Investigation of mesophilic *Aeromonas*: Response to hydrogen peroxide and role in false-positive Colilert reaction

by

Julien B. P. Landré

A thesis submitted to The Robert Gordon University in partial fulfilment for the requirement of the degree of Doctor of Philosophy

The Robert Gordon University

Faculty of Science and Technology

May 1999

Table of contents

Chapter 2. False-positive reaction induced by the presence of A. hydrophila in the Colilert defined-substrate technology system.

Chapter 3.The oxidative stress response of A. hydrophila.

Chapter 4. Stress response in A. hydrophila: the possible role for quorum sensing.

Chapter 5. General discussion

Chapter 6. Future Work

Chapter 7. Bibliography

Appendix

Published material

Table of Figures

Chapter 4

Table of Tables

* Species determined using genetic testing (e.g. AFLP).
** Species determined using phenotypic testing.

Acknowledgements

Abbreviations

A.: *Aeromonas*

AFLP: Amplified fragment length polymorphism

AHL: acyl homoserine lactone

AI: Autoinducer

BHL: N-butyryl-L-homoserine lactone

cfu: colony forming unit

ClO^- : hypochlorite ion

Er. carotovora: *Erwinia carotovora*

E.coli : *Escherichia coli*

En. faecalis: *Enterococcus faecalis*

GSH: Glutathione

GSSG: oxidised glutathione

HG: hybridisation groups

$HO^{\cdot}$: hydroxyl radical

$HOO^{\cdot}$: Peroxyl radical

H_2O_2 : hydrogen peroxide

K. oxytoca:: *Klebsiella oxytoca*

NA: nutrient agar

NB: nutrient broth

NCIMB : National Collection of Industrial and Marine Bacteria

O_2^-: Superoxide anion

*O*NP: ortho-Nitrophenol

*O*NPG: ortho-Nitrophenol-β-galactopyranoside

Ps. aeruginosa : *Pseudomonas aeruginosa*

ROS: Reactive oxygen species

R. solanacearum: *Ralstonia (Pseudomonas) solanacearum*

S. typhimurium: *Salmonella typhimurium*

Abstract

Investigation of mesophilic *Aeromonas*: Response to hydrogen peroxide and role in false-positive Colilert reaction

Mesophilic *Aeromonas* are opportunistic human pathogens which produce a wide range of virulence factors and have been isolated from both untreated and chlorinated drinking waters. The presence of these microorganisms in the distribution systems suggests that *Aeromonas* could display an adaptive response to oxidant present during water treatment. This adaptive response of *Aeromonas* would lead to interference in analysis for faecal coliforms used to determine the quality of potable drinking water, and be a potential source of intestinal disorders.

The Colilert defined substrate technology system was developed as a one-step detection of both coliforms and *E. coli* while suppressing non-coliform heterotrophic growth. *Aeromonas* species were previously shown to cause production of false-positive reaction at high cell densities (Edberg *et al.*, 1988). Similar results were obtained in our study when using fresh Colilert reagents. However, results obtained during this project showed *Aeromonas* to mediate false-positive reactions at low cell densities (10^1 cells/ml in presence of salt, 10^2 cells/ml in absence of salt) when using Colilert reagents within 4 weeks prior to shelf-life expiry. Increased incidence in false-positive reactions mediated by *Aeromonas* were shown to be dependent upon the stability of the Colilert reagent affected with age. Such *Aeromonas* interference would lead to over-estimation of coliforms and *E. coli* in potable drinking water supplies.

The ability of bacteria to adapt to a wide range of stress factors such as pH, heat shock, oxidants or starvation has been extensively studied. Little is known about the response of *Aeromonas* to such stress conditions. During this project, it has been demonstrated that mesophilic *Aeromonas* display an adaptive tolerance response to a lethal oxidative challenge through pre-treatment with a sub-lethal dose of oxidant. The stress adaptation process was demonstrated to occur through synthesis of stress proteins and modulation of pre-existing catalase. Of the species studied, *A. sobria* was most sensitive, whereas *A. caviae* and *A. hydrophila* displayed similar responses to oxidative stress. The hypersensitivity of *A. sobria* did not impair the adaptive response of the organism. During our investigations, stationary phase *Aeromonas* cells have been shown to be more resistant than their logarithmic counterpart and suggested that excreted molecules may play a role in protecting the cells. Re-suspension of fresh cells into spent medium from a stationary phase cells revealed a higher resistance of these cells compared to those re-suspended in minimal medium. This resistance was demonstrated to be mediated by non-proteinaceous, thermo-sensitive effector molecule. A potential candidate as the effector molecule, butyryl homoserine lactone, was synthesised and assayed. Preliminary data strongly suggest that this molecule has a role to play in the stress adaptation phenomenon and might be involved in stimulating synthesis of key stress proteins.

PhD aims

Mesophilic *Aeromonas* are aquatic microorganisms which have been isolated from both untreated and chlorinated drinking waters. Members of this group have also been recognised as opportunistic human pathogens leading to both extra-intestinal e.g. deep wound infections and meningitis, as well as intestinal infections e.g. gastro-enteritis. Mesophilic *Aeromonas* have been recovered from drinking water supplies despite a high chlorine residual. Chlorine is a strong oxidative agent which is used in water treatment due to its action on various cellular targets such as membranes, proteins and DNA and should therefore dramatically reduces bacterial numbers in treated waters. The presence of mesophilic *Aeromonas* in the distribution systems is of relevance for both the water industries and the public health authorities, as it can lead to interference during routine analysis of indicator organisms used to determine water contamination, and can be a potential source of gastro-enteritis due to production of a wide range of virulence factors. Chapter 2 describes the investigations of the potential of mesophilic *Aeromonas* to produce false-positive reactions at low cell densities in the Colilert defined-substrate technology system. This involved the study of potential degradation of the reagent during storage by testing different cell concentrations at different times intervals during the storage. Other parameters which might influence the false-positive reactions were investigated such as the NaCl content of the cells dilution medium. More importantly, study of the effect of nutrient limitation and chlorine is essential as they are important parameters when dealing with routine analysis of water samples. This chapter demonstrates the importance of learning more about *Aeromonas* species as they can affect results of routine analysis of water and therefore cast a shadow on the validity of the results concerning the quality of the drinking water distributed.

Adaptive tolerance response have been extensively studied in *Escherichia coli* and *Salmonella* spp., but very little work has been performed on the general stress response of *Aeromonas* spp. Recently it was demonstrated that *Aeromonas* spp. display an adaptive tolerance to pH (Karem et al., 1994). The fact that *Aeromonas* spp. can be recovered from chlorinated drinking water supplies suggested a possible resistance to oxidative stress. The work described in Chapter 3 relates experiments which would allow determination of whether *Aeromonas* displays an adaptive response to oxidative stress. For this purpose it was necessary to investigate the range of lethality of H_2O_2 using standard growth curve methods. From these results, appropriate lethal and sub-lethal doses would be determined and used for the adaptation study of some members of the *Aeromonas* genus. The next step of this project was to gain an insight on how *Aeromonas* would respond to oxidative challenges and investigating the relative time scale protection from a sub-lethal dose could provide to cells prior to lethal challenge. The hypothesis we wished to test envisaged that adaptation required several mechanisms such as fresh synthesis of key stress proteins and/or the modulation of pre-existing enzymes such as catalase. The latter part of this chapter dealt with the study of the effect of growth phase and culture age in the oxidative response as it is known that the cell metabolism is dissimilar at different stages of growth.

Data obtained during investigations on the stress response (detailed in chapter 3) have shown that the longer the cells were in stationary phase, the more resistant to the H_2O_2 lethal dose *Aeromonas* species were. Reports on cell-density dependant catalase production in *Rhizobium leguminosarium bv. phaseoli* (Crockford *et al.,* 1995) and papers on the isolation of acyl homoserine lactone compounds (bacterial signal molecule involved in cell-density mechanisms) in *Aeromonas* species have appeared in

the literature (Swift *et al.*, 1997). The aim of this study was therefore to investigate the possible role of cell-density and autoinducer molecules in modulating the oxidative stress response mechanism in *A. hydrophila*. Firstly, investigation on the nature of the molecule(s) involved in the mediation of the stress response was undertaken by re-suspension of non-stressed cells into supernatant of fresh cells and challenged with a known lethal dose of H_2O_2 without prior oxidative pre-treatment. Cells remained viable suggesting presence of excreted molecules playing a role in the protection of the cells. The next step was therefore to undertake a series of experiments to determine the nature of these molecules. The second part of this investigation was to synthesise the homoserine lactone derivative and assess the role of this potential candidate (BHL) in mediating the response of non-pre-treated *A. hydrophila*.

CHAPTER 1. Introduction

1.1. *Aeromonas* species.

*A*eromonas spp. are classified as members of the *Vibrionaceae* family (Richard *et al.*, 1991; Farmer *et al.*, 1992) along with *Vibrio*, *Plesiomonas*, and *Photobacterium*. However there is some evidence that the genus *Aeromonas* should be a separate family, the *Aeromonadaceae* (Colwell *et al.*, 1986; Holmes, 1992; Esteve, 1995). *Aeromonas spp.* are waterborne species, being Gram negative, oxidase positive and facultatively anaerobic (Popoff, 1984). Two well-separated groups constitute the genus *Aeromonas* (Popoff, 1984). The first group consists of a single psychrophilic, non-motile species called *Aeromonas salmonicida* divided into 4 biovars namely *A. salmonicida bv salmonicida*, *A. salmonicida bv achromogenes*, *A. salmonicida bv masoudia* and *A. salmonicida bv smithia* (Carnahan and Altwegg, 1996: Table 1-1). However the sub-species *masoudia* is included in subspecies *achromogenes* (Austin and Adams, 1996). *A. salmonicida* is an important fish pathogen and is the cause of furunculosis of salmon characterised by boil-like lesions (i.e. furuncles, internal haemorrhaging and necrosis). *A. salmonicida* mortality in fish appears to be due to localised growth in muscles rather than through septicaemia (Austin and Adams, 1996), but is not considered pathogenic for humans as it has not yet been isolated or incriminated in human diseases (Gosling, 1997). The second group constitutes the mesophilic and motile species, described by Popoff (1984). Some mesophilic strains are now known to be opportunistic human pathogens responsible for both intestinal and extra-intestinal infections (Namdari and Bottone, 1990a, Namdari and Bottone, 1990b; Wadstrom and Ljungh, 1991; Sloan, 1995).

1.1.1 Historical perspective and taxonomy of the genus *aeromonas*.

Aeromonas have been isolated from cold and warm-blooded animals for over a 100 years (Sanarelli, 1891) and from human beings since the early 1950's (Hill *et al.*, 1954). Over the past 100 years, the taxonomy of the genus *Aeromonas* has been very confused, the details of which will be considered here in a chronological fashion to ease comprehension. Zimmermann, in 1890, isolated from a drinking water supply bacteria which exhibited a typical dotted growth on gelatine agar, leading to the name *Bacillus punctatus* (definition from Altwegg and Geiss, 1989). In 1891, Sanarelli reported a species that he termed *Bacillus hydrophilus fuscus*. This bacteria was isolated from the blood and fluids of a cold blooded animal (i.e. a frog). This bacillus was proven, after injection, to be pathogenic to cold and warm-blooded animals which developed septicaemia (Sanarelli, 1891). In 1901, *Bacillus hydrophilus fuscus* was renamed as *Bacterium hydrophilus sanarelli* due to the affinity of this bacterium for water (Chester, 1901). For the next 60 years, *Aeromonas* have been part of several genera including *Aerobacter, Proteus, Pseudomonas,* and finally *Vibrio* (Carnahan and Altwegg, 1996).

In 1936, Kluyver and Van Niel proposed the genus *Aeromonas* on the basis of morphological and physiological characters, especially on the ground of carbohydrates fermentation with production of carbon dioxide and hydrogen (Kluyver and Van Niel, 1936). Further in 1957, Snieszko proposed four species for the genus *Aeromonas* namely *A. hydrophila, A. punctata, A. liquefaciens* for the motile species, and *A. salmonicida* for the non-motile species (Snieszko, 1957). All four species were at that time part of the *Pseudomonadaceae* Family.

During the 1960's several taxonomic studies were conducted and led to conflicting results. For example, Ewing and colleagues proposed *A. hydrophila* and *A. shigelloides* for the motile, mesophilic species, and *A. salmonicida* for the non-motile psychrophilic

2

Aeromonas (Ewing and Johnson., 1960; Ewing *et al.*, 1961). But Eddy and co-workers proposed two species for the motile *Aeromonas* namely *A. punctata* and *A. caviae* (Eddy, 1960; Eddy and Carpenter, 1964). A key study by Popoff and Véron in 1976 using numerical taxonomy (i.e. biochemical and physiological characteristics) demonstrated that the genus *Aeromonas* could be sub-divided into two defined groups (Popoff and Véron, 1976). The non-motile group was shown to be fairly homogenous with *A. salmonicida* as the main species (Janda and Duffey, 1988), the motile group was shown to be more diverse containing *A. hydrophila* as the major member (Janda and Duffey, 1988). Popoff and co-workers, in 1981 continued their work and Popoff published a chapter on the genus *Aeromonas* in the first edition of Bergey's Manual of Systematic Bacteriology in 1984 (Popoff, 1984) and described *Aeromonas* as *"straight cells that are rod shaped with rounded ends to coccoid. Their size vary from 0.3 to 1.0 µm in diameter and 1.0 to 3.5 µm in length. They occur singly, in pairs or short chains. They are Gram negative, generally motile by a single polar flagellum, metabolise glucose through both the respiratory and fermentative pathways. Carbohydrates are broken down to acid or acid and gas (CO_2, H_2). Nitrate is reduced to nitrite. They are both oxidase and catalase positive. They show resistance to the vibriostatic agent O/129. Their optimal temperature is between 22-28°C. The mol % G+C of their DNA is 57-63 (Bd, T_m)."*

Numerous taxonomic studies have since been performed leading to the recognition of an increasing number of species, primarily determined through hybridisation groups (HG). In 1991, 13 HG groups were known (Janda, 1991) whereas in 1996, a possible 16 HG groups were proposed (Carnahan and Altwegg, 1996; Table 1-1). This continuous increase in the number of HG groups demonstrates the diversity that exists in the motile *Aeromonas* group. A lot of work in this area still remains to be done as it is likely that more species remain to be discovered; for example a seventeenth species called *A. popofii* has recently been reported

(Huys *et al.,* 1997) to substitute the species *A. bestiarum* described by Ali and co-workers (Ali *et al.,* 1996) but not included by Carnahan in her review in 1996. Progress has still to be made on biochemical tests able to differentiate *Aeromonas* species. Some tests have been described by Carnahan in her review on *Aeromonas* taxonomy (Carnahan and Altwegg., 1996). However, results from existing tests are dependant upon the temperature the tests are performed at and to resolve this issue, other taxonomic methods were considered (Altwegg, 1996). In his review, two main groups of methods were discussed, being phenotypic and genotypic techniques. The subtyping methods are various due to problems to biochemically identify *Aeromonas* to their sub-species level and the lack of genetic homogeneicity within the mesophilic members of the genus. The methods have been developed using independently isolated strains rather than strains isolated from well investigated outbreak as none have been reported as yet and poses a problem in validating subtyping methods (Altwegg, 1996). It is therefore impossible to draw exact conclusions on the origin of the strain and to establish a link between clinical and environmental strains.

Table 1-1: Current genospecies[*] and phenospecies[**] within the genus *Aeromonas* (Data from Carnahan and Altwegg., 1996; Janssen *et al.*, 1996; Huys *et al.*, 1996, Huys *et al.*, 1997).

DNA HG	Type strain	Genospecies	Phenospecies
1	ATCC 7966	*A hydrophila*	*A. hydrophila*
2	ATCC 14715	*A. bestarium*	*A. hydrophila*
3	CDC 0434-84	*A. hydrophila*	*A. hydrophila*
3	ATCC 33658	*A. salmonicida* bv *salmonicida*	*A. salmonicida*
3	ATCC 27013	*A. salmonicida* bv *masoucida*	*A. salmonicida*
3	ATCC 33659	*A. salmonicida* bv *achromogenesa*	*A. salmonicida*
3	ATCC 49393	*A. salmonicida* bv *smithia*	*A. salmonicida*
4	ATCC 15468	*A. caviae*	*A. caviae*
5A	Huys *et al* collection	*A. caviae*	*A. caviae*
5B	CDC 0435-84	*A. media*	*A. media*
5B	Huys *et al* collection	*A. caviae*	*A. caviae*
6	ATCC 23309	*A. eucrenophila*	*A. eucrenophila*
7	CIP 74.33	*A. sobria*	*A. sobria*
8	CDC 0437-84	*A. veronii*	*A. veronii* bv *sobria*
8	ATCC 9071	*A. veronii*	*A. veronii* bv *sobria*
9	ATCC 49568	*A. jandei*	*A. jandei*
10	ATCC 35624	*A. veronii*	*A. veronii*
11	ATCC 35941	*A. veronii*	*Aeromonas sp.*
12	ATCC 43700	*A. schubertii*	*A. schubertii*
13	ATCC 43946	*A. trota*	*Aeromonas* group 501
14	ATCC 49657	*A. trota*	*A. trota*
15	CECT 4199	*A. allosaccharophila*	*A. allosaccharophila*
16	CECT 4342	*A. encheleia*	*A. encheleia*
17	Huys *et al* collection	*A. popoffii*	*A. popoffii*

[*] Species determined using genetic testing (e.g. AFLP).

[**] Species determined using phenotypic testing.

1.1.2. Ecology of *Aeromonas* species.

Members of the genus *Aeromonas* are waterborne species and can be found in almost all types of aquatic environment (Schubert, 1991; Holmes *et al.*, 1996). The aquatic environment can be divided into four main types of waters, these being freshwater environments, marine waters, sewage treatment, and potable water divided into two subgroups namely treatment and distribution (Holmes *et al.*, 1996). These four types will be individually described later on in this chapter. *Aeromonas spp.* have been recovered in

5

quite high numbers in both polluted and non-polluted waters (Schubert, 1991), and even in chlorinated drinking waters (Knochel, 1990; Gavriel *et al.*, 1998). The mesophilic *Aeromonas* have been implicated as pathogens in a series of different hosts ranging from poikilothermic aquatic animals, such as fish (Austin and Adams, 1996), to infection of warm-blooded animals, including human beings (Mathewson and Dupont, 1992). Such diseases were generally due to infection of deep wounds after contact with contaminated water containing strains of *Aeromonas spp.* (Seidler *et al.*, 1980). Suggestions have been made that drinking water supplies could be a source of *Aeromonas*-associated gastro-intestinal diseases (Sloan, 1995), but the nature of the clinical and the environmental strains isolated in different studies was proven to be different (Gosling, 1997). Water (Seidler *et al.*, 1980) is an example of the various sources of *Aeromonas* contamination which are only suggestions as no evidence of an *Aeromonas*-related outbreak or animal models has been reported. It seems that the distribution and number of mesophilic *Aeromonas* is linked to the level of pollution (Miranda and Castillo, 1996; Table 1-2) and displays a seasonal variation, having a greater number during warmer summer months (Pathak *et al.*, 1988; Holmes *et al.*, 1996).

Table 1-2: Typical numbers of mesophilic *Aeromonas* species in aquatic environments (Holmes *et al.*, 1996)

Aquatic environment	Typical counts (cfu/ml)
Domestic sewage sludge	$> 10^8$
Crude sewage	$10^6\text{-}10^8$
Treated sewage	$10^3\text{-}10^5$
Waste water	$10^2\text{-}10^7$
Rivers receiving sewage discharges	$10\text{-}10^4$
Clean rivers, lakes, storage reservoirs	$1\text{-}10^2$
Seawater	$10^{-2}\text{-}10^2$
Drinking water at treatment plant	$10^{-2}\text{-}10^1$
Drinking water in distribution system	$10^{-2}\text{-}10^3$
Ground waters	< 1

1.1.2.1. Freshwater environments.

This includes two types, being groundwaters and surface freshwaters. Groundwaters are nutrient poor, and the mineral content is determined by the type of rocks present. Some *Aeromonas* species have been recovered in very low number from deep groundwaters where no coliforms were detected (Havelaar *et al.*, 1990 a). Regarding the surface freshwaters, these are far more diverse in their nutrient contents than the groundwaters. These types of waters are by far the most studied as they provide the water for the majority of human consumption and recreational activities.

In 1978, Hazen and colleagues showed that *Aeromonas* could be isolated from a wide range of freshwater environments within a temperature range of 4-45°C, and to survive temperatures as high as 55°C (Rouf *et al.*, 1971; Palumbo *et al.*, 1985). pH did not seem to have an influence in the recovery process as long as the pH of the water was in the range 5.2-9.8. *Aeromonas* were not recovered from environments having pH exceeding a value

of 10.0 or less than 4.0. It was also reported that a model could be used to predict the number of *Aeromonas* present in a sample (Hazen, 1983). A negative correlation was found between dissolved oxygen and *A. hydrophila* population, and a positive correlation with the number of faecal coliforms and heterotrophic bacteria. However, other reports have stated that no correlation exists between the number of faecal coliforms and the number of *Aeromonas* species (Miranda and Castillo, 1996). Miranda and Castillo (1996) found a moderate correlation (r = 0.69) between *A. hydrophila* numbers and the heterotrophic flora in natural waters. This correlation increases when pollution levels increase. However Holmes and co-workers reported that it was difficult to identify other individual factors which would influence the *Aeromonas* population (Holmes *et al.*, 1996). The aquatic environments are of great variance, and many studies of these environments resulted in the conclusion that *Aeromonas* numbers are more dependent upon human activities than any seasonal variations (Havelaar *et al.*, 1990 a; Holmes and Niccolls, 1995). Nevertheless, seasonal effects have been demonstrated to play a major part in the fluctuation of the *Aeromonas* population (Pathak *et al.*, 1988, Gavriel *et al.*, 1998).

1.1.2.2. Marine waters.

The presence of members of the genus A*eromonas* in the marine environment has been investigated because of their potential pathogenicity to humans through contact with contaminated water or through consumption of seafood (Esteve *et al.*, 1995). Being haloduric, mesophilic *Aeromonas* can be found in the marine environment and also brackish waters (Monfort and Baleux, 1991; Holmes *et al.*, 1996). Aeromonads have been shown to be more numerous in marine environments than in freshwater ones (Hazen *et al.*, 1978). Seasonal variation have also been noted in Brackish waters (Hazen, 1983; Monfort and Baleux, 1991) and in marine environments (Seidler *et al.*, 1980).

1.1.2.3. Sewage treatment.

Aeromonas spp. are present in similar numbers to coliforms in sewage waters, but are not considered as normal inhabitants of the human gastro-intestinal flora (Holmes *et al.*, 1996). As shown with freshwaters and marine environments, a seasonal pattern has been noticed, that is an increased number of *Aeromonas* species present in sewage in the summer period ($\sim 10^6$ cfu/ml) while numbers indicates a marked decrease in the winter months (10^3 cfu/ml) (Monford and Baleux, 1990). Reduction in the number of *Aeromonas* present in sewage after treatment depends upon the process applied (Holmes *et al.*, 1996). However, two different studies (Boussaid *et al.*, 1991; Stecchini and Domenis, 1994) reported a $\sim$ 95% reduction in the number of mesophilic *Aeromonas* when comparing two different treatment processes (i.e. stabilisation pond or activated sludge).

1.1.2.4. Potable water treatment and distribution.

Water treatment is not aimed at obtaining a sterile water, but to eliminate pathogens and reduce the overall load of non-pathogenic microorganisms (Holmes *et al.*, 1996). Large numbers of microorganisms present in the water within distribution systems, downstream of the treatment can be due to two main factors:

1) Re-growth: *Aeromonas spp.* are known to be able to grow in low nutrient waters (Van der Kooije and Hijnen, 1988; Van der Kooije, 1991).

2) Biofilms: protection of inner microorganisms due to extracellular factors and species from the surface layers (Block *et al.*, 1992; Holmes and Niccolls, 1995).

The quality of drinking water is primarily evaluated based upon the results of screening for faecal coliforms and *Escherichia coli* as indicator organisms (Anon., 1994). Tests performed to assay the quality of potable water are usually done using the standard method of membrane filtration (Anon., 1994). However, new systems have appeared on the market using defined-substrate technology systems which are based upon a presence/absence

9

result for the test organisms (Edberg *et al.,* 1988; Edberg *et al.,* 1989). These new systems are more rapid in delivering results, and therefore offer some advantages over standard methods. However, these new systems have some drawbacks (Chapter 2 this volume).

More recently, attention has been driven towards biofilm studies, as they are known to be the main source of drinking water recontamination (Mackerness *et al.,* 1991). Disruption of the biofilm by the water flow leads to re-seeding of the water by microorganisms present in the biofilm potentiating down-stream re-growth to occur. Bacteria have been isolated from the main body of the water, but the vast majority are part of biofilms on the pipe walls (Block, 1992). Holmes and colleagues (Holmes *et al.,* 1996) have assumed that *Aeromonas* spp. enter a viable but non-culturable state as one of their survival strategies in order to survive stress conditions.

a) *Aeromonas* in drinking water.

Aeromonas species are known to be present in large amounts in some water sources such as reservoirs and might enter distribution systems as a result of ineffective water treatment (Holmes *et al.,* 1996). Studies on the effect of water treatment upon the reduction of *Aeromonas* spp. numbers has been addressed only recently with the first study performed by Mehus and Peeters (1989). In their study, different stages of the water treatment process were investigated and the data obtained shown in Table 1-3.

Table 1-3: Reduction of Aeromonads at different stages of a water treatment process (reproduced from Meheus and Peeters, 1989).

Following the treatment process	% reduction in *Aeromonas* numbers
Floculation/sedimentation	30-60
Rapid sand filtration	70-90
Granular activated carbon	80-90
Hyperchlorination/ direct filtration	99-100
Slow sand filtration	98-100

Holmes and colleagues (1996) noted a 90% reduction in numbers of *Aeromonas* species after coagulation and clarification, and no aeromonads were recovered after chlorination (level of free chlorine being 2.6 mg/l). However *Aeromonas* spp. were recovered from the activated carbon contained within the filters. It was suggested that the presence of *Aeromonas* spp. in the filters might provide an explanation for the re-seeding of the waters by aeromonads in a viable but non-culturable state (Holmes *et al.*, 1996). High numbers of aeromonads have been recovered in some effluent of rapid sand filters and activated carbon filters compared with the usual numbers obtained in most plants (Kersters *et al.*, 1995). The authors suggested that these higher numbers obtained for one plant compared to the three others were probably due to growth of *Aeromonas* on bio-degradable organic materials such as decomposed algae and/or bacteria (Kersters *et al.*, 1995).

b) *Aeromonas* in water distribution systems.

Aeromonas have been isolated from many water distribution systems (Havelaar *et al.*, 1990 a; Holmes and Nicolls, 1995; Huys *et al.*, 1995) in rather high numbers in some cases. 90% of domestic supplies in Cairo were found to contain *Aeromonas* spp. (Gahnem *et al.*,

1993), and 85% of samples analysed during a study by Krovacek and colleagues (1992) conducted in Sweden showed to be positive for *Aeromonas*. Growth of *Aeromonas* showed a seasonal pattern with a peak during the summer months (Havelaar *et al.*, 1990 a; Holmes *et al.*, 1996; Gavriel *et al.*, 1998). Temperature and free chlorine concentration have been shown to have an effect on the growth of *Aeromonas* spp. in drinking water supplies (Holmes *et al.*, 1996; Burke *et al.*, 1984). Although *Aeromonas* spp. are isolated during warm months, they still have the ability to grow throughout the year. Control of aeromonads can be performed by increasing disinfection as *Aeromonas* spp. appear to be more sensitive to chlorine and chlorine derivatives than other bacterial families e.g. *Escherichia* or *Pseudomonadaceae* (Knochel, 1991; Sisti *et al.*, 1998). The fact that, despite high chlorine doses, *Aeromonas* spp. are still recovered could be due to their tendency to be incorporated in mixed biofilms (Mackerness *et al.*, 1991).

1.1.3. Health significance of *Aeromonas* species.

The first accepted report of *Aeromonas* as a human pathogen was in 1954 when Hill and co-workers isolated *Aeromonas* from a woman who died from a fulminant septicaemia. The woman developed the disease after swimming in the sea and died two days after being admitted to hospital. Post-mortem examination showed necrosis of muscles and intestinal oedema (Hill *et al.*, 1954).

However the most common symptom of mesophilic A*eromonas* infections is gastro-enteritis due to ingestion of contaminated water (Gahnem *et al.*, 1993) or food (Fricker and Tompsett, 1989). Many reports have appeared in the literature raising concern over the fact that there seems to be an increase in the occurrence of gastro-enteritis caused by ingestion of contaminated water (Nichols *et al.*, 1996) or describing more severe diseases such as deep wound infections, and septicaemia which can be fatal (Gosling, 1997; Nichols *et al.*,

1997; Table 1-4). Both intestinal and extra-intestinal infections are considered to be the result of a wide range of extracellular factors produced especially cytotoxins (Janda, 1991).

Table 1-4: Type of infection associated with *Aeromonas spp.*(reproduced from Janda & Duffy, 1988)

Type of infection	Characteristics	Frequency*
Gastro-intestinal		
Secretory diarrhoea	Acute watery diarrhoea, often with vomiting.	Very common
Dysentric diarrhoea	Acute diarrhoea with blood and mucus.	Common
Chronic diarrhoea	Diarrhoea persisting for more than 10 days.	Common
Choleric diarrhoea	"Rice water" stools.	Rare
Cellulitis	Inflammation of connective tissue often resembling streptococcal cellulitis; occasionally seen as granulomatous ulcer.	Common
Systemic		
Myonecrosis with or without gas gangrene	Haemorrhage, necrosis and liquefaction of soft tissues (muscle); subcutaneous gas formation with muscle fibres separated and lysed. Often requires debridement or limb amputation, and septicaemic patients have high mortality.	Rare
Ecthyma	Ecthyma skin lesions with erythematous border and necrotic centre.	Uncommon
Gangrenosum	Associated with *Aeromonas* sepsis.	Uncommon
Septicaemia	Fever, chills and hypotension with high mortality.	Fairly common
Pneumonia	Pneumonia with septicaemia, sometimes with necrosis.	Rare
Osteomyelitis	Bone infection of the gall bladder.	Rare
Cholecystitis	Acute infection of the gall bladder.	Rare
Gynaecological infection	Fatal infection following incomplete abortions.	Rare
Ocular infection	Mild conjunctivitis, corneal ulcer and endophtalmitis.	Rare

* The frequency of occurrence relates to how common individual types of infection are among all cases of *Aeromonas* infection.

1.1.3.1. Gastro-intestinal infections.

Aeromonads are the cause of two main types of gastro-enteritis (Palumbo and Abeyta, 1992). The first type, which is the most common, is a cholera-like disease. The symptoms are similar to those of cholera in the sense that patients have watery stools with or without fever, abdominal pain or cramps, and vomiting in children aged less than 2 years old. The second type of infection, is less common and shows dysentery-like symptoms. This group

is characterised by bloody, mucoidal stools; vomiting is rare but abdominal pain is possible. The fact that aeromonads lead to enteric infections is due to the wide range of virulence factors produced by these microorganisms (Janda, 1991, Janda and Abbott, 1996; section 1.4).

1.1.3.2. Extra-intestinal systemic infections.

a) Wound infections.

This mode of infection has more serious implication than gastro-enteritis as far as human health is concerned. Most of the cases studied were fatal to the host if not treated in the early stages of infection (Janda and Abbott, 1996). Three main types of deep wound infections have been reported in the literature, where the host needed extreme treatment (e.g. heavy chemotherapy, amputation) for survival (Janda & Abbott, 1996). These type of wound infections can be rapid in onset and fatal.

From a base number of 100 wound infections reported, a profile of candidates more likely to succumb to these diseases have been drawn as follows (Janda & Abbott, 1996):

- Around 95% of infections are community acquired i.e. from the environment. 63% of patients had a soil or water-contaminated wound.

- Men are 3 times (76%) more likely to develop an *Aeromonas* infection than women (24%). It was suggested (Baddour, 1992) that *Aeromonas* adhesin might be affected by hormones or the metabolic conditions of the host.

- Age was suggested as a factor, but the results are conflicting (Janda & Abbott, 1996). It was reported that the majority (90%) of the infected people were aged over 10 years old. 70 % of the infections were in patients over 21 years of age. These findings were reported as being peculiar (Janda & Abbott, 1996) since the

penetrating trauma causing the infections are usually common in accidents involving young children.

b) Septicaemia.

This is a type of bacteremia which usually occurs as a complication after gastro-intestinal disorders or deep wound infections (Picard *et al.,* 1984; Table 1-5).

Table 1-5: Major categories of *Aeromonas* septicaemia (reproduced from Janda & Abbott, 1996)

Group	Age	Portal of entry	Immunocom-promised	Underlying disease	Fresh water exposure	Associated syndromes	Mortality rate (%)
I	Adults	GI	Yes	Yes	Uncommon	Occasional	30-50
II	Infants<2 years Adults>34 years	GI	Yes	Yes	Uncommon	Meningitis	37
III	All age	Wound	No	Common	Common	Myonecrosis	>85
IV	Adults	Unknown	No	No	Unknown	None	75

As shown in Table 1-5, septicaemia can be associated with underlying diseases as a consequence of a breakdown of the immune system. Four species of the mesophilic *Aeromonas* spp. have been reported as being responsible for septicaemia. These are *A. hydrophila* (58%), *A. veronii* (31.7%), *A. caviae* (6.3%), and *A. jandaei* (3.2%) (Janda & Abbott, 1996).

It is important to bear in mind however, that most studies listing the species type involved in disease are undoubtedly subject to a lack of rigorous determination, as the majority of these investigations were based on simple phenotypic analysis not reliable for species determination.

1.1.4. Virulence factors in *Aeromonas* spp.

From the 17 DNA hybridisation groups outlined earlier (Table 1-1), several species have emerged within the mesophilic group which were found to produce a wide range of

virulence factors such as extracellular enzymes (e.g. protease) and toxins (e.g. cytolytic toxins, enterotoxins). Examples included *A. hydrophila* (Chopra *et al.*, 1993); *A. veronii bv. sobria* (Champsaur *et al.*, 1982; Gosling *et al.*, 1993); and *A. veronii bv. veronii* (Karunakaran and Devi, 1994).

Not much is known on the pathogenesis of *Aeromonas* spp. The lack of an animal model to study *Aeromonas* pathogenicity implies that most virulence factors (except the S-layer in *A. salmonicida*) have been implicated to play a role in virulence by inference rather than by direct experimentation (Janda, 1991). This was possible as similar molecules have been shown to be linked to pathogenicity in other microorganisms such as *E. coli* (Janda, 1991). These virulence factors can be separated into two different groups, the first being the surface and cell-associated virulence factors and the second as being extracellular agents.

1.1.4.1. Surface associated virulence factors.

Attachment capabilities have been demonstrated to be a pre-requisite for a microorganism to infect a host and subsequently for disease to occur (Soto and Hulgren, 1999). This is achieved as a result of a range of structural features such as pili, the S-layer, lipopolysaccharides and outer membrane proteins.

a) Pili

In recent reviews (Janda, 1991; Gosling, 1996), two types of pili have been described for *Aeromonas*. They differ in size, shape and in their pattern of expression. The first was designated as straight pilus, and bears strong homology with the *E. coli* type I pilus (Soto and Hultgren, 1999). Its molecular weight varies between 17-18 kDa. Surprisingly it does not share immunologic cross-reactivity with *E. coli* strains expressing colonisation factor antigens type I (Janda, 1991). It is constitutively expressed under all conditions. The second type has been named as the flexible pilus due to its curvilinear shape or mini pilus

due to its molecular weight of 4 kDa. It shows no homology to any known pilus sequences. It seems to be environmentally regulated and expressed when an optimal temperature of 22 °C has been reached. It has been suggested that this environmental regulation is a pre-requisite for colonisation to occur (Janda, 1991).

b) Surface layer.

The Surface-layer (S-layer) was first reported for *A. salmonicida* and was called the A-layer for Additional layer (Janda, 1991). This layer is external to the cell wall and its loss was proven to increase the LD_{50} by a factor of 10^3 to 10^4 (Janda, 1991). This is one of the most important virulence factors along with the Glycerophospholipid:cholesterol acyl transferease-lipopolysaccharide layer complex (GCAT-LPS) (detailed in 1.5.1 b) which has been demonstrated to be linked to pathogenicity in fish (Austin and Adam, 1996). The S-layer seems to be composed of a single surface array of protein sub-units of 52 kDa, and confers hydrophobic characteristics to the bacterium (Dooley, *et al.*, 1988). This S-layer has since been identified in *A. hydrophila* and *A. veronii bv. sobria.* The role of the S-layer in human pathology remains unknown but may differ from the action of the S-layer from *A. salmonicida* (Janda, 1991). This was explained (Kokka *et al.*, 1991) by the fact that in *A. salmonicida*, the S-layer conferred hydrophobicity to the cells which resisted lysis and allowed attachement to macrophages. This S-layer was a requirement for *A. salmonicida* to be virulent. On the contrary, strains of *A. hydrophila* lacking S-layer were of variable virulence in outbread mice assay, and cells which could synthesise surface array proteins (SAP), but not attached were more pathogenic than the intact O:11 wild type counterpart (Kokka *et al.*, 1991).

c) Lipopolysaccharides.

Individual lipopolysaccharides are composed of three distinct molecular regions named from the innermost to the outermost, being lipid A, core polysaccharide, and a repeating motive of polysaccharide called O-side chains of lipopolysaccharides or O-antigen. The toxic portion of the lipopolysaccharide lies in the lipid A region. A series of studies reported by Janda in his review (1991) defined three chemotypes according to the variation of the core region of the lipopolysaccharide in certain monosaccharides. Those chemotypes were defined as follows:

- Chemotype I composed of *A. hydrophila* (HG 2)

- Chemotype II composed of mainly *A. caviae*-like species (*A. eucrenophila, A. media*) but also *A. hydrophila* belonging to HG 1

- Chemotype III composed of *A. sobria* (HG 7,8).

When the O-side chains of lipopolysaccharides were analysed on sodium dodecyl sulfate - polyacrylamide gel electrophoresis, they displayed three profiles (Chart *et al.*, 1983; Dooley *et al.*, 1985). The first one is similar to the pattern observed in many enteric bacteria (Hitchcock, *et al.*, 1986), the second being linked to the anchoring of the S-layer (Dooley *et al.*, 1985) and the third showing a lack of detectable side chains (Dooley *et al.*, 1985). No direct link to pathogenicity in human beings has been reported (Gosling, 1996).

1.1.4.2. Cell-associated virulence factors.

a) Invasins.

Studies on *Aeromonas* invasiveness have been made difficult by the fact that toxins such as haemolysin interfere with the assays by damaging the cell line studied (Janda, 1991). Until appropriate deletion mutants are available it will be difficult to verify the role of invasin in the mechanism of *Aeromonas* pathogenesis. It would appear that the gene responsible for

invasiveness of *Aeromonas* is located on the chromosome and is not plasmid borne (Gosling, 1996). It has been suggested (Gosling, 1996) that this factor may be the cause of certain diseases such as dysentery, but no evidence has yet appeared in the literature.

b) Plasmids.

Aeromonas species have been shown to be a carrier of one or more plasmids (Hedges *et al.,* 1985). These plasmids function mainly to mediate resistance to antibiotics such as ampicillin or kanamycin (Hedges *et al.,* 1985) which are considered as added wild type phenotypes. These would be a considerable advantage for a strain to have in order to increase its resistance to various factors such as antibiotics (Son *et al.,*1997). The plasmids can also harbour genes which will alter the strain phenotype such as modulation of gelatinase production (Gosling, 1996).

1.1.4.3. Extracellular virulence factors.

Aeromonas spp. are known to produce a wide range of toxins, many of which are proteins, especially enzymes (detailed below). The only known exceptions of extracellular virulence factors which are not of proteinaceous nature are the endotoxin and the lipopolysaccharide layer (Howard *et al.,* 1996). This diversity expressed by *Aeromonas* allows the bacteria, as a pathogen, to act in two ways. The first option is to destroy target cells using cytotoxins (such as haemolysins) in order for the bacterium to protect itself (Howard *et al.,* 1996). The second option is the use of the wide array of exogenous enzymes to provide new sources of nutrients (Howard *et al.,* 1996). In a way, killing of a another cell also provides the invading cell with nutrients analogous to the phenomenon of cryptic growth. Most, if not all, of these virulence factors are secreted through the general secretory pathway and released into the medium (Howard *et al.,* 1996).

a) Cytolytic toxins.

It has been observed that *A. hydrophila* infections can cause erythrocytes to lyse therefore suggesting the organism was synthesising haemolysins. Ljungh and Wadström (1986) have described two types of haemolysins, the α-and ß-haemolysins. The synthesis of these two haemolysins is temperature and growth-phase dependant. The α-haemolysin, which produces a weak zone of lysis, was synthesised during the late stationary phase (Janda, 1991), whereas ß-haemolysin (strong zone of lysis) was released during the logarithmic phase (Gosling, 1996).

i) α-haemolysin.

Little has been reported on α-haemolysin, with most research to date conducted on ß-haemolysin. Information available on the α-haemolysin is rather confusing as data relates to either crude extracts or partially purified toxin (Ljung and Wadström, 1986). A molecular weight of 65 kDa was stated for the purified toxin with an isoelectric point ranging from pH 5.1 to 5.7. The partially purified α-haemolysin was reported as being produced at higher yield at a temperature of 22°C than 30°C and with production being repressed at 37°C (Ljung and Wadström, 1986). The authors reported a dose and temperature-dependant cytotoxic effect where the toxin acts solely on the membrane with reversible effects when cells were re-suspended in fresh medium. The toxin was heat-sensitive (56°C for 10 min, but no pH stated) and degraded when in presence of proteolytic enzymes and it was therefore suggested it might be of enzymatic character (Ljung and Wadström, 1986).

ii) β-haemolysin.

The ß-haemolysin is, to date, the only well characterised extracellular toxin produced by *Aeromonas* spp.(Howard *et al.,* 1996). It has been reported under many names such as

aerolysin (Bernheimer and Avigad, 1974), cytotoxic factor (Chopra *et al.*, 1993) and ß-haemolysin (Ljung and Wadström, 1986). Its role is to kill target cells through damage to the membrane which ultimately causes lysis (Holmes *et al.*, 1996). Lysing cells is needed to either overcome an attack from the host immune system and/or to provide nutrient to the bacterium (Howard *et al.*, 1996). This toxin was found to have a molecular weight around 50 kDa and two isoelectric points pH 4.2 and 5.5 (Ljungh and Wadström, 1986) or pH 5.39 and 5.46 (Gosling, 1996). Ljungh and Wadström reported the toxin was heat-sensitive (50°C for 1 hour at pH 7). They also reported their data for the toxin stability was different from those obtained by Bernheimer and Avigad (1974), who found ß-haemolysin to be rather stable upon degradation when in presence of proteolytic enzymes such as trypsin or subtilin and even stabilised by papain. However Ljungh and Wadström (1986) found their preparation to be inactivated by papain and subtilin.

b) Enterotoxin.

A cytotonic toxin termed enterotoxin has been frequently mentioned in the literature (Ljungh and Wadström, 1986; Gosling, 1996; Howard *et al.*, 1996). This toxin was described as being stable in pH range from 4.5 to 10 and more active at alkaline pH (Ljungh and Wadström, 1986). Its molecular weight was between 15 to 20 kDa. Papain destroyed the toxin, but trypsin, for example, would lead to a decrease in activity of only 25 % (Ljungh and Wadström, 1986). It was also found to be stable at a temperature of 56°C, but not at 100°C (no time or pH stated) suggesting that this *Aeromonas* virulence factor has no relation to *E. coli* enterotoxin which is inactivated at both temperatures (Howard *et al.*, 1996). Ljungh and Wadström also reported the fact that strains which had lost their enterotoxic property could regain it when passaged through rabbit epithelial cells (Ljung and Wadström, 1986). They also suggested that enterotoxic activity might be under

chromosomal control (similar to *Vibrio spp.*) as no plasmid bearing this function has been isolated from enterotoxic strains.

c) Other virulence factors.

A wide range of additional toxins have been described in the literature such as cell-rounding factor (Champsaur *et al.*, 1982) or shiga-like toxin (Haque *et al.*, 1996). Champsaur and colleagues have reported that *A. sobria* caused a cholera-like illness. Heat treatment of 56°C for 30 minutes abolished haemolytic and proteolitic cytolytic activities, but did not abolish the cell-rounding properties of the cell-free filtrate. Even after treatment of the filtrate at 100°C for 30 minutes the cell-rounding properties remained. The authors reported that the biological significance of the cell-rounding factors was not fully known but was considered more cytotonic than degenerative in nature. Haque and co-workers (1996) showed that 3 out of 13 faecal isolates of *A. hydrophila* and 1 out of 7 environmental isolates of *A. caviae* reacted positively when in presence of IgG sensitised to shigella-like toxin 1 (SLT1) of *E. coli* O157:H7 (Haque *et al.*, 1996). The authors reported that this SLT1 was plasmid encoded both in *E.coli*O157:H7 and *Aeromonas* (Haque *et al.*, 1996). The fact that virulence factors produced by *Aeromonas* spp. strains are so numerous has made the study of these individual products very difficult and could lead to conflicting results when studying crude, partially purified and purified molecules.

1.1.5. Pathogenic mechanisms.

As discussed previously, *Aeromonas* spp. have been shown to produce a wide range of virulence factors, but no link between these virulence factors and pathogenicity have been clearly reported (Gosling, 1996). No animal model has been able to produce results to corroborate the hypothesised mechanisms of action elaborated on the different virulence factors produced by *Aeromonas* spp. (Gosling, 1996). Similarly, no strains have been

found to entirely fulfil the Koch's postulates (Nichols *et al.*, 1996) therefore leaving the debate on the opportunistic/primary pathogenicity of *Aeromonas* spp unresolved. Due to the diversity in the toxic factors that *Aeromonas* species produce, the pathogenic mechanisms are viewed as being complex, multifactorial and a model for non-inflammatory diarrhoeal disease have been proposed (Gosling, 1996).

1.1.5.1 Mechanisms of action of Aerolysin (ß-haemolysin) and Glycerophospholipid: cholesterol acyl transferase (GCAT).

The mechanisms of action of ß-haemolysin and a lipase/acyltransferase, a toxin with known effects proven in fish pathogenesis (Austin and Adam, 1996) will be considered to illustrate the manner by which they may mediate a pathogenic response.

a) Mechanisms of action of Aerolysin (ß-haemolysin).

Howard and Buckley (Howard and Buckley, 1985a, Howard and Buckley, 1985b) have isolated two proteins from *A. hydrophila* supernatant, one being identified as aerolysin, the other being of higher molecular weight. It was shown that the heavier protein resembled aerolysin at its N-terminus end and had an haemolytic activity 250-fold less than the ß-haemolysin and was therefore considered as inactive zymogen precursor. Treatment of the heavier protein with trypsin or with an *Aeromonas* protease resulted in an active aerolysin. This was the first report of *A. hydrophila* excreting a zymogen protoxin which after cleavage of a peptide at the C-terminal end resulted in active toxin (Howard *et al.*, 1996). In 1992, Buckley suggested a pathway for excretion, activation and action of aerolysin and subsequently provided the proaerolysin structure (Parker *et al.*, 1994).

Several reviews explaining the mechanisms of action of aerolysin are available (Van der Goot *et al.*, 1994; Parker *et al.*, 1996; Howard *et al.*, 1996), the details of which are summarised in Figure 1-1.

Figure 1-1: Suggested mechanism of action of aerolysin (reproduced from Howard *et al.*, 1996)

PREPROAEROLYSIN $\xrightarrow[\text{signal sequence removed}]{\text{SECRETION}}$ PROAEROLYSIN
(54 kDa)   (52 kDa)

$\downarrow$ ACTIVATION
C-terminal fragment
removed

Binding to the
cell membrane.

BOUND
TOXIN $\longleftarrow$ AEROLYSIN
(47.5 kDa)

$\downarrow$ OLIGOMERISATION

HEPTAMERIC $\xrightarrow[\text{Through the cell membrane}]{\text{INSERTION}}$ 1-2 nm CHANNELS
TOXIN

$\downarrow$

CELL DEATH
loss of permeability barrier
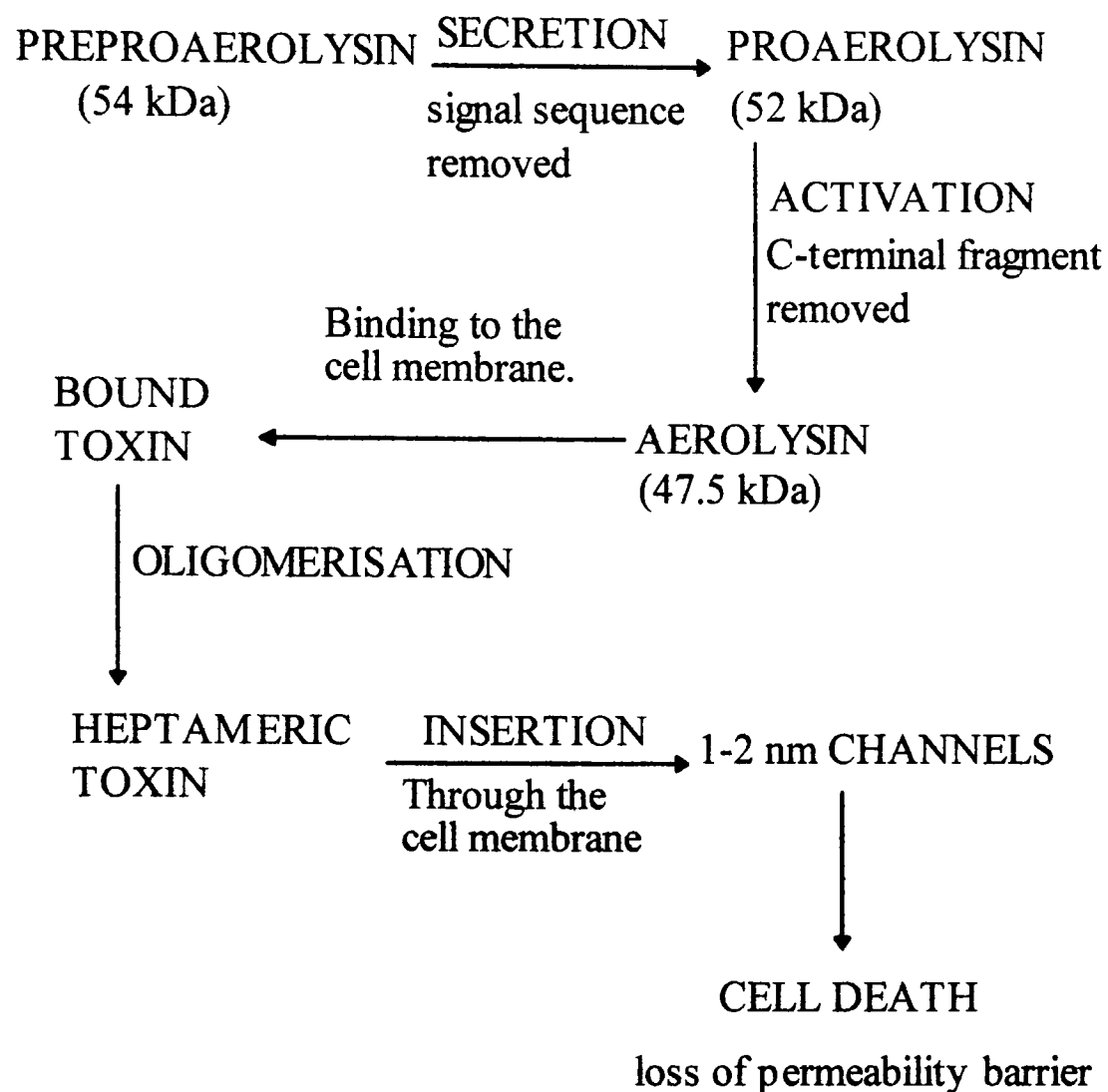
b) Mechanisms of action of Glycerophospholipid:cholesterol acyl transferase (GCAT).

This toxin, similarly to aerolysin, is excreted in a protoxin form by *Aeromonas* (Howard *et al.*, 1996). It is a monomer which is capable of lipid hydrolysis and acyl transferase activity (Howard *et al.*, 1996). It is activated by proteases such as trypsin and *Aeromonas* proteases. It was suggested that GCAT can associate with LPS to give a complex 10-100 times more active than the toxin itself (Howard *et al.*, 1996). This GCAT-LPS complex is regarded as being the major lethal toxin produced by *Aeromonas salmonicida* (Austin and Adams, 1996). LPS seems to provide protection to GCAT from proteolytic degradation, and enhance its specificity in targeting cells.

1.2. Bacterial stress.

Bacteria have to constantly face an incredible range of environmental stresses in order to survive. The stress phenomenon is different in nature depending on the environment surrounding the cells, but can generally be considered as nutrient starvation (Kjelleberg *et al.*, 1993; Gauthier and Clément., 1994), pH stress (Gorden and Small, 1994; Karem *et al.*, 1994), temperature stress (Klein *et al.*, 1995; Mager and De Kruijff., 1995), osmotic stress (Poirier *et al.*, 1998) and oxidative stress (Stortz *et al.*, 1990; Demple and Amabile-Cuevas, 1991; Demple, 1991).

1.2.1 Nutrient starvation.

When nutrients are available the cells have the ability to undergo rapid growth. On the other hand when the medium is depleted of these nutrients, the bacteria manage to remain viable by entering what could be considered a starvation-induced program, during which the bacteria are metabolically less active, but more resistant to environmental stresses. Non-sporulating starved cells are known to change their morphology, for example by reducing their size, and become more hydrophobic (Morita, 1993). They also have a decrease in their overall metabolism, but still retain some endogenous metabolism to allow low level production of ATP or other energy-containing compounds, and maintenance of the proton motive force across the membrane. This is necessary for the cell to be able to transport any possible nutrient encountered. Under nutrient limiting conditions it has been shown that a complex genetic regulation is activated to control:

(i) groups of structural genes which will allow more nutrient uptake if present, (Matin *et al.*, 1989).

(ii) the entry into stationary phase (Hengge-Aronis, 1996): σ factors, (i.e. RNA polymerase co-factors), and RNA polymerase holoenzyme co-ordinate the altered gene expression. σ factors and RNA polymerase holoenzyme mediate the

production of proteins which respond to the stress (Reeve *et al.*, 1984). Therefore, these changes in gene expression cause alteration in the cellular morphology and physiology (Hengge-Aronis, 1993).

Because microorganisms have only a limited capacity over the control of their environment, they have to change themselves in order to respond to environmental changes. This was termed the 'global system approach' to the physiology of starved cells (Nyström, 1993). These changes were considered as being of two orders. The first being physiological where drastic changes in the membrane lipid content were observed, DNA content of the cell decreased, along with a decrease in cell size (Morita, 1993). The second type of changes were genetic where an altered pattern of protein synthesis is initiated (Reeve *et al.*, 1984). Nyström reviewed three types of stress proteins. The first, being universal stress proteins, which are always expressed whatever stress the bacteria is facing. The second group is the stress specific proteins being expressed for a particular type of stress such as starvation or oxidative stress. The last group encompass the unique stress proteins synthesised for a unique type of stress. Taking oxidative stress as an example, stress proteins induced by the presence of H_2O_2 are different from those induced by O_2^-. The mechanism of regulation of the general control system for expression of these stress proteins is not known, but several hypothesis have been proposed such as passive control or gearbox promoters (Nyström, 1993). Passive control has been described as being a competition between promoters for a limited supply of free RNA polymerase (Jensen and Pedersen, 1990). During growth arrest or when growth rate decreases as a result of nutrient limitation, transcription elongation rates decrease therefore more RNA polymerase molecules are sequestered, leading to a decrease in the available RNA polymerase pool (Jensen and Pedersen, 1990). It was suggested that control could be at the promoter level where promoters for genes encoding stress proteins might easily be saturated, whereas the

genes responsible, for example, for rapid growth when nutrients are available, might possess a rapid clearing promoter region (Jensen and Pedersen, 1990). The gear-box promoters have two distinctive features (Bohannon *et al.*, 1991). Firstly, gene expression controlled by a gear-box promoter would be inversely proportional to the growth rate as Gear-box promoters expression is driven by the shift from exponential to stationary phase (Bohannon *et al.*, 1991). Secondly, the promoters have a highly conserved sequence around -10 and -35 regions (i.e. region for RNA polymerase fixation and initiation sites) (Bohannon *et al.*, 1991). Bohannon and co-workers have suggested two possible mechanisms of recognition for gear-box promoters. They hypothesised that different σ factors are involved in the recognition of different types of these promoters. The second suggestion was that they are transcribed by a form of RNA polymerase which increases in abundance with decrease in growth rate. They speculated that this form of RNA polymerase may contain σ^{70} (as -35 region of gear box promoter is identical to -35 consensus recognised by σ^{70}) and alter its specificity by binding a protein factor termed gear-box binding factor or a small metabolite. In addition, various positive activator proteins whose transcription would be dependent upon σ^{s} transcription may aid the transcription of these gear-box promoters (Bohannon *et al.*, 1991).

1.2.2 pH stress.

pH stress can occur when bacteria are present in waters such as sewage treatment ponds experiencing an algal bloom leading to an alkaline pH (Monford and Baleux, 1990) or when present in acidic foods (Salmond *et al.*, 1984). When invading an animal or human system, the bacteria have to face several pH-dependent defence mechanisms. If ingested, the first barrier the microorganism will face would be the low pH of the stomach. Indeed, the pH of the gastric system is around 2.0 to 3.0 (Goodson and Rowbury, 1989 b) and its role is to kill or drastically reduce the number of both harmless and potentially pathogenic

27

organisms. The small intestine is also acidic due to the lactic products produced by the bacterial flora present (Goodson and Rowbury, 1989 b). If bacteria invade a host by a route other than the digestive tract (e.g. wound infections), they will have to face the phagocyte which leads to acid and oxidative stress, the pH in phagocytes being around 3.5 to 5.0 (Goodson and Rowbury, 1989 a). Gram negative bacteria are known to be very sensitive to killing by acidic pH especially in the presence of organic acids (Salmond *et al.*, 1984). But it is known that bacteria can survive the extreme pH of the digestive tract, because many food and waterborne illnesses result from oral ingestion of pathogenic microorganisms (Gorden and Small, 1993). Several parameters have been shown to have an effect on resistance of microbes to pH, such as attachment to particles. It has also been shown that if bacteria were previously placed into an environment with sub-lethal pH, they could then withstand a normally lethal pH (Goodson and Rowbury, 1989 a; Goodson and Rowbury, 1989 b; Karem *et al.*, 1994).

1.2.3 Temperature shock.

Temperature stress, both heat and cold shock, has probably been the most studied in both eukaryotes and prokaryotes. All living organisms respond to stress, including temperature, by the synthesis of stress proteins (Lindquist, 1986). In the context of elevated temperature they have been termed heat shock proteins (Hsps). The temperature of induction of the Hsps in different organisms such as *Drosophila*, fish, plants, yeast and bacteria were compared and it was concluded that the induction of the stress protein is rapid, but that the maximum induction temperature varies with the organisms studied (Lindquist, 1986). For organisms growing over a broad range of temperatures this optimum was usually 10-15°C above their optimal growth temperature, whereas it was usually 5°C in organisms with a more restricted temperature range (Lindquist, 1986). Hsps are not only synthesised when the temperature of the environment elevates, but also when the organism faces other

stresses such as presence of reactive oxygen species. Hsps proteins are known to play a role in the response to heat stress, but are also constitutively expressed and play a role as molecular chaperones (Langer *et al.,* 1992; Becker and Craig, 1994). These proteins have been classified in families according to their molecular weight (e.g. Hsp 60, Hsp 70). Several good reviews have detailed the different Hsps families (Lindquist, 1986; Becker and Craig, 1994; Gross, 1996) and the chaperone action (Ellis and Van der Vies, 1991; Langer *et al.,* 1992) and their role will be reviewed below (1.2.6.1 Protein repair system).

1.2.4 Oxidative Stress.

Aerobic organisms employ oxygen as the terminal electron acceptor in the electron transport chain (Cadenas, 1989; Demple, 1991). These organisms need oxygen to live, but they must also deal with the side effect of oxygen (Demple, 1991), which participate in radical generating reactions when oxygen is reduced to water (Davies, 1995). Reactive oxygen species (ROS; $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$) are collectively referred to as oxidative stress, and can be produced by aerobic metabolism, environmental agents, oxidative burst in phagocytes, and various chemicals (Storz et al., 1990; Demple and Levin, 1991; see Figure 1-2).

Figure 1-2: Schematic overview of the main sources of reactive oxygen species and their biological effects: oxidation of bioconstituents, stimulation of repair mechanisms, and induction of proteins (reproduced from Cadenas, 1995).
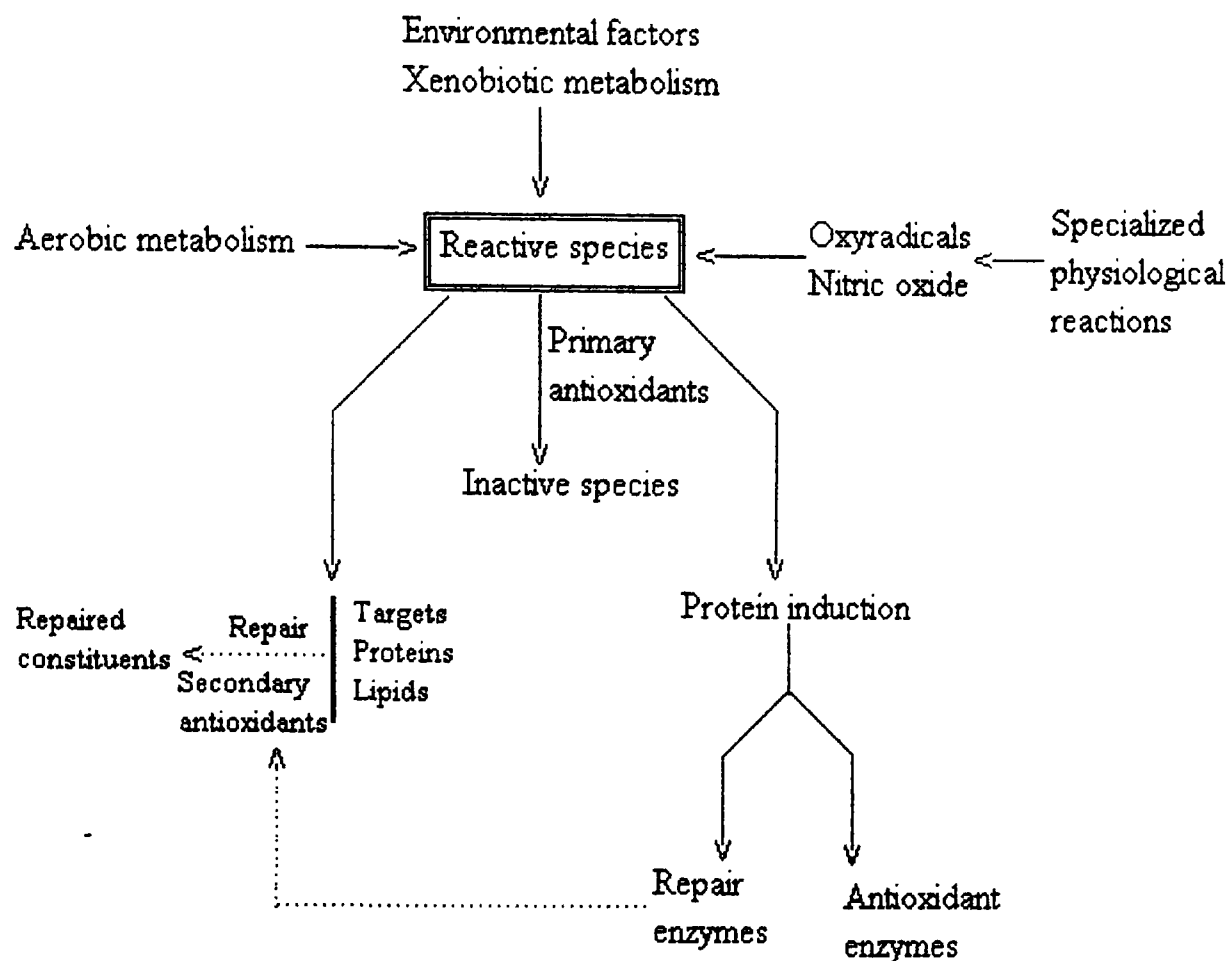
Oxidative stress arises when the concentration of ROS increases to a level over the basal level of the cells defence capacity (Demple and Amabile-Cuevas, 1991; Farr and Kogoma, 1991).

1.2.4.1 Formation of reactive oxygen species (ROS).

Molecular oxygen has two unpaired electrons on its outer orbitals which means this molecule cannot easily oxidise molecules and oxidation by O_2 is restricted to acceptance of electrons (Farr and Kogoma, 1991). This molecule is involved in a wide range of reactions (Cadenas, 1989; Storz *et al.*, 1990).

a) Superoxide anion (O_2^-).

This is both a one-electron transport electron reductant and a one-electron oxidant (Cadenas, 1995) that can pass through cell membrane via ion channels. It appears that

superoxide does not have direct toxic effects, but rather exerts its toxicity by penetration to important sites where it is converted to other ROS with hydrogen peroxide (H_2O_2), $OH^{\cdot}$, and singlet oxygen are the main types. In aqueous, neutral pH, $O_2^{\cdot-}$ decays to H_2O_2 through disproportionation (Equation 1-1).

$$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad\qquad \textbf{Equation 1-1}$$

Protonation of $O_2^{\cdot-}$ produces hydroperoxyl radicals (Equation 1-2).

$$O_2^{\cdot-} .+ H^+ \rightarrow HOO^{\cdot\cdot} \qquad\qquad \textbf{Equation 1-2}$$

$HOO^{\cdot}$ is the conjugated acid of $O_2^{\cdot-\cdot}$ which is a very reactive species as its pKa_a is close (2 pH units below) to the neutral pH value (Cadenas, 1989). $HOO^{\cdot}$ can cross the biological membranes and initiate lipid peroxidation (Cadenas, 1995). *In vivo* $O_2^{\cdot-}$ is though to cause toxicity through participation in $OH^{\cdot}$ formation via the Fenton reaction (Equation 1-3, 1-4 from Keyer *et al.*, 1995).

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad\qquad \textbf{Equation 1-3}$$

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow OH^{\cdot} + Fe^{3+} + H_2O \qquad\qquad \textbf{Equation 1-4}$$

Keyer and colleagues suggested that $O_2^{\cdot-}$ could not only have the role of transferring one electron to free iron as other biological molecules more abundant (e.g. NADPH) in the cell could fulfil this role (Keyer *et al.*, 1995). $O_2^{\cdot-}$ has been shown to oxidatively excise iron from Fe-S cluster containing dehydrases such as fumarase (Flint *et al.*, 1993). Keyer and

co-workers have therefore suggested that by estimating the rate of iron reduction by O_2^- and the content of O_2^- in cells, 10 hours would be needed for half the amount of iron to be reduced, time which is far too long to support rate of DNA damage they observed in cells stressed with H_2O_2. They hypothesised that O_2^- leaches iron from Fe-S cluster proteins or directly from iron storage proteins such as ferritin (Keyer *et al.*, 1995). Transition metals (e.g. iron) seems to have two roles. The first role being a protective one against $O2^{-\bullet}$ as the metal ligand is an essential part of some catalysing enzymes, whereas the second role appears to sensitise cells to the toxic effect of $O_2^{-\bullet}$ by reaction such as Fenton which generate hydroxyl radicals (Goldstein & Czapski., 1986).

b) Hydrogen peroxide (H_2O_2).

This is formed by the endogenous superoxide dismutases to detoxify the cell from superoxide or by reduction of the 2 electron charge that superoxide bears. H_2O_2 is not a radical as it bears no unpaired electrons and is relatively unreactive towards organic compounds. H_2O_2 can either cross freely the cell membrane, or lead to the formation of more active compounds such as hydroxyl radicals ($OH^\bullet$). Production of $OH^\bullet$ is rendered possible through decomposition of H_2O_2 when in the presence of transition metals in the Fenton reaction (equation 1-4). H_2O_2 acts to break DNA strands in presence of iron and is therefore a significant stress (Storz *et al.*, 1990).

c) Hydroxyl radical ($OH^\bullet$).

This is formed in several ways from which the Fenton reaction is the most important. This type of reaction involves both $O_2^{-\bullet}$ and H_2O_2 and is catalysed by transition metal chelate (e.g. Fe) The toxicity of $OH^\bullet$ results from its ability to serve as a strong one-electron

oxidant capable of abstracting electrons from a large variety of compounds with the formation of a new radical which can oxidise other substances via a chain reaction. OH˙ is very reactive and able to either add to DNA bases or abstract H atom from the DNA helix (Cadenas, 1995).

d) Singlet oxygen ($^1O_2$).

This can be produced in various ways. Chemically or biologically using the Haber-Weiss reaction (Khan and Kasha, 1994; equation 1-5).

$$O_2^- + H_2O_2 \rightarrow {}^1O_2(^1\Delta_g) + {}^{\cdot}OH + OH^- \qquad \textbf{Equation 1-5}$$

Singlet oxygen is an oxygen form whose electrons are at a higher energy level compared to the normal ground state (triplet oxygen). The singlet oxygen is highly reactive and when present in a biological system, produces a wide range of uncontrolled and unwanted oxidation reactions leading to the destruction of vital cell components.

1.2.5. Damage caused by oxidative stress.

Damage caused by oxidative stress can be separated into three groups depending on the nature of the target, being DNA, membrane, or protein components.

1.2.5.1. DNA damage.

DNA has been shown to be a prominent cellular target by the fact that strains bearing mutations in their DNA repair enzymes could not resist oxidative stress challenges and were shown to exhibit damaged DNA. There are as many different types of lesions reported as there are causes. Ionising, or chemical (Imlay *et al.*, 1988) reactions are two examples of the nature of these causes which affect all DNA components (Demple and Levin, 1991). DNA damage can be divided in two general classes. Damage which affect

the DNA sequence, but not its overall structure belong to the class of single base changes. The second class relates to structural distortion which impair replication or transcription through intrastrand cross-linking for example (Paragraph 1.2.6.2. b iii). Many of the effects of oxidative damage to DNA are subject to DNA repair pathways (Chapter 1 Paragraph 1.2.6.2).

1.2.5.2. Membrane damage.

The harmful effect of H_2O_2 damaging action is mediated through lipid peroxidation of the membrane that leads to altered membrane functions. Oxygen derived radicals disrupt the cellular integrity (Davies, 1995) leading to a loss of proton motive force, which reduces ATP formation and also nutrient uptake. Further membrane damage would eventually lead to cell lysis (Davies, 1995).

1.2.5.3. Protein damage.

Oxidative damage of various nature can yield non-functional proteins by disrupting the protein conformation. Two major types of protein damage exist. The first interferes with the three-dimensional structure of the protein and represents conformational damage which involves the breakage of non-covalent bonds such as ionic, hydrogen, or hydrophobic bonds. The second affects the secondary structure and is called covalent damage as it involves covalent bond breakage e.g. disulfide bridges. Both of these type of breakage are usually reversible. Oxidised proteins are recognised by proteases and replaced by newly synthesised proteins (Davies, 1995) involving the chaperone proteins (Chapter 1 Paragraph. 1.2.6.1 b). As for DNA, protein damage are subject to protein repair systems (Visick and Clarke, 1995; Davies, 1995).

1.2.6. Defence systems.

ROS encountered during either normal growth (by-products of the metabolism) or in the cells environment (oxidative burst in macrophages during the infection process), induce damage not only to DNA but to all cell components. Injuries to cell components are minimised by systems that recognise and correct the damages. This defensive response to stress has therefore two goals. The first and most obvious is to provide protection mechanisms for cell survival to a sudden onslaught of ROS. The second being maintenance of the genetic stability in aerobes producing ROS as by-products of their normal metabolisms. The repair mechanisms can be divided into two main groups. The first deals with proteins (Visick and Clarke., 1995) and the second with DNA (Demple and Harrison, 1994).

1.2.6.1. Protein repair system.

There are two general types of protein damage. As protein generation time is short, unlike DNA, it has been suggested that damaged proteins are degraded or replaced, with proteolysis as an option (Visick and Clarke., 1995). However some repair mechanisms also exist. It is known that after denaturation, it is possible to re-nature the protein. But for many proteins, folding or refolding is not necessarily rapid and requires either of two systems, i.e. enzymatic repair systems such as isomerases or chaperones, to avoid partially folded or misfolded proteins.

a) Isomerases.

Covalent damage caused by molecular oxygen leads to many forms of damage, but only one repair pathway is known in *Escherichia coli* (Visick and Clarke, 1995). In this repair system, methionine sulphoxide reductase recognises only methionine sulphoxide and reduces this abnormal residue back to methionine. However, if the damages occur at other

sites, they are probably difficult to recognise and repair as an abnormal residue may be converted to an ordinary residue but different from the one of the original sequence (Visick and Clarke, 1995). The fact that, unlike DNA repair mechanisms, no template bearing the original amino-acid sequence of a protein exists, shows a need to have preventive mechanisms such as catalase or superoxide dismutase in order to prevent damages before they occur.

b) Molecular chaperones.

As described in section 1.2.5.3 stress encountered by bacteria can lead to partially folded or misfolded proteins (i.e. denatured proteins). When a protein unfolds, regions contained within the protein become exposed and induce interactions with other proteins which therefore aggregate instead of going back to their native state (Becker and Craig, 1994). Cells respond to stress by the synthesis of key stress proteins of protective function being universal, general, specific or unique. Heat-shock proteins (Hsps) belongs to the universal group.

i) Hsps 70

Hsps 70 interact with nascent peptides and polypeptides in extended conformations. They are involved in maintaining proteins in unfolded states, dissociating protein aggregates and facilitating re-naturation (Langer *et al.*, 1992). It is suggested that Hsps 70 bind to polypeptides on its C-terminal end and binds ATP on its N-terminal end. However Hsps 70 possess a weak ATPase activity. In *E. coli* , Hsps 70 is DnaK and is required for thermo-tolerance (Becker and Craig, 1994). DnaK function is enhanced by interacting with two others Hsps being DnaJ (40 kD) and GrpE (21 kD). DnaJ appears to increase DnaK ATPase activity whereas GrpE in some cases enhance protein release and in other cases increase DnaK binding to polypeptides (Becker and Craig, 1994). DnaK-DnaJ-GrpE

complex is thought to prevent misfolding and aggregation of proteins prior to protein refolding by Hsps 60 (Langer *et al.*, 1992).

ii) Hsps 60

Hsps 60 are thought to recognise secondary structures and stabilise bonds formed in early folding (Langer *et al.*, 1992). Hsps 60 are involved in the refolding process itself. Hsps 60 are high-molecular weight structures composed of two inverted rings made of seven sub-units. In *E. coli*, Hsps 60 are GroEL and GroES (Gross, 1996). GroES binds to GroEL inducing a conformational change increasing the volume of the interacting ring. Protein folding occurs thanks to a coupled ATP hydrolysis by GroES and GroEL. The folding is hypothesised to occur through a series of release and re-binding of the polypeptide until no sites are recognised by GroEL (Becker and Craig, 1994).

The sequence of action of DnaK-DnaJ-GrpE complex and GroES-GroEL are summarised in Figure 1-3.

Figure 1-3: Hypothetical model for the chaperone-mediated folding pathway of newly synthesised proteins (reproduced from Langer *et al.*, 1992)
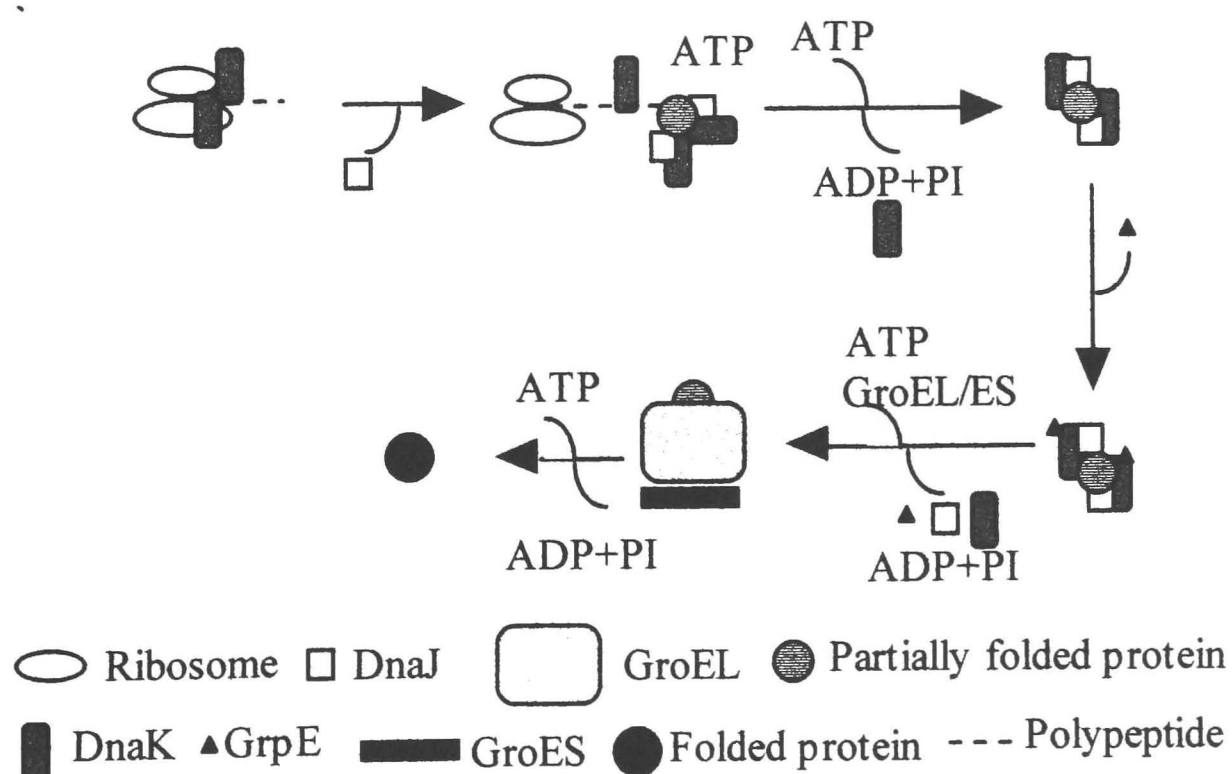
1.2.6.2. DNA repair system

The fact that aerobic organisms use O_2, this leads to creation of ROS (Demple and Harrison, 1994). These free radical forms of O_2 are toxic agents that induce cellular damage. Several defence systems have been developed for the cells' protection. Two main DNA repair mechanisms exist: a general DNA repair mechanism divided in three main categories (Chapter 1 Paragraph 1.2.6.2 a) and a more specific DNA repair pathway i.e. repair enzymes which help to counteract specific damage done by ROS.

a) General repair systems.

i) Nucleotide excision repair.

Several excision repair mechanisms exist such as the mismatch repair system (Murphy *et al.*, 1996) or *uvr* system. The general feature of all these excision repair mechanisms is that they recognise the mispaired or damaged bases in the DNA, excise the damaged sequence and synthesise a new stretch of DNA to replace the damaged one (Sancar and Sancar, 1988). This occurs in four stages. The first step is the recognition of the damaged sequence by an endonuclease which cleaves the strand on both sides of the damage. Then, a 5'→3' exonuclease removes the damaged stretch. Thirdly, DNA polymerase I synthesise a replacement sequence using the remaining sequence on the opposite strand as a template. And finally, DNA ligase links the 3' end of the newly synthesised sequence to the old stretch.

In *E. coli* the best characterised excision repair system is the *uvr* one. *uvr* A,B,C genes code for the repair endonuclease. Uvr AB locate the damage (e.g. pyrimidine dimers). UvrA dissociates from UvrB by ATP hydrolysis. UvrBC complex forms cut the DNA on both sides of the lesion. DNA helicase (UvrD) unwinds DNA. DNA polymerase and ligase

eliminate the damaged oligonucleotide and complete the repair (Demple and Harrison, 1994).

ii) Recombination mechanism.

In theory the recombination mechanism should repair any damage if an intact copy of the damaged region is available in the cell. At least two classes of oxidative damage makes the recombination mechanism crucial. The first one is the interstrand cross-links i.e. cross-links between groups in the same chain. The chemical nature and mechanisms are unknown, but two hypothesis on the nature of these cross-links have been suggested (Demple and Levin, 1991; Demple and Harrison, 1994). The first assumption is the formation of base dimers (e.g. pyrimidine dimers) induced by U.V and is considered as mutagenic. The second is the interstrand cross-link through presence of an heavy metal (e.g. *cis*-platinum) and is considered as being cytotoxic.

When DNA polymerase encounters a damaged nucleotide (e.g. pyrimidine dimer), it stops DNA replication, and starts again, 1000 bp down stream of the damage. The single-stranded gap formed will be recognised by RecA protein. RecA forms a complex between single-stranded and intact copy and promotes a strand exchange (Sancar and Sancar, 1988).

b) Specific DNA repair pathways.

Each pathway is specific to a certain type of damage and depends on the enzymes involved. The first pathway considered here involves glycosylases, and the second endonucleases. These two pathways could also be considered as being specific cases of the general excision repair pathway depicted in the previous sub-section (Paragraph 1.2.6.2 a).

i) Repair of damaged nitrogenous bases by DNA glycosylases.

These enzymes function to hydrolyse the N-C glycosylic bond (base-sugar bond) to release a damaged base from DNA therefore creating an AP site (Demple and Levin, 1991; Sancar and Sancar, 1988).

These enzymes are used for both oxidative and non-oxidative damage. One of these enzymes in *E. coli* is endonuclease III (a thymine glycol glycosylase). *E. coli* has developed enzymes of broad-specificity such as endonuclease III rather than many enzymes with a unique specific activity (Demple and Harrison, 1994).

ii) Repair of sugar damage by AP endonucleases.

AP endonuclease enzymes are involved in the repair of deoxyribose damage by attack of the AP sites or sugar fragment present in the DNA. In *E.coli*, two AP endonucleases have been well characterised being exonuclease III and endonuclease IV. These two enzymes are involved in the repair of oxidative damage caused to DNA. Both exonuclease III and endonuclease IV activate oxidised DNA templates allowing action of the DNA polymerase I. The two enzymes also work in concert in the repair of alkylation-induced AP sites and lesions produced by exposure to γ-rays (Demple and Harrison, 1994). Exonuclease III is considered as being the major AP endonuclease in *E. coli* . Strains deficient in exonuclease III (*xth*) are much more sensitive to H_2O_2 damage than wild-type strains (Demple and Levin, 1991). Strains deficient in endonuclease IV (*nfo*) are more sensitive to oxidative agents such as t-butyl hydroperoxide. The *nfo* mutation also increases the sensitivity to H_2O_2 damage of the *xth* strains (Demple and Levin, 1991).

1.2.6.3. Antioxidant defence.

Many molecules are available to the cell as antioxidants. These can be divided into two groups. The first is represented by molecules of a non-enzymatic nature (e.g. glutathione),

40

and the second, enzymes acting as antioxidants such as catalase, superoxide dismutase and glutathione peroxidase.

a) Non-enzymatic antioxidants.

These are primarily small molecules such as glutathione which are considered as chain breaking antioxidants (i.e. stop radicals chain reactions). They operate according to one of two modes of action. The first one is called the suicidal mode where the antioxidant molecule becomes oxidised when reacting with the radical. The second function is to prevent metal catalysed oxidation (e.g. Fenton reaction) by chelating metal ions present in the cell. One of the most important and thoroughly studied non-enzymatic antioxidants present in both prokaryotes and eukaryotes is glutathione (GSH) which is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) and is the most abundant cellular thiol (Meister and Anderson, 1983). GSH rapidly interacts with free radicals such as O_2^- and other oxidants such as H_2O_2 and forms an unstable GS˙ radical. This GS˙ needs to be stabilised and can either react with itself to form a stable dimer of oxidised glutathione (GSSG) or with other molecules such as O_2 therefore leading to toxic products within the cell (Cadenas, 1989). GSSG is then reduced to GSH by electron transfer performed by glutathione reductase. This reaction requires NADPH. With regard to the toxic products formed during GS˙ stabilisation, glutathione conjugates are degraded by a specific pathway called the glutathione conjugates pathway shown in Figure 1-4.

b) Enzymatic antioxidants.

The bacterial cell possesses several enzymes which act to detoxify ROS. Each enzyme degrades a specific substrate and three major groups of enzymes will be described in this section being glutathione-S-transferases, superoxide dismutases and catalases:

41

Figure 1-4: Glutathione conjugates pathway (reproduced from Pickett and Lu, 1989).

Gly
|
Cys——R
|
Glu

γ-Glutamyl
Transpeptidase

Glu

Gly
|
Cys——R

Cysteinyl Glycine
Dipeptidase

Gly

COOH
|
CH_2——CH
/   \
R——S NH_2

Cystein conjugate

N-Acetyl
Transferase

Deacetylase

Cystein Conjugate
β-lyase

COOH
|
CH_2——CH
/   \
R——S   NH
             |
             C==O
             |
            H_3C

Mercapturate

R——SH + Pyruvate + Ammonia

UDPG-Glucuronide
Transferase

S-Methyl
Transferase

R——S——Glucuronide

R——S——CH_3

Methylthio conjugate
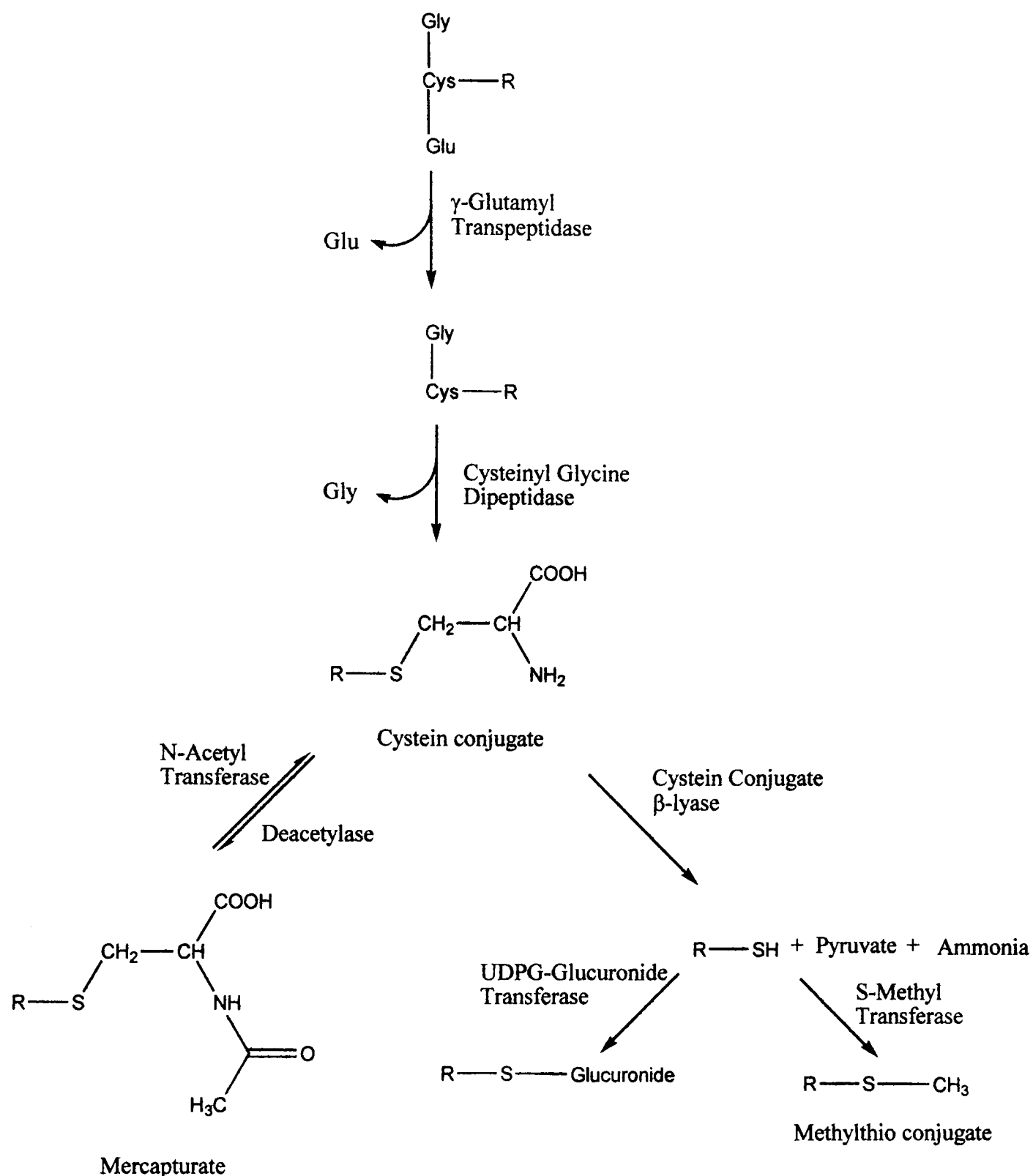
i) Glutathiones-S-transferases (GSTs).

Enzymes which catalyse the nucleophilic addition of the glutathione thiol group to various electrophiles such as alkyl halides of general formula R-X (Pickett and Lu, 1989), therefore forming glutathione conjugates which then enter the glutathione conjugates metabolism (Figure 1-4). However the major groups involved in the detoxification of the

cell from ROS are superoxide dismutase and catalases which will be described in more detail.

ii) Superoxide dismutases.

Superoxide dismutases (SOD) are enzymes which catalyse dismutation of O_2^- to H_2O_2. H_2O_2 as a final reaction product raised concern about the benefit of this reaction to a cell (Farr and Kogoma, 1991). The authors suggested 3 possibilities for cells which used superoxide dismutase. The first hypothesis was that O_2^- may be more harmful than H_2O_2. Molecules are unreactive with O_2^- as they resist univalent redox reactions (Keyer *et al.*, 1995). The second suggestion was that H_2O_2 was rapidly disproportionated to H_2O and O_2. The last proposition mentioned was the possibility that dismutation of O_2^- to H_2O_2 might be favoured over other routes leading to more toxic compounds such as glutathione radicals. Evidence that disproportionation of H_2O_2 is rapid ($\approx 10^9$ molecules/active site/second at 1 M H_2O_2), that it does not require an exogenous reducing source, nor ATP, are in favour of the second hypothesis (Farr and Kogoma, 1991).

iii) Catalases.

Catalases along with peroxidases belong to the group of hydroperoxidases. Catalases disproportionate H_2O_2 in two steps (Anon., 1997). In the first step, catalase ferric (Fe^{3+}) heme interacts with H_2O_2 leading to a catalase with an oxidation state being Fe^{5+} (Anon., 1997). The second step is the reaction of catalase in the latter exited state (Fe^{5+}) with a molecule of H_2O_2 to give native state catalase (Fe^{3+}), oxygen and water.

E. coli has been shown to produce two hydroperoxidases, HPI and HPII. HPI is produced when cells are exposed to H_2O_2 and is regulated by *oxyR* (Farr and Kogoma, 1991). HPII is produced in stationary phase under *katF* control (Farr and Kogoma, 1991) and is not induced by oxidative stress.

Most of these defence mechanisms can be induced by stressful conditions such as heat-shock proteins induced by heat or catalase (HPI) by presence of H_2O_2 (Farr and Kogoma, 1991). Induction of the response is rendered possible due to the presence of specific regulons containing genes which share genetic regulation. In the context of oxidative stress, two major regulons have been studied in both *E. coli* and *S. typhimurium* being SoxRS which responds to superoxide stress and OxyR regulons in facing an H_2O_2 insult (Demple and Amabile-Cuevas, 1991; Farr and Kogoma, 1991).

1.2.7. Oxidative stress regulons.

1.2.7.1. SoxRS regulon.

The SoxRS regulon requires two proteins SoxR and SoxS to be activated (Wu and Weiss, 1991; Nunoshiba, *et al.*, 1992). Activation of this regulon is thought to be a cascade of events (Figure 1-5).

Figure 1-5: Model of regulation of the *soxRS* regulon (reproduced from Demple and Amabile-Cuevas, 1991).

$O_2^{-\cdot}$ Redox activation

SoxR

Transcriptional activation

soxS

SoxS

Transcriptional activation

sodA, nfo, zwf, micF, etc
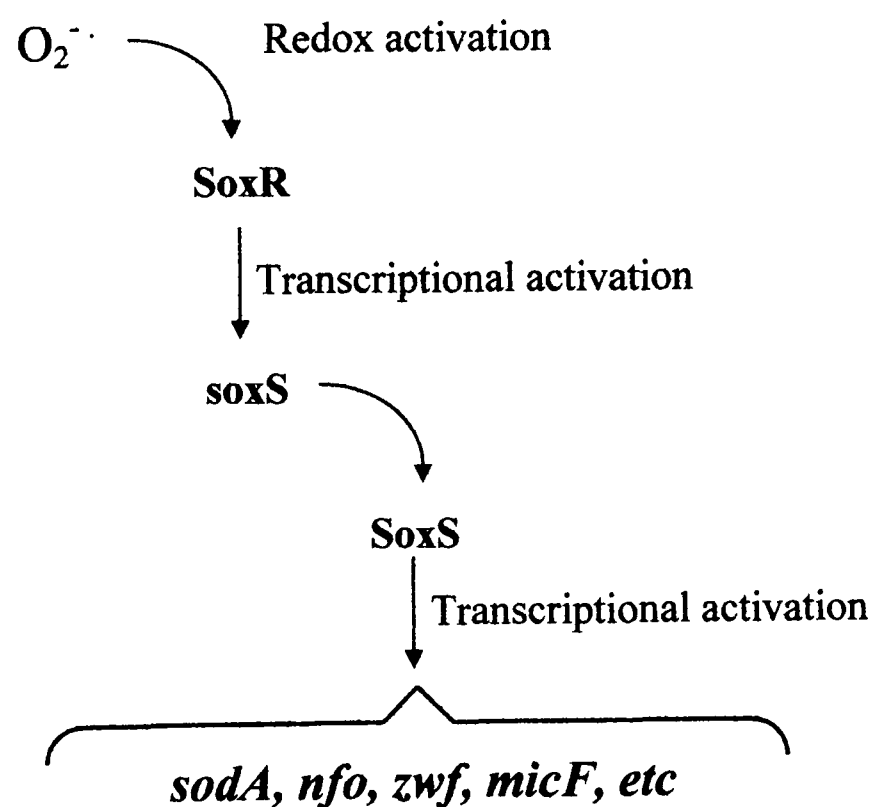
Superoxide radicals activate pre-existing SoxR proteins which then triggers expression of *soxS* gene leading to production of SoxS protein. SoxS protein then activates promoters of the regulon it controls, which triggers expression of several genes (Demple and Amabile-Cuevas, 1991). 2D-SDS PAGE analysis using labelled amino acids showed that the synthesis of at least 30 proteins was induced (Farr and Kogoma, 1991) under superoxide stress. Some of these have been identified such as *sodA* (Mn-superoxide dismutase), *nfo* (endonuclease IV), *zwf* (Glucose-6-phosphate dehydrogenase), although others remain unknown. Another interesting characteristic of SoxRS regulon activation is that it also increases resistance to antibiotics which are not involved in oxidative stress. It is suggested that activation of SoxRS regulon increases expression of *micF* (encoding an antisense RNA) which product inhibits *ompF* mRNA translation. *ompF* encodes OmpF porins reducing the membrane permeability to antibiotics (Farr and Kogoma, 1991).

1.2.7.2. OxyR regulon.

Only the OxyR protein is needed to activate the OxyR regulon. OxyR expression is controlled through a negative feedback loop (Storz *et al.*, 1990) and is growth-phase dependent (Gonzalez-Flecha and Demple, 1997). Toledano and colleagues (1994) demonstrated that OxyR acts both as a sensor and transducer of the stress signal. The authors also proposed that OxyR acts as a tetramer therefore binding DNA at four different sites. Reduced and oxidised OxyR have been shown to have different sites specificity (Toledano *et al.*, 1994). The authors found that upon oxidation, repositioning of the DNA footprints of OxyR occurred. OxyR was shown to bind to *katG*, *ahpC* and *gorA* promoters and activate them. Therefore it appears that OxyR senses oxidative stress by becoming oxidised, which brings about a change in conformation which in turn transduces the stress signal to RNA polymerase (Storz *et al.*, 1990). Synthesis induces around 30 proteins as

shown by 2D-SDS PAGE (Farr and Kogoma, 1991). Some of these have been identified such as *katG* (as HPI catalase), although others remain unknown.

The complexity of the stress response has been illustrated using the two examples detailed above. However many other regulons exist such as heat-shock and starvation regulons. Detailing every single regulons is not in the scope of this project however it is important to note that all these regulons have overlaps with each other. 2D-SDS PAGE analyses have shown that O_2^- can also induced some heat shock proteins such as GroEL, or catalase (belonging to H_2O_2 regulon) (Farr and Kogoma, 1991). Heat has been shown to induce SOS, *oxyR* and *soxRS* regulons (VanBogelen *et al.,* 1987) and starvation was shown to induce, for example, production of heat shock proteins (Jenkins, *et al.,* 1988). This adds to the complexity of the regulation of the stress response, but also favours a better defence against general stress conditions.

CHAPTER 2. False-positive reaction induced by the presence of *A. hydrophila* in the Colilert defined-substrate technology system.

2.1 Introduction.

Raw water destined for consumption originates from various sources such as rivers, and lakes but requires treatment to obtain water of hygienic quality prior to distribution. To ensure that the water entering the distribution system has reached official standards, a series of monitoring tests have to be performed, including basic microbiological analysis (Anon., 1994). Finding pathogenic microorganisms amongst the high number of heterotrophic and commensal bacteria from human and animal faeces found in water is a very lengthy, time consuming procedure and does not prove to be convenient as routine microbiological tests. This unsuitability is due to the fact that screening for pathogenic microorganisms would require several samples of large volumes of water, a wide range of selective media specific to the pathogens suspected to be present and would consequently prohibit rapid screening. Therefore, assessment of the microbiological quality of drinking water relies upon screening for specific indicator organisms, in particular coliforms and *E. coli* rather than for specific pathogens. Indicator organisms are almost always present in human and warm-blooded animal's faeces in high numbers and are easy to isolate and characterise. The indicator organisms selected must be representative of the water contamination bacterial flora and ideally should have the following characteristics (Anon., 1994):

1) Be abundant in faeces and sewage but not normally from other sources, or if so be in very low numbers.

2) Denote the potential presence of intestinal pathogens.

3) Have the capacity for an easy isolation, identification, enumeration and be unable to replicate in the aquatic environment.

Unfortunately, no known microorganisms display all these criteria at the same time, but *E. coli* and coliforms collectively fulfil most of them (Anon., 1994). Monitoring the presence of coliforms and *E. coli* is acknowledged to provide reliable information regarding the presence of faecal contamination of the water supply being investigated. Both standard methods and new methods to be validated must detect total coliforms and faecal coliforms according to the following definitions.

2.1.1 Definition of total coliforms.

Members of the genus or species within the family of the *Enterobacteriaceae* capable of growth at 37°C, oxidase negative and normally possessing ß-galactosidase (an enzyme responsible for the degradation of lactose into glucose). This revised definition (Anon., 1994), used as a working definition for water examination, has been proposed to eliminate the variable characteristics of lactose fermentation as it is not displayed by all coliforms.

2.1.2 Definition of faecal coliforms.

Bacteria showing a positive result for acidification of a lactose medium, growing at 44°C, and bearing the same characteristics as coliforms (i.e. oxidase negative) are considered as being presumptive faecal coliforms, as some thermo-tolerant coliforms are not of faecal origin, and some faecal coliforms are not thermo-tolerant (Anon., 1994).

2.1.3. Standard techniques for detection of *E. coli* and coliforms from water.

Examination for these organisms is normally performed by standard protocols using either the most probable number technique or by membrane filtration (Anon., 1994). The standard stipulates that coliforms should be absent from a 100 ml sample.

2.1.3.1 The most probable number technique (MPN).

This method is statistical and based on probabilities. A series of multiple dilutions of the sample is performed in quintuplet using an appropriate liquid differential medium in order to reach a point of extinction (no growth) where no organism from the sample has been deposited in the test medium and where growth is considered as being the positive criterion. The pattern of positive and negative results is then used to estimate the extent by which the sample has been contaminated by comparing the growth pattern obtained with a table of statistical probabilities. The medium recommended (Anon., 1994) for coliforms isolation is minerals modified glutamate medium into which an inverted Durham vial is placed to detect gas production. MacConkey medium was found to be inappropriate due to variation in the inhibitory properties of bile salt batches (Anon., 1994). Further confirmatory tests are required such as oxidase and indole tests if positive results are obtained (Anon., 1994).

2.1.3.2 Membrane filtration method.

This method involves the filtration of a defined volume of sample to be tested via a filtration apparatus utilising a membrane of defined pore size (usually 0.45 μm) in order to retain the microorganisms for further analysis. Upon removal from the filtration apparatus, the side of the membrane which has been in contact with the water sample is placed upward on the appropriate selective medium and incubated at the selected temperature for a defined length of time. Colony morphology and the change in colour of the indicator

present in the medium give a presumptive identification of the microorganisms being tested. Further confirmatory tests are required such as oxidase and indole tests if positive results are obtained (Anon., 1994).

2.1.4. Emerging techniques for the detection of *E. coli* and coliforms from water.

In recent years there has been a move towards development of new methods designed to resolve three major problems by:

1) Decreasing the time period before results are available.

2) Improving the degree of sensitivity.

3) Increasing method versatility.

Before any alternative method can be accepted, it must be shown to have at least the same detection limit as those currently in use. Several different methods have been described recently such as the use of polymerase chain reaction (PCR) (Fricker & Fricker, 1994) or defined-substrate medium (Edberg *et al.*, 1988; Anon., 1994).

2.1.4.1 Use of polymerase chain reaction (PCR) in monitoring microbiological water quality.

The PCR technique has been used to detect coliforms and *E. coli* using specific primers for these indicator organisms. Primers designed for *E. coli* (derived from *uidA* gene encoding for the β-glucuronidase enzyme) resulted in the appropriate detection of 100 % of the *E. coli* present in the samples tested. However primers designed for coliforms only detected 70 % of the coliforms tested (Fricker & Fricker, 1994). This was due to the fact that it is difficult to design primers only specific to coliforms as other non-coliform organisms can be closely related to coliforms. Further research is currently underway to find a suitable set of primers appropriate for detection of all coliforms. However it seems unlikely that PCR

will be used in the water industry as it detects both living and non-viable cells (Fricker & Fricker, 1994). This lack of discrimination comes from the fact that cells present in the sample have to be lysed by heat treatment, and the DNA released from the previously living, dead cells or non-viable cells will be present in the same solution without distinction.

2.1.4.2 Defined-substrate technology.

The second option being developed is the use of defined-substrate technology. In the Report of Public Health and Medical Subjects N° 71 (Anon., 1994), this type of technique is defined as being a presence-absence test for total coliform organisms. The definition given is that this technique is a modification of the most probable number technique where one large volume is used instead of the serial dilution. The recommended medium (minerals modified glutamate medium) is the same as for the MPN. Other defined medium have been compared in order to verify whether they facilitate the recovery of the indicator organisms, such as membrane lauryl sulphate broth or membrane lactose glucuronide agar (Walter *et al.,* 1994).

The auto analysis Colilert system is a different type of defined-substrate technology as it does not involve the use of a medium supporting growth of micro-organisms, but acts as a buffer which allows recovery of injured coliforms and *E. coli* . This system is one of the most prevalent kits on the market (Covert *et al.,* 1992). The system is based on the degradation of ortho-nitrophenyl-ß-galactopyranoside (ONPG) to give ortho-nitrophenyl (λ_{max} = 420 nm) by the enzyme ß-galactosidase produced by the coliforms and *E. coli* , and the degradation of 4-methyl-umbelliferyl-ß-D-glucuronide (MUG) by the enzyme ß-glucuronidase, produced by *E. coli,* leading to a fluorescent compound (i.e. 4-methyl-umbelliferone) detected at 366 nm. A study published by Edberg and colleagues (1988) stated that the goals of this system were :

• To have a detection limit of 1 total coliform per 100 ml in a maximum of 24 hours.

• To simultaneously detect *E. coli* and coliforms in the same test.

• The results should not be affected by the heterotrophic community.

• No confirmatory tests should be required as the enzymes were supposed to be constitutively produced only in coliforms and *E. coli*.

• To allow detection of injured coliforms.

• To be easy to inoculate and that the results should be easy to interpret.

The authors concluded that the auto analysis Colilert system met these criteria and gave results similar to the standard method studied (MPN) with a significant labour reduction (Edberg *et al.*, 1988). Nevertheless false-positive reactions were shown to occur (Edberg *et al.*, 1988) despite the fact that this system has been developed to suppress non-coliform interference. *Aeromonas* spp. were found to lead to a false-positive result at concentration above 10^4 cfu/ml (Edberg *et al.*, 1988). The aims of the present study was to assess the extent by which *Aeromonas* spp. led to false-positive reactions within the Colilert defined-substrate technology system. The effect of different parameters (i.e. age of reagents, dilution medium) on such false-positive reactions were also investigated.

2.2 Materials and methods.

2.2.1 Materials.

2.2.1.1 Reagents.

Reagents were of analytical grade and were purchased from either Fisons Scientific Equipment, Bishop, Loughborough, UK or BDH Laboratory Supplies, Poole, UK. The Colilert kits were purchased from IDEXX Laboratory, London UK.

2.2.1.2 Bacterial strains.

The following type strains were obtained from the National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, UK: *Aeromonas hydrophila* NCIMB 9240, *Klebsiella oxytoca* NCIMB 12849, *Escherichia coli* NCIMB 12819. *Pseudomonas aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection, Rockville, Maryland, USA. All strains were maintained on nutrient agar with frequent sub-culture every 3 to 4 weeks. Master plates were grown at 30°C for 24 h and stored at 4°C until use.

2.2.2 Methods.

2.2.2.1 Effect of Colilert reagent age upon false-positive reaction mediated *Aeromonas* spp.

A colony from the stock plate of each of the test organism and controls was used to inoculate 9 ml of M9 medium and the tube incubated aerobically at 30°C for 18-24 hours. M9 minimal buffer (M9 buffer) was prepared as follows (mM): Na_2HPO_4, (42); KH_2PO_4, (22); NH_4, (19); NaCl, (8.5); and the solution adjusted to pH 7.2. This solution was autoclaved at 121°C for 15 minutes. 75 ml of deionised water was added to 20 ml of 5xM9 buffer, and this autoclaved for 15 minutes at 121°C. 1 ml aliquots of the following sterile

solutions were added to this medium: 20% (w/v) glucose; 10 mM $CaCl_2$; 100 mM $MgSO_4.7H_2O$ to give M9 minimal medium (M9 medium). 1 ml of *A. hydrophila* suspension was used to prepare a 10-fold dilution series (down to 1×10^{-6}) using a sterile diluent of 1.0 % w/v saline. The Colilert reagent was reconstituted using 100 ml of sterile water and aliquoted into 9 ml and 10 ml volumes. A 1 ml aliquot from each dilution was used to inoculate 9 ml of Colilert previously reconstituted with sterile water. Both fresh and aged Colilert reagent were examined, with aged reagent being within 4 weeks of the shelf-life expiry date. 100 μL of overnight culture of *K. oxytoca, E. coli* (positive controls) and *Ps. aeruginosa* (negative control) were used to seed 10 ml of reconstituted Colilert reagent. An *A. hydrophila* positive control was also prepared in this manner. All tubes were incubated in a 35°C water-bath for exactly 24 hours. The production of *O*NP was determined by spectrophotometry, recording the absorbance at 420 nm using a Novaspec II spectrophotometer (Pharmacia Biotech). The remaining volume from each dilution, either 8 or 9 ml, was used to determine the viable cell count. These dilutions were filtered through 0.45μm 47 mm pre-sterilised membrane filters (Whatman, UK) which were transferred to tryptone soya agar medium. The plates were incubated at 30°C for 24 hours and the cell count from each plate established.

2.2.2.2 Effect of salt content of the dilution medium upon false-positive reaction mediated by *A. hydrophila*.

A colony from the stock plate of each of the test organisms was used to inoculate 9 ml of M9 medium and the tube incubated aerobically at 30°C for 18-24 hours. 1 ml of *A. hydrophila* suspension was used in a 10-fold dilution series (10^{-1} down to 10^{-6}) using sterile water as the diluent. The Colilert was reconstituted using 50 ml of sterile water. This double strength Colilert solution was aliquoted into 5 ml volumes and an increasing volume of 1% w/v saline was added to the different tubes (final concentration from 0.1%

54

to 0.5%), with each tube having a final volume of 10 ml. Controls were prepared as described in the previous experiment. All tubes were incubated in a 35°C water-bath for exactly 24 hours and production of ONP determined as previously mentioned.

2.2.2.3 Effect of Colilert reagent age upon false-positive reaction mediated by an *A. hydrophila* under increasing saline concentrations.

This experiment was performed following the protocol described in Chapter 2, paragraph 2.2.2.1., except that two series of dilutions were prepared instead of one. The first one used sterile 1.0 % saline and the other one used sterile deionised water as the dilution medium. This experiment was performed with both aged and fresh Colilert reagents.

2.2.2.4 Effect of nutrient limitation upon false-positive reaction mediated by *A. hydrophila.*

A colony from the stock plate of each of the test organisms was used to inoculate 9 ml of M9 medium and the tube incubated at 30°C for 18-24 hours. Cells were then washed, resupsended in 100 ml of M9 buffer (prepared as follows (mM): Na_2HPO_4, (42); KH_2PO_4, (22); NH_4, (19); NaCl, (8.5); and the solution adjusted to pH 7.2 with conc HCl. This solution was autoclaved at 121°C for 15 minutes. The suspension was incubated for 5 days at room temperature prior to the experiment in order to starve the cells. The rest of this experiment was performed following the protocol mentioned in Chapter 2 paragraph 2.2.2.1.

2.2.2.5 Effect of chlorine upon false-positive reaction mediated by *A. hydrophila.*

Two 100 ml samples of tap water were filter sterilised using Whatman 47 mm/0.45 µm membrane filters according to standard procedures (Anon., 1994). One of the volumes received 0.1 ml of a 1.8 % w/v sodium thiosulphate solution in order to neutralise any residual chlorine present. Both volumes were then spiked with 200 µl volume of an *A.*

hydrophila overnight culture grown in M9 medium. These two 100 ml volumes were left to incubate at room temperature for 5 days and examined as described in Chapter 2 paragraph 2.2.2.1.

2.3 Results.

2.3.1 Effect of age of the Colilert reagent upon false-positive reaction mediated by *A. hydrophila.*

The Colilert defined-substrate technology system was used according to the manufacturer's instructions with regards to reconstitution of the medium. A series of experiments were performed at two month intervals until the shelf-life expiry date was reached. Reconstituted Colilert was seeded using an overnight culture of *A. hydrophila*, incubated in a 35°C water-bath for exactly 24 hours and absorbance at 420 nm recorded using a spectrophotometer. The results are gathered in Figure 2-1. In order to ease the interpretation of the Figure, the positive controls (*K. oxytoca, E. coli*) and the negative control *P. aeruginosa* are not included in Figure 2-1, but absorbance data are shown in Table 2-1.

Table 2-1: Data from the time-scale experiment performed on the Colilert system. Expiry date of the kit was 3/9/97. Cell concentration in the positive and negative control tubes were of 10^7 cells/ml. Blanks were performed with reconstitued Colilert reagent only.

cell count A. hydrophila	OD$_{420nm}$ 25/4/97	OD$_{420nm}$ 3/6/97	OD$_{420nm}$ 13/8/97	OD$_{420nm}$ 3/9/97
10^1	0	0.006	0	0
0.005	0		0.03	0.25
10^3	0.001	0.006	0.08	0.635
10^4	0.007	0.007	0.568	0.821
10^5	0.035	0.024	1.46	1.721
10^6	0.093	1.35	2.374	2.287
10^7	0.431	2.316	2.408	2.317
E.coli*	**0.544**	**0.396**	**1.397**	**1.335**
K.oxytoca*	**0.157**	**0.492**	**2.378**	**2.367**
Ps.aeruginosa*	**0.11**	**0.37**	**0.503**	**0.481**

- Positive and negative controls are shown in bold

It is nevertheless important to state that *K. oxytoca* and *E. coli* displayed a positive result in all experiments, as would be expected, and that *P. aeruginosa* displayed a negative result.

Figure 2-1
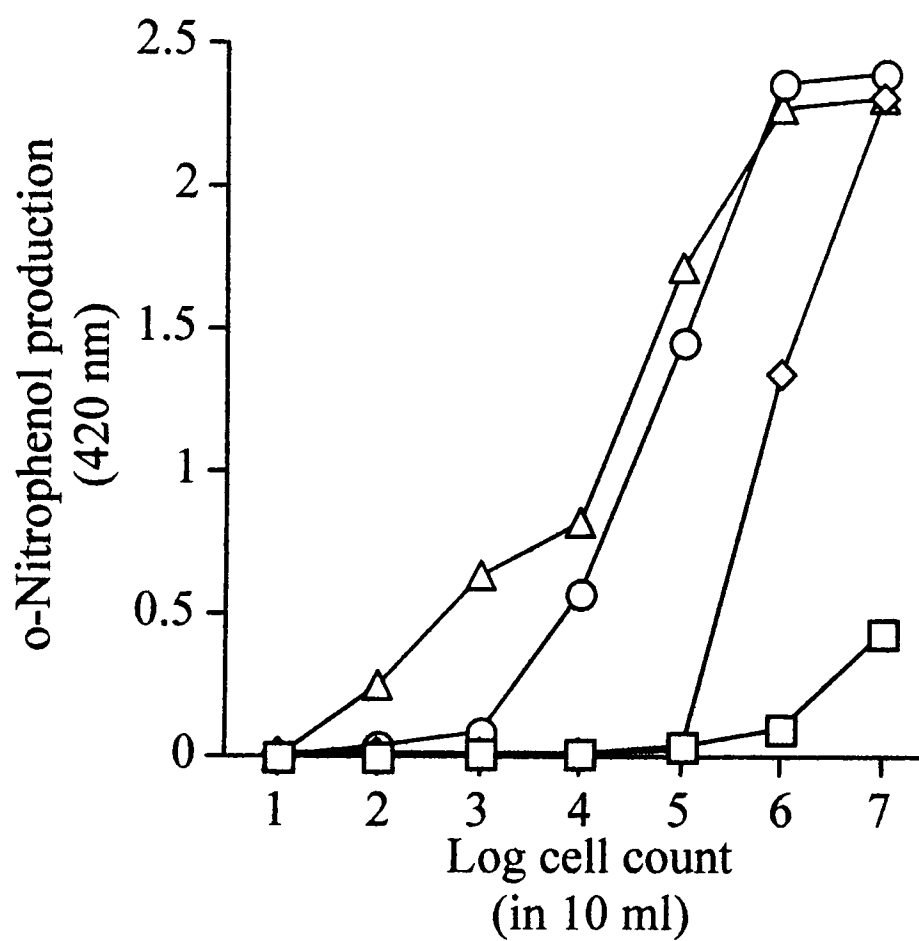
Effect of age of Colilert reagent upon false-positive reaction mediated by ***A. hydrophila.***
Reagent were 4 months prior to expiry date 25/4 (□), 2 months prior to expiry date 3/6 (◇), 1 week prior to expiry date 13/8 (○) and on the expiry date 3/9 (△).

False-positive reactions in the Colilert system have been reported previously with *A. hydrophila* (Edberg *et al.*, 1989: Cowburn *et al.*, 1994) when present at high cell density ($>10^4$ cfu/ml). Data obtained are in agreement with these earlier studies. Results in Figure 2-1 show a gradual decrease in the concentration of *A. hydrophila* required to cause a false-positive reaction correlated with the ageing of Colilert reagents. *A. hydrophila* has been considered unlikely to cause false-positive reactions when using the Colilert defined-substrate system to monitor drinking water samples, as they are usually present in low level in potable waters (Holmes and Nicolls, 1995). However the present study has raised concern about the stability of the Colilert reagents with time. Members of the genus *Aeromonas* are known to be haloduric, meaning they can grow when in the presence of a substantial amount of NaCl in the medium. One of the identification test for this genus is the growth in 1.5% NaCl. It was argued that results were influenced by NaCl from the 1% saline dilution buffer used (Fricker, personal communication). In order to verify whether results obtained were due to the presence of salt or if age of the Colilert reagents had indeed an effect, the following experiments were undertaken.

2.3.2 Effect of salt content of the dilution medium upon false-positive reaction mediated by *A. hydrophila*.

The next set of experiments were designed to investigate the effect of NaCl upon the false-positive reaction mediated by a low concentration of *A. hydrophila*. Increasing amounts of NaCl were added to tubes containing double strength Colilert medium. A cell concentration of 10^3 cells/ml were added to each tube. Tubes were then incubated in a 35°C water-bath for exactly 24 hours and absorbance recorded at 420 nm using a spectrophotometer. The results are shown in Figure 2-2, from which it can be seen that the

Figure 2-2
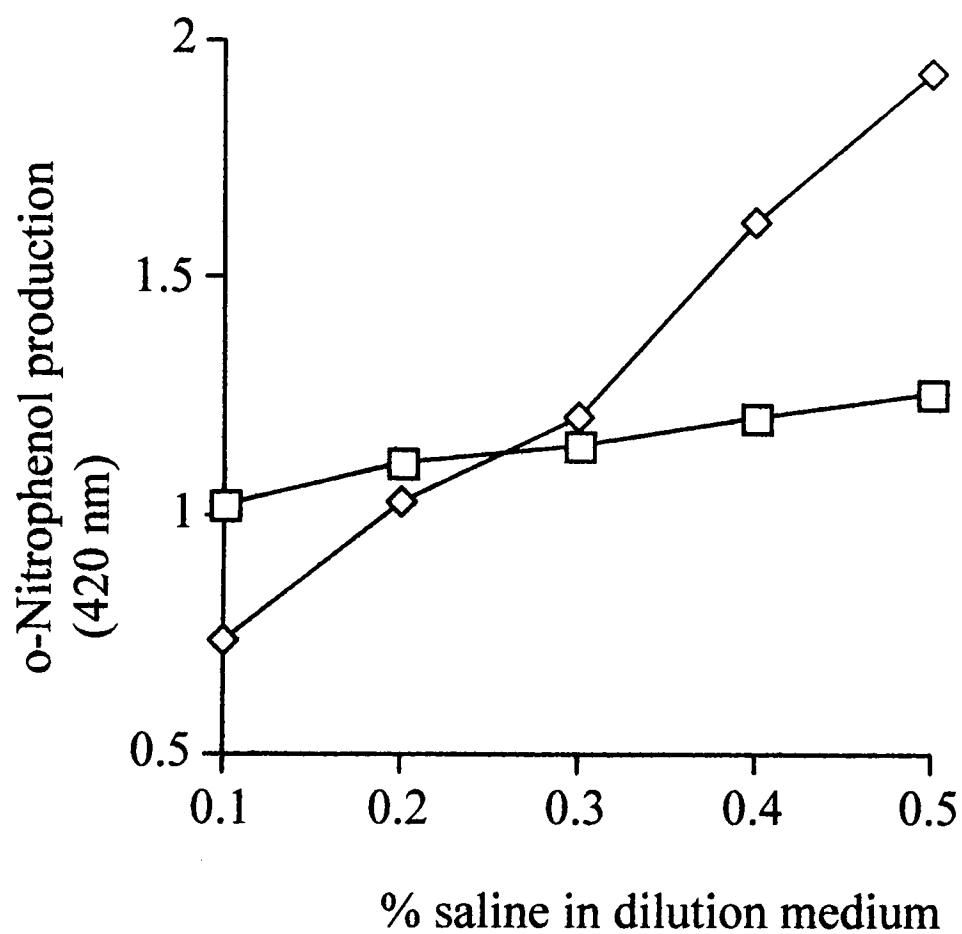
**Effect of salt content of the dilution medium upon false-positive reaction mediated by
A. hydrophila.**
Old kit date 13/8 (□), New kit date 25/4(◇).This was also the case when a dilution
medium containing NaCl was used with the old kit.

salt present within the dilution medium has a more significant effect when fresh Colilert kit is used. Cell counts of 10^3 cells/ml mediated a false-positive reaction. However it seems that the salt has a minor effect in the old kit compared to the fresh kit (Figure 2-2). This would suggest that whereas salt has a definite effect in the potential to mediate false-positive reaction in fresh kits, the effect of age of the Colilert reagents is not an artefact brought with the presence of salt as this has a negligible effect when the reagent is near shelf-life expiration. In order to verify this hypothesis, a second set of experiments was designed to investigate the role of reagent age with regards to the salt concentration.

2.3.3 Effect of age of the Colilert reagent upon false-positive reaction mediated by an *A. hydrophila* cell concentration of 10^3 cells/ml under presence or abscence of salt.

As demonstrated in Figure 2-2, the NaCl content of the 1 % saline dilution buffer has an effect on the occurrence of the false-positive reaction mediated by *A. hydrophila*. However a noticeable difference between fresh and old reagent was also observed. In order to clarify the role played by the ageing of the Colilert reagents in false-positive reactions, both fresh and aged Colilert systems were tested using cultures diluted in either water or 1% saline. Results are presented in Figure 2-3, where it can be seen that the false-positive reaction occurs at lower cell densities for the aged kit than for the new kit regardless of the medium used for the *A. hydrophila* dilution. The difference observed between the new and old kit when using water as a diluent for the cultures, eliminates the potential effect of salt and strongly suggests that age of the Colilert reagents is crucial for determining the cell count at which false-positive reactions occur. When deionised water was used as a dilution medium, the false-positive reaction occurred at 1×10^5 cfu/ml for the fresh kit whereas the cell density needed for a false-positive reaction to be displayed with the old kit was between 1×10^2 to 1×10^3 cfu/ml. Figure 2-3 clearly shows that the composition of the dilution medium plays a very important role alongside reagent age.

Figure 2-3
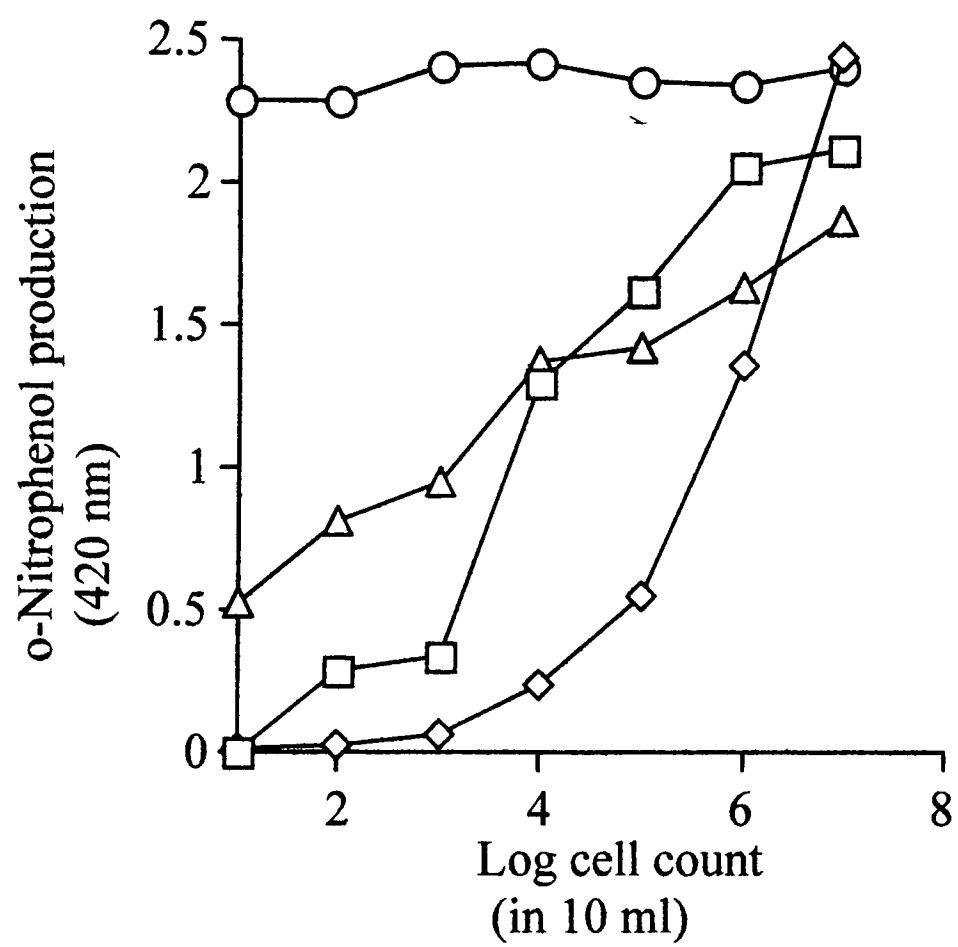
Effect of age of Colilert reagent and salt content of the dilution medium upon false-positive reaction mediated by *A. hydrophila*.
Old kit + H_2O (□), New kit + H_2O (◇), Old kit + 1% saline (O) and New kit + 1% saline (Δ).
Old kit is from the 13/8 and the new kit from 25/4.

If NaCl is present, the likelihood of a false-positive occurring appears to be greatly increased. Even the fresh kit shows false-positives at very low cell density (1×10^2 cfu/ml). Results obtained in Figure 2-3 therefore suggest that the presence of NaCl may interfere with the role of the growth inhibitors present in the Colilert reagents. This effect seems to be more significant with the fresh kit, whereas false-positive reaction in kits close to their expiry date might essentially be due to destabilisation and degradation of the chromophore reagent.

2.3.4 Response of *Aeromonas* spp. using both aged and fresh Colilert reagents.

The previous experimental investigations have established the role of *A. hydrophila* as an agent for mediating false-positive reaction within the Colilert system. It is understood that *A. hydrophila* is considered as the major human pathogen of the motile, mesophilic group, but as mentioned in the introduction, other members of this group have been implicated as potential human pathogens. It is therefore important to assess whether these potential pathogens also have the ability in mediating false-positive reaction in the Colilert system. Experiments using fresh and old Colilert reagents were undertaken using the same protocol as for *A. hydrophila* studies. Results are presented in Figure 2-4. These data show further evidence that very low amounts of *A. hydrophila* (1×10^1 cells/ml) do indeed mediate false-positive reaction in the Colilert system. Furthermore, Figure 2-4 also shows that *A. caviae* and to a lesser extent, *A. sobria* are also potential agents for mediating false-positive reaction when dilution medium used was 1 % saline with a Colilert kit within 4 weeks of the expiry date. This demonstrates that the genus *Aeromonas* not only produce β-galactosidase, therefore leading to false-positive reactions, but also that all members tested have the potential to prevent supression in the Colilert test medium despite the fact that growth inhibitory substances are present.

Figure 2-4
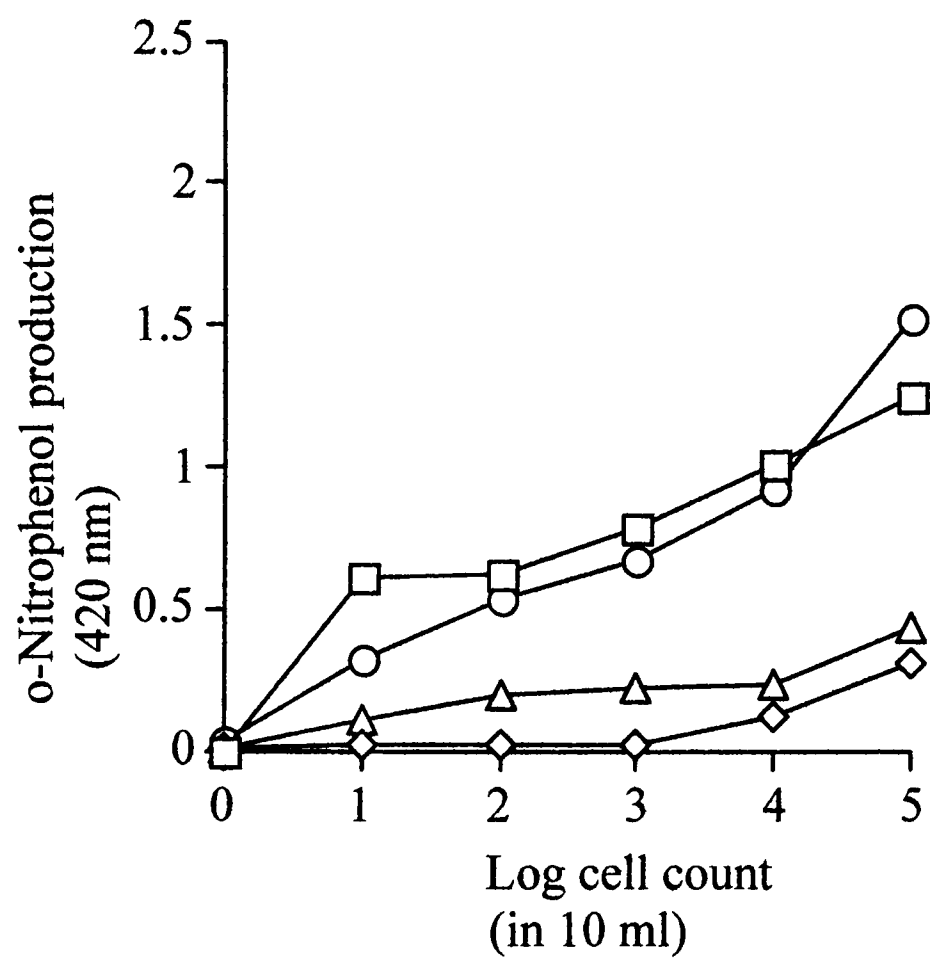
Response of *Aeromonas* spp. using both aged and fresh Colilert reagents.
A. hydrophila + old kit (□),*A. hydrophila* + new kit (◇),*A. caviae* + old kit (○) and *A. sobria* + old kit (△).
Old kit is from the 13/8 and the new kit from 25/4.

2.3.5 Effect of nutrient limitation upon false-positive reaction mediated by *A. hydrophila.*

All previous experiments were performed with bacteria grown under very favourable conditions regarding nutrient availability and temperature. Such conditions are not encountered in nature and might influence the response of the microorganisms to Colilert reagents. To investigate whether false-positive reactions encountered in Colilert were altered when cells faced a nutrient limited environment, *A. hydrophila* was grown overnight in 9 ml of M9 medium, washed in 1 % saline, re-suspended in M9 buffer and incubated at room temperature for 5 days prior to assessment with Colilert in order to simulate such poor nutrient conditions (Figure 2-5). The data clearly show that nutrient depleted environments do not affect the ability of *A. hydrophila* to mediate false-positive reactions at low cell levels. This suggests that the effect seen upon the false-positive reaction is due to the age of the reagents and the salt content of the dilution medium (1 % saline) and not upon any stages of growth or nutritional status of the microorganism.

2.3.6 Effect of chlorine upon false-positive reaction mediated by *A. hydrophila.*

Any potential interference that might have been caused by the presence of chlorine in the water was ruled out by undertaking the Colilert experiment using an old reagent and 2 samples of filter-sterilised tap water, one containing thiosulphate (Figure 2-6). Both samples, either treated or untreated with thiosulphate, display a similar profile at all cell densities tested. Thiosulphate did not appear to significantly increase or decrease the incidence of false-positive reactions mediated by *A. hydrophila* when using a fresh reagent. This suggests that the difference observed between the fresh and old kit (Figure 2-5) is only due to ageing reagents as no salt or chlorine were present during the experiments shown in Figure 2-6.

Figure 2-5
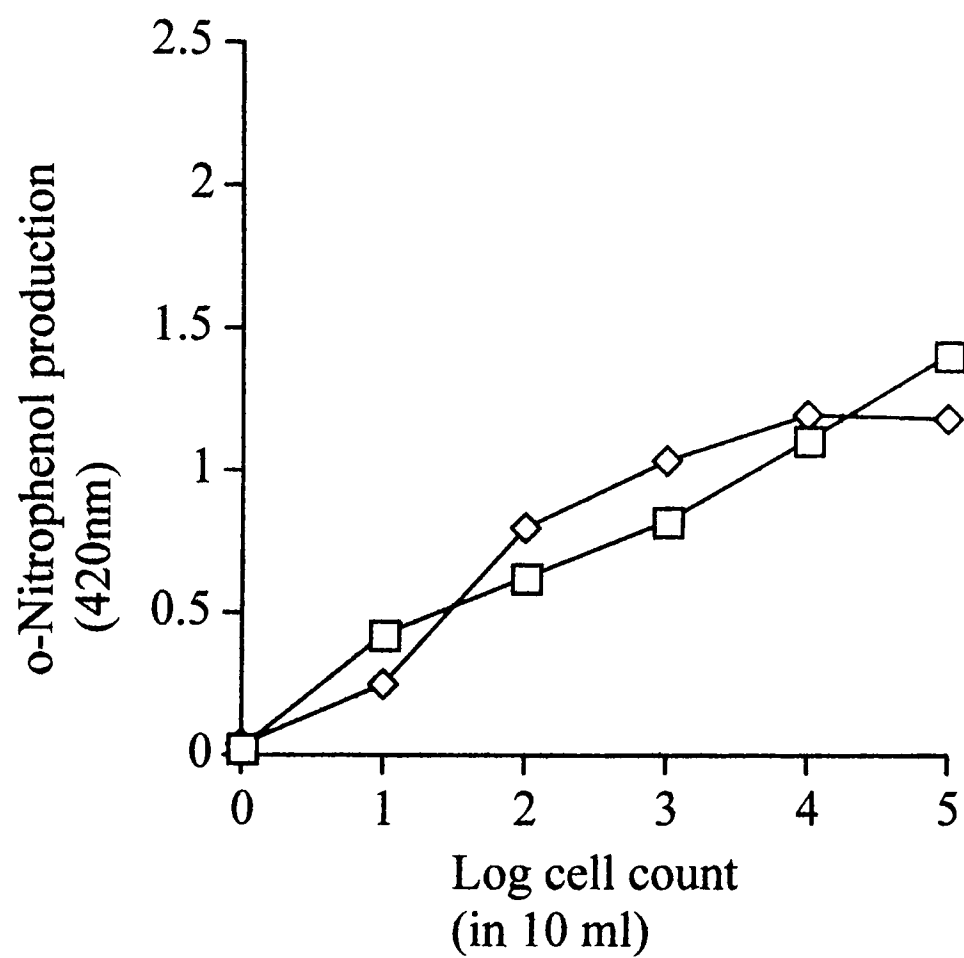
Effect of nutrient limited environment on the false-positive reaction mediated by _Aeromonas_ spp. using aged Colilert reagents.

Culture grown in M9 medium, washed in 1% NaCl and re-suspended in M9 buffer for 5 days (□),culture grown in M9 medium, re-suspended to a cell density of 10^6 cells/100 ml of filter sterilised tap water for 5 days (◇).

Figure 2-6
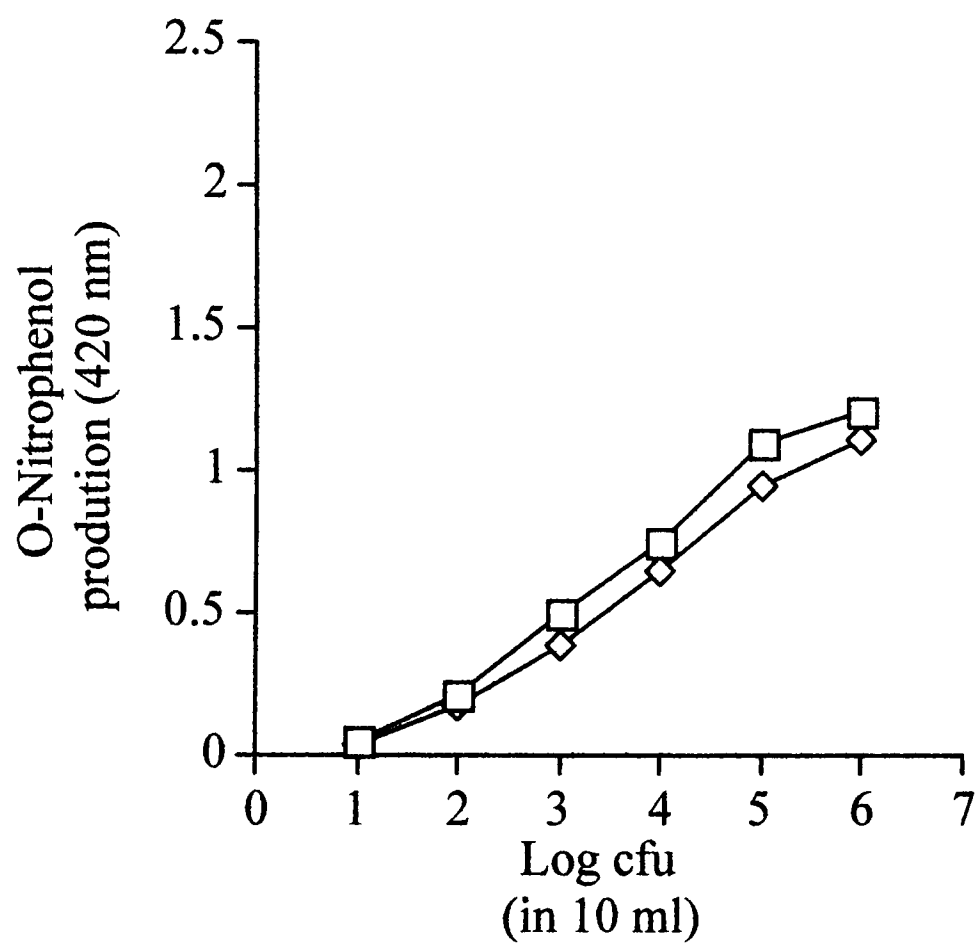
Effect of nutrient limited environment on the false-positive reaction mediated by
***Aeromonas* spp. using fresh Colilert reagents.**
Culture grown in M9 medium, re-suspended to a cell density of 10^6 cells/100 ml of filter
sterilised tap water for 5 days ($\square$),culture grown in M9 medium, re-suspended to a cell
density of 10^6 cells/100 ml of filter sterilised tap water for 5 days + addition of thiosulfate
($\diamondsuit$).

2.4 Conclusions.

Standard methods for the detection of pathogenic micoorganisms in drinking water supplies described in the introduction are usually time consuming and require confirmatory tests on presumptive positive. Recently, development of new methods to improve either the rapidity in obtaining the results or the sensitivity of the method itself has been a priority. The Colilert defined-substrate technology system was designed for this purpose and gives a presence/absence result within 24 hours. Nevertheless, false-positive reactions mediated by *Aeromonas* spp. were previously reported for cell concentrations >10^4 cfu/ml (Edberg *et al.*, 1988). Results from the current study agree with the general finding that the Colilert defined-substrate technology system can produce false-positive reactions due to non-coliform bacteria at rather high cell concentrations which do not generally meet the water quality standards of potable water. However, a closer inspection of the results gathered (Figure 2-1) showed a time-dependent increase in the occurrence of false-positive reaction mediated by the type strain *A. hydrophila* NCIMB 9240. This would suggest that some components of the Colilert kit become unstable with time. The composition of the Colilert medium is under patent and could not be revealed to allow further study. It can nevertheless be hypothesised that this increase in false-positives may be due to the failure of 2 types of compound. The first is the chromophore used i.e. *O*NPG. However degradation of *O*NPG to *O*NP should be visible when kit is reconstituted as the latter compound would display a strong yellow colour, the characteristic used for determination of the positive reaction. On extended ageing this phenomenon has been observed. The possibility remains that, with age, the molecule would become more sensitive to hydrolysis when in aqueous phase and at temperature higher than room temperature, leading to an increased hydrolysis during incubation at 35°C. This could be verified by reconstituting Colilert kits of increasing age, incubating the tubes at 35°C and recording at regular time

intervals the optical density using a spectrophotometer set at a wavelength of 420 nm. Plotting the absorbance data against time would allow determination of any correlation between ageing of the reagent (i.e. instability of $ONPG$) and increased rate of hydrolysis.

The second possibility leading to this increase in false-positive reactions could be the degradation of the inhibitory substance(s) that the Colilert reagent contains. Even if the nature of the growth inhibitory substance(s) is unknown, it has been shown in our laboratory (la Grange, personal communication) that *A. hydrophila* plate counts performed during the Colilert experiment are higher in samples tested on the old kits than for those tested on the fresh kits. This would favour the hypothesis regarding instability of the growth inhibitory substances as time elapses. The presence of low amounts of NaCl (0.1% w/v) in the sample was apparently sufficient to inactivate the effect of the inhibitory substance(s) present in the Colilert defined-substrate technology system and promote growth of *Aeromonas*, a microorganism shown to be NaCl tolerant from 0-4%.

It might also be possible a combination of both of the above possibilities.

2.4.1 Effect of salt content of the dilution medium upon false-positive reaction.

In order to investigate whether the age of the reagents of the kit was an experimental artefact or if it was indeed significant, further experiments were designed examining different salt concentrations in both fresh (2 months prior to expiry date) and old (1 week prior to expiry date) Colilert kits. Results obtained in Figure 2-2 showed that the NaCl concentration had an effect on the extent by which false-positive reaction occurred. However although the NaCl content had a dramatic effect when using the fresh reagent, this effect was relatively minor when results from the old reagent are examined. This could be explained by the fact that fresh reagents are stable and the 'initial background noise' produced by product decomposition is negligible. Consequently, when NaCl is present in the medium, the haloduric portion of the heterotrophic population, e.g. *Aeromonas* spp.,

encounter favourable growth conditions in the buffer where inhibitory molecules are inactivated or not in sufficient concentration to counteract the positive effect of NaCl. Whether these molecules react with the salt or the concentration becomes insufficient is unknown due to the lack of information upon the nature of these inhibitory molecules. Further investigation on different salts which might be present in water such as phosphate or nitrate salts would be worthwhile. Such studies would have to be done on individual salts, but also on different combination of these salts. This would allow determination of whether the combined effect of salts would mediate false-positive reactions, where the concentration of individual salts might be insufficient to mediate such a response.

2.4.2 Effect of age of the Colilert reagent upon false-positive reaction mediated by an *A.hydrophila* cell concentration of 10^3 cells/ml under an increasing saline concentration.

The fact that salt content with old reagent had a minor effect on the false-positive response suggest that inhibitory molecules are inactivated according to the length of storage, therefore making the inactivation of the inhibitory molecule by the presence of salt obsolete. Growth of *Aeromonas* would be possible at any salt concentration in the old reagent because of the lack of inhibitory molecule(s) whereas with new reagent growth would be dependent upon salt concentration.

In order to eliminate any doubt on the issue of reagents age dependence of false-positive reactions in the Colilert define substrate technology system, experiments were repeated using sterile water or 1 % saline as the diluent, therefore allowing direct comparison of results obtained for the 2 diluents (Figure 2-3). Results obtained for the experiments performed confirmed that NaCl has an effect in enhancing the false-positive reactions (Figure 2-2). Yet, results obtained when diluent was water showed that a higher cell concentration is needed for false-positive reactions to occur in both new and old reagent.

Results clearly show a difference between the new reagent displaying false-positive reactions for cell concentrations equals 10^4 cfu/ml whereas false-positive reactions for old reagent were displayed at very low concentrations of 10^1 cfu/ml when 1% saline was used as the diluent (Figures 2-3, 2-4 and 2-5) and 10^2 cfu/ml when diluent was sterile water (2-3 and 2-6). Therefore these results validate the previous hypothesis suggesting degradation of the inhibitory molecule(s) rather than degradation of the chromophore.

2.4.3 Response of *Aeromonas spp.* using both aged and fresh Colilert reagents.

In order to determine whether this capacity for mediating false-positive reactions was a unique feature of *A. hydrophila* or if this potential was widely spread among mesophilic members of the genus, the Colilert kit was also assessed using *A. caviae* and *A. sobria*. Results presented in Figure 2-4 showed that the potential of *A. sobria* to mediate a false-positive reaction is significantly weaker than either *A. hydrophila* or *A. caviae*. This different behaviour of *A. sobria* is recurrent in all the studies performed in our laboratory. *A. sobria* has been shown to be more sensitive to chlorine (Gavriel *et al.*, 1997) and to H_2O_2 (see chapter 3). The fact that *A. sobria* mediates a weaker false-positive response is remarkable for two reasons. The first is that *A. sobria* might be either more sensitive to the inhibitory molecule(s) present in the Colilert medium or produce less or a less efficient ß-galactosidase than the two other members under study. As the results obtained in Figure 2-4 relates to experiments performed with old Colilert reagent, it is reasonable to envisage that the inhibitory molecule(s) have been degraded during storage. It is therefore more probable that the difference observed between *A. sobria* and the group containing *A. hydrophila* and *A. caviae* is due to a reduced synthesis of or a less efficient ß-galactosidase in the former. The second reason is that along with other studies showing the different response of *A. sobria* (Gavriel *et al.*, 1997; Chapter 3), it emerges that besides the fact that

A. sobria belongs to the genus *Aeromonas* but they have evolved along a different evolutionary line.

2.4.4 Effect of nutrient limitation upon false-positive reaction mediated by *A. hydrophila.*

It could be argued that the potential conferred to *Aeromonas* species to mediate false-positive reactions in the Colilert defined-substrate technology system was due to the fact that the previous experiments were undertaken with physiologically healthy cells grown under optimal conditions. Simulation of nutrient limited environment was undertaken by harvesting cells of an overnight culture and resuspending them in 100 ml of filter-sterilised tap water and left to incubate for 5 days. No difference between the cells incubated for 5 days in M9 minimal medium and those incubated in water for the same period of time was observed (Figure 2-5).

2.4.5 Effect of chlorine upon false-positive reaction mediated by *A. hydrophila.*

However as it was demonstrated that the presence of salt had an effect in increasing the incidence of false-positive reactions, it remained possible that results observed for the salt-free sample were due to an artefact induced by the presence of residual chlorine from the tap water. A similar experiment was undertaken using old Colilert reagent with re-suspension of the cells in filter-sterilised tap water either with or without thiosulphate to neutralise chlorine present in one of the samples. Presence of thiosulphate did not increase or decrease the incidence of false-positive reaction compared to the M9 control. Data suggest that mediation of a false-positive response did not depend upon the growth state of the microorganisms studied or the presence of residual chlorine, but solely on the age of the reagent and the presence of salt.

The present study demonstrates that the Colilert defined-substrate technology is effective in suppressing growth of the heterotrophic flora when the reagents of the kit are fresh. However this study clearly illustrates that age of the reagents has a dramatic impact on the reliability of positive results obtained. The data indicates that the deficiency might be due to instability of the inhibitory molecule(s) upon storage.

CHAPTER 3 The oxidative stress response of *A. hydrophila.*

3.1 Introduction.

Aerobic microorganisms have to face a constant oxidative stress whether from internally generated radicals or from external factors (e.g. oxidative burst of phagocytes). When the concentration of reactive oxygen species (ROS) reaches levels above the cell's defence capacities, the microorganism is said to be under oxidative stress. ROS are numerous and have been reviewed in chapter 1. Oxygen is vital for any aerobically metabolising cell, being used as the terminal electron acceptor in the electron transport chain. However molecular oxygen is the precursor of many radicals which are potentially lethal to the cell if not dealt with by detoxification systems. To counteract the toxicity of the metabolic side-products of oxygen, bacteria have developed several elaborate defence strategies throughout evolution which can be summarised as protein and DNA repair systems (Chapter 1 paragraph 1.2.6). The oxidative stress phenomenon has been extensively studied in *E. coli* and *S. typhimurium* (Farr and Kogoma, 1991) where sub-lethal challenge have been shown to mediate resistance to a dose which ordinarily would be lethal. Studies in these microorganisms determined that prior sub-lethal exposure was necessary to allow production of stress proteins in order to bring adaptation. To date, no studies have been performed on the response of *Aeromonas* spp. to oxidative stress.

Ingestion of drinking water contaminated with *Aeromonas* spp. has been suggested to be a potential route for gastro-intestinal infections. Recovery of *Aeromonas* spp. from chlorinated water supplies have suggested that these microorganisms may partially adapt to the oxidising agent used to maintain good microbiological quality of the water. The

presence of oxidising agents in the environmental medium may potentially help the microorganisms to withstand onslaught by oxidative stress in macrophages via prior adptation to oxidative stress. When stimulated phagocytes engulf invading microorganisms, these microbes are exposed to lytic enzymes and a series of oxygen radicals and related oxidants (e.g. H_2O_2), used by the enzyme myeloxiperoxidase to oxidise chlorine ion to hypochlorous acid (McKenna and Davies, 1988). If bacteria face a non-lethal dose of a stress prior to infection, they would then be able to withstand higher doses with less damage compared to cells which have not had the opportunity of launching such an adaptive response. Therefore pre-exposed cells may have a better chance of survival within the host and have the potential of launching a pathogenic attack. This chapter will present a study on the response of *Aeromonas* spp., mainly *A. hydrophila*, to H_2O_2 and some parameters which affect the response.

3.2 Materials and Methods.

3.2.1 Materials.

3.2.1.1 Reagents.

Reagents were of analytical grade and were purchased from either Fisons Scientific Equipment, Bishop, Loughborough, UK., or BDH Laboratory Supplies, Poole, UK. Complex microbiological media such as tryptone soya agar, nutrient agar and nutrient broth were obtained from Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK. and chloramphenicol from Molecular Biology Sigma, Poole, UK.

3.2.1.2 Bacterial strains.

The following type strains were obtained from the National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, UK: *A. hydrophila* NCIMB 9240, *A. caviae* NCIMB 13016, *A. sobria* NCIMB 12065. The environmental strains used in certain experiments were supplied by Mr. Andros Gavriel (North of Scotland Water Authority, Turriff Water Treatment Works, Turriff, UK.).

All strains were maintained on nutrient agar with frequent sub-culture every 3 to 4 weeks. Master plates were grown at 30°C for 24 h and stored at 4°C until use.

3.2.2 Methods.

3.2.2.1 Evaluation of the effect of a range of concentration of H_2O_2 on *A. hydrophila* in complex medium.

1 ml from a 9 ml nutrient broth overnight culture was used to inoculate flasks containing 100 ml of nutrient broth. When the cultures had reached mid logarithmic phase of growth ($OD_{650\ nm}$ = 0.4) they were challenged with different amounts of H_2O_2 at a final concentration of: 3 mM, 15 mM, 30 mM, 150 mM, and 2.9 M. Using nutrient broth as a

blank, the samples were measured in a spectrophotometer set at a λ of 650 nm. At appropriate times, 1 ml of sample was removed from the experimental flasks and measured.

3.2.2.2 Evaluation of the effect of a range of concentration of H_2O_2 on *A. hydrophila* in minimal medium.

M9 Medium was prepared as described in Chapter 2 Paragraph 2.2.2.1. A 1 ml aliquot from a 9 ml M9 medium overnight culture was used to inoculate flasks containing 100 ml of M9 medium. When the cultures had reached mid-log phase of growth ($OD_{650\,nm}$ of 0.4), they were challenged with different amounts of H_2O_2 at a final concentration of: 50 μM, 250 μM, 500μM, 750μM, 1 mM, and 10 mM. Using M9 buffer as a blank, the samples were measured in a spectrophotometer set at a λ of 650 nm. At appropriate times, 1 ml of sample was removed from the experimental flasks and measured.

a) Determination of the sub-lethal and lethal dose of H_2O_2 to be used for adaptation studies.

A 9 ml M9 minimal medium overnight culture was grown to stationary phase ($OD_{650\,nm}$ = 0.8) and 1 ml of this overnight culture was used to inoculate flasks containing 100 ml of M9 buffer. The following concentrations of H_2O_2 were then added to separate flasks at time zero: 0, 50 μM, 250 μM, 500 μM, 750 μM, 1 mM and 10 mM. Samples were removed from the flasks at time: 0, 5, 15, 30, 60, 90, 120, 150, 180, 240, 300, and 1440 minutes. Viable counts were determined by the Miles and Misra plate count method (Chapter 3 paragraph 3.2.2.3).

b) Investigation into the response of *A. hydrophila* to oxidative stress.

i) Adaptation of *A. hydrophila*, *A. caviae*, *A. sobria* and environmental isolates stationary phase cells to H_2O_2.

M9 medium was used to prepare cultures for the stress experiments. M9 buffer was prepared as described previously. H_2O_2 being liable to react with the components of the complex medium, the adaptation of *A. hydrophila* to H_2O_2 was performed in M9 minimal medium. A 9 ml minimal medium M9 overnight culture was grown to stationary phase ($OD_{650 \, nm} = 0.8$) and 1 ml of this overnight culture was used to inoculate flasks containing 100 ml of M9 buffer. The sub-lethal dose (50 μM) of H_2O_2 was applied after 5 minutes, and the lethal dose (1 mM) after 60 minutes. Samples were removed from the flasks at time: 0, 5, 15, 30, 60, 90, 120, 150, 180, 240, 300 and 1440 minutes and viable counts were determined by the Miles and Misra plate count method (Chapter 3 paragraph 3.2.2.3).

ii) Relative time scale protection of *A. hydrophila* using 50 μM as sub-lethal dose prior to lethal challenge added at different times.

A 9 ml M9 minimal medium overnight culture was grown to stationary phase ($OD_{650 \, nm} = 0.8$) and 1 ml of this overnight culture was used to inoculate flasks containing 100 ml of M9 buffer. The sub-lethal dose (50 μM) of H_2O_2 was applied at time 0 minute, and the lethal dose (1 mM) at time of 30, 60, 120, 180, 240, 300 minutes. Samples were removed from the flasks at time: 0, 60, 120, 180 minutes after addition of the lethal challenge. Viable counts were determined by the Miles and Misra plate count method (Chapter 3 paragraph 3.2.2.3).

iii) Effect of Chloramphenicol upon the adaptation phenomenon of *A. hydrophila* stationary phase cells to H_2O_2 challenge.

A 9 ml M9 minimal medium overnight culture was grown to stationary phase ($OD_{650\ nm} = 0.8$) and 1 ml of this overnight culture was used to inoculate flasks containing 100 ml of M9 buffer. Chloramphenicol (10 µg/ml) was added at time 0 minute, the sub-lethal dose (50 µM) of H_2O_2 was applied after 5 minutes, and the lethal dose (1 mM) after 60 minutes. Samples were removed from the flasks at time: 0, 5, 15, 30, 60, 90, 120, 150, 180, 240, 300 and 1440 minutes and viable counts were determined by the Miles and Misra plate count method (Chapter 3 paragraph 3.2.2.3).

c) Investigation of the different parameters affecting the stress response.

i) Catalase assay on *A. hydrophila* cell extract.

Preparation of the cell extract.

A 5 ml M9 minimal medium overnight culture was grown to stationary phase ($OD_{650\ nm} = 0.8$). The following concentrations of H_2O_2: 50 µM, 500 µM or 1 mM, were added to the re-suspended cells for a period of 5, 30 and 60 minutes. A no addition control was performed following the same procedure as for the samples. After the appropriate incubation period cells were harvested after centrifugation at 13,500 rpm for 30 seconds. The supernatant was discarded and cells were re-suspended in 1 ml of fresh M9 buffer. Cells were washed 3 times with 0.5 ml of 0.1 M potassium phosphate buffer pH 7.4. Finally cells were resupsended in 1 ml of 0.1 M potassium phosphate buffer pH 7.4 and lysed using a sonicator (Heat systems ultra processor XL) with a repeat of 3 cycles of 30 seconds sonication, and 30 seconds on ice. The output of the sonicator was set at 28 % of the maximum power. After sonication, the cells were centrifuged at 13,500 rpm for 20

seconds. The cytosolic fraction was then transferred into a fresh sterile Eppendorf tube, and the solution kept on ice.

Catalase assay.

The catalase activity in the cell extract was measured via its ability to break down H_2O_2. 60 μl of a 1 % H_2O_2 solution was added to 1 ml of potassium phosphate buffer pH 7.4 and the absorbance measured to be around an $A_{240\ nm}$ of 0.7. 100 μl of cell extract was then added to the assay. Recording of the optical density was performed every 10 seconds starting 10 seconds. after addition until 210 seconds were reached. A final value was read 10 minutes after the assay was started.

ii) Effect of growth phase and culture age upon *A. hydrophila* oxidative stress response.

Two pre-cultures were obtained by growing cells of *A. hydrophila* in M9 medium at 30°C for 24 h to stationary phase ($OD_{650\ nm}$ = 0.8), one for the preparation of the logarithmic phase cells, the other for the stationary phase cells preparation. 4 ml of culture were removed from one of the cultures and used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase ($OD_{650\ nm}$ = 0.4). Experimental flasks used for the stationary phase study were seeded using 1 ml of overnight stationary phase cells into 100 ml of fresh M9 buffer. Experimental flasks used for the logarithmic phase study were seeded using 1 ml logarithmic phase cells in 100 ml of fresh M9 buffer. Subsequent to challenge with H_2O_2, samples were removed from the flasks at time: 0, 60, 120, 180, 240, 300 and 1440 minutes and viable counts were determined by the Miles and Misra plate count method (Chapter 3 paragraph 3.2.2.3).

3.2.2.3 The Drop Plate Count method (Miles and Misra)

At appropriate times, 1 ml of sample was removed from the experimental flasks and subject to a 10-fold dilution series using tubes containing 9 ml volumes of M9 buffer (i.e. 10^{-1} down to 10^{-4}). Pre-dried nutrient agar plates were divided into four quadrants. Five 20 µl volumes from each dilution i.e. 10^{-1} through to 10^{-4}, were applied on one of these quadrants. The plates were incubated at 30°C for 24 hours and the number of colony forming units enumerated.

3.3 Results.

3.3.1 Evaluation of the effect of a range of concentrations of H_2O_2 on *A. hydrophila* in complex medium.

A series of experiments were designed in order to assess the effect of H_2O_2 upon viability of *A. hydrophila*. Sensitivity to this oxidative agent was first assessed in complex medium (i.e. nutrient broth using a spectrophotometric technique) (Figure 3-1). From the data obtained it can be seen that relatively high concentrations of the oxidative agent were needed to kill the cells. The possibility existed that H_2O_2 might react with some components of the complex medium, thereby reducing its oxidising potential.

3.3.2 Evaluation of the effect of a range of concentrations of H_2O_2 on *A. hydrophila* in minimal medium.

To verify whether interactions between H_2O_2 and some components of the complex medium were indeed significant, a similar experiment was performed in a minimal salt medium i.e. Miller's M9 minimal medium (Figure 3-2). The data obtained confirmed that H_2O_2 had been reacting with components of the complex medium as lethal effects in M9 buffer were substantially more pronounced at equivalent concentrations. Results obtained from the experiment in minimal medium suggested that the spectrophotometric technique was not sufficiently sensitive to measure H_2O_2 bactericidal activity on *A. hydrophila*, as the method cannot differentiate between living cells and non-viable, but non-lysed organisms. The lack of sensitivity of this method is due to two major drawbacks. The first is that only through cell lysis will the optical density decrease, the second being that cells which are dead but non-lysed will still deflect the beam of light similarly to their living counterparts and thus be incorporated in the optical density reading displayed.
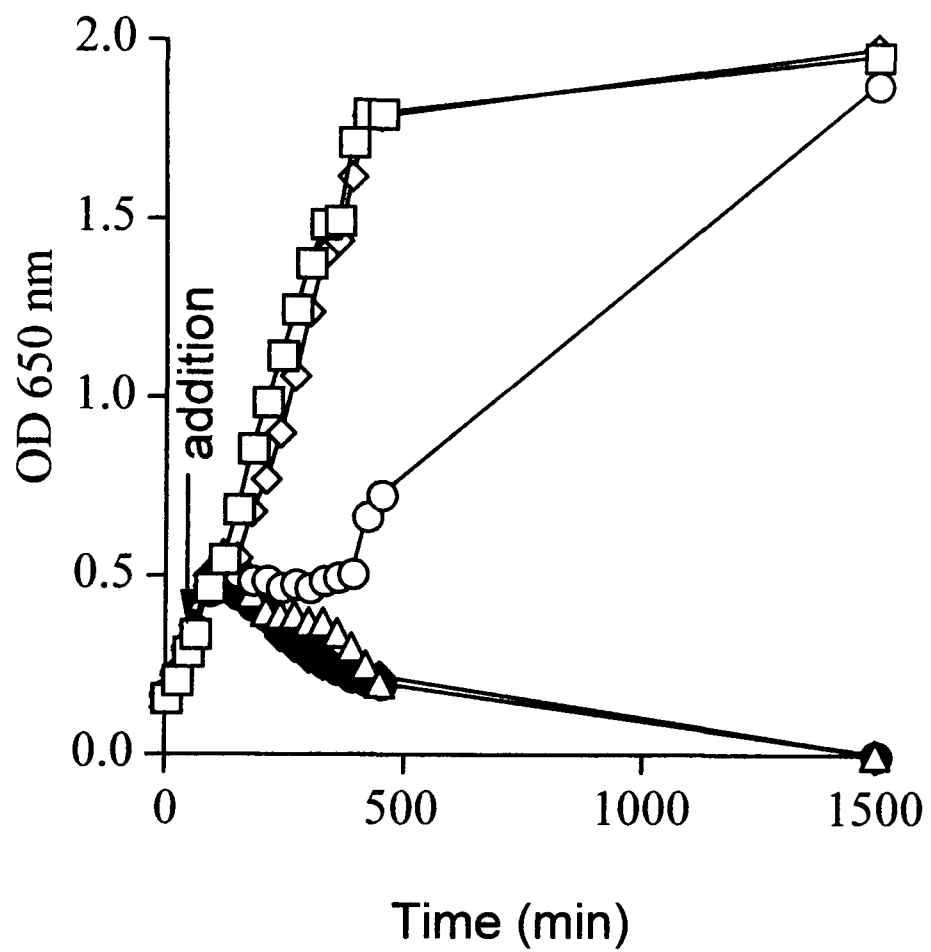
H_2O_2 challenge of *A. hydrophila* in NB complex medium.
Cells were grown in nutrient broth (NB) at 30°C at 150 rpm. No addition of H_2O_2 is shown
as a control (□). The addition of 3 mM (◇), 15 mM (○), 30 mM (△), 150 mM (■), 2.95 M
(◆) H_2O_2 was performed when the $OD_{650\ nm}$ reached 0.4 (Marked addition).

Figure 3-2
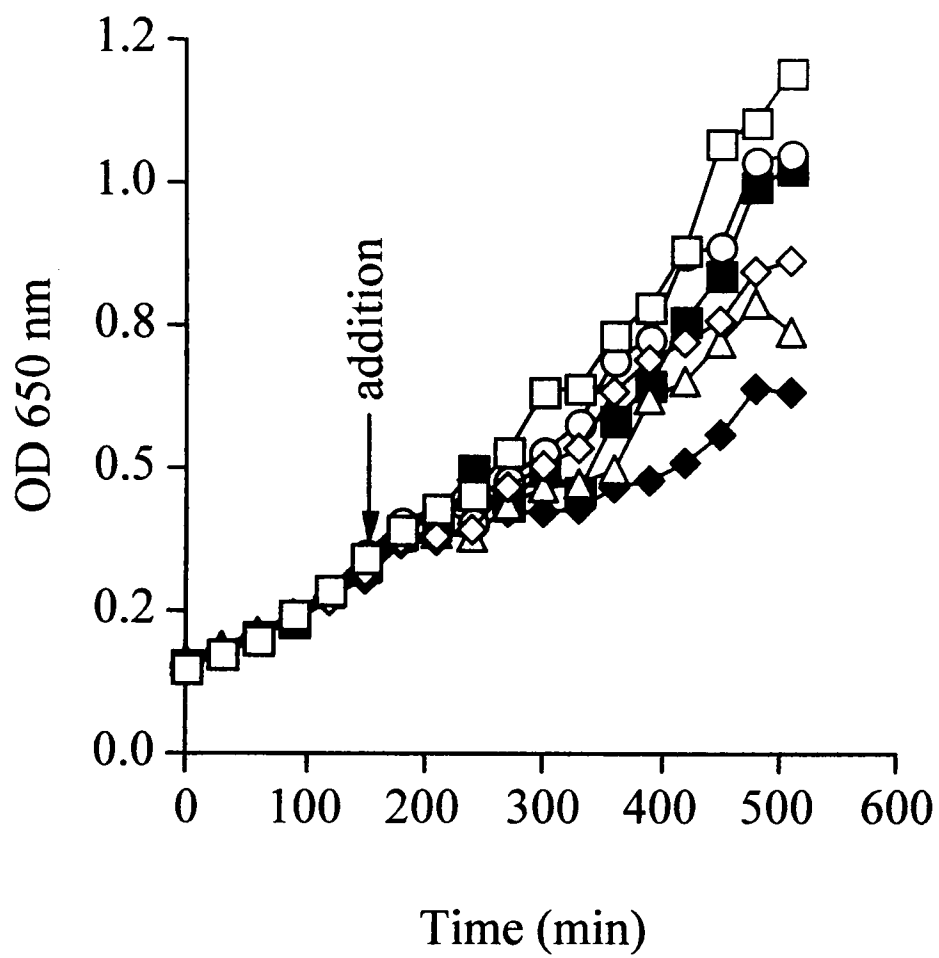
H₂O₂ challenge of *A. hydrophila* in M9 miller minimal medium.
Cells were grown in liquid minimal medium (M9) at 30°C at 150 rpm. No addition of H_2O_2 is shown as a control (□). The addition of 50 μM (◇), 250 μM (○), 750 μM (△), 1 mM (■), 10 mM (◆)H_2O_2 was performed when the $OD_{650\ nm}$ reached 0.4 (Marked addition).

Therefore a more rigorous and discriminatory approach was necessary to measure for more subtle effects of the oxidising agent. Methods suitable for assessment of viability using plate count techniques are more sensitive than spectroscopy and the Miles and Misra drop plate count procedure was employed as the most appropriate assay for cell viability.

3.3.3 Investigation into the response of *A. hydrophila* to oxidative stress. Adaptation of *A. hydrophila* stationary phase cells to H_2O_2.

Data from Figure 3-3 a showed 1 mM H_2O_2 to be the minimal bactericidal dose of oxidant where loss of viability is rapid (i.e. within 60 minutes). An appropriate sub-lethal dose was determined to be 50 µM (Figure 3-3 b). Therefore a preliminary experiment to assess the oxidative stress response of *A. hydrophila* was performed using 50 µM as sub-lethal dose and 10 mM as lethal challenge of H_2O_2. These values were chosen based upon results obtained from the previous experiments where 5 mM and 10 mM were shown to be definitely bactericidal. 10 mM H_2O_2 was chosen in order to assess to which extent *A. hydrophila* could display an adaptation response to that oxidant. From the experimental results obtained (Figure 3-4), there was no apparent differences in response between the 2 test flasks. Pre-exposure of cells to 50 µM had no influence in preventing onset of rapid loss of viability upon subsequent addition of 10 mM H_2O_2.

That no adaptation was observed during this experiment also strengthened the fact that *A. hydrophila* is more sensitive to oxidising agents than *E. coli* , which was previously shown to display an oxidative stress adaptive response to H_2O_2 concentrations up to 25 mM (Demple and Halbrook, 1983; Imlay et al., 1988).

Figure 3-3 a (300 minutes plot)
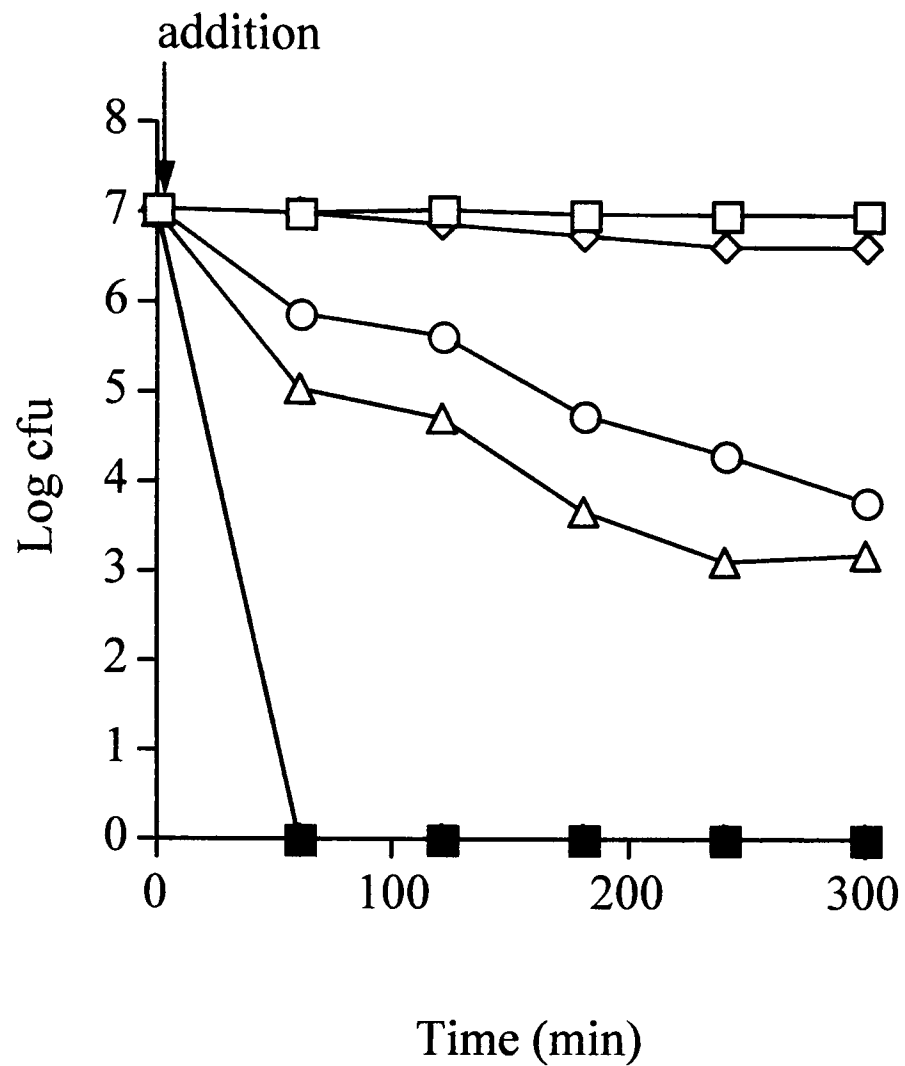
Determination of the effect of H_2O_2 upon *A. hydrophila*.
Cells were grown in liquid minimal medium (M9) at 30°C at 150 rpm. The addition of 50 μM (□), 250 μM (◇), 500 μM (O), 750 μM (Δ) 1 mM (■), 5 mM (◆), 10 mM (●)H_2O_2 was done at time 5 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using the Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

86

Figure 3.3 b (1440 minutes plot)
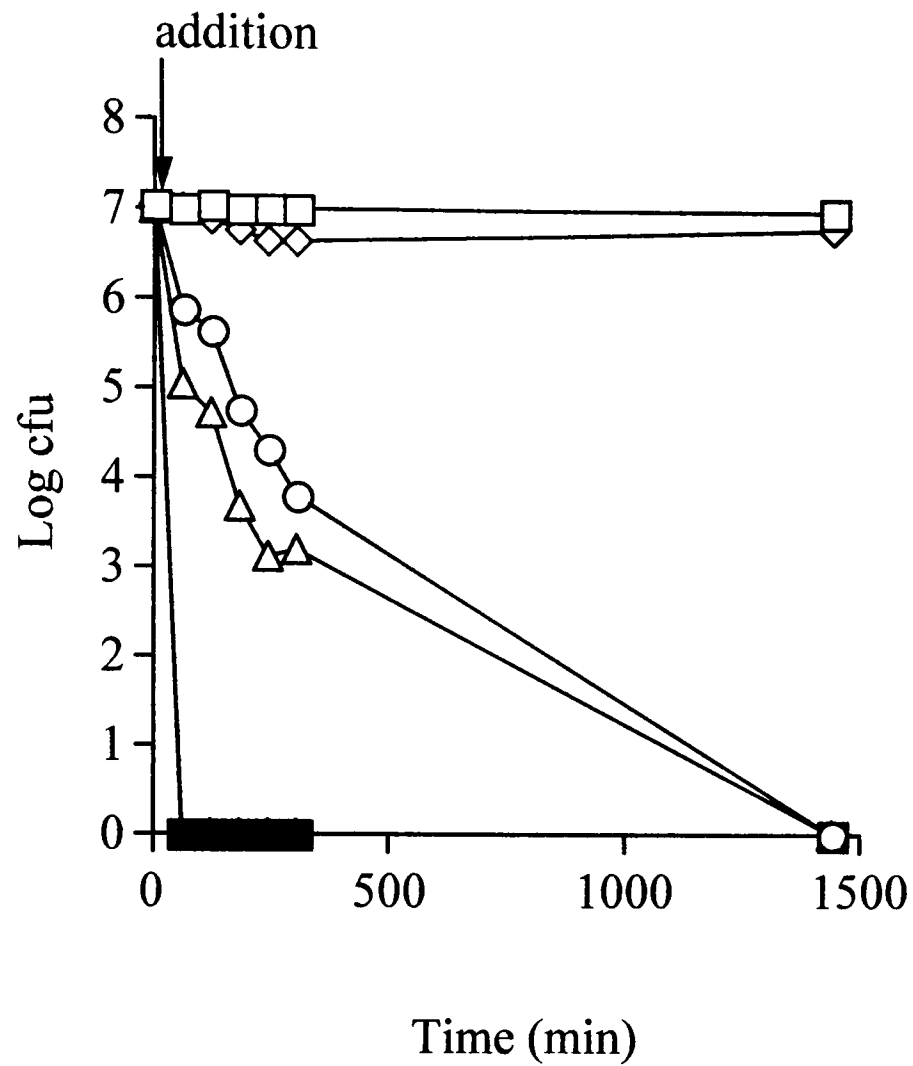
Determination of the effect of H_2O_2 upon *A. hydrophila*.

Cells were grown in liquid minimal medium (M9) at 30°C at 150 rpm. The addition of 50 μM (□), 250 μM (◇), 500 μM (○), 750 μM (Δ) 1 mM (■), 5 mM (◆), 10 mM (●)H_2O_2 was done at time 5 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using the Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 3-4
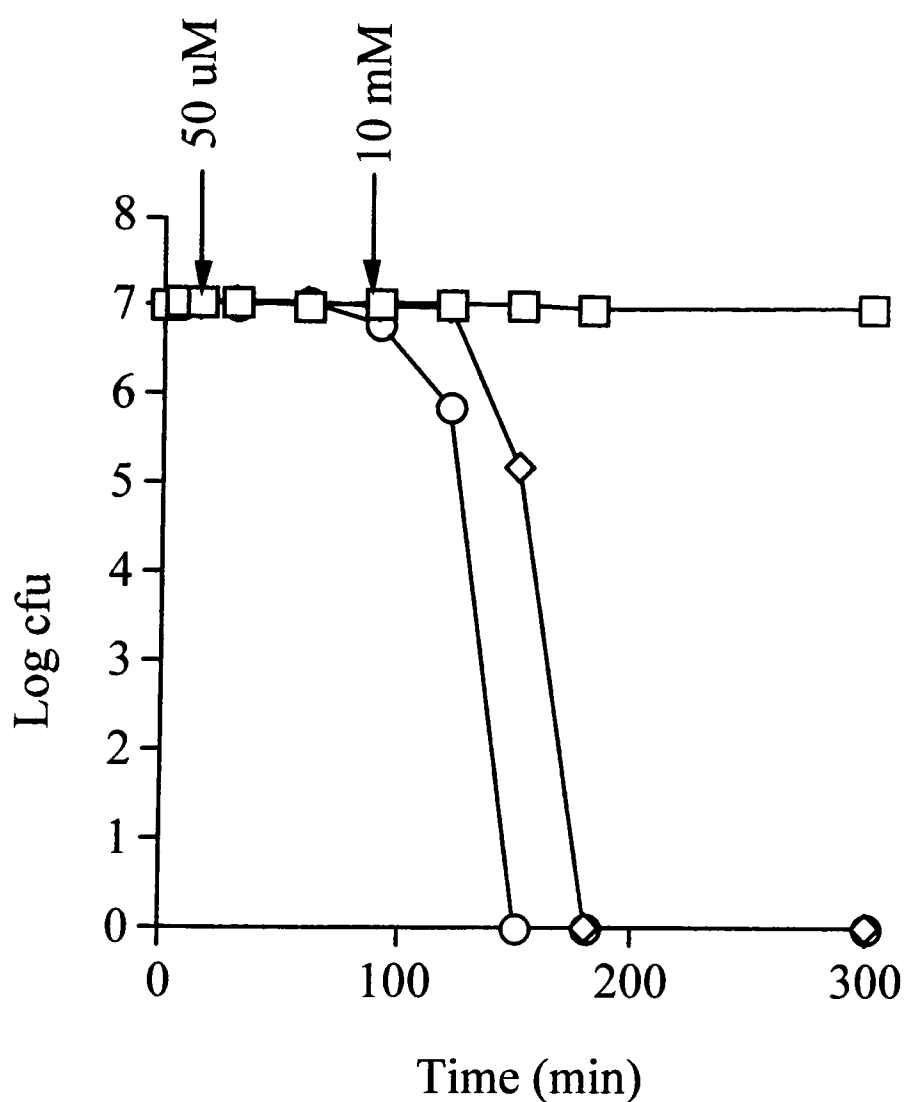
Adaptation of *A. hydrophila* to 10 mM H₂O₂ using 50 μM as a sub-lethal dose.

Cells were grown in liquid minimal medium (M9). No addition of H₂O₂ is shown as a control (□).The first addition (50 μM) was performed after 5 minutes in the adaptation flask. At time 60 minutes, 10 mM was added to both adaptation (◇) and untreated (○) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using the Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

This experiment was therefore repeated using a lower bactericidal dose as determined from Figure 3-3, i.e. 1 mM H_2O_2. When repeated using 1 mM H_2O_2 as the lethal dose a totally different result to that obtained previously was observed (Figures 3-5 a and b). When 1 mM H_2O_2 was the lethal dose, the sub-lethal concentration of 50 μM H_2O_2 was indeed shown to stimulate onset of a stress adaptive response. Figures 3-5 a and b show a definite difference in the response displayed by the pre-treated and non pre-treated flasks. In the latter flask, the cells rapidly lost viability until no cell could be recovered 60 minutes after addition of the lethal challenge, indicating that 1 mM was truly a lethal challenge for the cells. When cells were pre-treated with 50 μM H_2O_2, a 2 log reduction in the viability count was observed in the first 60 minutes with a further 1 log reduction in viability observed within the next 180 minutes after challenge. This reduction in the death rate and the recovery of viable cells 180 minutes after lethal challenge has demonstrated that cells pre-treated with a sub-lethal dose of an oxidant could withstand higher doses normally lethal to the cells.

As it was demonstrated that *A. hydrophila* could display an adaptive stress tolerance response to H_2O_2, it was important to study the relative time scale of the protection acquired through sub-lethal challenge in order to determine the potential role of stress adaptation in the infection mechanism of pathogenic microorganisms. The relative time scale for onset of protection was studied using the previously determined sub-lethal and lethal doses.

The sub-lethal dose was added at the same time to all of the flasks, while addition of the lethal dose was performed at a set increase of time between the different flasks. Results of this investigation are presented in Figure 3-6 and show that the degree of cellular adaptation to H_2O_2 challenge is time-dependent.

Figure 3-5 a (300 minutes plot)
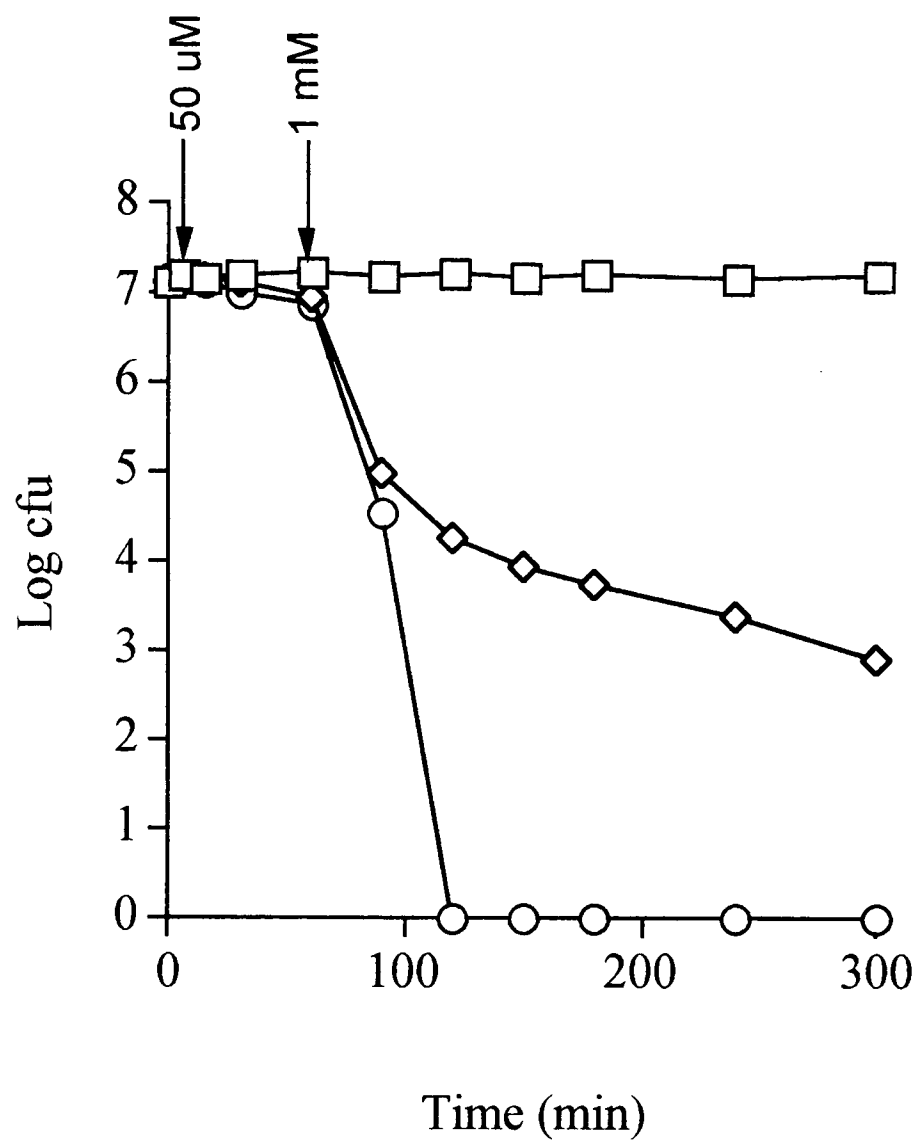
Axis labels: "50 uM" and "1 mM" (vertical labels with arrows), Y-axis "Log cfu", X-axis "Time (min)"

Adaptation of *A. hydrophila* to 1 mM H$_2$O$_2$ using 50 μM as a sub-lethal dose.

Cells were grown in liquid minimal medium (M9). Control (No H$_2$O$_2$ addition) (□).The first addition (50 μM) was performed after 5 minutes in the adaptation flask. At time 60 minutes, 1 mM was added to both adaptation (◇) and untreated (○) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 3-5 b (1440 minutes plot)
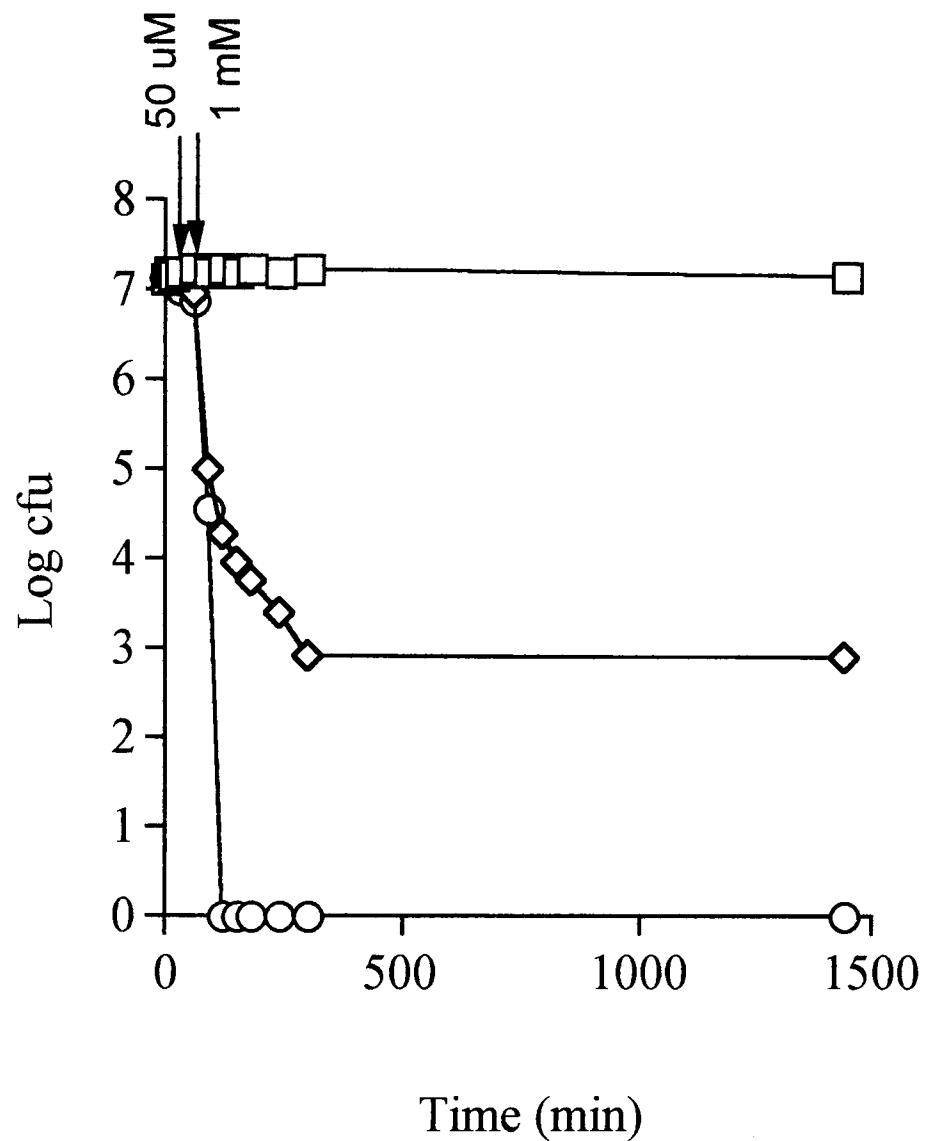
Adaptation of *A. hydrophila* to 1 mM H_2O_2 using 50 μM as a sub-lethal dose.

Cells were grown in liquid minimal medium (M9). Control (No H_2O_2 addition) (□).The first addition (50 μM) was performed after 5 minutes in the adaptation flask. At time 60 minutes, 1 mM was added to both adaptation (◇) and untreated (○) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Two trends seems to emerge (Figure 3-6). Firstly, a sub-lethal exposure time of 120 minutes prior to 1 mM H_2O_2 addition seems to be the optimal time for adaptation to occur during the first 60 minutes of the challenge. Secondly, after the first 60 minutes of the experiment, all curves tend to plateau. The longer the pre-treatment, the higher the viability count, therefore suggesting that the longer the sub-lethal exposure, the stronger the adaptation.

3.3.4. Investigation of the different parameters affecting the stress response of *A. hydrophila*.

The adaptive phenomenon of *A. hydrophila* to oxidative stress could be manifested through a variety of possible mechanisms. Firstly, increased synthesis of redox active cellular metabolites such as glutathione, triggered by the sub-lethal dose, could afford the cells protection to higher doses of oxidant by reducing the chemical more efficiently. Alternatively, the phenomenon of adaptation may be modulated through enhanced synthesis of proteins functioning to catalyse breakdown of the oxidant e.g. catalase or in protecting cellular targets and/or repairing cell lesions. Another possibility could involve the synthesis of a set of stress induced proteins such as heat-shock (Langer *et al.*, 1992) or the OxyR regulated proteins (Farr and Kogoma, 1991). In order to clarify among these possibilities, a set of experiment was designed to study some of these parameters i.e. catalase and *de novo* protein synthesis.

3.3.4.1 Role of catalase activity in mediating the adaptive tolerance to H_2O_2.

This set of experiments was designed to determine whether catalase had a role to play in the phenomenon of adaptation of *A. hydrophila* to H_2O_2 stress. Cells were grown overnight in M9 medium, and subsequently incubated in presence the of 50 μM, 500 μM or 1 mM H_2O_2 for 5, 30 and 60 minutes.

Figure 3-6
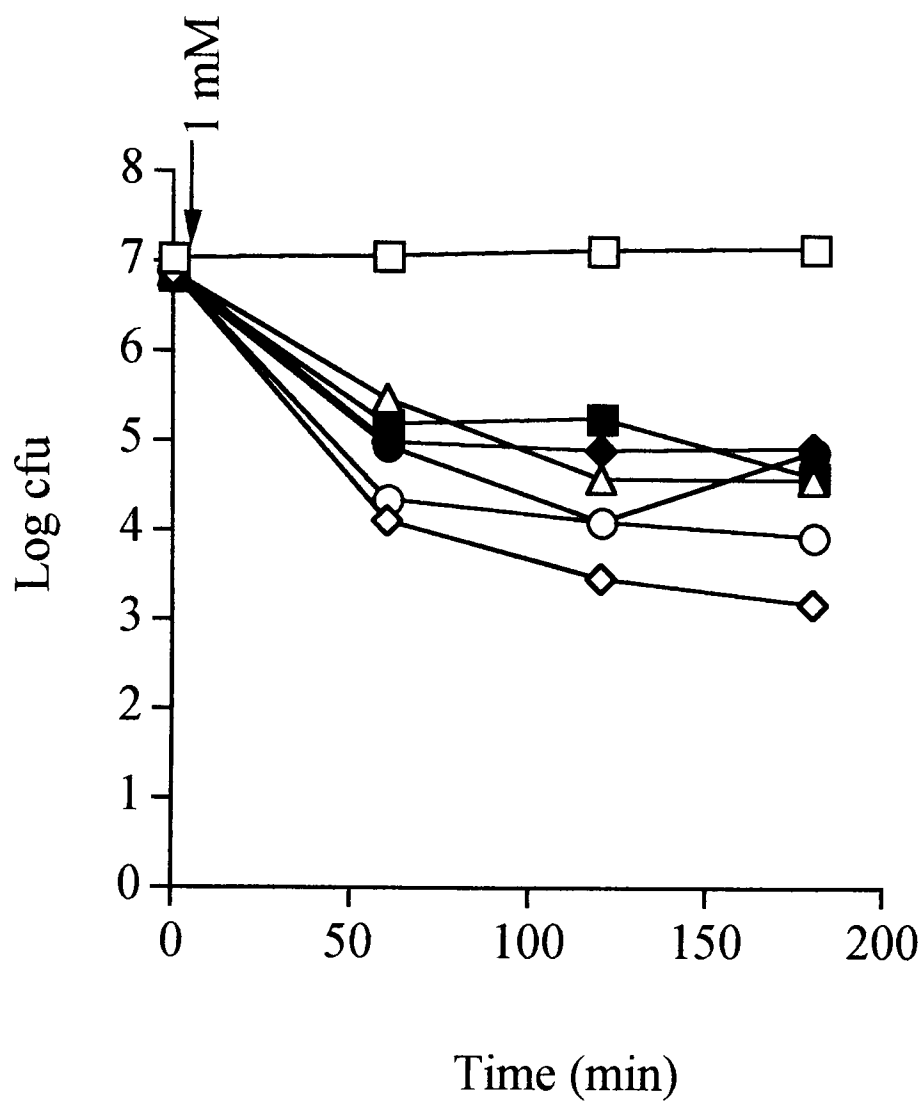
Relative time scale protection of *A.* hydrophila acquired through sub-lethal challenge (50 μM) prior to 1 mM H₂O₂.

Cells were grown in liquid minimal medium (M9), pre-treated with 50 μM H₂O₂ at the same time in all flasks, then challenged with 1 mM H₂O₂ at time (minutes): 30 (◇), 60 (○), 120 (△), 180 (■), 240 (◆), 300 (●). A Control receiving no H₂O₂ addition was performed (□). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

These cells were then harvested by centrifugation, re-suspended in potassium phosphate buffer pH 7.4 and lysed by sonication. Assessment of catalase activity was performed by monitoring breakdown of H_2O_2 at $A_{240\,nm}$. Results for both 50 μM and 1 mM H_2O_2 concentrations are shown in Table 3-1 and 3-2. The data from Table 3-1, 3-2, showed a common trend where H_2O_2 treated cells displayed enhanced catalase activity compared to the no treatment control.

Table 3-1: Measurement of catalase activity from *A. hydrophila* pre-treated with 50 μM H_2O_2. Crude cellular extract from *A. hydrophila* was assayed for ability to degrade H_2O_2. Cell extract prepared by sonication was diluted in phosphate buffer pH 7.4 and this assayed for catalase activity by monitoring decrease in absorbance of H_2O_2 at λ=240 nm. Catalase activity determined from the intial reaction rate, was calculated based upon total protein concentration of the crude extract.

Length of pre-treatment (minutes.)	Specific Activity of Catalase (absorbance change in catalase activity/μg of total protein/second)
0	4.11×10^{-6}
5	6.31×10^{-6}
30	9.88×10^{-6}
60	1.17×10^{-5}

Table 3-2: Measurement of catalase activity from *A. hydrophila* pre-treated with 1 mM H_2O_2. Crude cellular extract from *A. hydrophila* was assayed for ability to degrade H_2O_2. Cell extract prepared by sonication was diluted in phosphate buffer pH 7.4 and this assayed for catalase activity by monitoring decrease in absorbance of H_2O_2 at λ=240 nm. Catalase activity determined from the intial reaction rate, was calculated based upon total protein concentration of the crude extract.

Length of pre-treatment (minutes.)	Specific Activity of Catalase (absorbance change in catalase activity/μg of total protein/second)
0	1.33×10^{-5}
5	1.51×10^{-5}
30	1.89×10^{-5}
60	2.14×10^{-5}

Calculation of the initial rate of catalase reaction for 50 μM and 1 mM H_2O_2 challenge (Table 3-1 and 3-2) showed an increased catalase activity when H_2O_2 concentration increases. When looking at the data presented, the reaction rates of the controls and 5 minute pre-treatment appeared relatively similar suggesting that a 5 minute pre-treatment seems insufficient for the cells to exhibit strong catalase activity. Closer inspection of the data presented showed that the trend for 30 and 60 minutes pre-treatment were similar and markedly different from the group described previously (control and 5 minutes pre-treatment). From the times assayed, it appears that the minimal time requirement for an significant increase in catalase activity was 30 minutes. These results have demonstrated the potential role of catalase in the process of adaptation. The difference in the H_2O_2 degradation rate between 50 μM and 1 mM might be occuring for two different reasons with regards to catalase activity *in vivo*. The first being due to a dose dependent *de novo* protein synthesis of enzymes, whereas the second resulting from a dose dependent modulation of pre-existing catalase. Further investigation on whether catalase was either newly synthesised as a component of *de novo* protein synthesis or if the phenomenon observed was due to modulation of pre-existing enzymes was therefore undertaken. This

set of experiments was performed on *A. hydrophila* pre-treated with 500 μM H$_2$O$_2$ in presence or absence of 10 μg/ml chloramphenicol, a transcritptional inhibitory molecule which completely abolishes protein synthesis in bacteria. Results are presented in Tables 3-3, 3-4.

Table 3-3: Measurement of catalase activity from *A. hydrophila* pre-treated with 500 μM H$_2$O$_2$. Crude cellular extract from *A. hydrophila* was assayed for ability to degrade H$_2$O$_2$. Cell extract prepared by sonication was diluted in phosphate buffer pH 7.4 and this assayed for catalase activity by monitoring decrease in absorbance of H$_2$O$_2$ at λ=240 nm. Catalase activity determined from the intial reaction rate, was calculated based upon total protein concentration of the crude extract.

Length of pre-treatment (minutes.)	Specific Activity of Catalase (absorbance change in catalase activity/μg of total protein/second)
0	2.05×10^{-5}
5	1.61×10^{-5}
30	2.52×10^{-5}
60	2.52×10^{-5}

Table 3-4:Measurement of catalase activity from *A. hydrophila* pre-treated with 500 μM H$_2$O$_2$ + 10 μg/ml chloramphenicol. Crude cellular extract from *A. hydrophila* was assayed for ability to degrade H$_2$O$_2$. Cell extract prepared by sonication was diluted in phosphate buffer pH 7.4 and this assayed for catalase activity by monitoring decrease in absorbance of H$_2$O$_2$ at λ=240 nm. Catalase activity determined from the intial reaction rate, was calculated based upon total protein concentration of the crude extract.

Length of pre-treatment (minutes.)	Specific Activity of Catalase (absorbance change in catalase activity/μg of total protein/second)
0	1.66×10^{-5}
5	1.66×10^{-5}
30	2.50×10^{-5}
60	2.50×10^{-5}

Results obtained for cells pre-treated with 500 μM H$_2$O$_2$ (Table 3-3) showed similar rates for either the control and the 5 minutes pre-treatment or the 30 and 60 minute pre-treatment.

3.3.4.2 Role of *de novo* protein synthesis in mediating the adaptive response of *A. hydrophila* to oxidative stress.

An *A. hydrophila* culture grown overnight was used to seed experimental flasks containing 100 ml M9 buffer. One flask received 10 μg/ml chloramphenicol at time 0 minute. Addition of 50 μM and 1 mM H_2O_2 were performed at 5 minutes and 60 minutes, respectively. An ethanol control (100 μl of pure ethanol) was performed to determine whether any effect observed was solely due to chloramphenicol or if the solvent had any involvement in the potential to mediate an effect on the response of the microorganisms to oxidative stress. Results are presented in Figures 3-7 a and b. Data obtained for the ethanol control showed that it had no effect upon on the cellular viability. Therefore any effect observed would be solely due to chloramphenicol and the oxidising agent, when present. Control cells in presence of 10 μg/ml chloramphenicol only showed a viability loss of 1.5 log after the 24 hours. Cells pre-treated with chloramphenicol and 1 mM H_2O_2 showed a rapid loss in viability until no viable cells could be recovered 60 minutes after addition of oxidant. In the presence of chloramphenicol, pre-treating the cells with 50 μM H_2O_2 was ineffective at preventing rapid loss of viability when 1 mM H_2O_2 was introduced. These results demonstrate that in presence of 10 μg/ml chloramphenicol the oxidative stress resistance displayed by *A. hydrophila* has been abolished. This strongly suggests that *A. hydrophila* stress response is partially mediated through the fresh synthesis of key stress proteins.

3.3.4.3 Effect of growth phase and culture age upon *A. hydrophila* oxidative stress response.

In recognition that physiological responses of bacterial organisms alter with age, it was sought to investigate the influence of growth phase and length of storage of microorganisms on their solid medium upon the adaptive response of *A. hydrophila*.

Figure 3-7 a (300 minutes plot)

Effect of chloramphenicol (10 µg/ml) on the adaptation response of *A. hydrophila*.

Cells were grown overnight in liquid minimal medium (M9). As a control 100 µl of ethanol was added to non-treated cells (□). Chloramphenicol addition was performed at time 0 minute in all appropriate flasks. Chloramphenicol control (◇). Adaptation flask received addition of 50 µM H_2O_2 at time 5 minutes and 1 mM H_2O_2 at time 60 minutes. Death control flask (○) received 1 mM H_2O_2 at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 3-7 b (1440 minutes plot)

Time (min)

Effect of chloramphenicol (10 µg/ml) on the adaptation response of *A. hydrophila*.
Cells were grown overnight in liquid minimal medium (M9). As a control 100 µl of ethanol was added to non-treated cells ($\square$). Chloramphenicol addition was performed at time 0 minute in all appropriate flasks. Chloramphenicol control ($\diamondsuit$). Adaptation flask received addition of 50 µM H_2O_2 at time 5 minutes and 1 mM H_2O_2 at time 60 minutes. Death control flask ($\bigcirc$) received 1 mM H_2O_2 at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Test organisms were grown on NA plates for 3 days, 2 weeks and 12 weeks and two pre-cultures were grown for each of the different plates. One to stationary phase, the other one to mid-log phase. The resulting cultures were then used in an adaptation experiment where the sub-lethal and lethal doses of H_2O_2 were 50 μM and 1 mM, respectively. These experiments were designed to study the effect of storage length and growth phase at the same time to ease comparison between them, but also to obtain a broader picture where both parameters could be working in concert. Figure 3-8 presents data for the 3 days old culture, Figure 3-9 for the 2 weeks old culture and Figure 3-10 for the 12 weeks old culture.

a) Effect of growth phase upon *A. hydrophila* oxidative stress response.

Both logarithmic and stationary phase controls were almost identical in all the three sets of experiments performed allowing reliable comparison to be made between the experiments. In agreement with previous findings, 1 mM H_2O_2 was proven to be lethal for both the logarithmic and stationary phase of cells stored 3 days on NA (Figure 3-8). However after 2 weeks of storage on plates, a marked difference appeared between the 2 growth stages (Figure 3-9). Logarithmic phase cells showed a total loss of viability 120 minutes after lethal challenge was applied, whereas the stationary phase counterpart remained viable slightly longer with a total loss of viability occurring 240 minutes after H_2O_2 addition. With regards to the 12 weeks storage, both logarithmic and stationary phase cells displayed an impressive resistance to the lethal challenge, only showing loss of viability 24 hours after the start of the assay (data not shown). This suggests that additional protective mechanisms (e.g. late protein synthesis) to the involvement of catalase and early protein synthesis takes place during long storage for cells to become resistant to stress challenges. Cells stored on solid medium for 12 weeks display a greater resistance than younger cells pre-treated with 50 μM H_2O_2. When one looks at the adaptation data for both stationary

Figure 3-8
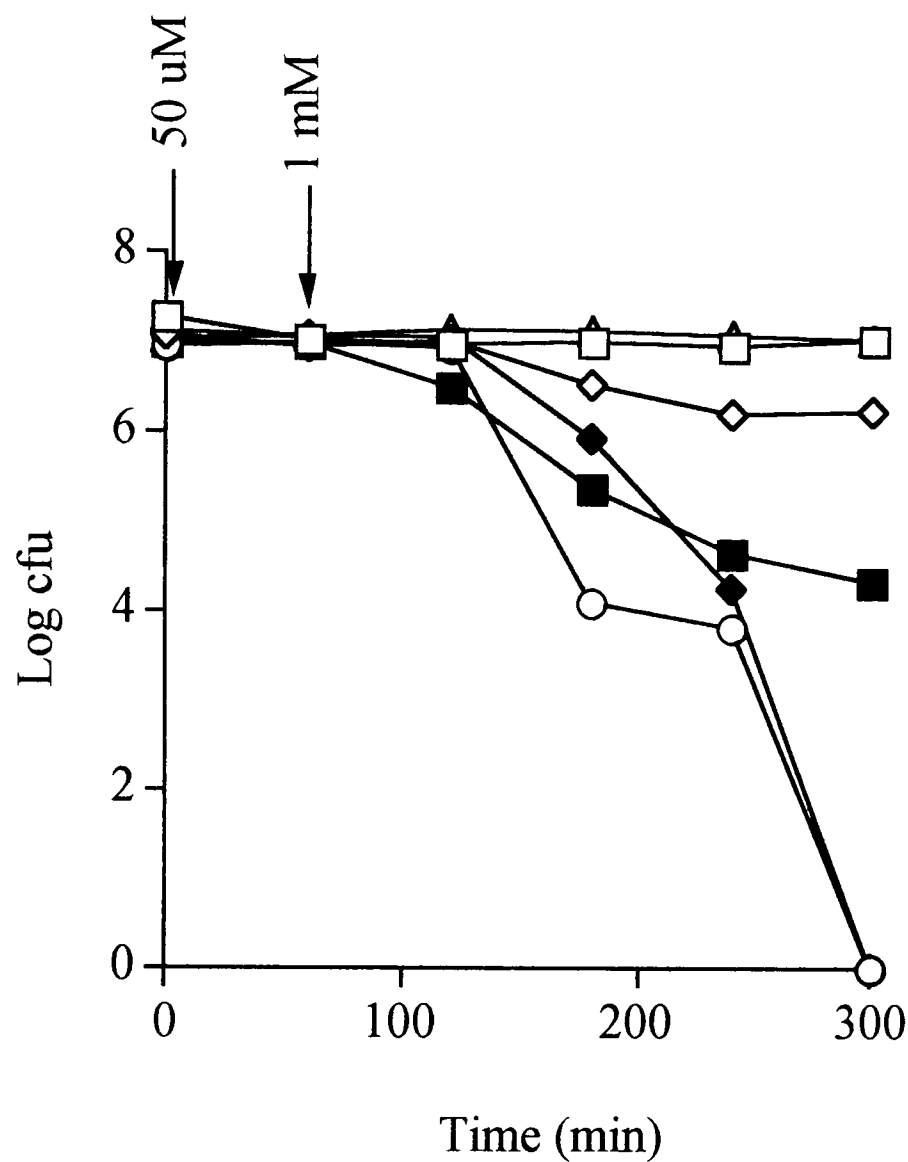
Effect of growth phase and age upon adaptation of 3 days old *A. hydrophila* to oxidative stress.

Two pre-cultures were obtained by growing cells overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights was used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary (□) and logarithmic (Δ) control. The first addition (50 μM) was performed after 5 minutes to both adaptation flasks. At time 60 minutes, 1 mM was added to stationary adaptation (◇), stationary lethal challenge (○), logarithmic adaptation (■), logarithmic lethal challenge (◆) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

101

Figure 3-9

Effect of growth phase and age upon adaptation of 2 weeks old *A. hydrophila* to oxidative stress.

Two pre-cultures were obtained by growing cells overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights was used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary (□) and logarithmic (Δ) control. The first addition (50 µM) was performed after 5 minutes to both adaptation flasks. At time 60 minutes, 1 mM was added to stationary adaptation (◇), stationary lethal challenge (○), logarithmic adaptation (■), logarithmic lethal challenge (◆) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 3-10

Effect of growth phase and age upon adaptation of *A. hydrophila* (12 weeks old) to oxidative stress.

Two pre-cultures were obtained by growing cells overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights was used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary (□) and logarithmic (Δ) control. The first addition (50 μM) was performed after 5 minutes to both adaptation flasks. At time 60 minutes, 1 mM was added to stationary adaptation (◇), stationary lethal challenge (○), logarithmic adaptation (■), logarithmic lethal challenge (◆) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

and logarithmic phase cells, it can be seen that logarithmic phase cells displayed a marginally weaker resistance to 1 mM H_2O_2 than cells in stationary phase.

b) Effect of culture age upon *A. hydrophila* oxidative stress response.

The ability of 3 days old logarithmic phase cells in displaying an adaptive response seems to be reduced as length of storage increases. When in logarithmic phase, cells stored for 2 and 12 weeks do not display any adaptation pattern, and loss of viability is similar to cells which have not been pre-treated prior to challenge with 1 mM H_2O_2. On the contrary, stationary phase cells pre-treated prior to lethal challenge, always display an adaptive response to the oxidising agent regardless of age of culture from the plate. Closer inspection of Figures 3-8, 3-9, 3-10 showed that cells stored for 3 days and pre-treated with 50 µM H_2O_2 display a stronger resistance to the lethal challenge than either of the 2 older cultures pre-treated with 50 µM H_2O_2. Viability loss is of 1 log for the 3 days stored bacteria, whereas the reduction in viable cells encountered by the two older samples is of around 2 log within 300 minutes of the assay.

It is interesting to note that whereas viability for the 3 days and 2 weeks old stabilised after 300 minutes into the assay, the 12 weeks old display an increase in viability between 300 to 1440 minutes (data not shown). This shows that although cells stored for 12 weeks on solid medium initially appear more sensitive to the 1 mM challenge than the 3 days old cells, they display similar viability to their younger counterparts after 24 hours. This would seem to suggest that alternate mechanisms of protection may be involved in the older cells. Another possible explanation for this increase in resistance is the involvement of cell memory due to maturation proteins prior to re-growth (Chapter 3 paragraph 3.4.3.4 b).

3.3.5 Adaptation of *A. caviae, A. sobria* and environmental *Aeromonas* isolates to H₂O₂.

All investigations which have been undertaken until now have been performed on the *A. hydrophila* type strain NCIMB 9240. As described in the introduction and further stressed in chapter 2, *A. hydrophila* is considered as being the major pathogenic species with regards to humans, but other sub-species such as *A. caviae* or *A. sobria* have emerged as potential agents for both intestinal and extra-intestinal diseases. It is therefore of importance to investigate whether the oxidative adaptation phenomenon demonstrated for *A. hydrophila* is unique to this species or whether it is more widely distributed among the genus. Therefore similar adaptation experiments to those described for *A. hydrophila* were performed on type strains of *A. caviae* (Figure 3-11) and *A. sobria* (Figure 3-12), and two non speciated environmental isolates determined by phenotyping to belong to the genus *Aeromonas* (Figures 3-13 and 3-14). 1 mM H₂O₂ was proven to be lethal towards all strains examined. However the susceptibility to the lethal challenge was shown to vary between the particular strain under investigation. *A. sobria* was shown to be more sensitive to 1 mM H₂O₂ than the environmental isolates whereas *A. caviae* showed a similar resistance pattern. The response of the type strains to the lethal challenge was shown to be more varied. Recovery of *A. caviae* was shown to be impossible 180 minutes after lethal addition, with *A. sobria* shown to be more sensitive than *A. caviae*, whereby no cells could be recovered 90 minutes after the 1 mM H₂O₂ challenge. Both the type strains and environmental isolates displayed an adaptive response to 1 mM H₂O₂ challenge when pre-treated with 50 µM of the same oxidant, suggesting that this phenomenon is widely spread among the *Aeromonas* genus. Further, the extent to which the cells remain viable in the presence of oxidative insult varies between the type strains and the environmental group,

PAGE

NUMBERING

AS ORIGINAL

Figure 3-13

Figure 3-13
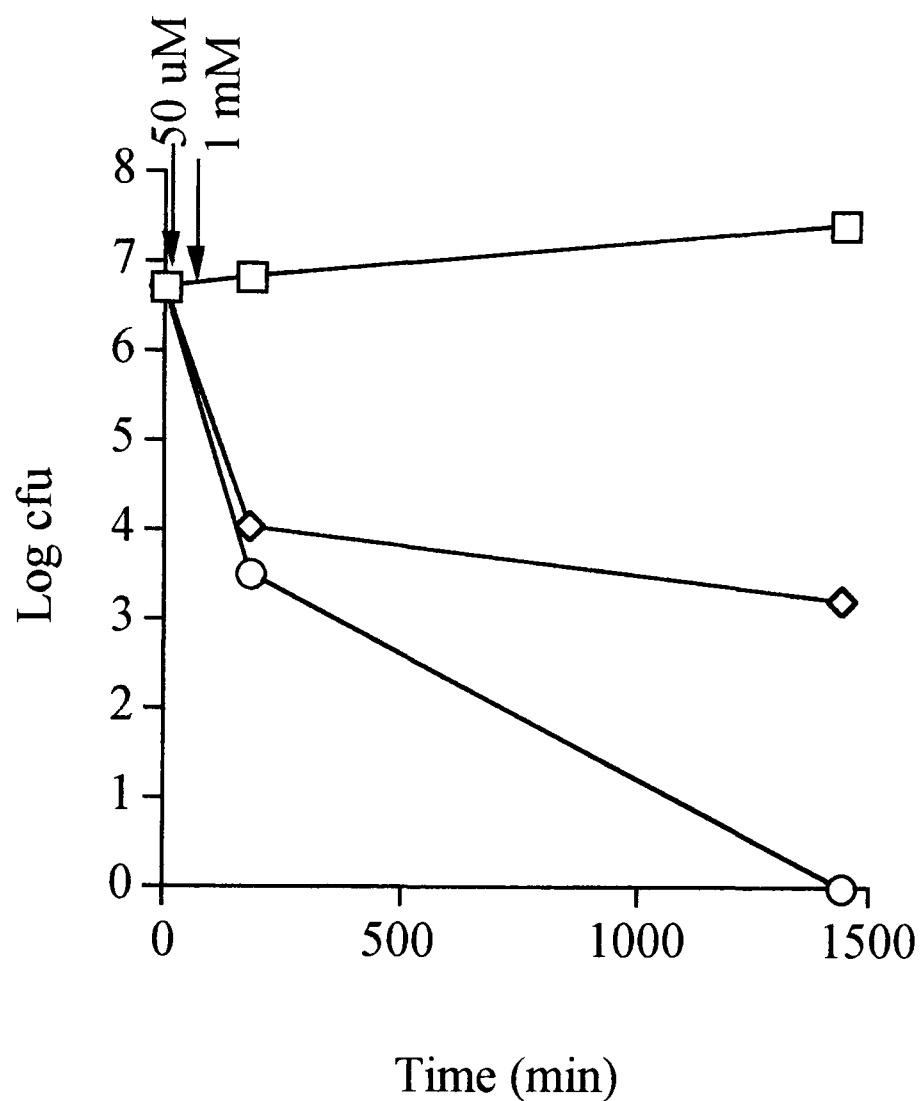
Adaptation of *Aeromonas* environmental isolate 14/8/96/1 to 1 mM H_2O_2 using 50 μM as a sub-lethal dose.

Cells were grown in liquid minimal medium (M9). No addition of H_2O_2 is shown as a control (□).The first addition (50 μM) was performed after 5 minutes in the adaptation flask. At time 60 minutes, 1 mM was added to both adaptation (◇) and death (○) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 3-14
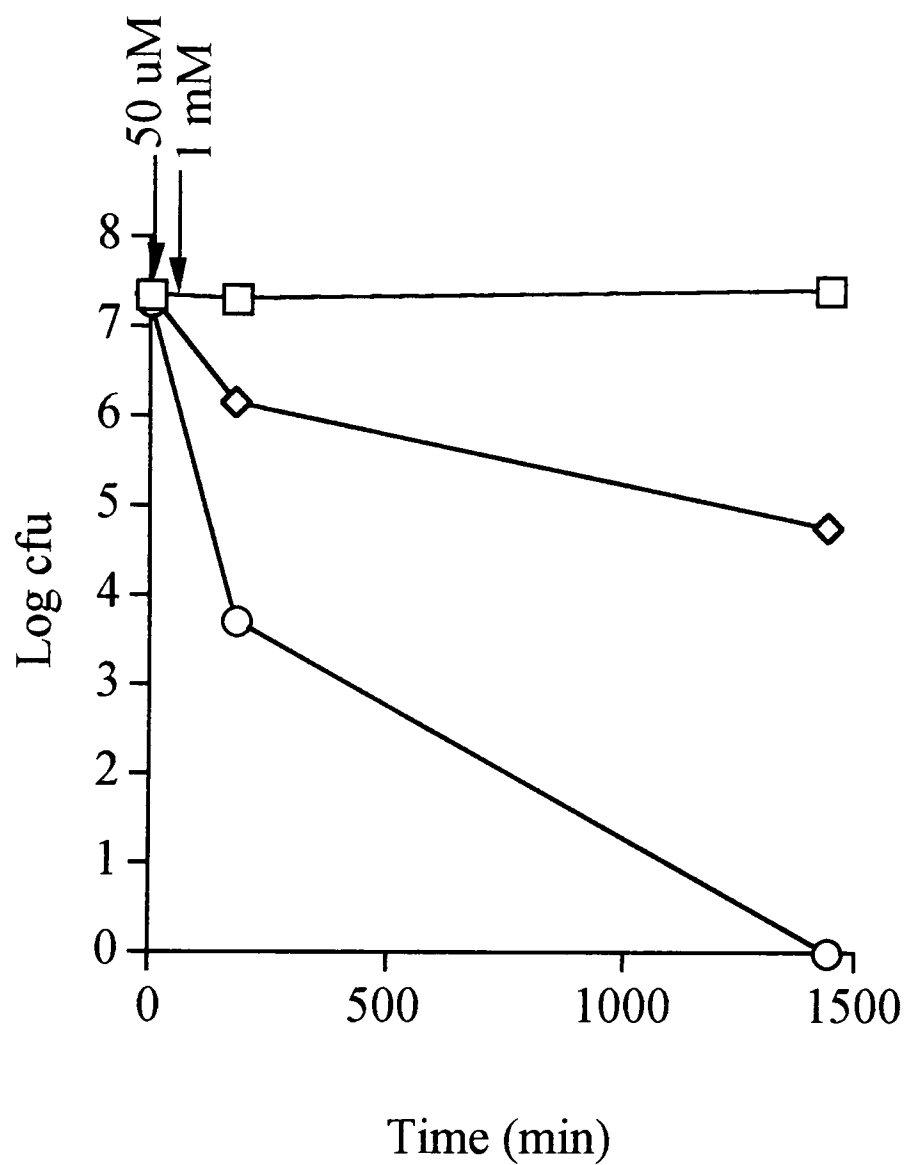
Adaptation of *Aeromonas* environmental isolate 14/8/96/5 to 1 mM H₂O₂ using 50 μM as a sub-lethal dose.

Cells were grown in liquid minimal medium (M9). No addition of H_2O_2 is shown as a control (□).The first addition (50 μM) was performed after 5 minutes in the adaptation flask. At time 60 minutes, 1 mM was added to both adaptation (◇) and death (○) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

but also within the groups themselves. A 1-2 log difference can be observed between the environmental isolates (Figures 3-13 and 3-14).

Phenotyping tests have shown that these environmental isolates belong to the *Aeromonas* genus, but their identity to subspecies level is not known as no genotyping test has been performed (A. Gavriel, unpublished data). It is probable they are two distinct subspecies which might explain their noticeable difference in response to the oxidative challenge. However this cannot be resolved until subspeciation has been performed. However cultivation in the laboratory using complex media might influence the response of these isolates to stress parameters.

With regards to the type strains, major differences can be noticed. Here, it is apparent that the difference are subspecies related. It is interesting to notice that whereas *A. sobria* was more sensitive to 1 mM H_2O_2 than *A. caviae*, its adaptation response when pre-treated with 50 μM appeared to be more efficient than for *A. caviae*. The difference between the control and the adaptation curve for *A. caviae* showed about a 3 log difference whereas *A. sobria* a difference of about 1 log. This surprising efficiency of *A. sobria* to better adapt than *A. caviae* might be a laboratory phenomenon, however the possibility of *A. sobria* having developed a more efficient protective mechanism cannot be discounted. It is feasible that *A. sobria*, being more sensitive than the other mesophilic members of the *Aeromonas* genus, had to develop better adaptative mechanisms to survive.

3.4 Discussion.

3.4.1 Determining the range of concentration in complex medium.

In order to establish the effect of different concentrations of H_2O_2 on *A. hydrophila* a series of growth experiments were undertaken initially using complex medium. From the results obtained for these experiments it was seen that relatively high concentrations of H_2O_2 were required to kill the cells. The minimum inhibitory concentration was observed to be 30 mM, with 15 mM being mildly bacteriostatic. This range of active concentration was very close to the values previously reported for Gram negative bacteria such as *E. coli* (Demple and Halbrook, 1983). In a study performed on *Enterococcus faecalis* (Flahaut *et al.*, 1998), this organism was reported to resist 45 mM H_2O_2 with a sub-lethal dose of 2.4 mM in brain heart infusion broth. Concentrations of H_2O_2 reported by Flahaut and co-workers were higher than any reported concentrations within the literature for both Gram negative bacteria (20 mM H_2O_2) and Gram positive (10-30 mM H_2O_2). Flahaut and co-workers (1998) attributed this enhanced resistance of *En. faecalis* to the high tolerance of the microorganisms when comparing with the lower resistance of other species. Importantly, most adaptation experiments reported in the literature (Demple and Halbrook, 1983; Imlay *et al.*, 1988) were performed in minimal medium when lower lethal doses reported were reported. This suggested that the higher resistance observed for *En. faecalis* (Flahaut *et al.*, 1998), and for *A. hydrophila* to H_2O_2 in complex medium should raise concern, as there was a possibility that H_2O_2 might have reacted with some components of the complex medium, thereby reducing its oxidising potential. H_2O_2 is considered as being a compound of relatively moderate chemical reactivity as it has no unpaired electrons (Cadenas, 1995). However, complex medium such as nutrient broth contains elements such as iron which could react with H_2O_2 and produced other more reactive oxygen species such as $HO^{\cdot}$ via

the Fenton reaction. These ROS would react with any component of the medium before coming into contact with bacteria. To verify whether this was indeed significant, a similar experiment was performed in M9 minimal medium which lacks organic material other than glucose as the metabolic substrate. The advantage in using a minimal medium is that the medium composition is well defined and that H_2O_2 might not be subjected to the same degree of interference postulated in complex medium, meaning that lower doses can be applied for the same or greater effect.

3.4.2 Determination of the lethal and sub-lethal doses of H_2O_2 for adaptation experiments in minimal medium.

It was shown previously that *E. coli* could withstand concentrations of H_2O_2 in the low mM region i.e. 5-20 mM when challenged in minimal medium (Demple and Halbrook, 1983; Imlay *et al.*, 1988). Previous work (Gavriel *et al.*,1997) demonstrated that *A. hydrophila* was a more chlorine-sensitive species than *E. coli* or *Ps. aeruginosa*. The use of a minimal medium was therefore considered at the view of the high lethal dose required in experiments performed in complex medium. A similar experiment to that undertaken in complex medium was performed. In this case a lower concentration range was used to study the effect of H_2O_2 upon *A. hydrophila* survival due to the reduction of interference compared to nutrient broth. Results obtained from this experiment showed that 10 mM H_2O_2 was only marginally bacteriostatic when added to a mid-log culture of *A. hydrophila* (Figure 3-2). From these data it was apparent that the spectrophotometer was not sufficiently sensitive for evaluating the extent of bactericidal activity of H_2O_2 on *A. hydrophila*. The problem with spectrophotometric analysis is that only lysis of the cells results in a decrease in the $OD_{650\ nm}$. This method will not discriminate between dead cells which have not been lysed, as they still absorb light, and living microorganisms which have survived. A more rigorous and discriminatory technique was necessary in order to

112

measure the more subtle oxidative effect of H_2O_2. Methods suitable for assessment of viability are more sensitive and the Miles and Misra drop plate count procedure (Chapter 3 paragraph 3.2.2.3) was employed as the most appropriate assay. Two factors entered in the selection of 10 mM as the lethal challenge. Firstly as 15 mM H_2O_2 was bacteriostatic in complex medium (Figure 3-1) and due to reduced interference in minimal medium a lower dose would be expected to be more active. Secondly *A. hydrophila* was found to maintain growth in the presence of 10 mM H_2O_2 at a reduced rate (Figure 3-2) when studied with the spectrophotometric technique. Switching to a more sensitive method suggested that this bacteriostatic dose might become bactericidal. Therefore the action of 10 mM H_2O_2 on stationary phase cells re-suspended in M9 was subsequently determined using the drop plate count procedure. It was found that *A. hydrophila* were very sensitive to 10 mM H_2O_2 with a rapid decrease in viability immediately after application of the challenge (Figure 3-3). After 60 minutes no viable cells could be recovered. 10 mM H_2O_2 was therefore shown to be a greater challenge than *A. hydrophila* could cope with. It appears that such a detrimental dose could not be fully eliminated by the catalase enzymes and scavenging molecules *A. hydrophila* produced, therefore leading to death of the bacteria. Evidence for this hypothesis was obtained subsequent studies. It was reported in the literature that pre-treatment of cultures using a sub-lethal dose of an oxidative agent could protect these organisms against lethal challenge of the same noxious agent. As a preliminary attempt to investigate the effect of pre-exposure to a sub-lethal challenge of H_2O_2, cells were pre-treated with 50 µM H_2O_2 prior to addition of the 10 mM dose. The 50 µM H_2O_2 pre-treatment dose was selected upon the fact that no loss of viability was recorded after a 24 h period (Figure 3-3 b). However pre-treatment with 50 µM H_2O_2 had little or no effect upon maintenance of viability when cells were subsequently challenged with 10 mM H_2O_2 (Figure 3-3 a). Many factors could have been the cause of the inability of *A. hydrophila* to

adapt to the 10 mM H_2O_2 challenge. The length of pre-incubation with the sub-lethal dose of H_2O_2 was the first parameter considered. Pre-incubation time is an important factor as it determines the extent to which the cells will switch on the genes responsible for their protection to oxidants such as *OxyR*, *SoxRS* or the genes responsible for the chaperone proteins. The second parameter considered was the growth phase stage of the cells. In the literature it was reported that resistance of *E. coli* to stress was dependent upon the growth phase. Logarithmic phase cells were more susceptible than their stationary phase counterpart (Siegele and Kolter, 1992). Another point taken into consideration was the sub-lethal dose. It might be possible that 50 μM H_2O_2 was insufficient to turn on the genetic machinery responsible for the cells to adapt. The last parameter which could be significant was the concentration of the lethal oxidative challenge. It was thought that the defence mechanisms of the cells under threat might not have been able to cope with such a concentration of H_2O_2, therefore leading to extensive damage to the cells followed by the rapid loss of viability displayed in the assay. Adjusting the lethal dose was thought to be the best option for the following reasons. First of all, the length of pre-incubation used during the experiment shown in Figure 3-4 was 2 hours. This length of time is physiologically substantial and should have been ample for the cells to turn on the appropriate genes required for adaptation. Also studying the effect of the growth phase was not considered as logarithmic phase cells would be more sensitive to the insult than the stationary phase cells assuming *A. hydrophila* behaves as other microorganisms such as *E. coli*. An alternative might have been the use of cells at a later stage into the stationary phase. But a study in our laboratory showed that *A. hydrophila* starved for 7 weeks could only resist doses up to 5 mM (C. A. Stirling, unpublished data). It was therefore considered that 10 mM H_2O_2 was probably too large an oxidative challenge since the cell densities in the viability assay, approximately 1×10^7 cells/ml, were considerably lower when compared

to the initial growth experiments where the values would have been 2 log higher. No adaptation being possible with 10 mM H_2O_2, it was decided to use 1 mM which was found to be the lowest concentration of H_2O_2 which killed *Aeromonas* within 300 minutes (Figure 3-4). Therefore for future investigations a lethal dose of 1 mM H_2O_2 was selected.

3.4.3 Investigation into the response of *A. hydrophila* to oxidative stress.

3.4.3.1 Adaptation of *A. hydrophila* stationary phase cells to H_2O_2.

A similar experiment as the one described above was performed in which cells were pre-exposed to 50 µM H_2O_2 for 60 minutes prior to addition of 1 mM H_2O_2. From data shown in Figure 3-5 a, this experiment clearly demonstrated that *A. hydrophila* can, in common with enteric species, adapt to oxidative stress. Cells treated only with 1 mM H_2O_2 died within 200 minutes whereas cells pre-treated with 50 µM maintained viability in the presence of 1 mM. Pre-exposed cells remained viable even after 24 hours incubation (Figure 3-5 b). This represents the first conclusive demonstration of adaptation to an oxidative stress challenge of the genus *Aeromonas*.

The efficiency of the protection afforded by the sub-lethal dose in relation to time was examined to gain an insight into the mechanisms involved in the stress response and whether they interact or are independent. The relative temporal protection was studied using the previously determined sub-lethal and lethal doses, being 50 µM and 1 mM H_2O_2, respectively. The sub-lethal dose was added at the same time in all of the flasks with the addition of lethal dose performed at a set increase of time. Results obtained demonstrate that the longer the delay between the sub-lethal and lethal addition, the stronger the adaptation (Figure 3-6). 1 log difference in viable count between cells left in presence of the sub-lethal dose for 30 minutes and the one pre-treated for 60 minutes was observed. This difference reduces to less than 0.5 log for cells pre-treated for 120 to 300 minutes.

Closer inspection of the data showed that 120 minutes seems to be the optimal time required for adaptation (Figure 3-6). During the first 60 minutes after lethal challenge has been applied, viability loss for 120 minutes pre-treatment was seen to be the minimal. It is interesting to note that for the first 60 minutes after lethal challenge viability does not reduce according to the length of time between the two additions. In other words, the longer the time that elapses this does not lead to enhanced protection. A 30 minutes pre-treatment was shown to protect the cells to a lesser extent than when similar cells were pre-treated for 60 minutes. On the contrary, a pre-treatment of 300 minutes was shown to afford less protection to the cells than if pre-treated for 240 minutes, which in turn was found to afford less protection than for cells in contact with the sub-lethal dose for than 180 minutes. These results, observed for the first 60 minutes of the experiment, were different for samples taken after the first 60 minutes of the experiments. The trend displayed in Figure 3-6 for the sampling time of 60 minutes gradually changed towards a pattern where the longer the delay between the sub-lethal and lethal addition, the stronger the adaptation. The data presented in Figure 3-6 shows a time-dependent pattern in resistance of *A. hydrophila* to H_2O_2. This alters if examined prior to or after the first 60 minutes of lethal challenge, which suggests that the first 60 minutes after lethal challenge are important for an immediate response to the stress and is a pre-requisite for an efficient longer-term stress resistance. This would imply that two individual pathways run in parallel in order to provide the proteins necessary for the resistance to short or long term-stress conditions. The fact that *A. hydrophila* stress resistance is time-dependent might involve a similar cascade pathway as the one described by Nyström (1993), and would be in agreement with published literature.

3.4.3.2 Role of catalase in *A. hydrophila* oxidative stress response. Fresh synthesis or modulation of an existing catalase pool?

Two groups of enzymes are involved in the degradation of H_2O_2, being peroxidases and catalases. They are both referred to as hydroperoxidases. Catalases are the most studied group as these are considered as being a special type of peroxidases (Anon., 1997). Catalases have both an oxidative and reducing role when in the presence of H_2O_2 and the reaction does not result in radical formation. Peroxidases, on the other hand, oxidise other intermediate compounds (both organic and inorganic) which results in the formation of reactive intermediates (i.e. free radicals) (Anon., 1997). *E. coli* posses 2 catalases: HPI which is induced by the presence of H_2O_2, and HPII which is growth phase regulated (Demple, 1991). HPI is encoded by *katG* and HPII encoded by *katE* (Farr and Kogoma, 1991).

In the current study, investigation of some of the mechanisms involved in the oxidative adaptation phenomenon lead to the study of the relationship between changes in catalase production and adaptation of the bacteria. Results reported in Tables 3-1 and 3-2 are for treatment of cells with 50 µM and 1 mM. Table 3-3 showed results for 500 µM, a concentration high enough to seriously challenge the cells and initiate any potential oxidative stress response, but not excessive, preventing total killing of the cells under study. Results obtained for the no pre-treatment control and the 5 minute pre-treatment displayed an almost identical trend for all the concentrations assayed, therefore indicating that a 5 minute pre-treatment period is not sufficient to trigger increased catalase activity. Comparing the different initial reaction rates calculated for each concentration tested seemed to support the latter hypothesis. Results for 30 and 60 minute pre-treatment displayed similar patterns and reaction rates which were different from the 5 minute pre-treated cells. From Tables 3-1 and 3-2 it can be seen that 30 minute pre-treatment seems to

be the minimum time period requirement for the cells to display a marked increase in catalase activity. It is possible that catalase is being freshly synthesised after onset of oxidative stress challenge. However, catalase has previously been considered to be part of a static mechanism independent of fresh protein synthesis (Davis, 1995). This statement raises the possibility that the increased catalase activity observed in *A. hydrophila* might possibly be due to modulation of a pre-existing form of the enzyme which might be converted to active catalase by a yet unknown factor synthesised or itself activated upon presence of H_2O_2 stress.

It was necessary to clarify whether catalase was newly synthesised as part of this cascade phenomenon or if it was a modulation of pre-existing enzymes as part of a different defence mechanism. This was made possible by the use of chloramphenicol, an antibiotic known to affect translation of the mRNA to proteins. This protein synthesis inhibitor is of very simple structure and binds specifically to the 50S sub-unit of the ribosome. This binding inhibits the activity of the enzyme peptidyl transferase. Peptidyl transferase is the enzyme which catalyses the reaction which uncouples the carboxyl end of the polypeptide chain from the previous tRNA (in the P-site) and joining the polypeptide chain to the next amino acid attached to the next amino acyl tRNA in the A-site. Chloramphenicol was added to the cell culture shortly before pre-treatment with H_2O_2. Performing the catalase assay on cells which were in presence or absence of chloramphenicol would indicate whether catalase was freshly synthesised during the exposure time or if it was present in the cell prior to H_2O_2 addition. The concentration of H_2O_2 chosen for this experiment was 500 μM. This concentration was shown to be non-lethal (Figure 3-4), but significant enough to be a serious challenge to the cell. When compared with catalase activity in non-chloramphenicol treated cells, very similar results were obtained. This result suggests that

a modulation of pre-existent catalase is the basis for an increased activity when the concentration of the noxious agent increase.

Cells were allowed to reach stationary phase cells prior to both chloramphenicol or sub-lethal H_2O_2 addition. Addition of chloramphenicol after overnight growth does not reduce catalase activity during the assay and would suggest that cells, when reaching stationary phase, would produce a set amount of HPII catalase via RNA polymerase-σ^s regulation. This amount would not increase with further stress unlike HPI catalase regulated by OxyR. Furthermore, HPI gene regulation by OxyR was rendered impossible with addition of chloramphenicol which prevent synthesis of fresh proteins. This would correlate with reports in the literature stating that a catalase activity is a cell-density phenomen, on known as quorum sensing, which regulates σ^s factor synthesis (Lafiti *et al.*, 1996), rather than a single cell defence mechanism (Crockford *et al.*, 1995). Another interesting point is that blockage of fresh protein synthesis by chloramphenicol, including HPI catalase, might lead to an increased activity (not amount) of HPII catalase to compensate the shut down of the second pathway. It can also be hypothesised that the sub-lethal oxidative stress will trigger the synthesis of both catalase and an unknown partially inhibitory molecule of catalase activity along with other proteins under the regulation of the *oxyR* regulon at the early stage of encounter of the oxidative stress. One scenario could be that when the cells face a more significant challenge, which the preventative mechanisms could not protect against, this unknown partially inhibitory molecule of catalase activity would act as a sensor of this failure by reacting with some of the oxidant molecules. A decrease in the concentration of this unknown factor would lift the partial inhibition of catalase. The increase of catalase activity may in turn act a signal for other members of the community which would therefore initiate the synthesis of other factors such as increased adherence properties allowing the cells to attach to particles or to aggregate, consequently being more stress

resistant. Further, the cells might respond to the increase in molecular oxygen released during degradation of H_2O_2 by catalase or by a signal molecule synthesised by the cells overloaded by the attack.

3.4.3.3 Effect of chloramphenicol on the adaptation of stationary phase cells of *A. hydrophila* to H_2O_2.

A. hydrophila has been shown to display an adaptive tolerant response to a lethal oxidative challenge through pre-exposure to a sub-lethal dose of this oxidising agent (Figure 3-5). Previous studies performed on *E. coli* (Demple and Halbrook, 1983) and *S. typhimurium* (Farr and Kogoma, 1991) for oxidative stress and starvation stress in *Vibrio* spp. (Nyström *et al.*, 1990) showed that such adaptive tolerance response phenomenon was linked to production of a set of various stress proteins. This was confirmed by abolition of the effect in the presence of the protein synthesis inhibitor chloramphenicol. Treatment of an *A. hydrophila* culture with chloramphenicol, added shortly before the sub-lethal dose, prevents any adaptation from occurring (Figures 3-7 a and b). This confirmed that protein synthesis was absolutely essential in achieving generation of tolerance to oxidative stress. However despite the fact that *A. hydrophila* displays the same requirements as *E. coli* for protein synthesis for the mediation of the adaptive response, *Aeromonas* has a remarkably lower level of resistance to H_2O_2 than *E. coli* . Adaptation of *A. hydrophila* was shown to occur to concentrations in the range of 1 mM H_2O_2 whereas *E. coli* displayed resistance at concentrations around 20 mM (Demple and Halbrook, 1983). From an evolutionary point of view, such a difference suggests that the niche in which the microorganism has evolved has played an important role . The natural aquatic habitat of *A. hydrophila* has minimised the likelihood for this bacterium to encounter a natural oxidative challenge until recently. However use of chlorine in water treatment has provided such a situation. In contrast, *E. coli* has been evolving in an environment rich in oxygen radicals formed through the host's

(i.e. animals and humans) defence mechanisms. This difference in environment raises the possibility that *A. hydrophila* stress proteins might be unrelated, less numerous and/or less efficient than those mediating the response in *E. coli* . Being a commensal species, the *E. coli* growth environment has been rich in ROS derived from oxygen for an extensive period of time. It is possible that this continuous pressure on the microorganisms lead to the selection of a very complex and efficient set of regulons tailored to the various stresses encountered by this species.

3.4.3.4 Effect of growth phase and length of storage of *A. hydrophila* in oxidative response.

a) Effect of growth phase of *A. hydrophila* in oxidative response.

Previous studies investigating the adaptive tolerance phenomenon in *E. coli* found that the response of the cells to stress varied with respect to the phase of growth. *E. coli* was observed to be more resistant to oxidative stress when in stationary phase, with log phase cells considerably less resistant. An analysis of the effect of growth phase upon the oxidative stress response of *A. hydrophila* was undertaken. Cells which had been grown to either mid-log or stationary phase were used to investigate viability following exposure to H_2O_2. It was essential that the total number of cells in the experimental flasks were equivalent when comparing the results obtained for logarithmic and stationary phase cells in order to link any potential differences to the phenomenon investigated rather than to the difference in cell number. It was also of importance to have an identical number of cells as some of the phenomena under study might be cell density-dependent. Figures 3-8, 3-9 and 3-10 showed that both logarithmic and stationary phase controls are steady and identical in pattern allowing comparison between the different experimental samples, but also between the three experiments themselves. Data obtained showed *A. hydrophila* logarithmic phase

cells to be more sensitive to H_2O_2 than the stationary phase cell counterpart. With regards to the stationary phase cells, the longer the storage period, the more efficient the adaptation.

b) Effect of length of storage of *A. hydrophila* in oxidative response.

Significantly, it was found that oxidative stress response in either logarithmic or stationary phase varies according to the length of storage of the test organisms on nutrient agar plates. The difference in resistance between logarithmic and stationary phase cells is a known phenomenon (Siegele and Kolter, 1992). However, no reference could be found in the literature with regards to the effect of the length of time of bacterial storage on master plates i.e. plates on which the bacteria are stored between experiments. Experiments performed during this project have demonstrated that bacterial storage time is an important parameter which affects the response of cells both in logarithmic and stationary phase. Logarithmic phase cells being less resistant than their stationary phase counterpart was shown to be independent of the length of time the bacteria were stored on nutrient agar plates. However, the degree of resistance of both logarithmic and stationary phase cells was proven to depend upon the length of time the strains spent stored on plate.

Studies performed on long-term starved *Vibrio* spp. found that bacteria maintain ability to rapidly metabolised nutrients by retaining certain functions such as the electron transport chain and associated membrane-bound ATPases (Kjelleberg *et al.*, 1993). Long-term starved *Vibrio* spp. produced what has been described as maturation proteins. The role of those maturation proteins is not clear, but it is hypothesised that they play an active role in degrading starvation inducible proteins which may become inhibitory to re-growth i.e. targeted proteases. The higher degree of resistance of the older cells, when subjected to oxidative stress after re-cultivation, might be explained by the fact that cells have a 'memory'. It is proposed here that memory would be mediated through maturation proteins

which do not degrade all the starvation induced proteins during initial re-growth. This could be explained by the fact that the length of the lag phase is dependent on the time of starvation (Albertson *et al.*, 1990; Morita, 1993). i.e. the longer the starvation, the longer the lag phase. The implication would be that for a period of time, more stress proteins would be degraded in cells for which length of starvation is shorter than for older cells. The remaining starvation proteins which have not been degraded by maturation proteins would confer some degree of resistance to the strain possessing them and would be diluted down with respect to further growth meaning that the bacteria would progressively become less resistant with respect to the increase number of generations.

This hypothesis is supported by the results obtained from the experiments with logarithmic phase cells of *A. hydrophila*. The logarithmic phase cells were obtained by growing cells of different storage length overnight and re-suspending part of this overnight in fresh M9 medium until logarithmic phase is attained. It was shown that the older the plate from which the initial cells were obtained the less resistant to oxidative stress the respective logarithmic phase cells were. This would be explained by the fact that the older cells have a longer the lag phase, meaning that the exponential phase is initiated later compared to young cells. It is important for the cells to have an active replication mechanism for proteins to be synthesised. During this active period, the sub-lethal dose can trigger the expression of the genes responsible for the stress proteins. The longer this period, the more proteins are synthesised. As the sub-lethal dose is added at the same time in all flasks, but that the extent of the lag phase is different for each strain assayed, the amount of stress protein synthesised would therefore be different. The older the cell, the less stress proteins, the more sensitive to stress. But because the cells are actively replicating when in exponential phase, they are more sensitive to oxidant as DNA for example is more

accessible and would not exhibit the same degree of resistance than the stationary phase counter-part.

3.4.4 Adaptation of stationary phase *A. caviae*, *A. sobria* and *Aeromonas* environmental isolates to H_2O_2.

In order to determine whether the phenomenon of adaptation was a unique feature to a specific strain or if it was a general response shown by other members of the genus, similar experiments were performed with type strains of both *A. caviae*, *A. sobria* and two environmental isolates (Figures 3-11, 3-12, 3-13 and 3-14). 1 mM H_2O_2 as a challenge was proven to be lethal for all strains tested. The fact that none of the type strains required higher lethal doses to display loss of viability, showed a common sensitivity of the genus to the oxidant compared to others such as *E. coli* and *Pseudomonas*. Results obtained from these experiments showed that adaptation to oxidative stress is a universal feature among members of the mesophilic *Aeromonas* as both type strains and environmental isolates displayed an adaptive tolerance response to the lethal challenged applied (Figures 3-11, 3-12, 3-13 and 3-14). Interestingly *A. sobria* consistently maintained a greater viability than either *A. hydrophila* or *A. caviae*. This result is rather surprising considering that *A. sobria* has been previously shown to be more sensitive than *A. caviae* or *A. hydrophila* to chlorine (Gavriel *et al.*, 1997). But when this result is viewed from an evolutionary perspective this bears no surprise. Being more vulnerable to stress than other members of the genus, *A. sobria* had to develop more efficient ways of survival in order for it to remain a viable part of the community. With regards to the heterogeneity of the genus, it is also a possibility that *A. sobria* has followed a different evolutory line within the genus; whereas *A. caviae* might be closer to the evolutionary line of *A. hydrophila*.

3.4.5 Conclusions.

The aim of the current study was to evaluate the effect of H_2O_2 onto mesophilic *Aeromonas* and gain an insight into the different mechanisms involved in the adaptive response to this agent. In summary, data presented in this chapter demonstrate that the use of complex medium was not suitable for our study as results suggested that H_2O_2 reacted with components of the complex medium such as iron and reduced H_2O_2 bactericidal activity. Therefore minimal medium was utilised to avoid such problems. It was initially found that pre-treatment with 50 μM H_2O_2 had little effect in protecting the cells against a 10 mM H_2O_2 challenge, which was probably too large a challenge for the cells defence mechanisms to cope with. Further experiments showed that resistance to 1 mM H_2O_2 challenge i.e. the minimal lethal dose, was rendered possible when cells were pre-treated with 50 μM H_2O_2. This is the first demonstration of an *Aeromonas* species to adapt to an oxidative challenge. Study of the temporal protection afforded to the cells by pre-treatment prior to lethal challenge showed a time-dependent pattern of the protection which alters as follows. Prior to 60 minutes after addition of 1 mM H_2O_2, the optimal pre-treatment time was 120 minutes. After 60 minutes, the trend showed that the longer the delay between the sub-lethal and lethal dose, the stronger the adaptation. The fact that protection is time-dependent appear to involve a cascade pathway similar to the one described by Nyström (1993). The role of defence mechanisms in the response was investigated by studying two specific aspects being catalase and the potential for protection by synthesis of key stress proteins. Catalase is known to be directly involved in the degradation of H_2O_2 present in the cell. Experiments showed that among the pre-exposure times assayed, 30 minutes seemed to be the minimum requirement for cells to display an increased catalase activity. Further study was undertaken to determine whether catalase was induced during stress or if the protection was due to modulation of pre-existing enzyme. Experiments performed in

the presence and absence of chloramphenicol showed protection to occur via modulation of pre-existing catalase as chloramphenicol did not abolished catalase activity. Fresh protein synthesis was shown to be essential when the presence of chloramphenicol abolished adaptation of pre-treated *A. hydrophila* to 1 mM H_2O_2 challenge. Despite the fact that *A. hydrophila* can adapt to lethal challenge through *de novo* protein synthesis, its increased sensitivity compared to *E. coli* suggests that the key stress proteins synthesised by *A. hydrophila* may be unrelated, less numerous and/or less efficient than those synthesised by *E. coli* . This difference might be due to the distinct environmental pressure these organisms have faced during their evolution.

Further investigation of the oxidative stress adaptation of *A. hydrophila* lead to the study of the effect of growth phase and length of storage on plates upon the response of the organism. It was shown that regardless of the length of storage, stationary phase cells were more resistant than their logarithmic phase counterpart. However, the degree of resistance of both stationary and logarithmic phase cells were dependent upon length of storage on plates. For stationary phase cells, the longer the storage, the more resistant. This increased resistance may be due to incomplete degradation of stress proteins by maturation proteins. Cells harbouring non-degraded stress proteins were therefore more resistant than cells not having stress proteins. This phenomenon of resistance due to incomplete degradation of stress proteins is termed cell memory. For logarithmic phase cells, the older the cells the less resistant they are. This was explained by the fact fresh proteins requires an active replication mechanisms and that the longer the storage, the longer the lag phase resulting in less proteins synthesised in a set period of time.

Further investigation with other members of the genus *Aeromonas* showed a common sensitivity of this genus to oxidative stress. Capability of adapting to H_2O_2 was demonstrated to be widely spread among mesophilic *Aeromonas*. However, despite these

universal features, *A. sobria* was found to maintain a greater viability than the two other members tested. This greater resistance remained unexplained as *A. sobria* was previously shown to be more sensitive to hypochlorite than *A. caviae* and *A. hydrophila* (Gavriel *et al.*, 1997). Such enhanced resistance was explained by the fact that *A. sobria* might have developed better protection to remain part of the community. We may envisage that *A. sobria* behaving differently from the two other strains tested, have followed a different evolutary line within the genus. Further data obtained when testing environmental isolates suggests that stress response is linked to the niche the organisms lived in and the cells history.

CHAPTER 4 Stress response in *A. hydrophila*: the possible role for quorum sensing.

4.1 Introduction.

Bacteria are by nature very simple in structure and have a limited capacity to control their environment, but bear the extraordinary potential to adapt rapidly to ever changing conditions (Losick and Kaiser, 1997). Until the early 1970's, the accepted view of microbiologists was that whilst bacterial cells were living in communities within colonies, they were looking out solely for themselves (Losick and Kaiser, 1997). It is fair to consider that communication between cells would be invaluable as they would be able to sense any variation within the community or warn the other members of any environmental changes, meaning that cells could co-ordinate their response with regards to these changes. Constraints from the environment the cells are living in, such as nutrient limitations, adverse pH or temperature, have led to selective phenotypes necessary for survival mediated via selective gene expression. In other words, cell to cell communication is one of the signal mechanisms through which environmental stimuli are transduced into gene expression (Swift *et al.*, 1996). This evolutionary point of view of survival of the most adaptable species is also true when the environment is a living host. A single bacterium bears minimal chance of survival if trying to overcome the host's immune defences. Cell communication would be a means to sense other members of the species and co-ordinate an effective attack (Robson *et al.*, 1997).

Cells produce low molecular weight compounds i.e. acyl homoserine lactones (AHL's) which seems to be constitutively produced and diffuse freely through the membrane due to their amphipatic properties (Kaplan and Greenberg, 1985; Robson *et al.*, 1997). The more

bacterial growth, the more AHL's produced. These compounds therefore accumulate in the milieu. However it requires a minimal population (quorum of bacteria) to be reached in order to attain the threshold concentration of signal molecule needed to lead to a population response (Swift *et al.*, 1996). This cell communication phenomenon is density dependent and has been termed quorum-sensing.

This phenomenon was first described in *Vibrio* by Nealson and Hasting in 1970. They reported an interesting finding whereby light emission in *Photobacterium (Vibrio) fischeri* did not match the bacterial growth curve, but started when cultures reached mid-log phase. At that point, the rate of bioluminescence increased in higher proportion relative to the increase in cell biomass. Light output was found to be doubling every 4 to 5 minutes. At peak of luminescence, luciferase, the enzyme responsible for luminescence, accounted for 2 to 5 % of the cell's soluble proteins. Four separate hypothesis were put forward to explain this phenomenon and subsequently assessed during their study. The first hypothesis was that luciferase was synthesised along side all the other enzymes and cellular components of the cell in concert with growth, but there was no activity because it was lacking a substrate. This was explained by the fact that reduced flavine mononucleotide ($FMNH_2$) is the substrate for bioluminescence (Equation 4-1; Engebrecht and Silverman, 1984) and that its regeneration to the reduced form when oxidised depends upon the cell reducing mechanisms:

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} light + RCOOH + H_2O + FMN \qquad \textbf{Equation 4-1}$$

Nealson and co-workers (1970) suggested that the absence of light emission might be due to a mechanism of electron channelling. Such a mechanism would drive the electron flow towards more preferred/needed molecules such as ATP, which would therefore not be

available to the cell to regenerate oxidised FMN. Being oxidised, FMN would not be available as a substrate. The hypothesis of luciferase being synthesised in concert with growth, but inactive due to substrate control, was ruled out by the fact that the first 3 to 4 hours of growth showed a constant decreased amount of extractable luciferase rather than an increase in production. The second hypothesis also suggested luciferase synthesis would parallel cell growth, the difference being that luciferase would be synthesised as an inactive zymogen precursor requiring proteolitic activation. Here the authors suggested that the precursor of luciferase should react with an antibody directed against luciferase. The reaction between the precursor enzyme and the antibody was termed cross-reacting material (CRM). This CRM should have been proportional to the cell mass if the above hypothesis was correct, but CRM was instead found to parallel the extractable luciferase activity, therefore refuting the second hypothesis. The third possibility suggested that mRNA was uniformly synthesised in the cells, not actively transcribed but accumulating. It was suggested that, at a specific growth phase, this mRNA would be called into activity resulting in the phenomenal boost in luciferase production seen at mid-log phase. If this was correct, only translational inhibitors would prevent luminescence. However, luminescence was proven to be inhibited both by transcriptional and translational inhibitors. Therefore the authors concluded that control of the light emission phenomenon catalysed by luciferase was due to transcriptional control. To strengthen their theory, they showed that luciferase, present at time of addition of the inhibitors, remained active when extracted so that inactivation of luminescence is not due to luciferase degradation.

The identity of a molecule involved in the transcriptional control of luciferase was reported in 1981 (Eberhard *et al.*, 1981) as being N-(3-oxohexanoyl)-L-homoserine lactone, now commonly known as *Vibrio fisheri* autoinducer I (VAI). Soon thereafter, isolation and identification of genes and gene products involved in bioluminescence in this species were

reported (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984). The autoinducers identified were small molecules which were found to be able to diffuse freely across the cytoplasmic membrane (Kaplan and Greenberg, 1985). Being constitutively expressed, AI concentration rose proportionally to the number of cells in the immediate environment. Once a critical biomass was achieved sufficient AI would be produced such that a cascade of events leading to light emission would occur. Crucially, production of this threshold concentration of AI required a minimum number of cells i.e. the cells had to be quorate for the necessary AI concentration to be produced. Once the threshold concentration of autoinducer is attained in the bacterium, the autoinducer activates a protein called LuxR which stimulates expression of genes controlled through the Lux operon (see Figure 4.1).

Figure 4-1: The *lux* Operon.

The Lux Operon
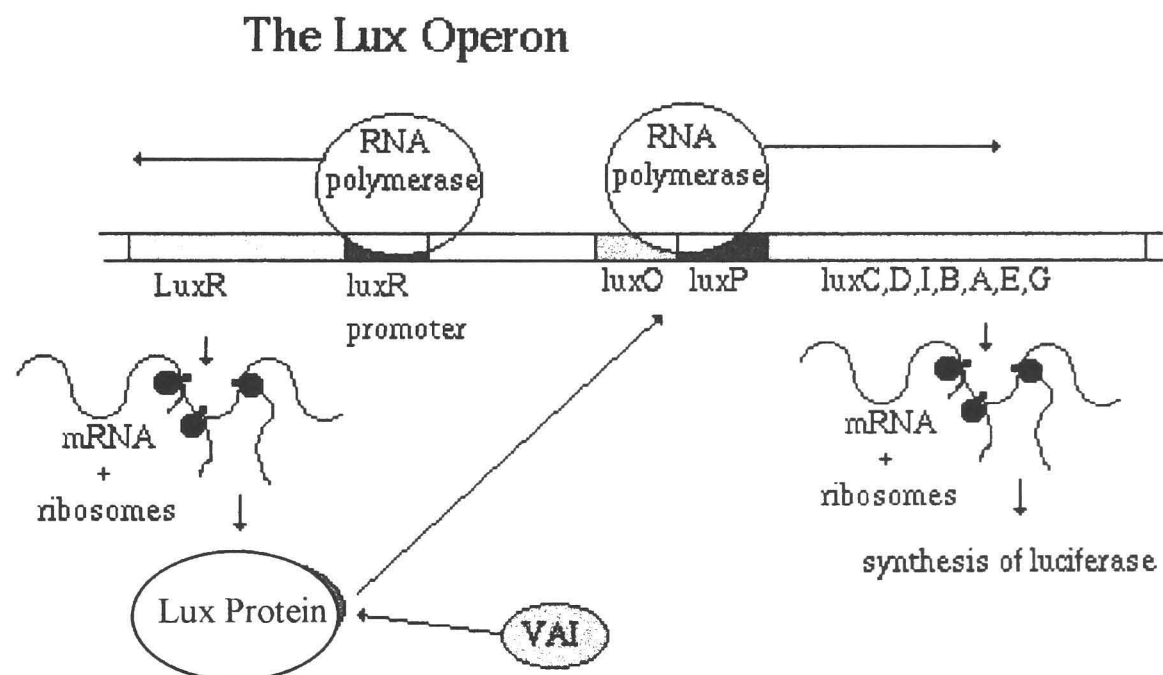
The LuxR protein is constitutively produced, its N-terminus contains an autoinducer binding site and its C-terminus, a DNA binding site (i.e. helix-turn-helix motif). Once LuxR has been activated by VAI binding, the resulting complex binds to *luxO*, the DNA binding site up-stream the promoter *luxP*. LuxR-VAI complex binding to *luxO* provoke a change in conformation of the DNA binding site allowing RNA polymerase to bind and

start the transcription of various structural genes leading to luciferase along with LuxI protein, involved in synthesis of autoinducer stimulating a positive feedback loop, hence the term autoinducer. The *luxR* gene is known to be transcribed divergently from *luxI* and identification of the existence of a central *cis-* control region i.e. a 20 nucleotide invert repeat called *lux* box, facilitates a co-ordinate regulation of both *luxI* and *luxR* (Fuqua *et al.*, 1996).

Various LuxR/I homologues have been identified in Gram negative species such as *Agrobacterium* spp. (Hwang *et al.*, 1994), *Pseudomonas* spp. (Lafiti *et al.*, 1995; Seed *et al.*, 1995), *Erwinia* spp. (Cui *et al.*, 1995; Macgowan *et al.*, 1995), *Rhizobium* spp. (Gray *et al.*, 1996), *Myxococcus* spp. (Kaiser and Losick, 1993) and *Serratia* spp. (Eberl *et al.*, 1996). Importantly *Aeromonas* spp. were also shown to produce homologues of these proteins (Swift *et al.*, 1997). *A. hydrophila* and *A. salmonicida* LuxR/I homologues termed *ahyR*/I and *asa*R/I, have been described, sequenced and the nature of the autoinducer determined as being N-butyryl-L-homoserine lactone (BHL, Swift *et al.*, 1997). Therefore quorum sensing appears to be a widespread phenomenon in Gram negative bacteria. Quorum sensing has been shown to have an effect on group behaviour where the autoinducers are known to play a role in the control of various pathways such as production of virulence factors in *Ps. aeruginosa* (Lafiti *et al.*, 1995), and antibiotics in *E. carotovora* (Chhabra *et al.*, 1993). A recent study on *Ralstonia (Pseudomonas) solanacearum* (Flavier *et al.*, 1998) showed that an RpoS (σ^S) homologue regulated production of the autoinducer. The fact that RpoS has been involved in the regulation of AHL's is of great interest as RpoS has been linked to stationary phase gene expression, but more significantly to bacterial stress response (Hengge-Aronis, 1993). Mutation in the RpoS homologue was shown to decrease autoinducer production causing sensitisation of *R.*

solanacearum to starvation and pH stress, but had no noticeable effects on heat shock or H_2O_2 stress (Flavier *et al.*, 1998).

A study into the potential role of cell density dependence and autoinducer molecules in modulating the overall oxidative stress response mechanisms in *A. hydrophila* was undertaken. Results from chapter 3 showed an increase in resistance to H_2O_2 lethal challenge the longer the cells were into stationary phase. It was sought to determine whether this increase in resistance was purely due to internal regulation of cell defence mechanisms or if some external regulatory factor could play a role in modulating protection of cells. The second aim of this work was to assess the role of a potential candidate (BHL) as an extracellular effector molecule in mediating the response of *A. hydrophila* non-pre-treated with sub-lethal challenge to H_2O_2.

4.2 Materials and Methods.

4.2.1. Materials.

4.2.1.1 Reagents.

Reagents were of analytical grade and were purchased from either Aldrich, Gillingham, UK., Sigma, Poole, UK., Lancaster Synthesis Ltd., Eastgate, Lancashire, UK., Fisons Scientific Equipment, Loughborough, UK, or BDH Laboratory Supplies, Poole, UK., Complex microbiological media such as tryptone soya agar, nutrient agar and nutrient broth were obtained from Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK., and chloramphenicol from Molecular Biology Sigma, Poole, UK.

4.2.1.2 Bacterial strains.

In the following experiments the strain used was *A. hydrophila* (NCIMB 9240) obtained from the National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, UK. The strain was maintained on nutrient agar with frequent subculture every 3 to 4 weeks. Master plates were grown at 30°C for 24 h and stored at 4°C until use.

4.2.1.3 Medium.

Millers M9 minimal medium was used to prepare cultures for the biological activity assays. M9 minimal buffer (M9 buffer) was prepared as stated in chapter 2 paragraph 2.2.1

4.2.2 Methods.

4.2.2.1 Synthesis of N-butyryl-L-homoserine lactone.

A mixture of α-amino-γ-butyrolactone hydrobromide (1g, 5.49 mmol) and triethylamine (2.44g, 24.1 mmol) in anhydrous dichloromethane (CH_2Cl_2: 45 ml) was stirred at room temperature for 30 minutes. A solution of butyryl chloride (0.59g, 5.49 mmol) in anhydrous CH_2Cl_2 (5 ml) was added dropwise to cooled (0°C), stirred reaction mixture and

134

this was allowed to mix at 0 °C for 2 hours and then at room temperature for an additional 24 hours. The solvent was then evaporated under reduced pressure and the residue was dried thoroughly *in vacuo* over P_2O_5. Ethyl acetate (EtOAc: 15 ml) was added to the residue and the suspension formed was left at room temperature for 15 minutes and then filtered through a Whatman N°1 filter paper. The precipitate was treated twice with EtOAc (15 ml), filtered, and filtrates were combined and evaporated *in vacuo* to give 0.92g of N-butyryl-L-homoserine lactone (98%) as white solid, mp. 80-82°C (EtOAc).

4.2.2.2 Spent medium experiments.

a) Effect of re-suspension of fresh cells into spent medium upon the stress response.

Three 100 ml M9 minimal medium cultures were grown overnight at 30°C to stationary phase. Cells were removed by centrifugation at 6,000 rpm for 10 minutes and the spent medium recovered for the next stage of the experiment. A 9 ml M9 minimal medium overnight culture was concurrently grown to stationary phase and 1 ml used to inoculate the flasks containing the 100 ml of previously recovered conditioned medium. The pre-determined lethal dose of 1 mM H_2O_2 was added after 60 minutes in the first experiment. This was repeated using different times of addition in the two separate flasks. One flask received addition at time 1 minute and the other at time 120 minutes. At selected times, typically 0, 60, 120, 150, 180, 240, 300 and 1440 minutes, the viable counts from these flasks were determined using the Miles and Misra plate count method as described in chapter 3 paragraph 3.2.2.3.

b) Effect of re-suspension of fresh cells into either spent medium treated with chloramphenicol or heat or pre-exposed to spent medium prior to re-suspension in M9 buffer.

The experiment described above was repeated with spent medium recovered as previously described. One flask was used as a control (no addition, no treatment). Experimental flasks received one of three treatments listed below according to the experiment undertaken with one flask used as a lethal dose control where 1 mM H_2O_2 was added at different times (except for b, see below):

a) A 10 µg/ml chloramphenicol addition at time 0 minute and the 1 mM H_2O_2 lethal dose was added after 60 minutes in both the treated and the lethal control flasks.

b) 100 ml of spent medium recovered from each of two separate cultures was boiled and allowed to cool down to room temperature prior to inoculation with 1 ml of an overnight culture of *A. hydrophila*. 1 mM H_2O_2 lethal dose was added after 60 minutes in one flask and after 120 minutes in the remaining one.

c) The cells were left for 2 hours in contact with the spent medium in one of the flasks, spun again at 6,000 rpm for 10 minutes and re-suspended into fresh M9 buffer. Lethal challenged was added straight after cell re-suspension into M9 buffer.

At selected times, typically 0, 60, 120, 150, 180, 240, 300 and 1440 minutes, the viable counts from these flasks were determined using the Miles and Misra plate count method described in chapter 3 paragraph 3.2.2.3.

c) Effect of re-suspension of fresh cells into enzymatically treated spent medium.

100 ml M9 minimal medium cultures were grown overnight to stationary phase. Cells were removed by centrifugation at 6,000 rpm for 10 minutes and one flask containing spent medium was boiled and allowed to cool down to room temperature prior to use for each of

the 2 experiments (i.e. treatment with trypsin or papain). Three flasks were used for treating the spent medium with different concentrations of papain (0.05,0.1,0.15 mg/ml solution), whereas one flask received 0.1 mg/ml trypsin. The remaining flask in both experiments was used as an untreated control. A 9 ml M9 minimal medium overnight culture was concurrently grown to stationary phase and 1 ml used to inoculate the flasks. Addition of the 1 mM H_2O_2 lethal dose was added after 60 minutes. Samples were taken as previously stated and the viable counts determined using the Miles and Misra plate count method described in chapter 3 paragraph 3.2.2.3.

4.2.2.3 Assay of butyryl homoserine lactone (BHL) biological activity.

a) BHL biological assay experiments in heat-treated spent medium or in M9 buffer with either presence or absence of chloramphenicol.

Assessment of the potential role of BHL to protect *A. hydrophila* against lethal H_2O_2 challenge was firstly performed in boiled spent medium with either presence or absence of 10 µg/ml chloramphenicol. A 9 ml M9 minimal medium overnight culture was grown to stationary phase and 1 ml was used to inoculate flasks containing 100 ml of treated spent medium recovered as previously described. At time 0 minute, the flasks received a selected amount of BHL (0.1,1,100 µg/ml) with or without 10 µg/ml chloramphenicol. 1 mM H_2O_2 was added after 60 minutes. At selected times, typically 0, 60, 120, 180, 240, 300 and 1440 minutes, the viable counts determined using the Miles and Misra plate count technique described in chapter 3 paragraph 3.2.2.3.

The above experiment was repeated with cells re-suspended in M9 buffer instead of spent medium following the same protocol.

b) Relative time scale protection of *A hydrophila* using 100 µg/ml BHL as sub-lethal dose.

A 9 ml M9 minimal medium overnight culture was grown to stationary phase and 1 ml used to inoculate flasks containing 100 ml of M9 minimal medium. 100 µg/ml BHL was applied at time 0 minute in all flasks, and 1 mM H_2O_2 at an increasing period of time, being 30, 60, 120, 180, 240 or 300 minutes. At selected times, typically 0 , 60, 120 and 180 minutes after addition of the lethal challenge, the viable counts were determined using the Miles and Misra plate count method.

c) Effect of growth phase and culture age upon *A. hydrophila* oxidative stress response.

Two pre-cultures were obtained by growing cells of *A. hydrophila* in M9 medium at 30°C for 24 h to stationary phase ($OD_{650\ nm}$ = 0.8). 4 ml of culture were removed from one of the overnights and used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase ($OD_{650\ nm}$ = 0.4). Experimental flasks used for the stationary phase study were seeded using 1 ml overnight stationary phase cells into 100 ml of fresh M9 buffer. Experimental flasks used for the logarithmic phase study were seeded using 1 ml logarithmic phase cells into 100 ml of fresh M9 buffer. 100 µg\ml of BHL was added at time 5 minutes. 1 mM H_2O_2 was added at time 60 minutes, the viable counts determined using the Miles and Misra plate count method.

4.3 Results.

4.3.1 Synthesis of N-butyryl-L-homoserine lactone.

The general reaction between α-amino-γ-butyrolactone hydrobromide and butyryl chloride is shown in Figure 4.2. The reaction follows a nucleophilic mechanism as illustrated in Figure 4.3.

Figure 4-2: General reaction between α-amino-γ-butyrolactone hydrobromide and butyryl chloride.
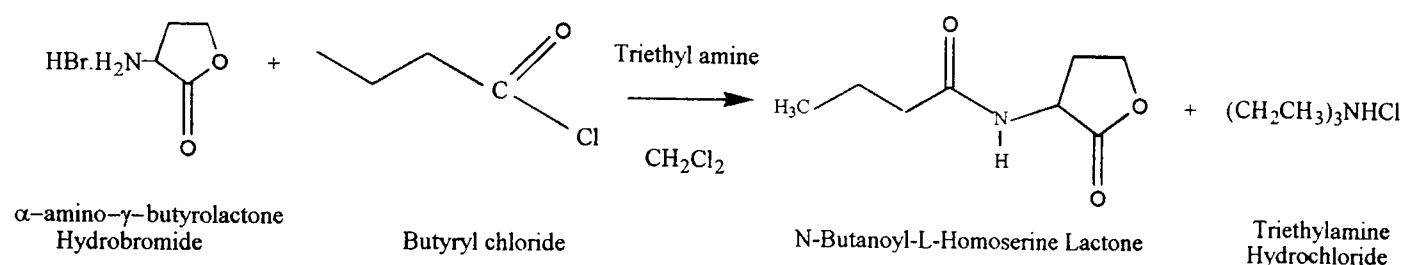
Figure 4-3: Reaction mechanism for the synthesis of N-butyryl-L-homoserine lactone.
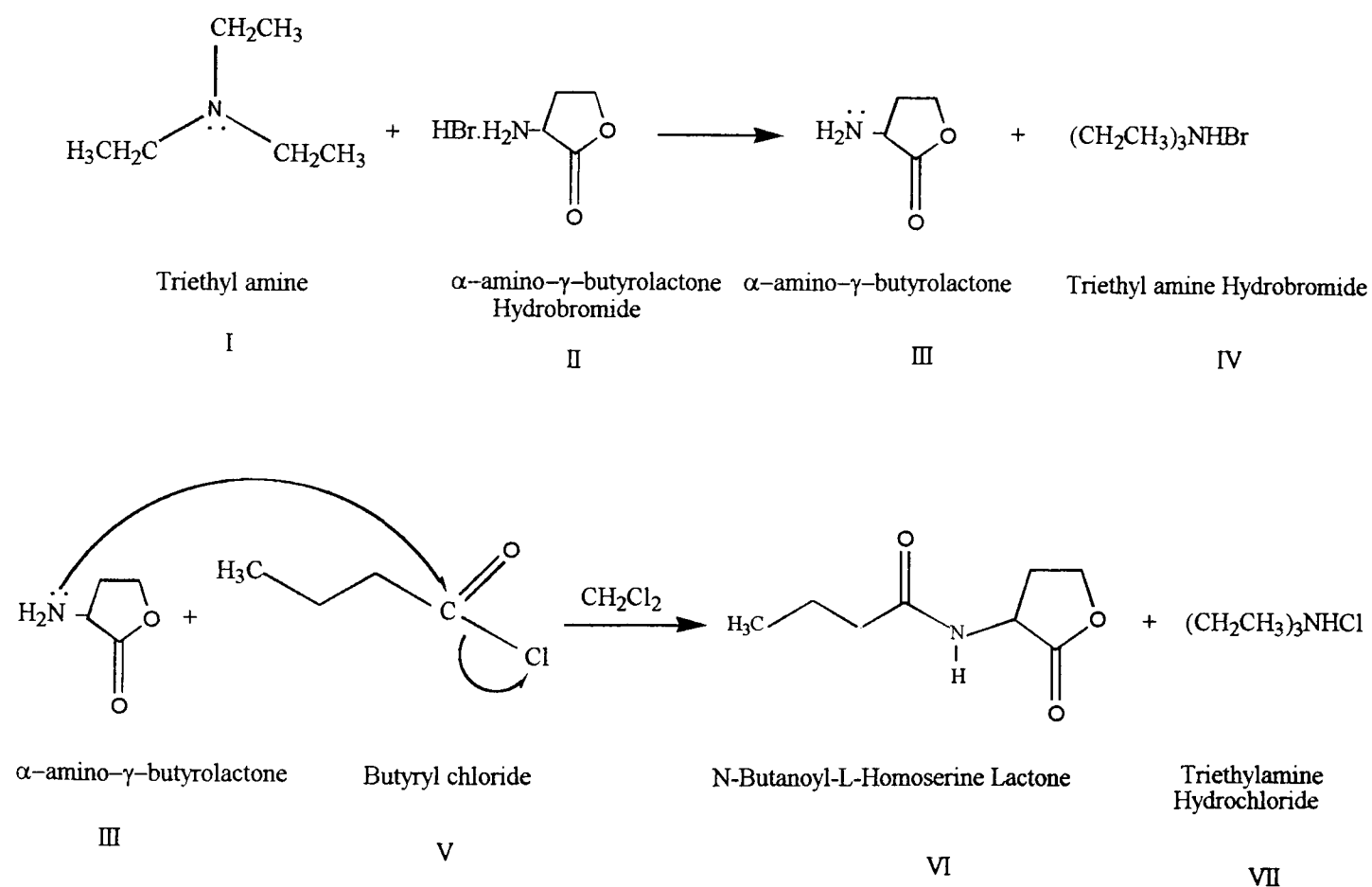
The first step of the reaction was the formation of the free α-amino- converting α- amino -γ-butyrolactone (III) from the α- amino-γ-butyrolactone hydrobromide salt (II) in presence of dichloromethane and triethylamine (I). This resulted in the formation of triethylamine

hydrobromide (IV) and the α-amino-γ-butyrolactone (III). The second step of the reaction was the nucleophilic substitution occurring between the amine of the α-amino-γ-butyrolactone (III) and butyryl hydrochloride (V) resulting in the formation of N-butyryl-L-homoserine lactone (VI) and triethylamine hydrochloride (VII), a by-product of the reaction. N-butyryl-L-homoserine lactone (VI) was purified by crystallisation from ethyl acetate. Confirmation of the identity of the final product after purification was performed by appropriate spectral analysis using IR, ^{1}H NMR, ^{13}C NMR, and MS spectra

4.3.1.1 IR results:

A sample for infra-red spectroscopy was prepared in nujol and bands at the following wave numbers were observed with the functionality given in parenthesis. A typical spectrum is presented in the appendix (Figure 4-4). All functional groups present in the compound BHL could be identified on the spectrum.

IR(nujol) v (cm^{-1}): 3400 (N-H), 1800 O $(C=O)$, 1640 NH $(C=O)$, 1550, 1270, 1220, 1170, 1010, 940.

4.3.1.2 ^{1}H NMR results

Peaks were identified using the reference spectra shown in the appendix (Figures 4-5 and 4-6). The ^{1}H NMR spectrum is shown in the appendix (Figure 4-7).

^{1}H NMR: δ = 6.66 (1H, NH), 4.13-4.68 (5H, lactone), 2.61-2.70 (1H, lactone), 2.02-2.38 (2H, lactone), 1.41-1.73 (2H, CH$_2$CO), 0.80-0.96 (3H,CH$_3$).

4.3.1.3 ^{13}C NMR results:

^{13}C NMR (CDCl$_3$): δ = 175.5 O $(C=O)$, 173.5 NH $(C=O)$, 65.7, 48.5 (tertiary C), 37.6, 29.1, 18.6 (3x CH$_2$), 13.3 (Alkyl C). The ^{13}C NMR spectrum is shown in the appendix (Figure 4-8).

4.3.1.4 GC-MS results:

Calculated ($C_8H_{13}NO_3$) 171.19. Found 171 [M^+].

The GC-MS spectrum is shown in the appendix (Figure 4-9).

All data gathered in the different spectra confirmed that the nature of the compound synthesised is the butanoyl derivative of homoserine lactone (i.e. BHL).

4.3.2 Characterisation of a non-proteinaceous, heat-sensitive effector molecule.

Experiments described in chapter 3 have shown that culture age and stage of growth of the bacterium are important parameters for *A. hydrophila* with regards to the stress response observed. Essentially, stationary phase cells exhibited a greater resistance than logarithmic phase cells to oxidative stress. It is known that when bacterial populations reach stationary phase, radical changes occur in the cell. Synthesis of primary metabolites necessary for cell growth and division are shut down to favour synthesis of secondary metabolites, which will prepare the cells for long-term starvation and also provide protection against a wide variety of stresses. Some of the secondary metabolites synthesised are excreted in the medium surrounding the bacterial community.

4.3.2.1 Effect upon the stress response of re-suspension of fresh cells into spent medium.

An investigation into the effect of resuspending fresh cells in spent medium was undertaken to determine whether any secreted molecules could act to facilitate development of a protective response against oxidative stress in non-pre-treated cells. Conditioned medium was prepared by growing *A. hydrophila* overnight in 100 ml M9 medium, with cells removed by centrifugation. 1 ml of a 9 ml *A. hydrophila* culture grown overnight was used to seed 100 ml of spent medium, giving a cell count of 1×10^7 per ml. Cells re-suspended in M9 buffer were shown to rapidly loose viability until no cells could

be recovered 120 minutes after 1 mM H_2O_2 challenge (Figures 4-10 a and b). This latter result is in agreement with those obtained in chapter 3. Significantly however, unlike results gained previously with M9 buffer where 1 mM H_2O_2 was shown to be a lethal challenge, cells re-suspended in spent medium displayed a significant increase in resistance to this level of oxidant (Figures 4-10 a, b). Cells re-suspended in spent medium showed a 2 log reduction in their viability 120 minutes after 1 mM H_2O_2 challenge. Subsequent to this 120 minutes period, cell viability increased until viability levels returned to the original level (Figure 4-10 b). The apparent resistance could be due to two factors. Either the direct action of antioxidants where antioxidant molecules would inactivate H_2O_2, or by a signal transduction process via a molecule present in the spent medium which enter re-suspended cells to activate antioxidant stress resistance.

In order to determine which of these was occuring, the same experiment was undertaken but with H_2O_2 addition at two different times. 1 mM H_2O_2 challenge was added either 1 minute or 120 minutes after re-suspension of cells (Figure 4-11). Data obtained showed that addition of 1 mM H_2O_2 challenge after 1 minute lead to an identical profile as the one obtained in Figure 4-10, where addition was performed after 60 minutes. This suggests that the response is not time-dependent. However, inspection of the data obtained for a 120 minutes addition reveals a rather different viability profile as cells incubated for 120 minutes prior to addition of lethal challenge showed no significant reduction in viability. Taken together these data showed that an increase in incubation time from 1 to 60 minutes does not afford the cells better resistance to the oxidant onslaught, but longer incubation-times (i.e. 120 minutes) appeared to be more effective in mediating a stronger resistance to

Figure 4-10 a (300 minutes plot)

Effect of re-suspension of *A. hydrophila* in spent medium upon the bacterium response to 1 mM H_2O_2.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. 1 ml of a fresh overnight culture was used to seed the experimental flasks. No addition control (□), cells re-suspended in spent medium (○) and cells re-suspended in M9 minimal medium(◇). Addition of 1 mM H_2O_2 was performed in the flasks at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the CFU (Colony forming unit) was plotted against time.

Figure 4-10 b (1440 minutes plot)

Effect of re-suspension of *A. hydrophila* in spent medium upon the bacterium response to 1 mM H_2O_2.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. 1 ml of a fresh overnight culture was used to seed the experimental flasks. No addition control (□), cells re-suspended in spent medium (⊃) and cells re-suspended in M9 minimal medium(◇). Addition of 1 mM H_2O_2 was performed in the flasks at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the CFU (Colony forming unit) was plotted against time.

Figure 4-11

Effect of re-suspension of *A. hydrophila* in spent medium upon the bacterium response to 1 mM H₂O₂.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. 1 ml of a fresh overnight culture was used to seed the experimental flasks. Growth control received no addition (□). Addition of 1 mM H_2O_2 was performed at time 1 minute (◇) or 120 minutes (○). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

the noxious agent. The first 60 minutes after challenge have previoulsy been reported as being crucial for fresh key stress protein synthesis (Nyström, 1993), however no difference in resistance pattern were observed between the 1 and a 60 minutes pre-treatment. It may therefore be possible that the resistance observed could be due to antioxidant molecules already present in the medium. It is tempting to speculate that during short incubation times degradation or complexation of H_2O_2 might be performed by extra-cellular components secreted by the cells previously grown in the conditioned medium (e.g. antioxidants) and that longer incubation times might allow further protection by the action of a signal molecule mediating fresh protein synthesis. However, more data need to be generated in order to test these hypothesis.

4.3.2.2 Effect of re-suspension of fresh cells into spent medium treated with 10 μg/ml chloramphenicol.

Degradation or complexation of H_2O_2 performed by extra-cellular components secreted by cells previously grown in conditioned medium was corroborated by the following experiment. 1 ml of cells grown overnight in 9 ml of M9 medium were re-suspended into 100 ml of spent medium, with 4 flasks prepared in this way. 10 μg/ml of chloramphenicol was added to one of the flasks at time 0 minute. Lethal challenge was applied after a 1 minute or 120 minutes incubation period. From Figure 4-12 it can be seen that both control and chloramphenicol treated flasks, which received the lethal addition after 1 minute, displayed identical profiles to those from previous experiment. With regards to the sample which received the 1 mM H_2O_2 challenge after 120 minutes, no significant loss of viability was observed. Bacteria remained viable even after 24 hours (Figure 4-12).

Figure 4-12

Effect of chloramphenicol (10 μg/ml) on the adaptation response of *A. hydrophila*.
Cells were grown overnight in liquid minimal medium (M9). Before any prior addition of H_2O_2, the cells were treated with 10 μg/ml chloramphenicol. Chloramphenicol was made up in ethanol. Ethanol (100 μl) addition to non-treated cells (□). Chloramphenicol addition to non-treated cells (△). Addition of 1 mM H_2O_2 was performed at time 1 minute (◇) or 120 minutes (○). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

4.3.2.3 Effect of exposure of fresh cells to spent medium and re-suspension in M9 buffer.

To confirm whether this resistance was solely due to degradation of H_2O_2 another experiment was undertaken. Spent medium was recovered by centrifugation, and 1 ml of cells grown overnight added, and the suspension subsequently incubated for 120 minutes. The cells contained in one of the flasks were then harvested by centrifugation followed by re-suspension in 100 ml of fresh M9 buffer. Oxidative challenge was added just after re-suspension of the cells. Results obtained showed that cells treated in this way withstand the lethal challenge (Figure 4-13).

The fact that cells incubated for 120 minutes in spent medium and then re-suspended in M9 buffer remained viable when in presence of the lethal challenge corroborates the hypothesis where a signal molecule might mediate synthesis of stress proteins. This 120 minute period of time appears to be sufficient to mediate an oxidative stress response in cells which have not been pre-treated with sub-lethal H_2O_2.

4.3.2.4 Effect of re-suspension of fresh cells into heat or enzymatically treated spent medium.

In bacteria, two component response regulators are commonly encountered. In order for signal transduction to occur, a signal molecule would be required to activate the response regulator. It was sought to further characterise the basic biochemical properties of the unpurified signal element in spent medium. Firstly, the thermal properties of the molecule were investigated. Loss of activity as a result of extensive heat treatment would suggest the effector to be proteinaceous in nature. Upon boiling of spent medium for 10 minutes this led to inactivation of stress tolerance response (Figures 4-14 a and b). Further it was shown that loss of viability was not time-dependent as both samples receiving lethal addition at 60

Figure 4-13
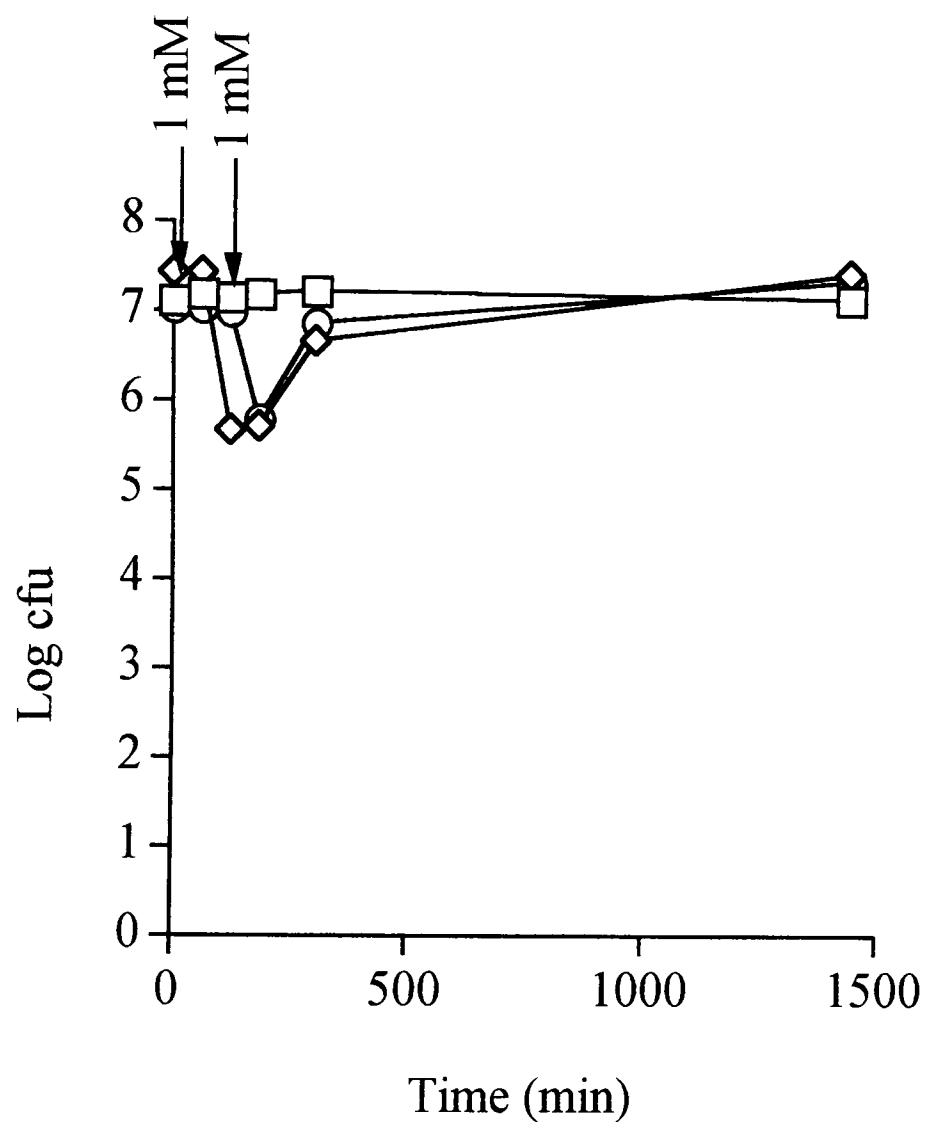
Effect of pre-treatment of *A. hydrophila* with spent medium prior to 1 mM H₂O₂ challenge.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. 1 ml of a fresh overnight culture was used to seed the experimental flasks. Growth control received no addition (□). Cells were incubated for 2 hours in spent medium, harvested by centrifugation and resupsended in 100 ml M9 buffer and challenged with 1 mM H₂O₂ (O). 1 ml of an overnight culture was used to seed 100 ml of spent medium and challenged with 1 mM H₂O₂ 1 minute after seeding (◇). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.
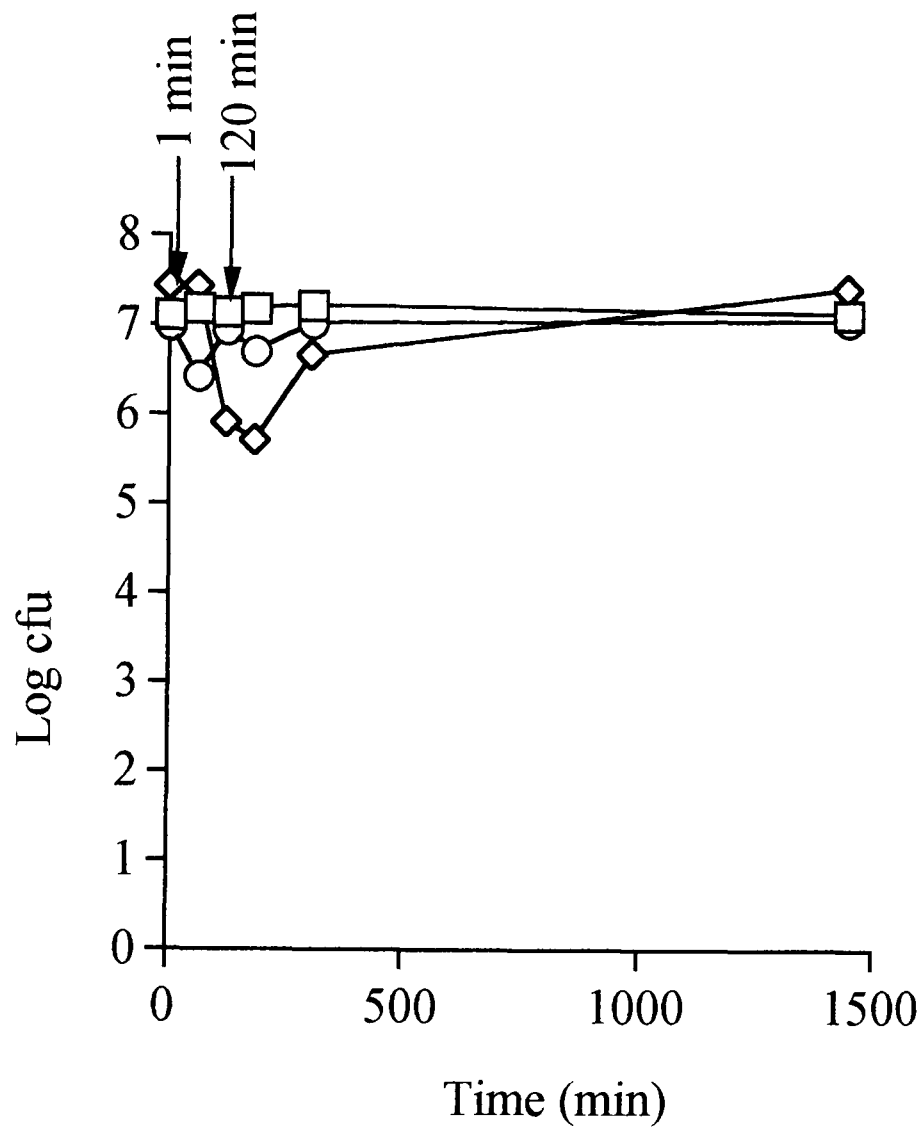
Figure 4-14 a (300 minutes plot)

Effect of heat treatment of the spent medium upon *A. hydrophila* adaptation response to 1 mM H_2O_2 challenge.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. Spent medium was heat treated for 10 minutes,and cooled down to room temperature. 1 ml of a fresh overnight culture was used to seed the experimental flasks. Boiled spent medium control (□), cells re-suspended in boiled spent medium and challenged at time 60 (◇), or 120 minutes (○). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-14 b (1440 minutes plot)

Effect of heat treatment of the spent medium upon *A. hydrophila* adaptation response to 1 mM H₂O₂ challenge.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. Spent medium was heat treated for 10 minutes,and cooled down to room temperature. 1 ml of a fresh overnight culture was used to seed the experimental flasks. Boiled spent medium control (□), cells re-suspended in boiled spent medium and challenged at time 60 (◇), or 120 minutes (○). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

or 120 minutes displayed identical viability reduction profiles. Both samples showed no viable cell counts 120 minutes after lethal addition. This confirmed that the agent mediating the stress response is thermolabile.

Due to the thermal instability observed, it was hypothesised that this molecule might be of proteinaceous nature. However, this was ruled out through a set of experiments where two proteases displaying differing catalytic specificity were assayed. Trypsin recognises positively charged lysine or arginine residues present on the substrate with optimal activity between pH 7.0 to 8.0. By contrast, papain preferentially cleaves peptide bonds between lysine and arginine residues like trypsin, but also the carboxyl side group of residues whose NH_2 group is linked to phenylalanine. Another unconventional property of papain is that the enzyme activity is pH independent between pH 4.0 to 8.0. Using enzymes with different activities ensured that cleavage of the effector molecule would occur if it was proteinous. No loss of viability was observed even at protease concentrations as high as 0.15 mg/ml papain or 0.1 mg/ml trypsin. (Figure 4-15 and 4.16). These results strongly suggest that the effector molecule mediating stress response in *A. hydrophila* is not of proteinaceous nature as pH of the spent medium measured during the experiment was 6.8 suggesting that trypsin (optimal pH 7.0-8.0) might have been inactivated, but not papain, known to have a pH-independent activity in the range pH 4.0-8.0. It was therefore hypothesised that mediation of the oxidative stress response in *A. hydrophila* was due to a non-proteinaceous, heat-sensitive molecule.

It was previously conjectured that two co-operative activities contributed to H_2O_2 resistance. Data obtained would suggest these to be antioxidant scavenging and non-proteinaceous heat-sensitive signal transduction molecules. The nature of the first protective element was attributed to antioxidants scavenging the oxidising molecules. The second pathway

Figure 4-15

Effect of papain treatment of the spent medium upon _A. hydrophila_ adaptation response to 1 mM H₂O₂ challenge.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. Spent medium received no enzymatic treatment (□), boiled followed by addition of 1 mM H_2O_2 (◇), boiling of spent medium, 0.05 mg/ml papain (○), 0.10 mg/ml papain (△), and 0.15 mg/ml papain (■). 1 ml of a fresh overnight culture was used to seed the experimental flasks. Addition of 1 mM H_2O_2 was performed at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-16
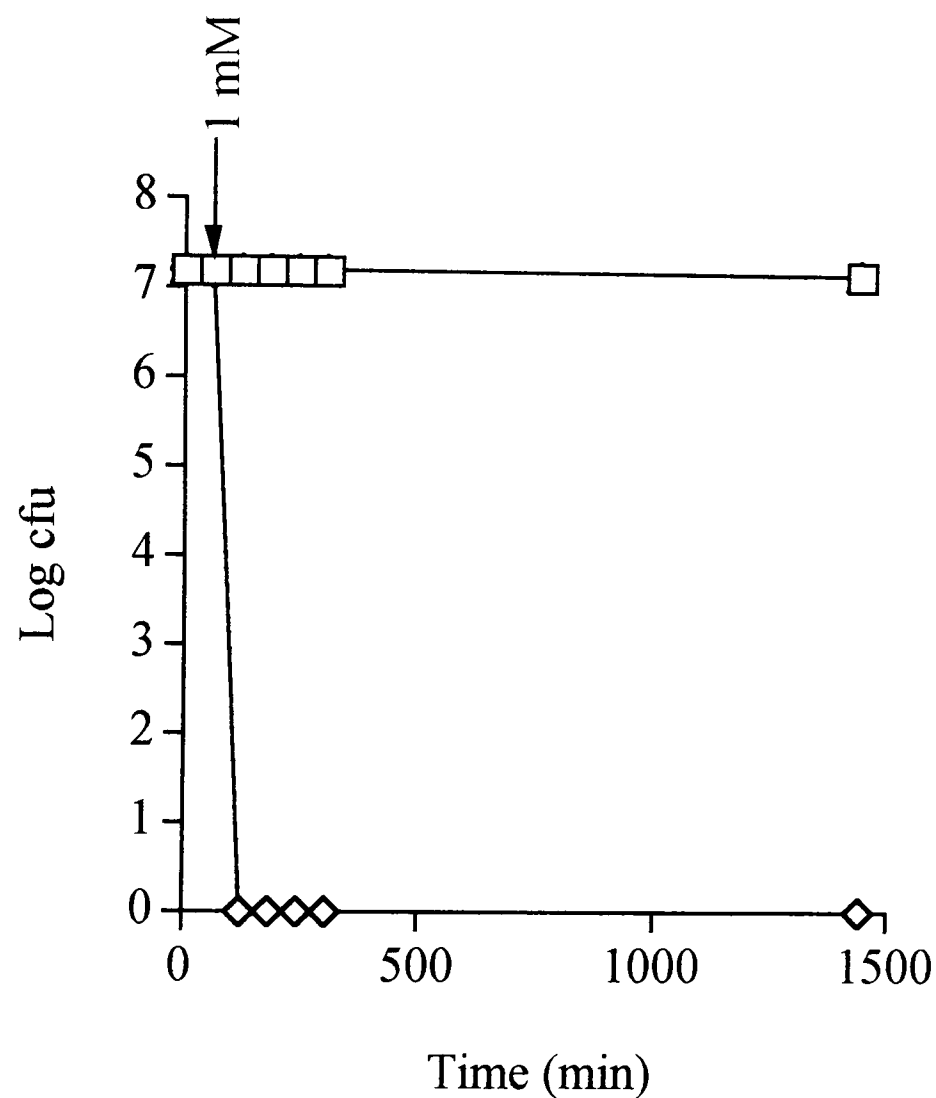
Effect of trypsin treatment of the spent medium upon *A. hydrophila* adaptation response to 1 mM H₂O₂ challenge.

Cells were grown in liquid minimal medium (100 ml M9). Cells were removed by centrifugation. Spent medium received no enzymatic treatment (□), boiling of spent medium (◇), 0.10 mg/ml trypsin (○). Addition of 0.10 mg/ml trypsin (△). 1 ml of a fresh overnight culture was used to seed the experimental flasks. Addition of 1 mM H₂O₂ was performed in the suitable flasks at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

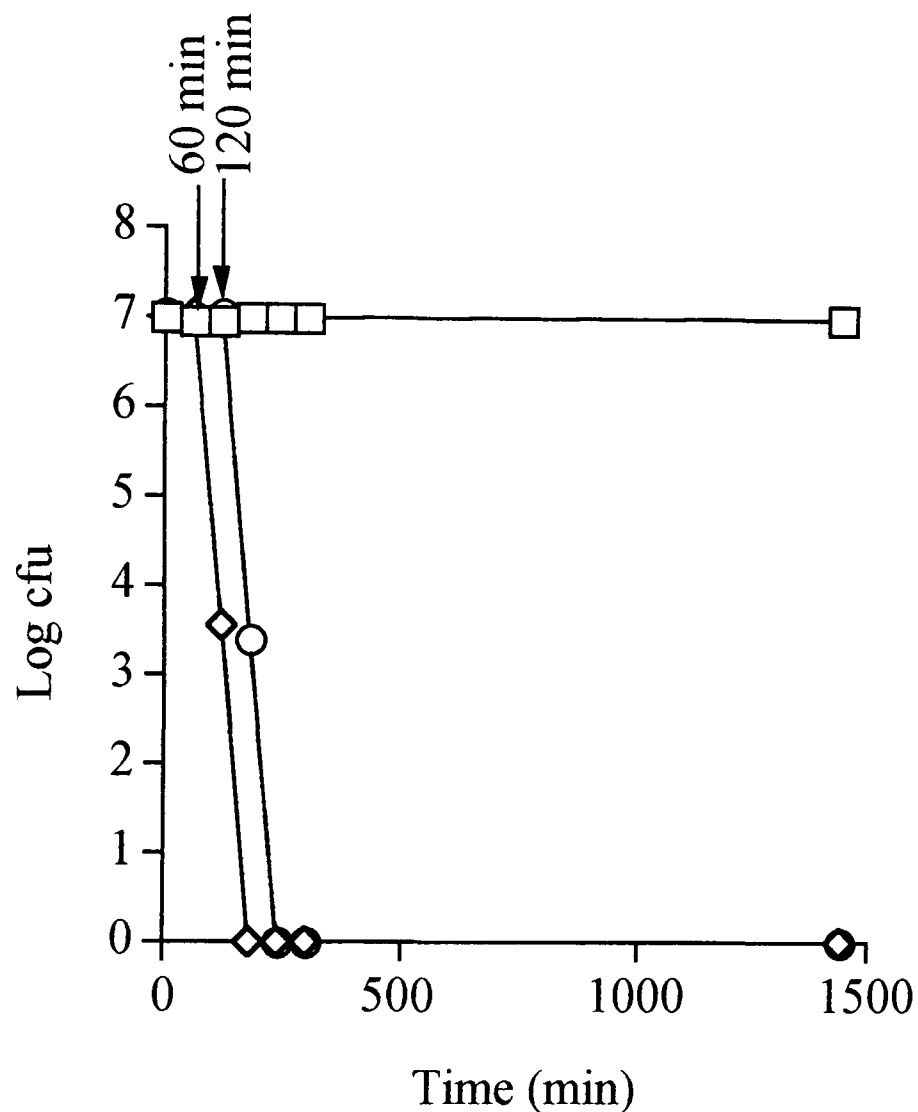involves heat-sensitive molecules of non proteinaceous nature which could play a role in the genetic regulation of oxidative stress regulons. Results obtained so far present some evidence towards this assumption, nevertheless, much work remains to be done to determine the validity of this hypothesis.

4.3.3 Investigation of the potential role of BHL in mediating the oxidative stress response in *A. hydrophila*.

4.3.3.1 Effect of addition of BHL in heat treated spent medium.

Recent discovery of the bacterial hormone BHL in *A. hydrophila* (Swift *et al.*, 1997) and the fact that regulation of catalase activity has been reported as being cell-density-dependent (Crockford, *et al.*, 1995) suggested that this molecule might play a role in the stress response of *A. hydrophila*. BHL was therefore synthesised and used in further experiments to assay any potential role in the mediation of the stress response of *A. hydrophila*. As shown previously, cells re-suspended in heat treated spent medium and facing a 1 mM H_2O_2 challenge were shown to rapidly lose viability (Figures 4-10 a and b). However addition of BHL (0.1, 1 or 100 µg/ml) prior to this lethal dose partially restored *A. hydrophila* adaptive tolerance response to the oxidative agent (Figure 4-17 a and b). Closer inspection of this data showed that the higher the BHL concentration, the more resistant the cells become. It appears that this oxidative stress tolerance response brought about by BHL is concentration dependent. In a normal physiological setting the concentration of BHL is dependent upon the cell population and it can be inferred that oxidative stress response is partly cell density-dependent. However in these experiments, concentrations of BHL greater than those biologically encountered were required to display the greatest protection.

Figure 4-17 a (300 minutes plot)

Adaptative response of _A. hydrophila_ to 1 mM H₂O₂ using different concentration of BHL as pre-adaptation dose in treated spent medium.
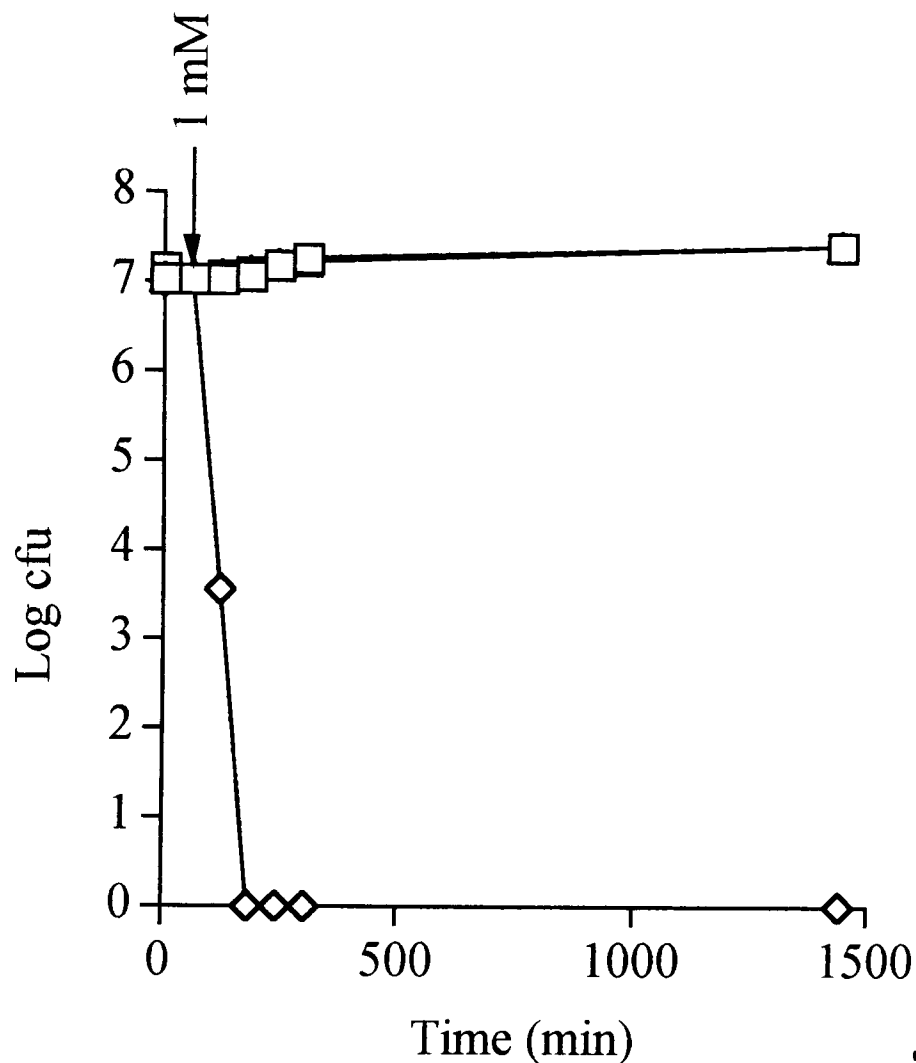
Cells were grown in liquid minimal medium (100 ml M9) and removed by centrifugation. Spent medium was heat-treated for 10 minutes and cooled down to room temperature. 1 ml of overnight culture was used to seed the experimental flasks. Boiled spent medium control (□). Cells re-suspended in boiled spent medium and challenged with 1 mM H₂O₂ at time 60 minutes (◇). Cells re-suspended in boiled spent medium, pre-treated with 100 μg/ml (○), 1 μg/ml BHL (Δ), or 0.1 μg/ml BHL (■) and challenged with 1 mM H₂O₂ at time 60 minutes. At appropriate times, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-17 b (1440 minutes plot)

Adaptative response of *A. hydrophila* to 1 mM H₂O₂ using different concentration of BHL as pre-adaptation dose in treated spent medium.

Cells were grown in liquid minimal medium (100 ml M9) and removed by centrifugation. Spent medium was heat-treated for 10 minutes and cooled down to room temperature. 1 ml of overnight culture was used to seed the experimental flasks. Boiled spent medium control (□). Cells re-suspended in boiled spent medium and challenged with 1 mM H₂O₂ at time 60 minutes (◇). Cells re-suspended in boiled spent medium, pre-treated with 100 μg/ml (○), 1 μg/ml BHL (△), or 0.1 μg/ml BHL (■) and challenged with 1 mM H₂O₂ at time 60 minutes. At appropriate times, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

4.3.3.2 Effect of chloramphenicol upon the adaptive response of *A. hydrophila* mediated by BHL in boiled spent medium.

In order to gain further insight into the mode of action of BHL upon the stress response of *A. hydrophila*, the latter experiment was repeated in the presence of chloramphenicol (Figures 4-18 a and b). Cells present in both the growth and chloramphenicol controls remained viable all through the experiment, showing no major lethal effect from either the spent medium or chloramphenicol during the span of the experiment. Cells in the presence of chloramphenicol subjected to a 1 mM H_2O_2 lethal challenge, lost this BHL mediation of the oxidative stress response. This would suggest that BHL plays a role in the activation of synthesis of proteins involved in the protection to oxidative stress.

4.3.3.3 Effect of addition of BHL in M9 buffer in presence or absence of chloramphenicol.

Similar experiments as the ones performed in spent medium were repeated using M9 buffer as the re-suspension medium. Figure 4-19 showed that 1 and 100 μg/ml BHL mediated a partial oxidative tolerance response to the H_2O_2 lethal challenge as cells pre-treated witht these concentrations remain viable 120 to 180 minutes respectively after addition of the lethal oxidative challenge. However, high non-physiological concentrations were required for a marked resistance to become apparent in the cells under stress, suggesting that BHL is not sufficient on its own to bring full protection to the cells. Addition of chloramphenicol in the assay abolished BHL-mediated stress response (Figure 4-20). Data obtained during assay of BHL would suggest that this lactone derivative might play a role in what was described earlier as the second pathway involving a secreted effector as the signal transduction molecule for mediation of the stress response. Further experimentation strengthen this hypothesis as follows. When 100 μg/ml of BHL was added

Figure 4-18 a (300 minutes plot)

Axis labels: y-axis "Log cfu" (0 to 8), x-axis "Time (min)" (0 to 300), arrow labeled "1 mM"

Effect of chloramphenicol (10 µg/ml) on the BHL mediated H_2O_2 adaptation response of *A. hydrophila*.

Cells were grown overnight in liquid minimal medium (M9). Before any prior addition of H_2O_2, the cells were treated with 10 µg/ml chloramphenicol. Ethanol (100 µl) addition to non-treated cells (□). Chloramphenicol addition to non treated cells (◇).Cells re-suspended in M9 buffer, pre-treated with 100 µg/ml (■), 1 µg/ml BHL (O), or 0.1 µg/ml BHL (Δ) and challenged with 1 mM H_2O_2 at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.
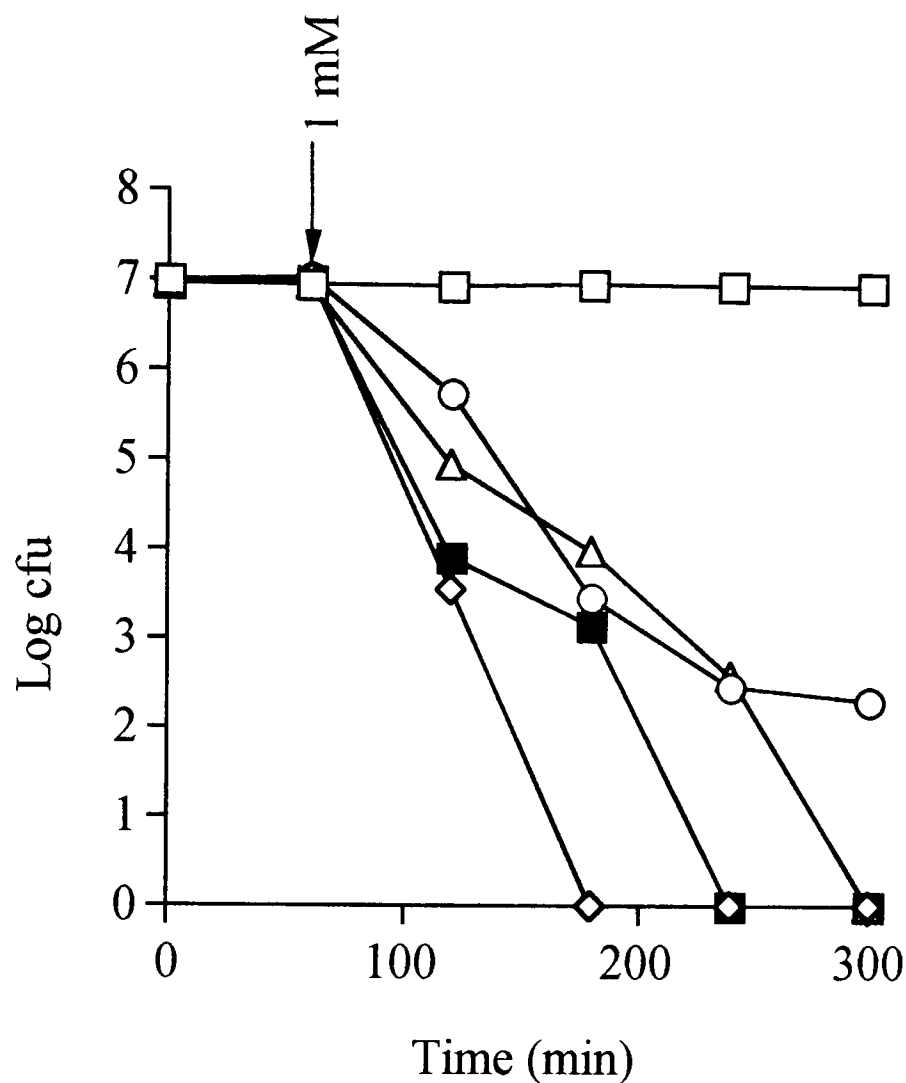
Figure 4-18 b (1440 minutes plot)

Effect of chloramphenicol (10 μg/ml) on the BHL mediated H_2O_2 adaptation response of *A. hydrophila*.

Cells were grown overnight in liquid minimal medium (M9). Before any prior addition of H_2O_2, the cells were treated with 10 μg/ml chloramphenicol. Ethanol (100 μl) addition to non-treated cells (□). Chloramphenicol addition to non treated cells (◇).Cells re-suspended in M9 buffer, pre-treated with 100 μg/ml (■), 1 μg/ml BHL (○), or 0.1 μg/ml BHL (△) and challenged with 1 mM H_2O_2 at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.
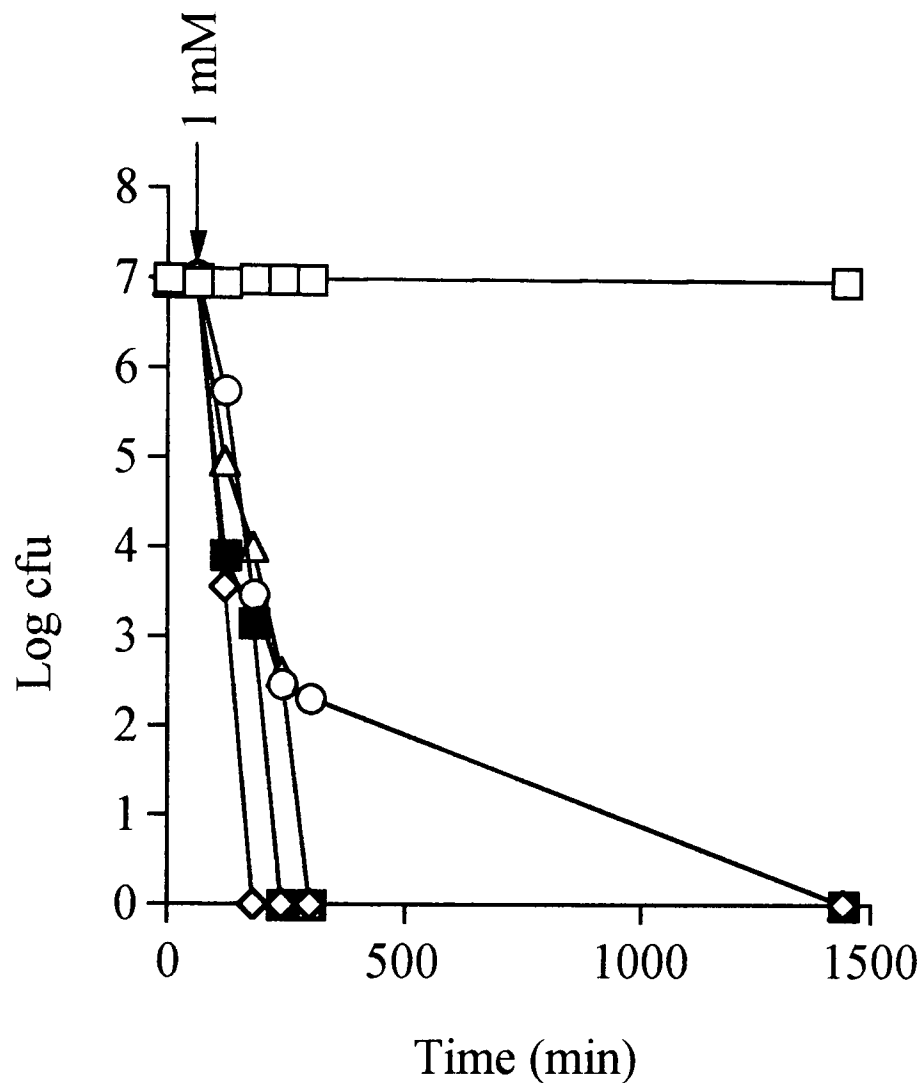
Figure 4-19
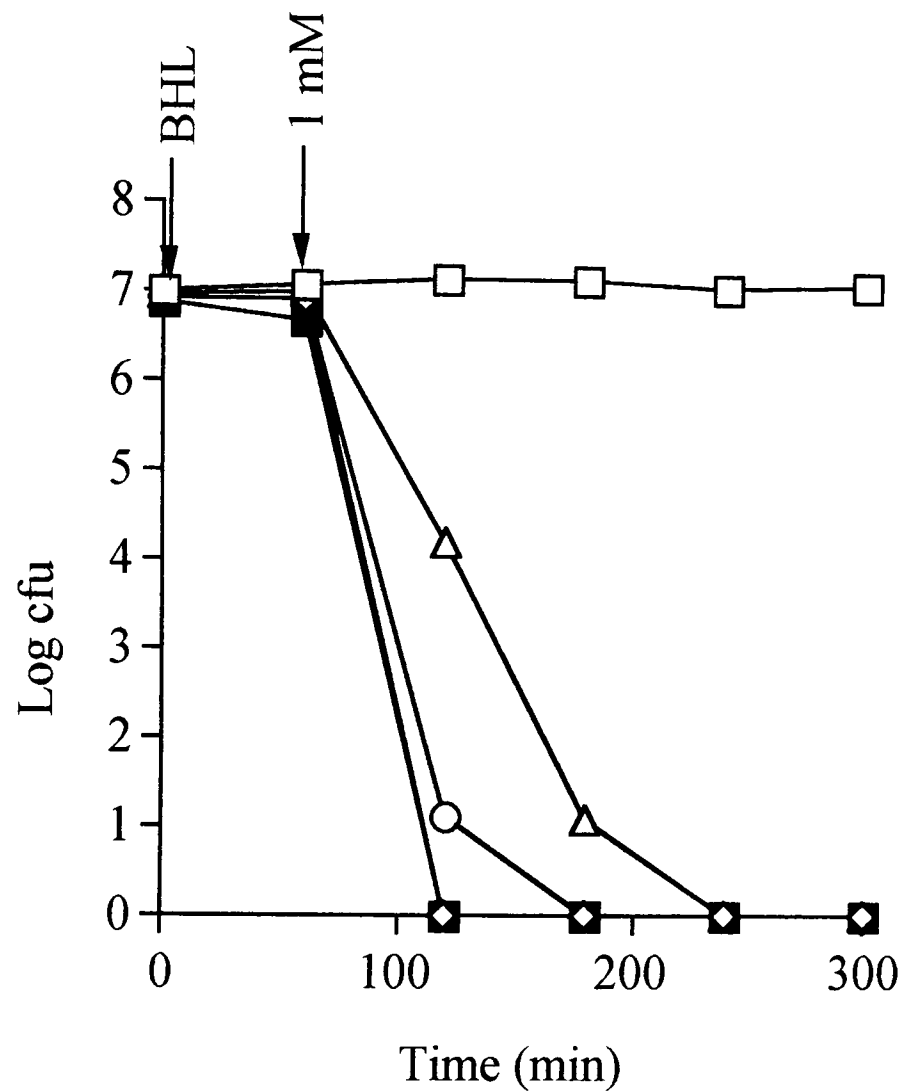
Adaptative response of *A. hydrophila* to 1 mM H₂O₂ using different concentrations of BHL as pre-adaptation dose in M9 buffer.

Cells were grown in liquid minimal medium (100 ml M9) and removed by centrifugation. Spent medium was heat-treated for 10 minutes, cooled down to room temperature. Boiled spent medium control seeded with 1 ml of overnight culture (□). 1 ml of overnight culture was used to seed flasks containing 100 ml M9 buffer, pre-treated with 100 μg/ml BHL(◇), 1 μg/ml BHL (○), or 0.1 μg/ml BHL (△), and challenged with 1 mM H₂O₂ at time 60 minutes. 100 ml M9 buffer seeded with 1 ml of overnight culture and challenged with 1 mM H₂O₂ at time 60 minutes (■). At appropriate times, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-20
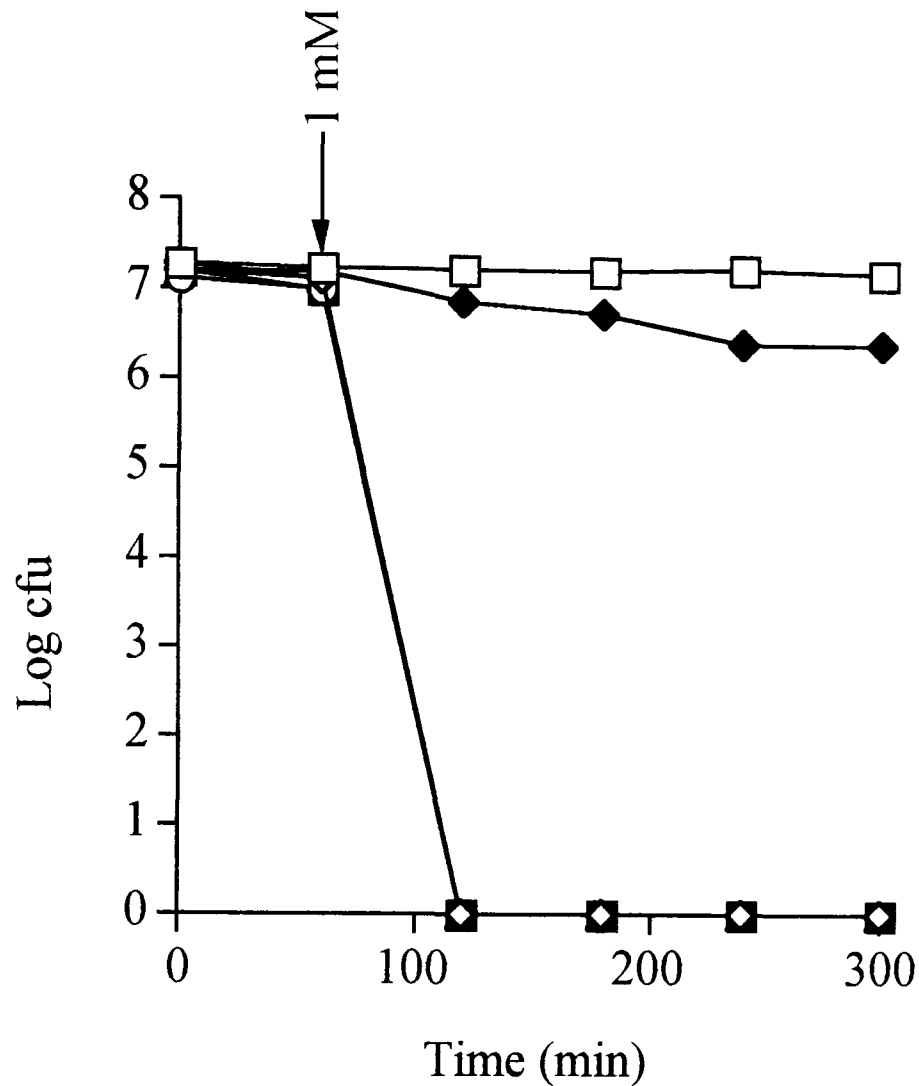
Effect of 10 μg/ml chloramphenicol upon the adaptative response of *A. hydrophila* to 1 mM H_2O_2 using different concentrations of BHL as pre-adaptation dose in M9 buffer. Cells were grown in liquid minimal medium (100 ml M9) and removed by centrifugation. Spent medium was heat-treated for 10 minutes, cooled down to room temperature. Chloramphenicol was made up in ethanol. Ethanol (100 μl) addition on non treated cells (□). Chloramphenicol control (◆) 1 ml of overnight culture was used to seed flasks containing 100 ml M9 buffer, pre-treated with 100 μg/ml (◇), 1 μg/ml BHL (○), or 0.1 μg/ml BHL (△), and challenged with 1 mM H_2O_2 at time 60 minutes. 100 ml M9 buffer seeded with 1 ml of overnight culture and challenged with 1 mM H_2O_2 at time 60 minutes (■). At appropriate times, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.
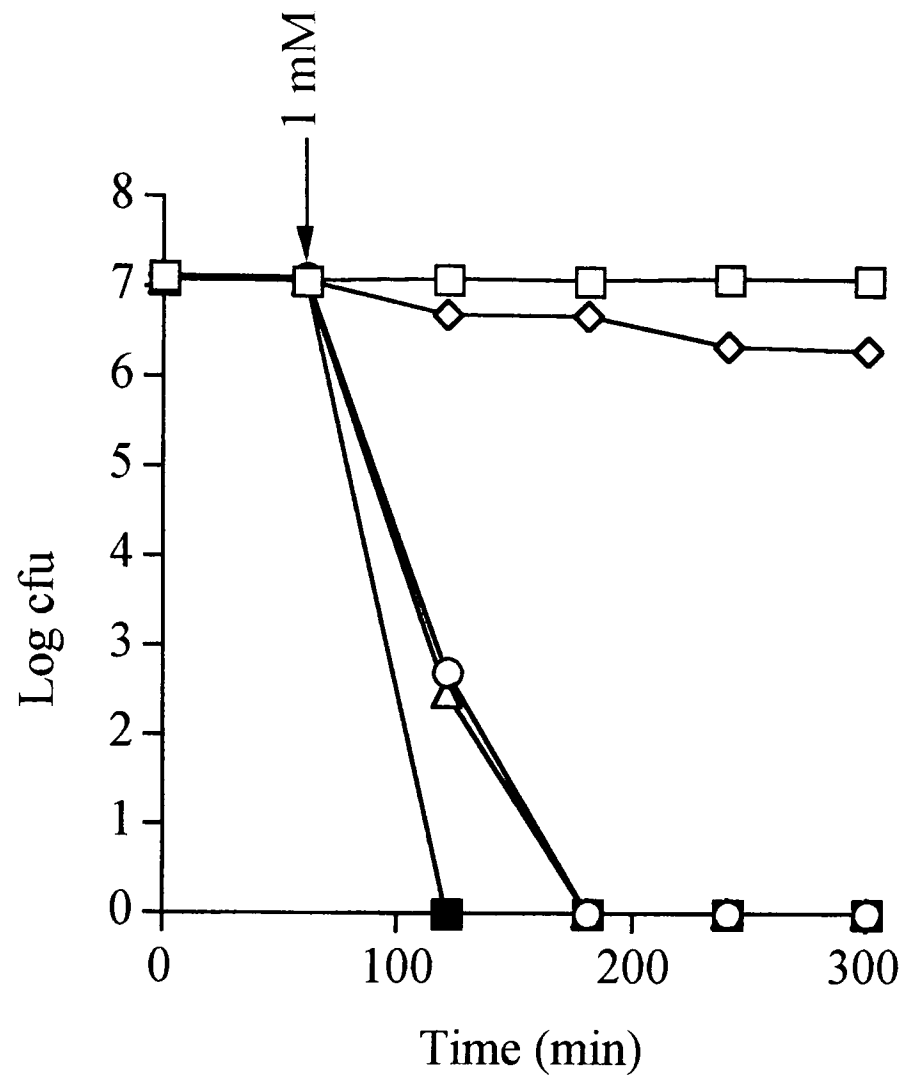
to 100 ml of M9 buffer and this subsequently boiled, cooled at room temperature and seeded with 1 ml of overnight grown cells and challenged with 1 mM H_2O_2, then rapid loss of *A. hydrophila* viability was observed (Fig 4-21).

No viable cells could be recovered 60 minutes after addition. The fact that heat treatment abolished *A. hydrophila* oxidative stress response demonstrates that BHL is thermolabile. As shown previously, boiling of spent medium abolished the protective response to oxidative stress.

It seems reasonable to envisage that BHL might be involved in the second protective pathway.

4.3.3.4 Relative time scale protection of *A. hydrophila* using 100 µg/ml BHL as pre-treatment dose.

Accepting that BHL mediates oxidative stress response in *A. hydrophila*, it was of interest to investigate the relative time scale for development of protection that this homoserine lactone (HSL) derivative stimulates. The sub-lethal dose of 50 µM H_2O_2 was replaced by the addition of 100 µg/ml BHL which was shown to mediate a certain degree of protection (Figure 4-17 a). BHL was added at the same time to all the experimental flasks with 1 mM H_2O_2 challenge, added at a set increase of time between the different flasks. Data obtained during this investigation are shown in Figure 4-22. These results indicates a time-dependency with regards to the degree of cellular adaptation to H_2O_2, correlating with results obtained in chapter 3 (Figure 3-6). Two trends seemed to emerge (Figure 4-22). In the first 60 minutes of H_2O_2 challenge, 120 minutes incubation time with BHL appears to be the optimal time for adaptation to occur. However, the second trend (i.e. 60 minutes after challenge) is rather different. Indeed, 60 minutes after challenge, the longer the period between exposure to BHL and lethal challenge does not produce stronger protection (Figure 4-22). It can also been noticed that the impressive initial protection (initial 60

Figure 4-21
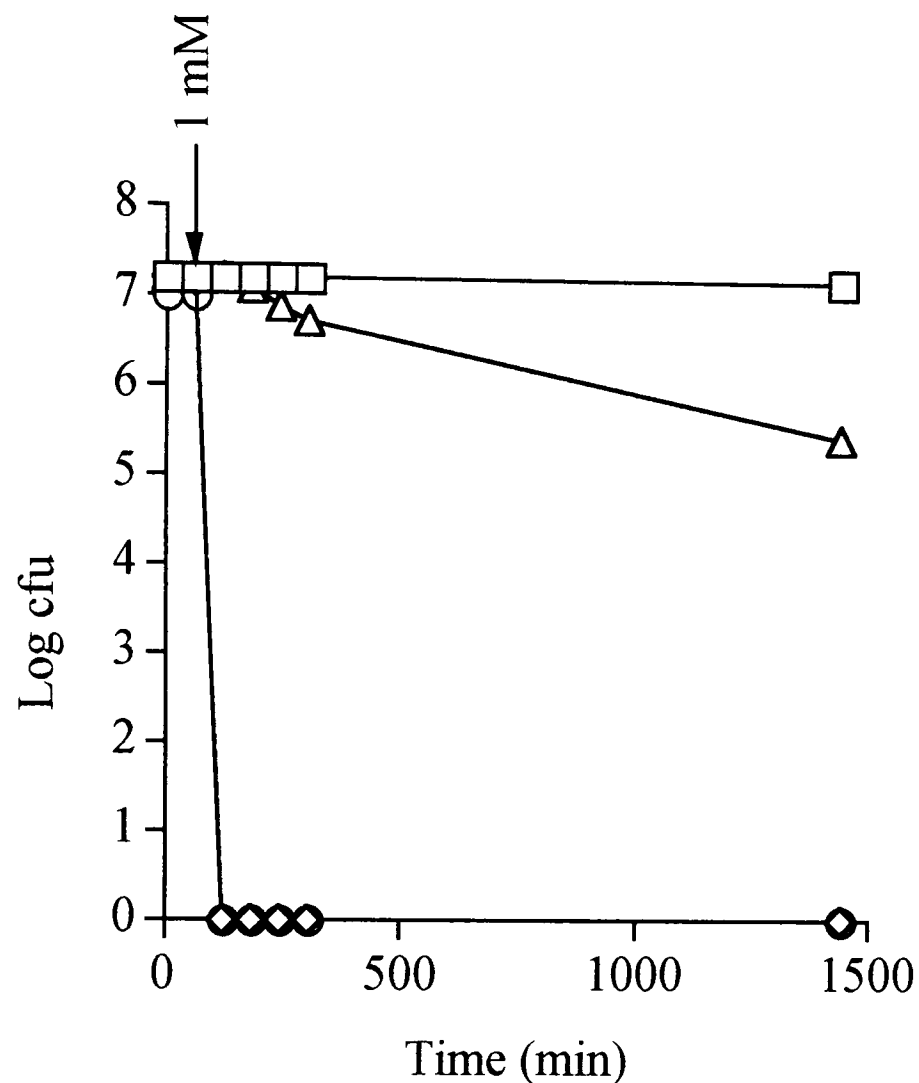
Effect of heat-treatment on BHL mediation of stress tolerance response in *A. hydrophila* in minimal medium.

Cells were grown overnight in liquid minimal medium (M9). Before any prior addition of H_2O_2, the cells were treated with 10 μg/ml chloramphenicol. Chloramphenicol was made up in ethanol. Ethanol (100 μl) addition on non treated cells (□). Chloramphenicol addition on non treated cells (△). Cells were re-suspended in boiled spent medium (◇) or M9 buffer pre-treated with 100 μg/ml then boiled (○), and challenged with 1 mM H_2O_2 at time 60 minutes. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-22
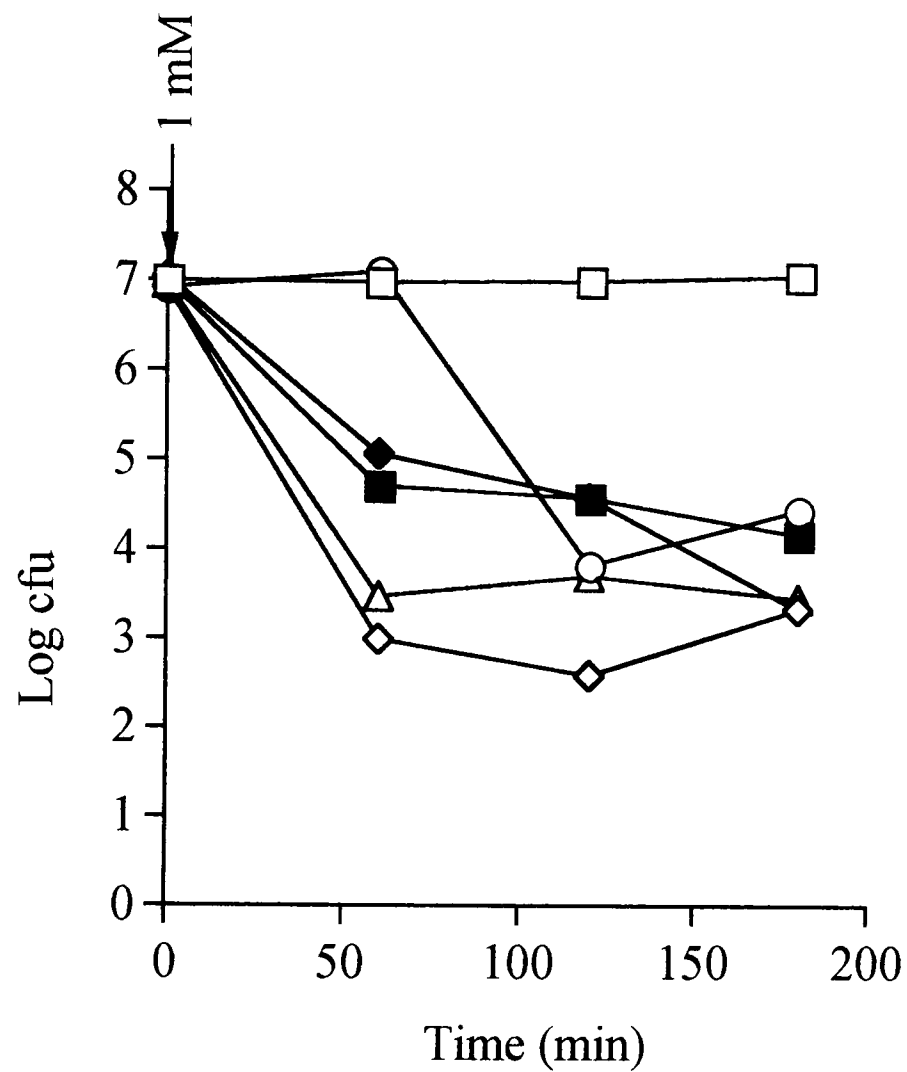
Relative time scale for protection of *A. hydrophila* to be acquired through adaptation to 100 μg/ml BHL prior to 1 mM H₂O₂ challenge.

Cells were resuspended in liquid minimal buffer (M9). The first addition (100 μg/ml BHL) was performed at time 0 in all flasks. No addition of H_2O_2 is shown as a control (□). 1 mM H_2O_2 was added at time: 30 (◇), 60 (△), 120 (○), 180 (■), 240 (◆). At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

minutes of the assay) afforded to the cells in contact with 100 μg/ml BHL for 120 minutes prior to lethal challenge with 1 mM H_2O_2 appears to be ineffective for protecting the cells with the same efficiency in the latter stages of the experiment (60 minutes atfer the start of the assay). Between 60 and 120 minutes after addition of the lethal challenge, the viability counts drop 3 log compared to the initial 60 minutes period of the experiment to reach the same protection level displayed by the other samples.

4.3.3.5 Effect of growth phase and culture age upon *A. hydrophila* oxidative stress response mediated by BHL.

As demonstrated in chapter 3, the oxidative stress response of *A. hydrophila* can be affected by factors such as the growth phase and/or the length of storage on solid medium of the test organism (Figures 3-12, 3-13 and 3-14). Test organisms were grown on NA plates for 3 days, 2 weeks and 12 weeks. Two cultures were grown for each of the different plates, one to stationary phase ($OD_{650\,nm} = 0.8$), the other one to mid-log phase ($OD_{650\,nm} = 0.4$), and these used in an adaptation experiment where pre-treatment was addition of 100 μg/ml BHL and the lethal dose was 1 mM H_2O_2. In order to compare results obtained for logarithmic and stationary phase cells, optical density of both cultures were measured to ensure equivalent amount of logarithmic or stationary phase bacteria were added. Experiments were designed to study both the effect of culture age and BHL at the same time, to ease comparison between them, but also to obtain a broader picture where both parameters act in concert. Both logarithmic and stationary phase controls were almost identical in all the three set of experiments performed, allowing comparison between the experiments (Figures 4-23, 4-24 and 4-25).

1 mM H_2O_2 was proven to be lethal for non BHL pre-treated cells in either logarithmic or stationary phase stored 3 days on NA (Figure 4-23 a and b). Non pre-treated cells in this series of experiments, where BHL was assayed, displayed identical results to those

Figure 4-23 a (300 minutes plot)

Time (min)

Effect of growth phase and age upon BHL mediated adaptation of _A. hydrophila_ (3 days old) to oxidative challenge.

Cells were grown overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights were used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary (□) and logarithmic (Δ) control. 100 µg/ml BHL addition was performed at time 0 minute. At time 60 minutes, 1 mM H_2O_2 was added to stationary adaptation (◇), stationary lethal challenge ie no BHL pre-addition (○), logarithmic adaptation (■), logarithmic lethal challenge (◆) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-23 b (1440 minutes plot)

Time (min)

Effect of growth phase and age upon BHL mediated adaptation of *A. hydrophila* (3 days old) to oxidative challenge.

Cells were grown overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights were used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary (□) and logarithmic (Δ) control. 100 μg/ml BHL addition was performed at time 0 minute. At time 60 minutes, 1 mM H_2O_2 was added to stationary adaptation (◇), stationary lethal challenge ie no BHL pre-addition (O), logarithmic adaptation (■), logarithmic lethal challenge (◆) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-24

Effect of growth phase and age upon BHL mediated adaptation of *A. hydrophila* (2 weeks old) to oxidative challenge.

Cells were grown overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights were used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary (□) and logarithmic (△) control. 100 μg/ml BHL addition was performed at time 0 minute. At time 60 minutes, 1 mM H_2O_2 was added to stationary adaptation (◇), stationary lethal challenge ie no BHL pre-addition(○), logarithmic adaptation (■), logarithmic lethal challenge (◆) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.

Figure 4-25

Effect of growth phase and age upon BHL mediated adaptation of *A. hydrophila* (12 weeks old) to oxidative challenge.

Cells were grown overnight in M9 medium to stationary phase. 4 ml of culture from one of the overnights were used to seed 6 ml of fresh M9 medium. The resultant cell suspension was incubated at 30°C for 4 hours to obtain cells in logarithmic phase. No addition of H_2O_2 is shown as a stationary ($\square$) and logarithmic ($\triangle$) control. 100 μg/ml BHL addition was performed at time 0 minute. At time 60 minutes, 1 mM H_2O_2 was added to stationary adaptation ($\blacksquare$), stationary lethal challenge ie no BHL pre-addition($\bigcirc$), logarithmic adaptation ($\diamondsuit$), logarithmic lethal challenge ($\blacklozenge$) flasks. At the appropriate time, 1 ml of sample was removed, diluted in M9 buffer, and plated using Miles and Misra technique. After 24 hours incubation at 30°C, the cells were counted, and the log of the cfu (Colony forming unit) was plotted against time.
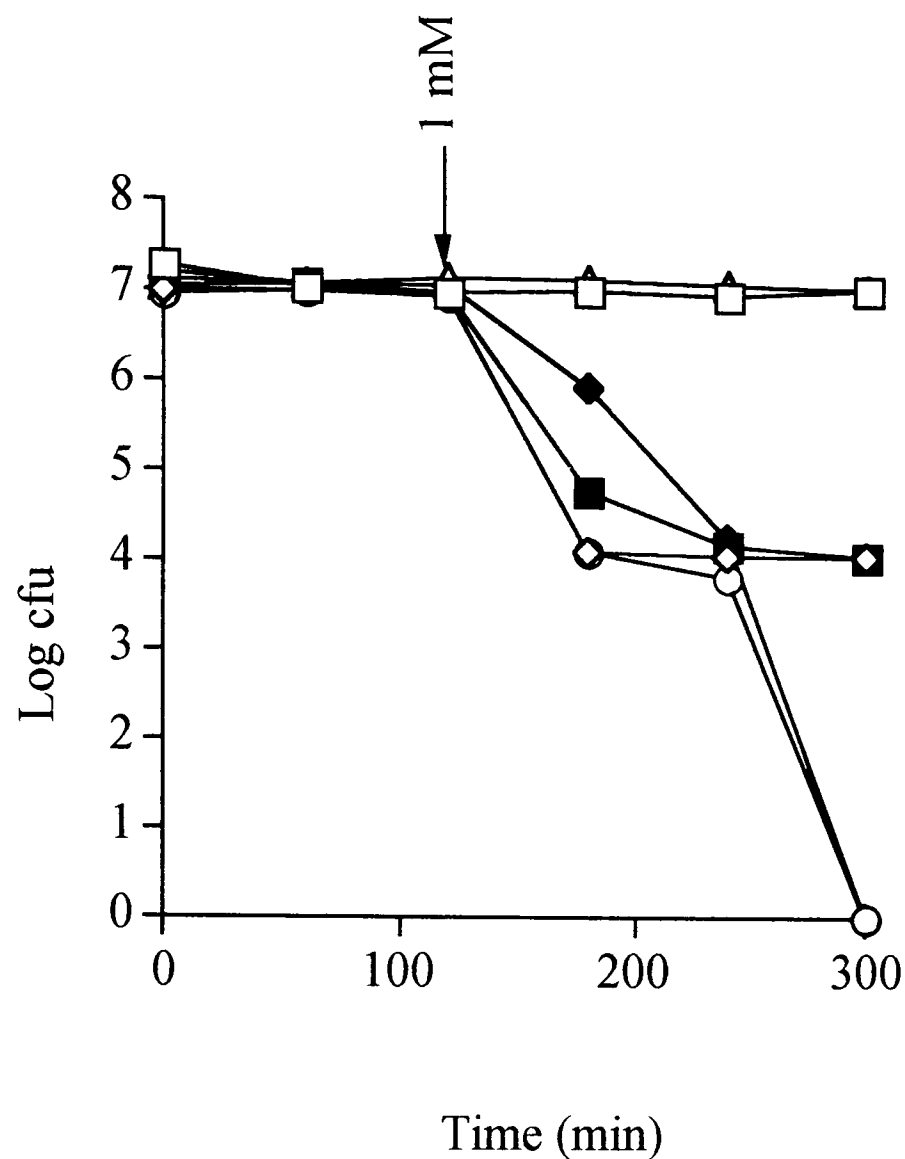
obtained when 50 µM H_2O_2 was used as a pre-treatment dose (Figures 3-12, 3-13 and 3-14). However, a 2 week storage on plates brought about a marked difference in the response to 1 mM H_2O_2 between logarithmic and stationary phase cells. 180 minutes were sufficient for logarithmic phase cells to display a total viability loss whereas stationary phase cells could still be recovered up to 300 minutes after addition of lethal challenge (Figure 4-24). Importantly however 2 week old cells were able to mount a BHL induced adaptation to H_2O_2. With regards to the 12 weeks old culture, cells in both logarithmic or stationary phase displayed an impressive resistance to the lethal challenge. For both non-BHL treated exponential or stationary phase cells, total loss of viability occurs only 24 hours after addition of lethal challenge. This strengthens the hypothesis proposed in chapter 3, where it would appear that mechanisms other than synthesis of stress proteins take place during long term storage enabling cells to become more resistant to stress challenge.

From data presented in Figures 4-23, 4-24, 4-25, where cells were pre-treated with 100 µg/ml BHL, it can be noticed that logarithmic phase cells displayed a weaker resistance to 1 mM H_2O_2 than the stationary phase counterpart, with the exception of 3 day old culture where the resistance displayed by both exponential and stationary cells is similar. This might be explained by the fact that young cells do not have such a memory (chapter 3 paragraph 3.4.3.4 b) as older cells, and the stress proteins content of 3 days old cells in both logaritmic and stationary phase is very similar, therefore implying a similar resistance to a stress.

Results obtained for logarithmic phase cells showed that for cells aged 3 days (Figure 4-23), 100 µg/ml BHL stimulates stronger protection to 1 mM H_2O_2 within the first 3 hours of challenge compared to the 2 week old cells (Figure 4-24). Whereas the viability count of the 3 day old cells drops until no cells could be recovered after 24 hours, the viability count

for the 2 weeks old cells showed an increase until it reached similar level to the one obtained with stationary phase culture. With regards to the logarithmic phase of the 12 week old culture (Figure 4-25), resistance to 1 mM H_2O_2 is definitely not due to presence of BHL, as the pattern displayed is identical to those of cells in both logarithmic and stationary phase which have not been pre-treated with BHL. Resistance would therefore be attributable to other mechanisms such as cell memory (see chapter 4 paragraph 4.4.2.4).

In view of the results, storage has proven to have a strong influence on the extent to which adaptation might occur.

In order to assess the repeatability of the effect of storage upon BHL mediation of an oxidative stress response, data from a number of experimental runs were analysed statistically where the average, standard deviation and error values were calculated between each set of data. This data is shown in Table 4-1. At first, stationary phase of fresh cells (i.e. 3 day old) displayed a similar resistance profile to their log phase counterparts. With ageing (2 weeks old), the cells displayed increasing resistance in stationary phase compared to logarithmic phase (Figure 4-24). When looking at much older cells (i.e. 12 week old), it appeared that during the first 300 minutes BHL sensitised stationary phase cells to 1 mM H_2O_2 challenge compared to their BHL treated logarithmic counterparts, where viability dropped 4 logs in 300 minutes (Figure 4-25). However, the viable count stabilises, whereas the logarithmic phase cells, at first more resistant, die off until no viability could be detected 24 hours after lethal challenge.

Table 4-1: Relative repeatability of sensitisation of old stationary phase *A. hydrophila* cells treated with 100 μg/ml BHL

Time (min)	Cont.1 stat (x1)	Cont.2 stat (x2)	Adapt.1 stat (x1)	Adapt.2 stat (x2)	Lethal 1 stat (x1)	Lethal 2 stat (x2)	Cont.1 log (x1)	Cont. 2 log (x2)	adapt1 log(x2)	Adapt 2 log (x1)	Lethal 1 log (x1)	Lethal 2 log (x2)
0.00	6.98	6.93	6.99	7.01	7.03	6.89	6.90	6.90	6.88	6.39	7.00	7.09
6.00	7.07	7.01	6.98	6.82	7.02	6.90	7.00	7.04	7.05	6.94	6.98	7.01
1.20	7.08	7.03	6.93	6.97	6.99	5.83	7.05	7.18	6.89	7.00	6.74	6.89
1.80	7.08	7.12	5.00	3.69	5.43	4.66	7.07	7.06	4.88	4.17	4.84	3.95
2.40	7.07	7.11	4.50	3.20	5.08	4.67	7.11	7.15	4.82	4.07	4.93	3.99
3.00	7.08	7.13	2.69	3.30	4.91	3.87	7.17	6.97	4.90	3.77	4.83	4.11
1.44	7.47	7.47	3.23	4.52	0.00	0.00	7.47	7.16	0.00	3.31	0.00	0.00
average	7.12	7.11	5.19	5.07	5.21	4.69	7.11	7.07	5.06	5.09	5.05	4.72
sdev	0.159	0.172	1.829	1.792	2.485	2.369	0.180	0.105	2.461	1.610	2.449	2.560
error [(x1-x2)/x1]x100	0.14		2.31		9.98		0.56		0.58		6.53	

4.4 Discussion.

Results obtained during the experimental work described in the previous chapter determined that stationary phase *A. hydrophila* exhibit a greater resistance than their logarithmic phase counterparts, in agreement with previous finding with other organisms (Kolter *et al.*, 1993). It is known that cells in exponential phase turn their resources towards growth and division, and do so by synthesising primary metabolites such as amino acids and structural proteins. But when the conditions in the environmental medium no longer favour growth, the cells change their pattern of gene expression by switching off genes that results in the synthesis of the primary metabolites to switching on genes whose products will prepare the cells for long-term nutrient starvation. The products involved during stationary phase are termed secondary metabolites. These products are varied in nature and can remain in the cells such as enzymes, be exported to the outer membrane such as capsule components, or excreted into the environmental medium such as virulence factors (Janda, 1991).

4.4.1. Characterisation of a non-proteinaceous, heat-sensitive effector molecule.

4.4.1.1 Effect of re-suspension of fresh cells into spent medium upon the stress response.

The potential role for extracellular factors to influence the oxidative stress response of *A. hydrophila* was investigated by the re-suspension of fresh cells into spent medium of cells previously grown to stationary phase (Figure 4-10). 1 mM H_2O_2 added to cells re-suspended in M9 buffer was shown to be lethal confirming results previously reported in chapter 3 (Figure 3-3). In contrast, addition of 1 mM H_2O_2 60 minutes after re-suspension of freshly grown cells into spent medium did not result in loss of viability. Further investigation was undertaken to study whether the length of the incubation period of cells

174

in spent medium might be influencing the oxidative stress response displayed by the organism. The assay was repeated but including two additional times for introduction of the oxidative challenge. The first addition was at 1 minute and the second being at 120 minutes. Loss of viability after addition of 1 mM H_2O_2 at 1 minute is about 2 log during the first 3 hours of challenge (Figure 4-11) followed by a 1 log increase in viability gained during the remaining time of the experiment. This profile displayed for H_2O_2 addition at 1 minute is similar to the profile of the 60 minutes addition. Two possibilities can be suggested to explain these data. The first one could be that extracellular factors such as antioxidants, already present in the medium, would immediately react with H_2O_2, therefore reducing its concentration to a level which is no longer lethal to the cells. The resulting more favourable conditions would then allow the cells repair mechanisms to cope with the damage. Surviving cells would then grow, feeding on the remenants of cells which died during the onslaught, a phenomenon known as necrophagia. The second possibility would be that additional mechanisms such as fresh protein synthesis might be activated through the effect of a signal molecule acting on gene regulation in order to complement the first line of antioxidant defence not sufficient on its own to protect the cells. The cells would then be allowed to grow thanks to nutrient present in the medium due to lysis of cells which have died during the onslaught, as shown by viability count recorded after 24 hours being identical to the initial count. The level of viability obtained for the experimental flask with addition of H_2O_2 after 120 minutes was relatively stable throughout the experiment. This would strengthen the hypothesis for a molecular effector. However it remains a possibility that H_2O_2 could have been either degraded or complexed by a component secreted into the supernatant by the original culture.

4.4.1.2 Effect of re-suspension of fresh cells into spent medium treated with 10 μg/ml chloramphenicol.

Addition of 10 μg/ml chloramphenicol did not prevent cells from surviving the lethal challenge (Figure 4-12) therefore suggesting protection via the action of antioxidant molecules. If protection mechanisms were solely due to gene regulation by a signal molecule, presence of chloramphenicol should have resulted in a significant loss of viability in the bacterial community. Addition of 1 mM H_2O_2 at 1 minute showed an identical pattern to results obtained without antibiotic (Figure 4-10). The decrease in viable count for the experimental flask containing chloramphenicol is 3 log after 24 hours. This can be explained by the fact that cells have lost their capacity to replicate due to the presence of the antibiotic and do not possess the ability to initiate protective mechanisms. That cells remains viable also indicates the presence of pre-existing protective molecules. The nature of these molecules remains unknown but it is be possible that they belong to the class of first line defence mechanisms (i.e. antioxidant such as GSH).

4.4.1.3 Effect of exposure of fresh cells to spent medium and re-suspension in M9 buffer.

The aim was to investigate whether mediation of resistance to oxidative stress was solely due to degradation of H_2O_2 or supported by the action of signal transduction effector molecules present in the medium. Figure 4-13 shows a comparison between fresh cells re-suspended in spent medium and challenged with 1 mM H_2O_2 at 1 minute and cells left in contact with the spent medium for 120 minutes, centrifuged and re-suspended in M9 buffer, with subsequent challenge with 1 mM H_2O_2. Data obtained showed a similar pattern of viability between both treatments. This suggested that spent medium contains molecules which mediate an adaptive response in cells prior to their re-suspension in M9 buffer. The incubation time of 2 hours is a critical, being identical to the time necessary to

176

bring optimal protection to cells pre-treated with 50 μM H_2O_2 prior to lethal challenge of 1 mM H_2O_2 (Figure 3-6). The fact that optimal protection is afforded when cells are incubated for 2 hours in the spent medium would suggest that the signal transduction effector molecules involved would have an influence on a cascade control mechanism (Nyström, 1993). In summary, this is a system of time-dependent protein synthesis divided into 3 stages: early; medium; and late protein synthesis, each dependant on the previously synthesised set for protection to starvation to be fully efficient. The role of the signal molecule in the cascade pathway described by Nyström would be an indirect one. Production of autoinducers within bacteria is already a cascade pathway in itself , as described for *Ps. aeruginosa* (Lafiti *et al.*, 1996). When a bacterial cell senses a stress, such as nutrient limitation, the catabolic repression is lifted. Catabolic repression (Kolb *et al.*, 1993) involves a set of operons which are regulated through the action of cAMP complexed to catabolite repression protein (CRP). Binding of cAMP leads to a conformational change in CRP enabling the cAMP-CRP complex to bind to DNA. This latter binding changes both conformation of the complex and the DNA, therefore modulating initiation of transcription by RNA polymerase. Several operons are known to be regulated by cAMP-CRP including the *lux* operon and its homologues in other species. After catabolic repression is lifted, synthesis of the first LuxR homologue and autoinducer are initiated. These are subsequently involved in synthesis of the second set of LuxR homologues and autoinducer 2 (AI 2), which in their turn initiate synthesis of σ^S factor (Lafiti *et al.*, 1996; Figure 4.26). σ^s is an alternative sigma factor synthesised during entry in stationary phase (Loewen and Hengge-Aronis, 1994). LuxR homologues are firstly synthesised in an unstable form. They are stabilised through the action of chaperon proteins such as GroES and GroEL, or through presence of a high concentration of their respective autoinducer e.g. LuxR homologue 1 stabilised through the presence of high

concentration of autoinducer 1 (Adar and Ulitzur, 1993). σ^S-RNA polymerase complex will then initiate synthesis of σ^S dependent pathways such as antioxidants, virulence factors, and HPII catalase.

4.4.1.4 Effect of re-suspension of fresh cells into either heat treated or enzymatically treated spent medium.

The nature of the stress effector molecules from *A. hydrophila* was further investigated. Initially the thermal stability characteristics of the molecules were examined. Figure 4-14 shows that boiling of the spent medium is sufficient to abolish resistance to lethal challenge, suggesting that the molecules are heat-labile. This thermo-sensitivity of the effectors involved suggests that the molecules might be of proteinaceous nature, but this hypothesis was ruled out by the following set of experiments. Two different proteases i.e. papain (Figure 4-15) and trypsin (Figure 4-16) were introduced and assayed independently, and lead to no loss of viability of cells re-suspended in conditioned medium, even at high protease concentrations. That site recognition, optimal pH range and catalytic mechanisms are different for both enzymes strongly suggests that the effector molecules is not of proteinaceous nature. It might have been possible that the pH of the spent medium was not appropriate for the action of trypsin (optimal pH 7.0-8.0), however papain is known to have a pH-independent activity in the range pH 4.0-8.0 and would therefore not been inactivated as pH of the conditioned medium measured during the experiment was 6.8.

4.4.2 Investigation of the potential role of BHL in mediating the oxidative stress response in *A. hydrophila*.

Taken together the results obtained throughout experiments performed with conditioned medium on stationary phase cells of *A. hydrophila* indicates that protection occurs partially due to a non-proteinaceous, heat-sensitive effector molecule(s).

Figure 4-26: Double quorum sensing cascade in the regulation of *rpoS* in *Ps. aeruginosa* (Lafiti *et al.*, 1996)

lasR *lasI*

(-)

(+)

LasR OdDH

LasR-OdDHL complex

(+) (+)

rhlR/vsmR *rhlI/vsmI*

(+)

RhlR BHL

RhlR-BHL complex

(+)

rpoS
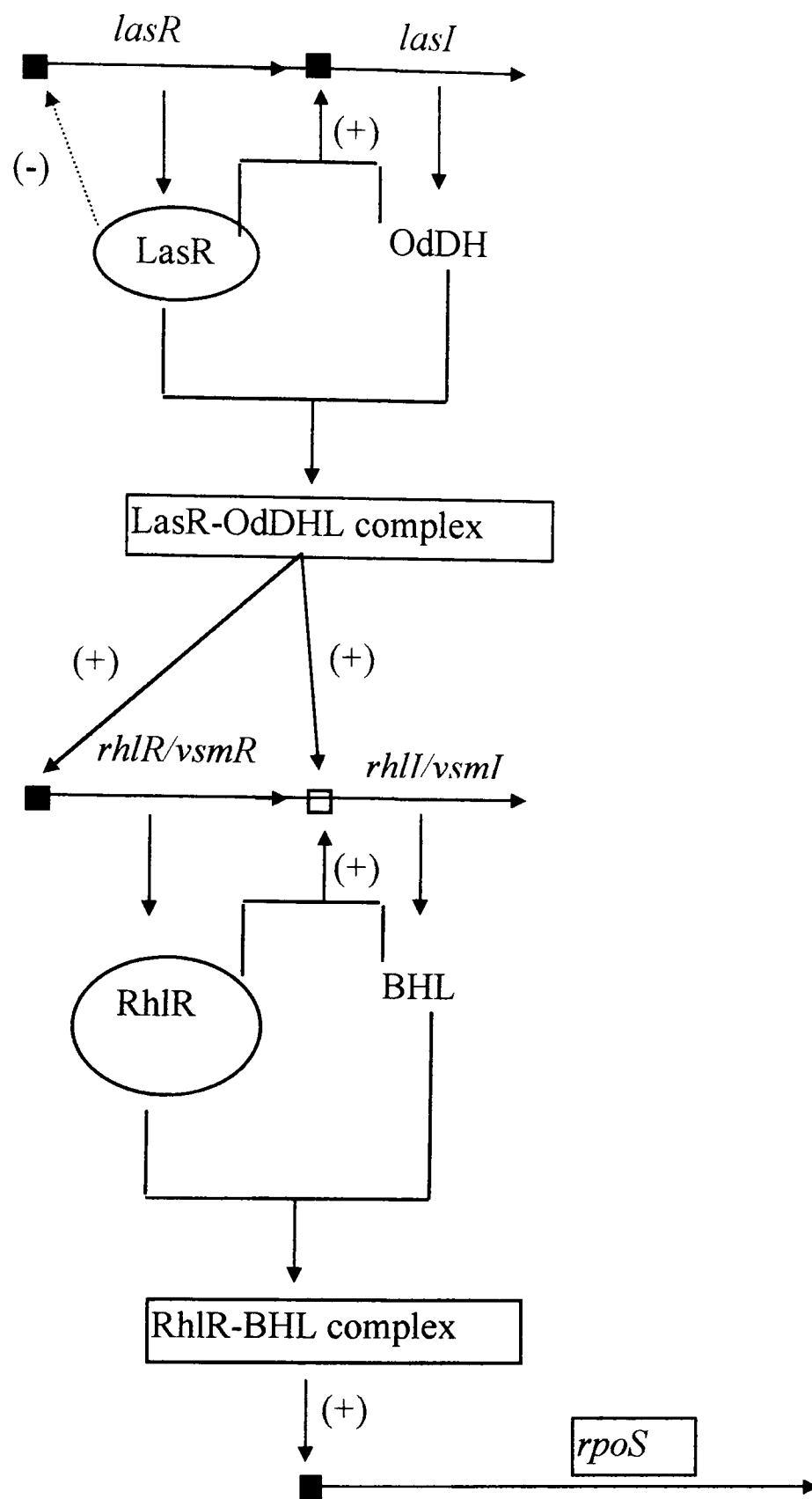
■ represent the promotor/operator regions. ☐ promotor/operator region containing an *lux* box-like element. The authors have omited *rhlI* and *lasI* gene products for clarity. Promotor activation and repression are indicated by + and – respectively. Essentially, LasR activates *lasI* to set up the first cascade such that LasR/OdDHL activates *rhlR*. RhlR/BHL sets up the second quorum sensing cascade driving expression of *rhlI* such that (i) more BHl is synthetised and (ii) *rpoS* is activated. The effects of LasR on *lasR* are less clear and so have been indicated by the dotted arrow.

Recently, two quorum sensing molecules have been reported for *A. hydrophila*, the major being N-butyryl-L-homoserine lactone (BHL), and have been isolated from *A. hydrophila* culture supernatant (Swift *et al.*, 1997). Cells are known to generate and respond to such acyl homoserine lactone (AHLs) signal molecules in order to adapt to environmental changes such as starvation (Huisman and Kolter, 1994). These bacterial 'hormones' have been implicated in the modulation of a wide variety of cellular and extracellular processes such as synthesis of virulence factors, haemolysin and RNA polymerase σ^s factor (Swift *et al.*, 1997). It would be advantageous for individual cells to be able to warn the rest of the community of potential oxidative stress threats, and for the population to be able to mediate an appropriate response to this changing environment. In order to study the potential role of these signal molecules in the phenomenon of oxidative stress response in *A. hydrophila*, BHL was synthesised and assayed in a series of experiments.

4.4.2.1 Effect of addition of BHL in heat treated spent medium in presence or absence of chloramphenicol.

BHL added to boiled spent medium restored the capability for the re-suspended cells to withstand lethal challenge (Figure 4-17). Oxidative stress response mediated by BHL appears to be concentration dependent. Concentration in the range of 0.1 to 1µg/ml BHL delayed the death rate of *A. hydrophila* challenged with 1 mM H_2O_2 but cells remained viable during a 5 hours period when in presence of 100 µg/ml BHL. It was demonstrated that BHL had a connection with *de novo* synthesis of key stress proteins as when the experiment was repeated in the presence of 10 µg/ml chloramphenicol, adaptation was totally abolished (Figure 4-18). This indicated that BHL might play an indirect role in the stress response, possibly by acting to positively control synthesis of fresh stress proteins. Positive control by autoinducers such as BHL in *A. hydrophila* (Swift *et al.*, 1997) or N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) in *Ps. aeruginosa* (Lafiti *et al.*, 1995) have

previously been implicated in the regulation of various gene expression such as virulence factors (e.g. elastase, protease). Also from the results gathered from the series of experiments using conditioned medium, the effector molecules involved in the mediation of oxidative stress resistance have been shown to be thermolabile and non-proteinaceous in nature.

4.4.2.2 Effect of addition of BHL in M9 buffer in presence or absence of chloramphenicol.

BHL was also found to mediate stress resistance of *A. hydrophila* exposed to 1 mM H_2O_2 in M9 buffer (Figure 4-19), mediation which was abolished in presence of chloramphenicol (Figure 4-20) or by heat treatment (Figure 4-21). This strengthens the hypothesis mentioned above regarding the potential role BHL in positively controlling fresh protein synthesis. However the response mediated by BHL in M9 buffer seems to be weaker than in spent medium and requires excessive concentration (probably non-physiological) for the effect to be markedly significant. It might be possible that *A. hydrophila* posesses a similar double quorum sensing cascade as the one described by Lafiti and co-workers for *Ps. aeruginosa*. The marked increase in resistance observed in M9 buffer could therefore be explained by the fact that BHL has stabilised its corresponding LuxR homologue by forming the LuxR homologue-autoinducer complex, therefore rendering σ^S synthesis possible and more rapid than through the stabilisation of LuxR homologue by chaperones (Figure 4-27). Chaperones (such as DnaK) are molecules involved in the folding and maintenance of proteins in living organisms (see chapter 1 paragraph 1.2.6.1 b). The fact that high autoinducer concentrations are needed to mediate full protection would also suggest that there are several pathways involved in the process of oxidative stress resistance and that the bacterial hormone BHL, although a likely candidate as one of the effector molecules, is not directly involved in the regulation but

goes through intermediates molecules such as σ^S factor of the RNA polymerase core enzyme in order to modulate this stress response.

Further work to determine the nature of the different type of molecules involved in this adaptation phenomenon is required, in particular, isolation and identification of the non-protein heat labile molecules from spent medium. Nevertheless, from the results gathered during this study, some preliminary hypothesis can be drawn. It is feasible that the bacteria synthesis some antioxidants which would be excreted and help the cells to tackle the H_2O_2 burst. The second possibility would be that BHL could positively regulate synthesis of key stress proteins such as , universal, general, specific, or unique proteins, or act as a signal for activation of the pre-existing catalase in the bacterial community. A single bacterium would not synthesis enough catalase to withstand the lethal challenge applied whereas catalase content within the community would help (Crockford *et al.*, 1995). This density-dependent phenomenon support a tempting hypothesis that catalase synthesis or its modulation might be regulated through autoinducers.

Figure 4-27: Role of stabilisation of LuxR homologues via action of chaperons or high concentrations of autoinduer in *rpoS* expression in *Ps. aeruginosa.* (Lafiti *et al.*, 1996; Adar and Ulitzur, 1993)
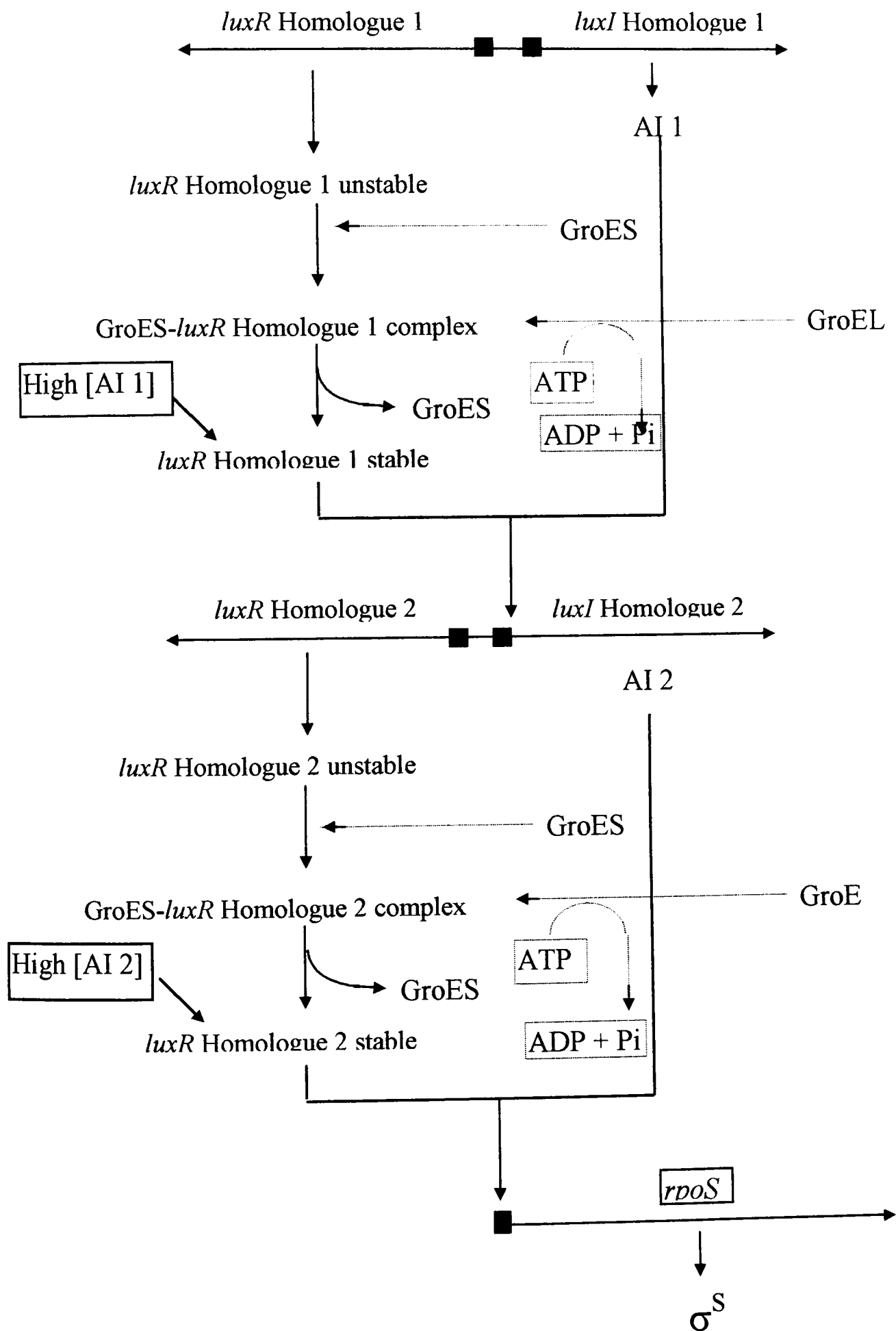
4.4.2.3 Relative time scale protection of *A. hydrophila* using 100 μg/ml BHL as pre-treatment dose.

Study of the relative time scale of protection granted by BHL addition was investigated using 100 μg/ml BHL as the adaptive dose and 1 mM H_2O_2 as the lethal challenge. BHL addition was performed at the same time in all the flasks. Oxidative challenge was performed at a set increase of time of 60 minutes between the flasks. Data (Figure 4-22) suggested that 120 minutes incubation provided optimal adaptation. The initial greater resistance afforded to the cells by presence of BHL is followed 60 minutes after 1 mM H_2O_2 challenge by a dramatic decrease in viability to stabilise at levels of protection displayed by samples tested with other incubation times.

4.4.2.4 Effect of growth phase and culture age upon *A. hydrophila* oxidative stress response.

The extent to which adaptation to H_2O_2 occurred due to pre-treatment with a sub-lethal dose of the same oxidant was shown to be dependent on the phase of growth and the length of time bacterial cells were stored on solid medium. After long-term starvation, *Vibrio* spp. were shown to produce maturation proteins during log phase which are thought to play a role in degradation of stress proteins therefore allowing re-growth. The higher degree of resistance of the older *A. hydrophila* cells could be due to a 'memory' phenomenon where stress proteins have not been fully degraded during initial re-growth. The non-degraded stress proteins would then confer some degree of resistance to the strain posessing them. Effect of growth phase and length of storage on plates, upon adaptation mediated through BHL, was investigated. Cells which had been grown to either mid-log or stationary phase were used to investigate viability following exposure to H_2O_2 after pre-treatment with 100 μg/ml BHL (Figures 4-23 a, 4-24 and 4-25). It was found that the oxidative stress response

in either logarithmic or stationary phase varies according to the length of storage of the test organism on NA plates of the test organisms.

In order to compare results obtained for logarithmic and stationary phase cells, optical density of both cultures were measured to ensure equivalent amounts of logarithmic or stationary phase bacteria were present. Similar to *A. hydrophila* treated with both sub-lethal and lethal doses of H_2O_2, cells pre-treated with 100 µg/ml BHL and subsequently challenged with 1 mM H_2O_2 were shown to be more resistant when in stationary phase than when in exponential phase. This difference in resistance was demonstrated to be independent of the length of time of the cells storage on plates. When comparing results a clear difference in pattern emerges between stationary phase cells and their exponential counterpart (Figures 4-23, 4-24 and 4-25). When cells are relatively young (3 days storage), the pattern of response is similar with cells from the different growth phases. As the length of storage increase, the exponential phase cells display a pattern closer to the pattern displayed by cells treated only with 1 mM H_2O_2. For the sample from 12 week old plates, the adaptation pattern between BHL treated and H_2O_2 treated cells are identical, probably due to late stationary phase protection mechanisms rather than BHL addition. Regarding the stationary phase cells, the adaptation phenomenon seems to correspond to the length of storage time, until a decrease in protection with extended storage is seen (i.e. the old cells begins to die). BHL is synthesised from mid-log to early stationary phase in a quorum sensing signal transduction purpose which, in this application, becomes obsolete when long term stress is applied. Results obtained during the study of the effect of growth phase and culture age upon *A. hydrophila* oxidative stress response suggested that *AhyR* might be negatively controlled when stationary phase is reached and that more specific mechanisms, such as antioxidant or stress proteins synthesis, are switched on. This hypothesis is strengthened by the data presented in chapter 3, where resistance to stress

was increased when specific oxidative stress defence mechanisms were stimulated through the sub-lethal challenge. This would suggest that *A. hydrophila* might have a similar hierarchical quorum sensing cascade as the one described by Lafiti and colleagues (1996) shown in Figure 4-26. LasR is the result of *lasR* gene expression in *Ps. aeruginosa*. This protein interacts with the *lasI* promoter and positively regulates synthesis of N-(3-oxododecanoyl)-$_L$-homoserine lactone (OdHL).

LasR/ OdHL complex is then involved in the activation of rhlR and rhlI leading to the synthesis RhlR and BHL. The complex RhlR/BHL increases BHL synthesis i.e. positive feedback, and activates expression of *rpoS*. RpoS stationary phase σ factor is known to be regulated by a complex regulatory cascade involving at least five regulators of different nature (Hengge-Aronis, 1994). It is worthwhile considering that growth related, cell density and starvation signals were suggested as possible regulators of RpoS (Hengge-Aronis, 1994). When bacterial cells enter stationary phase, *rhlR* promoter activity is almost abolished (Lafiti *et al*, 1996). This inactivation could be due to negative control exerted by overproduction of LasR. This protein will act in a negative feedback loop, reducing the amount of LasR produced, therefore reducing production of RhlR and BHL. It could also be possible that RpoS acts in a negative feedback loop to prevent BHL production when in stationary phase, as production of acyl homoserine lactone was demonstrated to be regulated by an RpoS (σ^S) homologue in *Ralstonia (Pseudomonas) solanacearum* (Flavier *et al.*, 1998). RpoS homologues generally activate gene expression in stationary phase, but in many bacteria the RpoS homologue has been shown to be maximally expressed in mid-log phase and production decreased during entry into stationary phase (Hengge-Aronis, 1996; Flavier *et al.*, 1997). Mutation in the *rpoS* gene lead to decrease in virulence factors and AHL production during stationary phase (Flavier *et al.*, 1997).

4.4.3 Conclusions.

Investigation of the potential role of BHL in *A. hydrophila* oxidative stress response has shown that cells re-suspended in conditioned medium display greater resistance to oxidative stress than the counterpart re-suspended in a M9 buffer. This increased resistance was not entirely dependent on the time of addition of oxidative challenge, but on molecules present in the spent medium. The nature of this molecule has not yet been fully established. Either antioxidants, increase in catalase activity, or quorum sensing autoinduction are all plausible candidates which may act in concert to bring full protection to the challenged microorganisms. However it was determined that protection occurs as a result of a non-proteinaceous, thermolabile effector molecule. Recently, N-butyryl-L-homoserine lactone (BHL) (Swift *et al.*, 1997) was shown to be the major autoinducer in *A. hydrophila*. BHL was synthesised and subsequently assayed for its potential role in mediating resistance to oxidative stress. Boiling of spent medium was proven to inactivate any molecules which might take part into the mediation of a stress tolerant response. Resuspending cells in such treated conditioned medium and subsequently challenged with 1mM H_2O_2 led to rapid loss of viability. However when repeated with addition of BHL cells remained viable but pre-treament with chloramphenicol abolishing this. This suggested that *de novo* synthesis of key stress proteins might be regulated through the effector molecule. Data suggest that BHL is not the only factor mediating this phenomenon of resistance to oxidative stress, as the level of viability displayed by cells re-suspended in minimal medium in the presence of only BHL and lethal dose is lower than the viability displayed by cells in the same conditions but re-suspended in spent medium. It was suggested that BHL might also have an action in the modulation of pre-existing catalase present in the medium. A pre-treatment period of 120 minutes was proven to be the optimum required for BHL mediated

adaptation to occur, which suggested that this phenomenon might be linked to a " cascade" phenomenon similar to that described by Nyström in his study of starvation in *Vibrio* spp.

Adaptation mediated through presence of the autoinducer BHL was proven to be dependent on two parameters being growth phase and time of storage of the microorganism on the solid medium. Cells in stationary phase were proven to be more resistant when incubated with BHL than their logarithmic phase counterparts, regardless of time of storage on agar plates. It was shown that the longer the storage, the more the oxidative tolerance response is due to a cell memory effect rather than the effect of BHL. Regarding the stationary phase cells, the adaptation phenomenon seems to be greater according to the length of time of storage, until a decrease in protection after extensive storage. Results suggested a potential hierachial quorum sensing regulation involving either a LuxR homologue feedback inhibition loop reducing BHL synthesis (Lafiti *et al.*,1996) or an RpoS feedback inhibition loop as previously reported in *Ralstonia (Pseudomonas)solanacearum*.

CHAPTER 5 General discussion

Few studies have been performed with regards to the investigation of the adaptive response mechanisms involved in the survival of *Aeromonas* when facing stressful conditions, such as those encountered during water treatment or when bacteria invade a host. This project was undertaken to study the effect of different environmental parameters involved in the regulation and modulation of the oxidative stress tolerance response of mesophilic members of the genus *Aeromonas*. Information from this project provided an insight to the various parameters which brought about an adaptive response to stress in members of the genus *Aeromonas*.

5.1 The oxidative stress response of mesophilic *Aeromonas*.

These microorganisms were known human pathogens, but the mechanisms by which they survived oxidising agents, encountered during water treatment or when invading a host, were unknown. Only one report had appeared in the literature concerning the stress response of *A. hydrophila* , but this was an examination of pH induced stress (Karem *et al.*, 1994). Results obtained from the current study offer an insight into the mechanisms involved in the oxidative stress response of the mesophilic *Aeromonas*. It was previously reported for *E. coli* that pre-treatment with a sub-lethal dose would bring resistance to a normally lethal challenge of the same oxidant (Demple and Halbrook, 1983). Adaptation studies were performed on *A. hydrophila* which showed that a pre-treatment with a sub-lethal challenge would bring resistance to a higher and lethal dose of the oxidative agent. It was further shown that adaptation was dependent upon catalase production and *de novo* protein synthesis. It was shown (chapter 3 paragraph 3.3.4.1) that cells required a minimal exposure time to 1 mM H_2O_2 of 30 minutes in order to display an optimum activity. Data

obtained are in agreement with the cascade hypothesis described by Nyström (1993) as a starvation induced stress phenomenon during an investigation on starved *Vibrio* spp. This cascade pathway can be described as a time-dependent synthesis of key stress proteins, where three stages were described. The first one being the first 0.5 hour, the second ranging from 0.5 to 6 hours and the last one from 6 to 100 hours. Different proteins were synthesised during these three stages. This investigation (Nyström, 1993) showed that the first 30 to 60 minutes of stress were very important, as it was demonstrated that starvation stress proteins were synthesised immediately after onset of a stressful stimulus. Furthermore, addition of inhibitors of protein synthesis at an early stage of the challenge (within 60 minutes) seriously reduced long-term survival of *Vibrio* (Nyström, 1993). Synthesis of these proteins is time-dependent and require the synthesis of the previous series to bring full protection to the microorganism. The degree of oxidative stress response of *A. hydrophila* was shown to be dependent upon the time delay between addition of the sub-lethal and lethal challenge, stage of growth and the length of storage of cells prior to examination. Data showed a minimal requirement of 120 minutes between addition of sub-lethal and lethal dose for optimal adaptation response to occur. In the first 60 minutes after the 1 mM H_2O_2 addition, the longer the time that elapses between sub-lethal and lethal additions does not lead to enhanced protection, but does in subsequent sample times. This would correlates with the cascade hypothesis described by Nyström (1993). To further strengthen this hypothesis of protective cascade pathway, it was shown that addition of an inhibitor of protein synthesis abolished the oxidative stress response, therefore preventing synthesis of protecting proteins.

It is interesting to notice that catalase and *de novo* protein synthesis have different time requirements for optimal activity to be reached. Results obtained during the catalase assay study showed that a 30 minute period was required for optimal activity, whereas fresh

protein synthesis required 120 minutes for optimal protection. This would suggest that they are each part of two separate pathways, both contributing to protection of the cell. This view can be further strengthened by the fact that control of catalase activity has been reported to be a cell density dependent phenomenon (Crockford et al., 1995) whereas individual cells would not be able to synthesis enough catalase to degrade high concentrations of oxidant. Therefore other more individualistic pathways have evolved in order to better protect single cells.

Logarithmic and stationary phase cells were found to have physiological differences when subject to oxidative challenges. These differences probably arise due to the different genes being expressed, therefore resulting in different products termed primary metabolites for logarithmic phase cells and secondary metabolites for the stationary counterpart. Secondary metabolites prepare and protect the cells against various stressful conditions. With regards to the effect of the growth phase upon the oxidative stress response, results are in agreements with the literature describing stationary phase cells as being significantly more resistant than the logarithmic phase counterpart (Siegele and Kolter, 1992). Results obtained during this project also demonstrated that increased resistance of stationary phase cells, compared to logarithmic cells, did not depend upon the length of time of the storage on plates. However the extent to which adaptive response occurs in both logarithmic and stationary phase cells have been shown to be dependent upon the length of time the microorganism have been stored. Effects of growth phase and length of storage upon adaptive response were shown to be independent of each other. The difference in resistance of A. hydrophila in logarithmic or stationary phase was shown to be similar regardless of the length of storage. However, the degree of resistance of both logarithmic and stationary phase cells was proven to be dependent on the length of storage. As length of storage increased stationary phase cells showed an increased resistance to the lethal challenge,

whereas logarithmic phase cells displayed a decrease in resistance. In a study on *Vibrio* spp. the role of proteins called maturation proteins which were hypothesised to degrade starvation inducible proteins and which were considered to inhibit fresh logarithmic growth. It is widely acknowledged that the longer the starvation, the longer the lag phase (Morita, 1993). Considering these points, less inhibitory starvation protein would be degraded by maturation proteins for a set period of time, in long-term starved cells than in short-term ones as the lag phase would be shorter for the latter. The remaining starvation proteins would confer a degree of resistance to the older cells compare to their younger counterpart. This partial degradation of inhibitory proteins is proposed here to be termed **cell memory**. The remaining starvation proteins would be diluting down with respect to further growth, leading to reduced resistance with increased number of generations, as shown by results concerning logarithmic phase cells. As described in chapter 3, logarithmic phase cells were obtained by growing cells of different storage time overnight and using the resulting culture to seed fresh M9 medium to obtain logarithmic phase cells. Results showed that the older the initial cells, the less resistant to oxidative challenge the resulting logarithmic phase cells. This was explained by the fact that the older the initial cells, the longer the lag phase, therefore the later the exponential phase was initiated. The adaptation dose was added at the same time in all the experimental flasks. The shorter the lag phase, the more stress proteins synthesised, the more resistant to lethal challenge the cells. Whereas some bacteria in the centre of the colony can encounter starvation when stored on nutrient agar plates when, others remain with plenty nutrients so do not starve. It is therefore more plausible that bacteria suffer from various stresses due to an increase in the toxic side products of their slow metabolism due to the low temperature of the storage conditions.

It is known that the activity of RNA Polymerase σ factors is related to the type of stress (e.g. σ^{32} for heat shock). The heat shock response have been shown to be controlled by the DnaK machinery introduced in chapter 1 (Figure 1-3) which sequesters σ^{32}, a phenomenon termed activity control (Narberhaus, 1999), or by what Naberhaus has termed stability controls involving degradation of σ^{32} by a protease called FtsH (Narberhaus, 1999). Due to the varied stresses and the knowledge that some regulons can be up-regulated by different type of stresses it is tempting to speculate that starvation proteins would belong to a more general regulon under σ^s regulation and would be synthesised or partially synthesised along with other key stress protein belonging to this group of genes.

This adaptation phenomenon has been shown to be widely spread among the mesophilic *Aeromonas*. However, differences in the degree of adaptation to oxidative challenge of both type strains and environmental isolates have been noticed. This greater resistance of *A. sobria* remains an unexplained phenomenon and requires further study.

The environmental isolates demonstrated a substantial resistance to 1 mM H_2O_2, being greater when compared to type strains. This enhanced resistance is probably due to two factors. The first one could be considered as the 'niche effect'. Five different types of habitats for aquatic species have been reported (Kjelleberg *et al.*, 1993) as being:

- the water body to which bacterial cells live in a free planktonic state

- suspended particles where the bacterial cells are attached

- at the air-water interface

- within the sediments

- the external (epiderm) and internal (gastro-intestinal tract) surfaces of higher aquatic species e.g. a fish.

Each of these habitats have a complex internal physicochemical structure. Sediments, for example, have a gradient of molecular oxygen and nutrient. Parameters such as

temperature, pH and nutrient limitation have been shown to play a major role in providing the cells with stimuli mediating changes favourable to enhance the cells survival. Due to their nature, aquatic ecosystems are prone to sustain unbalanced bacterial growth. For microorganisms this means a dynamic interplay between growth, starvation, recovery and re-growth (Kjelleberg *et al.*, 1993). Starved cells have some amazing assets such as increased adhesion or aggregation properties, but more interestingly the ability to respond rapidly to fresh nutrient availability which leads to an ordered pattern of protein synthesis during the lag phase, also known as the maturation phase (Kjelleberg *et al.*, 1993). As describe previously, maturation proteins could act as the memory of the cells affording them protection where conditions worsen.

The second factor to take into consideration is the history of the cells. This is closely linked to the previously mentioned 'niche effect', but bears some unique characteristics. Cells are rarely constrained to their niche. They either follow, for example, the flow of a river or animals in a lake, which make them change environments and therefore face new conditions (i.e. in temperature, nutrient availability or various stress such as chlorine, heavy metals, and the host immune system). Previous niche conditions will determine the chance of survival of the microorganisms in the new environment.

5.2 Stress response in *A. hydrophila*: a possible role for quorum sensing.

When cells reach stationary phase, secondary metabolites synthesis is favoured against primary metabolites necessary for cell growth and division. The nature of secondary metabolites is varied and comprise some non-excreted molecules such as enzymes, those exported to the outer membrane or be molecules excreted in the medium, such as antioxidants.

The phenomenon of increased resistance to H_2O_2 of stationary phase cells of *A. hydrophila* compared to the same cells in logarithmic phase lead to a series of experiments

investigating the effect of re-suspension of fresh stationary phase cells into spent or conditioned medium in order to determine whether some of these excreted molecules have an effect in mediating the oxidative stress response of *A. hydrophila*. It was found that such a treatment led to a resistance of the cells to H_2O_2 without any pre-treatment of any sort. Further investigation resulted in the discovery of effector molecules present in the medium. These were determined to be non-proteinaceous and thermolabile in nature. The nature of a couple of likely candidate molecules was either antioxidants, which would have degraded H_2O_2 before too much damage was caused to the cells, and/or a signal transduction molecule which could regulate gene expression of proteins needed for oxidative stress adaptation. The antioxidant nature of the unidentified molecules has been confirmed, but not fully characterised. However additional data suggested that two different mechanisms act in concert. A short incubation time would allow only the action of extracellular antioxidant components secreted by the cells, whereas longer incubation times would allow further protection to the cells through a specific signal transduction pathway.

In recent years behaviour of bacterial communities has been revolutionised by the concept of bacterial cell to cell communication. Prior to the early 1970's bacterial cells were seen as individualistic organisms, looking solely after themselves and having no contact with other members of the community (Losick and Kaiser, 1997). In 1970, Nealson and Hasting demonstrated that in the bacterium *Vibrio (Photobacterium) fisheri* production of a luminescent compound (luciferase) was density dependent and did not follow the growth curve pattern. Instead luciferase was produced massively when *V. fisherii* was in the mid-logarithmic growth phase and production plateaued when cells reach stationary phase (Nealson and Hasting, 1970). Luminescence occurred when a threshold (quorum) concentration of an autoinducer molecule, which stimulates synthesis of luciferase, the

195

enzyme catalysing luminescence was reduced (Engebrecht and Silverman, 1984). This was proven to be triggered by a small diffusible molecule belonging to the acyl homoserine lactone family (Kaplan and Greenberg, 1985). Homologues of the *lux* system have been found in many Gram negative bacteria. Cell communication is now thought to be a mechanism widely spread among Gram negative species (Fuqua *et al.*, 1996). Therefore quorum sensing is considered as being one of the mechanisms involved in the transduction of environmental stimuli into gene expression (Swift *et al.*, 1996).

Due to a recent publication on the existence and isolation of bacterial hormones in *A. hydrophila* (Swift *et al.*, 1997), which appeared to represent possible candidates as molecules mediating the increased resistance observed when cells were re-suspended in spent medium. Heat treatment of the spent medium was shown to abolish resistance of *A. hydrophila* to 1 mM H_2O_2 challenge. Addition of BHL was demonstrated to restore adaptation, albeit to a limited extent. Partial adaptation was also demonstrated in minimal M9 medium when BHL was added. However the response mediated by BHL in M9 buffer was shown to be weaker than when cells were re-suspended in conditioned supernatant, requiring high concentrations (100 μg/ml) for the effect to be pronounced. It was therefore suggested that BHL, whereas it brings a partial protection, is a likely candidate as an effector molecule mediating general stress response in *A. hydrophila*.

In a wider context the experimental data suggests that at least two pathways operate in concert for full protection to occur. Similar experiments to those performed for the investigation of the effect of an H_2O_2 sub-lethal dose in the oxidative stress response were performed with BHL as the pre-adaptive molecule. The extent to which adaptation to H_2O_2 occurred was shown to be dependent on the phase of growth and the length of time bacterial cells were stored on solid medium, suggesting an increased protection due to cell

memory as outlined above. Logarithmic phase cells showed a decrease in resistance according to the length of time of storage on solid medium until they finally displayed the same death pattern as non-BHL treated cells. Regarding stationary phase cells, adaptation seems to increase with the length of time of storage, until a decrease in protection is seen after extended periods of storage occurs. The fact that BHL protection becomes weaker with increased storage time could be explained by the fact that the longer the storage, the longer the lag phase. For set time span, an increased lag phase led to a decrease in the protection afforded by BHL due to a shorter time of activity of the fresh proteins synthesis mechanisms. The fact that *lux* genes in *V. fisheri* and *V. harveyi* were shown to be strongly activated in late exponential phase (Fuqua *et al.*, 1996), and that rhlR promoter activity is almost abolished upon entry into stationary phase (Lafiti *et al.*, 1996) suggests that autoinducer mediation of the stress response would become obsolete in long term starvation as other more specific pathways take over, such as long term starvation protein synthesis. Results suggested a potential hierachial quorum sensing regulation involving either a double quorum sensing cascade (chapter 4 Figure 4-26) as for *Ps. aeruginosa* (Lafiti *et al.*, 1996) or involvement of an RpoS-homologue required for expression of the LuxR homologue as previously reported for *Ralstonia solanacearum* (Flavier *et al.*, 1998).

5.3 Role of *Aeromonas* in mediating false positive Colilert reactions.

Results obtained, when using a kit within 4 weeks of the expiry date, showed that relatively high *A. hydrophila* cell concentrations ($>10^4$ cells/ml) were needed for false-positive reactions to occur. These results were in agreement with the published literature (Edberg *et al.*, 1988). However, when the kit used during experimental procedures was within four weeks of the expiry date, results were different to those reported in the literature with regards to the levels of cells required to mediate a false-positive reaction. It has been demonstrated here that the effect of the Aeromonads inhibitory reagents

197

contained by the Colilert system was influenced by the age of these reagents. The older the kit, the lower the amount of cells required to mediate false-positive reactions. Furthermore, results have shown that low cell numbers (10^1cfu/ml) of *A. hydrophila* were shown to be responsible for such a positive reaction when diluent used was 1 % saline whereas 10^2 cfu/ml were required when diluent was sterile water. The fact that data obtained during the study indicated that mesophilic *Aeromonas* , which constitute a significant fraction of the heterotrophic population, were not efficently supressed within four weeks of the shelf-life expiry of the Colilert reagent suggested two possible failures of the system. Firstly, the chromophore compound used as substrate for the β-galactosidase might, with time, be more prone to hydrolysis when reconstituted. Secondly, some of the inhibitory compounds used for preventing growth of the heterotrophic flora might decompose when time elapses. This was corroborated by a study performed in our laboratory where *A. hydrophila* plate counts were higher when performing the Miles and Misra drop plate count technique on samples from old Colilert reagents compared to those when accomplished on a fresh Colilert reagents. It was shown that this phenomenon of false-positive reactions was not restricted to *A. hydrophila* but was widely spread through the genus. It was interesting to notice that *A. sobria* displayed a weaker false-positive reaction than the other two members tested, suggesting that *A. sobria* seemed to have either a reduced production of or a less efficient β-galactosidase. When reviewing these results, it was concluded that reagent age had a crucial impact on the incidence of false-positive reaction mediated by mesophilic *Aeromonas*.

5.4 Conclusion.

The aims of this project were detailed at the beginning of this thesis and could be summarised as follow:

1) The parameters influencing the false-positive reactions mediated by mesophilic *Aeromonas* in the Colilert defined substrate technology system were to be determined.

2) Investigation of the effect of H_2O_2 and the potential of mesophilic *Aeromonas* to display a tolerance response to this oxidant when pre-treated with a sub-lethal dose of this noxious agent. The study of this potential resistance to an oxidant would imply determining some of the mechanisms implicated and parameters (e.g. growth phase) influencing the response.

3) The possible implication of extracellular effector molecules especially butyryl homoserine lactone i.e. BHL in mediating a stress tolerance response to H_2O_2 was investigated.

The Colilert define substrate technology system showed a time-dependant increase in false-positive reactions mediated by *A. hydrophila*. For fresh Colilert reagents, the cell concentration ($>10^4$ cells/ml) required for false-positive reactions was in agreement with the literature (Edberg *et al.*, 1988). However, when reagents used were within 4 weeks of shelf-life expiry, lower concentrations (10^1 cfu/ml when diluent used was 1% saline and 10^2 cfu/ml when diluent was sterile water) were necessary for false-positive reactions to occur. It was demonstrated that false-positive reactions due to ageing of the Colilert reagents (instability of reagents) was a genuine phenomenon proven to be growth phase-independent and not a chlorine artefact. The cause of this increase in false-positive reactions when reagents were ageing was imputed to the degradation of either the chromophore and/or the inhibitory substances. Mediation of false-positive reactions has been shown to be widely spread among the genus. However, *A. sobria* displayed a weaker potential. It was suggested that *A. sobria* might either be more sensitive to the inhibitory molecules present in the Colilert reagents or that *A. sobria* produces less or a less efficient β-galactosidase.

Selection of an appropriate medium and detection method has been crucial for assessing the oxidative stress response of mesophilic *Aeromas*. Complex medium was shown to reduce H_2O_2 oxidising potential, suggesting potential interactions between components of the medium and oxidant molecules. The spectrophotometric technique was insufficiently sensitive to measure viability. Miller M9 minimal medium and Miles and Misra drop plate count technique were therefore selected for further experimentation. Preliminary data showed *A. hydrophila* to be more sensitive to H_2O_2 than *E. coli* . Adaptation was rendered possible when using 50 μM H_2O_2 as a sub-lethal dose and 1 mM (i.e. the minimal lethal dose) as the challenge. 120 minutes pre-treatment with the sub-lethal dose was shown to be an optimal for adaptation to occur. Studies of the relative temporal protection showed that the longer the delay between the sub-lethal and lethal addition, the stronger the adaptation. This adaptation phenomenon was demonstrated to require fresh synthesis of key stress proteins, as chloramphenicol abolished the adaptive response. This would be in agreement with the cascade pathway described by Nyström. The second mechanism investigated was catalase. It was shown that 30 minute pre-treatment with H_2O_2 was a requirement for optimal activity. However, chloramphenicol did not abolish activity and suggested a modulation of catalase activity. *De novo* protein synthesis and catalase have different times for optimal protection which suggests that these 2 pathways are separate. The degree to which *A. hydrophila* adapt as been shown to be dependent upon delay between sub-lethal and lethal addition, the stage of growth and the length of storage. Stationary phase *A. hydrophila* were proven to be more resistant than their logarithmic counterpart regardless of the storage length. However, resistance of both stationary and logarithmic phase are time-dependent. This increased in resistance was suggested to occur via partial degradation of stress proteins by maturation proteins. This partial degradation was termed "cell memory". Data showed this tolerance response phenomenon to be widely spread among

the genus with *A caviae* displaying a similar response to *A. hydrophila*, whereas *A. sobria* manifested a surprisingly stronger tolerance response than both *A caviae* and *A. hydrophila*. It was therefore suggested that *A. sobria*, being more sensitive to the oxidant had to developped more efficient defence mechanisms to survive. It was hypothesised that *A. sobria* might have followed a different evolutary line within the genus, presumely to be due to difference in proteins which relates to the niche and history of the cells.

It was demonstrated that resuspension of freshly grown cells in spent medium resulted in an drastic increase of resistance to previously lethal H_2O_2 challenge. This resistance was shown to be optimal for a contact time prior to lethal challenge of 120 minutes. This suggested that the response mechanisms involved might be similar to either the cascade pathway reported by Nyström or the one reported by Lafiti and co-workers. The fact that chloramphenicol did not abolished the tolerance response indicates the presence of pre-existing antioxidant molecules in the medium. Further investigation of the spent medium indicated that the effector molecule present were non-proteinaceous heat-sensitive. The potential role of BHL in mediating the stress response was also assayed. Data showed that BHL did restore the stress response in heat treated spent medium. BHL also mediates the tolerance response when experiments were performed in M9 minimal medium, but the adaptation displayed was weaker than in spent medium and required high concentrations. Addition of chloramphenicol prior to BHL abolished the stress response in both heat-treated spent medium and minimal medium, suggesting that BHL to have an action on the fresh synthesis of key stress proteins. The longer the storage, the less efficient BHL protection, suggesting that BHL protection becomes obsolete for long-term growth arrest.

CHAPTER 6 Future Works

6.1 Colilert defined substrate technology system.

Aeromonas have been shown to cause false-positive reactions when using Colilert defined substrate technology system. These false-positive reactions were shown to be due to reagents instability with time. Re-evaluation of the shelf-life and temperature of storage are essential in order to determine a time interval and temperature ensuring 'freshness' of the kit i.e. interval of time and temperature where integrity of reagents is guaranteed. It is therefore essential to determine whether the unstable reagent is the chromophore and/or the inhibitory substances. Chromophore instability can be time dependent and/or temperature dependent. The former hypothesis can be investigated by the following series of experiments. Colilert reagents of increasing storage time at room temperature would be reconstituted with sterile water and incubated in a water bath at 35°C for 24 hours (temperature and length of incubation of the usual assay). $OD_{420\ nm}$ would be recorded using a spectrophotometer. $OD_{420\ nm}$ of all sample would then be compared with the value obtained for fresh reagent (i.e. control), and results showing a significant difference would indicate a degradation of the chromophore. From those results a minimal storage time for which chromophore would remain intact would be determined. In order to determine a potential effect of the storage temperature, a similar experiment could be performed, this time using different storage lengths and different temperatures of storage from 4°C (i.e. cold room temperature) to 25°C (i.e. room temperature). Colilert reagents would be reconstituted with sterile water and incubated in a water bath at 35°C for 24 hours and OD recorded at 420 nm. Comparison between the control (fresh reagent) and values obtained for the samples would allow determination of the optimal temperature of storage.

The nature of the inhibitory molecules present in the Colilert defined substrate technology system are unknown, however results obtained in our laboratory tends to lean towards the fact that their instability results in false-positive reactions. It would be useful to be able to know their nature and be able to study other more stable inhibitory molecules to replace the deficient ones. Nevertheless, even if the nature of the inhibitory molecules present in the Colilert system remains unknown, investigation of various *Aeromonas* inhibitory molecules could allow determination of a molecule which could complement the Colilert reagent. These inhibitory molecules would have to be tested on all type strains and some environmental isolates of *Aeromonas* due to the difference in mediating the response shown by *A. sobria* compared to *A. hydrophila* or *A. caviae*. It is important to ensure that addition of this complementary molecule would not lead to any interference or adverse effect on the colilforms and *E. coli* detection. The fact that *A. sobria* appeared less resistant in many experiments undertaken in our laboratory (Gavriel *et al*, 1997; chapter 3 this volume) would suggest that β-galactosidase is produced in lesser quantities or that this enzyme is less efficient in *A. sobria* compared to *A. hydrophila* or *A. caviae*. The validity of either of these hypothesis could be verified by a simple set of experiments. Firstly, all type strains could be grown overnight and recovered by centrifugation. An equivalent cell number of each of the type strains could be re-suspended and β-galactosidase extracted. Using HPLC, β-galactosidase could be identified, purified and its quantity determined for each sample. The second experiment would involve extraction, purification by HPLC of β-galactosidase in each of the type strains. Activity of a set concentration of the recovered enzyme would then be assayed to determine the rate by which it degrades *ONPG* into *ONP* and compared to determine the efficiency of β-galactosidase for each of the strains under study.

Investigation of the Colilert defined substrate technology system showed that false-positive reaction could be enhanced with presence of NaCl. This salt is not present in sufficiently high concentrations in potable drinking water to mediate false-positive reactions, however other more prevalent salts such as phosphate or nitrate salts might have an influence on the incidence of such false-positive reactions. It would be therefore important to investigate the potential role of various salts present in the drinking water supplies.

6.2. Oxidative stress response of *Aeromonas* spp. mediated by a sub-lethal pre-treatment of H_2O_2.

During experiments performed in complex medium, reactions between H_2O_2 and some components of the milieu have been suggested. It would be of interest to determine the nature and reaction mechanism of these interactions as for example presence of iron could, via the Fenton reaction, leads to hydroxyl radicals.

That bacteria are able to adapt and better resist to various stresses is a known phenomenon in many species such as *E. coli* or *S. typhimurium*. It has been suggested that some of these protective mechanisms would be of some help to the cells to better resists macrophage onslaught (Beaman and Beaman, 1984), however no experiments have been performed on *Aeromonas* survival to oxidative burst in macrophages. In order to elucidate this potential route for infections, the following set of experiments could be performed. *Aeromonas* cultures could be subjected to no-pre-treatment or various pre-treatment such as heat, starvation, H_2O_2 or BHL to name a few, put in presence of macrophages and allow phagocytose. Macrophages could be recovered by centrifugation, washed and lysed taking care not to lyse potentially viable *Aeromonas*. The resulting solution would then be used to prepare a dilution series and used in a Miles and Misra drop plate count assay and viability determined. Recovery and lysis of macrophages could be undertaken at various time

points, allowing determination of the extent of the protection afforded by various stress pre-treatment.

Data obtained during the course of this project have demonstrated that *Aeromonas* spp. have developed similar defence mechanisms as other microorganisms such as *E. coli* , *S. typhimurium* or *Vibrio* spp. which have allowed the presentation of a schematic summary of some of the pathways involved. Catalase is one of the defence mechanisms studied. By producing HPI, HPII and double catalase mutants and using complementation it could be possible to investigate the role of each of the catalases separately and their interactions in the degradation of H_2O_2. This would therefore give crucial information with regards to whether HPII is modulated or not in absence of HPI. This could also provide data on the maximal H_2O_2 concentration HPII can degrade prior to HPI intervention. Catalase modulation have been suggested to occur either via a partial inhibitory molecule or by direct action of H_2O_2. This could be determined by investigation of the cytosolic fraction of wild-type strains in presence or absence of chloramphenicol and the cytosolic fraction of a HPI deficient mutant by HPLC. Comparing the HPLC profiles would show either a disappearance of one peak (i.e. showing reaction between H_2O_2 and the inhibitory molecule) or change in the retention time (i.e. showing a potential oxidation). The eluted fraction for this peak could then be recovered and further analysed to determine the nature of the compound. This effect of this molecule could then be assayed using purified catalase in presence of H_2O_2.

While evolution has conferred *Aeromonas* spp. with a highly complex web of protective pathways, some differences with other species have arisen. It seems that they there might be some variance in either the quality or the quantity of the proteins involved in the protection. Two dimensional SDS electrophoresis data obtained for several species such as

E. coli , *S. typhimurium*, *Vibrio* spp and *A. hydrophila* would be of great help in order to gain an insight in the nature of the proteins by analysing the profile obtained for those different microorganisms. This would allow determination of whether, the same number of proteins are synthesised for a given stress, by comparing spot pattern or the quantity in which they are synthesised by comparing the spot intensity. The lower resistance of *A. hydrophila* compared to *E. coli* would suggest that *A. hydrophila* stress proteins might be unrelated to or less numerous and less efficient than those found in *E. coli* . It would be interesting to compare the protein patterns and sequences of both microorganisms when responding to the same stress, in order to determine the differences and their effect on the adaptive response of each genus to a particular stress. Sequences of unique proteins for each stress studied could be used as a stress marker (see below).

Another interesting point to address is how long does the protective effect of a sub-lethal challenge last. Is it permanent or does it fade with time? This could be investigated using pulse chase protein studies using radiolabelled amino-acids.

2D-SDS electrophoresis and pulse chase protein studies would also contribute in gaining an insight in the cascade pathway suggested to be involved in fresh protein synthesis by comparing results in presence and absence of chloramphenicol at different time intervals.

The niche and history of the cells are factors which influence the response of a microorganism to stress due to cell memory. It was suggested that memory was due to stress proteins which were not degraded by maturation proteins. This could be confirmed by investigation of the membrane composition of cells at various stages of storage or from different habitat. Sequencing the stress proteins could also give an indication of the type of

stress bacteria have faced (i.e. history of the cell) by comparing them to the sequence of each stress unique proteins.

6.3. Oxidative stress response of *Aeromonas* spp. mediated by BHL pre-treatment .

With regards to the supernatant studies, much more remains to be determined for the effector molecules. BHL has been demonstrated to be a likely candidate for an adaptive stress response to occur, but was shown not to be efficient enough for being the only candidate. It was suggested that antioxidants might be another class of molecules involved in the stress response by scavenging the toxic agent before any damage was done. Detailed study of the supernatant of cells grown to stationary phase using analytical techniques such as HPLC or mass spectrometry would give valuable information in the determination of the nature of the molecules involved. Different classes of molecules could then be separated, their structure determined and then individually tested in order to see if they would mediate an adaptive response.

Swift and colleagues (1997) have reported that *A. hydrophila* produced two AHLs being BHL and HHL which indicate the potential of a double cascade pathway mentioned in chapter 4. By producing strains deficient in either or both of these molecules by knock out of the genes responsible for BHL (*ahydIR*) and HHL (to be determined) production, their involvement in the previously mentioned double cascade could be investigated. Screening for a mutant overproducing HHL would allow determination of the gene involved in its synthesis.

Knock out of *ahydIR* boxes in *A. hydrophila* and two dimensional SDS electrophoresis data in the presence and absence of AHLs for a wide range of stresses would also give important information on the extent in which AHLs are involved in bringing an adaptation response. Comparison of both data obtained when studying either a specific stress or AHL effect would help to elucidate the connection between the different pathways described in

the literature. It would also give information on unknown pathways as some of the proteins involved in the various stress responses remain unknown. By producing the appropriate mutant strains or reporter fusion from a gene library, the effect of BHL on each protection mechanism such as catalase or SOS repair mechanism or on secondary metabolites synthesis such as virulence factors or antioxidants could be examined.

CHAPTER 7 Bibliography

Adar, Y.Y., Ulitzur, S., (1993), GroEL proteins facilitate binding of externally added inducer by LuxR protein-containing *E. coli* cells, J. Biolumin. Chelumin., **8**, 261-266.

Albertson, N. H., Nyström, T., Kjelleberg, (1990), Functional mRNA half-lives in the marine *vibrio sp.* S14 during starvation and recovery, J. Gen. Microbiol., **136**, 2195-2199.

Ali, A., Carnahan, A. M., Altwegg, M., Lüthy-Hottenstein, J., Joseph, S.W., (1996), *Aeromonas bestarium sp. nov.*(formaly genospecies DNA group *2 A. hydrophila*), a new species isolated from non-human sources, Med. Microbiol. Lett., **5**, 156-165.

Altwegg, M., Geiss, H.K., (1989), *Aeromonas* as a human pathogen, Crit Rev Microbiol, **16**, 253-286.

Altwegg M., (1996), 4. Subtyping methods for *Aeromonas* species, In Austin B., Altwegg M., Gosling P.J., Joseph S. (eds), The genus *Aeromonas*, John Wiley & Son, England, 109-125.

Anonymous, (1994), The microbiology of water, part 1-drinking water, report on public health and medical subjects n° 71, HMSO, London.

Anonymous (1997), Hydrogen peroxide, catalase, and peroxidases, In Josephy, P.D., Mannervik, B., Artiz de Montellano, P (ed.) Molecular toxicology, Oxford university press.

Austin, B., Adams, C., (1996), 8. Fish pathogens, In Austin B., Altwegg M., Gosling P.J., Joseph S.(eds), The genus *Aeromonas*, John Wiley & Son, England, 197-243.

Baddour, L. M., (1992), Extraintestinal *Aeromonas* infections-Looking for Mr.Sandbar, Mayo Clin. Proc., **67**, 496-498.

Beaman, L.,Beaman, B.L., (1984), The role of oxygen and its derivatives in microbial pathogenesis and host defense, Ann. Rev. Microbiol., **38**, 27-48.

Becker, J., Craig, A., (1994), Heat-shock proteins as molecular chaperones, Eur. J. Biochem., **219**, 11-23.

Bernheimer, A. W., Avigad, L., (1974), Partial characterization of Aerolysin, a lytic exotoxin from *Aeromonas hydrophila*, Infect. Immun., **9**, 1016-1021.

Block J.C., (1992), Biofilms in drinking water distribution systems, In Bott T.R., Melo L., Fletcher M., Capdeville B. (eds), Biofilms-Science and Technology, Dordrecht:Kluwer, 469-485.

Bohannon, D.E., Connell, N., Keener, J., Tormo, A., Espinosa-urgel, M, Zambrano, M.M., Kolter, R., (1991), Stationary-phase-inducible "gearbox" promoters: Differential effects of *katF* mutation and role of σ^{70}, J. Bact., **173**, 4482-4492.

Boussaid A., Baleux B., Hassani L., Lesne J., (1991), *Aeromonas* species in stabilization ponds in the arid region of Marrakesh, Morocco, and relation to fecal-pollution and climatic factors, Microb Ecol, **21**, 11-20.

Buckley, T.J.,(1992), Crossing three membranes channel forming aerolysin, FEBS, **307**, 30-33.

Burke V., Robinson J., Gracey M., Peterson D., Partridge K., (1984), Isolation of *Aeromonas hydrophila* from a metropolitan water supply:Seasonal correlation with clinical isolates, Appl Environ Microbiol, **48**, 361-366.

Cadenas E., (1995), Mechanisms of oxygen activation and reactive oxygen species detoxification, In Ahmad A. (Ed.), Oxidative stress and antioxidant defenses in biology, Chapman & Hall, England,1-61.

Cadenas E., (1989), Biochemistry of oxygen toxicity, Annu. Rev. Biochem., **58**, 79-110.

Carnahan A.M., Altwegg M.,(1996), 1. Taxonomy, In Austin B., Altwegg M., Gosling P.J., Joseph S. (eds), The genus *Aeromonas*, John Wiley & Son, England, 1-31.

Champsaur, H., Andremont, A., Mathieu, D., Rottam, E., Auzepy, P., (1982), Cholera-like illness due to *Aeromonas sobria*, J.Inf.Dis., **145**, 248-254.
Chester F. D., (1901), A manual of determinative bacteriology, New York: Macmillan, 235.

Chart, H., Shaw, D.H., Ishiguro, E.E., Trust, T.J., (1984), Structural and immunochemical homogeneity of *Aeromonas salmonicida* lipopolysaccharide, J.Bact., **158**, 16-22.

Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P. C., Stewart, G. S. A. B., Williams, P., Bycroft, B. W., (1993), Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of N-(3-oxhexanoyl)-$_L$-homoserine lactone, J. Antibiot., **46**, 441-454.

Chopra, A.K., Houston, C.W., Peterson, J.W., Jin, G.F.,(1993), Cloning, expression and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*, Can.J.Microbiol., **39**, 513-523.

Colwell, R. R., MacDonell, M. T., De Ley, J., (1986), Proposal to recognize the familly *Aeromonadaceae* fam. nov., Int. J. Syst. Bact., **36**, 473-477.

Covert, T. C., Rice, E. W., Johnson, S. A., Berman, D., Johnson, C. H., Mason, P., J., (1992), Comparing defined-substrate Coliform tests for the detection of *Escherichia coli* in water, J. AWWA, 98-104.

Cowburn, J.K., Goodall, T., Fricker, E.J., Walter, K.S., Fricker, C.R., (1994), A preliminary study of the use of Colilert for water quality monitoring, Lett. Appl. Microbiol., **19**, 50-52.

Crockford, A. J., Davis, G. A., Williams, H. D., (1995), Evidence for cell-desity-dependent regulation of catalase activity in *Rhizobium leguminosarum* bv. *phaseoli*, Microbiol., **141**, 843-851.

Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C. K., Chatterjee, A. K., (1995), Identification of a global repressor gene, *rmsA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, N-(3-oxhexanoyl)-$_L$-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp., J. Bact., **177**, 5108-5115.

Davies, K. J. A., (1995), Oxidative stress: The paradox of aerobic life, In Rice-ewans, C., Halliwell, B., Lunt, G. G. (eds), Free radicals and oxidative stress: Environment, drugs and food additives, Biochem. Soc. Symp. 61, Portland press, 1-31.

Demple, B., Halbrook, J., (1983), Inducible repair of oxidative DNA damage in *Escherichia coli*, Nature, **304**, 466-468.

Demple, B., and Amabile-Cuevas, C.F., (1991), Redox redux: the control of oxidative stress responses. Cell, **67**, 837-839.

Demple, B., (1991), Regulation of bacterial oxidative stress genes. Ann. Rev. Gen., **25**, 315-337.

Demple, B., Levin, J. D., (1991), Repair systems for radical-damaged DNA, In Sies H. (ed.), Oxidative stress: oxidants and antioxidants, New York: Academic press, 119-154.

Demple, B., and Harrison, L. (1994). Repair of oxidative damage to DNA: Enzymology and biology, Ann. Rev. Bioch., **63**, 915-948.

Dooley, J.S.G., McCubbin, W.D., Kay, C.M., Trust, T.J., (1988), Isolation and biochemical characterization of the S-layer protein from a pathogenic *Aeromonas hydrophila* strain, J.Bact., **170**, 2631-2638.

Dooley, J.S.G., Lallier, R., Shaw, D.H., Trust, T.J., (1985), Electrophoretic and immunochemical analyses of the lipopolysaccharides from various strains of *Aeromonas hydrophila*, J.Bact., **164**, 263-269.

Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H., Oppenheimer, N.J., (1981), Structural identification of autoinducer of *Photobacterium fisheri* luciferase, Biochem., **20**, 2444-2449.

Eberl, L., Winson, M. K., Sternberg, C., Stewart, G. S. A. B., Christiansen, G., Chhabra, S. R., Bycroft, B. Williams, P., Molin, S., Givskov, M., (1996), Involvement of N-acyl-$_L$-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*, Mol. Microbiol., **20**, 127-135.

Edberg, S. C., Allen, M. J., Smith, D. B., the national collaborative study, (1988), National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: Comparison with the standard multiple tube fermentation method, Appl. Env. Microbiol., **54**, 1595-1601.

211

Edberg, S. C., Allen, M. J., Smith, D. B., the national collaborative study, (1989), National field evaluation of a defined substrate method for the simultaneous detection of total Coliforms and *Escherichia coli* from drinking water: Comparison with the presence-abscence techniques, Appl. Env. Microbiol., **55**, 1003-1008.

Eddy B.P., (1960), Cephalotrichous, fermentative Gram-negative bacteria: The genus *Aeromonas*, J Appl Bacteriol, **23**, 216-249.

Eddy B.P., Carpenter K.P., (1964) Futher studies on *Aeromonas*. II. Taxonomy of *Aeromonas* and C27 strains, J Appl Bacteriol, **27**, 96-109.

Ellis, R. J., Van der Vies, S. M., (1991), Molecular chaperones, Ann. Rev. Biochem., **60**, 321-347.

Engebrecht, J., Nealson, K., Silverman, M., (1983), Bacterial bioluminescence: Isolation and genetic analysis of functions from *Vibrio fisheri*, Cell, **32**, 773-781.

Engebrecht, J., Silverman, M., (1984), Identification of genes and gene products necessary for Bacterial bioluminescence, Proc. Natl. Acad. Sci. USA, **81**, 4154-4158.

Esteve,C., Amaro, C., Garay, E., Santos, Y., Toranzo, A.E., (1995), Pathogeneicity of live bacteria and extracellular products of motile *Aeromonas* isolated from eels, J. Appl. Bacteriol., **78**, 555-562.

Esteve,C, (1995), Numerical taxonomy of *Aeromonadaceae* and *Vibrionaceae* associated with reared fish and surrounding fresh and brackish water, System. Appl. Microbiol., **18**, 391-402.

Ewing W.H., Johnson J.G., (1960), The differentiation of *Aeromonas* and C27 strains from *Enterobacteriaceae*, Int Bull Bacteriol Numen Taxon, **10**, 223-230.

Ewing W.H., Hugh R., Johnson J.H., (1961), Studies on the *Aeromonas* group, Atlanta GA: Centers for disease control, 1-37.

Farmer, J.J., Arduino, M.J., Hickman-Brenner, F.W., (1992), The genera *Aeromonas* and *Plesiomonas*. In Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H., (eds), The Prokaryotes, second edition, vol 3. New York: Springer verlag, 1992: 3012-3045.

Farr, S. B., Kogoma, T., (1991), Oxidative stress response in *Escherichia coli* and *Salmonella typhimurium*, Miocrobiol. Rev., **55**, 561-585.

Flahaut, S., Laplace, J-M., Frère,J., Auffray, Y., (1998), The oxidative stress response in *Enterococcus faecalis*: relatioship between H_2O_2 tolerance and H_2O_2 stress, Lett. Appl. Microbiol., **26**, 259-264.

Flavier, A. B., Ganov-Raeva, L. M., Schell, M. A., Denny, T. P., (1997), Hierarchical autoinduction in *Ralstonia solanacearum*: Control of Acyl-homoserine lactone production by a novel autoregulatory sustaem responsive to 3-Hydroxypalmitic methyl ester, J. Bact., **179**, 7089-7097.

Flavier, A. B., Schell, M. A., Denny, T. P., (1998), An RpoS (σ^S) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*, Mol. Microbiol., **28**, 475-486.

Flint, D.H., Tuminello, J.F., Emptage, M.H., (1993), The inactivation of Fe-S cluster containing hydro-lyases by superoxide, J. Biol. Chem., **268**, 22369-22376.

Fricker, E. J., Fricker, C.R., (1994), Application of the polymerase chain reaction to the identification of *Escherichia coli* and coliforms in water, Lett. Appl. Microbiol., **19**, 44-46.

Fricker, C. R., Tompsett, S., (1989), *Aeromonas* spp. In foods: A significant cause of food poisoning?, Int. J. Food Microbiol., **8**, 17-23.

Fuqua, C., Winans, S., Greenberg, E. P., (1996), Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators, Ann. Rev. Microbiol., **50**, 727-751.

Gahnem E.H., Mussa M.E., Eraki H.M.,(1993), *Aeromonas*-associated gastro-enteritis in Egypt, Zentralbl Mikrobiol, **148**, 441-447.

Gauthier, M.J., Clément, R.L., (1994), Effect of a short period of starvation in oligotrophic waters on the resistance of enteric bacterial pathogens to gastric pH conditions, FEMS microbiol.ecol., **14**, 275-284.

Gavriel, A.A., Landré, J.B.P., Lamb, A.J., (1998), Incidence of mesophilic *Aeromonas* within a public drinking water supply in the north east Scotland, J. Appl. Microbiol., **84**, 383-392.

Gavriel, A.A., Landré, J.B.P., Lamb, A.J., (1997), Oxidative stress response of mesophilic *Aeromonas*, In Morris R, Gammie A.(eds.), Proceedings of the 2 nd UK symposium on Health related water microbiology, 157-169.

Goldstein, S., Czapski, G., (1986), The role and mechanism of metal ions and their complexes in enhancing damages in biological systems or in protecting these systems from the toxicity of O_2^-, J. Free Rad. Biol. Med., **2**, 3-11.

Gonzalez-Flecha, B., Demple, B., (1997), Transcriptional regulation of *Eschcerichia coli* oxyR gene as a function of cell growth, J. Bact., **179**, 6181-6186.
Goodson, M., Rowbury, R.J., (1989a), Habituation to normally lethal acidity by prior growth of *Escherichiae coli* at a sublethal acid pH value, Lett. Appl.Microbiol., **8**, 77-79.

Goodson, M., Rowbury, R.J., (1989b), Resistance of acid-habituated *Escherichiae coli* to organic acids and its medical and applied significance, Lett. Appl.Microbiol., **8**, 211-214.

Gorden , J., Small, P.L.C., (1993), Acid resistance in enteric bacteria, Infect.immun, **61**, 364-367.

Gosling, P., (1997), Public health significance of *Aeromonas* species in drinking water, In Morris R, Gammie A.(eds.), Proceedings of the 2 nd UK symposium on Health related water microbiology, 56-64.

Gosling, P., (1996), In Austin B., Altwegg M., Gosling P.J., Joseph S.(eds), The genus *Aeromonas*, John Wiley & Son, England, 245-265.

Gosling,P., Turnebull, P.C.B., Lightfoot, N.F., Pether, P.V.S., Lewis, R.J., (1993), Isolation and purofication of *Aeromonas sobria*cytotonic enterotoxin and ß-haemolysin, J. Med. Microbiol., **38**, 227-234.

Gray, K. M., Pearson, J. P., Downie, A., Boboye, B. E. A., Greenberg, E. P., (1996), Cell-to-cell signalling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: Autoinduction of stationary phase and rhizosphere-expressed genes, J. Bact., **178**, 372-376.

Gross, C. A., (1996), 88.Function and regulation of the heat-shock proteins, In *Escherichia coli* and *Salmonella* cellular and molecular biology, Neihardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Brooks Low, K., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E. (eds.), Washington D. C.: ASM Press, **1**, 1382-1512.

Haque, Q.M.,Sugiyama, A., Iwade, Y., Midorikawa, Y., Yamauchi, T., (1996), Diarrheal and environemtal isolates of *Aeromans* spp. produce a toxin similar to Shiga-like toxin 1,Cur. Microbiol., **32**, 239-245.

Havelaar A.H.,Versteegh J.M.F., During M., (1990), The presence of *Aeromonas* in drinking water supplies in the Netherlands, Zentralbl Hyg, **190**, 236-256.

Hazen T.C., (1983), A model for the density of *Aeromonas hydrophila* in Abermarle Sound, North Carolina, Microb Ecol, **9**, 137-153.

Hazen T.C., Fliermans C.B., Hirsch R.P., Esch G.W., (1978), Prevalence and distribution of *Aeromonas hydrophila* in the United States, Appl Environ Microbiol, **36**, 731-738.

Hengge-Aronis, R., (1996), Back to log phase: σ^{S} as a global regulator in the osmotic control of gene expression in *Escherichia coli*, Mol. Microbiol., **21**, 887-893.

Hengge-Aronis, R., (1993), Survival of hunger and stress: The role of *rpoS* in early stationary phase gene regulation in *E. coli* , Cell, **72**, 165-168.

Hill K.R., Caselitz F.H., Moody L.M., (1954), A case of acute metastasic myosistis caused by a new organism of the family *Pseudomonadaceae*: a preliminary report, W Ind Med, **3**, 9-11.

Hitchcock, P.J., Leive, L., Mäkelä, P.H., Rietschel, E.T., Strittmatter, W., Morrison, D.C., (1986), Lipopolysaccharide nomenclature-past, present and future, J.Bact., **166**, 699-705.

Holmes B., (1992), International committee on systematic bacteriology subcommitte on taxonomy of *Vibrionaceae*, Intern. J. Syst. Bact., **42**, 1, 199-201.

Holmes P., Nicolls L.M., Sartory D.P.,(1996), 1. The ecology of mesohilic *Aeromonas* in the aquatic environment, In Austin B., Altwegg M., Gosling P.J., Joseph S.(eds). The genus *Aeromonas*, John Wiley & Son, England, 127-150.

Holmes P., Niccolls L.N., (1995), Aeromonads in drinking water supplies-their occurrence and significance, J Chartered Inst Water Environ Manage, **5**, 464-469.

Howard, P.S.,Macintyre, S., Buckley, T.J., (1996), Toxins, In Austin B., Altwegg M., Gosling P.J., Joseph S.(eds), The genus *Aeromonas*, John Wiley & Son, England, 267-286.

Howard, P.S., Buckley, T.J., (1985a), Activation of the hole-forming toxin aerolysin by extracellular processing, J. Bacteriol,**163**, 336-340.
Howard, P.S., Buckley, T.J., (1985b), Protein export by a Gram negative bacterium: Production of Aerolysin by *Aeromonas hydrophila*, J. Bacteriol,**161**, 1118-1124.

Huisman, G. W., Kolter, R., (1994), Sensing starvation: A homoserine lactone-dependent signalling pathway in *E. coli* , Science, **265**, 537-539.

Huys G., Kämpfer, P., Altwegg, M., Kersters, I., Lamb, A., Coopman, R., Lüthy-Hottenstein, J.,Vancanneyt, M., Janseen, P., Kersters, K., (1997), *Aeromonas popoffii sp. nov.*, a mesophilic bacterium isolated from drinking water production plants and reservoirs, Int. J. Syst. Bact., **47**, 1165-1171.

Huys G., Coopman R., Janssen P., Kersters K., (1996), High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting, Int. J. Syst. Bact., **46**, 572-580.

Huys G., Kersters I., Vancanneyt M., Coopman R., Janssen P., Kersters K., (1995), Diversity of *Aeromonas sp.* in Flemish drinking water production plants as determined by gas-liquid chromatographic analysis of cellular fatty acid methyl esters (FAMEs), J Appl Bacteriol, **78**, 445-455.

Hwang, I., Li, P-L., Zhang, L., Piper, K. R., Cook, D., Tate, M. E., Farrand, S. K., (1994), TraI, a LuxR homologue, is responsible for the production of conjugated factor, the Ti plasmid N-acylhomoserine lactone autoinducer, Proc. Natl. Acad. sci. USA, **91**, 4639-4643.

Imlay, J. A., Chin, S. M., Linn, S., (1988), Toxic damages by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*, Science, **240**, 640-642.

Janda, M.J., Abbott, S.L., (1996), 6. Human Pathogens, In Austin B., Altwegg M., Gosling P.J., Joseph S.(eds), The genus *Aeromonas*, John Wiley & Son, England, 151-173.

Janda, J.M., (1991), Recent advances in the study of the taxonomy, pathogeneicity, and infectious syndromes associated with the genus *Aeromonas*, Clin Microbiol Rev, **4**, 397-410.

Janda, J., Duffey, P. S., (1988), Mesophilic Aeromonads in human disease: Current taxonomy, laboratory identification, and infectious disease spectrum, Rev. Infect. Dis., **10**, 980-997.

Jensen, K. F., Pedersen, S., (1990), Metabolic growth rate control in *Escherichia coli* may be a consequence of saturation of the macromolecular biosynthetic apparatus with substrates and catalytic components, Micorbiol. Rev., **54**, 89-100.

Kaiser, D., Losick, R., (1993), How and why bacteria talk to each other, Cell, **73**, 873-885.

Kaplan H.B., Greenberg, E.P., (1985), Diffusion of autoinducer is involved in regulation of the *Vibrio fisheri* luminescence system, J. Bact., **163**, 1210-1214.

Karem, K.L., Foster, J.W., and Bej, A.K., (1994), Adaptive acid tolerance response (ATR) in *Aeromonas hydrophila*, Microbiology, **140**, 1731-1736.

Karunakaran, T., Devi, B.G., (1994), Characterisation of haemolytic activity from *Aeromonas caviae*, Epidemiol.infect.,**112**, 291-298.

Kersters I., Huys G., Janssen P., Kersters K., Verstraete W., (1995), Influence of temperature and process technology on the occurrence of *Aeromonas sp.* and hygenic indicator organisms in drinking water production plants, 5th International *Aeromonas-Plesiomonas* Symposium, Edinburgh, Scotland, April 1995.

Keyer, K., Strohmeier, G., Imlay, J.A., (1995), Superoxide and the production of oxidative DNA damage, J. Bact., **177**, 6782-6790.

Khan, A. U., Kasha, M., (1994), Singlet molecular oxygen in the Haber-Weiss reaction, Proc. Natl. Acad. Sci. USA, **91**, 12365-12367.

Kjellberg, S., Flärdh, K.B.G., Nyström, T., Moriarty, J.W., (1993), Growth limitation and starvation of bacteria, In Kjellberg, S (ed.) Starvation in bacteria, Plenum press, N.Y., 289-320.

Klein, G., Walczak, R., Kranowska, E., Blaszczak, A., lipinska, B., (1995), Characterisation of heat-shock response of the marine bacterium *Vibrio harvyi*, Mol. Microbiol., **16**, 801-811.

Kluyver A.J., van Niel C.B., (1936), Prospects for a natural system of classification of bacteria, Zentralbl Backteriol Parasitenk Infectionsk Hyg Abt II, **94**, 369-403.

Knochel, S, (1990), Growth characteristics of motile *Aeromonas spp.* isolated from different environments, Inter. J. Food Microbiol., **10**, 235-244.

Knochel, S, (1991), Chlorine Resistance of motile *Aeromonas spp.*, Water Science Technology, **24**, 2, 327-330.

Kokka, R.P., Vedros, N.A., Janda, M.J., (1991), Characterisation of classic and atypical serogroup O:11 *Aeromonas*: evidence that the surface array proteun is not directly involved in mousse pathogenicity, Micro.Path., **10**, 71-79.

Kolb, A., Busby, S., Buc, H., Garges, S., Adhya, S., (1993), Transcriptional regulation by cAMP and its receptor protein, Ann. Rev. Biochem., **62**, 749-795.

Kolter, R., Siegele, D. A., Tormo, A., (1993), The stationary phase of the bacterial life circle, Ann. Rev. Microbiol., **47**, 855-874.

Krovacek K., Faris A., Baloda S.,B., Lindberg T., Peterz M., Mansson I., (1992), Isolation and virulence profile of *Aeromonas* spp. from different municipal drinking water supplies in Sweden, Food microbiol, **9**, 215-222.

Lafiti, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S. A. B., Lazdunski, A., Williams, P., (1995), Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PA01, Mol. Microbiol., **17**, 333-343.

Lafiti, A., Foglino,.Tanaka, K., Williams, P., Lazdunski, A, (1996), A hierarchical quorum sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS, Mol. Microbiol., **21**, 1137-1146.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., Hartl, U. F., (1992), Successive action of DnaK, DnaJ, and GroEL along the pathway of chaperone-mediated protein folding, Nature, **356**, 683-689.

Lindquist, S., (1986), The heat-shock response, Ann. Rev. Biochem., **55**, 1151-1191.

Ljungh,A., Wadström, T., (1986), 16. *Aeromonas* Toxins, In Pharmacology of bacterial toxins, Dorner, F., Drews, J. (eds.), New York: Pergamon, 289-305.

Losick, R., Kaiser, D., (1997), Why and How bacteria communicates, Scientific American, 52-57
Loewen P.C., Hengge-aronis, R., The role of the sigma factor σ^S (KatF) in bacterial global regulation, Ann.Rev. Microbiol., **48**, 53-80.

Matin, A., Auger, E.A., Blum, P.H., Schultz, J.E., (1989), Genetic basis of starvation survival in nondifferentiating bacteria, Ann. Rev. Microbiol., **43**, 293-316.

MacKerness, C. W., Colbourne, J. S., Dennis, P. L. J., Rachwal, T., Keevil, C. W., (1993), Formation and control of Coliform biofilms in drinking water distribution systems, In Society for Applied Bacteriology Technical Series ed. Deyner, **30**, 217-227.

McGowan, S., Sebaihai, M., Jones, S., Yu, B., Bainton, N. Chan, P.F., Bycroft, B., Stewart, G.S.A.B., williams, P., Salmond, G.P.C., (1995), Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator, Microbiol., **141**, 541-550.

McKenna, S. M., Davies, K. J., A., (1988), Bacterial killing by phagocytes: Potenyial role (s) of hypochlorous acid and hydrogen peroxide in protein turnover, DNA synthesis, and RNA synthesis, Basic life Sci., **49**, 829-832.

Mager W.H., De Kruijff, A. J. J., (1995), Stress-Induced Transcriptional Activation, Microbiol. Rev., **59**, 506-531.

Mathewson J.J., Dupont H.L., (1992), *Aeromonas* Species: role as human pathogens. In Remington J.S., Swartz M. N., (eds) current clincal topics in infectious Diseases, Vol 12. Cambridge: Blackwell scientific, 26-36.

Mehus J., Peeters P., (1989), Preventive and corrective actions to cope with *Aeromonas* growth during treatment, Water supply, 7, 10-1-10-4.

Meister, A., Anderson, M. E., (1983), Glutathione, Ann. Rev. Biochem., 52, 711-760.

Miranda, C.D., Castillo, G., (1996), Isolation and characterisation of motile Aeromonads from Chilian freshwater and their potential use as water quality indicators, Env. Toxicol. Wat. Qual., 11, 91-98.

Monfort P., Baleux B., (1991), Distribution and survival of motile *Aeromonas spp.* In brackish water recieving sewage treatment effluent, App. Env. Microbiol., 57, 2458-2467.

Monfort P., Baleux B., (1990), Dynamics of *Aeromonas hydrophila, Aeromonas sobria, Aeromonas caviae* in sewage treatment pond, Appl Environ Microbiol, 56, 1999-2006.

Morita, R. Y., (1993),1. Bioavailability of energy and the starvation state, In S.Kejelleberg (ed.), Starvation in bacteria, Plenum press, N.Y.,1-23.

Murphy, H. S., Palejwala, V. A., Rahman, M. S., Dunman, P. M., Wang, G., Humayn, M. Z., (1996), Role of mismatch repair in the *Escherichia coli* UVM response, J. Bact., 178, 6651-6657.

Namdari H., Bottone E.J., (1990a), Microbiologic and clinical evidence supporting the role of *Aeromonas caviae* as a pediatric enteric pathogen, J.Clin. Microbiol., 28, 837-840.

Namdari H., Bottone E.J., (1990b), Cytotoxin and enterotoxin production as factors delineating enteropathogeneicity of *Aeromonas caviae*, J.Clin. Microbiol., 28, 1796-1798.

Naberhaus, F., (1999), negative regulation of bacterial heat-shock genes, Mol. microbiol., 31, 1-8.

Nealson, K. H., Platt, T., Hasting, J. W., (1970), Cellular control of the synthesis and activity of the bacterial luminescencent system, J. Bact., 104, 313-322.

Nichols, G. L., Lightfoot, N. F., de Louvois, J., Monsey, H., (1996), Health significance of bacteria in distribution systems, Review on *Aeromonas*, UK water Industry Research Limited, 1-37.

Nichols, G. L., Lightfoot, N. F., de Louvois, J.,Adak, G. K., (1997), Prevalence study of specific bacterial infections- Geographical distribution of *Aeromonas* and *Yersinia* illness in the UK, UK water Industry Research Limited, 1-19.

Nunoshiba, T., Hidalgo, E., Amabile-cuevas, C. F., Demple, (1992), Two stage control of an oxidative stress regulon: The *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene, J. Bact., 174, 6054-6060.

Nyström, T., Flärdh, K., Kjelleberg, S., (1990), Response to multiple-nutrient starvation in marine Vibrio sp. strain CCUG 15956, J. Bact., **172**, 7085-7097.

Nyström, T., (1993), 6.Global systems approach to the physiology of the starved cell, In S.Kejelleberg (ed.), Starvation in bacteria, Plenum press, N.Y., 129-150.

Palumbo S.A., Morgan D.R., Buchanan R.L., (1985), Influence of temperature, NaCl, and pH on the growth of Aeromonas hydrophila, J Food Sci, **50**, 1417-1421.

Palumbo S.A., Abeyta, C., (1992), 30. Aeromonas hydrophila group, In Vanderzant, C., Splittstoesser, D. F., (eds.), Compremedium of methods for the microbiological examinations of foods, 3rd edition, American Public Health Association, Washington D. C., 497-511.

Parker, M.W., Buckley,T.J., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F., Tsernoglou, D., (1994), Structure of the Aeromonas toxin proaerolysin in its soluble and membrane-channel states, Nature, **367**, 292-295.

Parker, M.W., Van der Goot, F.G., Buckley,T.J., (1996), Aerolysin-the ins and outs of a model channel-forming toxin, Mol. Microbiol., **19**, 205-212.

Pathak S.P., Bhattacherjee J.W., Kalra N., Chandra S., (1988), Seasonal distribution of Aeromonas hydrophilain river water and isolation from river fish, J. Appl. Bacteriol., **65**, 347-352.

Picard, B., Arlet, G., Goullet, P., (1984), Septicémies à Aeromonas hydrophila, aspects épidémiologiques. Quinze observations, La Press Méd., **13**, 1203-1205.

Pickett, C. B., Lu, A. Y. H., (1989), Glutathione S-transferases: gene structure, regulation, and biological function, Ann. Rev. Biochem., **58**, 743-764.

Poirier, I. Maréchal, P.A., Evrard, C., Gervais, P., (1998), Escherichia coli and Lactobacillus plantarum resposnes to osmotic stress, Appl. Microbiol. Biotechnol., **50**, 704-709.

Popoff M., (1984), Genus III. Aeromonas.Kluyver and van Niel 1936,398^{AL}. In Krieg N.R., Holt J.G., (eds), Bergey's Manual of Systematic Bacteriology, **1**, Baltimore, MD: William and Wilkins, 545-552.

Popoff M., Coynault C., Kiredjian M., Lemelin M., (1981), Polynucleotide sequence relatedness among motile Aeromonas species, Curr Microbiol, **5**, 109-114.

Popoff, M., Véron, M., (1976), A taxonomic study of the Aeromonas hydrophila-Aeromonas punctata group, J Gen Microbiol, **94**, 11-22.

Reeve, C.A., Amy, P.S., Matin, A., (1984), Role of protein synthesis in the survival of carbon-starved Escherichia coli K-12, J. Bact., **160**, 1041-1046.

Richard D.C. (1991), Cours de microbiologie des Boissons, Produits Agricoles et Produits de la Mer: *Vibrio cholerae*, *Vibrio* de Toxi-infection Alimentaire, *Pleisiomonas*, *Aeromonas*: Taxonomie et identification, Institut Pasteur de Lille.

Robson, N. D., Cox, A. R. J., McGowan, S. J. Bycroft, B. W., Salmond, G. P.C., (1997), Bacterial N-acyl-homoserine-lactone-dependent signalling and its potential biotechnological applications, TIBTECH., **15**, 458-464.

Rouf M.A., Rigney M.M., (1971), Growth temperatures and temperature characteristics of *Aeromonas*, Appl Microbiol, **22**, 503-506.

Salmond,C.V., Kroll, R.G., Booth, I.R., (1984), The effect of food preservatives on pH homeostatis in *Escherichiae coli*, J.Gen.Microbiol., **130**, 2845-2850.

Sanarelli G., (1891), Über einen neuen Mikroorganismus des wassers, welcher für Thiere mit veränderlicher und konstanter Temperatur pathogen ist, Zentralbl Bakt Parasitenk, **9**, 9-11.

Sancar, A., Sancar, G.B., (1988), DNA repair enzymes, Ann. Rev. Biochem., **57**, 29-67.

Schubert R.H.W. (1991), Aeromonads and their significance as potential pathogens in water, Journal of Applied Bacteriology Symposium Supplement, **70**, 131S-135S.

Seed, P. C., Passador, L., Iglewski, B. H., (1995), Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and *Pseudomonas* PAI: an autoinduction regulatory hierachy, J. Bact., **177**, 654-659.

Seidler, R. J., Allen, D. A., Lockman, H., Colwell, R. R., Joseph, S. W., Daily, O. P., (1980), Isolation, enumeration, and characterization of *Aeromonas* from polluted waters encountered in diving operations, Appl. Env. Microbiol., **39**, 1010-1018.

Siegele, D.A., Kolter R., (1992), Life after log, J. Bact., **174**, 345-348.

Sisti, M., Albano, A., Brandi, G., (1998), Bactericidal effect of chlorine on motile *Aeromonas spp.* in drinking water supplies and influence of temperature on disinfection efficacy, Lett. Appl. Microbiol., **26**, 347-351.

Sloan, D., (1995), The epidemiology of aeromonads infections in Scotland in 1994-more questions than answeres, Scot. Cent. Infect. Env. Helth Week. Rep., **29**, 134-136.

Snieszko S.F., (1957), Genus IV. *Aeromonas* Kluyver and van Niel, 1936. In Breed R.S., Murray E.G.D., Smith N.R. (eds), Bergey's Manual of Determinative Bacteriology, 7 th Edn. Baltimore: Williams and Wilkins, 189.

Son, R., Rusul, G., Sahilah, A. M., Zainuri, A., Raha, A. R., Salmah, I., (1997), Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish *Telapia (Telapia mossambica)*, Lett. Appl. Microbiol. **24**, 479-482.

Soto G.E., Hultgren, S.J., (1999), Bacterial adhedins: Common themes and variations in achitecture and assembly, J.Bact., **181**, 1056-1071.

Stecchini M.L., Domenis C., (1994), Incidence of *Aeromonas* species in influent and effluent of urban wastewaters purification plants, Lett Appl Microbiol, **19**, 237-239.

Storz, G., Tartaglia, L.A., Farr, S.B., Ames, B.N., (1990), Bacterial defenses against oxidative stress, TIG, **6**, 363-368.

Swift, S., Throup, J. P., Williams, P., Salmond, G. P. C., Stewart, G.S.A.B., (1996), Quorum sensing: A population density component in the determination of bacterial phenotype, TIBS, **21**, 214-219.

Swift, S., Karlyshev, A.V., Fish, L., Durant, E., L., Winsdon, M., K., Chhabra, S., R., Williams, P., Macintyre, S., Stewart, G.S.A.B., (1997), Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: Identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules, J. Bact., **179**, 5271-5281.

Toledano, M. B., Kullik, I., Trinh, F., Baird, P. T., Schneider, T. D., Storz, G., (1994), Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: A mechanism for differential promoter selection, **78**, 897-909.

VanBogelen, R.A., Kelley, P.M., Neidhart, F.C., (1987), Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Esherichia coli*, J. Bact., **169**, 26-32.

Van der Goot,G.F., Pattus, F., Parker, M., Buckley, T.J., (1994), The cytolytic toxin aerolysin: from the soluble form to the transmembrane channel, Toxicol., **87**, 19-28.

Van der Krooije D., Hijnen W.A.M., (1988), Nutritional versabilty and growth kinetics of an *Aeromonas hydrophila* strain isolated from drinking water, Appl Env. Microbiol, **54**, 2842-2851.

Van der Krooije D., (1991), Nutritional requirements of aeromonads and their multiplication in drinking water, Experientia, **47**, 444-446.

Visick, J. E., Clarke, S., (1995), Repair, refold, recycle: How bacteria can deal with spontaneous and environmental damage to proteins, Mol. Microbiol., **16**, 835-845.

Wadstrom T., and Ljungh, A., (1991), *Aeromonas* and *Plesiomonas* as Food-and waterborne pathogens, Int. J. Food Microbiol., **40**, 145-148.

Walter, K. S., Fricker, E. J., Fricker, C. R., (1994), Observations on the use of a medium detecting β-glucurosinadsase and lactose fermentation for the simultaneous detection of *Escherichia coli* and coliforms, Lett. Appl. Microbiol., **19**, 47-49.

Wu, J., Weiss, B., (1991), Two divergently transcribed genes, *soxR* and *soxS* control a superoxide response regulon of *Escherichia coli*, J. Bact., **173**, 2864-2871.
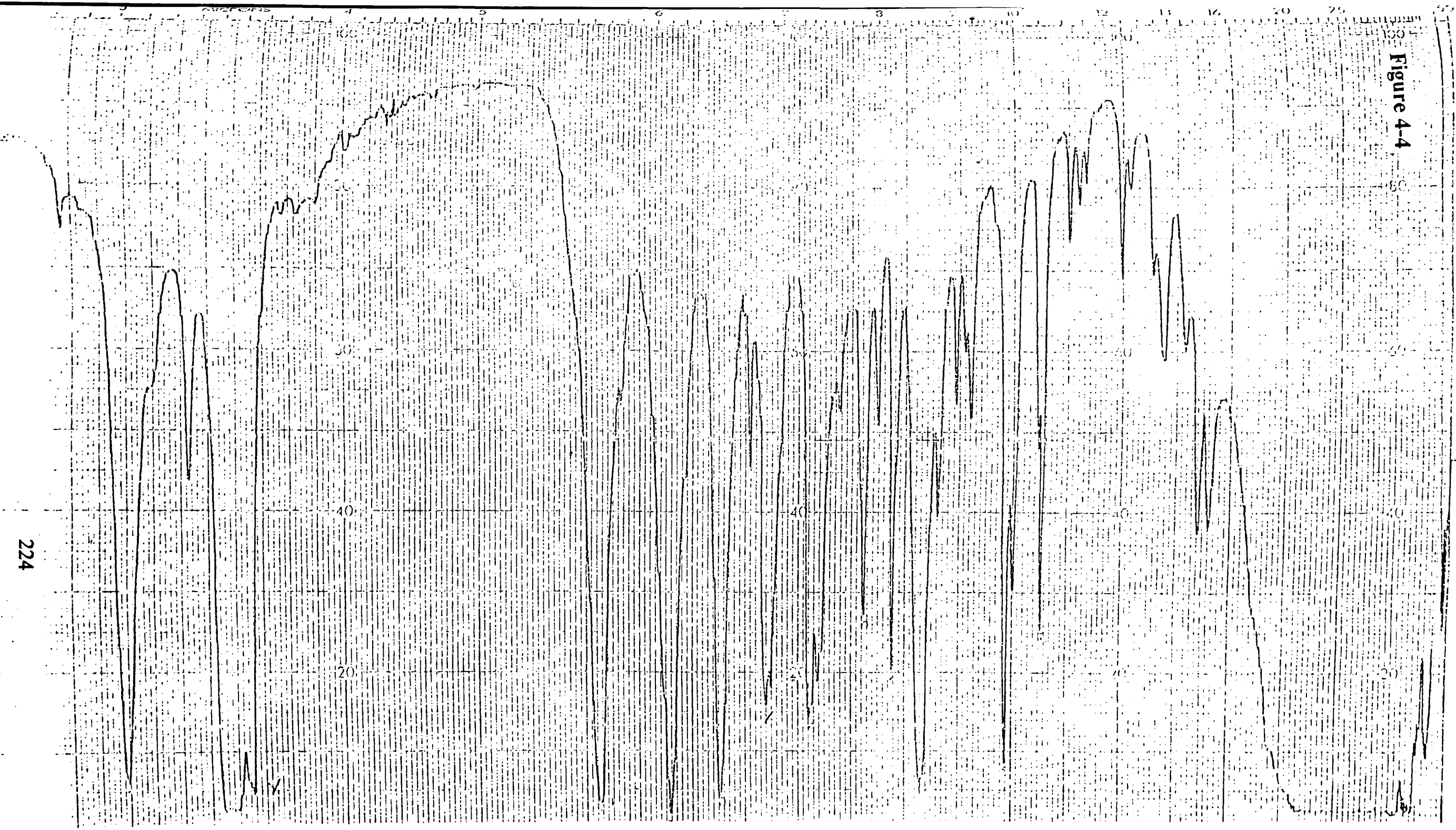
APPENDIX

IR Spectrum of BHL

BHL was grounded in Nujol. The resulting mixture was applied onto NaCl discs to obtain

a single layer film. Scanning was performed from 3500 to 200 nm.

Figure 4-5

Aldrich A4,450-9   CAS [6305-38-0] $C_4H_7NO_2$ 60 MHz: 1, 592D

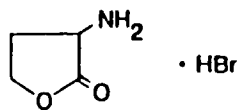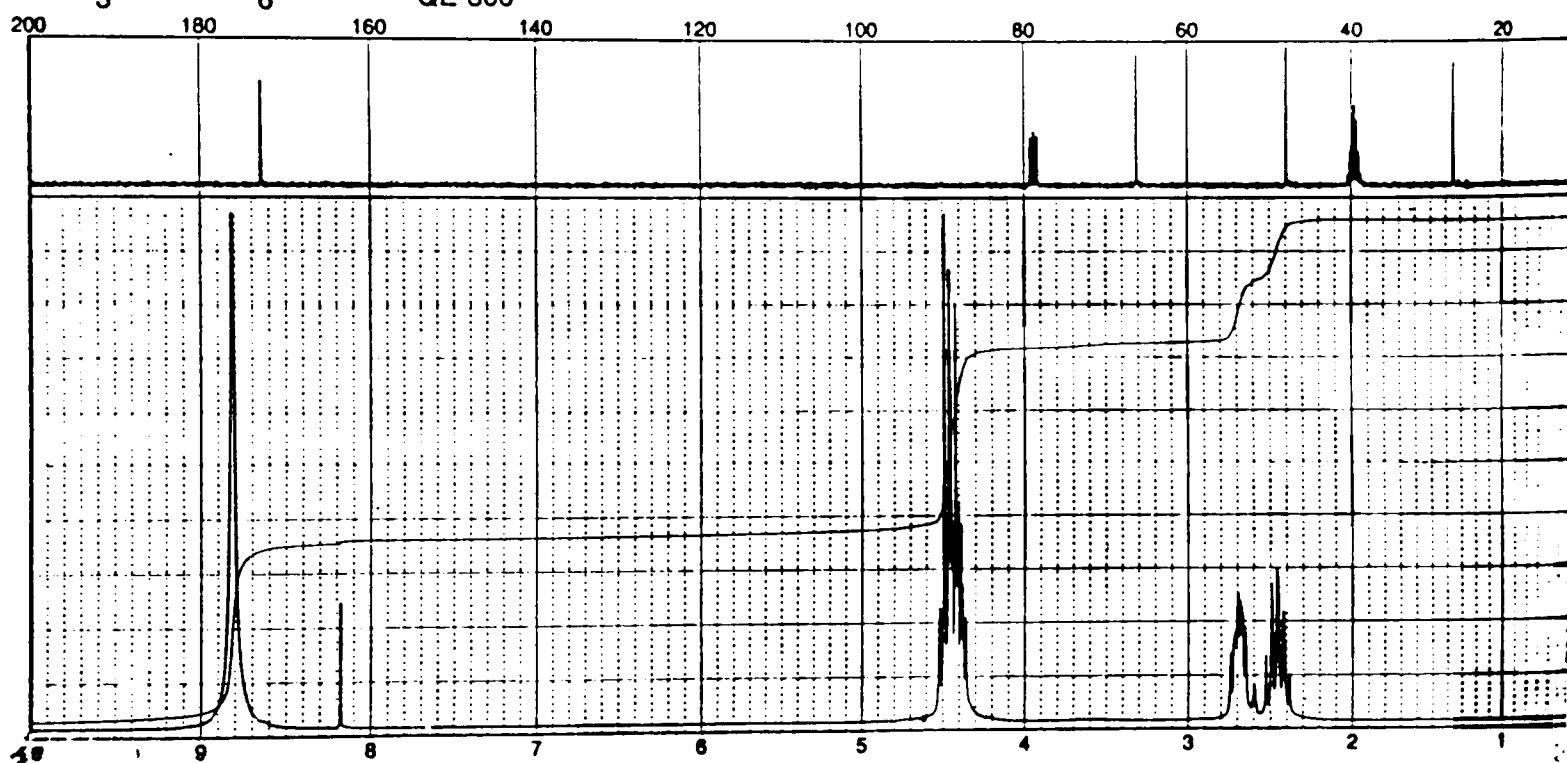(±)-α-Amino-γ-butyrolactone hydrobromide, 99%   FW 182.02   FT-IR: 1, 700C   mp 221°C d.
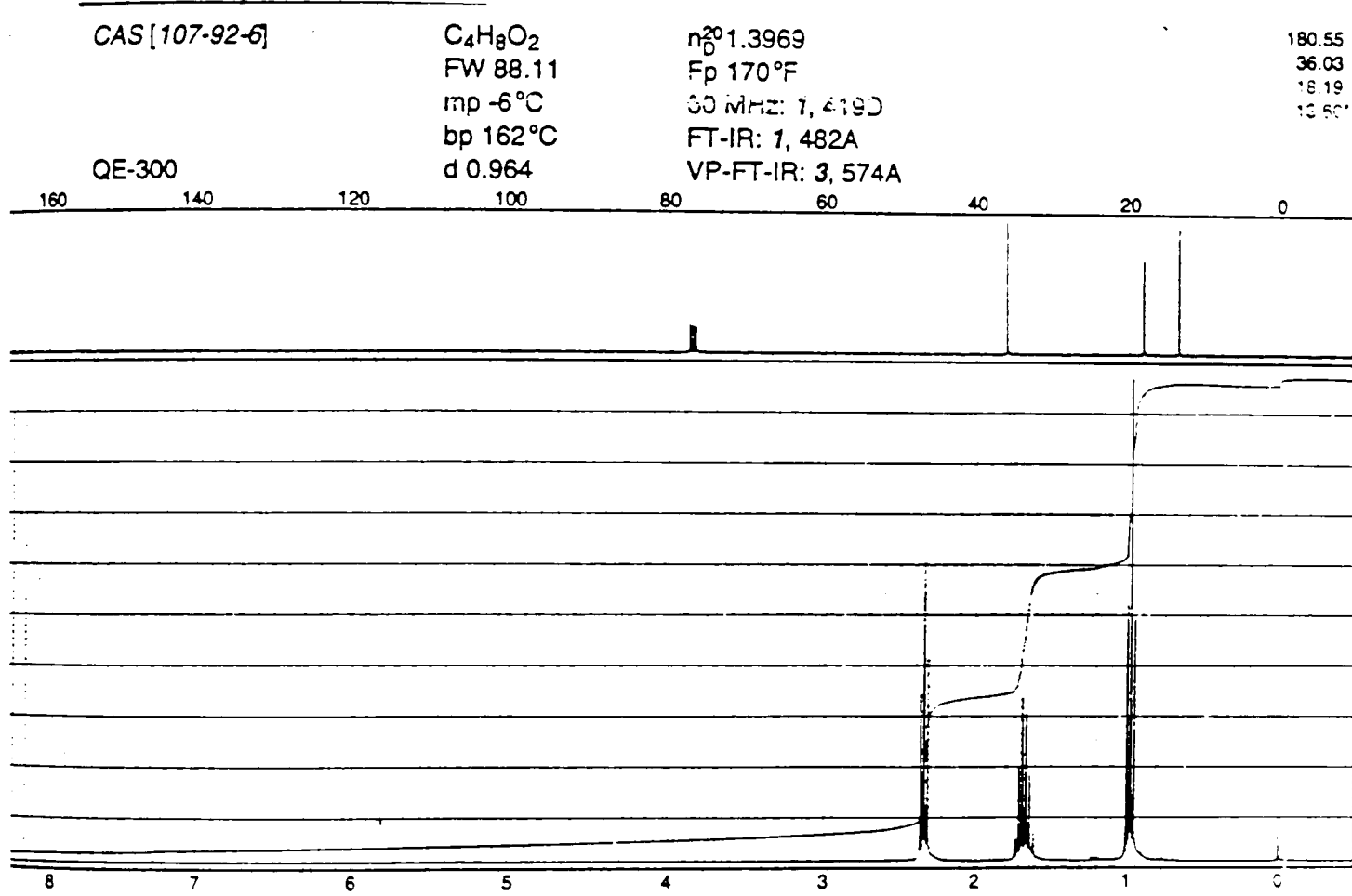
CDCl3 + DMSO-d_6 QE-300

NMR reference spectrum of α-Amino-γ-butyrolactone hydrobromide.

From The Aldrich Library of ^{13}C and ^1H FT NMR spectra, Volume 1, by Pouchet, C. J., Behnkes, Aldrich, 1993.
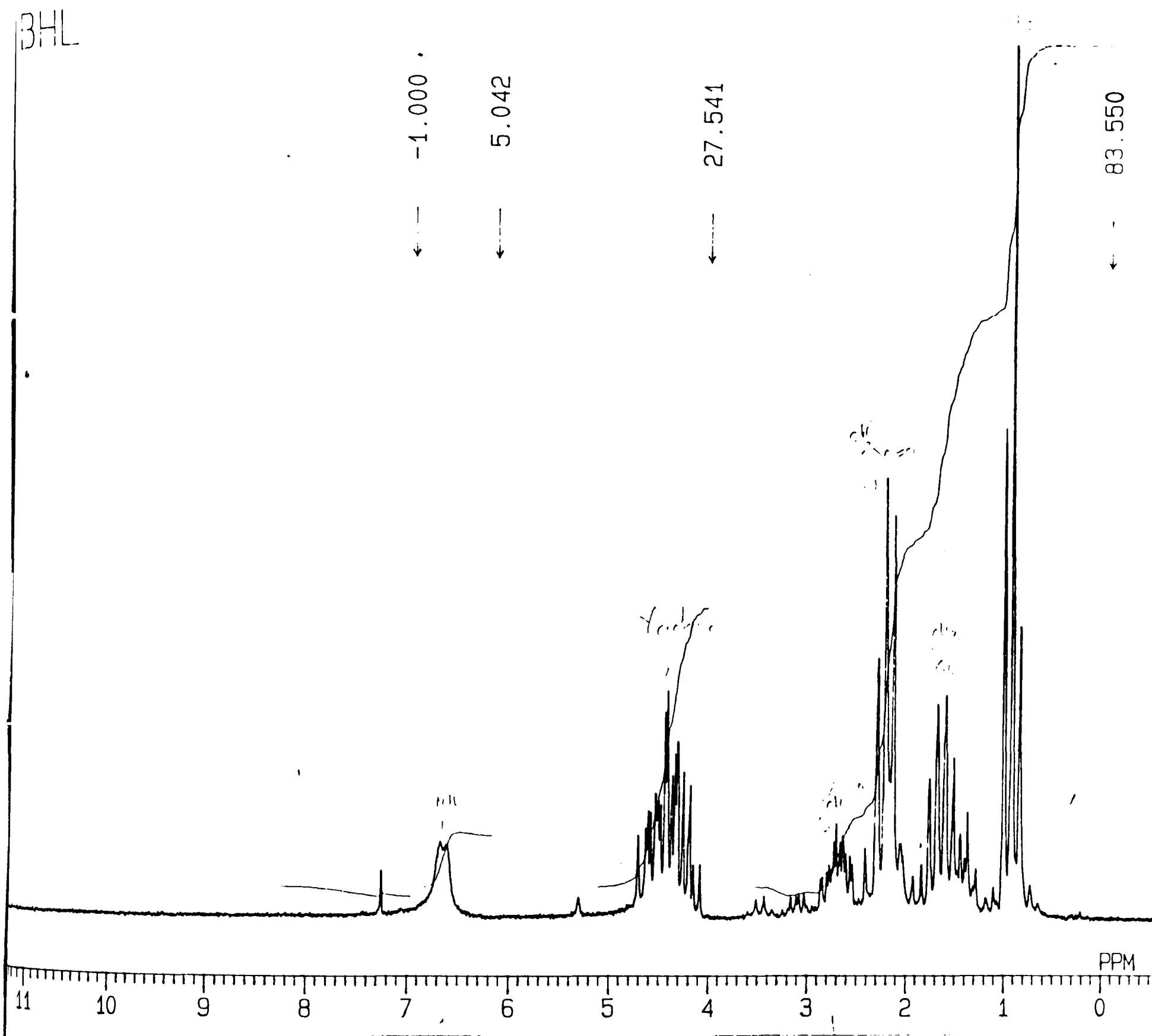
Figure 4-6

CAS [107-92-6] $C_4H_8O_2$ n_D^{20} 1.3969   180.55
                   FW 88.11   Fp 170°F   36.03

QE-300   mp -6°C   30 MHz: 1, 419D   18.19

                   bp 162°C   FT-IR: 1, 482A

                   d 0.964   VP-FT-IR: 3, 574A

NMR reference spectrum of Butyric acid.

From The Aldrich Library of ^{13}C and ^{1}H FT NMR spectra, Volume 1, by Pouchet, C. J., Behnkes, Aldrich, 1993.
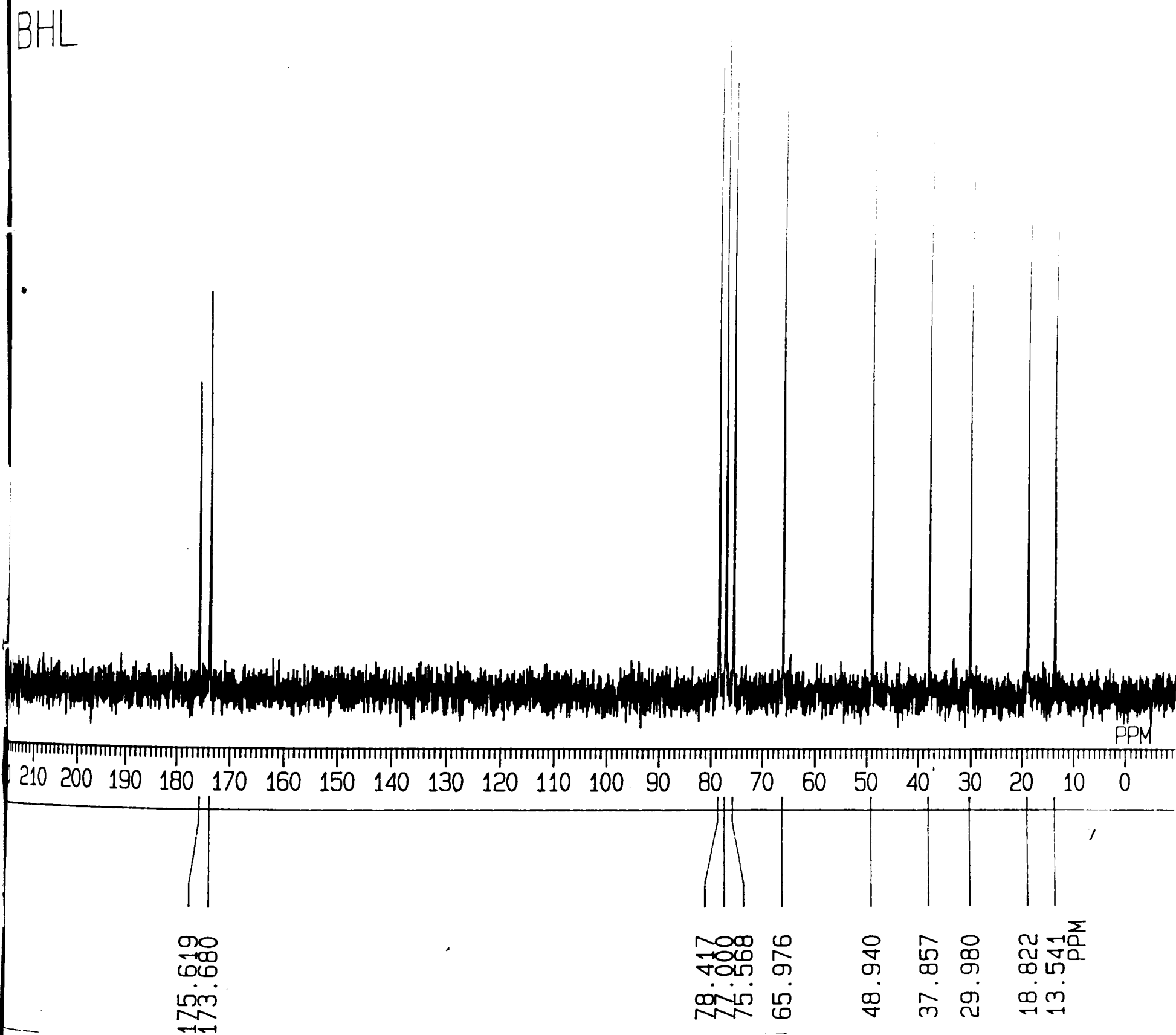
226

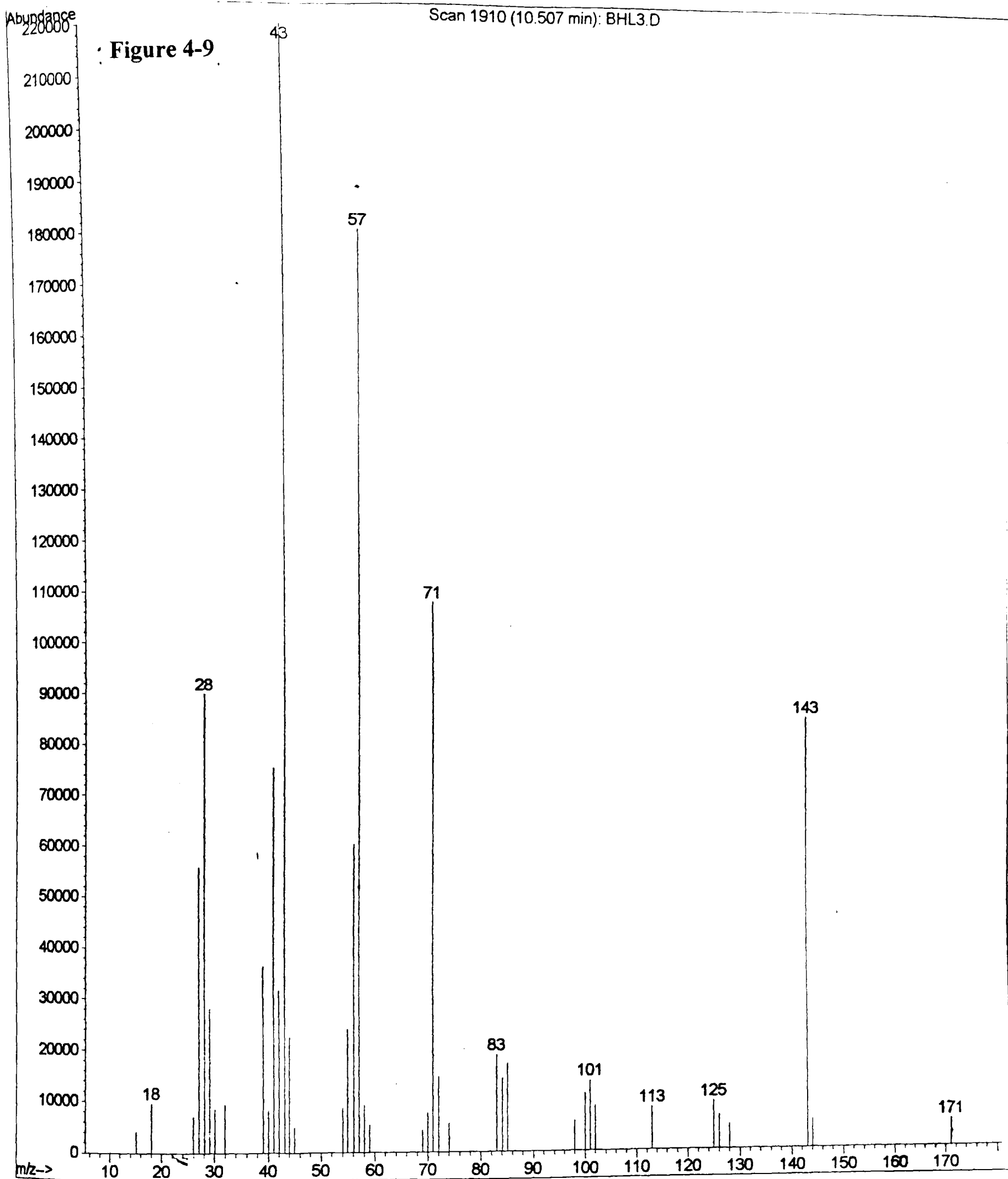Figure 4-7

¹H NMR spectrum of butyryl homoserine lactone.

50 mg of BHL was resuspended in CDCl₃ and the scan performed between 0 to 12 ppm at

a frequence of 1800.5 Hz.

Figure 4-8

^{13}C NMR spectrum of butyryl homoserine lactone.

50 mg of BHL was resuspended in CDCl$_3$ and the scan performed between 0 to 12 ppm at

a frequence of 5500.6 Hz.

Figure 4-9

GC-MS spectrum of butyryl homoserine lactone.

1 μl of a concentrated BHL solution was injected. Decomposition of BHL gave rise to

several ions between 0 to 180 m/z.

Published Material

False-positive coliform reaction mediated by *Aeromonas* in the Colilert defined substrate technology system

J.P.B. Landre, A.A. Gavriel[1]* and A.J. Lamb

School of Applied Sciences, The Robert Gordon Univeristy, Aberdeen and [1]The North of Scotland Water Authority, Turriff Water Treatment Works, Turriff, UK

J.P.B. LANDRE, A.A. GAVRIEL AND A.J. LAMB. 1998. The Colilert defined substrate technology system allows specific, one-step detection of both coliforms and *Escherichia coli* while claiming to suppress the influence of non-coliform heterotrophs. The Colilert assay was examined in order to determine whether organisms from the genus *Aeromonas* could interfere and cause production of a false-positive coliform result as aquatic *Aeromonas* are known to constitute a fraction of the heterotrophic population found in drinking water. Results obtained clearly demonstrate that *Aeromonas* sp. can elicit a positive coliform type reaction at very low densities. Cell suspensions as low as 1×10^1 cells 10 ml^{-1} were observed to yield a positive reaction using Colilert reagent 4 weeks short of shelf-life expiry. Use of aged Colilert for monitoring water quality could lead to over-estimation of coliforms as *Aeromonas* have been identified in many treated drinking water supplies.

INTRODUCTION

Assessment of the microbiological quality of drinking water relies on screening for specific indicator organisms rather than for specific pathogens. The most common indicator organisms to be screened are the coliform group and *Escherichia coli*. This approach is acknowledged to provide reliable information regarding the presence of faecal contamination of the water supply being investigated. Examination for these organisms is normally performed by standard protocols using either the most probable number method, or by membrane filtration (Anon. 1994). The main problem with this approach is that up to 48 h can be required to obtain confirmatory results on presumptive positives.

In recent years, there has been a move towards development of new methods designed either to decrease the time period before results are available, improve the degree of sensitivity or increase method versatility. One system recently introduced to increase versatility is the Colilert defined sub-

strate technology system which proported to give accurate results, either presence/absence or fully quantitative, within 24 h or less (Edberg *et al.* 1988, 1989; Berger 1991; Gale and Broberg 1993). The basis for the method is the specific conversion of separate chromogenic and fluorogenic substrates, *o*-nitrophenol-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG), into either a detectable colour or fluorescence; the conversions are catalysed by the enzymes β-galactosidase and β-glucuronidase, respectively. Coliforms contain only β-galactosidase which converts ONPG into readily detectable yellow *o*-nitrophenol, whereas *E. coli* contains both enzymes, with β-glucuronidase catalysing formation of the fluorogenic product 4-methylumbelliferone.

The essence of the Colilert system relies on the fact that only the coliform group of bacteria contain these particular enzymes. However, it has been shown that false-positive results can occur, with these attributed to non-coliform organisms replete with these enzymes (Edberg *et al.* 1989; Katamay 1990). In particular, the aquatic *Aeromonas* have been found to evoke a positive coliform response in the Colilert system at cell densities exceeding 1×10^4 ml^{-1} (Edberg *et al.* 1989; Katamay 1990; Cowburn *et al.* 1994). In the following study, an evaluation was undertaken of the

Correspondence to: Dr Andrew Lamb, School of Applied Science, The Robert Gordon University, St Andrew Street, Aberdeen AB25 1HG, UK (e-mail: a.lamb@rgu.ac.uk).
*Present address: Essex and Suffolk Water, Hanningfield Treatment Works, South Hanningfield, Essex CM3 5HS, UK.

potential for false-positive reactions mediated by low cell densities of *Aeromonas* in the Colilert system. In particular, age of the Colilert reagent was examined to determine whether the incidence of *Aeromonas* mediated positives is potentiated with aged Colilert reagent.

MATERIALS AND METHODS

Bacterial strains used in this study were type cultures obtained from either NCIMB, Aberdeen, UK or ATCC, Rockville, MD, USA. Strains used were *Aeromonas hydrophila* NCIMB 9240, *Aeromonas caviae* NCIMB 13016, *Aeromonas sobria* NCIMB 12065, *Klebsiella oxytoca* NCIMB 12819 and *Pseudomonas aeruginosa* ATCC 27853. All media constituents were obtained from Unipath, (Oxoid, Basingstoke, UK) and chemical reagents were of analytical grade and obtained from either BDH (Poole, UK) or Sigma (UK). Strains were maintained either on Tryptone Soya agar or Nutrient agar. Colilert was obtained from IDEXX Laboratories, UK.

In the following series of experiments, cultures grown in M9 minimal medium (Miller 1972) at 30 °C for 18–24 h were used. An overnight culture of the *Aeromonas* test strain was used to prepare a 10-fold serial dilution in 1·0% NaCl. A 1 ml volume from each dilution was used to inoculate 9 ml of Colilert, previously reconstituted with sterile deionized water. *Klebsiella oxytoca* and *Ps. aeruginosa* were used as positive and negative controls, respectively, with 100 μl of overnight culture used to inoculate 10 ml of reconstituted Colilert. An *Aeromonas* sp. control was also prepared in this manner. All inoculated Colilert tubes were incubated at 35 °C for 24 h prior to examination. After this period, the Colilert samples were examined for *o*-nitrophenol production by measuring their absorbance at 420 nm.

The remaining 9 ml volumes from the *Aeromonas* dilution series were subjected to membrane filtration (Whatman 47 mm/0·45 μm membrane filters) according to standard procedures (Anon. 1994). Plate counts were determined after 24 h of incubation at 35 °C on Tryptone Soya agar. All the experiments described above were performed in duplicate with results shown representing typical data.

RESULTS AND DISCUSSION

It had been reported previously that *Aer. hydrophila* could cause a false-positive coliform reaction in the Colilert test when present at high cell densities with counts exceeding 1×10^4 cfu ml^{-1} (Edberg *et al.* 1989; Katamay 1990; Cowburn *et al.* 1994). Such values are only found in untreated raw water, with potable waters containing substantially lower levels (Holmes and Nicolls 1995; Gavriel *et al.* 1998). Consequently, the likelihood of an aeromonad causing a false-positive in drinking water was considered negligible. In the

current study, age of Colilert reagent has been found to be fundamental in determining the cell count at which false-positives occur. Cell counts greater than 10^5 cfu ml^{-1} were required to produce a positive reaction with new reagent (Fig. 1); this is in agreement with data published previously (Edberg *et al.* 1989; Katamay 1990; Cowburn *et al.* 1994). However, it was found here that levels of *Aer. hydrophila* as low as 1×10^1 cfu 10 ml^{-1} were sufficient to give a positive result when using Colilert reagent within 4 weeks of shelf-life expiry (aged Colilert; Fig. 1). Similar data were obtained when *Aer. caviae* was examined with aged Colilert whereas *Aer. sobria* displayed weaker positive reaction at all cell densities tested (Fig. 1).

The previous experiments were undertaken with physiologically healthy cells grown under optimal conditions. Aquatic organisms must survive in nutrient depleted environments which might influence the response of the organism to aged Colilert reagent. To ascertain whether extended incubation in a nutrient limited environment altered the aeromonad response, *Aer. hydrophila* was grown for 24 h in M9 medium, washed, and resuspended in M9 buffer followed by 5 days of incubation at room temperature prior to analysis. Results obtained clearly demonstrate that poor nutritional status does not significantly reduce the ability of *Aer. hydrophila* to give a positive reaction in aged Colilert at low cell counts (Fig. 2). To demonstrate that the false positive phenomenon with aged Colilert was not artificially induced by laboratory conditions,
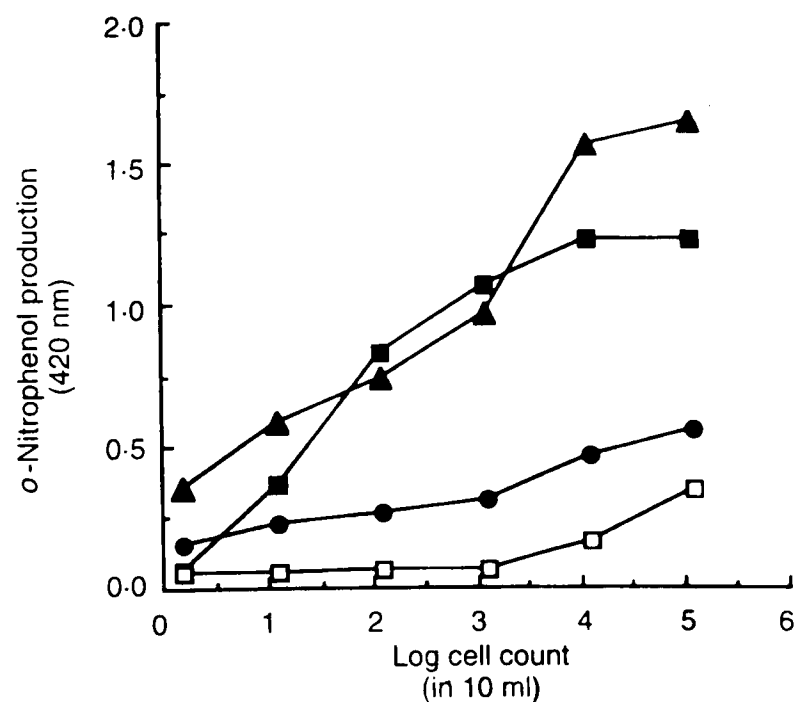

Fig. 1 Response of *Aeromonas* spp. using both fresh and aged Colilert reagent. The aged Colilert reagent used was within 4 weeks of shelf-life expiry. Response of *Aeromonas hydrophila* to fresh (□) and aged (■) Colilert reagent. Response of *Aeromonas caviae* (▲) and *Aeromonas sobria* (●) examined using aged Colilert reagent
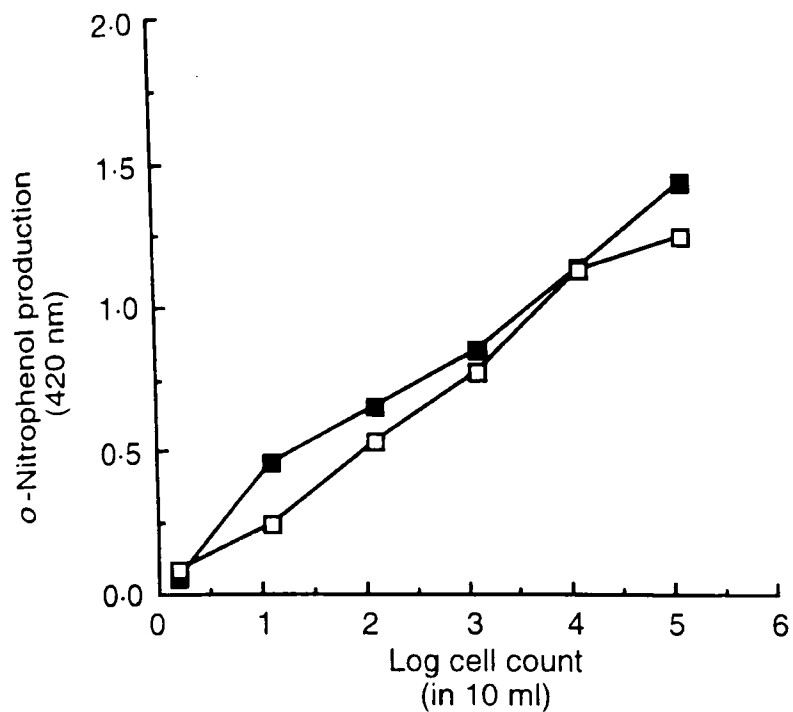
Fig. 2 Response of *Aeromonas hydrophila* using aged Colilert reagent. The Colilert reagent used was within 4 weeks of shelf-life expiry. Culture grown in M9 minimal medium, washed in 1% NaCI and resuspended in M9 buffer for 5 days (■); culture grown in M9 minimal medium, resuspended to a cell density of 1×10^6 cell 100 ml^{-1} filter-sterilized tap water for 5 days (□)

an experiment was undertaken in which 100 ml of filter-sterilized tap water was spiked with *Aer. hydrophila* to approximately 10^6 cells ml^{-1}. The sample was incubated at room temperature for 5 d prior to examination. Data obtained corroborate results of the previous experiments, with a false-positive coliform reaction resulting at low cell numbers (Fig. 2). Similar results were obtained when sodium thiosulphate was present, which confirmed that residual chlorine had not caused interference (data not shown).

It has previously been stated that Colilert can suppress the influence of heterotrophic organisms and allow coliform or *E. coli* identification in a sample containing a background of up to 7×10^5 bacteria ml^{-1} (Edberg *et al.* 1988). The data presented here indicate that aeromonads, which are known to constitute a fraction of the aquatic heterotrophic population, are not efficiently suppressed by Colilert reagent within 4 weeks of shelf-life expiry. Further, it appears that very low levels of aeromonads are capable of giving a false-positive reaction with this aged reagent. Taken together, the results of the current study demonstrate that reagent age has a fundamental impact on the potential for obtaining an

Aeromonas mediated false-positive Colilert reaction when examining drinking water samples.

REFERENCES

Anon. (1994) *The Microbiology of Water. Part 1 Drinking Water.* London : HMSO.

Berger, S.A. (1991) Ability of the Colilert method to recover oxidant-stressed *Escherichia coli. Letters in Applied Microbiology* 13, 247–250.

Cowburn, J.K., Goodall, T., Fricker, E.J., Walter, K.S. and Fricker, C.R. (1994) A preliminary study of the use of Colilert for water quality monitoring. *Letters in Applied Microbiology* 19, 50–52.

Edberg, S.C. (1988) National field evaluation of a defined-substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water : comparison with the standard multiple-tube fermentation method. *Applied and Environmental Microbiology* 54, 1595–1601.

Edberg, S.C. *et al.* (1989) National field evaluation of a defined-substrate method for the simultaneous detection of total coliforms and *Escherichai coli* from drinking water : comparison with presence-absence techniques. *Applied and Environmental Microbiology* 55, 1003–1008.

Gale, P. and Broberg, P.J. (1993) Evaluation of a rapid, defined substrate technology method for enumeration of total coliforms and *Escherichia coli* in chlorinated drinking water. *Letters in Applied Microbiology* 17, 200–203.

Gavriel, A.A., Landre, J.P.B. and Lamb, A.J. (1998) Incidence of mesophilic *Aeromonas* within a public drinking water supply in north-east Scotland. *Journal of Applied Microbiology* 84, 382–392.

Holmes, P. and Nicolls, L.M. (1995) Aeromonads in drinking water supplies : their occurrence and significance. *Journal of Chartered Institute of Water and Environmental Management* 5, 464–469.

Katamay, M.M. (1990) Assessing defined-substrate technology for meeting monitoring requirements of the total coliform rule. *Journal of the American Water Works Association* Part 9, 83–87.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY : Cold Spring Harbor Laboratory Press.

Incidence of mesophilic *Aeromonas* within a public drinking water supply in north-east Scotland

A.A. Gavriel, J.P.B. Landre[1] and A.J. Lamb[1]

The North of Scotland Water Authority, Turriff Water Treatment Works, Turriff and [1]*School of Applied Sciences, The Robert Gordon University, Aberdeen, UK*

A.A. GAVRIEL, J.P.B. LANDRE AND A.J. LAMB. 1998. The motile mesophilic *Aeromonas* are ubiquitous to a wide variety of aquatic environments including drinking water distribution systems. Concern over the presence of mesophilic *Aeromonas* in public drinking water supplies has been expressed in recent years as it has been regarded as a pathogenic organism of importance in gastroenteritis. A major drinking water distribution system in north-east Scotland was monitored over a 12 month period to determine the prevalence of mesophilic *Aeromonas*. These data were examined in relation to chlorine concentration, pH, temperature, rainfall and the standard bacteriological indicators of water quality. *Aeromonas* were isolated to varying degrees from 21 of the 31 reservoirs investigated. The maximum recovery observed during the study was 605 cfu in 300 ml. The probability of isolation generally decreased with increasing levels of chlorination, although this oxidant was found to be ineffective in many reservoirs. Certain reservoirs with poor chlorination profiles yielded very few isolates, whereas some highly chlorinated sites liberated *Aeromonas* frequently and in relatively high numbers. A seasonal pattern in the incidence of *Aeromonas* emerged with infrequent isolation during the winter period increasing to a peak during the summer, with most isolates recovered when water temperature was > 12 °C. An association was demonstrated between the pattern of *Aeromonas* isolations and that of rainfall. No relationship was apparent between incidence of *Aeromonas* and total heterotrophic plate counts.

INTRODUCTION

Bacteria from the genus *Aeromonas* are ubiquitous in aquatic environments and have been found in a wide variety of conditions including oligotrophic upland waters, eutrophic lowland rivers, sewage effluents and estuarine and marine waters (Allen *et al.* 1983; Nakano *et al.* 1990; Poffe and Op de Beeck 1991; Ashbolt *et al.* 1995). Species taxonomy is complex and many studies investigating *Aeromonas* have attempted speciation using limited phenotypic analysis. However, due to the heterogeneity of the members of this genus, phenospeciation methods are unreliable, with accurate genospeciation requiring time consuming molecular methods (Miyata *et al.* 1995; Huys *et al.* 1996). As a working compromise, it has been common practice for members of the genus to be divided into the non-motile psychrophiles and the

motile mesophiles. Members of the non-motile group, commonly referred to as *Aeromonas salmonicida*, appear to be obligate fish parasites (Austin and Austin 1985). The motile mesophilic group represents a more diverse collection, most frequently classified as *Aer. hydrophila*, *Aer. sobria* and *Aer. caviae*. These mesophilic species have been recognized as causative agents of human gastroenteritis, wound infections, septicaemia, and a number of additional opportunistic conditions (Gracey *et al.* 1982; Altwegg and Geiss 1989; Namdari and Bottone 1990; Merino *et al.* 1995). Pathogenicity is associated with the liberation of virulence factors and cell associated endotoxin (Ljungh and Wadstrom 1986; Cahill 1990; Vadivelu *et al.* 1995).

The potential for pathogenicity mediated by mesophilic *Aeromonas* has caused concern to public health authorities and probably accounts for significantly more cases of gastroenteritis than is apparent at present. Indeed, a large proportion of cases of gastroenteritis are still being classified with

Correspondence to : Dr Andrew Lamb, School of Applied Sciences, The Robert Gordon University, St Andrew's Street, Aberdeen AB25 1HG, UK.

unknown or unidentified aetiology. Drinking water supplies have previously been shown to be a source of mesophilic *Aeromonas*, their presence attributable to ineffective disinfection at the treatment plant, post-treatment infiltration, or as a result of aftergrowth within the distribution system (LeChevallier *et al.* 1982; Burke *et al.* 1984; Knochel and Jeppesen 1990; Havelaar *et al.* 1990). Biofilms might, in part, account for aftergrowth of bacteria in such environments and *Aeromonas* has been shown to inhabit such biofilms (Block *et al.* 1993; Holmes and Niccolls 1995). Public drinking water supplies could therefore represent a potential public health risk with regard to gastroenteritis, although no evidence currently exists to support this. Indeed, although typing studies have been unable to match isolates from drinking water and faecal samples (Havelaar *et al.* 1992), the potential risk has been acknowledged in many countries, with the Netherlands stipulating indicative maximum values for *Aeromonas* densities in domestic water supplies (van der Kooji 1991).

A recent survey investigating the epidemiology of *Aeromonas* gastrointestinal infections in Scotland revealed that of 364 cases reported in 1994, 82% were from the Grampian area in the north-east of the country (Sloan 1995). This survey raised the question of whether aeromonads in drinking water supplies could contribute to these cases of gastroenteritis. The work presented here represents an investigation into the incidence of *Aeromonas* in a major public drinking water distribution system in the Grampian area of Scotland, and of the relationship with chlorine concentration, pH, temperature, rainfall and standard bacteriological indicators of water quality.

MATERIALS AND METHODS

Treatment plant and water distribution system

The water distribution system investigated is served by a major treatment plant in north-east Scotland. Abstracted lowland river water is treated by storage reservoir settlement, coagulation settlement by aluminium sulphate, rapid gravity sand filtration, and chlorine disinfection using chlorine gas. The final treated water is fed to a holding reservoir for 24 h where total chlorine is maintained at levels between 0·3 and 0·7 mg l^{-1}. From this point, water passes into a distribution network consisting of three geographical zones, i.e. A, B and C (Fig. 1). Each zone represents a series of reservoirs, extending over a wide geographic area, that directly supplies local consumers. Secondary disinfection of these reservoirs is accomplished by the application of sodium hypochlorite directly into the reservoirs. In addition, reservoirs 18, 28 and 30 are also subject to u.v. disinfection of inlet water. Secondary disinfection was carried out on a routine basis, but particularly during servicing of the reservoir or associated mains, and also in the event of a significant bacteriological failure of the supply as assessed by official indicators of water quality (coliform/*Escherichia coli* counts). All reservoirs are fitted with sample taps and these represent official sampling points for the monitoring of water quality; they were used for obtaining samples during the survey.

Sampling

In this study, 31 reservoirs, being a representative selection from each of the three zones, were examined on a weekly basis. Samples were obtained using sterile bacteriological sample bottles containing sodium thiosulphate (capable of neutralizing up to 5 mg l^{-1} of chlorine) following standard protocols. Chlorine residuals of sample waters were also determined at the time of sampling using a colorimetric assay with N,N-diethyl-p-phenylenediamine.

Bacteriological examination

Samples were maintained between 8 and 15°C and processed within 6 h of collection. Examination for mesophilic *Aeromonas* was carried out using a membrane filtration procedure (Gelman GN-6 Metricel 47 mm/0·45 μm membrane filters) using 300 ml sample volumes. The selective medium used for isolation was ampicillin dextrin agar (Havelaar *et al.* 1987; Gavriel and Lamb 1995) with incubation at 30 °C for 24 h. All presumptive *Aeromonas* were confirmed by Gram stain, oxidase test, Hugh and Leifson's O-F test and susceptibility of 0/129 vibriostatic agent. To ensure that pure cultures were obtained for these confirmatory tests, presumptive aeromonad colonies were subcultured on tryptone soy agar to obtain at least two consecutive pure cultures.

Examination for total coliforms and *Escherichia coli*, and total heterotrophic plate counts, were carried out following standard methods (Anon 1994). Membrane filtration through 0·45 μm filters was performed for total coliform/*E. coli* determination using 100 ml sample volumes. The selective isolation medium was membrane lauryl sulphate broth and samples were, after 4 h pre-incubation at 30 °C, incubated at 37 °C and 44 °C for 14 h to determine total coliforms and *E. coli*, respectively. Total heterotrophic plate counts were obtained by the pour plate method using 1 ml samples added to yeast extract agar with incubation at both 22 °C for 72 h and 37 °C for 24 h.

RESULTS

Incidence of mesophilic *Aeromonas* in the water distribution system

An extensive survey of a potable water distribution system examining incidence of mesophilic aeromonads in north-east Scotland revealed a significant seasonal prevalence of these
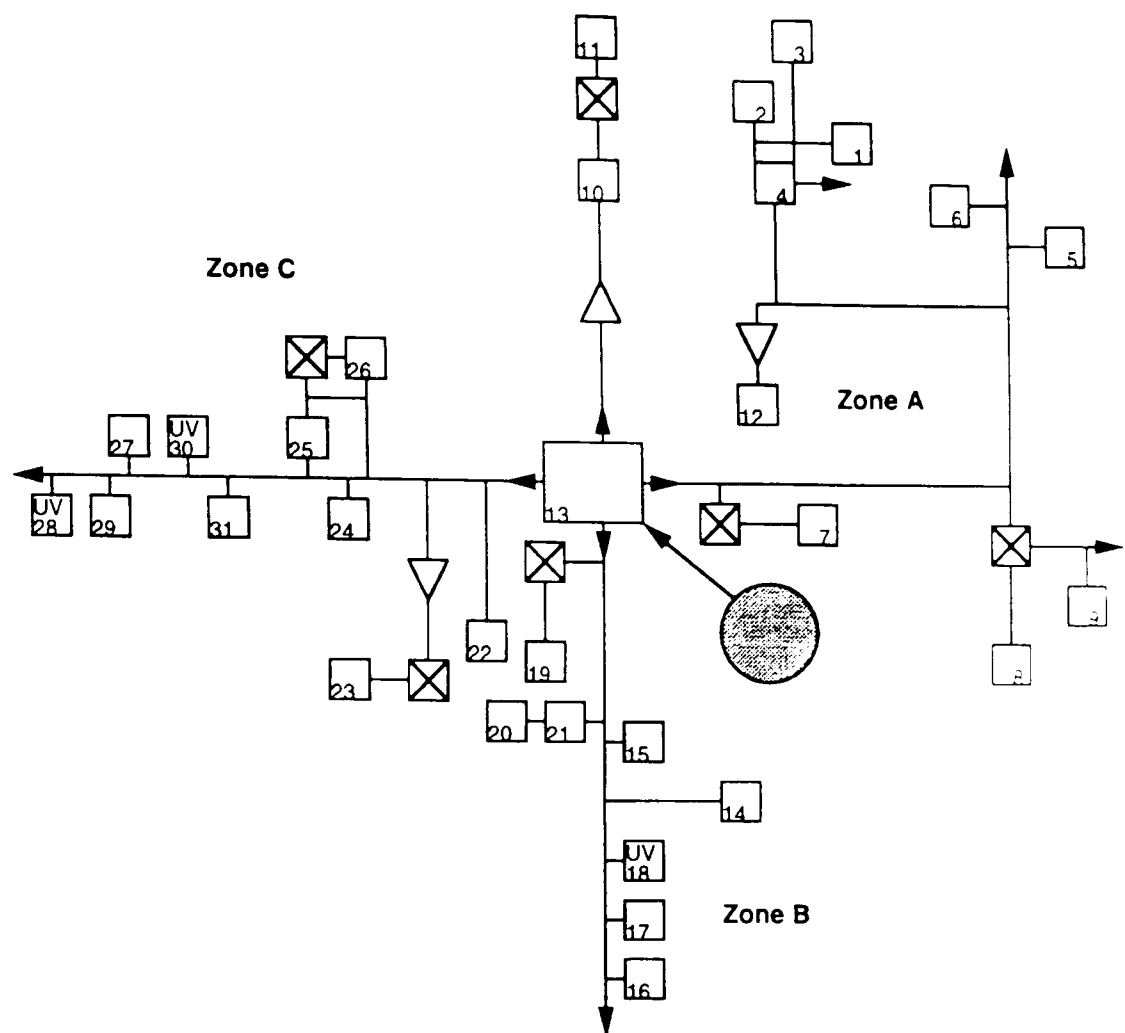
Fig. 1 Schematic of the sections of the water distribution system examined (not fully to scale). Zone A consists of reservoirs 1–12, zone B reservoirs 14–21 and zone C reservoirs 22–31. Reservoir 13 is the main service reservoir. ●, Water treatment Plant; ☐, service reservoir; ▷, pumping station; ☐, reservoir; ☒, reservoir not examined

organisms. Of 31 reservoirs monitored weekly during the 12 month period between May 1995 to May 1996, 21 yielded *Aeromonas* in at least one sample (Fig. 2). In contrast, *Aeromonas* were never recovered either from samples taken at the water treatment plant downstream of chlorination immediately prior to entry into the distribution system, or from the main service reservoir (no. 13; Fig. 2). Those reservoirs giving recoveries did so to varying degrees, both in the number of isolates obtained and in the frequency of positive samples, with the highest recovery being 605 aeromonads in 300 ml from reservoir 19 (Figs 2 and 3a). Unlike previous studies, it was found that distance from the main service reservoir had no significant bearing, as several closely situated reservoirs gave aeromonad recoveries while certain outlying reservoirs remained negative for this organism. The free and total available chlorine concentrations were also monitored for all the reservoirs investigated, with the determinations being made when the weekly samples were collected. The spectrum of mean concentrations in these reservoirs ranged from 0.05–0.5 mg l^{-1} free chlorine up to 0.1–0.6 mg l^{-1} total chlorine (Fig. 4). During the survey period, treated water in distribution maintained a relatively constant pH with values ranging between 7.8 and 8.9 (Fig. 5). This constant pH would ensure minimal fluctuation in the ionization status of the chlorine species within the distribution network. In support of the lack of association between relative reservoir dis-

tance and incidence of aeromonad isolation, it was observed that chlorine levels did not uniformly decrease as distance from the main service reservoir increased (Figs 1 and 4). Of the 10 reservoirs negative for *Aeromonas* recovery, all except reservoir 28 maintained a total chlorine residual above 0.2 mg l^{-1} (Fig. 4). Lack of recovery from this reservoir probably results from the u.v. disinfection which the inlet water received at this location.

Although chlorination is a proven bactericidal treatment for drinking water supplies, data from this study demonstrate that maintenance of chlorine within the distribution network is insufficient on its own to control the levels of aeromonads. Of the four reservoirs which were positive for *Aeromonas* in more than 10% of samples, only reservoir 3 had a total chlorine concentration below 0.2 mg l^{-1} (Figs 2 and 4). In addition, from the three reservoirs lacking a residual chlorine in more than 15% of samples, this only appeared significant in reservoir 3, with reservoirs 4 and 9 being negative for *Aeromonas* in 98% of samples tested (Fig. 2). Also, of the 17 reservoirs that maintained a chlorine residual in all samples tested, 10 were found to be positive for *Aeromonas* on at least one occasion. Another observation found three reservoirs to be negative for *Aeromonas*, despite lacking a chlorine residual in one sample. Clearly these results taken together suggest that continual presence of a chlorine residual is not sufficient to ensure prevention of *Aeromonas* recovery.
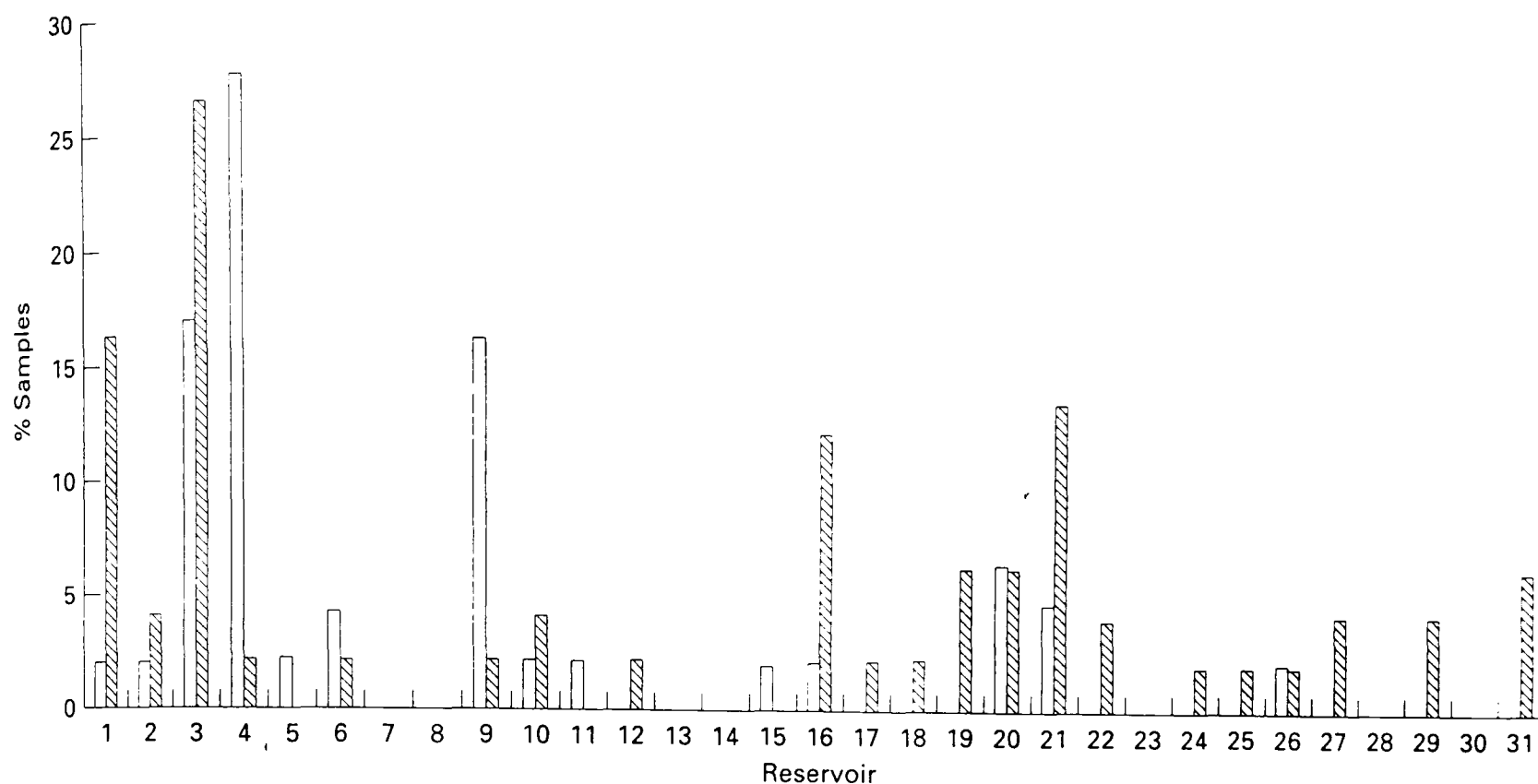
Fig. 2 Percentage of individual reservoir samples positive for *Aeromonas* recovery (hatched bars) and negative for total chlorine residual (clear bars)

Seasonal incidence of mesophilic *Aeromonas*

A distinct seasonal pattern in recovery of *Aeromonas* was observed where 95% of all isolates were recovered between the period from mid-June to the end of September 1995 (Fig. 3b). This corresponded to the period where the water temperature was maintained above 12 °C (Fig. 5). An apparent association between the level of rainfall and the total number of *Aeromonas* isolates recovered from all reservoirs was observed. The very heavy and prolonged period of rainfall from weeks 35 to 37 coincided with a much increased incidence of *Aeromonas* when the maximum recovery of *Aeromonas* derived from reservoirs 19 and 29 occurred (Figs 3a and 6). Increased recovery during periods of moderate to heavy rainfall could be explained by the combined effect of a number of factors. As rainfall increases, the organic load in abstracted water for treatment has previously been shown to increase significantly. During post-treatment chlorination, organics present will react with chlorine to form less reactive chloramines. Although these chloramines display a prolonged action, the disinfectant potential is reduced. The increased organic material will also potentiate the aftergrowth activity of both planktonic and biofilm-associated organisms. In addition, post-treatment plant infiltration of the distribution system during periods of rainfall probably influences the water quality with potential for *Aeromonas* contamination existing.

Association between mesophilic *Aeromonas* and other microbiological parameters of water quality

Examination of the standard bacteriological indicators of water quality have shown no obvious relationship with mesophilic *Aeromonas* levels. Although aeromonads are known to constitute a considerable fraction of the heterotrophic population of raw water, heterotrophic plate counts at either 22 or 37 °C did not demonstrate any notable association to the recovery of *Aeromonas* (Figs 3b and 7). During the 12 month test period, the bacteriological water quality was of a high standard with only an occasional positive for either coliforms or *E. coli*. With so few data available, no valid interpretation could be made between the incidence of aeromonads and that of either coliforms or *E. coli*.

DISCUSSION

This study, investigating the presence of mesophilic *Aeromonas* in final drinking water supplies in north-east Scotland, has demonstrated that substantial numbers are present, with a distinct seasonal pattern of recovery noted. Both overall values and the seasonal pattern for recovery of isolates are in agreement with results obtained previously (LeChevallier *et al.*, 1982; Knochel and Jeppesen 1990; Havelaar *et al.* 1990; Holmes and Niccolls 1995). The probability of detecting *Aeromonas* in the reservoirs examined generally increased
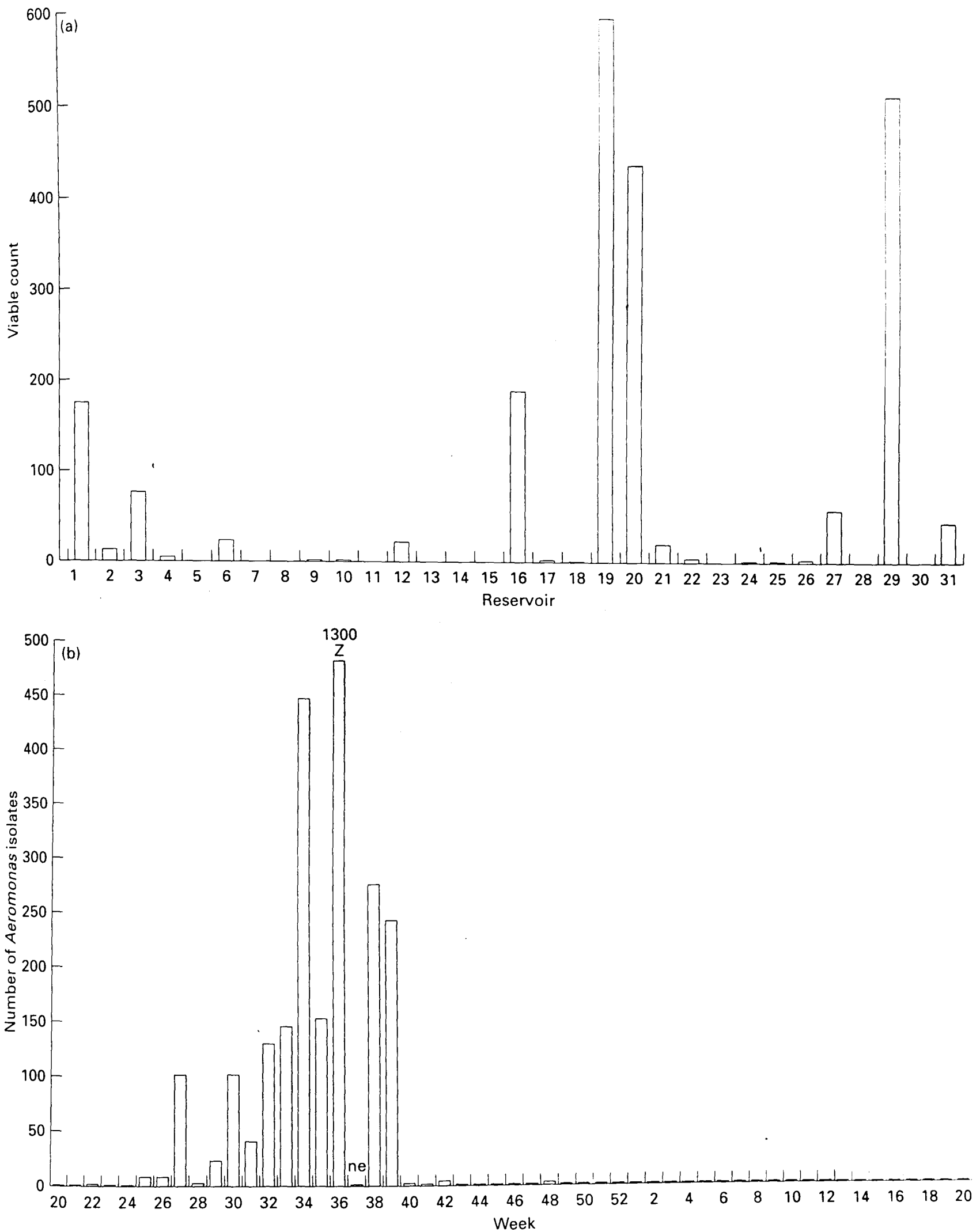
Fig. 3 Viable counts of mesophilic *Aeromonas*. (a) Maximum viable count obtained from a 300 ml sample from each reservoir; (b) total number of isolates recovered per week from all 31 reservoirs. During week 37, samples were not obtained for examination (ne)
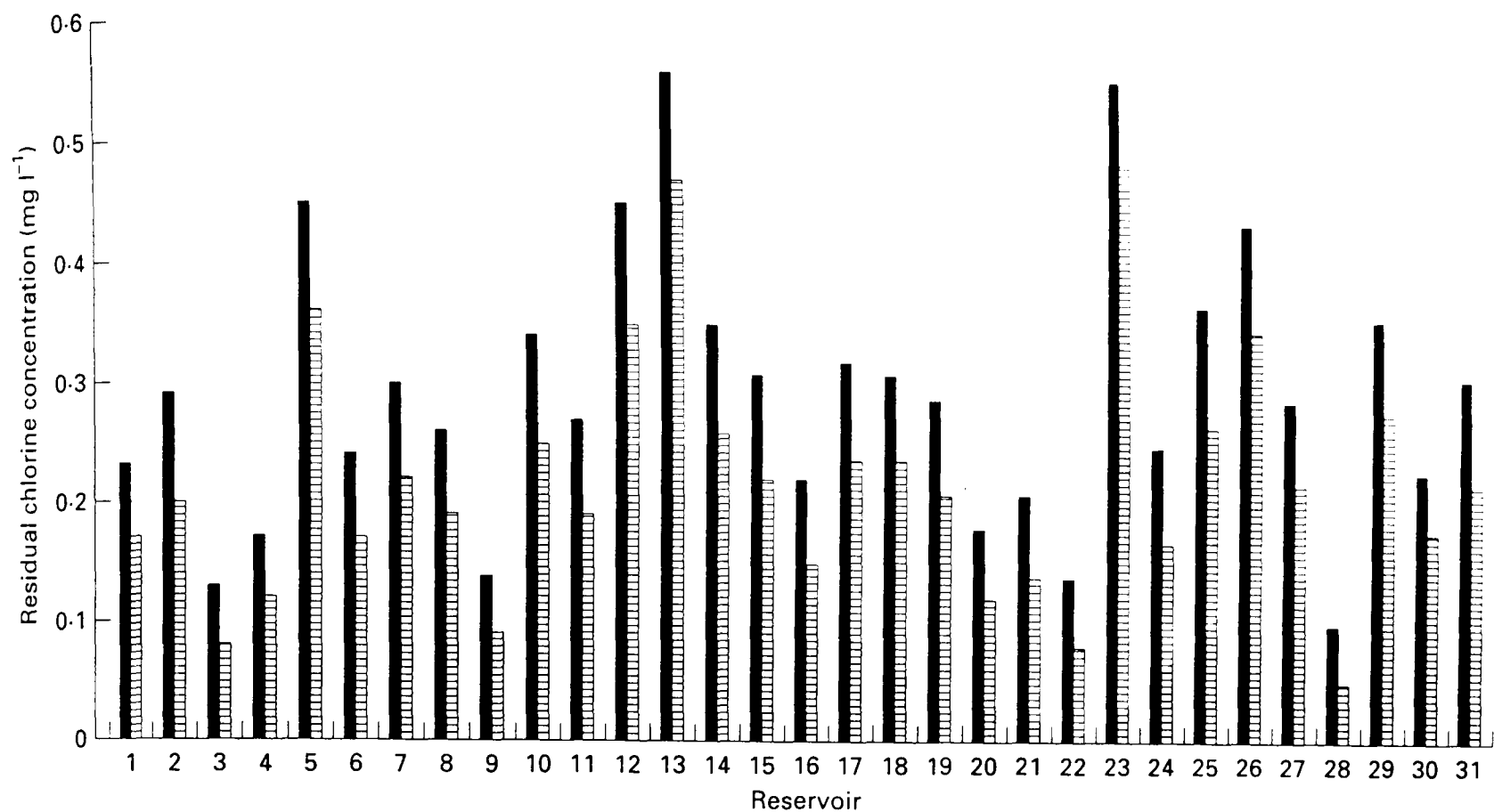
Fig. 4 Chlorine concentrations in the service reservoirs. Free and total chlorine concentrations (mg l^{-1}) are shown in the hatched and shaded bars, respectively
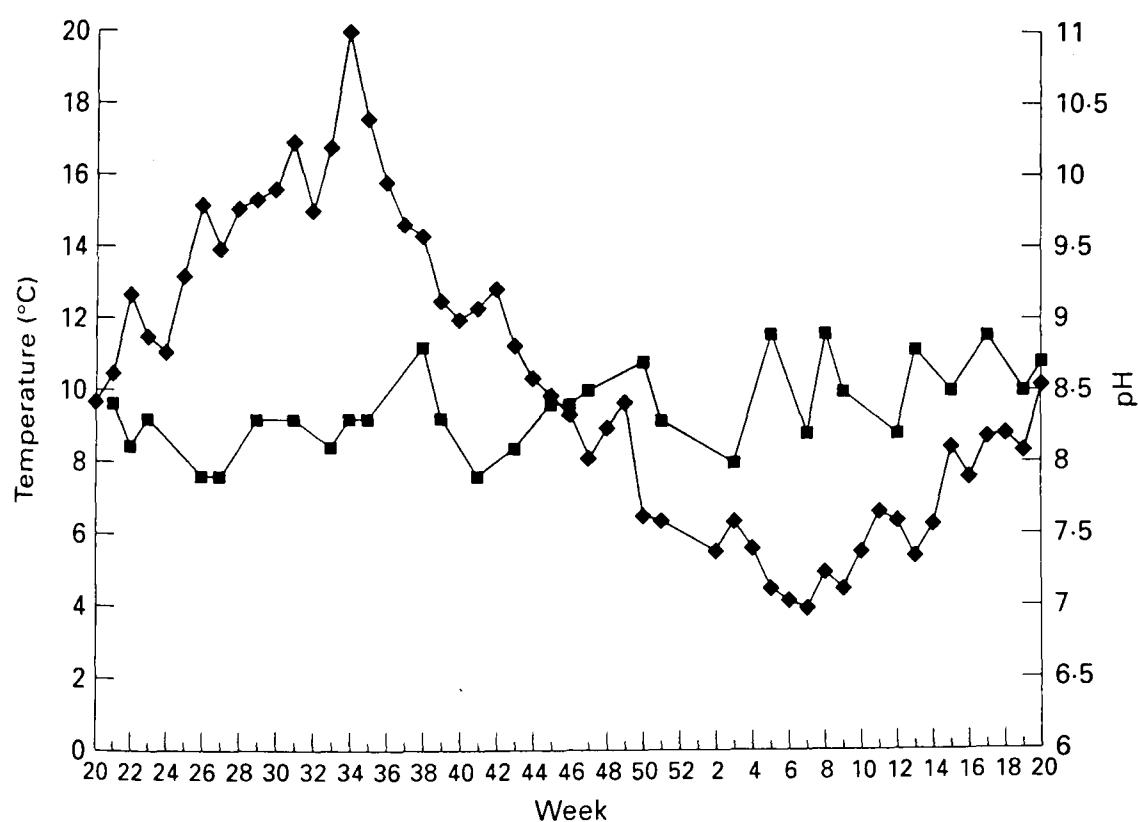

Fig. 5 Average temperature (°C ◆) and pH (■) of water from supplies in all three zones of the distribution network

with decreasing levels of chlorination. However, reservoirs exposed to sustained, substantial dosing of chlorine were also observed to yield *Aeromonas* occasionally. These results corroborate findings of similar investigations where *Aeromonas* were recovered from chlorinated supplies (LeChevallier *et al.* 1982; Holmes and Niccolls 1995). Given the relative sensitivity of mesophilic aeromonads to this oxidizing agent (Knochel 1991), the data would indicate that these organisms are subject to some form of protection. There are two possible explanations to account for survival of aero-
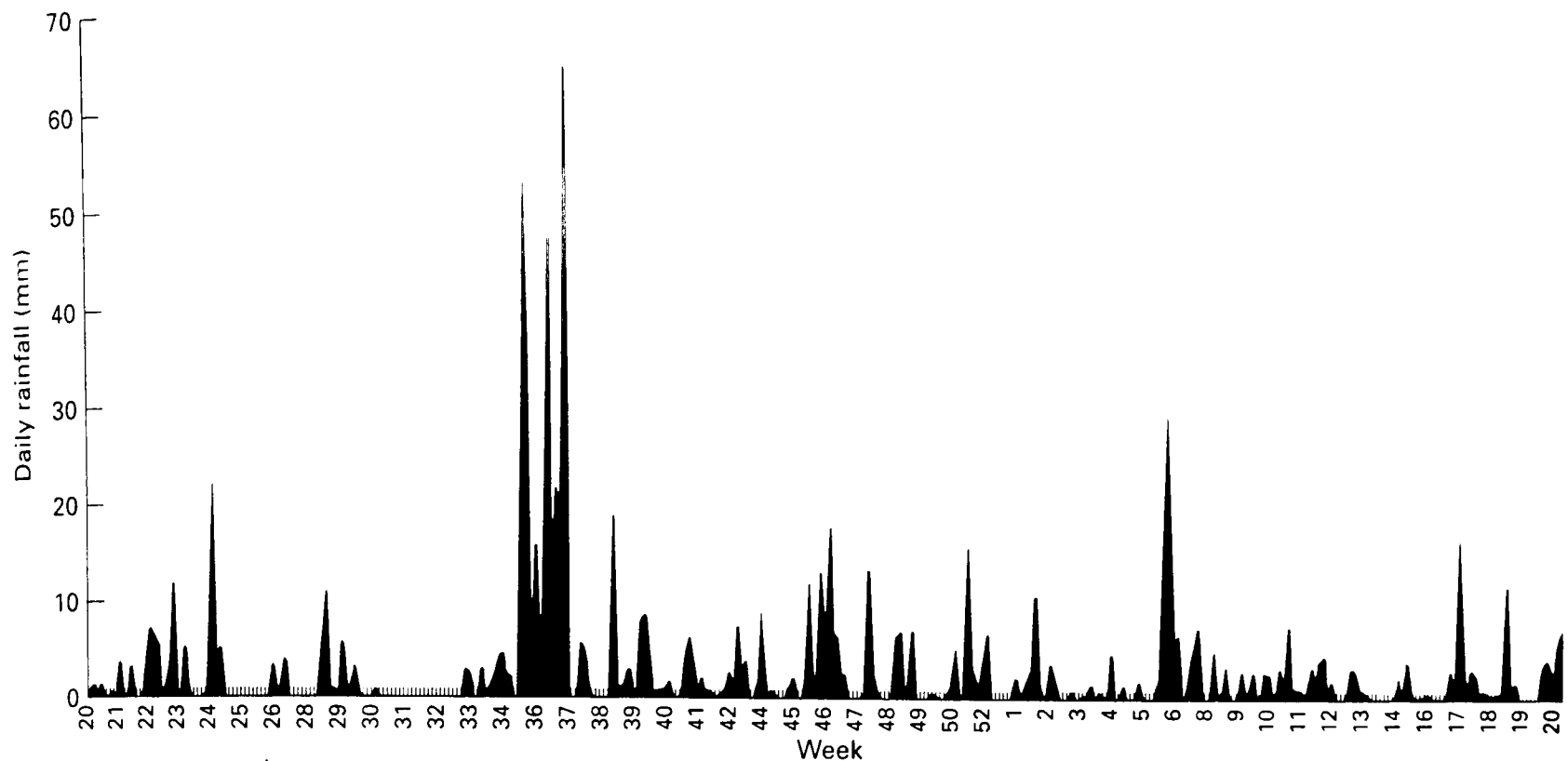
Fig. 6 Rainfall (mm) monitored daily at water treatment plant facility

monads under conditions encountered within the distribution network. Biofilm formation within water distribution networks is known to occur (Block *et al.* 1993). These biofilms provide a protective organic matrix within which the embedded cells are shielded from the effects of chlorine. In addition to protecting inhabitants, biofilms also accumulate nutrients to facilitate maintenance of viability and aftergrowth. Aeromonads have been shown to be inhabitants of these biofilms (Holmes and Niccolls 1995). Isolates liberated from the biofilm community are probably coated in an extracellular matrix derived from the biofilm which would afford protection to the effects of chlorine. The possibility that distribution network biofilms contribute significantly to the recovery of *Aeromonas* is supported by the results from particular reservoirs which yielded a high proportion of these organisms compared to reservoirs with nearly identical chlorination patterns.

An alternative explanation could result from the acquisition of tolerance to the oxidizing effects of chlorine action through physiological adaptation. Previous studies with *E. coli* have demonstrated the formation of tolerance to lethal concentrations of oxidants, either through prior exposure to sub-lethal concentrations, or through cell starvation (Demple and Halbrook 1983; Jenkins *et al.* 1988). Exposure of aeromonads, either planktonic or biofilm-associated to fluctuating levels of chlorine might induce a similar response where adapted cells maintain viability even when challenged with a chlorine concentration at a level normally sufficient to kill. It is highly feasible that biofilm-associated cells would be exposed to reduced, sub-lethal chlorine doses and that such

exposure could trigger the onset of this adaptive phenomenon. In support of this hypothesis, Karem and colleagues demonstrated that *Aer. hydrophila* can induce an adaptive tolerance to acid stress (Karem *et al.* 1994) and preliminary data obtained in our laboratory have shown that *Aer. hydrophila* displays a reduced sensitivity to hypochlorite challenge when pre-exposed to a sub-lethal dose of this oxidant (data not shown). Holmes and Niccolls (1995) indicated that maintenance of chlorine at or above 0.2 mg l^{-1} should provide adequate control of *Aeromonas*. Results presented here clearly demonstrate that these organisms can be recovered from reservoirs which maintain chlorine levels as high as 0.45 mg l^{-1}. It would appear that additional factors, such as temperature or organic content of the water, have an important influence in the control of aeromonads.

The apparent association between rainfall and levels of *Aeromonas* in the water distribution system probably results from a combination of the following factors. Initially, aeromonads could enter into the distribution system by infiltration through breeches in the systems integrity, primarily through cracks or corrosion. This ingress of contaminating material will occur normally but will be exacerbated during periods of sustained rainfall. Another contributory factor would be an enhanced nutrient concentration available to the organisms as a result of the nutrient rich water entering the system. This would allow organisms in the distribution system, whether planktonic or biofilm, to proliferate. In addition, the free residual chlorine in the system would be significantly reduced through the formation of combined chlorine with organic material, thereby allowing the organ-
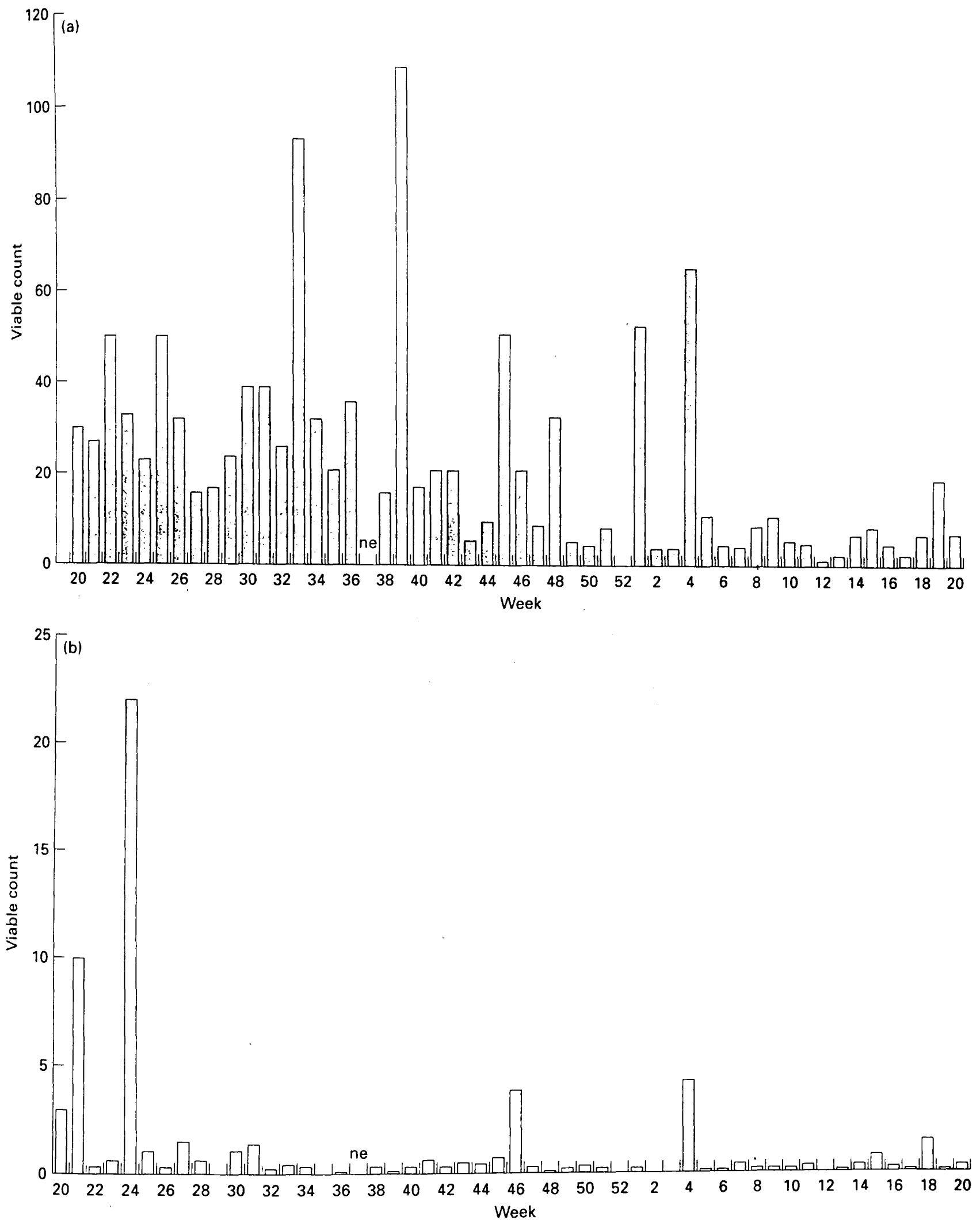
Fig. 7 Average total heterotrophic plate counts per week from all 31 reservoirs determined (a) at 22 °C, (b) at 37 °C

isms to survive. It is also possible that during conditions of very heavy and prolonged rainfall, when particularly poor quality water is being abstracted for treatment by the water plant, some breakthrough into the distribution system may occur because of the severe challenge to the treatment process. The organisms could pass through the high chlorine disinfection environment shielded by the particulate matter present under these circumstances. However, in the present investigation, none of the samples taken immediately downstream of the treatment plant yielded *Aeromonas*, suggesting that infiltration, increased nutrient supply and decreased chlorine activity are the contributory reasons for elevated *Aeromonas* counts during periods of heavy rainfall.

In summary, this study has shown that treated water supplies in north-east Scotland can harbour substantial numbers of *Aeromonas* with isolates primarily recovered over the summer period. This pattern of isolation corresponds to the pattern of aeromonad mediated gastroenteritis observed during 1994 (Sloan 1995). Although no evidence exists to support a role for direct waterborne transmission of *Aeromonas* in gastrointestinal disorders, at the present time, such a possibility cannot be discounted.

ACKNOWLEDGEMENTS

REFERENCES

Allen, D.A., Austin, B. and Colwell, R.R. (1983) *Aeromonas media*, a new species isolated from river water. *International Journal of Systematic Bacteriology* 33, 599–604.

Altwegg, M. and Geiss, H.K. (1989) *Aeromonas* as a human pathogen. *CRC Critical Reviews in Microbiology* 16, 253–286.

Anon. (1994) *The Microbiology of Water. Part 1 Drinking Water*. London: HMSO.

Ashbolt, N.J., Ball, A., Dorsch, M. *et al.* (1995) The identification and human health significance of environmental aeromonads. *Water Science and Technology* 31, 263–269.

Austin, B. and Austin, D.A. (1985) Bacterial pathogens of fish. *Journal of Applied Bacteriology* 58, 483–506.

Block, J.C., Haudidier, K., Paquin, J.L., Miazga, J. and Levi, Y. (1993) Biofilm accumulation in drinking water distribution systems. *Biofouling* 6, 333–343.

Burke, V., Robinson, J., Gracey, M., Peterson, D. and Partridge, K. (1984) Isolation of *Aeromonas hydrophila* from a metropolitan water supply: seasonal correlation with clinical isolates. *Applied and Environmental Microbiology* 48, 361–366.

Cahill, M.M. (1990) Virulence factors in motile *Aeromonas* species. *Journal of Applied Bacteriology* 69, 1–16.

Demple, B. and Halbrook, J. (1983) Inducible repair of oxidative DNA damage in *Escherichia coli. Nature* 304, 466–468.

Gavriel, A.A. and Lamb, A.J. (1995) Assessment of media used for selective isolation of *Aeromonas* spp. *Letters in Applied Microbiology* 21, 313–315.

Gracey, M., Burke, V. and Robinson, J. (1982) *Aeromonas* associated gastroenteritis. *Lancet* 2, 1304–1306.

Havelaar, A.H., Durning, M. and Versteegh, J.M.F. (1987) Ampicillin dextrin agar medium for the enumeration of *Aeromonas* species in water by membrane filtration. *Journal of Applied Bacteriology* 62, 279–287.

Havelaar, A.H., Versteegh, J.F.M. and During, M. (1990) The presence of *Aeromonas* in drinking water supplies in the Netherlands. *Zentralblatt für Hygiene und Umweltmedizin* 190, 236–256.

Havelaar, A.H., Schets, F.M., van Silfhout, A., Jansen, W.H., Wieten, G. and van der Kooji, D. (1992) Typing of *Aeromonas* strains from patients with diarrhoea and from drinking water. *Journal of Applied Bacteriology* 72, 435–444.

Holmes, P. and Niccolls, L.M. (1995) Aeromonads in drinking water supplies: their occurrence and significance. *Journal of Chartered Institute of Water and Environmental Management* 5, 464–469.

Huys, G., Coopman, R., Janssen, P. and Kersters, K. (1996) High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *International Journal of Systematic Bacteriology* 46, 572–580.

Jenkins, D.E., Schultz, J.E. and Matin, A. (1988) Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli. Journal of Bacteriology* 170, 3910–3914.

Karem, K.L., Foster, J.W. and Bej, A.K. (1994) Adaptive acid tolerance response (ATR) in *Aeromonas hydrophila. Microbiology* 140, 1731–1736.

Knochel, S. (1991) Chlorine resistance of motile *Aeromonas* spp. *Water Science and Technology* 24, 327–330.

Knochel, S. and Jeppesen, C. (1990) Distribution and characteristics of *Aeromonas* in food and drinking water in Denmark. *International Journal of Food Microbiology* 10, 317–322.

LeChevallier, M.W., Evans, T.M., Seidler, R.J. *et al.* (1982) *Aeromonas sobria* in chlorinated drinking water supplies. *Microbial Ecology* 8, 325–333.

Ljungh, A. and Wadstrom, T. (1986) *Aeromonas* toxins. In *Pharmacology of Bacterial Toxin* ed. Dorner, F. and Drews, J. pp. 289–305. New York: Pergamon.

Merino, S., Rubries, X., Knochel, S. and Tomas, J.M. (1995) Emerging pathogens: *Aeromonas* spp. *International Journal of Food Microbiology* 28, 157–168.

Miyata, M., Aoki, T., Inglis, V., Yoshida, T. and Endo, M. (1995) RAPD analysis of *Aeromonas salmonicida* and *Aeromonas hydrophila. Journal of Applied Bacteriology* 79, 181–185.

Nakano, H., Kameyama, T., Venkateswaran, K., Kawakami, H. and Hashimoto, H. (1990) Distribution and characterisation of hemolytic, and enteropathogenic motile *Aeromonas* in aquatic environment. *Microbial Immunology* 34, 447–453.

Namdari, H. and Bottone, E.J. (1990) Microbiologic and clinical evidence supporting the role of *Aeromonas caviae* as a paediatric enteric pathogen. *Journal of Clinical Microbiology* 28, 837–840.

Poffe, R. and Op de Beeck, E. (1991) Enumeration of *Aeromonas*

hydrophila from domestic wastewater treatment plants and surface waters. *Journal of Applied Bacteriology* **71**, 366–370.

Sloan, D. (1995) The epidemiology of aeromonad infections in Scotland in 1994—more questions than answers. *Scottish Centre for Infection and Environmental Health Weekly Report* **29** (95/25), 134–136.

Vadivelu, J., Puthucheary, S.D., Phipps, M. and Chee, Y.W. (1995) Possible virulence factors involved in bacteraemia caused by *Aeromonas hydrophila*. *Journal of Medical Microbiology* **42**, 171–174.

van der Kooij, D. (1991) Nutritional requirements of aeromonads and their multiplication in drinking water. *Experientia* **47**, 444–446.

OXIDATIVE STRESS RESPONSE OF MESOPHILIC AEROMONAS

AA Gavriel[1], JPB Landre[2] & AJ Lamb[2]

[1]The North of Scotland Water Authority, Turriff Water Treatment Works, Turriff AB53 7PL, UK; [2]School of Applied Sciences, The Robert Gordon University, Aberdeen AB25 1HG, UK

ABSTRACT

Aeromonas are ubiquitous within the aquatic environment and have been reported to be present within drinking water distribution systems subjected to chlorination. In recent years the mesophilic Aeromonas are increasingly being linked to human gastrointestinal disorders and are now viewed as opportunistic pathogens. Type strains of Aeromonas were treated with sodium hypochlorite to evaluate the sensitivity and physiological response of these organisms to this oxidising agent. All species examined were observed to be more sensitive to this bacteriocide than were Escherichia coli or Pseudomonas aeruginosa. However, Aeromonas were observed to be capable of acquiring tolerance to lethal concentrations of hypochlorite through prior exposure to sub-lethal concentrations of this agent. Acquisition of tolerance to oxidative stress was found to occur through an adaptive physiological process. These results demonstrate that, although being more susceptible to the oxidising effects of hypochlorite than are other aquatic bacteria, mesophilic Aeromonas possess a mechanism which can afford protection to disinfection agents. This provides a plausible explanation to account for the finding that mesophilic Aeromonas are periodically recovered from treated drinking water supplies.

KEYWORDS

Aeromonas; disinfection; tolerance; drinking water.

INTRODUCTION

The genus Aeromonas represents a collection of heterotrophic bacteria commonly divided into the motile mesophilic free-living organisms and the non-motile psychrophilic obligate parasites; both are ubiquitous in freshwater and marine aquatic environments (Chowdhury et al, 1990; Holmes, Niccolls & Sartory, 1996). Mesophilic Aeromonas are the largest of the two groups both in terms of prevalence and variety of different species/strains (Carnahan & Altwegg, 1996)). These free-living organisms constitute a significant proportion of the bacterial population in natural freshwater environments as trophically diverse as upland lakes and lowland rivers and, in water-industry

environments, as disparate as drinking-water distribution systems and sewage waters (Holmes & Niccolls, 1995). In such environments aeromonads have been observed to be both planktonic and biofilm associated. Speciation of mesophilic *Aeromonas* remains difficult due to the physiological and genetic diversity of the group (Huys *et al*, 1996). Phenotypic approaches are numerous and can yield up to 13 species while genotypic identification currently reveals 17 hybridisation groups (Carnahan & Altwegg, 1996; Huys *et al*, 1997).

The presence of mesophilic *Aeromonas* in the drinking-water supply is relevant to both the water industry and public health authorities as it can interfere in the analysis for detection of faecal indicator organisms and also because it is a recognised opportunistic human pathogen (Janda, 1991; Ashbolt *et al*, 1995; Mwerino *et al*, 1995). Most clinical isolates are from cases of gastroenteritis (Janda & Abbott, 1996) and, therefore, ingestion of *Aeromonas* in contaminated drinking water may represent a potential risk although to date there is no clear evidence of a waterborne outbreak or for an epidemiological link (Holmes & Niccolls, 1995). Studies of chlorinated public drinking-water supplies have shown *Aeromonas* to be present in significant numbers (Holmes & Niccolls, 1995; Gavriel, Landre &· Lamb, 1997). *Aeromonas* was found to be concentrated at particular sites in one system, with a seasonal pattern for isolations showing a late summer peak (Gavriel, Landre & Lamb, 1997). Interestingly, *Aeromonas* was frequently recovered despite the existence of high chlorine residuals.

Chlorination of drinking-water supplies in the UK is achieved by dosing with chlorine gas or hypochlorite solution. The pH, temperature and concentration of ammonia nitrogen or organic nitrogen will determine which forms of chlorine develop in the water. In the absence of a chlorine demand from nitrogenous compounds a pH and temperature dependent equilibrium between HOCl and OCl⁻ (free chlorine) exists. In the presence of nitrogenous compounds the free forms, being highly reactive, produce chloramines (combined chlorine). Dosing to satisfy chlorine demand results in a free as well as combined residual, with the total residual being the sum of these. The bacteriocidal effect of chlorine is attributable to its oxidising effect resulting ultimately in cell death from membrane damage affecting permeability and transport mechanisms, protein alteration and fragmentation including inactivation of enzymes, reaction with nucleotides and possibly DNA (Demple, 1991; Dukan & Touati, 1996).

The current study was undertaken to evaluate the sensitivity of *Aeromonas* to the effect of oxidation induced by hypochlorite. The existence in certain bacteria of physiological mechanisms, triggered by a variety of stress factors such as pH, temperature, and oxidising agents that confer tolerance to higher levels of stress has been extensively reported (Demple, 1991; Yura, Nagai & Mori, 1993; Karem, Foster & Bej, 1994; Dukan & Touati, 1996). This adaptive response was demonstrated with *E coli* where pre-exposing with H_2O_2 was found to induce an adaptive response to both H_2O_2 and HOCl whereas pre-exposing with HOCl gave an adaptive response only to H_2O_2 and not to HOCl

(Demple, 1991; Dukan & Touati, 1996). In this paper we examined the response of *Aeromonas* to oxidative stress challenge by HOCl over both short and long term periods.

MATERIALS & METHODS

Bacterial strains and media - the type strains employed for this study were *Aeromonas hydrophila* NCIMB 9240, *Aeromonas caviae* NCIMB 13016, *Aeromonas sobria* NCIMB 12065, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. They were all grown on tryptone soya agar (TSA) at 30°C and maintained on this medium at 4°C until use. Millers M9 minimal medium was used to prepare cultures for the stress assays. All media were obtained from Oxoid or Difco and chemicals from Merck.

Oxidative stress assay - a colony from a fresh overnight TSA plate was used to inoculate 20mL of Millers M9 minimal medium. This was incubated at 30°C for 24h and 1mL used to inoculate 20mL of fresh M9 medium and this culture incubated at 30°C for 24h. 1mL of the resultant culture was used to seed the test vessel which contained 250mL of the test suspension medium. When washed cells were employed these were prepared by two cycles of centrifugation at 3,500 rpm using the test medium for resuspension. The test media used in these assays were Miller's M9 minimal medium, M9 without glucose, ammonium-free M9 (no NH_4Cl or glucose), and 1/4 strength Ringer's solution, with pH of these being between 7.0-7.4. Upon inoculation of the test media the cells were challenged with oxidant, prepared as described below. Incubation of the test vessels was at room temperature 18-22°C, shaking only on addition of inoculum, oxidising agent, and prior to removing aliquots to determine the concentration of viable (culturable) cells.

Determination of cell count - this was performed using the Miles & Misra drop plate count method with TSA. A ten-fold dilution series from the test medium was made by adding 1mL of the test suspension to 9mL of diluent (same medium as the test suspension medium). Between 5 and 12 aliquots of 20μL were used and determinations were performed in duplicate. Incubation of plates was at 30°C for 24h (further incubation did not increase recoveries). Non oxidant-dosed control vessels were set up for each experiment.

Oxidising agent - hypochlorous acid was prepared from 12% sodium hypochorite solution ('Everchlor', Ellis & Everard, England) and the working solution used for dosing in the experiments was 20mg/L chlorine. This was introduced into the test vessel as a single dose each time. Determination of chlorine residual was by the use of the Hach model DR/700 colorimeter with the assay dependent on the reaction of free chlorine with N,N-diethyl-p-phenylenediamine and total chlorine measurement additionally requiring potassium iodide for the combined chlorine component.

RESULTS

Chlorine sensitivity of mesophilic Aeromonas - a range of concentrations of hypochlorite were used to examine the relative chlorine sensitivity and showed *Aeromonas* to be the most sensitive to this oxidant in comparison with *E coli* and *P aeruginosa*. A total chlorine residual of 0.35mg/L was enough to prevent recovery of *A hydrophila* and *E coli* after 24h but *P aeruginosa* was still viable (Figure 1A) and at higher doses remained significantly the most resilient of the three organisms (Figure 1B).
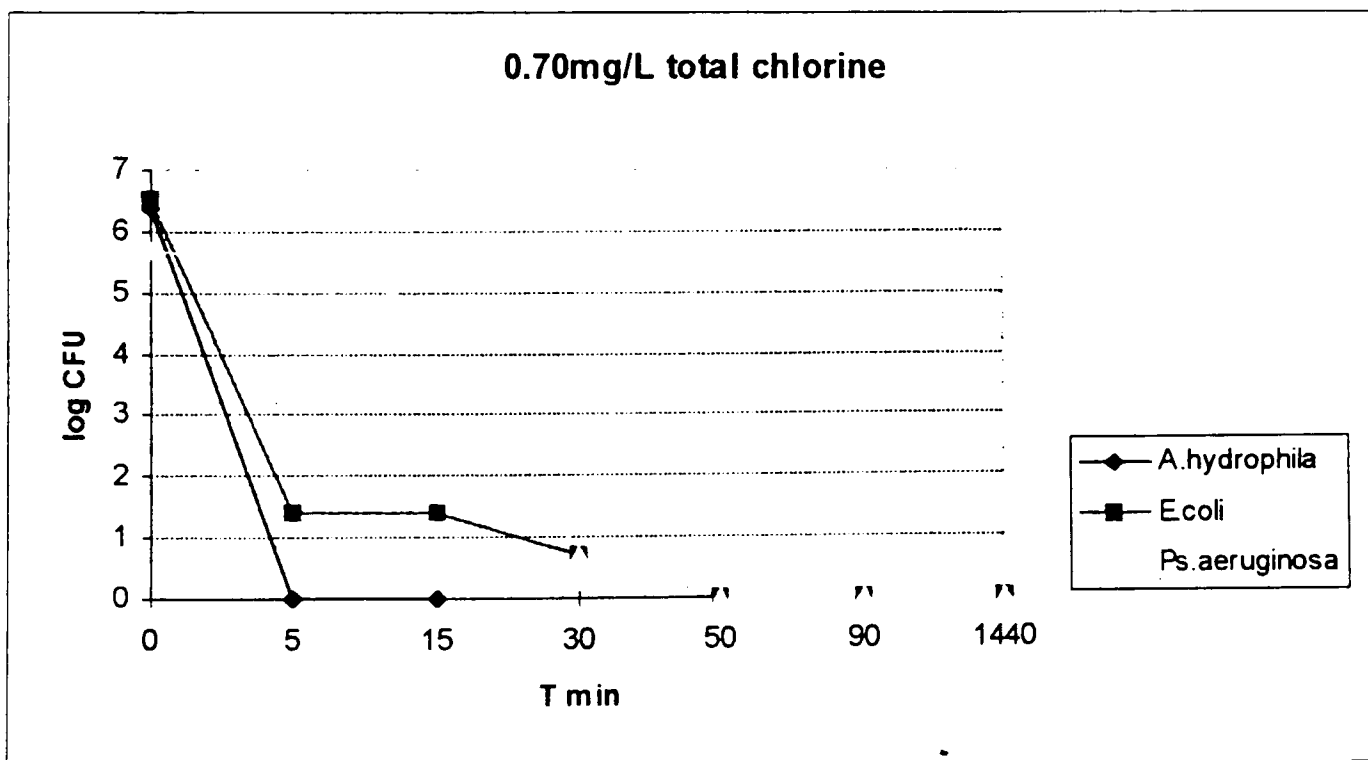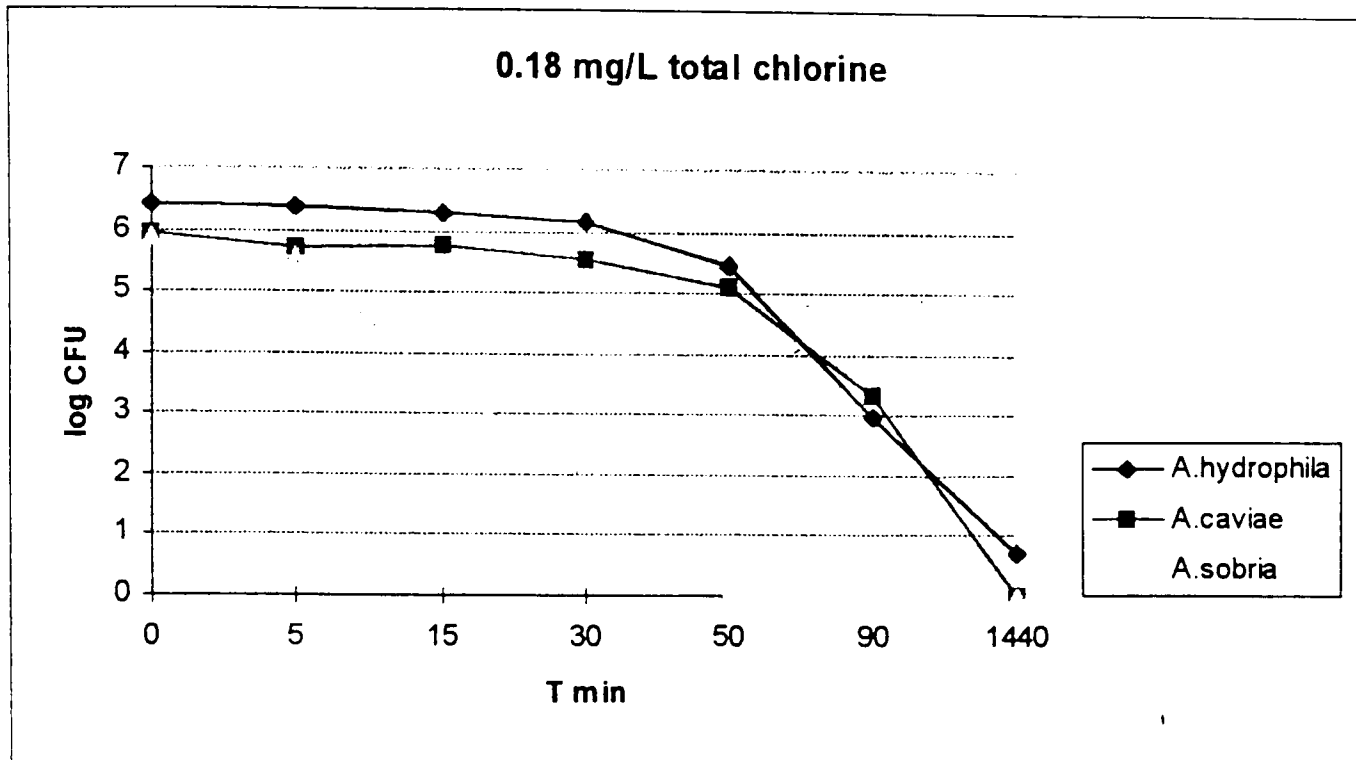
A
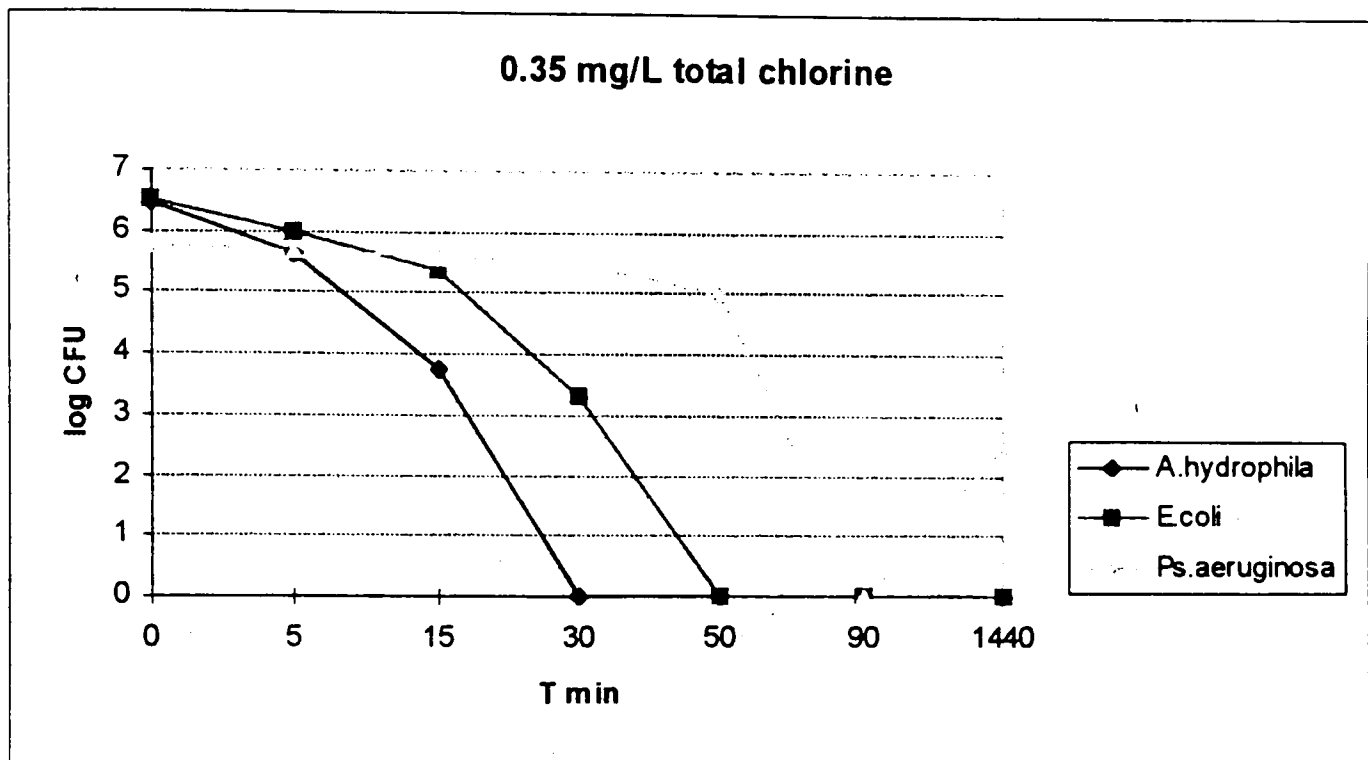
B

**Figure 1:   Recoveries of type strains after dosing in
1/4 strength Ringer's at 0.35mg/L (A) and 0.70mg/L (B) total chlorine**

Comparison of *A hydrophila* with two other species of mesophilic *Aeromonas, A caviae* and *A sobria*, showed *A sobria* to be particularly sensitive to chlorine whilst *A caviae* resembled closely *A hydrophila* (Figures 2A,B).

A

0.18 mg/L total chlorine

log CFU

7
6
5
4
3
2
1
0

0   5   15   30   50   90   1440

T min

—◆— A.hydrophila
—■— A.caviae
A.sobria

B

0.35 mg/L total chlorine

log CFU

7
6
5
4
3
2
1
0

0   5   15   30   50   90   1440

T min

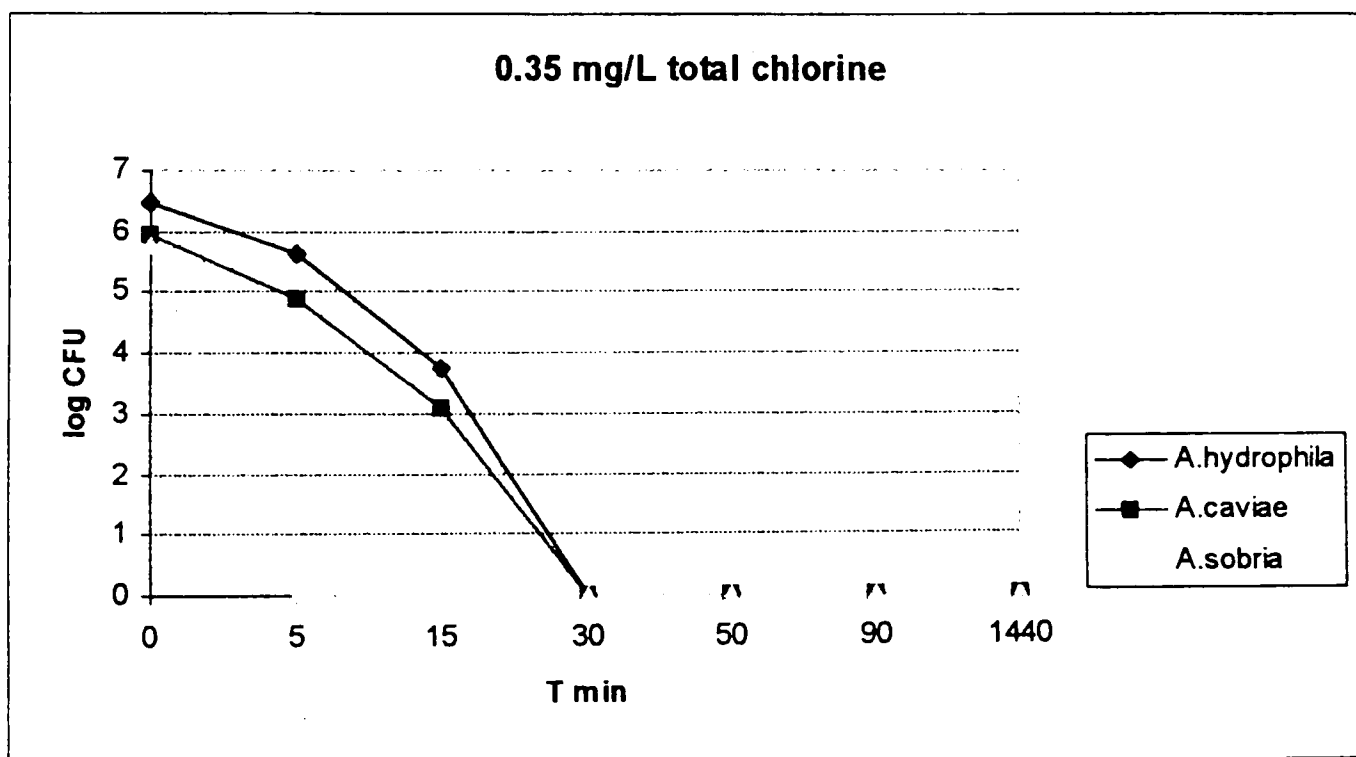—◆— A.hydrophila
—■— A.caviae
A.sobria

Figure 2: Recoveries of type strains after dosing in 1/4 strength Ringer's at 0.18mg/L (A) and 0.35mg/L (B) total chlorine

1/4 strength Ringer's solution was used as the test medium in this experiment and, being a mineral buffer medium, would contain no components that chlorine would be reactive with. The use of unwashed cells for the 1mL inoculum would, however, introduce ammonium salt from the M9 culture medium which in combination with hypochlorite solution would form chloramines. Under these conditions no free chlorine, in the form of HOCl or

161

OCl⁻, was found to be present in the Ringer's test medium (data not shown). Similarly, when uninoculated M9 was added to the Ringer's test medium, no free chlorine was detectable. The results of the previous experiments represent the effect of chloramines and possibly other unknown chlorinated entities, rather than free chlorine, on the organisms tested. When repeated using washed culture inocula a much smaller chlorine dose was effective in reducing viability, as would be expected because the absence of ammonium nitrogen would allow free chlorine to affect the suspended cells. Free chlorine exerts a significantly stronger bactericidal effect than combined chlorine. However, the relative susceptibilities of *A hydrophila*, *E coli*, and *P aeruginosa* remained the same as before (Figure 3).
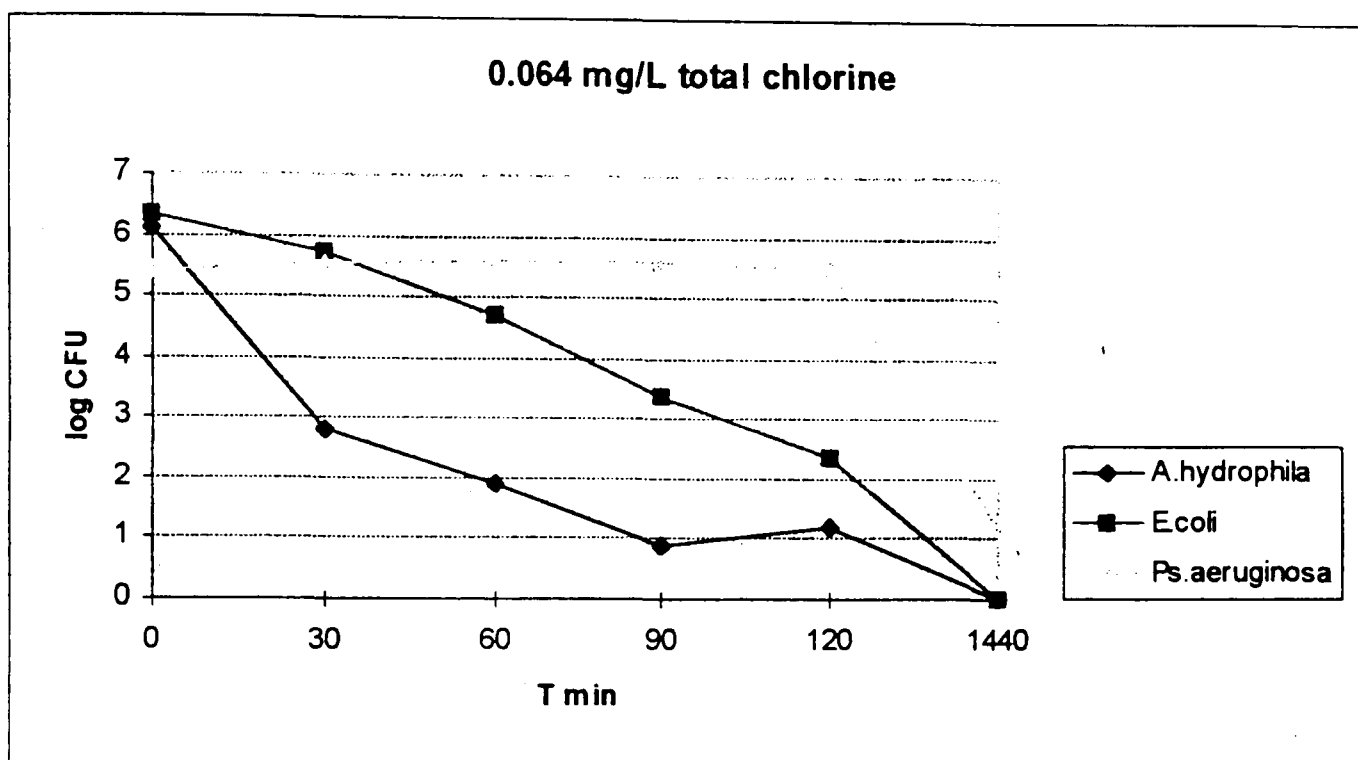

0.064 mg/L total chlorine

Figure 3: Recoveries of type strains after dosing in 1/4 strength Ringer's at 0.064mg/L total chlorine

Effect of test medium upon sensitivity of mesophilic Aeromonas - to investigate any effect of the test medium in influencing the inherent sensitivity of the organisms a number of different solutions were analysed using unwashed culture inoculum. Examination of a range of doses of hypochlorite showed *A hydrophila* to be most susceptible to the effects of chlorine in 1/4 strength Ringer's solution and least susceptible in M9 medium without glucose (Figures 4A-C).
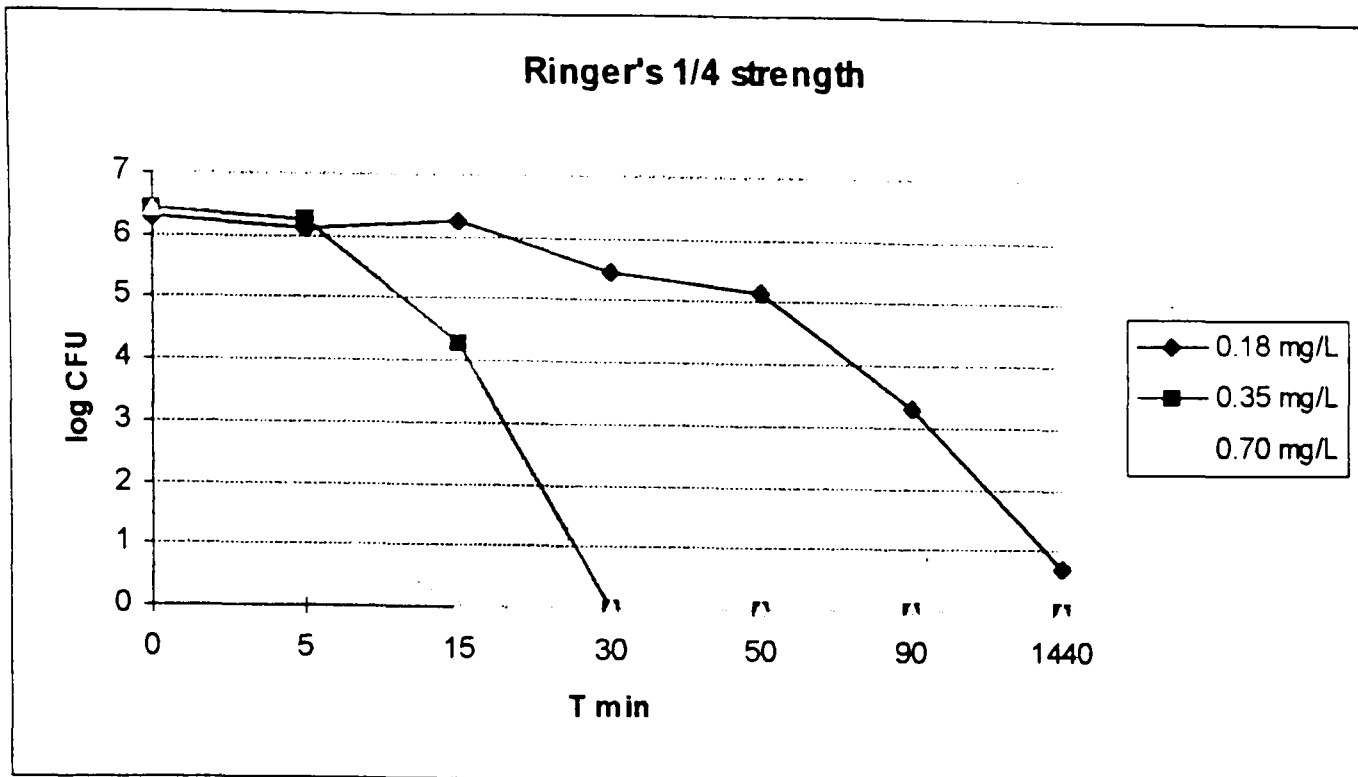
162

Figure 4A: Recoveries of _A hydrophila_ in 1/4 strength Ringer's at 0.18, 0.35, & 0.70mg/L total chlorine
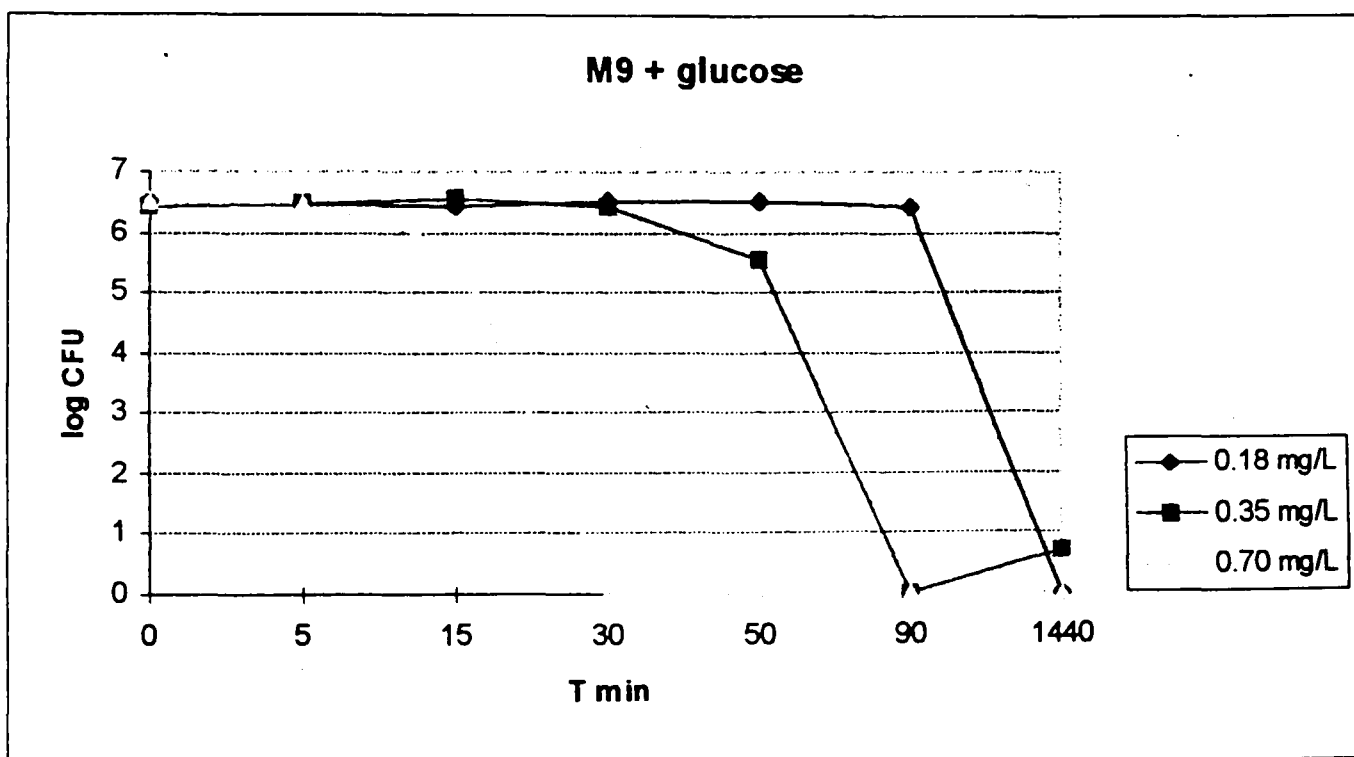

Figure 4B: Recoveries of _A hydrophila_ in M9 + glucose at 0.18, 0.35, & 0.70mg/L total chlorine
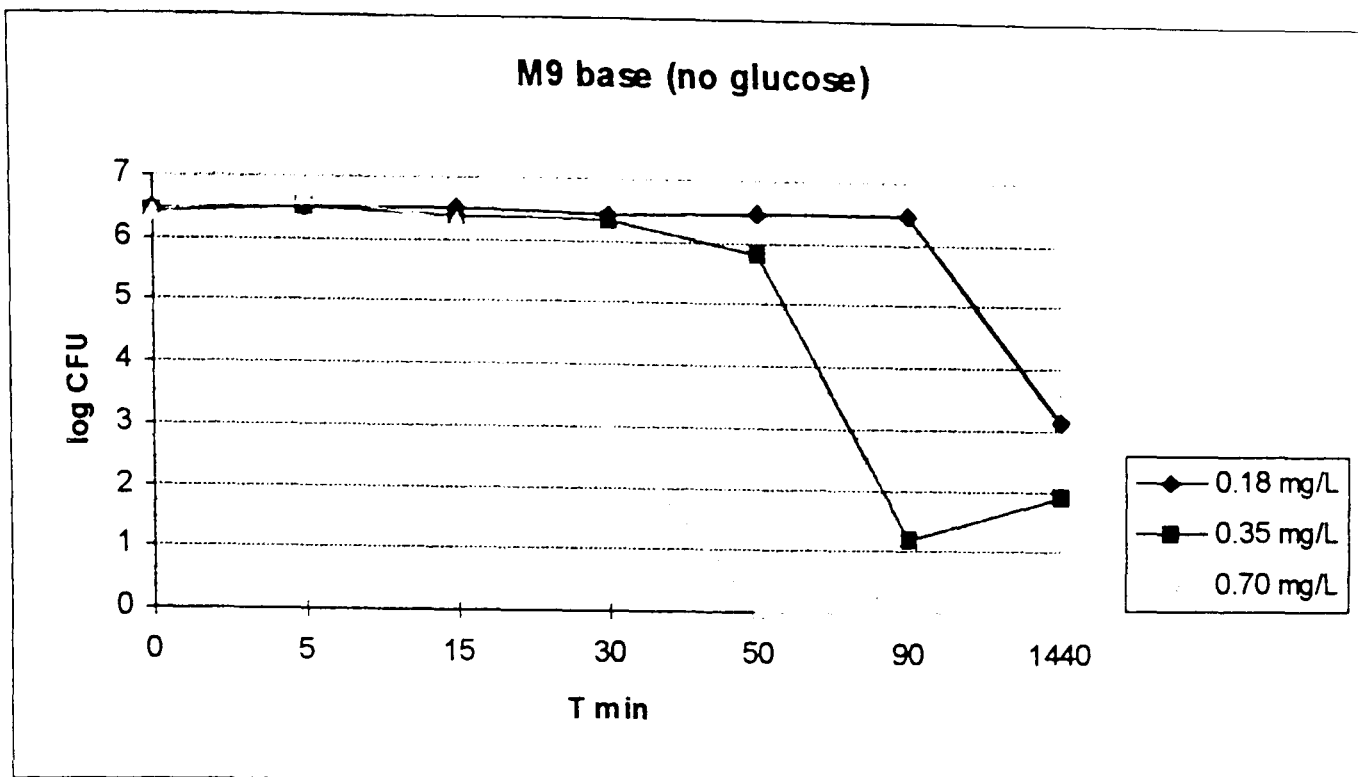
Figure 4C: Recoveries of *A hydrophila* in M9 without glucose at 0.18, 0.35, & 0.70mg/L total chlorine

As no ammonium salts are present in the formulation of Ringer's solution any chlorine added to this medium would be in a free form. However, as unwashed culture inoculum was used this would contribute ammonium nitrogen to the test medium so that the bactericidal effect seen here can be attributed mainly to chloramines and possibly other forms of combined chlorine. The effect of chlorine dosing is less severe with both M9 media where the concentration of ammonium nitrogen is relatively very high. Dosed chlorine would probably produce combined forms instantly before any free form could reach and affect the suspended cells. Interestingly, the presence of glucose in M9 results in lower viability and could be attributable to greater metabolic activity of the cells in this environment thus speeding up exposure of cellular mechanisms (membrane transport and enzymatic systems) to chlorine resulting in greater damage.

Adaptation of mesophilic Aeromonas to chlorine - in these experiments washed cultures were used with 1/4 strength Ringer's test medium. Cell viability was determined at time zero prior to addition of the sub-lethal dose, 0.004mg/L, and after 30min immediately before addition of the higher dose, (Figures 5A-C).
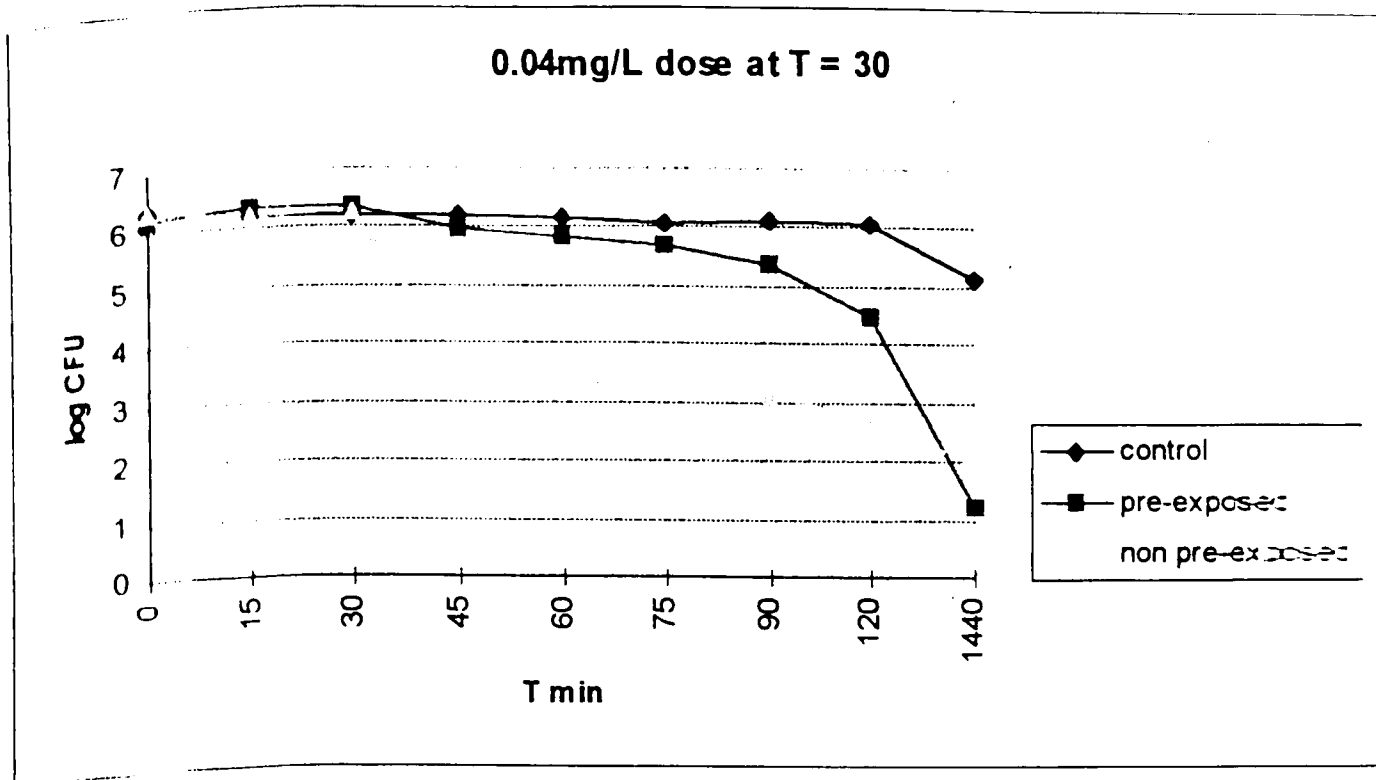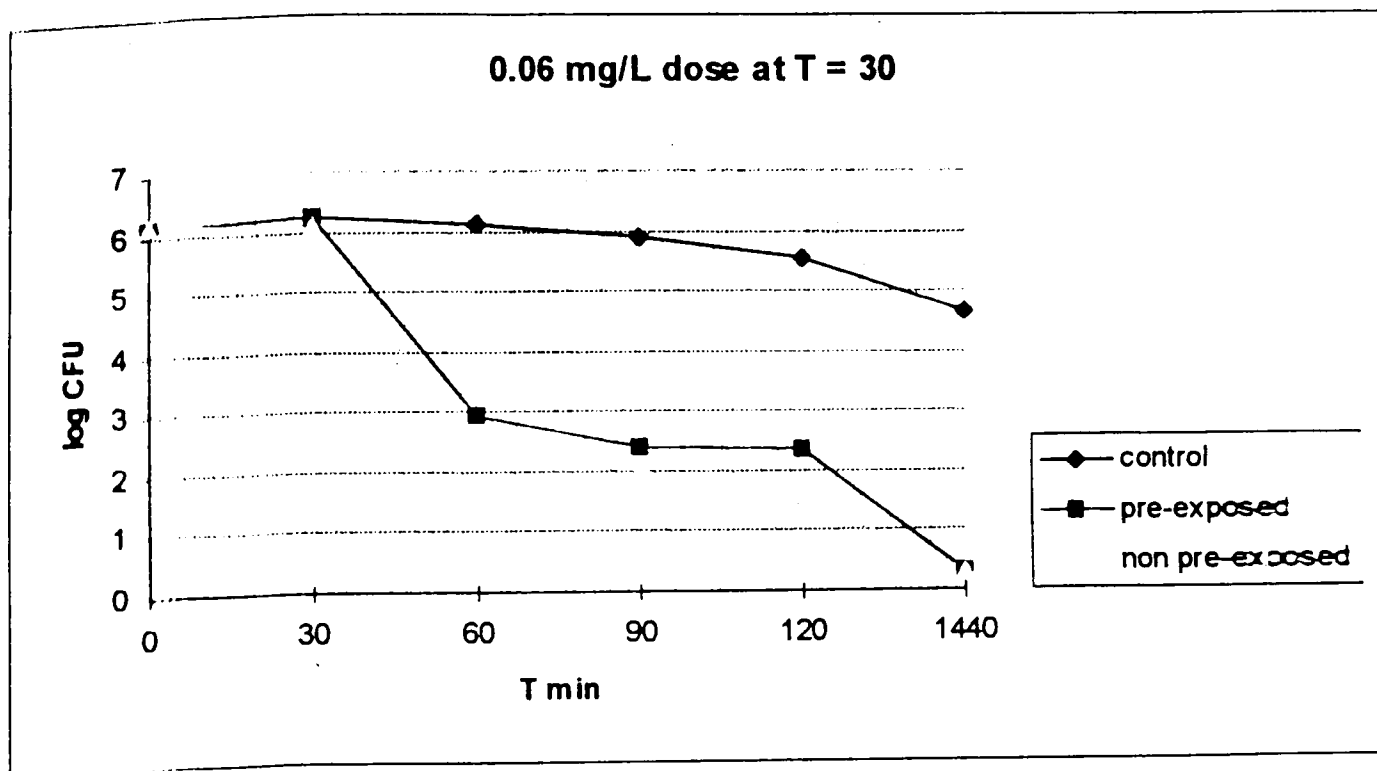
164

Figure 5A: Pre-exposure of *A hydrophila* to 0.004mg/L; subsequent dose 0.04mg/L (total chlorine)

Figure 5B: Pre-exposure of *A hydrophila* to 0.004mg/L; subsequent dose 0.06mg/L (total chlorine)
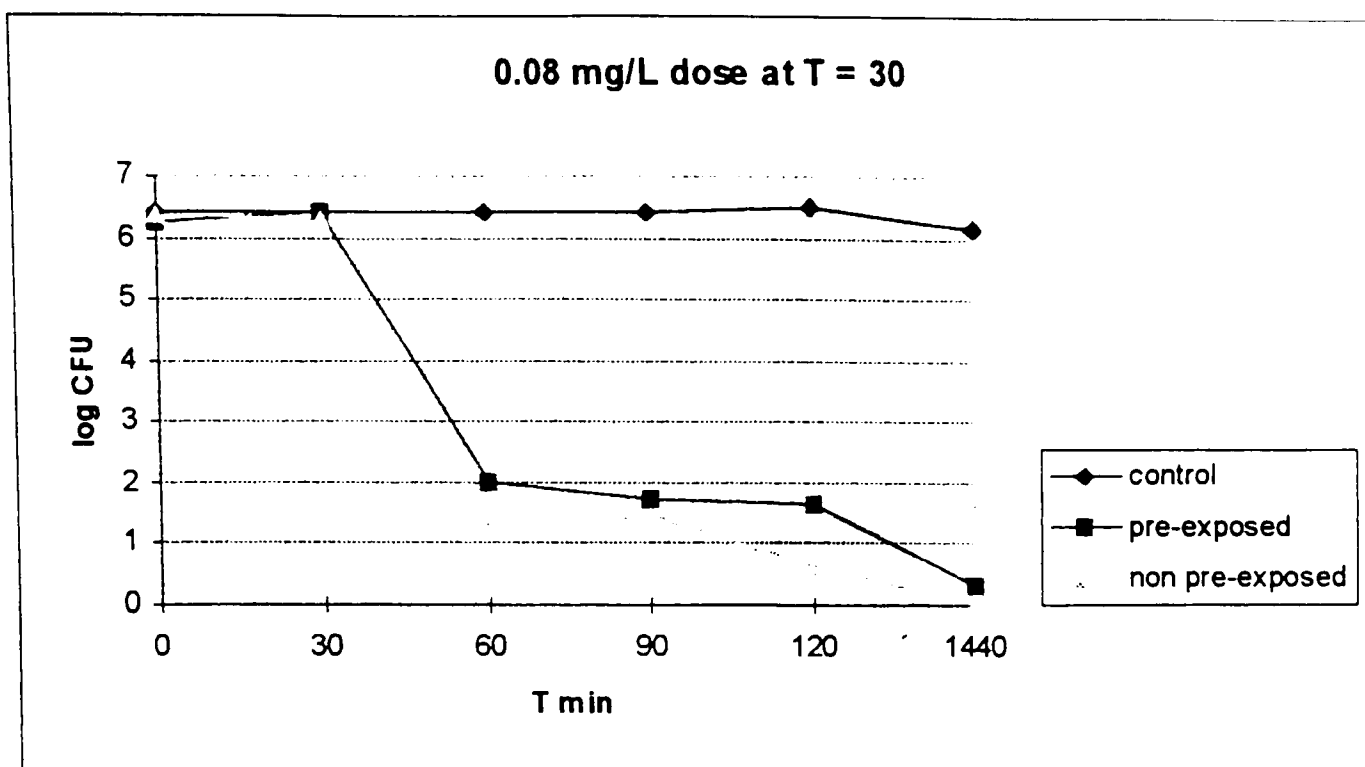
0.08 mg/L dose at T = 30

Figure 5C: Pre-exposure of *A hydrophila* to 0.004mg/L; subsequent dose 0.08mg/L (total chlorine)

The results indicate that the pre-exposure to a very low chlorine concentration, 0.004mg/L, which was previously found to have no effect on reducing recoveries over the test period, confers greater tolerance to subsequent higher concentrations for a period of several hours. This was particularly apparent for the 0.04mg/L dose. However, the reproducibility of this adaptive phenomenon has been difficult to achieve and results from similar experiments gave a cumulative effect of chlorine dosing on recoveries where the greater the sum total dose, the greater the lethal effect.

Effect of long term exposure of mesophilic Aeromonas to chlorine - this experiment evaluated the effect of repeated chlorine dosing upon a suspension of *A hydrophila* over a period of several weeks in order to more closely simulate exposure to this oxidant in a water distribution system. M9 minus the ammonium chloride component was used to eliminate ammonium nitrogen and the cell inoculum was washed. A dose equivalent to 0.08mg/L total chlorine was added at day 13, 25 and 54 subsequent to an initial dose at day 0 of 0.016, 0.048 or 0.080mg/L (Figure 6). Over the experimental period the unchlorinated control remained stable with no decrease in recoveries after the first 30 days (6.6 $\log_{10}$ CFU) and by day 80 remained at a substantial level (4.9 $\log_{10}$ CFU).
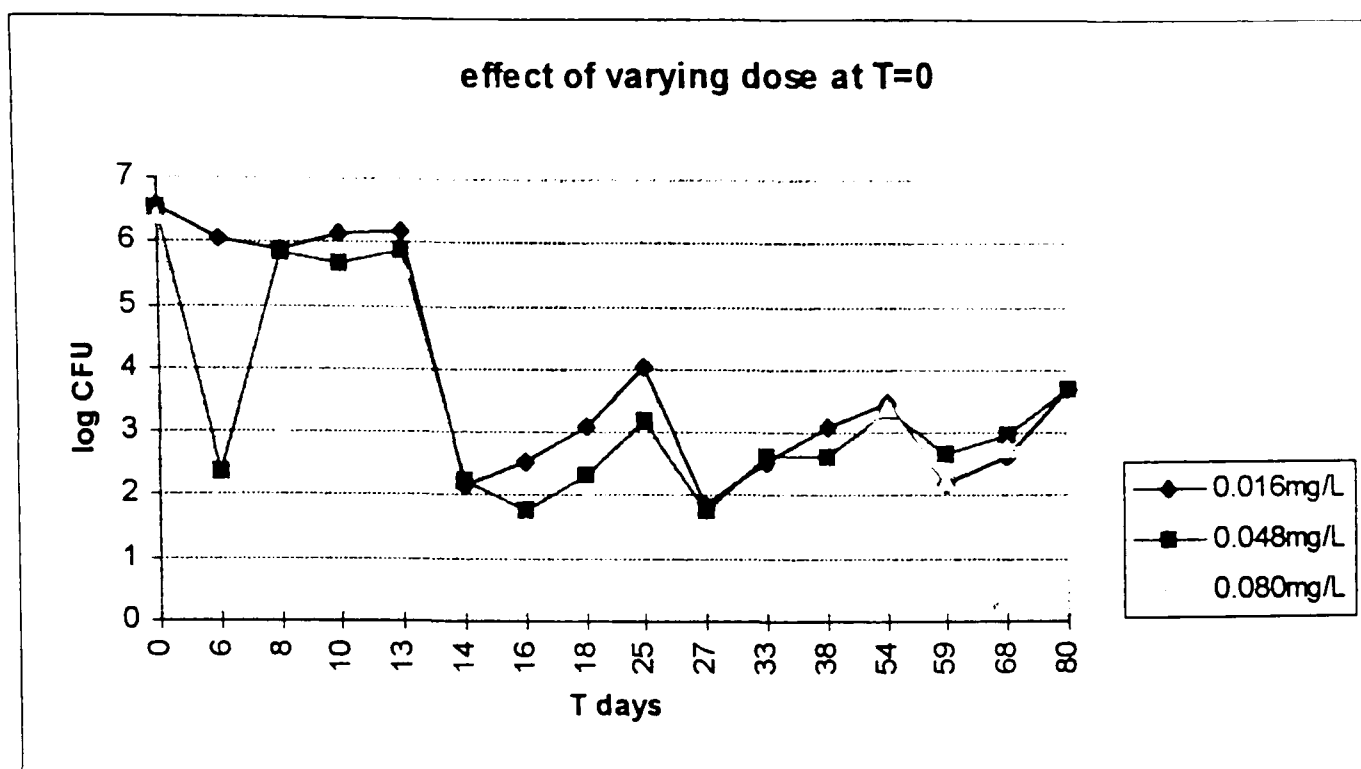
effect of varying dose at T=0

Figure 6: Recoveries of _A hydrophila_ in M9 minus ammonium; initial dose (T = 0) varied; subsequent periodic doses of 0.08mg/L total chlorine

After the initial chlorine dose at day 0 the cell concentration decreased substantially but recovered to the original level after 2 weeks. The initial dose appeared to have little impact upon either the initial loss of viability or the subsequent recovery. The subsequent dose of 0.08mg/L on day 13 of incubation reduced recoveries to 1-2 $\log_{10}$ but viability increased by 2 $\log_{10}$ after a further 12 days of incubation, when the next dose was added at day 25. Again viability was reduced but not completely lost with a slow increase of 2 $\log_{10}$ CFU after an additional 29 day incubation. Interestingly, the final dose on day 54 hardly reduced recovery of the population, viability only dropping by 1 $\log_{10}$.

DISCUSSION

In spite of isolating _Aeromonas_ from drinking water supplies in the presence of high levels of chlorine it was found to be the most sensitive to the effects of this oxidant in our comparison with _E coli_ and _P aeruginosa_. Of the three species of _Aeromonas_ examined _A sobria_ showed particular sensitivity to chlorine. This species is, however, among those commonly reported to be isolated from chlorinated drinking-water systems.

The phenomenon of bacterial adaptation to stress factors (such as temperature, pH and starvation) have been investigated extensively, primarily in the Enterobacteriacea (Demple, 1991; Yura, Nagai & Mori, 1993). Previously it was established that _A hydrophila_ is capable of acquiring tolerance to pH through an adaptive process (Karem, Foster & Bej, 1994).

However, little work has been undertaken to examine the effect of chlorine upon bacteria due to the highly reactive nature of this oxidant.

One study performed recently with *E coli* (Dukan & Touati, 1996) demonstrated that the same mechanisms involved in conferring protection to hydrogen peroxide are involved in providing resistance against chlorine. However, they were unable to demonstrate a chlorine-induced adaptation to subsequent treatment with chlorine. In contrast we have shown that *A hydrophila* can develop a chlorine resistant phenotype when pre-exposed to small, non-lethal concentrations of this agent. Given the reactive nature of this oxidant, repeatability of this response has proven to be difficult to achieve. On the basis of the data obtained this response is likely to contribute to the recoverability of aeromonads from chlorinated drinking water supplies.

The results of the experiments on long-term survival with repeated dosing were interesting although interpretation of these results is complex. The fall in recoveries after the initial dose was proportional to the size of the dose, as would be expected. With all three doses the initial cell density was re-established by day 13 at a rate inversely proportional to the dose. With subsequent treatment, for all three test vessels at 0.08mg/L, the rate of recovery appears to be the same whilst the final count reached seems to be related to the initial level of chlorine used. With further treatment the difference in final count between the three populations is minimal. Initially then, the rate of recovery is inversely proportional to the concentration added but independent of cumulative exposure whereas the final count achieved is inversely proportional to cumulative exposure.

There are a number of possible explanations for the pattern seen in these long-term repeated dosing experiments.

(i)   The repeated exposure eventually results in the death of most of the population. This leaves behind a more resistant sub-population aided in its survival by nutrients provided by the lysed dead cells and shielded from the effects of chlorine due to mopping of the oxidant by the cellular debris from the dead population.

(ii)   (ii)Alternatively, we could be seeing the results of cryptic growth where the death of most of the population leaves behind survivors which, aided by the nutrients obtained from the dead cells and their chlorine mopping effect, are growing and multiplying. If growth and multiplication of this sub-population is ruled out, then the gradual increase in recoveries could be attributed to the time required to recover from the stress i.e. the sub-population may be in a viable but non-culturable (vbnc) state.

(iii)   Another possible related explanation could be that most of the population is not killed at this level of chlorination but, due to the effects of chlorine stress and starvation, the cells are in a vbnc state. The gradual increase in counts could then be attributable to gradual

recovery from the dormant, non-culturable state some time after contact with chlorine.

However it is liable to be that a combination of the factors of cryptic growth, vbnc state, and adaptive tolerance account for the pattern of viability observed.

In summary we have demonstrated that mesophilic aeromonads, whilst displaying high levels of sensitivity to chlorine, can develop the ability to maintain viability in the presence of substantial amounts of this bactericidal agent.

REFERENCES

Ashbolt, NJ, Ball, A, Dorsch, M, Turner, C, Cox, P, Chapman, A & Kirov, SM (1995). The identification and human health significance of environmental aeromonads. *Wat Sci Tech*, **31**, 263-269.

Carnahan, AM & Altwegg, M (1996). Taxonomy. In "The Genus *Aeromonas*" edited by B Austin, M Altwegg, PJ Gosling & S Joseph, John Wiley & Sons Ltd.

Chowdhury, MAR, Yamanaka, H, Miyoshi, S & Shinoda, S (1990). Ecology of mesophilic *Aeromonas* spp in aquatic environments of a temperate region and relationship with some biotic and abiotic environmental parameters. *Zlb Hyg*, **190**, 344-356.

Demple, B (1991). Regulation of bacterial oxidative stress genes. *Ann Rev Genetics*, **25**, 315-337.

Dukan, S & Touati, D (1996). Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage and comparison with hydrogen peroxide stress. *J Bact*, **178**, 6145-6150.

Gavriel, AA, Landre, JPB & Lamb, AJ (1997). Incidence of mesophilic *Aeromonas* within a public drinking water supply in north east Scotland. *J Appl Microbiol*, in press

Holmes, P & Niccolls, LM (1995). Aeromonads in drinking water supplies: their occurrence and significance. *J Chart Inst Wat Environ Manag*, **5**, 464-469.

Holmes, P, Niccolls, LM & Sartory, DP (1996). The ecology of mesophilic *Aeromonas* in the aquatic environment. In "*The Genus Aeromonas*" edited by B Austin, M Altwegg, PJ Gosling & S Joseph, John Wiley & Sons Ltd.

Huys, G, Coopman, R, Janssen, P & Kersters, K (1996). High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int J Syst Bacteriol*, **46**, 572-580.

Huys, G, Kampfer, P, Altwegg, M, Kersters, I, Lamb, AJ, Coopman, R, Luthy-Hottenstein, J, Vancanneyt, M, Janssen, P & Kersters, K (1997). *Aeromonas popoffii* sp nov, a mesophilic member of the genus *Aeromonas* isolated from drinking water plants and reservoirs. *Int J Syst Bacteriol*, submittted.

Janda, JM & Abbott, SL (1996). Human pathogens. In "*The Genus Aeromonas*" edited by B Austin, M Altwegg, PJ Gosling & S Joseph, John Wiley & Sons Ltd.

Janda, JM (1991). Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clin Microbiol Rev*, **4**, 397-410.

Karem, KL, Foster, JW & Bej, AK (1994). Adaptive acid tolerance response (ATR) in *Aeromonas hydrophila*. *Microbiology*, **140**, 1731-1736.

Merino, S, Rubries, X, Knochel, S & Tomas, JM (1995). Emerging pathogens: *Aeromonas* spp. *Int J Food Microbiol*, **28**, 157-168.

Yura, T, Nagai, H & Mori, H (1993). Regulation of the heat shock response in bacteria. *Ann Rev Microbiol*, **47**, 321-350.