The PerA mutant (in stationary phase) survived low (500 μM) dosages of H_2O_2 (Fig. 4.7). This indicates that other mechanisms of protection against H_2O_2 might be expressed by *V. cholerae*. Firstly, another catalase gene, *katB*, is encoded by the genome of *V. cholerae*, (although we did not observe another band of catalase on zymograms, except when the cultures were induced by the oxidant [see Chapter 3]). The reason for the absence of the other catalase on the zymogram might be that the *katB* catalase is produced in quantities too low to be detected by this specific zymogram stain, or alternatively, that the KatB is membrane-bound and is removed with other cell debris upon centrifugation of crude cell extracts. The *katB* gene is found on the genome of *V. cholerae* adjacent to *sodA* (encodes for superoxide dismutase) and *ankB* (encoding for an ankyrin type protein). The arrangement of these three genes¹ is similar to that found in *Pseudomonas aeruginosa*. In the latter bacterium, the 3 genes are transcribed as an operon. AnkB is an ankyrin-like protein and it is hypothesized that AnkB “anchors” KatB to the cell membrane (Howell *et al.*, 2000), aiding the orientation of the catalase on the membrane and therefore contact with the substrate. Thus, since a similar genetic arrangement is present in *V. cholerae* N16961, and assuming that the “ankyrin” hypothesis is true, it is possible that KatB might have been removed through centrifugation with the rest of the cell debris (membranes, cell wall etc.). A third reason that might explain the absence of the other band, is the fact that we only actually observed another band in cells of *V. cholerae* that were exposed to non-lethal dosages of H_2O_2 (Chapter 3). This other catalase might therefore be produced only when induced by exposure to the oxidant.

The negative effect of melanin on cell numbers has been described in Chapter 2, both in *V. cholerae* and *E. coli* cells over-expressing melanin. We demonstrated here that this negative effect was enhanced in the absence of PerA and that the numbers of *perA* mutant cells declined severely after 48 hours, whereas the numbers of mutant cells was comparable with wild type cells before the onset of melanogenesis). To test whether it was indeed the presence of the melanin that caused a decline in mutant cell numbers, we cultured the mutant under conditions of increased or decrease aeration (more air = more pigment and *vice versa*), or alternatively, added an inhibitor of melanogenesis (L-proline) to the cultures. These experiments confirmed that the presence of the melanin indeed caused a decline in mutant cell numbers. Non-melanised, two-day old cultures of mutant cells had higher cell numbers (at least 10^8 cfu/ ml), whereas the cell numbers from melanised cultures of the same age declined to 10^4 cells per ml of culture.

Regarding the sensitivity of the PerA mutant to the presence of melanin, this result confirmed the results of Chapter 2, i.e. the presence of melanin had a detrimental effect on *V. cholerae* cell numbers (see Chapter 2) and indicates that PerA is essential to the bacterium during melanin

¹ Gene loci VC1583, VC1584 and VC1585, www.tigr.org.

production. The need for an active catalase might be explained by the fact that H₂O₂ generated *in vitro* during autopolymerization of homogentisate (HGA), caused damage of genomic DNA (Hiraku et al., 1998). In the study by Hiraku et al. (1998) DNA damage was prevented if the autopolymerisation of HGA was performed in the presence of catalase, indicating that the source of DNA damage was H₂O₂. Generation of oxidative radicals, including H₂O₂, had also been demonstrated in the process of DOPA melanin formation (Kruk et al., 1999). More recently, it was demonstrated that *oxyR* mutants of *E. coli* were sensitive to growth in the presence of various phenolic substances, including homogentisic acid [the precursor of *V. cholerae* pyomelanin] (Urios et al., 2003). The OxyR protein is a redox-sensitive regulator of bacterial cells, regulating the expression of H₂O₂-inducible genes such as *E. coli katG*, the gene encoding for the *E. coli* catalase-peroxidase. Upon the presence of oxidizing agents, such as H₂O₂, OxyR undergoes a conformational change, leading to the transcriptional induction of various genes, including catalases (Aslund et al., 1999). In the study by Urios and co-workers (2003), the *oxyR* mutation caused a greater degree of mutagenicity of the bacterium when grown on media containing various phenolic substances. In this study, we showed that the absence of the catalase-peroxidase severely affected *V. cholerae* cell numbers when cultured under melanin-inducing conditions. Our work thus demonstrates, for the first time, a potential role for a bacterial catalase as a means of protection against H₂O₂ formed during the process of melanization. The severe decline in cell numbers observed in the PerA mutant upon melanization, confirms the observation made in Chapter 2, that is, that melanin production has a negative impact on cell numbers. Although we could not prove that melanization led to oxidative DNA damage (Chapter 2), oxidative damage of other cellular targets can occur. It might also be that the damage occurs as the pigment or its precursor homogentisic acid is secreted and crosses the bacterial membrane.

All experiments in this study were performed with liquid cultures of *V. cholerae*. However, future work should include experiments with *V. cholerae* biofilms, since this bacterium exists in its natural environment both in free-living form (planktonic) and in biofilms, or attached to various aquatic plant and animal surfaces (see Literature Review). It is known that the rugose colony of *V. cholerae* is more resistant to oxidative stress and increased salinity (Wai et al., 1998). This rugose form of *V. cholerae* colonies is induced by starvation and promotes biofilm formation due to the presence of an extracellular matrix (Wai et al., 1998). It should therefore be interesting to see how biofilms of the wild type and PerA mutant behave when cultured under pigment-inducing conditions. Of greater interest, is the fact that pigmentation and biofilm formation in another vibrio, *V. anguillarum*, is modulated by the same regulator, namely VanT (Croxatto et al., 2002). The gene responsible for biofilm formation in *V. cholerae*, *vpsR*, has a homologue in *V. anguillarum* that is also regulated by VanT (Yildiz et al., 2001). All these observations, combined with the fact that

pigmentation in *V. cholerae* is induced by osmotic stress (Coyne and Al-Harthi, 1992) and starvation (Ruzafa et al, 1995; Sanchez-Amat et al., 1998), indicates that there may be a close relationship between osmotic stress, pigmentation, biofilms and oxidative stress in *V. cholerae*.

In conclusion, the PerA catalase-peroxidase of *V. cholerae* is an important defence mechanism for the bacterium against H_2O_2 , since the mutation rendered *V. cholerae* sensitive to externally added H_2O_2 . The mutation rendered *V. cholerae* cells sensitive to the presence of melanin, thereby confirming results obtained in Chapter 2, that reactive oxygen intermediates form during melanin formation in *V. cholerae*. It is further hypothesised that (1) the reactive oxygen species responsible for the decline in cell numbers is H_2O_2 , and (2) PerA scavenges the H_2O_2 formed during melanin formation, thereby removing this oxidative threat before damage to cellular components might occur. This catalase-peroxidase thus seems to play an important role in *V. cholerae* to protect against endogenous and exogenous H_2O_2 .

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CHAPTER 5

**THE ROLE OF *V. CHOLERAE* MELANIN AS
PROTECTION AGAINST HEAVY METAL AND
HYPOCHLORITE TOXICITY**

University of Cape Town

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Summary

Pigmentation in microbes provides a number of advantages, particularly as defence against ultraviolet light, heavy metals and host-induced oxidative defence mechanisms. *V. cholerae* produces pyomelanin under stress conditions and since pigmented marine bacteria are known to be more resistant to heavy metal toxicity, the ability of pyomelanin to protect *V. cholerae* against heavy metals and an oxidizing reagent was investigated. Bacteria grown under melanin-inducing conditions were exposed to CuCl_2 , FeSO_4 , AgNO_3 , or NaOCl . Melanin provided protection against CuCl_2 , but not against FeSO_4 , AgNO_3 or NaOCl . From these results, it was concluded that melanin confers protection to the bacterium against certain, but not all, exogenous stresses.

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5.1 Introduction.

V. cholerae is a successful marine bacterium and human pathogen. In the marine environment, as well as the human host, the bacterium encounters a number of adverse conditions such as osmotic stress, oxidative stress and starvation conditions. In addition, toxic metals are found in increasing quantities in aquatic environments (Comber et al., 2002). Exposure of bacteria to metal pollutants has led to the development of increased tolerance to toxic heavy metals (Nair et al., 1992). Marine bacteria also become increasingly tolerant after exposure to metals in water, such as was found with *V. cholerae* isolates from a coastal region on the Indian sub-continent (Choudhury and Kumar, 1996). A similar phenomenon was also described in other marine bacteria (Sabry et al, 1997). In addition, it seems that exposure of bacteria to heavy metals is linked to increased antibiotic resistance, perhaps because genes encoding for metal resistance are acquired via plasmids that carry antibiotic resistance genes (Choudhury and Kumar., 1996; Stepanauskas et al., 2005). This short review will focus on the interaction between bacteria and various exogenous threats.

5.1.1 Microorganisms and heavy metals

Heavy metals are both useful and deleterious to living organisms. Metals such as iron (Fe), manganese (Mg) and copper (Cu) are essential for certain enzyme activities (e.g. Cu-Zn superoxide dismutases), and act as catalysts in essential metabolic reactions (Puig and Thiele, 2002). In contrast, metals such as copper and silver are bactericidal against microbes such as *Mycobacterium avium* (Lin et al, 1998) and *V. cholerae* (Dibrov et al., 2002).

Due to the fact that various metals are essential in protein function and as co-factors in some enzyme reactions, bacteria possess mechanisms to obtain essential metal elements from the environment, as well as mechanisms to combat metal toxicity. Firstly, metals such as iron and copper are bound and taken up by bacteria through iron chelators such as the siderophores (Page, 1995), or active transport mechanisms in the form of protein pumps (Odermatt, 1995). Secondly, excess metals are stored by bacteria in specific storage proteins, eg. iron in bacterioferritin (Ma et al., 1999) and copper in CopA (Rensing and Grass, 2003; Odermatt and Solioz, 1995). Storage in proteins like bacterioferritin and Cop A ensures that there is not a free pool of these metals in the cytoplasm, because free metal ions, such as iron, can interact with cellular metabolites to generate oxidative radicals (Touati, 2000).

For the same reason, the acquisition (and storage) of metals is tightly controlled by bacteria. For example, uptake and metabolism of ferrous (Fe^{2+}) iron is under tight control of the ferric uptake regulator (Fur) (De Lorenzo et al., 1988; Griggs and Konisky, 1989; Horsburgh et al., 2001). Fur regulates a number of genes involved in uptake, metabolism and storage of iron (Escolar et al. 1999;

Escolar *et al.*, 2000; Bsat *et al.* 1998). Another example of tightly controlled regulation, is the copper uptake and efflux systems of *E. coli* (Rensing and Grass, 2003) and *Enterococcus* (Odermatt, 1995). In *Enterococcus*, 2 ATPases (CopA and CopB) are responsible for uptake and efflux upon depletion or an excess of Cu inside the cell (Odermatt, 1993; Odermatt, 1995). The processes of uptake and efflux are regulated by a transcriptional repressor protein, CopY, as well as an antirepressor protein, CopZ (Odermatt, 1995).

V. cholerae possesses a number of genes encoding for products involved in iron acquisition, storage and metabolism (Table 5.1). These include genes encoding for the ferric uptake regulator, various siderophores and iron storage proteins. The large number of genes in *V. cholerae* that encode for iron uptake, utilization and storage, is due to the fact that iron is essential for the growth and the virulence of the bacterium (Henderson and Payne, 1994; Wyckoff *et al.*, 2004). A smaller number of genes with a potential role in copper metabolism were also identified in the genome of *V. cholerae* (www.tigr.org). Included amongst these genes is a putative copper ATPase efflux pump (VC2215).

The toxicity of heavy metals is ascribed to:

- their ability to interact with oxidative radicals to generate highly reactive hydroxyl radicals and cause oxidative damage to bio-molecules (Touati, 2000; Ercal *et al.*, 2001),
- their ability to bind to cysteine groups in proteins (Schutzendubel and Polle, 2002), and
- their ability to replace essential core metals needed for enzymatic activity (Schutzendubel and Polle, 2002; Rivetta *et al.*, 1997).

These reactions cause inactivation of enzyme activity. Another mechanism explaining the bactericidal activity of a metal was described in *V. cholerae*. The bacterium is sensitive to silver nitrate applied in micromolar dosages. The bactericidal effect of silver was ascribed to a loss of membrane potential and membrane transport, thereby causing cell death (Dibrov *et al.*, 2002).

5.1.2 Resistance mechanisms to heavy metals.

Bacteria mediate resistance against heavy metals with the aid of plasmid encoded resistance factors, efflux pumps and cation/anion antiporters (Silver, 1996). Plasmid-encoded resistance factors have been described amongst a number of marine bacteria (Ravel *et al.*, 1998). Heavy metals bind to polymers, such as humic acid (Lores *et al.*, 1999) and melanin from microbes such as *Shewanella algae* (Turick *et al.*, 2003) and *Cryptococcus neoformans* (Jacobson, 1997). Binding of heavy metals by pigments such as melanin confers certain advantages to microorganisms. In the marine environment, it was found that pigmented marine bacilli were more resistant to heavy metals than non-pigmented bacteria (Hermannson *et al.*, 1987; Nair *et al.*, 1992). In the case of the opportunistic human pathogenic yeast, *Cryptococcus neoformans*, melanin limited the toxicity of silver (Ag^+)

ions to the organism (Garcia-Rivera et al., 2001). In another example, the binding of toxic organotins by melanin-producing *Aureobasidium pullulans* was postulated to aid the survival of the organism, at the expense of competitive bacteria (Gadd, 2000). Organotins are components of paints specifically used in the shipping industry. As organotins are toxic, accumulation of this substance in melanin thus presents a threat to microbial neighbours, whereas *Aureobasidium* itself is not affected by the accumulated toxin.

Table 5.1. Subset of genes from *V. cholerae* involved in iron metabolism.

Locus	Gene	Name/function
VC0365	<i>bfr</i>	bacterioferritin
VC0078	<i>btn</i>	ferritin
VC2078	<i>feoA</i>	ferrous iron transport protein A
VC2077	<i>feoB</i>	ferrous iron transport protein B
VC0779	<i>viuC</i>	vibriobactin and enterobactin ABC transporter, ATP-binding protein
VC0776	<i>viuP</i>	vibriobactin and enterobactin ABC transporter, periplasmic ferric vibriobactin/enterobactin-binding protein
VC0777	<i>viuD</i>	vibriobactin and enterobactin ABC transporter, permease protein
VC2211	<i>viuA</i>	ferric vibriobactin receptor
VC2106	<i>fur</i>	ferric uptake regulation protein

Data obtained from www.tigr.org. This table represents only a few of the iron binding genes present in the *V. cholerae* genome.

5.1.3 Bacterial resistance to hypochlorite

Chlorine containing compounds are widely used to provide safe water to households. However, in geographical areas with a lack of potable water, cholera and other diarrhoeal diseases are a frequent occurrence due to a lack of sanitation and contamination. An effective disinfectant against pathogens such as *V. cholerae* is thus essential for the prevention of cholera outbreaks. In order to prevent cholera outbreaks in areas with a lack of potable water (eg. KwaZulu-Natal, South Africa, where cholera outbreaks occur annually), people are advised to add commercially available

household chlorine-containing liquids to water before consumption (<http://www.umgeni.co.za/worldofwater/cholera/>; Mughero et al., 2001).

Hypochlorites are both bactericidal and sporicidal. The active component of chlorine compounds is hypochlorous acid (HClO) and its cellular targets are thought to be bacterial membranes and cytoplasmic compounds (Russel and Chopra, 1996). The bactericidal action of chlorine compounds is postulated to involve the generation of reactive oxygen species (Dukan et al., 1999). *E. coli* exposed to the oxidant under aerobic conditions is killed by reactive oxygen species generated in the presence of the oxidant. In contrast, exposure to the oxidant under anaerobic incubation prevented cell death (Dukan et al., 1999). The lethal damage caused by these species occurs even after the hypochlorite was no longer present. It was further demonstrated that low amounts of hypochlorite caused a lowering of *E. coli* catalase-peroxidase HPI and glucose-6-phosphate dehydrogenase activity, and a change in the redox status of the cell (demonstrated by a drop in the amount of reduced glutathione). At increased hypochlorite concentrations, *E. coli* HPII catalase and SOD were also inactivated (Dukan et al., 1999). In a different study, it was demonstrated that free cellular iron, derived from oxidized haem-containing proteins, increases in the presence of hypochlorite, thereby aiding the generation of potential Fenton reactions (Rosen and Klebanoff, 1982). From other studies it is known that the presence of oxidative radicals cause a rise in free iron, due to leaching of iron from haem-containing proteins (See Literature Review).

In *E. coli* it was shown that resistance to HOCl is due to genes involved in H₂O₂ resistance, such as the catalase genes, as well as the gene encoding for Dps, a DNA-binding protein that protects DNA against oxidative damage (Dukan, 1996). In another report, a resistant strain of *Salmonella* showed a lesser degree of DNA damage after hypochlorite treatment, compared to a more sensitive strain. The resistance mechanism most probably involved increased DNA protection or increased repair mechanisms (Mokgatla et al., 2002). Melanin was also indicated as another potential mechanism of protection against hypochlorite when it was shown that melanin from black fungi served as an antioxidant and prevented killing by hypochlorite (Jacobson et al., 1995).

In the case of *V. cholerae*, it was reported that the bacterium became more resistant to chlorine treatment when it assumed a rugose colony form (Rice et al., 1993). This colony form emerges during starvation conditions and is characterised by the presence of an extracellular matrix (Mizunoe et al., 1999; Wai et al., 1998). Since *V. cholerae* produces melanin, and hypochlorite is an important factor in the prevention of cholera, the potential to resist hypochlorite might represent a problem, especially since melanin is formed under osmotic and starvation stress conditions, such as those found to occur in its natural environment.

5.1.4. Aim of the chapter

Since a large number of marine bacteria, including *V. cholerae*, produce pigments such as melanin, melanogenesis might have certain biological implications in terms of the survival of *V. cholerae* in its natural environment. The aim of this chapter was to investigate the potential role of melanin to protect *V. cholerae* against heavy metal and hypochlorite toxicity. The latter is relevant, particularly because it is used world-wide as a tool to provide water fit for human consumption. To elucidate the function of melanin as a mechanism of protection against these toxic compounds, the bacterium was grown for two days under pigment-inducing conditions before exposure to copper, silver, iron or a hypochlorite-containing compound.

5.2 Materials and methods

V. cholerae strains 569B (melanin producing) and Mut32 (melanin-deficient) were used to investigate the function of the pigment in this bacterium. These strains have been described in Chapter 2.

5.2.1. Growth of *V. cholerae* strains under pigment-inducing conditions.

Tryptone broth, containing 1% tryptone, 4% NaCl, 5mM L-tyrosine and 5 μ M CuCl₂, were inoculated with *V. cholerae* strains 569B or Mut32. The optical densities (at 600nm) of all cultures at the start of incubation was approximately 0.05. Cultures were incubated at 37°C for two days before exposure to a stress. After two days of growth, the pigment-producing culture (wild type strain 569B) was standardised to an OD of approximately 1, whereas the melanin mutant was standardized to an OD of approximately 0.8. This standardisation was performed to ensure that an equal number of culturable cells (approximately 10⁸ cells, as determined by plate counts) were present¹ in each culture before exposure to the various stresses. Standardised cultures were divided into 5ml aliquots in sterile universals, before cells were exposed to a specific stress (heavy metal or sodium hypochlorite). Preliminary experiments were performed with each stress factor to determine the appropriate concentrations to be used per experiment.

5.2.2. Exposure to abiotic stresses

A. Exposure to CuCl₂. CuCl₂ was added to 5 ml cultures (in sterile universals) to obtain final concentrations ranging from 0, 250, 500 to 1000 μ M. Cultures were incubated, on a shaker, for 30

¹ It was previously observed that melanin negatively affected the culturability of the bacterium, with an increase in melanin correlating to a decrease in culturability. For this reason the non-melanised cells were standardized to a higher optical density to obtain equal amounts of culturable cells at time = 0 minutes of exposure (See Chapter 2 for data on culturability).

minutes at 37°C, before 100 µl aliquots were removed from each culture. Dilutions were made and plated, in triplicate, on tryptone agar. Plates were incubated overnight at 37°C before enumeration of colonies. The mean colony-forming units per ml of culture (CFU/ml), was calculated from two experiments.

B. Exposure to AgNO₃. AgNO₃ was added to 5ml cultures to obtain final concentrations ranging from 0, 25, 50 to a 100 µM. Cultures were incubated, on a shaker, for 30 minutes at 37°C, before 100 µl aliquots were removed from each culture. Dilutions were made and plated, in triplicate, on tryptone agar. Plates were incubated overnight at 37 °C before enumeration of colonies. The experiment was repeated once. The mean colony-forming units per ml of culture (CFU/ml), was calculated from two experiments.

C. Exposure to FeSO₄. FeSO₄ was added to 5ml cultures to obtain final concentrations ranging from 0, 100, 1000 to a 10 000 µM. Cultures were incubated, on a shaker, for 30 minutes at 37°C, before 100 µl aliquots were removed from each culture. Dilutions were made and plated, in triplicate, on tryptone agar. Plates were incubated overnight at 37 °C before enumeration of colonies. The mean colony-forming unit per ml of culture (CFU/ml) was calculated from two experiments.

D. Exposure to NaOCl. A commercially available solution of NaOCl (Commercial name: Jik, 3.5% w/v) was used in this experiment. Dilutions of NaOCl were prepared in phosphate buffer. Hypochlorite was added to 4 universals, each containing 5ml of culture, in concentrations ranging from 0, 0.025, 0.05 to a 0.1 mg per ml of culture. Cultures were incubated for 5 minutes at room temperature, before 100 µl aliquots were removed from each culture. Hypochlorite is a very effective bactericidal agent, even at room temperature, and lysis and killing of cells occur within 10 minutes of exposure. The exposure time of each broth to this substance was therefore limited to 5 minutes. Dilutions were made and plated, in triplicate, on tryptone agar. Plates were incubated overnight before enumeration of colonies. The mean colony-forming unit per ml of culture (CFU/ml) was calculated from 2 experiments.

5.3. Results

Exposure to CuCl₂. Melanised *V. cholerae* cells survived exposure to CuCl₂, whereas the cell numbers in the melanin-deficient mutant declined significantly in the presence of 500 and 1000 µM of the metal (Fig. 5.1). Exposure of the wild type cells to this metal caused a decline in cell numbers from 100% (0 CuCl₂) to 85.13% (at 250µM), 78.45% (at 500 µM) and 76.03% (at 1 000µM). In contrast to that, the cell numbers of the melanin mutant declined from 100% before exposure to 96.13, 22.87 and 21.21% in the presence of increased amounts of copper.

Exposure to AgNO₃. Silver was found to be highly bactericidal against *V. cholerae*, even in very small quantities, ranging from 25 µM to 100 µM (Fig. 5.2). Although *V. cholerae* survived to a greater degree in the absence of melanin, compared to when it was present (the wild type strain), less than 0.01% of both strains remained.

Exposure to FeSO₄. Melanised *V. cholerae* cell numbers declined from 100% to 82.8 %, 71.2% and 59.8% in the presence of increasing amounts of Fe (Fig. 5.3). In contrast, more than 71% of the mutant cells still grew after exposure to 10 mM of the metal. The non-melanised cells therefore survived slightly better than the wild type cells.

Exposure to NaOCl. Preliminary tests showed that, under the conditions used in this experiment, 50% of *V. cholerae* cells were killed by NaOCl at a concentration of 0.05 mg/ml. In this experiment therefore, the survival of melanised and non-melanised cells were examined at a concentration of 0.025 mg/ml, 0.05 mg/ml or 0.1 mg/ml of hypochlorite. There was no difference in survival between the 2 strains tested at a concentration of 0.025 mg/ml of hypochlorite (Fig. 5.4). However, at a concentration of 0.1 mg/ml of the oxidant, a sharp decline in cell numbers was observed for both strains. Less than 0.01% of the melanised cells survived exposure to NaOCl, whereas the cell numbers of the mutant declined to approximately 1%.

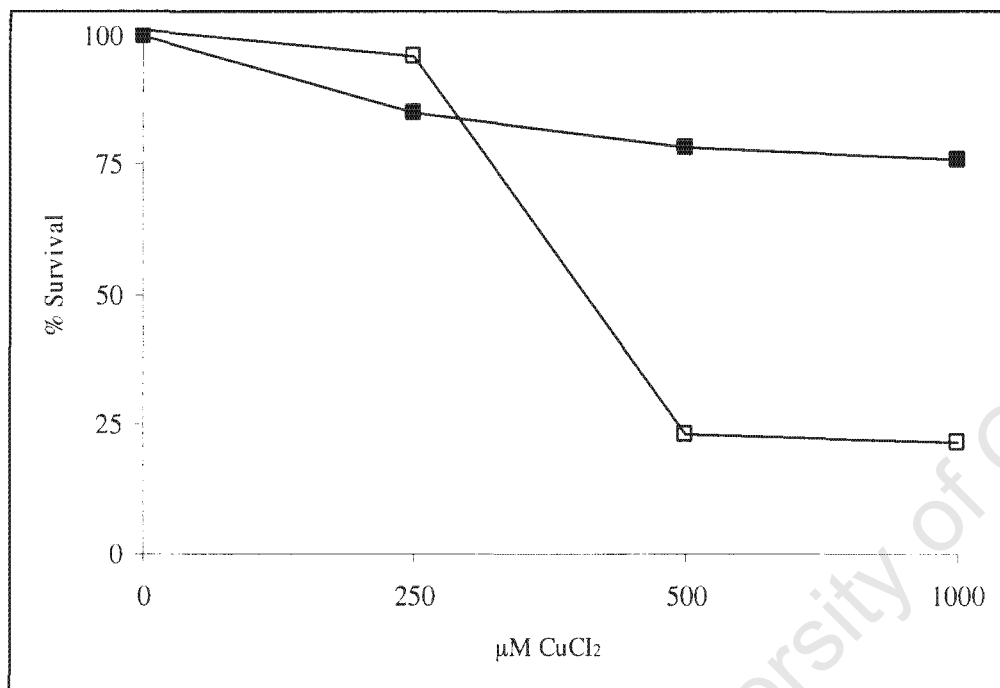


Fig 5.1. Survival of *V. cholerae* upon exposure to CuCl_2 . Cells were grown under pigment-inducing conditions before exposure to the heavy metal. Solid symbol, melanin-producing *V. cholerae* 569B; Open symbol, melanin-deficient *V. cholerae* Mut 32.

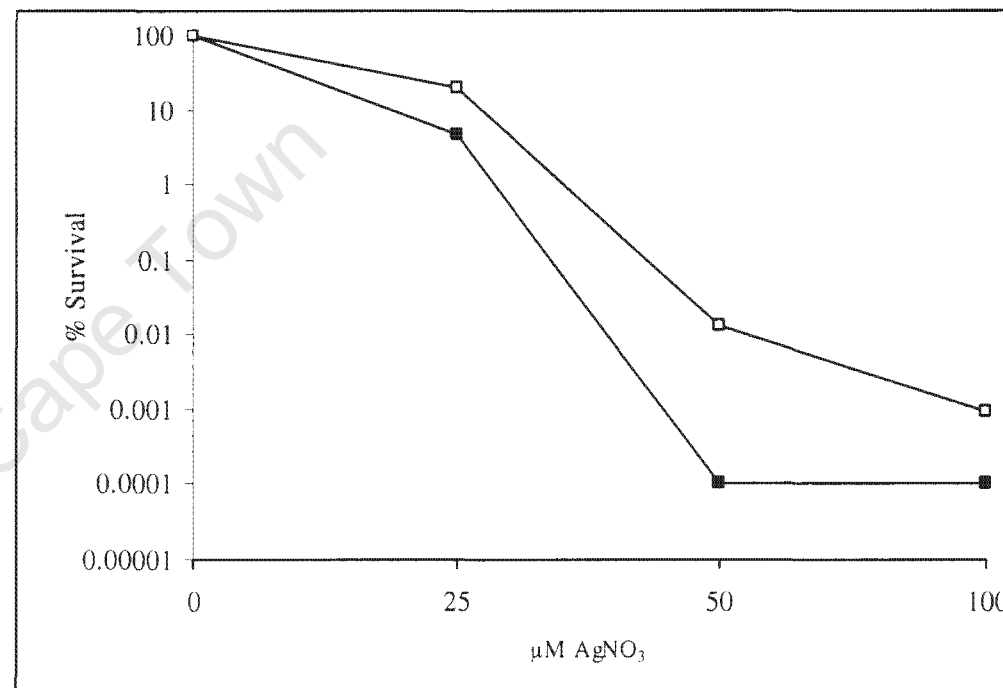


Fig 5.2. Survival of *V. cholerae* upon exposure to AgNO_3 . Cells were grown under pigment-inducing conditions before exposure to the heavy metal. Solid symbol, melanin-producing *V. cholerae* 569B; Open symbol, melanin-deficient *V. cholerae* Mut 32.

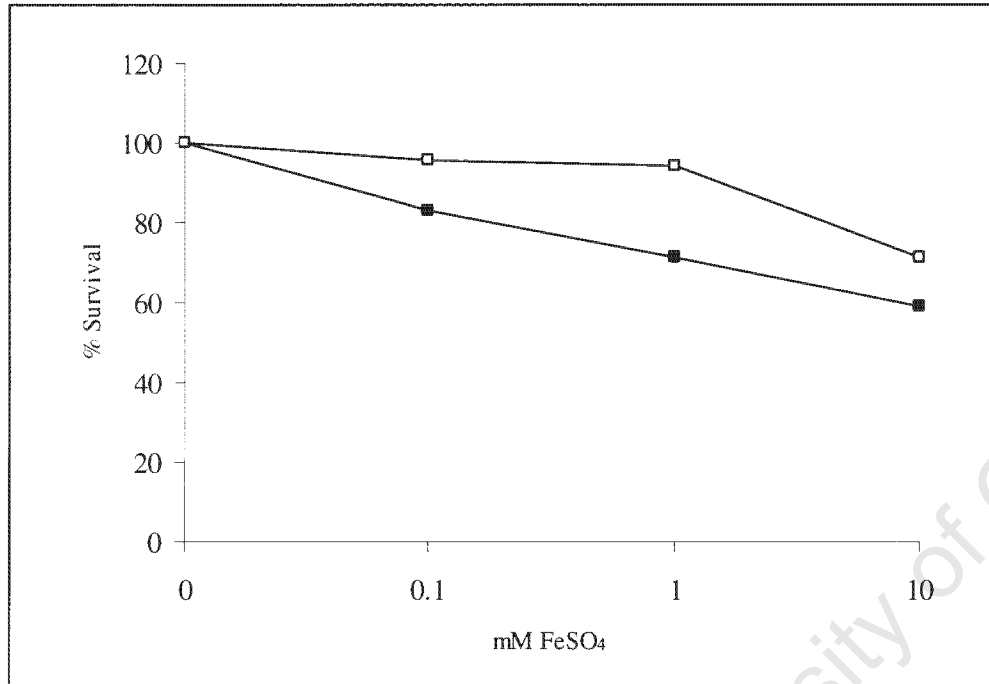


Fig. 5.3 Survival of *V. cholerae* upon exposure to FeSO₄. Cells were grown under pigment-inducing conditions before exposure to heavy metal. Solid symbol, melanin-producing *V. cholerae* 569B; Open symbol, melanin-deficient *V. cholerae* Mut 32.

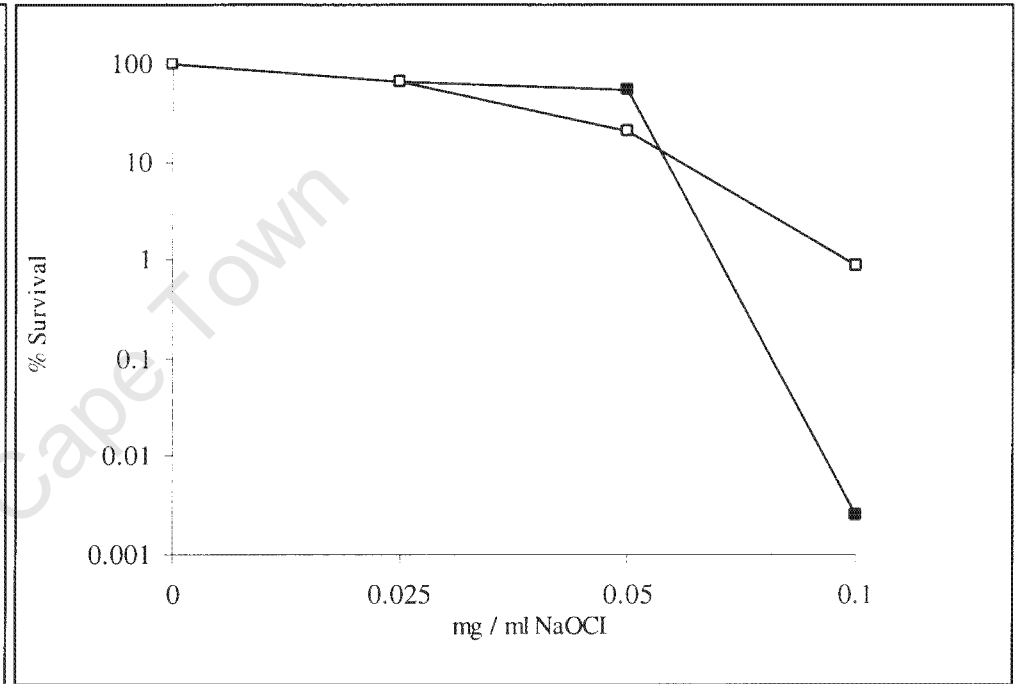


Fig. 5.4. Survival of *V. cholerae* upon exposure to NaOCl. Cells were grown under pigment-inducing conditions before exposure to the oxidant. Solid symbol, melanin-producing *V. cholerae* 569B; Open symbol, melanin-deficient *V. cholerae* Mut 32.

5.4 Discussion

Pollution by heavy metals is observed increasingly in the marine and other aquatic environments. These heavy metals occur naturally (e.g. metals in rocks), or are introduced into the environment by human activities such as mining and herbicidal treatment of crops (Liu et al., 2003; Comber, 2002; Gimeno-García et al., 1996; Carabias-Martínez et al., 2003). This affects the marine ecology, causing diseased animals, animals unfit for human consumption, as well as a change in microbial patterns (Labare et al., 1997; Tawari et al., 2001). Marine bacteria therefore need mechanisms to defend themselves against potentially toxic heavy metals. One of the mechanisms observed is the development of increased metal tolerance, as was demonstrated with marine bacteria (including *V. cholerae*) isolated from polluted aquatic environments (Choudhury and Kumar, 1997, Sabry et al. 1997).

This study demonstrated that melanin protected the marine bacterium *V. cholerae* from the toxic effect of copper. Copper is a known bactericidal agent, with potential use as a sterilizing agent. For example, its bactericidal action against two opportunistic pathogens, *Mycobacterium avium* and *Legionella pneumophila*, potentially can be utilized to sterilize hospital hot water systems which are frequently contaminated by these pathogens (Goetz et al., 1998). A copper-containing substance, copper oxychloride, is also effectively used in South America to eliminate *V. cholerae* from harvested lemons before export (de Castillo et al., 1998). Unfortunately, copper is also a component of pesticides, the latter being used to prevent crop damage. Aqueous environments, such as groundwater, rivers and estuaries might become contaminated by pesticides. Aquatic bacteria therefore are exposed to copper and the potential thus exists for the development of increased tolerance to this metal. In a previous study, by Choudhury and Kumar (1996), enteric bacteria and *V. cholerae* isolates that were isolated from a coastal region of India exhibited increased tolerance to copper, zinc, silver and a few other heavy metals. This region is subject to heavy metal pollution due to human activities. The observed increase in metal tolerance is an indication that *V. cholerae* is able to adapt to increased copper levels in the water.

The protection provided by *V. cholerae* pyomelanin against copper toxicity might be due to exclusion of the metal from the bacterial cell, since melanin is able to bind copper effectively (Fogarty and Tobin, 1996). This effective binding might therefore prevent copper from entering bacterial cells. Although no literature could be found regarding copper exclusion to bacterial cells due to melanin, one report showed the exclusion of copper from a marine bacterium, *Vibrio natriegens*, and two ciliates by the presence of humic substances (Lores et al., 1999) in the aquatic

environment. Both melanin and humic acid contain polyphenolic, redox-reactive groups that are able to bind copper (Menter and Willis, 1997; Newman and Kolter, 2000). If melanin functions in a similar manner, it might well prevent copper uptake by sequestering it from the environment.

Copper, when bound to melanin, causes a change in the structure and aggregation of melanin (Chodurek et al., 2002; Gallas et al., 1999). The physiological consequences of these changes are not known. However, when copper and melanin or melanin precursors (L-Dopa, DHI, DHICA, homogentisic acid) interact *in vitro* in the presence of nucleic acid, it leads to DNA degradation, as well as cytotoxic and clastogenic events, through the generation of oxidative radicals (Hiraku et al., 1998; Kortytowski and Sarna, 1990; Snyder, 1998). These reactions are thought to involve the heavy metal and quinone moieties of the melanin. However, melanized *V. cholerae* cells survived exposure to copper to a greater degree, compared to non-melanised *V. cholerae* Mut32. We hypothesise that the added copper was excluded from entering *V. cholerae* cells by the presence of melanin, thereby preventing potential cytotoxic interactions between copper and homogentisic acid intracellularly.

Unlike copper, melanin provided no protection against silver ions. In fact, it seemed that the presence of melanin increased killing by Ag ions. A report by Dibrov, et al. (2002), demonstrated that micromolar amounts of Ag ions were bactericidal to *V. cholerae*. Our study confirmed that this bacterium is killed at these low concentrations of Ag, irrespective of the presence or absence of melanin. The study by Dibrov et al. (2002) showed that exposure of *V. cholerae* membrane vesicles to micromolar amounts of Ag ions caused abolition of the proton-motive force across membranes. It was therefore postulated that the Ag ions modify certain membrane proteins, potentially causing proton leakage through either these damaged proteins or the membrane itself. In our study, both the melanin proficient and mutant cells were effectively killed by low amounts of silver ions, although the bactericidal effect of Ag was enhanced in the presence of the melanin, through an unknown mechanism. This result is the opposite than that found in *C. neoformans*, where the presence of melanin protected the microbe from Ag (Garcia-Rivera et al., 2001.). Although the melanin from *V. cholerae* 569B is derived from homogentisic acid (Kotob et al., 1995), whereas *C. neoformans* (Williamson, 1997) melanin is derived from L-DOPA, this difference in melanin might not entirely explain the difference in protection. Reasons for the difference in survival might also be due a difference in culture medium, the application of osmotic stress to induce melanogenesis in *V. cholerae* (but not in *Cryptococcus*) and the lack of a cell wall or thick, extra-cellular capsule in *V. cholerae* (unlike the black capsule of *C. neoformans*).

The presence of melanin did not affect *V. cholerae* cell numbers severely, both in the presence and absence of iron (Fe). In fact, quite high amounts (10 mM) of this metal were needed to affect *V. cholerae* cell numbers (at 10 mM concentration, more than 60% of both melanised and non-melanised cells still survived). Like Ag, the bacterium survived better in the absence of melanin, although only slightly better. It is known that melanin precipitates at high concentrations of Fe ions, and therefore the millimolar amounts used could have had an effect on the outcome of the experiment. The reason for the lack of killing by lower quantities of Fe might be because *V. cholerae* possesses adequate mechanisms to obtain and assimilate Fe (see Table 5.1). Included amongst the uptake, storage and metabolic mechanisms are the siderophores (vibriobactin and enterobactin), iron transport proteins and bacterioferritin storage proteins (Table 5.1). This large number of iron uptake and assimilation genes might be enough for the organism to effectively assimilate high concentrations of iron without damage to the organism. In addition, melanin is known to bind Fe (Page and Shivprasad, 1995; Shivprasad and Page, 1995), and facilitates electron transfer in *Shewanella algae* (Turick et al., 2003) and *C. neoformans* (Nyhus et al., 1997, Jacobson et al., 1997). The presence or absence of melanin might therefore be irrelevant to the bacterium in terms of protection against Fe toxicity.

The result of this study showed that melanin does not protect *V. cholerae* against the bactericidal action of hypochlorite, but its presence instead lead to a reduction in cell numbers when exposed to a dose of 0.1 mg/ml hypochlorite. This result might be explained by the target (bacterial membranes, enzymes and other cytoplasmic components) and mechanism of action of chlorine-releasing compounds (the generation of reactive oxygen species) (Dukan et al., 1999). Firstly, inactivation of cellular antioxidant enzymes occurs upon exposure to hypochlorite (Dukan et al., 1999). In addition, both hypochlorite and homogentisic acid are known to cause the formation of oxidative radicals (Dukan et al., 1999; Hiraku et al., 1998). In Chapter 2 of this study, it was demonstrated that bacterial cell numbers of both *V. cholerae* and *E. coli* declined in the presence of increasing amounts of melanin, indicating that melanin, in excess, negatively affected cell viability. This negative effect was even more pronounced in a catalase mutant of *V. cholerae* (see Chapter 4), indicating that oxidative stress might be responsible for the decline in cell numbers in the presence of melanin. In addition, a previous report indicated that H₂O₂ forms during the process of autopolymerisation of homogentisic acid (Hiraku et al., 1998). This generation of H₂O₂ in the presence of homogentisic acid and copper caused oxidative DNA damage, but was prevented in the presence of catalase or bathocuprein (a chelator of copper). If damage of biomolecules occurs as a result of the production, secretion and/or autopolymerisation of homogentisic acid, then the addition of another DNA damaging oxidant (such as hypochlorite) would cause further generation of

radicals (coupled with inactivation of important scavenger enzymes by hypochlorite), thereby exacerbating prior melanin-induced damage.

In conclusion, we demonstrated that melanin provides protection against certain abiotic stresses, while it seems to enhance the bactericidal effects of others. The fact that melanin only protected against certain metals, but not others, might be the result of different interactions with different metals. Future work to elucidate the reasons for the lack of protection against certain stresses could focus on the effect of homogentisic acid on the bacterial membrane and other cellular components, in order to establish whether damage to cellular components occurs due to the interaction between melanin or homogentisic acid and applied stresses. Previous research showed various types of negative consequences of melanin or other quinone-containing pigments on microbial cells or cellular components (Fava et al., 1993, Hiraku et al., 1998). These negative consequences included lipid peroxidation, DNA damage and cell death. Further studies should therefore focus specifically on lipid peroxidation, protein and DNA damage.

This study demonstrated that melanin is both detrimental and advantageous to *V. cholerae*. Most importantly though, is the fact that melanin does not confer protection against a chlorine-containing compound, but instead, enhanced the bactericidal effect of hypochlorite. This is an important result, since chlorine containing compounds are essential in the fight against cholera in geographical regions with a lack of potable water.

CHAPTER 6
GENERAL DISCUSSION

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The survival of microbes such as *V. cholerae* is dependent on the ability of the bacteria to overcome adverse conditions such as starvation, osmotic pressure and oxidative stress. The presence of additional defense mechanisms, such as antioxidant metabolites, is therefore useful to bacteria, providing additional protection to that provided by cellular scavenger enzymes such as catalases and superoxide dismutases. Melanin is such a by-product of cellular metabolism, and functions as an antioxidant scavenger in various microbes, thereby increasing the survival of bacteria. This thesis aimed to elucidate the role of *V. cholerae* pyomelanin as a potential antioxidant defence mechanism. The ability of pyomelanin to defend the bacterium against stresses such as H₂O₂, NaOCl, and various metals was therefore evaluated in *V. cholerae* 569B and a melanin-deficient mutant, as well as in the presence and absence of a stationary phase catalase-peroxidase.

Melanin protected *V. cholerae* 569B against H₂O₂, whereas a *V. cholerae* melanin mutant showed increased susceptibility to the oxidant. This protection was also demonstrated in *E. coli* cells transformed with a low copy number plasmid carrying a functional copy of the *ppdA* gene. However, this protection was not observed when bacteria (both *V. cholerae* and *E. coli*) were forced to overproduce the pigment (from a high copy number plasmid carrying a copy of the *ppdA* gene), but instead showed enhanced killing by H₂O₂. In line with the above observation, we made another observation, i.e. that the presence of the pigment coincided with a general decline in bacterial cell numbers. This effect was seen without the addition of H₂O₂, and was also reproduced in *E. coli* cells transformed with plasmids containing the *ppdA* gene. This decline in cell numbers was not as severe in the non-melanised *V. cholerae* mutant, and this, together with the observation that excessive melanin production enhanced killing of bacteria by H₂O₂, implicated melanin as the culprit potentially responsible for the decline in numbers. A search of available literature indicated that, while melanins and their pre-cursors (L-DOPA, DHICA, homogentisic acid, DOPA melanin, and black fungal melanins) are able to provide protection against oxidants to a number of microbes, it might also be responsible for oxidative DNA damage, lipid peroxidation, damaging bacterial cells, as well as inhibition of hyphal development. From this study, it was clear therefore that the presence of pyomelanin, although able to confer protection to *V. cholerae*, was also detrimental to bacterial survival.

A clue towards understanding this negative effect of melanin on bacterial cells was when we mutated the *perA* gene of *V. cholerae*. Initially, this mutation was generated in order to study the protective effect of melanin against externally added H₂O₂ in the absence of catalase (which is an effective scavenger of the oxidant in bacteria). We found instead that the decline in bacterial numbers, as observed in *V. cholerae* overproducing melanin, was enhanced in the *perA* catalase-peroxidase mutant upon melanogenesis, and that the melanised PerA mutant did not survive at all

when exposed to H_2O_2 . A number of experiments were performed with the *perA* mutant, ranging from addition of an inhibitor of melanogenesis, to changing the rate of aeration so that the degree of pigmentation varied. These experiments all confirmed that cell numbers were severely diminished in the presence of melanin. The fact that this severe decline was observed in a catalase mutant suggests that the target of catalases, i.e. H_2O_2 , is potentially the cause of the decline in bacterial numbers. This latter theory is supported by published studies that showed that H_2O_2 is formed during homogentisic acid autopolymerisation and cause, at least *in vitro*, oxidative damage to DNA (see Literature Review, also Chapter 2). Unfortunately we could not demonstrate DNA damage as the cause of the decline in cell numbers in the presence of *V. cholerae* pyomelanin. Future studies should use more sensitive techniques to investigate potential oxidative DNA, protein and membrane damage in order to define the nature of the damage incurred during melanogenesis in *V. cholerae*.

A number of conclusions were drawn from this study, namely:

1. Melanin protected *V. cholerae* from H_2O_2 when the oxidant was added exogenously.
2. H_2O_2 is a by-product of melanin production in *V. cholerae*, posing a threat to bacterial viability as observed through a decline in cell numbers.
3. The bacteria utilizes the catalase-peroxidase PerA as a scavenger of the H_2O_2 formed during melanin production.

The exact site of this H_2O_2 formation is unknown, although it most probably is formed when homogentisic acid consumes oxygen and polymerises to form pyomelanin.

Some unanswered questions remain, namely:

1. Is the site of H_2O_2 formation (when homogentisic acid consumes oxygen) inside the bacterial cell, or is it produced when homogentisic acid crosses the bacterial membrane, or lastly, is it formed extracellularly?
2. What is the nature of the damage caused by H_2O_2 : DNA damage, lipid peroxidation in the bacterial membrane or is it protein damage through oxidation?

As a minor part of this thesis, the role of melanin as a mechanism of protection against various heavy metals and hypochlorite was investigated. We found that melanin conferred protection against some, but not all heavy metals. The reason why melanin afforded protection against some, but not all metals, was not investigated. However, from the published literature it is clear that melanin interacts differently with different metals, and this potentially might explain these differences.

We found that melanin enhanced the bactericidal effect of hypochlorite against *V. cholerae*. This is indeed encouraging news, as this substance is used world-wide for sanitation purposes. In addition, in some geographical regions lacking proper facilities to provide potable water (therefore a high cholera risk), hypochlorite is supplied to people as a means to disinfect water obtained from rivers and dams. Hypochlorite, like H_2O_2 , causes oxidative damage to biomolecules, and it perhaps should come as no surprise that melanin enhanced the toxic effect of hypochlorite towards *V. cholerae*.

The original aim of this thesis was to elucidate the role of the pyomelanin produced by *V. cholerae* as a potential means of protection against oxidative stress. We conclude that melanin conferred some level of protection to the bacterium only in the presence of a functional catalase-peroxidase. The latter enzyme seems to remove damaging H_2O_2 formed during the process of melanization against exogenously added H_2O_2 . *V. cholerae* pyomelanin further protects the bacterium against some, but not all, externally added abiotic stresses. We conclude therefore that *V. cholerae* pyomelanin serves as an antioxidant, and plays a protective role against some, but not all heavy metals.

APPENDIX A

SOLUTIONS USED

CONTENTS

- A.1 Growth media
- A.2 Antibiotic solutions
- A.3. Enzyme solutions
- A.4. Stock solutions
- A.3. Solutions for chromosomal DNA extractions
- A.4. Solutions for RNA extraction.
- A.5. Solutions for mini-scale isolation of plasmid DNA
- A.6. Solutions for native acrylamide page and catalase activity
- A.7. Solutions for Southern hybridization analysis
- A.8. Solutions for agarose gel analysis
- A.9. Solutions for Bradford assays

A.1 GROWTH MEDIA

Media were prepared with deionised water, unless indicated otherwise.

All growth media were autoclaved before use, unless indicated otherwise.

Tryptone broth

10 g tryptone
10 g NaCl
H₂O to 1000 ml

Luria broth

10 g tryptone
5 g yeast extract
5 g NaCl
H₂O to 1000 ml

Tryptone broth for melanin-induction

10 g tryptone
40 g NaCl
5g L-tyrosine (Sigma)
5 mg CuSO₄
H₂O to 1000 ml

For agar: Add 15 g of agar per 1000 ml of broth.

A.2 ANTIBIOTIC SOLUTIONS

Ampicillin (100 mg / ml stock solution)

Dissolve 1g of ampicillin powder in 10 ml of H₂O. Filter sterilize. Store at 4°C.

Dilute to concentration of 100 µg/ ml in media

Rifampicin (50 mg / ml)

Dissolve 1g into 20 ml of methanol. Filter sterilize. Store at 4°C.

Dilute to concentration of 50 µg/ ml in media.

A.3. ENZYME SOLUTIONS

Proteinase K (20mg / ml)

Dissolve 20 mg in 1ml sterile HO. Store at - 20 °C.

RNase A (10mg/ml)

Dissolve 100 mg of RNase A in 10mM Tris-HCl, 15mM NaCl (pH7.5)

Heat for 15 minutes at 100 °C to remove DNase activity. Cool to room temperature. Store as aliquots at -20 °C.

Lysozyme (80 mg/ml)

Dissolve 0.8 g into 10 ml sterile H₂O.

Aliquot and store at -20 °C.

A.4. STOCK SOLUTIONS

Ammonium acetate (7.5 mM)

Dissolve 262.84 g in 400 ml of H₂O. pH to 7.4 using glacial acetic acid. Make up to 500 ml.

Autoclave.

CaCl₂ (1 mM)

Dissolve 14.7 g (CaCl₂·H₂O) into 100 ml H₂O.

DEPC treated H₂O.

Add 1ml of DEPC to 1 liter of H₂O. Allow to stand overnight. Autoclave to remove all traces of DEPC. DEPC supplied by Sigma.

EtOH (70%)

Mix 70 ml of absolute ethanol and 30 ml of sterile H₂O. Store at - 20° C.

EDTA (0.5 M)

Add 400 ml of H₂O to 93.05 g of EDTA. Add 10g of NaOH pellets. Adjust to pH 8.

Adjust volume to 500 ml. Autoclave.

MgCl₂

Dissolve 20.3 g MgCl₂

Adjust volume to 500 ml. Autoclave.

NaCl (5M)

Dissolve 29.22 g into 100ml H₂O. Autoclave.

Tris-HCl (1M)

Dissolve 12 g of Tris in 80 ml of H₂O. Adjust to pH with HCl.

Make up to final volume of 100 ml with H₂O

Tris-EDTA (TE)

Mix appropriate volumes of Tris and EDTA from stock solutions to prepare a solution of TE containing 10 mM Tris (pH 8) and 1mM EDTA. Adjust solution to 100 ml with sterile H₂O.

A.3. SOLUTIONS FOR CHROMOSOMAL DNA EXTRACTIONS**10 % SDS (Sodium dodecyl sulphate)**

Dissolve 10 g in 100 ml H₂O. Stir on warm plate, but do not overheat.

CTAB / NaCl

Dissolve 41 g NaCl in 80 ml H₂O. Add 10g of CTAB (hexadecyltrimethyl ammonium bromide) slowly to solution. Heat while stirring slowly. Adjust to final volume of 100 ml with H₂O.

Chloroform:Isoamyl alcohol (24:1)

Mix 240 ml of chloroform and 10 ml of isoamyl alcohol. Store at 4⁰C.

Saturated NaCl

Dissolve 40 g of NaCl in 100 ml of H₂O. Stir until solution reaches saturation.
Autoclave.

Phenol:Chloroform: Isoamyl alcohol (25:24:1)

Mix at ratio of 25:24:1

Store at 4 °C.

A.4. SOLUTIONS FOR MINI-SCALE ISOLATION OF PLASMID DNA

TENS Lysis solution

1 x TE, pH 8

0.5 % SDS

0.1 M NaOH

For midi scale preparations of plasmid, the Qiagen Plasmid extraction kit was used.

Potassium acetate (KOA)

3M KOA

pH using glacial acetic acid. Autoclave.

A.5. SOLUTIONS FOR NON-DENATURING (NATIVE) PAGE ANALYSIS

4 X Resolving Buffer (1.5 M Tris-Cl, Ph 8.8)

Dissolve 36.3 g Tris base in 150 ml of H₂O. Adjust pH to 8.8. with HCl. Adjust to final volume of 200 ml with H₂O.

Electrophoreses Buffer

Tris- base 12.1 g

Glycine 57.6 g

Make up to 4 liters with water. No need to adjust pH.

TEMED

Supplied by Sigma

Ammoniumpersulphate (10%)

Dissolve 1g Of APS in 10ml of H₂O. Dispense in aliquots of 100 µl. Store frozen at -20 °C.

Activity stain for catalase

Dissolve 2 g of potassium ferricyanide and 2 g of ferrichloride in 100 ml H₂O. Prepare fresh on day of use.

30 mM H₂O₂ stock

340 µl of 30% stock

Make up to 100 ml with H₂O.

A.6. SOLUTIONS FOR SOUTHERN HYBRIDIZATION ANALYSIS

20 X SSC

NaCl	17.5 g
Tri-NaCitrate	8.82 g
H ₂ O	80 ml

Adjust pH to 7.4 with NaOH. Make up to final volume of 100 ml with H₂O. Autoclave.

25% SDS

Dissolve 250 g in 1 000 ml of H₂O. Stir while heating. Do not overheat.

0.25 M HCl

Mix 21.35 ml of 30% HCl and H₂O to final volume of 1 000 ml

0.4 M NaOH

Dissolve 16 g in 1000 ml H₂O.

0.4 M NaOH / 1 M NaCl

NaOH	16 g
NaCl	58.44 g

Dissolve and adjust the volume to 1 000 ml with H₂O

PB stock (1M Na₂HPO₄, pH 7.2)

Na ₂ HPO ₄ ·7H ₂ O	134 g
H ₃ PO ₄ (85%)	4 ml

Make up to 1 000 ml with water. Autoclave.

Church hybridization buffer

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

Make up to 100 ml with H₂O.

Wash Buffer A (WBA)

PB stock solution	20 ml
EDTA (0.5M)	1 ml
SDS(25%)	100 ml
Make up to 500 ml with H ₂ O.	

Wash Buffer B (WBB)

PB stock solution	40 ml
EDTA (0.5M)	2 ml
SDS(25%)	40 ml
Make up to 1000 ml with H ₂ O.	

STE (NaCl- TE)

TE buffer containing 0.1 M NaCl

Sephadex G-50

Slowly add 30 g of Sephadex G-50 to 250 ml STE Buffer. Autoclave. Store at 4 °C.

Tracking dye

Dissolve Dextran Blue to final concentration of 3% in 50 mM NaCl. Dissolve Orange G in this solution to final concentration of 1%.

A.7. SOLUTIONS FOR AGAROSE GEL ANALYSIS

Tracking Dye

Bromophenol Blue	62.5 g
Sucrose	10 g
EDTA	1 ml

Make up to volume of 25 ml with H₂O. Autoclave.

50 X TAE (Tris-acetate Buffer)

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

Make up to 1 000 ml with H₂O. Autoclave.

Ethidium Bromide (10 mg/ ml)

Dissolve 0.1 g in 10 ml H₂O. Shake well to dissolve. Wear gloves.

A.8. SOLUTIONS FOR BRADFORD ASSAYS

Bovine serum albumin (BSA, 1 mg/ ml)

Dissolve 10 mg in 10 ml sterile H₂O. Store at -20 °C.

NaCl (0.15 M)

Dissolve 880 mg in 100 ml H₂O. Autoclave.

Coommassie Brilliant Blue Solution

Dissolve 100mg in 50 ml of 95 % ethanol. Add 100 ml of 85 % phosphoric acid.

Make up to 1 000 ml with H₂O. Filter through Whatman No.1 paper.

Store at 4 °C.

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APPENDIX B

STANDARD METHODS

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- B.1 Preparation of bacterial competent cells
- B.2 Transformation of competent cells
- B.3 Large scale preparation of bacterial genomic nucleic acids
 - 1. Isolation of DNA
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 - 1. Capillary transfer of DNA to a nitrocellulose membrane
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 - 1. Preparation of crude bacterial cell extracts by sonication
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B.1 Preparation of bacterial competent cells

Preparation of *E.coli* competent cells (Dagert and Ehrlich, 1979)

1. Inoculate a single colony of *E.coli* (fresh overnight plate) into 5 ml of LB and shake overnight 37°C.
2. Inoculate this 5ml culture into 100 ml of prewarmed LB. Grow at 37°C until OD (600 nm) reaches approximately 0.35 - 0.4.
3. Transfer the culture to a GSA centrifuge tube and centrifuge, at 4°C, for 5 minutes at 5 000 rpm.
4. Decant the supernatant fraction. Resuspend the pellet into 100 ml ice cold 100 mM MgCl₂. Leave on ice for 1 minute.
5. Collect cells as before and resuspend in 50 ml 0.1 M CaCl₂. Incubate on ice for 2 hours.
6. Collect cells as before and resuspend in 10 ml 0.1 M CaCl₂.
7. Aliquot 100 µl aliquots into 1.5 ml microcentrifuge tubes and store at -70°C.

B.2 Transformation of competent cells (Dagert and Ehrlich, 1979)

1. Add 1 to 50 ng of plasmid DNA to 100 µl of competent cells.
2. Leave on ice for 10 minutes.
3. Heat shock cells at 37°C for 5 minutes.
4. Add 900 µl LB and incubate for 30 - 60 minutes at 37 °C.
5. Plate 100 µl of cells on LA containing the appropriate antibiotic.
6. Incubate plates overnight at 37°C.

B.3 Large scale preparation of bacterial nucleic acid

1. Isolation of genomic DNA (Ausubel et al. , 1989, unit 2.4)

1. Pellet 100 ml of cells from overnight culture by centrifugation at 4 000 rpm for 10 minute.
2. Discard supernatant and resuspend cells in 9.5 ml of TE buffer (Appendix A)
3. Add 500 µl of 10% SDS solution and 50 µl of proteinase K (20mg / ml stock), mix and incubate for 1 hour at 37°C.
4. Add 1.8 ml of 5 M NaCl (Appendix A.3) and mix thoroughly.
5. Add 1.5 ml of CTAB/ NaCl (Appendix A.3) and mix well.
6. Incubate at 65°C for 20 minutes.
7. Extract with an equal volume of chloroform : isoamyl alcohol (Appendix 3)

8. Centrifuge at 6 000 rpm for 10 minutes, at room temperature.
9. Transfer the aqueous phase to a clean tube.
10. Precipitate DNA by 0.6 volumes of isopropanol.
11. Centrifuge at 10 000 rpm for 10 minutes.
12. Wash pellet with 1 ml 70% EtOH.
13. Resuspend in 1ml TE.
14. Measure DNA concentration at 260 nm on a spectrophotometer.

2. Isolation of bacterial genomic RNA (Ausubel et al. , 1989, unit 4 .4)

1. Grow bacteria in the appropriate liquid media.
2. Harvest cells by centrifugation at 10 000 rpm for 10 minutes.
3. Resuspend cells in 10 ml of protoplasting buffer.
4. Add 80 μ l of 50 μ g/ml lysozyme and incubate on ice for 15 minutes.
5. Collect the resulting protoplasts by centrifuging at 7 000 rpm for 5 minutes.
6. Resuspend the protoplast pellet in 500 μ l lysing solution. Add 15 μ l DEPC, mix gently and transfer to microcentrifuge tube.
7. Incubate on at 37 °C for 5 minutes.
8. Chill on ice.
9. Add 250 μ l salt-saturated NaCl. Mix by inversion.
10. Incubate on ice for 10 minutes.
11. Centrifuge for 10 minutes. Remove the supernatant to 2 clean microcentrifuge tubes.
12. Add 1 ml absolute ethanol to each tube and precipitate for 30 minutes on dry ice or overnight at -20 °C.
13. Centrifuge at 4 °C for 15 minutes.
14. Rinse with 70% ethanol and dry pellet.
15. Dissolve pellet in DEPC-treated water.
16. Add 10 U of DNaseI to RNA, in a volume of 50 μ l.
17. Incubate at 37 °C for one hour.
18. Purify RNA further by extraction with an equal volume of phenol: chloroform:isoamylalcohol (25:24:1).
19. Centrifuge at 14 000 rpm for 10 minutes.
20. Transfer aqueous phase to clean tube.
21. Precipitate RNA with 5 μ l of sodium acetate (5 M) and 150 μ l absolute ethanol.
22. Centrifuge at 14 000 rpm for 10 minutes.

23. Resuspend pellet in DEPC -treated water.
24. Quantify RNA by reading the absorbance at 260 nm.

B.4. Mini-scale isolation of plasmid DNA. (Zhou, 1990)

1. Spin 1.5 ml of cells from an overnight culture
2. Remove supernatant fraction, leaving 100 μ l behind. Resuspend cells in this fraction.
3. Add 300 μ l of TENS lysing solution. Vortex 2 - 3 seconds.
4. Add 150 μ l of 3 M potassium acetate (pH 5.4)
5. Spin for 5 minutes at 14 000 rpm.
6. Remove the supernatant fraction into clean tube.
7. Add 900 μ l absolute ethanol.
8. Spin for 5 minutes.
9. Wash the pellet with 70% ethanol.
10. Resuspend in 30 μ l TE, containing RNase. Use 3 μ l of this preparation on a slide agarose gel.

B5. Restriction endonuclease digestions (Ausubel et al. , 1989, Unit 3.1)

1. Pipette 100 ng to 4 μ g of DNA into a sterile microcentrifuge tube.
2. Add 2 μ l of the appropriate restriction enzyme buffer (supplied with enzymes by manufacturer).
3. Add sterile H₂O to volume of 18 μ l.
4. Add 1 - 5U of enzyme per μ g of DNA.
5. Incubate for 1 to 2 hours at the appropriate temperature.
6. For multiple digestions, first cleave with one restriction enzyme, precipitate with ammonium acetate precipitation, then cleave with the second enzyme.

B.6 Agarose gel electrophoreses (Ausubel et al. , 1989, Unit 2.5)

1. Prepare agarose in 1 X TAE by heating in microwave oven.
2. Add ethidium bromide (Appendix A.8) to a final concentration of 0.5 μ g/ ml.
3. Cool the agarose to approximately 55 oC before pouring onto gel casting tray.
4. Insert gel comb, ensuring no bubbles are trapped underneath comb.
5. When gel has hardened, remove the gel comb and place the gel tray in the electrophoreses tank.
6. Add sufficient 1 x TAE to cover gel.
7. Mix DNA with loading dye and load into the wells.
8. Attach leads to tank so that DNA migrate towards the anode.
9. Electrophoreses is performed at 1 - 10 V/ cm until the dye reach the end of the gel.

B7. Quantitation of DNA

Dilute the DNA or RNA in TE or sterile H₂O. Measure the absorbance of nucleic acids by doing a scan between 220 and 310nm with in the presence of ultraviolet light. 1 OD at 260 nm is equivalent to 50 µg/ ml of double stranded DNA or 40 µg/ ml single stranded RNA.

For determination of nucleic acid concentration with ethidium bromide:

1. Prepare lambda DNA standards with known concentrations of 5 ng /µl, 10 ng /µl, and 20 ng /µl.
2. Mix 10 µl from each standard with 2.5 µl of tracking dye and load into the wells of a thin 1% TAE agarose gel.
3. Prepare several dilutions of DNA samples of unknown concentration in 10 µl. Add 2.5 µl of tracking dye and load into the wells next to the standards.
4. Electrophores at 100 V for 5 minutes.
5. Visualise bands at 254 nm using an UV transilluminator.
6. Determine the concentration of the DNA sample by comparing the band intensities with those of the standards.

B.8. Electroelution of DNA fragments from agarose gels (Ausubel et al. , 1989, Unit 2.6).

1. After electrophoreses of digested DNA, cut selected band of DNA out of gel under long ultraviolet light (310 nm). Use a sterile blade.
2. Rinse dialysis tubing with 1 X TAE buffer. Tie of one end of tubing with a dialysis clip.
3. Slide the gel into open end of the tube. Add 500 µl of TAE (1 X) to the bag.
4. Seal the open end with another dialysis clip.
5. Place sealed tube into electrophereses tank and fill with 1X TAE. till bag is covered. Orientate the bag that it is parallel with the electrodes. Take care that the gel slice is against the side of the bag facing the anode.
6. Electroelute at a constant voltage of 2V / cm overnight.
7. Recover DNA using ammonium acetate precipitation.

B.9 Ammonium acetate precipitation (Coyne et al., 1996)

1. Add half the volume of 7.5 M ammonium acetate (Appendix A.1) to the DNA suspension.
2. Incubate at room temperature for 15 minutes.
3. Centrifuge at 14 000 rpm for 15 minutes.
4. Transfer supernatant to a clean microcentrifuge tube.
5. Add 2.5 volumes of 100% ethanol. Incubate at room temperature for 30 minutes.

6. Centrifuge at 14 000 rpm for 30 minutes at room temperature.
7. Wash pellet with 70% ethanol.
8. Resuspend DNA IN 10µl of TE.
9. Determine DNA concentration with the aid of lambda standards.

B.10. Generating blunt-ended fragments (Ausubel et al., 1989, Unit 3.5)

1. Add 1 µl of dNTPs (0.5 mM stock) to 20 µl DNA (digested with restriction enzymes) containing 1 - 4 µg of restriction enzyme digested DNA.
2. Add 1µl of Klenow and incubate at 30 °C for 30 minutes.
3. Stop the reaction by heating to 75 oC for 10 minutes or by adding 1 µl of EDTA (0.5 M stock).

B.11. Ligations (Coyne et al., 1996)

For intramolecular ligations:

1. Use approximately 1 pmol of DNA.
2. Add 2 µl ligation buffer (supplied by manufacturer).
3. Make up to 18 µl with sterile water.
4. Add 2 µl of enzyme containing 2U of T4 ligase.
5. Incubate reaction mixture at 15°C overnight.

For intramolecular ligations:

The ligation mixture in total contains a maximum of 10 pmol of DNA.

The ratio of vector : insert DNA is 1:1 to 1:4.

1. Add the linearised vector and the target fragment to a sterile microcentrifuge tube.
2. Add 2 µl of 10 x ligation buffer.
3. Add 2 U of T4 ligase.
4. Add sterile H₂O to a final volume of 20 µl.
5. Ligation of DNA fragments with cohesive ends is incubated at 15°C, whereas blunt-ended fragments are incubated at room temperature. For the latter fragments, also add 10 x more ligase enzyme.

B12. Southern hybridisation procedure (Reed and Mann, 1985)

1. Southern transfer of DNA to a nitrocellulose membrane

1. After electrophoreses, soak the gel in 2 x volumes of 0.25 M HCl for 5 minutes at room temperature.
2. Rinse the gel in deionised H₂O.
3. Saturate 10 sheets of Whatman 3MM paper with 0.4 M NaOH.
4. Place the sheets on top of an inverted gel casting tray which has been placed inside a tray. Add enough of the 0.4 M NaOH / 1 M NaCl solution to the tray so that the ends of the Whatman paper is submerged.
5. Invert the gel and place on top of the saturated Whatman paper. Ensure that no air bubbles are trapped beneath gel.
6. Cut Hybond N+ nylon membranes the size of the gel.
7. Wet the membrane with deionised H₂O and place on gel, once again ensuring that no air bubbles remain.
8. Cover the edges with Saran wrap.
9. Place 3 sheets of Whatman 3 MM paper over the membrane. On top of this, place a 10 cm stack of dry absorbant paper towel.
10. Place a glass plate on top of the towels. Complete the blotting system by placing a weight of maximum 400 g on top of the glass plate.
11. Blot for 4 hours minimum to overnight.
12. When removing the membrane after blotting, mark the wells of the gel on the membrane.
13. Rinse the membrane with 2x SSC for 5 minutes at room temperature.
14. Air dry the membrane. Store between 2 sheets of Whatmann 3MM paper until used.

2. Labelling linearised DNA fragments with random prime labelling.

1. Random prime labelling was performed according to the protocol prescribed by the manufacturer (Roche) .
2. Denature 25 ng of the DNA fragment by heating at 95°C for 10 minutes. Immediately place on ice when removing from heating block.
3. Add 3 µl of the dATP, dGTP, dTTP mixture supplied in the kit, as well as 2 µl of the hexanucleotide reaction mixture (supplied in kit).
4. Add 5 µl [α - ³² P] dCTP (3 000 Ci/ mmol) and H₂O to final volume of 19 µl.
5. Add 1 µl of Klenow enzyme and incubate at 37 °C for 30 minutes.
6. Stop the reaction by heating to 65 °C for 10 minutes.
7. Before using the radiolabelled fragment, it has to be separated from unincorporated nucleotides using a spin column procedure.

3. Spin column procedure to separate radioisotope labelled DNA from unincorporated nucleotides (Ausubel et al., 1989, Unit 3.4).

1. Plug the bottom of a sterile 1 ml disposable syringe with a small amount of sterile glass wool.
2. Prepare a Sephadex G-50 column with a 0.9 ml bed volume in the syringe.
3. Wash the column with 100 µl of STE.
4. Add 10 µl of tracking dye and 40 µl of STE to the single stranded, labelled probe.
5. Place a sterile 1.5 ml microcentrifuge tube inside a bigger centrifuge tube (volume of 10 ml). Place the column inside the microcentrifuge tube so that the separated probe will empty inside the microcentrifuge tube.
6. Load the DNA onto the column and centrifuge for 4 minutes at 14 000 rpm.
7. The labelled probe moves with the Dextran blue dye, whereas the unincorporated nucleotides move with the Orange G. The latter remains on the column.
8. Add 1 µl of probe in 2 ml scintillation fluid and determine the specific activity of labelled DNA with the aid of a scintillation counter. Specific activity = counts per minute (cpm) / µg DNA.

4. Prehybridization, hybridisation and washing of Southern blots (Church and Gilbert, 1984).

1. Seal the membrane containing DNA in a plastic bag to which $200 \mu\text{l} / \text{cm}^2$ of pre-hybridisation CHB was added.
2. Incubate the sealed bag at 65°C for 1 hour with agitation.
3. Denature the probe by heating at 100°C for 10 minutes and place immediately on ice.
4. Remove the pre hybridisation mixture from the bag. Add Church hybridisation buffer (CHB) to the bag ($50 \mu\text{l} / \text{cm}^2$), as well as 1×10^6 cpm / ml of labelled probe.
5. Remove the bubbles and heat seal the bag.
6. Hybridise overnight overnight at 65°C , with agitation.
7. Wash the membrane with wash buffer A (WBA), and wash buffer B (WBB), for 10 minutes each at 65°C . MONITOR THE RADIOACTIVITY IN-BETWEEN WASHES.
8. Stop the washing once the counts on the membrane reaches 200 - 500 cpm on the membrane.
9. Seal inside a new bag. Ensure that no bubbles are trapped inside the bag.
10. Place bag inside an X-ray cassette containing enhance screens.
11. Expose the membrane to an X-ray film and develop in an automatic X-ray film processor.

B13. DETERMINING CATALASE ACTIVITY OF BACTERIAL EXTRACTS.

1. Preparation of crude bacterial cell extracts by sonication

1. Grow cells in appropriate media (LB for *E. coli*, TB for *V. cholerae*).
2. Pellet 20 - 50 ml of cells by centrifugation for 10 minutes at 5 000 rpm.
3. Wash cells once in phosphate buffer.
4. Resuspend cells in 1/10 th volume of the original volume with phosphate buffer.
5. Transfer cells to sterile glass container before sonication.
6. Prepare an ice-water bath by mixing ice and ethanol.
7. Sonicate the cells while cells are kept cold in the ice-water bath. **Sonication** : 4 bursts of 20 seconds each at 25% output. Between each 20 second burst, there is a 20 second pause in the sonication to ensure that extracts remains ice-cold and prevent denaturing of proteins.
8. Remove cell debris by centrifugation AT 14 000 rpm for 15 minutes at 4°C .
9. Extracts are stored at -20°C till analysed.

2. Preparation of acrylamide gel and electrophoreses.

For 8% non-denaturing acrylamide gel, mix together:

- 2ml of acrylogel mix (40% stock, supplied by BDH Chemicals)
- 2.5 ml of resolving buffer
- 5.445 ml of water
- 50 μ l APS (10% stock)
- 5 μ l of TEMED.

3. Activity stain for catalase activity (Wayne and Diaz, 1986).

1. After electrophoreses, the gel is rinsed briefly in H₂O.
2. The gel are immersed in a solution containing 5 mM H₂O₂ for 20 minutes.
3. Thereafter the gel are immersed in a solution containing 1% ferric chloride and 1% potassium ferricyanide. The stain was removed is removed when gel turns green.
4. Areas containing yellow bands represent the catalase.

4. Determination of specific catalase activity (Beers, et al., 1951, adapted by Visick and Clarke, 1997).

(Method essentially as described by references above)

1. *Mix inside a quarts cuvette:* An aliquot of the crude protein extracts containing 100 μ g of protein mixed with phosphate buffer in a maximum volume of 2 ml.
2. The cuvette is placed inside a spectrophotometer.
3. The reaction is started by adding 1ml of H₂O₂ to the mixture. The concentration of H₂O₂ should be 10 mM (thus add 1 ml from a 30 mM stock).
4. Record the change of UV absorbance at 240 nm every 15 minutes for 60 seconds. The starting optical density should be close to 0.5. The change in absorbance over 15 seconds should not be more than 0.1, but not less than 0.02.
5. Calculate the specific activity of H₂O₂ with the following formulae:

$$\frac{1\ 000 \times \Delta A_{240\text{nm}} / \text{minute (average)}}{43.6 \times \text{mg of protein/ml of reaction mixture}}$$

where $\Delta A_{240\text{nm}}$ = difference in absorbance at 240 nm;

43.6 = the extinction coefficient of H₂O₂ at 240 nm.

B14. Alkaline gel electrophoreses

Prepare 0.5% agarose gel in neutral buffer. Then soak it in alkaline electrophoreses buffer for one hour. Add alkaline tracking dye to to DNA sample and load gel. DNA is electrophoresed in alkaline electrophoreses buffer, at very low voltage (maximum 50 volts in a big tank). After running, neutralize gel in 0.1M Tris buffer, Ph 8, then stain with ethidium bromide.

Neutral buffer

2.92g NaCl

2 ml EDTA (0.5 M stock)

Prepare 1 liter buffer in water. Autoclave.

Alkaline electrophoreses Buffer

1.2g NaOH

4 ml EDTA (0.5 M stock)

Make up to 1 litre with water. Autoclave

Alkaline tracking dye

12 mg bromocresol green

2.5 ml glycerol

Make up to 9 ml with water. Autoclave. Add 1 ml of 5M NaOH, just before using.

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