INDUCIBLE ACID RESISTANCE
IN ESCHERICHIA COLI

by
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DEDICATION

This thesis is dedicated to my parents

and

nephews namely Umer, Ali, Usama,

Imran and Junaid
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ABSTRACT

The acid sensitivity of *Escherichia coli* K-12 strains was studied. The introduction of ColV, I-K94 into the cells, made them more sensitive to acid and hydrogen peroxide treatments than cells without ColV plasmids.

The organisms tended to habituate when they were grown at pH 5.0, a sub-optimal value, and showed resistance when exposed to acid and H₂O₂ whereas the non-habituated cells, which were grown at the optimal pH of 7.0, were significantly more sensitive to acid conditions.

Habituation induced the synthesis of some proteins, which conferred on the cells resistance against acid treatments, and these were either synthesised in lower amounts in non-habituated cells or not at all, at 37 °C, when studied by one dimensional and two dimensional SDS polyacrylamide gel electrophoresis. Some stress-related proteins which induced at 37 °C and 44 °C were formed in very small amounts or not at all at 24 °C growth temperature.

Autoradiographic studies showed that the induction of stress-related proteins occurred within 5 min of an acid shift from pH 7.0 to pH 4.3. Addition of 10 mM phosphate buffer in NB of pH 4.3 inhibited induction of habituation in P678-54 but not in P678-54 ompA.

One probable site of acid injury was DNA. It was shown that for habituated organisms the DNA was less damaged and repaired better, after
acid treatments, compared with non-habituated cells when examined through transformation experiments using biological activity of pBR 322 as a transformation index. The probable biochemical basis of DNA repair was studied using mutants altered in _uvrA_, _recA_ and _polA_. 
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Abbreviations

Length

\begin{align*}
\text{mm} & \quad \text{nanometer} \\
\mu \text{m} & \quad \text{micrometer} \\
\text{nm} & \quad \text{millimeter}
\end{align*}

Mass

\begin{align*}
\mu \text{g} & \quad \text{microgram} \\
\text{mg} & \quad \text{milligram} \\
\text{g} & \quad \text{gram}
\end{align*}

Volume

\begin{align*}
\mu \text{l} & \quad \text{microlitre} \\
\text{ml} & \quad \text{millilitre} \\
\text{l} & \quad \text{litre}
\end{align*}

Time

\begin{align*}
\text{S} & \quad \text{second} \\
\text{min} & \quad \text{minute} \\
\text{hr} & \quad \text{hour} \\
\text{d} & \quad \text{day}
\end{align*}

Media

\begin{align*}
\text{NA} & \quad \text{nutrient agar} \\
\text{NA+D} & \quad \text{nutrient agar + 0.5\% sodium deoxycholate} \\
\text{NB} & \quad \text{nutrient broth}
\end{align*}

Chemicals

\begin{align*}
\text{Ca Cl}_2 & \quad \text{calcium chloride} \\
\text{DNA} & \quad \text{deoxyribonucleic acid} \\
\text{EDTA} & \quad \text{ethylenediaminetetraacetic acid} \\
\text{HCl} & \quad \text{hydrochloric acid} \\
\text{H}_2\text{O}_2 & \quad \text{hydrogen peroxide} \\
\text{LPS} & \quad \text{lipopolysaccharide} \\
\text{NaCl} & \quad \text{sodium chloride}
\end{align*}
NaOH sodium hydroxide
RNA ribonucleic acid
SDS sodium dodecyl sulphate

Miscellaneous

A ampere
mA milliampere
ca approximately
ccc covalently closed circular
oc open circular
°C degree Celsius
Ci curie
μCi microcurie
EAE C enteroadherent E. coli
EHEC enterohaemorrhagic E. coli
EIIEC enteroinvasive E. coli
EPSEC enteropathogenic E. coli
ETEC enterotoxigenic E. coli
D H₂O double distilled water
IEF iso electric focusing
kDa kilodaltons
LT heat labile toxin
ST heat stable toxin
p⁻ ED1829 E. coli strain
p⁺ ED1829 ColV, I-K94 E. coli strain
pH hydrogen ion concentration
pH₀ external pH
pHᵢ internal pH
μM micromolar solution
nM millimolar solution
M molar solution
MW molecular weight
OD optical density
UV ultraviolet
UTI urinary tract infection
w/v weight to volume
V volt
mV millivolt
1. Introduction.

1.1. Plasmids and their properties.

Plasmids are extrachromosomal genetic elements made up of double stranded DNA and exist predominantly in covalently closed circular (ccc) form which can be stably inherited. Some plasmids are capable of integrating into the host chromosome and are known as episomes.

Plasmid replication is either under stringent or relaxed control. In stringent control the replication is strictly related to chromosomal replication and 1 - 2 plasmid copies exist per cell. A large self-transferable plasmid like ColV shows stringent replication and requires cell to cell contact for transfer of genetic material from donor to the recipient organisms. Under relaxed replication control, up to 50 plasmid copies per chromosome are present and here the replication of the plasmids is independent of the control on chromosomal DNA replication, although host components are needed for the copying of the plasmid. Small or intermediate-size, multicopy, non-conjugative plasmids fall into this category.

Two or more compatible plasmids can co-exist in a bacterial cell whereas incompatible plasmids cannot exist together and after a few generations of bacterial growth one or the other is lost. Incompatible plasmids probably either compete for an attachment site on the host membrane or produce the same repressor that inhibits initiation of replication.
A number of characteristics are plasmid-specified such as the following:

1. Colicinogenic plasmids encode the ability to produce protein toxins, i.e. colicins.

2. Virulence plasmids code for increase in the pathogenicity of the invading organism within a host, like ColV plasmids which code for;

   a. Increased serum resistance.
   b. Enhanced iron uptake.
   c. Enhanced adherence to host cell surfaces.

3. The resistance or R factors (and some other plasmids) can carry resistance to antibiotics, inorganic ions and other bacteriocidal agents.

4. Degradative plasmids code for the ability to synthesise catabolic enzymes.

5. Some plasmids code for fermentation of certain sugars.

Plasmids are very useful tools for genetic engineers in cloning DNA technology. Many eukaryotic genes can be incorporated in plasmid vectors, for the synthesis of eukaryotic gene products.
1.2. ColV plasmid-specified properties:

1.2.1. Bacteriocins and colicins.

Bacteriocins are the compounds produced by some bacterial strains capable of killing the sensitive cells of closely related species and are protein in nature (Hardy, 1975). Chiefly they are produced by Gram-negative organisms like members of the enterobacteriaceae. Colicins are the very widely studied bacteriocins reported from *Escherichia coli* and *S. sonnei*. However, Gram-positive organisms can produce certain substances which are analogous to bacteriocins such as those produced by *Staphylococcus* and *Streptococcus* (Hardy, 1975).

1.2.2. Colicinogenic plasmids:

The colicinogenic plasmids, like other plasmids, are supercoiled covalently closed circular molecules. They code for colicinogeny and are named for the kind of colicin they produce such as E, K, I, B, A and V etc., colicins.

1.2.3. Grouping of Col plasmids:

The Col plasmids fall into two distinct groups depending upon their molecular weights (Hardy et al., 1973).

Group 1: contains small plasmids of molecular weight approximately 5x10^6. They are non-conjugative, their replication is
under relaxed control and up to 50 copies per cell are present; the colicins produced by them are mostly cell free, exemplified by ColA, ColE and ColK plasmids.

Group 2: contains large plasmids with molecular weights between $61 \times 10^6$ to $113 \times 10^6$. They are self-transmissible, conjugative, show stringent replication and are present in 1 - 2 copy number per chromosome, their colicin is cell bound as exemplified by ColV, Coll and ColB.

1.2.4. Characteristics of ColV plasmids:

ColVs are large F-like plasmids, as exemplified by ColV,I-K94 which is a self-transmissible, conjugative plasmid with stringent replication control and confers 1 - 2 copy number per chromosome (Hardy, 1975). It codes for the synthesis of colicins V and 1a and its immunity factors. It has a molecular weight of $94 \times 10^6$ (Clowes, 1972). It falls into incompatibility (IncF1) group with the transfer properties which are under derepressed control coded by the tra region.

1.2.5. Similarity between ColV factor and the F plasmid:

Marked homology exists between ColV factors and the F plasmid (Sharp et al., 1973) which is evident from the following facts:

1. Electron microscopy heteroduplex studies demonstrate that ColV,I-K94 and the F plasmid share extensive polynucleotide sequence similarities and the transfer region of F is homologous to ColV,I-K94 (Sharp et al., 1973)
except for the surface exclusion genes.

2. The sex pili of ColV,I-K94 and the F plasmid are essentially identical when studied through serological techniques (Hardy, 1975).

3. The finO gene product can repress the transfer of F and ColV,I-K94.

4. Both belong to the IncF1 group (Lane, 1981).

1.2.6. Colicin V synthesis:

Colicin V which is a small molecular weight, dialyzable, protein capable of killing the sensitive cells of closely related species, was discovered first by Gratia in 1925, following isolation of \textit{E.coli} V strain from a rabbit infection (Gratia, 1932). Maximum production of colicin V takes place during the late exponential phase of the culture and the production is temperature dependent as colicin ceases to be synthesised at 25 °C or lower. Purification of colicin V seems to be more difficult than the purification of other colicins but purified material has been obtained and molecular weight is approximately 4000 d (Frick \textit{et al.}, 1981; Yang and Konisky, 1984). Killing action of colicin is a two step event, where the colicin must bind to receptors in the outer membrane of the sensitive cells (stage I) and subsequent events (stage II) are energy dependent; the cells treated with uncoupling agents remain susceptible to rescue by trypsin which probably destroys the colicin adsorbed to the receptors of the target cells (Hardy \textit{et al.}, 1973).

1.2.7. Immunity to colicin V:

The Col factor also codes for the immunity factor whose gene product,
molecular weight 6500 d (Frick et al., 1981), renders \text{Col}^+ \text{ cells}
resistant to the lethal activity of their own colicin and certain other
\text{type V} \text{ colicins}. \text{Immunity} conferred by \text{ColV}, can be overcome by a high
concentration of colicin.

\text{Immunity} protein, is coded by a gene (\text{cvi}), closely linked with colicin
\text{production} gene (\text{cva}), and is \text{constitutively} \text{synthesised}.

1.2.8. \text{Plasmid incompatibility}:

\text{Incompatibility} is the phenomenon through which two closely related
plasmids cannot coexist in the bacterial cell stably. If two plasmids of
the same \text{incompatibility} group are introduced into a bacterial cell then
after a few generations most of the daughter cells will contain only one
of the plasmid kinds. \text{Incompatibility} is thought to be a consequence of
\text{the} \text{mechanism} controlling \text{plasmid} \text{replication} and \text{segregation} at cell
division.

\text{The} \text{phenomenon} may be due to the fact that plasmids of the same
\text{incompatibility} group compete for the \text{attachment} \text{sites} at the host
membrane. The \text{attachment} \text{site} may be important either for \text{initiation} of
\text{replication} or \text{essential} for \text{plasmid} \text{segregation} at the time of cell
division.

\text{ColV, I-K94} exists in nature as a \text{cointegrate} \text{plasmid} having two \text{replicons}:
\text{Rep1}, which is similar to \text{RepA} \text{replicon} of \text{IncFII} \text{plasmids} and \text{Rep2}
(Fig. 1), which is \text{homologous} to the \text{secondary} \text{replication} \text{region} of \text{F}
factors \text{belonging} to \text{IncF1}. \text{Inspite} of the fact that \text{ColV, I-K94} \text{plasmid}
has a \text{Rep-A} like \text{replication} \text{region}, it does not generally \text{behave} like
\text{IncFII} \text{plasmids}.  

- 26 -
Fig. 1. Map of the ColV,1-K94 :: Tn 903 derivative pWS12. Coordinates are in kilobases. Rep 1 and Rep 2: replication regions, incD: incompatibility locus, oriT: origin of transfer, $x_1$, $x_2$ and $x_3$: inverted repeats (adapted from Weber et al., 1984; Weber and Palchaudhuri, 1985).
Weber et al., (1984) demonstrated that the colicin-producing plasmids pWS12, pWS16, Tn903 derivatives of ColV,I-K94 showed incompatibility with both IncFI and IncFII plasmids. Whereas pWS15 and pWS17, EcoRI digests of pWS12, plasmids expressed only IncFII incompatibility but not IncFI incompatibility of their parental ColV plasmid. Though pWS12 has an IncFII replicon, RepI, it does not normally express IncFII incompatibility due to the presence of second replicon, Rep2. When Rep2 is deleted then ColV behaves as an IncFII plasmid.

1.2.9. Plasmid replication:

Replication starts at a specific site on the DNA termed as oriV, origin of vegetative replication, by adding deoxyribonucleotides through DNA polymerase to the RNA primer. The replication regions of ColV,I-K94 are represented in Fig. 1, ColV,I-K94 uses only Rep1 for replication since removal of Rep2 as in pWS16 has no effect on the stability of the plasmid. Moreover, inactivation of Rep2 by introducing the plasmid into a polA mutant strain does not affect the stability and replication of pWS16 (Weber et al., 1984).

Initiation of replication takes place by producing a nick at one of the DNA strands at oriV followed by unwinding of the DNA for synthesis of the new complementary strand. Electron micrographic studies show the replication of F plasmid is bidirectional and symmetrical. The direction of replication for ColV,I-K94 is unknown.

For replication of F, as for other plasmids, host factors are required (Scott, 1984). These include dnaB, dnaC and dnaE (polymerase III). Large
plasmids like F can replicate in dnaA mutants of Escherichia coli since they produce their own DnaA gene product because dnaA is integrated into the plasmid DNA. The DnaB is the replicative helicase of E. coli (Park, 1987). Moreover, RNA synthesis is required during the initiation of plasmid replication which acts to prime DNA synthesis of new strand.

1.2.10. Sex pili:

These are elongated, filamentous structures, essential for conjugation, present on the surface of F+, Hfr and certain other p+ bacteria belonging to the genera Escherichia, Shigella, Salmonella and Proteus. Their numbers are dependent upon environmental conditions, increasing during logarithmic growth phase and reaching maximum during late exponential phase. The transfer-proficient cells synthesise more sex pili after rapid washing, addition of antibodies specific to pili or decreasing the internal concentration of 3' 5' cyclic AMP. Stationary phase and amino acid starved cultures have no sex pili (Tomoeda et al., 1975).

The proximal tra cistrons like J,A,L,E,K,B,V,W,C,U,F,H and probably G are responsible for the synthesis and assembly of pili. A mutation in tra loci which code for the synthesis of pili renders the plasmid containing cell unable to conjugate. Similarly when the pili are sheared by blending or treated with antipilus antibodies, conjugation cannot take place.

Antibodies raised against F pili shows cross reactivity for ColV-specified sex pili suggesting that the sex pili encoded are essentially identical. F-like sex pili have a tube like structure, made up of repeated subunits.
of pilin which are arranged in a helical manner around the central pore. Bacteriophages like R17 and MS2 adsorb to the side of the F pili whereas M13 binds to the pilus tip.

The role of sex pili in conjugation is not fully understood. The two proposed functions are: 1. to provide a hollow tube for transfer of DNA from donor to the recipient, or 2. to bring donor and recipient together by retraction establishing close wall to wall contact after the pilus tip has become attached to the recipient (Manning and Achtman, 1979). The transfer of plasmid DNA is possible by both methods (Ou and Anderson, 1970).

1.2.11. Surface exclusion:

The phenomenon of surface exclusion is mediated through the gene products of \textit{tra S} and \textit{tra T} and is a mechanism to ensure that plasmid-containing strains do not mate with the cells bearing the same or closely related plasmids. The \textit{tra S} and \textit{tra T} gene products confer an additive effect during this process and a mutation in either gene abolishes surface exclusion.

The protein coded by \textit{tra T} is localized in the outer membrane of F\textsuperscript{*} cells and prevents the stable connections between donor and recipient. Its molecular weight is 25 kDa and there are approximately 29,000 copies per cell. The \textit{tra S} gene product, 18 kDa, occurs in the inner membrane and prevents DNA transfer after the establishment of stable connections between donors and recipients (Manning and Achtman, 1979) acting in an unknown way.
1.2.12. Plasmid specified conjugation:

Since ColV, I-K94 and the F factor share extensive homology in the \textit{tra} region, therefore, the events in transfer of plasmid DNA of one, may apply for the other. The \textit{tra} region (Fig. 2), comprising of 1/3 genome or 33 kilobases of F plasmid (Willetts and Wilkins, 1984), codes for functions necessary for transfer of plasmid DNA and is shown in Table 1.

Table 1. Functions of \textit{tra} region genes.
(From Willetts and Skurray, 1980; Hardy, 1981; Willetts and Wilkins, 1984)

<table>
<thead>
<tr>
<th>\textit{tra} gene(s)</th>
<th>functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>J</td>
<td>controls \textit{tra} operon switching on</td>
</tr>
<tr>
<td>A and J</td>
<td>involved in pilin production</td>
</tr>
<tr>
<td>Y and Z</td>
<td>involved in nicking of DNA strand for transfer</td>
</tr>
<tr>
<td>I</td>
<td>DNA helicase involved in unwinding of plasmid DNA strand</td>
</tr>
<tr>
<td>S and T</td>
<td>Surface exclusion</td>
</tr>
<tr>
<td>G and N</td>
<td>Involved in forming stable mating aggregates</td>
</tr>
<tr>
<td>D,I and G</td>
<td>involved in pilus retraction</td>
</tr>
</tbody>
</table>

Generally, in repressed plasmids like \textit{fin}^{+}, \textit{finO} and \textit{finP} gene products act together at an operator \textit{traO} (Finnegan and Willetts, 1973), which inhibit the transcription of \textit{traJ} gene coding for a product (J) required to switch on transcription of \textit{tra} operon. In contrast, ColV and F are derepressed plasmids since their \textit{tra} operon is expressed constitutively and they are able to transfer themselves into recipient cells.
Fig. 2. The map of F plasmid. oriT: origin of transfer, inc: incompatibility region, oriV: origin of vegetative replication.
1.3. Cell envelopes of Gram-negative bacteria: components and characteristics.

The envelope of the Gram-negative cell is composed of the innermost cytoplasmic membrane surrounded by two further layers: a thin peptidoglycan layer and the outer membrane (Fig. 3), which give an appearance of a trilaminar "railroad track" in electron micrographs. The envelope gives structural integrity and offers a protective barrier against antibiotics and hydrolysing enzymes. These layers are discussed in the following section of this chapter.

1.3.1. Cytoplasmic membrane:

The cytoplasmic membrane acts as an osmotic barrier between the cell and its surroundings and is composed of phospholipids and proteins. The phospholipids are amphipathic molecules consisting of a hydrophilic polar region and long hydrophobic lipid chains. In an aqueous environment, the phospholipids tend to aggregate in a fashion to bury their hydrophobic tails and leave their hydrophilic heads exposed to water constituting a thin bilayer. Some proteins are buried in the membrane, some cross it and are exposed at both sides.

Various physiological functions are attributable to the cytoplasmic membrane which include transport of solutes into the cell, electron transport and oxidative phosphorylation, biosynthesis of lipids, synthesis of peptidoglycan, DNA replication, protein secretions, motility and chemotaxis.
Protein catalyzing specific, facilitated diffusion

OmpA

Lipopoly saccharides

Trimers of porin protein

8 nm

5.7 nm

Murein lipoprotein
Phospholipid
Peptidoglycan (Murein)

Fig. 3. Schematic representation of the structure of Escherichia coli outer membrane-peptidoglycan complex (adapted from Nikaido and Vaara, 1985).
The cytoplasmic membrane forms a hydrophobic permeability barrier to most hydrophilic molecules. Aside from a few molecules that seem to diffuse freely e.g water, entry of most molecules in the cell requires specific proteins that mediate their uptake.

Most substances are transported across the membrane by three methods which are namely: active transport, carrier-mediated transport or facilitated diffusion and group translocation. In active transport, carrier proteins are involved in the transport of substances uphill against the electrochemical gradient which is energy dependent and the energy is obtained by hydrolysis of ATP by membrane bound ATPase. In carrier-mediated transport the passage of substances takes place through the carrier proteins without any expenditure of energy, therefore, it is called facilitated or passive transport or facilitated diffusion. Whereas in group translocation the solutes undergo chemical modification exemplified by the phosphotransferase system of glucose and mannose in E.coli. A small carrier cytoplasmic protein, HPr, with a molecular weight of 9 kDa is phosphorylated which transfers the P to a specific substrate on the outer surface, transports the phosphorylated sugar to the inner surface and releases it into the cytoplasm.

The electron transport carriers and cytochrome system of the respiratory chain are embedded in the cytoplasmic membrane. Pairs of electrons from various donors are transferred to NADH or a flavoprotein and then passed
from one carrier to another till the ultimate electron acceptor \((O_2)\) resulting in generation of ATP and free energy. Transferance of a pair of electrons from NADH to \(O_2\) results in generation of 3 ATP molecules from ADP and \(P_i\).

The cytoplasmic membrane is a seat of a number of biosynthetic processes. These include the synthesis of phospholipids, lipopolysaccharides and peptidoglycans. During lipopolysaccharide synthesis, the subunits of polysaccharide are assembled on a lipid carrier for ultimate transfer to the lipid A core. Similarly the N-acetylmuramic acid pentapeptide attaches to the carrier which receives N-acetylglucosamine from UDP to form the disaccharide for transferring to the growing cell wall.

The cytoplasmic membrane as well is involved in the division of bacteria through binary fission where the cytoplasmic membrane at the equatorial zone starts growing inwardly till the completion of a transverse septum for dividing the bacterium into two equal daughter cells.

Bacterial chemotaxis is another cytoplasmic membrane function associated with transport. By specifically sensing the concentration gradient of attractants or repellents, \textit{Escherichia coli} can modify the flagellar response and move toward or away from substances. Both the proteins responsible for sensing the chemical gradient (sensory transducers) and the proteins that drive the flagella (the flagellar motor) are integral membrane proteins.
1.3.1.1. Integral membrane methyl-accepting receptors/transducers:

These proteins are not involved in transport but do act as specific receptors for chemotactic effectors. Unlike the periplasmic binding proteins (see section 1.3.3), they recognize a signal and undergo methylation and are, therefore, known as transducers as well as receptors (Macnab, 1987b).

There are four well-characterized proteins of this sort: Tsr (taxis to serine and repellents including acids; also called MCPI), Tar (taxis to aspartate and repellents; MCPII), Trg (taxis to ribose and galactose; MCPIII), and Tap \( E \) (taxis associated protein; MCPIV \( _E \)); a fifth, Tip \( S \) (taxis-involved protein), is less understood.

These proteins have similar molecular weight (ca. 60 kDa) and firstly they act as conventional receptors, binding specific chemoeffectors with high affinity. Secondly, they act as secondary receptors for sugars, which they recognize indirectly by periplasmic binding proteins. Thirdly, they act as detectors of environmental parameters such as pH and temperature. Fourthly, they are "receptor" for a variety of hydrophobic compounds but little is known of the mechanism by which they sense the compounds (Tso and Adler, 1974).

1.3.2. Peptidoglycan layer:

The peptidoglycan layer which is attached to the outer membrane is made up of the repeated units of N-acetylmuramic acid tetrapeptide and N-acetylglucosamine linked with \( \beta-1,4 \) bonds, as shown in Figure 4,
Fig. 4. Building blocks of the *E. coli* peptidoglycan.
constituting the backbone of the bacterial cell responsible for maintaining the shape and integrity of the cell. In Gram-negative bacteria the polysaccharide chains are cross linked by direct peptide bonds between tetrapeptides.

1.3.3. Periplasmic space:

The space between the cytoplasmic and outer membranes is referred to as the periplasmic space which may be filled with periplasmic gel, a term coined by Hobot et al., (1984) and is responsible for maintaining the distance between the two membranes. The periplasmic gel is made up of a spongy peptidoglycan; the holes in the sponge contain an aqueous solution of the periplasmic proteins, oligosaccharides, monosaccharides, and other small solutes. The periplasmic proteins, are grouped into at least 3 distinct classes depending upon their functions.

Periplasmic binding proteins are water soluble proteins having specific binding sites for many active sugars, amino acids etc. They are likely to be involved in passing the bound solute to specific transmembrane transport proteins.

Three periplasmic proteins, products of the mglB, rbsB_E/rbsP_s, and mal_E genes, bind sugars (glucose/galactose, ribose, and maltose, respectively) with high affinity and specificity and transfer them to membrane-associated transport system (Macnab, 1987b). When associated with their ligands, they can also bind to integral membrane proteins that are members of the chemotaxis system, namely, the methyl-accepting chemotaxis proteins Trg (for MglB and RbsB) and Tar_E (for Mal_E).
Detoxifying enzymes: involved in detoxification or degradation of antimicrobial agents, e.g. $\beta$-lactamase present in some Gram-negative organisms, reacts with penicillin thus making it harmless for the microorganism.

Scavenging enzymes: involved in degradation of substances which are too large or highly charged to pass through cytoplasmic membrane into a form able to be translocated across the membrane such as alkaline phosphatase and 5'-nucleotidase (Hammond et al., 1984).

1.3.4. Outer membrane:

The outer membrane (Fig. 3), which is a coarse molecular sieve, consists of a bilayer of amphipathic molecules, with interspersed protein molecules. It is an additional layer in Gram-negative organisms constituting a permeability barrier to many environmental inhibitors and antibiotics. The outer membrane endows *Escherichia coli* with an effective barrier against the action of bile salts, lysozyme, and complement and affects phagocytosis.

The outer membrane is composed of three major constituents: phospholipids, lipopolysaccharides and proteins, which are discussed here.

1.3.4.1. Phospholipids:

In *Escherichia coli* the phospholipids (Figure 5) of the outer membrane are quite similar to those of cytoplasmic membrane. The
A. General structure of phospholipids, R: fatty acid chain.

B. Structure of the major types of phospholipids.

Fig. 5: A. General structure of phospholipids, R: fatty acid chain.

B. Structure of the major types of phospholipids.
phosphatidylethanolamine is enriched in outer membrane whereas phosphatidylylglycerol and diphosphatidylylglycerol are enriched in the cytoplasmic membrane. The phosphatidylethanolamine content of E. coli is slightly higher than that of S. typhimurium.

1.3.4.2. Lipopolysaccharide:

Lipopolysaccharide (LPS) is considered to be the endotoxin, with lipid A (Fig. 6) retaining the toxicity and the polysaccharide components conferring the antigenic specificity. LPS is composed of three parts: the proximal, hydrophobic lipid A region; the distal, hydrophilic O antigen polysaccharide region which protrudes out; and the core polysaccharide region which connects the two (Figure 7). The lipid A is made up of β-1, 6-D-glucosamine disaccharide, and is substituted with six or seven saturated fatty acids (Nikaido and Vaara, 1987). The proximal end of the core region is rich in negatively charged groups. The phosphate, pyrophosphate and COOH of KDO confer a strong negative charge to the surface of an enterobacterium which can be reduced by substitution with ethanolamine. The properties of mutants synthesizing incomplete LPS molecules reflect the biological functions of the LPS of outer membrane. Loss of O antigen results in loss of virulence. E. coli K-12 completely lacks the O antigen whereas B strain lacks in addition the more distal part of the core. Furthermore, the loss of more proximal part of core region, as in Re to R₁, result in "deep rough mutants" which are sensitive to hydrophobic agents (Figure 7).
Fig. 6. Structure of lipid A from the LPS of *Salmonella typhimurium*.
**Fig. 7. Structure of LPS.** (A) LPS from *S. typhimurium* LT2 and its mutants. Re through Rd refer to the chemotypes of LPS the mutants produced. (B) LPS from *E. coli* K12. (C) LPS from *E. coli* B. Dotted lines represent partial substitution. In B and C, dotted area (==) shows structural similarity with *S. typhimurium* LPS. In *E. coli* K-12, the core portion is different having rhamnosyl residue on one side of KDO. Moreover, lipid A carries 2 fatty acids chains instead of three. Abbreviations: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, galactose; OAc, O-acetyl; Glc NAc, N-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-mannoseheptose; KDO, 2-keto-3-deoxyoctonic acid; EtN, ethanolamine; P, phosphate; Glc N, D-glucosamine; Ara NH₃, 4-aminoarabinose.
1.3.4.3. Proteins:

About half the weight of outer membrane is made up of "major" proteins which include, murein lipoprotein, OmpA protein and porins. Relatively few enzymes are located in the outer membrane, like phospholipase A₁ and proteases, contrary to the cytoplasmic membrane (Salton, 1987).

1.3.4.3.1. Murein lipoprotein:

Lipoprotein is a small molecular weight (7.2 kDa) protein and there are approximately $7 \times 10^5$ copies in each cell. About one third of them are covalently linked with the peptidoglycan layer through the NH₂ group of its C-terminal lysine while the rest are free. Their polypeptides are mostly in the alpha-helical form and show cross linking (Lambert, 1988).

1.3.4.3.2. OmpA proteins:

The OmpA protein is a major constituent of the outer membrane (Fig. 3), with a molecular weight of ca 35 kDa, similar to porins, but behaves rather differently upon solubilization in SDS at low temperature being extracted at room temperature unlike porins. It is present in abundance like porins in outer membrane and its copy number approaches to $10^5$ per cell. It can be cross-linked chemically to underlaying peptidoglycan. The OmpA protein is rich in β-sheet structure and appears to span the thickness of the membrane. It is heat-modifiable and antisera raised against the OmpA protein of Escherichia coli cross react with an equivalent protein in Salmonella typhimurium. The OmpA proteins have two
important functions; 1. to stabilize the outer membrane and help in maintaining shape of the cell (Reithmeir and Bragg, 1977), 2. to act as receptors in conjugation in association with LPS (Lugtenberg and Van Alphen, 1983).

1.3.4.3.3. Porins:

The proteins coded by _ompF_, _ompC_ and _phoE_ genes are termed porins which are water filled pores or diffusion channels for the passage of hydrophilic molecules up to ca 600 D across the outer membrane (Fig. 3). The characteristics of these porins are listed in Table 2. They constitute major outer membrane proteins with ca $10^4$ copies/cell, the exact number depending upon the growth conditions. Their main function is to allow diffusion of nutrients across the outer membrane but antibiotics and other inhibitors can also use them. The OmpF and OmpC porins favour neutral and cationic compounds whereas the PhoE protein favours anions.

1.3.4.3.3.a. Porin channels in bacterial physiology:

Enteric bacteria have to survive in an environment that is full of powerful detergents (the bile salts), and the properties of porin channels are ideal for excluding these large, negatively charged, hydrophobic compounds (Nikaido and Vaara, 1987). The synthesis of OmpF porin is known to be repressed at high osmolarity and temperature and some strains under conditions that mimic the interior of animal body switch from larger OmpF channel to narrow OmpC channels and lose 50% or so of the permeability toward small nutrients with molecular weights of 100 to 200, while permeability toward larger, more hydrophobic, or negatively charged
Table 2: Porins of *Escherichia coli* K-12.

<table>
<thead>
<tr>
<th>Name</th>
<th>Alternative names</th>
<th>Estimated diam (nm)</th>
<th>Receptor for phage/colicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmpF</td>
<td>Ia, O-9, b</td>
<td>1.16</td>
<td>Tula, colicin A</td>
</tr>
<tr>
<td>CmpC</td>
<td>Ib, O-8, c</td>
<td>1.08</td>
<td>Tulb, Mel,</td>
</tr>
<tr>
<td>PhoE</td>
<td>Ic, e, E</td>
<td>1.16</td>
<td>TC23, TC45</td>
</tr>
</tbody>
</table>
compounds is drastically reduced because of the restrictive properties of OmpC channels (Nikaido and Vaara, 1985). On the other hand, the OmpF porin is probably beneficial when bacteria are out of the animal body.

1.3.4.3.3.b. Diffusion through porin channels:

The outer membrane of _E.coli_ acts as a molecular sieve that allows the passage of small hydrophilic molecules through porins. Diffusion of solutes through these channels is affected greatly by the physicochemical properties of the solutes. Three major factors that influence the penetration of these solutes are the size, electrical charge and hydrophobicity of the solute molecules (Nikaido and Vaara, 1987).

Size of a solute: the chances of the solute molecule to successfully enter the channel by the process of random collisions is greatly influenced by the size. The rate of diffusion of disaccharides through OmpF and OmpC is nearly two orders of magnitude lower than the rate of diffusion of a pentose.

Hydrophobicity of the solute: permeability through OmpF channels in _E.coli_ is affected negatively by the hydrophobicity of the solute.

Electrical charge: OmpF and OmpC porins have a preference for cations and uncharged molecules and diffusion of uncharged sugars through these channels was several times faster than that of corresponding negatively charged sugar acids.
1.3.4.3.4. Proteins involved in specific diffusion processes:

The proteins involved in specific diffusion processes are listed in Table 3. The gene product of lamB is involved in the passage of maltose and maltodextrins through the outer membrane. When lamB is fully induced very large copy numbers of protein are produced, comparable to those of porins. This protein resembles porins in the sense that it forms tightly associated trimers stable in SDS, is rich in β-sheet structure, and is associated with murein. It also act as a receptor of lambda phage (Nikaido and Vaara, 1987).

The gene product of tsx is the receptor for phage T6 and required for diffusion of nucleosides. These functions are not dependent on the TonB product. Apart from LamB and Tsx, the proper functions of other proteins require the TonB product (Postle and Good, 1983). The TonB is assumed to be involved in "energy coupling" but its precise role is not clear yet. A number of outer membrane minor proteins (Table 3) are involved in transport of various chelates of ferric ion.
Table 3: Outer membrane proteins involved in specific transport.

<table>
<thead>
<tr>
<th>Name</th>
<th>Mol Wt in kDa</th>
<th>Receptor for phages/colicins</th>
<th>Ton B requirement</th>
<th>Solute transported</th>
</tr>
</thead>
<tbody>
<tr>
<td>LamB</td>
<td>ca. 47</td>
<td>lambda</td>
<td>-</td>
<td>Maltose, maltodextrin</td>
</tr>
<tr>
<td>Tsx</td>
<td>&quot; 26</td>
<td>T6, colicin K</td>
<td>-</td>
<td>Nucleosides</td>
</tr>
<tr>
<td>BtuB</td>
<td>&quot; 66</td>
<td>BF23, E-colicins</td>
<td>+</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
</tr>
<tr>
<td>Cir</td>
<td>&quot; 74</td>
<td>Colicins I and V</td>
<td>?</td>
<td>Ferric iron ?</td>
</tr>
<tr>
<td>FhuA (TonA)</td>
<td>&quot; 78</td>
<td>T1, colicin M</td>
<td>+</td>
<td>Ferrichrome</td>
</tr>
<tr>
<td>FecA</td>
<td>&quot; 80.5</td>
<td></td>
<td>+</td>
<td>Ferric citrate</td>
</tr>
<tr>
<td>FepA</td>
<td>&quot; 81</td>
<td>Colicin B</td>
<td>+</td>
<td>Enterochelin</td>
</tr>
</tbody>
</table>

Adapted from Nikaido and Vaara, (1987).
1.4. Motility and chemotaxis of E.coli:

Motility and chemotaxis play an important role in the survival of Escherichia coli and numerous other organisms that are motile. The motility and taxis systems of Escherichia coli and Salmonella typhimurium resemble each other closely and both the organisms have a quite extensive range of stimuli to which they respond by moving in the direction of increasing stimulus intensity (in case of attractant) or decreasing stimulus intensity (repellent). The mechanism involves sensing temporal gradients of stimulus and responding accordingly by means of the locomotory machinery of the cell (Macnab, 1987b).

1.4.1. Flagellum:

Movement of E.coli involves the rotation of a flagellum (Fig. 8) which consists of an external filament, a short curved segment known as hook and a complex structure of rings and rods called the basal body (Macnab, 1987a).

1.4.1.1. Filament:

The filament is the propulsive component of the flagellum, it does no chemomechanical work but is passively driven by a motor at its base and performs the function of movement by rotation and not by bending. It is significant clinically in terms of its variability in antigens (Macnab, 1987a).
Fig. 8. Structure of the Escherichia coli flagellum.
Characteristics of filament:

The flagellar filament is composed of a protein called, flagellin, whose molecular weight is 55 kDa. The filament length ranges between 5 to 10 μm and has a constant diameter of approximately 20 nm throughout the length. Isolated flagellar filaments can be readily depolymerized into monomeric flagellin by mild acid or heat (Asakura, 1970). Flagellin and flagellar filaments are potent antigens and this has provided a basis for distinguishing strains.

1.4.1.2. Hook:

The filament is connected to the cell by the hook, which is structurally homologous to the filament but composed of a distinct hook protein whose molecular weight is ca. 42 kDa. The hook is approximately 80 nm long and is believed to act as a flexible coupling or joint between the cell and filament (Macnab, 1987a).

1.4.1.3. Basal body:

The hook is connected to a complex structure of rings and rods embedded in the cell surface. This consists of a set of four rings; the outer two rings (known as L and P) are spaced apart but joined through cylindrical wall to the inner two rings (S and M) as shown in the schematic diagram Fig. 8. The MotA and MotB proteins, which are integral cytoplasmic proteins, are necessary for motor rotation both counterclockwise (CCW) and (CW) clockwise (Macnab, 1987a).
1.4.2. Chemotaxis:

Motile bacteria exhibit a chemotactic response after tasting their environment by means of specific chemoreceptors (see section 1.3.1.1). They show positive chemotaxis towards attractants like sugars and amino acids and negative chemotaxis from repellants such as phenol, acid or alkali (Tso and Adler, 1974; Leive and Davis, 1980; Macnab, 1987b).

1.4.2.1. Mechanism of sensory transduction process:

A schematic model for sensory transduction of E.coli and S.typhimurium is shown in Fig. 9. The MCP-type receptors bind specific chemicals such as aspartate or maltose complexed to periplasmic binding protein (see section 1.3.3) and cause excitation (Macnab, 1987b). The excitation signal (S_{excit}) causes enhanced CCW rotation of the flagellar motor if the stimulus is an increase in attractant concentration. The signal probably comprises a change in the association state and small-molecule-binding state of various chemotactic proteins (CheY, CheZ, CheA, CheW). The adaptation signal (S_{adapt}) progressively cancels the excitation signal and the motor returns to its unstimulated CCW/CW ratio; the process involves a change in the methylation of the MCPs, mediated by a methyltransferase and a methylesterase. Binding of CheZ and CheY to the flagellar switch places it in CCW and CW state respectively.
Fig. 9. Process of sensory transduction of *E.coli* and *S.typhimurium*. Specific chemicals (solid circle), change in the association state and small-molecule binding state (solid square). Abbreviations: $S_{\text{excit}}$, excitation signal; $S_{\text{adapt}}$, adaptation signal; MCP, methyl-accepting receptors/transducers; CheY, CheZ, CheA and CheW, chemotaxis proteins (adapted from Macnab, 1987b).
1.5. Public health importance of *Escherichia coli*:

The pathogenesis of *E.coli* is classified into 2 broad groups; 1. extraintestinal diseases, 2. intestinal diseases.

1.5.1. Extraintestinal diseases:

1.5.1.1. Infection in man:

*E.coli* is an important aetiological agent for urinary tract infection (UTI). The UTI being most common in females (Sonnenwirth, 1980) and increasing in incidence with age in both sexes (Sussman, 1985). Various types of urinary tract infection are caused by *Escherichia coli*, among them covert bacteriuria (CBU) or asymptomatic bacteriuria is the most common. It is now recognized that the silent phase of infection may be punctuated by symptomatic episodes which lead to infection limited to the bladder (acute cystitis) or kidney (pyelonephritis).

The pathogenicity of *Escherichia coli* strains responsible for urinary tract infection may rest upon the production of different virulence factors; amongst them are capsular (K) antigen, somatic (O) antigen and haemolysin whilst the presence of colonizing factor (leading to increased adherence to epithelial membrane) may allow *E.coli* to establish themselves in the urinary tract and resist the wash-out effects of urine flow (Reid and Sobel, 1987; Sussman, 1985).

In septicaemia, *Escherichia coli* enter the blood circulation in large numbers, over a period of time, either from an infected tissue lesion,
after invasive penetration from the intestine or most commonly from a urinary tract infection. In the case of septicaemia, like bacteraemia, the micro-organisms are removed by the phagocytic system from circulation but it is the endotoxin (LPS) released from the organisms in the blood which cause the symptoms (Sussman, 1985).

Meningitis due to *Escherichia coli* is an infection of the newborn and 80% of the causative strains contain the K1 antigen (McCraken *et al.*, 1974). The *E. coli* isolated from cerebrospinal fluid of a newborn baby with meningitis cross reacted with the serum raised against *N. meningitidis* (Grados and Ewing, 1970) indicating structural homology of K antigen with the meningococcal group B polysaccharide (Liu *et al.*, 1971).

Wound infections due to *Escherichia coli* are common following surgical operations such as appendicectomy.

1.5.1.2. Infection in animals:

1.5.1.2.a. Systemic colibacillosis:

*E. coli* can cause colisepticaemia in farm animals, where the organisms pass through the mucosa of the alimentary tract and enter the blood stream causing generalized infection. It affects calves that fail to absorb gamma globulin from colostrum after their birth. It is a potentially fatal infection often leading to death of the calves within a few days of birth. It is characterized by enlargement of the spleen and haemorrhages on the surfaces of heart, peritoneum and blood vessels (Linton and Hinton, 1988). It was reported that of 31 *Escherichia coli* strains isolated from generalized infection of calves, 25 produced colicin V (Smith, 1974).
ColV⁺ strains are highly pathogenic and this property can easily be lost by curing the plasmids while on its reintroduction the virulence can be restored. The virulence of the Col⁺ strains is not due to the toxicity of colicin V but due to their ability to survive in the blood and internal organs of the infected host. Studies of orally fed colostrum-deprived calves administered Col⁺ and Col⁻ forms revealed that the calves given the ColV⁻ form were normal in appearance after 24 hr whereas ones administered with ColV⁺ were seriously ill with high concentrations of organisms in blood, kidneys and lungs (Smith and Huggins, 1976). Similar results were observed in chickens injected intramuscularly with ColV⁺ and Col⁻ forms, confirming the pathogenicity of ColV⁺ strains (Smith and Huggins, 1976).

Systemic colibacillosis is common in 2 - 3 week old lambs and the infection may be either generalized or localized (Linton and Hinton, 1988). Colissepticaemia, an important cause of losses in the poultry industry, occurs in chickens aged 5 - 12 weeks characterized by fibrinous pericarditis, air sacculitis and synovitis (Linton and Hinton, 1988).

1.5.1.2.b. Bovine mastitis:

The incidence rate for bovine mastitis caused by Escherichia coli has risen rapidly. The pre-disposing factors like use of intramammary tube, increase in herd size, changes in methods of housing and decreased time on pasture, teat dipping and dry cow therapy have rendered the mammary gland susceptible to opportunist E.coli. The infection is characterized by responses which range from mild to acute reactions. In acute cases, the organisms multiply rapidly without any check and cause toxaemia resulting
in severe damage to the udder; death may occur within 24 hr (Linton and Hinton, 1988).

1.5.2. Intestinal diseases:

Certain E. coli strains exhibit pathogenicity and cause diarrhoea including, enterotoxigenic, enteropathogenic, enteroinvasive, enteroadherent, and enterohaemorrhagic types (Levine, 1987).

1.5.2.a. Enterotoxigenic strains: ETEC strains are responsible for causing disease ranging from the mild traveller's diarrhoea to the severe cholera-like syndrome and infant diarrhoea in less developed countries. ETEC diarrhoea is less common in USA and other developed countries.

ETEC infection is acquired by ingesting contaminated food or water. The bacteria adhere to the host tissue through hair like filamentous organelles called fimbriae and colonize the proximal small intestine. The fimbriae are rigid structures of 6 - 7 nm diameter encoded by plasmids; many but not all, mediate haemagglutination of certain erythrocytes which is unaffected by mannose.

The enterotoxigenic strains produce a heat-labile enterotoxin (LT), or heat-stable (ST) one, or both. The heat-labile toxin is quite similar to the cholera toxin, with a molecular weight of 80 kDa, has serological specificity and activates adenyl cyclase. The heat-stable toxin, on the other hand, is 8.5 kDa and activates guanyl cyclase. The clinical features of the ETEC infection are watery diarrhoea, nausea, abdominal cramps and
low grade fever.

1.5.2.b. Enteropathogenic strains: EPEC were found in epidemics of severe diarrhoea in infants. The strains have transmissible plasmids and _Shigella_-like invasiveness. When EPEC strains lacking LT, ST, and invasiveness were fed to adult volunteers they caused definite diarrhoea (Levine et al., 1978).

EPEC strains cause a peculiar ultrastructural histopathologic lesion in human intestine (visible by electron microscopy) that is not observed with ETEC strains. The lesions involve destruction of microvilli by EPEC bacteria without any further destruction of host cells (Polotsky et al., 1977).

The clinical features of EPEC infection are fever, malaise, vomiting, and diarrhoea with mucus but without blood. The EPEC illness seems more severe than other diarrhoeal infections.

1.5.2.c. Enteroinvasive strains: EIEC strains are, of serotypes distinct from ETEC and EPEC, and are similar to _Shigella_ in certain respects. They cause an invasive, dysenteric form of diarrhoeal illness in volunteers. EIEC penetrate, proliferate within the epithelium of the large intestine (less frequently the small intestine) and eventually cause the death of the host cell without any systemic phase. The invasiveness of EIEC and _Shigella_ is dependent upon plasmid coded outer membrane protein. Clinically, EIEC illness is characterized by fever, severe abdominal cramps, malaise, toxaemia, and watery diarrhoea followed by gross dysentery with stools containing blood and mucus (Levine, 1987).
1.5.2.d. Enteroadherent strains: EAEC are identified by their property of adherence to Hep-2 cells (Mathewson et al., 1985), and cause mild diarrhoea in human volunteers. EAEC do not produce LT, ST, or increased level of Shiga-like toxin or invade epithelial cells.

1.5.2.e. Enterohaemorrhagic strains: Escherichia coli 0157:57 was a serotype and not recognized as a causative agent of diarrhoeal disease, until isolated in a USA outbreak of haemorrhagic colitis in 1982. 0157:H7 has emerged as an enteric pathogen of public health importance when associated with outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome, and diarrhoea in nursing homes, day care centres and schools in Canada and the United States. The clinical features of the disease are bloody but copious diarrhoea, these features distinguish it from dysentery by Shigella or EIEC.

1.5.3. Virulence characteristics of ColV+ organisms.

1.5.3.1. Enhanced adhesion to epithelial surfaces.

Bacterial colonization factors, surface components that enhance colonizing ability, influence the complex interaction between bacteria and host animal tissues such that greater numbers gain access to potential new niches to proliferate rapidly at particular sites. Invasive bacteria must normally colonize animal mucosae before crossing the epithelial linings to enter into sterile regions of the body so colonization factors are very important components for invasion (Williams et al., 1988).
E. coli strains produce adhesins (colonization factors) to colonize the mucosal surfaces of the host and e.g. enable themselves to overcome firstly the clearing mechanism of the peristaltic movement and secondly the presence of the inhibitory mucus which is attached to the epithelium. Production of adhesins is encoded by various plasmids, and enables the bacteria to adhere to the mucosal surfaces e.g. of the intestinal tract and by elimination of those plasmids the bacteria lose the property of adhering to the surface of epithelial membranes.

Strains of E. coli K-12 which harbour ColV\(^+\) adhered \emph{in vitro} 2 - 3 times better to discs of mouse intestinal tissue than isogenic ColV\(^-\) strains (Clancy and Savage, 1981). This observation was confirmed by Tewari (1986) by demonstrating that ColV\(^+\) cells adhered in greater numbers than a strain lacking the ColV plasmid. Hydrophobic interactions are important in adhesion to mucous surfaces. The hydrophobicity of the two strains was investigated, and surfaces of ColV\(^+\) were much more hydrophobic than the ColV\(^-\) cells. The ColV plasmid-encoded sex pili were responsible for the increased hydrophobicity. Hydrophobicity was decreased when the cultures were grown at 21 °C rather than at 37 °C and by mechanical shearing. This demonstrates a relationship between the hydrophobicity and sex pili (Tewari \emph{et al.}, 1985).

1.5.3.2. Autoagglutination and motility inhibition:

The presence of virulence plasmids ColV,I-K94 or ColV-K30 in \emph{E. coli} produces some envelope changes. Tewari \emph{et al.}, (1986) demonstrated that introduction of ColV,I-K94 or ColV-K30 into \emph{E. coli} strains produced
derivatives which had a motility lesion if grown without shaking at 37 °C. Although most ColV+ organisms from shaken cultures were motile, 80 - 90% of free unclumped organisms from static cultures were flagellate but non-motile. This plasmid effect was temperature-dependent with only ColV+ organisms grown at 37 °C being affected; ColV+ organisms grown at 30 °C or below were predominantly motile. A similar phenomenon of autoagglutination/clumping was observed by Rowbury et al., (1985) where ColV plasmid induces clumping for static cultures at 37 °C.

Autoagglutination and motility inhibition may be potential virulence factors. In the intestine, the organisms overcome the clearing mechanism of peristaltic movements and the inhibitory action of mucus, by colonization; in this situation, motility and hydrophobicity are advantageous. However, on reaching the blood stream the motility may be disadvantageous for a motile pathogenic Gram-negative organism (Tomita et al., 1981). It is presumed that ColV+ organisms might lose motility and autoagglutinate (clump) in this static condition thus reducing susceptibility to phagocytosis.

Autoagglutination and motility inhibition seem to be governed by the same factors. Organisms regained their motility on incubation with buffered detergent solution suggesting that envelope changes might be responsible for this altered behaviour. The motility lesion occurs in the presence of transfer factor and colicin components (Tewari et al., 1986).

1.5.3.3. Increased serum resistance:

Pathogenic enteric bacteria on gaining access to the blood, by overcoming
the numerous defense mechanisms, confront the bacteriostatic and
bacteriocidal activities of serum. Immunoglobulins and non-specific
factors like lysozyme and complement can lead to phagocytosis and/or
bacterial killing (Waters and Crosa, 1991). Bacterial killing by serum
occurs when the five terminal proteins of the complement cascade,
organized as a macromolecular assembly known as the membrane attack
complex (MAC), are deposited on and inserted into the bacterial outer
membrane, destroying its integrity.

Resistance to serum killing is multifactorial and complex and has been
attributed to different surface components, such as lipopolysaccharides,
capsules and surface proteins, most of which are chromosomally encoded
(Waters and Crosa, 1991). Plasmid-mediated factors have also been
identified as responsible for increased serum resistance (Binns et al.,
1979).

Earlier findings of Smith (1974) demonstrated that a ColV+ strain
survives better than its counterpart ColV- in animal sera. Later on
Binns et al., (1979) observed 100-fold reduction in LD50 of chicks after
injecting strains carrying plasmid derivatives containing a cloned
BamHI-generated fragment of ColV,I-K94; the (iss) locus was responsible
for increased survival in serum. Plasmid derivatives having iss genetic
determinants enhanced the pathogenicity of the organisms in chickens
indicating that increased survival in serum is associated with virulence.

The iss gene maps well outside the tra operon and does not hybridize with
the traT gene (the product of this gene from several plasmids also
enhances serum resistance) so the two genes are distinct; the two gene
products are also antigenically different (Binns et al., 1982). The \textit{traT} gene product is a 25 kDa outer membrane protein, whereas the identity of \textit{iss} gene product is unknown but it may be, like TraT, an envelope protein.

It is probable that \textit{iss} and \textit{traT} gene products function in the same manner, but the mechanism is not fully understood. Binns et al., (1982) proposed that the terminal complement complex is not blocked by both the gene products, but the action of this complex on bacterial surfaces is abolished in the presence of \textit{iss} and \textit{traT} genes.

1.5.3.4. Enhanced iron uptake:

Availability of free iron in nature is very low, because it occurs in insoluble forms. In the animal body, iron occurs in bound form with the red blood corpuscles (intracellularly). In the serum and other body fluids it is found (extracellularly) in bound state in iron binding proteins like transferrin and lactoferrin respectively.

Ability of invasive organisms to multiply in host tissues is greatly influenced by the availability of iron. \textit{E.coli} synthesize a low molecular weight siderophore, capable of competing with host iron-binding proteins for iron, called enterochelin. Enterochelin is produced endogenously and secreted into the medium where it binds iron and transports it back into the bacterial cell through specific receptors on the outer membrane.

It has been demonstrated that strains of \textit{E.coli} carrying ColV plasmids are more highly pathogenic than their counterpart plasmid free strains. This
is attributed to their ability to survive in the host tissues (Smith and Huggins, 1976).

In 1979, Williams designed a classical experiment where strains with and without pColV-K30 were inoculated into pairs of mice intraperitoneally. One animal of each pair received iron. Without the coinjection of iron, the ColV strain was recovered at a much higher proportion than that in the administered dose. However, with added iron, there was no enhanced recovery of ColV bearing E.coli. He demonstrated that some ColV plasmids encode an iron-sequestering system which plays a key role in survival of the bacteria under iron-limited conditions. This mechanism was encoded by pColV-K30 and all tested ColV plasmids from bacteraemic strains but not by ColV,I-K94 plasmid. He also reported that some colicin V-producing cells (but not ColV,I-K94+ ones) grew normally despite addition of the serum iron-binding protein transferrin to media, whereas cells lacking the ColV plasmid had a significantly decreased growth due to conversion of the free iron to an unavailable form. This inhibitory effect could be reversed by addition of excess iron.

The ColV plasmid-mediated iron-sequestering mechanism involves production of a siderophore called aerobactin, and an outer membrane protein that forms at least part of the ferric-aerobactin receptor. This receptor has a molecular weight of 74 kDa and is observed in cir mutants which lack the 74 kDa Cir protein (Bindereif et al., 1982). The property of synthesizing aerobactin is an added advantage to a bacterial strain which is already capable of synthesizing enterochelin; since the enterochelin iron-uptake system is energetically expensive as enterochelin cannot be recycled, on
the other hand aerobactin can be recycled. Moreover, the production of anti-enterochelin and anti-receptor antibodies in serum of animals may limit the effectiveness of enterochelin-mediated iron-sequestering system and favours the aerobactin-mediated iron transport (Griffiths, 1985).

1.5.3.5. Colicin production:

E.coli harbouring ColV,I-K94 code for the synthesis of both colicin V and Ia and immunity factors which confer immunity to the bacterial cell for their own action. The molecular interaction associated with this immunity becomes the basis for identification of subtypes. Although, colicins Ia and Ib adsorb to the same receptor and share homology in physical and chemical properties yet the strains having the ColIa plasmids are immune to the colicin Ia but not to colicin Ib and vice versa. As far as immunity to colicin Ia is concerned, it is mediated by plasmid coded immunity protein of 14.5 kDa located in the cytoplasmic membrane to which the colicin Ia interacts and this renders the bacterial cell immune to its action (Konisky, 1982).

The colicin V is synthesized in low amount within the bacterial cell and is not released from the cells by means of cell lysis (the typical mechanism for most colicins) but rather appears to be exported (Gilson et al., 1987). Four plasmid genes required for colicin V synthesis, export and immunity, have been defined within a 4.2-kb region of DNA cloned from pColV-K30 by Gilson et al., (1987). They designated the immunity determinant cvi, the genes required for colicin V export cvaA and cvaB and the structural gene for the synthesis of colicin V cvaC. Mutation in cvaA
and cvaB prevents any detectable activity in the supernatants of bacterial cells whereas colicin V activity can be observed in lysed cells. On the other hand, any mutation in cvaC gene results in abolishing of colicin V activity in both the supernatants and the cells (Gilson et al., 1987).

Smith (1974) showed 67% of E.coli strains causing bacteraemic infections in poultry produced colicin V. It was therefore thought that the colicin molecule itself might have some role in pathogenicity of the ColV+ organism. However, in 1979, Quackenbush and Falkow used transposon Tn1 mutagenesis to show that bacteria carrying a ColV plasmid with a mutated colicin V structural gene (cvaC) were more lethal to mice (than Col- strains) when injected intraperitonially suggesting that colicin V is not essential for increased virulence.

Colicin V action is initiated by its adsorption to specific receptors on the surfaces of sensitive bacteria which allow the colicin to overcome the barrier properties of outer membrane. Colicins vary in their mode of action which is summarized in Table 4. The primary target of colicin V is the cytoplasmic membrane. It has been demonstrated that partially purified colicin V inhibited the active transport across the membranes of colicin V sensitive cells but not in colicin resistant cells (Yang and Konisky, 1984). This inhibition of active transport was not a result of general leakiness of the cytoplasmic membrane but due to collapsing of the membrane potential of the treated cells. In the same way, colicin la induces formation of ion-permeable channels in the cytoplasmic membrane, resulting in dissipation of membrane potential and leakage of low
Table 4: Colicins with known mode of action.

<table>
<thead>
<tr>
<th>Colicin</th>
<th>Mode of action</th>
<th>From SDS-PAGE</th>
<th>Mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Membrane depolarization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Membrane depolarization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>Membrane depolarization</td>
<td></td>
<td>69 kDa</td>
</tr>
<tr>
<td>la</td>
<td>Membrane depolarization</td>
<td></td>
<td>79 kDa</td>
</tr>
<tr>
<td>lb</td>
<td>Membrane depolarization</td>
<td></td>
<td>80 kDa</td>
</tr>
<tr>
<td>E2</td>
<td>DNA endonuclease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>Ribosome inactivation</td>
<td></td>
<td>60 kDa</td>
</tr>
<tr>
<td>DF13</td>
<td>Ribosome inactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>prevent membrane potential of treated sensitive cells</td>
<td></td>
<td>4 kDa</td>
</tr>
</tbody>
</table>

Adapted from Luria and Suit, 1987.
molecular weight substances.

1.5.3.6. VmpA protein:

Most Col\(^+\) strains produce VmpA, a major outer membrane protein (Rowbury et al., 1985). Changes in cell envelope due to production of this plasmid specified protein may cause increased virulence (Moores and Rowbury, 1982). The VmpA protein is synthesized from a precursor of approximate molecular weight 34.5 kDa and shares homology with OmpA protein (1) immunologically (Deeney et al., 1986), and in being (2) a transmembrane component of the outer membrane, (3) trypsin sensitive in membrane preparation, (4) not murein associated, (5) soluble in 2\% SDS at 60 °C, (6) molecular weight 33 kDa. However, they differ in certain respects such as VmpA cannot replace OmpA as receptor for phages K3 and Tull\(^*\) and as a component in colicin L sensitivity but nevertheless it weakly replaces OmpA protein in stabilisation of conjugal aggregates (Moores and Rowbury, 1982). Its synthesis is dependent upon cultural conditions. VmpA is produced by Col\(^V+\) strains when grown at 37 °C with shaking rather than statically. Incorporation of magnesium and calcium (0.005 - 0.05 M) in the growth media inhibit the synthesis of the protein (Deeney et al., 1986).
1.6. Adaptive responses to stress:

1.6.1. Adaptive response to alkylating agents:

The physiological response through which microbial cells overcome the threat of alkylating agents like N-methyl-N’-nitro-N’-nitrosoguanidine (MNNG), (a toxic agent capable of producing O⁶-methylguanine (O⁶-meG) which can be read as adenine during DNA replication and transcription therefore causing GC - AT transitions), involves induction of a repair system hence developing resistance in the stressed cells. This is referred to as an adaptive response.

Low levels of alkylating agents like N-methyl-N’-nitro-N’-nitrosoguanidine (MNNG) or ethyl methanesulfonate (EMS) induce resistance in *E. coli* against the mutagenic and lethal effects of higher doses of these agents (Samson and Cairns, 1977). The adaptive response is chloramphenicol sensitive i.e requires de novo protein synthesis. The adapted cells tend to remove enzymatically (O⁶-meG) from their DNA by O⁶-alkylguanine-DNA alkyltransferase (Mitra *et al.*, 1982) that is an *ada* gene product. The *ada* group of genes also code for 3-methyladenine-DNA glycolase capable of releasing 3-methylguanine, 7-methylguanine and 7-methyladenine from methylated DNA (Karran *et al.*, 1982), in polymerase I dependent manner.

1.6.2. Adaptive response to oxidative stress:

Oxygen is very toxic to anaerobic organisms and its metabolic products like superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals are toxic to aerobes as well (Fridovich, 1978). It would be
expected that facultative organisms such as *Escherichia coli* and *Salmonella typhimurium* which undergo shifts from anaerobic to aerobic conditions might possess an adaptive response to overcome oxidative stress. Hydrogen peroxide adaptation by *E. coli* and *S. typhimurium* increases the chances of their survival in response to the respiratory burst components $\text{H}_2\text{O}_2$ and superoxide anion associated with phagocytosis by activated granulocytes. The respiratory bursts are responsible for generating millimolar concentrations of hydrogen peroxide a means of elimination of the potential pathogens (Root and Cohen, 1981).

*Salmonella typhimurium* becomes resistant to killing by hydrogen peroxide and other oxidants when pretreated with a non-lethal concentration of hydrogen peroxide. During this process of adaptation to hydrogen peroxide, 30 proteins are induced (Christman *et al.*, 1985). Out of these 30 proteins, 9 are constitutively overexpressed in dominant hydrogen peroxide-resistant oxyR mutants. Mutant oxyR1 is resistant to different oxidizing agents and overexpresses at least 5 enzyme activities involved in defences against oxidative damage.

Another study also reveals that hydrogen peroxide induces 30 different stress related proteins in *Salmonella typhimurium* (Morgan *et al.*, 1986). Five of these proteins are also synthesised by heat shock, including the very conserved DnaK protein. The synthesis of one of these heat shock proteins is dependent on oxyR, a positive regulator of hydrogen peroxide-inducible genes, while the rest are induced independent of oxyR.

Similarly, pretreated *Escherichia coli*, with 30 uM $\text{H}_2\text{O}_2$ for 30 min,
survived 20 mM hydrogen peroxide challenge. Consistently 10 - 20 fold increase in resistance was observed in E.coli K-12 strain already exposed to hydrogen peroxide in different growth media (Demple and Halbrook, 1983). Induction of peroxide resistance failed to increase the survival of Escherichia coli exposed to N-methyl-N'-nitro-N-nitrosoguanidine and UV light, distinguishing this phenomenon from the ada and the recA+ -dependent SOS responses.

Other oxidative stresses induced some proteins in E.coli as studied through two-dimensional gel electrophoresis (Walkup and Kogoma, 1989). Nine proteins are constitutively produced over wild-type levels in superoxide dismutase (sodA, sodB) double mutants. Exposure to redox cycling agents like paraquat and plumbagin at different concentrations led to synthesis of 13 proteins in wild-type organisms. Out of these 13 proteins, 5 were also constitutively induced in the sodA and sodB double mutants. Addition of micromolar concentrations of paraquat and plumbagin to the media of superoxide dismutase mutants led to the synthesis of an additional 14 proteins, out of which 5 are associated with stress responses, consisting of endonuclease IV (Nfo), three oxyR regulated proteins and one heat shock protein.

1.6.3. Adaptive response to starvation stress:

Bacteria frequently go from a feast situation, of living in a host, to a period of famine, of living in natural environments like open oceans where the starvation conditions may last for months at a time. The bacteria survive during the harsh conditions of starvation by production of some proteins which specifically increase their starvation resistance.
Starvation stress induces the synthesis of certain peptidases which render the microbial cell more able to overcome the stress. The survival of *Escherichia coli* and *Salmonella typhimurium* depends upon peptidase activities during carbon starvation (Reeve *et al*., 1984). A mutant of *Escherichia coli* K-12, CM89 which lacks five distinct peptidase activities, on starvation for glucose loses its viability very rapidly compared with the wild type. Similar results are obtained for *Salmonella typhimurium* LT2 and LT2 derived mutants lacking different peptidase functions. *Escherichia coli* synthesises new polypeptides in response to phosphate or nitrogen starvation (Groat *et al*., 1986). Some of these polypeptides are unique to a given starvation regimen, but at least 13 appear to be synthesised regardless of the type of nutrient deprivation causing the starvation.

The induction of new polypeptides following a starvation threat is important and cross protects the bacterial cells against thermal and oxidative stresses; induction can be inhibited by chloramphenicol. Glucose or nitrogen-starved cultures of *Escherichia coli* demonstrate resistance to heat (57 °C) or hydrogen peroxide (15 mM) challenge, in comparison with logarithmically grown counterpart controls (Jenkins *et al*., 1988). The extent of resistance is enhanced with the time for which the cultures are starved before the challenge. Maximal resistance is offered by cultures starved for 4 hr.
1.6.4. Heat-shock response:

The heat-shock response is an inducible, cellular response to sudden temperature upshift or other environmental stresses by strongly and rapidly inducing the synthesis of a small set of proteins called heat shock proteins and is exhibited by almost all living organisms. The rate of protein synthesis increases transiently within a few minutes after a thermal upshift and reaches a new steady state value within 30 minutes (Yamamori et al., 1978).

When *Drosophila melanogaster* larvae or cultured cells are transiently exposed to high temperature a number of heat-shock polypeptides are induced (Tissieres et al., 1974). The heat-shock response has been very widely studied in eucaryotes and procaryotes such as; human beings, hamster, chicken, *Drosophila melanogaster*, tobacco, nematodes, chick embryo fibroblast, *Escherichia coli*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Staphylococcus*, *Mycoplasma capricolum*. The phenomenon of heat-shock response is not only highly conserved during evolution but individual components of the heat-shock response belong to families of proteins which are very conserved in nature.

The heat-shock response is controlled by a positive regulatory locus called *htpR* or *hin* or *rpoH* and this controls the induction of over 13 heat-shock proteins upon temperature upshift. Such proteins are members of the HTP (high temperature production) regulon (Neidhardt et al., 1983).

Both eucaryotic and procaryotic cells are equally capable of responding to
environmental stresses by synthesis of heat-shock proteins. Mycoplasmas which are the smallest free living cells (about 1/6 to 1/3 the genetic capacity of \textit{Escherichia coli}) have the capacity to synthesise heat shock proteins. The resemblance of the heat-shock response of mycoplasmas to that of other organisms suggests that there has been selective pressure for retention of the heat-shock system during a major decrease in genomic complexity involved in mycoplasma evolution. The conservation of the heat shock system during mycoplasma phylogeny suggests that the response is vital for cell physiology (Dascher et al., 1990).

1.6.4.1. Heat-shock genes:

At least nine heat-shock genes have been mapped, they are scattered around the oriC proximal half of the \textit{Escherichia coli} chromosome (Fig. 10). There are two bicistronic operons i.e \textit{dnaKJ} and \textit{groESL}, whereas the remaining five genes are separate from each other while \textit{rpoD} lies between those for ribosomal proteins S21 (\textit{rpsU}) and DNA primase (\textit{dnaG}). Nucleotide sequences of a few of these genes show consensus among their promoter regions (Neidhardt and VanBogelen, 1987).

1.6.4.2. Effectors:

The \textit{htpR} gene codes for a single protein, HtpR or sigma-32, which shares a similarity for amino acid sequences with the \textit{rpoD} gene product i.e sigma-70. During a heat-shock response, somehow, the activity or level or both of the positive effector, HtpR (sigma-32) must be raised for the transcription of heat-shock genes. Unfortunately the precise mechanism is not clear yet.
Fig. 10. Location of heat-shock genes on the *Escherichia coli* linkage map.

The position of oriC is shown for reference only.
Conditions that increase the amounts of sigma-32 relative to sigma-70 in the cell cause the induction of heat-shock response. When the concentration of sigma-70 is lowered this causes the induction of heat-shock proteins. Conversely, a mutant that overproduces sigma-70 synthesises lowered levels of heat-shock proteins which suggests a competitive interaction at some level between the two sigma factors.

1.6.4.3. Regulation of heat-shock response:

The induction of heat-shock proteins is controlled by a positive regulatory locus known as *htpR* or *hin* (Yamamori and Yura, 1982), located at 76 min on the *E.coli* chromosome (Neidhardt et al., 1983). The *hin* gene codes for a positive regulatory protein which induces the transcription of many heat-shock genes following a thermal shock. For example, following a stimulus there is an increased synthesis of *groE* mRNA in a cell which is accompanied by an increased synthesis of *groE* proteins; synthesis of both can be inhibited by rifampicin (Yamamori and Yura, 1980). The rate and extent of heat-shock induction is directly proportional to *hin* gene expression and the amount of *hin* gene product determines both the rate and extent of heat-shock protein synthesis (Yamamori and Yura, 1982).

Amino acid compositions and secondary structures of HtpR and sigma factors show a certain degree of homology (Neidhardt and VanBogelen, 1987). It is now thought that the gene product of *htpR* is a sigma factor which recognises the promotors of heat-shock genes. It is termed sigma-32 and the gene has been called *rpoH* in recognition of its involvement in the synthesis of a sigma factor.
1.6.5. Induction of the heat-shock or related stress system:

A wide variety of agents or agencies can trigger the synthesis of heat shock proteins or stress related proteins in a number of organisms from human beings to Drosophila melanogaster. Among such agents are heat shift, ethanol, viral infection, U.V. treatment and alkaline or acidic shift which are capable of induction of stress related proteins in procaryotes.

1.6.5.1. Induction by temperature upshift and by ethanol:

Two groups of proteins induced by temperature upshift have molecular weights of approximately 70 and 90 kDa (also known as hsp 70 and hsp 90) and are among the most highly conserved proteins in nature. The importance of some heat-shock proteins is obvious in Escherichia coli, since its dnaK null mutants grow more slowly than the wild type at low temperatures, produce filaments after a shift to 42 °C, are unable to synthesise dnaK protein and lose their viability on incubation for 2 hr at 42 °C. Introduction of a plasmid carrying the dnaK+ gene into these mutants restores normal cell growth, synthesis of dnaK protein and cell division at 42 °C (Paek and Walker, 1987).

Procaryotic cells respond to temperature upshift and exposure to ethanol by inducing a specific set of proteins. Two major heat-shock proteins with apparent molecular weights of 76 and 61 kDa are recorded among the 17 proteins synthesised at 45 °C by Pseudomonas aeruginosa which show immunological cross reactivity with the E.coli DnaK and GroEL proteins. In contrast, seven proteins are synthesised on exposure to ethanol, three of
which are heat-shock proteins (Allan et al., 1988).

Similarly the heat-shock response of Bacillus subtilis is studied at 50 °C, which is characterised by induction of 26 proteins, after 3 min, whereas a similar set of proteins are synthesised on treatment with ethanol but rather slowly. Among them are 3 major heat-shock proteins and they are immunologically related to Escherichia coli DnaK, Lon and groEL (Arnosti et al., 1986).

1.6.5.2. Induction of stress related proteins by viral infection:

Bacteriophage lambda infection of Escherichia coli triggers a complex response in the cell, in which the rate of synthesis of most, if not all, host proteins are affected. This leads to the induction of 21 stress related polypeptides and an increase in rate of synthesis to above the preinfection rates as observe with groE, dnaK and SSP proteins. Moreover the pattern of protein synthesis is similar with amino acid starvation but different from heat shock (Drahos and Hendrix, 1982). The induction of stress related proteins after lambda infection contradicts an earlier finding of Cohen and Chang, (1970) which claimed that the infection resulted in depression of host DNA, RNA and protein synthesis; their investigation was confined to synthesis of β-galactosidase which in fact decreased fivefold.

Subsequently the induction of stress related proteins following the lambda infection of Escherichia coli was confirmed by recording twofold stimulation in rate of synthesis of one of the products of groE (Kochan and Murialdo, 1982).
1.6.5.3. Induction of stress related proteins by alkaline and acidic shift:

Alkaline shift like many other environmental stresses induces the synthesis of stress related proteins in _E.coli_; a sixfold increase in production of _dnaK_ and _groE_ gene products is observed within 5 to 10 min, which is _htpR_ dependent, whereas acidic shift from pH 8.0 to pH 6.0 is not effective in inducing any stress related protein (Taglicht _et al._, 1987). Alkalization of intracellular pH (_pHi_) causes an increase in U.V resistance in _E.coli_ (Schuldiner _et al._, 1986).

Subsequently an important discovery reveals that an acidic shift to pH 5.0 results in increased synthesis of 13 stress-related proteins to 1.5 - 4 fold and 19 stress-related proteins were increased 2 to 14 time in _E.coli_ and _Salmonella typhimurium_ respectively, whereas in _E.coli_ 7 polypeptides are synthesised only at low pH (Hickey and Hirshfield, 1990) which contradicts the earlier findings of Taglicht and coworkers perhaps because the response was studied at pH 6.0. Another study confirmed the induction of 20 stress related proteins in _E.coli_, including 9 polypeptides which are exclusively produced by an acid shock, 30 - 45 min, at pH 4.3, called acid shock proteins (ASP) and concomitantly 4 well known proteins, GroEL, DnaK, HtpG and HtpM (Heyde and Portalier, 1990). The synthesis of one polypeptide out of nine acid shock proteins is _htpR_ (_RpoH_) dependent.

1.6.5.4. Induction of stress related proteins by UV light:

The SOS response which looks very complex is induced by agents and
conditions that either damage DNA or interfere with DNA replication. Thus, UV light, nalidixic acid and mitomycin C, can switch on the SOS network which is controlled by two regulatory proteins, the lexA and recA proteins (Little and Mount, 1982). The response seems even more complex since agents like UV light and nalidixic acid induce GroEL, DnaK and other heat shock proteins in Escherichia coli in a htpR dependent manner (Krueger and Walker, 1984).

1.6.6. The heat-shock response and thermotolerance:

Introduction of most stress responses leads to resistance to higher levels of the stress for example in the adaptive response to alkylating agents (Samson and Cairns, 1977) and to hydrogen peroxide (Demple and Halbrook, 1983). Heat-shocked organisms also gain increased thermotolerance (Yamamori and Yura, 1982; Mackey and Derrick, 1986; Mackey and Derrick, 1987).

Mackey and Derrick, (1987) demonstrated that the development of thermotolerance in Salmonella typhimurium was rapid and remained at its maximum level for at least 10 hr. This differs from the earlier report of Yamamori and Yura, (1982), where transiently induced thermoresistance of Escherichia coli, lasted for 60 min. The reason of this difference is not known, perhaps it is related to differences in growth media or phase of growth before heat shock. Additionally, an alkaline shift in S.enteritidis induces thermotolerance.

1.6.7. Biochemistry of stress related response:

Several stress related proteins are induced by microorganisms, under the
influence of a specific threat like starvation, viral infection, u.v treatment, alkalinization and acidification. Some of these proteins are similar in function and immunologically related to heat-shock proteins of E.coli while others are less well understood. Extensive work has been done on mapping of E.coli heat shock response genes and some of their locations have been worked out (Neidhardt and VanBogelen, 1987). Thus 17 heat-shock proteins of Escherichia coli induced following a thermal stress are shown in Table 5 and Figure 10.

1.6.7.1. Characteristics of Lon protein:

The Lon protein, 94 kDa, also known as protease La has an ATP-dependent protease activity. It can hydrolyse α-casein and globin in vitro. The Lon protein is encoded by the _lon_ gene whose function is regulated by _htpR_ gene product and this protein is induced under stressful conditions like elevated non-permissive temperature or exposure to ethanol (Goff et al., 1984). The _htpR_ mutants have low rate of _lon_ transcription at permissive temperatures, lower level of ATP-dependent protease activity hence decreased capacity for hydrolysis and cleaving of abnormal proteins (Phillips et al., 1984).

In Escherichia coli aberrant proteins, are rapidly degraded by the Lon protein in an ATP-dependent fashion. The rates of hydrolysis of anomolous proteins by the _lon_ mutants are 2 - 3 times slower than in the wild-type cells. On the other hand, if cells harbour multicopy plasmids carrying the _lon_ gene, then they produce protease La in several times more than normal level and abnormal protein degradation by those cells is very rapid.
Table 5: Some heat-shock proteins of *Escherichia coli*.

<table>
<thead>
<tr>
<th>Protein number</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GrpE</td>
<td>grpE</td>
<td>23,300</td>
</tr>
<tr>
<td>2</td>
<td>GroEL</td>
<td>mopA (groEL)</td>
<td>62,883</td>
</tr>
<tr>
<td>3</td>
<td>DnaK</td>
<td>dnaK</td>
<td>69,121</td>
</tr>
<tr>
<td>4</td>
<td>Sigma-70</td>
<td>rpoD</td>
<td>70,263</td>
</tr>
<tr>
<td>5</td>
<td>htpE</td>
<td></td>
<td>14,700</td>
</tr>
<tr>
<td>6</td>
<td>GroES</td>
<td>mopB (groES)</td>
<td>10,670</td>
</tr>
<tr>
<td>7</td>
<td>htpG</td>
<td></td>
<td>71,000</td>
</tr>
<tr>
<td>8</td>
<td>htpI</td>
<td></td>
<td>48,500</td>
</tr>
<tr>
<td>9</td>
<td>Lysyl-tRNA Synthetase form II</td>
<td>lysU</td>
<td>60,000</td>
</tr>
<tr>
<td>10</td>
<td>htpK</td>
<td></td>
<td>10,100</td>
</tr>
<tr>
<td>12</td>
<td>htpL</td>
<td></td>
<td>21,500</td>
</tr>
<tr>
<td>13</td>
<td>htpM</td>
<td></td>
<td>84,100</td>
</tr>
<tr>
<td>14</td>
<td>htpN</td>
<td></td>
<td>13,500</td>
</tr>
<tr>
<td>15</td>
<td>htpO</td>
<td></td>
<td>21,000</td>
</tr>
<tr>
<td>16</td>
<td>Lon</td>
<td>lon</td>
<td>94,000</td>
</tr>
<tr>
<td>17</td>
<td>DnaJ</td>
<td>dnaJ</td>
<td>40,975</td>
</tr>
</tbody>
</table>
1.6.7.2. Characteristics of DnaK protein:

DnaK, a major heat-shock protein in _E.coli_, is homologous to the hsp70 of _Drosophila melanogaster_ and humans and is ca. 70 kDa in molecular weight. The DnaK protein is coded by the _dnaK_ gene, located at 0.3 min on the genetic map; synthesis is controlled by _htpR_ gene product and on induction comprises upto 4.3% of total cellular proteins after temperature upshift and other environmental stresses. The null mutants of _E.coli_ which produce _dnaK_ protein at basal level at low temperature are unable to form colonies at 42 °C and show filamentation but can revert to have the normal functions on introduction of a plasmid carrying _dnaK*_ gene (Paek and Walker, 1987). This gene product is essential for lambda and M13 in vitro DNA replication system, autophosphorylates at a threonine residue and has a weak DNA-dependent ATPase activity.

1.6.7.3. Characteristics of GroE protein:

The _groE_ gene, located on 94 min of _E.coli_ chromosome, contain two functionally related genes _groEL_ and _groES_, coding for ca. 63 and 11 kDa polypeptides respectively (Tilly et al., 1981), mutation in both of these genes exert similar effects on assembly of phage and microbial growth at nonpermissive temperatures (Wada and Itikawa, 1984).
1.6.8. DNA repair:

DNA repair is a corrective mechanism of all living organisms and serves to reduce the level of spontaneous and induced mutation; a balance is set between genetic change and adaptation and strict maintenance of existing genotypes. Cells lacking repair mechanisms are liable to undergo mutation or die when lesions occur in their DNA. The DNA damage in E.coli is categorised in two main classes on the basis of the processing necessary to cause mutation: 1; this class includes mutations which take place due to mispairing during DNA replication following the action of methylating agents; it is caused by the production of adducts like O\(^6\)-methylguanine, 2; this class requires the involvement of special inducible "SOS" processing (referred to also as "error-prone repair", "SOS repair", or "misrepair") to cause mutation, exemplified by UV mutagenesis (Walker, 1984).

1.6.8.1.a. Class 1. Adaptive response:

This adaptive response falls into the first class, is recA independent and is induced by alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea. This is governed by the ada gene product, a 37 kDa protein which acts as a positive regulatory element as well as an O\(^6\)-methylguanine-DNA methyltransferase. The production of O\(^6\)-meG by alkylating agents switches on transcription of at least four genes; ada, alkA, alkB, and aidB (Volkert and Nguyen, 1984).

The alkA gene codes for a broad-spectrum DNA glycolase known as 3-methyladenine-DNA glycosylase II having an approximate molecular weight of
Fig. 11. DNA repair by N-glycosylase. A portion of genome shown; R altered nitrogenous base. N-glycosylase recognise the altered base and apurine/apyrimidinic site is cleaved by apurine/apyrimidinic endonuclease. The sugar phosphate is removed by a 5'-3' exonuclease by DNA polymerase I. DNA polymerase I synthesise the cross-hatched nucleotides and DNA ligase forms the final phosphodiester bond (adapted from Kushner, 1987).
30 kDa and is induced 20 fold during an adaptive response. The enzyme is capable of excising several methylated bases.

1.6.8.1.b. Mechanism of adaptive response:

The altered nitrogenous bases of DNA caused by the alkylating agents are recognised by N-glycosylase and apurinic/apyrimidinic sites are cleaved by apurinic/apyrimidinic endonuclease either at the 5' or 3' end as diagrammed schematically in Fig. 11. The sugar phosphate is removed by a 5'-3' exonuclease and DNA polymerase I replaces the damaged DNA nucleotides and subsequently DNA ligase connects the new nucleotides with the preexisting old strand to complete the repair process (Karran et al., 1982).

1.6.8.2. Class 2. Error-prone (inducible) repair:

The error-prone repair, most commonly called "SOS" repair, is operative when the DNA is either damaged or its replication is threatened by some mutagenic agents or some conditions. The SOS response results in an increased capacity for DNA repair, cessation of respiration, DNA degradation, induction of stable DNA replication, and induction of prophages (Kushner, 1987).

1.6.8.2.a. Model for SOS regulation:

The SOS regulatory network is controlled by the involvement of RecA and LexA proteins. In the normal state of the cell, the _lexA_ gene product acts as a repressor of a considerable number of unlinked genes including _lexA_ and _recA_...
and binds with the operator regions of each gene (Walker, 1984).

On induction of the SOS system, a signal is generated which reversibly activates the RecA protein. The RecA protein proteolytically cleaves (1) LexA protein, at an -Ala-Gly- peptide near the middle of the protein, and (2) repressors of bacteriophages; lambda, P22, and 434 (Walker, 1984) and other repressors, hence increasing the transcription of a number of genes including \textit{uvrA}, \textit{uvrB}, \textit{uvrD}, \textit{recA}, \textit{lexA}, \textit{ssb}, \textit{ruv}, \textit{recN}, \textit{umuC}, \textit{umuD} and probably \textit{uvrC} (Kushner, 1987).

If the inducing signal is pretty strong, more molecules of RecA protein are activated resulting in a declining level of Lex protein which leads to maximised transcription of even those genes whose operators are tightly bound with LexA. When the cell starts to recover from the effect of DNA damaging agents, RecA molecules begin to decline in concentration thus enhancing the transcription of LexA. Therefore, the level of LexA rises which shuts off SOS system and the cell returns to the repressed state in an uninduced condition.

1.6.8.2.b. Lex A cleavage:

The molecular mechanism by which activated RecA, cleaves LexA and lambda repressor is not fully understood. It is proposed that activated RecA after interacting with LexA may conform the LexA molecule in such a way which enhances the susceptibility of -ala-gly- bond either to hydrolysis or to autodigestion. Little, (1984), suggested that RecA may not be necessary for the cleavage of LexA since it can take place, in the absence
of RecA, under mild alkaline conditions in the presence of divalent cation.

1.6.8.2.c. Biochemical aspects of SOS regulation:

Genetical analysis of the SOS family of genes shows that they share sequence homology in their operator regions. The strength with which LexA binds with the operator regions of the SOS genes determines the expressions of SOS genes, at a basal level, in uninduced state of the cell. LexA shares a functional similarity with lambda repressor and both the molecules are cleaved at -ala-gly- region.

1.6.9. Comparison of the SOS, heat-shock and adaptive regulatory systems:

The responses exhibited by Escherichia coli to some DNA damaging agents are summarized in Fig. 12. There are three independent regulatory systems, inducible by their own inducers. Some inducers of SOS system, like UV and nalidixic acid, can induce the groEL and dnaK heat shock genes (Krueger and Walker, 1984) and on the otherhand some agents inducing the adaptive response like MNNG (low doses) switch on the synthesis of SOS proteins at higher rate.

These three networks are positively controlled by their own regulatory elements; RecA, HtpR and Ada respectively. The Ada element differs from the RecA in a sense that its overproduction can induce the expression of adaptive response but on the otherhand overproduction of RecA does not induce SOS response.
Fig. 12. Three regulatory networks of E.coli that can be induced by agents that damage DNA.
1.7. The pH homeostasis of the bacterial cell:

In nature, there are a wide range of pH levels, extending from pH 1 - 2 in acidic sulphur springs to pH 11 in soda lakes. Microorganisms live and grow in all these pH values, though some species have evolved to grow in extreme acidic environments (acidophilic) and some in extreme alkaline conditions (alkalophilic) while others which tolerate only mid range pH values of 6 - 8, are referred to as neutrophilic; *E. coli* is a representative of this class. Proton concentration is an important parameter in governing a number of biological reactions like solvolysis, ionization and oxidoreduction; as a result, biological molecules function optimally at a narrow pH range, mostly close to neutrality. All bacteria have evolved an effective mechanism of coping with change in external pH (pH<sub>o</sub>); by a mechanism poorly understood they maintain constant internal pH (pH<sub>i</sub>) values: 6.5 - 7.0 for acidophiles, 7.5 - 8.0 for neutrophiles and 8.4 - 9.0 for alkalophiles (Ingraham, 1987).

*Escherichia coli*, like other neutrophiles, has evolved a mechanism of maintaining constant internal pH. This was studied by Slonczewski *et al.*, (1981), in a classical experiment using <sup>31</sup>P nuclear magnetic resonance. They measured pH<sub>i</sub> of non-growing cells when external pH was lowered from 7.55 to 5.6 for a time period of 45 min and then increased to 8.7 over a period of 70 min. During these changes, pH<sub>i</sub> remained close to the pH crossover (pH<sub>co</sub>) the pH at which pH<sub>o</sub>=pH<sub>i</sub> value, varying by the relationship (pH<sub>i</sub> - pH<sub>co</sub>) = k (pH<sub>o</sub> - pH<sub>co</sub>). The pH<sub>i</sub> changes only slightly by the lowering or raising of external pH.
Maintenance of internal pH is essential for growth of *Escherichia coli*. When, due to an environmental stress, pH$_i$ drops to 7.2, growth rate is reduced by a factor of 2; growth stops completely on further decreasing the internal pH to 6.6 - 6.8 (Booth, 1985).

1.7.1. Perturbation of pH$_i$:

Generally the internal pH is quite stable and remains unperturbed due to the properties of the cytoplasmic membrane which is impermeable to protons and offers low permeability to cations. Even when the driving force is large, net influx of protons is limited by the capacity for cation extrusion.

The major sources of cytoplasmic pH perturbation are: 1. the value of pH$_o$, 2. the acidic or basic end products of metabolic processes and growth, and 3. the selective utilization by the cell of the cation or anion component of the salt present in the medium as for instance, utilization of the nitrate anion from the medium causes pH$_o$ to become more alkaline (Ingraham, 1987; Booth, 1985).

In fermentative organisms, the production and accumulation of acidic fermentative products leads to perturbation of internal pH because pH$_i$ does not remain constant if pH$_o$ is reduced in the presence of weak acids (Baronofsky et al., 1984). This problem may be so acute that the organisms have to evolve some mechanism to convert the acidic products into neutral compounds to overcome the toxic effects of weak acids in the cytoplasm.
1.7.2. Buffering capacity of the cell:

The buffering capacity of the bacterial cell is classified into two components; $B_o \text{, operative on the surface of the intact cell in suspension and } B_i \text{ is a derivative value that is the difference between total buffering capacity (} B_t \text{) of permeabilized cells (treated, e.g., with 5\% n-butanol or 10\% Triton X-100) and } B_o \text{ (Ingraham, 1987).}$

1.7.3. Components of pH homeostasis system:

Possible components of the cytoplasmic pH regulation system include preexisting cytoplasmic buffers and biochemical production of $H^+$ or $OH^-$. The cytoplasmic buffers can avert a limited amount of acidification or alkalization, on the contrary the production of $H^+$ or $OH^-$ from metabolism is a neglected area, moreover there is very little evidence that they constitute components of pH homeostasis system.

1.7.4. Mechanism of maintaining pH homeostasis:

The precise mechanism by which the cell maintains its pH homeostasis is not fully understood but possibly a number of factors are responsible for setting the $pH_i$. Among these are the buffer capacity of the cell, the outward transport of protons associated with respiration or hydrolysis of ATP, and electroneutral antiport systems that exchange protons for certain cations, particularly $Na^+$ and $K^+$. 
1.7.4.a. A Na⁺/H⁺ antiporter in regulation of internal pH:

An electroneutral Na⁺/H⁺ antiporter (NHA) will be driven by the \( \Delta \text{pH} (\Delta \text{pH} = \text{pH}_i - \text{pH}_o) \) initially formed by the proton pumps: Na⁺ will be extruded and H⁺ will be returned to the cell. If the rate of such an antiporter increases with pH it could account for the decrease in the \( \Delta \text{pH} \) observed. At external pH 7.6, it can explain the zero \( \Delta \text{pH} \), if the back leak of protons via the antiporter equates the primary proton extrusion rate (Padan et al., 1976).

1.7.4.b. A K⁺/H⁺ antiporter in regulation of cytoplasmic pH:

The K⁺/H⁺ antiport (KHA) appears electroneutral over pH 7-9 and is less specific than the Na⁺/H⁺ antiporter since it antiports K⁺, Na⁺, Li⁺, Rb⁺ and Tl⁺. The activity of KHA is optimal at pH 8 (Brey et al., 1980). It has been suggested that the pronounced alkaline pH optimum of KHA will regulate its function in accordance with the external pH.

The NHA and KHA are the major candidates for affecting controlled acidification of cytoplasm and thereby homeostasis of pH in E.coli. Though there is indirect evidence that each of these system participate in controlling cytoplasmic pH (Booth, 1985), there is no proof that either of them is the controlling mechanism (Ingraham, 1987).

1.7.5. Sensitivity of Enterobacteria at acidic pH:

Enterobacteria, such as Escherichia coli, face many physical and chemical
environmental stresses and acidity is amongst them. In the body, gastric acid (ca pH 2 - 3) reduces the number of ingested bacteria both harmless and potentially pathogens. Secondly, the upper intestinal tract is generally mildly acid and this in conjunction with weak fatty acids can kill enterobacteria (Lee and Gemmell, 1972). Thirdly, the organisms can be damaged or probably killed by acid, in the range of 3.5 - 5.0, of the phagolysosome (Mims 1972; Jensen and Bainton, 1973). Finally, potential pathogens have to resist acid in the vagina, where pH values of approximately 5.0 - 5.5 are produced by commensal Lactobacilli, and in urine, where similar pH values are found.

Outside the animal body, bacteria are exposed to acidic conditions in acid mine water, in rivers and lakes affected by acid rain (Double and Bissonette, 1980), and in sewage plants receiving certain wastes. Such exposures can also occur in some food processing treatments (Okrend et al., 1986; Roth and Keenan, 1971; Freese et al., 1973; Eklund, 1980) and, for some organisms, in acid foods (Minor and Marth, 1972).

It has been reported that ColV bearing strains of E.coli were more sensitive to acid pHs of 2.5 - 3.5 than their counterpart ColV^- cells (Cooper and Rowbury, 1986). Moreover, when the organisms (ColV^+ and ColV^-) were adhering to glass beads, they showed greater resistance to the killing effect of pHs 2.5 - 3.5 than their corresponding unattached cells (Poynter et al., 1986). The increased resistance of attached organisms to pH 2.5 may allow the survival of organisms attached to the food in gastric acid.
1.7.6. Sensitivity to organic acids/preservatives:

Chemical food preservatives (sorbate, benzoate, propionate, acetate, and lactate) have been used widely during the greater part of this century. Most of these food additives inhibit the growth of unwanted organisms, usually without killing them and are ingested by humans without any apparent toxicity (Freese et al., 1973).

In bacteria, the weak acid traverses the membrane in its undissociated form and dissociates in accordance with the intracellular pH, liberating a proton in the cytoplasm. When ΔpH is large, the amount of acid dissociating in the cytoplasm is correspondingly larger. Consequently, the potential effect of the weak acid accumulation on intracellular pH increases as pH₀ is lowered (Salmond et al., 1984).

Toxicity of organic acids was studied by Sinha, (1986), where the wild type strain and four repair-deficient strains (uvrA6, uvrB5, recA56, and polA1) of E.coli, in their stationary growth phase, were exposed to organic acids (acetic acid, lactic acid and p-aminobenzoic acid) at pH 3.5. The sensitivity exhibited by the polA strain was much higher than its isogenic pol⁺ derivatives.

1.7.7. Mechanism of inhibition:

Exposure to lethal acidity, in the presence or absence of weak acids, may damage or kill by affecting several sites in the bacterial cell. Sheu and Freese (1972), showed that bacterial oxygen uptake and growth were
inhibited by weak acids. In 1973, Freese et al., demonstrated that weak acid at low pH inhibits microbial growth and they traced the inhibition to a failure of active transport. Secondly, enterobacterial cells exposed to inorganic acid or to a range of weak organic acids at low pH appear to suffer outer membrane damage, becoming sensitive to a range of agents which fail to cross the outer membrane of untreated cells (Roth and Keenan, 1971; Przybylski and Witter, 1979). A third major factor in killing by acid pH, in the presence or absence of weak acids, appears to be damage to DNA (Sinha, 1986; Raja et al., 1991b).

1.7.8. Sensitivity to hydrogen peroxide:

Hydrogen peroxide is a strong oxidizing agent and is able to inactivate a wide variety of micro-organisms. Brief exposure (10 min) of Escherichia coli to low concentrations of hydrogen peroxide (0.01 M), at 25 °C results in induction of a substantial number of single stranded (SS) deoxyribonucleic acid breaks (Ananthaswamy and Eisenstark, 1977). Subsequently, Hagensee and Moses, (1986), reported DNA damage of intact Escherichia coli by hydrogen peroxide.

1.7.9. Recovery of injured cells:

Presently, frequent usage of organic acids alone or in combination with other physical and chemical agents in food industry, cause either damage to the existing microbial population or make conditions unsuitable for unwanted micro-organisms. In a situation when sublethal (acid) stress is applied to a bacterial population, this may cause injury to the
microbial cells. The injured organisms may recover, if the acid-stress is removed, and they may form colonies on low stress-media but be unable to form colonies on stressing-media (Przybylski and Witter, 1979).

When Escherichia coli K-12 cultures were treated with acidic 0.3 M sodium acetate buffer, the extent of both death and injury of acid-treated cells increased with decreasing pH. Injured cells were able to recover their colony-forming ability in the recovery medium. The recovery was not affected by the presence of inhibitors of protein, cell wall, DNA, or RNA synthesis (Przybylski and Witter, 1979).

Alkali-injured cells were found to recover when incubated at 37 °C for 2 hr in 0.05 M phosphate buffer of pH 8.0, whereas no such liquid holding recovery was observed in recA and lexA mutants. Recovery in phosphate buffer was not affected by metabolic inhibitors (Musarrat and Ahmad, 1988).

1.7.10. Repair response of acid/H₂O₂-damaged E. coli:

The lesions induced in certain repair mutant strains of Escherichia coli, caused by organic acids, were not efficiently repaired, which resulted in greater killing of cells in contrast with the wild type. Moreover, the increased sensitivity of the polA mutant compared with wild-type Escherichia coli suggested that polA gene product was necessary for repair response (Sinha, 1986).

The hydrogen peroxide induced single stranded breaks in Escherichia coli
were repaired only to a small extent in polA, recA recB and recA mutants. The repair is polA+ and recA+ dependent (Ananthaswamy and Eisenstark, 1977).

The repair of Escherichia coli DNA, damage by hydrogen peroxide, takes place by an incision repair pathway that requires DNA polymerase I (Hagensee and Moses, 1986).

1.7.11. Habituation to acid stress:

Exposure to certain stresses at a sub-lethal level (an adaptive dose) habituates the organism allowing it subsequently to withstand lethal levels of the stress (the challenge dose). Such phenomena have been best studied with respect to oxidative stress (Demple and Halbrook, 1983) and heat stress (Yamamori and Yura, 1982; Mackey and Derrick, 1987) although similar phenomena may occur for ethanol and metal ion stress (Khazaeli and Mitra, 1981; Michel and Starka, 1986).

Enterobacteria can face exposure to extremes of acidity in many situations in the natural environment, in food or in the animal body. In the natural environment, exposure to acidity from industrial or agricultural sources is common and can occur in water or in soil (Rowbury et al., 1989). Because such exposures usually involve the periodic release of wastes or slurries, organisms frequently face acidity which gradually increases to a potentially lethal maximum. The gradual increase in acidity can allow induced acid resistance (habituation to acid) to occur which permits habituated organisms to survive subsequent exposures which are lethal to
non-habituated cells (Rowbury et al., 1989). Pre-exposure to mild acidity in water or soil may allow organisms to subsequently survive in acid foods or in animal body.

Habituation involves a phenotypic change in organisms exposed to mild acidity which gain acid resistance after a few minutes exposure to pH 5.0 (Raja et al., 1991a). Such induction of acid resistance is dependent on protein synthesis and at least 14 proteins are induced at pH 5.0 (Raja et al., 1991a).
1.8. Aims of this study:

*Escherichia coli* is a multipotent pathogen that has evolved the ability to cause not only gastrointestinal tract infections but also extraintestinal infection of humans (see sections 1.5.1.1 and 1.5.2). The organisms can cause Public Health problems on entering into food chain as a contaminant. The significance of such food contamination depends upon bacterial pathogenicity and a major factor which increases pathogenicity is plasmid carriage (Smith, 1974). The presence of weak acid at low pH inhibit growth and survival of enterobacteria and accordingly, naturally acid foods or acidulated ones should be free from pathogens.

Therefore, this project was undertaken to investigate the following:

a. Susceptibility of ColV+ and ColV- strains to acid inhibitors.

b. Acid sensitivity of ED1829ColV,I-K94 habituated organisms compared with that of non-habituated cells.

c. Possible factor responsible in development of acid-resistance of habituated organisms.

d. Acid-sensitivity of DNA from habituated and non-habituated cells.

e. Inhibition of acid-habituation.
2. Materials and Methods

2.1. Bacterial strains:

The genotypes of bacterial strains and plasmids used during the study are presented in Tables 6 and 7. The cultures were maintained on nutrient agar (NA) at 4 °C and subcultured at monthly intervals.

2.2. Media:

Cxtoid Nutrient Broth No.2 (NB) was used at 25 g/l as a growth medium in most of the experiments. Its pH was adjusted by the addition of HCl, to required values, where needed. In some cases, this medium was made up in 0.1 M pH 7.5 phosphate buffer, for studies of recovery of injured organisms.

Solid medium was prepared by the addition of 2% Difco Bacto Agar to the NB. Where appropriate, sodium deoxycholate was added to NA at 0.5% for selection of undamaged organisms. Medium selective for antibiotic resistant organisms was made by the addition of 20 μg/ml tetracycline and 150 μg/ml ampicillin; these additions were made to the molten sterilized agar after cooling to 55 °C.

2.3. Growth conditions:

Overnight cultures were prepared by growing the bacterial cells in NB of required pH, at 37 °C under shaking conditions. For exponential cultures, the overnight grown cells were diluted into fresh NB of required pH and incubated with shaking, at 37 °C, for 2 - 3 hr.
Table 6: Characteristics of *Escherichia coli* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/properties</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>thr, leu, thi, ara, str&lt;sup&gt;r&lt;/sup&gt; arg, xyl, lac Y, his</td>
<td>Bachmann (1987)</td>
</tr>
<tr>
<td>ED1829</td>
<td>trp</td>
<td>Finneggan and Willetts (1971)</td>
</tr>
<tr>
<td>DP1152</td>
<td>trp, rha, lac Z, str polAl&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Dr. R. Pinney</td>
</tr>
<tr>
<td>AB2463</td>
<td>thr, leu, pro, his, thi, arg, rec-13</td>
<td>Howard-Flanders, P. (1968)</td>
</tr>
<tr>
<td>P678-54</td>
<td>thr, leu, thi, lac Y, ara, xyl, minicell producer</td>
<td>Adler et al., (1967)</td>
</tr>
<tr>
<td>P678-54 cmrA 2101 cmrA mutant of P678-54 (RJR.200)</td>
<td>This laboratory.</td>
<td></td>
</tr>
<tr>
<td>GW1000</td>
<td>recA441, sulA11, lacU169, thr-1, leu-5, his-4, argE3, ilv(ts), gAI2, rpsL31</td>
<td>Kenyon and Walker (1980).</td>
</tr>
</tbody>
</table>

str; streptomycin, ara; arabinose, arg; arginine, gal; galactose, his; histidine, lac; lactose, leu; leucine, lys; lysine, thi; thiamine, trp; tryptophan, rha; rhamnose, xyl; xylose
<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plasmid</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Plasmids used and their properties.
2.4. Measurement of optical density:

Optical density of the cultures in NB was measured with a Hilger photoelectric colorimeter using a 550 nm filter. A reading of 0.1 corresponded to ca. 100 μg dry weight organisms/ml.

2.5. Measurement of viable count:

The viable cell number was determined by plating 0.1 ml of appropriate dilution of a sample, prepared by serial dilution in 0.85% (w/v) saline, on a suitable medium surface. The colonies were counted after 24 - 48 hr incubation at 37 °C. Percentage survival of the cell population treated with the inhibitory agents was determined by using the formula below;

\[
\% \text{ survival} = \frac{\text{Viable cell numbers of treated population}}{\text{Viable cell counts for untreated population}} \times 100
\]

2.6. Habituation of E.coli to acid pH:

ED1829ColV,I-K94 cells, grown overnight in NB of pHs 7.0 and 5.0, were diluted respectively into fresh NB of pHs 7.0 and 5.0, and grown to logarithmic phase for a period of 2 - 3 hr at 37 °C, under shaking conditions, till their O.D reached between 0.2 - 0.3. The mild acid pH led
to habituation, therefore, pH 5.0 grown cells were referred to as habituated and pH 7.0 cells as non-habituated. These organisms were used later on for undertaking further experimentation for investigation of various aspects of this phenotypically expressed phenomenon.

2.7. Effect of habituation on resistance of *E. coli* to acid pH:

The habituated and non-habituated cells (see above 2.6) were exposed to an appropriate acid pH value, with (where required) a suitable concentration of an inhibitor, in NB, with aeration at 37 °C. Samples were withdrawn at intervals and at the end of the treatment time, neutralized in 0.02M Tris-HCl buffer pH 7.0, and plated on NA, after serial dilution of samples in 0.85% (w/v) NaCl. The plates were incubated at 37 °C for 24 hr and the viable count was used for estimation of percentage survival of acid treated populations compared with untreated controls as explained in section 2.5.

2.8. Colicin production test:

The strains to be tested for colicin production were streaked on NA plates containing 0.065 M phosphate (pH 7.4) following the method of Rowbury and Hicks (1987) and incubated at 37 °C overnight. The plates were exposed to chloroform vapour for 15 min and the vapour was allowed to evaporate for 15 min. After overlaying with 5 ml of soft agar containing 0.2 ml of
overnight culture of sensitive cells, plates were incubated for 18 - 24 hr before examining zones for growth inhibition (Figure 13) by the ColV\(^{+}\) strain.

2.9. Sensitivity of the organisms to putative inhibitory agents:

ED1829 and ED1829ColV,I-K94 were grown exponentially for 2 - 3 hr and then treated with various inhibitory agents at a required pH for an appropriate length of time with shaking, at 37 °C. The treated cultures were neutralized into fresh NB and percentage survival was estimated as explained in section 2.5.

2.10. Sensitivity to hydrogen peroxide:

Acid habituated and non-habituated cells of exponential phase (see section 2.6) were subjected to various concentrations of hydrogen peroxide, at 37 °C with aeration for an appropriate length of time. In the same way, the p\(^{+}\) and p\(^{-}\) organisms were exposed to the putative agent. The treated cultures were neutralized with fresh NB containing 10 μg/ml catalase and percentage survival was estimated as described in section 2.5.

2.11. Sensitivity to urea:

ColV\(^{+}\) and ColV\(^{-}\) strains of exponential phase were treated with urea at 37 °C in NB with aeration for different time lengths and percentage survival was determined (see section 2.5).
Fig. 13. Zone of clearance of sensitive cells shown by colicin producing 14 ColV strain whereas its mutant strain was unable to kill the sensitive cells.
2.12. Sensitivity to weak acids:

Percentage survival of the acid habituated and non-habituated and of the ColV⁺, versus ColV⁻ organisms was studied after treating the exponentially grown cells with 50 mM concentration of the following fatty acids; butyric acid, acetic acid, lactic acid and succinic acid in NB, at pH 4.5, for 2.5 and 3.5 hr, at 37 °C, with shaking. Exposure to propionic and formic acids was done in the same way except that concentrations were 60 and 65 mM respectively. The treated cells were neutralized by diluting with fresh NA for estimation of percentage survival on NA as described in section 2.5.

2.13. Motility:

The motility of exponentially grown habituated and non-habituated organisms was studied in microslide capillaries (Cam lab., Cambridge). The exponential cultures of ED1829ColV,I-K94 were obtained as described in section 2.6, while the pHs of the organisms were adjusted to 3.0, 3.5 and 4.0 by appropriate addition of NB of lowered pH values. The organisms were drawn into the microslide of path length 0.2 mm, through capillary action and motility was observed at intervals at the liquid/air interface, under X40 objective using the phase contrast microscope, at room temperature, for both types of culture. Percentage motility was checked by counting the motile bacteria out of at least 100 cells.

2.14. Negative chemotaxis:

The negative chemotactic response, of exponentially grown habituated and
non-habituated ED1829ColV,I-K94, was determined after the cultures were prepared by the method explained in section 2.6. The cells were washed twice with 0.85\% (w/v) NaCl at room temperature, suspended evenly in agar at 50 °C (0.3\% in phosphate buffer pH 7.0 + 10 mM EDTA) according to Tso and Adler, (1974). Petri dishes were poured and after setting, wells were dug in the agar with a corkborer at an equal distance. Plugs, cut from Gilson filter tips to a thickness of the poured medium, were soaked with buffer pHs 3.0, 3.5 and 4.0 for treatments and 7.0 for untreated controls.

These plugs were placed in the wells of 2 sets of plates for habituated and non-habituated organisms. The plates were left undisturbed at room temperature for 1.5 hr to observe the zone of clearance around the plugs and were photographed using Ilford FP4 film.

2.15. Respiration of habituated and non-habituated ED1829ColV,I-K94 cells:

A Clark-type oxygen electrode of Rank Brothers, Cambridge, England was used to determine the rate of respiration of exponentially grown habituated and non-habituated ED1829ColV,I-K94 cells, with the cultures being prepared according to the method described in section 2.6. The rate of respiration of both cultures was studied at pH 3.0, for various temperatures, by incorporating 2 ml of each culture (approximately of same O.D) in the incubation chamber, taking care not to trap any air bubbles at the time of placing the perspex disc. The study was carried out at 10 mV, 36 uA and a chart speed of 1 cm/min, which remained constant throughout
The experiments.

The standard curve shown in Figure 14 represents the amount of $O_2$ present in fully-saturated water at a particular temperature. The rate of $O_2$ consumption was calculated by the equation given below;

$$X \cdot \frac{V}{\text{time}},$$

where $X = O_2 \ \mu\text{mol/ml}$ at a particular temperature in °C, $V = \text{volume of the sample}$, and the units were expressed in μmoles of $O_2$/ml/min.

2.16. Release of cellular constituents:

Habituated and non-habituated cells of ED1829ColV,I-K94 (ca. same C.D) from stationary and exponential phases were harvested, treated with pH 3.0 saline 0.75% (w/v) or pH 7.0 saline with aeration at 37 °C and the untreated and acid treated samples withdrawn at 5, 10 and 15 min time intervals spun down at 16000 rev/min and supernatants read at 260 nm for any possible absorbance.

2.17. Recovery of injured organisms:

The broth grown ED1829ColV,I-K94 organisms from exponential phase were treated with putative inhibitory agents for a set time, with aeration at 37 °C. Samples (1 ml) were withdrawn and diluted into 99 ml of the recovery medium with and without 0.1 M phosphate buffer of pH 7.5 as explained in section 2.2, and the cultures were incubated statically at room temperature or 37 °C for 2 hr prior to plating 0.1 ml of appropriate dilutions on NA plates to determine percentage recovery.
Fig. 14. Total amount of dissolved $O_2$ in aqueous solution (adapted from Wise and Naylor, 1985).
2.18. Induction of stress-related proteins:

2.18.1. Protein analysis of habituated and non-habituated cells:

2.18.1.1. Sample preparation:

Protein analysis of habituated and non-habituated broth grown cells of ED1829ColV,1-K94 from late exponential and early stationary phase was studied by one dimensional and two dimensional SDS polyacrylamide gel electrophoresis. The organisms were washed twice with 0.85% (w/v) saline, suspended in sonication buffer following the method of O'Farrell, 1975, frozen in liquid nitrogen and thawed (freezing-thawing was repeated six times) and then sonicated for 16x15 S, at 1.5 A in an M.S.E ultrasonicator while samples were kept at 0 °C in an icebath. The supernatant fluids after centrifugation at 20,000 rev/min in a Sorval RC-5B for 20 min at 4 °C were then concentrated in dialysis tubing with polyethyleneglycol 15-20 K molecular weight at 4 °C for 2 hr. To this concentrate, 9 molar (dry) urea was added, dissolved at room temperature and the samples were then prepared for one dimensional and two dimensional gel electrophoresis.

2.18.1.2. Polyacrylamide gel electrophoresis:

The comparative polypeptide composition of habituated and non-habituated cells was examined by polyacrylamide gel electrophoresis using vertical slabs of 12.5% SDS polyacrylamide running in one dimension, by the method
of Laemmli (1970) and Chua and Bennoun (1975). The two dimensional gels were run following the method of O'Farrell (1975).

2.18.1.3. One dimensional SDS polyacrylamide gel electrophoresis:

2.18.1.3.a. Pouring of gel:

The separating gel was a 12.5% (w/v) acrylamide, 0.32% (w/v) bis acrylamide slab gel containing 0.4% (w/v) SDS and 0.375 M Tris- HCl, pH 8.8, polymerised with 0.06% (w/v) freshly made ammonium persulphate and 100 µl N,N,N',N'-tetramethylethlenediamine (TEMED). A 1-2 cm 6% (w/v) acrylamide stacking gel, containing 0.16% (w/v) bis acrylamide, 0.4% (w/v) SDS and 0.125 M Tris-HCl, pH 6.8 was polymerised in the same manner onto the separating gel.

2.18.1.3.b. Loading of gel:

Sample was added (1:1) to lysis buffer and the mixture was incubated in an icebath for 10 min and then samples were solubilised in 4% (w/v) SDS, 5% mercaptoethanol, 5% glycerol, 0.2% bromophenol blue and heated at 90 °C for 10 min. These treated samples (40 µl) were loaded on gels and electrophoresed. The running buffer used consisted of 0.375 M Tris-HCL, 2M glycine and 0.1% (w/v) SDS pH 8.3. The gels were run at 25 mA for 5.5 hr and were stained with Coomassie Brilliant Blue R250 overnight following the method of Deeney et al., 1986; destaining was with 10% methanol and 10% acetic acid.
2.18.2.1. Two dimensional SDS polyacrylamide gel electrophoresis:

Proteins were separated, based upon their isoelectric point, by isoelectric focusing (IEF) in the first dimension using a disc electrophoresis apparatus for 2D gels of Shandon, England, and, according to their molecular weights, by 12.5% SDS polyacrylamide gel electrophoresis in the second dimension.

2.18.2.2. Sample preparation:

Samples were prepared as described in the section 2.18.1.1. To 1 part sample was added 1 part lysis buffer, without mercaptoethanol according to the method of O'Farrell, 1975; the mixture was incubated in an icebath for 10 min, then heated at 90 °C for 10 min and loaded onto the electrofocusing gel.

2.18.2.3. Setting up and running of Iso Electric Focusing:

Glass tubes (120 x 1.5 mm internal diameter) were soaked in chromic acid for 2 hr, rinsed with double distilled water (D H₂O) then left in 50% EtOH: 50% 0.1 M KOH for 30 min, were further rinsed with D H₂O and dried. Their bases were sealed with two layers of parafilm and they were filled with IEF gel mix from the bottom to 110 mm using a longneck pasteur pipette. The IEF gel mix contained; urea 5.5 g, 1.33 ml of 30% acrylamide, 2 ml of 10% nonidet P40, 300 µl of ampholine pH 3.5-10, 200 µl of ampholine pH 5.0-7.0, 2 ml of D H₂O, 20 µl of 10% ammonium persulphate (freshly prepared) and 10 µl TEMED. The top was overlayered with D H₂O
and the gel was allowed to polymerise for 1 hr. The bottom reservoir of the IEF tank was filled with lower electrode buffer, which contained 1.11 ml of 85% phosphoric acid/l of degassed D$_2$O. The overlay and the parafilm were removed from the tubes and they were loaded into the IEF tank, sealing the joints with 4% agarose. The top reservoir was filled with upper electrode buffer, NaOH 800 mg/l D$_2$O and the gels were prefocused according to the following schedule: a) 200 V for 15 min; b) 300V for 30 min; c) 400 V for 30 min. Then the samples were loaded and overlayed with 10 ul of sample overlay (1 part solution A + 2 part D$_2$O). The solution A was composed of 20 mM Tris-HCl pH 7.6, 2% (w/v) nonidet P40, 2% (w/v) ampholine (60% pH 3-10 and 40% pH 5-7), 9.5 M urea, 5% (w/v) 2-mercaptoethanol. The samples were run for IEF at a constant 2 mA and 400 V for 16.5 hr and 800 V for 3 hr. The gels were removed from the tubes using a high pressure air line and stained in Laemlli (1970) sample buffer for 25 min at room temperature.

2.18.2.4. Setting up and running the proteins in the second dimension:

For electrophoresis in the second dimension, a 12.5% SDS-PAGE gel (see 2.18.1.3.a) was poured and the stained gel then applied to its top and the join sealed with 1% agarose. The gel was run for 6.5 hr at 25 mA, stained with Coomassie Brilliant Blue R250 and processed as described earlier in the section 2.18.1.3.b.
2.19. Induction of stress-related proteins studied by pulse labelling:

2.19.1. Time course study for the induction of stress-related proteins:

P678-54 and P678-54 \textit{ompA} were used in the pulse labelling experiments. The organisms were grown overnight in NB of pH 7.0, diluted 1/25 into fresh NB of pH 7.0 and incubated with aeration at 37 °C for 2.5 hr, to obtain exponential phase cultures. The cells were harvested by centrifugation at room temperature and resuspended in NB of pH 7.0 and pH 4.3 and the cultures were incubated for a further 5 min at 37 °C, with aeration before the addition of \( ^{35} \text{S} \) methionine. The radioactively labelled methionine, from Amersham International was added as follows: 3 \( \mu \text{Ci/ml} \) and 30 \( \mu \text{Ci/ml} \) for exponentially grown cultures of P678-54 in NB of pH 7.0 and pH 4.3. Similarly to P678-54 \textit{ompA}, 3\( \mu \text{Ci/ml} \) and 45 \( \mu \text{Ci/ml} \) of \( ^{35} \text{S} \) labelled methionine was added to the NB of pH 7.0 and pH 4.3. The cells were labelled for 10 min and then neutralized and harvested for sonication. The cell extracts were prepared essentially as described in section 2.18.1.1. and samples electrophoresed unidimensionally on 12.5% SDS polyacrylamide gels followed by autoradiography.

2.19.2. Inhibition of habituation by \textit{E. coli} at pH 4.3 by the addition of phosphate:

Exponential phase cultures of P678-54 and its \textit{ompA} mutant were prepared as for 2.19.1. The cells were then centrifuged and pellets were resuspended in NB of pH 7.0, pH 4.3 or pH 4.3 + 10 - 25 mM phosphate buffer and the cultures were again incubated at 37 °C for 5 min, with
aeration before adding of $^{35}$S methionine as per following schedule: 3 $\mu$Ci/ml, 30 $\mu$Ci/ml and 15 $\mu$Ci/ml for logarithmically grown cultures of P678-54 in the NB of pH 7.0, 4.3 and 4.3 + 10 - 25 mM phosphate buffer respectively. Similarly for P678-54 ompA, 3 $\mu$Ci/ml, 45 $\mu$Ci/ml and 25 $\mu$Ci/ml of $^{35}$S radioactively labelled methionine was added to the NB of pH 7.0, 4.3 and 4.3 + 10 - 25 mM phosphate buffer. The organisms were labelled for 10 min and then harvested for sonication to prepare cell extracts essentially following the method in section 2.18.1.1. The cell extracts were run on 1 D polyacrylamide gels followed by autoradiography.

2.20. Drying the polyacrylamide gels for autoradiography:

The gels were stained with Coomassie Brilliant Blue R250, destained, acetic acid removed in D$_2$O for 30 min before intensifying with 1 M sodium salicylate pH 6.0 for 30 min as described by Chamberlain, 1979. The gels were placed on 3 mm Whatmann filter paper, covered with saran and dried in a Bio Rad slab dryer model 483 for 3 hr at 60 °C.

2.21. Autoradiography:

The dried gels were placed in a 8"x10" cassette with 7"x9.5" Fuji X-ray film and stored in a freezer at -70 °C for an appropriate time. The X-ray film was processed and developed with X-ograph Compact X2.

2.22. Sizing of plasmid:

Overnight cultures were prepared from ED1829ColV,1-K94, E.coli marker
(39R 861) and 14 ColV strains in NB with shaking at 37 °C. The next day, plasmids were isolated essentially by the method of Kado and Liu, 1981; The cells were lysed with lysis solution pH 12.52 - 12.56 and brief mixing was followed by heating for 45 min at 55 °C, before addition of freshly prepared phenol/chloroform. Samples (50 µl) were loaded on agarose gel for electrophoresis (see section 2.26). The sizing of the plasmids was carried out by following the formula below:

\[
\text{Mobility of plasmid} = \frac{\text{Distance travelled by a plasmid}}{\text{Distance travelled by standard plasmid}} \times \text{mobility of standard plasmid}
\]

The marker strain carried 4 plasmids with the following specification.

Table 8: Specification of marker plasmids.

<table>
<thead>
<tr>
<th>M.W.</th>
<th>Mobility</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>98x10^6</td>
<td>5.9</td>
<td>cap, suf, tet</td>
</tr>
<tr>
<td>42x10^6</td>
<td>8.6</td>
<td>--</td>
</tr>
<tr>
<td>23.9x10^6</td>
<td>10.0</td>
<td>cap, kan, str, suf</td>
</tr>
<tr>
<td>4.2x10^6</td>
<td>17.6</td>
<td>suf, tet</td>
</tr>
</tbody>
</table>

Then the size of each ColV was determined by calculating its mobility and checking the mobility X-axis against molecular weight on Y-axis from the curve drawn for the marker strain, shown in Figure 15.

The electrophoretic migration of ColV plasmids (Fig. 16) is shown in the lanes 2, 4 and 9. The distance travelled by ColV plasmid was approximately 5.5 mm while the distance travelled by standard plasmid was ca. 6.0 mm. The calculated molecular weight of ColV plasmid as per formula comes to be 94 x 10^6.
Fig. 15. Standard curve for sizing of M1 plasmids.
Fig. 16. Sizing of ColV plasmid. Tracks 1 and 11, lambda Hind III digest; tracks 3, 7 and 10, M1; tracks 2 and 4, ED1829ColV, l-K94; track 9, 14 ColV⁺; track 5, ED1829; tracks 6 and 8, ED1829ColV, l-K94 and 14 ColV⁺, respectively, treated with SDS and further incubated at 44 °C, for 48 hr.
2.23. Curing of plasmids:

The plasmid bearing strains were grown overnight at 37 °C with shaking to stationary phase. The overnight cultures were then diluted 1/10 in fresh NB containing 0.5% SDS, further incubated for 48 hr, at 37 °C with shaking. These cultures were then diluted 1/100 in fresh NB and incubated statically at 44 °C for 48 hr. Dilutions were plated on NA plates and colonies were tested for colicin production.

This was confirmed by running on agarose gel electrophoresis (Figure 16) after plasmid extraction. The tracks 6 and 8 represented the cell extracts from ED1829ColV,I-K94 and 14 ColV+ cells treated with 0.5 % SDS and incubated 44 °C for 48 hr, and did not indicate any sign of ColV plasmid since the SDS and high temperature cured the ColV plasmids.

2.24. Preparation of competent cells and their transformation:

Cultures of ED1829ColV,I-K94 grown overnight in NB, pH 7.0 were diluted 1/100 in fresh NB of pH 7.0 and grown with aeration at 37 °C to an optical density of 0.6 at 600 nm. The cells were treated with 0.1 M CaCl₂ to make them receptive for the uptake of foreign DNA by the method of Lederberg and Cohen, 1974. The competent cells were dispensed in 0.5 ml lots in 0.1 M CaCl₂ + 16 % glycerol and stored at -20 °C. Just before transformation experiments, the competent cells were quickly thawed out, spun briefly to free them of any endonuclease from the supernatant as described by Baltz and Matsushima, 1981, resuspended in 0.1 M CaCl₂ and added to plasmid DNA (pBR 322 from Sigma, UK) in TEN buffer,
[0.02M Tris (hydroxymethyl) aminomethane (pH 8.0) - 1 mM EDTA (pH 8.0) - 0.02 M NaCl]. The competent cells along with pBR 322 DNA were incubated on ice for 60 min then given a temperature shock by transferring to 37 °C for 10 min. The cells were cooled in an icebath for 2 min and then diluted in fresh prewarmed NB of pH 7.0 and incubated at 37 °C with aeration overnight. The transformants, selected for growth on NA containing 20 μg/ml tetracycline and 150 μg/ml ampicillin at 37 °C for 48 hr, were later used in further experiments.

Competent cells from habituated and non-habituated cultures of ED1829ColV, I-K94 were prepared in the same manner as described above. Since the ED1829ColV, I-K94 cells grew slower at pH 5.0, they were pooled to get an approximate optical density of 0.6 at 600 nm, before making them competent for accepting the foreign DNA. The recipient cells were transformed with pBR 322 DNA (80 μl) isolated from treated and untreated habituated and non-habituated organisms (see section 2.25) and the transformation was carried out as mentioned above.

Similarly competent cells from pH 7.0 broth cultures of strains; GW1000, GW1060, AB1157, DP1152 and AB2463, were transformed to determine the probable gene products responsible for DNA repair after acid treatment.

2.25. Sensitivity of ED1829ColV, I-K94-pBR 322 to pH 3.0:

Habituated and non-habituated 37 °C broth-grown cells of ED1829ColV, I-K94-pBR 322, from exponential phase were treated at pH 3.0 in NB, at 37 °C, with vigorous shaking for an appropriate period of time.
The sensitivity of treated and untreated cultures was studied by isolating their DNA and measuring its biological activity (in transformation) and its behaviour on agarose gels.

2.26. Isolation of pBR 322 plasmid DNA for agarose gel electrophoresis:

A comparative study was performed to check the extent of damage to pBR 322 plasmid DNA isolated from exponentially grown ED1829ColV,I-K94-pBR 322, habituated and non-habituated organisms after acid treatment. The pBR 322 DNA was isolated from (1) organisms which were grown in NB of pH 7.0 and pH 5.0 and then treated in broth at pH 3.0, and 37 °C, with vigorous shaking, for various time periods and (2) from untreated controls following the method of Kado and Liu, 1981. Cells were harvested by using 0.2 μm, 47 mm (diameter) Pall membrane filters and were washed off the filters using 10 mM Tris-HCl buffer pH 7.0 to neutralise the effect of acid. Then the treated cells and untreated controls were spun down in the MSE 21 centrifuge at 4 °C for 15 min. The pellets were resuspended in TE buffer for isolation of plasmid using essentially the method of Kado and Liu, 1981, with the exception that 50 μg/ml preheated RNAse was incorporated in TE buffer and alkaline lysis was carried out at 70 °C for 12 min (Kieser, 1984). After the addition of freshly prepared phenol chloroform, vortexing and centrifugation gave rise to two clear phases. Plasmid DNA samples obtained from the aqueous phase of the treated and their counterpart untreated controls were loaded on 0.7% agarose gels, electrophoresed in TBE buffer pH 7.9 having 89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA for 3.5 to 4 hr, at 140 V. The gels were stained with 700 μl/ml of ethidium bromide for
30 - 40 min, rinsed with \( \text{D}_2\text{H}_2\text{O} \) and observed with a C-63 transilluminator of Ultraviolet Products Inc, USA. Photographs were taken on Polaroid 665 film.

2.27. Isolation of plasmid DNA from habituated and non-habituated cells for transformation purposes:

Plasmid DNA was isolated from exponentially grown habituated and non-habituated cells of ED1829ColV;I-K94-pBR 322 which had been acid treated or untreated. The isolation was as in 2.26 except that DNA was subjected to more purification to get rid of toxic traces of phenol and chloroform which might have inhibited the competent cells, in the transformation experiments. Purification was as follows: added 10 % volume of sodium acetate (4 M, pH 6.0) and 1 volume of isopropanol to the aqueous supernatant of section 2.26, mixed by inversion and kept at room temperature for 5 min. Centrifuged the contents, decanted the supernatant and pellet was washed by 70 % alcohol by gentle inversion which was followed by another washing with absolute alcohol. The pellet was dried and then redissolved in T.E buffer to be used for transformation experiments.
3. Results:

3.1. Studies on acid-sensitivity of non-habituated ColV$^+$ and ColV$^-$ cells:

3.1.1. Comparison between the sensitivity of non-habituated ColV$^+$ and ColV$^-$ cells to fatty acids.

Enterobacteria can cause Public Health problems due to their introduction into food for example from contaminated water, fruits or vegetables used in manufacture or from animal products used as food constituents. The significance of such food contamination depends on bacterial pathogenicity and a major factor which increases pathogenicity is plasmid carriage (Smith, 1974).

The presence of weak acids, especially at acid pH, is an important inhibitory influence on growth and survival of enterobacteria and accordingly, naturally acid foods or acidulated ones should be free of putative pathogens. Previous work conducted in this laboratory demonstrated that certain strains of *E.coli* were very sensitive to acidic pH when they harboured ColV plasmids (Cooper and Rowbury, 1986).

A comparative study was performed to examine the degree of sensitivity of non-habituated ED1829ColV$^+$I-K94 and its isogenic strain ED1829, a ColV plasmid bearing and ColV$^-$ non-plasmid containing strain respectively, to various concentrations of weak organic acids at pH 4.5 in NB; the results are presented in Table 9.
The results shown in Table 9 illustrate that all the fatty acids killed both $p^+$ and $p^-$ organisms but the extent of killing in $p^+$ cells was much more pronounced than in the corresponding $p^-$ strain, at the tested time periods. Among the fatty acids tested, butyric acid was the strongest inhibitor for $p^+$ and $p^-$ organisms whereas propionic and lactic acids were the mildest.

In ED1829 the ColV,I-K94 plasmid caused 12 and 26 fold further decrease in survival, after 2.5 and 3.5 hr respectively, on treatment with butyric acid compared to plasmid-free organisms and 6 and 15 times less survival, after 2.5 and 3.5 hr respectively, when treated with acetic acid compared with the ColV$^-$ organisms. Survival percentage of the $p^+$ strain was 5 and 24 fold down compared with their counterpart $p^-$ strain when exposed to lactic acid for 2.5 and 3.5 hr. Less survival of 12 and 17 times was observed for the ColV$^+$ organisms compared with the ColV$^-$ cells on exposure to succinic acid. Propionic acid treatment yielded survival down 5 and 9 fold for the ColV,I-K94 bearing strain in contrast with the isogenic ColV$^-$ strain for the time 2.5 and 3.5 hr, respectively. Similarly, the ColV,I-K94$^+$ organisms showed 5 and 13 times decrease in survival compared with ColV$^-$ cells, for the stated time periods respectively, on treatment with formic acid.

The results mentioned above show that the plasmid-bearing organisms are markedly more sensitive to organic acids than the plasmid-free cells. This may suggest that presence of plasmid increases the permeability of cell wall which might enhance the sensitivity of the ColV$^+$ organisms.
<table>
<thead>
<tr>
<th>Time</th>
<th>pH</th>
<th>No.</th>
<th>Treatment</th>
<th>Control</th>
<th>PH 4.5</th>
<th>E. Coli</th>
<th>Lact. acid</th>
<th>Acetic acid</th>
<th>Butyric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>3.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.33</td>
<td>-</td>
<td>-</td>
</tr>
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<td>4.01</td>
<td>4.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>189</td>
<td>5.0</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>7.78</td>
<td>-</td>
<td>-</td>
</tr>
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<td>21.2</td>
<td>5.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.96</td>
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<td>-</td>
<td>-</td>
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<td>NA</td>
<td>0.26</td>
<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>0.87</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.44</td>
<td>5.0</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
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<td>NA</td>
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<td>2.03</td>
<td>5.0</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>0.24</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 9: Effect of lactic acids on E. coli strains from exponential phase when treated in NB at pH 4.5, at 37°C, with aerations.
Each experiment was repeated four times with consistent results. Mean % survival values represent the percentage of initial numbers.

<table>
<thead>
<tr>
<th>Date</th>
<th>pH</th>
<th>Formic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Table 9 continued</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.018</td>
<td>1.96</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>206</td>
<td>185</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.23</td>
<td>9.52</td>
<td>--</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>213</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>3.13</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>176</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>14.8</td>
<td>60</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>220</td>
<td>189</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.011</td>
<td>0.23</td>
<td>50</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>188</td>
<td>171</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.19</td>
<td>2.82</td>
<td>50</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>243</td>
<td>209</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.1.2. Comparison between the sensitivity of non-habituated ColV\(^+\) and ColV\(^-\) cells to hydrogen peroxide.

Sensitivity of non-habituated ColV\(^+\) and ColV\(^-\) organisms was also checked with hydrogen peroxide. Plasmid containing and plasmid-free cells were subjected to various concentrations of hydrogen peroxide for treatment periods of 10 and 20 min, at pH 7.0, with aeration at 37 °C for enumeration of percentage survival of the cells on both NA and NA + 0.5 % sodium deoxycholate. Any damaged cells normally failed to form colonies on NA + 0.5% sodium deoxycholate medium.

The results presented in Table 10 indicate that, in general, the death rate of plasmid containing organisms was much faster than the non-plasmid bearing strain. The ColV,I-K94 plasmid bearing strain was markedly more sensitive to the hydrogen peroxide, compared with the plasmid-free strain, in terms of their viability on NA.

The treated organisms were also plated on NA + 0.5% sodium deoxycholate for determination of injured cells; data shown in Table 10 reveals also the percentage injury of both the cells.

Bacteriocidal activity of 7.5 mM concentration of hydrogen peroxide was also tested for determining the sensitivity of the ColV\(^+\) and ColV\(^-\) strains at pH 4.5 (Table 11). The plasmid bearing strain, plated on NA, showed 3 and 6 fold reduced viability compared to the plasmid free parent
Each experiment was repeated three times with consistent results.

<table>
<thead>
<tr>
<th></th>
<th>20 min</th>
<th>10 min</th>
<th>5 min</th>
<th>2 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>60.05</td>
<td>5</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>50</td>
<td>18</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>35</td>
<td>21</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>19</td>
<td>36</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>25</td>
<td>40</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>15</td>
<td>47</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>25</td>
<td>59</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**% Injury**: % Injury of strain E. coli NA treated for 10 min. **Survival**: Survival on NA + D after 10 min. **p**: E. coli strain 1 - K-94, p: E. coli 18499, NA + D; nutrient agar + 0.5% NA deoxycholate.

**p**: E. coli K-94, NA + D; nutrient agar + 0.5% NA deoxycholate.

**p**: E. coli K-94, NA + D; nutrient agar + 0.5% NA deoxycholate.

Table 10: Effect of hydrogen peroxide on E. coli strains from exponential phase.
Table 11: Effect of hydrogen peroxide on *E. coli* strains from exponential phase when treated in NB of pH 4.5 with aeration at 37 °C.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Concentration of hydrogen peroxide (mM)</th>
<th>Treated for</th>
<th>Mean % survival on NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-</td>
<td>7.5</td>
<td>8 min</td>
<td>69</td>
</tr>
<tr>
<td>p+</td>
<td>7.5</td>
<td>16 min</td>
<td>47</td>
</tr>
<tr>
<td>p-; E1829ColV, I-K94, p+; E1829, NA; nutrient agar</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean % survival; values represent the % of initial cell numbers.

The experiment was performed four times with consistent results.
for the exposure times of 8 and 16 min, respectively.

The data presented above clearly shows that the ColV,I-K94 bearing organisms are much more sensitive than their corresponding ColV\(^-\) cells to hydrogen peroxide. The presence of ColV,I-K94 plasmid may increase permeation through cell wall of H\(_2\)O\(_2\) which in turn enhances the sensitivity of organisms.

3.1.3. Comparison between the sensitivity of non-habituated ColV\(^+\) and ColV\(^-\) organisms to urea:

Antibacterial activity of 10 and 25 mM concentration of urea was checked against logarithmically grown ColV\(^+\) and ColV\(^-\) organisms, in NB at pH 5.0 for the time periods of 120 and 180 min (Table 12). The survival percentage on NA for the treated organisms belonging to both strains did not manifest any remarkable difference in sensitivity to either of the concentrations of urea.

3.2. Recovery of non-habituated injured cells:

The presence of phosphate in the recovery media, as explained in Materials and Methods, enhanced the recovery of the injured ED1829ColV,I-K94 cells which were treated with hydrogen peroxide and weak acids like acetic and formic acids.

The recovery of organisms, injured by exposure to 10 mM concentration of hydrogen peroxide, was 4 times more in the recovery medium with phosphate
Table 12: Effect of urea on *E.* coli strains from exponential phase when treated in NB with aeration at 37 °C.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Concentration of urea</th>
<th>*Mean % survival on NA Treated for (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>180</td>
</tr>
<tr>
<td>$p^-$</td>
<td>10 mM</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.D</td>
</tr>
<tr>
<td>$p^-$</td>
<td>25 mM</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>$p^+$</td>
<td>10 mM</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>$p^+$</td>
<td>25 mM</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
</tr>
</tbody>
</table>

$p^-; ED1829, p^+; ED1829ColV, I-K94, N.D; not determined.

*Mean % survival; values represent percentage of initial cell numbers.

The experiment was repeated thrice with consistent results.
than in medium without phosphate, at room temperature (Table 13). Similarly the organisms injured by acetic acid recovered twice as well, at room temperature, plus phosphate than those of cells from medium without supplementation with phosphate (Table 14). A similar trend was exhibited by formic acid injured organisms (Table 15).

The results gave a clear indication that phosphate might have some stimulatory effects on the recovery of injured organisms.


3.3.1. Comparison between the sensitivity of habituated and non-habituated organisms to fatty acids.

It has previously been demonstrated in this laboratory that acid-habituated *Escherichia coli* ColV,I-K94 cells resisted the antibacterial activity of inorganic acid (HCl, pH 3.0) compared with its control non-habituated organisms (Goodson and Rowbury, 1989; Rowbury et al., 1989).

Logarithmically grown ED1829ColV,I-K94 organisms which were habituated by growing them at suboptimal pH of 5.0 and non-habituated cells grown at optimal pH value of 7.0, were subjected to 50 - 65 mM of the following fatty acids; butyric acid, acetic acid, lactic acid, succinic acid, propionic acid and formic acid for checking the sensitivity of both types of organisms, in terms of percentage survival on NA. Both types of cells
Experiment was repeated thrice with consistent results.

Recovery medium: NB prepared in 0.1 M phosphate buffer of pH 7.5.

NB: nutrient agar; NB: nutrient broth.

<table>
<thead>
<tr>
<th>Recovery medium</th>
<th>NB, pH 7.5</th>
<th>0 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery in medium</td>
<td>10 min H2O2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated cells</td>
<td>Prior to plating on NA</td>
<td>10 min H2O2</td>
<td></td>
</tr>
<tr>
<td>G Recovery after incubation</td>
<td>&amp; Survived on NA for</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 13: Effect of incubation in different media on recovery of ED182902V1-K94
Each experiment was repeated four times with consistent results.

Recovery medium: NB prepared in 0.1 M phosphate buffer of pH 7.5.

Table I: Effect of incubation in different media on recovery of ED182900Y1-K94

<table>
<thead>
<tr>
<th>Medium</th>
<th>0 min</th>
<th>63 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.A. Ph 7.5</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Recovery medium</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

Cells after 65 min exposure to acetic acid at pH 4.5.

Cells treated with 50 mM acetic acid prior to plating on NA.

NA; nutrient agar, NB; nutrient broth.
Experiment was repeated three times with consistent results.

Recovery medium: NB prepared in 0.1 M phosphate buffer of pH 7.5.
NA: nutrient agar, NB: nutrient broth pH 7.5.

<table>
<thead>
<tr>
<th>92</th>
<th>39</th>
<th>14</th>
<th>100</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>N.B Ph 7.5</th>
<th>Recovery medium in medium for 2 hr at 37°C (prior to plating on NA)</th>
<th>0 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>cellis</td>
<td>untreated</td>
<td>treated with 80 mM formic acid</td>
<td>mean % survival on NA for cells after 60 min of exposure to formic acid at pH 4.5.</td>
</tr>
</tbody>
</table>

Table 15: Effect of incubation in different media on recovery of ED182G01V,1-K94
exhibited some degree of sensitivity against the tested acids. Remarkably the death rates of pH 7.0 grown cells were far more rapid than the death rates of pH 5.0 grown organisms.

The non-habituated organisms demonstrated 19 and 70 fold reduced viability against the bacteriocidal activity of butyric acid for the treatment times of 2.5 and 3.5 hr respectively, compared with the viability of habituated organisms (Table 16) and 7 and 26 times less survival when exposed to acetic acid than their habituated counterpart cells at the same treatment times. Likewise the non-habituated cells showed 5 and 13 fold reduction in survival on treatment with lactic acid in contrast with the control habituated cells and 26 and 42 fold less viability for non-habituated organisms in comparison with corresponding habituated cells on exposure to succinic acid. The propionic acid treatment yielded 4 and 7 times less viability for non-habituated organisms compared with habituated cells at 2.5 and 3.5 hr time intervals, respectively. Similarly the loss in viability shown by pH 7.0 grown organisms against formic acid was 5 and 14 fold more than the loss exhibited by the pH 5.0 grown organisms for the exposure time of 2.5 and 3.5 hr respectively.

All the above mentioned results clearly confirmed that pH 5.0 grown organisms acquired phenotypically some resistance against the antimicrobial activity of the inhibitors like weak organic acids in contrast with the pH 7.0 grown organisms, the non-habituated cells (Table 16).
Table 16: Effect of fatty acids on ED1829ColV, I-K94, habituated and non-habituated cells in NB at pH 4.5, at 37°C, with aeration.

<table>
<thead>
<tr>
<th>pH 4.5 control</th>
<th>Mean &amp; survival on NB</th>
<th>Concentration of fatty acid (mm)</th>
<th>Acetic acid</th>
<th>Butyric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>13.0</td>
<td>50</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>2.22</td>
<td>185</td>
<td>Yes</td>
<td>--</td>
<td>--</td>
</tr>
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<td>0.6</td>
<td>2.5</td>
<td>50</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>2.01</td>
<td>119</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2.05</td>
<td>5.00</td>
<td>50</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>2.02</td>
<td>175</td>
<td>Yes</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.08</td>
<td>0.71</td>
<td>50</td>
<td>7.0</td>
<td>7.0</td>
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<tr>
<td>1.06</td>
<td>125</td>
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<td>--</td>
</tr>
<tr>
<td>0.21</td>
<td>0.85</td>
<td>50</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>1.94</td>
<td>172</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.03</td>
<td>0.046</td>
<td>50</td>
<td>7.0</td>
<td>7.0</td>
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<td>1.08</td>
<td>188</td>
<td>Yes</td>
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<td>--</td>
</tr>
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</table>
Each experiment was repeated four times with consistent results.

<table>
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<tr>
<th>pH</th>
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<th>Formic acid</th>
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<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>197</td>
<td>173</td>
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<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.38</td>
<td>74.67</td>
<td>65</td>
<td>Formic acid</td>
<td>7.0</td>
<td>0.0</td>
</tr>
<tr>
<td>189</td>
<td>164</td>
<td>--</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6.57</td>
<td>15.80</td>
<td>60</td>
<td>Propionic acid</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>193</td>
<td>169</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>5.0</td>
</tr>
<tr>
<td>0.98</td>
<td>4.07</td>
<td>60</td>
<td>Propionic acid</td>
<td>0.0</td>
<td>7.0</td>
</tr>
<tr>
<td>204</td>
<td>178</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>7.0</td>
</tr>
<tr>
<td>0.76</td>
<td>3.13</td>
<td>50</td>
<td>Succinic acid</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>203</td>
<td>180</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>5.0</td>
</tr>
<tr>
<td>0.08</td>
<td>0.12</td>
<td>50</td>
<td>Succinic acid</td>
<td>0.0</td>
<td>7.0</td>
</tr>
<tr>
<td>188</td>
<td>159</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Table 16 continued
3.3.2. Comparison between the sensitivity of habituated and non-habituated cells to hydrogen peroxide.

A comparative study was performed to check the sensitivity of habituated versus non-habituated organisms to hydrogen peroxide. The results in Table 17 show that the habituated cells resisted the antibacterial activity of hydrogen peroxide better, therefore, the death rate of the habituated organisms was far slower than the death rate of the non-habituated ones. The pH 7.0 grown organisms exhibited 4 and 11 fold greater decrease in percentage survival than that of pH 5.0 organisms, when exposed for 12 and 18 min, respectively. The resistance showed by the habituated cells against the bacteriocidal activity of hydrogen peroxide was far more at the exposure period of 18 min in comparison with 12 min treatment time.

Antibacterial activity of hydrogen peroxide was also studied at pH 3.0, where 10 mM concentration of the agent was tested against the habituated and non-habituated cells, at 37 °C, with aeration for time periods of 5 min and 10 min. The results presented in Table 18 show that the habituated cells exhibited 3 and 8 times more percentage survival versus the percentage survival of their corresponding non-habituated organisms.

These results indicate that the acid-habituated cells acquired some resistance in contrast with non-habituated counterparts against the antimicrobial activity of hydrogen peroxide.
Table 17: Effect of hydrogen peroxide on ED1829ColV, I-K94, habituated and non-habituated cells in NB of pH 7.0 with aeration at 37 °C.

<table>
<thead>
<tr>
<th>Growth pH of the cells</th>
<th>Concentration of hydrogen peroxide</th>
<th>*Mean percentage survival on NA Treated for</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>15 mM</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>15 mM</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.1</td>
</tr>
</tbody>
</table>

NA; nutrient agar

* Mean % survival; values represent % of initial cell numbers.

The experiment was performed thrice with consistent results.
Table 18: Effect of hydrogen peroxide on ED1829ColV, I-K94, habituated and non-habituated, cells in NB of pH 3.0 with aeration at 37 °C.

<table>
<thead>
<tr>
<th>pH of E. coli strain</th>
<th>Concentration of H₂O₂</th>
<th>pH 3.0 control</th>
<th>* Mean % survival on NA Treated for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>--</td>
<td>Yes</td>
<td>38</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>10 mM</td>
<td>--</td>
<td>23</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>--</td>
<td>Yes</td>
<td>74</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>10 mM</td>
<td>--</td>
<td>65</td>
</tr>
</tbody>
</table>

NA: nutrient agar.

* Mean % survival; values represent the % of initial cell numbers.

Experiment was repeated thrice with consistent results.
3.3.3. Comparison between the acid sensitivity of ED1829ColV,I-K94 cells grown at 44 °C and 37 °C.

Bacteriocidal response to fatty acids, was checked. The results are presented in Table 19 which demonstrates that the death rate of 44 °C grown organisms was relatively slower than the death rate of 37 °C grown cells, at their exposure to such acids.

Cells grown at 37 °C demonstrated 9 and 42 times less survival than that exhibited by 44 °C grown cells at the exposure times of 2.5 and 3.5 hr respectively, on treatment with butyric acid (Table 19) and 4 and 10 fold decrease in viability compared with that of 44 °C grown cells on treatment with acetic acid. Similarly on exposure to lactic acid, the 37 °C organisms exhibited 3 and 6 times reduced viability compared to 44 °C grown cells at the exposure times of 2.5 and 3.5 hr respectively.

These results suggested that some sort of resistance was acquired at 44 °C against exposure to weak acids which made them less prone to the bacteriocidal activity of the inhibitor compared with the sensitivity manifested by the 37 °C grown cells.

3.4. Effect of acid on the respiration of habituated and non-habituated, ED1829ColV,I-K94 cells at pH 3.0:

The rate of respiration for the habituated and non-habituated cells at pH 3.0 was determined by using an oxygen electrode to measure the amount of dissolved oxygen in the NB. Respiration rates for the non-habituated
- 147 -

Each experiment was performed in triplicate with consistent results.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Nutrient Agar</th>
<th>Lactic acid</th>
<th>44°C</th>
<th>Acetic acid</th>
<th>44°C</th>
<th>Butyric acid</th>
<th>44°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.00</td>
<td>11.00</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>37.00</td>
<td>8.30</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>44.00</td>
<td>2.39</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>37.00</td>
<td>12.54</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>44.00</td>
<td>0.24</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>44.00</td>
<td>0.93</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>37.00</td>
<td>5.2</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
<tr>
<td>44.00</td>
<td>0.022</td>
<td>0.09</td>
<td>50</td>
<td>0.06</td>
<td>50</td>
<td>0.02</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 19: Effect of growth temperatures on sensitivity of ED1829C01V.1-K94 organs to fatty acids in NB, at pH 7.0 with aeration.
(pH 7.0 grown) cells and habituated (pH 5.0 grown) organisms were 0.272 μmoles/ml/min and 0.252 μmoles/ml/min, respectively, at their respective growth pHs. The consumption of oxygen fell for both types (non-habituated and habituated) at acid pH. Although this was so, the extent in reduction of oxygen consumption was markedly more pronounced in the non-habituated cells compared with the corresponding acid-habituated ones (Table 20).

These observations suggest strongly that respiration is an acid-sensitive process and the oxygen uptake by non-habituated cells is more susceptible to acid in contrast with that of acid-habituated organisms. It might be correlated with the sensitivity to acid killing exhibited by both types of organism.

3.5. Effect of acid on motility and chemotaxis of habituated and non-habituated organisms:

The ColV,I-K94+ Escherichia coli strains are motile in shaken cultures at 37 °C or at 21 - 30 °C in static or shaken condition (Tewari et al., 1986). Study was undertaken to establish the effect of acid on motility and chemotaxis.

3.5.1. Effect of acid on the motility of habituated and non-habituated, ED1829ColV,I-K94 organisms.

A comparative study on the effect of acid pH (3.0, 3.5, 4.0) on the percentage motility of logarithmically grown, organisms of ED1829ColV,I-K94, was undertaken. The results are presented in Table 21.
Table 20: Effect of acid on respiration of ED1829ColV, I-K94, habituated and non-habituated, cells.

<table>
<thead>
<tr>
<th>Growth pH of cells</th>
<th>Rate of oxygen consumption in (μ moles/ml/min) before addition of acid</th>
<th>Rate of oxygen consumption in (μ moles/ml/min) after addition of acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>0.272</td>
<td>0.027</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>0.252</td>
<td>0.066</td>
</tr>
</tbody>
</table>

Experiment was performed six times with consistent results.
and show that both types of cells exposed to acidic pH lost their motility very rapidly. Generally the lower the pH value the faster the organisms lost their motility.

When the motility of habituated and non-habituated organisms was tested at pH 3.0, both lost their motility readily. At this pH the percentage motility of habituated cells was 3 times more than that of non-habituated organisms at 2 min, and at 3 min the percentage motility of pH 5.0 grown cells was 7 times higher (as indicated in Table 21) than the motility of pH 7.0 grown organisms.

When the motility of both types of cells was checked at pH 3.5, both showed a relatively higher percentage motility than that exhibited by the organisms exposed to pH 3.0. The proportion of habituated organisms remaining motile was twice as great as that for non-habituated organisms.

The cells were even less sensitive at pH 4.0, with the percentage motility of habituated cells being again twice as great as that of non-habituated organisms.

3.5.2. Effect of acid on chemotaxis of habituated and non-habituated ED1829ColV,1-K94 cells:

Exponentially grown, habituated and non-habituated, organisms responded differently with respect to chemotaxis from plugs containing acid. Both types of organisms (pH 7.0 and pH 5.0 grown) when exposed to pH 4.0 did not exhibit any negative chemotactic response and remained unaffected at that pH value at room temperature. Similar behaviour was shown by the habituated organisms when treated at pH 3.5; on the other hand the
Table 21: Effect of acid on motility of habituated and non-habituated ED1829ColV, I-K94 cells, at room temperature.

<table>
<thead>
<tr>
<th>Growth pH of the cells</th>
<th>Acid treatment at pH</th>
<th>Percentage motility at time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>pH 3.0</td>
<td>N.D</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>pH 3.0</td>
<td>N.D</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>pH 3.5</td>
<td>9</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>pH 3.5</td>
<td>16</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>pH 4.0</td>
<td>N.D</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>pH 4.0</td>
<td>N.D</td>
</tr>
</tbody>
</table>

Initial motility of both cells; 100%

N.D; not determined
non-habituated organisms demonstrated slight repulsion indicated in terms of a small zone of clearance on treatment at pH 3.5 which is illustrated in the Figure 17 a and 17 b. When the organisms grown at pH 7.0 and pH 5.0 were treated at pH 3.0, at room temperature, there was a zone of clearance seen only for pH 7.0 grown cells in all the experiments while comparing with the corresponding pH 5.0 grown cells as depicted in Figure 17 c and 17 d. This might imply that the non-habituated organisms had the tendency to show negative chemotactic response at acid pH values whereas pH 5.0 grown cells remained unaffected at that pH value in contrast with the untreated organisms at neutral pH value. Negative chemotaxis was poorly exhibited by ED1829ColV,I-K94 cells when treated with 0.1 % sodium sulphide, a strong repellent, at room temperature in comparison with the Pseudomonas aeruginosa when treated under the similar conditions as shown in figure 17 e and 17 f.

The above results indicate that the effect of acid on bacterial motility and negative chemotactic response is influenced by growth pH. Organisms grown at pH 7.0 showed greater sensitivity in their motility and more negative chemotactic response to acid compared with that of acid-habituated cells.

3.6. Effect of acid on release of cellular constituents:

Leakage of some cellular constituents, absorbance 260 nm, from ED1829ColV,I-K94 cells into the treatment medium after exposure to acid was observed. Such release from stationary and exponentially grown,
Fig. 17. Negative chemotactic response of habituated and non-habituated, ED1829ColV,1-K94, cells to acidic values. a, pH 7.0 grown cells treated at pH 3.5; b, pH 5.0 grown cells treated at pH 3.5.
Fig. 17. Negative chemotactic response of habituated and non-habituated, ED1829ColV1-K94, cells to acidic values. c, pH 7.0 grown cells treated at pH 3.0; d, pH 5.0 grown cells treated at pH 3.0.
Fig. 17. Negative chemotactic response. e, ED1829ColV, l-K94 treated with 0.1% sodium sulphide; f, P. aeruginosa treated with 0.1% sodium sulphide.
habituated and non-habituated, cells was studied.

3.6.1. Leakage of cellular constituents from stationary phase cells.

Acid treatment of stationary phase cells caused the release of some components which absorbed at 260 nm. Both habituated and non-habituated cells leaked compounds into the environment but the difference in the extent of leakage between the habituated and non-habituated cells was remarkable. Table 22 indicates that the non-habituated cells leaked 10 times as much in terms of absorbance at 260 nm as their counterpart habituated cells when acid exposed for 5 min. The amount of leaked materials was 7 times higher from non-habituated cells than from habituated cells at the treatment period of 10 min whereas on further prolonging the treatment to 15 min, 6 times more released materials were observed from the non-habituated cells than their corresponding habituated cells.

3.6.2. Leakage of cellular constituents from exponential phase cells.

When exponentially grown, habituated and non-habituated, organisms were treated with acid at pH 3.0, cellular leakage was observed (Table 23). The non-habituated cells showed more leakage than the habituated cells at a treatment time of 5 min whereas at a treatment time of 10 min there was only a slight difference in the amount of the released cellular constituents from the non-habituated and habituated cells. On prolonging the treatment to 15 min, the amount of released materials from the non-habituated cells was almost twice higher than from the habituated
Table 22: Effect of acid on stationary phase ED1829ColV, I-K94 cells; release of cellular constituents.

<table>
<thead>
<tr>
<th>Growth pH of the cells</th>
<th>Time of acid treatment at pH 3.0 in min</th>
<th>Medium absorbance at 260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>5</td>
<td>0.204</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>5</td>
<td>0.021</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>10</td>
<td>0.217</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>10</td>
<td>0.033</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>15</td>
<td>0.181</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>15</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Experiment was performed thrice with consistent results.
Table 23: Effect of acid on exponential phase ED1829ColV,1-K94 cells; release of cellular constituents.

<table>
<thead>
<tr>
<th>Growth pH of the cells</th>
<th>Time of acid treatment at pH 3.0 in min</th>
<th>Medium absorbance at 260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>5</td>
<td>0.163</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>5</td>
<td>0.108</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>10</td>
<td>0.097</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>10</td>
<td>0.079</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>15</td>
<td>0.146</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>15</td>
<td>0.087</td>
</tr>
</tbody>
</table>

Experiment was performed thrice with consistent results.
3.7. Induction of stress-related proteins at pH 5.0:

3.7.1. Protein synthesis at 37 °C for cells grown at pH 7.0 and pH 5.0.

Resistance exhibited by pH 5.0 grown cells against killing by acid might be due to the induction of some stress proteins. This was studied by one-dimensional polyacrylamide gel electrophoresis. The organisms grown at 37 °C, pH 5.0 showed increased levels of several cytoplasmic proteins (Table 24 and Figure 18) compared to pH 7.0. Additionally, one protein at least (K, ca 63 kDa) appeared to be induced only at pH 5.0 and one (L, ca 35 kDa) was repressed at this pH.

Subsequently induction at 37 °C of cytoplasmic proteins in pH 5.0 cells was reconfirmed when the cell extracts from pH 5.0 and pH 7.0, grown organisms were subjected to electrophoresis by 2-dimensional SDS-polyacrylamide gels. The results are presented in Table 25 and Figure 19 which illustrates that pH 5.0 grown organisms, at 37 °C, showed increased synthesis of several cytoplasmic proteins compared with pH 7.0 grown organisms.

3.7.2. Protein synthesis of 24 °C grown pH 7.0 versus pH 5.0 organisms.

A comparative study of the synthesis of cytoplasmic proteins at 24 °C was performed by electrophoresing the cell extracts, of pH 7.0 and pH 5.0
Table 24. Polypeptides synthesised in ED1829ColV,I-K94 cells grown at pH 7.0 and pH 5.0, at 37 °C, studied by unidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>ca M.W in kDa</th>
<th>Synthesis at pH 5.0</th>
<th>Synthesis at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>73</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>40</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>37</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>18</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
<td>16</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>K</td>
<td>63</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L</td>
<td>35</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Representative of four gels.

+; low level
++; high level
-; not present
Fig. 18. Proteins induced in ED1829ColV,1-K94 at pH 5.0 and 37 °C, studied by unidimensional gel. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lanes 1 and 3, proteins from organisms grown at pH 5.0; lanes 2 and 4, proteins from organisms grown at pH 7.0.
Table 25. Polypeptides synthesised in ED1829ColV, I-K94 cells grown at pH 7.0 and pH 5.0, at 37°C, studied by bidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>ca M.W in kDa</th>
<th>Synthesis at pH 5.0</th>
<th>Synthesis at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>73</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>40</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>37</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>18</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
<td>16</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>K</td>
<td>63</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L</td>
<td>32</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>M</td>
<td>49</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>N</td>
<td>45</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>O</td>
<td>38</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>57</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Q</td>
<td>50</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Representative of three gels.
+; low level
++; high level
-; not present.
Fig. 19. Proteins induced in ED1829ColV, I-K94 at pH 5.0, 37°C study by bidimensional gel. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. A, proteins from organisms grown at pH 5.0; B, proteins from organisms grown at pH 7.0.

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Fig. 19. Proteins induced in ED1829ColV,I-K94 at pH 5.0, 37 °C study by bidimensional gel. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. A, proteins from organisms grown at pH 5.0; B, proteins from organisms grown at pH 7.0.
organisms. Organisms grown at pH 7.0 had increased levels of certain proteins (Table 26 and Figure 20), compared with pH 5.0 grown organisms at 24 °C. In some experiments, protein A, ca 95 kDa was induced only in pH 7.0 grown cells.

When the cell extracts of pH 7.0 and pH 5.0 grown organisms were subjected to polyacrylamide 2 dimensional gel electrophoresis, the pH 7.0 grown organisms showed increased synthesis of some proteins (Table 27 and Figure 21). Additionally protein A (in some experiments but not all), with an approximate molecular weight of 92 - 95 kDa, which was either exclusively synthesised by pH 7.0 grown cells or induced in very very low quantity in pH 5.0 organisms, was comparable to Lon protein.

3.7.3. Protein synthesis of organisms grown at 44 °C and pH 7.0 or pH 5.0.

When the cell extracts were subjected to unidimensional electrophoresis on SDS-polyacrylamide gel, pH 7.0 grown organisms showed increased synthesis of some proteins (compared with pH 5.0, 44 °C grown organisms) as indicated in Table 28 and Figure 22. There was an additional protein (C, ca. 72 - 73 kDa) which seemed to be induced only in organisms grown at pH 7.0, 44 °C.

3.7.4. Time course for induction of stress-related proteins at 37 °C by pH 7.0 grown versus pH 4.3 transferred organisms.

Though induction of acid-resistance (habituation) occurred in NB pH 5.0
Table 26. Polypeptides induced in ED1829ColV, I-K94 cells grown at pH 7.0 and pH 5.0, at 24 °C, study through unidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>ca M.W in kDa</th>
<th>Synthesis at pH 5.0</th>
<th>Synthesis at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>73</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Z</td>
<td>79</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 27: Polypeptides synthesised in ED1829ColV, I-K94 cells grown at pH 7.0 and pH 5.0, at 24 °C, study by bidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>ca M.W in kDa</th>
<th>Synthesis at pH 5.0</th>
<th>Synthesis at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C</td>
<td>73</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Z</td>
<td>79</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>R</td>
<td>65</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Representative of four gels.
+/- ; no to very low level
+ ; low level
++ ; high level
Fig. 20. Proteins induced in ED1829ColV, I-K94 at pH 5.0, 24 °C study by unidimensional gel. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lane 1, proteins from organisms grown at pH 5.0; lane 2, proteins from organisms grown at pH 7.0.
Fig. 21. Proteins induced in ED1829ColV,1-K94 at pH 5.0, 24 °C study by bidimensional gel. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. A, proteins from organisms grown at pH 5.0; B, proteins from organisms grown at pH 7.0.
Fig. 21. Proteins induced in ED1829ColV,1-K94 at pH 5.0, 24 °C study by bidimensional gel. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. A, proteins from organisms grown at pH 5.0; B, proteins from organisms grown at pH 7.0.
Table 28: Polypeptides synthesised in ED1829ColV, I-K94 cell grown at pH 7.0 and pH 5.0, at 44 °C, studied by unidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>ca M.W in kDa</th>
<th>Synthesis at pH 5.0</th>
<th>Synthesis at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>G</td>
<td>40</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>H</td>
<td>37</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>I</td>
<td>18</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>X</td>
<td>21</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>K</td>
<td>63</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>L</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>72</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>W</td>
<td>47</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Representative of four gels.
+/−: no to very low level
+: low level
++: high level
Fig. 22. Proteins induced in ED1829ColV, I-K94 at pH 5.0, 44 °C. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lane 1, proteins from organisms grown at pH 5.0, 44 °C; lane 2, proteins from organisms grown at pH 7.0, 44 °C; lane 3, proteins from organism grown at pH 5.0, 37 °C; lane 4, proteins from organism grown at pH 7.0, 37 °C; lane 5, protein standard markers.
within 5 to 10 min (Raja et al., 1991a; Table 1) yet previously synthesis of acid-induced proteins was studied, through PAGE, from the cells of late exponential to early stationary phase (Raja et al., 1991a). Therefore, time course for induction of stress-related proteins was carried out by autoradiography.

Cell extracts were prepared from pH 7.0 grown and pH 4.3 transferred organisms which had been pulse labelled for 10 min with $^{35}$S methionine. These were then subjected to electrophoresis by unidimensional polyacrylamide gel. Acid-shock (pH 4.3) cells from both the strains induced the synthesis of some proteins which were either missing or produced in reduced amount in pH 7.0 grown organisms and the results are illustrated in Table 29 and Figures 23 and 24. Protein T, ca 20 - 22 kDa, was observed in some experiments.

3.8. Phosphate effect on new protein synthesis by E.coli at pH 4.3.

Habituation involves the synthesis of stress-related proteins in NB of pH 5.0 at 37 °C (Raja et al., 1991a). This study was conducted to observe any impact of phosphate on synthesis of stress-related proteins at pH 4.3.

The cell extracts prepared from P678-54 and P678-54 ompA strains, (as explained in Materials and Methods) were subjected to polyacrylamide unidimensional gel electrophoresis for autoradiography. Several proteins are synthesised in much larger amounts in the first few minutes after a shift to pH 4.3 than at pH 7.0 prior to shift by both the strains (Table 29 and Figure 23 and 24). Most of these induced proteins are produced at pH 4.3 + 10 - 25 mM phosphate but in reduced amount in P678-54 ompA (Fig. 25), but not in P678-54 (Fig. 26). This suggests that
Table 29: Polypeptides synthesised in P678-54 and P678-54 ampA at pH shift from pH 7.0 to 4.3, at 37 °C, studied by autoradiography of unidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>ca M.W in kDa</th>
<th>Synthesis at pH 4.3</th>
<th>Synthesis at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>73</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>L</td>
<td>35</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>S</td>
<td>30</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>T</td>
<td>20</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Representative of four gels.
*+; low level
++; high level
Fig. 23. Proteins induced at acid-shift from pH 7.0 to 4.3 in P678-54. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lane 1, proteins from organisms grown at pH 7.0; lane 2, proteins from organisms on acid shift at pH 4.3.
Fig. 24. Proteins induced at acid-shift from pH 7.0 to 4.3 in P678-54 ompA. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lane 1, proteins from organisms grown at pH 7.0; lane 2, proteins from organisms on acid shift at pH 4.3.
Table 30: Polypeptides synthesised in P678-54 and P678-54 Mohammad A on acid shift at pH 4.3 and pH 4.3 + 10 mM phosphate studied by autoradiography of unidimensional gel.

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>ca M.W in kDa</th>
<th>Induction in P678-54 Mohammad A at pH 4.3</th>
<th>Induction in P678-54 Mohammad A at pH 4.3 + 10 mM P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>73</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>D</td>
<td>68</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>L</td>
<td>35</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>S</td>
<td>30</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>T</td>
<td>20</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Representative of four gels.
+/-; no to very low level
+; low level
++; high level
Fig. 25. Proteins induced at acid-shift from pH 7.0 to 4.3, in P678-54 ompA, and at pH 4.3 + 10 mM phosphate. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lane 1, proteins from organisms grown at pH 7.0; lane 2, proteins from organisms on acid shift at pH 4.3; lane 3, proteins from organisms on pH 4.3 + 10 mM phosphate.
Fig. 26. Proteins induced at acid-shift from pH 7.0 to 4.3, in P678-54, and at pH 4.3 + 25 mM phosphate. Protein standards (arrowed) are of molecular weights: 66 kDa, no. 1; 45 kDa, no. 2; 36 kDa, no. 3; 29 kDa, no. 4; 24 kDa, no. 5; 19 kDa, no. 6. Lane 1, proteins from organisms grown at pH 7.0; lane 2, proteins from organisms on acid shift at pH 4.3; lane 3, proteins from organisms on pH 4.3 + 25 mM phosphate; lane 4, proteins from organisms on acid shift at pH 4.3.
acid-habituation can be inhibited by addition of phosphate at low pH.

3.9. Plasmid DNA from acid-treated organisms:

Since biological properties of the DNA could be studied through transformation, pBR 322 plasmid DNA was used to study how transformation ability was affected by acid-exposure of ED1829ColV, I-K94.pBR 322 cells. DNA damage, of acid-exposed cells, was also studied directly by electrophoretic migration on agarose gels.

3.9.1.a. Transforming ability of pBR 322 from acid-treated organisms:

The habituated and non-habituated donor cells, harbouring pBR 322 plasmid, were treated with acid for variable lengths of time and their plasmid DNAs were isolated for transformation purposes. The percentage transformation of habituated and non-habituated organisms gave an indication of the extent of DNA damage done by the acid.

10 min acid-treated plasmid DNA from habituated and non-habituated organisms did not transform pH 7.0 grown competent cells at all (Table 31 and Figure 27) compared to the untreated controls. These results are in accord with the results of agarose gel where no covalently closed circular (ccc) or open circular (oc) plasmid DNA was seen in the extracts of 10 min treated habituated and non-habituated organisms (Fig. 27). This probably means that 10 min treatment might be too injurious for the donor DNA to
Table 31: Biological activity of plasmid DNA from acid-treated (10 min) cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Growth pH of plasmid at pH 3.0 for 10 min</th>
<th>Acid treatment of pH 7.0 grown cells</th>
<th>Percentage transformation of pH 7.0 grown cells by plasmid DNA</th>
<th>Ratio transformation by plasmid DNA from treated / untreated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PH 5.0</td>
<td>No</td>
<td>0.32</td>
<td>0.0300</td>
</tr>
<tr>
<td></td>
<td>PH 7.0</td>
<td>Yes</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Note: The experiment was repeated twice with consistent results.
transform the recipient cells in contrast with the untreated controls.

The plasmid DNA from 7 min acid-treated cells of both types transformed pH 7.0 grown recipients. The percentage transformation of the acid-treated plasmid DNA from habituated cells was markedly more than that of the treated non-habituated organisms (Table 32). This suggests that plasmid from pH 5.0 grown organisms is less damaged by acid than than that from organisms grown at pH 7.0.

This was in accord with the physical properties exhibited by the 7 min pretreated DNA on agarose gel where the habituated DNA was damaged less in comparison with non-habituated organisms (Fig. 28). The ratios of transformation by plasmid DNA from treated/untreated cells for the habituated organisms were 3 - 5 fold more than for non-habituated cells as depicted by these representative experiments.

3.9.1.b. Increased repair by habituated cells:

Plasmid isolated (after acid treatment) from organisms grown at pH 5.0 or pH 7.0 was used to transform recipients grown at the two pH values. Both types of acid-damaged plasmid (i.e from organisms grown at pH 5.0 and pH 7.0 respectively) produced more transformants in habituated than in non-habituated competent organisms (Table 33). This observation strongly suggests that acid-habituated cells can repair acid-damaged plasmid better than non-habituated ones.
<table>
<thead>
<tr>
<th>Number</th>
<th>untransformed cells</th>
<th>transformed by plasmid DNA</th>
<th>number of plasmid DNA from pH 7.0 grown transformation</th>
<th>percentage of cells containing plasmid for 7 min at pH 3.0</th>
<th>report of acid treatment experiment</th>
<th>Growth pH into organisms grown at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.43</td>
<td>0.92</td>
<td>Yes</td>
<td>0.95</td>
<td>Yes</td>
<td>0.58</td>
<td>5.0</td>
</tr>
<tr>
<td>0.47</td>
<td>0.89</td>
<td>No</td>
<td>0.90</td>
<td>Yes</td>
<td>0.58</td>
<td>5.0</td>
</tr>
<tr>
<td>0.15</td>
<td>0.12</td>
<td>No</td>
<td>0.17</td>
<td>Yes</td>
<td>0.70</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Table 32: Biological activity of plasmid DNA from acid-treated (7 min) cells transformed.
Table 33: Increased repair of acid-damaged plasmid DNA in acid-habituated cells.

<table>
<thead>
<tr>
<th>Source of plasmid DNA</th>
<th>pH 7.0</th>
<th>pH 5.0</th>
<th>pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-treated plasmid</td>
<td>4.47</td>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>Ph 7.0 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unacid-treated plasmid</td>
<td>3.55</td>
<td>0.57</td>
<td>0.53</td>
</tr>
<tr>
<td>pH 7.0 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid-treated plasmid</td>
<td>3.87</td>
<td>0.35</td>
<td>0.31</td>
</tr>
<tr>
<td>pH 5.0 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unacid-treated plasmid</td>
<td>5.68</td>
<td>0.36</td>
<td>0.34</td>
</tr>
<tr>
<td>pH 5.0 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.9.1.c. Biochemical basis of DNA repair.

Plasmid DNA was isolated from 5 min acid-treated organisms to transform pH 7.0 grown competent cells belonged to GW1000, GW1060, AB1157, DP1152 and AB2463 to know the biochemical basis for DNA repair. The results are presented in Table 34 which suggests that polA, recA and uvrA are required for DNA repair.

3.9.2. Electrophoretic mobility of acid-treated pBR 322 plasmid DNA:

ED1829ColV,7-K94,pBR 322, habituated and non-habituated, cells were acid-treated for variable periods of time. Isolated plasmid pBR 322 DNA was subjected to electrophoresis, to observe directly any damage to its acid-treated DNA, through agarose gels (as explained earlier in the Materials and Methods).

The extent of DNA damage by 10 min of acid treatment is shown in Figure 27, which indicates that exposure might be too injurious to show any (ccc) or (oc) DNA bands from treated cells compared with untreated controls.

The DNA damage exhibited by acid for 7 min treatment time is shown in Figure 28, which illustrates that no covalently closed circular (ccc) plasmid was observed on agarose gels when extracts from acid treated non-habituated cells at pH 3.0 was examined in all the experiments. On the other hand in several experiments (but not all), covalently closed circular (ccc) plasmid was seen in extracts from acid treated organisms grown at pH 5.0. Traces of open circular (oc) plasmid DNA were also seen
Experiment was repeated thrice with consistent results.

<table>
<thead>
<tr>
<th>pH</th>
<th>0.0</th>
<th>0.05</th>
<th>0.037</th>
<th>0.015</th>
<th>0.005</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>0.10</th>
<th>0.27</th>
<th>0.18</th>
<th>0.54</th>
<th>0.37</th>
<th>0.61</th>
<th>0.37</th>
<th>0.51</th>
<th>0.25</th>
<th>0.46</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DC1152</td>
<td>DC1152</td>
<td>AB2463</td>
<td>AB1157</td>
<td>AB1157</td>
<td>AB1157</td>
<td>AB1157</td>
<td>AB1157</td>
<td>CM1060</td>
<td>CM1060</td>
</tr>
</tbody>
</table>

Table 34: Biological activity of plasmid DNA from acid-treated cells transformed into

untransformed cells by plasmid DNA from treated/acid-treated or PH 7.0 cells by treated plasmid DNA

Recipient growth at pH 7.0

<table>
<thead>
<tr>
<th>Percentage of the acid treatment</th>
<th>Recipient growth at PH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 27. Plasmid DNA from acid-treated (pH 3.0 for 10 min) pBR 322 E. coli. Tracks 1 and 4, plasmid DNA from untreated pH 5.0 cells; tracks 2 and 3, plasmid DNA from acid-treated pH 5.0 cells; tracks 5 and 8, plasmid DNA from untreated pH 7.0 cells; tracks 6 and 7, plasmid DNA from acid-treated pH 7.0 cells.
Fig. 28. Plasmid DNA from acid-treated (pH 3.0 for 7 min) pBR 322+E.coli. Tracks 1 and 4, plasmid DNA from untreated pH 5.0 cells; tracks 2 and 3, plasmid DNA from acid-treated pH 5.0 cells; tracks 5 and 8, plasmid DNA from untreated pH 7.0 cells; tracks 6 and 7, plasmid DNA from acid-treated pH 7.0 cells.
in the extracts from acid treated organisms grown at pH 5.0. Whereas good plasmid bands were observed in the tracks of untreated, habituated and non-habituated, organisms.

When 7 min acid-treated pBR 322 plasmid bearing, habituated and non-habituated, organisms were incubated for a period of 30 min (as explained in Materials and Methods) after exposure followed by plasmid extraction and electrophoresis, as indicated in Figure 29, both types (habituated and non-habituated) contained ccc DNA (even if none were isolated immediately after treatment) but there was always more in the cells grown at pH 5.0. This may mean that acid-habituated cells repair better than non-habituated organisms.

The damage done by acid to the pBR 322 plasmid DNA for 5 min treatment is presented in Figure 30, which demonstrates that the plasmid DNA in (ccc) form was observed in the tracks of both types of treated cells in contrast with untreated cells but there was always more covalently closed circular (ccc) DNA from the treated organisms grown at pH 5.0 compared with the extracts of the organisms grown at pH 7.0.
Fig. 29. Repair of acid-damaged plasmid after acid-treatment of pBR 322<sup>+</sup>
E.coli. Tracks 1 and 6, plasmid DNA from untreated pH 5.0 cells; tracks 2
and 3, plasmid DNA from acid-treated pH 5.0 cells, grown further for 30
min prior to plasmid isolation; tracks 4 and 5, plasmid DNA from
acid-treated pH 5.0 cells. Tracks 7 and 12, plasmid DNA from untreated pH
7.0 cells; tracks 8 and 9, plasmid DNA from acid-treated pH 7.0 cells;
tracks 10 and 11 plasmid DNA from acid-treated pH 7.0 cells further
incubated for 30 min prior to plasmid isolation; CCC, covalently closed
circular pBR 322.
Fig. 30. Plasmid DNA from acid-treated (pH 3.0 for 5 min) pBR 322* E. coli. Tracks 1, 6 and 12, lambda Hind III digest; tracks 2 and 5, plasmid DNA from untreated pH 7.0 cells; tracks 3 and 4, plasmid DNA from acid-treated pH 7.0 cells; tracks 7 and 11, plasmid DNA from untreated pH 5.0 cells; tracks 8, 9 and 10, plasmid DNA from acid-treated pH 5.0 cells.
4. Discussion

4.1. Acid effect on ColV,1-K94* cells:

The acid sensitivity of *Escherichia coli* is important for survival in the natural environment. In aquatic habitats the organisms may be subjected to acidic pHs resulting from acid rain and acid snow melts; acid run-off from fields may arise because of agricultural use of acid slurries or acid generating fertilizers, whilst chemical processes produce a range of acid effluents. Organisms in aquatic environments may also encounter weak organic acids such as butyrate, propionate and acetate in the vicinity of sediments. Acid exposure may also occur in the animal body. In order to reach and multiply in the intestine, ingested microorganisms must survive exposure to gastric acid (ca. pH 2.0 - 3.0) and they may also be exposed to a number of weak acids present in ingested food. Mild acid pH together with weak acids also occurs in the upper intestine, in the phagolysosomes and in the urinary tract. Exposure to acid pH and weak acid may also occur for contaminating organisms in acid foods.

4.1.1. Acid sensitivity of non-habituated cells:

Gram-negative bacteria, such as *Escherichia coli* are quite sensitive to acid pH and their sensitivity is enhanced by addition of a weak organic acid. Killing and damage by acid may be greater if plasmids are present. ColV plasmids confer or enhance several bacterial properties that may be related to virulence but also increase sensitivity to some agents. Such increased killing by inhibitory agents (Rowbury et al., 1985) depends
mainly upon derepressed transfer properties. Several observations suggested the involvement of these components in acid sensitivity. A derivative of ED1829 carrying a mutant form of the plasmid ColV,I-K94, namely ColV-M 50(1) which encodes normal colicin components but no transfer components, showed acid-susceptibility quite similar to ED1829, i.e., loss of transfer components led to resistance. On the contrary, the presence of plasmid ColV-M40(5), which confers normal transfer properties but no colicin components, made the ED1829 strain as acid-sensitive as ED1829ColV,I-K94 (Cooper and Rowbury, 1986; Table III).

The results presented here (see Tables 9, 10 and 11) demonstrate that ColV bearing strains are significantly more sensitive to hydrogen peroxide and weak organic acids than their isogenic plasmid free strains. The increased acid sensitivity of ColV+ strains might depend on the presence of transfer components. The transfer components may confer acid sensitivity by allowing hydrogen peroxide and weak acids to pass the outer membrane more quickly. The tight packing of the lipopolysaccharide (LPS) molecules maintains the impermeability of the outer membrane to many environmental agents and insertion of transfer components into the outer membrane may weaken LPS-LPS linkages, as has been suggested for compounds with strong cationic regions (Hancock, 1984) and allow increased penetration of e.g. organic acids and hydrogen peroxide. This could be tested by examining the sensitivity of 1829ColV-M40(5) and 1829 ColV-M50(1) to hydrogen peroxide and weak acids or by testing sensitivity in ED1829 derivatives carrying ColV,I-K94 and another plasmid which represses the transfer properties of the ColV.
4.1.2. Sensitivity of acid-habituated cells:

Water pollution has been an increasing problem in recent years and many pollutants are responsible for acidification or alkalisation of natural waters of rivers, streams and estuaries. Acidification has become a particular concern because it results from a wide range of polluting conditions; acid rain, acid snow melts, acid mine and chemical wastes, acid sewage, run-offs from fields treated with acid-generating fertilisers and acid agricultural wastes play an important role in causing it.

Acidification usually occurs due to periodic discharges of effluents or waste at particular sites. With diffusion from these sites, there will be gradual pH changes at nearby locations from neutrality to a maximum extreme of pH and back to neutrality, due to dilution and neutralisation. Acidification affects the aquatic flora and fauna but extreme acidic pH may also influence the nature and abundance of micro-organisms. Such gradual increases in acidity can allow induced acid resistance (habituation to acid) to occur which permits habituated organisms to survive subsequent exposures which are lethal to non-habituated organisms (Goodson and Rowbury, 1989; Rowbury et al, 1989).

The phenomenon of habituation (induced resistance) to a stress is quite common in procaryotes. When bacteria are exposed to a sub-lethal dose (an adaptive dose) of a particular stress they acquire resistance phenotypically and resist damage or killing by a higher dose (a challenge dose) of that stress which is normally very damaging for the unexposed organisms. Such induced resistance occurs for organisms exposed to heat
(Mackey and Derrick, 1987), oxidative stress (Demple and Halbrook, 1983) and metal ion stress (Khazaeli and Mitra, 1981).

It has previously been demonstrated in this laboratory that ColV− and ColV,I-K94+ strains of *Escherichia coli*, when grown at pH 7.0, fail to grow after relatively short periods of exposure to pH 3.0 or 3.5. However, the growth of cells cultured at pH 5.0 was almost unaffected by exposure to such acid values (Goodson and Rowbury, 1989). Subsequently Rowbury et al., (1989) observed that the ColV,I-K94+ strain of *Escherichia coli* initially grown at pH 4.6 or 5.0 (acid-habituated) resisted long exposures to pH 3.0 and was much more resistant than pH 7.0-grown organisms (non-habituated) at this pH value.

Habituation to acid for ED1829ColV,I-K94 cells occurred in NB of pH 5.0 during incubation at 37 °C and habituated organisms were much more resistant to the bacteriocidal activity of organic acids such as butyric, succinic, acetic, lactic, formic and propionic acids than pH 7.0 grown (non-habituated) organisms, at acid pH 4.5 (see Table 16).

Similarly the acid-habituated (pH 5.0 grown) ColV,I-K94+ strain of *Escherichia coli* demonstrated significantly more resistance against the antibacterial activity of hydrogen peroxide compared with their counterpart non-habituated (pH 7.0 grown) cells, if exposure was for relatively short periods of time at both the tested pHs of 7.0 and 3.0 (see Tables 17 and 18).

The results mentioned above (Tables 16, 17 and 18) are for organisms grown
in NB at sub-optimal pH 5.0 for several hours. However, it has been demonstrated that habituation can occur very rapidly in broth culture but more slowly in glucose minimal media at 37°C. The ColV,I-K94+ strain of E.coli gained partial resistance in NB of pH 5.0 in 5 min and was fully resistant in 10 min, whereas the organisms took 30 - 60 min to achieve acid-resistance in glucose minimal media at 37°C and this was dependent on the presence of a nitrogen source and tryptophan but not glucose (Raja et al., 1991a). The induction of acid-resistance was dependent on continuing protein and RNA synthesis but unaffected in the presence of nalidixic acid, an inhibitor for DNA synthesis. Moreover, acid resistance was long lasting; organisms grown at pH 5.0 retained most of their resistance after 2 hr growth at pH 7.0.

Since induction of acid-resistance in Escherichia coli takes place rapidly relative to the generation times in NB and glucose minimal media, this strongly suggests that the process of habituation involves phenotypic gain of resistance to the stress and rules out the selection of resistant mutants since these time periods would not allow resistant mutants to outgrow acid sensitive strains.

The findings that habituation is dependent on presence of a nitrogenous source in glucose minimal media, and upon continuing protein and RNA synthesis in NB strongly suggests that induction of acid-resistance may be associated with the synthesis of stress related proteins which may reduce damage by the stress challenge dose or allow increased repair of stress damaged cells. Such induction of stress related proteins in response to a stress is quite a common phenomenon among procaryotes and is observed with
respect to heat stress (Yamamori and Yura, 1982), and metal ion stress (Khazaeli and Mitra, 1981).

Subsequently, induction of acid-stress related proteins was observed in habituated organisms (Raja et al., 1991a) which might confer some protective role in preventing acid damage in them.

Accordingly, Escherichia coli which is a frequent water pollutant and an indicator of water quality, can habituate to acid and this may affect its survival and abundance in waters subject to periodic extremes of pH. It may also affect its ability to resist acid in other locations such as in acid foods and in the animal body.

4.2. Acid sensitivity after temperature upshift:

It has previously been demonstrated that the ColV,I-K94 plasmid markedly increased the heat sensitivity of Escherichia coli K12. The plasmid effect on strain K-12 appeared to result from ColV-encoded transfer and colicin components possibly due to their effects on membrane properties (Ghazaleh et al., 1989). Subsequently, when ColV,I-K94+ organisms were subjected to a gradual rise in temperature from 34 to 50 °C, they acquired thermotolerance and exhibited marked resistance to killing (failure to form colonies on NA) on exposure to 60 °C (Ghazaleh, 1990). Thermotolerance shown by the organisms might be attributed to induction of heat-shock proteins which confer resistance to killing at 60 °C.

Amongst other effects, thermotolerance results from ability of cells to
repair heat damage better. Such repair activities might also protect acid exposed cells and, therefore, the thermotolerance acquired by the ColV,1-K94* strains of \textit{Escherichia coli} during growth at 44 °C in NB of pH 7.0 might be responsible for the resistance against the bacteriocidal activity of some organic acids observed at neutral pH (see Table 19) compared with their counterpart grown at 37 °C in NB of pH 7.0. Such cross resistance might be similar to the earlier findings of Jenkins et al., (1988), where glucose or nitrogen starved cultures of \textit{Escherichia coli} exhibited increased resistance to heat or hydrogen peroxide challenge and the degree of resistance was time dependent.

Procaryotes respond to environmental or chemical stresses by inducing specific sets of proteins. In some cases, proteins associated with one stress-stimulon can be induced during other stresses. For example, various heat-shock proteins in \textit{E. coli} are also produced when cells are exposed to \( \text{H}_2\text{O}_2 \) (VanBogelen et al., 1987) and starvation (Jenkins et al., 1988). Induction of synthesis of some stress proteins by several stimuli is common in procaryotes which leads to cross protection of the micro-organisms for an unrelated stress. Accordingly, the heat-shock proteins induced at 44 °C might confer some resistance against the antibacterial activity of organic acids (Table 19). Though heat-shock is a transient response and is likely to decline after some time (Mackey and Derrick, 1986) yet it may provide some cross-protection.

Cellular components like outer membrane, cytoplasmic membrane, proteins, DNA, RNA, and ribosomes are vulnerable to heat. It may be possible that
heat-shock proteins are involved in repairing damage to some or all these sites or some or all may be protected by induced heat-shock proteins or the vulnerability of these sites reduced which consequently increases the survival chances of the exposed organisms at high temperature. Some heat-shock proteins are associated with ribosomes (e.g. GroEL); on interaction they may prevent heat induced unfolding of ribosomal subunits and hence protect rRNA molecules from degradation; they may also protect other proteins from heat-inactivation or reverse heat-denaturation. Some heat-shock proteins like GrpE, GroES and DnaK are involved in nucleic acid synthesis at high temperature; they may have polymerase activities or DNA repair activities for heat induced DNA breaks hence enhancing DNA stability. If acid caused similar effects to heat then heat-shock proteins might be involved in repair or in reducing the extent of acid damage.

The DnaJ gene product is located in the cell envelope and is induced by temperature shift (Neidhardt and VanBogelen, 1987). This protein has large hydrophobic regions (Zylicz et al., 1985) and may contribute to stabilising the outer membrane structure by substituting for lipopolysaccharide released by heating at high temperature, resulting in re-creation of hydrophobic bilayers which might prevent the entry of inhibitory substances thus lowering the sensitivity of exposed cells.

Several heat-shock proteins are molecular chaperones which are believed to control protein folding (Ellis and Saskia M, 1991). Such components are believed to be able to renature heat-inactivated proteins and might play a similar role for acid-denatured ones.
4.3. Effect of acid on motility and chemotaxis:

It was observed that the motility of acid-habituated and non-habituated ColV,1-K94+ organisms was adversely affected at acidic pH (see Table 21). However, the acid-habituated organisms were relatively more motile at acidic pHs than their corresponding non-habituated cells. This may indicate a higher internal pH for habituated cells at acidic pH, allowing motility to occur in a higher proportion of cells.

Since the motility of both types of organism is very sensitive to an acidic environment (Table 21), it is possible that flagellar filaments may be depolymerized at the acid pHs tested (Asakura, 1970) therefore, their motility would be sensitive to a pH range from 3 - 4 even for a very short period of time. However, the habituated cells showed relatively less susceptibility to inhibition of motility and this might be due to the fact that their viability is less sensitive to acid in contrast with non-habituated cells.

The failure of habituated cells to be repelled by acid (Fig. 19, b and d) may indicate that at acid pH, they maintain a higher pH than non-habituated ones. It is a fall in pH which induces negative chemotaxis from acid (Repaske and Adler, 1981). Accordingly, if habituated cells keep pH high, chemotaxis would not be switched on and repulsion would not occur.
4.4. Effects of acid pH on release of cellular constituents:

Leakage of 260 nm absorbing material (such as amino acids and nucleotides) was observed in the acid treated ColV,I-K94 bearing strain of E. coli. Both habituated and non-habituated organisms from exponential and stationary phase of growth showed the release of substances (see Tables 22 and 23). The acid treated non-habituated (pH 7.0 grown) organisms from logarithmic and stationary growth phase released much more material than their corresponding acid-habituated (pH 5.0 grown) cells strongly suggesting that envelopes of the non-habituated cells are more vulnerable to acid than the acid-habituated organisms. It seems likely that there is greater damage by acid to the outer and/or cytoplasmic membranes of non-habituated cells than habituated ones. It would be interesting to examine acid effects specifically on the outer membrane. This could be done by (a) studying acid-induced leakage of periplasmic proteins and (b) examining outer membrane damage by acid by following ability to plate on NA + deoxycholate (versus NA). Organisms which are viable (and plate on NA) but have damaged outer membrane let through deoxycholate and, therefore, fail to plate on NA + deoxycholate.

It has previously been demonstrated in this laboratory that the non-habituated cells of ED1829ColV,I-K94+, are much more sensitive than the habituated cells on exposure to pH 3.0 or 3.5 for short time based upon their colony forming ability on complete media (Goodson and Rowbury, 1989; Rowbury et al., 1989). Moreover, the earlier findings of Cooper and Rowbury, (1986), suggest that the presence of transfer components increases permeability of ColV,I-K94+ organisms for acid inhibitors.
These studies indicated that the outer membrane plays a key role in determining the penetration of acid inhibitors but by this notion, the outer membranes of habituated and non-habituated organisms might be equally permeable to the acid inhibitors but this could be studied as described above. It is possible that acid inhibitors only damage the cytoplasmic membrane and this causes the leakage of 260 nm absorbing material, as the most common observation after any damage to the cytoplasmic membrane is the loss of 260 and 280 nm absorbing material (Hurst, 1977). The findings, see below, on effect on respiration may also indicate cytoplasmic membrane damage (Table 20). In other experiments (Table 32) it was observed that acid may cause less DNA damage in habituated organisms than in the non-habituated cells. Therefore, it is possible that the leakage of 260 nm absorbing material from the non-habituated cells may be more than that from the habituated cells even if there is no difference in envelope properties. There may be another possibility, namely that acid-stress related proteins induced in the habituated organisms (Raja et al., 1991a) may have some protective role in preventing acid damage to the cell or in DNA repair. The gene product of DnaJ is located in the membrane. It is induced during stress and may confer some stability to the membrane of stressed organisms (Zylicz et al., 1985), reducing release of cell constituents from habituated cells.

4.5. Recovery of injured cells:

A stress may not be severe enough to destroy all bacteria present in a system but may, instead, inflict sublethal injury to a proportion of the
population. These injured cells can form colonies on low stress media but not on stressing media. However, if these injured organisms are removed from stressful environments they may revive so as to be able to form colonies on all media.

It has previously been reported by Przyblski and Witter (1979), that cells of *Escherichia coli* K-12, injured by sub-lethal acidification tend to recover in trypticase soy broth and in potassium phosphate buffer. Almost complete recovery (95%) was obtained after 120 min of incubation in 0.04 M potassium phosphate buffer of pH 8.0.

Subsequently, in a recent study, Musarrat and Ahmad (1988), observed that alkali-injured cells could recover on incubation in 0.05 M potassium phosphate buffer of pH 8.0, at 37 °C, after 2 hr. Recovery in phosphate buffer was not affected by metabolic inhibitors.

Recovery of injured ColV1-K94+ cells was observed, on incubation, in a complete medium like nutrient broth to a small extent; however, the process of recovery of the injured cells was somehow expedited in NB prepared in 0.1 M potassium phosphate buffer of pH 7.5 (see Tables 13, 14 and 15). The recovery medium used in the present study was totally supportive to growth and contained an energy source unlike the above mentioned previous two studies. It appears that phosphate buffer somehow supports the recovery of injured cells but its mechanism is not clear.
4.6. Effect of acid on respiration:

Respiration rate for acid-habituated and non-habituated ColV, I-K94+ cells was affected at acidic pH; it dropped in both types of cells (see Table 20) indicating that respiration is a pH sensitive process. However, the decrease in respiration rate of non-habituated cells was significantly greater than for the acid-habituated organisms. This observation might be correlated with the acid-sensitivity of habituated and non-habituated cells (colony forming ability on NA) which strongly suggests that former are resistant to the antibacterial activity of acid.

The respiratory system of *Escherichia coli* is associated with the cytoplasmic membrane and shows sensitivity to acid, indicated by a decrease in oxygen consumption (Table 20). This observation was also made in *Bacillus subtilis*, an obligately aerobic organism, by Sheu and Freese, in 1972. They demonstrated that the oxygen uptake rate of whole cells without any acid inhibitors, at 37 °C, was 293 nmoles/min whereas cells treated with fatty acids had reduced oxygen uptake. Ahmed and Booth (1983), reported the respiratory rate of *Escherichia coli* ranging between 160 to 210 nmol O₂ consumption (mg dry wt)⁻¹ min⁻¹ at pH 6.1.

Though fatty acids inhibited cellular growth with reduction in oxygen uptake and concentration of ATP (Sheu and Freese, 1972), yet they did not inhibit NADH oxidation by isolated membranes (Sheu et al., 1972) or the cytochrome-linked electron transport system. The reduction in oxygen uptake in whole cells could be from the deficiency of compounds, e.g., amino acids, entering the cell due to failure of active transport.
(Freese et al., 1973). It is more likely, however, that the great reduction in oxygen uptake observed plus acid (Table 20), reflects damage either to the cytoplasmic membrane or to the respiratory components attached to it. The greater resistance of habituated organisms could be due to slower penetration of $H^+$ (e.g. through the outer membrane) or to better repair of acid-damaged structures in the cytoplasmic membrane.

4.7. Effect of acid pH on synthesis of stress-related proteins:

A previous study has indicated that an alkaline but not an acid shift in external pH ($pH_0$) induces a stress related response in *Escherichia coli* and synthesis to over sixfold of *dnaK* and *groE* gene products (Taglicht et al., 1987). Subsequently two reports (Goodson and Rowbury, 1989; Rowbury et al., 1989), on acid sensitivity of pH 5.0 grown (acid-habituated) and their respective pH 7.0 grown (non-habituated) *E. coli* cells, have demonstrated that an acid-shift during growth of the organisms confers acid-resistance which might be due to induction of some stress related proteins. Also, Heyde and Portalier (1990) have shown that stress related proteins, referred to as acid-shock proteins, were induced on acid shift from pH 6.9 to 4.3 in *Escherichia coli*. Among the proteins induced by an acid shift, 4 were heat-shock proteins including GroEL, DnaK, HtpG and HtpM while 7 were new proteins which had not been described previously to be induced by any stress other than acid shift.

These different observations are perhaps associated with different experimental conditions as Taglicht et al., (1987) used acid shift from pH 8.0 to pH 6.0 for 5 to 10 min, whereas Heyde and Portalier (1990),
observed the response by dropping the pH from 6.9 to 4.3 for a period of 30 - 45 min.

However, a report from this laboratory demonstrates that stress related proteins are synthesised by late log-early stationary phase ColV, I-K94+ cells at an acid shift from pH 7.0 to pH 5.0, at 37 °C (Figure 1 of Raja et al., 1991a). The process of habituation, as observed here, is associated with protein synthesis and at least 14 proteins are induced by acid shift (Table 24 and Figure 18). This observation was subsequently confirmed by two dimensional gel electrophoresis (Figure 19 and Table 25). Among the proteins synthesised by an acid shift, several might be heat-shock proteins including Lon, GroEL and DnaJ (see Tables 24 and 25 and Figures 18 and 19) depending upon their electrophoretic mobilities. If some of these proteins produced at pH 5.0 are heat-shock proteins (hsp5), they could play a role in acid resistance since both heating at 52 °C (Neidhardt et al., 1984) and acid treatment (Sinha, 1986) cause damage to DNA. Possibly the protein A which is synthesised at pH 5.0, with an apparent molecular weight of approximately 95 kDa and clear separation from the other proteins, may be the Lon protein. This is a heat-shock protease which destroys abnormal or damaged proteins and could play this role for acid-damaged ones. Similarly induction, of DnaJ (ca. 40 kDa) which is localized in outer membrane may play a role in stabilizing the membrane during exposure to acid. GroEL may be associated with the DNA of Escherichia coli after a brief exposure to heat and, accordingly, after mild stress it may play a role in preventing subsequent DNA damage or in repair of already damaged DNA (Pellon and Gomez, 1981).
Subsequently it has been observed that some of the stress-related proteins induced at 37 °C, pH 5.0 are also synthesised at 44 °C (Table 28 and Figure 22). This may mean that some of the acid-induced proteins are heat-shock proteins. In contrast, few polypeptides are induced at pH 5.0, when the organisms are grown at 24 °C (Tables 26 and 27 and Figures 20 and 21).

Autoradiographic studies (Figures 23 and 24 and Table 29) have demonstrated that several proteins are induced in relatively much larger amounts in the first few minutes after a shift to pH 4.3 than at pH 7.0 prior to the shift. Two of these polypeptides i.e C and D with apparent molecular weights of 73 and 68 kDa respectively, might correspond to the electrophoretic migration patterns of HtpG and DnaK, the well known heat-shock proteins. This confirms that habituation is associated with induction of new stress-related proteins in relatively short periods of time (10 min) and is in accord with the earlier findings of Heyde and Portalier, (1990); Hickey and Hirshfield, (1990); Raja et al., (1991a). Confirmation that the 68 kDa protein induced at pH 4.3 is the DnaK gene product could be obtained by shifting the dnaK mutant NRK156 to pH 4.3 and examining proteins synthesised.

4.8. Effect of phosphate on the synthesis of proteins induced at acid pH:

Habituation requires protein synthesis with several proteins being induced at low pH (Raja et al., 1991a). Autoradiographic studies indicate (Table 29 and Figures 23 and 24) that several proteins are synthesised in much larger amounts in the first few minutes after a shift to pH 4.3 than
at pH 7.0 prior to the shift. Most of these induced proteins are produced at pH 4.3 + 10 mM phosphate, in reduced amount in P678-54 ompA but not at all in P678-54 (Table 30 and Figures 25 and 26). This observation may be related to the fact that phosphate prevents habituation in P678-54 but has no effect on its ompA mutant (Rowbury et al., 1992).

The outer membrane plays an important role in protecting *Escherichia coli* from extreme acidity and factors which increases its permeability, such as Ips mutations and ColV,1-K94 plasmid, enhance acid sensitivity (Cooper and Rowbury, 1986).

At low pH, organisms with normal outer membrane must maintain a pH differential between periplasm and outside which could be detected by a sensor to initiate the habituation response. Alternatively, the sensor could detect periplasmic pH (rather than a differential).

Phosphate prevents habituation at low pH most likely by competing with hydrogen ions at the PhoE pore and preventing their reaching the habituation sensor on the cytoplasmic membrane or in the periplasm. It is most likely that hydrogen ions use the PhoE porin as the major outer membrane penetration route. Polyphosphate, phosphates and citrate are the most effective inhibitors of habituation, polyphosphate being most effective among them (Rowbury et al., 1992). Such anions strongly inhibit the passage of other compounds through the PhoE pore (Overbeeke and Lugtenberg, 1982). The failure of phosphate to inhibit habituation of the P678-54 ompA mutant and to stop stress protein synthesis in this strain (Table 30 and Figure 25) also agree with such a mechanism of action for
anions, and rules out the possibility of phosphate acting directly on the sensor, because in this strain (P678-54 ompA) the PhoE pore is not the major route of hydrogen ion penetration (Rowbury et al., 1992).

If phosphate and polyphosphate stop $H^+$ from reaching the habituation sensor, they may, at suitable concentrations also stop damaging $H^+$ effects on pH 7.0 cells e.g. on the cytoplasmic membrane (and, therefore, on oxygen uptake), on leakage, on motility and on chemotaxis. It would be interesting to examine phosphate and polyphosphate effects on these parameters.

4.9. Effect of acid on DNA of habituated organisms:

The results of transformation experiments (see Table 32) suggest that the organisms grown at pH 5.0 can repair acid-damaged DNA better than those grown at pH 7.0. The greater number of transformants formed in habituated (compared with non-habituated) competent organisms when pBR 322 DNA from acid-damaged cells was used confirms that habituated organisms have enhanced ability to repair acid-damaged DNA. The repair process responsible may require the gene products of uvrA, recA and polA (Table 34).

Habituated organisms also showed less DNA damage by acid (Table 32 and Figures 28, 29 and 30). Isolated pBR 322 DNA was predominantly in the form of ccc DNA after extraction from untreated pBR 322+ cells whether they were habituated or not. In contrast, extracts from habituated cells generally contained ccc plasmid after exposure to pH 3.0 for 7 min whereas
none was observed in extracts from non-habituated cells. Traces of oc plasmid were also frequently noted when habituated p+ cells were acid-treated. If acid treatment causes single stranded (ss) nicks in the DNA, slight damage would lead to oc form but further damage (i.e. with several ss nicks per plasmid) would give rise to a plasmid form which might be repairable in vivo but which might be completely degraded by the plasmid isolation procedures used or not immediately recognizable as a plasmid DNA.

There are several possible mechanisms by which habituated cells might protect their DNA from acid-damage. Firstly, pH\textsubscript{i} might be maintained closer to neutrality at very low pH\textsubscript{o} values either by the active extrusion of protons or by the production of basic compounds to neutralize internal acidity. Alternatively, the pH\textsubscript{i} might behave similarly, with response to very low pH\textsubscript{o} values, in habituated and non-habituated cells but the former might contain high levels of DNA-binding proteins or other DNA binding components which protect the acid-susceptible regions of DNA. The previous findings (Raja et al., 1991a) of high level of a ca 63 kDa protein in the acid-habituated organisms may be significant here. The GroEL protein (molecular weight 62883) appears to associate with the DNA of Escherichia coli after a brief exposure to heat and, accordingly, after mild stress it may play a role, either in preventing subsequent DNA damage or in the repair of already damaged DNA (Pellon and Gomez, 1981).
References


