INDUCIBLE TOLERANCE AND SENSITIVITY TO STRESS RESPONSES IN *Escherichia coli* WITH PARTICULAR REFERENCE TO COPPER AND pH

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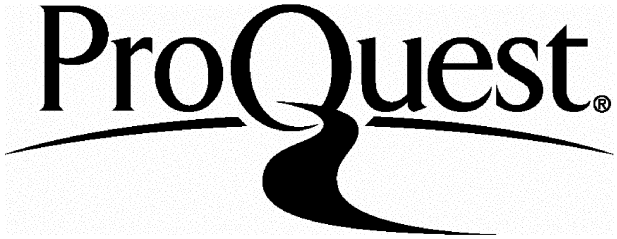

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For Bapak

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ABSTRACT

Stress responses to copper and alkali were studied in *Escherichia coli*. *E. coli* 1829 and its derivatives, were able to tolerate lethal doses of $CuSO_4$ (58.92 μg/ml and 117.84 μg/ml) after pre-exposure to sublethal doses of $CuSO_4$ (14.73 μg/ml and 29.46 μg/ml). The observed copper tolerance was due to a phenotypic change induced during the pre-exposure period which depends on *de novo* protein synthesis. Cytoplasmic membrane proteins of molecular weights 26 and 24.5 kDas and outer membrane proteins of molecular weights 16.5, 18, 31.5 and 65 kDas were overexpressed in the copper-induced cells. The DNA from the copper-induced cells was also less damaged than that from the uninduced cells. Pre-exposure to 14.73 μg/ml $CuSO_4$ also confers cross-protection to heat, acid, alkali and cadmium sulphate but not to hydrogen peroxide .

Pre-exposure to mildly acidic pH which would normally induce acid tolerance was shown to also induce alkali sensitivity. When *E.coli* 1829 cells were transferred from pH_0 7.0 to pH_0 5.5 for one hour they became alkali sensitive upon challenge with pHs 9.5 and 9.75 for 30 minutes. Substantial induction also occurs at pH 6.0 but there was less at pH_0 5.0 and practically none at pH_0 6.5. The response was triggered by cytoplasmic acidification by protons entering the cells possibly via OmpC, LamB, PhoE, NhaA and NhaB. The induction of alkali sensitivity also depends on *de novo* protein synthesis of components involved in alkali sensitization. Cytoplasmic membrane proteins of molecular weights 14 and 18 kDas were overexpressed in the pH 5.5 induced cells. The induction of the alkali sensitization components is not subject to catabolite repression nor affected by deletion in *rpoS* but appeared to be under the control of Fur, RelA, CysB and Lrp. Mutants with a deletion in *tonB* showed derepressed alkali sensitivity; the response being observed in pH 7.0 induced cells instead. The expression of the alkali sensitization components also appeared to be affected by changes in the DNA supercoiling and is influenced by HimA, HimD and H-NS.

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LIST OF ABBREVIATIONS

Ala	Alanine
Ap	Ampicillin
ATP	Adenosine Triphosphate
CAP	Chloramphenicol
CBU	Covert Bacteriuria
CFU	Colony Forming Units
cpm	Counts Per Minute
$CuCl_2$	Copper(II) chloride (cupric chloride)
$CuSO_4$	Copper(II) sulphate (cupric sulphate)
Da	Dalton
DNA	Deoxyribonucleic Acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
g/L	Gramme per Litre
Glu	Glucose
HCl	Hydrochloric Acid
IPTG	Isopropyl-β-D-Thiogalactosidase
Kan	Kanamycin
kDa	Kilodalton
KDO	3-deoxy-D-manno-2-octulosonic acid
KOH	Potassium hydroxide
K_2SO4	Potassium sulphate
LPS	Lipopolysaccharide
Lys	Lysine
M	Molarity
mA	Milliampere
MDO	Membrane-Derived Oligosaccharide
mg/L	Milligramme per Litre

mins	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
MM	Minimal media
MMA	Minimal Media Agar
N	Normality
NA	Nutrient Agar
NaOH	Sodium Hydroxide
NB	Nutrient Broth
nm	Nanometre
OD	Optical Density
OmpA	Outer membrane protein A
OmpC	Outer membrane protein C
OmpF	Outer membrane protein
pH_o	External pH
pH_i	Internal pH
P_i	Inorganic Phosphate
R	Resistance
Rif	Rifampicin
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
Tet	Tetracycline
TNA	Tryptone Sodium Chloride
UTI	Urinary Tract Infection
UV	Ultra Violet
V	Volume
v/v	Volume per Volume
w/v	Weight per Volume

Symbols

°C	Degree Celsius
%	Percentage
Δ	Deletion
λ	Lambda
$\Delta\psi$	Electrical Potential Difference
Δp	Proton Motive Force (PMF)
ΔpH	pH Difference
$\Delta\mu H^+$	Electrochemical Potential Difference for Protons
µg	Microgramme
µci	Microcurie
µl	Microlitre
µM	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Prologue

Facultative bacteria such as the potential enteric pathogen *Escherichia coli*, can exist in widely diverse environments and thus have to cope with a variety of stresses. The ability of these bacteria to overcome some of these stresses has been a subject of intense research in recent years. It is now known that for most, tolerance to a stress can be induced and maximised if the bacterial cells have prior exposure to sublethal conditions of the same stress. Such inducible tolerances have been demonstrated to, among others, temperature (**Yamamori and Yura, 1982**), DNA-damaging agents (**Samson and Cairns, 1977**; **Jeggo *et al.*, 1977**) oxidative stress (**Demple and Halbrook**), acid pH (**Goodson and Rowbury, 1989a; Foster and Hall, 1990**) and alkaline pH (**Goodson and Rowbury, 1989b**) in *Escherichia coli* and *Salmonella typhimurium.* Frequently, exposure to sublethal condition of one stress can also alter responses to other stresses thus inducing cross-responses (**Morgan *et al.*, 1986; Jenkins *et al.*,, 1988; Jenkins *et al.*, 1990; Goodson and Rowbury, 1990**). One of the significant implications of inducible tolerance responses and cross-responses is the possible effects of subsequent survival of pre-stressed potential pathogens in food and water.

Although bacterial responses to a number of chemical and physical stresses have been studied, there is not much known about stress-responses to common polluting heavy metals such as copper, which is used in water treatment processes. Likewise, while tolerance to low acid pH could be induced in bacterial cells that have had prior exposure to sublethal acid pH, not much is known of the response of these pre-exposed bacteria to high alkaline pH which is frequently encountered in parts of the body and in polluted waters. In light of this, the study of responses to copper and pH stresses in *Escherichia coli* was undertaken.

Before introducing the relevant current background knowledge to this study, the following section will discuss the public health importance of *Escherichia coli.*

1.2 *Escherichia coli*- its importance in public health

Escherichia coli belongs to the Enterobacteria and is the type species of the genus *Escherichia* (**Ewing, 1985**). This short, non-sporing, facultative anaerobic Gram-negative bacillus is usually fimbriated and motility is by peritrichous flagella. It often has a capsule or microcapsule and in a few strains profuse polysaccharide slimes are produced (**Sussman, 1985**). The primary habitat of *Escherichia coli* is the gastro-intestinal tract and when found in nature, it is usually derived from the primary habitat due to faecal contamination (**Sussman, 1985**). As a member of the commensal flora of the gut *Escherichia coli* does not cause harm and can play a nutritional role by synthesizing vitamins particularly vitamin K (**Sussman, 1985**). However some types of *Escherichia coli* are known to produce severe diseases in man and animals. The pathogenicity of *Escherichia coli* involves a complex of virulence factors namely, bacterial adhesion, invasion of the host defenses, production of toxins and maintenance of bacterial growth under adverse nutritional conditions, for example starvation (**Finlay and Falkow, 1989**). Pathogenic *Escherichia coli* is multipotent and can cause infections and diseases in several body systems.The infections and diseases caused by *Escherichia coli* in man and animals can be categorised as either intestinal or extraintestinal.

1.2.1 Intestinal infections and diseases in man and animals due to *E.coli*

a) Gastrointestinal diseases. In man, *E. coli* has been shown to be the causative agent of gastrointestinal diseases or diarrhoeal diseases through ingestion of contaminated water and food. The *E.coli* strains that cause diarrhoeal diseases in man have the ability to adhere or invade and colonize the epithelial cells of the intestinal walls. Some are known to produce enterotoxins (Enterotoxigenic *E.coli*) whilst others do not (Enteroinvasive *E.coli*); or only do so in some serotypes (Enteropathogenic *E.coli*) (**Sussman, 1985**; **Levine 1987**). The enterotoxigenic *E.coli*, (ETEC) can cause diarrhoea in adults and children and is also the causative agent of traveller's diarrhoea which is common in Europeans and North Americans travelling to developing countries (**Sussman, 1984, Ewing, 1985**). The clinical features of ETEC infection are watery diarrhoea, nausea, abdominal cramps and fever (**Levine, 1987**). The enteropathogenic *E.coli* (EPEC) can cause severe or chronic infantile diarrhoea (**Sussman, 1985**; **Levine 1987**). They can

cause histopathologic lesions in the intestine resulting in loss of microvilli and thickening of the cell surface at site of the attachment (**Sussman, 1985**; **Levine 1987**). The infection of EPEC is followed by fever, malaise, vomiting and diarrhoea with mucus (**Levine, 1987**). The enteroinvasive (EIEC) *E.coli* is capable of producing bacillary dysentery (**Sussman,1985**). The EIEC is capable of penetrating, proliferating within the epithelium of the large intestine resulting in eventual death of host cells. The features of EIEC infection are fever, severe abdominal cramps, malaise, toxaemia and watery diarrhoea with blood and mucus (**Levine, 1987**). The enterohaemorrhagic *E.coli* (EHEC) is non-invasive and belongs to the single serotype O157:H7 (**Sussman, 1985**; **Levine 1987**). It is associated with haemorrhagic colitis which is characterised by sudden severe abdominal colic and grossly bloody diarrhoea (**Sussman, 1985**). A group of potent toxins that are related to the Shiga toxin is reported to be involved (**Sussman, 1985**).

b)Enteric colibacillosis. *E.coli* infections in animals are usually referred to as colibacillosis (**Sussman, 1985**). Enteric colibacillosis is one of the two forms of colibacillosis that infect young animals. Enteric colibacillosis can be grouped into two syndromes namely colibacillary diarrhoea and colibacillary toxaemia which include oedema disease of pigs (**Sussman, 1985**). Colibacillary diarrhoea is the most common form of enteric colibacillosis and is due to enterotoxin produced in *E.coli* (**Sussman, 1985**). The diarrhoea due to the enterotoxins produced occurs frequently in newborn calves, lambs and piglets (**Sussman, 1985**). Colibacillary toxaemia is associated with endotoxin producing *E.coli* and frequently occurs in newborn calves, lambs and particularly weaning piglets (**Sussman, 1985**).

1.2.2 Extraintestinal infections and diseases in man and animals due to *E.coli*

a) *Urinary tract infections.* *E.coli* is also an aetiological agent for urinary tract infections (UTI). There are several types of UTI, the most common is covert bacteriuria (CBU) or asymptomatic bacteriuria (**Sussman, 1985**). The silent phase of this infection is interrupted with symptomatic events leading to acute cystitis (infection of bladder) or acute pyelonephritis (infection of kidney) (**Sussman, 1985**). CBU in pregnancy has been associated with excess risk of toxaemia, prematurity, low birth weight and still birth

(**Sussman, 1985**). The production of haemolysin, the presence of the capsular K antigen and production of adhesins (MR-HA fimbriae) that enable the organism to colonize the urinary tract and resist the wash out effects of urine flow are the virulence traits of this strain (**Sussman, 1985**).

b)Haemolytic-uraemic syndrome. This is the commonest cause of acute renal failure in children and was first described in 1955 (**Sussman, 1985**). It consists of an acute febrile (fever) illness followed by acute renal failure and intravascular haemolysis. In most of the sporadic cases reported, *E.coli* strains that produce verotoxin are involved (**Sussman, 1985**).

c)Meningitis in newborn is predominantly due to *E.coli* and the strains responsible for this carry the KI capsular antigen (**Sussman, 1985**).

d)Septicaemia. The entry of *E.coli* into the blood stream in large numbers over a period of time through discrete or diffuse tissue lesion or more commonly through urinary tract infection results in septicaemia (**Sussman, 1985**). Although the organisms are rapidly removed from the circulation by actions of phagocytes and/or complement, the symptoms of septicaemia are due to effects of endotoxin released from the organisms. The effects include damage to blood vessels, increases in vascular permeability and dilatation leading to some of the haemodynamic changes that constitute endotoxic shock (**Sussman, 1985**). The endotoxin may also affect the lungs, kidney and liver (**Sussman, 1985**)

e)Systemic colibacillosis. Systemic colibacillosis occurs frequently in calves, lambs and poultry (**Sussman, 1985**). The invasive bacteraemic strains of *E.coli* enter the blood stream through the mucosa of the alimentary or respiratory tract resulting in either generalized infection or a localized infection such as meningitis and/or arthritis in calves and lambs or air sacculitis and pericarditis in poultry (**Sussman, 1985**).

f) Mastitis. In adult cattle, coliform mastitis is associated with *E.coli* infection. Coliform mastitis affects lactating cows particularly during the winter months and is caused by serum resistant *E.coli* (**Sussman, 1985**). Coliform mastitis is often severe in early lactation and this normally coincides with changes from grazing to winter housing and feeding (**Sussman, 1985**). Contamination of bedding with *E.coli* mastitis have been demonstrated (**Sussman, 1985**). The infection is usually confined to the epithelium of the teat sinus, lactiferous sinus and large ducts (**Sussman, 1985**). The responses to the

infection depend on the stages of lactation and the number of infected quarters. With a very mild reaction, there is a transient increase in number of *E.coli* followed by a rapid elimination of this organism (**Sussman, 1985**). The mild response to the infection include symptoms such as clots, milk discolouration and swelling of the gland (**Sussman, 1985**). If the multiplication of *E.coli* is unchecked severe damage to the udder, general toxaemia and death within 24 hours of illness may result (**Sussman, 1985**).

1.3 The Cell Envelope Of *Escherichia coli* - structures and functions

The cell envelope of *E.coli* and all Gram-negative bacteria consists of essentially three layers namely outer membrane, peptidoglycan layer (or murein) and cytoplasmic membrane (**Lugtenberg and van Alphen, 1983**). The outer membrane is anchored to the peptidoglycan layer which occupies the space between the outer and cytoplasmic membranes (**Figure 1-1**). This space is called the periplasm or periplasmic space (**Lugtenberg and van Alphen, 1983**; **Stock *et al.*, 1977**). The outer and cytoplasmic membranes are interconnected in parts by zones of adhesions or Bayer junctions (**Lugtenberg and van Alphen, 1983**). A capsular layer (K-antigen) which is a polysaccharide containing material can be present outside the outer membrane (**Lugtenberg and van Alphen, 1983**). Appendages such as flagella, fimbriae and pili are also anchored in the cell envelope (**Lugtenberg and van Alphen, 1983**).

1.3.1 Outer membrane

Chemical analysis of isolated outer membrane revealed that it consists of lipopolysaccharides (LPS), phospholipids and proteins (**Nikaido and Nakae, 1979; Lugtenberg and van Alphen, 1983**). The outer membrane is asymmetric as a result of the distribution of phospholipids and LPS; the LPS being exclusively located only in the outer leaflet of the membrane and the phospholipids being predominantly located in the inner leaflet (**Nikaido and Vaara, 1985**). The following describes the components of outer membrane.

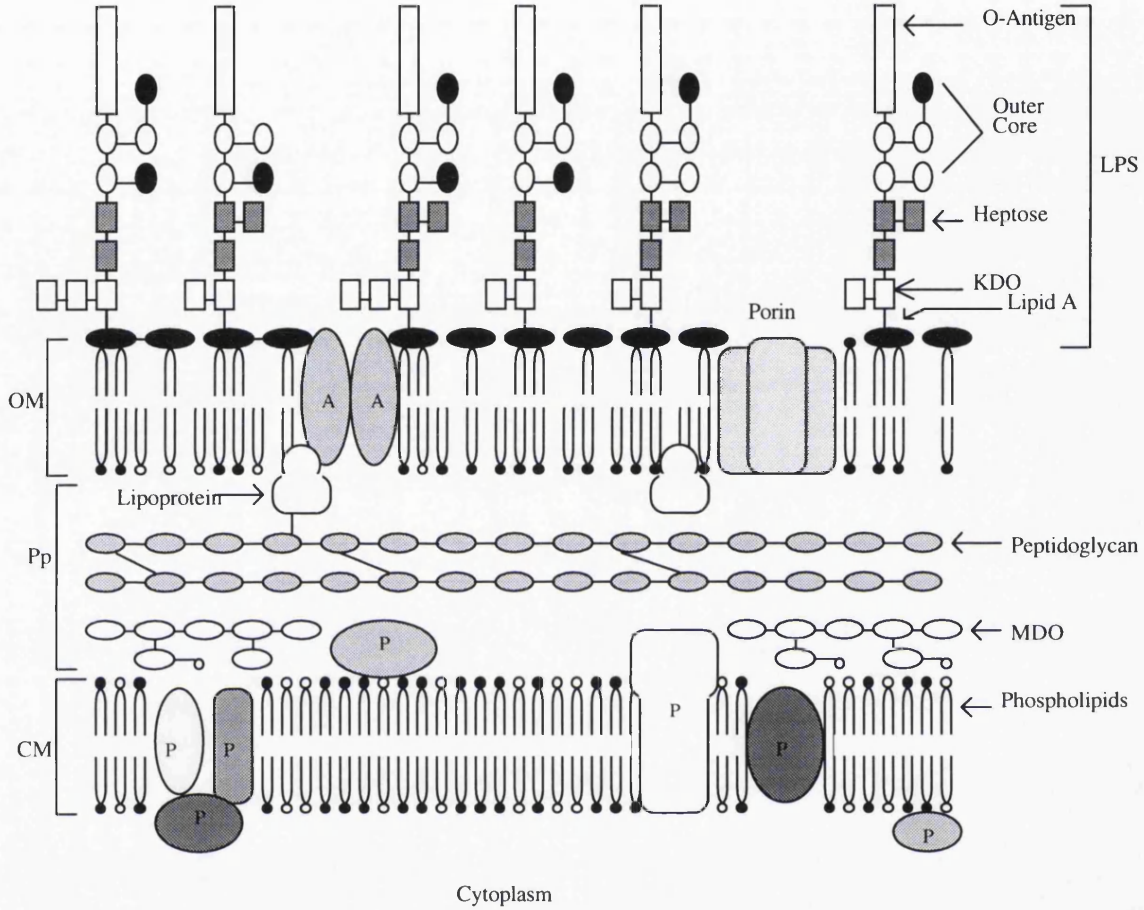


Figure 1-1 Schematic molecular representation of the *Escherichia coli* cell envelope (after **Raetz, 1990**). Ovals and rectangles depict sugar residues; circles represent polar head groups of phospholipids. KDO and heptose make up the inner core LPS.

Abbreviations: A- outer membrane protein A (OmpA); CM- Cytoplasmic membrane; KDO- 3-deoxy-D-manno-2-octulosonic acid; LPS- Lipopolysaccharides; MDO- membrane-derived oligosaccharides; OM- Outer membrane; P- protein ; Pp- Periplasm

1.3.1.1 Lipopolysaccharide

LPS is characteristic of Gram-negative bacteria and exclusively occupies 40% of the outer leaflet of the outer membrane (**Benz, 1985**). LPS is an amphipathic molecule consisting of three general regions; a hydrophilic portion represented by the highly variable O-antigenic polysaccharide which is linked to the hydrophobic, biologically active endotoxin (Lipid A) via the core oligosaccharide (**Figure 1-2**) (**Lugtenberg and van Alphen, 1983**; **Benz, 1988**) The lipid A moiety is a β, 1-6 linked disaccharide of glucosamine to which between five and seven acyl side chains are linked via ester and amide bonds (**Lugtenberg and van Alphen, 1983**). LPS is attached to the outer membrane in part, by these acyl side chains and this attachment is fully stabilized by its interaction with divalent cations and with proteins in the outer membrane (**Lugtenberg and van Alphen, 1983**). The LPS core contains the unique sugars 2-keto-3-deoxyoctulosonate (KDO) and L-glycero-D-mannoheptose and a number of common sugars including glucose, galactose and glucosamine (**Osborn, 1969**). The core region can be distinguished into backbone or inner core and outer core (**Figure 1-2**) .The inner core is made up of L-glycero-D-mannoheptose, KDO, phosphate and ethanolamine and the outer core contains glucose, galactose and glucosamine which provide the sites for attachment of the O-specific side chains (**Osborn, 1969**). The O-antigen which provides the basis for serological classification of the enterobacteriaceae according to the Kauffmann-White scheme, can consist of more than 40 repeating units containing 3 to 6 sugar residues (**Lugtenberg and van Alphen, 1983**). A large diversity of sugars can be found in the repeating units of the O-antigen which can vary from none to more than 40 (**Lugtenberg and van Alphen, 1983**). The O-antigen is not present in all strains. Strains with absent or reduced O-polysaccharide are called "rough" mutants and such strains include the *E.coli* laboratory strains K-12, B, and C (**Lugtenberg and van Alphen, 1983**).

The molecular weight of individual LPS molecules can vary from 8,000 to 54,000 Da according to the lack or presence of variable numbers of repeating saccharide units that comprise the O-antigen polysaccharide (**Hancock, *et al.*, 1994**). Certain Gram-negative bacteria such as *Neisseria sp., Haemophilus influenza* and *Bordetella pertussis* do not produce long O-polysaccharide but a chain of ten monosaccharides called lipoligosaccharides (LOS) (**Wu *et al.*, 1987**).

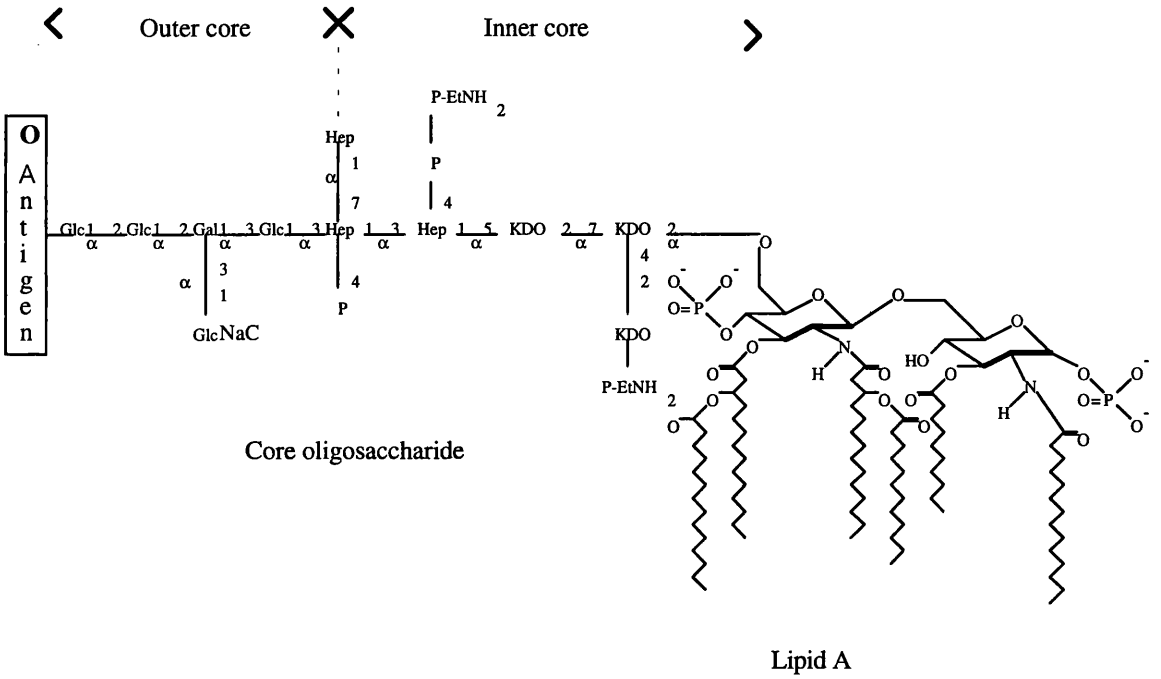


Figure 1-2 The structure of LPS from *Escherichia coli* showing the three regions; Lipid A, core oligosaccharide (inner and outer core) and the O-antigen (reproduced from **Hancock, *et al.*, 1994**).

Abbreviations: Glc-glucose; Gal-galactose; GlcNac-acetyl glucosamine; Hep-heptose; $EtNH_2$- ethanolamine.

LPS is responsible for endowing the outer membrane with strong hydrophilicity which is important in evading phagocytosis, acquiring resistance to complement and avoiding specific immune attack (**Nikaido and Vaara, 1985**). The LPS also forms a strong barrier preventing diffusion of hydrophobic molecules through the outer membrane. This diffusion barrier is attributable to the long oligosaccharide chains and to the close association of the LPS molecules in the outer monolayer via van der Waals interactions between the side chains and via ion bridges between charged groups located at the polysaccharide moiety (**Benz, 1985**). Mutants with drastically reduced length of oligosaccharides ("rough" mutants) very often have an increased permeability to the outer membrane for hydrophobic compounds (**Benz, 1985**).

1.3.1.2 Phospholipids

Phospholipids are amphipathic molecules having a negatively charged hydrophilic phosphate group and a non polar hydrophobic lipid portion. There are three main species of phospholipids in the outer membrane of *E.coli* namely phosphatidylethanolamine, phosphotidylglycerol and cardiopilin (**Raetz, 1978**). The phospholipids play a structural role in the outer membrane (**Cronan and Vagelos, 1972**). The phospholipids are exclusively located in the inner leaflet of the outer membrane. The outer membranes of *E.coli* and *S.typhimurium* are enriched with phosphatidylethanolamine which is a neutral lipid (**Osborn *et al.*, 1972; Jones and Osborn, 1977**). The enrichment of outer membrane with phosphatidylethanolamine could be because it forms stable bilayers with LPS (**Lugtenberg and van Alphen, 1983**).

1.3.1.3 Proteins

There are several types of proteins present in the outer membrane of the Gram-negative bacteria; some appear to play a role in the stabilization of the membrane, some are involved in transport of solutes, some acts as receptors to phages while others are only inducible under certain growth conditions. The following describes the types of proteins present in the *E.coli.*

1.3.1.3.1 Lipoprotein

The lipoprotein is the most abundant membrane protein in *E.coli* (**Lugtenberg and van Alphen, 1983**). Nine types of lipoprotein have been described in *E.coli* (**Lugtenberg and van Alphen, 1983**). The Braun lipoprotein was the first outer membrane protein to be isolated and purified (**Lugtenberg and van Alphen, 1983**). It is a small protein (molecular weight of 7,200 Da) with 58 amino acids in which no glycine, histidine, proline, phenylalanine and tryptophan are present (**Lugtenberg and van Alphen, 1983**). One third of this lipoprotein is covalently bound to the peptidoglycan layer (murein lipoprotein) while the remaining two-thirds exists in a free form (**Inouye *et al.*, 1972**). The murein lipoprotein is also anchored to the outer membrane by the fatty acyl chains of the N-terminus (**Braun and Wu, 1994**)

Another class of lipoprotein which is called peptidoglycan-associated lipoprotein or PAL has been described in *P.mirabilis* , *Ps. aeruginosa* and *E.coli* (**Lugtenberg and van Alphen, 1983**). These lipoproteins are closely associated but are not covalently linked to the peptidoglycan. In addition to Braun lipoprotein and PAL, seven other lipoprotein (NPL) in *E.coli* have been described; four (NPL1, NPL2, NPL5 and NPL7) were found localized in the outer membrane, two (NPL3 and NPL4) in the cytoplasmic membrane while localization of one species (NPL6) was unknown (**Ichihara *et al.*, 1981**).

The murein lipoproteins are also anchored to the outer membrane and thus may have a role in the stabilization of the outer membrane (**Lugtenberg and van Alphen, 1983**). Studies with lipoprotein deletion mutants showed that they suffer increased production of outer membrane vesicles, increased sensitivity to EDTA and leakage of periplasmic enzymes (**Lugtenberg and van Alphen, 1983**) In mutants with deletions in both lipoprotein (*lpp*) and *ompA* genes, inability to grow in the rod form, abundant blebbing and loss of peptidoglycan outer membrane connections were observed (**Lugtenberg and van Alphen, 1983**).

1.3.1.3.2 OmpA protein

The OmpA is present in high copy number in the outer membrane (10^5 per cell) and is encoded by the *ompA* gene. It is also known as a heat modifiable protein because of the change of its apparent molecular weight after heating of the porin samples in SDS

(**Schnaitman, 1973; Nakamura and Mizushima, 1976**). The protein has an apparent weight of 28,000 Da at room temperature and 35,000 Da when heated (**Table 1-1**) (**Schnaitman, 1973; Hall and Silhavy, 1981**). It is thought to exist as a monomer pore with a C-terminal periplasmic associated domain and an N-terminal outer membrane associated domain spanning the membrane eight times as β-sheets (**Vogel and Jahnig, 1986**; **Klose *et al.*, 1988**) with an estimated pore size of 0.6-0.7 nm (**Saint *et al.*, 1993**). OmpA is needed for maintaining the integrity of the cell envelope (**Hall and Silhavy, 1981**). Recently it has been proposed that OmpA functions as an outer membrane diffusion channel but with low activity (**Sugawara and Nikaido, 1992; Saint *et al.*, 1993**). From experiments carried out by Saint *et al.* (**1993**), OmpA appeared to be anion selective. OmpA also functions as a receptor for some phages (**Table 1-1**) and is also required for activity of colicins K and L. It is also required in F-plasmid mediated conjugation to stabilize the mating aggregates (**Lugtenberg and van Alphen, 1983**).

1.3.1.3.3 Porins

Porins or non-specific channels are a class of transmembrane oligomeric proteins which form water-filled pores that mediate non-specific passive diffusion of ions and small hydrophilic solutes and efflux of waste products (**Nakae, 1976; Osborn and Wu, 1980; Lugtenberg and van Alphen, 1983**). The "classical porins" of *E.coli* K-12 (**Nikaido and Vaara, 1985**) are as follows.

1.3.1.3.3.a OmpC and OmpF. The OmpC (outer membrane protein C) and OmpF (outer membrane protein F) are two of the major porins of outer membrane of *E.coli* K-12. OmpC and OmpF have molecular weights of 37,205 and 36,000 Da respectively and are encoded by *the ompC* and *ompF* genes (**Table 1-1**). These porins are usually tightly associated with LPS and peptidoglycan through ion bridges (**Nakae, 1976**). OmpC and OmpF are organized as trimers of three identical subunits with each subunit forming a water-filled channel with pore sizes of 1.1 and 1.2 nm respectively (**Osborn and Wu, 1980; Nikaido and Rosenberg, 1983; Nikaido, 1992; 1994**). The trimer is stabilized by hydrophobic and hydrophilic interactions between the subunits (**Schultz, 1994**). Studies on the tertiary structures of these porins showed that the subunits

Table 1-1 The major outer membrane proteins in *E.coli* (adapted from **Hall and Silhavy, 1981; Osborn and Wu, 1980)**

Protein	Other names	Genes (map position)	Mol wt.	Receptor for:	
				Phage	Colicin
OmpF	1a,Ia,b,O-9	*ompF* (21)	37,205	TuIa,T2 TP1,TP2,TP5	A,E2,E3,K L,N,S4
OmpC	1b,Ib,c,O-8	*ompC* (47)	36,500	PA-2,TuIb,T4 MeI,434,SS1, TP2,TP5,TP6	
PhoE	NmpAB,1c, e,E	*phoE* (6)	36,782	TC23,TC45, E2,E3	
LamB	maltoporin	*lamB* (91)	47,393	λ, K10,TP1 TP5,SS1	
OmpA	3a,II*,d,O1O	*ompA* (21)	35,000	K3,TuII*,OX2	L

of the trimers are organized as hollow cylinders formed by 16-antiparallel β-barrels (**Cowan, *et al.*, 1992; Nikaido, 1992; 1994**). The pore of the OmpF has been shown to be basically one channel with three openings facing the external surface (**Engel *et al.*, 1985**).

The OmpF and OmpC porins allow passive diffusion of hydrophilic solutes up to a mass of approximately ca 600 Da but exclude all lipophilic solutes (**Osborn and Wu, 1980**). They also act as receptors to a number of phages and colicins (**Table 1-1**). The receptor functions of these porins to various phages and colicins have been exploited to isolate mutants that fail to express the targeted porin. The OmpF and OmpC porins prefer neutral molecules and cations (**Benz *et al.*, 1979**). This is influenced by the net negative charge inside the pores (**Benz, 1988**).

The relative amounts of these two proteins are influenced by the cultural medium (**Lugtenberg *et al.*, 1976**), osmolarity of the medium (**van Alphen and Lugtenberg, 1977**), pH of medium (**Heyde and Portalier, 1987; Heyde *et al.*, 1988**), the nature of the carbon source (**Schnaitman, 1974**), levels of cAMP (**Schnaitman, 1974; Thomas and Booth, 1992**) and temperature (**Lugtenberg, *et al.*, 1976; Nikaido and Vaara, 1985**). The ratio of OmpF to OmpC is high in cells grown in minimal media, intermediate in complex media without sugar and low in complex media with sugar (**Lugtenberg *et al.*, 1976**). OmpF is preferentially expressed in media of low osmolarity whereas OmpC is preferentially expressed in media of high osmolarity (**van Alphen and Lugtenberg, 1977**). The genes responsible for the osmoregulation of the porins are the two-component regulatory system *envZ/ompR* (coding for a "sensor" that is located in the periplasm and "regulator" that is located in the cytoplasmic membrane) (**Mizuno *et al.*, 1982**). Since OmpC produces a channel that is slightly smaller than OmpF, this probably benefits *E.coli* when present in the intestinal tract as it will decrease the influx of cationic bile salts. The high osmolarity of the intestinal secretions is probably used as a signal to sense the change in environment (**Nikaido 1992**). Evidence from SDS-PAGE and β-galactosidase activity of LacZ-*ompC/ompF* fusions showed that at acid pH, there is more of the OmpC protein and at basic pH there is more OmpF (**Heyde and Portalier, 1987; Heyde *et al.*, 1988; Thomas and Booth, 1992**). Heyde and co-workers (**1988**) showed that the level of OmpC was reduced gradually when the pH of the growth medium was increased from 5.5

to 8.0 concomitantly with the increase of OmpF levels (**Heyde and Portalier, 1987**). In the presence of succinate or glycerol, expression of OmpF but not OmpC was observed and in the presence of glucose, expression of OmpC but not OmpF was observed (**Thomas and Booth, 1992**). The increase in expression of OmpC in the presence of glucose is also associated with low cAMP levels and strong catabolite repression (**Schnaitman, 1974; Thomas and Booth, 1992**). The expression of OmpC and OmpF is also affected by temperature; at high temperature, expression of OmpC is increased whereas expression of OmpF is reduced (**Nikaido and Vaara, 1985**). Changes in the growth conditions may also result in formation of a chimeric trimer formed by two OmpC and one OmpF or *vice versa* (**Sen and Nikaido, 1991**).

1.3.1.3.3.b PhoE protein. The PhoE porin or phosphoporin exists as a trimeric tight complex of identical subunits, forming a 16 stranded anti-parallel β-barrel containing pore (**Lugtenberg and van Alphen, 1983;Cowan *et al.*, 1992**). PhoE is encoded by the *phoE* gene and has a molecular weight of 36,782 (**Table 1-1**) (**Lugtenberg and van Alphen, 1983**). In wild-type cells, the PhoE porin or phosphoporin is induced by phosphate limitation (**Osborn and Wu, 1980; Tommassen and Lutenberg, 1980**). In *E.coli*, adaptation to hyperosmotic conditions also reduce the amount of PhoE porin (**Meyer, *et al.*, 1990**). Although PhoE shares many properties with OmpC and OmpF it however, shows a general preference for anionic solutes including phosphates, polyphosphates, sulphates and chlorides (**Benz *et al.*, 1984**). This preference is due to the presence of the positively charged lysine residues (**Hancock *et al.*, 1986; Bauer *et al.*, 1988**). The PhoE porin is a component of the high affinity phosphate (Pho) uptake sytem and its expression is regulated by PhoR (sensor and repressor) and PhoB the positive regulator (**Wanner, 1987**).

1.3.1.3.4 Specific diffusion channels

Specific diffusion pores can also form water-filled channels which contain specific binding sites. These channels enable the diffusion of nutrient molecules that are too large to penetrate through the porins and also those that penetrate the porins too slowly. The following describes some of the known specific diffusion channels.

1.3.1.3.4.a LamB. The LamB proteins or maltoporins of *E.coli* form aqueous pores and have specific binding sites for maltose and maltodextrin and facilitate specific diffusion of these substrates (**Szmelcman and Hofnung, 1975; Brass *et al.*, 1985; Dargent *et al.*, 1987**). However, the channel also allows the diffusion of nonmaltose saccharides, amino acids (**Luckey and Nikaido, 1980; Nakae, 1979**) and alkali metal ions (**Boehler-Kohler *et al.*, 1979**) although with less efficiency (**Brass *et al.*, 1985; Nakae, 1986**). Like the general porins, the LamB protein is a trimeric transmembrane protein. It is also a receptor for the lambda phage and hence so named. The LamB protein is encoded by *lamB* and has a molecular weight of approximately 47,000 Da (**Table 1-1**) (**Nikaido and Vaara, 1985**). The LamB protein has similar tertiary structure to that of the general porins despite a lack of significant homology with them (**Werts *et al.*, 1992**). LamB shows a weak interaction with peptidoglycan (**Hall and Silhavy, 1981**). LamB-proteins are inducible in the presence of maltose and their expression is regulated as part of the maltose regulon (**Raibaud and Schwartz, 1980**). The levels of LamB are also influenced by pH; levels of LamB are lower at acid pH than at alkaline pH (**Heyde *et al.*, 1987**).

1.3.1.3.4.b Tsx. The Tsx protein of the outer membrane, forms a specific pore for nucleosides (**Hantke, 1976; van Alphen *et al.*, 1978; Maier *et al.*, 1988**). Strains lacking *tsx* have impaired uptake of all nucleosides except cytidine and deoxycytidine (**Hantke, 1976; Krieger-Brauer and Braun, 1980**). The Tsx channel can discriminate between the closely structurally related pyrimidine nucleosides, cytidine and thymidine (**Hantke, 1976; Krieger-Brauer and Braun, 1980**). Tsx protein is also a receptor for colicin K and bacteriophage T6 (**Manning *et al.*, 1977**).

1.3.1.3.4.c TolC. The outer membrane protein, TolC forms a channel selective for peptides (**Webster, 1991**). It has a molecular mass of 52 kDa and is involved in permeation of colicin E1 through the outer membrane and secretion of haemolysin and colicin V (**Wandersman and Delepelaire, 1990; Webster, 1991**).

1.3.1.3.5 High -affinity receptors - TonB-dependent transport proteins

The transport of iron-chelator complexes (siderophores) and vitamin B12 is carried out

by this class of proteins (**Crosa, 1989; Nikaido, 1992**). The receptor proteins of this transport system includes FepA, FhuA, FhuE, IutA, FecA, Cir and BtuB (**Crosa, 1989**). These outer membrane proteins bind to their respective substrates with high-affinity (**Table 1-2**). The bound Fe^{3+}-siderophores and vitamin B12 are transported across the membrane by an energy requiring step transduced via the cytoplasmic membrane protein, TonB. The transduction of energy causes TonB to change its conformation and this change allows it to interact with the outer membrane receptor with concomitant release of Ca^{2+} and bound ligands into the periplasmic space (**Figures 1-3**) (**Postle, 1990**). Once taken up by the outer membrane, the iron is dissociated from the siderophore via chemical breakdown of the siderophore or a reduction step of the ferric ion in the cytoplasm (**Crosa, 1989**). The TonB protein is encoded by *tonB* and has an apparent molecular mass of 36-40 kDa on SDS-PAGE (**Crosa, 1989; Postle, 1990**). The hydrophobic amino terminus of this protein is anchored to the cytoplasmic membrane, stabilized by an auxiliary protein ExbB, and its hydrophilic central region spans the periplasmic space where its C-terminus interacts with the receptor protein during the transport of the siderophores and vitamin B12 (**Figure 1-3**) (**Postle and Skare, 1988; Postle, 1990**). The transduced energy used to drive the siderophores across the outer membrane is believed to derived from the interaction of the TonB protein with the cytoplasmic proteins involved in generating and maintaining the proton-motive force (**Silver and Walderhaug, 1992**). Ton is reported to have a functional half-life of 15-30 minutes (**Postle, 1990**).

All the proteins involved, in the passage of iron across the outer membrane, appear to be induced during conditions of iron starvation (**Neilands, 1982**). The transcription of TonB and other proteins involved in iron uptake is negatively regulated by the *fur* (ferric uptake regulator) gene (**Crosa, 1989**). The iron transport systems and the uptake of vitamin B12 in *E.coli* depend on the TonB protein function and on aerobic conditions (**Braun, 1985; Postle, 1990**). Under anaerobic conditions, the ferrous ion is soluble and is transported into the cells in a TonB-independent manner unless iron is limited by the presence of iron chelators (**Postle, 1990**). Under these conditions too, the vitamin B12 is probably synthesized endogenously by the bacterial cells (**Postle, 1990**). A number of antibiotics such as microcins (**Pugsley *et al.*, 1986**), rifamycin derivatives (**Pugsley *et al.*, 1987**), and catechol-substitute cephalosporins (**Watanabe *et al.*, 1987; Curtis *et al.*, 1988**)

Table 1-2 Outer membrane proteins which have high-affinity for Fe^{3+}-siderophores and vitamin B12 (adapted from **Lutenberg and van Alphen, 1983; Crosa, 1989**)

Proteins (other names)	Conditions optimal expression	Substrates	Receptor for: phage/colicin
FhuA (TonA)	Fe^{3+} limitation	Fe^{3+}-Ferrichrome	T1,T5,ϕ80,ColM
FhuE	Fe^{3+} limitation	Fe^{3+}-Rhodotolurate Fe^{3+}-coprogen	
IutA	Fe^{3+} limitation	Fe^{3+}-aerobactin	
FecA (Cit)	presence of citrate	Fe^{3+}-dicitrate	
FepA (FeuB)	Fe^{3+} limitation	Fe^{3+}-enterobactin	ColB,D
Cir	Fe^{3+} limitation	Fe^{3+}-catechols complexes	ColI,V,E1,E2,E3
BtuB (Bfe)	Vitamin B12 limitation	Vitamin B12	

Ferrichrome and coprogen are fungal siderophores; enterobactin and aerobactin are produced by *E.coli.*

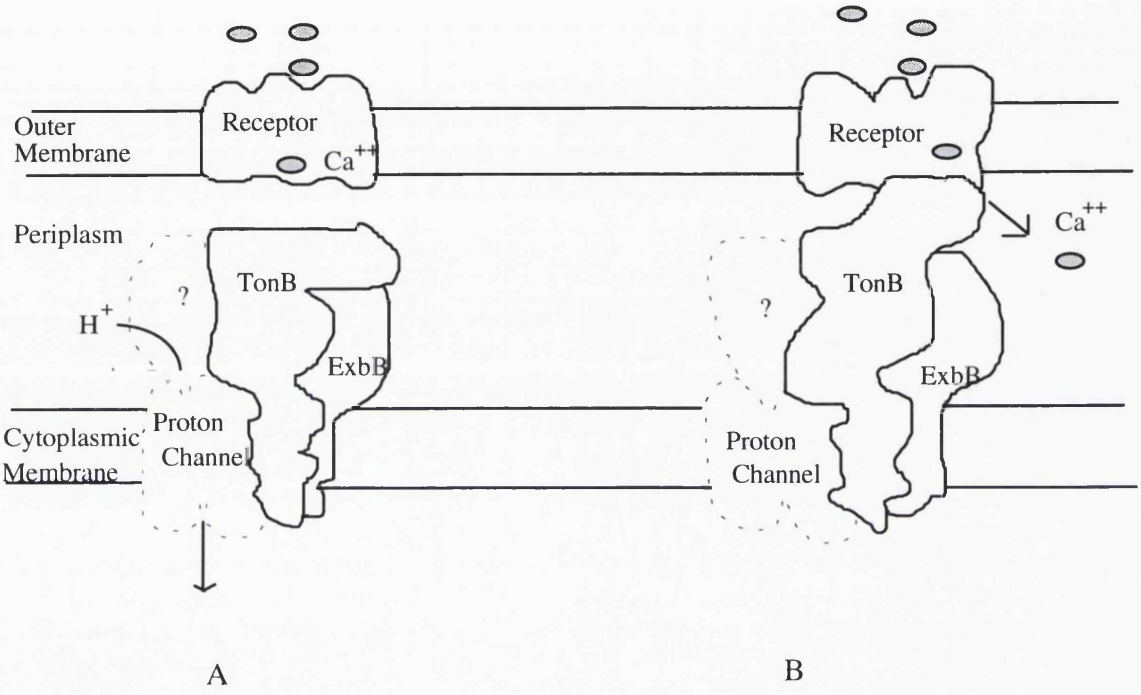


Figure 1-3 A model for TonB-dependent energy transduction and active transport across the outer membrane of *Escherichia coli* (after **Postle, 1990**)

A The movement of a proton down its concentration gradient causes a conformational change to be transmitted to TonB. **B**. The conformational change causes TonB to interact with the outer membrane receptor such that Ca^{2+} and bound ligands are released into the periplasmic space. The existence of the proton channel is tentative as shown by the broken lines. The protein labelled with question mark represents other proteins that might interact with TonB. Ovals represent transport ligands.

are also known to require a functional *tonB* gene to efficiently enter the Gram-negative bacteria (**Postle, 1990**). Mutants with lesions in *tonB* are resistant to phage T1, i.e T-one, ϕ80, and colicin M (**Postle, 1990**)

1.3.2 Peptidoglycan

The peptidoglycan (or murein or mucopeptide) is a heteropolymer consisting of glycan strands cross-linked by peptides. The glycan portion consists of linear polysaccharide strands of alternating units of N-acetyl-D-glucosamine and N-acetylmuramic acid (3-O-lactyl-N-acetyl-D-glucosamine) in β-1,4 linkage (**Osborn, 1969**) (**Figure 1-4a**). The peptide portion is composed of tetrapeptide subunits of H_2N.L-Ala-D-Glu-L-Lys (or meso-DAP)-D-Ala.COOH (**Figure 1-4a**). The glycan strands are arranged parallel to each other and run around the circumference of the cell perpendicular to the axis. Neighbouring strands are linked to each other via the tetrapeptide subunits (**Figure 1-4b**). The tetrapeptide subunits are linked to the glycan chains by amide linkages between the amino groups of L-alanine and the lactyl carboxyl groups of muramic acid residues. The tetrapeptide subunits are also partially linked to each other with the C-terminal D-alanine residue of one being linked to the free amino group of lysine (or DAP) in a second unit either directly or through an oligopeptide bridge (**Figure 1-4b**). The peptidoglycan is associated with the outer membrane via covalently linked lipoprotein (**Braun, 1975**) and via tight association with the some of the outer membrane proteins (**Rosenbush, 1974**).

The peptidoglycan forms a bag-like structure (sacculus) around the cell and thus has been regarded as an "exoskeleton" playing a role in determining the shape of the cell. It is also vital in protecting the cell from osmotic lysis. In addition, the presence of peptidoglycan is essential in the cell division cycle. The formation of septa at cell division involves synthesis of new peptidoglycan at a precise location to form the poles of the daughter cells and the absence of peptidoglycan results in difficulty in cell division (**Park, 1987**).

1.3.3 Cytoplasmic membrane

The cytoplasmic membranes of *E.coli* and *S.typhimurium* contain 75% of the cellular phospholipid. Three species of phospholipid are present in the cytoplasmic membrane. The

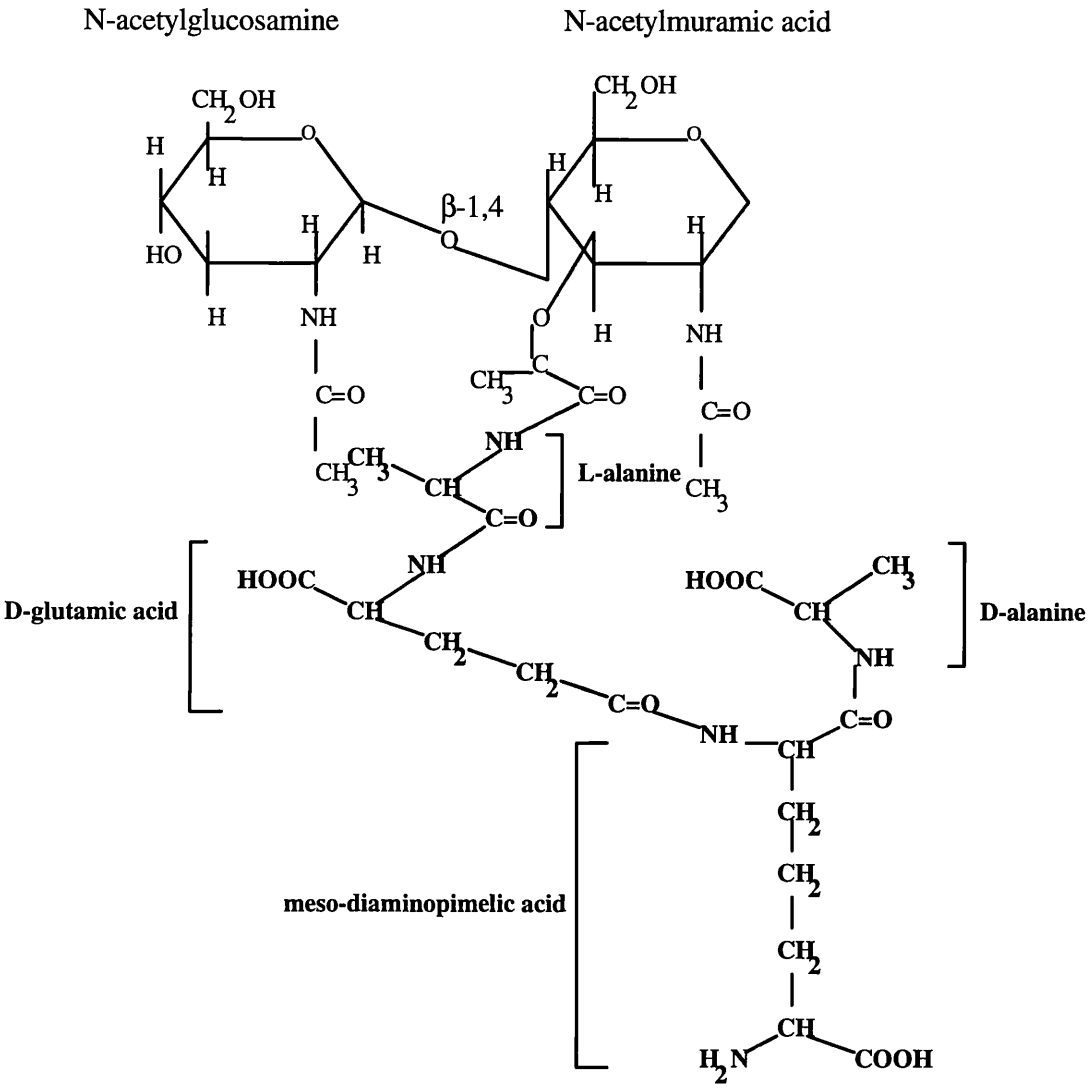


Figure 1-4a The polymerization of the two glycan subunits (N-acetylglucosamine and N-acetylmuramic acid) by β-1,4 glycosidic bond, and the linkage between the muramic acid and the tetrapeptides via carboxyl group of the muramic acid and the amino group of L-alanine (Reproduced from **Park, 1987**).

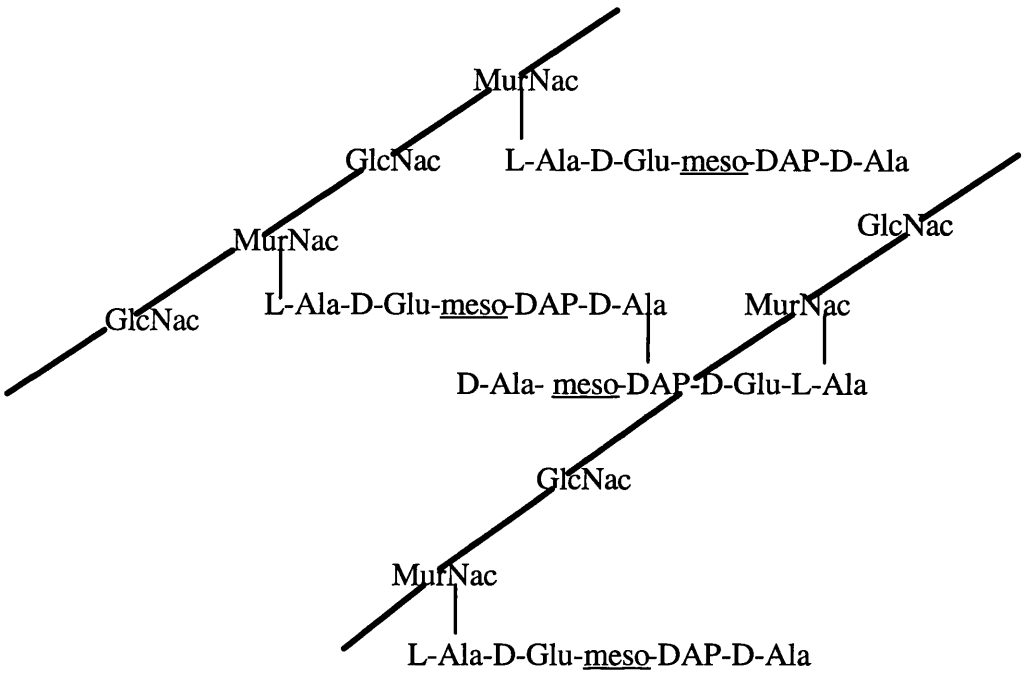


Figure 1-4b The linkage of parallel glycan strands and a peptide bond between the carboxyl group of the D-alanine residue of one tetrapeptide and a free amino of meso-DAP of the other tetrapeptide.

bacterial cytoplasmic membranes have a bilipid structure. The phospholipids form two opposing layers with their hydrophobic ends orientated towards each other while the hydrophilic ends point away from each other. Different species of membrane proteins are also inserted in the bilayer; some are tightly bound (integral proteins), some are loosely bound (peripheral proteins) while others are thought to interact only transiently with the membrane (**Cronan *et al.*, 1987**). These membrane proteins include, among others, those in the electron transport chain, the F_1F_0 component of the ATPase or ATP synthetase assemble and solute transporters.

1.3.3.1 Functions of the cytoplasmic membrane

(i) Hydrophobic barrier. The hydrophobic core of the lipid bilayer creates an effective barrier to entry of charged species (cations and anions). The bilayer also has a high electrical resistance and can withstand very high electrical fields. Besides small hydrophobic molecules such as medium chain fatty acids, the cytoplasmic membrane is permeable to a variety of uncharged species such as water, oxygen, carbon dioxide and undissociated weak acids and bases (**Maloy *et al.*, 1981; Melchoir and Carruthers, 1983**). The barrier function however, depends on the physical state of the lipid bilayer. The phospholipid bilayers can undergo reversible changes of state depending on temperature and lipid composition (phase transition); at low temperature, the acyl chains are closely packed and the bilayer is quite solid whereas at high temperature the acyl chains are in a disordered state resulting in the thinning of the bilayer due to greater movement of the acyl chains (**Cronan *et al.*, 1987**).

(ii) Energy transduction. The cytoplasmic membrane is the site of the primary energy source for the cell as it is where the catalysing of electron transport in the respiratory chain and synthesis of ATP by oxidative phosphorylation occurs. According to the accepted chemiosmotic hypothesis proposed by Mitchell (**1966**), protons are extruded out of the cytoplasmic membrane as a result of electron transport. The components of the electron transport chain are embedded and orientated in the lipid bilayer in an asymmetric manner with some being accessible from the inside and some from outside of the cytoplasmic membrane to enable the separation of protons from electrons during the transport (**Hinkle and McCarty, 1978**) (**Figure 1-5a**). Since the cytoplasmic membrane

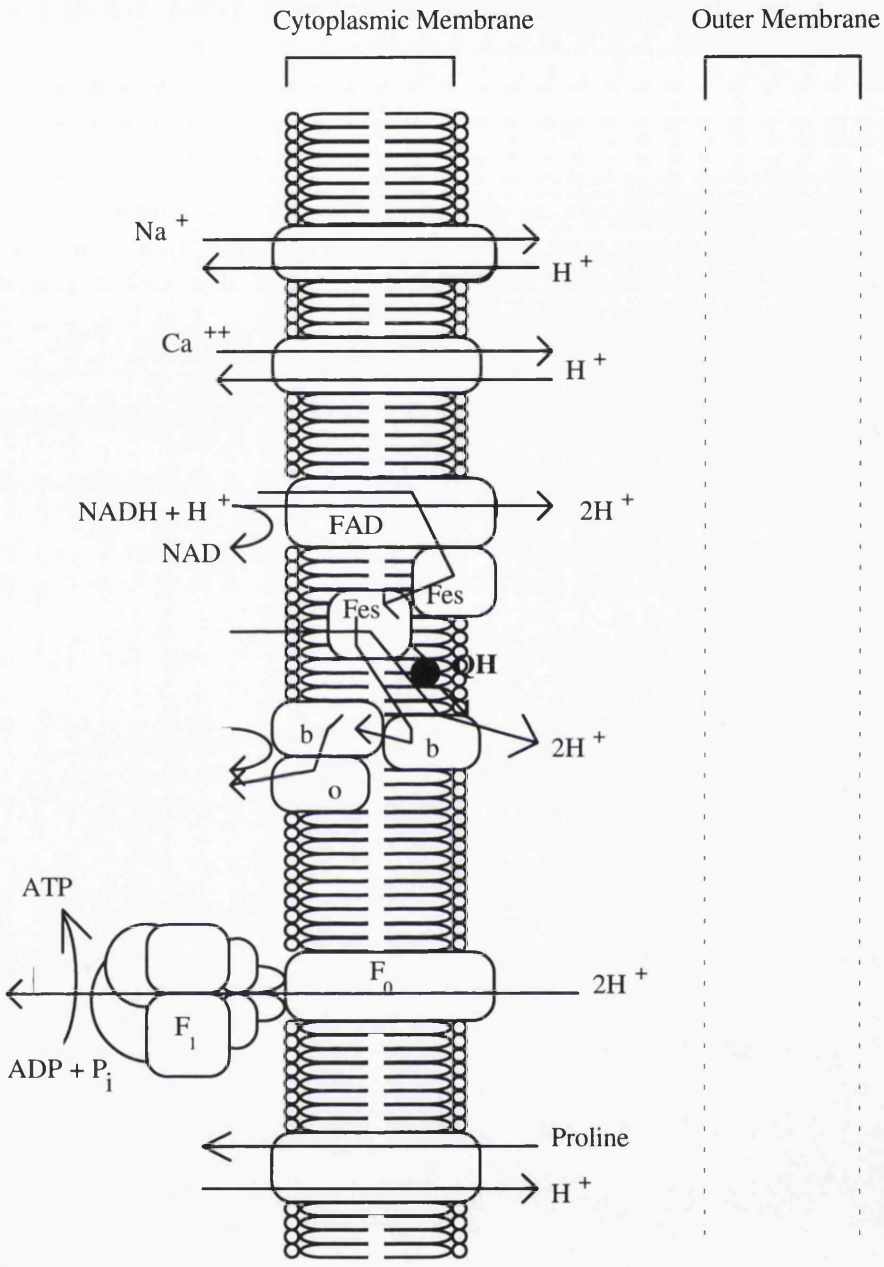


Figure 1-5a Diagrammatic representation of the cytoplasmic membrane of *Escherichia coli* with the electron transport chain proteins. ATPases (F_1F_0) and some of the transport proteins (adapted from, **Hinkle and McCarty, 1978**).

is impermeable to protons and hydroxyl ions, proton extrusion during the electron transport processes (i.e when hydrogen atoms are removed from NADH and $FADH_2$ and when electrons are being shuttled between coenzyme Q and cytochrome b), leads to generation of an electrochemical proton gradient ($\Delta\mu H^+$) across the membrane with the cytoplasm being electrically negative and alkaline and the outside of the membrane being positively charged and acidic (**Hellingwerf and Konings, 1985**). The electrochemical proton gradient is thus composed of an electrical component, the electrical potential $\Delta\psi$ ($\Delta\psi_{in}$- $\Delta\psi_{out}$), and a pH or chemical gradient, ΔpH (pH_{out} minus pH_{in}) (**Hellingwerf and Konings, 1985**). The inwardly directed force that is exerted by the electrochemical proton gradient on the protons is expressed as the proton motive force (Δp) (**Hellingwerf and Konings, 1985**). Mitchell (**1966**) in his chemiosmotic hypothesis proposed that the established proton motive force, drives the membrane-bound ATP synthetase to synthesise ATP. The ATP synthetase consists of two multipeptide assemblies, F_0 which is the hydrophobic and intrinsic part of the membrane and the F_1 complex which is situated on the inside of the membrane (**Hinkle and McCarty, 1978; Hellingwerf and Konings, 1985**). The F_0 provides passage for external protons to gain access to the catalytic F_1 complex which then catalyses the formation of ATP and water from ADP and P_i (**Figure 1-5a**). The ATPase also catalyses the hydrolysis of ATP with concomitant extrusion of $2H^+$ to the outside of the membrane and thus can also contribute towards generating the proton gradient across the membrane (**Hellingwerf and Konings, 1985**).

The proton motive force generated can then be used by the cell to drive a variety of energy-linked processes including uptake of solutes against a concentration gradient, cell motility and driving a reverse flow of electrons through the respiratory chain to reduce NAD when the supply of $NADH_2$ is inadequate (**Hellingwerf and Konings, 1985**).

(iii) Macromolecule Biosynthesis. The cytoplasmic membrane is also the location for some enzymes and carriers involved in the biosynthesis of components of the bacterial cell envelopes such as peptidoglycan and lipopolysaccharides and capsules (**Raetz, 1978**; **Lugtenberg and van Alphen, 1983**). It is also the site for synthesis of its own phospholipids and glycolipids (**Raetz, 1978**). Furthermore, it contains membrane bound ribosomes which are involved in the synthesis of periplasmic and outer membrane proteins (**Randall and Hardy, 1977**).

(iv) Solute transport. The cytoplasmic membrane is only permeable to water and small hydrophobic molecules. Weak undissociated acids and bases, can also cross the cytoplasmic membrane passively without the involvement of specific carrier proteins (**Hellingwerf and Konings, 1985**). The uptake of the majority of solutes however, is carried out by specific carrier molecules or transport proteins that are embedded in the cytoplasmic membrane. Many of these transport systems are coupled to the input of energy and can be classified according to the nature of their energy source (**Nikaido and Saier, 1992**). These classes of transporters include facilitated diffusion systems, secondary active transport systems, group translocation systems and the periplasmic binding protein-dependent active transport systems (**Figure 1-5b**) (**Nikaido and Saier, 1992; Poolman and Konings, 1993**).

In **facilitated diffusion**, solute transport is mediated by carrier proteins without any expenditure of energy (**Heller *et al.*, 1980**). This mode of transport, transfers only single species of ion or molecule across the membrane. In *E.coli*, glycerol uptake is the only known example of facilitated diffusion (**Heller *et al.*, 1980**). The glycerol facilitator protein, GlpF, forms a specific pore in the cytoplasmic membrane which catalyses the diffusion of glycerol and related polyols across the membrane until the internal and external glycerol concentrations are in equilibrium (**Heller *et al.*, 1980**).

The **secondary active transport systems** or ion-coupled transport systems, link uphill solute transport to downhill electrochemical ion gradients of H^+, Na^+, Cl^- ,K^+ or phosphate (**Nikaido and Saier, 1992; Heidger 1994**). Three types of ion-coupled transport are recognized and are classified as symporters, antiporters and uniporters (**Nikaido and Saier, 1992; Heidger 1994**).

Symporters transport two different molecules simultaneously in the same direction by the same carrier while antiporters transport two different molecules simultaneously in opposite direction by the same carrier. The solute-cation symports in *E.coli* generally use protons and sodium ions as coupling ions for uptake of sugars, amino acids and other nutrients (**Table 1-3**) (**Nikaido and Saier, 1992; Heidger 1994**).

The antiporter systems in general, are well suited for the excretion of undesired solutes or products from the cytoplasm. The efflux of solute is generally directly linked to proton or sodium influx. Some of the known antiporters in bacteria include the

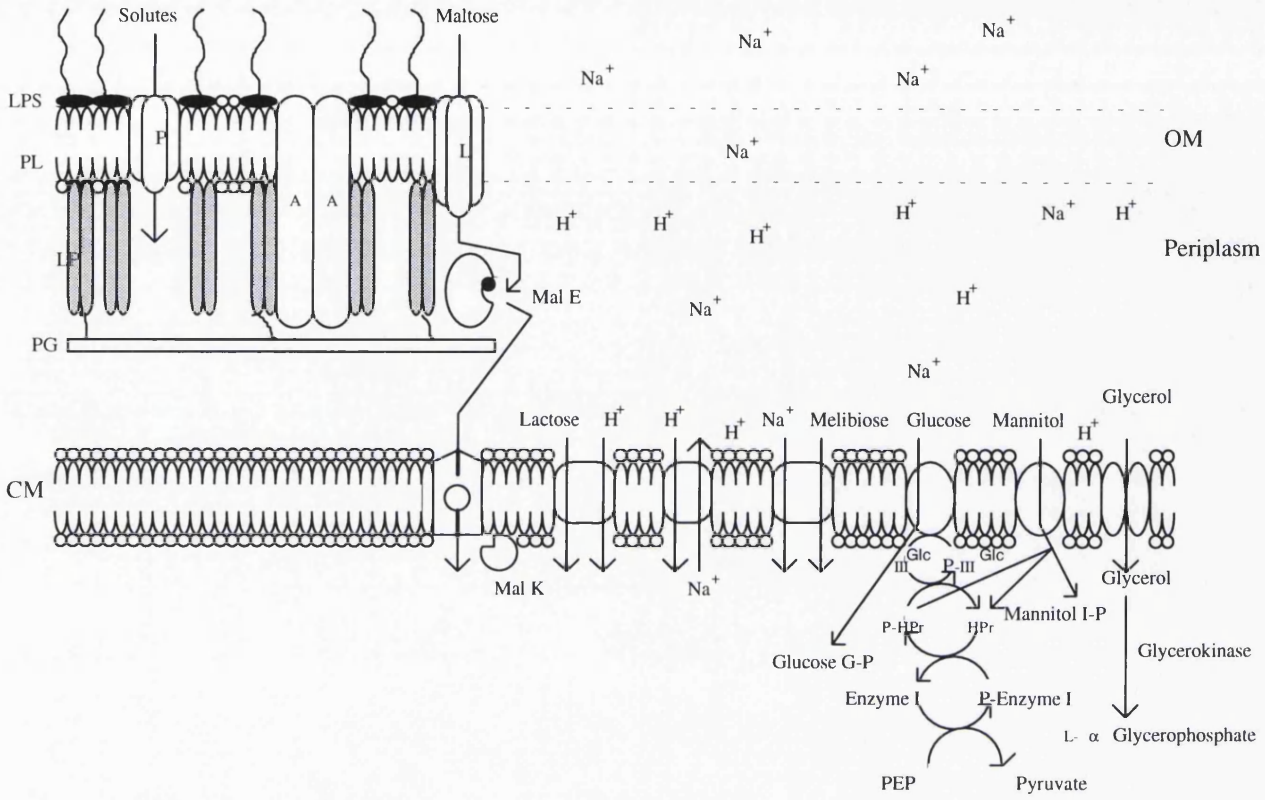


Figure 1-5b A schematic outline of various types of transporters in the cell-envelope of Gram-negative bacteria depicting the solute-cation symporters (e.g.,H^+/lactose; Na^+/melibiose), sodium/proton antiporter, facilitated diffusion, group translocation and binding protein-dependent active transport.

Abbreviations: LPS-lipopolysaccharides; PL-phospholipid, LP-lipoprotein, PG-peptidoglycan, P-porin, A-outer membrane porin A, L-LamB, OM-outer membrane, CM-cytoplasmic membrane

TABLE 1-3 Substrates transported by secondary active transport mechanism in *E.coli* (modified from **Poolman and Konings, 1993**)

Mechanism	Substrates	co-substrate	Transporter
Symport	Lactose	H^+	LacY
Symport	Melibiose	H^+, Na^+, Li^+	MelB
Antiport	Sugar-phosphate	phosphate	UhpT
Antiport	Oxalate	Formate	OxPT
Symport	Proline	Na^+, Li^+	PutP
	Proline	H^+	ProP
Antiport	Metal-tetracycline	H^+	TetA
Symport	galactose	H^+	GalP
Symport	arabinose	H^+	AraE
Symport	xylose	H^+	XylE
Symport	Glutamate, Serine	Na^+	GltS
	Glutamate	H^+	GltP
Antiport	Na^+, Li^+	H^+	NhaA

sodium/proton (Na^+/H^+) (**West and Mitchell, 1974; Beck and Rosen, 1979**), calcium/proton (Ca^{2+}/H^+) (**Ivey *et al.*, 1993**) and potassium/proton (K^+/H^+) (**Plack and Rosen, 1980**) antiporters. Another class of antiporter catalyses the uptake of solute (precursor, substrate) in a coupled exchange with another solute (product) (**Table 1-3**) (**Poolman and Konings, 1993**). The Δp or one of its components, can affect the translocation process through protonation or deprotonation of the substrates and/or through altering the differential charge on individual substrates (**Poolman and Konings, 1993**). The symporters and antiporters may be electroneutral or electrogenic depending upon whether the proton translocated is accompanied by the movement of a compensating anion or by a neutral solute. Uniporters however ferry only one solute in one direction. The uptake of a cation through one uniporter is normally followed by the efflux of an anion through another uniporter in response to the membrane potential (**Poolman and Konings, 1993**). Facilitated diffusion can be considered as a passive uniport of a neutral compound (**Poolman and Konings, 1993**).

Group translocation couples transport of substrate to chemical modification, resulting in the release of a chemically modified substrate (usually as its phosphate ester) into the cell (**Hellingwerf and Konings, 1985**). The most thoroughly studied group translocation systems in bacteria are the phosphoenolpyruvate (PEP) dependent sugar phosphotransferase systems (PTS) (**Hellingwerf and Konings, 1985**). A distinctive feature of these systems is that an integral membrane polypeptide specific for a particular sugar (generally called enzyme II) binds the sugar from the external surface and phosphorylates using PEP before releasing the modified sugar into the cytoplasm (**Nicholls, 1992**). This system mediates the uptake of several hexose and hexitol sugars such as glucose and mannitol principally in anaerobic and facultative anaerobic bacteria (**Nicholls, 1992**).

The **binding-protein-dependent active transport systems**, involve soluble periplasmic binding proteins which have high affinity for substrates. The bound substrates are then transferred to the cytoplasmic membrane-associated transporter complexes (**Nikaido and Saier, 1992**). The cytoplasmic membrane-associated transporter complexes are made up of two hydrophobic proteins which presumably form the diffusion channel and two hydrophilic subunits which bind ATP and ATP analogues and function as

ATPases (**Nikaido and Saier, 1992**). Energy from ATP hydrolysis is used to drive the translocation of the solute across the membrane (**Dean *et al.*, 1989; Davidson and Nikaido, 1990; Mimura *et al.*, 1990**). In *E.coli* and *Salmonella typhimurium*, ions, sugars, amino acids and vitamins are transported in this manner (**Boos, 1974; Nicholls, 1992; Heidger, 1994**). The best studied binding protein-dependent transport systems are the maltose uptake system in *E.coli* (**Dean *et al.*, 1989**), the potassium ion TrkA transport system in *E.coli* (**Rhoads and Epstein, 1977**) and the histidine uptake system in *Salmonella typhimurium* (**Ames and Higgins, 1983**)

iv) Environmental "sensors"/Signal transducers. Some of the cytoplasmic proteins can monitor and detect changes in the environment and signal the presence of these changes to the 'response regulators' in the cells via phosphorylation processes (**Ronson *et al.*, 1987; Stock *et al.*, 1989**). The N-terminus of these proteins are very often extended into the periplasmic space. Some of these include the sensor components of the chemotaxis response, such as methyl-accepting chemotaxis proteins (MCPs) and CheA, the sensor component of the osmoregulation, EnvZ, the sensor component of the phosphate regulation PhoR, and the sensor component of oxygen limitation CpxA (**Ronson *et al.*, 1987; Stock *et al.*, 1989**).

1.3.4. Zones of adhesions

Two distinctive types of structures that bridge the periplasm were revealed in plasmolysed *E.coli* cells namely discrete domains of contact between cytoplasmic and outer membrane (zones of adhesion) and plasmolysis bays (**Bayer and Bayer, 1994**). At the site of adhesion, the cytoplasmic membrane is closely associated with the inner contour of the outer membrane (**Bayer and Bayer, 1994**). There are about 100 to 200 of such connections in *E.coli* cells (**Bayer and Bayer, 1994**). The size of the contact varies from approximately 20 nm to over 200 nm (**Bayer and Bayer, 1994**).

The zones of adhesion have been implicated as sites of: (a) penetration of phage DNA into the cell (**Lugtenberg and van Alphen, 1983**), (b) translocation of newly synthesized LPS (**Mulhradt and Golecki, 1975**), (c) synthesis and translocation of some outer membrane proteins and (d) insertion of F-pilus (**Lugtenberg and van Alphen, 1983**). These domains have also been reported to be enriched in phospholipase activities

(**Bayer *et al.*, 1982**).

1.3.5 Periplasmic space

The periplasmic space is the cell compartment between the outer and cytoplasmic membranes. This space comprises of 20 to 40 % of the total cell volume (**Stock *et al.*, 1977**). The periplasm that fills the periplasmic space, is comprised of gel-like solutions of proteins and oligosaccharides and is isotonic with the cytoplasm (**Lugtenberg and van Alphen, 1983; Bayer and Bayer, 1994**) Besides these resident molecules, the periplasm also houses those which are temporarily present during their import into the cell and those during their export to the outer membrane before they are assembled to form multimeric products such as fimbriae, protein pores and receptors for viruses, vitamins and colicins (**Bayer and Bayer, 1994**).

There are two polymer species which are permanent residents in the periplasm namely peptidoglycan and ***m***embrane ***d***erived ***o***ligosaccharides (MDOs) (**Van Golde *et al.*, 1973**) (**Figure 1-1**). The MDOs have molecular weights of approximately 2,400 Da and consist of glucose as sole sugar substituted with phosphoglycerol, ethanolamine and succinyl ester groups. Their synthesis is controlled by the osmolarity of the environment of the cell with low osmotic pressure enhancing their synthesis significantly (**Kennedy, 1982**). They may also play a role in osmoregulation of the periplasm (**Kennedy, 1982**). The MDOs exhibit variable negative charges and have been described as affecting the shutter functions of the outer membrane pores (**Delcour *et al.*, 1992**).

The periplasmic proteins can be grouped as ***(i) binding proteins*** for amino acids (e.g, arginine and leucine), sugars (e.g galactose/glucose, arabinose), ions (e.g, phosphate, sulphate) and vitamins (e.g, thiamine, vitamin B12) (**Lugtenberg and van Alphen, 1983; Neidhardt *et al.*, 1990**), ***(ii) degradative enzymes*** such as phosphatases, nucleases and proteases which are involved in degradation of solutes, that are too large or too highly charged, to a form more permeable to the cytoplasmic membrane (**Lugtenberg and van Alphen 1983; Oliver, 1987**) and ***(iii) detoxifying enzymes*** that inactivate certain antibiotics, for example inactivation of penicillin and kanamycin by β-lactamase and aminoglycoside-phosphorylating enzyme respectively (**Oliver, 1987**).

The periplasmic space of an intact bacterium is accessible from the cell's environment via 3 classes of transport pathways: (i) the non-specific pores or porins (**1.3.1.3.3**) of the outer membrane which allow passage of small hydrophilic solutes, (ii) via specific protein channels (**1.3.1.3.4**) and (iii) high affinity, energy-dependent transport systems (**1.3.1.3.5**).

1.4 Biological Role Of Copper In *Escherichia coli*

Copper, zinc and nickel are a few of the heavy metals which are essential for growth in trace amounts but toxic in excess. Copper can undergo redox reactions between Cu(I) and Cu(II) and forms the essential prosthetic group in the synthesis of some metalloproteins which include mainly oxygenases and electron transport proteins (**Brown *et al.*, 1992**). As metalloproteins, they can therefore act as electron donors and acceptors in the electron transport chain and in redox-active enzymes which use oxygen as a substrate.

1.5 Toxicity Of Copper In *Escherichia coli*

Copper is toxic in its free ionic form (Cu^{2+}). Copper(II) salts are acidic when in solution but at high pH values it precipitates as hydroxides or oxides and thus reducing the availability of free Cu^{2+} (**Hughes and Poole, 1991**). The acidity of copper salts in solution increases with increasing copper concentration. Zevenhuizen *et al.*, (**1979**) found that the pH of casitone yeast extract-glycerol medium was lowered to pH 4.0 when the copper concentration was increased up to 10^{-2}M.

In excess of physiological levels, copper can catalyse adverse redox reactions in the cell giving rise to hydroxyl radicals (**Brown *et al.*, 1992**):

$$Cu^{+} + H_2O_2 = Cu^{2+} + OH^{-} + OH^{\cdot}$$

Hydroxyl radicals can cause a number of deleterious reactions which include: (a) peroxidation of lipids, that can lead to membrane disruption and (b) oxidation of proteins that leads to their inactivation. Microscopic examination of copper-stressed cells revealed cell-wall damage and many lysed cells (**Zevenhuizen *et al.*, 1979**). Copper can also bind and modify functional groups in proteins, nucleic acids, polysaccharides and lipids causing

alterations in the structure and/or functions of these macromolecules. The histidine and cysteine residues of enzymes which are important in enzymic catalytic reactions are particularly vulnerable to inactivation by copper action of covalent modification. Besides macromolecules, copper also binds to low molecular weight compounds and thus alters the biological function and the bioavailability of these micromolecules.

1.6 Sources Of Copper Stress Exposures

Copper is widely found in the environment in the form of ores such as chalcopyrite (CuFeS) and chalcocite(CuS_2) and has been mined since early times (**Brown *et al.*, 1992**). Geological releases of weathered minerals can give rise to soluble cupric and cuprous salts such as carbonates and chlorides. Copper is widely used as bactericides and fungicides for agricultural crop plants and also as growth promotants in pigs (**Cooksey, 1987**). It is also commonly found in potable water due to its wide usage in the manufacture of pumps, valves, pipings and other plumbing accessories (**Domek *et al.*, 1984**) Copper is introduced as copper sulphate in water treatment processes to eliminate algae (**Domek *et al.*, 1984**). In a survey of 44 drinking samples in Montana U.S.A, copper concentrations in water was found to be in the range of 0.07 mg/L and 0.54 mg/L (**Domek, *et al.*, 1984**).

1.7 Metabolism Of Copper In *Escherichia coli*

The proposed model for copper metabolism in *E.coli* involves at least four processes based on the analysis of copper transport mutants *(cutA-cutF)* (**Rouch *et al.*, 1989a**; **Brown *et al.*, 1992**). The model is also based on *a priori* assumptions of the properties of a homeostatic mechanism (**Figure 1-6**). The proposed processes involved in copper metabolism are described below:

(i) Uptake. Since copper is required for the synthesis of metalloproteins, uptake of copper from the environment is essential. Thus there must exist channels and /or transport systems in the cell envelope membrane of *E.coli* that will allow the passage of copper cations. Copper ions permeate the outer membrane of *E.coli* using the relatively non specific pores formed by OmpF and OmpC (**Luktenhaus 1977; van Alphen *et al.*,**

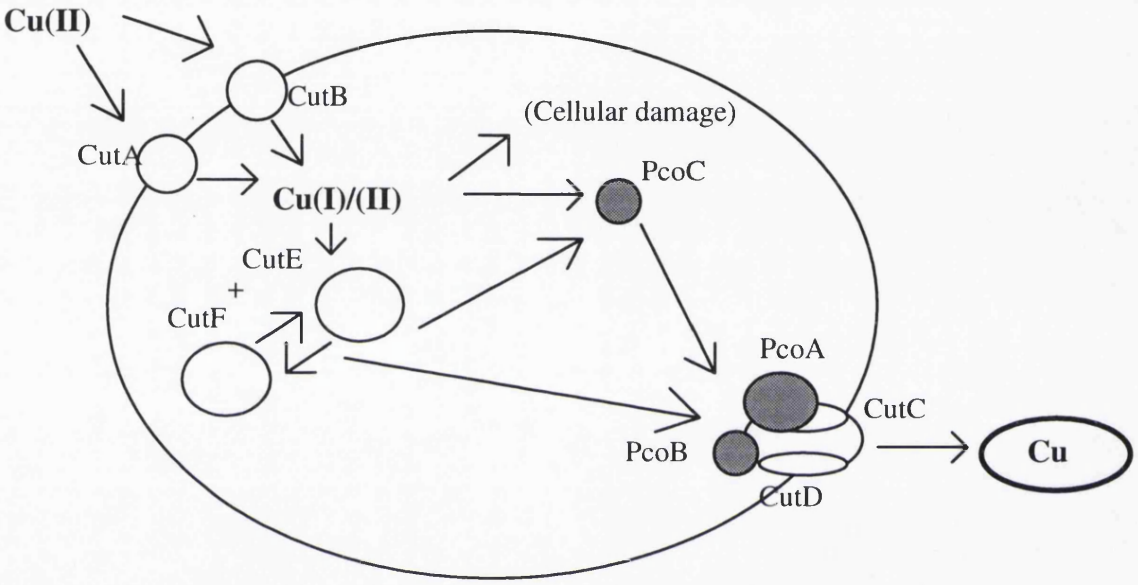


Figure 1-6 Model for the mechanism of copper resistance and metabolism in *E.coli* containing plasmid pRJ1004 (after **Brown *et al.*, 1992**). The metabolic (chromosomal) genes encode the systems for uptake of copper (*cutA*, *cutB*), for storage and intracellular transport of copper (*cutE*, *cutF*) and for export of copper (*cutC*, *cutD*). The resistance (plasmid) genes alter the intracellular storage of copper (*pcoC*), increase the export of copper, and change the speciation of copper (*pcoA* and *pcoB*)

1978; Benz, 1985). The importance of OmpF and OmpC in copper uptake was demonstrated in the OmpF, OmpC double mutants of *E.coli* K-12; these mutants not only showed reduced copper accumulation and resistance to copper but also required elevated levels of copper for growth (**Luktenhaus, 1977**). Rouch *et al.*, (**1989a**) identified two chromosomal genes *cutA* and *cutB* that mediate uptake into the cytoplasm.

(ii) Transport/storage. Once copper ions get into the cytoplasm, they must be delivered to the sites of metalloprotein synthesis. The intracellular movement of the copper must be controlled so as to prevent damage to intracellular constituents through free-radical reactions. Thus if copper escapes, control systems should be able to nullify its toxicity. Storage facilities are necessary to provide copper, for copper-dependent enzymes, and to act as a buffer against any intracellular changes in levels of the metal. Cytoplasmic carrier proteins (CutE and CutF) are postulated to be involved in transferring copper from the membrane to the storage protein and also from the membrane to both storage and efflux system (**Rouch *et al.*, 1989a**).

(iii) Efflux. The existence of a membrane efflux system for excess copper is vital in controlling intracellular levels of copper. The removal of excess copper is thought to be via an energy-dependent system as demonstrated by the inhibition of copper ion efflux by the metabolic inhibitor valinomycin in *E.coli* (**Rouch *et al.*, 1989a**).The authors have identified two chromosomal genes *cutC* and *cutD* as genes for the efflux proteins. Rouch *et al.*, (**1989a**) suggest that chemical modification might be involved prior to extrusion of copper from the cell so that it would be no longer recognised by the copper uptake systems thus preventing re-entry. This modification process might be inducible when extracellular levels of copper are high (**Rouch *et al.*, 1989a**).

(iv) Regulation. Control of the intracellular levels of copper is essential for the copper homeostasis of the cell. In order to provide essential supplies of copper to the metalloprotein biosynthetic machinery while simultaneously protecting the cells against high and toxic levels of copper, the cellular components involved in uptake, storage/transport and efflux have to be coordinately regulated. A chromosomal locus *cutR* which has a trans-acting role in the maintenance of homeostasis at normal external copper concentrations has been identified (**Rouch *et al.*, 1989a**). A mutation designated as *cutS* has been identified as the "sensor" assuming that *cutR* and *cutS* make up a two-component

regulating system (**Brown *et al.*, 1992**).

1.8 Copper Resistance In Gram-Negative Bacteria

There are no defined standard concentrations of copper used to distinguish between copper-resistance and copper-sensitivity in bacteria (**Trevors *et al.*, 1985**). The response to copper is complicated by the effects of media components, pH and cultural conditions used which have the capability of influencing the toxicity of the metal (**Trevors *et al.*, 1985**). Thus different responses are being reported on the effect of different concentrations of copper on bacteria.

Domek *et al.*, (**1984**) found that copper concentrations of 0.025 and 0.05 mg/L in inorganic carbon buffer (pH 7.0) caused 90 % injury in coliforms after 2 and 6 days respectively. In contrast, non-encapsulated *Klebsiella aerogenes* was inhibited at a copper chloride concentration of 10 ppm (10 mg/L) in distilled water at pH values between 5.5-6.0 whereas the capsulated strain showed a 2.5 log higher viable cell count than the sensitive strain (**Bitton and Freihofer, 1978**). Zevenhuizen *et al.*, (**1979**) investigated the inhibitory effects of free Cu^{2+} concentrations on bacteria and found that copper sensitive strains of *K. aerogenes* were inhibited by 10^{-8} to 10^{-6}M $CuSO_4$ (ca. 0.25-0.002 mg/L $CuSO_4$) in inorganic salts media. However, in complex media at pH 7.0, higher Cu^{2+} concentrations were tolerated because copper becomes bound to medium components. Tetaz and Luke (**1983**) showed that sensitive strains of *E.coli* K-12 were unable to form colonies on nutrient agar containing more than 4 mM copper sulphate (998.7 mg/L) whereas transconjugant strains of *E.coli* K-12 which contain the copper-resistance plasmid, pJR1004, could tolerate 20mM of copper sulphate (4.99 g/L $CuSO_4$). The marine bacterium *Vibrio alginolyticus* was inhibited at copper sulphate concentrations of 2.1 μM (0.52 mg/L $CuSO_4$ in minimal medium pH 7.1) and 6.4 μM (1.59 mg/ml $CuSO_4$ in minimal medium pH 7.1) in anaerobic and aerobic conditions respectively (**Schreiber, *et al.*, 1984**). In *Pseudomonas syringae*, the minimal inhibitory concentration (MIC) ofcopper sulphate ranges from 0.1 mM (24.96 mg/L $CuSO_4$ in mannitol-glutamate yeast agar) in copper-sensitive strains to 0.6 mM (149.76 mg/L $CuSO_4$ in mannitol-glutamate yeast agar) in strains displaying low level copper resistance. In a strain carrying the copper resistance

plasmid the MIC is 2.0 mM (499.2 mg/L $CuSO_4$ in mannitol-glutamate yeast agar) copper sulphate (**Lim and Cooksey, 1993**).

1.8.1 Mechanism of copper resistance in *Escherichia coli*

Since copper is required in trace amounts but is toxic in excess, resistance to copper would present the cell with a problem. Therefore the cells need a mechanism that is responsive to the essential requirements of the cell while at the same time reducing cytoplasmic copper at potentially toxic levels (**Lee *et al.*, 1990**). The mechanism of copper resistance in *E.coli* is based on the study of the plasmid pRJ1004 by Rouch *et al.*, (**1989b**). This conjugative plasmid was first isolated by Tetaz and Luke (**1983**) in *E.coli* from a porcine source. The plasmid-borne resistance genes allow the cells to tolerate up to 20 mM $CuSO_4$ under conditions where plasmid-free strains can tolerate only up to 4 mM $CuSO_4$ (**Tetaz and Luke, 1983**). Rouch *et al.*, (**1985**) later demonstrated that the copper resistance on this plasmid can be induced by pregrowth in presence of 0.04 mM $CuSO_4$. This prior exposure to copper resulted in a shorter lag in growth when copper concentrations were subsequently raised to 12 mM. They also showed that the higher the concentrations of $CuSO_4$ used in the pre-growth, the shorter the lag, and that resistance to copper was markedly higher in late exponential and stationary phase than early or mid-exponential phase cells. At 12 mM $CuSO_4$, no growth of plasmid-free strain occurs irrespective of whether or not the cells have been grown previously in 0.04 mM $CuSO_4$. Genetic studies on the plasmid pRJ1004 using transposon mutagenesis have shown that the pco (***p***lasmid-borne ***co***pper) resistance determinant consists of four genes, *pcoARBC* (**Figure 1-6**) (**Rouch *et al.*, 1989b**). At high copper concentrations, the *pcoC* gene products were proposed to modify the action of the proteins for normal copper metabolism, increase the efflux of copper in an energy-dependent manner and modify the copper so that it is not biologically available. The *pcoC* gene product, an inducible binding protein, is proposed to act as a cytoplasmic copper transport and/or storage protein while *pcoA* and *pcoB* proteins are responsible for the modification of copper and its export from the cell. The DNA sequence of the *pcoR* shows that it is a member of the two-component kinase-transphosphorylation family of regulatory gene pairs (**Brown *et al.*, 1992**). Regulation of plasmid-determined resistance genes appears to be coupled to

regulation of chromosomal genes as mutation in the plasmid-borne activator of the pco genes, *pcoR,* can be complemented by chromosomal gene *cutR* which has an analogous function in regulation of the cut genes (**Rouch *et al.*, 1989b**). Furthermore, the *pcoA* and *pcoB* gene products seem to interact with the *cutC* and *cutD* gene products to build a four-component efflux pump. Thus the copper resistance in *E.coli* is a complex system of plasmid-mediated intracellular copper binding, exclusion by enhanced efflux and chemical modification that is tightly coupled to chromosomally encoded functions required for normal copper uptake and regulation.

1.8.2 Mechanism of copper resistance in other Gram-negative bacteria

The *pco*-like determinant has also been demonstrated in *Citrobacter freundii* and *Salmonella sp.* (**Williams *et al.*, 1993**). In *Klebsiella aerogenes*, the copper resistance is thought to be due to high levels of extracellular polysaccharides production (**Bitton and Freihofer, 1978**). Copper resistance in *Vibrio alginolyticus* has been shown to constitutively involve the production of extracellular copper-binding proteins (CUBP). Two copper binding proteins have been identified, (CUBP1 and CUBP2) and these proteins are involved in complexing and detoxifying copper in the growth medium (**Harwood-Sears and Gordon, 1990**). Copper resistance in *V.alginolyticus* is not inducible in the wild type or in the copper resistant mutant (**Harwood and Gordon, 1994**).

Resistance to copper has also been described in *Pseudomonas syringae* pv. tomato (**Bender and Cooksey, 1986**). The plasmid-borne copper resistance consists of four genes *copABCD* which are induced specifically by copper (**Mellano and Cooksey, 1988a,b; Cooksey, 1993**). The proposed mechanism of resistance is different from that proposed in *E.coli.* Analysis of *cop* genes suggests that the mechanism of resistance is by sequestration of copper by periplasmic and outer membrane proteins (**Cha and Cooksey, 1991**) thus preventing copper ion from entering the cytoplasm. CopB is an outer membrane protein and is probably involved in copper binding although there is no direct evidence yet (**Cooksey, 1993**). However, the periplasmic proteins CopA and CopC have been shown to bind to copper. One molecule of CopA binds to approximately 11 atoms of copper and one molecule of CopC binds to 0.6 atoms of copper (**Cha and Cooksey 1991**). CopC and CopD are also thought to be involved in transporting adequate supplies of copper for

copper-dependent enzymes (**Cooksey, 1993**). Regulation of copper resistance in *P.syringae* was proposed to involve the interaction between plasmid and chromosomal regulatory genes. The resistance was also proposed to be negatively regulated by a chromosomally encoded repressor.

1.9 pH Stress Exposures

Lethal effects of pH include damages to outer membrane, ribosomes, cellular enzymes, DNA, proteins and pH sensitive metabolites. Bacteria can be subjected to pH stresses in the natural environments, in foods and in hosts environments such as in the human and animal bodies. In the natural environments, bacteria can be subjected to acidification and alkalization of natural waters due to pollutants from acidic or basic wastes and run-offs from naturally acidic or alkaline soils. In foods, pH stresses are encountered in fermented foods, foods that contain organic acids as preservatives, and egg whites which have pH values of 9.0-9.2 (**Minor and Marth, 1972; Humphrey *et al.*, 1991**). In human and animal bodies, bacteria are exposed to acid pH stress in the stomach (pH 2-3), the intestine, the urinary tract and vagina (pH 5.0-5.5) (**Goodson and Rowbury, 1989a**). The bacteria are also exposed to alkaline secretions (pH 9 and higher) from the pancreatic duct in the upper part of the small intestine just below the pylorus (**Small *et al.*, 1994**). In the phagolysomes, the bacteria are exposed to both acid and alkaline phases (**Segal *et al.*, 1981**).

1.10 pH Homeostasis In *Escherichia coli*- a mechanism of pH stress management

Although bacteria encounter considerable pH variations in their extracellular environments, some however, have evolved to grow in extreme pH environments such as in sulphur springs which have pH values of 1-2 (acidophiles) and in soda lakes which have pH values of ca 11 (alkalophiles). Meanwhile, most thrive in mid-range pH values of between 4 and 9 (neutrophiles) (**Padan *et al.*, 1981; Padan and Schuldiner, 1986**). In addition to perturbations from external sources, these bacteria also have to cope with pH perturbations from internal sources such as the acidic and basic end products formed from metabolic

processes (**Booth, 1985**). All these groups of bacteria adapt to their external and internal pH by very efficient constitutive housekeeping pH homeostasis systems that maintain relatively constant internal pH values over the range of permissive external pH values (**Padan *et al.*,1981; Booth, 1985; Padan and Schuldiner, 1987**). The internal pH (pH_i) values exhibited by neutrophiles, acidophiles and alkalophiles are respectively 7.5-8.0, 6.5-7.0 and 8.4-9.0 The variance in these pH_i values show that these bacteria have evolved enzyme systems with optimum function and stability within these narrow pH ranges. Cytoplasmic pH therefore plays a role as a metabolic regulator signalling the specific state of the cell to pH sensitive cellular processes. A tight regulation of the cytoplasmic pH in varying conditions is therefore vital for bacterial cell metabolism and growth.

E.coli, a representative of the large neutrophile class, grows optimally between external pH values (pH_o) of 6.0 and 8.0 and more slowly at pH values of one unit or so beyond these limits (**Ingraham, 1987**). Respiring *E.coli* cells have been shown to maintain a pH_i of 7.6±0.2 when measured using ^{31}P NMR over an extracellular pH range of 5.5 to 9.0 with the extreme pH_i values being 7.4 and 7.8 at pH_o of 5.5. and 9.0 respectively (**Slonczewski, *et al.*, 1981**). Zilberstein *et al.*, (**1984**) showed that electrochemical gradient of protons ($\Delta\mu_{H+}$) over pH_o 6-8.5 is relatively constant. To maintain such narrow physiological pH_i, a mechanism that can sense changes in both pH_o and pH_i and adjust the perturbed pH_i to its regulated state by either controlled acidification or alkalization of cytoplasm must be developed (**Booth, 1985**).

1.10.1 Components of pH homeostasis in *Escherichia coli*

The mechanism of pH homeostasis in *E.coli* is still under investigation. It has been suggested that the concentration of the proton gradient or the pH difference ΔpH (pH_i-pH_o) can be sensed within the periplasm by the sensory transducing proteins or *m*ethyl accepting *c*hemoreceptor *p*roteins (MCPs) involved in chemotaxis (**Padan and Schuldiner, 1986**). While a limited amount of acidification and alkalization can be offset to some extent by the preexisting cytoplasmic buffers (**Booth, 1985**), major acidification or alkalization require active removal of excess H^+ or OH^- from the cell by either direct or indirect pumping processes. The identity of the systems that are involved in major acidification and alkalization of the cytoplasm in pH homeostasis have been extensively

studied and systems based on the modulation of ΔpH and Δψ created by primary proton pumps are favoured to play these major roles (**Padan *et al.*, 1981; Padan and Schuldiner, 1987**). Since the proton electrochemical gradient is made up of membrane potential difference (Δψ) and pH gradient ΔpH (**1.3.3.1**), a change in one of the components is often accompanied by a compensatory change in the other (**Zilberstein *et al.*, 1984**). Thus within the pH limit of growth in *E.coli*, as pH_o increases, ΔpH decreases and Δψ increases to compensate for the decreased ΔpH, and as pH_o decreases, ΔpH increases and Δψ decreases to compensate for the increased ΔpH (**Padan *et al.*, 1981**).

The mechanism of the modulation of the ΔpH and Δψ in pH homeostasis is still unknown but there is increasing evidence of collaborative functioning of primary proton pumps with K^+ in alkalization of cytoplasm at acidic pH_o and with cation/proton antiporters in acidification of cytoplasm at alkaline pH_o (**Zilberstein *et al.*, 1984**). The roles of the K^+/ATPase and cation/proton antiporter in pH homeostasis are described as follows:

1.10.1.1 Electrogenic K^+/ATPase uptake system

The uptake of K^+ was suggested to play a role in raising pH_i when pH_o is acidic (**Kroll and Booth, 1983; Rosen, 1986**). This alkalization of the cytoplasm would involve electrogenic uptake of K^+ accompanied with concomitant extrusion of H^+ by hydrolysis of ATP thus increasing and restoring cytoplasmic pH to its homeostatic state (**Kroll and Booth, 1983; Rosen, 1986**). The process by which this alkalization would be triggered is still poorly understood although ΔpH and Δψ are thought to be involved.

1.10.1.2 Potassium/ proton (K^+/H^+) antiporters

Since the K^+/H^+ antiporter (KhaA) could couple the electroneutral extrusion of K^+ to the uptake of H^+, it was suggested to have a role in pH homeostasis at alkaline pH_o (**Brey *et al.*, 1980**). The influx of H^+ would acidify and lower the intracellular pH, thus returning it to its regulated state (**Brey *et al.*, 1980**). The involvement of this antiporter was suggested after it was found that K^+/H^+antiporter mutants grew very poorly at pH_o 8.3 with a doubling time of five-fold higher than the wild-type (**Plack and Rosen, 1980**).

The K^+/H^+ antiporter also extrudes Na^+, Li^+, Rb^+, Tl^+ in exchange for H^+ although

the net efflux is minimal (**Brey *et al.*, 1978; Rosen, 1986**). The K^+/H^+ antiporter has an optimum activity at pH 8 and is inactive below pH 7 (**Brey *et al.*, 1980**).

1.10.1.3 Calcium/proton (Ca^+/H^+) antiporters

The Ca^+/H^+ antiporter (ChaA) has recently been suggested to be involved in pH homeostasis at alkaline pH (**Ivey *et al.*, 1993; Ohyama *et al.*, 1994**). The ChaA, is encoded by the structural gene *chaA* that maps at 27 min on the chromosome. It has a molecular weight of 39,200 Da and a pH-independent activity (**Ivey *et al.*, 1993**). The ChaA gene product can extrude both Na^+ and Ca^+ at alkaline pH coupled with the uptake of H^+ and thus acidifying the cytoplasm to neutrality (**Ohyama *et al.*, 1994**).

1.10.1.4 Sodium /proton (Na^+/H^+) antiporters

The Na^+/H^+ antiporters are cytoplasmic membrane proteins that are widely found in bacterial, animal and plant cells (**Padan and Schuldiner, 1994a**). The existence of the Na^+/H^+ antiporter in *Escherichia coli* was first demonstrated by West and Mitchell (**1974**). Such antiporters have also been described in *Streptococcus faecalis* (*Enterococcus hirae*) (**Harold and Papineau, 1972; Kakinuma, 1987**), *Salmonella typhimurium* (**Tokuda and Kaback, 1977**), *Vibrio alginolytic*us (**Nakamura *et al.*, 1992**) and *Bacillus* species (**Krulwich *et al.*, 1986**).

In Escherichia coli, two specific Na^+/H^+ antiporter systems encoded by two unlinked genes namely *nhaA* and *nhaB* that map at 0.3 min and 25.6 min on the bacterial chromosome respectively have been described (**Goldberg *et al.*, 1987; Karpel *et al.*, 1988**; **Pinner *et al.*, 1992**). These structural genes encode single polypeptides and their amino acid sequences show extensive α-helical structures spanning the membrane as expected for transport proteins (**Taglicht *et al.*, 1991; Padan and Schuldiner 1994a**). The *nhaA* gene encodes a cytoplasmic membrane protein of 41,316 Da which is electrogenic with a stoichiometry of $2H^+$ for every Na^+ at both neutral and alkaline pH (**Taglicht *et al.*, 1991; Taglicht *et al.*, 1993**). The *nhaB* which encodes a 47,000 Da cytoplasmic membrane protein is also electrogenic with a stoichiometry of $3H^+$ for every $2Na^+$ (**Pinner *et al.*, 1994**).

Since the Na^+/H^+ antiporters couple the electrogenic extrusion of Na^+ with the H^+

uptake, they therefore play a role in the acidification of cytoplasm at alkaline external pH (**Schuldiner and Fishkes, 1978; Booth, 1985; Padan *et al.*, 1976;1981; Zilberstein *et al.*, 1982;1984**). However, studies using Δ*nhaA* and Δ*nhaA*Δ*nhaB* strains of *E.coli* showed that *nhaA* and *nhaB* are required for growth at alkaline pH only in the presence of Na^+; the mutants were able to grow up to pH 8.6 as long as Na^+ was withheld from the medium (**McMorrow *et al.*, 1989; Padan *et al.*, 1989; Padan and Schuldiner, 1994a,b**). This suggests that another system might be involved in pH homeostasis at alkaline pH in the absence of Na^+ (**Padan and Schuldiner 1994a**). The study with the Δ*nhaA* mutant also showed that tolerance to Na^+ is linked closely to pH; the sensitivity to sodium increases at alkaline pH; at pH 7.5 the cell can survive up to 500 mM NaCl but at pH 8.6, 100 mM of NaCl is inhibitory (**Padan *et al.*, 1989**). Based on the above, Padan and Schuldiner (**1994a**) suggested two possibilities for the importance of *nhaA* at alkaline pH. The first possibility is that *nhaA* is only required to alleviate the Na^+ toxicity but not for pH homeostasis at alkaline pH. Alternatively, the *nhaA* is involved in the regulation of both $[Na^+]$ and pH_i; the activation in *nhaA* at external alkaline pH suggesting that it might be involved in the acidification of the cytoplasm back to the resting internal pH.

When activities of both NhaA and NhaB were compared it was found that NhaB is only vital when: (a) the level of NhaA activity is growth limiting, (b) *nhaA* is not sufficiently induced or not activated (i.e at acidic pH and low $[Na^+]$) (**Pinner *et al.*, 1993**). The NhaB also has a higher affinity for Na^+ but lower affinity for Li^+ whereas NhaA has a higher affinity for Li^+ than Na^+ (**Padan *et al.*, 1989; Pinner *et al.*, 1993; 1994**). The activity of NhaB is not affected by pH whereas NhaA is markedly affected by pH (**Pinner *et al.*, 1992; Thelen *et al.*, 1991; Karpel *et al.*, 1991**). Ohyama *et al.*, (**1994**) showed that NhaB functioned at neutral pH and that its activity at high pH (pH 8.5) is low, and not sufficient for net Na^+ extrusion whereas NhaA showed high activity at high pH (pH 8.5). The inducibility of *nhaA* also increases with increasing pH (**Padan *et al.*, 1989; Padan and Schuldiner, 1994a,b**) The NhaB is sensitive to amiloride, an inhibitor of the eukaryotic antiporter whereas NhaA is insensitive (**Padan and Schuldiner, 1994b**).

1.10.1.4.1 Other physiological roles of Na^+/H^+ antiporters in E.coli

As mentioned above, the Na^+/H^+ antiporters are required for growth at high concentrations of sodium; the extrusion of Na^+ by the antiporters is important in maintaining low intracellular concentration of Na^+ as high intracellular Na^+ is inhibitory to essential enzymes (**Padan *et al.*, 1989**). The *nhaA* is also required for growth in the presence of lithium (>10mM) (**Padan *et al.*, 1989**). The extrusion of Li^+ via the antiporter is important in its detoxification (**Schuldiner and Fishkes, 1978**). The Na^+/H^+ antiporter has an important role in the bioenergetics of the cell; the extrusion of Na^+ by the antiporters is important in maintaining a sodium concentration gradient that is directed inwards (i.e $[Na^+]_{out} > [Na^+]_{in}$) which serves as driving force for the uptake of some metabolites via the sodium/substrate symport system (**Schuldiner and Fishkes 1978; Beck and Rosen, 1979; Zilberstein *et al.*, 1980**). In *E.coli*, substrates such as melibiose (**Tsuchiya *et al.*, 1977**), proline (**Stewart and Booth, 1983**), serine (**Ishikawa, 1987**) and glutamate (**MacDonald *et al.*, 1977**) have been shown to be co-transported with the sodium symport. A mutant having a deletion in both *nhaA* and *nhaB* was unable to grow on metabolites symported with Na^+ (**Pinner *et al.*, 1993**). Since in many bacteria the Na^+ gradient is in a steady state of equilibrium with the proton gradient it may well serve as an energy buffer also (**Schuldiner and Fishkes, 1978; Brown *et al.*, 1983**).

1.10.1.4.2 Regulation of transcription of Na^+/H^+ antiporters

Of the two antiporter genes, more is known on the regulation of *nhaA*. The expression of *nhaA* is positively regulated by Na^+ and Li^+ through a LysR-type regulatory gene *nhaR* located downstream of *nhaA* (**Karpel *et al.*, 1991; Rahav-Manor *et al.*, 1992**). Also the magnitude of expression induced by these ions increases markedly at alkaline pH and the activity of NhaA was found to increase by 2000-fold at pH 8.5 relative to neutral pH (**Taglicht *et al.*, 1991**). In the absence of these ions, there is no increase in expression when pH change of medium was between pH 6.5 and 8.6 (**Karpel *et al.*,1991**). The histidine 226 (His-226) has been suggested to be the "pH sensor" domain of NhaA (**Gerchman *et al.*, 1993**) and the Glu-134 has been speculated to be the "Na^+ sensor" of the NhaR (**Padan and Schuldiner 1994a**). Thus the NhaA is regulated both at transcription (**Karpel *et al.*, 1991**) and activity levels (**Taglicht *et al.*,1991**).

1.11 Inducible Stress Tolerance In *Escherichia coli*

Tolerance if any, to some physical or chemical stresses exhibited by bacteria may be constitutive, or due to selection of mutants or due to presence of plasmids or extrachromosomal DNA encoding the resistance traits. Frequently however, tolerance can be induced if the bacteria had prior exposure to sub-lethal doses of chemical or physical stresses. These tolerance mechanisms which are encoded by groups of chromosomal genes enable the exposed (habituated or adapted or induced) cells to survive level of stress which would otherwise be lethal to the non-habituated ones. Such known stress-tolerance induction systems are described in the following sections.

The terms "pre-exposed to stress", "induced", "habituated to stress" and "adapted to stress" will be used synonymously. Similarly the terms "induced tolerance", "acquired tolerance", "habituation" and "adaptive tolerance response" will also be used synonymously.

1.11.1 Inducible Thermotolerance

The lethal effects of high temperatures in bacterial cells include strand breaks in DNA, inactivation of enzyme activities and macromolecules synthesis, and membrane damage (**Neidhardt, 1984**; **Ingraham , 1987**). Yamamori and Yura (**1982**) showed that transient, non-heritable acquisition of tolerance to normally lethal temperature can be induced by short prior exposure to a non-lethal temperature shock. Their findings showed that *E.coli* cells that had prior exposure to a sub-lethal temperature, 42°C had a slower rate of death when challenged with lethal temperature, 55°C, than cells that were incubated at 37°C. The thermotolerance exhibited by the *E.coli* is small and transient, reaching a maximum protection at 30 minutes and disappearing after 60 minutes (**Yamamori and Yura, 1982**). In contrast, thermotolerance induced in *S.typhimurium* remained at its maximum level for 10 hours (**Mackey and Derrick, 1987**) and this was probably due to the stationary phase cells used in this experiment as opposed to the exponential phase cells used with the *E.coli* experiments. Cells of many strains in a steady state of growth at 45-46°C have a permanently increased thermal resistance compared to those grown at 30°C (**Neidhardt and VanBogelen, 1987**).

Thermotolerance has also been demonstrated in thermophiles. *Bacillus caldolyticus*

grown at 60°C and heat shocked at 69°C for 10 minutes showed thermotolerance to 74°C. Similarly *Sulfolobus shibatae* grown at 70°C heat-shocked at 88°C for 60 mins showed thermotolerance to 95°C (**Trent *et al.*, 1994**).

1.11.2 Inducible tolerance to alkylating agents

Alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrourea which can modify DNA bases by methylation are toxic and mutagenic to bacterial cells. The methylation gives rise to O^6-methylguanine, which is a miscoding base, and 3-methyladenine which is a cell-killing lesion (**Lindahl and Sedgwick, 1988**). However bacteria could resist lethal effects of high doses of MNNG if they had prior exposure for 30 minutes to low levels of MNNG (**Samson and Cairns (1977); Jeggo *et al.*, 1977**). The resistance was shown to involve an inducible DNA repair pathway which required *de novo* protein synthesis (**Samson and Cairns, 1977**).

1.11.3 Inducible tolerance to oxidative stress agents

Active oxygen species such as hydroxyl radicals (OH·) and superoxide radicals ($O_2^{-}\cdot$) can cause damage to DNA, RNA, protein and lipids in the bacterial cells (**Demple and Halbrook, 1983**). These active oxygen species can arise from several sources that include normal aerobic metabolism, hydrogen peroxide (H_2O_2), which can generate hydroxyl radicals and chemicals such as paraquat and plumbagin which can generate superoxide radicals (**Farr and Kogoma, 1991**). The presence of these active oxygen species in the bacterial cells is dealt with by protective enzymes such as the constitutively produced superoxide dismutases (SODs) such as the FeSOD and catalases (**Walkup and Kogoma, 1989**). The SODs, dismutate $O_2^{-}\cdot$ to H_2O_2 which is then further broken into H_2O and O_2 by the catalases (**Farr and Kogama, 1991; Demple, 1991**). Sometimes the active species of oxygen concentration rises above the basal level of the scavenging capacity of the bacterial cell and this leads to oxidative stress condition.

Demple and Halbrook (**1983**) showed that *E.coli* cells were able to survive lethal levels of (5mM-25mM) H_2O_2 if they were pretreated with low levels (5μM-50μM) of H_2O_2. Their results also showed that DNA repair activities that were induced during the H_2O_2 pretreatment enable the cells to survive lethal levels of H_2O_2 on subsequent

exposure. Similarly Farr *et al.*, (**1985**) showed that pre-exposure with a non-lethal dose of plumbagin conferred resistance upon exposure to a challenge dose and the ability for this survival was attributable to the ability of the pre-exposed cells to repair DNA.

1.11.4 Inducible tolerance to low pH

Lethal effects of acid below pH 4.0 could be attributable to intracellular damage of macromolecules such DNA, protein and acid sensitive metabolites (**Hall *et al.*, 1995**). Goodson and Rowbury (**1989a**) had shown that *E.coli* cells that had prior exposure at pH 5.0 showed greater resistance to normally lethal acid pH (pHs 3.0 and 3.5) than the cells that were grown at pH 7.0. This phenomenon of induced acid resistance was termed "acid habituation" by the authors. Habituated organisms showed increased resistance to DNA damage by acid and better DNA repair of acid DNA damage (**Raja *et al.*, 1991**).

Similarly, Foster and Hall (**1990**) showed that *S. typhimurium* cells that were pre-exposed to pH 5.8 for one doubling were more resistant at pH 3.3 than cells that were shifted from pH 7.6 to pH 3.3. The authors termed this phenomenon as "adaptive acid tolerance response" (ATR). Studies with two-dimensional proteins showed that 12 proteins were overexpressed during the induction period (**Foster, 1991**). In a separate report, Foster and Hall (**1991**) also showed that the adapted cells exhibit a ΔpH of 0.5 to 0.9 units higher than the unadapted when challenged at pH 3.3. They proposed that the ATR system may be an additional pH homeostasis system induced during the adaptation period since inhibiting protein synthesis did not prevent the housekeeping pH homeostasis systems from functioning above pH 4.0 whereas the addition of chloramphenicol 15 minutes prior to exposure eliminated pH_i enhancement and survival. Alternatively the enhanced survival in ATR system could be due to the pre-existing or constitutive pH homeostasis mechanisms being protected by the proteins induced during the adaptation period (**Foster and Hall, 1991**).

Induced acid tolerance has also been described in *Listeria monocytogenes* (**Kroll and Patchett, 1992**) *Aeromonas hydrophila* (**Karem *et al.*, 1994**) and a range of potentially pathogenic Gram-negative bacteria (**Nojoumi *et al.*, 1995**).

1.11.5 Inducible tolerance to high pH

At lethal alkaline pH, that is above pH 9.0, exposed organisms show damage to outer membrane, ribosomes, cellular enzymes and DNA (**Rowbury, 1994**). The ability to survive the exposure depends on the ability of the exposed cells to maintain a relatively neutral pH_i and repair damage that is caused by the alkali. Goodson and Rowbury (**1989b**) have shown that *E.coli* cells that had prior exposure at pH 9.0 for 60 minutes were more resistant to extreme alkaline pH (10.5-11.0) than cells that were pregrown at pH 7.0. The habituation to alkali was due to a phenotypic change during pre-exposure at pH 9.0 (**Goodson and Rowbury 1989b**).

1.12 Inducible Cross-Tolerance To Stress In *Escherichia coli*

Frequently bacterial cells that are pre-exposed to a sub-lethal dose of one stress not only show tolerance to lethal doses of the same stress but also to other stresses. This cross-tolerance or cross-protection has been described by several authors (**Table 1-4**). Pardasani and Fitt (**1989**) showed that *E.coli* cells that had prior exposure at 42°C for 30-45 minutes showed enhanced resistance to UV radiation. Enhanced resistance to UV radiation was also conferred in *E.coli* cells after prior exposure to 1mM H_2O_2 (**Fitt *et al.*, 1992**), 2.5mM H_2O_2 (**Asad *et al.*, 1994**), acid pH (**Goodson, and Rowbury, 1991**) and pH 9.0 (**Goodson and Rowbury, 1990**). This increased resistance to UV after pretreatment with pH 9.0 appeared to be RecA-independent (**Goodson and Rowbury, 1990**). Prior exposure at 42°C did not only confer thermotolerance at 50°C but also tolerance to ethanol (**Neidhardt and VanBogelen, 1987**). Tolerance to heat (56°C) and acid (pH 2.6) were exhibited by *Salmonella enteriditis* PT4 after a shift from 20°C to temperatures of between 37-46°C for a maximum duration of 60 minutes (**Humphrey *et al.*, 1993a**).

Humphrey *et al.* (**1993b**) also showed that prior treatment with alkaline pH values of between 8.0-9.75 resulted in enhanced resistance to heat (54°C) in *S. enteriditis* PT4 cells with maximum heat resistance achieved after pre-exposure at pH 9.5. Enhanced resistance to inactivation at 50°*C* as well as to other chemical oxidants were also gained by *Salmonella typhimurium* cells after prior exposure to an adaptive dose of H_2O_2 (**Christman *et al.*, 1985**). Acid-adapted (with prior exposure at pH 5.8) *Salmonella*

Table 1-4 Inducible cross-protection to stress responses in *E.coli* and *Salmonella sp.*

Inducing agents	Cross-protection	References
Heat (42°C)[a]	UV radiation	Pardasani and Fitt, 1989
Heat (42°C)[a]	Ethanol	Neidhardt and Vanbogelen, 1987
Heat (37-46°C)[b]	Heat (56°C), pH 2.6	Humphrey *et al.*, 1993a
H_2O_2[a]	UV radiation	Fitt *et al.*, 1992; Asad *et al.*, 1994
H_2O_2[c]	Heat (50°C), chemical oxidants	Christman *et al.*, 1985
Acid pH[a,c]	H_2O_2, heat (50°C), NaCl (2.5 M), crystal violet dye (25 mg/L), polymyxin B sulphate (10 mg/L), activated lactoperoxidase system, UV radiation	Foster and Hall, 1990 Leyer and Johnson, 1993 Goodson and Rowbury, 1991
Alkali (pH 9.0)[a]	UV radiation	Goodson and Rowbury, 1990
Alkali (pH 8-9.75)[b]	Heat (54°C)	Humphrey *et al.*, 1993b
Glucose/nitrogen[a] starvation	Heat (57°C), H_2O_2	Jenkins *et al.*,1988

[a] *E.coli* strains; [b] *Salmonella enteridits* PT4; [c] *Salmonella typhimurium*

typhimurium also showed increase in tolerance to 10 mM H_2O_2 (**Foster and Hall, 1990**), heat (50°C), salt (2.5M NaCl), crystal violet dye (25 mg/L), polymyxin B sulphate (10 mg/L) and activated lactoperoxidase system (**Leyer and Johnson, 1993**).

Glucose or nitrogen starved *E.coli* cells were also reported to show enhanced resistance to heat (57°C), and H_2O_2 (15 mM) (**Jenkins *et al.*, 1988**)

1.13 Inducible Cross-Sensitivity To Stress In *Escherichia coli*

Prior exposure to sublethal doses of one stress, can also induce sensitivity to other stresses. This cross-sensitivity has been described by Rowbury *et al.*, (**1993**). The authors showed that *E.coli* cells that had prior exposure to pH 9.0 showed more marked sensitivity to pH 3.0 than those that were pre-exposed at pH 7.0. This response was fully in place after 15 minutes of pre-exposure to pH 9.0 37°C. This response was termed acid sensitivity induction (ASI) by the authors.

1.14 Stress Response Systems In *Escherichia coli*

The mechanisms that enable bacteria to adapt to changes in their environments have been a subject of immense interest and intense research. Various systems induced in response to stress stimuli in bacteria, have been recognized and are well characterized in *E.coli.* These stress response systems include the heat-shock response, SOS response, alkylation response, oxidative response, catabolite repression and stringent response (**Table 1-5**) (**Neidhardt, 1987; Neidhardt *et al.*, 1990**). These stress response systems, promote cellular changes that enable the bacteria to adapt and survive the stress conditions.

The cellular changes induced in presence of the stress stimuli, involve changing the pattern of expressions of groups of genes or operons which encode proteins that promote adaptive responses. The modulations of gene expressions are carried out by regulators which are usually protein activators or repressors that recognize specific sequences in the controlling regions of the operons. In certain cases, alternative sigma factors were found to act as the regulators (**Neidhardt, 1987**). The member operons in some stress systems, are under the control of a common regulator while in others, the

Table 1-5 Stress response regulons in *E.coli* (adapted from **Neidhardt, 1987; Neidhardt *et al.*, 1990**)

Stimulus	System	Regulatory gene(s)	Regulated genes	Type of regulation
Carbon limitation	Catabolite repression	*crp/cya*	Catabolite repressed genes	Activation of CRP-cAMP complex
Amino acid limitation	Stringent response	*relA/spoT*	Ribosomal and other translational proteins and biosynthetic enzymes	Unknown
UV and other DNA damage agents	SOS response	*lexA/recA*	~20 genes for repair of UV-damaged DNA	Repression by LexA protein
Alkylation of DNA	Alkylation (Alkylation response)	*ada*	Four genes for removal of alkylated bases from DNA	Activation by Ada protein
H_2O_2 or similar oxidants	Oxidative stress response	*oxyR*	~ 12 genes for protection from $H_2O_2$and similar oxidants	Repression by OxyR protein

Continue overleaf

Table 1-5 *Continued.*

Stimulus	System	Regulatory gene(s)	Regulated genes	Type of regulation
Shift to high temperature	Heat-shock response	*htpR* (*rpoH*)	~17 genes for proteins involved in macromolecule synthesis, processing and degradation	Induction by σ^{32}
High osmolarity	Porin	*envZ/ompR*	*ompF,ompC*	Complex
Starvation/ Stationary phase	Stationary phase	*rpoS* (σ^{s})	Hundreds of genes	Induction of σ^{s}

member operons are governed by individual regulatory proteins, in addition to, a common pleiotropic regulatory protein (**Neidhardt, 1987**). The first of these operon networks are known as regulons and the latter are known as modulons (**Neidhardt, 1987**). In *E.coli* about 20 regulons are recognized and some are listed in **Table 1-5**. Very often the operons of a regulon are physically scattered around the bacterial genome and encode proteins of separate pathways or processes and accordingly, they are designated as global regulons (**Gottesman, 1984**).

A given regulon might be induced by more than one environmental condition and a given environmental condition might induce more than one regulon. In addition, a given stimulus might induce only a subset of the operons in a regulon (**Neidhardt, 1987**). Operons, irrespective of the same or different regulons, that respond together to a common stimulus are referred to as stimulons (**Neidhardt, 1987**).

Other components of the stress response systems include the "sensors" or cellular targets which detect the presence of the stress stimuli, and the transducers which transduce stress signals from the sensors to the regulators which then modulate gene expressions to promote adaptive responses (**Neidhardt, 1987**) (**Figure 1-7**). The stress response systems also have additional mechanisms for reverse induction that enable the bacteria to return to the prestimulus state once the stress stimulus is no longer present. This is normally achieved via regulation of the synthesis of the regulatory protein itself (**Gottesman, 1984**).

The following describes some of the specific stress response systems.

1.14.1 Heat shock response

The cellular changes exhibited by organisms following temperature upshifts are referred to as the heat-shock response. The heat shock response was first discovered in *Drosophila melanogaster*. In 1962, Ritossa, observed that specific puffs in the polytene chromosome were formed after the organism was exposed to a brief heat treatment (37°C). Tissieres *et al.*, (**1974**) later showed that heat exposure of *D.melanogaster* salivary glands to 37°C led to a dramatic increase in the rate of synthesis of seven polypeptides with concomitant inhibition of synthesis of most other cellular proteins. Analogous heat-shock responses have also been described in chick embryonic fibroblasts (**Kelly and Schlesinger, 1978**), chinese hamster ovary cells (**Bouche *et al.*, 1979**), yeast (**Miller *et al.*, 1979; McAlister**

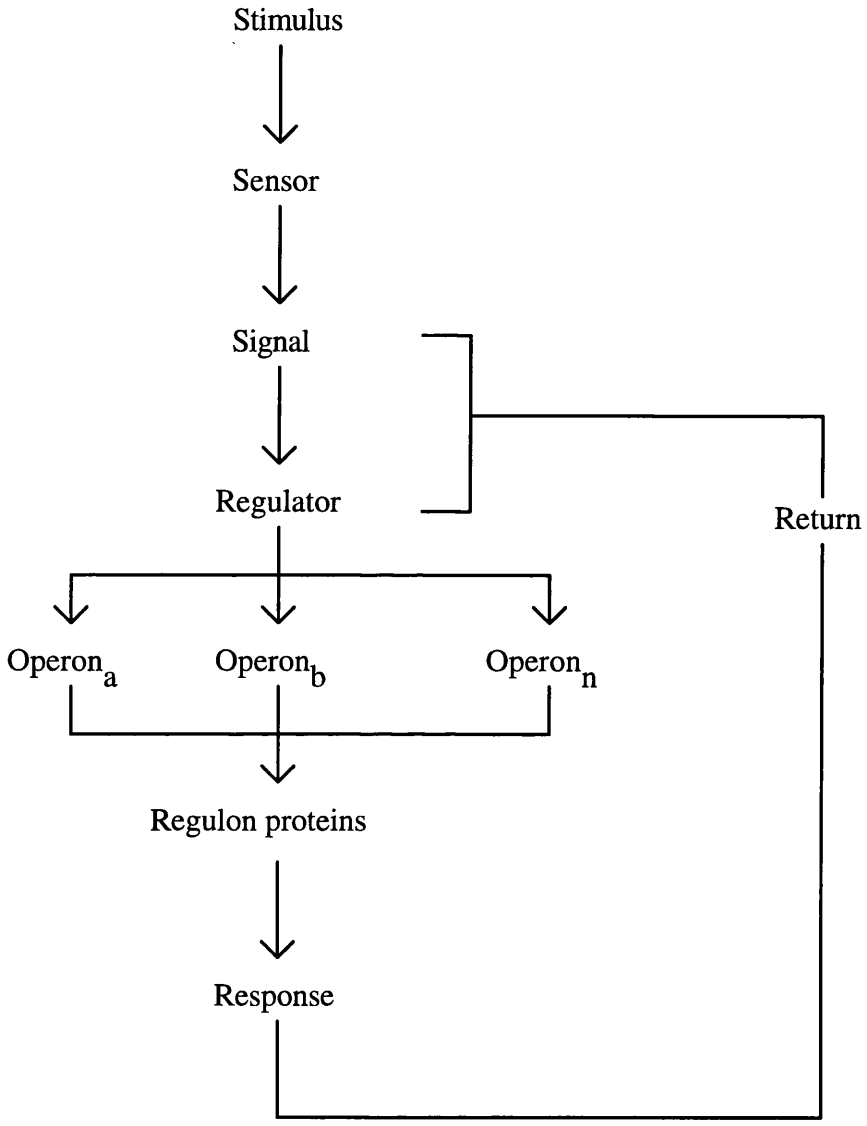


Figure 1-7 Regulon depicted as a stimulus-response pathway (after **Neidhardt, 1987**)

and Finkelstein 1978), in *Tetrahymena* (**Fink and Zeuthen, 1978**) and subsequently in other species including plants (**Barnett *et al.*, 1980**). The heat-shock response in *E.coli* was discovered in the late 1970's by Yamamori and Yura (**1978**) and Lemeaux *et al.*, (**1978**). Since *E.coli* has a wider range of growth temperatures than most higher organisms, the heat-shock response in this bacterium can be observed over a wide range of temperatures, for example, this response can be detected upon shifts from temperatures between 20-37°C to temperatures between 42-46°C (**Neidhardt, 1984**; **Neidhardt and VanBogelen, 1987**). This response can even be observed upon shifts from low temperature (e.g, 28°C) to that, below the optimal growth temperatures (e.g, 33°C or 36°C) (**Neidhardt, 1984**).

The universal cellular response of heat-shock is the transient exclusive synthesis of a number of proteins at high rates (**Neidhardt, 1984**). Among these proteins induced by high temperature in *E.coli*, is a set of 17 proteins collectively referred to as heat-shock proteins (hsps) which are positively regulated by the σ^{32} subunit of RNA polymerase (RNAP) (**Table1-6**) (**Neidhardt, 1984**; **Neidhardt and VanBogelen, 1987**). The σ^{32} or RpoH which is encoded by *rpoH* (*htpR* or *hin*) confers the core RNAP the specificity to transcribe heat-shock genes (**Neidhardt, 1984; Neidhardt and VanBogelen, 1987**). The sigma-32 subunit is the only subunit of RNAP that is heat-inducible unlike the alpha and beta subunits which are transiently repressed by shift to high temperature (**Neidhardt and VanBogelen, 1987**). The synthesis of σ^{32} increases by 11-fold within 2-4 minutes of a temperature upshift (30 to 42°C) and then declines during the adaptation period to a new steady state level that is sixfold higher than at 30°C (**Straus *et al.*, 1987**).

The presence of heat-shock proteins can be detected as early as 20 seconds after a shift in temperature, with maximum increase in protein synthesis after 5-7 minutes before subsiding to a steady state by 20 minutes (**Neidhardt and VanBogelen, 1987**). The sigma-32 has a stable life-span of 4-5 minutes following a temperature upshift, thus maximum accumulation of protein synthesis was observed in this duration (**Yura *et al.*, 1993**). Although referred to as heat-shock proteins most of these proteins are expressed at rather significant levels under normal growth conditions, varying less than two-fold during growth at 37°C in media with different composition (**Neidhardt and VanBogelen,**

Table 1-6 Heat-shock proteins of *E.coli* (adapted from **Neidhardt and VanBogelen, 1987**)

Number	Alphanumeric designation	Molecular weight	Abundance (α',10^3)[a]	Protein names[b]	Gene[c]
1	B25.3	25,300	1.44	GrpE	*grpE* (57')
2	B56.4	62,883	16.47	GroEL (Hsp60)	*groEL*(94')
3	B66.0	69,121	14.09	DnaK (Hsp70)	*dnaK*(5')
4	B83.0	70,263	2-3	Sigma	*rpoD*(67')
5	C14.7	14,700	0.87	-	*htpE*
6	C15.5	10,670	2.61	GroES	*groES* (94')
7	C62.5	71,000	2.61	-	*htpG*(11')
8	D33.4	33,400	1.0-2.0	-	*htpH*
9	D48.5	48,500	1.0-2.0	-	*htpL*
10	D60.5	60,500	0.18	Lysyl-tRNA synthetase II	*lysU*(93.5')
11	F10.1	10,100	<0.1	-	*htpK*
12	F21.5	21,500	<0.2	ClpP	*clpP* (10')
13	F84.1	84,100	<0.73	ClpB	*clpB* (57')
14	G13.5	13,500	<0.2	-	*htpN*
15	G21.0	21,000	<0.1	-	*htpO*
16	H94.0	94,000	1.61	Lon,La	*lon* (10')
17	H26.5	40,975	<0.2	DnaJ	*dnaJ* (0.5)

[a] Expressed as α', the weight fraction of each protein relative to total protein, in glucose-rich medium of 37°C.

[b] names in parentheses are the homologous counterpart in eukaryotes

[c] values in parentheses are locations of genes on the *E.coli* linkage map

1987). The unlinked genes that code for these proteins together with their common regulator, σ^{32}, form the high temperature production (HTP) regulon (**Neidhardt and VanBogelen, 1987**). The *E.coli* heat-shock regulon serves as a paradigm for analysis of the pathways that convert stress to a cellular response (**Figure 1-8**).

1.14.1.1 Functions of heat-shock proteins

The primary structure of most hsps seems to be highly conserved suggesting that they serve similar functions in all organisms (**Yura *et al.*, 1993**). The heat shock proteins of *E.coli* are diverse in respect to size, net charge, cellular abundance and the extent of inducibility to heat as defined by two-dimensional gel electrophoresis (**Neidhardt and VanBogelen, 1987**). Molecular size ranges from 10,000 to 94,000 Da. Some, like GroEL and DnaK, are among the most abundant proteins in the cell at 37°C while others escape detection until induced (**Neidhardt and VanBogelen, 1987**). Most of the major *E.coli* heat shock proteins such as DnaK, DnaJ, GrpE, GroEs, GroEL, Lon, ClpP, ClpB and HtpG have homologous eukaryotic counterparts (**Yura *et al.*, 1993**).

Heat-shock proteins are essential for cellular growth at all physiologically relevant temperatures since they are also present under non-stressed conditions (**Yura *et al.*, 1993**). The GroEL, GroES, DnaK and DnaJ proteins are found to be essential for *E.coli* growth at all temperatures (**Fayet *et al.*, 1989; Georgopoulos and Welch, 1993**). Their transient increase in rates of synthesis upon upshift in temperature represents a protective and homeostatic mechanism to cope with physiological stresses caused by heat. They are involved in protein folding, repair and degradation (**Table1-7**). The ability of some hsps (DnaK, DnaJ, GrpE, GroEL, GroES and HtpG) to assist in protein folding lead to their designation as molecular chaperones (**Ellis, 1987**). When overproduced, these proteins protect various other proteins from heat inactivation (**Yura *et al.*, 1993**). The DnaK, DnaJ and GrpE proteins also collectively known as the DnaK chaperone machine, can exert a negative-feedback control of the synthesis of heat-shock proteins following initial induction through direct interaction with the transcription factor sigma-32 thus releasing it from RNA polymerase core and therefore inactivating the transcription of the heat shock protein genes (**Figure 1-8**) (**Tilly *et al.*, 1983; Georgopoulos and Welch, 1993**). The Lon protein, a product of the *lon* gene, is an ATP-dependent proteolytic enzyme (**Neidhardt**

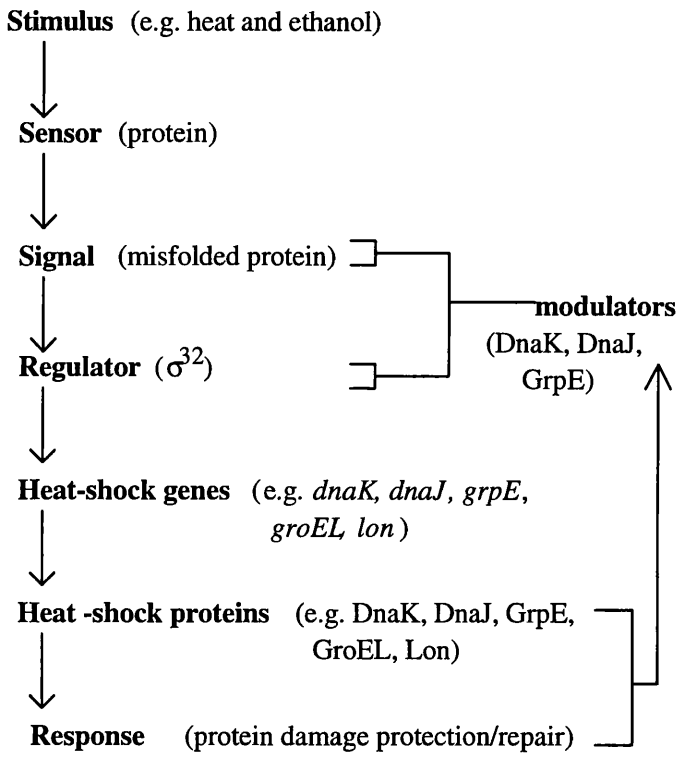


Figure 1-8 Feedback regulation in the heat-shock response. The model, based on the general stimulus-response scheme as given by Neidhardt (**1987**) (**Figure 1-7**), shows the elements that convert stress into heat-shock response .DnaK, DnaJ and GrpE provide a negative feedback regulation; they repair damaged proteins and they are the negative modulators of σ^{32}-dependent heat-shock gene expression. Damage repair by these chaperones and other heat-shock proteins alleviates the signal which then allow DnaJ, DnaK and GrpE to shut off the heat-shock response.

Table 1-7 Known functions of heat-shock proteins in *E.coli* (after **LaRossa and Van Dyk, 1991**)

Protein	Classical function	New Function
ClpP	Proteolytic component of the Cip protease	-
DnaJ	λ phage DNA replication	Polypeptide folding
DnaK	λ phage DNA replication	Polypeptide folding
GroEL	Phage morphogenesis	Polypeptide folding
GroES	Phage morphogenesis	Polypeptide folding
GrpE	λ phage DNA replication	Polypeptide folding
Lon	Protease, degradation of abnormal and other short-lived proteins	-
Lysyl-tRNA synthetaseII	Translation, second minor isozyme	-
Sigma	Transcription, selection of 'housekeeping' promoters	-

and VanBogelen, 1987). It plays an important role in removal of abnormal proteins and incompletely folded proteins (**Goff and Goldberg, 1985**). Lon is not essential for cell survival and growth under normal conditions but its deficiency can lead to a variety of phenotypic abnormalities. Lon is also essential in the degradation of SulA, the SOS protein that inhibits cell division (**Neidhardt and VanBogelen, 1987**). Other heat shock proteins with proteolytic functions include protein F84.1(ClpB or HtpM) and F21.5 (ClpP or HtpL).

1.14.1.2 Inducers, sensors and signals for the heat-shock regulon in *E.coli*

The synthesis of various heat-shock proteins in *E.coli* is induced by a variety of other stress factors besides temperature upshifts (**Table1-8**). These factors include physico-chemical factors such as pH, osmolarity changes and UV irradiation; metabolically harmful substances such as ethanol, antibiotics, heavy metals and DNA damaging agents ; and complex metabolic processes such as carbon and amino acid starvation, oxidative stress and viral infections (**Neidhardt and VanBogelen, 1987**). The inducing conditions vary with respect to the kinetics, level of induction, and specific subsets of hsps induced (**Bukau, 1993**). Among the inducers, only ethanol and depletion of 4.5S RNA have been reported to induce heat-shock response exclusively in a temperature upshift-like manner (**VanBogelen *et al.*, 1987; Bourgaize *et al.*, 1990**). Ethanol induces all 17 heat-inducible proteins in *E.coli* (**Neidhardt and VanBogelen, 1987**) although more gradually, with maximum induction reached after 30 to 60 minutes following a concentration upshift compared to that of 5-7 minutes following a temperature upshift (**Neidhardt, 1984**). Exposure of cells to cadmium chloride and hydrogen chloride, respectively induced six and, one of 17 heat-shock proteins found in *E.coli* (**VanBogelen *et al.*, 1987**). GroEL and DnaK were induced in cells that were pre-exposed to UV (irradiated at 100 J/m2 for 20 min) and nalidixic acid (40 μg/ml for 10 minutes). The maximum induction of these proteins took 15 minutes for nalidixic acid treatment and 20-25 minutes for UV irradiation compared to 5-7 minutes for temperature upshift (**Krueger and Walker 1984**). The basis for the induction of the heat-shock response by these inducers is not fully understood.

The induction of the heat-shock response by many stress conditions besides

Table1-8 Agents that induce heat-shock response in *E.coli* (modified after **Neidhardt and VanBogelen, 1987; VanBogelen *et al.*, 1987**)

Inducing agents[a]	Effects of inducing agents on cell structures and functions	Number of Heat-shock proteins induced
Shift from 28 to 42°C	Growth rate doubles, transient inhibition of cell division;general transient change in gene expression; increase ppGpp accumulation	15
Shift from 28 to 50°C	Single and double stranded breaks in DNA; inactivation of many enzymes and macromolecular synthesis; damage to cytoplasmic membrane; degradation of ribosomes and rRNA; unfolding of nucleiod followed by cell proteins aggregating to it	15
Ethanol	Mistranslation; disruption of transmembrane transport and translocation; increase in ppGpp accumulation	15
Puromycin	Premature chain termination and release of peptidyl puromycin from ribosomes	6

Continue overleaf

Table 1-8 *Continued*

Inducing agents[a]	Effects of inducing agents on cell structures and functions	Number of Heat-shock proteins induced
Viral Infection	Inhibition of host RNA, DNA and protein synthesis; decreased accumulation of cAMP; increase ppGpp accumulation	2
Nalidixic acid	Inhibition of DNA gyrase	10
Methylating/ alkylating agents	Inactivation through modification of nucleic acids and proteins	?
Cadmium chloride	Single strand breaks in DNA; inactivation of proteins	5
Hydrogen peroxide	Direct damage to DNA; general activation of proteins	3
ACDQ[b]	Inhibition of leucyl-tRNA synthetase	1
Amino acid restriction	Increased accumulation of ppGpp and decreased synthesis of stable RNA and protein	8

[a]Agents are listed in order of effectiveness as inducer; [b] 6-amino-7-chloro-5,8-dioxoquinoline

temperature upshift has led to the idea that, there may be multiple cellular targets or sensors that could generate the inducing signals. The potent inducers of the heat shock response such as temperature upshifts and ethanol affect structures of proteins, DNA and membranes whereas others have a specific immediate effect (**Table 1-8**). This has led to several proposals of possible sensors and inducing signals. These proposals however do not address the partial responses to various other stimuli.

Since many of the inducers are known to damage DNA, DNA was thus suggested as the sensor, and DNA damage as the signal for inducing the heat-shock response (**Neidhardt, 1984**). Proteins were also suggested to serve as sensors and aberrant or damaged proteins as signals that induced the heat-shock response (**Goff and Goldberg, 1985**). This hypothesis is strongly supported by the demonstration that the production of mutant proteins or the introducing of denatured protein fragments induces heat-shock response in *E.coli* (**Goff and Goldberg, 1985**). The ability of the cells to "sense" the accumulation of abnormal proteins and subsequently initiate transcription remains unanswered. This hypothesis however does not account for the heat-shock response induced by agents that damage DNA. Although there is increasing evidence for the role of aberrant proteins as signals, the existence of other additional sensors/signals such as membrane components, ribosomes (**VanBogelen *and Neidhardt,* 1990**) and perturbation of intracellular pH (**Travers and Mace 1982**) is not excluded.

1.14.1.3 Heat-shock response and thermotolerance

The relationship between heat-shock response and induced thermotolerance in *E.coli* is at present not fully understood. The synthesis of heat-shock proteins seems to play a critical role for their survival at high temperature as cells lacking the *rpoH* gene failed to acquire thermotolerance under the same conditions (**Travers and Mace 1982**). However it has been shown that the synthesis of the full spectrum of heat-shock proteins is not required for thermotolerance (**VanBogelen *et al.*, 1987**). It was suggested that the role of heat shock proteins that function as molecular chaperones in stressed cells is to aid recovery from the stress as opposed to helping the cells to withstand the stress by repairing either partially denatured and misfolded proteins or solubilized aggregated proteins back into their native conformation (**Watson, 1990; Georgopoulos and Welch, 1993**). The

proteolytic activity of the Lon protein may also protect the cells from lethal effects of abnormal proteins. Thus the synthesis of heat-shock proteins represents a part of the cellular adaptation mechanism for survival at high temperature by providing the cells with the capability to cope with the burden of increasing amounts of abnormal folded proteins. Although the protective roles of heat-shock proteins at lethal temperature is perfectly plausible, there is however, no correlation between the time taken for maximum thermotolerance to be induced (30 minutes at 42°C) and the time taken for maximum induction of heat shock proteins (5-7 minutes at 42°C). These observations have led to suggestions that thermotolerance acquired may be either related to some secondary effect of the response or that thermotolerance and heat-shock are two distinct inducible states; the former being a transient state associated with resistance to high lethal temperature while the latter being a more permanent state essential for physiological adjustment to growth at a high but non-lethal temperature (**Watson, 1990**).

1.14.2 Oxidative Stress Response

As previously mentioned, reactive oxygen species such as superoxide anion ($O_2^{-}\cdot$) hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{-}\cdot$) that occur as mutagenic byproducts of aerobic metabolism can cause damage to almost all cell components including DNA, lipid membranes and proteins (**Greenberg and Demple, 1989**). They can also mediate the effects of diverse environmental agents; for example H_2O_2 can react with Fe^{2+} or Cu^{2+} to generate $OH^{-}\cdot$ (**Farr and Kogoma, 1991**). *E.coli* responds to oxidative stress by invoking two distinct stress regulons, namely the OxyR-regulon, triggered by H_2O_2 and the SoxRS regulon triggered by superoxide generating agents (**Walkup and Kogoma, 1989; Tsaneva and Weiss, 1990; Farr and Kogoma, 1991; Nunoshiba *et al.*, 1992**). The following describes the OxyR regulon.

1.14.2.1 Peroxide stress response-OxyR regulon

An adaptive response to H_2O_2 is seen in *E.coli* and *S.typhimurium* in which at least 30 proteins are overexpressed in response to low doses of H_2O_2, enabling them to survive subsequent lethal doses of H_2O_2 and other peroxidizing agents (**Christman *et al.*, 1985; Demple and Halbrook, 1983; Demple, 1991; Farr and Kogoma, 1991**). The expression

of eight of the induced proteins in *E.coli* and nine in *S.typhimurium*, are under the control of the *oxyR* gene (**Walkup and Kogoma, 1989**). *E.coli* strains carrying *oxyR* deletions are unable to induce these proteins and are hypersensitive to hydrogen peroxide and other peroxidizing agents (**Walkup and Kogoma, 1989**). Several of the proteins whose expression is regulated by *oxyR* have been identified and these include glutathione reductase (encoded by *gor*), catalase-hydroperoxidase 1 (HP1; encoded by *katG*) and alkyl hydroxyperoxide reductase (Ahp; encoded by *ahp*) (**Walkup and Kogoma, 1989**; **Greenberg and Demple, 1989**). The role of the HP1 catalase is to reduce levels of H_2O_2 and the Ahp activity also reduces the levels of peroxides and peroxidized cellular components (**Farr and Kogoma, 1991**). The *oxyR* regulon does not appear to include a DNA repair pathway and this regulon may be a part of the broader peroxide stimulon (**Farr and Kogoma, 1991**). Unusual nucleotides ApppN (N= A,C,U or G) have been suggested to be the "alarmones" signalling onset of the oxidative stress regulon (**Lee *et al.*, 1983**)

OxyR exists in both oxidized and reduced forms and conversion between these two forms of OxyR is rapid and reversible (**Storz *et al.*, 1990**). In its oxidized form it activates the transcription of the oxidative stress genes (**Storz *et al.*, 1990**). The oxidation of OxyR protein brings about a conformational change by which OxyR transduces an oxidative stress signal to RNA polymerase to activate transcription (**Storz *et al.*, 1990**).

The OxyR regulon can also induce subsets of proteins that belong to heat shock and SOS regulons (**Goerlich *et al.*, 1989; Farr and Kogoma, 1991**)

1.14.3 The stringent response

The stringent response is a term used to describe the elaborate set of adjustments that a cell makes in response to starvation for amino acids (**Cashel and Rudd, 1987**). The two main classical changes observed in this response were: (i) a rapid shutdown of the synthesis of stable RNA (rRNA and tRNA), ribosomal proteins and other translational components, and (ii) a corresponding accumulation of two unusual guanine nucleotides (formerly called magic spots I and II), which are derivatives of GDP and GTP bearing the 3' pyrophosphate residues commonly abbreviated as ppGpp (guanosine 5'-diphosphate-3'-diphosphate) and pppGpp (guanosine 5'-triphosphate-3'-diphosphate), respectively or

(p)ppGpp collectively (**Cashel and Rudd, 1987**). Evidence has indicated that the elevated levels of (p)ppGpp in response to either amino acid starvation or to other nutritional adversities are directly involved in inhibiting the transcription of tRNA and rRNA genes probably through direct interaction with the β subunit of the RNA polymerase (**Cashel and Rudd, 1987; Reddy *et al.*, 1995**). When starvation from amino acid is alleviated, the levels of (p)ppGpp are degraded to their basal level by a 79-kDa protein ((p)ppGpp 3'-pyrophosphohydrolase) encoded by the gene *spoT* (**Cashel and Rudd, 1987; Mertzer *et al.*, 1989**).

The synthesis of (p)ppGpp during amino acid starvation is catalyzed by the RelA protein (also known as the stringent factor) in response to a decrease in the ratio of aminoacylated or charged tRNA to non aminoacylated or uncharged tRNA in the cell (**Condon *et al.*, 1995**). The RelA protein is a 84-kDa ribosome bound protein identified as (p)ppGpp synthetase I , and is encoded by *relA* which maps at 59 min on the *E.coli* chromosome (**Cashel, 1975; Pao and Dyess, 1981**). The designation *relA* is derived from the term "relaxed" which referred to mutants that are defective in the stringent control of their RNA synthesis under condition of amino acid starvation (**Cashel and Rudd, 1987**). Other mutations that affect the metabolism of (p)ppGpp synthetase I or alter the stringent response have also been mapped at loci *relB*, *rel*C and *rel*X located at 34 min, 90 min and 59.4 min on the *E.coli* genetic map respectively (**Pao and Gallant, 1978; Cashel and Rudd, 1987**).

The stringent response enables the cells to conserve resources which would be otherwise pointlessly used in synthesis of RNA and ribosomes. Besides the inhibition in tRNA, ribosomes and overall protein syntheses, a cascade of changes in other metabolic activities were also observed as a result of shortage in amino acids (**Cashel and Rudd, 1987**). These include, among others, inhibition of initiation of DNA replication (replication of DNA however continues at replication forks that exist at time of amino acid starvation), reduction in the rates of phospholipid, carbohydrate and murein syntheses and in the synthesis of their respective building blocks (nucleotides, sugars, carbohydrate intermediates and fatty acids), reduction in the synthesis of polyamines and in the uptake of purines and pyrimidines, induction of some heat-shock proteins (Lon, DnaK, GroEl, F84.1 and C62.5), increased rate of proteolysis and increased rates of synthesis of

enzymes in the amino acid biosynthetic pathway (**Cashel, 1975; Stephens *et al.*, 1975; Cashel and Rudd, 1987; Neidhardt *et al.*, 1990**). The compensatory cellular adjustments made during the stringent response, in order to adapt to conditions of amino acid starvation and other nutritional adversities, are truly global in character. The stringent response can be considered as a response network in which the stimulus, is a decrease in supply of nutrients; the sensor being the RelA-containing ribosome with an uncharged tRNA in its A site , the signal being (p)ppGpp; the regulator may be RNA polymerase itself; and the response is the turning off, of the transcription of genes coding for rRNA, tRNA and other translational machinery thus diverting the valuable resources to greater use to the cell during the starvation conditions (**Neidhardt *et al.*, 1990**). The cell returns to pre-stimulus state when (p)ppGpp is degraded by SpoT enzyme once starvation is alleviated (**Cashel and Rudd, 1987**).

1.14.3.1 Other inducers of stringent response

Besides amino acid starvation, stringent response is induced when growth rate is disturbed due to nutritional restriction such as a shift from rich to minimal medium, a shift to a poorer carbon and energy source or to a poorer nitrogen source (**Cashel and Rudd, 1987**). Similarly toxic agents that interfere directly or indirectly with energy transduction or amino acid biosynthesis and agents such as oxidants, solvents and other protein denaturing agents could also induce the stringent response in addition to their own specific adaptive response (**Neidhardt *et al.*, 1990**).

1.15 Transmembrane Signal Transduction

Many of the adaptive response systems, consist of a two-component regulatory system that handles stimulus detection, signal processing and production of appropriate cellular responses (**Table 1-9**) (**Ronson *et al.*, 1987; Stock *et al.*, 1989**). One of the components acts as an environmental "sensor" which detects and transmits "stress signals" to the second component which acts as a regulator, which mediates adaptive response by modulating the expression of the genes involved (**Ronson *et al.*, 1987; Stock *et al.*, 1989**; **Parkinson, 1993**). Nucleotide sequence analysis of the sensors and regulators in these

Table 1-9 Two-component regulatory systems in *E. coli* (**Ronson *et al.*, 1987; Stock *et al.*, 1989**)

Environmental stimulus	Adaptive response	Sensor	Response regulator
'attractants and repellants'	Chemotaxis	CheA	CheB/CheY
Changes in osmolarity	Osmoregulation	EnvZ	OmpR
Oxygen limitation	Oxygen regulation	CpxA*	ArcA
Phosphate limitation	Phosphate regulation	PhoR	PhoB
Nitrogen limitation	Nitrogen regulation	NR_{II}*	NR_I

* located in the cytoplasm

responses, shows a remarkable degree of conservation among them (**Ronson *et al.*, 1987**). The "sensor" component of the regulatory pair is a kinase that uses ATP to phosphorylate itself at a histidine residue (**Stock *et al.*, 1989; Stock *et al.*, 1990**). The phosphoryl group from the histidine protein kinase (HPK) is transferred to an aspartic acid side chain within the conserved domain of the second component, the response regulator (RR) which modulates the appropriate cellular responses (**Stock *et al.*, 1989; Stock *et al.*, 1990; Parkinson, 1993**). Many of the histidine kinases are transmembrane proteins, with an N-terminal periplasmic domain interacting with stimulatory ligands in the periplasmic space, and a C-terminal cytoplasmic domain that mediates the kinase activities (**Ronson *et al.*, 1987; Stock *et al.*, 1989**). The kinase activities in the C-terminal domain are under the control of the transmembrane signals from the N-terminal domain (**Stock *et al.*, 1989**). The response regulators are cytoplasmic proteins with a conserved N-terminal domain which acts as receptor of signals and the remainder of the protein which functions in DNA binding and interaction with RNA polymerase (**Ronson *et al.*, 1987**)

In *E.coli*, this conserved signal transduction mechanism has been demonstrated in the regulation of outer membrane porin synthesis in response to changes in medium osmolarity (**Ronson *et al.*,1987; Stock *et al.*, 1989**). The EnvZ acts as the sensor and a signal transducer and the OmpR acts as the response regulator (**Stock *et al.*, 1989**). The *envZ* and *ompR* genes belong to the *ompB* locus which is mapped at 74 min on the *E.coli* genetic map (**Hall and Silhavy, 1981**).

1.15.1 Regulation of *ompF* and *ompC* expression by EnvZ/OmpR *in E.coli*

In *E.coli*, the porin proteins OmpF and OmpC are regulated reciprocally such that the total amount of the porin proteins remains constant (**Lugtenberg *et al.*, 1976; Schaitman, 1974**). Studies on porin regulation have been mainly focussed on osmolarity effects (**van Alphen and Lugtenberg, 1977; Kawaji *et al.*, 1979; Mizuno *et al.*, 1982; Wurtzel *et al.*, 1982; Mizuno and Mizushima, 1986,1990; Forst *et al.*, 1987; Mizuno *et al.*, 1988**) although their regulation has been shown to be affected by pH (**Heyde *et al.*, 1988; Heyde and Portalier, 1987; Todt, *et al.*, 1992; Thomas and Booth, 1992**), temperature (**Manning and Reeves, 1976; Lundrigan and Earhart, 1984**), carbon source (**Schnaitman, 1974**), and procaine (**Granett and Villarejo, 1982**).

The genes *ompF* and *ompC* encoding the OmpF and OmpC respectively are located at 21 min and 48 min on the *E.coli* genetic map (**Hall and Silhavy, 1981**). Expression of these genes is regulated by the EnvZ/OmpR two-component system in response to osmolarity changes in the medium (**Silhavy and Hall, 1981**). The EnvZ is a cytoplasmic membrane protein which possesses a periplasmic N-terminal domain and a cytoplasmic C-terminal domain (**Forst *et al.*, 1987**). The periplasmic domain is involved in monitoring or sensing osmolarity changes in the periplasmic space although it is still not known how this is achieved (**Igo and Silhavy, 1988; Stock *et al.*, 1989**). This information is then transmitted via phosphotransfer events between the C-terminal domain of the EnvZ and the N-terminal domain of the OmpR (**Figure 1-9**) (**Tokishita *et al.*, 1992**). The C-terminal domain of the EnvZ, besides having a kinase activity and transferring a phosphate group to OmpR, is also a phosphatase, in that it can dephosphorylate the OmpR-phosphate and can also undergo autophosphorylation (**Aiba and Mizuno, 1990; Tokishita *et al.*, 1990,1992; Pratt and Silhavy, 1994;1995**). OmpR also has two functional domains, the N-terminal domain interacts with the C-terminal domain of EnvZ during phosphotransfer, and the C-terminal domain which binds to the upstream regions of the *ompF* and *ompC* promoters (**Maeda and Mizuno, 1990; Nakashima *et al.*, 1991; Rampersaud *et al.*, 1994; Huang *et al.*, 1994**). EnvZ modulates the function of OmpR through phosphorylation and dephosphorylation (**Aiba and Mizuno, 1990; Tokishita *et al.*, 1990**)

The regions upstream of both the *ompF* and *ompC* promoters have multiple OmpR binding sites (**Figure 1-10**) (**Maeda and Mizuno, 1990, Rampersaud *et al.*, 1994; Huang *et al.*, 1994**). The OmpR binding sites upstream the *ompF* promoters have different affinities to the phosphorylated OmpR. The high affinity sites or activating sites are occupied when levels of OmpR-P are low and the low affinity sites or deactivating sites are occupied when levels of OmpR-P are high (**Huang *et al.*, 1994**). The phosphorylated OmpR is a transcriptional activator of *ompC* but is either a repressor or activator at *ompF* (**Slauch and Silhavy, 1989**).

Under conditions of low osmolarity, the low levels of phosphorylated OmpR present binds preferentially to the activating sites upstream of the *ompF* promoter thus resulting in the F^+C^- phenotype (**Slauch and Silhavy, 1989; Russo and Silhavy, 1991;**

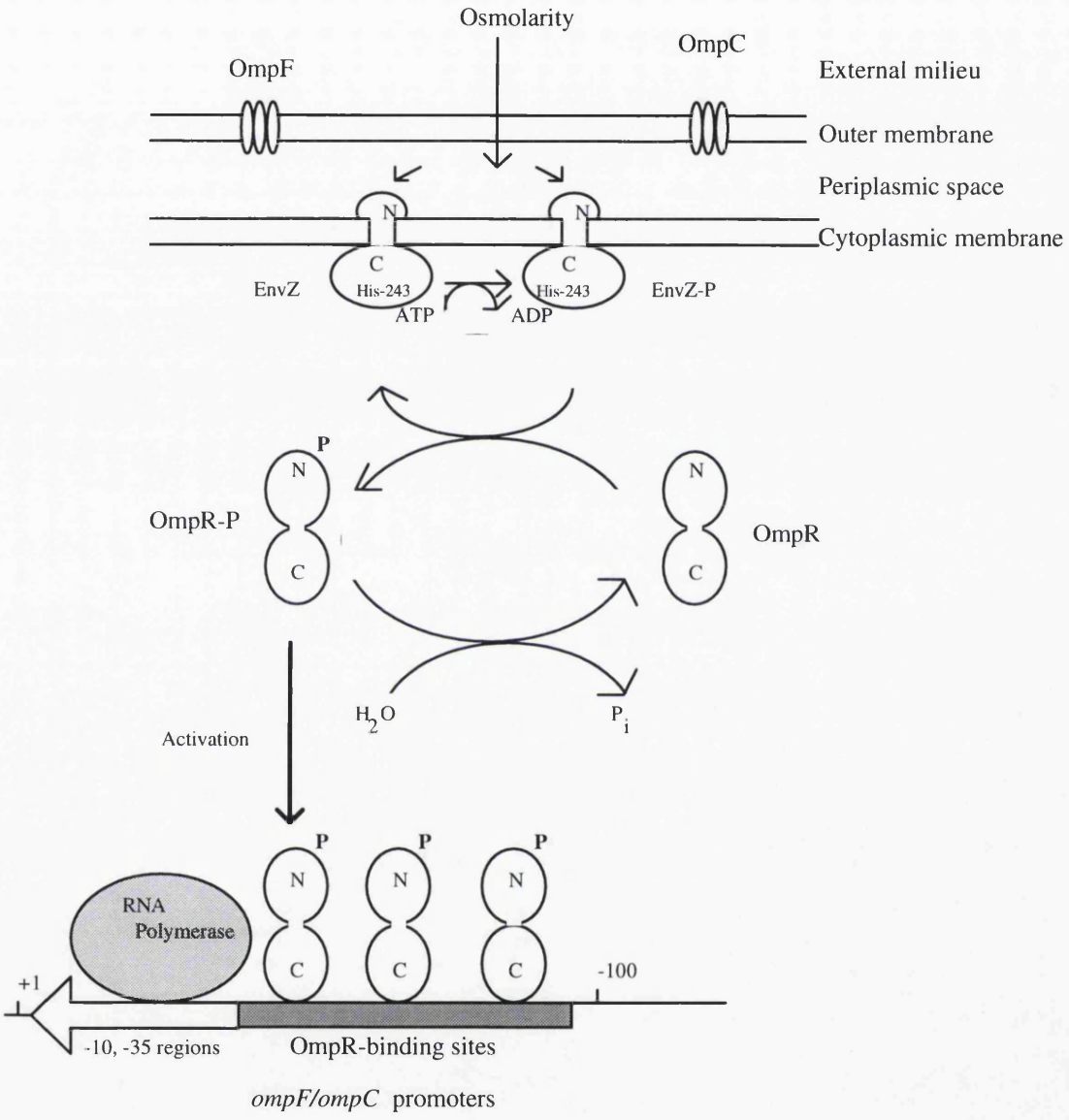


Figure 1-9 Scheme showing signal transduction and regulation of the *ompF* and *ompC* genes through phosphorylation of two regulatory components, EnvZ and OmpR, in response to an environmental osmotic signal (modified after **Stock *et al.*, 1989; Mizuno and Mizushima, 1990**)

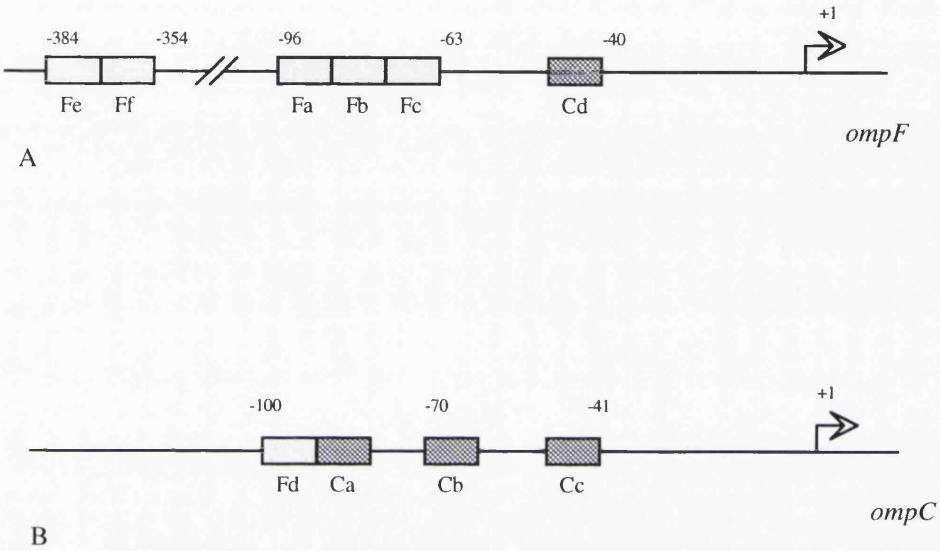


Figure 1-10 Schematic representation of multiple OmpR-binding sites in the regulatory regions of *ompC* and *ompF* (modified after **Pratt and Silhavy, 1995**). In panel A, the regions Fe, Ff and Cd are the deactivating OmpR-binding sites and Fa, Fb, and Fc are the activating OmpR-binding sites. In panel B, the regions, Ca, Cb, and Cc are the OmpR-activating sites and Fd is the *ompF* consensus OmpR-binding site.

Huang *et al.*, 1994). Under conditions of high osmolarity, the high levels of phosphorylated OmpR bind to the sites of upstream of *ompC* promoter and also the deactivating sites upstream of the *ompF* promoters thus resulting in F^-C^+ phenotype (**Slauch and Silhavy, 1989; Forst *et al.*, 1990, Mizuno and Mizushima, 1990; Russo and Silhavy, 1991; Huang *et al.*, 1994**). In the absence of phophorylated OmpR neither porin is expressed (**Russo and Silhavy, 1991**). Studies of the effects of pH on porin expression in *E.coli* have shown that the fluctuations in porin ratios induced by pH variations in growth medium are partly controlled by EnvZ protein (**Heyde and Portalier, 1987**)

1.16 Global Regulatory Proteins in *Escherichia coli*

Some of the regulatory proteins involved in the stress response systems are involved in the regulation of various genes coding for proteins of related functions and proteins of unrelated functions. Below are some of the examples of these global regulatory proteins.

1.16.1 The Sigma Factor S (RpoS)

The *rpoS* gene is induced as cells enter the stationary phase or when they are starved (**Hengge-Aronis, 1993**). There is considerable evidence that *rpoS* is primarily expressed in stationary phase as the *rpoS* transcriptional expression in cells growing in rich medium was low in early exponential phase and increased gradually two to three fold during exponential phase and to a substantial 20-fold above basal levels after the transition into stationary phase (**Mulvey *et al.*, 1990; Lange and Hengge-Aronis, 1991a**). Mulvey *et al.* (**1990**) also reported that transferring cells from rich to minimal medium resulted in a 10-fold increase in *rpoS* expression during the lag period and the expression was elevated throughout the exponential phase with only a slight increase upon entry into the stationary phase. However results by Lange and Hengge (**1991a**) indicate that there was no *rpoS* expression in minimal medium except in a Δ*cya* (adenylate cyclase mutants) background in which *rpoS* expression was significantly increased in the early exponential phase which was thought to be due to limiting but sufficient nutrients allowing the expression of *rpoS*. Mulvey *et al.*, (**1990**) found that starvation for carbon resulted in limited expression of *rpoS* whereas starvation for nitrogen or phosphate result in maximal expression.

Anaerobiosis reduced growth rates and thus stimulated *rpoS* expression during exponential growth in Luria-Bertani medium. Aromatic acids such as benzoic acid and other weak acids such acetate and propionate could induce expression of *rpoS* in minimal medium suggesting that *rpoS* expression is modulated by internal pH of the cell (**Mulvey *et al.*, 1990; Schellhorn and Hassan, 1988**). The starvation-inducible genes in *S.typhimurium* were found to be positively regulated by guanosine tetraphosphate (ppGpp) during both carbon and nitrogen but not phosphate starvation suggesting a link between ppGpp control and the general starvation phenomenon involving *rpoS* (**Spector and Cubitt, 1992**).

The *rpoS* gene product has a striking homology to the main or housekeeping sigma factor (σ^{70}, in *E.coli*) and promotes specificity of binding to the promoter-sequence recognition site of RNA polymerase and thus was indicated as the alternative sigma factor, sigma S (σ^{S}) (**Mulvey and Loewen, 1989**). The designations 'σ^{S}' and '*rpoS*' were given by Lange and Hengge-Aronis (**1991a**) as the gene product and its gene respectively where 'S' stands for stationary phase or starvation. The *rpoS* homologues have also been identified in other enteric bacteria such as *S. typhimurium* (**Fang *et al.*, 1992**) and *Shigella flexneri* (**Small and Falkow, 1992**).

Several alleles of *rpoS* conferring different phenotypes were isolated before the gene product was identified and hence the *rpoS* gene is also known by other names such as *katF* the regulatory gene for *katE* (encoding catalase HPII) (**Loewen and Triggs, 1984**) and for *xthA* (encoding exonuclease III) (**Sak *et al.*, 1989**), *nur* a gene locus conferring near-UV resistance (**Sammartano *et al.*, 1986**), *appR* the regulatory gene of *app*A (encoding acidic phosphatase), *csi-2* a carbon starvation inducible gene (**Lange and Hengge-Aronis, 1991a**) and *abrD* a regulatory gene involve in adaptive response to methylation (**Loewen and Hengge-Aronis, 1994**).

The σ^{S} positively regulates and controls a regulon of 30 or more genes that are selectively expressed in response to starvation and during the transition into stationary phase in *E.coli* (**Table 1-10**) (**Groat *et al.*, 1986; Hengge-Aronis, 1993**). Its synthesis is controlled transcriptionally and posttranscriptionally by as yet undefined mechanisms that are active well into the stationary phase (**Loewen and Hengge-Aronis, 1994**). In *E.coli* when these genes are being expressed as the bacteria enter a stationary phase or in starvation, they undergo significant physiological and

Table 1-10 *rpoS*-controlled genes in *E.coli* (after **Hengge-Aronis, 1993**)

Gene or operon	Map position (min)	Gene product	Function
appY	13.0	Regulatory gene	Control of cyxAB-*appA* expression
bolA	10.0	Regulatory gene	Morphogen
csgA	23.1	Curli subunit protein	Fibrinogen binding
csi-5 (osmY)	99.3	periplasmic protein	?
cyxAB-appA	22.4	Third cytochrome oxidase, acid phosphatase	?
dps	18	DNA-binding protein	DNA-protection; control of gene expression
glgS	66.6	GlgS	glycogen synthesis
katE	37.2	Catalase HPII	H_2O_2 resistance
osmB	28.0	Lipoprotein	?
otsBA	41.6	Trehalose-6P-	Trehalose synthesis; osmoprotection; thermotolerance
treA(osmA)	*26.0*	Periplasmic trehalase	Uptake of trehalose in high osmolarity medium
xthA	38.0	Exonuclease III	DNA repair; H_2O_2resistance

molecular changes that allow them to survive a wide variety of environmental stresses such as prolonged nutrient limitation (**Groat *et al.*, 1986; Matin *et al.*, 1989**), near-UV radiation (**Sammarano *et al.*, 1986**), hydrogen peroxide (**Jenkins *et al.*, 1988; Lange and Hengge-Aronis, 1991a**), elevated temperature (**Jenkins *et al.*, 1988**) and high salt (**Jenkins *et al.*, 1990; Hengge-Aronis *et al.*, 1993**). Some of these changes include the accumulation of glycogen (encoded by *glgS*; **Okita *et al.*, 1981; Hengge-Aronis and Fischer, 1992**) and trehalose (encoded by *otsBA*; **Hengge-Aronis *et al.*, 1991**), changes in cell morphology (encoded by *bolA*; **Aldea *et al*; 1989; Lange and Hengge-Aronis, 1991b**), syntheses of catalase HPII (encoded by *katE*; **Loewen *et al.*,1985**), exonuclease III (encoded by *xthA*; **Sak *et al.*, 1989**), acidic phosphatase (*appA*; **Touati *et al.*, 1987**) and DNA-binding protein (*dps;* **Almiron *et al.*, 1992**).

The thermotolerance and osmotolerance exhibited by stationary cells have been attributed to the synthesis of trehalose (**Kaasen *et al.*, 1992; Hengge-Aronis *et al.*, 1991**) whose membrane and protein protective properties help in counteracting heat shocks and the high salt concentrations (**Van Laere, 1989**). The synthesis of glycogen as storage compound enabled the cells to survive in prolonged nutrient limiting conditions (**Okita *et al.*, 1981; Groat *et al.*, 1986**). The lethal effects of H_2O_2 on the stationary phase cells are overcome by the induced production of catalase HPII which degrades the H_2O_2 (**Loewen *et al.*,1985**), the induced expression of exonuclease III which repairs damaged DNA (**Sak *et al.*, 1989**) and the induced expression of *dps* that encodes a novel histone-like protein which probably protects DNA against attack by H_2O_2 (**Almiron *et al.*, 1992**).

The *rpoS* gene has also been reported to mediate virulence; the expression of the *Salmonella* plasmid virulence genes (*spv*) increases as cells enter the stationary phase and is *rpoS*-dependent (**Norel *et al.*, 1992; Fang *et al.*, 1992**). In *S. flexneri*, a *rpoS* mutation makes the cells much more sensitive to acid and much less infective probably because the cells can no longer survive passage through the gut to the intestine (**Small and Falkow 1992**). In both these cases *rpoS* enables the cells to tolerate low pH conditions at stationary phase (**Gorden and Small 1993**). The *rpoS* is also required for *E. coli* to induce expression of fibronectin-binding organelles or curli upon reaching the stationary phase (**Olsen *et al.*,1989**). These fibers play a role in binding in fibronectin and laminin for adherence to host intestinal tissues (**Olsen *et al.*, 1989**).

1.16.2 Ferric uptake regulatory (Fur) protein

Fur is a 17 kDa histidine rich protein which is encoded by the gene *fur* located at 15.5 min on the *E. coli* genetic map (**Hantke, 1987; Crosa, 1989; Saito *et al.*, 1991**). A mutation at this locus was first generated by Ernst *et al.*, (**1978**) in *S. typhimurium* which resulted in constitutive expression of several high-affinity iron assimilation systems and thus was designated *fur* (ferric iron uptake). Fur autoregulates its own expression by binding to its own promoter (**Crosa, 1989**). Its expression is also regulated through the cyclic adenosine monophosphate-catabolite gene activator protein system (**De Lorenzo *et al.*, 1988**).

Fur is a DNA-binding repressor protein which requires Fe^{2+} as activator (**Bagg and Neilands, 1987a,b**). Other divalent metal ions such as Mn^{2+},Co^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} have also been shown to act as cofactors to Fur (**Bagg and Neilands, 1987a,b**). Interestingly, Hantke (**1987**) found that manganese-resistant mutants of *E.coli* were also *fur* mutants. The histidine residues in Fur are likely to be involved in the binding of divalent metal cations and the binding of the cations to these residues shows an unusual pH dependence with pKa values in the range of pH 6-9 (**Saito *et al.*, 1991**; **Saito and Williams, 1991**). Thus Fur not only monitors iron concentration but also the intracellular pH as well (**Saito *et al.*, 1991**).

Fur represses transcription by binding to a specific DNA sequence or "iron box" at the operator regions of Fur-regulated genes (**Bagg and Neilands, 1987; Crosa, 1989; Silver and Walderhaug, 1992**). The DNA binding site on the Fur is relatively close to a cation binding site on the protein (**Saito and Williams, 1991**). Le Cam *et al.*, (**1994**) showed that Fur binding does not bend DNA. Unlike other DNA-binding regulatory proteins, Fur lacks a concensus "helix-turn-helix" motif characteristic (**Hennecke, 1990**).

Fur, is regarded as a global regulator, in that, it is not only involved in the regulation of various bacterial genes related to iron (III) and iron (II) transport systems (**Silver and Walderhaug, 1992**) but also in other *E.coli* genes that are not directly related or even related to iron transport such as a variety of toxins and virulence determinants (**Calderwood and Mekalanos, 1987; Goldberg *et al.*, 1991**), superoxide dismutase genes (**Niederhoffer, *et al.*, 1990; Tardat and Touati, 1991**) and some pH-regulated genes (**Foster and Hall, 1992**). Thus Fur regulation suggests a correlation between the

modulation of iron uptake and the general metabolism of the cell.

1.16.2.1 Regulation of internal iron concentration by Fur

The Fur protein has a vital role in controlling the internal concentration of iron of many bacterial species (**Braun, 1985**). Although iron is an essential component of membrane-bound enzymes in the electron transport chains and a cofactor of many soluble enzymes (**Braun, 1985**), it can also have deleterious effects through reacting with reduced forms of oxygen to generate free radicals if presence in excess (**Baggs and Neilands, 1987b**). Thus control involves strict regulation of assimilation and storage of iron to ensure adequate iron supply and at the same time protecting cells from iron toxicity. This control is achieved through regulating the transcription of the genes involved in iron assimilation and storage systems by Fur (**Braun, 1985**).

In *E.coli*, the iron uptake systems consist of low molecular weight Fe(III)-chelators or siderophores, their specific outer membrane receptors and the transmembrane protein TonB which acts as an energy transducer (**1.3.1.3.5**) (**Braun, 1985; Crosa, 1989**). These genes are negatively regulated by Fur (**Hantke, 1982; Stojiljkovic *et al.*, 1994**). Thus when the internal concentration of iron reaches a critical limit, Fur forms a complex with Fe^{2+} and blocks transcription of these genes by binding to their specific operator sequences (iron box); in contrast, low internal concentrations of iron lead to activation of these genes (**Crosa, 1989; Silver and Walderhaug, 1992**). In many bacteria excess iron is deposited in iron stores, and in *E. coli*, bacterioferritin, a hemoprotein, is known to be associated with this function (**Andrews *et al.*,1989**). The bacterioferritin gene (*bfr*) however is indirectly regulated by *fur* probably through repression of an antisense promoter by *fur* (**Andrews *et al.*,1989**).

1.16.2.2 Involvement of Fur in pH-regulated genes

The effect of Fur on pH regulated genes has been studied in *S. typhimurium* by Foster and Hall (**1992**). They have shown that *S.typhimurium* mutants harbouring *fur* mutations, fail to exhibit an effective acid tolerance (ATR) and consequently are very sensitive to acid. Analysis on two-dimensional polyacrylamide gel electrophoresis showed a significant overlap between iron-Fur and pH-regulated protein synthesis. Seven of the genes which

are members of the ATR modulon were found to be dually influenced by pH and iron and under the control of *fur*. One or more of these low-pH and Fur-controlled genes may be of integral importance to ATR.

1.16.3 Leucine responsive regulatory protein

The leucine responsive regulatory protein (Lrp) governs the expression of a diverse group of genes known as the leucine/Lrp regulon (**Table 1-11**) (**Calvo and Matthews, 1994; Newman *et al.*, 1992; Newman and Lin, 1995**). The Lrp is a small moderately abundant (3000 molecules per cell) dimeric basic protein with monomeric molecular mass of 18.8 kDa (**Calvo and Matthews, 1994; Newman and Lin, 1995**). It is encoded by the *lrp* gene located at 20 min on the *E.coli* chromosome and was first identified as a locus *(livR)* that affects the transport of branched chain amino acids (**Anderson *et al.*, 1976**). Mutations in *lrp* suggested that it might have a general role in amino acid metabolism probably similar to the role played by *crp* (cAMP receptor protein) in carbohydrate metabolism. Lrp importance in cellular function is indicated by its remarkably high degree of conservation in other microorganisms (**Newman and Lin, 1995**; **Calvo and Matthews, 1994**). The Lrp molecule consists of three domains; a DNA-binding domain in the N-terminal, a transcription activation domain and a leucine-responsive domain (**Newman and Lin, 1995**). The interaction between Lrp and DNA was shown to induce bending within the DNA (**Wang and Calvo, 1993**).

Lrp is a transcriptional regulator acting as either activator or repressor of the operons of the leucine/Lrp regulon (**Calvo and Matthews, 1994; Newman and Lin, 1995**). The action of Lrp as a transcriptional regulator is intensified, and even dependent on, the presence of exogenous leucine at some operators, while at others it is reduced by leucine and in some cases it is independent of leucine (**D'Ari *et al.*, 1993; Calvo and Matthews, 1994; Newman and Lin, 1995**). Studies on the patterns of regulation of Lrp suggest that leucine is an allosteric effector of Lrp and that some promoters might respond to free Lrp, some to bound Lrp and some to either (**Newman and Lin, 1995**). The leucine effect is usually less severe than mutational inactivation of Lrp although the operon *leuABCD* is the only one known to be affected by exogenous leucine in the absence of Lrp (**Newman and Lin, 1995**).

Table 1-11 Operons regulated by Lrp and the effect of leucine in *E.coli* (modified from **Newman *et al.*, 1992; Calvo and Matthews, 1994; Newman and Lin, 1995**).

Lrp Effect	Leucine Effect	Operons	Product
Activator	Antagonist	*ilvIH*	Isoleucine;valine;leucine; biosynthesis
		leuABCD	Enzymes involved leucine biosynthesis
		serA	Serine biosynthesis
		gltBDF	Glutamate synthetase
		fanABC	K99 pili
Activator	Enhancer	*sfaA*	S pili
Activator	none	*gcvTHP*	Glycine cleavage pathway
		papBA	P pili
		daaA-E	F1845 pili
Activator	?	*ompF*	Outer membrane porin F
Repressor	Antagonist	*sdaA*	L-serine deaminase
		tdh/kbl	Threonine dehydrogenase; CoA ligase;2-amino-3 ketobutarate
		oppA-F	Binding protein and membrane components for oligopeptide transport
		lysU	Lysyl-tRNA synthetase II

Continue overleaf

Table 1-11 *continued*

Lrp Effect	Leucine Effect	Operons	Product
Repressor	Enhancer	*livJ*	Binding protein for isoleucine, leucine and valine transport
Repressor	None	*lrp*	Leucine responsive regulatory protein
		fae	K88
Repressor	?	*livKHMGF*	Uptake of branched amino acids
		ompC	Outer membrane porin C
		micF	Antisense RNA
		osmY	Periplasmic protein

Lrp was also suggested to have a role as a chromosome organizer due to its small, basic DNA-bending nature and the large number of these molecules present in the cell (**Newman and Lin, 1995**)

1.17 pH-Induced Stimulons

External pHs have been shown to be the inducer of the expressions in a number of genes and regulons in *E.coli* and in other enteric bacteria (**Table 1-12**) (**Olson, 1993; Hall *et al.*, 1995**). In *E.coli* and *S.typhimurium*, external acid induces acid-tolerance gene products (**Hyde and Portalier, 1990; Foster, 1991; Raja, 1992**). The levels of OmpF and OmpC are also regulated by pH (**Heyde and Portalier, 1987**). Alkaline pH-inducible transcription is observed for sodium proton antiporter gene, *nhaA* (**Karpel *et al.*, 1991**) and for induced-alkaline resistance gene, *alx* (**Bingham *et al.*, 1990**). In *E.coli* SOS (**Schuldiner *et al.*, 1986**) and heat-shock (**Taglicht *et al.*, 1987**) responses are also induced at alkaline pH.

This ability of bacteria to respond metabolically to changes in pH has long been recognized since the pioneering experiments by Gale and Epps (**1942**) on the effect of pH of growth medium on enzymic activities in *E.coli* and *Micrococcus lysodeikticus.* They showed that both *E.coli* and *M. lysodeikticus* generate amino-acid decarboxylases at low external pH and deaminases at high external pH. In each case, these adaptive enzymes act as a neutralization mechanism to modify the acidity and alkalinity of the external environment of the cell.

One of the biodegradative carboxylases induced at low pH is arginine decarboxylase which is encoded by the *adiA* gene that maps between 89 and 2 min (**Auger *et al.*, 1989**). Arginine decarboxylase is capable of increasing the surrounding pH by removing acidic carboxyl groups and releasing CO_2 from its substrate, arginine (**Stim and Bennet, 1993**). The *adiA* gene is under the positive control of the CysB regulator which is a tetrameric protein of identical 36 kDa subunits (**Ostrowski *et al.*, 1987; Shi and Bennet, 1994**). The CysB protein is a member of the LysR family of activator proteins and is also involved in the positive regulation of most of the genes involved in the cysteine biosynthesis pathway (**Kredich, 1992**) CysB binds upstream of the -35 region

Table 1-12 Genes regulated by pH in *E. coli* (after **Hall *et al.*, 1995**)

Gene/protein	Process/function	Regulator	Other inducers(s)
adiA	Arginine decarboxylase	CysB	Arginine, other amino acids, iron, anaerobiosis
alx	Induced by alkaline pH	?	-
aidB	Akylation-induced DNA-damaged		Alkylating agents
cadA, *cadB*	Lysine decarboxylase lysine/cadavarine transporter	CadR	lysine, anaerobiosis
groEL *dnaK, htpM* *htpG, grpE*	Stress proteins	HtpR	Heat
inaA	Induced by membrane -permeable weak acids	InaR	-
lamB	Maltose transport	MalT	Maltose

Continue overleaf

Table 1-12 *Continued*

Gene/protein	Process/function	Regulator	Other inducers(s)
lysU	Lysyl-tRNA synthetase	LysR	Heat, ethanol, puromycin
malE	maltose -binding protein	MalT	Maltose
nhaA	Na^+/H^+ antiporter	NhaR	NaCl
nmpC	Outer membrane proteins	cAMP/Crp	Osmolarity
ompF, *ompC*	Porins	EnvZ/OmpR	Temperature, osmolarity
polA	DNA polymerase 1	-	-
rpoS	Stationary phase sigma factor	?	Stationary phase/ starvation
SOS genes	DNA repair	LexA/RecA	DNA-damaging agents

of its positively regulated promoters. Kredich (**1992**) showed that the binding of the inducer molecules, O-acetylserine and N-acetylserine to CysB is required for the activation of CysB regulated-*cysB* promoters. DNA gyrase inhibitors which decrease the expression of *cysB* reduces *adiA* expression (**Shi and Bennet, 1994**).

1.18 The Role Of Antisense RNA In Posttranscriptional Regulation Of Gene Expression

Until the early 1980's, the regulation of gene expression was assumed to be mediated by protein-nucleic acid interactions until accumulated evidence of interactions between nucleic acids had been reported (**Laporte, 1984**). A particular example of this interaction is provided by the antisense RNAs which regulate gene expression at translational level (**Laporte, 1984**). The term "antisense" indicates that these RNAs have sequences that are complementary to those of the mRNAs whose translation is regulated (**Laporte, 1984**). The antisense RNA forms a duplex with the complementary target mRNA thereby preventing translation (**Laporte, 1984**). In *E.coli* cells replication of the ColE1 plasmid and regulation of Tn10 are believed to be regulated by a short antisense transcript (**Aiba *et al.*, 1987**). A similar mechanism was proposed by Mizuno *et al.*, (**1984**) as the third regulatory mechanism for the osmoregulatory reciprocal expression of *ompF* and *ompC*.

1.18.1 Involvment of MicF antisense RNA in posttranscriptional repression of *ompF*

MicF RNA (mRNA-interfering complementary RNA) is a small RNA molecule which is encoded by the gene *micF* (**Mizuno *et al.*, 1984**). The MicF RNA is a 4.5 S RNA with 93 nucleotides (**Andersen *et al.*, 1989**). The sequences on the 3' end of the *micF* RNA are complementary to the sequences on the 5' end of the *ompF* mRNA and interfere with the translation of *ompF* message by forming a stable RNA-RNA hybrid in the presence of another factor which has yet to be identified (**Mizuno *et al.*, 1984; Misra and Reeves, 1987; Andersen and Delihas, 1990; Esterling and Delihas, 1994**). MicF RNA is part of the global response as its activation is stimulated by various environmental stimuli and it is also under the control of several regulons (**Delihas, 1995**).

The *micF* RNA is peculiar as an antisense RNA, in that, its gene which is located at 48 min on the *E.coli* genetic map, is distal from its target gene, the *ompF* gene which is mapped at 21 min on the genome; whereas other antisense RNAs originate from the 5' ends of the target genes (**Aiba *et al.*, 1987**; **Delihas, 1995**). Instead, *micF* is located to the right upstream of the *ompC* gene and its transcription is directed opposite to that of *the ompC* gene from a promoter that appears to share some regulatory elements with the *ompC* promoter (**Figure 1-11**) (**Aiba *et al.*, 1987**). Since *micF* is upstream of *ompC*, it shares the same regulatory region which includes the binding sites for OmpR and IHF (integration host factor) (**Figure 1-11**) (**Esterling and Delihas, 1994**). Like *ompC*, the expression of *micF* is under the control of EnvZ/OmpR regulatory proteins (**Matsuyuma and Mizushima, 1985**). The *micF* RNA was shown to be the principal and possibly sole mediator of osmoregulation of OmpF when cells are grown in conditions of low-to-moderate levels of osmolarity (**Ramani *et al.*, 1994**). At high osmolarity the control by OmpR becomes the primary mechanism for the strong negative regulation of OmpF (**Ramani, *et al.*, 1994**).

Besides suppressing the expression of *ompF* in osmoregulation, *micF* was also shown to be necessary for the suppression of *ompF* as a result of a mutation in *tolC* (*tol*erance to *c*olicin E1) (**Misra and Reeves, 1987**). The mutation at the *mar* locus which leads to *m*ultiple *a*ntibiotic-*r*esistance significantly reduces levels of OmpF correlating partially with increased *micF* expression (**Cohen *et al.*, 1988**).

Andersen *et al.*, (**1989**) also showed that *micF* RNA plays a major role in the thermal regulation of OmpF. At high temperature and in presence of agents such as ethanol and copper, that induce heat shock, increased level of *micF* RNA suppressed the *ompF* expression (**Andersen *et al.*, 1989**). The regulation *of micF* expression with temperature increase is however not under the control of the *htpR* heat shock regulon (**Anderson *et al.*, 1989; Coyer *et al.*, 1990**) nor was it dependent on OmpR (**Coyer *et al.*, 1990**). It was previously reported that the *envY* gene is involved in thermoregulation of OmpC and OmpF porins in the deletion strain of *E. coli* UT5600 (**Lundrigan and Earhart, 1984**). However other mutant strains lacking the *envY* do have normal porin thermoregulation and in separate experiments *micF* RNA levels analyzed at 24 and 37°C

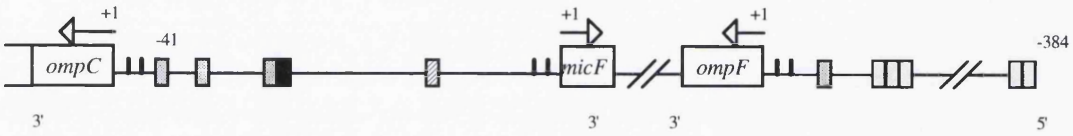


Figure 1-11 Schematic representation of location and direction of gene transcription of *ompC*, *micF*, and *ompF* (modified from **Esterling and Delihas, 1994; Pratt and Silhavy, 1995**). The OmpR-binding sites are shown as shaded and dotted boxes; solid black box represents the *ompF* concensus OmpR-binding site; the hatched box represents the IHF binding site on the shared regulatory region of *ompC* and *micF*. Vertical solid bars are the -10 and -35 promoter sites. *micF* interferes with the translation of *ompF* by forming stable RNA-RNA hybrid.

in mutant strain UT5600 (*envY*) showed no differences from the levels in the parental strain RW193 (**Coyer *et al.*, 1990**).

Chou and co-workers (**1993**) showed that *micF* also mediates the repression of *ompF* exerted by the *soxRS* system in response to oxidative stress. The SoxS protein of the *soxRS* locus has been shown to bind upstream of the promoter of the *micF* gene (**Chou *et al.*, 1993**). The leucine-responsive regulatory protein also binds upstream of the *micF* and appears to be a repressor of *micF* transcription (**Ferrario *et. al.*, 1995**) .

1.19 The Effects Of Glucose On Gene Expression In *E.coli*

The repression of the synthesis of catabolic enzymes is the major inhibitory effect of glucose on gene expression. The glucose effect is exerted on the operons of the cAMP-CAP (cyclic adenosine monophosphate-cAMP receptor protein) regulon and given that the presence of glucose can lower the intracellular levels of cyclic adenosine monophosphate (cAMP), the transcription of operons that require the cAMP-CRP complex as activator is thus repressed (**Magasanik and Neidhardt, 1987, Neidhardt *et al.*, 1990; Kolb *et al.*, 1993**). The reduction in intracellular concentration of cAMP in the presence of glucose, is due to the reduced activity of adenylate cyclase (encoded by *cya*) which catalyzes the synthesis of cAMP (**Neidhardt *et al.*, 1990**). For adenylate cyclase to be active it has to be phosphorylated by the enzyme III which is a component of the phosphoenolpyruvate (PEP):phosphotransferase system (PTS) that mediates glucose transport (**Neidhardt *et al.*, 1990**). In the presence of glucose, the sugar is preferentially phosphorylated, adenylate cyclase is dephosphorylated and is less active and thus rates of cAMP synthesis decrease (**Neidhardt *et al.*, 1990; Botsford and Harman, 1992**).

It is now known that the cAMP-CRP regulatory complex is not only involved in the positive regulation of operons encoding sugar catabolic enzymes but also is required in a much wider regulatory network activating and repressing the expression of many genes that are not directly related to catabolism (**Botsford and Harman, 1992**). For example, the regulation of *ompF* and *ompC* expression is affected by the conditions that changes the level of cAMP; high levels of cAMP increase expression of *ompF* and decrease the expression of *ompC* (**Scott and Harwood, 1980; Botsford and Harman,**

1992). It has been shown that the *ompB* locus which regulates the expression of *ompF* and *ompC* has both activating and inhibiting sites for the CRP-cAMP complex (**Huang *et al.*, 1992**). A deficiency in cAMP has also been shown to result in decreased growth rate with increasing pH (6-7.8) in *E.coli* K-12 (**Ahmad and Newman, 1988**). The *fur* gene expression has also been shown to be influenced by a second control system, the CRP-cAMP system which raises the possible correlation between iron regulation and metabolic status (**De Lorenzo *et al.*, 1988**).

1.20 The Influence Of DNA Topology On Gene Expression

The regulation of gene expression in bacteria, besides being modulated by regulatory proteins acting as either repressors or activators, can also be mediated by changes in the topological state of the DNA (**Brahms *et al.*, 1985**). Changes in supercoiling and localized conformational variations in the DNA such as bending and looping have been shown to affect gene expression (**Menzel and Gellert; 1983; Higgins *et al.*, 1988; Graeme-Cook *et al.*, 1989;Rouviere-Yaniv, 1990; Hsieh *et al.*, 1991**).

1.20.1 Influence of supercoiling on gene expression in *Escherichia coli*

In *E.coli*, the chromosomal DNA is compacted into a structure called a nucleoid in which the DNA is structured and ordered in topologically independent domains of negatively supercoiled DNA (**Krawiec and Riley, 1990; Rouviere-Yaniv, *et al.*, 1990**). The negative supercoils twist the DNA about its axis in the opposite direction from the clockwise turns of the right-handed double helix (**Bauer *et al.*, 1980**). The chromosome of growing *E.coli* cells is segregated into 43±10 domains of supercoiling per genomic equivalent of DNA (**Sinden and Pettijohn, 1981**). The number of domains can vary with growth rates and growth medium (**Sinden and Pettijohn, 1981**). *In vivo* the extent of negative supercoiling of DNA is approximately one negative turn for every 200 base pairs (**Sinden and Pettijohn, 1981; Kraweic and Riley, 1990**). The arrangement of the chromosome into separate domains enables different levels of superhelical tension to be maintained in different parts of the chromosome and also allows introduction of localized

unwinding for replication or transcription without effecting the bulk of the chromosome (**Sinden and Pettijohn, 1981**). The introduction of negative supercoils, creates a torsional stress in the DNA. This superhelical tension in the DNA, favours processes that will relieve its tension and these include processes that involve strand separations such as transcription, replication and recombination (**Drew *et al.*, 1985; Goldstein and Drlica, 1984**). Negative supercoiling also facilitates formation of structures such as the cruciform and Z-form DNA, loop formation and also allows the DNA to wrap around proteins such as the histone-like proteins and also during formation of protein-DNA complexes associated with specific recognition of nucleotide sequences by proteins (**Drlica, 1990**).

The level of negative supercoiling in *E.coli* is maintained at optimal levels by a balanced action between DNA gyrase (topoisomerase II), encoded by *gyrA* and *gyrB* genes, and topoisomerase I, encoded by *topA* gene (**Cozzarelli, 1980; Gellert *et al.*, 1982; Menzel and Gellert, 1983; Krawiec and Riley, 1990**). The former introduces negative supercoils in presence of ATP and the latter prevents excessive supercoiling (**Krawiec and Riley, 1990; Rouviere-Yaniv *et al.*, 1990; Drlica, 1992**). DNA gyrase could also exhibit DNA-relaxing action in the absence of ATP (**Drlica, 1984**). Negative supercoiling can be increased by mutations in *topA*, by transcription, and by low concentrations of gyrase inhibitors; at high concentrations, the gyrase inhibitors cause relaxation of chromosomal supercoils (**Drlica *et al.*, 1990**). Mutations in the DNA gyrase subunits or presence of gyrase inhibitors such as coumermycin A1, novobiocin, nalidixic acid and oxilinic acid result in reduced supercoiling (**Gellert *et al.*, 1982; Menzel and Gellert, 1983; Drlica, 1984**). The levels of DNA gyrase and topoisomerase 1 are under homeostastic control in that, their syntheses respond to current levels of DNA supercoiling (**Menzel and Gellert, 1983; Kraweic and Riley, 1990**).

In *E.coli*, the changes in supercoiling, have been shown to affect gene expression. *In vivo* experiments with inhibitors of DNA gyrase such as coumermycin and novobiocin, or mutants defective in DNA topoisomerase 1, have shown that the transcriptions of various genes respond in different and characteristic manner. While some transcriptions are repressed when DNA is relaxed, others were activated or unaffected (**Pruss and Drlica, 1989; Thompson *et al.*, 1990**). This was clearly demonstrated from the genetic studies of the leucine operon in *E.coli* and *S.typhimurium* (**Pruss and Drlica, 1989**). In

this study, deletions in *topA* (which increase supercoiling) activate the mutant *leu*500 promoter but this effect is reversed by addition of coumermycin (which relaxed supercoiling) (**Pruss and Drlica, 1989**).

Changes in supercoiling levels due to transcription influence gene expression in a different manner in that, it can not only, cause local variations but also influence the transcription of another promoter (**Drlica *et al.*, 1990; Dove and Dorman, 1994**). This is due to the generation of both negatively coiled, and relaxed or positively coiled domains, as a result of the movement of transcriptional complexes move along the DNA template (**Wu *et al.*, 1988; Dove and Dorman, 1994**). The domains of positively coiled or relaxed domain is formed ahead of the complex and the domains of negatively supercoiled DNA is formed behind the complex. The involvement of transcription indicates that topoisomerase is probably involved in relieving superhelical tension associated with the movement of the transcription complex along the DNA.

Although negative supercoiling of DNA is maintained at constant levels by the tight regulation of opposing activities of DNA gyrase and DNA topoisomerase1, there is now increasing evidence that environmental signals can also alter supercoiling.

1.20.1.1 Influence of environmental factors on supercoiling

Environmental signals such as osmolarity, temperature, depletion of certain types of nutrition, anaerobiosis, entry into stationary phase and transition from one carbon source to another, have been shown to alter DNA supercoiling in bacteria (**Table 1-13**) (**Graeme-Cook, *et al.*, 1989; Drlica *et al.*, 1990; Hsieh *et al.*, 1991**) These changes in the level of supercoiling appear to be at least in part responsible for regulating gene expression in response to these signals (**Higgins *et al.*, 1988; Balke and Gralla, 1987; Dorman *et al.*, 1988; Drlica *et al.*, 1990**). These changes in supercoiling in response to environmental stresses suggest that the topoisomerases involved in maintaining the supercoiling, are also affected by environmental changes through changes in their gene expressions. These environmentally induced changes in DNA supercoiling are of similar magnitude to those induced by gyrase inhibitors or *topA* mutations (**Hulton *et al.*, 1990**).

Negative supercoiling increases at high osmolarity and in anaerobiosis (**Dorman *et al.*, 1988; Graeme-Cook, *et al.*, 1989**). The expression of *tonB* promoter of *E.coli*

Table 1-13 Perturbation of DNA supercoiling (after **Drlica, 1992**)

Perturbations	Relevant Genotype	Effect on supercoiling
Mutations		
	gyrA	Relaxation
	gyrB	Relaxation
	himA	Relaxation
	topA	Increase
	hupA hupB	Relaxation
	hns	Allele specific
		Increase or decrease
Environmental factors		
Shift to high temperature		Relaxation
Shift to low temperature		Decrease
High osmolarity		Increase
Anaerobic shock		Transient relaxation
Anaerobiosis		Increase
Nutrient downshift		Relaxation
Nutrient upshift		Transient relaxation
Other factors		
Stationary phase		Relaxation

which is repressed under anaerobic growth, was found to be sensitive to increases in DNA superhelicity (**Dorman, *et al.*, 1988**). In conditions of amino acid starvation when there is an increased level of ppGpp there is a reduction in the levels of supercoiling and this reduction is even greater upon carbon starvation (**Ohisen and Gralla, 1992**). The mechanisms by which environmental stimuli influence cellular DNA topology are obscure. Changes in DNA supercoiling in response to osmolarity and anaerobiosis are rapid and do not require protein synthesis (**Drlica *et al*, 1990**). It has been suggested that, the external stimuli may influence the interactions of DNA with its chromatin components, and thus altering the topology and hence gene expression (**Higgins *et al.*, 1990a**). Another possible mechanism is the modulation of topoisomerase 1 or DNA gyrase by the environmental signals which influence DNA topology and hence gene expression (**Hulton *et al.*, 1990**). Hsieh *et al.*, (**1991**) have shown that the ratio of ATP to ADP concentrations might be involved since shifting *E.coli* cells from aerobic to anaerobic growth reduced the ATP:ADP concentrations and supercoiling.

It has also been suggested that environmentally induced changes in DNA supercoiling may play a role in the control of bacterial virulence coordinating the cell response to the multiple stresses encountered during transition from free living to a host-associated state thus enabling the required adjustments in gene expression and physiology (**NiBhriain *et al.*, 1989; Dorman *et al.*, 1990; Higgins *et al.*, 1990b**). This is supported by the observation that many genes that play a role in bacterial virulence are supercoiling-sensitive including the outer membrane porins (**Dorman *et al.*, 1989; Graeme-Cook *et al.*, 1989**), the *vir* genes of *S.flexneri* (**Dorman *et al.*, 1990**) and the invasion genes of *S.typhimurium* (**Galan and Curtiss HI, 1990**). Also, environmental signals known to influence DNA supercoiling including osmolarity, anaerobiosis, temperature and nutrient status are exactly those encountered when bacterial cells interact with host. It is well known that many virulence genes are regulated by those environmental stresses that affect supercoiling and that 'stress'-regulated genes are important for bacterial virulence (**Miller *et al.*, 1989; Buchmeier and Heffron, 1990**).

1.20.2 Influence of histone-like proteins on gene expression in *E.coli*

Histone-like proteins which associate with the DNA have been implicated in having a role

in compacting the chromosomal DNA (**Falconi *et al.*, 1988; Drlica and Rouviere-Yaniv, 1987**). They are also involved in restraining the tension present in the supercoiled DNA (**Rouviere-Yaniv *et al.*, 1990; Drlica, 1992**). Several histone-like proteins are associated with the bacterial nucleiod; the two most abundant of these are HU (also known as, B2, HD, NS) and H-NS (also known as B1, H1) which are present at 60-100x10^3 and 20x10^3 copies per cell respectively (**Drlica and Rouviere-Yaniv, 1987; Falconi *et al.*, 1988; Higgins *et al.*, 1990b**). Unlike the histones that are associated with the eukaryotic DNA, the histone-like proteins of the prokaryotic nucleoid do not form regular compact nucleosome structures, but instead are readily disassociated from the DNA (**Higgins *et al.*, 1990**). Thus the prokaryotic DNA is only partially constrained by bound proteins such that a net free energy of negative supercoiling is still available as torsional stress to influence processes such as DNA replication and transcription (**Higgins *et al.*, 1990b**). Some of the histone-like proteins are known to act as accessory factors in facilitating other protein-DNA interactions by inducing bending in the DNA. The following considers the histone-like proteins H-NS and IHF in *E.coli* and their influence on gene expression.

1.20.2.21 H-NS

H-NS is the second most abundant nucleoid protein after HU. H-NS is a 15.4 kDa protein which displays properties of histone-like proteins such as, it is heat stable and binds tightly to dsDNA and more weakly to ssDNA, rRNA and tRNA (**Falconi *et al.*, 1988**). It also increases thermal stability of DNA and inhibits *in vitro* transcription of phage lambda or *E.coli* DNA (**Falconi *et al.*, 1988**). Unlike HU and other histone like proteins, H-NS is neutral rather than basic with pI of 7.5. The H-NS is a homodimeric protein comprising of 136 amino acids and binds to DNA nonspecifically thus introducing significant compaction of DNA (**Falconi *et al.*, 1988**). In *E.coli* the gene coding for H-NS, *hns* (*osmZ*), is located at 27.5 min on the *E.coli* chromosome (**Higgins *et al.*, 1990b; Yasuzawa *et al.*, 1992**). The mutation in the gene encoding H-NS has been isolated based on a variety of selections and thus many genetic designations have been given to this locus (*osmZ*, *bglY*, *drdX*, *pilG*, *viR*) (**Table 1-14**) reflecting the range of phenotypes of mutations at this locus (**Higgins *et al.*, 1990b**). Mutations in *hns* are pleiotropic; it affects the expression of many bacterial genes that have no apparent link, either in their locations,

Table 1-14 Effects of lesions in *hns* (after **Higgins *et al.*, 1990b**)

Gene designations (all affecting *hns*)	Phenotypes
osmZ	Derepression of osmoregulated gene expression
	Altered ratio of *ompF* and *ompC*
	Mucoid; non-motile
drdX	Derepression of temperature-regulated *pap* pili synthesis
pilG	Altered frequency of *fimA* phase inversion
bgly	Increased frequency of chromosome deletions
hns	Encoding H-NS

functions or in the environmental signals that regulate their expression (**Hulton *et al.*, 1990; Hinton *et al.*, 1992**). The fact that these genes are regulated by very different stimuli and are scattered around the genome demonstrates that the effects on chromosomal DNA topology are global (**Hulton *et al.*, 1990**).

Many of the H-NS dependent loci are environmentally regulated and are sensitive to changes in supercoiling. The *hns* gene product appears to play an underlying role in the control of DNA topology and gene expression in response to environmental signals such as osmolarity, temperature and oxygen availability (**Hulton *et al.*, 1990**). H-NS is also part of the cold shock regulon of *E.coli*; its expression being stimulated 3 to 4 fold during the lag that follows a shift from 37 to 10°C (**Teana *et al.*, 1991**) This led to the suggestion that H-NS can alter gene expression through changes in nucleoid organization and/or DNA topology (**Higgins *et al.*, 1988; Dorman *et al.*, 1990; Hulton, *et al.*,1990**). In most cases, mutation in *hns* causes an increase in transcription suggesting that H-NS acts as a negative regulator of transcription. While many genes are derepressed in *hns* mutants, some are repressed while many others are relatively unaffected (**Hulton *et al.*, 1990**). The *hns* mutations derepressed expression of a number of osmotically-regulated genes (**Higgins *et al.*, 1988**), several temperature regulated genes (**Dorman *et al.*, 1990**) and genes that are influence by unknown stimuli (**Higgins *et al.*, 1990a,b**).

Besides affecting transcription, *hns* mutations also increase frequencies of deletion formation and site specific recombination events (**Higgins *et al.*, 1988**). Changes in DNA supercoiling and transcriptional silencing of chromosomal DNA have been proposed as the mechanisms underlying global gene regulation by H-NS (**Gorannson *et al.*, 1990; Higgins *et al.*, 1990a,b**).

Many if not all of the of the functions altered on *hns* mutants are sensitive to changes in supercoiling. Most importantly, *hns* mutations also alter DNA supercoiling *in vivo* and these changes in DNA topology are probably associated with phenotypes of *hns* mutations (**Higgins *et al.*, 1990a,b**). Higgins *et al.*, (**1990a**) suggested that the changes in supercoiling in *hns* mutants are due to the direct consequences of altered interactions between H-NS and DNA or alternatively the possibility of H-NS altering the DNA topology indirectly by interacting or displacing of other histone-like proteins. However there is no evidence that H-NS interacts directly with HU although H-NS has a higher

affinity for double-stranded DNA than HU and thus could potentially displace it. H-NS was also suggested to function as a general "silencer" of transcription rendering parts of chromosomes transcriptionally inactive (**Gorannson *et al.*, 1990**).

H-NS also preferentially recognizes curved DNA and shows enhanced affinity for it (**Yamada *et al.*, 1990**). Interaction of H-NS at DNA curves in the vicinity of *proU* promoter appears to play an important role in determining the repression of the expression of this locus (**Owen-Hughes *et al.*, 1992**). Tanaka (**1991**) however suggested that the binding of H-NS to curved DNA sequences often found upstream of *E.coli* promoters represses the expression of the genes concerned. The *hns* promoter region also exhibits a curved DNA structure and H-NS acts as a repressor of its own transcription by binding to its promoter region (**Ueguchi *et al.*, 1993**). The autoregulation of *hns* is dependent on growth phase i.e autoregulatory expression is derepressed at stationary phase and consequently levels of H-NS appear to increase in this phase of growth which causes altered nucleoid compaction and leads to transcriptional repression or silencing of some genes in a growth-phase dependent manner (**Ueguchi *et al.*, 1993**). There is also evidence to suggest *hns* plays an important role in the mechanism underlying the regulation of the cellular content of σ^s particularly at posttranscriptional level (**Yamashino *et al.*, 1995**). Under physiological conditions the level of H-NS has to be properly regulated as overproduction of H-NS has a harmful effect on cells (**Hulton *et al.*, 1990**).

1.20.2.2 Integration host factor (IHF)

The IHF of *E.coli* is a small basic DNA-binding protein with a molecular weight of 21.8 kDa (**Freudilch *et al.*, 1992**). It is composed of two non identical subunits encoded by the unlinked *himA* and *himD (hip)* genes which map at 38 min and 21 min on the genetic map of the *E.coli* chromosome respectively (**Drlica and Rouviere-Yaniv, 1987**). It is also one of the most abundant DNA-binding proteins in *E.coli* (20,000-100,000 molecules per cell) and has a large number of binding sites all over the genome (**Freundlich *et al.*, 1992**). IHF is regarded as a member of the histone like family of proteins because it can compact DNA and also because each of its subunits shows strong amino acid sequence homology with HU (**Freundlich *et al.*, 1992**). Unlike HU, binding of IHF to DNA is strongly sequence specific (**Schmid, 1990**). IHF , often but not always occupies an A-T

rich region of the DNA and chemical footprinting analysis suggested that the principal contacts of IHF are made within the minor groove of the DNA (**Goosen and van de Putte, 1995**).

The IHF proteins were originally discovered as host-factors required for *in vitro* site-specific recombination for phage lambda integration in which the IHF proteins organize the DNA and the L integrase proteins into a tight complex termed an "intasome" (**Schmid 1990**). The role of IHF however, not only involves formation of L integration complex but includes a variety of site-specific recombination events, phage packaging and partition, DNA replication and formation of protein-DNA complexes necessary for initiation of transcription of a number of genes (**Friedman, 1988; Hoover *et al.*, 1990; Freundlich *et al.*, 1992**). IHF binding sites are located in markedly different places within the promoter regions (**Freundlich *et al.*, 1992**). In many of these cases, IHF was found to influence gene expression by inducing bends or kinks in the DNA thus facilitating protein-protein interactions between upstream activators and RNA polymerase which is critical for the regulation of gene expression (**Craig and Nash, 1984**; **Friedman, 1988; Schmid, 1990; Freundlich, *et al.*, 1992; Goosen and van de Putte, 1995**). For example, in the transcription of the *glnHPQ* operon, which encodes for products involved in the uptake glutamine, IHF, brings together the activator NR_I and the σ^{54} of the RNAP by forming a loop (**Freundlich *et al.*, 1992**). Similarly, formation of loop by IHF is also involved in the activation of the *narGHJI* operon encoding nitrate reductase (**Freundlich *et al.*, 1992**; **Goosen and van de Putte, 1995**). There is also evidence that, in some of the cases, IHF function is influenced by DNA supercoiling and that supercoiling can also be altered by IHF (**Friedman *et al.*, 1988**). In several operons of *E.coli*, IHF has been shown to inhibit transcription by forming a repressive loop, for example, in the repression of the *ompF* expression under high osmolarity. IHF facilitates the formation of this loop by aiding the OmpR-OmpR interactions at sites between -56 and -41 and at sites between -384 and -351 upstream of the *ompF* promoters (**Figure 1-12**) (**Huang *et al.*, 1994**). Some of the effects of IHF are also through direct interaction with *ompR* which has IHF binding sites (**Huang *et al.*, 1994; Freundlich *et al.*, 1992**).

In *E.coli*, IHF has also been shown to directly stimulate transcription in some operons. For example in the expression of *ilvGMEDA* operon which encodes for products

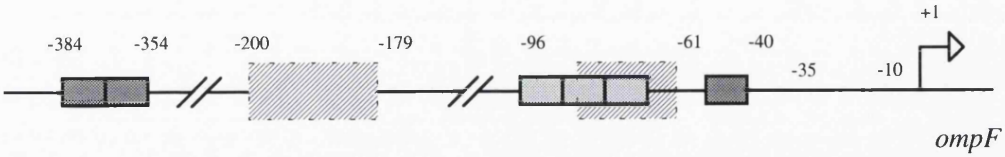


Figure 1-12 A schematic representation of OmpR and IHF binding sites in the *ompF* region. The OmpR binding sites are shown as shaded boxes, the darker of the two shades being the OmpR-deactivating sites, and the lighter shade being the OmpR-activating sites. The IHF binding sites are shown as hatched boxes (modified after **Huang *et al.*, 1994**).

that are involved in the uptake of isoleucine and valine, IHF which has a binding site upstream from the promoter region facilitates the formation of a closed-complex by bringing sequences located further upstream from the IHF binding site into contact with the RNA polymerase (RNAP).

There is also evidence that, in some cases, IHF function is influenced by DNA supercoiling and that supercoiling can also be altered by IHF (**Friedman *et al.*, 1988**). IHF has also been reported to increase as *E.coli* enters stationary phase by five to ten-fold (**Freundlich *et al.*, 1992**).

1-20 Aims of Study

Bacteria are often faced with continual fluctuations of environmental insults during their lifetime and still show a remarkable ability to tolerate and survive these hostilities. For some, it has been recognized that tolerance can be induced if they had prior exposure to a sublethal condition of the stress as has been described to heat, oxidative stress agents and pH. Sometimes exposure to one stress can alter responses to another. Of interest in the recent years are the responses of polluting enterobacteria to chemical and physical stresses in the natural environment due to their increasing occurrence through sewage pollution and more importantly, since they include a number of major potential pathogens.

Copper and pH stresses are two of the many stresses encountered by bacteria in their environment. This study was undertaken to study the responses of *E.coli* strains to copper and alkali. The aims of this study were to investigate the following:

A. *Tolerance to copper*; specifically the following were investigated:

1. Sensitivity of *E.coli* 1829 to copper sulphate

2. Effect of pre-exposure to sublethal doses of copper on subsequent challenge to lethal doses of copper

3. Kinetics of copper habituation

4. Effect of a protein synthesis inhibitor on copper habituation

5. Comparison of the ability of copper-habituated and non-habituated cells to synthesize proteins and RNA after a challenge with lethal dose of copper

6. Effect of copper challenge on DNA of copper habituated and non-habituated cells

B. *Responses of copper-habituated and non-habituated cells to other stresses*

C. *Alkali sensitivity of cells grown at* pH_o *7.0 and* pH_o *5.5;* specifically the following were investigated:

1. Effect of prior exposure to pH 5.5 on subsequent challenge to lethal alkaline pH

2. Effect of protein and DNA synthesis inhibitors on alkali sensitivity induction

3. Comparison of the abilities of pH 5.5 and pH 7.0 induced cells to synthesize

proteins after an alkali challenge

4. Effect of alkali challenge on DNA in pH 5.5 and pH 7.0 induced cells

5. Effect of induction of alkali sensitivity in strains carrying lesions in known regulatory lesions

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains And Phages.

The bacterial strains used in this study are listed in **Table 2-1**. The bacteriophages used in this study were P1vir, Me1, K3 and λgti.

2.2 Maintenance Of Bacterial Strains

For long-term storage the bacteria strains were maintained on porous beads (Microbank^{Tm} Pro-Lab Diagnostics) at -70°C. For short-term storage they were maintained on NA (nutrient agar) slopes at 4°C and subcultured every three months. For day-to-day use, the strains were maintained on NA plates at 4°C and subcultured every week.

2.3 Growth Media

Nutrient Broth (Oxoid No:2 25 g/L) was used as the main growth medium in this study. Where appropriate the broth was acidified with hydrochloric acid (HCl) or alkalinized with 1N sodium hydroxide (NaOH) or 1N potassium hydroxide (KOH). In the copper tolerance study, the broth was supplemented with appropriate concentrations of cupric sulphate ($CuSO_4.5H_2O$; BDH). Where necessary, other chemical agents or antibiotics were added to the media as described in the following sections. For solid media, the Nutrient Broth (NB) was solidified with 2% (w/v) agar (Difco) to give Nutrient Agar (NA). Semi-solid nutrient agar or soft agar was prepared by adding 1% (w/v) agar to the NB.

The Minimal medium used was that of Davis and Mingioli (**1950**) which was made up using following ingredients:

Dipotassium hydrogen phosphate (K_2HPO_4; BDH)	7.0g/L
Potassium dihydrogen phosphate (KH_2PO_4; BDH)	3.0g/L
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$; BDH)	0.5g/L
Ammonium sulphate ($(NH_4)_2SO_4$; BDH)	1.0g/L
Magnesium sulphate ($MgSO_4.7H_2O$; BDH)	0.1g/L

Table 2-1 Strains of *Escherichia coli* used in this study

Strain	Genotype	Source
1829	Δ*trp*, *lac*	This laboratory
1829 F'Lac	1829 F*lac*$^+$	This laboratory
1829 ColV, I-K94 pBR322		This laboratory
CF1648	*rel A*$^+$	Dr. M.Cashel
CF1652	CF1648 Δ*relA*	Dr. M.Cashel
6482	*ara-14,leuB6,azi-6, tonA23,lacY1,proC83, tsx-67,purE42,supE44, galK2,trpE38,rfbD1, arg77,his-208,rpsL109, xyl-5,mtl-1,ilvA681, metA160,thi-1*	Dr. B.Bachmann
7157	6482, Δ*katF3*,Δ*xthA*15, Δ*mgl*-51,Δ*glp*R201,Δ*katG*16	Dr. B.Bachmann
W3110-1	W3110 *Tna-2*, Δ(*argF,lac*) U169	Dr. JB Neilands

Continue overleaf

Continued (**Table 2-1**)

Strain	Genotype	Source
W3110-2	W3110-1 *fur*::Tn5 U169	Dr. JB Neilands
AB2847	*tsx,malT,aroB*	Dr. K.Hantke
H2300	AB2847 Δ*tonB*	Dr. K.Hantke
TA15	*melBLid,rpsL,* Δ*lacZY*	Dr. S.Schuldiner
NM81	TA15 Δ*nhaA 1* Kan^R	Dr. S.Schuldiner
1157	*thr,leu,proA* 2 Δ(ProA *phoE,gpt)his,thi,argE lacY,galK,xyl,rpsl*	Dr. N.E Gillies
1157-4	1157 $proA^+$,$phoE^+$	This Laboratory
HN678	*galK2,supO,strR,*Δ*himA*, Tet^R	Dr. H.Nash
HN1069	*galK2,supO,strR,*Δ*himD* Cap^R	Dr. H.Nash
PD32	Δ*hns*,Ap^R	Dr. E.Bremer

Continue overleaf

Continued (**Table 2-1**)

Strain	Genotype	Source
SM3001	Δ*micF*,Kan^R	Drs. H.Aiba and T.Mizuno
CV975	*lrp*$^+$,*ara, thi,* Δ[*lac, pro*] ilvH::MudI 1734	Dr. J.M Calvo
CV1008	Δ*lrp*::Tn10	Dr. J.M Calvo
JA199	CV 975 *cysB*$^+$, *trpE5*, *leu-6*, *thi, hsdR, hsdM*$^+$	Dr. N. Kredich
NK1	JA199 Δ*cysB*	Dr. N. Kredich
MC4100pALS10	*envZ*$^+$, *ara D139*, Δ[*argF, lac*]169, *rps150, flbB5301,deoC1,ptsF25,rbsR*	This Laboratory
AT142pALS10	MC4100, Δ*envZ*	This Laboratory
1829-HNS	1829, Δ*hns*	This study
1829-MicF	1829,Δ*micF*	This study
1829-NhaA	1829, Δ*nhaA*	This study
1829-Fur	1829, Δ*fur*	This study

Continue overleaf

Continued (**Table 2-1**)

Strain	Genotype	Source
1829-HimA	1829, Δ*himA*	This study
1829-HimD	1829, Δ*himD*	This study
1829-OmpA	1829, *ompA*	This study
1829-OmpC	1829, *ompC*	This study
1829-LamB	1829, *lamB*	This study

Where necessary, any nutritional requirements were also added. For carbon source, glucose was added to a final concentration of 0.2% (w/v) to the sterilized broth. For solid media, the minimal medium (MM) was supplemented with 2% (w/v) agar to give minimal media agar (MMA).

Transduction experiments were performed using TNA broth which was made up with the following ingredients:

Tryptone(Oxoid)	10.0g/L
NaCl	8.0g/L
Glucose	1.0g/L
$CaCl_2$ $2H_2O$ (5mM)	0.735g/L

TNA solid media and TNA soft agar were made by adding 2% (w/v) and 1% (w/v) agar respectively to the TNA broth medium. For selection of transductants, appropriate antibiotics were added to the TNA agar.

All media were sterilized at 15lb.(121°C) for 20 minutes.

2.4 Antibiotics

All antibiotics used in this study were purchased from Sigma Chemical Company. Appropriate concentrations of antibiotics were added either to sterilized broth or molten agar media which were cooled to 55°C.

2.5 Chemicals

All chemicals used were of analytical grade, purchased from BDH, Sigma Chemical Company or Fisons.

2.6 Growth Conditions

Bacterial cells were initially grown overnight in NB (pH 7.0) at 37°C in a shaking water bath. The overnight cultures were then diluted into fresh NB (pH_o 7.0) and grown exponentially to an optical density of ca 0.3. These exponential phase cells were used in

all experiments unless otherwise stated.

2.7 Measurement Of Optical Density

The optical density of cell cultures was measured using a Hilger photoelectric colorimeter. Filters used were 490nm for minimal medium cultures and 520 nm for broth cultures.

2.8 Measurement Of Viable Cells

The number of viable cells in cultures was estimated by plating samples diluted in 0.75% sodium chloride (NaCl) on a suitable surface. Colony forming units were counted after 24-36 hours incubation at 37°C. The percentage of the bacterial population that survived treatment was determined by the following expression:

$$\% \text{ Survival} = \frac{\text{number of CFU at time of sampling}}{\text{number of CFU prior to treatment}} \times 100$$

2.9 External And Internal pH

All pH values given in this study are external pH (pH_o) values unless otherwise stated.

2.10 Sensitivity To Copper(II) Sulphate

Exponential phase cultures of *E.coli* 1829, were harvested by centrifugation in an MSE Microcentaur at 3,500 rpm for 10 minutes. They were then washed once in NB pH 5.5 and resuspended in (NB) containing 0.0 μg/ml copper sulphate (pH 5.5), NB plus 14.73 μg/ml copper sulphate (pH 5.5), NB plus 29.46 μg/ml copper sulphate (pH 5.5), NB plus 58.92 μg/ml copper sulphate (pH 5.5) and NB plus 117.84 μg/ml copper sulphate (pH 5.5) to give an initial O.D of 0.03. They were then grown at 37°C for a duration of 3 hours and at intervals, the optical densities and the viability of cells in the samples were measured as described in sections **2.7** and **2.8** respectively.

2.11 Recovery Of Copper-Treated Cells

Exponential phase cultures were harvested, washed and resuspended in NB containing copper sulphate as stated in **2.10.** After growing for one hour at 37°C, they were harvested, washed, resuspended in NB pH 7.0 and grown for a further 3 hours. At intervals, samples were taken for optical density measurement as described in **2.7**.

2.12 Sensitivity To Copper(II) Chloride

Exponential cultures of *E.coli* 1829, were harvested and washed as in **2.10** and resuspended in NB plus 0.0 μg/ml $CuCl_2$ (pH 5.5), NB plus 10.06 μg/ml $CuCl_2$ (pH 5.5), and NB broth plus 80.47 μg/ml $CuCl_2$ (pH 5.5). At intervals, for a duration of 3 hours viability of the cells was measured as described in **2.8**. The $CuCl_2$ concentrations, 10.06 μg/ml $CuCl_2$ and 80.47 μg/ml $CuCl_2$ used, correspond to the copper concentrations of 14.73 μg/ml $CuSO_4$ and 117.84 μg/ml $CuSO_4$ respectively.

2.13 Sensitivity To Potassium Sulphate

Exponential cultures of *E.coli* 1829, were harvested and washed as described in section **2.10** and resuspended in NB plus 0.0 μg/ml K_2SO_4 (pH 5.5), NB plus 10.28 μg/ml K_2SO_4 (pH 5.5) and nutrient broth plus 82.24 μg/ml K_2SO_4 (pH 5.5). At intervals, for a duration of 3 hours viability of the cells was measured as described in **2.8**. The concentrations of K_2SO_4 used, i.e, 10.28 μg/ml and 82.24 μg/ml correspond to the sulphate concentrations of 14.73 μg/ml and 117.84 μg/ml $CuSO_4$ respectively.

2.14 Induction Of Tolerance To Copper(II)Sulphate

2.14.1 Pre-exposure (induction) period

Exponential cells of *E.coli* 1829 grown in NB pH 7.0 were pre-exposed to NB plus 0.0 μg/ml $CuSO_4$ (pH 5.5), NB plus 0.0 μg/ml $CuSO_4$ pH 7.0, NB plus 14.73 μg/ml $CuSO_4$ (pH 5.5) and NB plus 29.46 μg/ml $CuSO_4$ (pH 5.5) for one hour at 37°C.

2.14.2 Test of copper tolerance

The pre-exposed cultures described in **2.14.1** were harvested and washed with NB pH 5.5 and resuspended in NB (pH 5.5), NB plus 58.92 µg/ml $CuSO_4$ (pH 5.5) and NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5). At intervals, for a duration of one hour, dilutions from the challenged cultures were plated on nutrient agar for viable counts.

2.15 Tests Of Copper Tolerance In Copper(II) Chloride And Potassium Sulphate Pre-Exposed Cells

Exponential cells of *E.coli* 1829, were pre-exposed in nutrient broth plus 10.06 µg/ml $CuCl_2$ (pH 5.5) and NB plus 10.28 µg/ml K_2SO_4 pH 5.5 for one hour at 37°C. As controls, the exponential cells were also pre-exposed to NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5). The pre-exposed cells were then challenged in NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for one hour. At intervals, dilutions from the challenged samples were plated on NA for viable counts.

2.16 Determination Of Whether Copper-resistant Mutants Arose During Induction To Copper(II)Sulphate

Exponential cells were grown for a further one hour at 37°C in NB plus 0.0 µg/ml $CuSO_4$ pH 5.5 and 14.73 µg/ml $CuSO_4$ (pH 5.5). Samples from the cultures were then diluted and plated on NA and incubated for 18 to 24 hours. Ten colonies from each treatment were randomly picked and grown overnight in NB (pH 7.0) and diluted the next day into fresh NB pH 7.0 and grown exponentially to an O.D of 0.3. The cultures were harvested, washed in NB pH 5.5 and resuspended in NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) and incubated at 37°C. After 30 minutes of treatment, diluted samples from the cultures were plated on NA plates and incubated at 37°C for 18-36 hours. The percentages of viable cells were determined as described in **2.8**.

2.17 Induction To Copper Tolerance At 30°C

Exponential cells were washed and resuspended in NB plus 0.0 µg/ml $CuSO_4$(pH 5.5) and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and grown for a further hour at 30°C. The pre-exposed cells were then challenged with NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for another hour at 37°C. At intervals, dilutions of samples were plated on NA and incubated at 37°C for 18-36 hours to measure viability.

2.18 Kinetics Of Induction Of Copper Tolerance

Exponential cells were pre-exposed in NB plus 0.0 µg/ml $CuSO_4$ (pH 5.5) for 30, 45 and 60 minutes. The cells that were pre-exposed for 30 and 45 minutes were harvested washed and were further grown for 30 and 15 minutes respectively in NB plus 14.73 µg/ml $CuSO_4$ so that all the test cultures would received a total pre-exposure duration of one hour. The pre-exposed cells were then challenged with NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for further one hour. The dilutions from the challenged cultures were then plated on NA and incubated for 18-24 hours for viable counts.

2.19 Kinetics Of Tolerance Induced In Copper Habituated Cells

Exponential cells were pre-exposed in NB pH 5.5 and NB plus 14.73 µg/ml $CuSO_4$ for one hour. The cells then were harvested, washed and resuspended in NB pH 5.5 and further grown for one and two hours after which the cells were challenged to NB plus 117.84 µg/ml $CuSO_4$. At the end of the challenge, dilutions of samples were plated on NA and incubated for 18-24 hours for viable counts.

2.20 Measurement Of Protein Synthesis Using Radiolabelled Isotopes In Copper Habituated And Non-Habituated Cells

2.20.1 Incorporation of isotopes into cells

Exponential cells of *E.coli* 1829, were pre exposed with NB plus 0.0 µg/ml $CuSO_4$ (pH 5.5) and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) for one hour and were then challenged

with NB plus 0.0 µg/ml $CuSO_4$ (pH 5.5) and NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for a further one hour. The cultures were then harvested, washed and resuspended in NB pH 5.5. containing 0.5 µci/ml of ^{14}C phenylalanine and NB pH 5.5 containing 0.5 µci/ml ^{14}C uracil.

2.20.2 Preparation of samples for radioassay

At intervals, samples (0.1 ml) were removed and pipetted onto Whatman 3MM filter papers (25mm diameter). After allowing the liquid to soak in, the filters were immersed into 5% ice cold trichloroacetic acid (TCA) and were left overnight at 4°C.

After an overnight fixing in TCA, the filters were washed for 10 minutes with two changes of ice cold 5% TCA to remove any "free" radioactivity. This was followed by another wash in cold ether-ethanol (1:1) mixture for 15 minutes and two washes with cold ether for 15 minutes to remove any traces of water which would prevent wetting of filters with toluene. The papers were then air dried at room temperature and placed in scintillant vials with 10 ml scintillant (0.6% Butyl PBD in 90% sulphur free toluene and 10% methanol) and the radioactivity was then counted in an LKB 1211 Minibeta scintillation liquid counter.

2.21 Induction Of Tolerance To Copper In The Presence of Chloramphenicol

Exponential cells of *E.coli* 1829, were pre-exposed with NB plus 0.0 µg/ml $CuSO_4$ (pH 5.5), NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and NB plus 29.46 µg/ml $CuSO_4$ (pH 5.5) with and without 200µg/ml chloramphenicol for one hour. These cultures were then harvested washed and challenged with NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for a further one hour. At the end of the challenge, dilutions of culture samples were plated on NA plates for viable counts.

2.22 Cross-Tolerance Responses In Copper-Induced Cells

Exponential cells that were given an hour pre-exposure in NB plus 0.0 µg/ml $CuSO_4$ (pH 5.5) and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) were used in the following cross-response

studies:

2.22.1 Heat

Pre-exposed cells were harvested,washed and resuspended in 20 ml NB (pH 5.5) and grown shaken at 50°C. At intervals, dilutions of samples were plated on NA and incubated at 37°C for 18-36 hours.

2.22.2 Hydrogen peroxide

Pre-exposed cells were resuspended in NB pH 5.5 and NB plus 15 mM H_2O_2 (pH 5.5). At intervals, 1 ml samples of the cultures were neutralised in 9 ml of 0.75% (w/v) NaCl containing $1.5x10^3$ units/ml of catalase (BDH). Dilutions of the neutralised samples were plated on NA for viable counts.

2.22.3 Cadmium sulphate

Pre-exposed cells were resuspended in NB (pH 5.5), NB plus 1.6 mg/ml $CdSO_4$ (pH 5.5) and NB plus 2.0 mg/ml $CdSO_4$ (pH 5.5). At intervals, dilutions of the culture samples were plated on NA for viable counts.

2.22.4 Acid

Pre-exposed cells were resuspended in NB pH 3.5 and at intervals 1 ml samples were neutralised in 9 ml of NB pH 7.0 followed by dilutions in 0.75% (w/v) saline. Dilutions of the samples were plated on NA and after 18-36 hours of incubation, the plates were counted for colony forming units.

2.22.5 Alkali

Pre-exposed cells were harvested and resuspended in NB pH 9.75. At intervals 1 ml samples were neutralised in 9 ml of NB pH 7.0 followed by dilutions in 0.75% (w/v) saline. The diluted samples were plated on NA pH 7.0 and incubated at 37°C. After 18-36 hours of incubation the plates were counted for colony forming units.

2.23 Induction Of β-Galactosidase In Copper Habituated And Non-Habituated Cells After Copper Tolerance Tests.

E.coli 1829,F'Lac$^+$ cells were pre-exposed in NB pH 5.5, NB plus 14.73 μg/ml $CuSO_4$ (pH 5.5) and NB plus 29.46 μg/ml $CuSO_4$ (pH 5.5). They were then challenged with NB plus 117.84 μg/ml $CuSO_4$ (pH 5.5) for one hour.

The challenged cells were then harvested, washed and resuspended in NB pH 5.5 and the β-galactosidase inducer, IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 10^{-3} M. The cultures were incubated shaken at 37°C for 30 minutes.

2.24 Assay For β-Galactosidase Production

The IPTG induced cultures (10.0 ml) were harvested, washed and resuspended in 10 ml of 150 mM sodium phosphate (Na_2HPO_4) pH 7.0. The O.D readings of the cell suspensions were taken prior to treatment with 100 ul toluene. The toluene treated suspensions were mixed by vortexing for 10 seconds and the toluene was allowed to evaporate by placing the tubes of cell suspensions in a shaking water bath at 37°C for 15 minutes with their tops opened.

The toluene treated suspensions (1.0 ml) were added to 1.0 ml of 150 mM sodium phosphate pH 7.0 and 0.4 ml of 12 mM ONPG and these assay mixtures incubated at 37°C . The activity of the enzyme was measured spectrophotometrically by measuring the release of *o*-nitrophenol (yellow compound) from *o*-nitrophenyl-β-D-galactoside (ONPG), a colourless compound. The reaction was stopped by adding 2.0 ml of 1M Na_2CO_3 solution when sufficient yellow colour had developed. The cells were pelleted and the amount of o-nitrophenol in the supernatant was measured at 420 nm using a Cecil spectrophotometer. The β-galactosidase activity was expressed in Miller (**1972**) units using the following formula:

$$\text{Miller units} = \frac{10^3 \times O.D_{420}}{t \times v \times O.D_{500}}$$

where, $O.D_{500}$ - cell density prior to assay

$O.D_{420}$ - reaction mixture reading

t - time taken for development of yellow colour

v - volume of culture used for assay

10^3 - fully induced culture grown on lactose has approximately 1000 units and uninduced culture has one unit

2.25 PLASMID DNA ISOLATION

Two methods of plasmid DNA isolation were used. The first, was the lysis by alkali method, a modification of that of Birnboim and Doly (**1979**) developed in this laboratory The second, was also the lysis by alkali method, which is a modification of that of both Birnboim and Doly (**1979**) and Ish-Horowicz and Burke (**1981**) (**Sambrook *et.al* 1989**).).

2.25.1 Method 1

The plasmid DNA from habituated and non-habituated cells of *E.coli* 1829 ColV,I-K94 pBR322 that had been challenged with NB (pH 5.5) and NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for one hour was isolated.

The challenged cells were concentrated to a final volume of 10.0 ml with an O.D of 0.8-1.0. The cells were pelleted for 10 minutes in a bench centrifuge (MSE Centaur 2) at 4,000 rpm. The pellets were then resuspended in 500 µl lysis buffer I (Glucose, 15 mM; EDTA, 10 mM; Tris-HCl, 25 mM; lysozyme, 2 mg/ml) and this first stage of lysis was continued on ice for 30 minutes after which freshly prepared lysis buffer II (0.2 M NaOH, 1% SDS) was added and lysis was further continued for 10 minutes. The lysate was then neutralised by adding 300 µl ice-cold solution 4 M sodium acetate and maintained on ice for 40 minutes after a thorough mix by inversion. The precipitate in the mixture was pelleted and the supernatant was decanted into a fresh tube. A 1.0 ml 50% (w/v) PEG was then added, to precipitate the DNA in the supernatant. The DNA precipitation was performed overnight at 4°C, pelleted the next day and resuspended in a mixture of TE (1 ml) (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)), 5 M NaCl (1 ml) and ethanol (2 ml) to get rid of traces of PEG. This mixture was then pelleted for 15 minutes and the final DNA pellet was then resuspend in 50 µl TE containing 20 µg/ml Dnase-free RNase and

stored at -20°C until use.

2.25.2 Method 2

The plasmid DNA from habituated and non-habituated cells of *E.coli* 1829 ColV,I-K94 pBR322 that had been challenged with NB (pH 5.5) and NB plus 117.84 µg/ml $CuSO_4$ (pH 5.5) for one hour was isolated.

The challenged cells were concentrated to a final volume of 1.5 ml with an O.D of 0.8-1.0. The cells were pelleted in an Eppendorf tube by centrifugation (MSE Microcentaur) for 10 minutes at 12,000 rpm. The cell pellet was washed once with STET buffer (Tris-HCl, 10 mM pH 8.0; NaCl 100 mM; EDTA 1 mM pH 8.0) and then resuspended in 100 µl ice-cold solution I (50 mM glucose, 25 mM Tri-HCl (pH 8.0), 10 mM EDTA (pH 8.0). The cells were then lysed by adding 200 µl of freshly prepared solution II (0.2N NaOH, 1% SDS). Lysis was performed on ice for 5 minutes after thorough mixing by gentle inversions. The lysate was neutralised by adding 150 µl ice-cold solution III (potassium acetate, 3 M) and maintained on ice for 10 minutes after a thorough mix by inversion. The precipitate in the mixture was pelleted and the supernatant was decanted into a fresh tube. An equal volume of phenol-chloroform was added to the supernatant and was emulsified by vortexing followed by 2 minutes of centrifugation to separate the phases. The upper aqueous phase was transferred into a fresh tube and DNA was precipitated by adding 2 volumes of ethanol. The mixture was allowed to stand at room temperature for 2 minutes. The DNA was pelleted by centrifugation at 12,000 rpm for 5 minutes. The supernatant was removed and the DNA pellet was rinsed with 1 ml 70% ethanol. The DNA pellet was then dried and was dissolved in 50 µl TE pH 8.0 (Tris-HCl, 10 mM pH 8.0; EDTA, 1 mM pH 8.0) containing 20 µg/ml DNase-free pancreatic RNase and stored at -20°C until use.

2.26 Agarose Gel Electrophoresis

All agarose gel electrophoresis was performed on a horizontal gel apparatus. Plasmid DNA samples isolated in **2.25** were mixed with sample buffer (bromophenol blue, 0.25%; xylene cyanol, 0.25%, ficoll type 400, 15%;in H_2O) and were loaded on 0.7%

agarose gel slabs and run at 5V/cm for 1.5-2 hours in TBE buffer (Tris -HCl, 89mM; boric acid, 89mM; EDTA, 2.5 mM) pH 8.0. The gels were stained with ethidium bromide (0.5 µg/ml), examined on a UV light transilluminator (UV Products Inc.) and photographed onto Polaroid 665 film or onto thermal prints (Seikosha VP-15052 Type H).

2.27 Analysis OF Cell Envelope Proteins In Habituated and Non-Habituated Cells

2.27.1 Isolation and separation of cell envelope

Cell envelope proteins were isolated and separated according to the method of Rossouw and Rowbury (**1984**) with modifications (see below).

Copper habituated cells and exponential cells grown for one hour in NB pH 7.0 and NB pH 5.5. were concentrated in 10 ml.volumes to give an O.D of 0.6-0.8. The cells were then pelleted for 15 minutes at 3,500 rpm in a bench centrifuge (MSE Centaur 2). They were washed once in 5 ml of 0.9% saline and then resuspended in 1.4 ml of Tris-HCL, 50 mM pH 8.5, EDTA, 2 mM. The suspensions were frozen at -70°C for 15 minutes and thawed at room temperature. The thawed suspensions were lysed by sonicating for 15x10 seconds bursts at 1.5 A in a MSE Sonic Oscillator. The sonicated samples were spun for 2 minutes at 12,000 rpm in a MSE Microcentaur to pellet unbroken cells and large pieces of debris. Aliquots of the supernatants containing the whole cell envelopes were stored at -20°C until analysed.

The remaining supernatants were transferred to a fresh tube and respun at 12,000 rpm for 15 minutes. The pellet containing outer membrane materials was resuspended in 20 µl 2 mM Tris. The supernatant was transferred to a fresh tube and the solubilized cytoplasmic membranes were precipitated with 2 volumes of absolute ethanol and allowed to stand overnight at -20°C. Cytoplasmic membranes were collected by centrifuging at 3,500 rpm for 15 minutes in a MSE Centaur 2 bench centrifuge. The resulting pellet was resuspended in 1 ml of 2 mM Tris.

2.28 Analysis Of Proteins By SDS-Polyacrylamide Gel Electrophoresis

The modified Laemmli (**1970**) gel system, was used for comparative analysis of

polypeptides from whole cell envelopes and the separated cell envelopes. Vertical slabs of 10.5% polyacrylamide gel were cast between two glass plates. The separating gel was a mixture of 14 ml of acrylamide stock solution (acrylamide,30%; bis-acrylamide,0.8%), 20 ml of 0.75M Tris pH 8.8, 0.4 ml 10% SDS, 3.6 ml H_2O, 2 ml of 10 mg/ml ammonium persulphate and 10 µl TEMED. The stacking gel contained 3 ml of acrylamide stock, 10 ml of 0.25M Tris pH 6.8, 0.2 ml of 10% SDS, 5.8 ml of H_2O, 1ml of 10 mg/ml ammonium persulphate and 5 µl TEMED.

Samples were denatured by mixing 10 µl aliquots with 40 µl of sample buffer (Tris-HCl, 100 mM pH 6.8; SDS 3%; glycerol, 16%; 2-mercaptoethanol, 8%; bromophenol blue, 0.0016%) and boiled at 100°C for 5 minutes. Samples containing a mixture of known molecular weight standards (Dalton Mark VII-L™) were also prepared in sample buffer. The denatured samples (40 ul) were loaded onto the gels and electrophoresed in SDS-glycine (glycine, 200 mM; Tris-HCl, 25 mM pH 8.8; SDS 0.1%) buffer at 30 mA constant current at room temperature. Electrophoresis was stopped when the dye front was 1 cm away from the edge of the separating gel.

The gels were stained overnight in solution containing 0.1% Coomassie Brilliant Blue R250, 10% acetic acid, 50% methanol and destained in many changes of 42% methanol-10% acetic acid destaining solution.

2.29 Isolation Of Outer Membrane Protein Mutants

The utilization of outer membrane proteins as receptors by viruses has been exploited to select the mutants. The OmpC, OmpA and LamB can act as receptors for the phages MeI, K3 and λgti respectively. Overnight cultures of *E. coli* 1829 (0.1 ml) were mixed with 0.1 ml suspension of the above phages in 5 ml of soft agar. The bacterial-phage mixture in the soft agar was then overlaid onto NA and left to solidify before incubating at 37°C for 18-24 hours. Bacterial colonies which formed on clear areas of the plates (i.e where the lawn had been cleared by the phage growth) were picked and purified several times. The 1829-phage resistant strains were tested for resistance to the respective phages and their protein compositions were analysed on SDS-PAGE as described in **2.28** to confirm the resistance.

2.30 Isolation Of *E.coli* 1829 Strains Carrying Known Deletions By Phage P1vir Transduction The *himA*, *himD*, *fur*, *nhaA*, *hns* and *micF* mutations were introduced into *E.coli* 1829 by P1vir transduction using the associated antibiotic resistance genes as the selective marker.

2.30.1 Preparation of P1 lysate.

The transducing lysates were produced by infecting exponential cultures of *E.coli* HN678-Tet^R(*himA*), *E.coli* HN1069-Cap^R(*himD*), *E.coli* W3110-Kan^R(*fur*), *E.coli* NM81-Kan^R(*nhaA*), *E.coli* PD32-Ap^R(*hns*) and *E.coli* SM3001-Kan^R (*micF*) grown in TNA broth at 37°C with 1x10^8 phage P1vir/ml. The mixtures were incubated for 10 minutes at 37°C for phage adsorption. This was followed by a ten-fold dilution in 10 ml TNA broth to dilute out unadsorbed phages. The diluted mixtures were then incubated shaken at 37°C for 2-3 hours or until complete lysis occured. The cell debris and uninfected cells in the lysate were then pelleted at 3,500 rpm for 10 minutes in a MSE Centaur2 bench centrifuge. The supernatants were carefully removed and then chloroform-treated (by shaking with 0.1 ml chloroform per 5 ml supernatant). The titres of the lysates were then determined using the agar overlay technique for plaque assay. The lysates were kept at 4°C until used.

2.30.2 Transduction experiments

Exponential cultures of *E.coli* 1829, in TNA broth were harvested and resuspended in 5 ml of diluent buffer (NaCl, 8 g/l; $CaCl_2$ $2H_2O$, 0.1 mM; TNA broth, 4 ml). Aliquots of 0.5 ml and 1.0 ml of phage lysates were added to 1.0 ml aliquots of bacterial suspensions and incubated for 2 hours static at 37°C. The cells were then pelleted for 10 minutes at 12,000 rpm in a microcentrifuge and resuspended in 5 ml TNA broth and grown shaken for one hour to allow the expression of the newly transferred genes. The bacterial suspensions were then pelleted and resuspended in 0.1 ml of dilution buffer and plated on MMA plus tryptophan containing the appropriate selective antibiotics (Amp-50 µg/ml, Cap-30 µg/ml, Kan-30 µg/ml, Tet-15 µg/ml). Appropriate controls were set up to test the presence of bacterial contaminants in the lysates and the resistance of the recipient strain to the selective antibiotics.

Transductants formed on the selective plates after 24-48 hours incubation at 37°C were picked and purified onto MMA and the appropriate MMA-antibiotic selective plates and incubated for 24-48 hours at 37°C. The transductants that successfully grew on these plates were used in subsequent experiments. Transductants that were resistant to kanamycin however, were restreaked onto non-selective plates (MMA) and then restreaked to the appropriate selective plates again to check for the stability of antibiotic resistance as kanamycin resistant strains can sometimes be unstable.

2.31 Induction Of Alkali Sensitivity At pH 5.5

Exponential cells of *E.coli* 1829 grown at pH 7.0,were harvested and resuspended in NB pH 7.0 and NB pH 5.5. Cells resuspended in NB pH 5.5 were washed once in NB pH 5.5 before the resuspension. The cells were grown shaken for one hour at 37°C.

2.32 Tests For Sensitivity To Alkali

Cells induced at pH 7.0 and pH 5.5 for one hour, were harvested and resuspended in NB alkalinized by 1N NaOH to pH 9.5 or pH 9.75 and grown shaken for 30 minutes at 37°C. At the end of the treatment, dilutions of the alkali challenged cells were plated on NA and incubated for 18-24 hours at 37°C.

2.33 Tests For Alkali Sensitivity In Nutrient Broth Alkalinized By Potassium Hydroxide

Cells induced at pH 7.0 and pH 5.5 for one hour, were harvested and and resuspended in NB alkalinized by 1N KOH to pH 9.5 and grown shaken for 30 minutes at 37°C. At the end of the treatment, dilutions of the alkali challenged cells were plated on NA and incubated for 18-24 hours at 37°C.

2.34 Induction Of Alkali Sensitivity At pH 5.5 In The Presence Of Putative Inhibitors

2.34.1 Protein synthesis inhibitors.

Exponential cells of *E.coli* 1829 grown at pH 7.0,were harvested and resuspended in NB pH 7.0 and NB pH 5.5. containing tetracycline (15μg/ml) and chloramphenicol (30 ug/ml). Cells resuspended in NB pH 5.5 were washed once in NB pH 5.5 before the resuspension. The cells were grown shaken for one hour at 37°C. The induced cells were then challenged to NB pH 9.5 for 30 minutes. Dilutions of the challenged samples were plated on NA for viable counts.

Unless otherwise stated, nutrient broths used at alkaline pH in subsequent experiments were alkalinized by 1N NaOH.

2.34.2 RNA polymerase or DNA gyrase inhibitors

Exponential cells of *E.coli* 1829 were induced for one hour at pH 7.0 and pH 5.5 in the presence of each of the followingantibiotics: rifampicin (30 μg/ml), novobiocin (2.5 μg/ml; 5.0 μg/ml), nalidixic acid (10 μg/ml; 20 μg/ml), coumermycin A1 (30 μg/ml). Sensitivity to alkali was tested as described in **2.32**.

2.34.3 Sodium ion pore inhibitor

Amiloride was added to a final concentration of 1 mM during the induction of *E.coli* 1829 in NB pH 7.0 and NB pH 5.5. After one hour of induction, sensitivity to alkali was tested as described in **2.32**.

2.34.4 Catabolite repressor

Glucose was added to NB 7.0 and NB pH 5.5 to a final concentration of 1% during the induction of *E.coli* 1829 in NB pH 7.0 and NB pH 5.5. After one hour of induction sensitivity to alkali was tested as described in **2.32**.

2.35 Induction Of Alkali Sensitivity In The Presence Of Other Agents

2.35.1 Phosphate

Exponential cells of *E.coli* 1829 were induced at pH 7.0 and pH 5.5 in presence of 10 mM phosphate (0.15 ml of 1.3M potassium phosphate buffer was added to 20 ml of the above media). After one hour of induction the cells were tested for sensitivity to alkali (**2.32**).

2.35.2 Ferric chloride

Exponential cells of *E.coli* 1829 were induced at pH 7.0 and pH 5.5 in the presence of 1mM ferric chloride ($FeCl_3$). After one hour of induction the cells were tested for sensitivity to alkali (**2.32**).

2.35.3 Ferrous sulphate

Exponential cells of *E.coli* 1829 were induced at pH 7.0 and pH 5.5 in the presence of 1mM ferrous sulphate ($FeSO_4$). After one hour of induction the cells were tested for sensitivity to alkali (**2.32**).

2.35.3 Sodium chloride

Exponential cells of *E.coli* 1829 were induced at pH 7.0 and pH 5.5 in the presence of 200 mM sodium chloride (NaCl). After one hour of induction the cells were tested for sensitivity to alkali (**2.32**).

2.35.4 L-Leucine

Exponential cells of *E.coli* 1829 were induced at pH 7.0 and pH 5.5 in the presence of L-leucine (50 µg/ml and 150 µg/ml). After one hour of induction the cells were tested for sensitivity to alkali (**2.32**).

2.35.5 Acetate and Benzoate

Exponential cells of *E.coli* 1829 were induced at pH 7.0 and pH 5.5 in presence of either sodium acetate (15 mM and 30 mM) or benzoic acid (10 mM). After one hour of

induction the cells were tested for sensitivity to alkali (**2.32**).

2.36 Induction Of Alkali Sensitivity In *E.coli* Strains Carrying Known Regulatory Lesions

Exponential *E.coli* cells of CF1648 *relA*$^+$, CF1652 Δ*relA*, 6482 *rpoS*$^+$, 7157 Δ*rpoS*, W3110-2 *fur*$^+$, W3110-1 Δ*fur*$^-$, AB2847 *tonB*$^+$, H2300 Δ*tonB*, TA15 *nhaA*$^+$*nhaB*$^+$, NM81 Δ*nhaA*, EP431 Δ*nhaB*, 1157-4 *phoE*$^+$, 1157 Δ*phoE*, N99 *himA*$^+$;*himD*$^+$, HN678 *himD*Δ*himA*, HN1096 *himA* ΔhimD, CV975 *lrp*$^+$, CV1008 Δ*lrp*, JA199 *cysB*$^+$, NK1 Δ*cysB*, MC4100 pALS10 *envZ*$^+$, AT142 pALS10 Δ*envZ*, 1829, 1829 Δ*himA*, 1829 Δ*himD*, 1829 Δ*hns*, 1829 Δ*nhaA*, 1829 Δ*fur*, 1829 *ompA*, 1829 *ompC*, 1829 *lamB* were induced in NB pH 7.0 and pH 5.5 for one hour. The induced cells were then tested for alkali sensitivity as described in **2.31**.

2.37 Induction Of β-Galactosidase In Alkali-Treated Cells

Exponential cells of *E.coli* 1829 F'*lac*$^+$, were grown for one hour in NB pH 7.0 and NB pH 5.5 at 37°C. The cells were then challenged with NB pH 9.5 for 30 minutes. The challenged cells were then harvested, washed and resuspended in NB pH 5.5 and IPTG was added to a final concentration of 10^{-3} M. The cultures were incubated shaken at 37°C for 30 minutes. β-Galactosidase activity was then determined as described in **2.24**.

2.38 Comparative Analysis Of Plasmid DNA From Alkali-Treated Cells

The plasmid DNA from pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 ColV,I-K94 pBR322 that had been challenged with NB pH 7.0 and NB pH 9.5 for one hour was isolated as described in **2.25.** The plasmid DNA extracts were analysed by agarose gel electrophoresis (**2.26**).

2.39 Comparative Analysis Of Cell Envelope proteins In pH 7.0 and 5.5 Induced Cells

The cell envelopes of *E.coli* 1829 cells grown in NB pH 7.0 and NB pH 5.5 were isolated and separated as described in **2.27.1.** The proteins from the isolated cell envelopes and the separated components were analysed on SDS-PAGE as described in **2.28.**

CHAPTER 3

RESULTS AND DISCUSSION

3.1 INDUCTION OF TOLERANCE TO COPPER (HABITUATION TO COPPER) IN *E.coli* 1829

Copper-resistant microbes have been described in several bacterial species from various sources (**Rouch *et al.*, 1985; Cha and Cooksey, 1991; Dressler *et al*, 1991; Williams *et al.*,1993; Yang *et al.*,1993**) The resistances to copper described, are sometimes constitutive and sometimes there has been a selection of mutants. The majority of copper-resistant isolates however, carry an inducible ability to resist copper determined by plasmids (**Rouch *et al.*, 1989a; Trevors 1987; Brown *et al.*,1992**).

The primary aim of this section of the study, was to determine whether tolerance to lethal doses of copper could be induced in bacteria following a period of pre-exposure to sublethal concentrations (adaptive doses) of this stress agent. The induction of tolerance to copper here, was carried out in *E.coli* 1829, a strain that is plasmidless. Sublethal and lethal doses of copper(II) sulphate ($CuSO_4$) were first determined before the induction to copper tolerance in *E.coli* 1829 was tested. The Cu^{2+} being the inhibitory component of the $CuSO_4$, used as the source of copper in this study was also confirmed. This study also investigated the kinetics of copper habituation and effect of protein synthesis inhibitors on copper habituation. A comparative study on the ability of copper-induced and non-induced cells to synthesize proteins and RNA after the challenge was also carried out. Effect of copper challenge on DNA in copper-habituated and non-habituated cells was also examined. Preliminary examination of protein samples from the outer membrane and cytoplasmic membrane was also carried out.

3.1.1 Sensitivity of *E.coli* 1829, to copper(II) sulphate.

Figure 3-1 and **Table 3-1** show the results of the effect of copper(II) sulphate ($CuSO_4$) in *E.coli* 1829. Four concentrations of $CuSO_4$ (each representing a 2-fold increase over the previous) were used. The appropriate $CuSO_4$ concentrations were added to NB (nutrient broth) and adjusted to pH of 5.5. This pH was chosen because $CuSO_4$ in solution is naturally mildly acidic and at neutral pH forms a precipitate. Moreover the pHs of many

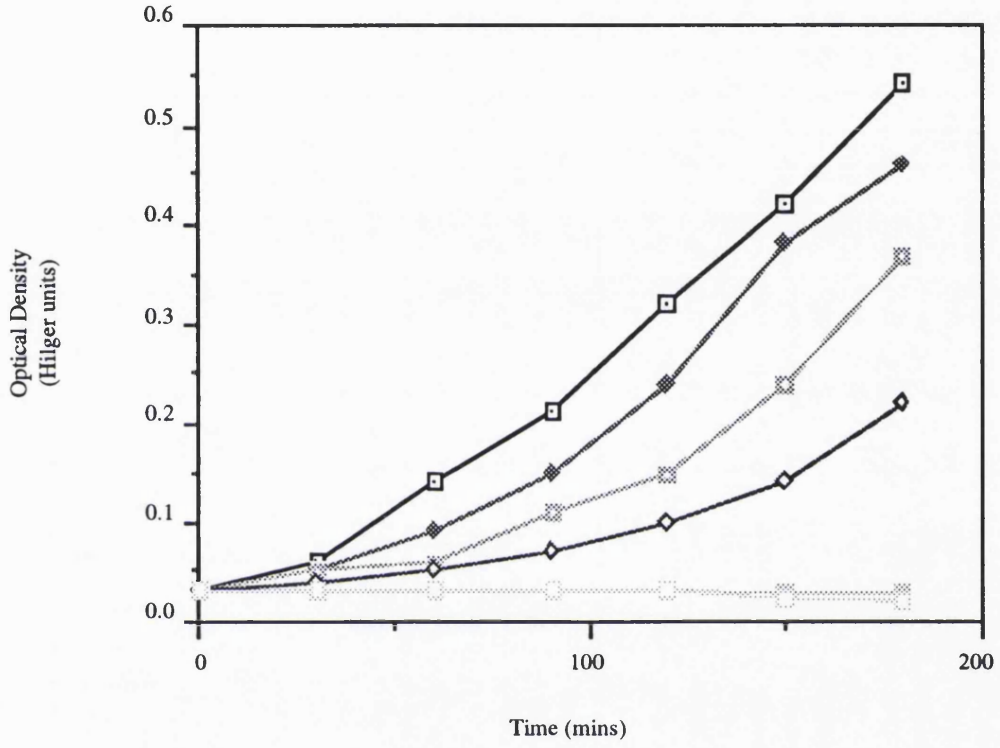


Figure 3-1 Effect of copper(II) sulphate on growth in *E. coli* 1829. Exponential phase cells of *E. coli* 1829, were incubated shaken at 37°C in NB pH 7.0 (——▫——), NB pH 5.5 (.....◆.....), NB plus 14.73 µg/ml copper(II) sulphate, pH 5.5 (.....▩.....), NB plus 29.46 µg/ml copper(II) sulphate pH 5.5 (——◇——), NB plus 58.92 µg/ml copper(II) sulphate, pH 5.5 (.....▩.....) and NB plus 117.84 µg/ml copper(II) sulphate, pH 5.5 (.....▫.....). At intervals, optical densities were measured from samples of cultures. Results shown are of a single experiment. Experiments were repeated three times with consistent results.

Table 3-1 Effect of copper(II) sulphate on viability of *E.coli* 1829.

Concentration of copper(II) sulphate (μg/ml) in NB pH 5.5	Percentage of colony forming units (mean±S.E.M) after treatment with copper(II) sulphate for (mins):			
	30	60	120	180
0.0 (pH 7.0)	225.0 ±10.50	480.0 ±12.20	1820.0 ±60.50	2520.0 ±150.80
0.0	183.60 ±12.60	313.80 ±15.30	1450.80 ±88.80	2044.50 ±212.10
14.73	136.40 ±4.30	222.20 ±10.20	899.90 ±44.20	1616.80 ±154.90
29.46	117.50 ±6.0	176.40 ±8.90	466.30 ±8.20	795.00 ±13.60
58.92	0.08 ±0.04	0.01 ±0.006	0.01 ±0.005	0.0
117.84	0.03 ±0.02	0.01 ±0.002	0.0	0.0

Exponential phase cells of *E.coli* 1829, were incubated shaken at 37°C in NB pH 5.5 without copper and NB plus the stated cupric sulphate concentration at pH 5.5. At intervals culture dilutions were plated on NA and incubated for 18-24 hours at 37°C. The survivals of the cells were expressed as a percentage of CFU/ml prior to treatment. Results shown are mean values of three experiments.

natural waters fall between pHs 5.0 and 6.0.

E.coli cells grew well at pH 5.5. Their turbidity increased by 3-fold after 60 minutes, 8-fold after 120 minutes and 15.7-fold after 180 minutes whilst for those grown at pH 7.0 the corresponding increases in turbidity were 4.7-fold, 10.6-fold and 18-fold (**Figure 3-1**). The growth at pH 5.5 is 1-1.5 times slower than that at pH 7.0. The mean percentage of colony forming units at pH 5.5 increases by 1.8-fold after 30 minutes, 3.1-fold after 60 minutes, 14.5-fold after 120 minutes and 20.4 fold after 180 minutes whilst at pH 7.0 the counts increased by 2.25-fold, 4.8-fold, 18.2-fold and 25.22-fold after 30, 60, 120 and 180 minutes respectively (**Table 3-1**). The mean percentage of colony forming units at pH 5.5 is 1.2-1.5 times lower than that at pH 7.0.

The presence of 14.73 μg/ml $CuSO_4$ has a slight effect on the growth; optical density increased by only 1.7-fold after 60 minutes, 5-fold after 120 minutes and 12.3-fold after 180 minutes (**Figure 3-1**). This is 1.2 to 1.8 times slower than growth in NB pH 5.5 without copper. The viable counts expressed as a mean percentage survival of colony forming units, increased by 2.2-fold after 60 minutes, 8.9-fold after 120 minutes and 16.2-fold after 180 minutes which is 1.3 to 1.6 times lower than counts in NB pH 5.5 without copper.

Cultures grown in NB containing 29.46 μg/ml $CuSO_4$ show more effect on growth than those at 14.73 μg/ml $CuSO_4$ although optical density increased by 1.6-fold after 60 minutes, 3.3-fold after 120 minutes and 7.3-fold after 180 minutes. The growth is 1.9 to 2.4 times slower than that in NB pH 5.5 and 1.2 to 1.7 times slower than that at 14.73 μg/ml $CuSO_4$. Increases in the mean percentage survival were still observed at this concentration; 1.2-fold after 30 minutes, 1.8-fold after 60 minutes 4.7-fold after 120 minutes and 7.9-fold after 180 minutes. These increases are 1.5 to 3 times lower than that in pH 5.5 and 1.2 to 2.1 times lower than that in NB plus 14.73 μg/ml $CuSO_4$.

In contrast, the presence of 58.92 μg/ml and 117.84 μg/ml $CuSO_4$ had strong lethal and inhibitory effects on the *E.coli* cells. Optical density remained the same in the first 120 minutes before a reduction was observed (**Figure 3-1**). The viability was greatly reduced in the first 30 minutes and after 60 and 180 minutes in the presence of 58.92 μg/ml $CuSO_4$ an average of only 1 in 10^5 and 1 in 10^6 cells survived respectively (**Table 3-1**). Likewise, in the presence of 117.84 μg/ml $CuSO_4$ an average of 1 in $1.4x10^5$ cells

survived after 60 minutes and none survived after 180 minutes.

In a separate experiment, (**Table 3-2**) cells treated in NB plus 117.84 μg/ml $CuSO_4$ for one hour, failed to recover even after being transferred to NB pH 7.0 for three hours. In contrast, cells treated in NB plus 14.73 μg/ml and NB plus 29.46 μg/ml $CuSO_4$ for one hour recovered well at pH 7.0 (optical density increased by 15.3- and 13.3 -fold respectively after three hours in NB pH 7.0).

Based on the results above, the $CuSO_4$ concentrations of 14.73 μg/ml and 29.46 μg/ml were chosen as the sublethal concentrations for pre-exposure since they have a slight effect on growth without being markedly inhibitory and the $CuSO_4$ concentrations 58.92 μg/ml and 117.84 μg/ml were chosen as the challenge concentrations since they are lethal to the cells.

Copper is toxic in the form Cu^{2+}. To ascertain that it is the Cu^{2+} and not the SO_4^{2-} that is exerting the inhibitory effect, a comparison study on the sensitivity of *E.coli* to $CuSO_4$ and that to copper(II) chloride and potassium sulphate was carried out.

3.1.2 Comparison of sensitivity of *E.coli* 1829, to copper(II) sulphate, copper(II) chloride and potassium sulphate

The concentrations of Cu^{2+} in 10.06 μg/ml and 80.47 μg/ml copper(II) chloride ($CuCl_2$) (**Table 3-3**) are equivalent to those present in 14.73 μg/ml and 117.84 μg/ml of $CuSO_4$ respectively. Similarly, the concentrations of SO_4^{2-} in 10.28 μg/ml and 82.24 μg/ml potassium sulphate (K_2SO_4) are equivalent to those present in 14.73 μg/ml and 117.84 μg/ml $CuSO_4$.

Table 3-3 shows the mean percentage survival of *E.coli* 1829, after growing in above mentioned concentrations of $CuSO_4$, $CuCl_2$ and K_2SO_4 at intervals of time for a duration of 3 hours. After 60 minutes in 14.73 μg/ml $CuSO_4$ there is a 2.4 fold increase in counts compare to the 5.1 fold increase in 10.06 μg/ml $CuCl_2$ and 5.8-fold increase in 10.28 μg/ml K_2SO_4. The counts in 14.73 μg/ml $CuSO_4$ are 2.1 to 2.4 times lower than those in 10.06 μg/ml $CuCl_2$ and 10.28 μg/ml K_2SO_4. At the end of 180 minutes however, there is a 20.6-fold increase in viable counts in 14.73 μg/ml $CuSO_4$, 23.1-fold increase in 10.06 μg/ml $CuCl_2$ and 27-fold increase in 10.28 μg/ml K_2SO_4 and statistically there is no significant differences in these increases. Thus at the end of 3 hours *E.coli* seemed to

Table 3-2 The recovery of copper-treated and non-treated *E.coli* 1829 cells in NB pH 7.0

Concentration used for $CuSO_4$ treatment (µg/ml)	Optical density readings (Hilger units) during recovery in NB pH 7.0 for (mins):				
	30	60	120	150	180
0.0 (pH 7.0)	0.06	0.15	0.34	0.42	0.53
0.0 (pH 5.5)	0.05	0.13	0.30	0.41	0.51
14.73	0.04	0.09	0.23	0.36	0.46
29.46	0.04	0.07	0.18	0.27	0.40
117.84	0.028	0.025	0.02	0.02	0.02

E.coli cells were grown to exponential phase in NB pH 7.0 at 37°C and, after being treated for one hour in NB plus the the stated copper concentrations at pH 5.5, were harvested and resuspended in NB pH 7.0 to an initial O.D of 0.03. At the stated intervals, optical densities of the samples were measured. The results shown above are for a single experiment.

Table 3-3 Comparison of sensitivity to copper(II)sulphate, copper(II)chloride and potassium sulphate in *E.coli* 1829.

Treatment	Concentration (µg/ml)	Percentage of colony forming units (mean±S.E.M) after treatment for (mins):			
		30	60	120	180
NB pH 5.5	0.0	197.90 ±6.86	648.70 ±47.50	1282.90 ±47.00	2679.65 ±294.80
NB pH 5.5 plus copper(II) sulphate	a) 14.73	149.50 ±44.90	243.20 ±14.40	888.70 ±77.80	2059.80 ±115.30
	b) 117.84	0.01 ±0.001	0.01 ±0.002	0.00	0.00
NB pH 5.5 plus copper(II) chloride	a) 10.06	266.25 ±66.30	515.70 ±35.20	950.50 ±80.50	2315.80 ±410.30
	b) 80.47	0.105 ±0.06	0.1 ±0.005	0.09 ±0.02	0.09 ±0.03
NB pH 5.5 plus potassium sulphate	a) 10.28	233.10 ±53.80	588.70 ±64.30	1526.50 ±218.60	2679.65 ±242.60
	b) 82.24	227.40 ±23.30	534.20 ±50.20	1677.50 ±42.90	2570.20 ±279.20

Exponential phase cells of *E.coli* were incubated shaken at 37°C in NB pH 5.5 and NB containing the stated copper salts and potassium sulphate at pH 5.5. At intervals dilutions of cultures were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml prior to treatment. Results shown are mean values of three experiments.

be growing equally well in all three treatments.

The presence of 117.84 μg/ml $CuSO_4$ and 80.47 μg/ml $CuCl_2$ was lethal to the cells; the number of surviving cells was markedly reduced in the first 30 minutes. On the contrary, the cells survived very well in presence of 82.24 μg/ml K_2SO_4; after 30 minutes in this treatment, the survival increased by 2.27-fold and at the end of 180 minutes, there was an increase of 25.7-fold compared to a 10^5-fold and $1.06x10^3$-fold decrease after treatment with 117.84 μg/ml $CuSO_4$ and 80.47 μg/ml $CuCl_2$ respectively. Since Cl^- and K^+ do not inhibit bacterial growth, the results showed that Cu^{2+} is the inhibitory component of $CuSO_4$. Of the copper salts, $CuSO_4$ is slightly more inhibitory than $CuCl_2$. This could be due to its chemical configuration.

Having established that Cu^{2+} is the inhibitory component of $CuSO_4$ and having chosen the concentrations of $CuSO_4$ for pre-exposure and for challenge, the tolerance to the lethal doses of copper in *E.coli* after a pre-exposure period in the chosen sublethal concentrations $CuSO_4$ was determined.

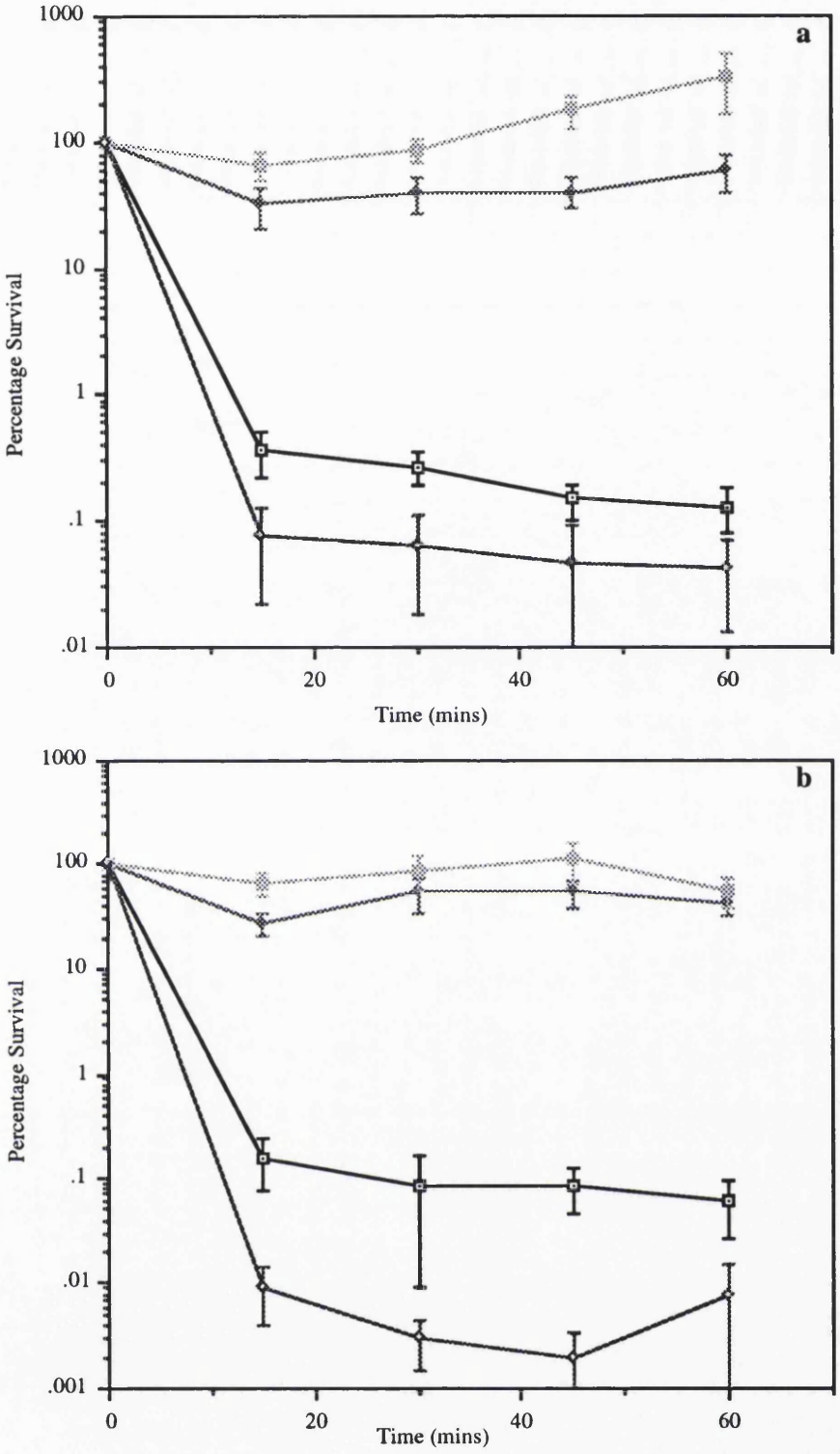
3.1.3 Induced tolerance to copper in *E.coli* 1829.

E.coli 1829 cells were pre-exposed to 14.73 μg/ml and 29.46 μg/ml $CuSO_4$ for a duration of one hour and subsequently challenged to 58.92 μg/ml and 117.84 μg/ml $CuSO_4$ for a further hour. The results shown in **Table 3-4** and illustrated in **Figures 3-2(a)** and **(b)** are means of 10 experiments. *E.coli* cells that have been pre-exposed to either NB pH 7.0 or NB pH 5.5 without copper showed no tolerance to lethal doses of copper (**Table 3-4, Figure 3-2a and 3-2b**). In contrast, the cells that have been pre-exposed to sublethal concentrations of copper showed significantly greater (99% confidence) tolerance to the lethal doses of copper; for cells that have been pre-exposed to 14.73 μg/ml and 29.46 μg/ml $CuSO_4$, a mean of 58.85 and 335.10 % respectively were able to form colonies after a challenge for one hour with 58.92 μg/ml $CuSO_4$ and a mean of 41.32 and 55.83 % respectively survived after a challenge with 117.84 μg/ml $CuSO_4$. The cells pre-exposed to 29.46 μg/ml $CuSO_4$ show more tolerance (99% confidence) than those pre-exposed with 14.73 μg/ml $CuSO_4$ after a challenge with 58.92 μg/ml $CuSO_4$. However, the difference in tolerance in these pre-exposed cells is not significant when challenged with 117.84 μg/ml $CuSO_4$. Thus it cannot be generalised that the degree of tolerance is dependent on

Table 3-4 Induced tolerance to copper in *E.coli* 1829

Pre-exposure conditions of	Concentration copper(II) sulphate used for challenge (µg/ml)	Percentage of colony forming units (mean±S.E,M) after challenge with Cu^{2+} for (mins):			
		15	30	45	60
NB pH 7.0 No copper(II) sulphate	58.92	0.08 ±0.05	0.07 ±0.05	0.05 ±0.04	0.04 ±0.03
	117.84	0.01 ±0.005	0.00	0.00	0.01 ±0.007
NB pH 5.5 No copper(II) sulphate	58.92	0.37 ±0.15	0.37 ±0.08	0.15 ±0.05	0.13 ±0.05
	117.84	0.16 ±0.08	0.09 ±0.07	0.09 ±0.04	0.06 ±0.03
NB plus 14.73 µg/ml copper (II) sulphate (pH 5.5)	58.92	32.19 ±11.69	39.75 ±12.98	40.20 ±10.78	58.85 ±19.26
	117.84	27.24 ±6.59	54.49 ±19.74	54.35 ±16.71	41.32 ±9.20
NB plus 29.46 µg/ml copper (II) sulphate (pH 5.5)	58.92	64.72 ±16.24	85.91 ±16.47	176.70 ±52.83	335.10 ±169.52
	117.84	166.99 ±17.42	88.57 ±32.26	115.14 ±17.90	55.83 ±17.87

E.coli cells were grown to exponential phase in NB pH 7.0 and, after being pre-exposed for one hour under the stated conditions with or without cupric sulphate, they were challenged with 58.92 or 117.84 µg/ml copper(II) sulphate in NB pH 5.5 for the stated times. Culture dilutions were plated on NA and incubated at 37°C for 24 hours. Results shown are mean values of 10 experiments expressed as a percentage of CFU/ml at time zero (i.e after pre-exposure but before challenge).



Figures 3-2a,b Increase in copper resistance after growth with sublethal concentrations of copper(II) sulphate in *E. coli* 1829. *E. coli* cells were grown to exponential phase in NB pH 7.0 and, after pre-exposure for one hour in NB pH 7.0 (—◇—), NB pH 5.5 (—□—), NB plus 14.73 µg/ml copper(II) sulphate, pH 5.5 (···◇···), NB plus 29.46 µg/ml copper(II) sulphate pH 5.5 (·····), were challenged with either (**a**) NB plus 58.92 µg/ml copper(II) sulphate, pH 5.5 or (**b**) NB plus 117.84 µg/ml copper(II) sulphate, pH 5.5 . At intervals, dilutions of samples were plated on NA and incubated at 37°C for 18-24 hours. The survival values of cells were expressed as a percentage of CFU/ml just prior to challenge. Results shown are mean values of 10 experiments.

the concentration of copper used for pre-exposures.

The results above indicated that cells that were pre-exposed to the low levels of copper gained ability to resist lethal levels of copper. This ability is probably a phenotypic effect i.e an effect that does not involve any genetical change. On the other hand, the observed results could be due to copper-resistant mutants that might be present in broth cultures of the strain 1829 being selected during the pre-exposure period. However, this was not so, as far less than 1% of resistant cells were present in both cultures that have been pre-exposed to pH 7.0 or pH 5.5 since nearly all of the original cells were killed by the challenge doses of $CuSO_4$. Even if these few mutants were to multiply 8-fold in the presence of the sublethal concentrations of $CuSO_4$, the ratio of their numbers to that of the normal cells would be very low as both 14.73 μg/ml and 29.46 μg/ml $CuSO_4$ allowed substantial growth and moreover the tolerance shown was considerably higher than if it is just solely due to the selected mutants. To ascertain this experimentally, besides basing on the observed results, 10 colonies from each pre-exposure with NB pH 5.5 without copper and NB plus 14.73 μg/ml copper (pH 5.5), were randomly picked from culture dilutions that had been plated on NA and incubated at 37°C. To abolish any phenotypically-gained copper tolerance, the chosen colonies were grown overnight and exponentially in NB pH 7.0. The sensitivity of these exponential cells to 58.92 μg/ml $CuSO_4$ was then tested (**Table 3-5**). The copper-sensitivities of the exponential pH 7.0 cells were slightly less than before, this might have been attributed to the slight modification to the formulation of Nutrient Broth No:2 by Oxoid in the new batch of broth received. The culture sensitivities however, were unaffected by whether they had been pre-exposed to low copper concentration or not. The 10 colonies randomly picked from the pre-exposured culture gave a mean survival percentage of 0.71±0.056 after a challenge with 58.92 μg/ml copper whereas 10 colonies isolated from the non-exposed cultures gave a mean survival percentage of 0.74±0.09 after the same challenge (**Table 3-5**) and statistically there is no significant difference. Hence the increased copper tolerance after a pre-exposure to low copper levels, presumably represents a phenotypic change in the majority, or in all of the pre-exposed cells. Accordingly, the cells that have gained the ability to survive lethal levels of copper after a prior exposure are considered to be habituated.

Table 3-5 Determination of whether copper resistant mutants of *E.coli* 1829, arose during induction of tolerance to copper(II) sulphate

Conditions of isolations of "mutant" colonies	Percentage survival (CFU/ml)(mean ±S.E.M) after copper treatment for 30 minutes
pH 5.5 without copper(II) sulphate	0.74±0.056
pH 5.5 plus 14.73 μg/ml copper(II) sulphate	0.71±0.09

Ten colonies isolated randomly from each of the stated conditions were grown to exponential phase in NB pH 7.0 and then challenged with NB plus 117.84 μg/ml copper(II) sulphate (pH 5.5) for 30 minutes. Dilutions of cultures at the end of the treatment were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml at time zero i.e prior to challenge. The results shown are means of results given by 10 colonies.

Tolerance to copper was also induced in *E.coli* 1829 *F'lac* and 1829 ColV I-K94, pBR322 following prior exposure to the sublethal concentrations of copper (**Tables 3-6a,b,**). *E.coli* 1829 *F'lac* cells (**Table 3-6a**) that were pre-exposed to 14.73 µg/ml and 29.46 µg/ml $CuSO_4$ showed a mean percentage survival of 229.7±87.3 and 483.4±59.7 respectively after a challenge with 117.84 µg/ml $CuSO_4$ for one hour. In contrast, the pH 5.5 induced cells only showed a mean percentage survival of 3.21±1.62 after the same challenge. The cells that were induced at 14.73 µg/ml and 29.46 µg/ml $CuSO_4$ are 71-fold and 150-fold more able to survive the lethal dose of copper. Thus prior exposure to sublethal concentrations of copper in this strain can confer tolerance to lethal dose of copper.

Table 3-6b shows a representative result of the effect of copper challenge on growth in *E.coli* ColV I-K94, pBR322 cells following prior exposures to 14.73 µg/ml and 29.46 µg/ml $CuSO_4$. After a challenge for 180 minutes with 58.92 µg/ml and 117.84 µg/ml $CuSO_4$, an increase of 16-fold and 15-fold respectively in growth was observed in cells that were pre-exposed to 14.73 µg/ml $CuSO_4$. Similarly, an increase of 13-fold and 10.6-fold of growth was observed in cells that were pre-exposed to 29.46 µg/ml $CuSO_4$ after the same respective challenge. In contrast cells that were induced at pH 5.5 in absence of copper appeared to cease growing. Accordingly, tolerance to copper was induced in this derivative of 1829.

The ability to resist lethal concentrations of cupric sulphate above, was due to pre-exposures to Cu^{2+} and not SO_4^{2-}, this was ascertained by pre-exposing exponential cells to cupric chloride and potassium sulphate as described below..

3.1.4 The effect of pre-exposures to copper(II) sulphate, copper(II) chloride and potassium sulphate on tolerance to copper in *E.coli* 1829

The results in **Table 3-7** show that cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ and 10.06 µg/ml $CuCl_2$ were able to tolerate 117.84 µg/ml $CuSO_4$on challenge; a mean percentage of 113.9±5.4 and 82.4±12.2 respectively were able to form colonies after 60 minutes challenge with the lethal copper dose. In contrast, cells that were pre-exposed to 10.82 µg/ml K_2SO_4 showed no tolerance when challenged with 117.84 µg/ml $CuSO_4$. After a challenge for 60 minutes at this lethal dose, only 0.013% were able to form

Table 3-6a Induced tolerance to copper in *E.coli* 1829 *F'lac*

Conditions of pre-exposure	Percentageof colony forming units (mean± S.E.M) after a challenge with 117.84 µg/ml copper(II) sulphate for 60 mins
NB pH 5.5without copper(II) sulphate	3.21±1.62
NB plus 14.73 µg/ml copper(II) sulphate(pH 5.5)	229.70±87.3
NB plus 29.46 µg/ml copper(II) sulphate(pH 5.5)	483.40±59.70

E.coli 1829 *F'lac* were grown to an exponential phase in NB pH 7.0 and, after pre-exposure for one hour under the stated conditions with or without copper(II) sulphate, they were challenged with NB plus 117.84 µg/ml copper(II) sulphate (pH 5.5) for 60 minutes. Cultures dilutions were plated on NA and incubated at 37°C for 24 hours. Results shown are mean values of three experiments.

Table 3-6b Induced tolerance to copper in *E.coli* 1829 ColV I-K94, pBR322

Conditions of pre-exposure	Concentration of copper(II) sulphate used for challenge (μg/ml)	Optical density readings (Hilger units) after a challenge with Cu^{2+} for:		
		60	120	180
NB without copper(II) sulphate	58.92	0.025	0.022	0.02
	117.84	0.025	0.02	0.02
NB plus 14.73 μg/ml copper(II) sulphate (pH 5.5)	58.92	0.12	0.29	0.49
	117.84	0.09	0.23	0.46
NB plus 29.46 μg/ml copper(II)sulphate (pH 5.5)	58.92	0.07	0.18	0.40
	117.84	0.06	0.14	0.32

E.coli 1829 ColV, I-K94 pBR322 cells were grown to an exponential phase in NB pH 7.0 and, after pre-exposure for one hour under the stated conditions were challenged to NB plus 58.92 or 117.84 μg/ml copper(II) sulphate for the stated times. The initial O.D at the onset of challenge was 0.03. At intervals of time, optical density from samples were measured using a Hilger photoelectric colorimeter with a 520 nm filter. Experiment was repeated three times with consistent results.

Table 3-7 Comparison of the effect of pre-exposure to copper(II)sulphate, copper(II) chloride and potassium sulphate on induction of tolerance to copper in *E.coli* 1829

Pre-exposure conditions	Percentage of colony forming units (mean±S.E.M) after challenge with 117.84 µg/ml copper(II) sulphate for(mins):	
	30	6 0
NB pH 5.5 without copper(II) sulphate	0.11±0.02	0.08±0.02
NB pH 5.5 plus 14.73 µg/ml copper(II) sulphate	97.00±15.10	113.90±5.40
NB pH 5.5 plus 10.06 µg/ml copper(II) chloride	49.70±4.74	82.40±12.20
pH 5.5 plus 10.82 µg/ml potassium sulphate	0.03±0.01	0.01±0.0

E.coli cells were grown to exponential phase in NB pH 7.0 at 37°C and, after pre-exposure for one hour under the stated conditions in NB with or without copper(II) sulphate, copper(II) chloride or potassium sulphate, they were challenged with NB plus 117.84 µg/ml copper(II) sulphate (pH 5.5) for the stated times. Culture dilutions were plated on NA and incubated for 18-24 hours. Results shown are mean values of three experiments and expressed as percentage of CFU/ml at time zero (i.e after pre-exposure but before challenge).

colonies. Similarly, those cells that were pre-exposed to pH 5.5 only, showed no tolerance to the lethal dose of copper. After a challenge for 60 minutes only 0.08±0.023% survived.

The Cu^{2+} concentration in 10.06 µg/ml cupric chloride is equivalent to that in 14.73 µg/ml $CuSO_4$ and the concentration of SO_4^{2-} in 10.82 µg/ml K_2SO_4 is equivalent to that in 14.73 µg/ml $CuSO_4$. From the results above it is ascertained that it is pre-exposure to Cu^{2+} and not to SO_4^{2-} that enable the cells to gain the ability to tolerate lethal doses of the cupric sulphate. Thus pre-exposure to Cu^{2+} is necessary before tolerance to lethal doses of Cu^{2+} is acquired.

Interestingly, cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ are 2 times (95% confidence) and 1.4 times (90% confidence) more tolerant to copper than cells that were pre-exposed to $CuCl_2$ after 30 minutes and 60 minutes respectively of challenge with the lethal dose of copper. This is probably due to $CuSO_4$ being slightly more inhibitory than cupric chloride (**3.1.2**) and it would seem that the degree of sublethality at pre-exposure has an influence on the degree of tolerance at challenge. This similar pattern was seen in **3.1.3** when cells pre-exposed to 29.46 µg/ml $CuSO_4$ were more tolerant (5.7 times) than cells pre-exposed to 14.73 µg/ml $CuSO_4$ when challenged with 58.92 µg/ml cupric sulphate after 60 minutes. Although a similar pattern was not observed when the pre-exposed cells were challenged with 117.84 µg/ml $CuSO_4$, the degree of tolerance to lethal concentrations of copper could to some extent depend on the degree of sublethality at pre-exposure. Cells that were pre-exposed to pH 5.5 only are 6.1 times (90% confidence) more able to form colonies than those pre-exposed to 10.82 µg/ml K_2SO_4 after 60 minutes of challenge with the lethal dose of copper. It would seem that pre-exposure to potassium sulphate sensitised the cells even more to challenge with the lethal dose of copper.

The duration of pre-exposure to sublethal concentrations of copper was so far carried out for 60 minutes. The following experiment was carried out to determine the rate of induction to copper tolerance in *E.coli* 1829.

3.1.5 Induction of copper tolerance in *E.coli* 1829 at 30 °C

Table 3-8 shows that cells that were pre-exposed to NB plus 0.0 µg/ml $CuSO_4$ 30°C showed a mean survival of 0.05±0.01% and those that were at 37°C showed a survival of 0.17±0.09% after a challenge with 117.84 µg/ml $CuSO_4$ (pH 5.5) at 37°C. There is no

Table 3-8 Induction of copper tolerance in *E.coli* 1829, at 30°C

Concentration of copper(II) sulphate in NB pH 5.5 used for pre-exposure (µg/ml)	Pre-exposure temperature (°C)	Percentage of colony forming units (mean ±S.E.M) after copper challenge for one hour
0.0	37.0	0.05±0.01
	30.0	0.17±0.09
14.73	37.0	131.10±11.60
	30.0	218.50±10.50
29.46	37.0	139.40±25.90
	30.0	282.20±41.30

E.coli cells were grown to exponential phase in pH 7.0 at 37°C and, after pre-exposure for one hour under the stated conditions with or without copper at 37°C or 30°C were challenged with NB plus 117.84 µg/ml copper (pH 5.5) for one hour at 37°C. Dilutions of challenged cultures were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge. The results shown were mean values of three experiments.

significant differences between the survival at these two different temperatures.

Cells that were pre-exposed to 14.73 µg/ml (pH 5.5) at 30°C gave a survival of 218.5±10.5% whereas when the same pre-exposure was carried out at 37°C only 131.1±11.6% cells were able to form colonies after a challenge with 117.84 µg/ml $CuSO_4$ (pH 5.5) at 37°C. Similarly, when the cells were pre-exposed in NB plus 29.46 µg/ml $CuSO_4$ (pH 5.5) at 30°C 282.2±41.30% of the cells were able to form colonies after a challenge with 117.84 µg/ml $CuSO_4$ (pH 5.5). In contrast, when the pre-exposure was done at 37°C, 139.4±25.90% of the cells survived after the same challenge.

The results suggest that the induction to copper tolerance is enhanced at 30°C; cells having a prior pre-exposure to NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and 29.46 µg/ml $CuSO_4$ (pH 5.5) at 30°C, are 1.7-fold (99% confidence) and 2-fold (95% confidence) more able to survive the lethal concentration of $CuSO_4$ than cells having their pre-exposure at 37°C. However pre-exposures at these two temperatures in NB pH 5.5 showed no significant difference.

3.1.6 Kinetics of induced tolerance to copper in *E.coli* 1829

Table 3-9 shows that a mean of 238.65±47.05% cells that had a 15 minute pre-exposure in 14.73 µg/ml $CuSO_4$ were able to form colonies after a challenge with 117.84 µg/ml $CuSO_4$ for one hour. Those that were pre-exposed at this sublethal concentration for 30 and 60 minutes showed mean survival values of 208.95±16.40% and 209.25±23.26% respectively after the same challenge. On the contrary, cells that did not received any pre-exposure in 14.73 µg/ml $CuSO_4$ showed little tolerance to the lethal dose of $CuSO_4$ after being challenged for one hour.

The results above showed that tolerance to copper (117.84 µg/ml $CuSO_4$) was induced in the first 15 minutes of pre-exposure to 14.73 µg/ml $CuSO_4$ and there were no significant differences in tolerance induced in these cells and in those having pre-exposure periods of 30 or 60 minutes at this sublethal concentration. The results above indicate that the maximum tolerance to copper was induced in the first 15 minutes of pre-exposure and since it has been verified that the induced tolerance to copper is due to phenotypic changes, these changes must have taken place in this first 15 minutes of the pre-exposure to sublethal concentration of copper. It is noted that the values of survival in **Table 3-9**

Table 3-9 Kinetics of induced tolerance to copper in *E.coli* 1829.

Duration of pre-exposure to:		Percentage of colony forming units (mean±S.E.M) after challenge with copper for one hour
NB plus no copper (mins)	NB plus 14.73 µg/ml copper(II) sulphate pH 5.5 (mins)	
60.0	0.0	3.10±1.54
45.0	15.0	238.65±47.05
30.0	30.0	208.95±16.40
0.0	60.0	209.25±23.26

E.coli cells were grown to exponential phase in NB pH 7·0 at 37°C and were pre-exposed to NB pH 5.5 with and without copper for stated durations at 37°C. Cells that were pre-exposed to NB pH 5.5 without copper for 30 and 45 minutes were harvested and resuspended in NB plus 14.73 µg/ml copper (pH 5.5) and pre-exposure was continued for a further 30 and 45 minutes respectively so that all the cultures received a total pre-exposure duration of one hour. The pre-exposed cells were then challenged with 117.84 µg/ml copper for one hour. The survivals after copper challenge were expressed as a percentage of CFU/ml at time zero i.e prior to challenge. The results shown above are mean values of five experiments.

are higher than those in earlier experiments (**Table 3-3**). As mentioned earlier (**3.1.3**) this could be attributed to the modified formulation of the Nutrient Broth No:2 (Oxoid).

Since induced tolerance to copper is due to phenotypic and not mutational changes occurring during the pre-exposure periods, this effect would be expected to be a transient one. The following experiment was designed to determine this .

3.1.7 Induced tolerance to copper following further exposure in NB pH 5.5 after pre-exposure with adaptive doses of copper

The results from **Table 3-10** showed that *E.coli* 1829 cells that were pre-exposed to 14.73 μg/ml $CuSO_4$ showed a mean survival of 150.15±22.70% after being challenged with 117.84 μg/ml $CuSO_4$ for one hour. When pre-exposure at this adaptive dose was followed by further exposure in NB pH 5.5 for one and two hours mean percentages of 55.97±13.30 and 32.41±8.44 of the cells respectively were able to form colonies. The survival of these cells that had the extra exposure of one and two hours in NB pH 5.5 is 2.7-fold (95% confidence) and 4.6-fold (99% confidence) lower respectively than those that did not. Controls with cells having an extra one and two hours in NB plus 14.73 μg/ml $CuSO_4$ (pH 5.5) were not carried out for comparison since it was found that the minimum period required for maximum tolerance to copper was 15 minutes (**3.1.5**) thus the extra pre-exposure in this adaptive copper dose would not give any significant differences in survival to those pre-exposed for one hour.

Cells that were pre-exposed to NB pH 5.5 plus 0.0 μg/ml $CuSO_4$ showed a survival of 0.29±0.06% after a challenge with 117.84 μg/ml $CuSO_4$ for one hour. When pre-exposure to NB pH 5.5 plus 0.0 μg/ml $CuSO_4$ was followed by a further exposure of one and two hours in NB pH 5.5, 0.46±0.15% and 0.41±0.12% of the cells respectively were able to form colonies after a challenge with the lethal dose of $CuSO_4$. The differences in survival in the cells having the extra pre-exposures in NB pH 5.5 and those that did not are statistically not significant.

The results above indicate that the ability to tolerate lethal concentration of $CuSO_4$ is diluted out following further pre-exposure in NB pH 5.5. In the one and two hours of further pre-exposure, 2 to 3 doublings and 4 to 6 doublings respectively would have taken place. This showed that the phenotypic changes that occurred during the pre-exposure

Table 3-10 The kinetics of tolerance to copper in habituated cells of *E.coli* 1829.

Condition of pre-exposure	Further growth in NB (pH 5.5) for (hours)	Percentage colony forming units(mean±S.E.M) after copper challenge for one hour
NB pH 5.5 without copper(II) sulphate	0.0	0.29±0.06
	1.0.	0.46±0.15
	2.0	0.41±0.12
NB plus 14.73 µg/ml copper(II) sulphate	0.0	150.15±22.70
	1.0	55.97±13.30
	2.0	32.41± 8.44

E.coli cells were pre-exposed to NB pH 5.5 without copper and NB plus 14.73 µg/ml copper (pH 5.5) for one hour. They were then harvested and resuspended in NB pH 5.5 and further grown for the stated times before challenging with NB plus 117.84 µg/ml copper pH 5.5 for one hour at 37°C. At the end of the challenge dilutions of samples were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge. The results shown are mean values of three experiments.

period with 14.73 μg/ml $CuSO_4$ was not fully transferred to the progeny during the cell doublings that took place in the extra one and two hours of pre-exposure in NB pH 5.5 and hence only a proportion of the cell population was able to tolerate the lethal dose of $CuSO_4$. The effects from the pre-exposure would therefore be expected to be eventually diluted out following repeated cell doublings and accordingly, the phenotypic effect is only a transient one.

The phenotypic changes that occur during the pre-exposure period presumably enable the cells to gain the ability to tolerate lethal doses of $CuSO_4$. Similar changes have been shown to involve synthesis of new proteins during adaptive period to heat (**Christman *et al.*, 1985**), hydrogen peroxide (**Demple and Halbrook, 1983**) and starvation (**Jenkins *et al.*, 1988**). The involvement of protein synthesis in habituation to copper was investigated in the following section.

3.1.8 Effect of chloramphenicol on induction to copper tolerance in *E.coli* 1829

Table 3-11 shows that when chloramphenicol, a protein synthesis inhibitor, was added at the onset of pre-exposure to $CuSO_4$, these pre-exposed cells did not gain the ability to withstand the lethal dose of $CuSO_4$. Cells pre-exposed to 14.73 μg/ml $CuSO_4$ plus chloramphenicol showed a mean survival of 0.04±0.04% whereas those that were pre-exposed in the absence of chloramphenicol showed a mean survival of 190.4±27.48% after a challenge with 117.84 μg/ml $CuSO_4$ for one hour. Similarly cells that were pre-exposed to 29.46 μg/ml $CuSO_4$ plus chloramphenicol gave a mean survival of 0.002±0.001% after a challenge with 117.84 μg/ml $CuSO_4$ for one hour whereas cells pre-exposed without the presence of chloramphenicol (CAP) showed a mean survival of 133.40±13.85% after the same challenge. In a control experiment, cells that were pre-exposed to 0.0 μg/ml $CuSO_4$ plus CAP showed a mean survival of 0.02±0.01% and in the absence of the protein synthesis inhibitor gave a mean survival of 5.43±1.72%.

The above results indicate that during the pre-exposure periods *de novo* protein synthesis must have taken place to enable the pre-exposed cells to withstand lethal concentration of cupric sulphate upon challenge. The protein/s induced presumably confer some protection during challenge with lethal concentration of cupric sulphate. The

Table 3-11 Effect of chloramphenicol on induction to copper tolerance in *E.coli* 1829.

Concentration of copper(II) sulphate in NB pH 5.5 used for pre-exposure (µg/ml)	Chloramphenicol (200 µg/ml)	Percentage of colony forming units (mean ±S.E.M) after copper challenge for one hour.
0.0	-	5.430±1.72
	+	0.02±0.01
14.73	-	190.40±27.48
	+	0.04±0.04
29.46	-	133.40±13.85
	+	0.002±0.001

E.coli cells grown to exponential phase in NB pH 7.0 at 37°C were pre-exposed for one hour in NB with or without copper at pH 5.5 in the presence or absence of chloramphenicol (200 µg/ml) and then challenged with NB plus 117.84 µg/ml copper pH 5.5 for a further one hour. Dilutions of the challenged cultures were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge. The results shown are mean values of five experiments.

presence of chloramphenicol during the pre-exposure period would inhibit the synthesis of the protein/s and hence cells pre-exposed in presence of sublethal concentration of $CuSO_4$ and chloramphenicol showed no tolerance to the lethal concentration of $CuSO_4$ when challenged for one hour unlike its counterpart. Thus the results suggest that habituation to copper is protein synthesis dependent.

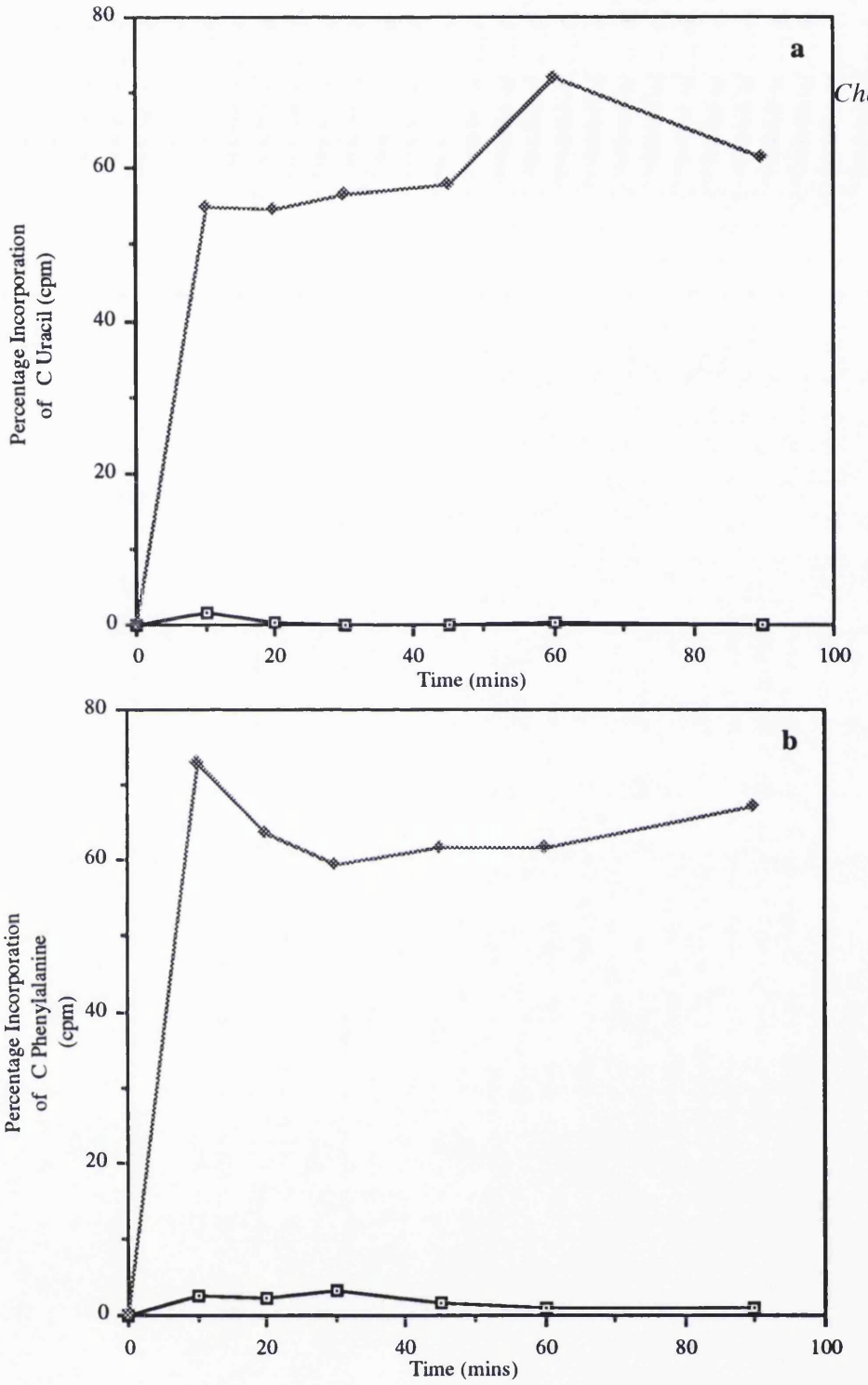
The protection gained during the pre-exposure period to the sublethal concentrations of $CuSO_4$ must have enabled the cells to perform normal metabolic reactions in the presence of challenge doses of Cu^{2+}. The following section describes the effects of copper challenge on some metabolic processes in habituated and non-habituated cells.

3.1.9 Effect of copper challenge on protein and RNA synthesis in copper habituated and non-habituated *E.coli.*

The ability of pre-exposed cells to synthesise proteins and RNA after a copper challenge was investigated by studying a) the incorporation of radiolabelled uracil (^{14}C uracil) and phenylalanine (^{14}C phenylalanine) and b) the induction of β-galactosidase. The incorporation of radiolabelled uracil, a component of RNA, and phenylalanine should give measures of RNA and protein synthesis respectively whilst formation of β-galactosidase should indicate whether the ability of the cells to synthesize depends on both mRNA and protein synthesis .

3.1.9.1 The effect of copper challenge on the incorporation of radiolabelled ^{14}C uracil and ^{14}C phenylalanine in habituated and non-habituated *E.coli* 1829 cells

Figures 3-3a and 3-3b show the incorporation of ^{14}C uracil and ^{14}C phenylalanine into cells that had been pre-exposed in NB pH 5.5 plus 0.0 µg/ml $CuSO_4$ and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and then challenged with 117.84 µg/ml $CuSO_4$ (pH 5.5) for one hour. After pre-exposure and then challenge, the cells were harvested, washed and resuspended in NB pH 5.5 containing 0.5 µci/ml of ^{14}C phenylalanine and also in NB containing 0.5 µci/ml ^{14}C uracil, and at intervals samples were removed for radioassay. The incorporation values of ^{14}C uracil or ^{14}C phenylalanine into the habituated cells were



Figures 3-3a,b Effect of copper challenge on protein synthesis in copper habituated and non-habituated *E. coli* 1829. Incorporation of ^{14}C uracil (**a**) and ^{14}C phenylalanine (**b**) in non-habituated cells (—□—) and copper habituated cells (.....◆.....) after a challenge with NB plus 117.84 µg/ml copper(II) sulphate, pH 5.5 for one hour, was measured at intervals of time for a duration of 90 minutes. The results were recorded as a percentage of incorporation into control cultures i.e. non-habituated and habituated cultures that were challenged with NB pH 5.5. Habituated cells were pre-exposed for one hour in NB plus 14.73 µg/ml copper(II) sulphate pH 5.5. The results are of a single experiment. Experiments were repeated three times with consistent results.

expressed as a percentage of ^{14}C uracil or ^{14}C phenylalanine incorporated in copper pre-exposed cells that were challenged with NB pH 5.5 plus 0.0 µg/ml $CuSO_4$. Likewise the incorporation of ^{14}C uracil or ^{14}C phenylalanine into the non-habituated cells was expressed as a percentage of ^{14}C uracil or ^{14}C phenylalanine incorporated into cells that were pre-exposed to NB pH 5.5 plus 0.0 µg/ml $CuSO_4$ and challenged with NB pH 5.5.

Figure 3-3a shows that approximately 55-57% of the control value of ^{14}C uracil was incorporated in the habituated cells during the first 45 minutes whereas hardly any ^{14}C uracil was being incorporated in the non-habituated cells. By the end of 90 minutes, 61% ^{14}C uracil and only 0.03% ^{14}C uracil were being incorporated into the habituated and non-habituated cells respectively.

Figure 3-3b show that in the first 10 minutes 73% of the control value of ^{14}C phenylalanine was incorporated in the habituated cells. After 45 and 90 minutes, 62% and 68% respectively were taken up by these cells. In contrast, only 3% of the control value of ^{14}C phenylalanine was incorporated in the first 10 minutes and after 45 and 90 minutes only 1.6 % and 1% of the control values of ^{14}C phenylalanine were being taken in by the non-habituated cells.

The differences in incorporation of ^{14}C uracil, between habituated and non-habituated cells, indicate that RNA synthesis can occur after treatment with a potentially lethal dose of copper in habituated cells but not in the non-habituated ones. Similarly the uptake of the radiolabelled phenylalanine after copper challenge, indicates that nascent polypeptides can be synthesized by the habituated cells but not by the non-habituated ones. The results suggest that after treatment with a lethal dose of copper, habituated cells are able to synthesize proteins required for their viability but this ability is not present in those not habituated. The ability of the habituated cells in taking up the ^{14}C uracil and ^{14}C phenylalanine suggests that the cells' integrity is protected from damage by the lethal dose of $CuSO_4$. Since the information for the cell's proteins is encoded in the DNA, presumably one other main protection gained during the pre-exposure period to sublethal concentrations of $CuSO_4$ may be against damage to the DNA.

3.1.9.2 Induction of β-galactosidase in copper habituated and non-habituated *E.coli* 1829 *F'lac* cells.

β-galactosidase was induced *in E.coli* 1829 *F'lac* which was shown to have the ability to induce copper tolerance (**Table 3-6a**). The induction of β-galactosidase in the presence of IPTG was carried out after copper habituated and non habituated cells were challenged with NB pH 5.5, NB plus 58.96 μg/ml $CuSO_4$ pH 5.5 and NB plus 117.84 μg/ml $CuSO_4$ pH 5.5. The enzymic activity induced after the challenges is illustrated in **Figure 3-4.**

When β-galactosidase was induced after a challenge with NB pH 5.5 (**a**), cells that were pre-exposed to NB pH 5.5, NB plus 14.73 μg/ml $CuSO_4$ (pH 5.5) and NB plus 29.46 μg/ml $CuSO_4$ (pH 5.5) gave enzymic activities of 1551.8±236.4, 564.8±47.2 and 425.6±33.2 Miller units respectively (**Table 3-12**).

The β-galactosidase induced after a challenge with NB plus 58.96 μg/ml $CuSO_4$ pH 5.5 (**b**), gave activities of 35.32±7.1, 421.6±58.03 and 483.0±58.40 Miller units when measured from cells that were pre-exposed to NB pH 5.5, NB plus 14.73 μg/ml $CuSO_4$ pH 5.5 and NB plus 29.46 μg/ml $CuSO_4$ pH 5.5 respectively.

When the enzyme was induced after a challenge with NB plus 117.84 μg/ml $CuSO_4$ pH 5.5 (**c**), cells that were pre-exposed to NB pH 5.5, NB plus 14.73 μg/ml $CuSO_4$ and 29.46 μg/ml $CuSO_4$ gave activities of 13.32±0.43, 248.6±86.6 and 356.2±66.1 Miller units respectively.

The results from the control experiment **Figure 3-4** (**a**) (**Table 3-12**) show that the presence of the sublethal concentrations of copper had an effect on the subsequent induction of β-galactosidase. The cells pre-exposed to 14.73 μg/ml $CuSO_4$ and 29.46 μg/ml $CuSO_4$ show a 2.7-fold and 3.6-fold decrease in the enzyme activity compared to those that were pre-exposed to NB pH 5.5. However after challenges with the lethal doses of copper (**b**) and (**c**) the cells that have prior exposure to sublethal concentrations of copper were more able to express the β-galactosidase compared to the cells that were pre-exposed to NB pH 5.5 plus 0.0 μg/ml $CuSO_4$. The cells that received prior exposure to 14.73 μg/ml $CuSO_4$ and 29.46 μg/ml $CuSO_4$ were 12 times and 13.4 times more able to express the induced enzyme respectively than the cells that were pre-exposed to NB pH 5.5 after a challenge with 58.92 μg/ml $CuSO_4$. After a challenge with 117.84 μg/ml $CuSO_4$, cells that were pre-exposed to 14.73 μg/ml $CuSO_4$ and 29.46 μg/ml $CuSO_4$ were

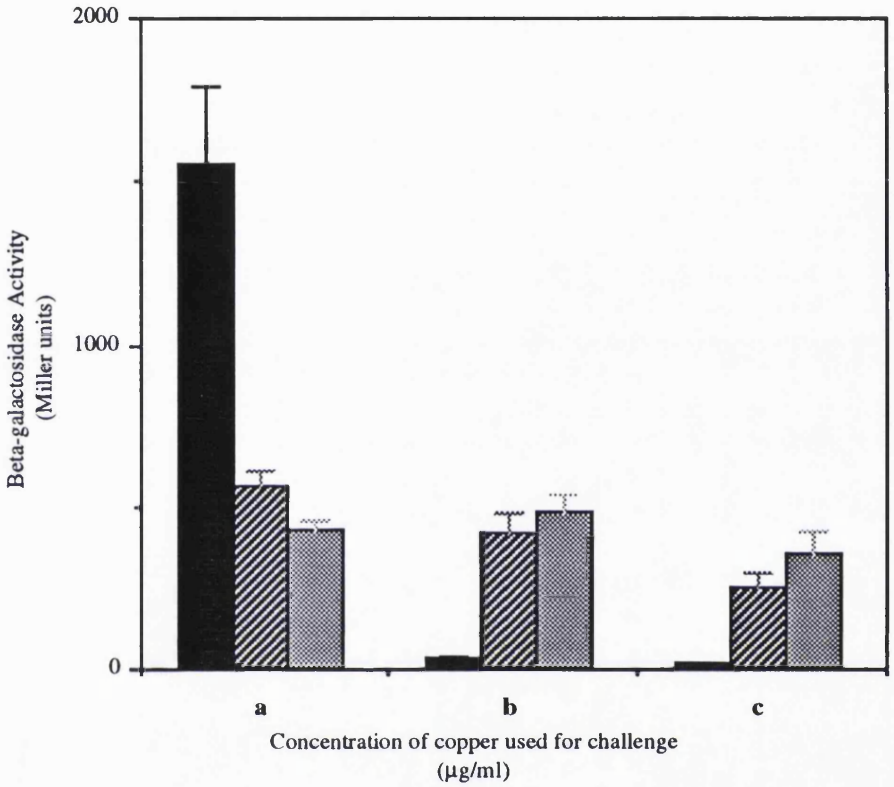


Figure 3-4 Induction of beta-galactosidase activity in copper habituated and non-habituated *E. coli* 1829. Beta-galactosidase activity was induced in non-habituated (■) and copper habituated cells (▨ pre-exposed to NB plus 14.73 µg/ml copper(II) sulphate, pH 5.5 and ▩ pre-exposed to NB plus 29.46 µg/ml copper(II) sulphate, pH 5.5) after one hour of challenge with NB pH 5.5 (**a**), NB plus 58.92 µg/ml copper(II) sulphate, pH 5.5 (**b**) and NB plus 117.84 µg/ml copper(II) sulphate, pH 5.5 (**c**). The enzyme was induced in the presence of 1 mM IPTG. Results are mean values of three experiments.

Table 3-12 Induction of β-galactosidase activity in copper habituated and non-habituated cells after a copper challenge

Pre-exposure conditions	Copper(II)sulphate concentrations used for challenge (μg/ml)	β-galactosidase activity (Miller units) (mean±S.E.M)
NB pH 5.5	0.0	1551.8±236.40
	58.92	35.32±7.10
	117.84	13.32±0.43
NB plus 14.73 μg/ml copper(II) sulphate (pH 5.5)	0.0	564.80±47.20
	58.92	421.60±58.03
	117.84	248.60±50.10
NB plus 29.46 μg/ml copper(II) sulphate (pH 5.5)	0.0	425.60±33.20
	58.92	483.0±58.40
	117.84	356.20±66.10

β-galactosidase was induced in copper habituated and non-habituated cells after a challenge with stated copper(II) sulphate concentrations for one hour. The treated cultures were induced for β-galactosidase in the presence of IPTG for a duration of 30 minutes. The results shown are mean values of three experiments.

19 times and 27 times more able to express the induced enzyme than those pre-exposed to NB pH 5.5. These results indicate that prior exposure to the sublethal concentrations of copper have conferred on the cells some protection during the challenge period that enable them to subsequently express the β-galactosidase on induction of the enzyme after the challenges. There is no significant difference in enzyme activity induced when cells that were pre-exposed to the sublethal concentrations of copper were challenged with 58.92 μg/ml $CuSO_4$ and 117.84 μg/ml $CuSO_4$.

When the enzyme activities of copper treated pH 5.5 induced cells were compared with untreated pH 5.5 induced cells, only a mean 2.2±0.46% and 0.86±0.02% of the activity from those treated with 58.92 μg/ml and 117.84 μg/m $CuSO_4$ respectively were expressed. In contrast cells that were pre-exposed in NB plus 14.73 μg/ml $CuSO_4$ were able to express 74.6±10.3% and 44.0±8.9% after a challenge with 58.92 μg/ml $CuSO_4$ and 117.84 μg/ml $CuSO_4$ respectively when compared with the untreated induced cells. Similarly cells that were pre-exposed to 29.46 μg/ml $CuSO_4$ were able to express 113.5±13.7% and 83.7±15.5% of the enzyme after a challenge with 58.92 μg/ml $CuSO_4$ and 117.84 μg/ml $CuSO_4$ respectively when compared with the untreated induced cells. The cells that were pre-exposed to 14.73 μg/ml $CuSO_4$ and 29.46 μg/ml $CuSO_4$ were 34 and 52 times more able to express the enzyme than the cells that were pre-exposed to NB pH 5.5 after a challenge with 58.92 μg/ml $CuSO_4$. Similarly these habituated cells are 51 and 97 times more able to express the β-galactosidase after a challenge with 117.84 μg/ml cupric sulphate than the non-habituated ones. The enzyme induced in cells pre-exposed to 29.46 μg/ml $CuSO_4$ is 1.5-fold and 1.9-fold higher (90% confidence) than cells pre-exposed to 14.73 μg/ml $CuSO_4$ after a challenge with 58.92 μg/ml and 117.84 μg/ml $CuSO_4$ respectively.

The induction of β-galactosidase involves the dissociation of the repressor-operator complex, transcription of specific mRNA which is translated into β-galactosidase monomers which are then assembled into active oligomers (**Miller and Reznikoff 1978**). These molecular processes have to be carried out by the cells in order for the active form of the enzyme to be synthesised. Any damage occurring to the DNA or RNA or the ribosomes which are all involved in the synthesis of the β-galactosidase will prevent the cells from making this protein. The formation of this enzyme in the habituated cells

indicates that these components are not markedly damaged during the challenges with the lethal concentrations of copper. One or all of these components in the cells that were pre-exposed with NB pH 5.5 presumably is/are damaged during the challenges with the lethal doses of cupric sulphate. Thus the molecular protection is conferred on some or all of these protein synthesis components in the habituated cells but not in the non-habituated cells.

Of all the components involved, the state of DNA in habituated and non-habituated cells was examined as described in the following..

3.1.10 Effect of copper challenge on DNA in copper-induced and non-induced cells .

Figure 3-5 shows the effect of copper challenge on DNA in *E.coli* 1829 ColV I-K94, pBR322 cells that were pre-exposed in NB pH 5.5 (Lanes 6 and 8) and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) (lanes 5 and 7). The results show that DNA from copper induced cells was less damaged than that from pH 5.5 induced cells after a challenge with 58.92 µg/ml $CuSO_4$ (Lane 7) and 117.84 µg/ml $CuSO_4$ (Lane 5). It appears only the ColV DNA in these cells are slightly damaged. In contrast DNA isolated from pH 5.5 cells after challenges with 58.92 µg/ml $CuSO_4$ (Lane 8) and 117.84 µg/ml $CuSO_4$ (Lane 6) were markedly damaged; the ColV and chromosomal DNA are completely damaged after a challenge with 117.84 µg/ml $CuSO_4$ (Lane 6) and approximately half of the monomeric and dimeric pBR322 DNA forms was also damaged. Similarly the DNA isolated from these cells after a challenge with 58.92 µg/ml $CuSO_4$ (Lane 8) were also markedly damaged; the ColV is completely damaged and traces of degrading chromosomal DNA can be seen. Also more than half of the dimeric form of pBR322 was also damaged.

These results suggest that DNA form habituated cells are more resistance to able deleterious effects of Cu^{2+} than the non-habituated ones and the presence of this resistance probably enhanced their survival during the the treatment with lethal concentration of copper.

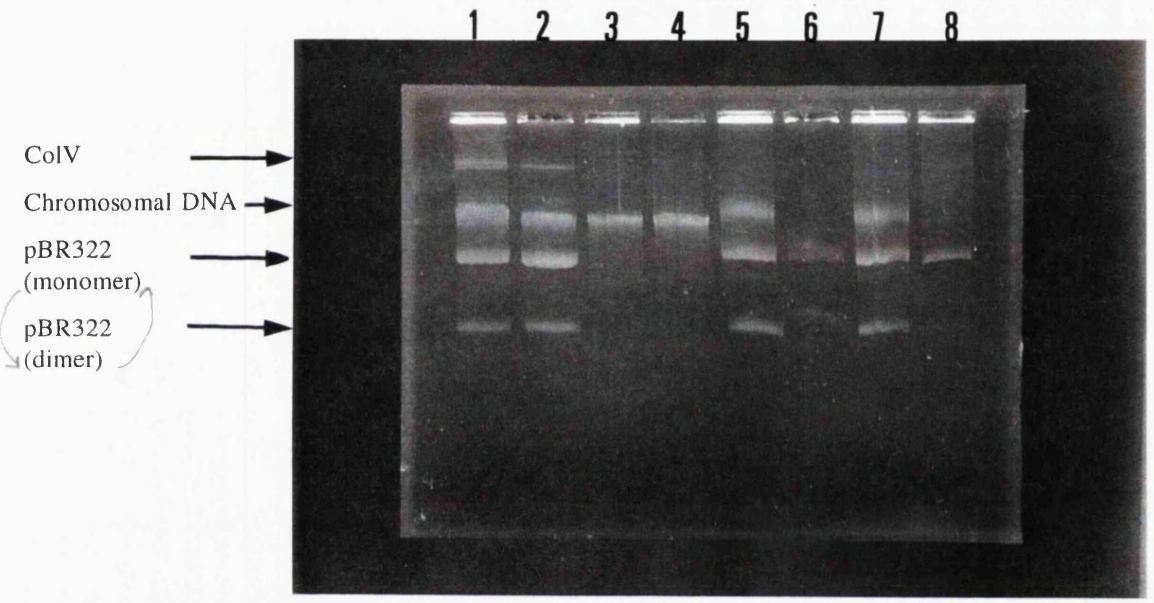


Figure 3-5 Effect of copper challenge on DNA in copper-induced and non- induced *E.coli* 1829 ColV, I-K94 pBR322 cells. **Lane 1**: DNA from *E.coli* 1829 I-K94, ColV pBR322 cells grown in NB pH 7.0; **Lane 2:** DNA from *E.coli* 1829 V-K94, pBR322 cells grown in NB pH 5.5; **Lane 3:** DNA from *E. coli* I-K94 ColV cells grown in NB pH 7.0; **Lane 4:** DNA from *E.coli* 1829 grown in NB pH 7.0; **Lane 5:** DNA from *E.coli* 1829 I-K94, ColV pBR322 cells that were pre-exposed to NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and challenged with 117.84µg/ml $CuSO_4$; **Lane 6:** DNA from *E.coli* 1829 I-K94, ColV pBR322 cells that were pre-exposed to NB (pH 5.5) without copper and challenged with 117.84µg/ml $CuSO_4$; **Lane 7:** DNA from *E.coli* 1829 I-K94, ColV pBR322 cells that were pre-exposed to NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and challenged with 58.92µg/ml $CuSO_4$; **Lane 8:** DNA from *E.coli* 1829 I-K94, ColV pBR322 cells that were pre-exposed to NB (pH 5.5) without copper and challenged with 58.92 µg/ml $CuSO_4$.

3.1.11 Comparison of cytoplasmic membrane and outer membrane proteins from copper habituated and non-habituated cells

Cytoplasmic membranes and outer membrane proteins were extracted from cells that were pre-exposed to NB pH 7.0 , NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) and NB plus 29.46 µg/ml $CuSO_4$ (pH 5.5) for one hour. **Figure 3-6a** shows the analysis of the extracted cytoplasmic membrane proteins on SDS polyacrylamide gel electrophoresis. Lanes 1 and 2 show the cytoplasmic membrane proteins that were isolated from cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ (pH 5.5) and NB plus 29.46 µg/ml $CuSO_4$ (pH 5.5) respectively. Two polypeptides of molecular weights 24.5 kDa and 26 kDa (arrows) were overexpressed compared to the pH 5.5 (Lane 3) and pH 7.0 (Lane 4) pre-exposed cells the former being expressed more markedly than the latter.

When outer membrane proteins from the copper induced cells were compared with the uninduced cells, four species of polypeptides appeared to be overexpressed (arrows) (**Figure 3-6b**). These polypeptides have molecular weights of 18 kDa, 16.5 kDa, 31.5 kDa and 65 kDa (Lanes 4 and 5).

These proteins that were induced during the pre-exposure periods probably have a role in aiding the cells to tolerate lethal doses of copper. As with most known stress proteins, these copper stress proteins probably act as chaperones involving in repairing denatured proteins and assisting in folding misfolded proteins.

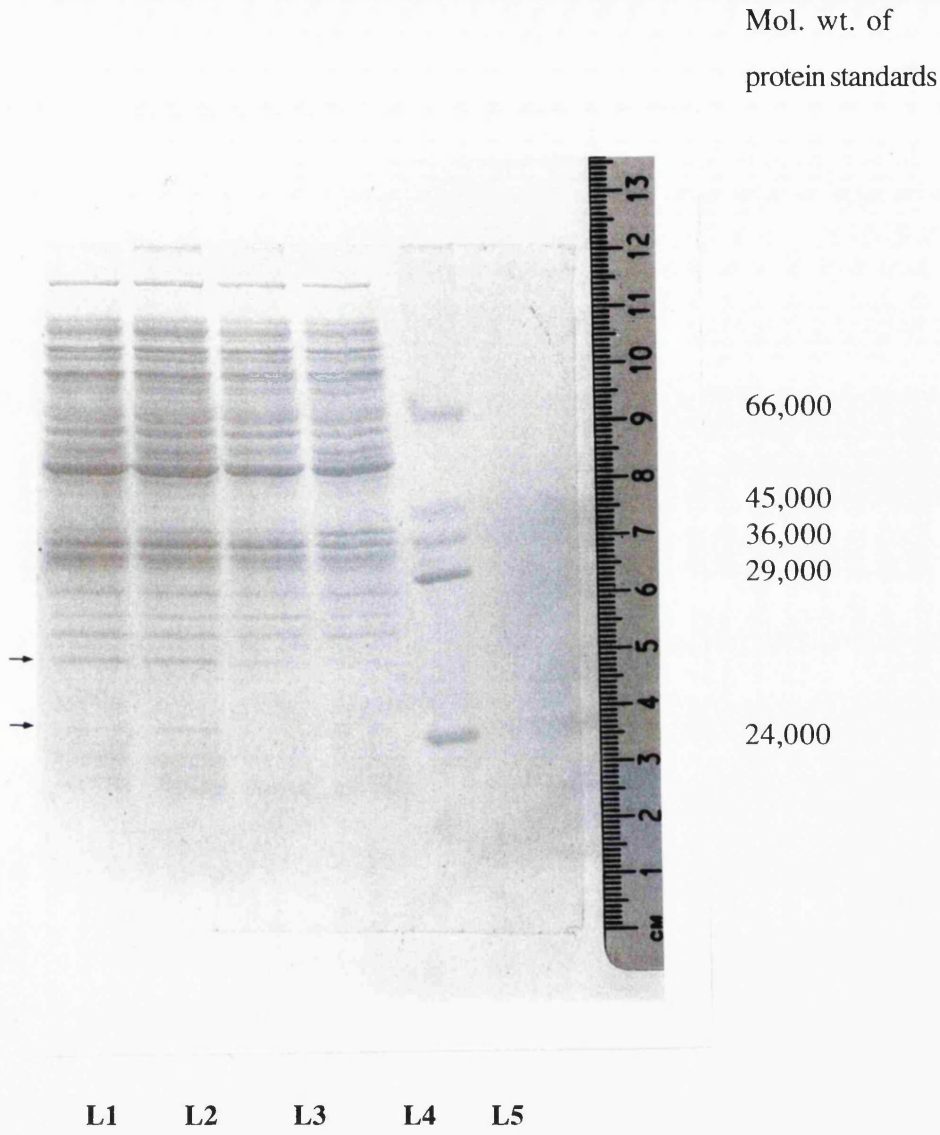


Figure 3-6a SDS polyacrylamide gel electrophoresis of cytoplasmic membrane proteins from copper induced and uninduced *E.coli* 1829 cells. **L1** Cytoplasmic membrane proteins from *E.coli* 1829 cells that pre-exposed in NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) for one hour; **L2** Cytoplasmic membrane proteins from *E.coli* 1829 cells that pre-exposed in NB plus 29.46 µg/ml $CuSO_4$ (pH 5.5) for one hour; **L3** Cytoplasmic membrane proteins from *E.coli* 1829 cells that were pre-exposed to in NB pH 5.5 without copper for one hour; **L4** Cytoplasmic membrane proteins from *E.coli* 1829 cells that were pre-exposed in NB pH 7.0 without copper for one hour; **L 5** Molecular weight protein standards

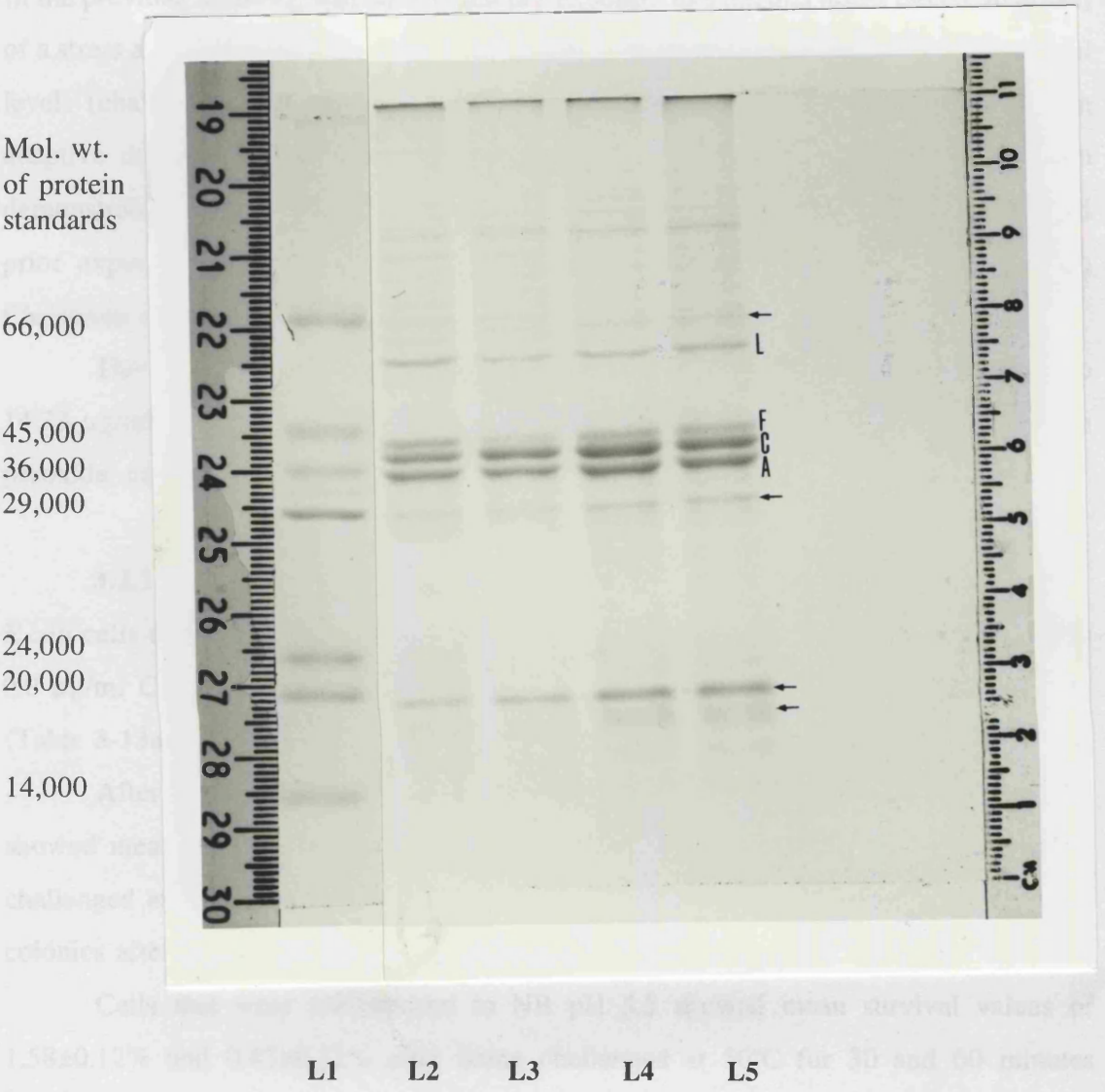


Figure 3-6b SDS polyacrylamide gel electrophoresis of outer membrane proteins from copper induced and uninduced *E.coli* 1829 cells. **L1** Molecular weight protein standards **L2** Cytoplasmic membrane proteins from *E.coli* 1829 cells that pre-exposed in NB pH 7.0 without copper for one hour **L3** Cytoplasmic membrane proteins from *E.coli* 1829 cells that pre-exposed in NB pH 5.5 without copper for one hour; **L4** Cytoplasmic membrane proteins from *E.coli* 1829 cells that pre-exposed in NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) for one hour; **L5** Cytoplasmic membrane proteins from *E.coli* 1829 cells that pre-exposed in NB plus 29.46 µg/ml $CuSO_4$ (pH 5.5) for one hour. L- LamB; F- OmpF; C-OmpC; A-OmpA

3.2 EFFECT OF PRE-EXPOSURE TO ADAPTIVE DOSE OF COPPER ON TOLERANCES TO OTHER STRESS AGENTS

In the previous section it was shown that pre-exposure to sublethal doses (adaptive doses) of a stress agent (copper) could confer protection against subsequent exposure to the lethal levels (challenge doses) of the same stress. Sometimes cells that are exposed to an adaptive dose of a stress agent develop tolerance to other stress agent/s. It has been demonstrated that *E.coli* and *S.typhimurium* could resist high temperatures when they had prior exposure to an adaptive dose of hydrogen peroxide (**Vanbogelen *et al.*, 1987; Christman *et al.*,1985; Morgan *et al.*,1986**).

This section examines the ability of *E.coli* 1829 cells that had prior exposure to 14.73 µg/ml $CuSO_4$ to resist lethal levels of other stress agents namely heat, hydrogen peroxide, cadmium sulphate, acid and alkali.

3.2.1 Induced tolerance to heat in cells pre-exposed to copper.

E.coli cells that were pre-exposed to NB pH 7.0 plus 0.0 µg/ml $CuSO_4$, NB pH 5.5 plus 0.0 µg/ml $CuSO_4$ and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) were challenged to 50°C (**Table 3-13a**) (**Figure 3-7a**) and 52°C (**Table 3-13b**) (**Figure 3-7b**) in NB pH 5.5.

After 30 and 60 minutes at 50°C, cells that were pre-exposed to NB pH 7.0 showed mean survival values of 1.36±0.20% and 0.23±0.012% respectively and when challenged at 52°C, only 0.3±0.01% and 0.04±0.003 % of these cells were able to form colonies after 30 and 60 minutes respectively.

Cells that were pre-exposed to NB pH 5.5 showed mean survival values of 1.58±0.12% and 0.43±0.22% after being challenged at 50°C for 30 and 60 minutes respectively. When challenged at 52°C, these cells gave a mean survival of 0.3±0.09% and 0.05±0.006% after 30 and 60 minutes.

When cells that were pre-exposed in NB plus 14.73 µg/ml $CuSO_4$ were challenged at 50°C, a mean survival of 14.7±2.5% and 4.1±0.58% was observed after 30 and 60 minutes respectively. After being challenged at 52°C for 30 and 60 minutes these cells showed mean survival values of 7.9±0.04% and 2.1±0.32% respectively.

The results above show that cells that were pre-exposed to the adaptive dose of copper were 18-fold and 9.5-fold more able (99% confidence) to survive temperature of

Table 3-13a Cross tolerance to 50° C in copper habituated *E.coli* 1829

Conditions of pre-exposure	Percentage of colony forming units (mean±S.E.M) after a challenge at 50°C for (mins):			
	15	30	45	60
pH 7.0 without copper(II) sulphate	2.90±0.60	1.36±0.20	0.36±0.06	0.23±0.01
pH 5.5 without copper(II) sulphate	3.07±0.96	1.58±0.12	1.13±0.15	0.43±0.22
pH 5.5 plus 14.73 µg/ml copper(II) sulphate	28.50±1.0	14.7±2.50	5.30±0.50	4.10±0.58

E.coli cells were grown in NB pH 7.0 to exponential phase and after one hour of pre-exposure under the stated conditions, were harvested and resuspended in NB pH 5.5 and challenged to 50°C for the stated times. Dilutions of samples at interval of times were plated on NA and incubated at 37°C for 18-24 hours. The survival values of the cells were expressed as a percentage of CFU/ml prior to challenge to heat. The results shown are mean values of three experiments.

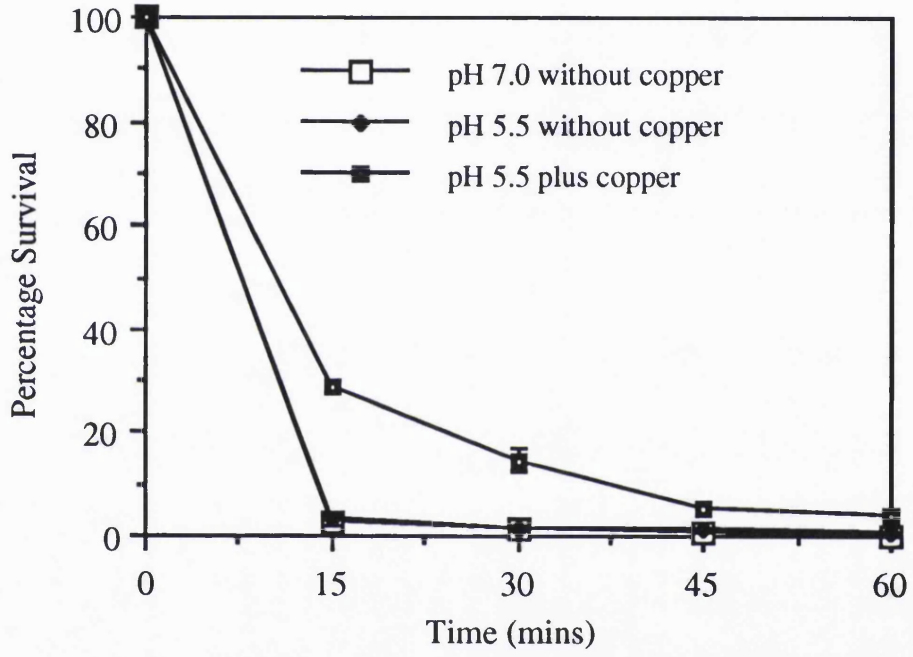


Figure 3-7 a Cross tolerance to 50 °C in copper habituated *E.coli* 1829 cells. Exponential phase *E.coli* cells were pre-exposed in NB pH 7.0 without copper, NB pH 5.5 without copper and NB plus copper (14.73 µg/ml $CuSO_4$) pH 5.5 for one hour at 37°C and then challenged in NB pH 5.5 at 50°C. At intervals of time samples were diluted and plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml prior to challenge with heat.

Table 3-13b Cross tolerance to 52°C in copper habituated *E.coli* 1829

Pre-exposure conditions	Percentage of colony forming units (mean±S.E.M) after challenge to 52°C for (mins):			
	15	30	45	60
pH 7.0 without copper(II) sulphate	1.40±0.01	0.30±0.01	0.07±0.003	0.04±0.003
pH 5.5 without copper(II) sulphate	1.70±0.15	0.30±0.09	0.12±0.02	0.05±0.01
14.73 µg/ml copper(II) sulphate (pH 5.5)	14.40±0.4	7.90±0.40	2.10±0.25	2.10±0.32

E.coli cells were grown in NB pH 7.0 to exponential phase and after one hour of pre-exposure under the stated conditions, they were harvested and resuspended in NB pH 5.5 and challenged to 52°C for the stated times. Dilutions of samples at intervals of times were plated on NA and incubated at 37°C for 18-24 hours.The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge to heat. The results shown are mean values of three experiments.

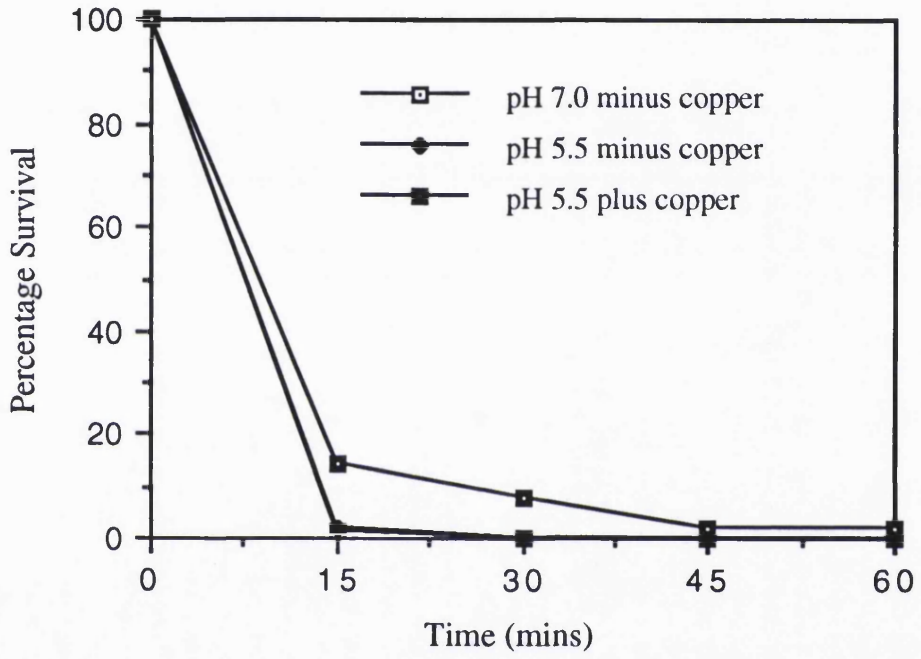


Figure 3-7 b Cross tolerance to 52 °C in copper habituated *E.coli* 1829 cells. Exponential phase *E.coli* cells were pre-exposed in NB pH 7.0 without copper, NB pH 5.5 without copper and NB plus copper (14.73 µg/ml $CuSO_4$) pH 5.5 for one hour at 37°C and then challenged in NB pH 5.5 at 52°C. At intervals of time samples were diluted and plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml prior to challenge with heat.

50°C after 60 minutes of challenge than cells that were pre-exposed to NB pH 7.0 and NB pH 5.5 respectively after the same challenge. There is no significant difference in survival at 50°C or 52°C in cells that were pre-exposed to NB pH 7.0 and NB pH 5.5 after a challenge for 30 and 60 minutes. Cells pre-exposed to copper survived 52 times and 42 times better at 52°C after 60 minutes of challenge than those cells that were pre-exposed to NB pH 7.0 and NB pH 5.5 after the same challenge. The results thus suggest that cells that received prior exposure to the sublethal dose of $CuSO_4$ exhibited enhanced tolerance to heat (50°C and 52°C) whereas cells that did not receive this pre-exposure hardly survive the heat.

3.2.2 The effect of pre-exposure to copper at 42°C on thermotolerance

E.coli cells that were pre-exposed to NB pH 7.0 plus 0.0 µg/ml $CuSO_4$, NB pH 5.5 plus 0.0 µg/ml $CuSO_4$ and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) at 42°C or 37°C, were challenged with 52°C for a duration of 60 minutes (**Table 3-14**) (**Figure 3-8**). Cells that were pre-exposed in NB pH 7.0 at 42°C showed mean survival values of 40.20±4.30%, 6.3±0.94%, 3.80±0.93% and 0.90+0.5% after a challenge for 15, 30, 45 and 60 minutes respectively whereas when the pre-exposure was carried out at 37°C only 1.40±0.12%, 0.3±0.02%, 0.07±0.003% and 0.04±0.003% cells were able to form colonies after the same challenge at the respective times. When cells that were pre-exposed to NB pH 5.5. at 42°C were given the same challenge for 15, 30, 45 and 60 minutes, 4.10±0.12%, 3.40±0.55%, 2.97±0.29% and 1.96±1.13% were able to survive respectively whereas those pre-exposed at 37°C showed mean survival values of 1.70±0.15%, 0.3±0.09%, 0.12±0.02% and 0.05±0.006% after the same challenge at the respective times. Cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ at 42°C showed survival values of 25.20±3.50%, 16.0±2.90%, 11.63±2.0% and 6.50±2.24% after a challenge at 52°C for 15, 30, 45 and 60 minutes respectively and when the pre-exposure was carried out at 37°C, 14.40±3.50%, 7.9±0.4%, 2.10±0.25% and 2.1±0.32% of the cells survived after the same challenge at the respective times.

The results above show that in the first 15 minutes of challenge, cells that were pre-exposed at 42°C in NB pH 7.0 show a 10-fold (99% confidence) and 1.6-fold increase (95% confidence) in survival than cells that were pre-exposed in NB pH 5.5 and

Table 3-14 Effect of copper habituation at 42°C on thermotolerance to 52°C in *E.coli* 1829

Conditions of pre-exposure		Percentage of colony forming units(mean±S.E.M) after a challenge at 52°C for (mins):			
		15	30	45	60
NB pH 7.0 without copper(II) sulphate at:	37°C	1.40±0.12	0.30±0.02	0.07±0.003	0.04±0.003
	42°C	40.20±4.30	6.30±0.94	3.80±0.93	0.90±0.50
NB pH 5.5 without copper(II) sulphate at:	37°C	1.70±0.15	0.30±0.09	0.12±0.02	0.05±0.006
	42°C	4.10±0.12	3.40±0.55	2.97±0.29	1.96±1.13
NB plus 14.73 µg/ml copper(II) sulphate (pH 5.5) at:	37°C	14.40±0.42	7.90±0.40	2.10±0.25	2.10±0.32
	42°C	25.20±3.50	16.20±2.90	11.63±2.0	6.50±2.24

E.coli cells were grown in NB pH 7.0 to exponential phase and after one hour of pre-exposure under the stated conditions,at 37°C or 42°C they were harvested and resuspended in NB pH 5.5 and challenged to 52°C for the stated times. Dilutions of samples at interval of times were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge to heat. The results shown are mean values of three experiments.

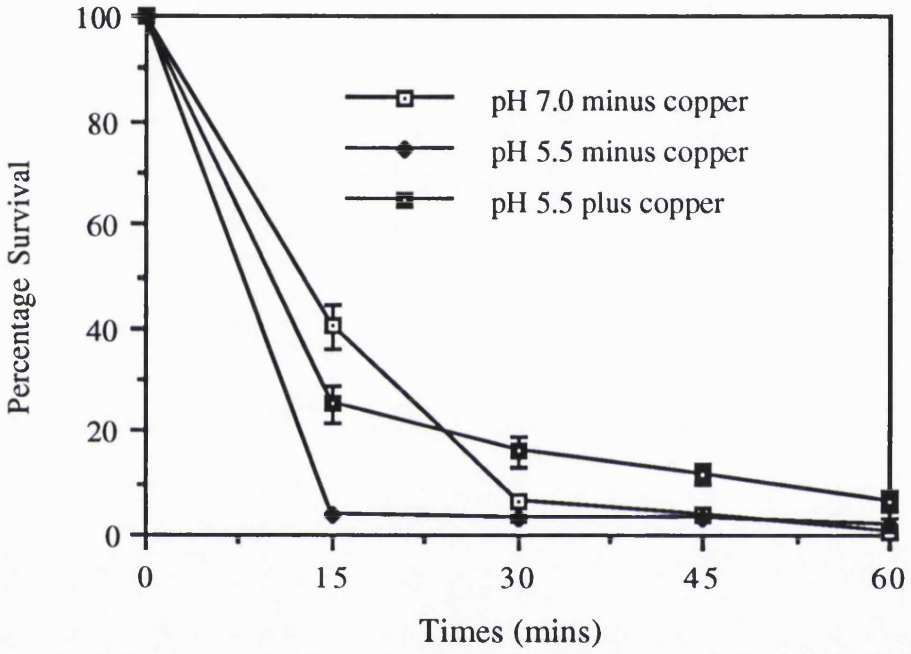


Figure 3-8 Effect of pre-exposure to copper at 42°C on thermotolerance to 52°C. Exponential phase *E.coli* cells were pre-exposed in NB pH 7.0 without copper, NB pH 5.5 without copper and NB plus copper (14.73 µg/ml $CuSO_4$) pH 5.5 for one hour at 42°C and then challenged in NB pH 5.5 at 52°C. At intervals of time samples were diluted and plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml prior to challenge with heat.

NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) respectively. However, after 30 minutes of challenge, cells that were pre-exposed in NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) at 42°C were 2.6-fold and 5-fold more able (95% confidence) to survive than cells that were pre-exposed to NB pH 7.0 and NB pH 5.5 at 42°C respectively. After 30 minutes of challenge the copper adapted cells were 3-fold and 4-fold more able (95% confidence) to survive than cells that were pre-exposed in NB pH 7.0 and NB pH 5.5 at 42°C respectively. At the end of the challenge, cells that were pre-exposed to copper at 42°C were 7-fold and 3-fold more able to survive than those that were pre-exposed to NB pH 7.0 and NB pH 5.5 at 42°C respectively.

In spite of the differences in the ability of the cells to survive the heat challenge, pre-exposure at 42°C enhanced the survival of the cells more than that at 37°C during the heat challenge; this is irrespective of whether they received prior exposure to copper or not. In cells that were pre-exposed to NB pH 7.0 the survival during the challenge is enhanced by 21-fold to 54-fold. In cells that were pre-exposed to NB pH 5.5 the survival is enhanced by 2-fold to 39-fold during the challenges. The survival in cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ is enhanced by 2-fold to 5-fold. This is notably much less than those cells that were pre-exposed in NB pH 7.0 and NB pH 5.5. When survival of copper adapted cells at 37°C were compared with those of the cells that were pre-exposed in NB pH 7.0 and NB pH 5.5 at 42°C after 30-60 minutes of challenge at 52°C, there was no significant difference between them. The thermotolerance exhibited by the copper adapted cells at 37°C is similar to that exhibited by cells that were pre-exposed in NB pH 7.0 and NB pH 5.5 at 42°C. Since it has been established that tolerance to lethal temperature is induced in cells that had prior exposure to 42°C (**Neidhardt and VanBogelen, 1987; Yamamori and Yura, 1982**) and this is conferred by heat shock proteins induced during the pre-exposure, the thermotolerance exhibited by the copper adapted cells at 37°C, could be due to similar proteins as these proteins are also known to be induced when cells encounter other stressful changes in the environment (**Christman *et al.*, 1985; Goff and Goldberg, 1985; Grossman *et al.*, 1985; Krueger and Walker, 1984;**). If the proteins induced during the pre-exposure period to copper at 37°C are actually heat shock proteins, then there should not be any difference in tolerance to the lethal temperature in the cells that were pre-exposed at 42°C in presence or absence of

copper. Since there are differences in survival in these cells, this is probably attributed to not only the heat shock proteins that were induced anyway at 42°C but also to the proteins that were induced due to the presence of the sub-lethal concentration of cupric sulphate. Another possibility would be, some proteins which are induced in the presence of the sublethal concentration of $CuSO_4$ are common to those induced during the temperature upshift.

3.2.3 Induced cross-tolerance to hydrogen peroxide in cells pre-exposed to copper

E.coli 1829 cells were challenged with 15 mM hydrogen peroxide (H_2O_2) after a pre-exposure period in NB pH 7.0 plus 0.0 µg/ml $CuSO_4$, NB pH 5.5 plus 0.0 µg/ml $CuSO_4$ and NB plus 14 73 µg/ml $CuSO_4$ (pH 5.5) for one hour at 37°C (**Table 3-15**). After a challenge for 10 and 20 minutes with H_2O_2, cells that were pre-exposed to NB pH 7.0 showed mean survival values of 36.12±3.73% and 1.71±0.57% respectively. Cells that were pre-exposed to NB pH 5.5, showed mean survival values of 54.90±9.5% and 3.05±0.76% respectively after the same challenge at the respective times. The cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ showed a mean survival of 69.30±2.6% and 4.55±1.30% after 10 and 20 minutes of challenge respectively with H_2O_2.

The results showed that in the first 10 minutes, cells that were pre-exposed in NB pH 5.5 and in NB plus copper were 1.5-fold (90% confidence) and 2-fold (99% confidence) more able to tolerate 15 mM H_2O_2 than cells that were pre-exposed to NB pH 7.0. However there is no significant difference in tolerance exhibited by cells that were copper adapted and cells that were pre-exposed in NB 5.5. After 20 minutes of challenge in H_2O_2 , there is no significant differences in the tolerances in all the cells . The cross tolerance to H_2O_2 was observed in the first 10 minutes of the challenge only in cells that were pre-exposed in NB pH 5.5 and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5). The tolerance to this oxidative agent disappears after this time. Since there is no significant difference in the tolerance showed by cells that were either pre-exposed in NB pH 5.5 or in NB 14.73 µg/ml $CuSO_4$ (pH 5.5) the cross tolerance exhibited by these cells was probably due to the mildly acidic pH of the medium they were pre-exposed in and not due to the presence of sublethal concentration of copper. Thus pre-exposure to sublethal concentrations of copper appears not to induce cross-tolerance to hydrogen peroxide.

Table 3-15 Comparison of tolerance to hydrogen peroxide in copper-habituated and non-habituated *E.coli* 1829.

Conditions of pre-exposure	Percentage of colony forming units (mean±S.E.M) after a challenge to 15mM H_2O_2 for (mins):	
	10	20
pH 7.0 without copper(II) sulphate	36.12±3.73	1.71±0.57
pH 5.5 without copper(II) sulphate	54.90±9.5 0	3.05±0.76
14.73 µg/ml copper(II) sulphate (pH 5.5)	69.30±2.60	4.55±1.30

E.coli cells were grown in NB pH 7.0 to exponential phase and after one hour of pre-exposure under the stated conditions, at 37°C they were harvested and resuspended in NB pH 5.5 containing 15 mM H_2O_2 and challenged for 20 minutes at 37°C.At intervals 1.0 ml samples of the cultures were neutralised with 8 µl of $1.89x10^5$ units/ml of catalase. Dilutions of samples at interval of times were plated on NA and incubated at 37°C for 18-24 hours.The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge. The results shown are mean values of four experiments.

3.2.4 Effect of copper pre-exposure on cross-tolerance to cadmium sulphate

E.coli 1829 cells that were pre-exposed to NB pH 5.5 and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) were challenged with NB plus 1.6 mg/ml and 2.0 mg/ml cadmium sulphate (pH 5.5) for a duration of 60 minutes (**Table 3-16**). Cells that were pre-exposed to NB pH 5.5 showed survival values of 7.84±0.42% and 2.90±0.32% after 30 and 60 minutes of challenge with 1.6 mg/ml cadmium sulphate and 1.73±0.64% and 0.44±0.10% after a challenge with 2.0 mg/ml cadmium sulphate for 30 and 60 minutes respectively. In contrast, when cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ were challenged with 1.6 mg/ml cadmium sulphate, 31.10±3.0% and 19.09±1.24% were able to form colonies after 30 and 60 minutes respectively. After a challenge with 2.0 mg/ml cadmium sulphate for 30 and 60 minutes, 20.53±1.19 % and 8.67±1.08% of these cells survived. The results showed that cells that were pre-exposed to copper were 3.9 and 6.6 times more able to survive the challenge with 1.6 mg/ml cadmium sulphate after 30 and 60 minutes respectively compare to those that were pre-exposed to NB pH 5.5. These cells were also 11.9 and 19.7 times more able to survive the challenge with 2.0 mg/ml cadmium sulphate after the respective times compare to those that were pre-exposed to NB pH 5.5.

Cadmium and solutions of its salts are toxic to bacterial cells (**Vallee and Ulmer, 1972**) and known to cause single-strand breakage in *Escherichia coli* DNA (**Mitra and Bernstein, 1978**). Unlike copper it has no known biological functions. The results above suggest that pre-exposure to sublethal concentration of cupric sulphate protects the cells from lethal effects of cadmium sulphate. The protection conferred as a result of pre-exposure to a sublethal concentration of $CuSO_4$ probably protects the cells' DNA.

3.2.5 Induced cross-tolerance to acid in copper induced and uninduced *E.coli* 1829, cells

E.coli cells that were pre-exposed in NB pH 7.0, NB pH 5.5 and NB plus 14.73 µg/ml cupric sulphate (pH 5.5) were challenged with NB pH 3.5 (**Table 3-17**). After 10, 20 and 30 minutes of challenge, cells that were pre-exposed in NB pH 7.0 showed mean survival values of 1.98±0.60% , 0.21±0.05%, and 0.017±0.001% respectively. Cells that were pre-exposed in NB pH 5.5 showed a mean survival of 7.50±1.04%, 2.60±0.35% and 1.26±0.46% after the same challenge at the respective times. When cells pre-exposed to

Table 3-16 Comparison of tolerance to cadmium sulphate in copper-habituated and non-habituated *E.coli* 1829

Conditions of pre-exposure	Cadmium sulphate concentrations used for challenge (mg/ml)	Percentage of colony forming units (mean±S.E.M) after a challenge to Cd^{2+} for (mins):	
		30	60
pH 5.5 without copper(II) sulphate	1.6	7.84±0.42	2.90±0.32
	2.0	1.73±0.64	0.44±0.10
14.73 μg/ml copper(II) sulphate (pH 5.5)	1.6	20.53±1.19	19.09±1.24
	2.0	20.53±1.19	8.67±1.08

E.coli cells were grown in NB pH 7.0 to exponential phase and, after one hour of pre-exposure under the stated conditions,at 37°C were challenged with NB plus 1.6 and 2.0 mg/ml cadmium sulphate (pH 5.5) for one hour at 37°C. Dilutions of samples at the stated intervals were plated on NA and incubated at 37°C for 18-24 hours.The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge. Results shown are mean values of three experiments.

Table 3-17 Comparison of tolerance to acid in copper-habituated and non -habituated *E.coli* 1829

Conditions of pre-exposure	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 3.5 for (mins):		
	10	20	30
pH 7.0 without copper(II) sulphate	1.98±0.60	0.21±0.05	0.017±0.001
pH 5.5 without copper(II) sulphate	7.50±1.04	2.60±0.35	1.26±0.46
14.73 µg/ml copper(II) sulphate (pH 5.5)	25.1±7.2	17.3±2.66	6.06±2.23

E.coli cells were grown in NB pH 7.0 to exponential phase and, after one hour of pre-exposure under the stated conditions, at 37°C were challenged with NB pH 3.5 at 37°C for a duration of 30 minutes. Dilutions of samples at the stated intervals were plated on NA and incubated at 37°C for 18-24 hours.The survival values of the cells were expressed as a percentage of CFU/ml prior to challenge. Results shown are mean values of three experiments.

14.73 µg/ml $CuSO_4$ were challenged with NB pH 3.5 a mean of 25.1±7.2%, 17.3±7.2% and 6.06±2.20% were able to form colonies after 10, 20 and 30 minutes of challenge respectively.

When ability of cells to survive the challenge at pH 3.5 were compared, cells pre-exposed in NB pH 5.5 were 3.4-fold (99% confidence), 12.3-fold (99% confidence) and 74.1-fold (95% confidence) more able to form colonies than cells pre-exposed in NB pH 7.0 after 10, 20, 30 minutes of the challenge.Cells that were pre-exposed to 14.73 µg/ml cupric sulphate were 12.7-fold (95% confidence), 82.4-fold (99% confidence) and 356.4% (90% confidence) more able to survive than cells pre-exposed in NB pH 7.0 after 10, 20, 30 minutes of challenge with pH 3.5. The copper induced cells are also 3.3-fold (90% confidence), 6.6-fold (99% confidence)and 4.8 fold (90% confidence) more able to form colonies than cells that were pre-exposed in NB pH 5.5 after the same challenge at the respective times.

The results above showed that pre-exposure to NB pH 5.5 alone induced tolerance to pH 3.5 and the tolerance is enhanced (3.3 to 6.6-fold) in presence of 14.73 µg/ml $CuSO_4$. The effect of prior exposure to mild acidic pH on tolerance to lethal acidic pH has been first reported in *Escherichia coli* by Goodson and Rowbury (**1989a**) and in *Salmonella typhimurium* by Foster and Hall (**1990**). Thus cells that were pre-exposed to 14.73 µg/ml cupric sulphate exhibit cross tolerance to pH 3.5. The tolerance shown by these cells may be attributed to the mild acidic nature of $CuSO_4$ in solution and also to the presence of Cu^{2+}.

3.2.6 Induced cross tolerance to alkali in copper induced and uninduced cells

E.coli 1829 cells that were pre-exposed to NB pH 7.0 plus 0.0 µg/ml $CuSO_4$, NB pH 5.5 plus 0.0 µg/ml $CuSO_4$ and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) were challenged with NB pH 10.0 (**Table 3-18**). After 30 minutes of challenge, cells that were pre-exposed to NB pH 7.0, NB pH 5.5 and NB pH plus 14.73 µg/ml $CuSO_4$ (pH 5.5) showed mean survival values of 10.96±1.04%, 0.03±0.006% and 2.58±0.46% CFU/ml respectively. The cells that were pre-exposed to pH 7.0 were 356 and 4.2 times more able to form colonies than cells that were pre-exposed to NB pH 5.5 and NB plus 14.73 µg/ml $CuSO_4$ (pH 5.5) respectively. The cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ were 86 times more

Table 3-18 Comparison of tolerance to alkali in copper-habituated and non-habituated *E.coli* 1829

Conditions of pre-exposure	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 10.0 for 30 minutes
NB pH 7.0 without copper(II) sulphate	10.96±1.04
NB pH 5.5 without copper(II) sulphate	0.03±0.01
NB plus 14.73 µg/ml copper(II) sulphate (pH 5.5)	2.58±0.46

E.coli cells were grown in NB pH 7.0 to exponential phase and, after one hour of pre-exposure under the stated conditions,at 37°C were challenged with NB pH 10.0 at 37°C for a duration of 30 minutes. Dilutions of samples at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survivals of the cells were expressed as a percentage of CFU/ml prior to challenge. Results shown are mean values of three experiments.

able to form colonies than cells that were pre-exposed to NB pH 5.5 only.

The results indicate three things, firstly, cells that were pre-exposed to 14.73 µg/ml $CuSO_4$ were more tolerant to lethal alkaline pH when compared to cells that were pre-exposed to NB pH 5.5 only. Secondly, cells that were pre-exposed to pH 7.0 were more able to resist lethal alkaline pH than cells pre-exposed at pH 5.5 with or without copper and thirdly pre-exposure to pH 5.5 which induces acid tolerance appears to also induce sensitivity to lethal alkaline pH.

3.3 INDUCTION OF ALKALI SENSITIVITY IN *ESCHERICHIA COLI*

3.31 Introduction

The maintenance of intracellular pH within physiological values at varying external pH is vital for bacterial growth. However, tolerances to lethal pH values can be induced if bacterial cells were given the opportunity to adapt or habituate to the sublethal pH values prior to exposure to the lethal pH values and these inducible tolerances to lethal acid and alkaline pH values have been demonstrated in *E.coli* (**Goodson and Rowbury, 1989a; 1989b**) and *S.typhimurium* cells (**Foster and Hall, 1990**). The pre-exposure to mildly acidic or alkaline pH does not only induce tolerance to lethal acid and alkaline pH values respectively but also induces cross-tolerances to other stresses such as H_2O_2, heat, high concentration of NaCl, and UV radiation (**Goodson and Rowbury, 1990; Foster and Hall 1990; Goodson and Rowbury, 1991; Leyer and Johnson, 1993**). However, Rowbury *et al.*, (**1993**) have shown that prior exposure to sublethal alkaline pH value (pH 9.0) which induces tolerance to lethal alkaline pH can also induce cross-sensitivity to lethal acidic pH. Since pre-exposure to mildly acidic pH can induce tolerance to lethal acidic pH, the response of these acid induced cells to lethal alkaline pH was investigated. This section describes the study on alkali sensitivity induction (ALSI) in *Escherichia coli.*

3.3.2 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829

Exponential cells of *E.coli* 1829 in NB pH 7.0 were transferred to NB pH 7.0 and NB pH 5.5 and grown for a further one hour. After these pre-exposures the cells were challenged with NB pHs 9.5, 9.75,and 10.0. After 30 minutes of challenges with pHs 9.5, 9.75 and 10.0, cells that were pre-exposed to pH 7.0 showed mean survival values of 68±2.32%, 22.0±2.10% and 10.96±1.04% respectively whereas cells that were pre-exposed to pH 5.5 showed mean survival values of 2.10±0.55%, 0.73±0.41% and 0.03±0.006% after these respective challenges (**Table 3-19**). The cells that were pre-exposed to pH 5.5 were markedly sensitised by 32-fold, 30-fold and 365-fold when compared with cells that were pre-exposed at pH 7.0 after challenges with pHs 9.5, 9.75 and 10.0 respectively. The sensitization is significant at 99% confidence. Pre-exposure to pH 5.5 has therefore induced sensitivity to alkaline pH and since this pre-exposure was only for one hour, the induced alkali sensitivity response observed here is most probably due to phenotypic effect

Table 3-19 Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5

Pre-exposure pHs	Percentage of colony forming units (mean±S.E.M) after a challenge for 30 minutes with pH :		
	9.5	9.75	10.0
7.0	68.10±2.32	22.0±2.10	10.96±1.04
5.5	2.10±0.55	0.73±0.41	0.03±0.006

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and pH 5.5 at 37°C, were challenged with NB pHs 9.5, 9.75 and 10.0 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

as the duration of pre-exposure was too short for selection of alkali sensitive mutants.

3.3.3 Comparison of sensitivity to alkali in cells induced at pHs 5.0. 5.5 and 6.0

Exponential cells of *E.coli* 1829, in NB pH 7.0 were transferred to NB pHs 5.0, 5.5 and 6.0 and grown for a further hour. As a control, the cells were also transferred to NB pH 7.0 and given this pre-exposure for one hour. After these pre-exposures the cells were challenged with NB pH 9.5 for 30 minutes. The cells that were pre-exposed to pHs 5.0, 5.5 and 6.0 showed mean survival values of 9.60±0.43%, 2.10±0.55% and 3.23±0.70% respectively after the alkali challenge (**Table 3-20**). The survival showed by these respective cells were 7-fold, 32-fold and 21-fold (99% confidence) less than those pre-exposed at NB pH 7.0. The cells that were pre-exposed at pH 5.0 were 4.6-fold and 3-fold (99% confidence) more able to survive the lethal alkaline pH than cells that were pre-exposed at pHs 5.5 and 6.0 respectively. The results showed that the sensitization to alkaline pH was very marked at pH 5.5 and 6.0 but was less at pH 5.0. The reason as to why cells that were pre-exposed to pH 5.0 did not so strongly conform to the trend observed is not known; probably, sensitization to alkali is inducible only in a narrow range of mildly acidic pH.

3.3.4 Kinetics of the induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829

Table 3-21 shows the rate at which alkali sensitivity is induced. After 15, 30 and 60 minutes of pre-exposure at pH 5.5, followed by challenges with pH 9.5, 36.70±4.8%, 12.10±0.98% and 2.10±0.98% of the cells were able to form colonies. The ability to form colonies dropped by 3-fold and 17-fold after 30 and 60 minutes of pre-exposure at pH 5.5 respectively. On the contrary, there are no significant differences in the survival in cells that were pre-exposed at pH 7.0 at the respective times. Cells that were pre-exposed at pH 5.5 for 60 minutes showed a 32-fold reduction in survival when compared with cells that were pre-exposed at pH 7.0 after the alkali challenge. The results thus indicate that cells that are pre-exposed at pH 5.5 are fully sensitised after a pre-exposure of 60 minutes.

Table 3-20 Comparison of induction of sensitivity to alkali at pHs 5.0, 5.5 and 6.0 in *E.coli* 1829

Pre-exposure pHs	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	68.10±2.32
6.0	3.23±0.70
5.5	2.10±0.55
5.0	9.60±0.43

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB at stated pHs at 37°C, were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments

Table 3-21 Kinetics of induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829

Pre-exposure pHs	Pre-exposure time (mins)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	15.0	51.40±4.10
	30.0	56.00±1.90
	60.0	68.10±2.30
5.5	15.0	36.70±4.80
	30.0	12.10±0.98
	60.0	2.10±0.55

E.coli cells were grown to exponential phase and, after pre-exposure in NB pHs 7.0 and 5.5 at 37°C for the stated times, were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

3.3.5 Comparison of the effects of NaOH and KOH as alkalinising agents for alkali challenge in pH 5.5 induced cells of *E.coli* 1829

The effects of using NaOH and KOH as alkalinising agents were compared (**Table 3-22**). Cells that were pre-exposed to pHs 7.0 and 5.5 were challenged with NB pH 9.5 that was alkalinised with either NaOH or KOH. There were no significant differences in the survival of the cells that were pre-exposed to pH 7.0 after a challenge with pH 9.5 alkalinised with either NaOH or KOH; results for six experiments showed mean survival values of 53.45±4.60% and 52.90±2.40% after a challenge with NB pH 9.5 that was alkalinised with either NaOH or KOH respectively. Sensitivity was observed in cells that were pre-exposed to pH 5.5 irrespective of whether the challenge medium was alkalinised with NaOH or KOH. These cells were 8 and 2-fold (99% confidence) more sensitised when compared with the pH 7.0 induced cells after a challenge with NB pH 9.5 that was alkalinised with NaOH and KOH respectively. The pH 5.5 induced cells that were challenged with NB pH 9.5 alkalinised with NaOH, were 3.5-fold less able (95% confidence) to form colonies than those that were challenged with NB pH 9.5 alkalinised with KOH. The presence of Na^+ in NB pH 9.5 proved to be more lethal to the cells that were pre-exposed to pH 5.5 than the presence K^+ at the challenge pH. Thus the inability to cope with presence of Na^+ at alkaline pH is probably one of the main reasons for alkali sensitization in pH 5.5 induced cells. This inability could be due to the lack of sodium antiporters in these acid-induced cells

3.3.6 Effect of acetate and benzoate on induction of alkali sensitivity at pH 6.5 in *E.coli* 1829

Benzoic acid and sodium acetate in solution are weak organic acids that will only cross the membranes in their neutral, protonated forms. Once in the cell, these acids will equilibrate between the protonated and ionized states releasing H^+ thus lowering the internal pH (pH_i). To investigate whether internal acidification is involved in the induction of alkali sensitivity, sodium acetate or benzoic acid was added during the induction at pH 6.5. The internal pH of these cells in the presence of these weak acids should approximately equal that of the external pH which is lower than the physiological pH values (pH 7.0-7.4).

Table 3-22 Comparison of the effects of NaOH and KOH as alkalizing agents for alkali challenge in pH 5.5 induced *E.coli* 1829

Pre-exposure pHs	Alkalinizing agent	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	NaOH	53.45±4.60
	KOH	52.90±2.40
5.5	NaOH	6.48±1.73
	KOH	22.80±5.22

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C, were challenged for 30 minutes with NB pH 9.5 alkalized either by NaOH or KOH . Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of six experiments.

Tables 3-23a and **b** show the effects of acetate and benzoate respectively on the induction of alkali sensitivity. The results show that cells pre-exposed to pH 6.5 did not exhibit any sensitization to alkali, that is, there is no significant difference in survival between cells pre-exposed to pH 6.5 and cells pre-exposed to pH 7.0. The presence of acetate however, markedly sensitized the cells to alkali; the sensitization was increased by 1.4 (95% confidence) and 2.2-fold (99% confidence) after pre-exposures in the presence 15.0 and 30.0 mM sodium acetate respectively. Similarly, the presence of 10.0 mM benzoic acid sensitized the cells considerably by 7-fold (99% confidence).

The effect of these weak acids on alkali sensitization indicates that internal acidification is required before sensitization to alkali is induced. Thus the presence of protons in the cytoplasm is a prerequisite for induction of alkali sensitivity. This can be further confirmed by carrying out the pre-exposure at pH 5.5 in the presence of phosphate which has been shown to competitively inhibit the entry of protons into the cells.

3.3.7 Effect of phosphate on induction of alkali sensitivity at pH 5.5 in *E.coli* 1829

E.coli cells grown to exponential phase were pre-exposed in NB pHs 7.0 and 5.5 in the presence or absence of phosphate before challenging with NB pH 9.5. The results from **Table 3-24** show that pre-exposure in the presence of phosphate at pH 7.0 has a slight effect on survival at pH 9.5. The cells pre-exposed in this condition are 1.3 times less able (99% confidence) to survive than cells pre-exposed at pH 7.0 without phosphate. Cells pre-exposed at pH 7.0 in the presence of phosphate are 1.9-fold more able to survive (99% confidence) in pH 9.5 than cells pre-exposed at pH 5.5 in presence of phosphate. In the absence of phosphate at pH 7.0 cells were 25-fold more able to survive than cells pre-exposed to pH 5.5 under the same conditions. The presence of phosphate during pre-exposure at pH 5.5 however, decreased the sensitization to alkali by 9.9-fold (99 % confidence) when compared to the pH 5.5. cells that were pre-exposed in the absence of phosphate.

The indication that phosphate reduces sensitization of pH 5.5 cells implies that hydrogen ions are needed for induction of sensitivity to alkali and this is in accordance with the requirement of H^+ for the induction (**3.3.7**). The presence of phosphates thus stopped the H^+ from reaching the induction sensor that could be either in the periplasmic

Table 3.23a Effect of acetate on the induction of alkali sensitivity at pH 6.5 in *E.coli* 1829

Pre-exposure pHs	Acetate (mM)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	0.0	60.80±6.50
6.5	0.0	54.20±3.94
	15.0	38.00±4.30
	30.0	24.20±5.22

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 6.5 at 37°C , in absence or presence acetate, were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3.23b Effect of benzoate on the induction of sensitivity to alkali at pH 6.5 in *E.coli* 1829

Pre-exposure pHs	Benzoic acid (10 mM)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	52.10±5.25
6.5	-	50.50±4.50
	+	7.24±3.46

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 6.5 at 37°C in absence or presence of benzoate,were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-24 Effect of phosphate on the induction of alkali sensitivity at pH 5.5 in *E.coli* 1829

Pre-exposure pHs	Phosphate (10 mM)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	60.50±1.70
	+	47.20±4.20
5.5	-	2.43±0.99
	+	24.10±5.90

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in absence or presence of phosphate,were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of six experiments.

space or cytoplasmic membrane and consequently reducing the sensitization to alkali. It has been shown that phosphates competitively inhibit the passage of the H^+ at the PhoE pore (**Rowbury *et al.*, 1992**). The 1.9-fold reduction in survival of the alkali treated pH 7.0 cells that were pre-exposed to phosphates, could be due to the phosphates competitively inhibiting the passage of a co-repressor that uses this pore by phosphates.

3.3.8 Effect of alkali treatment on the β-galactosidase activity in *E.coli* 1829 *F'lac*

Since β-galactosidase can be easily assayed, it is a useful indicator in testing the effect of alkali treatment on enzymic activity. The effect of alkali treatment on β-galactosidase activity in pHs 7.0 and 5.5 cells were studied in the 1829 derivative that harbours the *lac* gene. Exponential cells of *E.coli* 1829 *F'lac* were pre-exposed to pHs 7.0 and 5.5 and after 30 minutes of the pre-exposures, β-galactosidase was induced by the addition of 1mM IPTG and pre-exposures in the presence of the inducer were continued for another 30 minutes before challenging with pH 9.75. Results from three experiments showed that the mean β-galactosidase activity present in pH 7.0 and pH 5.5 induced cells after the challenge with pH 9.75 were 430.87±13.78 and 235.7±12.32 Miller units respectively (**Table 3-25**). Assuming that the enzyme activity present prior to alkali challenge to be 100% activity, only 50.87±1.76 and 30.04±1.87% of the enzyme in the pH 7.0 and pH 5.5 induced cells respectively were still active after the alkali challenge. The results suggest that enzymic activity and probably other non-structural proteins of pH 5.5 induced cells are more prone to damage than pH 7.0 induced cells.

If the enzyme is induced after pH 7.0 and 5.5 induced cells had the alkali treatment it could indicate the damage incurred in the macromolecules involved in transcription and translation of this enzyme. The following section describes the effect of alkali challenge on β-galactosidase activity induction.

3.3.9 Induction of β-galactosidase activity in alkali treated *E.coli* 1829 F'Lac cells.

Exponential cells of *E.coli* 1829 F'*lac* cells in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging with NB pHs 7.0 or 9.5. After 30 minutes of the challenges, the cells were harvested, washed, resuspended in NB pH 7.0 and IPTG was added to induce the expression of β-galactosidase. The β-

Table 3-25 Effect of alkali challenge on β-galactosidase activity in pH 5.5 induced cells of *E.coli* 1829 *F'lac*

pH at induction	β-galactosidase activity (Miller units) (mean±S.E.M):	
	prior to challenge	after challenge at pH 9.75 for 30 minutes
7.0	846.55 ±2.69	430.87±13.78
5.5	787.60±8.77	235.70±12.32

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 in the presence of IPTG were challenged with NB pH 7.0 or NB pH 9.75 for 30 minutes. β-galactosidase activity were measured before and after challenge to assess damage by alkali treatment to this enzyme. Results shown are mean values of three experiments.

galactosidase activity measured after 30 minutes of induction in presence of IPTG (**Table 3-26**) showed that the enzyme activities induced after the challenge with pH 9.5 in cells that were pre-exposed to pHs 7.0 and 5.5 were 228.10±11.49 and 58.17±21.12 Miller units respectively. The results showed that after the alkali challenge, cells that were pre-exposed to pH 7.0 were 3.9-fold (99% confidence) more able to express the induced β-galactosidase than cells that were pre-exposed to pH 5.5 cells. Assuming that the β-galactosidase expressed in the respective pH 7.0 challenged cells represents 100% enzyme activity, the enzymic activity expressed in pH 7.0 and pH 5.5 induced cells after the alkali challenge were 82.86% and 17.66% of total enzyme activity respectively.

Since β-galactosidase was induced after the alkali challenge, the enzyme activity expressed reflects the extent of the DNA, RNA or ribosomes damage since the induction of β-galactosidase involves transcription of specific mRNA which is translated into β-galactosidase monomers which are then assembled into active oligomers (**Miller and Reznikoff, 1978**). The results therefore indicate that all or one of these components (i.e DNA, mRNA or ribosomes) are markedly damaged during the alkali challenge in pH 5.5 induced but only slightly damaged cells in the pH 7.0 induced cells.

3.3.10 Effect of alkali challenge on DNA in pH 5.5 induced cells of *E.coli* 1829 ColV I-K94, pBR322.

Exponential cells of *E.coli* 1829 ColV I-K94, pBR322 in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging with NB pHs 7.0 and 9.5 for 30 minutes. The plasmid DNA from the challenged cultures was extracted and electrophoresed on an agarose gel (**Figure 3-9a**). Lanes 1 and 2 show the DNA extracted from pH 7.0 induced cells after a challenge with pH 7.0 (control) and pH 9.5 respectively. There is no apparent difference in the intensity of the plasmid DNA bands of the ColV and pBR322 between the control and the alkali challenged cells. Lanes 3 and 4 show the DNA extracted from pH 5.5 induced cells after a challenge with pH 7.0 (control) and pH 9.5 respectively. As can be seen, the plasmid DNA ColV and pBR322 of the alkali challenged cells were totally destroyed and markedly damaged respectively unlike the pH 7.0 alkali challenged cells.

For a quantitative comparison, a densitometer scanning of the negative of the

Table 3-26 Induction of β-galactosidase activity in alkali challenged cells of *E.coli* 1829 *F'lac*

pH at induction	β-galactosidase activity (mean±S.E.M of Miller units) induced in cells challenged at pH:	
	7.0	9.5
7.0	275.27±4.57	228.10±11.49
5.5	329.37±22.30	58.17±21.12

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 7.0 or NB pH 9.5 for 30 minutes. The challenged cells were then induced for β-galactosidase activity using IPTG. Results shown are mean values of three experiments.

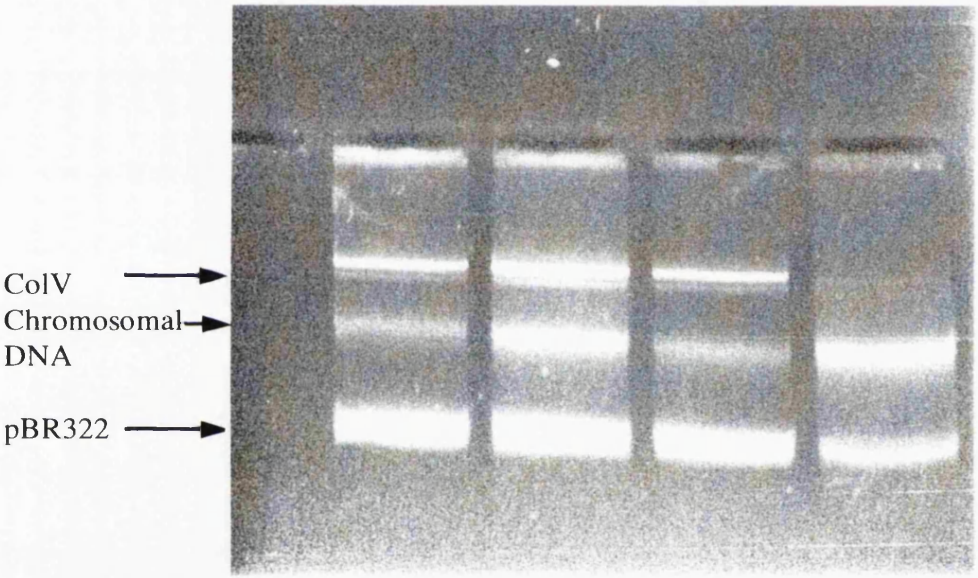


Figure 3-9a Effect of alkali challenge on DNA in pHs 7.0 and 5.5- induced cells of *E.coli* I-K94, ColV pBR322 cells. **Lane 1**: DNA from pH 7.0 induced cells that were treated with NB pH 7.0 for 30 minutes; **Lane 2** DNA from pH 7.0 induced cells that were treated with NB pH 9.75 for 30 minutes; **Lane 3** DNA from pH 5.5 induced cells that were treated with NB pH 7.0 for 30 minutes; **Lane 4** DNA from pH 5.5 induced cells that were treated with NB pH 9.75 for 30 minutes. DNA was extracted using Method 2 (**2.25.2**)

photographed gel was performed (**Figure 3-9b**). The area of each peak in relation to total area of peaks in each lane determined by the densitometer is shown in **Table 3-27**. It can be seen that the relative area of pBR322 peaks in the pH 5.5 induced cells that were challenged with pHs 7.0 (lane 3) and 9.5 (lane 4) were 60.8% and 35.6% respectively whereas the relative area of this peak in the pH 7.0 induced cells that were challenged with pHs 7.0 (lane 1) and 9.5 (lane 2) were 70.3 and 51.9% respectively. The relative area of the ColV,I-K94 peaks in the pH 7.0 induced cells that were challenged with pHs 7.0 (lane 1) and 9.5 (lane 2) were 20.0% and 18.8% respectively whereas the relative area of this peak in the pH 5.5 induced cells that were challenged with pHs 7.0 (lane 3) and 9.5 lane 4) were 19.4 and 0.1%. Thus the alkali treatment in pH 7.0 induced cells destroyed 26.17 and 6.0% of the pBR322 and ColV respectively whereas in pH 5.5 induced cells the alkali treatment destroyed 41.12% and 100% of pBR322 and ColV respectively.

Although the method of DNA extraction employed was for plasmid DNA (method 2; **2.24.2**), there were also traces of chromosomal DNA in the extraction samples as can be seen in **Figure 3-9a**. Interestingly, the alkali treated samples have the most traces of chromosomal DNA compared to the non-alkali treated samples; 29.3% and 64.3% of DNA extracted from the alkali challenged pH 7.0 and pH 5.5 induced cells respectively were chromosomal DNA compare with the respective 9.7 and 19.7% of DNA extracted from pH 7.0 challenged cells. The trace amounts of chromosomal DNA present in the pH 7.0 challenged cells is due to some of the large fragments of chromosomal DNA nicked during lysis were further cut during the lysing incubation period and owing to their small size were not pelleted out with other large fragments of chromosomal DNA that were nicked or still attached to the cytoplasmic membrane and other cell debris. The presence of large traces of chromosomal DNA in the alkali challenged cells especially the pH 5.5 induced cells which has 1.7-fold more chromosomal DNA present than pH 7.0 induced cells could be due to a combination of small proportion of small DNA fragments being generated during lysis and larger proportion being generated during the alkali challenge.

The results thus show that the DNA of the pH 5.5 induced cells is more susceptible to damage by alkali than the DNA of pH 7.0 induced cells. This is also in accordance with the results obtained in **3.3.9**

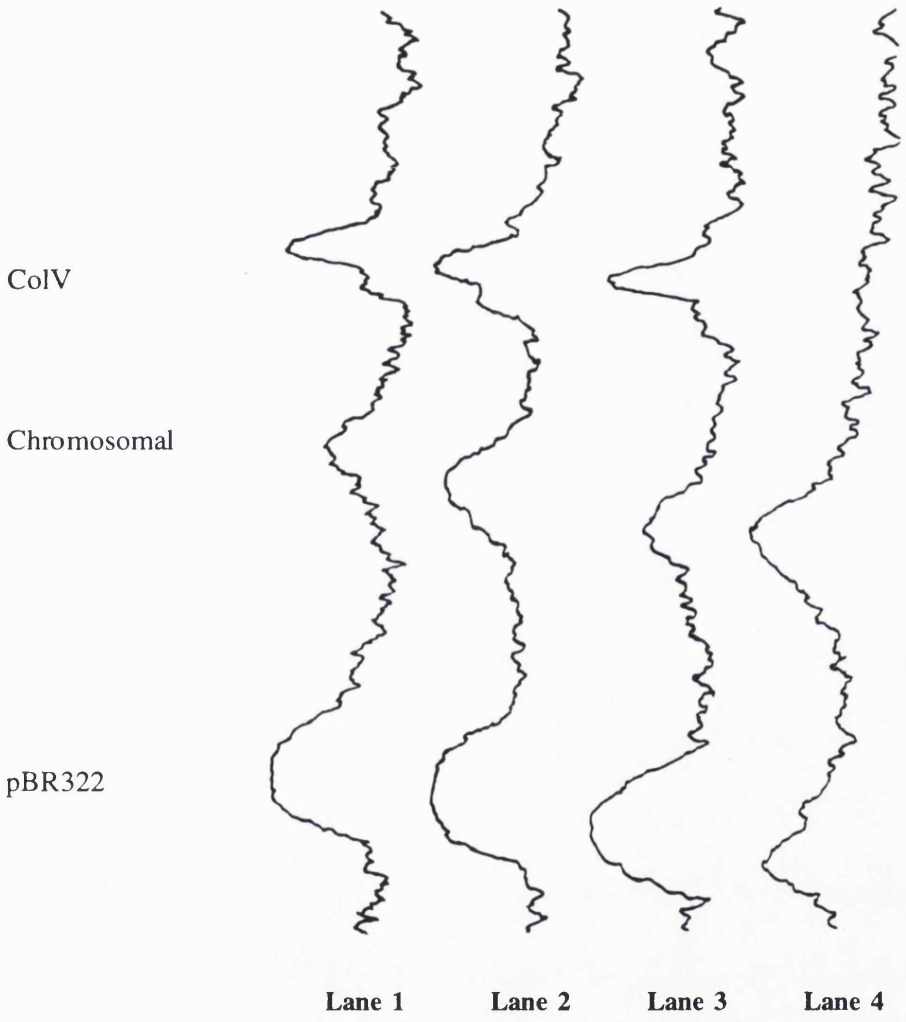


Figure 3-9b A densitometer scanning of DNA bands from **Figure 3-9b**. The peaks in each lane represent the different DNA as shown above. **Lane 1**: DNA from pH 7.0 induced cells that were treated with NB pH 7.0 for 30 minutes; **Lane 2** DNA from pH 7.0 induced cells that were treated with NB pH 9.75 for 30 minutes; **Lane 3** DNA from pH 5.5 induced cells that were treated with NB pH 7.0 for 30 minutes; **Lane 4** DNA from pH 5.5 induced cells that were treated with NB pH 9.75 for 30 minutes.

Table 3-27 Analysis of relative area of DNA bands using the densitometer scanner

Lane	Treatment	DNA	Relative area (%)
1	DNA from pH 7.0 induced cells	ColV Chromosomal pBR322	20.0 9.7 70.3
2	DNA from pH 7.0 induced cells treated with alkali	ColV Chromosomal pBR322	18.8 29.3 51.9
3	DNA from pH 5.5 induced cells	ColV Chromosomal pBR322	19.4 19.8 60.8
4	DNA from pH 5.5 induced cells treated with alkali	ColV Chromosomal pBR322	0.1 64.3 35.6

DNA samples from the above treatments were electrophoresed on agarose gel and photographed. Quantitative analysis of the bands were carried out using a densitometer. Relative area represents area of each peak as percentage of the total area of the three peaks in each lane (**Figure 3-9a**).

3.3.11 The effect of protein synthesis inhibitors on the induction of alkali sensitivity in pH 5.5 induced cells of *E.coli* 1829

The effects of chloramphenicol and tetracycline on the induction of alkali sensitivity at pH 5.5 respectively were investigated. Exponential cells of *E.coli* 1829 were pre-exposed at pHs 7.0 and 5.5 in the presence or absence of chloramphenicol or tetracycline before challenging with pH 9.5. After 30 minutes of alkali challenge, the cells that were induced in pHs 7.0 and 5.5 in the absence of chloramphenicol showed mean percentage survival values of 65.70±1.50 and 1.45±0.21 CFU/ml respectively (**Table 3-28a**). The pH 5.5 cells were sensitised by 45-fold when compared with the pH 7.0 induced cells after the alkali challenge. In the presence of chloramphenicol, the sensitization in the pH 5.5. cells was totally abolished; the pH 5.5 cells were instead 1.2-fold (99% confidence) more able to survive compare to the pH 7.0-induced cells.

Similarly, when tetracycline was present during the pre-exposure period, the sensitization to alkali in pH 5.5 was also totally abolished (**Table 3-28b**). There was no significant difference between the pHs 7.0 and 5.5 cells that were induced in the presence of tetracycline after the alkali challenge whereas in the absence of this protein inhibitor, the pH 5.5 cells were sensitized by 31-fold when compared to the pH 7.0 induced cells. Chloramphenicol and tetracycline inhibit protein synthesis by preventing the binding of aminoacyl-tRNA to the A site of the active ribosome (**Bryan, 1982**). The former also inhibits peptide bond formation. The results indicate that proteins synthesized during the pre-exposure at pH 5.5 confer sensitivity to the cells when challenged with the lethal alkaline pH and in the presence of chloramphenicol and tetracycline, which inhibit the synthesis of these proteins, the sensitization is abolished. Thus *de novo* synthesis of alkali sensitization components is required for the induction of alkali sensitivity response and this synthesis is being induced as a result of pre-exposure to pH 5.5. These alkali sensitization components probably enhanced the cells' sensitivity to pH 9.5. The 1.8-fold reduction in survival in cells pre-exposed to pH 7.0 in the presence of chloramphenicol cells suggest that some of the alkali sensitization repressor components may also be synthesized during the pre-exposure period at pH 7.0 although results with tetracycline suggest otherwise.

Table 3-28a Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5; effect of the presence of chloramphenicol

Pre-exposure pHs	Chloramphenicol (200.0 µg/ml)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	65.70±1.50
	+	36.40±1.20
5.5	-	1.45±0.21
	+	45.40±1.20

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in presence or absence of chloramphenicol were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments

Table 3-28b Induction of sensitivity to alkali in *E.coli* 1829, at pH 5.5; effect of the presence of tetracycline

Pre-exposure pHs	Tetracycline (5.0 µg/ml)	Percentage of colony forming units (mean ±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	64.30±2.50
	+	55.0±7.64
5.5	-	2.05±0.50
	+	40.40±2.30

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB at the stated pHs at 37°C, in the presence of 5.0 µg/ml tetracycline were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

3.3.12 Effect of RNA polymerase and DNA gyrase inhibitors on induction of alkali sensitivity in pH 5.5 induced cells of *E.coli* 1829

The effects of the presence of rifampicin, nalidixic acid, novobiocin and coumermycin A1 on the induction of alkali sensitivity in pH 5.5 induced cells were investigated (**Table 3-29 a-d**). Cells were pre-exposed at pHs 7.0 and 5.5 in the absence or presence of the RNA polymerase and DNA gyrase inhibitors and then challenged with pH 9.5.

When rifampicin, the RNA polymerase inhibitor, was present during the pre-exposure to pH 5.5, the cells were 23 times more able to survive the challenge than the pH 5.5 cells that were pre-exposed in the absence of this inhibitor (**Table 3-29a**). The presence of rifampicin during exposure to pH 7.0 however did not have any effect on the survival at pH 9.5. The results show that sensitization at pH 9.5 in cells pre-exposed to pH 5.5 was dependent on transcription of the alkali sensitivity genes as in presence of rifampicin, a RNA polymerase inhibitor, sensitization was abolished.

The presence of 10 μg/ml and 20 μg/ml nalidixic acid, a DNA gyrase inhibitor, during the pre-exposure at pH 5.5 (**Table 3-29b**) enabled the cells to survive the challenge 8.8-fold and 33-fold more, respectively, when compared with the pH 5.5 cells that were induced in the absence of this inhibitor. The presence of 10 μg/ml and 20 μg/ml nalidixic acid during pre-exposure at pH 7.0 reduces survival by 2.1-fold and 3.1-fold respectively (99% confidence) when compared to the pH 7.0 cells that were induced in the absence of this inhibitor after the alkali challenge. Pre-exposure to pH 5.5 in the presence of 2.5 μg/ml and 5.0 μg/ml novobiocin, another DNA gyrase inhibitor, enabled the cells to survive 11.6-fold and 22-fold better, respectively, than those pre-exposed at pH 5.5 without the inhibitors after the same challenge (**Table 3-29c**). The pre-exposure at pH 5.5 in the presence of coumermycin, also a DNA gyrase inhibitor, increased the survival at pH 9.5 by 10.7-fold when compared to the pH 5.5 cells that were pre-exposed in the absence of this inhibitor (**Table 3-29d**). The presence of this inhibitor at pH 7.0 appears to reduce the survival at pH 9.5 by 1.6-fold when compared to those pH 7.0 cells that were induced in the absence of this inhibitor.

Nalidixic acid, novobiocin and coumermycin A1 inhibit DNA gyrase activity which is involve in introducing negative supercoiling in DNA and changes in supercoiling have been shown to affect gene expression. The results suggests that DNA in its supercoiled

Table 3-29a Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5; effect of the presence of rifampicin

Pre-exposure pHs	Rifampicin (30.0 μg/ml)	Percentage of colony forming units(mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	60.90±4.90
	+	48.50±7.50
5.5	-	2.70±1.60
	+	62.60±7.70

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C, in presence or absence of rifampicin were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-29b Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5; effect of the presence of nalidixic acid

Pre-exposure pHs	Nalidixic acid (μg/ml)	Percentage of colony forming units (mean ±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	0.0	51.00±6.40
	10.0	24.30±2.95
	20.0	18.70±2.75
5.5	0.0	1.30±0.30
	10.0*	11.50±2.28
	20.0	43.10±9.10

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C, in presence nalidixic acid at the stated concentrations were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of six experiments.
* Results are mean values of four experiments.

Table 3-29c Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5; effect of the presence of novobiocin

Pre-exposure pHs	Novobiocin (μg/ml)	Percentage of colony forming units (mean ±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	0.0	49.70±1.40
	2.5	65.60±5.20
	5.0	65.2±5.40
5.5	0.0	0.98±0.13
	2.5	11.45±3.8
	5.0	21.70±0.86

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in absence or presence of nalidixic acid were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-29d Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5; effect of the presence of coumermycin A1

Pre-exposure pHs	Coumermycin A1 (30 µg/ml)	Percentage of colony forming units (mean ±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	70.70±3.50
	+	44.95±1.96
5.5	-	4.05±2.40
	+	43.30±5.50

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in the presence or absence of coumermycin A1, were challenged with NB pH 9.5 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

state must be maintained for the expression genes that code for the alkali sensitization components. The presence of these inhibitors either reduces the supercoiling in DNA or relaxes it completely. However the transcription genes that repress alkali sensitivity at pH 7.0 are only mildly affected by these changes in supercoiling.

3.3.13 Comparison of cytoplasmic membrane proteins from pHs 7.0 and 5.5 induced cells

Cytoplasmic membrane protein samples from pH 7.0 and pH 5.5 induced cells were isolated and analysed on a monodimensional SDS-PAGE electrophoresis. The contaminating major outer membrane porins present in these extracts showed that higher levels of OmpC are present in pH 5.5 than in pH 7.0 in accordance with the results of Heyde and Portalier (**1987**) (**Figure 3-10**). Further inspection of the gel also showed two polypeptides of molecular weights 18 kDa and 14 kDa were overexpressed (arrows) in pH 5.5 induced cells but not pH 7.0, the former more markedly expressed than the latter. Further work on two-dimensional protein analysis will have to be done to determine the presence of other proteins involved in alkali sensitization that were not detected on the one-dimensional protein analysis system

3.3.14 The involvement of the outer membrane components in the induction of alkali sensitivity at pH 5.5

The roles of outer membrane components in the induction of alkali sensitivity were investigated by using mutants of outer membrane proteins that were isolated as described in **2.28**

3.3.14.1 Induction of alkali sensitivity at pH 5.5 in E.coli strain lacking the PhoE pore

Exponential phase cells of *E.coli* 1157-4 *phoE*$^+$ and *E.coli* 1157 *phoE* that were grown in NB pH 7.0 were transferred to NB pH 7.0 and NB pH 5.5 and grown for a further one hour before challenging with NB pH 9.75 for 30 minutes. The results from 3 experiments (**Table 3-30**) show that the pH 7.0 and pH 5.5 induced cells of *E.coli* 1157-4 *phoE*$^+$ gave mean survival values of 3.06±2.12% and 8.90±3.40% respectively after the alkali

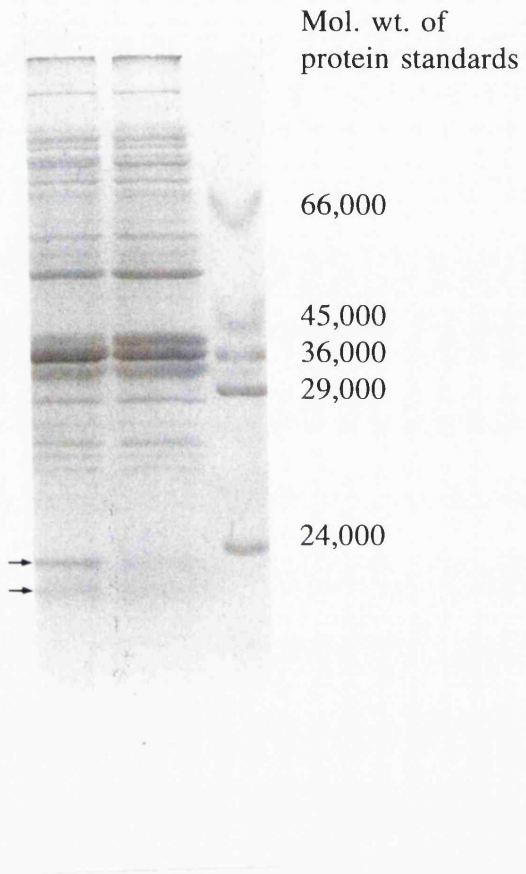


Figure 3-10 SDS polyacrylamide gel electrophoresis of cytoplasmic membrane proteins from pHs 7.0 and 5.5 induced *E.coli* 1829 cells. **L1** Cytoplasmic membrane proteins from *E.coli* 1829 cells that were pre-exposed in NB pH 5.5 for one hour; **L2** Cytoplasmic membrane proteins from *E.coli* 1829 cells that were pre-exposed in NB pH 7.0 for one hour; **L3** Molecular weights of protein standards.

Table 3-30 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1157-4 *phoE*$^+$ and *E.coli* 1157 *phoE*

pH at induction	Regulatory component (genotype)	Percentage Survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*phoE*$^+$	3.06±2.12
	phoE	8.90±3.40
5.5	*phoE*$^+$	4.88±1.90
	phoE	7.86±1.89

E.coli 1157-4 *phoE*$^+$ and *E. coli* 1157 *phoE* cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

challenge. When pH 7.0 and pH 5.5 induced cells of *E.coli* 1157 *phoE* were given the same challenge, 8.90±3.40% and 7.86±1.89% of the cells respectively were able to form colonies.

The results above suggest that alkali sensitivity was not induced in the pH 5.5 pre-exposed cells of *E.coli* 1157-4 *phoE*$^+$ and 1157 *phoE*. Indeed there is no significant difference in the survival of pH 7.0 and pH 5.5 induced cells of *E.coli* 1157-4 *phoE*$^+$ after the alkali challenge. Likewise, pH 7.0 and pH 5.5 cells of *E.coli* 1157 *phoE* showed no significant differences in sensitivity to alkali.

The absence of induced alkali sensitivity in the *E.coli* strain that lacks the *phoE* gene contradicts the results in **3.3.7** which showed that the presence of phosphate which competitively inhibits the passage H^+ through the PhoE pore abolishes the sensitization. From the results in **3.3.7**, it was concluded that presence of H^+ ions intracellularly is needed for the induction of alkali sensitivity as it was implicated in **3.3.6** that the alkali sensitization is induced due to a fall in the internal pH. Thus it should follow that a mutation in the PhoE pore should abolish the alkali sensitivity. The fact that this was not the case does not invalidate the findings in **3.3.6** and **3.3.7** as the results above could be attributed to the genetic background of the *phoE*$^+$ and *phoE* strains used. This could have been overcome by transducing the deletion into *E.coli* 1829 or selecting *phoE* mutants by using phages that utilise PhoE as receptors. Unfortunately neither suitable strains carrying this deletion nor phages utilising this pore as receptors, were available during the course of this work. This would be more comparable with the findings in **3.3.6** and **3.3.7** obtained by using *E.coli* 1829.

3.3.14.2 Induction of alkali sensitivity in E.coli 1829 lacking the outer membrane porin C (OmpC)

Exponential cells of *E.coli* 1829 *ompC*$^+$ and *E.coli* 1829 *ompC* were transferred to NB pHs 7.0 and 5.5 NB pHs and grown for a further one hour before challenging with NB pH 9.75. **Table 3-31** shows that the pH 7 and 5.5 induced cells of *E.coli* 1829 *ompC*$^+$ gave mean survival values of 24.46±2.37% and 0.62±0.21% respectively after a challenge with pH 9.75 for 30 minutes. When pH 7.0 and pH 5.5 induced cells of *E.coli* 1829-*ompC* were challenged with pH 9.75 for 30 minutes, 23.60±4.22% and 7.18±0.95% of the cells

Table 3-31 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *ompC*$^+$ and 1829 *ompC*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge for 30 minutes at pH 9.75
7.0	*ompC*$^+$	24.46±2.37
	ompC	23.60±4.20
5.5	*ompC*$^+$	0.62±0.21
	ompC	7.18±0.95

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml attime zero. Results shown for challenge with pH 9.75 are mean values of three experiments.

respectively were able to form colonies. The results show that the pH 5.5 induced cells of *E.coli* 1829-*ompC*$^+$ and *E.coli* 1829 *ompC* were sensitised by 39.5-fold and 3.3 fold respectively when compared to the pH 7.0 induced cells. Thus the absence of OmpC in pH 5.5 cells reduced the sensitization by 11.6-fold when compared with pH 5.5 cells that were OmpC$^+$. As OmpC allows non-specific passage of cations across the outer membrane (1.3.1.3.3a), these results suggest that OmpC is probably one of the routes used for proton passage for the induction of alkali sensitivity.

3.3.14.3 Induction of alkali sensitivity at pH 5.5 in E coli 1829 lacking the porin LamB

Exponential cells of *E.coli* 1829 *lamB*$^+$ and *E.coli* 1829 *lamB* grown in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging with NB pH 9.75 for 30 minutes. The pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 *lamB*$^+$ showed mean survival values of 17.93±1.69% and 1.25±0.32% respectively after the alkali challenge for 30 minutes (**Table 3-32**). When pH 7.0 and pH 5.5 induced cells of *E.coli* 1829-*lamB* were given the same challenge, 16.12±2.32% and 3.29±0.52% of the cells were able to form colonies. The results showed that the pH 5.5 induced cells of *E.coli* 1829 *lamB*$^+$ and *E.coli* 1829 *lamB* were sensitised by 14.3-fold (99% confidence) and 4.9-fold (99% confidence) respectively by the alkali challenge when compared with the respective pH 7.0 induced cells. The absence of the LamB in pH 5.5 cells reduced the sensitization by 2.9-fold when compared with pH 5.5 LamB$^+$ cell (95% confidence) suggesting that the mutation of the *lamB* gene reduces the sensitization slightly. Since LamB is a diffusion channel it is possible that protons that are required for the alkali induction can also use this pore to cross the outer membrane.

3.3.14.4 Induction of alkali sensitivity at pH 5.5 in E coli 1829 lacking the outer membrane protein A (OmpA)

Table 3-33 shows the alkali sensitivity in pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 *ompA*$^+$ and *E.coli* 1829 *ompA*. After a challenge with pH 9.75 for 30 minutes the pH 7.0 and pH 5.5 induced cells of *E.coli* 1829-*ompA*$^+$ showed mean survival values of 20.19±2.12% and 2.32±0.20% respectively. The pH 7.0 and pH 5.5 induced cells of *E.coli*

Table 3-32 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *lamB*$^+$ and 1829 *lamB*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*lamB*$^+$	17.90±1.69
	lamB	16.0±2.32
5.5	*lamB*$^+$	1.25±0.32
	lamB	3.29±0.5

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18 to 24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-33 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *ompA*$^+$ and 1829 *ompA*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*ompA*$^+$	20.19±2.12
	ompA	7.51±1.12
5.5	*ompA*$^+$	2.30±0.20
	ompA	0.86±0.37

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 9.75 in the absence or presence of 1mM of amiloride for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18 to 24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of five experiments.

1829-*ompA* showed mean survival values of 7.51±1.120% and 0.86±0.37% respectively after the same challenge. The results show that the pH 5.5 induced cells of *E.coli* 1829 *ompA*$^+$ and *E.coli* 1829 *ompA* were sensitized by 8.8-fold and 8.7-fold (99% confidence) respectively when compared with respective pH 7.0 induced cells. However the absence of OmpA porin appeared to enhance sensitization to alkali in pH 5.5 induced cells by 2.7-fold (95% confidence) when compared with pH 5.5 *ompA*$^+$ cells. The lack of OmpA also reduced the survival at pH 9.75 by 2.7-fold (99% confidence) when compared with pH 7.0 *ompA*$^+$ cells. Since OmpA is involved in maintaining the integrity of the cell envelope, the enhancement of alkali sensitization is probably due to the lack of this integrity which results in excess Na^+ and OH^- crossing the outer membrane thus increasing the lethal effects of these ions.

3.3.15 Involvement of the *envZ* gene product in the induction of alkali sensitivity at pH 5.5

The EnvZ protein is known to be involved in sensing changes in osmolarity and also pH in the periplasm.and transmitting these changes to a regulator via phosphorylation and dephosphorylation processes. The involvement of this gene product as a pH sensor was investigated. Exponential cells of *E.coli* MC4100 pAlS10 *envZ*$^+$ and AT142 pALS10 *envZ* were induced in pH 5.5 and pH 7.0 and then challenged with pH 9.75 for 30 minutes. The pH 7.0 and pH 5.5 *envZ*$^+$ induced cells showed mean survival values of 54.6±0.7% and 15.1±1.53% respectively (**Table 3-34**). The deletion in *envZ* in pH 7.0 and pH 5.5 induced cells, gave mean survival values of 65.17±3.55% and 15.33±1.08% respectively. The results showed that the *E.coli envZ*$^+$ and *envZ* induced at pH 5.5 were sensitized by 3.6-fold and 4.25-fold respectively when compared with the respective pH 7.0 induced cells. Also there is no significant difference between the alkali sensitization in pH 5.5 *envZ*$^+$cells and pH 5.5 *envZ* cells. Since the *envZ* cells have no effect on alkali sensitization it appears that EnvZ is not involved as the pH sensor in the induction of alkali sensitization. The results also indicate that either other sensors might be involved in detecting changes in pH in the periplasm or pH changes in the periplasm do not act as the signal for induction of alkali sensitization. The results with acetate and benzoate suggest that the latter is the case.

Table 3-34 Induction of alkali sensitivity at pH 5.5 in *E.coli* MC4100 pALS10 *envZ*$^+$ and *E.coli* AT142 pALS10 *envZ*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*envZ*$^+$	54.60±0.70
	envZ	65.17±3.55
5.5	*envZ*$^+$	15.1±1.53
	envZ	15.33±1.08

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18 to 24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

3.3.16 Involvement of the cytoplasmic membrane protein TonB in the induction of alkali sensitivity at pH 5.5.

The TonB protein acts as an energy transducer especially in the uptake of Fe^{3+} - siderophores and vitamin B12. Possible TonB involvement in the induction of alkali sensitivity was investigated using *E.coli* H2300 a strain that carries a deletion in this gene, The results from **Table 3-35** show that pH 7.0 and pH 5.5 induced cells of *E.coli* AB2847 *tonB*$^+$ gave mean survival values of 44.53±4.09% and 1.22±0.67% respectively after a challenge with pH 9.75 for 30 minutes. The pH 5.5 cells were markedly sensitised by 36.5-fold when compared to the pH 7.0 induced cells. When pH 7.0 and pH 5.5 induced *E.coli* H2300 *tonB* cells were challenged with pH 9.75, 3.79±1.82% and 9.90±4.66% respectively of the cells were able to form colonies. There is no significant difference in survival between the pH 7.0 and pH 5.5 induced *tonB* cells. The results showed that deletion of *tonB* gene derepressed the sensitization by 11.7-fold (99% confidence) in the pH 7.0 induced cells. However the deletion in this gene did not abolish alkali sensitization in pH 5.5 induced cells. The results suggest that TonB is repressing the alkali sensitivity induction in pH 7.0 cells or more likely that TonB is involved in energising the uptake of a component acting as a co-repressor of this induction.

3.3.17 Involvement of sodium antiporters in the induction of alkali sensitivity at pH 5. 5

The Na^+/H^+ antiporter has been implicated in the regulation of cytoplasmic pH at alkaline pH since electrogenic extrusion of Na^+ is coupled with H^+ uptake and therefore there is the potential to neutralise the cytoplasm to its physiological pH values (**Booth, 1985; Zilberstein *et al.*, 1984; McMorrow *et al.*,1989**). The involvement of these antiporters were studied in *E.coli* strains carrying deletions in these genes.

3.3.17.1 Effect of deletion in nhaA gene on induction of alkali sensitivity at pH 5.5

Exponential cells of *E.coli* TA15 *nhaA*$^+$, NM18 *nhaA*, 1829 *nhaA*$^+$ and 1829 *nhaA* were transferred to NB pHs 7.0 and 5.5 and further grown for one hour. After this induction period, they were challenged with NB pH 9.75 for 30 minutes. The *E.coli* strain TA15

Table 3-35 Induction of sensitivity to alkali at pH 5.5 in *E.coli* AB2847 *tonB*$^+$ and *E.coli* H2300 *tonB*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*tonB*$^+$	44.53±4.09
	tonB	3.79±1.82
5.5	*tonB*$^+$	1.22±0.67
	tonB	9.90±4.66

E.coli strains stated above were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments

nhaA$^+$ that was induced at pHs 7.0 and 5.5 showed mean survival values of 21.16±1.82% and 1.59±0.56% respectively (**Table 3-36**). The NM81 *nhaA* cells that were induced at pHs 7.0 and 5.5 showed mean survival values of 45.78±4.59% and 17.23±1.95% respectively. The results showed that *E.coli* TA15 *nhaA*$^+$ that were induced at pH 5.5 were sensitized by 13.3-fold (99% confidence) when compared to those induced at pH 7.0 after the alkali challenge. The deletion in the *nhaA* in the NM81 pH 5.5 cells however reduced the sensitization by 10.8 fold when compared to TA15 pH 5.5 cells. The deletion in *nhaA* also increased the mean percentage survival by 2.2-fold in pH 7.0 induced cells. When *nhaA* deletion is transduced into *E.coli* 1829 strain, alkali sensitization in the pH 5.5 induced cells was reduced by 9.9-fold when compared with pH 5.5 *nhaA*$^+$. The deletion also increased survival in the pH 7.0 cells by 2.1-fold (**Table 3-36**)

If sensitization to alkali in pH 5.5 induced cells is due to lack of NhaA antiporters, then the deletion in *nhaA* would enhance the sensitization in these cells and also reduce the survival in the pH 7.0 induced cells but instead, this deletion reduces the alkali sensitization in the pH 5.5 cells and increases survival in the pH 7.0 induced cells in both *E.coli* NM81 and 1829 strains. Thus the sensitization in pH 5.5 cells does not appear to be due to lack of the NhaA antiporter. Since cytoplasmic acidification is required for induction of alkali sensitization, (**3.3.6**), it is very likely that NhaA is involved in the passage of protons across the cytoplasmic membrane during the induction and it follows that in the absence of this antiporter alkali sensitization is abolished.

3.3.17.2 Effect of deletion of the nhaB gene on induction of alkali sensitivity at pH 5.5.

Exponential cells of *E.coli* TA15 *nhaB*$^+$ and *E.coli* EP431 *nhaB* grown in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging with NB pH 9.75 for 30 minutes. The pH 7.0 and pH 5.5 induced cells of TA15 showed mean survival values of 16.75±1.0% and 2.01±0.37% respectively after a challenge with pH 9.75 for 30 minutes (**Table 3-37**). When pH 7.0 and pH 5.5 induced cells of EP431 were challenged with pH 9.75 mean values of 36.93±6.02% and 16.95±3.41% respectively of the cells were able to form colonies. The results show that the pH 5.5 induced cells of TA15 were sensitized by 8.3-fold (99.9% confidence) by the alkali treatment when

Table 3-36 Induction of sensitivity to alkali at pH 5.5 in *E.coli* strains lacking the sodium antiporter NhaA

pH at induction	Strain	Regulatory component (genotype)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	TA15	*nhaA*$^+$	21.16±1.82
	NM81	*nhaA*	45.78±4.59
	1829	*nhaA*$^+$	18.5±1.54
	1829	*nhaA*	38.10±4.37
5.5	TA15	*nhaA*$^+$	1.59±0.56
	NM81	*nhaA*	17.23±1.95
	1829	*nhaA*$^+$	1.96±0.47
	1829	*nhaA*	19.40±3.07

E.coli strains stated above were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments .

Table 3-37 Induction of alkali sensitivity at pH 5.5 in *E.coli* TA15 *nhaB*$^+$ and *E.coli* EP431 *nhaB*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*nhaB*$^+$	16.75±1.0
	nhaB	36.93±6.02
5.5	*nhaB*$^+$	2.01±0.37
	nhaB	16.85±3.41

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18 to 24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of four experiments.

compared with the pH 7.0 induced cells . The deletion in *nhaB* in pH 5.5 induced cells reduced the sensitization by 8.4 fold when compared with the pH 5.5 *nhaB*$^+$cells. The deletion of the *nhaB* gene also increases the survival of pH 7.0 induced cells by 2.2-fold (95% confidence). Like NhaA, NhaB is also implicated in the regulation of cytoplasmic pH at alkaline pH although its activity is lower than NhaA at high pH. Since the absence of NhaB enhances survival in both pH 5.5 and pH 7.0 cells this suggests that the sensitization is not due to the lack of this antiporter. The abolishment of alkali sensitization at pH 5.5 in the absence of NhaB is probably due to the reduced acidification of the cytoplasm by protons and this is only possible if the NhaB is used by protons to cross the cytoplasmic membrane. The involvement of NhaB can be further confirmed using amiloride which competitively inhibits the exchange of sodium ions for H^+ at the NhaB pore.

3.3.17.2.1 *Effect of amiloride on induction of sensitivity to alkali at pH 5.5 in E.coli 1829*

The effect of amiloride on induction of alkali sensitivity was examined. Exponential cells of *E.coli* 1829, were pre-exposed for one hour in NB pHs 7.0 and 5.5 in presence or absence of amiloride before challenging with NB pH 9.5 for 30 minutes. There is no significant difference in survival after a challenge with pH 9.5 in cells that were pre-exposed to NB pH 7.0 in absence or presence of amiloride (**Table 3-38a**). The mean survival for five experiments were 61.46±3.13% and 56.60±3.60% for cells pre-exposed in NB pH 7.0 in presence and absence of amiloride respectively. Similarly there are no significant differences in survival after a challenge with pH 9.5 between cells that were pre-exposed at pH 5.5 in the presence or and in the absence of amiloride; the mean survival values being 1.77±0.77% and 0.99±0.70% for pH 5.5 cells pre-exposed in presence and absence of amiloride respectively. The results indicate that the reduced activity of NhaB by amiloride has no effect on the induction to alkali sensitivity. This contradicts with the results obtained in **3.3.17.2** which suggest that NhaB is involved in the passage of protons. If NhaB acts as a passage for protons, then, in the presence of amiloride the sensitization should be reduced, but this was not the case. The amiloride may indicate that NhaB is not involved in the proton passage and the discrepancy in the

Table 3-38a Effect of amiloride on the induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829

Pre-exposure pHs	Amiloride (1.0 mM)	Percentage of colony forming units(mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	61.46±3.13
	+	56.60±3.60
5.5	-	1.77±0.77
	+	0.99±0.70

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C in absence or presence of amiloride, were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of five experiments.

results could be due to genetic background of the EP431 *nhaA*. The transduction of the *nhaB* gene into the *E.coli* 1829 would have to be carried out to confirm the role of NhaB in the induction of alkali sensitivity at pH 5.5 .

3.3.17.2.2 *The effect of presence of amiloride during alkali challenge in pH 5.5 induced cells*

In a separate experiment, the effect of the presence of amiloride during alkali challenge in cells induced at pH 5.5 was studied. Exponential cells of *E.coli* 1829 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour. These cells were then challenged with NB pH 9.75 plus or minus amiloride. Results from seven experiments (**Table 3-38b**) showed that after 30 minutes of challenge with NB pH 9.75 minus amiloride, cells that were induced at pH 7.0 showed a mean survival value of 22.70±2.6% whereas cells that were induced at pH 5.5 showed a mean survival value of 0.26±0.04%. When the challenge was carried out in presence of amiloride, cells that were induced at pH 7.0, showed a mean survival value of 6.08±1.95% whereas cells that were induced at pH 5.5 showed a mean survival value of 0.38±0.05%. In the absence of amiloride at challenge, cells that were induced at pH 5.5 were 128-fold more sensitised than with cells that were induced at pH 7.0. In the presence of amiloride at challenge, cells that were induced at pH 5.5 were only 16-fold more sensitised (99% confidence) than cells induced at pH 7.0. However, cells that were induced at pH 7.0 were sensitised by 3.7 -fold in the presence of amiloride at challenge (99% confidence) whereas cells that were induced at pH 5.5 showed no significant difference.

The results suggest that NhaB is required to reduce the lethal effects of alkaline pH as the lack of this pore sensitized the pH 7.0 induced cells. Thus it is probable that the lack of this antiporter in pH 5.5 cells is one of the causes of the induction of the alkali sensitization and accordingly the presence of amiloride at challenge did not have any significant effect. However these results contradict with those in **3.3.17.2** which suggest that the alkali sensitization at pH 5.5 was due to the presence of NhaB. The differences in results could be due to either the genetic background of the *E.coli* EP431 *nhaA* or that there are of other unidentified antiporters which are affected by amiloride that are involved in the induction of alkali sensitivity at pH 5.5. However as mentioned above, a

Table 3-38b Effect of the presence of amiloride at alkali challenge in pH 5.5 induced *E.coli* 1829 cells

Pre-exposure pHs	Percentage of colony forming colony units (mean ±S.E.M) after a challenge with pH 9.75 for 30 minutes	
	minus amiloride	plus amiloride
7.0	23.00±1.07	8.38±1.70
5.5	0.18±0.04	1.40±0.80

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH5.5 at 37°C were challenged with NB pH 9.75 in the absence or presence of amiloride for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of seven experiments.

deletion of *nhaB* will have to be transduced into *E.coli* 1829 to confirm these results.

3.3.17.3 Effect of NaCl on alkali sensitivity at pH 5.5

The sodium antiporters can be induced in the presence of NaCl **(Padan and Schulidiner, 1994a,b).** The effect of the induction of these antiporters by NaCl on alkali sensitivity was investigated. **Table 3-39** shows the results of the effect of NaCl (200 mM) on induction of alkali sensitivity at pH 5.5. In the absence of NaCl the pHs 7.0 and 5.5 induced cells showed mean survival values of 25.58±0.88% and 3.02±0.24% after the alkali challenge with the pH 5.5 induced cells being sensitised by 8.5-fold (99% confidence). However when NaCl is present during induction the sensitization to alkali is completely abolished; there is no significant difference in alkali sensitivity between when pHs 7.0 and 5.5 cells were induced in the presence of NaCl. The presence of NaCl may induce sodium/proton antiporters which may aid survival at alkaline pH or NaCl may induce an adaptive response that will cross-protect the pH 5.5 cells to lethal effects of pH 9.75.

3.3.18 The effect of glucose on the expression of alkali sensitization components at pH 5.5 in *E.coli* 1829.

Exponential cells were pre-exposed at pHs 7.0 and 5.5 in presence or absence of 1% glucose before challenging with pH 9.5. The results in **Table 3-40** show that pre-exposure in the presence of 1% glucose at pH 7.0 has only a slight effect on the survival after a challenge with pH 9.5. These cells were 1.4-fold less able (90% confidence) to survive than cells pre-exposed at pH 7.0 without glucose. On the contrary, the presence of glucose during pre-exposure at pH 5.5 did not have any significant effect on the induction of alkali sensitivity. Glucose brings about catabolite repression and in its presence the syntheses of a variety of unrelated enzymes are inhibited due to the low levels of cAMP. Cyclic AMP has been shown to be a key element of a variety of control systems in bacteria as it promotes the binding of RNA polymerase to some promoter sites on the DNA. The results indicate that the alkali sensitivity genes that are transcribed during pre-exposure at pH 5.5 are not affected by glucose and therefore not under the control of cAMP.

Table 3-39 Effect of sodium chloride (NaCl) on the induction of alkali sensitivity at pH 5.5 in *E.coli* 1829

Pre-exposure pHs	NaCl (200mM)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	-	25.58±0.88
	+	26.32±1.25
5.5	-	3.02±0.24
	+	23.17±1.98

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in absence or presence of 200 mM NaCl, were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA andincubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-40 Induction of sensitivity to alkali in *E.coli* 1829 at pH 5.5 in the presence of glucose

Pre-exposure pHs	Glucose (1%)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	-	56.10±7.20
	+	41.50±4.09
5.5	-	4.60±2.20
	+	6.90±2.20

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in absence or presence of glucose were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of five experiments.

3.3.19 The involvement of global regulatory proteins in the induction of alkali sensitivity at pH 5.5

Some of the regulatory proteins involve in the stress responses are involve : in the regulation of various genes coding for proteins of related and unrelated functions. The role of some of the known global regulatory proteins was investigated as discussed below.

3.3.19.1 Involvement of Fur protein in induction of alkali sensitivity at pH 5.5

Table 3-41 show the effect of deletion in the *fur* gene on the induction of alkali sensitivity. The pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 *fur*$^+$ showed mean survival values of 30.20±2.52% and 1.25±0.35% respectively after a challenge with pH 9.75 for 30 minutes The *fur*$^+$ cells that were induced at pH 5.5 were sensitised by 24.2-fold (95% confidence) after the challenge when compared with the pH 7.0 cells. The pH 7.0 and pH 5.5 induced cells having a deletion in the *fur* gene showed mean survival values of 29.0±2.21% and 6.46±2.20% respectively after 30 minutes of challenge with pH 9.75. The deletion in this gene reduced the sensitization in pH 5.5 cells by 5.2-fold (95% confidence) when compared with the pH 5.5 *fur*$^+$ cells. The results suggest that *fur* acts as an activator of the expression of some of the alkali sensitization components and accordingly, in the absence of *fur* the sensitization is reduced. Fur generally requires metal ions such as Fe^{2+} as co-factors for its activation. The effect of the presence of $FeCl_3$ and $FeSO_4$ on induction of alkali sensitivity at pH 5.5 were studied in *E.coli* 1829 *fur*$^+$

3.3.19.1.1 Effect of ferric chloride on induction of alkali sensitivity at pH 5.5 in E.coli 1829

Table 3-42 shows the effect of ferric chloride on induction of alkali sensitivity in *E.coli* 1829. *E.coli* cells grown to an exponential phase, were pre-exposed in NB pHs 7.0 and 5.5 before challenging with NB pH 9.75 for 30 minutes. After the alkali challenge, cells that were induced in pHs 7.0 and 5.5 in the absence of $FeCl_3$ show mean survival values of 20.2±1.13% and 1.79±0.26% respectively. When cells that were induced in pHs 7.0 and 5.5 in presence of $FeCl_3$, were challenged with pH 9.75, 16.46±0.82% and 5.06±0.42% of the cells respectively were able to survive.

The results show that the pH 5.5 cells that were induced in the absence of $FeCl_3$

Table 3-41 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *fur*$^+$ and *E.coli* 1829 *fur*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*fur*$^+$	30.20±2.52
	fur	29.0±2.21
5.5	*fur*$^+$	1.25±0.35
	fur	6.46±2.20

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of four experiments.

Table 3-42 Effect of ferric chloride on the induction of alkali sensitivity at pH 5.5 in *E.coli* 1829

Pre-exposure pHs	Ferric chloride (1.0 mM)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	-	20.20±1.13
	+	16.46±0.82
5.5	-	1.74±0.26
	+	5.06±0.42

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in absence or presence of ferric chloride,were challenged with NB pH 9.75 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA andincubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

were sensitised by 11.6-fold (99% confidence) when compared to pH 7.0 cells under the same conditions but in the presence of $FeCl_3$ the sensitization in the pH 5.5 cells was reduced by 3.5-fold (99% confidence). The results suggest the $FeCl_3$ may be involved in repressing the expression of some of the alkali sensitive components by inactivating the function of Fur. Thus in the presence of excess Fe, the function of Fur as an activator of alkali sensitivity genes is repressed.

3.3.19.1.2 *Effect of ferrous sulphate on induction of alkali sensitivity at pH* 5.5 in *E.coli* 1829

Table 3-43 shows the effect of ferrous sulphate on induction of alkali sensitivity at pH 5.5 in *E.coli* 1829. In the absence of this salt, pH 7.0 and pH 5.5 cells showed mean percentage survival values of 26.41±0.65 and 3.55±0.22 respectively. In the presence of this salt the mean percentage survival values were 25.2±0.65 and 10.7±1.8 for pH 7.0 and pH 5.5 induced cells. When compared with pH 7.0 cells, the pH 5.5 cells were sensitized by 7.4-fold in the absence of ferrous sulphate but in the presence of this salt the sensitization in these cells was reduced by 3-fold (95% confidence). The results suggest that ferrous sulphate is involved in repressing the expression of the alkali sensitization components probably by acting as an inhibitor to the Fur activator protein.

3.3.19.2 Involvement of RpoS protein in the induction of alkali sensitivity at pH 5.5

Table 3-44 shows that after a challenge with pH 9.75 for 30 minutes, the pH 7.0 induced *E.coli* 6482 *rpoS*$^+$ cells showed a mean survival value of 26.0±5.0% whereas the pH 5.5 induced cells showed a mean survival value of 12.60±2.90%. The pH 5.5 induced cells of *E.coli* 6482 *rpoS*$^+$ was sensitised by 2-fold (95% confidence). When pH 7.0 and pH 5.5 induced *E.coli* 7157 *rpoS* cells were challenged with pH 9.75, 19.50±9.60% and 1.56±0.41% of the cells respectively were able to form colonies. The pH 5.5 induced *E.coli* 7157 *rpoS* were sensitised by 12.5-fold (90% confidence) when compared with the pH 7.0 induced cells. There are however no significant differences in survival in pH 7.0 induced cells of *E.coli* 6482 *rpoS*$^+$ and *E.coli* 7157 *rpoS*. However the pH 5.5 induced cells of *E.coli* 7157 *rpoS* showed enhanced sensitization (8-fold) than pH 5.5 induced cells

Table 3-43 Effect of ferrous sulphate on the induction of alkali sensitivity at pH 5.5 in *E.coli* 1829

Pre-exposure pHs	Ferrous sulphate (1.0 mM)	Percentage of colony forming units (mean±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	-	26.41±0.65
	+	25.20±0.70
5.5	-	3.55±0.22
	+	10.70±1.80

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pHs 7.0 and 5.5 at 37°C , in absence or presence of ferrous sulphate, were challenged with NB pH 9.75 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA andincubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-44 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 6482 *rpoS*$^+$ and *E.coli* 7157 *rpoS*

pH at induction	Regulatory component (genotype)	Percentage Survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*rpoS*$^+$	26.00±5.00
	rpoS	19.50±9.60
5.5	*rpoS*$^+$	12.60±2.90
	rpoS	1.56±0.41

E.coli 82 *rpoS*$^+$ and *E. coli* 7157, *rpoS* cells were grown to exponential phase and, after hour one of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of five experiments.

of *E.coli* 6482 *rpoS*$^+$. This is significant at 99% level of confidence.

The *rpoS* gene encodes a sigma factor, sigma S (σ^S), which is normally induced in nutrient-limiting conditions and upon entry into the stationary phase. The σ^S positively regulates a number of genes which are selectively expressed in order to allow starved and stationary phase cells to survive various stresses. Anaerobiosis and reduction in pH_i caused by weak acids have also been reported to induce *rpoS* expression (**Mulvey *et al.*, 1990; Schellhorn and Stones 1992**). The results show that the absence of the *rpos* gene does not only abolish the sensitization but instead enhanced the sensitization of the pH 5.5 induced cells. This suggests that the components under the control of *rpoS* may confer some protection to the pH 5.5 cells during alkali challenge and the deletion in this gene enhances the sensitization even more.

3.3.19.3 Involvement of RelA protein in the induction of alkali sensitivity at pH 5.5

Table 3-45 shows the effect or a *rel* deletion on induction of alkali sensitization at pH 5.5. Results from six experiments show that the pHs 7.0 and 5.5 induced cells of *E.coli* 1652 *relA*$^+$ gave mean survival values of 65.50±5.89% and 16.9±6.5% respectively after a challenge with pH 9.5 for 30 minutes. When the pH 7.0 and pH 5.5 induced cells of *E.coli* 1652 *relA* were challenged with pH 9.5 for 30 minutes, 68.50±4.07% and 33.80±6.50% of the cells respectively were able to form colonies. These results show that pH 5.5 induced cells of *E.coli* 1652 *relA*$^+$ and *relA* were sensitised by 3.9-fold and 2.0-fold respectively (99% confidence) after the alkali challenge suggesting that the deletion in *relA* reduces the sensitization in pH 5.5 by 2-fold (95% confidence). This shows that *relA* may have some effect on induction of alkali sensitivity. RelA is a stringent factor which is involved in the synthesis of the guanine nucleotides (p)ppGpp in response to amino acid starvation and also to carbon and nitrogen source limitation. This response entails induction of cellular adjustments in order to cope with the mentioned adverse conditions. The results suggest that the RelA is involved in inducing some form of compensatory cellular adjustment at pH 5.5 which enhances sensitization to alkaline pH and deletion in this gene reduces the sensitization. These cellular adjustments may include synthesis of the alkali sensitization components.

Table 3-45 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1652 *relA*$^+$ and *E.coli* 1652 *relA*

pH at induction	Regulatory component (genotype)	Percentage Survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	*relA*$^+$	65.50±5.89
	relA	68.50±4.07
5.5	*relA*$^+$	16.90±6.50
	relA	33.80±6.50

E.coli 1652 *relA* and 1652 *relA*$^+$ cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.5 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of six experiments.

3.3.19.4 Involvement of MicF in induction of alkali sensitivity at pH 5.5

Exponential cells of *E.coli* 1829 *micF*$^+$ and *E.coli* 1829 *micF* grown in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging to NB pH 9.75. The pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 *micF*$^+$ showed mean survival values of 30.80±1.90% and 1.55±0.12% respectively after a challenge with pH 9.75 for 30 minutes (**Table 3-46**). The pH 5.5 induced cells were sensitized by 19.8-fold (99% confidence). When pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 *micF* were challenged with pH 9.75, 29.10±2.0% and 7.60±2.60% of the cells respectively were able to form colonies. The deletion of *micF* reduces the sensitization by 4.9-fold (90% confidence) in pH 5.5 cells. Since *micF* shares some of the regulatory elements in the *ompC* promoter region, a deletion in *micF* may have affected the expression of *ompC*. As a result, low levels of OmpC may be present in this *micF* deletion strain. The reduced levels of OmpC may then result in reduced proton entry and thus reduced cytoplasmic acidification and hence reduced alkali sensitization.

3.3.19.5 Involvement of CysB protein in the induction of alkali sensitivity at pH 5.5

The results from **Table 3-47** show the effect of deletion in *cysB* on alkali sensitivity at pH 5.5. After a challenge with pH 9.75 for 30 minutes, the pHs 7.0 and 5.5 induced cells of *E.coli* JA199 *cysB*$^+$ showed mean survival values of 79.6±4.26% and 4.8±0.33% respectively. The induction at pH 5.5 has sensitized the cells by 16-fold when compared with the pH 7.0 cells. The deletion in this gene however reduces the sensitization by 8.5-fold (99% confidence). This suggests that *cysB* gene product acts as an activator of the transcription of the alkali sensitization components and the deletion of this gene reduces the sensitization.

3.3.19.6 Involvement of Lrp in the induction of alkali sensitivity at pH 5.5

The effect of deletion in the *lrp* gene on alkali sensitization was tested. After a challenge with pH 9.75, the pHs 7.0 and 5.5 induced cells of *E.coli* CV *lrp*$^+$ showed mean survival values of 55.2+±7.02% and 23.87±2.66% respectively (**Table 3-48**). The pH 5.5 induced cells showed a 2.3-fold sensitization after the treatment (95% confidence). The

Table 3-46 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *micF*$^+$ and 1829 *micF*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*micF*$^+$	30.80±1.90
	micF	29.10±2.00
5.5	*micF*$^+$	1.55±0.12
	micF	7.60±2.60

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-47 Induction of alkali sensitivity at pH 5.5 in *E.coli* JA199 *cysB*$^+$ and *E.coli* NK1 *cysB*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*cysB*$^+$	79.60±4.26
	cysB	71.10±4.44
5.5	*cysB*$^+$	4.80±0.33
	cysB	40.80±2.33

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18 to 24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-48 Induction of sensitivity to alkali at pH 5.5 in *E.coli* CV975 *lrp*$^+$ and *E.coli* CV1008 *lrp*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	*lrp*$^+$	55.20±7.02
	lrp	48.90±4.90
5.5	*lrp*$^+$	23.87±2.66
	lrp	42.90±1.22

E.coli cells were grown to exponential phase and, after one hour of pre-exposure at 37°C in NB pH 7.0 and in NB pH 5.5 were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18 to 24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

deletion of this gene however abolishes this sensitization. This suggests that the *lrp* gene product is involved in the positive regulation of the alkali sensitization components. Since leucine can influence the action of Lrp as a transcriptional regulator its effect on the induction of alkali sensitivity was tested as described below.

3.3.19.6.1 *Effect of leucine on induction of alkali sensitivity at pH 5.5 in E.coli 1829*

Exponential cells of *E.coli* 1829 in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour in presence or absence of L-leucine before challenging with NB pH 9.75. After 30 minutes of alkali challenge, the pH 7.0 induced cells showed a mean survival value of 20.40±0.62% in the absence of leucine and in presence of 50 and 150 µg/ml leucine 21.1±1.2% and 28.1±4.20% of the cells respectively were able to survive (**Table 3-49**). When the cells were induced in pH 5.5 in absence of leucine they showed a mean survival of 1.56±0.27% after the alkali challenge whereas in the presence of 50 and 150 µg/ml leucine, 5.05±0.49 and 7.22±1.15 of the cells respectively were able to form colonies. The results show that the presence of 50 and 150 µg/ml leucine during induction at pH 5.5 reduces the sensitization to alkali by 3.2-fold (99% confidence) and 4.6-fold (95% confidence) respectively. This suggests that leucine might be involved in repression of the alkali sensitization components. Since leucine can influence the action of Lrp as a transcriptional regulator, the repression of the alkali sensitization components by leucine is probably by antagonizing the action of Lrp.

3.3.20 Involvement of histone-like proteins on the induction of alkali sensitivity at pH 5.5

The histone-like proteins such as H-NS and IHF have been implicated in influencing gene expression through changes in the DNA topology. Their influence in the induction of the expression of alkali sensitization components was investigated.

3.3.20.1 Involvement of H-NS proteins in the induction of alkali sensitivity at pH 5.5

Exponential cells of *E.coli* 1829 *hns*$^{+}$ and *E.coli* 1829 *hns* in NB pH 7.0 were transferred

Table 3-49 Effect of leucine on the induction of alkali sensitivity at pH 5.5 in *E.coli* 1829

pH at induction	L-Leucine (µg/ml)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.75 for 30 minutes
7.0	0.0	20.40±0.62
	50.0	21.10±1.12
	150.0	28.10±4.26
5.5	0.0	1.56±0.27
	50.0	5.05±0.49
	150.0	7.22±1.15

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C in absence or presence of leucine, were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown for the effect of 50 and 150 µg/ml L-leucine are mean values of four and three experiments respectively.

to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging with NB pH 9.75. **Table 3-50** shows that the pHs 7.0 and 5.5 induced cells of *E.coli* 1829-*hns*$^+$ gave mean survival values of 20.10±1.95% and 1.27±0.52% respectively after 30 minutes of alkali challenge. The pH 5.5 induced cells was sensitised by 15.8-fold (99% confidence) by the alkali. When the pH 7.0 and pH 5.5 induced cells of *E.coli* 1829 *hns* were challenged with pH 9.75, 59.0±20.2% and 33.9±7.2% of the cells respectively were able to form colonies. The results show that the deletion in *hns* reduces the sensitization by 26.7-fold (99% confidence).

H-NS is a histone-like protein that is involved in restraining the tension present in the supercoiled DNA and a deletion in *hns* can cause supercoiled DNA to relax. The results thus suggest that when supercoiled DNA is relaxed due to a deletion in *hns*, the expression of the alkali sensitization components is repressed thus supercoiling is involved in the induction of alkali sensitization components.

3.3.20.2 Involvement of integration host factor (IHF) in the induction of alkali sensitivity at pH 5.5

Exponential cells of *E.coli* 1829 *himA*$^+$ and *E.coli* 1829 *himA* in NB pH 7.0 were transferred to NB pHs 7.0 and 5.5 and grown for a further one hour before challenging with NB pHs 9.5 and 9.75. After 30 minutes of challenge with pH 9.5, the pHs 7.0 and 5.5 induced cells of *E.coli* 1829 *himA*$^+$ showed mean survival values of 53.60±4.90% and 2.75±0.40% respectively and after a challenge with pH 9.75, the pHs 7.0 and 5.5 induced cells of *E.coli* 1829 *himA*$^+$ showed mean survival values of 23.90±1.60% and 0.23±0.07% respectively (**Table 3-51**). When compared to pH 7.0 induced cells, the pH 5.5 induced cells were sensitized by 19.5-fold (99.9%) and 104-fold (99.9%) after the challenge with pHs 9.5 and 9.75 respectively.

When pHs 7.0 and 5.5 induced cells of *E.coli* 1829 *himA* were challenged with pH 9.5 they showed mean survival values of 56.50±6.22% and 24.50±4.72% respectively and after a challenge with pH 9.75 of 24.0±2.90% and 2.70±1.30% of the cells respectively were able to survive. The deletion in *himA* reduces the sensitization in pH 5.5 by 2.3-fold (99% confidence) and 8.8-fold (99% confidence) when challenged with pHs 9.5 and 9.75 respectively.

Table 3-50 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *hns*$^+$ and *E.coli* 1829 *hns*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge for 30 minutes with pH 9.75
7.0	*hns*$^+$	20.10±1.95
	hns	59.00±20.2
5.5	*hns*$^+$	1.27±0.52
	hns	33.90±7.20

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pH 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of three experiments.

Table 3-51 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *himA*$^+$ and *E.coli* 1829 *himA*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge for 30 minutes with pH:	
		9.5	9.75
7.0	*himA*$^+$	53.60±4.90	23.90±1.60
	himA	56.50±6.22	24.00±2.90
5.5	*himA*$^+$	2.75±0.40	0.23±0.07
	himA	24.50±4.72	2.70±1.30

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pHs 9.50 and 9.75 for 30 minutes. Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown for challenges with pHs 9.5 and 9.75 are mean values of four and three experiments respectively.

Similar results were also obtained with *E.coli* 1829 *himD* deletion mutants (**Table 3-52**). The pHs 7.0 and 5.5 induced cells of *E.coli* 1829 *himD*$^+$ showed mean survival values of 53.40±1.90% and 9.40±2.50% respectively after a challenge with NB pH 9.5; the pH 5.5 cells being sensitized by 5.7-fold. When pHs 7.0 and 5.5 induced cells of *E.coli* 1829 *himD* were challenged with NB pH 9.5, 61.40±10.10% and 33.90±8.20% of the cells were able to survive; the deletion in *himD* reduces the sensitization by 3.6-fold.

The results from the *himA* and *himD* deletion mutants suggest that both the gene product of the IHF subunits, *himA* and *himD*, are involved in the regulation of the expression of the alkali sensitization components probably by inducing DNA bends or loops to facilitate the interactions between promoters of alkali sensitization components and RNA polymerase.

Table 3-52 Induction of sensitivity to alkali at pH 5.5 in *E.coli* 1829 *himD*$^+$ and *E.coli* 1829 *himD*

pH at induction	Regulatory component (genotype)	Percentage survival (% mean of CFU/ml±S.E.M) after a challenge with pH 9.5 for 30 minutes
7.0	*himD*$^+$	53.40±1.90
	himD	61.40±10.10
5.5	*himD*$^+$	9.40±2.50
	himD	33.90±8.20

E.coli cells were grown to exponential phase and, after one hour of pre-exposure in NB pH 7.0 and NB pH 5.5 at 37°C were challenged with NB pHs 9.50 for 30 minutes.Dilutions of culture at the end of the challenge were plated on NA and incubated at 37°C for 18-24 hours. The survival of the cells was expressed as a percentage of CFU/ml at time zero. Results shown are mean values of four experiments.

CHAPTER 4

GENERAL DISCUSSION

4.1 Introduction

The bacteria are often subjected to continual fluctuations of environmental stress conditions and yet show a remarkable ability to tolerate these hostilities. It is known that, for some, tolerance can be induced if the bacterial cells were given prior opportunity to adapt to sublethal levels of the same stress. In recent years there have been increasing interests in the responses of polluting enterobacteria to chemical and physical stresses due to their increasing occurrence in natural waters as a result of increasing sewage pollution. More importantly, since enterobacteria include a number of major potential pathogens capable of causing a wide range of human and animal diseases subsequent survival of these bacteria in foods and water used for human consumption after prior exposures to sublethal conditions of the same stresses would be of major public health concerns. Thus it is important to understand how responses to the various stresses encountered by bacteria influence their subsequent survival. This work was undertaken to study the effect of pre-exposure to copper on their subsequent survival in lethal doses of copper concentration and also to other stresses that may be encountered and secondly is to study the effect of prior exposure to mild acid pH on survival at alkaline pH.

4.2 Induction of Tolerance to Copper (Habituation to Copper)

Copper is one of the heavy metals which is necessary for growth in traces amount but is toxic in excess. It is a potentially important class of inhibitor due to its bactericidal effects and common occurrence in natural and potable waters. This study has shown that tolerance to lethal levels of copper could be gained if bacterial cells had prior exposure to sublethal concentrations of this trace element, as was demonstrated by *E.coli* 1829 and its derivatives (**Tables 3-4, 3-6a,b**). Such inducible tolerance was also demonstrated recently to cadmium chloride (**Inbar and Ron, 1993**). The induction of copper tolerance can be fully induced either at 37°C or 30°C. This ability to resist lethal levels of copper was due to a phenotypic effect rather than to a selection of copper-resistant mutants during the induction period as copper-induced cells lost the ability to survive the lethal doses of

copper after an overnight growth in a copper-free medium suggesting that no genetical changes had taken place (**Table 3-5**). The phenotypic changes that enable the cells to survive lethal doses of copper take place during first 15 minutes of the induction period and these changes are only transient, being maintained only as long as the cells are subjected to sublethal concentrations of copper (**Tables 3-8 and 3-9**). After being in a copper-free medium for 2 hours the copper-induced cells have lost their tolerance by 4.6-fold (**Table 3-9**). When compared to the non-habituated cells, copper-induced cells that had no further growth in copper-free medium were 517-fold more able to survive the copper challenge whereas the copper-induced cells that were further grown in copper-free medium were 79-fold more able to survive the challenge. The inducible tolerance to copper requires *de novo* protein synthesis; in the presence of chloramphenicol, a protein synthesis inhibitor, copper-exposed cells were unable to survive the subsequent copper challenge. The *de novo* protein synthesis appears to be a common theme in nearly all the inducible tolerance responses documented to stress agents such as heat (**Neidhardt and VanBogelen, 1987**), acid (**Foster, 1991; Raja, 1992**) and oxidative agents (**Christman *et al.*, 1985; Farr and Kogoma, 1991**). In these responses, the induced stress proteins are postulated to be involved in repairing macromolecules such as DNA, RNA and proteins during challenge with lethal levels of the stresses thus enabling them to survive. Preliminary analysis of protein samples of copper habituated and non-habituated cells on monodimensional gel electrophoresis showed that 4 proteins from the outer membrane extracts with molecular weights of 65,000, 31,500, 18,000 and 16,500 daltons and two from the cytoplasmic membrane with molecular weights of 26,000 and 24,500 were overexpressed (**Figures 3-6a,b**). These proteins are probably involved in protecting the cells from the deleterious effects of the lethal doses of copper sulphate. It is very likely that many other proteins that are involved in inducing tolerance to copper cannot be detected in this preliminary analysis. Further work using radiolabelled samples or analysis on two-dimensional PAGE need to be done to determine their existence.

One of the causes of cell death in non-habituated cells upon copper-challenge is damage to DNA. The damage to the DNA was observed in non-habituated derivatives of *E.coli* 1829 that harbour the plasmid DNA of ColV and pBR322 (**Figure 3-5**). Unlike the non-habituated cells, the habituated ones appeared to be able to protect their DNA; the full

damage is observed only in the ColV DNA but less in the monomeric or dimeric forms pBR322 DNA (**Figures 3-5**). The ability to protect DNA and RNA from damage was indirectly demonstrated by inducing the expression of β-galactosidase genes in non-habituated and habituated cells after copper challenge (**Figure 3-4**). As the induction of β-galactosidase involves transcription of specific mRNA which are then translated into β-galactosidase monomers and assembled as active oligomers, it is an important tool in indicating whether DNA and the components involved in translation of the transcribed genes are damaged. In the non-habituated cells, hardly any (0.02%) β-galactosidase activity was induced after a challenge with NB plus 117.84 μg/ml $CuSO_4$ when compared to those non-habituated cells that were challenged in copper-free medium (**Figure 3-4**). On the other hand, after the same challenge, about 44% and 83% of β-galactosidase activity was induced in those that had been pre-exposed to NB plus 14.73 μg/ml $CuSO_4$ and NB plus 29.46 μg/ml $CuSO_4$ compared to those habituated cells that were challenged in a copper-free medium. The results shown by the non-habituated cells are in accordance with the results obtained from the DNA agarose gel electrophoresis which indicate that damage to DNA is probably the main cause of cell death. The ability of copper-habituated cells to synthesize RNA and proteins were further confirmed by the ability of these cells to incorporate more than 50% of the radiolabelled ^{14}C uracil and ^{14}C phenylalanine after the copper challenge (**Figures 3-3a,b**) whereas the non-habituated cells were hardly able to incorporate any of the radiolabelled uracil or phenylalanine.

The ability of habituated cells to survive lethal levels of copper upon subsequent exposure, is probably due not only to their ability to protect the vital macromolecules such as DNA and RNA from deleterious effects of the excess Cu^{2+} but also perhaps to their ability in maintaining the intracellular levels of Cu^{2+} at its physiological levels but at the same time reducing the potentially lethal levels in the cytoplasm. The inducible ability to do this is most probably analogous to the mechanism postulated by Brown *et al.*, (**1992**) for the constitutive copper resistance mechanism in which excess levels of copper are controlled at levels of uptake, transport and storage and efflux and by a regulator (**1.6**). The uptake of Cu^{2+} across the outer membrane is usually via the OmpF and OmpC porins, however, copper habituated cells appeared to have high levels of both porins (**Figure 3-6b**). This may indicate the regulation of copper uptake may not be

involved in inducible copper tolerance. However this can only be confirmed if accurate measurement of internal and external copper concentrations are carried out. Similarly the occurrence of copper efflux during copper challenge in habituated cells may be detected if such measurement were carried out. The involvement of global regulatory components in this response has yet to be tested. Detailed studies on all the proteins induced in habituated cells and the effect of their genes being deleted on inducible tolerance to copper have to be determined to establish the existence of the postulated mechanism.

The periodic releases of copper contaminants in natural waters may affect subsequent survival of potential bacteria that may escape to potable water systems and this may have some public health implications. From the documented inducible stress tolerance responses it would appear that bacteria such as *E.coli* can adapt to almost any stress if they were given the opportunity of pre-exposure to sublethal concentrations of the same stress. The ability of bacteria to evolve inducible mechanisms in "emergencies" probably contribute to their success in colonizing a variety of hostile niches. It would be interesting to postulate the similarity between the inducible tolerance responses in bacteria and the immune response in higher organisms.

4.3 Cross-Tolerance Responses

The complexity of stress responses is evident from the fact that bacteria are subjected to different kinds of stresses simultaneously rather to one stress at a time. Thus one of the aims of this study was to investigate the influence of pre-exposure to copper on response to other stresses commonly encountered by bacteria in their environments. The ability of bacteria to resist other stresses after a pre-exposure to one stress has been documented in the cross-tolerance to UV radiation after a pre-exposure to 42°C (**Parsadani and Fitt, 1989**), hydrogen peroxide (**Fitt *et al.*, 1992; Asad *et al.*, 1994**), alkali (**Rowbury and Goodson, 1990**) and acid (**Goodson and Rowbury, 1991**); to heat after a pre-exposure to hydrogen peroxide (**Christman *et al.*, 1985**), alkali (**Humphrey *et al.*, 1993b**) and glucose and nitrogen starvation (**Jenkins *et al.*, 1988**) and to acid after a pre-exposure to heat (**Humphrey *et al.*, 1993a**).

This study showed that pre-exposure to copper enhanced the survival of the cells

at temperatures of 50°C and 52°C compared to those that did not receive the pre-exposure. Although no increase in growth was observed the pre-exposure to heat enabled the cells to tolerate the heat longer than the non-habituated ones. To ascertain whether changes involved during copper adaptive period are the same to those during adaptive period to sublethal temperature (42°C), thermotolerance in copper adapted cells at 37°C and 42°C were carried out (**Table 3-14**). Pre-exposing exponential cells that were grown at 37°C to copper at 42°C, a temperature that normally would induce heat-shock response, induces thermotolerance to 52°C irrespective whether they were pre-exposed to copper or not as expected. Also the thermotolerance exhibited by copper adapted cells at 37°C is similar to that exhibited by cells that were pre-exposed in NB pHs 7.0 and 5.5 at 42°C. It is known that the thermotolerance conferred in cells grown at 42°C, is due to the induction of heat-shock proteins during the adaptive period to act as molecular chaperones (**Neidhardt and VanBogelen, 1987**). Comparison of thermotolerance in copper adapted cells at 37°C and 42°C showed that the thermotolerance in the former is related to copper-induced proteins and the latter related to a combination of both heat-shock and copper-induced proteins. It is highly likely that some of the heat-shock proteins especially the chaperonins such as the DnaK, GroEL and GroES may also be induced by copper as these chaperonins have been reported to be induced by other stresses such as ethanol, hydrogen peroxide and cadmium chloride (**VanBogelen, *et al.*, 1989**). The effect of pre-exposure to 42°C on tolerance to copper would have to carried out in future to find out whether heat-shock proteins could confer tolerance to lethal doses of copper. Additionally the overlap in proteins induced during the copper adaptive period and pre-exposure to 42°C would be interesting to study.

Copper-induced cells were also protected against toxic effects of cadmium sulphate. One of the deleterious effects of Cd^{2+} is damage to DNA by strand breakage. The protection conferred by pre-exposure to copper may be due to the ability of these cells to repair both damaged DNA and probably denatured proteins caused by Cd^{2+}.

Cross-tolerance to low acid pH (pH 3.5) was also conferred in copper pre-exposed cells. As copper exposure was performed at pH 5.5, this mild acidity partly contributes to the tolerance to pH 3.5 as it is known that pre-exposure to mild acid pH in *E.coli* induces tolerance to lethal acid pHs (pH 3.0 and 3.5) (**Goodson and Rowbury 1989a**) and this

tolerance is partly due to the ability of the acid-induced cells to repair DNA (**Raja *et al.*, 1991**). The presence of copper during the adaptive period further enhances the survival at the lethal acid pH (**Table 3-17**).

The pre-exposure to copper however did not enhance the survival of these cells to hydrogen peroxide (**Table 3-15**). There is no significance difference in tolerance between copper habituated and non-habituated cells at pH 5.5. However there is a difference in survival between pH 5.5 cells and pH 7.0 cells. This protection conferred against H_2O_2 is due to mild acidity of the medium used. Moreover, pre-exposure to mild acid pH has been reported to confer tolerance to H_2O_2 in *S.typhimurium* (**Foster and Hall, 1990**).

Pre-exposure to copper also confers tolerance to pH 10.0. Cells that were pre-exposed to copper were 95-fold more able to form colonies compared to cells that were preexposed to pH 5.5. Thus it would seem that the components induced during the copper pre-exposure period enable the copper-habituated cells against survive the lethal effects of alkali better than the non- habituated one (**Table 3-18**).

Thus the copper tolerance components induced during the copper adaptive period could confer tolerance to other stresses such heat, acid, alkali and cadmium chloride suggesting that the presence of overlap in inducible tolerance response components. These components may represent the general stress proteins induced in the presence of any stress agents. The abilities of copper-induced cells to survive heat and acid suggest the possibility that when potentially pathogenic cells are in a potable water source, they may survive food treatment processes and also able to thrive in acidic foods and in the host body. Future aspects of the study could include studying the identity of these overlapping components and studying cross-tolerances to other stress such as to chlorine as both chlorine and copper are used in the water treatment processes.

4.4 Induction of Alkali Sensitivity in *Escherichia coli*

The ability of bacteria to survive external lethal pH stresses relies at least partially on them being able to maintain intracellular pH at physiological values. The constitutive pH homeostasis mechanism enables the bacteria to survive external pH values within a certain limit, for example, the constitutive pH homeostasis mechanism in neutrophiles allow them

to survive external pH of within 4-9 (**Padan *et al.*, 1981**). However, the ability to induce tolerance to lethal acid or alkaline pH after a pre-exposure to mild acid and alkaline pH respectively, led to the possible existence of an inducible pH homeostasis systems (**Foster, 1991**). Thus it would appear that bacterial cells may have two types of sytems for pH homeostasis, one type being constitutive and one inducible in times of stress. Since response to one stress is known to alter responses to another either by inducing cross-protection or even enhancing sensitivity as in the alkali-induced acid sensitivity response (**Rowbury *et al.*, 1993**), the effect of prior exposure to mild acid on sensitivity to alkaline pH was investigated.

This study shows that pre-exposure of *E.coli* cells to mildly acidic conditions does not only induce acid tolerance as previously demonstrated (**Goodson and Rowbury, 1989a**), but also induces sensitivity to lethal alkaline pH upon subsequent challenge (**Table 3-19**). The sensitization to alkali is likely to be due to a phenotypic effect as the complete response was acquired after one hour of pre-exposure period thus ruling out any selection of alkali sensitive mutants (**Table 3-21**). The process that induces acid habituation is unlikely to be the same as that which induces alkali sensitivity, because alkali sensitivity is markedly induced at pH_0 5.5 and 6.0 but much less at pH_0 5.0 (**Table 3-20**) whereas acid habituation is markedly induced at pH 5.0 and even at pH_0 4.5 (**Rowbury, 1994**). Thus cellular changes induced only at pH_0 5.5. and 6.0 are involved in alkali sensitization. These cellular changes occurring during the pre-exposure or sensitization period are likely to involve synthesis of new proteins or overexpression of proteins already present or both since induction of alkali sensitivity was markedly reduced when protein synthesis inhibitors such as chloramphenicol and tetracyline were present during the pre-exposure period (**Table 3-28a,b**). Hence sensitization to alkali requires *de novo* protein synthesis. Sensitization to alkali was also completely abolished when rifampicin, a RNA polymerase inhibitor, was present during the induction period. This was presumably due to inhibition of the transcription of genes encoding the newly synthesized sensitization proteins. Thus the induction of alkali sensitivity appeared to be a protein-dependent phenomena. Monodimensional SDS-PAGE electrophoresis of cytoplasmic membrane proteins showed that two polypeptides with molecular weights of 18,000 and 14,000 were overexpressed in cells that were pre-exposed at pH 5.5 (**Figure 3-10**). The functions of these proteins are

not known; most probably they are involved in changes which lead to to enhancement of alkaline damage. Interestingly, the *sulA* gene product, an inducible component of the SOS response and whose function is to inhibit septation is also a 18,000 dalton protein (**Walker, 1984**). Whether these two proteins are the same is yet to be determined. The possibility of the presence of other overexpressed or new proteins during this induction period should not be ruled out and further work on two-dimensional SDS-PAGE electrophoresis of protein samples will have to be performed to determine the existence of other overexpressed or new proteins.

Clearly, one of the causes of alkali sensitivity in pH 5.5 pre-stressed cells is increased susceptibility to damage to macromolecules such as DNA. Agarose gel electrophoresis of alkali treated DNA samples of pH 5.5 and 7.0 pre-exposed cells (**Figure 3-9a**) showed that the DNA of the pH 5.5 pre-stressed cells was more damaged than than that from the pH 7.0 pre-exposed cells. In pH 7.0 pre-exposed cells, 26.17 and 6.0% of the pBR322 and ColV DNA respectively were damaged whereas in pH 5.5 pre-exposed cells 41.0 and 100% of the pBR322 and ColV DNA were damaged respectively. The damage to DNA and also to the components involved in protein synthesis such as RNA and ribosomes were shown by the reduced ability of alkali-treated pH 5.5 pre-stressed cells to induce β-galactosidase as the induction of this enzyme involves both transcription and translation of β-galactosidase genes. There was only 17.71% β-galactosidase activity induced in pH 5.5 cells after the alkali challenge whereas the pH 7.0 pre-exposed cells 82.31% of the enzymic activity was induced (**Table 3-26**). Protein damage by alkali was also observed; inducing the enzyme β-galactosidase before alkali challenge showed that about 70 % of the enzyme activity is inactivated after the alkali challenge in the pH 5.5 induced cells compared to 50% in pH 7.0 pre-exposed (**Table 3-25**) suggesting that the pH 5.5 pre-stressed cells are more susceptible to protein damage.

Unlike acid habituation, the induction of alkali sensitivity at pH 5.5 involves acidification of the cytoplasm. Collapsing the ΔpH and $\Delta\psi$ with weak acid especially with benzoate at pH 6.5, a pH where alkali sensitivity is not induced, induces the sensitization (**Table 3-23b**). The acidification of the cytoplasm therefore must be sensed and then signalled for appropriate cellular responses. Although at pH_o 5.5 it is known that internal pH homeostasis is maintained at ca 7.4 (**Slonczewski *et al.*, 1981**), the initial

transient drop in pH_i before homeostasis is achieved, probably serves as an intracellular signal for induction of the appropriate cellular response. The sensor is probably located in the cytoplasm since acidified cytoplasm is being sensed rather than difference in pH between cytoplasm and periplasm. It is also probable that the sensor is part of a two-component regulatory system in which the sensor senses the drop in pH_i and transmits a stress signal to a regulator or regulators such as demonstrated by the histidine protein kinases and their associated regulators.

Although most of the members of the two-component systems have their sensors spanning across the cytoplasmic membrane into the periplasm, a few such as the CheW and NR_{II} proteins which are involved in chemotaxis and nitrogen limitation responses respectively are located in the cytoplasm (**Stock *et al.*, 1989**). Furthermore deletion in *envZ* gene which can sense periplasmic pH, did not have any effect on alkali sensitization confirming that, in the induction of alkali sensitivity, acidified cytoplasm is being sensed rather than changes in periplasmic pH. Deletion of *envZ* in *E.coli* 1829 would have to be tested to further confirm this.

Like acid habituation, protons for induction of alkali sensitivity most probably cross the outer membrane via PhoE since phosphates which have been shown to inhibit H^+ passage through PhoE (**Rowbury *et al.*, 1992**), reduced the alkali sensitization response (**Table 3-24**). A reduction in alkali sensitization however was not observed in the *E.coli* 1157 strain bearing a *phoE* deletion. In fact, alkali sensitivity was not induced at all and interestingly this strain was also the most sensitive to alkali of all the *E.coli* strains used in this study. This may be attributable to the presence of unknown deletions that affect the alkali sensitization response in the PhoE strains. As the phages that use PhoE as receptors were not available, mutants of *E.coli* 1829, lacking PhoE could not be isolated and therefore the effect of a *phoE* deletion in *E.coli* was not tested. Also there were no suitable strains available from which the deletion in *phoE* can be transduced. Although PhoE is cited to be induced only during phosphate starvation, basal levels of it present in rich medium probably suffice to allow passage of H^+. Indeed unpublished results of *phoE-lacZ* fusions showed that levels of *phoE* are 2.1-fold higher at pH 5.5 than 7.0 (**Lazim and Rowbury, 1995**).

The possibility of H^+ using some other routes besides PhoE porin to cross the outer

membrane should not be ruled out. Since phosphates can also cross the outer membrane via OmpC and OmpF but with less efficiency, phosphate may competitively inhibit the passage of H^+ through these porins too. Since the transcription of *ompC* is induced at acid pH, an *ompC* mutant of *E.coli* 1829 was tested for alkali sensitization. The alkali sensitivity after a pre-exposure at pH 5.5 was markedly reduced by 11.5-fold in *ompC* mutants compared to that in the *ompC*$^+$ strain (**Table 3-31**). The results suggest that alkali sensitization only occurs if OmpC is present. The fact that OmpC allows non-specific diffusion of positively charged ions, and also is the most abundant outer membrane protein at acid pH besides OmpA, it is possible that protons that induce the alkali sensitization response might use this porin to cross the outer membrane (**Figure 3-8b;** lane 3). A strain with a deletion in *micF* also showed a 5-fold reduction in alkali sensitization (**Table 3-46**). Since *micF* shares some of the regulatory elements in the *ompC* promoter region, a deletion in *micF* might affect the transcription of *ompC*. However the sensitization in the *micF* mutants is about 2-fold less than that in *ompC* mutants; this difference could be attributable to the presumably increased levels of OmpF in the *micF* mutants which may allow the passage of more H^+ than it normally does in the OmpC$^+$ OmpF$^+$ strain at pH 5.5. *lamB* whose expression is also affected by pH was also tested; a reduction in alkali sensitization was also observed in 1829 *lamB* mutants after pre-exposure at pH 5.5. However, *lamB* mutants showed a 2.6-fold reduction in sensitization whereas *ompC* mutants showed a 11.5-fold reduction (**Tables 3-32, 3-31**). This is probably due to less of LamB being present than OmpC at pH 5.5 (**Figure 3-6b**; lane3). Thus it is possible that PhoE, OmpC, OmpF and LamB are all used for H^+ penetration to the sensor with OmpC being the major H^+ passage as sensitization is reduced more in *ompC* mutants (**Figure 4-1**).

On the other hand, mutants of OmpA that were pre-exposed to pH 5.5, showed marked increase in alkali sensitivity compared the to parent, even those that were pre-exposed at pH 7.0. Since OmpA is involved in maintaining the integrity of the cell, the lack of membrane integrity allows excess leaks of Na^+ and OH^+ into the cells thus reducing numbers of surviving cells. There was also no alkali sensitization in the *tonB* mutants that were pre-exposed at pH 5.5 but this response was derepressed in the pH 7.0 pre-exposed cells (**Table 3-35**). This suggests that TonB is involved in repressing this

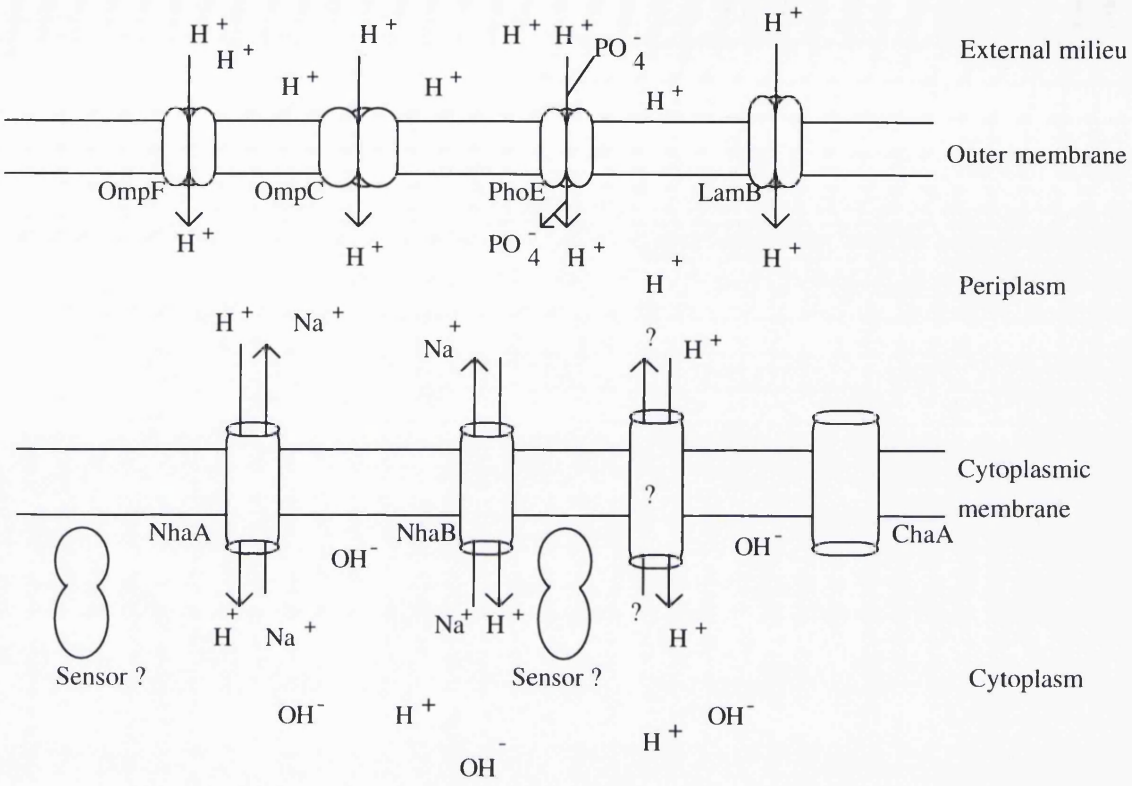
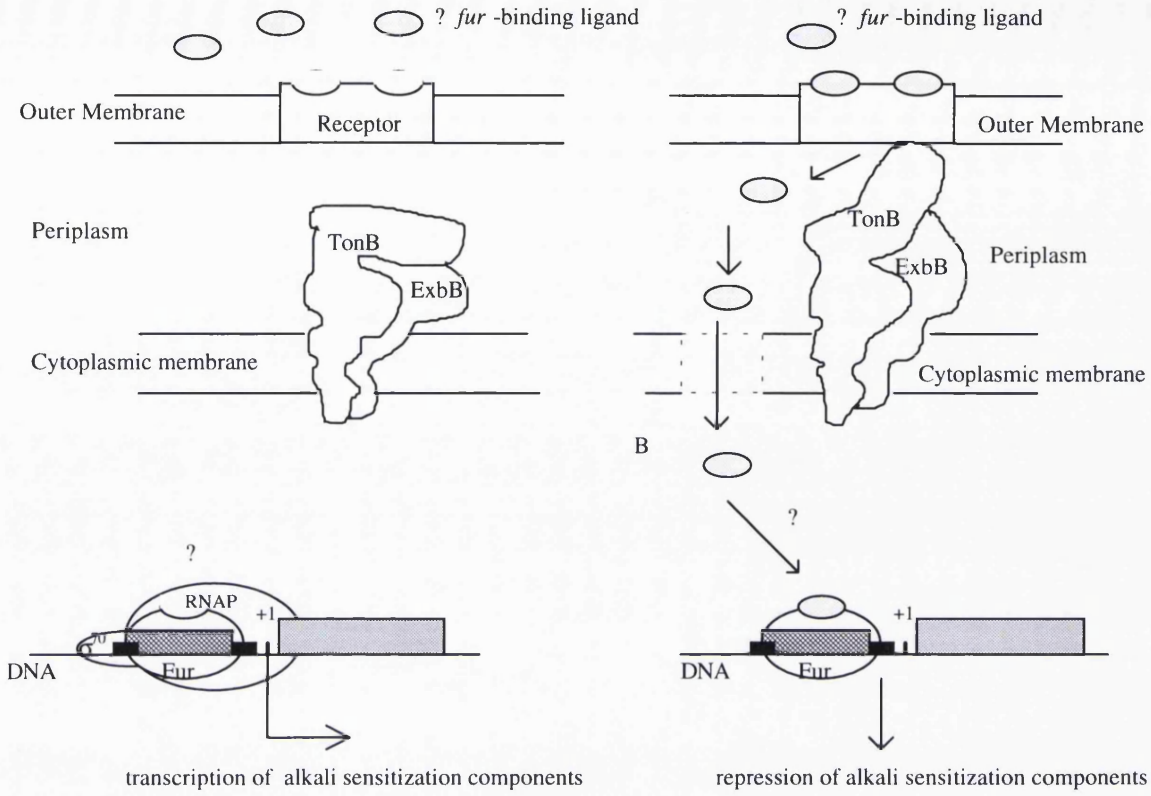


Figure 4-1 Diagrammatic representation of the possible routes of protons entry into the cytoplasm.

response at pH 7.0 or more likely that a component that uses TonB as an energizer to cross the outer membrane probably act as a co-repressor of this response at pH 7.0 (**Figure 4-2**). The co-repressor is likely to use the Fe^{3+}-siderophores receptors as they are TonB-dependent. It is also possible that the co-repressor is co-transported with the Fe^{3+}-siderophores and enhances the repression of alkali sensitzation components. Since TonB and the receptor proteins that are involved in Fe^{3+}-siderophores uptake are under the control of Fur (**Crosa, 1989**), most probably the repression by the co-repressor involves Fur (**Figure 4-2**). As to why the receptor proteins for the Fe^{3+}-siderophores and TonB are being induced at pH 7.0, is probably due to Fe being present in an insoluble state at pH 7.0 and thus requires the induction of these proteins for its uptake. Furthermore it is known that the solubility of Fe in solution is a function of pH, and that Fe exists as the insoluble Fe^{3+} complex at neutral to alkaline pH and as the soluble Fe^{2+} at acidic pH. The uptake of the latter is TonB and Fe^{3+}-siderophore receptors independent. Further studies on the effect of lesions in receptor proteins that use TonB as an energy transducer on alkali sensitivity may shed a light on the identity of this component.

One of the possible routes used by protons to cross the cytoplasmic membrane is via the NhaA antiporter since it can couple the uptake of H^+ with Na^+ extrusion (**Figure 4-1**). Since alkali sensitization requires cytoplasmic acidification by protons, and since alkali sensitizations were reduced by 9.8-fold and 10.8-fold in strains lacking *nhaA* (**Table 3-36**), this antiporter could therefore be used by protons to cross into the cytoplasm. The presence of amiloride, during the induction period suggests that NhaB was not used by protons to cross the cytoplasmic membrane as amiloride is known to inhibit NhaB activity. However the reduction in alkali sensitivity exhibited by *nhaB* mutants suggests otherwise (**Table 3-37**). The explanation for this is not known; probably it is due to the presence of some unidentified cytoplasmic component whose activity is preferentially inhibited by amiloride. Thus results from experiments with strains lacking *nhaB* suggest that H^+ may also use NhaB to cross the cytoplasmic membrane (**Figure 4-1**).

The survival at alkaline pH may depend on the ability of the cells to maintain their pH_i at physiological level appropriate for maintenance and growth. The sodium ions present in the NaOH-alkalinized medium proved to be one of the inhibitory components



A Induction at pH .5.5 **B** Induction at pH 7.0

Figure 4-2 A postulated role for TonB in pH 7.0 and 5.5 induced cells. (**A**) The TonB activity is not induced in pH 5.5 cells probably due to Fe being present in the soluble form (Fe^{2+}) at acidic pH and thus the co-repressor is unable to gain entry into cytoplasm and therefore transcription of alkali sensitization components is not repressed. (**B**) TonB probably acts as an energy transducer for the uptake of a co-repressor for alkali sensitivity induction in pH 7.0 cells. The TonB is induced probably due to the presence of insoluble form of Fe (Fe^{3+}) in the media at pH 7.0. The co-repressor presumably gains entry into the cytoplasm via the receptors for the Fe^{3+}-siderophores. The binding of this co-repressor with Fur or other yet to be identified regulatory protein, represses the transcription of the alkali sensitization components. The black boxes in the diagram represent the -35 and -10 boxes; the shaded area between these boxes is the 'iron box' and the dotted boxes represent the genes for the alkali sensitization components. RNAP is RNA polymerase and σ^{70} is the housekeeping sigma factor.

affecting the survival of the cells at alkaline pH. The extent of alkali sensitization exhibited by pH 5.5 cells was 8-fold greater in medium alkalinized with NaOH and 2-fold greater in medium that was alkalinized with KOH. This is probably due to the inability to cope with a high intracellular Na^+ presence suggesting the lack or absence of sodium antiporters which would be required to neutralize the pH_i. The addition of amiloride, a NhaB sodium antiporter inhibitor, during alkali challenge showed that NhaB is required for alkali tolerance at alkaline pH in pH 7.0 pre-exposed cells and that the lack of this antiporter in pH 5.5 cells may lead to alkali sensitization. Since results showed by a strain carrying the *nhaB* deletion contradict this finding it is possible that unidentified antiporters which are also affected by amiloride and are active at alkaline pH, are instead involved in alkali sensitization. One such antiporter could be ChaA which can extrude both Na^+ and Ca^+ at alkaline pH coupled with H^+ uptake (**Ivey *et al.*, 1993; Ohyama *et al.*, 1994**). However there are no reports on the effect of amiloride on ChaA antiporter and since strains bearing this deletion were not available, this effect could not be tested. However, the presence of sodium chloride during induction appeared to abolish alkali sensitization. Although alkali sensitization in pH 5.5 cells does not appear to be due to the loss of NhaA or NhaB, induction of the former in the presence of Na^+ at pH 5.5 probably aided the survival of the pH 5.5 pre-exposed cells at alkaline pH. Alternatively, the elimination of alkali sensitivity could be due to an induction of an adaptive response by Na^+ which confers cross-tolerance to alkali.

The formation of alkali sensitization components is not subjected to catabolite repression as the presence of 1% glucose during the induction at pH 5.5 has no effect on the sensitivity to alkali (**Table 3-40**). Thus the induction of these components is not under the control of the *crp/camp* regulon. Similarly, the formation of alkali sensitization components is also independent of sigma factor S (RpoS) control, as strains having deletions in the respective genes did not show any significant changes in alkali sensitization. In the case of the *rpoS* mutants, the alkali sensitization was enhanced instead and this suggests that the presence of the alkali sensitization components and the absence of *rpoS* regulated components (which may confer some protection at alkaline pH) increases killing by alkali (**Table 3-44**).

The alkali sensitization was reduced somewhat in the *relA* deletion mutant

suggesting that some of the alkali sensitization components are under the control of the stringent response factor RelA (**Table 3-45**). This can be further confirmed by the adding of (p)ppGpp which would enhance the sensitization.

Like the acid tolerance response (ATR) in *S.typhimurium* (**Foster and Hall, 1992**), *fur* appeared to be involved in the formation of some of the alkali sensitization components as a deletion in *fur* reduced the sensitization by 5.2-fold. Since Fur can act as an activator, these components are thus positively regulated by this protein (**Figure 4-3**). As *fur* is known to have its regulatory properties modified by iron salts, the effect of excess iron on alkali sensitization was tested. In the presence of Fe_2SO_4 and $FeCl_3$, alkali sensitizations were reduced suggesting that the transcription of the alkali sensitive components is induced by *fur* at acidic pH but this induction can be partially prevented in the presence of iron. Since classically *fur* is involved in the regulation of genes that are involved in iron transport and assimilation, it is possible that the *fur*-regulated alkali sensitive components are members of the iron transport group. If these components are involved in iron transport then they would also be induced under conditions of iron starvation. This could be determined by comparing protein samples from pH 5.5 pre-exposed cells and pH 5.5 iron-starved cells. An interesting observation was shown by effect of copper on alkali sensitivity (**Table 3-18**) in which the presence of copper reduced the alkali sensitization significantly. Since Cu^{2+} could act as co-factors to Fur, the presence of copper thus repressed the transcription alkali sensitization components (**Figure 4.3(C)**). This further confirms the involvement of Fur in the regulation of alkali sensitization conponents . It has also been suggested that Fur also monitors changes in intracellular pH (**Saito *et al.*, 1991**). This could mean that the transcription of alkali sensitive components may be activated when Fur detects a change in pH_i and accordingly, in the absence of Fur a reduction in alkali sensitivity might be observed. Thus Fur may act as one of the sensors as well as regulators in the induction of alkali sensitivity.

The leucine responsive regulatory protein (Lrp) also appears to govern the expression of perhaps some of the alkali sensitive components as a strain lacking this gene showed a reduction in alkali sensitivity (**Table 3-48**). The Lrp has a DNA-binding domain (at the N-terminus) which can induce bending, an activating domain and a leucine-binding domain (C-terminus) (**Figure 4-4**). Since the action of *lrp* as a transcriptional regulator can

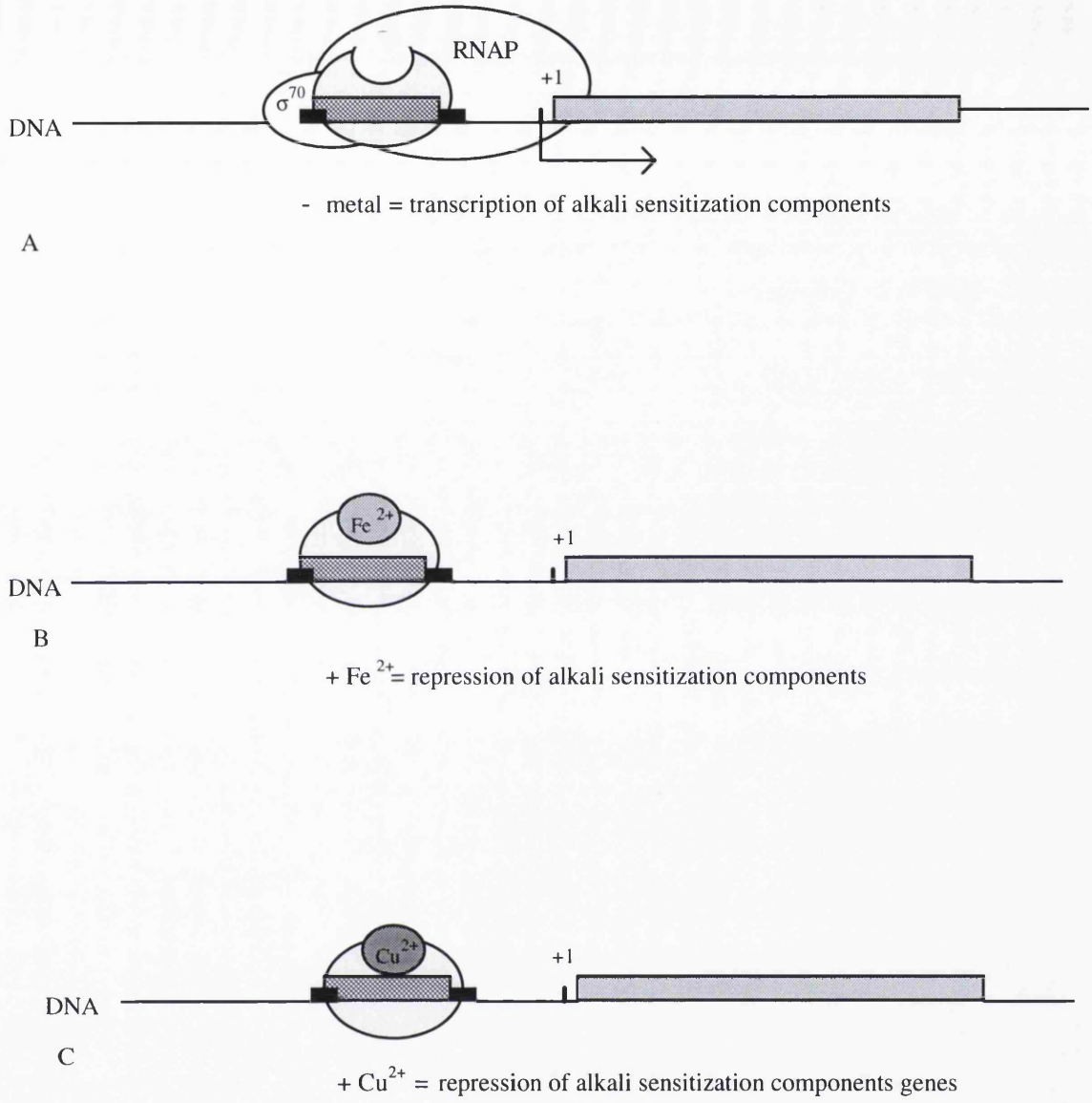
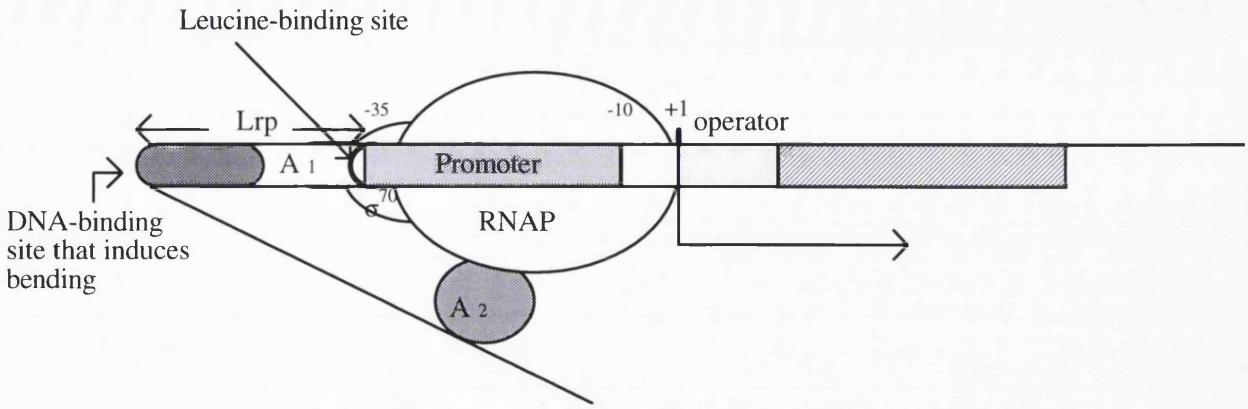
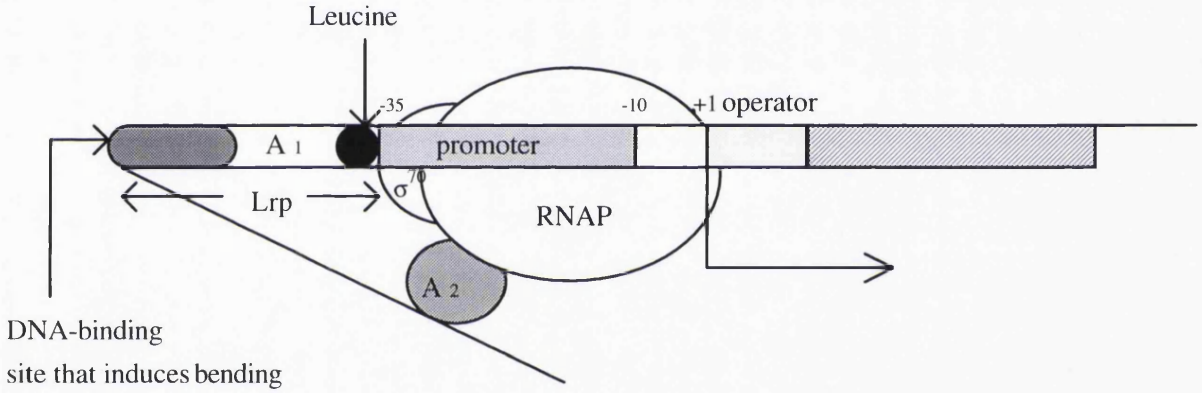


Figure 4-3 The role of Fur in the regulation of the transcription of alkali sensitization components. (**A**) Fur acts as an activator in the absence of Fe^{2+}, Fe^{3+} or Cu^{2+} ions. (**B**) In the presence of Fe^{2+} or (**C**) Cu^{2+} Fur acts as a repressor. The action with Fe^{3+} is not shown as Fe^{3+} is converted into Fe^{2+} once it enters the cell and thus binds to Fur in the form of the latter. The black boxes in the diagram represent the -35 and -10 boxes; the shaded area between these boxes is the 'iron box' and the area shaded grey represent the genes for the alkali sensitization components. RNAP is RNA polymerase and σ^{70} is the housekeeping sigma factor.



A minus leucine = transcription of alkali sensitization components



B plus leucine = partial repression of alkali sensitization components

Figure 4-4 The postulated role of Lrp (Leucine responsive protein) in alkali sensitivity induction at pH 5.5. (**A**) In the absence of leucine the activator part of the Lrp (A_1) binds to the RNAP and its σ^{70} factor and transcription of the alkali sensitization components is activated. The DNA binding site of Lrp (shaded dark grey) induces bending and this further allows a second activator to interact with the RNAP at the promoter and enhance the transcription of the alkali sensitivity components. (**B**) In the presence of leucine the interaction between activator (A_1) and RNAP is blocked but not the interaction between A_2 and RNAP since the DNA bending is still present and thus alkali sensitization components are partially repressed.

be influenced by exogenous leucine, the addition of leucine during induction in *lrp*$^+$ strain was tested (**Table 3-49**). The reduction in sensitization suggests that the expression of the alkali sensitive components by Lrp can be partly antagonized by presence of leucine as leucine did not fully stop the induction (**Figure 4-4**). It could postulated that the binding of leucine prevents the activating domain from interacting with the RNA polymerase and since induction is not fully abolished this may mean that other activator or activators may also be involved in the expression if these components and the binding of the leucine does not affect their interaction with the RNAP (**Figure 4-4**). Thus some of the alkali sensitization components belong to the leucine/lrp regulon.

CysB which is involved in the transcriptional regulation of *adiA* (arginine decarboxylase) also appeared to act as an activator of the transcription of alkali sensitization genes. Among the global regulatory proteins studied here, CysB probably control the induction of the majority of the alkali sensitization components as deletion in *cysB* reduces the sensitization by 8.5-fold compared to the 5.2-fold, 2-fold and 1.8-fold reduction in sensitization that was observed in the strains carrying the *fur*, *relA* and *lrp* deletions respectively.

The regulation of the expression of the alkali sensitization components besides being under the control of *fur*, *lrp* and *cysB* (**Figure 4-5**) also appeared to be affected by changes in DNA supercoiling. The level of negative supercoiling in *E.coli* is maintained at optimal levels by the balanced actions of DNA gyrase and topoisomerase I with DNA gyrase introducing negative supercoils and topoisomerase I preventing excessive supercoiling. The introduction of DNA gyrase inhibitors (which reduce supercoiling) such as nalidixic acid, novobiocin and coumermycin during induction at pH 5.5 (**Tables 3-29b-d**) reduced the sensitization by 11.6-fold, 8.8-fold and 10.7-fold respectively. This suggests that the expression of the alkali sensitive components is repressed when DNA is relaxed. In the supercoiled state, the DNA probably promotes interactions between activators of alkali sensitization components and RNA polymerase and in the relaxed state there is a reduction in these interactions. This can be further confirmed if strains having deletions in *gyrA* or *gyrB* and *topA* are used. It would be expected that sensitization to alkali sensitivity would be reduced in *gyrA* and *gyrB* deletion mutants and unaffected or enhanced in the *topA* deletion mutant.

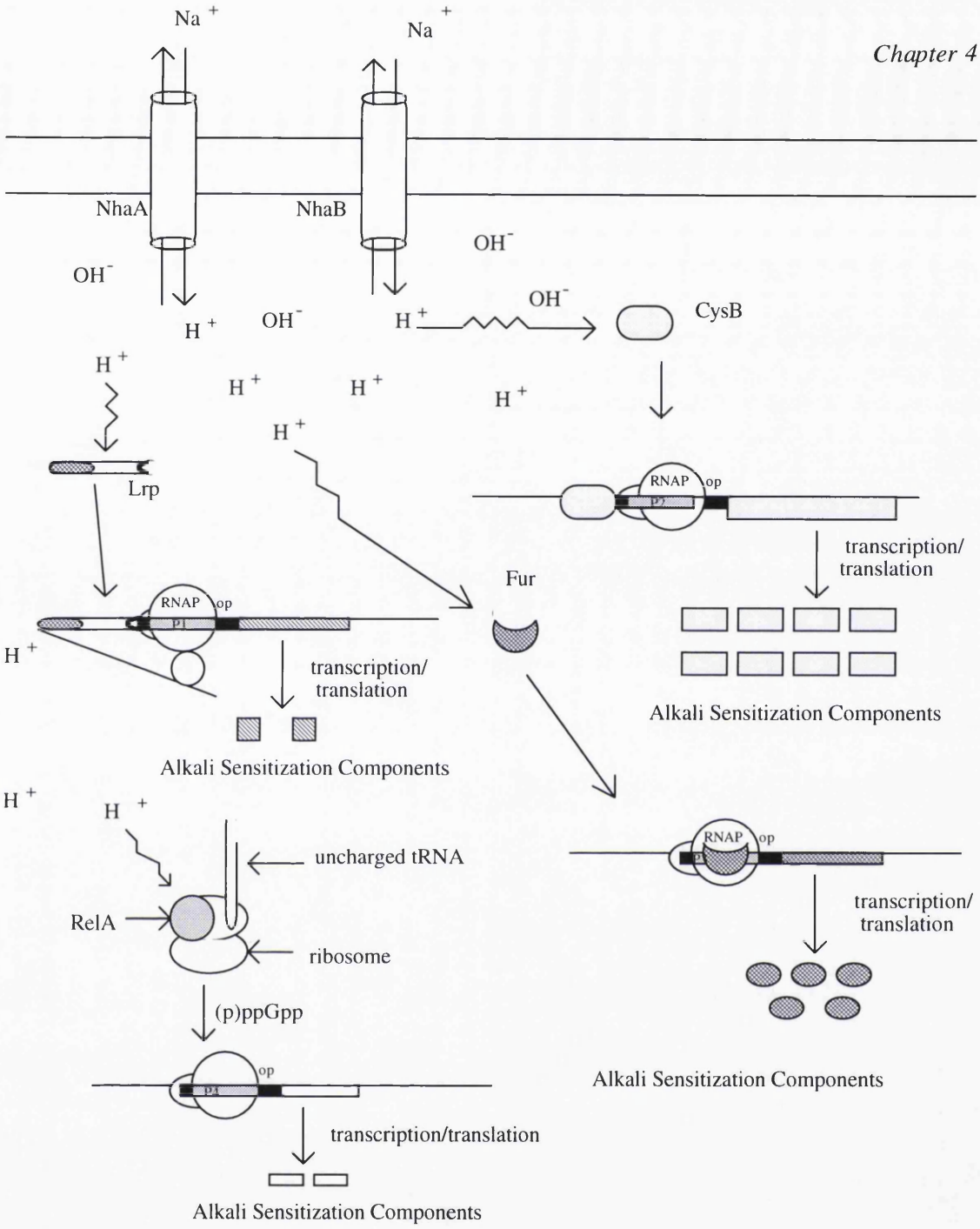


Figure 4-5 Diagram depicting the positive regulation of alkali sensitive components by Fur, Lrp, RelA and CysB at pH 5.5. These regulators presumably stimulated by the presence of H^+ in the cytoplasm bind to the appropriate site upstream of the promoters(P1, P2, P3) and activate the transcription of the alkali sensitive components. Of all these regulators, CysB is probably involved in the regulation of the majority of the alkali sensitization components as induction is almost abolished in strain having deletion in *cysB*. RNAP- RNA polymerase; Op-operator; P1, P2, and P3- promoters.

It has also been suggested that, external stimuli may influence the interactions of DNA with its chromatin components thus affecting topology and hence gene expression. In strains with deletion in *hns*, a gene encoding the histone-like protein, H-NS, the alkali sensitization was markedly reduced (**Table 3-50**). Similar effects were observed in strains having deletions in either *himA* or *himD*, the subunits of the integration host factor (IHF) (**Tables 3-51, 3-52**). Since mutants in *himA* and *hns* can cause relaxation in supercoiling (**Drilica, 1992**) the results obtained are in agreement with those obtained from the DNA gyrase inhibitors. The results suggest that the expression of alkali sensitive components is repressed in these deletion mutants. Thus these chromatin components appeared to play a role in the transcription of the alkali sensitization genes. Since H-NS acts as a histone-like protein and preferentially recognizes and shows enhanced affinity to curved DNA, the interaction of H-NS in the vicinity of the alkali sensitization components promoters may aid in the transcription of these genes by stabilizing the supercoiling thus enhancing activator-RNAP interactions. The involvement of IHF in the expression of these genes is probably through facilitating protein-protein interactions by inducing bends or loops between activators that are present upstream of the promoter region of genes encoding for the alkali sensitization components, and RNA polymerase (**Figure 4-6**). Thus, unlike acid habituation components, the expression of alkali sensitization components are affected by changes in the DNA topology.

Based on the results obtained from this study, a tentative model of stimulus-response pathway of acid-induced alkali sensitivity is outlined in **Figure 4-7**. Two possible pathways are proposed. In (**Figure 4-7 (A)**), Fur is proposed as the sensor of acidification of the cytoplasm and the cytoplasmic acidification serves as a signal to stimulate appropriate activators or regulators such as Fur, Lrp, RelA and CysB to transcribed the alkali sensitive components. In this pathway Fur acts both as a sensor and a regulator similar to the two-component regulatory systems. In the second pathway (**Figure 4-7 (B)**), the acidified cytoplasm is both the sensor and the signal and Fur, Lrp, RelA and CysB acts as the regulator for the activation of alkali sensitization components.

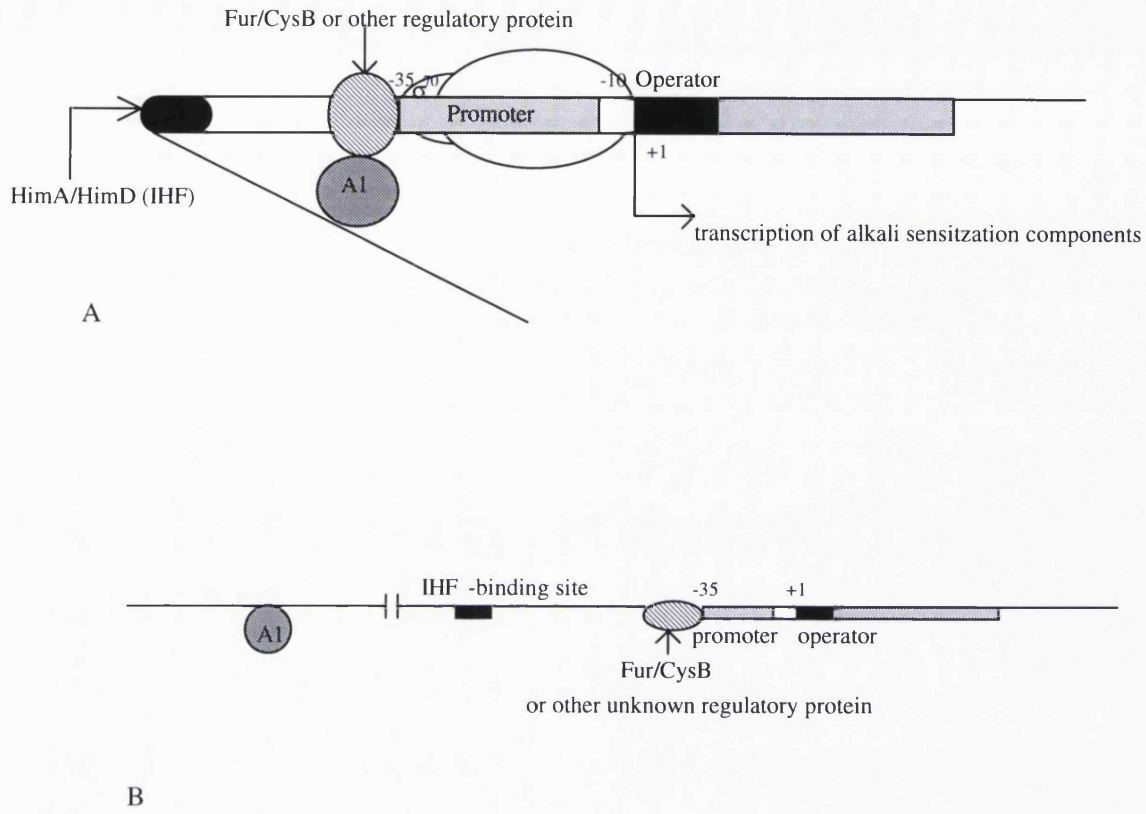


Figure 4-6 The role of IHF (HimA and HimD) in the regulation of the induction of alkali sensitive components at pH 5.5. (**A**) HimA and HimD can induce bending in DNA and thus can facilitate the interaction of upstreams activators (CysB or Fur) and RNA polymerase (RNAP) for the activation of alkali sensitization components. The deletion in *himA* or *himD* (**B**), prevents interaction between upstream activators and RNAP and thus repressed the transcription of alkali sensitization components.

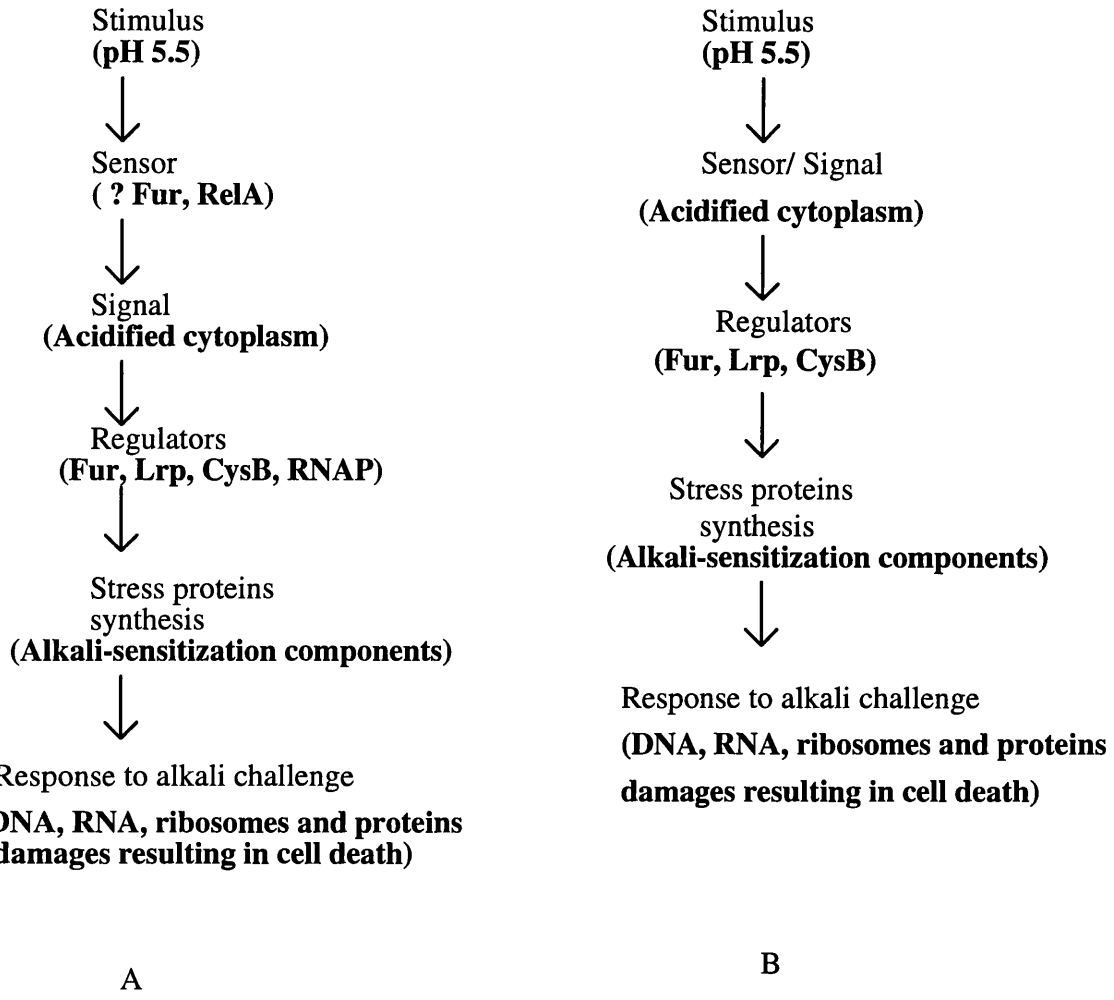


Figure 4-7 An outline of two possible stimulus-response pathways in acid-induced alkali sensitivity induction

4.5 Concluding Remarks

The aims of this study have been largely satisfied. Prior exposure to sublethal concentrations of copper could induce tolerance to lethal doses of copper on subsequent exposure. The copper -induced cells were also able to tolerate other stresses such as heat, acid and alkali pH and lethal concentrations of cadmium chloride. However copper exposure confers very little protection to hydrogen peroxide. Pre-exposure to mild acid pH which normally would confer tolerance to lethal acid pH, was found to induce sensitivity to lethal alkaline pH. In both inducible tolerance to copper and inducible sensitivity to alkali *de novo* protein synthesis was required. In the former, the newly synthesized or copper-induced components partly aided subsequent survival by protecting DNA and RNA and perhaps other macromolecules from the excess Cu^{2+}. The involvement of inducible mechanisms for effluxing and storing excess Cu^{2+} is also probable. Cells that were shifted from pH_o 7.0 to 5.5 were shown to be more sensitized to subsequent alkali challenge than those that were shifted from pH_o 7.0 to pH_o 7.0. The induction of these components requires acidification of the cytoplasm by H^+ crossing the outer membrane via PhoE, OmpC and to an extent LamB, and the cytoplasmic membrane via NhaA and NhaB. The induction of these components also seemed to be under the control of several global regulatory proteins such Fur, Lrp, RelA and CysB.

Although this study has shed a light on the fundamental processes involved in inducible tolerances to copper and inducible sensitivity to alkali further studies at the molecular levels of the components involved need to be undertaken to provide better understanding of these responses.

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