EFFECT OF COLV PLASMIDS ON THE HEAT SENSITIVITY OF *ESCHERICHIA COLI*

SUBMITTED FOR PH.D EXAMINATION BY

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Abstract

The heat sensitivity of *E. coli* K12 strains harbouring the ColV,I-K94 plasmid was examined after exposure to 52°C or 60°C. The presence of this plasmid rendered the cells more sensitive to heat, and the production of both colicin and transfer components by the $ColV^+$ cells was involved in this increased heat sensitivity. Exposure to high temperature damaged the outer and cytoplasmic membranes of both $ColV^+$ and $ColV^-$ cells with a greater effect on the $ColV^+$ strains. Heating at 60°C resulted in release of lipopolysaccharide into the heating medium. It reduced the interactions between some of the proteins of the cell envelope and other constituents of the envelope causing weakening of the outer membrane structure as observed by analysis of the proteins of the cell envelopes of heated and unheated strains. Also, the permeability of the outer membrane of $ColV^-$ and $ColV^+$ cells to hydrophobic compounds was increased in heated strains compared with the unheated ones. Heating also caused leakage of internal constituents of p^- and ColV plasmid-bearing cells, and the amount released from $ColV^+$ cells was twice that released from the p^- cells. In addition to these effects, damage to other cytoplasmic compounds and DNA damage occur in heated cells. The thermal sensitivity of a *polA* strain was much higher than that of the parental strain, but the presence of the ColV,I-K94 plasmid in

this polA strain did not enhance its heat sensitivity.

Some of the conditions influencing the thermal resistance of ColV$^+$ cells were examined. Growth of these cells in magnesium enriched medium increased their heat resistance and greatly reduced the difference between the heat sensitivity of the p$^-$ and the ColV$^+$ cells. Exposure to a gradual rise in temperature from 34 to 50^{o}C before heating at 60^{o}C resulted in marked increase in the percentage of survival of the heated ED1829 and its ColV$^+$ derivative, and this increase was much greater in the ColV plasmid-bearing cells.

The incidence and properties of wild type ColV$^+$ strains isolated from samples obtained from chicken processing plants were studied. A high proportion of these strains was resistant to cotrimoxazole and tetracycline. About 30% and 40% of the strains were resistant to chloramphenicol and ampicillin, respectively; and multiple antibiotic resistance was frequently observed in the isolates. Forty six percent of the E. coli strains produced colicin(s), and 31% of these produced colicin V and Ia and harboured plasmids resembling the ColV,I-K94 plasmid in mobility on agarose gels. Three of the five ColV$^+$ strains produced VmpA protein.

Contents

List of Tables

List of Figures

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1. Introduction

1.1. Properties of ColV plasmids:

1.1.1. Bacteriocins and colicins:

Bacteriocins are proteinaceous compounds produced by certain strains of bacteria and kill only bacterial cells which are closely related to the strains that produced them (Hardy 1978). These are mainly produced by Gram-negative bacteria but some Gram-positive bacteria are reported to produce bacteriocins, such as staphylococcins and streptococcins produced by some strains of Staphylococcus and Streptococcus, respectively (Reeves 1972). The colicins, the most extensively studied group of bacteriocins, are produced by some strains of Escherichia coli and Shigella sonnei.

1.1.2. Colicinogenic plasmids:

Plasmids are extrachromosomal molecules of DNA that are maintained separate from the chromosome. They are composed of covalently closed circles (CCC) of double stranded DNA. In the cell, plasmids are present as CCC DNA, sometimes associated with the open circular form.

Plasmids can determine a wide range of functions, such as resistance to antibiotics and metals, ability to ferment certain sugars,production of enterotoxins,production of co lonization adhesins(e.g.K88,K99),or synthesis of colicins(Jawetz

et al. 1984, Hardy 1986). The latter are termed colicinogenic plasmids and are given a name which specifies the colicin type, such as B, E or I, and the name of the first strain which was shown to produce the colicin. Thus, ColV-K30 is a type V colicin plasmid which was originally detected in E. coli strain K30.

1.1.3. Types of Col plasmids:

The colicin factors can be divided into two major groups according to their molecular weight (Hardy et al. 1973). Group I: are small plasmids with average molecular weight of 5 X 10^6, non-conjugative but can be transferred into other bacteria by self transmissible plasmids. They show relaxed replication where 10-50 copies of the plasmid are observed per chromosome equivalent. ColE, ColK and ColA factors are examples of this group. At least half of the amount of colicin produced by plasmids of this group is released into the medium, causing the appearance of distinct lacunae around bacterial colonies overlaid with sensitive indicator strains. Group II: large plasmids, their molecular weight ranges from ca. 40-ca. 100 X 10^6. They are usually conjugative and show stringent replication where only 1 or 2 copies are present per chromosome equivalent. ColB, I and V are members of this group of Col factors. Almost all colicin , produced by this group, is cell bound, causing the production of barely visible lacunae upon colicin testing.

Table 1: Properties of representative conjugative and non-conjugative plasmids

Plasmid	Molecular weight	Copy No.	Incompatibility group	Self transferability	Antibiotic resistance	Colicin production
F	63×10^6	1-2	FI	+ (derepressed)	-	-
R100	58×10^6	1-2	FII	+ (repressed)	Te,C,Fa Hg,S,Su	-
ColV,I-K94	85×10^6	1-2	FI	+ (derepressed)	-	Colicins V&Ia
ColB-K98	70×10^6	1-2	FIII	+ (repressed)	-	Colicins B&M
ColE1-K30	4.3×10^6	~15	N.D.	-	-	Colicin E1

N.D.: not determined

Te: tetracycline, C: chloramphenicol, Fa: fusidic acid, Hg: mercuric ions, S: streptomycin, Su: sulphonamide.

The main Col factors of this group can be further divided into F-like and I-like plasmids based on the nature of the sex pilus produced (Hardy 1975). F-like plasmids include ColB- K98, ColV- K30 and ColV, I- K94 factors, which code for pili that resemble the F-(fertility) pilus. I-like plasmids include Colb-P9 and determine I-like pili. F- and I- pili are distinguishable immunologically and by adsorption of specific phages (Smith 1982). Table 1 demonstrates some of the properties of conjugative and non-conjugative plasmids.

1.1.4. Homology between ColV plasmid and F:

ColV, I-K94 is a large conjugative F-like plasmid that encodes for production of colicins V and Ia and their immunity components. Electron microscopy studies have revealed that ColV, I-K94 and F plasmids share extensive polynucleotide sequence homology, particularly in the *tra* region, *inc D* region (47.6 F - 49.4 F), and the secondary replication region of F (32.6 F - 35.4 F) (Lane 1981). The *tra* region of F and ColV, I-K94 is completely homologous except in the genes implicated in surface exclusion (*traS* and *traT*). This confirms the observation that ColV, I-K94 belongs to a surface exclusion group distinct from that of F (Willetts and Maule 1973). On the other hand, ColV, I-K94 does not show homology with the primary replication region of F (42.5 F - 46.7 F) (Weber *et al*. 1984).

Weber and Palchaudhuri (1985) were able to construct a

physical and genetic map of the ColV, I-K94 plasmid using restriction endonuclease analysis and electron microscope heteroduplex studies of ColV, I-K94 and F plasmids. Figure 1 represents the map of pWS12, a derivative of ColV, I-K94 that has all ColV plasmid properties. It is 134 kb, in length, of which 3.2 kb are contributed by transposon Tn 903. The transfer region (60 V - 93 V), replication region, and positions of the inverted repeats X_1, X_2, X_3 are indicated.

ColV, I-K94 has been shown to be a naturally occurring cointegrate plasmid possessing genetic features of IncF II as well as IncF I plasmids. It contains Rep 1, which is homologous to the RepA replicon of IncF II plasmids, and Rep 2, which is homologous to the secondary replication region of F and other IncF I plasmids (Weber et al. 1984).

1.1.5. ColV plasmid incompatibility:

Incompatibility is the inability of two plasmids to coexist stably in the cell. The plasmid which is not maintained due to incompatibility is not destroyed by restriction enzymes, but simply fails to replicate or segregate into daughter cells. This phenomenon occurs between closely related plasmids that possess either a shared segregation (partitioning) mechanism or a shared replication control system (Novick and Hoppensteadt 1978). Thus, incompatible plasmids either compete for the attachment site on the host membrane or produce the same repressor that inhibits

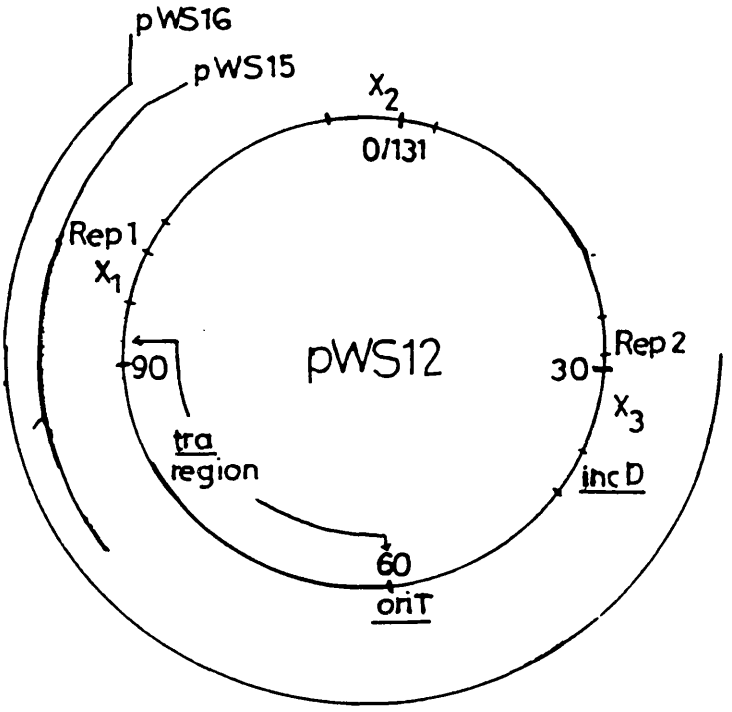


Fig 1: Map of the ColV,I-K94::Tn903 derivative pWS12. Coordinates are in kilobases. Rep 1 and Rep 2: replication regions, incD: incompatibility locus, oriT: origin of transfer, x_1,x_2 and x_3: stem-loop structures(From Weber et al. 1984 & Weber and Palchaudhuri 1985).

initiation of replication.

F and ColV, I-K94 plasmids belong to the IncF I incompatibility group. They share a common 3-kilobase region (39 V - 42 V) (see section 1.1.4 and fig 1) that includes incD locus and a region essential for partitioning of new copies of the plasmid into daughter cells during cell division.

Although ColV, I-K94 has a RepA-like (Rep 1) replication region, it does not behave as an IncF II plasmid. The masking of IncF II expression in ColV can be explained by the cointegrate nature of this plasmid. Its Rep 1 replication region is silenced by the replication control repressors from the IncF II plasmid with which they coexist. In this situation, ColV plasmid can replicate using the Rep 2 replication region, thus incompatibility between the IncFII plasmids and ColV is avoided. Experimental evidence to this conclusion was presented by Weber et al. (1984). They reported that removal of Rep 2 in the mini ColV plasmid pWS16 resulted in acquisition of IncFII incompatibility.

1.1.6. Plasmid replication:

Replication is initiated at a specific site on the DNA molecule designated origin of vegetative replication (oriV). This site is defined as the position of the first deoxyribonucleotide added by DNA polymerase to the RNA primer. The replication regions of ColV plasmid are shown in figure 1.

ColV, I-K94 uses only Rep 1 for replication because removal of Rep2 as in pWS16 has no effect on stability of the plasmid. Furthermore, 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). On the other hand, ColV can use its alternate replicon under certain conditions.

Replication begins with endonucleolytic cleavage at the $oriV_1$ (origin of vegetative replication) site to allow unwinding of the supercoiled DNA. Synthesis of the new strands proceeds either unidirectionally or bidirectionally depending on the type of plasmid. Some plasmids, such as ColE1, replicate unidirectionally where the new strand grows in one direction around the circular molecule so that the replication fork terminates at the origin. Other plasmids, such as F, replicate bidirectionally. The new strand proceeds in two directions until the terminus is reached at 180° from the origin (Hardy 1986). The direction of replication of ColV,I-K94 is not known.

Gene products of both host and plasmid are required for plasmid replication. Scott (1984) reported that the host functions dnaB, dnaC, dnaG and dnaE are needed for replication of the IncFII group plasmids. The E. coli dnaA function and DNA polymerase I are not required. Large plasmids can replicate normally in dnaA mutants of E. coli which fail to

initiate chromosome replication. These plasmids produce their own DnaA gene product because they can integrate into the chromosome of dnaA strains, correct the initiation lesion and allow normal chromosome replication. In addition to the above mentioned functions, RNA synthesis is needed during the initiation of plasmid replication. It acts to prime DNA synthesis of the new strand (Rowbury 1977).

Elongation of the DNA strand is by a discontinuous mechanism in which Okazaki fragments are formed. Short RNA segments at the 5' termini are inserted through the action of DnaG gene product (RNA polymerase). Deoxyribonucleotides are added to the RNA primers by DNA polymerase III (product of dnaE gene) in replicating large plasmids, or by DNA polymerase I (product of polA gene) when replicating small plasmids. Then, primer removal and ligation of the termini of the newly synthesized strand take place. The plasmids are segregated into daughter cells by the aid of par gene products. These proteins specifically interact with DNA and with sites in the membrane such that the attached plasmids are segregated into daughter cells (Hardy 1986).

1.1.7. Conjugal transfer of plasmids:

Many plasmids are conjugative and able to transfer themselves and other non-conjugative plasmids from one bacterium to another. A large proportion (about one-third in F) of plasmid DNA constitutes the tra region which codes for

proteins essential for transfer (fig 2). The functions of these gene products will be discussed in the following sections. Most of the studies were done on F. However, the tra regions of F and ColV plasmids are homologous (see section 1.1.4) and almost all the events in the transfer process will presumably, therefore, apply to both plasmids.

a. Sex pili:

These structures are filamentous appendages present on the surface of transfer-proficient strains (e.g. strains containing F or other derepressed conjugative plasmids) but not on plasmid-free strains or strains carrying repressed conjugative plasmids (Datta et al. 1966). The proximal tra genes, e.g. traA, -L, -E, -K, -B, -P, -V, -C, -U, -N, -F, -Q, -H, are responsible for pilus synthesis and assembly. Sex pili are composed of sub-units called pilin. The plasmid traA gene encodes propilin, a precursor protein, which is, apparently, cleaved to pilin by the product of the traQ gene (Hardy 1986).

The synthesis of sex pili is affected by environmental conditions. It increases during the exponential phase of cell growth, reaches a maximum during the late exponential phase and decreases after the culture enters the stationary phase (Tomoeda et al. 1975). Also, starvation of cells for certain amino acids causes loss of sex pili.

Sex pili have an essential role in conjugation. Several observations confirmed that pili are involved in the transfer

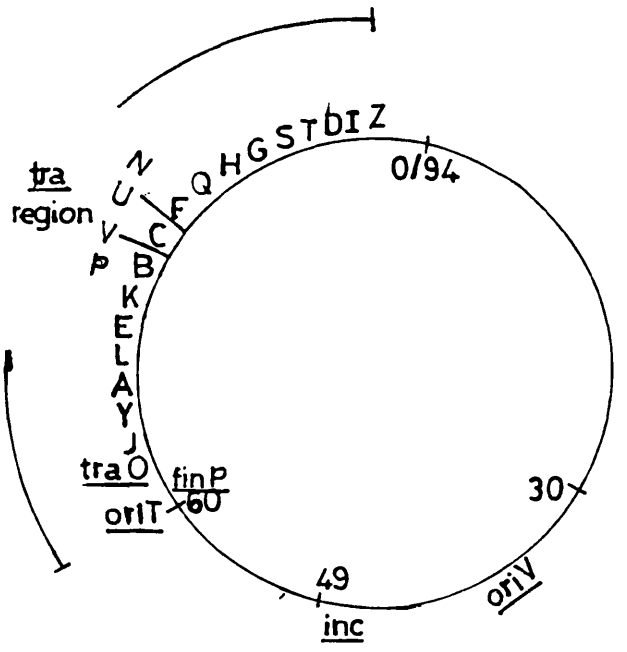


Fig 2: The map of F plasmid. oriT: origin of transfer, inc: incompatibility region, oriV: origin of vegetative replication. Coordinates are in kilobases.

process. Cells containing conjugative plasmids are unable to transfer the plasmid if they fail to produce sex pili either because of a mutation in the tra gene responsible for pili formation or due to repression of the tra operon. Removal of sex pili by shearing or blending renders the cells unable to act as donors until new pili are formed (Manning and Achtman 1979). Treatment of donor cells with specific pilus antibodies or with male - specific phages inhibits conjugation.

Although the above observations confirmed that sex pili play an important role in the conjugal transfer of plasmids, their exact function is not yet fully understood. Several observations indicate that they can act as a conjugation tube, to provide a hollow tube through which DNA passes from donor to recipient. Other experiments show that sex pili can act to bring donor and recipient into wall to wall contact through retraction of pilus after the pilus tip has become attached to the recipient (Manning and Achtman 1979). However, a combination of both functions may occur in nature. Pili may retract, then form a short channel through which DNA passes.

b. Surface exclusion:

This term describes the phenomenon that cells carrying a conjugative plasmid are reduced in their ability to act as recipients with other donor cells carrying identical or closely related plasmids. Genetic analyses have revealed that

products of traS and traT genes are responsible for this phenomenon. It results from the additive effect of the two gene products since mutation of either gene partially abolishes surface exclusion. The traT gene product is responsible for the reduced ability to form stable donor - recipient aggregates, thus leading to reduced DNA transfer within the cell population. The TraT protein may bind to a specific receptor needed for stable mating aggregate formation (Achtman et al. 1977). The product of the traT gene is localized in the outer membrane of bacteria carrying transmissible plasmids. It has a molecular weight of 25,000 daltons and it is present at about 29,000 copies per cell.

The traS gene product has a minor effect in inhibiting formation of mating aggregates but reduces DNA transfer in another unknown way. It is present in the inner membrane of the bacterial cell envelope and has a molecular weight of 18,000 daltons (Manning and Achtman 1979).

c. Transfer of plasmid DNA:

Transfer of DNA is initiated at a specific site on the plasmid termed origin of transfer (oriT). A schematic representation of the transfer process is demonstrated in figure 3. A nick is introduced at this site in a specific strand, the heavier DNA strand in F. Nicking at oriT requires the products of traY, traZ and traJ genes. The role of TraJ protein is indirect since it has a regulatory role in

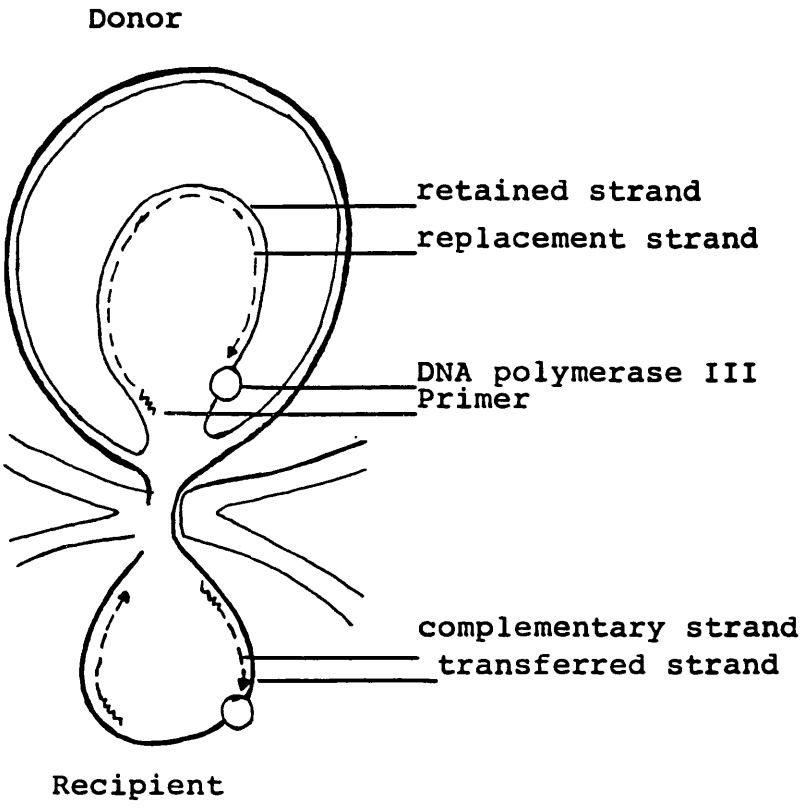


Fig 3:Diagram of the conjugative transfer of F(Willetts & Wilkins 1984)

transcription of the whole tra operon. The traY and -Z gene products have endonuclease activity (Willetts and Wilkins 1984). oriT sequences are highly specific. It was observed that they differ among conjugative plasmids of the same incompatibility group and there is a specificity of interaction between these sequences and the products of traY and -Z genes. oriT sequences differ among non-conjugative plasmids, and these also differ from oriT sequences of conjugative plasmids able to mobilize them (Willetts and Wilkins 1984). Accordingly, the traYZ endonuclease is highly specific. For example, the traYZ gene product of F cannot function in transfer of the non-conjugative plasmid ColE1. The mobilization genes of ColE1 may provide the necessary nicking function.

After nicking at oriT, a single DNA strand is transferred into the recipient cell with the tra region transferred last. The two DNA strands are unwound by DNA helicase I, the product of the traI gene, to allow separation of the duplex and transfer of the single stranded DNA. This enzyme is located in the cytoplasm and possesses a DNA - dependent ATPase activity. It binds near the 5' terminus of the nicked DNA strand and unwinds it in the 3' direction using the energy of ATP hydrolysis (Abdel-Monem et al. 1983). Further investigations have revealed that interaction of this enzyme with DNA is specific, the F traI product for example cannot complement mutants of the closely related IncFII plasmids.

Another protein is required to bind to the single stranded DNA and aid the unwinding process. It was observed that F and other conjugative plasmids specify single stranded DNA binding proteins. They are encoded by a gene (denoted ssf) located in a segment of F that is outside the tra region (Kolodkin et al. 1983).

Transfer of a single strand of plasmid DNA is associated with synthesis of a replacement strand in the donor cell and of a complementary strand in the recipient. As observed in DNA replication, RNA primers are synthesized, then deoxyribonucleotides are added to these primers by the action of the host DNA polymerase III. RNA primers are generated by plasmid DNA primases. Existence of these primases was suggested by the observation that some plasmids (e.g. plasmids belonging to IncI_{α} group) can partially suppress the effect of dnaG mutations in E. coli. In contrast to vegetative plasmid replication which was not affected in primase - deficient mutants, primase - defective strains showed about 80% deficiency in conjugative DNA synthesis (Chatfield et al. 1982).

Synthesis of complementary strand in the recipient does not involve expression of plasmid genes in the recipient cell, and the required plasmid - encoded products are supplied by the donor cell (Willetts and Wilkins 1984). Synthesis of RNA primers for complementary strand formation

in the recipient is mediated by primase supplied by the donor.

After synthesis of complementary strands in both donor and recipient cells, ligation of 5' and 3' termini of the DNA strands and formation of covalently closed circular DNA molecules take place. Upon nicking at oriT, the 5' terminus is linked to a protein, and after completion of DNA transfer this protein recognizes the 3' terminus and ligates it to the 5' end. It was reported that this protein is the traYZ gene product in F transfer (Everett and Willetts 1980).

d. Control of the transfer properties:

The majority of conjugative plasmids are repressed and cannot transfer themselves into recipient cells. However, some plasmids (e.g. F, ColV) are naturally derepressed and express their transfer properties constitutively. Many repressed plasmids are not only able to reduce the transfer properties of themselves but also repress the transfer of F in F^+ strains. These plasmids are termed fin^+ (fertility inhibition positive).

In general, regulation of the tra operon of conjugative plasmids is brought about by a repressor which acts at an operator to inhibit transcription of the tra operon. It was observed that fin^+ plasmids specify repressors which repress their own tra operon and act in trans to repress the F tra operon (Hardy 1986). fin^+ plasmids possess

two genes, *finO* and *finP*, that act together at an operator (*traO*). The *finP* gene product is more specific in its effects than the product of *finO* gene. Thus, the *finP* gene product of a repressed plasmid (e.g. R 100) does not act on F nor vice versa. The FinO product of fin^+ plasmid interacts with FinP product of F or with its own FinP product to stop transcription of *traJ* gene which codes for a product (J) required to switch on transcription of the *tra* operon (fig 4). Using this hypothesis, F is described as a naturally occurring derepressed plasmid that does not code for an active repressor but has an operator which can be acted upon by repressors specified by fin^+ plasmids. Therefore, F is characterized as $finO^-$, $finP^+$, $traO^+$. FinO product is absent, leading to continuous production of TraJ product and expression of the transfer properties.

Repression of plasmid transfer does not become effective immediately after entry of a plasmid into the recipient. The FinP product is slowly synthesized by the newly acquired plasmid, allowing transfer and rapid spread of the plasmid among bacterial cells. The high frequency of transfer is observed for a few generations before the conjugative ability of the plasmid become repressed.

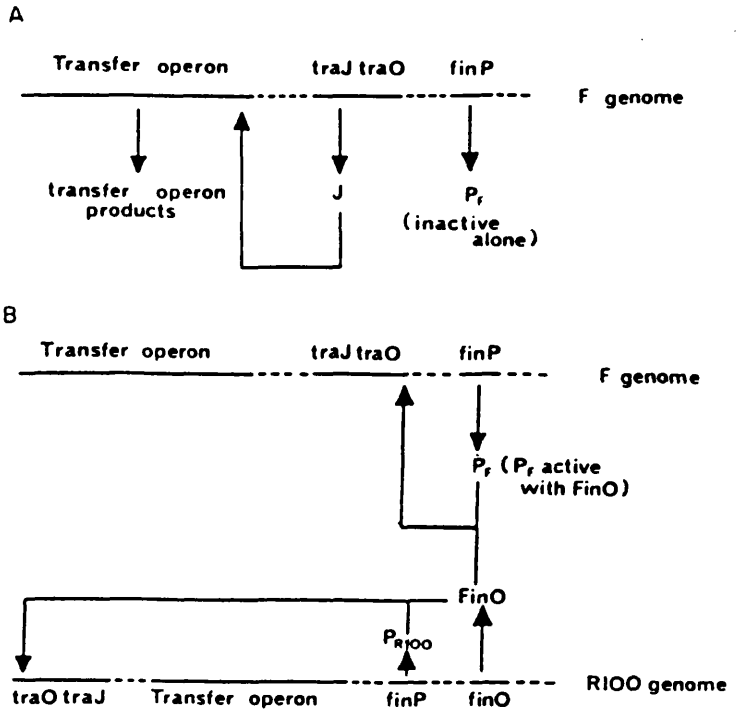


Fig 4: Regulation of the transfer properties of conjugative plasmids . A. part of the F plasmid. B. repression of F transfer by fin$^+$ plasmid(R100) (Rowbury 1977) .

1.2. Cell envelope of Gram-negative bacteria: components and characteristics

The term "cell envelope" refers to the layers that surround the cytoplasm. It is a complex structure consisting of three layers, the cytoplasmic membrane (inner membrane), the peptidoglycan in the periplasmic space, and the outer membrane. These layers are discussed in the following sections, with detailed description of the outer membrane structure and properties.

1.2.1. Cytoplasmic membrane:

It is composed of phospholipids and proteins in about equal amounts. The proteins are embedded in a phospholipid bilayer forming a typical "unit membrane". Electron micrographs of thin sections of E. coli showed a double "unit membrane" structure surrounding the cell corresponding to the cytoplasmic and outer membranes (Jawetz et al. 1984).

The cytoplasmic membrane has diverse functions. It possesses specific proteins involved in transport of solutes across the membrane, the components involved in electron transport and oxidative phosphorylation, and enzymes which function in the biosynthesis and assembly of various cell envelope components (Salton 1978) together with components involved in the export and secretion of proteins.

The passage of substances through the cytoplasmic membrane is usually achieved by three different processes. These include

group translocation, facilitated diffusion, and active transport. In group translocation, chemical modification of the transported molecule takes place. The best known example of group translocation is the phosphotransferase system for sugar transport. A carrier protein is first phosphorylated in the cytoplasm, then this protein binds the sugar at the exterior face of the cytoplasmic membrane and transports it into the cytoplasm releasing it as sugar-phosphate. On the other hand, facilitated diffusion does not involve modification of the solutes. In this process, transport occurs downhill from the more concentrated medium to the cytoplasm, using specific carrier proteins located in the cytoplasmic membrane. In active transport, specific proteins are also involved in carrying solutes across the membrane. During this process accumulation of nutrients takes place against a concentration gradient. Energy is required and is obtained by ATP hydrolysis through the membrane-bound ATPase (Rogers et al. 1980).

The cytoplasmic membrane contains the cytochromes and other electron transport carriers of the respiratory chain. Pairs of electrons are transported from the ultimate electron donor (NADH) and passed sequentially from one electron carrier to another until they reach the ultimate electron acceptor (O_2). This electron transport is coupled to ATP generation, and the free energy change accompanying passage of a pair of electrons from NADH to O_2 is sufficient to

generate three molecules of ATP from ADP and Pi.

The cytoplasmic membrane possesses biosynthetic functions. It has been reported that the reactions involved in phospholipid biosynthesis are located in this membrane. Moreover, the cytoplasmic membrane is the site of the carrier lipids on which the subunits of cell wall components are assembled. During synthesis of peptidoglycan, N-acetylmuramic acid-pentapeptide is attached to the carrier and receives a molecule of N-acetylglucosamine from UDP to form the disaccharide subunit of peptidoglycan. Then the oligomeric intermediate is transferred to the growing end of the peptidoglycan in the cell wall. Similarly, in lipopolysaccharide synthesis the subunits of O-chain polysaccharide are assembled on the lipid carrier in the cytoplasmic membrane, then transferred to the lipid A-core moiety (Hammond et al. 1984).

1.2.2. Peptidoglycan layer:

Peptidoglycan comprises 10-20% of the weight of the wall of Gram-negative bacteria. It consists of a network of linear amino sugar chains cross-linked by peptide linkages. The linear polysaccharide chain is composed of alternating residues of N-acetylglucosamine and N-acetylmuramic acid joined by a 1,4-β glycosidic linkage. A chain of four amino acids is attached to each N-acetylmuramic acid residue (Fig 5). In most Gram-negative bacteria, the polysaccharide chains are

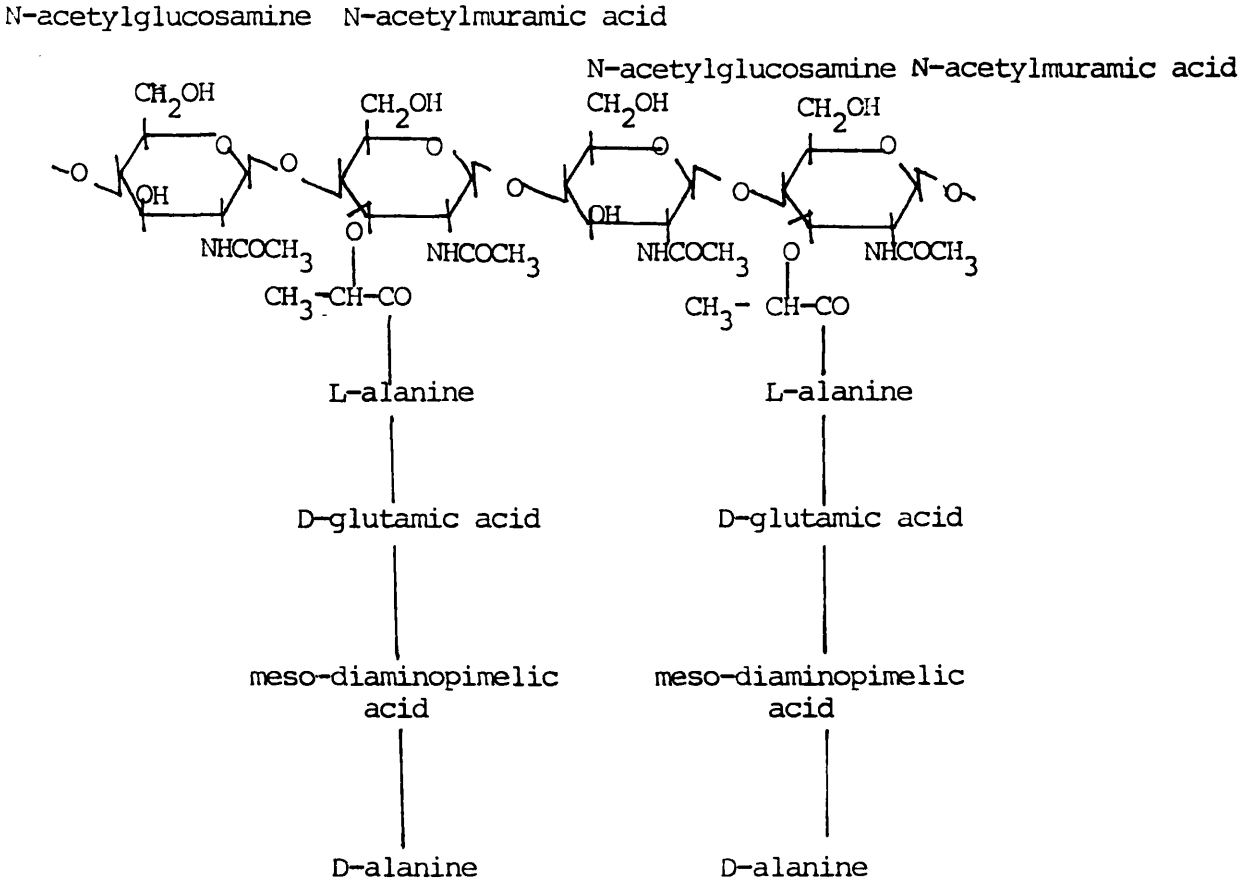


Fig 5 : Structure of a segment of peptidoglycan. Chemical composition of the building blocks (disaccharides) is shown for E.coli.

cross-linked by a direct peptide linkage between the carboxyl group of the fourth amino acid on one chain and the amino group of the third amino acid on an adjacent chain (Rogers et al. 1980). This unique structure of peptidoglycan is responsible for maintaining the shape and integrity of the cell. The cytoplasmic membrane has little intrinsic strength, and the rigidity of peptidoglycan enables the cell to withstand the high osmotic pressure of the cytoplasm. Degradation of peptidoglycan (e.g. by lysozyme) results in lysis of the cell as a consequence of swelling caused by the flow of water into the cytoplasm; this demonstrates the role of this layer in maintaining the integrity of bacterial cells.

1.2.3. Periplasmic space:

It is located between the cytoplasmic and outer membranes. The space contains a unique series of proteins and oligosaccharides. According to their functions, three classes of periplasmic proteins are distinguished:

Detoxifying enzymes: involved in degradation or modification of the antimicrobial agent in such a way as to render the agent harmless to the bacterial cell. β-lactamase which degrades penicillin is a common periplasmic protein in Gram-negative bacteria. Similarly, enzymes capable of inactivating aminoglycoside antibiotics (e.g. kanamycin, gentamicin) by phosphorylation or acetylation are present in the periplasmic space of the antibiotic resistant strains.

Scavenging enzymes: involved in degradation of compounds which are too large or highly charged to pass through cytoplasmic membrane into a form amenable for translocation across the membrane. Alkaline phosphatase and 5'-nucleotidase belong to this class of periplasmic proteins (Hammond et al. 1984).

Nutrient-binding proteins: have high affinity for solutes such as sugars, amino acids or ions. Sulphate and phosphate-binding proteins are single polypeptides which bear binding sites for these ions. These proteins are essential for transport of the ions and are found to be important components of the transport system of the specific ion (Hammond et al. 1984). A wide variety of amino acids and sugars (e.g. maltose, arabinose, ribose) also possess specific periplasmic binding proteins. These proteins serve as the first step in the specific transport pathways. Solutes able to penetrate the outer membrane are rapidly bound by the periplasmic binding proteins. This lowers the free solute concentration in the periplasmic space, facilitating further solute diffusion. Furthermore, direct interaction of the periplasmic binding proteins with outer membrane porins has been observed in uptake of some solutes. Penetration of maltose and maltodextrins through the cell envelope involves cooperation of the periplasmic binding protein with the LamB porin. Heuzenroeder and Reeves (1980) reported that there is a physical association between the LamB protein and maltose

binding protein such that the binding protein is readily available for the incoming maltose. The maltose-binding protein, upon binding maltose, would be dissociated from the LamB porin due to conformational changes, thus making maltose available to the permease in the cytoplasmic membrane. Also, binding proteins for thiamin and vitamin B12 were purified and found to be components of the specific transport system of these solutes.

Membrane-derived oligosaccharides are also present in the periplasmic space. These are composed of glucose as the sole sugar. Some of the glucose residues are substituted with glycerolphosphate and phosphatidylethanolamine which are derived from the polar head groups of membrane phospholipids (Kennedy 1982), hence the name membrane-derived oligosaccharide. There is some evidence (Hobot et al. 1984) that the peptidoglycan fills the periplasmic space as a gel, with large pores in which periplasmic constituents are found.

1.2.4. Outer membrane:

The outer membrane is an important part of the envelope of Gram-negative bacteria, present at the exterior of the peptidoglycan layer. It constitutes a barrier making the surface of Gram-negative bacteria less permeable than that of Gram-positive organisms to a variety of molecules (such as lysozyme and hydrophobic antibiotics). Also, in enteric Gram-negative bacteria, the outer membrane acts as an effective

barrier protecting cells from the detergent action of bile salts.

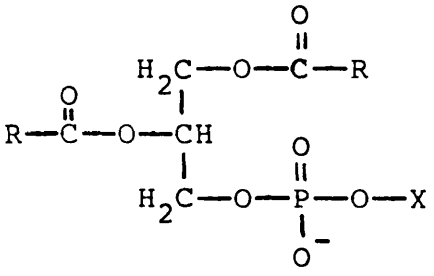
E. coli and other Enterobacteriaceae can survive in a wide range of environments, including the alimentary tract of animals and humans, surface water and food. Moreover, specific types of these bacteria are able to survive in the blood stream, cerebrospinal fluid or urinary tract. Therefore, the outer membrane of these bacteria has developed efficient systems to take up nutrients which vary both qualitatively and quantitatively from one habitat to another (see next sections).

Analysis of the outer membrane reveals that it consists of 3 major components; lipopolysaccharide, proteins and phospholipids. It has been reported that there are 10^5 molecules of lipopolysaccharide, 10^5 molecules of protein and 10^6 molecules of phospholipid in 1 μm^2 of outer membrane (Braun 1978). The structure of these constituents is discussed in the following sections.

a. Phospholipids:

All phospholipids of E. coli are located in the cell envelope. The outer membrane is enriched in phosphatidylethanolamine whereas cytoplasmic membrane is enriched in the other two major species, phosphatidylglycerol and diphosphatidylglycerol. The former type may form stable bilayers with lipopolysaccharide, thus causing its enrichment

A.



B. Phosphatidylethanolamine X: $-CH_2-CH_2-NH_2$

Phosphatidylglycerol X: $-CH_2-CH(OH)-CH_2OH$

Diphosphatidylglycerol

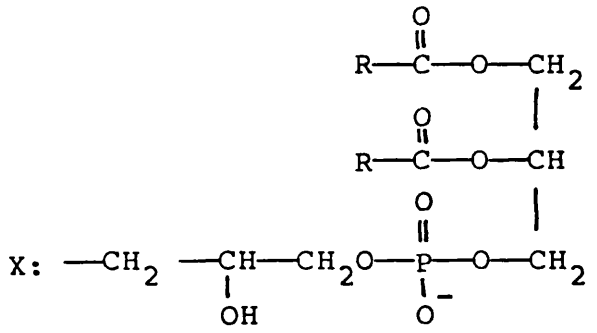


Fig 6 : A. General structure of phospholipids, R: fatty acid chain.

B. Structure of the major types of phospholipids.

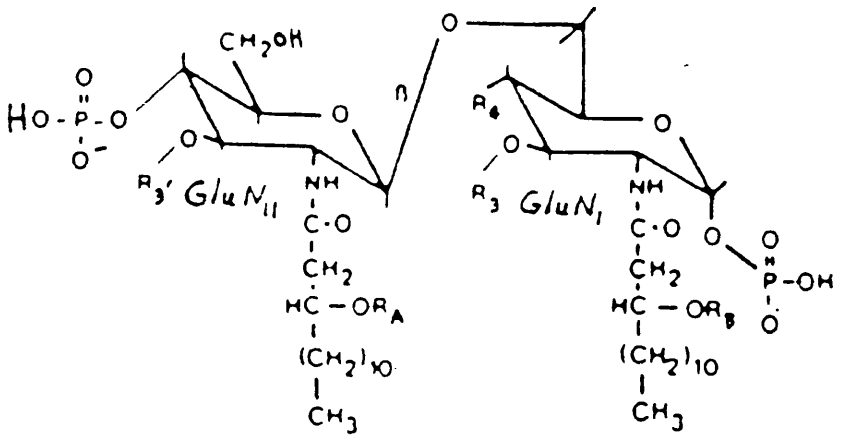
in the outer membrane. The phospholipids of outer membrane(Fig 6) have slightly higher content of saturated fatty acids in contrast to those of the cytoplasmic membrane which are enriched in unsaturated and cyclopropane fatty acids. This arrangement is independent of changes in the fatty acid composition by altered growth conditions (Cronan 1979).

b. Lipopolysaccharide:

It is an amphipathic molecule with a hydrophobic part, lipid A, and a hydrophilic polysaccharide chain. Lipid A region is integrated into the outer membrane bilayer, whereas the core region consisting of a short carbohydrate chain and the longer carbohydrate polymer, O-side chain, are extending outwards. Among Enterobacteriaceae, the structure of lipid A-core region is highly conserved. Fig 7 represents the structure of lipopolysaccharide in E. coli K12 strain. Lipid A is composed of a β-(1→6) linked glucosamine disaccharide which carries phosphate residues in positions 1 and 4'. Six or seven fatty acyl chains are attached to the disaccharide, two of which are amide-bound (Fig 7). In contrast to phospholipids, all of the fatty acids are saturated and some are present as hydroxy-fatty acids (Nikaido and Vaara 1985).

The core region contains the unique sugars 3-deoxy-D-manno-octulosonic acid (KDO) and heptose, in addition to other more common sugars. KDO region is indispensible since its

A



B

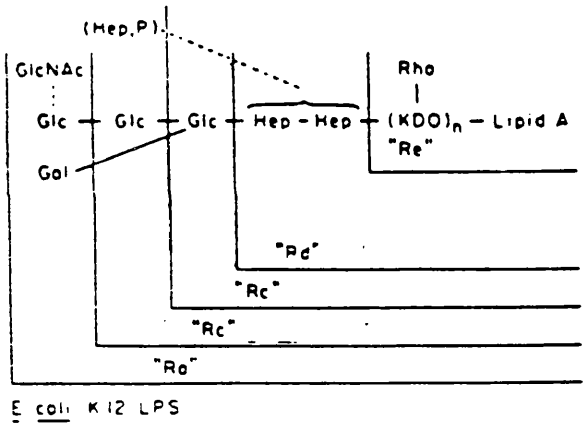


Fig 7: Chemical composition of lipopolysaccharide. A. Structure of the glucosamine disaccharide backbone of lipid A region. R_A, R_B, R_3, $R_{3'}$, R_4: fatty acid chains. B. Stucture of the lipopoly-saccharide of E.coli k12. R_a through R_e refer to the chemotypes of the mutant LPS produced. GlcNAc: N-acetyl-glucosamine, Glc: glucose, Gal: galactose, Hep: heptose, P: phosphate, Rha: rhamnose.

absence was found to be lethal to the cell. Mutants defective in the synthesis of other regions of LPS have been isolated, usually by selection for resistance to a certain bacteriophage or to the antibiotic polymyxin. Ra mutants are defective in the biosynthesis of O-antigen, and Re mutants lack all heptose residues (Fig 7). Other types of mutants defective in synthesis of various sugars of the core region are also identified in Fig. 7.

The O-side chain polysaccharide consists of repeating oligosaccharide units. The structure of these units is subject to great diversity which accounts for the large number of O-serotypes among single species. The O-antigen is not present in all strains; it is lacking in the E. coli laboratory strains K-12, B and C (Prehm et al. 1976).

c. Proteins:

The outer membrane contains a relatively limited number of proteins compared with the cytoplasmic membrane. The protein pattern of the outer membrane usually consists of a few "major" proteins. These include the porins, the OmpA protein and the lipoprotein. However, other types of proteins can be detected in appreciable amounts if bacterial cells are grown under certain environmental conditions. The organisation of the proteins and other components of the outer membrane is represented in Fig 8.

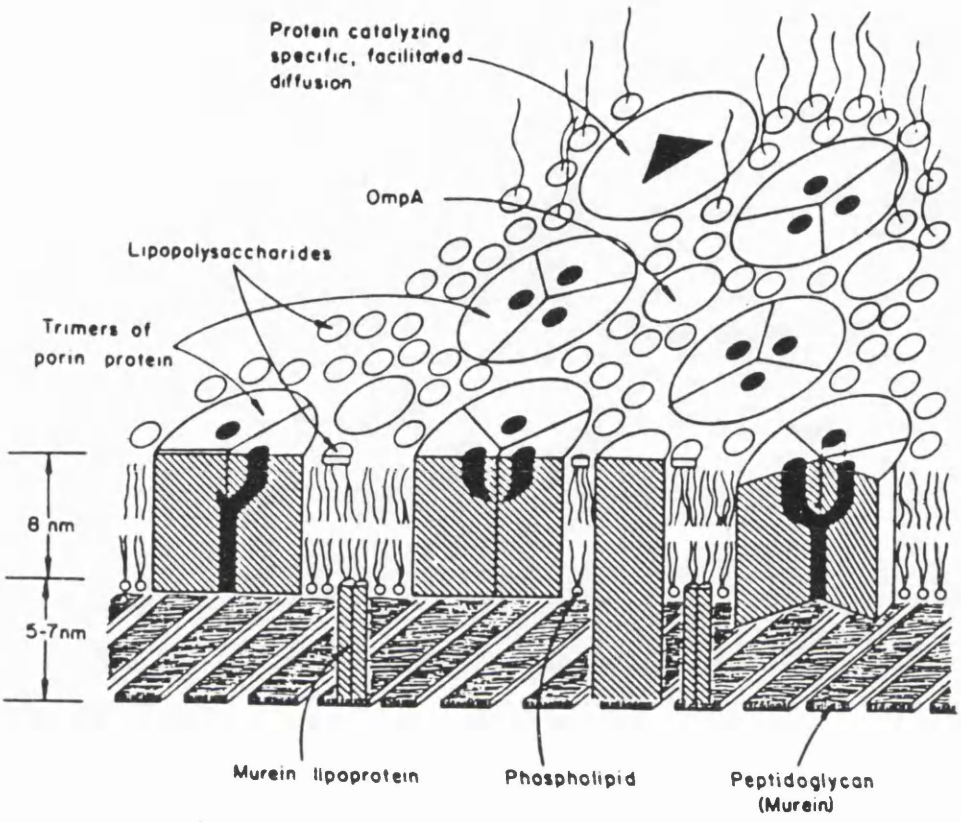


Fig 8: Organisation of the components of the outer membrane.

i. Lipoprotein:

It is a small protein (7.2 Kd), composed of 58 amino acid residues in which the N-terminal residue is covalently linked to a lipid moiety. Lipoprotein is the most abundant protein in the bacterial cell, where its estimated number of copies per cell is $\sim 7.5 \times 10^5$. One-third of these molecules is covalently bound to the peptidoglycan layer, whereas the rest are in the free form (Braun and Hantke 1974).

Isolation of lpo mutants showed that lipoprotein is not essential for growth and division of the bacterial cell. However, these mutants had increased production of blebs and vesicles, increased sensitivity to EDTA and leakage of periplasmic enzymes (Suzuki et al. 1978). These observations demonstrated the important role of lipoprotein in stabilisation of the outer membrane by anchoring it to the peptidoglycan layer.

Lipoprotein is not exposed on the cell surface, since it does not function as a receptor for any known phage and does not interact with lipoprotein-specific antibodies in wild type cells (Braun 1978).

ii. OmpA protein:

The OmpA protein, like the porins, is rich in β-sheet structure, where this unique conformation enables the molecule to span the thickness of the membrane. OmpA protein is exposed

at the external face as well as the periplasmic face of the outer membrane. It acts as receptor for phages K3 or TuII* and for colicin L, indicating the presence of sites exposed at cell surface (cited in Cole et al. 1983). Studies on mutants that produce the OmpA protein but are unable to function as phage receptors revealed the positions of these regions. DNA sequence analysis showed that these mutants were altered near or at residues 25, 70, 110 and 154 of the mature OmpA protein (Morona et al. 1985). Recently, a model for the structure of the NH_2-terminal half of the OmpA protein was developed. This part of the protein consisted of eight amphipathic membrane-spanning β-strands and four large loops exposed to the extracellular side corresponding to the receptor regions mentioned above(Vogel and Jähnig 1986). The carboxy-terminal half of the protein extends into the periplasmic space, since cleavage of the protein by trypsin occurs only when the inner surface of the membrane is made accessible to the protease (ie when outer membrane preparations are treated with trypsin).

OmpA protein is involved in maintaining the structural integrity of the outer membrane. Abundant blebbing was observed in the double mutant, ompA and lpo, lacking both OmpA protein and lipoprotein. The outer membrane tends to detach from peptidoglycan layer, thus forming blebs and vesicles. OmpA protein is also required for stabilisation of mating aggregates. Mutants lacking OmpA are defective in

conjugation with donors carrying F or F-like plasmids. *ompA* mutants are reduced in their recipient ability by ~5000-fold compared with their *ompA*$^+$ parents (Manning and Achtman 1979). Mutants in *ompA* also show growth defects and inhibitor sensitivities (Manning *et al.* 1977, Deeney *et al.* 1986, Reakes *et al.* 1988).

iii. Porins:

These are non-specific water filled channels or pores that allow passive penetration of low molecular weight hydrophilic compounds across the outer membrane. The type of porins varies among different species of Gram-negative bacteria and between the strains of the same species. For example, *E. coli* K-12 cells contain OmpF and OmpC porins when grown under normal cultural conditions whereas *E. coli* B cells contain OmpF porin only (Lutkenhaus 1977). PhoE porin can be induced in both strains when cells are grown in phosphate-limited medium.

Porins are rich in β-sheet structure and cross the outer membrane many times using these stretches of β-sheets. They are also exposed at the surface of cell envelope, where OmpF acts as receptor for phage TuIa and OmpC as receptor for phages TuIb and Me1.

The biologically active form of the porin is a trimer. Each porin contains a triplet of holes which are fused in

Table 2: Major outer membrane proteins of *E. coli* K-12

Protein	Molecular weight	Receptor for phage or colicin	Function
Lipoprotein	7.2 Kd	-	Stabilisation of the outer membrane
OmpA	33 Kd	K3, TuII*, colicin L	Stabilisation of mating aggregates and maintenance of outer membrane integrity
OmpC	36 Kd	TuIb, Me1	General diffusion pore for small hydrophilic solutes
OmpF	37.2 Kd	TuIa, colicin A	General diffusion pore for small hydrophilic solutes

the middle of the outer membrane and exist on the periplasmic face of the outer membrane as a single central channel (Nikaido and Vaara 1985). The diffusion rate of solutes through the porins is influenced by the size, charge and hydrophobicity of the solute. Liposome-swelling experiments showed that OmpF and OmpC porins of E. coli are permeable to compounds of molecular weight 600-700 daltons or less. Cations penetrate through these porins more easily than anions. Solutes carrying negative charges diffuse more slowly than their uncharged counterparts, and those with double-negative charges diffuse even more slowly. However, it should be noted that in contrast to the above mentioned porins, the PhoE porin has a preference for penetration of negatively charged compounds.This can be explained by the presence of recognition or binding sites for these solutes.

The diffusion rate is inversely proportional to the hydrophobicity of the compound.A possible explanation of this exclusion is that entrance of hydrophobic molecules through the porin channel requires breaking of relatively strong hydrogen bonds between the water molecules inside the channel and the groups lining the walls of the channel.

The OmpC pore is slightly narrower than the OmpF pore. This produces only a small difference in permeability of small solutes, but a larger difference in penetration rates of

larger or hydrophobic compounds. E. coli mutants lacking OmpF protein acquire low-level resistance to tetracycline and chloramphenicol due to the low penetration rate through the narrow OmpC porin (Pugsley and Schnaitmann 1978).

The functional part of OmpC porin has been identified. The pore domain of the OmpC protein resides in the first one-third of the protein (Misra and Benson 1988). Charged residues are important in determining channel properties of the pore. Mutations in the DNA region coding for the first 110 amino acids of the mature OmpC lead to increase in the size of the channel. Thus, compounds of molecular weight higher than the exclusion limit of the wild type porin can penetrate across the outer membrane.

The relative amounts of OmpF and OmpC proteins are controlled by cultural conditions such as growth medium and growth temperature. High osmotic pressure (such as presence of 0.9% NaCl or 10% sucrose in the growth medium) or high growth temperature (~42^{o}C) represses the production of OmpF porin. Cells grown in minimal medium have high OmpF to OmpC protein ratio (b to c protein in the old nomenclature), and cells grown in complex media have low OmpF to OmpC ratio (Lugtenberg et al. 1976). Thus, when bacteria are present in the bodies of animals, where temperature and osmotic pressure are high, they produce the narrower OmpC protein.

The major purpose of this process is presumably protection of bacteria from some of the inhibitory substances in the bodies of animals. OmpF porin is produced when enteric bacteria are outside the bodies of animals (e.g. in rivers). The wider diameter of the OmpF pore accelerates penetration of nutrients from the dilute environments. The regulation of porin expression is mediated by the ompB operon. This operon consists of two genes ompR and envZ. The ompR gene product is a soluble protein and regulates the synthesis of the porins. The EnvZ protein resides in the envelope, "senses" the environment and regulates OmpR synthesis (cited in Lundrigan and Earhart 1984).

iv. Specific diffusion proteins:

Several proteins are involved in diffusion of specific compounds across the outer membrane. These include the proteins Tsx, LamB, BtuB and other receptors involved in uptake of iron-chelating compounds.

The Tsx protein (or T6 receptor) is required for diffusion of nucleosides. It is a channel-forming protein, and its function does not require the presence of TonB product. Some of the proteins involved in specific transport processes bind their substrates tightly, in contrast to the very loose binding in LamB and Tsx proteins. Functional TonB product in these cases is necessary, where it is assumed to energize

the cytoplasmic membrane thus facilitating diffusion (Braun 1978).

The BtuB protein is a receptor for phage BF23 and E-colicins. This protein also facilitates uptake of vitamin B12 and requires TonB function for this process. The btuB gene product binds vitamin B12 with a high affinity to the extent that it protects the cell against the killing action of colicins and phages. Inhibition of BtuB synthesis results in the rapid loss of sensitivity to E-colicins, followed later by resistance to the BF23 phage whereas vitamin B12 uptake continues. This suggests that only newly synthesized protein is functional as a receptor for colicins and phages. Upon aging, changes in the conformation of the envelope may lead to loss of the optimum orientation of the BtuB protein for colicin uptake. Another explanation is that newly synthesized BtuB protein emerges on the cell surface at adhesion sites where it is an effective receptor for colicins. As the protein diffuses from the adhesion site it cannot bind phage or colicin but retains its affinity for the vitamin (Braun and Hantke 1981).

Some of the outer membrane proteins are involved in transport of iron. Growth of E. coli cells in media of low concentration of free iron induces synthesis of a number of proteins and other compounds. Siderophores (low molecular

weight chelators) are produced by bacteria and have high affinity for iron. Different types of chelators are used; these include enterochelin, ferrichrome and citrate. The iron-chelator complex is transported across the outer membrane through specific receptors on the cell envelope. Five different receptors, induced under conditions of limiting iron, have been identified. TonA (or FhuA) protein has a molecular weight of 78,000 and mediates the transport of ferrichrome. FepA protein is required for the transport of ferric enterochelin. FecA protein is a receptor for ferric citrate complex, and is induced in iron limited media containing high concentrations of citrate. Other outer membrane proteins (Cir and 83K proteins) are induced by iron starvation but their exact role in iron transport is unknown.

LamB protein plays an important role in uptake of maltose and maltodextrins. Presence of these substrates in the growth medium induces the synthesis of LamB and other proteins involved in transport of these compounds across the outer and the cytoplasmic membranes. The functional LamB protein, like porins, is composed of a trimer and rich in β-sheet structure. The accumulation of maltose and maltodextrins is dependent upon the presence of the maltose binding protein in the periplasmic space. LamB interacts physically with this protein such that the penetrating solutes are readily bound to the maltose binding protein. This provides rapid penetration and

Table 3: Minor outer membrane proteins of *E. coli* K12

Protein	Molecular weight	Conditions for optimal expression	Receptor for phage or colicin	Function
LamB	~47 Kd	Presence of maltose	λ	Pore for maltose and maltodextrins
Tsx	26 Kd	Constitutive	T6, colicin K	Pore for nucleosides
PhoE	40 Kd	Phosphate limitation	TC23	Pore for anionic solutes
BtuB	60 Kd	Vitamin B12 limitation	BF23, E-colicins	Uptake of vitamin B12
Cir	74 Kd	Iron limitation	Colicins I and V	Uptake of iron
TonA	78 Kd	Iron limitation	T1, colicin M	Uptake of ferrichrome
FecA	80.5 Kd	Iron limitation	-	Uptake of ferric citrate
FepA	81 Kd	Iron limitation	Colicin B	Uptake of Fe^{3+}-enterochelin
83K protein	83 Kd	Iron limitation	-	Uptake of iron

further diffusion of these substrates.

1.2.5. Organisation of the outer membrane:

The exposure of certain component on the cell surface can be detected by different methods. The observation that phage or bacteriocin resistant mutants lack an outer membrane protein and do not bind the phage or bacteriocin shows that the protein is exposed at the cell surface. Proteolytic enzymes are also used as tools for detecting surface localization of proteins. However, this method is not always reliable since some proteins may be shielded from the action of proteolytic enzymes. In intact cells of E. coli, none of the outer membrane proteins are degraded by these enzymes although some are exposed at the cell surface. The natural environment of Enterobacteriaceae is rich in such enzymes, and the proteins must be oriented such that the susceptible site is not accessible to the enzyme. Interaction with mono-specific antibodies is a third method that is used to determine exposure at the cell surface. Using one or all of these methods shows that most of the outer membrane proteins (i.e. the porins, OmpA, PhoE and LamB proteins) have sites exposed at the cell surface.

Studies using antibodies against the O-antigenic portion of the lipopolysaccharide indicate that LPS is located in the outer leaflet of the outer membrane. Also, it is reported that sugar chains of the LPS in whole cells are oxidized by

exogenous galactose oxidase. Phospholipids are located at the inner leaflet of the outer membrane. In intact wild type cells, phospholipids are not degraded by exogenous phospholipases A_2 and C. This shows that they are not exposed at the cell surface since they are not accessible to the degrading enzymes.

Interaction between the various constituents of the outer membrane has been observed. OmpA protein-LPS interactions are required in vivo for the phage receptor function of the OmpA protein, where the lipid A portion of the LPS is the active component in this situation. LPS is also necessary for the in vitro biological activities of outer membrane proteins, such as phage receptor activity and pore function (Lugtenberg and van Alphen 1983). Mutants defective in the lipopolysaccharide contain decreased amounts of outer membrane proteins. OmpF is hardly present in outer membrane of heptose-less LPS mutants (Havekes et al. 1976). The phage receptor activity of LamB protein is decreased in these mutants too. In this case, the amount of LamB protein in the outer membrane may be decreased. Furthermore, the interactions of porins with LPS molecules influence the function of the porin. Porins produce unstable channels in the absence of LPS.

The lipopolysaccharides and phospholipids are almost completely segregated, forming the two layers of the outer membrane. In the presence of Mg^{2+}, the LPS-LPS interaction is very strong in comparison with the LPS-phospholipid

interaction, thus stabilising this segregation (Nikaido and Vaara 1985). Cations play an important role in the organisation of the LPS leaflet. A portion of the saccharide chain of LPS has negatively charged groups. This results in a strong electrostatic repulsion between the neighbouring molecules, which is overcome through binding cations. Removal of divalent cations with chelators confirms the important role of these ions in the integrity of the outer membrane (as will be discussed in the following section).

1.2.6. Barrier properties of the outer membrane:

The outer membrane of Gram-negative bacteria, especially members of the Enterobacteriaceae, is impermeable to hydrophobic compounds. This property is important for survival of enteric bacteria, since their normal habitat, the intestinal tract of higher animals, is rich in hydrophobic substances such as bile salts and free fatty acids. Most Gram-negative bacteria are resistant to hydrophobic antibiotics (e.g. erythromycin, novobiocin), detergents and hydrophobic dyes (e.g. eosine, methylene blue). These molecules are too large or too hydrophobic to penetrate through the porin channels and cannot penetrate through the tightly packed lipopolysaccharide region of the outer membrane (Hancock 1984).

In an enteric bacterium, the uptake of gentian violet, a hydrophobic dye, is low (20%) in wild type (S-form) and in rough strains of Ra or Rc LPS type. Loss of the next few sugar

residues to produce the Rd LPS mutant highly increases the penetration of the hydrophobic agent, where the uptake of gentian violet is more than 60% (Stan-Lotter et al. 1979). Several hypotheses have been proposed to explain the increased permeability of the deep rough LPS mutants to hydrophobic compounds. The most accepted one indicates that deep rough mutants contain phospholipid molecules in the outer leaflet of the outer membrane in contrast to wild type cells. The created phospholipid bilayer domains allow penetration of these hydrophobic substances. Nikaido (1979) observed that the phospholipid content of the outer membrane is similar in wild type strains and in Rc LPS mutants, but significantly increases in deep rough mutants (Rd and Re types). In agreement with the above mentioned observations, wild type cells and Rc LPS mutants were able to grow in the presence of bile salts and sodium dodecyl sulfate (SDS), whereas Re mutants and pore protein deficient mutants were sensitive to these agents. Lugtenberg and van Alphen (1983) reported that cells of the sensitive strains have increased amounts of phospholipid which are located in the outer leaflet of the outer membrane. The use of exogenous phospholipases or chemical labelling of cells with CN Br-activated dextran confirms this conclusion. Phospholipids of wild type cells and Rc mutants are resistant to the degradation by phospholipases and are not accessible to the labelling compound. In contrast, phospholipids in cells of Rd and Re strains can be coupled to the labelling agent, indicating

their exposure at the cell surface.

The second hypothesis indicates that a strong LPS-LPS interaction is essential in preventing penetration of hydrophobic compounds. Such a strong lateral interaction is lacking in the deep rough LPS mutants. These mutants are missing most of the core sugars and phosphate residues which are involved, in the presence of divalent cations, in formation of tight interactions between neighbouring LPS molecules.

The treatment of wild type cells with chelating agents such as ethylenediaminetetracetate (EDTA) sensitises cells to hydrophobic substances. Leive (1974) showed that EDTA, in the presence of Tris buffer, released about one - half of the LPS. Mg^{2+} and Ca^{2+} ions are chelated by EDTA, causing disorganisation of the lipopolysaccharide layer. LPS contain negatively charged groups and interaction with divalent cations is essential for maintaining LPS in the outer membrane. After addition of EDTA, the released LPS is replaced by phospholipids, thus phospholipid bilayer regions are formed and the cell's permeability to hydrophobic agents is increased. In addition, LPS-LPS interactions may be weakened by removal of divalent cations, allowing penetration of hydrophobic molecules.

1.3. Characteristics of ColV$^+$ *Escherichia coli* strains

1.3.1. *E. coli*, including ColV$^+$ strains, in human diseases

E. coli strains associated with disease in man are classified into three groups.

a. Enterotoxigenic *E. coli* (ETEC): causes diarrhoea in travellers and affects both adults and children. These strains produce enterotoxins, either heat - labile or heat - stable or both. Enterotoxins induce secretion of water and electrolytes from the small intestine, causing diarrhoea. ETEC strains also possess fimbrial colonisation factors that allow bacterial cells to adhere to the intestinal mucosa and secrete the enterotoxins.

b. Enteroinvasive *E. coli* (EIEC): causes a dysentery - like syndrome, where invasion of the epithelial cells of the intestine is the essential feature. A large outbreak involving this strain was reported in U.S.A.; the pathogenic organisms were isolated from imported cheese (Sussman 1985). Enteroinvasive strains penetrate and multiply inside intestinal cells resulting in inflammation and ulceration of the intestine. In addition, EIEC strains cause increased secretion of water and salts from intestine. Recent data report that elevated levels of adenylate cyclase activity is observed after mucosal penetration. This increases the level of cAMP, thus secretion of water and electrolytes to the lumen of the intestine is increased. The increase in

adenylate cyclase results from stimulation by prostaglandins which are synthesized locally in the mucosal inflammatory reaction (Formal et al. 1976). Also, EIEC strains can enter the blood stream causing septicaemia.

c. Enteropathogenic E. coli (EPEC): affects infants and young children.

Diarrhoea is one of the major causes of children's deaths in the world. It is among the ten leading causes of death in children of 1-4 years in North American and European nations (Rohde and Northrup 1976). In developing countries the incidence is higher where diarrhoea accounts for more fatalities than any other disease. Thirty percent of childhood deaths were attributed to diarrhoea in Latin Ameria. In India and Indonesia, it was reported that during the first three years of life 100-200 attacks of diarrhoea per hundred children occurs annually. These attacks were fatal in 1-4% of the cases, yielding a death rate from diarrhoea of 20-55 per 1000 children annually (cited in Rohde and Northrup 1976).

In animals, several types of disease can be produced by E. coli. These include:

a. Enteric colibacillosis: where bacterial cells remain in the intestine and affect young animals such as poultry, calves, lambs and pigs. Infection by E. coli comprised 35% of all

the diarrhoea - causing organisms in pigs between 1975 and 1980. In calves and lambs the incidence was 26% and 17% respectively (Morris and Sojka 1985).

b. Systemic colibacillosis: characterized by invasion and septicemia, where E. coli cells pass through the mucosa of the alimentary tract and enter the blood stream causing generalized infection. This form of colibacillosis occurs frequently in animals especially poultry. It affects 5-12 weeks old broiler chickens with a maximum incidence at 6-9 weeks. Respiratory distress associated with lesions in the lower respiratory tract and severe green diarrhoea are the main clinical symptoms. A high proportion of E. coli strains associated with septicaemia in animals produce colicin V. It was reported that of 31 E. coli strains isolated from generalized infection in calves, 25 produced colicin V (Smith 1974). Colicin V - producing strains are more pathogenic than non-producing forms, and this property was attributed to the presence of the ColV plasmid. 'Curing' of the ColV plasmid reduced the pathogenicity of the host cells, whereas its re-introduction caused restoration of the virulence. The greater virulence of the $ColV^+$ form is not associated with a toxic effect of colicin V itself but with the greater ability of $ColV^+$ strains to survive in blood and internal organs of infected animals. The effect of ingestion of $ColV^+$ or $ColV^-$ strains on colostrum - deprived calves was monitored by Smith and Huggins (1976). The calves given the

ColV$^-$ form were normal in appearance after 24 hr whereas the ones given the ColV$^+$ form were nearly dead with high concentrations of organisms in blood, kidneys and liver. A similar result was obtained when ColV$^+$ or ColV$^-$ forms were given intramuscularly to chickens, confirming the greater pathogenicity of ColV$^+$ strains.

Contaminated food is the main source of E. coli infection in man. Organisms are introduced into the intestine by improper cooking or through cross-contamination where fresh uncooked food is implicated. Efficient control procedures during rearing and slaughtering of animals are important to provide bacteria-free meat to consumers. At poultry farms, pathogenic bacteria are initially introduced in contaminated feed and may spread from faeces of infected birds to external surfaces of other healthy birds. Infected birds contaminate the environment inside the pens such as bedding, feed or water (Clinton et al. 1981) which causes the spread of pathogens to other birds or flocks. Infected chickens are also an important factor in transmission of pathogenic bacteria to eggs and consequently to newly hatched chicks (Barbour and Nabbut 1982), or in contamination of eggshells which introduces pathogens to egg consumers.

Therefore, many chickens entering the processing plant may be contaminated. The first step in chicken processing is scalding; however, this step is a major site of cross-contamiantion between carcasses (cited in Humphrey and Lanning 1987). Defeathering machines and eviscerators may also be cross-contaminated which spread pathogenic bacteria to other processed carcasses that were originally bacteria-free (Rigby et al. 1980).

To prevent or decrease the spread of pathogens in poultry, sterilization of feed is necessary since infection of animals and birds with enteric pathogens is attributable to consumption of contamianted feed in most of the cases (Jones et al.1982, Yoshimura et al. 1979). Also, eradication of enteric pathogens by competitive exclusion has been applied recently. One day old chicks are treated with intestinal microflora of mature chickens which increases the resistance of chickens to infection (Soerjadi et al. 1982, Pivnick et al. 1981). To eliminate pathogenic bacteria from processed carcasses, addition of chlorine or lactic or succinic acid to the washing water (Mossel 1983) is applied.

1.3.2. Virulence characters; enhanced iron uptake

Iron is an essential growth element for bacteria. However, the amount of free iron in nature is very low. In the environment, ferric salts are found as highly

insoluble aggregates. In man and animals, most of the iron is found intracellularly (e.g. in red blood cells as haem) or extracellularly in serum or other body fluids, where it is bound to high affinity iron-binding proteins. These binding proteins include transferrin in serum and lactoferrin in milk and mucosal secretions. Accordingly, the level of free Fe^{++} and Fe^{+++} ions in body fluids etc. is very low.

One of the important characteristics of pathogenic bacteria is their ability to multiply in host tissues. This property is influenced by the availability of iron, therefore bacteria have evolved specific mechanisms that enable them to solubilize ferric iron and transport it back into the cell. Under conditions of iron starvation, microorganisms produce low molecular weight iron-chelating compounds, designated siderophores. Enterochelin is produced endogenously by many bacteria; it is secreted into the medium where it binds iron and transports it into bacterial cells through specific receptors on the outer membrane. The different types of receptors for iron chelates are described in section 1.2.4.c; some take up Fe enterochelin. Also, bacteria are able to use exogenous siderophores which are not synthesized by the organism itself but produced by other microorganisms, such as ferrichrome produced by fungi. Bacteria and other micro-organisms can

also produce and / or utilize as iron chelators less specific agents such as citrate.

Strains carrying ColV plasmid have increased virulence in human and animal infections compared with the plasmid-free strains (see section 1.3.1). This can be attributed to the enhanced survival of ColV$^+$ strains in bodies of animals. Williams (1979) reported that most of the ColV$^+$ strains encode an iron-sequestering mechanism which may play an important role in survival of bacteria under the iron-limited conditions. This system was encoded by pColV-K30 and all tested ColV plasmids from bacteremic strains but not by the ColV, I-K94 plasmid. Generally, the presence of free iron promotes infection. Animals injected with iron are more susceptible to infection with various types of bacteria compared to untreated ones (Elwell and Shiply 1980). This was also observed in pColV-bearing strains but administration of iron compounds in experimental infections resulted in nearly equal survival of ColV$^+$ and ColV$^-$ strains in host tissues. The iron chelating property of ColV plasmids appears to be one factor in increased virulence. Addition of transferrin to a defined bacterial growth medium limited the growth of plasmid-free strains due to conversion of the free iron to an unavailable complexed form. This inhibitory effect could be reversed by addition of excess iron.

In contrast, ColV$^+$ strains were not inhibited by transferrin. The presence of the ColV plasmid conferred on bacteria the ability to acquire iron. Further studies showed that, firstly, growth of cured derivatives of these strains was reduced by transferrin. Secondly, transfer of the ColV plasmid from the bacteremic strains into *E. coli* K12 rendered the recipient cells unaffected by the presence of iron-binding protein (transferrin) in the growth medium (Williams 1979).

The ColV plasmid-mediated iron uptake system involves production of a siderophore designated aerobactin, and an outer membrane protein that forms at least part of the ferric-aerobactin receptor. This receptor has a molecular weight of 74K and is clearly observed in *cir* mutants which lack the 74K Cir protein (Bindereif *et al*. 1982). The ability to make aerobactin provides selective advantage to organisms already capable of synthesizing enterochelin. This can be attributed to the fact that enterochelin-mediated iron transport is energetically expensive since enterochelin is used only once for transporting iron whereas aerobactin can be recycled. Also, the presence of anti-enterochelin and anti-receptor antibodies in serum of animals may restrict the effectiveness of enterochelin-mediated iron uptake and favours the aerobactin-mediated iron transport (Griffiths 1985).

1.3.3. Virulence characters; serum resistance

It was obserevd that ColV$^+$ strains survive better in animal sera than do ColV$^-$ ones. Twenty four hours after intravenous injection of chickens with viable organisms of the two strains, significantly higher concentrations of ColV-bearing strains than plasmid-free strains were found in blood (Smith 1974). Fresh samples of serum were inoculated with a mixture of ColV$^+$ and ColV$^-$ organisms to confirm that the increased number of ColV$^+$ cells in blood is attributed to their ability to resist the bactericidal action of serum. After incubation at 37^{o}C for 1-2 hr, most of the mixture consisted of ColV$^+$ cells. Moreover, use of serum inactivated by heating to 65^{o}C for 30 min resulted in equal survival percentages for ColV$^+$ and ColV$^-$ strains. It has been demonstrated that plasmid ColV,I-K94 carries determinants for resistance to the bactericidal activity of serum (Binns et al. 1979). The genetic locus has been termed iss (increased survival in serum). Plasmid derivatives that have this locus enhance the pathogenicity of the bacterial cells for chickens indicating that the phenomenon of serum resistance is associated with virulence.

Serum is an important host defence mechanism against infections by various types of bacteria. It possesses bactericidal activity that results from the action of

the complement system on bacterial surfaces. Activation of the complement sequence of reactions occurs either by the "Classical"pathway triggered by formation of antigen-antibody complex, or by the "alternative" pathway which does not require antigen-antibody reaction. The complement system is composed of several proteins (Fig 9). C1 exists in serum as an aggregate of three proteins: C1q, C1r and C1s. C1q combines with the antibody causing conversion of the proenzyme C1r to $C1\bar{r}$. The cleavage of C1s to $C1\bar{s}$ is catalyzed by $C1\bar{r}$. Then $C1\bar{s}$ cleaves C4 and C2 to generate the C3-cleaving enzyme C4b,2a. This enzyme cleaves C3 into two fragments; C3b binds to C4b,2a to generate the "C5-convertase" enzyme. The cleavage of C5 results in generation of C5b fragment which has the ability to bind C6 and C7, thus forming a trimolecular complex C5b 67. This complex is able to bind to membranes where it complexes with factors C8 and C9. The assembly of this complex on the bacterial surface causes membrane leakage. It has been proposed that the complex penetrates through the membrane forming a channel that destroys the integrity of the membrane and eventually kills bacteria.

The "alternative" pathway of complement can be activated by a variety of substances such as polysaccharides and lipopolysaccharides. The reactions involved are represented in Fig 9. The water-induced cleavage of the

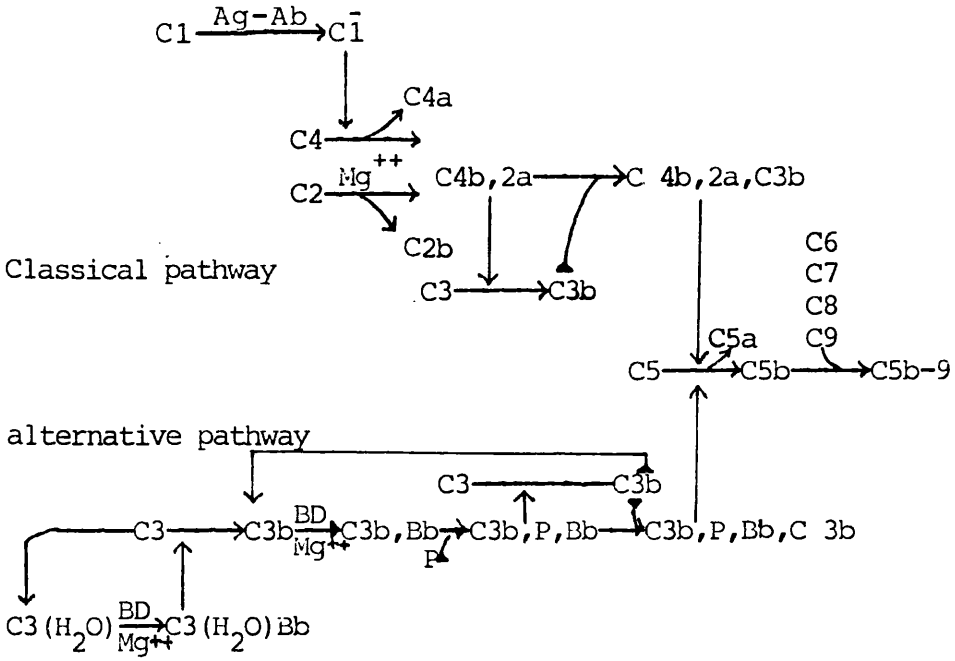


Fig 9: Diagrammatic representation of the classical and alternative pathways of complement activation.

thioester bond in C3 allows C3 molecules to react with factors B and D to generate an enzyme that cleaves C3. When activators of the"alternative" pathway are present (e.g. polysaccharids), some of the C3b becomes deposited on the surface of the activator. The surface-bound C3b interact with factors B and D to form the enzyme C3b Bb which is able to cleave very large amounts of C3. Considerable amounts of this newly generated C3b are deposited on the surface of the activator, interact with additional factors B and D and form more C3bBb. In the presence of factor P, the C3bBb enzyme molecules bind to it forming a more efficient C3-cleaving enzyme. Many of the C3b molecules generated by the surface-bound C3bBb or C3bPBb enzymes bind to the surface of the activator in close proximity to these enzymes. This results in formation of modified enzymes, C3bBbC3b or C3bPBbC3b, which are able to cleave C5 and initiate the membrane attack mechanism. The formation of C5b-C9 complex proceeds in a similar manner to that described for the "classical" pathway (Cooper 1984).

In addition to ColV,I-K94, certain conjugative multiple antibiotic resistance plasmids increase the resistance of bacteria to the complement action. The serum resistance determinant in the latter plasmids is the traT gene product. It is an outer membrane protein of molecular weight of 25,000 and part of the tra operon

of conjugative plasmids. However, the iss gene of pColV,I-K94 maps outside the transfer region of the plasmid. The product of this gene is also immunologically different from traT gene product, indicating that the two genes are different. The identity of the iss gene product is unknown but it may be, like TraT protein, an outer membrane protein. Both proteins function in a similar way. Formation of the terminal complement complex is not blocked by both gene products, but the action of this complex on bacterial surfaces is abolished in the presence of iss or traT genes. Consumption of the terminal components of complement, C6-C9, is the same whether E. coli contained the iss or traT genes or not (Griffiths 1985).

1.3.4. Increased adhesion to epithelial cells

Adhesion to the mucosal surface of the intestinal tract is an important feature in the pathogenesis of infection with E. coli and other members of the Enterobacteriaceae. Adhesins (or colonization factors) allow bacterial cells to establsih themselves at the mucosal surfaces of eukaryotic hosts. Thus, pathogens overcome the clearing mechanism of the peristaltic movement of the intestine. Production of adhesins is encoded by different types of plasmids. The most prevalent are those that encode the production of K88, K99, or CFA adhesins observed in pathogenic

E. coli strains isolated from pigs, calves and lambs, or humans, respectively. Removal of these plasmids rendered organisms unable to cause enteritis. The important function of colonization factors in promoting adhesion to epithelial cells was confirmed by immunofluorescence examination of tissues from infected pigs. For example, the K:88^{+} bacteria adhered to the mucosa of the small intestine whereas K88^{-} cells were distributed in the lumen (cited in Parry and Rooke 1985).

A similar observation was demonstrated in ColV^{+} bacteria. Strains of E. coli K-12 possessing the ColV plasmid adhered in vitro to discs of mouse small bowel tissue in threefold greater numbers than isogenic strains lacking ColV. Curing of ColV plasmid was accompanied by a decrease in numbers of bacteria adhering to tissue discs (Clancy and Savage 1981). The decreased adhesion of ColV^{-} cells was attributed to loss of sex pili as evidenced from electron microscopic studies. This observation was confirmed in a recent study. ColV^{+} cells adhered in greater numbers to epithelial cells than strains lacking ColV plasmid (Tewari 1986). Hydrophobic interactions are important in mediating the adhesion of bacteria to eukaryotic host cells. The hydrophobicity of the two strains was investigated, and the surfaces of ColV^{+} cells were significantly more hydrophobic than ColV^{-} ones. The ColV plasmid-encoded

sex pili were responsible for the increased hydrophobicity. Several observations are in accord with this conclusion, growth at low temperature (21°C) abolishes the effect of ColV on bacterial hydrophobicity, correlating with the finding that sex pili are not produced at low temperatures. Also, removal of sex pili from the ColV$^+$ cells by shearing reduces the hydrophobicity and subsequently decreases the adherence of bacteria to epithelial cells (Tewari et al. 1985).

1.3.5. Colicin production

Some colicin V- producing strains (e.g. ColV,I-K94$^+$) code for production of both colicin V and Ia while others (e.g. ColV-K30$^+$) code for colicin V synthesis only. Cells that synthesize a particular type of colicin are immune to its action. The molecular interaction involved in this immunity is highly specific, and is the basis for identification of colicin subtypes. For example, colicins Ia and Ib have similar chemical and physical properties and adsorb to the same receptor, yet strains carrying the ColIa plasmid are immune to colicin Ia only and sensitive to colicin Ib and vice versa. The immunity system involves the interaction of a plasmid-encoded gene product and the colicin. It has been reported that immunity to colicin Ia is mediated by an inner membrane protein of molecular weight of 14,500. In this case, immunity results from association of the colicin with

the immunity protein at the cytoplasmic membrane, thus preventing colicin action (Konisky 1982).

Colicin V is produced in low amounts, and its release from the bacterial cell does not require the presence of a lysis gene product. It is observed that three genes, designated cvaA , cvaB and cvaC are required for colicin production. cvaC is responsible for synthesis of the colicin polypeptide whereas cvaA and cvaB gene products facilitate export of the colicin. Mutations in these two genes prevents any detectable activity in the supernatant of the bacterial culture whereas colicin V activity can be detected in lysed cells. However, mutation in cvaC gene eliminates colicin V activity in both the supernatant and the cells (Gilson et al. 1987).

Colicin V has a small size. Analysis of the products of the colicin V structural and immunity genes revealed that colicin V has a molecular weight of ca 4,000 and its immunity protein is about 6,500 daltons (Frick et al. 1981). Colicin action is initiated by adsorption of colicin molecules to specific receptors on the bacterial surface. Resistance to colicins results from loss of ability of whole cells to adsorb colicin. Receptors provide a means whereby colicins are able to overcome the barrier properties of the outer membrane. For

example, colicin Ia-mediated de-energization of membrane-vesicles prepared from resistant cells was observed, indicating that direct access of colicin to its target abolishes the need for receptors. Different types of colicins have variable modes of action, some degrade DNA or RNA, others interact with the cytoplasmic membrane. The primary target of colicin V is the cytoplasmic membrane. E. coli cells that are treated with colicin V are unable to carry out active transport of nutrients. This inhibition of active transport is not a result of generalized leakiness of the cytoplasmic membrane but due to collapsing of the **energized membrane state**(Yang and Konisky 1984). Similarly, colicin Ia induces formation of ion-permeable channels in the cytoplasmic membrane, causing dissipation of membrane energy and leakage of ions and low molecular weight compounds.

1.3.6. VmpA protein production:

Most ColV$^+$ strains encode the production of a new major outer membrane protein designated VmpA. The properties of this protein were investigated by Moores and Rowbury. The VmpA protein resembles the OmpA protein in some respects. Both proteins have a similar apparent molecular weight (ca 33,000d) as deduced from analysis of cell envelopes by SDS-polyacrylamide gel electrophoresis. VmpA, like OmpA protein, is not

peptidoglycan - associated since it can be solubilized in 2% SDS at 60°C (Moores and Rowbury 1982). The new protein, like other major outer membrane proteins, is synthesized from a precursor of about 34,500d (Rowbury et al. 1985). The VmpA protein is located in the outer membrane as evidenced by analysis of cytoplasmic and outer membrane fractions from ColV$^-$ and ColV$^+$ cells(Deeney et al. 1986).

The VmpA protein, like the OmpA protein, spans the outer membrane with segments protruding on both sides. This protein is degraded if membrane preparations are treated with trypsin but exposure of whole cells to the enzyme does not cleave VmpA protein suggesting that a segment extends inwards into periplasmic space. A portion of VmpA protein is also exposed to the outside. Treatment of whole cells with cyanogen bromide activated dextran and examination of membrane preparations by SDS-PAGE showed greatly reduced amount of VmpA protein appearing on the gel (Deeney et al. 1986). VmpA protein reacts with the non-penetrating activated dextran indicating its exposure to the outside. Furthermore, OmpA and VmpA proteins are immunologically related. VmpA protein reacts specifically with serum raised against OmpA protein and vice versa. However, other outer membrane proteins (e.g. porins) do not react with sera raised against VmpA or OmpA proteins (Deeney et al. 1986).

Although VmpA and OmpA proteins share some similar properties, they differ in many other characteristics. VmpA protein cannot replace the OmpA protein as a receptor for phages k3 and TuII* and for colicin L. Also, it only weakly replaces OmpA protein in stabilisation of mating aggregates (Moores and Rowbury 1982). The VmpA protein can, however, replace OmpA protein in allowing high temperature minimal medium growth and resistance to hydrophobic and cationic agents (Deeney et al. 1986, Reakes et al. 1988).

Production of VmpA protein is influenced by cultural conditions. Growth of ColV$^+$ organisms at 37^{o}C static instead of shaken abolishes formation of VmpA protein. Presence of magnesium or calcium ions (0.005-0.05 M) in the growth medium inhibits synthesis of this protein (Deeney et al. 1986).

1.4. Effect of elevated temperature on the bacterial cell:

Temperature is one of the most important environmental factors that affect growth and viability of bacteria. Death and several forms of injury are observed in bacterial cells subjected to heating above their maximum growth temperature. Upon exposure to high temperature, cells may be dead, very injured, slightly injured or unaffected. Cells are classified as injured rather than dead if they possess the capability to regain a normal physiological state and initiate growth and cell division when they allowed to recover in an appropriate medium and optimal conditions. Injured cells may be metabolically or structurally injured or both, where exposure to sublethal heating can result in the loss of the ability of the microorganism to grow under conditions that are suitable for growth of unheated cells. Metabolically injured cells are unable to grow and multiply on a solid minimal medium but retain colony-forming capabilities when recovered in non-selective complex medium. Structurally injured cells are unable to proliferate in a selective medium. Injured cells may be altered in the entry of inhibitors (increased penetration) or more susceptible to any penetrating inhibitor. Therefore, injured bacteria may have enhanced sensitivity to dyes (e.g. brilliant green and crystal violet), bile salts, sodium chloride, lysozyme, Tris, hydrogen peroxide, and antibiotics(Russell 1984).They may also exhibit increased lag time, slower growth rate

and even the inability to divide until an adequate resuscitation period has occurred (Mossel and VanNetten 1984).

The primary site at which heat acts is not clearly determined but there are several sites in the microbial cell that are vulnerable to thermal damage. Failure to repair the damaged sites may result in death of the bacterial cell. The severity of the damage or injury depends on the heating temperature and the length of the exposure time. Consequently, death may occur when injures are so severe that the cell cannot repair the damage.

The damage to the various components of the bacterial cell is discussed in the following sections.

1.4.1. Heat effects on cell envelope:

The outer membrane of Gram-negative bacteria is damaged when cells are exposed to thermal stress. Heat-injured cells showed increased sensitivity to selective media such as eosin methylene blue agar and Mac Conkey agar (Ray and Speck 1973). This was attibuted to the heat-induced damage to the outer membrane, causing reduction in its permeability barrier properties. It was reported that about 20% of the lipopolysaccharide was released into the heating medium when cells of E. coli K-12 were subjected to mild heating (48^{o}C) for 1 hour (Hitchener and Egan 1977).

Ninety percent of the cells could not grow on the above selective media after this treatment; the LPS loss could be responsible, allowing dyes or deoxycholate in the media to penetrate and stop growth.

Although heating at 48°C damages part of the outer membrane structurally and functionally, this damage is not a direct cause of cellular death but may be one contributing factor. Heating cells in media containing EDTA causes a larger increase in the rate of thermal injury but does not significantly affect the thermal death rate (Hitchener and Egan 1977). Combining heating with EDTA treatment removes a larger portion of the lipopolysaccharide leading to increased damage to the cell wall. These results were for heating at 48°C and heat damage to the outer membrane caused by higher temperatures might cause death.

The cytoplasmic membrane is also affected by heating. Intracellular constituents such as pentoses, potassium ions, amino acids and materials absorbing at 260nm leaked from E. coli cells upon heating at temperatures of 50-60 °C, indicating membrane damage (Beuchat 1978). Undamaged cytoplasmic membrane controls the passage of solutes into and out of the cells, and the heat-induced leakage of cellular constituents correlates with the loss of cellular viability (Russell and Harries 1968). The heat induced membrane damage may deprive the cell of the essential

nutrients and components necessary for growth. Accordingly, thermal damage to the cytoplasmic membrane may cause death.

However, the damage to cytoplasmic membrane may only contribute, along with changes induced in other sites in the cell, to the loss of viability. Thus non-viable E. coli cells, induced by heating, are able to relatively maintain the concentration gradient across the membrane indicating that death may precede extensive membrane damage (Grau 1978). Also, E.coli spheroplast suspensions in sucrose are not lysed by exposure to high temperature but become only irregular in shape.

Accordingly, damage to both the outer and cytoplasmic membranes occurs on heating. Either or both may contribute to cell death but this is not yet established with certainty.

1.4.2. Heat effects on ribosomes and rRNA:

Ribosomes are composed of rRNA and proteins, where rRNA constitutes about 63% of the weight of the ribosome. Inter- and intramolecular interactions are observed between the rRNA and ribosomal proteins and are involved in maintaining the proper function of the ribosome. Magnesium ions are also needed for proper ribosome assembly. In prokaryotes, the 70S ribosome is composed of two subunits. The 30S subunit consists of one 16S rRNA molecule and 21

protein molecules. The 50S subunit contains one 23S rRNA molecule, one 5S molecule and 31 protein molecules (Darnell et al. 1986).

Ribosome degradation occurs in heat injured cells. In Salmonella typhimurium, the 30S subunit is totally degraded upon heating cells for 30 minutes at 48°C. Sedimentation analysis of crude extracts of normal and injured cells on 5-20% sucrose gradients showed that extracts from unheated cells had 50S and 30S ribosomal subunits while heated cells had only one type of particle with a sedimentation coefficient of 47S (Tomlins and Ordal 1976). A similar observation was reported in heat injured Staphylococcus aureus cells; the 50S subunit remained intact whereas the 30S subunit was absent. Heating at 50°C for 7 minutes caused 90% destruction of the 30S subunits while there was only 10% loss in the 50S subunits (cited in Pierson et al. 1978). Polyacrylamide gel electrophoresis of rRNA from normal and heated cells revealed that the 16S rRNA was completely degraded whereas the 23S rRNA was only partially degraded.

RNA and protein synthesis are required for recovery of heat- injured cells. Incubation of heated cells in a medium containing actinomycin D or rifampin prevented the recovery of cells from thermal injury. Incorporation of uracil - 6 - ^{3}H and ^{14}C - leucine into heat injured Salmonella typhimurium during recovery was studied (Tomlins

and Ordal 1971). RNA synthesis increased linearly during the first four hours of recovery after which a plateau was reached. RNA synthesis again began to increase after five hours of recovery indicating cellular growth. Maturation of precursor rRNA occurs during the plateau period. Heat-injured S. typhimurium that was allowed to recover for 4 hours had 17S, 16S, 23S, and 24S rRNA, where the 17S and 24S molecules represented precursor rRNA. The maturation of these species required protein synthesis since their processing was abolished in injured cells recovered in the presence of chloramphenicol. Accordingly, it is clear that during thermal injury, there is damage to and degradation of the 30S ribosome sub-unit and some of its components and recovery from such injury involves ribosome regeneration.

Ribosomes are the sites at which protein synthesis occurs and functional ribosomes are required for growth and division of the cells. The re-synthesis of ribosomal components is dependent on the presence of a functional DNA molecule able to provide the necessary genetic information and a functional protein-synthesis machinery able to form the enzymes involved in processing of the rRNA. The results reported in this section were for cells heated at 50^{o}C for a short time; however, heating cells at a higher temperature or for a longer time may cause extensive ribosomal damage and / or DNA damage resulting

in the inability to recover from heat injury, consequently death occurs.

1.4.3. Heat effects on proteins:

The effect of thermal injury on the enzymes of glycolysis and tricarboxylic acid cycle were investigated (Tomlins et al. 1971). There was minor loss in specific activities of fructose diphosphate aldolase, lactate dehydrogenase and fumarate hydratase of Salmonella typhimurium cells exposed to heating at 48°C for 30 minutes. The efficiency of the TCA cycle or glycolysis was not affected, suggesting that enzyme inactivation is not the primary target of this damaging but not killing heat action. However, during recovery from thermal injury, protein synthesis may occur causing resynthesis of heat inactivated enzymes.

On the other hand, structural proteins or other types of enzymes may be damaged when cells are subjected to high temperature. It was observed that protein synthesis is required for recovery of injured cells; addition of chloramphenicol to the recovery medium inhibited repair of heat injured S. typhimurium. Protein synthesis is required for recovery of denatured enzymes and / or other heat inactivated proteinaceous compounds (cited in Hurst 1984). Also, protein synthesis is essential for production of functional ribosomes during recovery from heat injury

(see section 1.4.2.). The failure to resynthesise the damaged proteins either due to non-functional DNA molecule or to extensive protein damage may cause death of bacteria.

1.4.4. Heat effects on DNA:

Various biochemical and genetic evidences suggest the involvement of DNA in thermal damage. DNA single-strand breaks were observed after heating E. coli cells at 52°C, where their induction was correlated with loss of cell viability. Using the alkaline sucrose-gradient technique, a decrease in the molecular weight of the DNA of heated cells was reported suggesting the presence of several DNA-strand breaks. These breaks occur as a consequence of attack by deoxyribonucleases stimulated upon exposure to high temperature (Sedgwick and Bridges 1972). Also, double strand breaks were reported in DNA of heated bacteria. Repair of these breaks can occur; addition of specific DNA synthesis inhibitors to heated cells prevents cell recovery providing further evidence for the presence of DNA damage during heating and indicating that DNA repair synthesis is required for maintaining DNA integrity and viability after heating.

Genetic evidence for heat induced DNA damage is demonstrated by studies on DNA repair-deficient strains. Ligase-defective E.coli mutants are more sensitive than

the wild type upon heating at 52°C. The viability of the wild type strain was 31% whereas that of the mutant was only 0.1% (Pauling and Beck 1975). Single strand breaks occur in both strains but in the wild type these are repaired by the action of DNA ligase whereas in the ligase-defective mutant the breaks cannot be repaired. Other DNA repair-deficient strains, such as *rec* and *uvr* mutants of *E. coli* and *Salmonella typhimurium* show increased heat sensitivity compared with the wild type parents (Russell 1984). These mutants are also more sensitive to u.v. radiation, suggesting that the products of *rec* and *uvr* genes may function in the repair of the heat induced damage to DNA in a similar way to that observed after u.v. irradiation. The *uvr*$^+$ genes are involved in excision repair, where an endonuclease recognises regions of distortion in the DNA helix and makes two cuts in the vicinity of each region. The excised DNA segment is replaced with a new strand by the action of DNA polymerase I. Then, joining of the newly synthesised segment takes place by DNA ligase.

The *rec*$^+$ genes are involved in recombination repair where a functional DNA molecule is reconstructed from undamaged fragments. During replication of partially damaged DNA, the replication fork is temporarily stopped when DNA polymerase III reaches a region of DNA distortion. However, DNA synthesis can be initiated beyond these regions resulting in daughter strands with large gaps. The

gaps are repaired by recombination, where a single-stranded segemnt is excised from one of the parental strands and inserted into the gap. The RecA product is required for this process. (Freifelder 1987).

Repair of thermally-induced DNA strand breaks is affected by the type of medium in which heated bacteria recover. Bacteria injured by heating have a reduced ability to grow on complex but not on minimal agar. However, incubation in liquid minimal medium after heat treatment increases the survival of heated cells on complex agar medium indicating that repair took place in the minimal medium. Incubation of heated bacteria in complex liquid medium results in very limited repair. The molecular weight of the DNA of heated cells recovered in complex medium was lower than that of unheated cells, whereas the integrity of the DNA of heated cells exposed to minimal medium after heat treatment was not affected (Pierson *et al*. 1978).

The double-stranded helical form of DNA is the predominant form in bacteria grown at physiological temperatures. This DNA structure is stabilised by hydrogen bonds between the bases of the opposite strands, hydrophobic interactions between bases of the same strand, and electrostatic forces between the phosphate groups. Exposure of DNA to high temperature *in vitro* causes

breakage of the hydrogen bonds and denaturation of DNA. However, association of DNA with other molecules such as polypeptides or polyamines increases the stability of DNA secondary structure. In E. coli binding of polyarginine results in DNA melting temperature (Tm) of 92°C whereas Tm of the free DNA is 50-52°C (Pellon and Sinskey 1984).

The effects of heat on DNA tertiary structure (the nucleoid or the supercoiled chromosome) were investigated by Pellon et al. (1982). Nucleoids isolated from cells heated at 50°C had low sedimentation coefficient indicating unfolding of the DNA and DNA breakage. Repair of the nucleoid damage is reported and includes two consecutive steps. The first involves the association of the nucleoids with a specific type of proteins resulting in the formation of fast-sedimenting structures. The second step involves dissociation of these proteins resulting in nucleoids with the same sedimentation coefficient as the ones isolated from unheated cells. These proteins seem to be the same proteins synthesised upon exposure of bacteria to heat shock (see the next section). In addition, in vitro association of DNA with proteins stabilises the DNA tertiary structure. Spermidine, a positively charged polyamine ., was reported to increase 5000 fold the renaturation of the unfolded DNA (Pellon et al. 1982).

The fraction of nucleoids that are repaired and acquire

their normal sedimentation coefficient after exposure to high temperature correlates with the percentage of cells that survive the heat treatment, indicating that DNA is, most probably, the primary target of heat action. Failure to repair DNA damage results in cellular death, while other damaged cellular components can be replaced with newly synthesised ones if the DNA is functional and provides the necessary genetic information.

1.4.5. The heat-shock response:

This phenomenon involves the transient induction of synthesis of a specific set of proteins, while synthesis of other proteins is decreased or inhibited, upon a shift from low to high temperature. The *htpR* or *hin* (heat-shock induction) gene controls induction of these proteins. It was reported that *hin* mutants are able to grow at 30 ^{o}C but not at 42^{o}C. Transfer of these mutants from 30^{o}C to 42^{o}C causes only little induction of the various heat-shock proteins, in contrast to *hin*$^{+}$ cells which show a marked induction of these proteins after exposure to a high temperature (Yamamori *et al.* 1982). Heat-shock induction is affected by the level of *hin* gene expression. The amount of heat-shock proteins synthesized is proportional to the level of *hin* gene activity suggesting that *hin* codes for a positive regulatory protein that induces the transcription of several heat-shock genes at high temperature.

Control of heat-shock response in bacteria is at the transcriptional level. It was observed that this response is rifampicin sensitive. Also, measurement of the mRNAs of the different heat-shock proteins revealed that the amount of these mRNAs increased upon transfer of cells to high temperature. The increase in the level of the specific mRNAs is observed within 15 seconds of a shift from 30 to 42°C and their maximum concentration is reached by ~3.5 min. This correlates with the rate of synthesis of heat-shock proteins where maximum synthesis of these proteins occurs after 5-7 min of exposure to a shift to higher temperature (Neidhardt et al. 1982). The nucleotide sequence of htpR(or hin) has a significant similarity with rpoD, the gene that encodes production of sigma (part of the RNA polymerase involved in recognition of promoters under normal cultural conditions). Comparison of the amino acid composition of HtpR and sigma factor reveals about 67% similarity between the two proteins. Similarities in their secondary structures are also reported. Therefore, HtpR has been designated sigma -32 and the normal sigma factor, sigma -70 (Neidhardt and VanBogelen 1987).

It is proposed that the htpR gene product acts in a manner analogous to that of sigma -70 where HtpR protein recognizes the promoters of the heat-shock genes and allows their transcription. Firstly, there is structural similarity between HtpR and sigma -70. Secondly, a close physical

association of HtpR and the RNA polymerase was observed. Thirdly, RNA polymerase containing HtpR allows transcription of heat-shock genes in vitro. Alteration of the relative levels of sigma -32 and sigma -70 in the cell affects the induction of the heat-shock response. Synthesis of heat-shock proteins is increased in a mutant that produces low levels of sigma -70. Also, it was reported that a mutant that overproduces sigma -70 forms reduced amounts of heat-shock proteins (Yamamori et al. 1982). Cells that overproduce sigma -32 show increased induction of the heat-shock response, suggesting a competitive interaction between the two sigma factors. It has been postulated that the transcription and translation of the htpR gene and consequently the heat-shock genes is autoregulatory, where the available concentration of sigma -32 controls the transcription of this gene. Factors that bind or denature this protein cause immediate induction of the heat-shock genes (cited in Ashburner 1982).

Some of the E. coli heat-shock genes have been mapped. The locations of dnaKJ, lon, htpG, grpE, rpoD, groESL and lysU are known (Neidhardt and VanBogelen 1987). These are scattered along the chromosome with htpR gene at 76 min. Seventeen heat-shock proteins were identified in E. coli (Table 4). Their exact function in the heat-shock response is not known, but mutants deficient in these proteins are unable to grow at high temperature. The GroEL

Table 4: Heat shock proteins of *E. coli*

Protein number	Protein name	Gene	Induction[a] ratio	Molecular weight
1	GrpE	*grpE*	9.5	25,300
2	GroEL	*mopA* (*groEL*)	7.9	62,883
3	DnaK	*dnaK*	13	69,121
4	Sigma-70	*rpoD*	ND	70,263
5		*htpE*	74	14,700
6	GroES	*mopB* (*groES*)	19	10,670
7		*htpG*	26	71,000
8		*htpH*	11	33,400
9		*htpI*	ND	48,500
10	Lysyl-tRNA sythetase form II	*lysU*	10	60,000
11		*htpK*	45	10,100
12		*htpL*	6.4	21,500
13		*htpM*	10	84,100
14		*htpN*	56	13,500
15		*htpO*	25	21,000
16	Lon	*lon*	12	94,000
17	DnaJ	*dnaJ*	ND	40,975

a. Determined by dividing the amount of (^{35}S) methionine incorporated into a given protein spot from 3 to 8 min after a shift from 28 to 42°C by the amount incorporated in a 5-min period before the shift. N.D.: not determind.

protein has a weak ATPase activity and sometimes it was found in association with ribosomes. Most groEL mutants show restricted DNA and RNA synthesis accociated with temperature sensitive growth (Wada and Itikawa 1984). DnaK protein has a 5' -nucleotidase activity and a weak ATPase activity. DnaJ protein has been purified and reported to reside in the cell envelope. The grpE mutant cells fail to grow at high temperature and show reduced nucleic acid synthesis (Ang et al. 1986). Lon protein has an ATP- dependent protease activity . Sigma- 70 is a heat inducible protein with a regulatory function. Although the heat-shock response is inversely related to the cellular level of sigma- 70, the induction of synthesis of this protein upon exposure to high temperature controls the expression of the heat-shock response by preventing continuous synthesis of the heat-shock proteins.

The exact role of the heat-shock response is not fully understood. Several observations associated this phenomenon with increased cellular resistance upon exposure to high temperature, protein degradation, macromolecular synthesis and cell division. Cells exposed to 42^{o}C for a short period before being subjected to heating to lethal temperature (55^{o}C) have a slower death rate than cells directly heated to 55^{o}C indicating that heat-shock response provides cellular thermotolerance. However, this resistance to high temperature is transient

and disappears after about 60 min. Accumulation of abnormal proteins in the cell may induce synthesis of the heat-shock response. It was reported that the heat-shock proteins are involved in degradation of intracellular proteins. The rate of proteolysis increased when E. coli cells were shifted to 42°C and this activity was abolished in htpR mutants. This proteolytic activity was attributed to induction of Lon and other proteins of the heat-shock system; double mutants of htpR and lon have less proteolytic activity than lon mutants (Neidhardt and VanBogelen 1987). Also, it was demonstrated that mutants deficient in the heat-shock response are incapable of cell division at 42°C. Cell division is inhibited in htpR mutants after a shift to 40°C (Zhou et al. 1988).

1.5. Aims of this study

Pathogenic strains of E.coli are associated with several types of enteric disease and are a common cause of diarrhoea in both adults and children (Formal et al.1976). In children less than 5 years old pathogenic types of E.coli are implicated, as the causative agent of diarrhoea, in 23% of the cases (Yam et al.1987). Various types of pathogenic E.coli are involved, these include enteropathogenic, enterotoxigenic, and enteroinvasive E.coli. Although gastroenteritis is usually a self-limited disease, it can develop into more complicated disease especially among debilitated people, infants, elderly and malnourished. For example, enteroinvasive E.coli strains which penetrate the epithelial cells of the intestine causing haemorrhagic colitis may enter the blood stream and infect other organs (e.g.kidneys) (Chart et al.1989). E.coli strains harbouring the ColV plasmid are more pathogenic than the isogenic p^- strains and they possess enhanced ability to survive in blood and body tissues(section 1.3.).

Contaminated animals and animal food products are the main source of infection of man with food borne diseases. Efficient meat processing is an important factor in providing bacteria-free meat to consumers. Heat is frequently used during food production (e.g.during slaughtering, cooking); consequently,the heat sensitivity of bacteria is an important element in their survival

after meat processing.

Therefore, this study is carried out to investigate the following:

a. The heat sensitivity of ColV-bearing strains compared with that of isogenic strains lacking the ColV plasmid, and an investigation of the sites of heat action on both strains.

b. Some of the conditions that may affect the thermal resistance of $ColV^+$ and $ColV^-$ cells.

c. The incidence of $ColV^+$ bacteria in samples from chicken processing plants and a study of their properties and heat resistance.

Because most of the organisms on or in chicken carcasses are likely to be in the stationary growth phase, organisms from this growth phase have been tested in most detail.

2. Materials and Methods

2.1. Bacterial strains and plasmids:

The properties of the strains and plasmids used in this study are presented in Tables 5 and 6. The characteristics of isolates from the chicken-processing plant are discussed in the Results. Bacterial strains were maintained on nutrient agar slants, stored at $4^{o}C$ and subcultured regularly every 2 months.

2.2. Media:

Oxoid nutrient broth No.2 was used at a concentration of 25 g/l as a growth medium in most of the experiments. In some experiments, L-broth was used. It is composed of bacto tryptone 10.0 g, yeast extract 5.0 g , NaCl 5.0 g and glucose 2.0 g/ litre. MacConkey agar was used as a selective medium and consists of peptone 20.0 g, lactose 10.0 g, bile salts 5.0 g, NaCl 5.0 g, neutral red 0.075 g and agar 12 g/ litre. The minimal medium used was that of Davis and Mingioli (1950). It contains the following / litre: K_2HPO_4 7.0 g, KH_2PO_4 3.0 g, sodium citrate 0.5 g, $MgSO_4.7H_2O$ 0.1 g and $(NH_4)_2SO_4$ 1.0 g. Glucose or galactose were added at a concentration of 0.2% and DL-lysine at a concentration of 200 µg / ml where required. For solidification of media, 2% Difco bacto agar was added. Soft agar consists of nutrient broth 2.5 g, $MgCl_2.6H_2O$ 0.5 g, NaCl 0.75 g and agar 1.9 g /250 ml. Phosphate buffer

Table 5: Characteristics of *Escherichia coli* strains

Strain	Genotype and properties	Source or reference
ED1829	*trp*	Finnegan&Willetts(1971)
P678-54	*thr*, *leu*, *thi*, *lacY*, S^R *ara*, *xyl*,	Adler *et al.* (1967)
LE1	*lys*, unable to use galactose as sole source of carbon	Rothfield & Pearlman-Kothencz (1969)
AB1157	*thr*, *leu*, *thi*, *ara*, S^R *arg*, *xyl*, *lacY*, *his*	Bachmann (1987)

S: Streptomycin

ara: arabinose, *arg*: arginine, *his*: histidine, *leu*: leucine, *lys*: lysine, *thi*: thiamine, *thr*: threonine, *trp*: tryptophan, *xyl*: xylose

Table 6: Plasmids used and their main properties.

Plasmid	Properties
ColV-K30	IncFI, colicin V (& immunity), derepressed F-like transfer, VmpA protein
ColV,I-K94	IncFI, colicins V and Ia (&immunity), derepressed F-like transfer, VmpA protein
ColV-M40(5)	Derivative of ColV, I-K94, no colicin production or immunity
ColV-M50(1)	Derivative of ColV, I-K94, tra$^-$
F*lac*	IncFI, derepressed F-like transfer
R 124	IncFIV, repressed transfer, Te^R
R483 ColIa	IncI, repressed transfer, colicin Ia, S^R, Tp^R

S: streptomycin, Te: tetracycline, Tp: trimethoprim

(1.3 M, pH 7.4) was used in some tests. It was composed of K_2HPO_4 46.7 g and KH_2PO_4 6.0 g/ 250 ml. The solution was diluted 1:20 to obtain 0.065 M phosphate buffer.

2.3. Growth and preheating conditions:

2.3.1. General conditions:

Experiments were performed using bacteria in the stationary or exponential phases. To obtain organisms in the stationary phase, bacterial strains were grown in 100 ml flasks containing 20 ml nutrient broth for 16-18 hr at 37°C or 25°C with shaking. For exponential phase organisms, one ml of this stationary bacterial culture was added to 30 ml fresh nutrient broth and incubated shaken for $1\frac{1}{2}$ hr at 37°C or for $2\frac{1}{2}$ hr at 25°C. The aeration of bacteria was at the same rate in all experiments; the speed of the shaking water bath was maintained at constant rate of 45 strokes / min.

2.3.2. Growth in magnesium - depleted or enriched media:

For culturing bacteria in magnesium - depleted medium, cells were grown overnight at 37°C with shaking in nutrient broth containing 0.065 M phosphate buffer (pH 7.4). Phosphate ions interact with magnesium to form an insoluble phosphate causing depletion of the Mg^{++} of the medium. For growth in magnesium-enriched medium, cells were grown in nutrient broth containing 0.05 M $MgSO_4 . 7H_2O$ overnight at 37°C with shaking. Cells were

then subjected to high temperature as described in section 2.4.

2.3.3. Exposure to gradual rise in temperature before heating:

One ml of the appropriate bacterial culture was diluted into 10 ml nutrient broth. The bacterial suspension was exposed to gradual rise in temperature from 34-50^{o}C at a rate of 1.3^{o}C / min. One ml of this bacterial suspension was added to 10 ml preheated (65^{o}C) nutrient broth and heated at 60^{o}C for 5 min. One ml samples were removed after 0 and 5 min heating and diluted into 9 ml nutrient broth at room temperature. The bacteria were recovered as described in section 2.5.1.

In control experiments, bacterial suspensions were exposed to rise in temperature from 34-37^{o}C prior to heating. The bacterial cultures were heated to 60^{o}C and the percentages of survival and injury were measured using the procedure mentioned above.

2.4. Heat treatment:

Heating was carried out in 100 ml flasks containing 10 ml nutrient broth preheated to 55^{o}C or 65^{o}C . One ml of the appropriate bacterial culture at 20^{o}C was added to this medium and incubated in a static water bath at 52^{o}C or 60^{o}C. One ml samples were removed at intervals

and immediately diluted into 9 ml nutrient broth at room temperature.

2.5. Recovery conditions:

2.5.1. General conditions:

After heating cells by the method described in section 2.4., samples were diluted in 0.75% sodium chloride at room temperature. Samples(0.1 ml)of the appropriate dilutions were spread on solid complex media such as nutrient agar for measuring the percentage of survival, and nutrient agar containing 0.5% sodium deoxycholate for assessing the percentage of injury. The colonies were counted after incubation of the plates at 37°C for 18-24 hr.

2.5.2. Recovery on media supplemented with catalase or DL-pantoyllactone:

Plates of nutrient agar containing catalase were used as a recovery medium in some experiments. A stock solution of catalase was sterilized separately by filtration, and a suitable volume was spread on the surfaces of 20 ml dry nutrient agar plates such that a final concentration of 3,000 units of catalase / plate (150 units / ml) was obtained. For recovery on media supplemented with DL-pantoyllactone, this compound was added to sterile nutrient agar at a concentration of 0.05 M. The appropriate dilutions of heated bacterial suspensions were spread on these media and colonies were

counted after incubation at 37°C for 18-24 hr.

2.5.3. Storage in minimal medium:

Cells were heated to 60°C as described in section 2.4. One ml samples of the heated bacterial cultures were removed at intervals and diluted into 9 ml liquid minimal medium without carbon source. The bacterial suspension was maintained in minimal medium for 120 min at 20°C. Then, cells were plated on nutrient agar and deoxycholate nutrient agar and colonies were counted after incubation for 18-24 hr at 37°C. In control experiments, one ml samples of the heated bacterial cultures were added to 9 ml liquid minimal medium without carbon source and plated immediately on nutrient agar and deoxycholate nutrient agar.

2.6. Estimation of materials absorbing at 260 nm:

Bacterial cultures were heated as described in section 2.4. in 0.75% sodium chloride at 60°C. Samples were removed at intervals and the cells were sedimented by centrifugation. The absorption of the supernatants of experimental and control cultures was then measured at 260 nm using a Cecil spectrophotometer.

2.7. Estimation of gentian violet uptake:

The procedure of Gustafsson et al. (1973) was used with some modifications. Bacterial strains were heated as

described previously (section 2.4.). One ml samples were removed after 0,5,10 and 15 min heating at 60°C and added to 9 ml fresh nutrient broth. Gentian violet was added to the bacterial suspensions to a final concentration of 5µg / ml and the flasks were incubated at 37°C with shaking for 15 min. After sedimenting cells by centrifugation at room temperature, the amount of gentian violet remaining in the supernatant was measured at 590 nm using a Cecil spectrophotometer.

2.8. Measurement of lipopolysaccharide release:

The amount of the lipopolysaccharide was estimated using the method of Hitchener and Egan (1977) with some modifications. LE1 and LE1 ColV,I-K94 strains were used; these cells incorporate low concentrations of exogenous galactose solely into the lipopolysaccharide, thus LPS can be specifically labelled and its release estimated. The strains were grown in nutrient broth + 0.3% yeast extract overnight at 37°C with shaking. The bacterial cultures were then diluted into fresh medium to optical density of 0.06 (Hilger photoelectric colorimeter; filter, 52). 0.02 mM D-(1-^{3}H) galactose (1.0 mCi / ml) was added to the cultures, and incubation was continued until turbidity reached 0.3 O.D. One ml of this bacterial culture was added to 10 ml nutrient broth (preheated to 65°C) and the mixture was incubated at 60°C. One ml samples were removed at intervals and cooled to room temperature.

For estimattion of the total lipoplysaccharide in the suspension of heated and unheated cells, 0.2 ml from each sample was pipetted onto a filter paper which was dropped into 2 ml cold 5% trichloroacetic acid (TCA). The cells were removed from the remainder of the 1 ml samples by centrifugation and 0.2 ml of the supernatant was added to 0.8 ml cold 5% TCA to estimate the amount of the released LPS. The mixtures were maintained at 4°C for ~30 min, then TCA was decanted and the filter papers were dried and placed in scintillation vials containing 2 ml of the scintillation reagent (0.6% butyl-PBD in toluene). The radioactivity was then measured in a liquid scintillation counter.

2.9. Electron microscopy:

The bacterial strains were heated as described in section 2.4. Samples were removed at 0 and 5 min, diluted (1:5) into fresh nutrient broth and grown at 37°C with shaking until marked increase in optical density was observed ($2\frac{1}{2}$ hr for samples removed after 0 min heating or $3\frac{1}{2}$ - $4\frac{1}{2}$ hr for samples heated for 5 min). For examination under the electron microscope, the bacterial cultures were centrifuged, washed twice with distilled water, and resuspended in distilled water. Then, a drop of each cell suspension was placed on a carbon-formvar coated grid and mixed with one drop of 2% phosphotungstic acid (pH 7.2). The excess was removed with filter paper and the mixture

was allowed to dry before examination under the electron microscope (Siemens).

2. 10. Assessment of bacterial growth after heating:

Bacteria were heated as described previously (see section 2.4.). Samples were removed after 0 and 2 min heating and diluted (1:5) into nutrient broth, nutrient broth containing 0.5 % sodium deoxycholate or containing 0.01M ethylenediaminetetra-acetic acid. The bacterial suspensions were then grown for 5-6 hr at 37°C with shaking, samples were removed at intervals and optical density was measured using a Hilger photoelectric colorimeter (filter 52).

2.11. Assay of stability of the ColV plasmid after heating:

$ColV^+$ cells were heated at 60°C for 15 min, samples were removed after 0 and 15 min heating, diluted and plated on nutrient agar. The resulting colonies were tested for colicin V production and the percentage of $ColV^+$ cells was calculated.

2.12. Identification of strains from the chicken processing plant:

The isolates were obtained from the chicken carcass scalding tanks. The strains were streaked on MacConkey agar to differentiate lactose negative and positive strains. Colonies of lactose-fermenting strains (appear red on this

medium) were subjected to further confirmatory tests using the API 20E identification system. In this system, identification of bacteria from a single colony can be performed rapidly (18-24 hr). The identification involves monitoring the reaction of a specific strain to 21 biochemical tests and comparing the results with the identification table.

2.13. Assay of colicin production:

The tested strain was streaked on nutrient agar containing 0.065 M phosphate (pH 7.4). After incubation overnight at 37°C, the strain was exposed to chloroform vapour for 10-15 min. From treated plates, the vapour was allowed to evaporate for 10 min, then the treated strain was overlaid with 4 ml soft agar containing 0.3 ml of overnight grown indicator strain. After incubation for 16-18 hr at 37°C, the plates were examined for appearance of zones of growth inhibition.

This method can also be used for testing the immunity of a particular strainto a certain colicin.In this study, for example, ED 1829 ColV-K30, ED 1829 R483 ColIa, or ED 1829 ColV,I-K94 were streaked on nutrient agar + 0.065 M phosphate (pH 7.4), treated as previously described and overlaid with the tested strain to detect the immunity to colicin V, colicin Ia or colicins V and Ia respectively. Phosphate (0.065 M) enhances colicin production

by some strains (Rowbury & Hicks 1987).

2.14. Antibiotic sensitivity testing:

The strains were tested for antibiotic sensitivity by the disc diffusion method. The bacteria were grown overnight at 37°C with shaking. 0.3 ml of this culture was added to 4 ml soft agar and overlaid on nutrient agar plates. The antibiotic discs were then placed on the surface of the inoculated plates aseptically; the plates were incubated at 37°C for 16-18 hr. The cells were considered sensitive to a particular antibiotic if a zone of growth inhibition appeared around the antibiotic disc and resistant if the zone of growth inhibition was not observed.

2.15. Transfer of plasmids:

2.15.1. Conjugation:

Two methods were used

a. Liquid medium matings:

The appropriate recipient and plasmid-bearing strains were grown to the exponential phase at 37°C with slow shaking. Then, the recipient and donor cells were added in 2:1 ratio to 10 ml fresh nutrient broth and incubated at 37°C for $1\frac{1}{2}$ - 2 hr with slow shaking. The bacterial mixture was washed in saline, diluted in 0.75% sodium chloride and plated on the appropriate selective medium. The colonies were then picked in duplicate onto

nutrient agar + 0.065 M phosphate plates and tested for colicin V production.

b. Membrane-filter matings:

The donor and recipient strains were grown to the exponential phase at 37°C with slow shaking. One ml of the donor and 1 ml of the recipient culture were filtered through a 0.22 µm membrane filter. The filter was placed on the surface of a nutrient agar plate and incubated at 37°C overnight. The cells were then dislodged into 9 ml of 0.75% saline and dilutions of this bacterial suspension were plated on the appropriate selective media. The resulting colonies were tested for colicin V production as described previously.

The selective media should counter-select the donor cells and, if possible, distinguish recipients from transconjugants. In this study, selection against the donor strain was achieved by addition of an antibiotic to which the donor cells are sensitive or plating on a minimal medium that does not support growth of donor cells. In the transfer of the ColV plasmid from ED 1829 ColV,I-K94 to LE1 strain, this medium was minimal agar containing glucose (0.2%), galactose (0.2%) and L-lysine (200 µg/ml). In transferring the same plasmid from ED 1829 ColV,I-K94 to a wild type E. coli isolate (strain 14), the selective medium was MacConkey agar where colonies of the donor

strain appear yellow and colonies of the recipient appear red. In transferring the ColV plasmid from ED 1829 ColV, I-K94 to AB 1157 , dilutions of the bacterial mixture were plated on nutrient agar containing streptomycin (200 mg / l). In all transfer experiments the recipient and donor strains were plated on the selective media to ensure their ability or inability, respectively, to grow on such media.

2.15.2. Transformation:

a. Isolation and purification of plasmid DNA:

The plasmid DNA was extracted from bacterial cells by the procedure described in section 2.16. The DNA was then separated, according to its molecular weight, by electrophoresis through agarose gels. The required DNA band was recovered using the method of Maniatis et al. (1982) with some modifications. This method involves electroelution of the specific DNA band into troughs cut into the gel. The DNA was visualized using a long wavelength U.V. light, and using a sharp scalpel a trough was cut directly in front of the leading edge of the band and ca 2 mm wider than the band on each side. A dialysis tube (of the same width as the trough) was inserted into the trough.The trough was then filled with electrophoresis buffer and electrophoresis was resumed. After the DNA band had moved into the dialysis tube, the polarity of the current was reversed for 2 min to release the DNA from

the wall of the dialysis tube, and the solution (containing the DNA) was recovered with micropipette.

DNA was purified by extraction with phenol / chloroform to remove ethidium bromide, then with chloroform. One ml of ethanol was added and DNA was recovered by centrifugation. The DNA pellet was resuspended in 100 µl of 10 mM Tris-acetic acid- 2mM EDTA (pH 8.0).

b. Permeabilisation of bacterial cells:

A 50 ml bacterial culture, grown to the exponentional phase, was harvested by centrifugation (9000 rpm, 15 min, 4°C) and resuspended in 25 ml of 0.1 M $CaCl_2$. The suspension was maintained at 0°C for 20 min; then cells were collected (9000 rpm, 15 min, 4°C), resuspended in 3ml 0.1 M $CaCl_2$ and stored in 0.2 ml aliquots at 4°C for 18 hr to increase formation of highly competent cells. The purified plasmid DNA was added and the mixture was 'heat-shocked' at 42°C for 2 min. The suspension was returned to 0°C for 5min, then 2 ml of fresh nutrient broth was added. The mixture was incubated for 2 hr at 37°C static to allow plasmid establishment. Then, dilutions were plated on the appropriate selective medium.

In this study, transformation of ED 1829 F<u>lac</u> with ColV plasmid DNA from a wild type <u>E.coli</u> strain was attempted. ColV plasmid is incompatible with and dominant

to F*lac* , therefore the introduction of the ColV plasmid into ED 1829 F*lac* cells causes the appearance of a Lac^- phenotype in the recipient strain. Accordingly, dilutions of the bacterial suspension were plated on a medium containing lactose (e.g.MacConkey agar). The plates were incubated at 37°C overnight and examined for appearance of yellow colonies.

2.16. Plasmid isolation:

The rapid procedure of Takahashi and Nagano (1984) was used. All the solutions were prepared in double distilled water; the stock lysing solution consisted of 4% sodium dodecyl sulphate in 100 mM Tris, stored at room temperature, and was mixed with an equal volume of freshly prepared 0.4M NaOH immediately before use. Stock buffer A was composed of 400 mM Tris - acetic acid and 20 mM disodium EDTA (pH 8.0); for use, it was diluted 10 times. Buffer B was composed of 3M sodium acetate - acetic acid (pH 5.5). Buffer C consisted of 10 mM Tris - acetic acid and 2mM disodium EDTA (pH 8.0). All buffers were autoclaved at 115°C for 10 min and stored at 4°C.

The bacterial cultures were harvested by centrifugation at room temperature at 9000rpm for 10min, and 200µl of buffer A was added to cell pellet. The pellet was suspended thoroughly and transferred to a polypropylene centrifuge tube. 400 µl of the lysing solution was added to the cell suspension and

the tube was gently inverted 5-10 times and allowed to stand at room temperature for 5 min. Then, 300µl of cold (4°C) buffer B was added and gently inverted 10-20 times to neutralize the mixture. The tube was maintained at 0°C for 5 min, centrifuged at room temperature and maintained again at 0°C for 10 min. Salt-precipitated material was centrifuged at room temperature, and the supernatant was transferred to another tube. An equal volume of chloroform was added and mixed by inversion 5-10 times. The tube was then centrifuged at room temperature and 500µl of the upper aqueous phase was carefully transferred to another tube. One ml of cold (-20°C) ethanol was added and inverted 5-10 times. The tube was maintained at 0°C for 5 min and the precipitated DNA was collected by centrifugation at room temperature. The pellet was dissolved in 100µl of buffer C and stored at -20°C.

2.17. Agarose gel electrophoresis:

Electrophoresis was carried out in a horizontal minigel apparatus. Agarose type II (Sigma) was used at a concentration of 0.7% and melted in Tris-borate buffer by heating at 110°C for 5 min in an autoclave. After cooling to 50°C, the agarose was poured into a sealed glass plate (7.5 x 5 cm) and allowed to set (30-45 min at room temperature). Then, electrophoresis buffer (Tris-borate:0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA)

was added to cover the gel to a depth of ca 1 mm . Samples were prepared for analysis by mixing 25 µl aliquots with 10 µl of the loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 15% Ficoll in water) and loaded into the wells of the submerged gel. Electrophoresis was performed at 14 V/ cm for 50 min or until the bromophenol blue migrates almost the full length of the gel. For staining, the gel was immersed in electrophoresis buffer containing 0.5 µg/ml ethidium bromide for 45 min at room temperature, and washed with distilled water. The DNA bands were then visualized under u.v. light.

2.18. Plasmid curing:

Two methods were used

a. Curing by high temperature and SDS:

Bacterial strains, grown to the stationary phase, were diluted to ca 10^5 cells / ml into fresh nutrient broth containing 1% sodium dodecyl sulphate, and incubated at 37°C for 48 hr with shaking. 0.1 ml of this culture was added to 10 ml fresh nutrient broth and incubated static or shaken at 44°C for 48 hr. Dilutions of the bacterial culture were then plated on nutrient agar, and the resulting colonies were tested for loss of colicin production.

b. Curing by rifampicin:

The method of Bazzicalupo & Valentini (1972) was used.

Exponentially-grown bacterial strains were diluted to ca 10^3 cells / ml in nutrient broth containing 5 µg/ml or 8 µg / ml rifampicin and incubated overnight at 37°C with gentle shaking. The bacterial culture was then diluted in 0.75% saline and plated on nutrient agar and the resulting colonies were tested for loss of colicin production.

To confirm the loss of the ColV plasmid, DNA from the non-colicin producing (presumably cured) cells was isolated and subjected to agarose gel electrophoresis.

2.19. Isolation of cell envelopes:

Bacteria, grown to the stationary phase, were harvested by centrifugation at room temperature (9000 rpm, 15 min) and washed in 5 ml 0.9% sodium chloride. The cells were then resuspended in 1.4 ml of 50 mM Tris-HCl (pH 8.5), 2 mM EDTA and frozen at -70°C for 15 min. The suspension was thawed at room temperature and sonicated six times (15s each) using an MSE ultrasonic power unit at a current of 1.5 amps. Unbroken cells and debris were removed by centrifugation for 3 min at 9000 rpm in an MSE microcentrifuge. The supernatant was then centrifuged at 12000rpm for 15 min to collect the cell envelopes. The pellet was suspended in 20 µl 2 mM Tris and stored at -20°C.

2.20 Polyacrylamide gel electrophoresis:

The vertical gel apparatus of Laemmli (1970) was used. The separating gel consisted of 14 ml stock acrylamide (30% acrylamide and 0.8% bis-acrylamide), 20 ml 0.75 M Tris (pH 8.8), 0.4 ml 10% SDS, 3.6 ml distilled water, 2 ml of freshly prepared 10 mg / ml ammonium persulphate and 12 µl TEMED (N, N, N', N' - tetramethylene diamine). The stacking gel consisted of 3 ml stock acrylamide, 10 ml 0.25 M Tris (pH 6.8), 0.2 ml 10% SDS, 5.8 ml distilled water, 1 ml 10 mg / ml ammonium persulphate and 6 µl TEMED.

Samples are prepared for electrophoresis by mixing 10 µl aliquots with 40 µl sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS,10% glycerol, 0.001% bromophenol blue and 5% 2-mercapto-ethanol) and incubating the mixture either at 100^{o}C for 5 min or at 37^{o}C for 15 min. For estimation of the apparent molecular weights, a mixture of standard proteins (4 mg / ml) of known molecular weights were added to the sample buffer (1:4 ratio); bovine serum albumen (68000d), ovalbumen (43000d) and lysozyme (14000d) were used.

Electrophoresis was run at 25 mA for 5-6 hr using an electrode buffer composed of 25 mM Tris, 200 mM glycine and 0.1% SDS. The gels were stained overnight in 10% glacial acetic acid, 50% methanol and 0.1% Coomassie

Brilliant Blue R250 and destained in several changes of a solution containing 10% glacial acetic acid and 40% methanol.

2.21 Lysozyme lysis:

The appropriate bacterial strains were grown to the stationary phase at 37°C with shaking, and heated at 60°C for 2 min using the procedure described in section 2.4. Samples were removed after 0 and 2 min heating and bacteria were collected by centrifugation, washed with 0.033 M Tris-HCl (pH 8.0) and suspended in the same buffer to optical density of 0.3- 0.4. Lysozyme at 0.05 mg/ ml (made up in 0.033 M Tris-HCl (pH 8.0)) was added to all bacterial suspensions, and EDTA (270 µg / ml) was added where required. The mixtures were incubated at 37°C static and the optical density of the suspensions was measured at intervals using Hilger photoelectric colorimeter.

2.22 Assay for β-galactosidase:

Stationary phase grown cells were diluted (1:2) into fresh nutrient broth. To induce the production of β- galactosidase, isopropyl-β-D- thiogalactoside was added at a final concentration of 10^{-3} M and the mixture was incubated shaken at 37°C for 30min. The bacterial suspension was then heated at 60°C for 5 min and toluene treated and untreated samples were assayed for

β- galactosidase.

The activity of the enzyme was quantitated spectrophotometrically by measuring the release of o-nitrophenol from o-nitrophenyl-β-D-galactoside (ONPG). The assay mixture consisted of 1 ml bacterial culture, 0.4 ml 12 mM ONPG and 1 ml nutrient broth. The mixture was incubated at 37°C for 15 min, then 1 ml of 1M Na_2CO_3 was added to terminate the reaction. The cells were removed by centrifugation and the amount of o-nitrophenol was measured at 420nm using a Cecil spectrophotometer.

3. Results

3.1 Studies on the heat sensitivity of ColV,I-K94-plasmid bearing cells.

3.1.1. Comparison between the heat sensitivity of ColV$^-$ and ColV$^+$ cells.

Exposure of food (during processing or cooking) to high temperature is one of the major methods used in eliminating bacteria. During poultry processing, the first step is immersion of birds in hot water to remove bacteria adherent to the external surfaces of birds and prevent cross- contamination of other birds that are originally free from bacteria. Therefore, efficient destruction of bacteria during this step is important in controlling the spread of bacteria among chicken carcasses. Subsequently, thorough cooking of the carcasses serves to eliminate any surviving surface bacteria, as well as those buried in the carcass.

The temperature of the scalding tanks is usually either 52^{o}C or 60^{o}C. The heat sensitivity of the ColV plasmid bearing bacteria was,therefore, examined at these two temperatures. Although internal temperatures during cooking should be higher than this, this is often not so and the lower temperatures, therefore, will reflect those during light cooking also. Initially organisms grown at 37^{o}C were tested. Figure 10 shows the survival curves of ColV$^+$ and ColV$^-$ strains after heating at 60^{o}C.

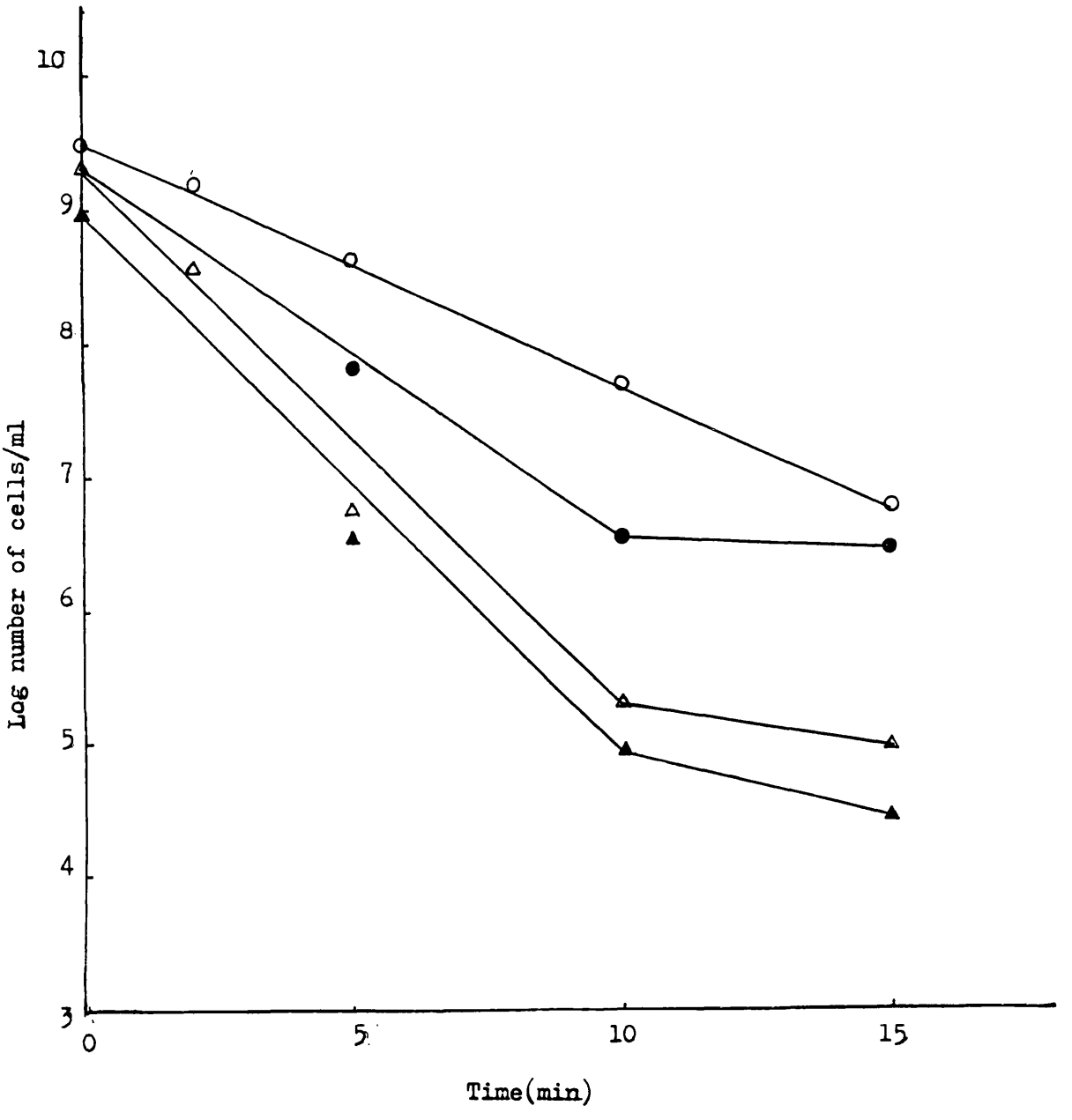


Fig 10: Effect of the ColV plasmid on survival at 60°C. Bacteria were grown to the stationary phase in broth with shaking at 37°C, heated to 60°Cand plated on nutriet agar. △ED1829ColV,I-k94, o ED1829, ▲ P678-54ColV,I-k94, ● P678-54.

In strains ED1829 and P678-54 the introduction of the ColV,I-K94 plasmid resulted in enhanced sensitivity of these strains to heat. In strain ED1829 the exponential rate of decrease in number of cells is constant throughout the heating period. The death rate of the other strains is constant during the first 10 min of heating after which a slower death rate is observed. This may be because the compounds released from dead cells (e.g. nucleotides and amino acids) can be utilized by the viable cells, thus readily metabolisable nutrients are available to the cells causing a decrease in their death rate. Alternatively there may be a small number of cells which are phenotypically more resistant e.g. because of their stage in the cell cycle. Or there may be resistant mutants (although the numbers of more resistant cells seems rather high for this).

Table 7 presents the percentages of survival and injury of $ColV^+$ and $ColV^-$ cells after heating at 60^oC. In strain ED1829, the presence of the ColV,I-K94 plasmid caused a 43 fold decrease in survival of bacterial cells after heating at 60^oC for 5 min. After heating for 10 min, 160-fold decrease in the survival of the $ColV^+$ strain was observed. Also, introduction of the ColV plasmid into another *E. coli* K-12 strain (P678-54) enhanced the sensitivity of that strain to heat. After heating for 15 min at 60^oC, P678-54 ColV,I-K94 cells

Table 7: Effect of the ColV plasmid - encoded components on the host's heat sensitivity

Strain	Exposure to heat for 2 min		Exposure to heat for 5 min		Exposure to heat for 10 min		Exposure to heat for 15 min	
	%survival*	%injury**	%survival	%injury	%survival	%injury	%survival	%injury
P678-54	ND	ND	3.5	95	0.3	93	0.1	94
P678-54ColV, I-K94	ND	ND	0.7	98	0.01	96	0.003	98
ED1829	70	78	13	92	1.6	97	0.2	98
ED1829ColV, I-K94	44	86	0.3	82	0.01	98	0.004	99
ED1829ColV-M40(5)	67	89	21	94	9.5	97	0.9	96
ED1829ColV-M50(1)	73	82	22	99	2	99.6	0.1	99.6
ED1829 cured	ND	ND	17	71	10	91	1	91
ED1829 R124	ND	ND	27	61	10	79	6	94
ED1829ColV, I-K94,R124	ND	ND	35	94	14	97	2	98

Table 7 continued

Organisms were grown to the stationary phase in broth at 37°C, heated at 60°C and treated as described in Materials and Methods.

* Values represent percentages of the initial cell number.

** Values represent percentage of injured cells with respect to the number of cells survivingat the corresponding time (i.e. %injury= $\frac{\text{no. of injured cells at certain time}}{\text{no. of cells survivingat that time}}$ X 100).

N.D.: not determined.

Each experiment was performed three times with consistent results.

were 33 times more sensitive than the isogenic strain lacking the plasmid. The thermal sensitivity of the cured strain was also tested. Table 7 shows that this strain has similar heat sensitivity to the parental ColV$^-$ strain although the percentage survival after 1o min was higher. This indicates that the plasmid was not introduced into a variant cell that was originally heat sensitive and confirms the effect of the ColV plasmid on the enhanced heat sensitivity of the ColV$^+$ cells.

The heat sensitivity of ColV$^+$ and ColV$^-$ cells was also tested after exposure to 52^{o}C. The ColV$^+$ strain was 6 times more sensitive than the ColV$^-$ one after heating at this temperature for 30 min. More than half of the initial population of ED1829 were able to form colonies on nutrient agar whereas only 9% of the initial ColV$^+$ population survived the heating at 52^{o}C for 3o min (Table 8A). There appeared to be a population of organisms (ca 10%) in the ColV$^+$ culture which was much more resistant to 52^{o}C exposure than the rest.

In this study, enumeration of heat stressed bacteria is performed by plating on nutrient agar and nutrient agar containing deoxycholate to assess the percentages of survival and injury. However, it has been suggested that the presence of hydrogen peroxide in media used

Table 8 : Effect of the ColV, I-K94 plasmid on survival at 52°C

Strain	Growth temperature	Exposure to heat for 10 min		Exposure to heat for 20 min		Exposure to heat for 30 min	
		%survival*	%injury**	%survival	%injury	%survial	%injury
A. ED1829	37	81	97	79	98	54	99
ED1829ColV, I-K94	37	10	79	10	99	9	97
B. ED1829	25	56	ND	47	ND	34	ND
ED1829ColV, I-K94	25	54	ND	44	ND	28	ND

Organisms were grown to the stationary phase in broth at 37°C (A) or 25°C (B), heated to 52°C and treated as described in Materials and Methods.

* Values represent percentages of the initial cell number.

** Values represent percentage of injured cells with respect to the number of cells survivingat the corresponding time (i.e. %injury=

$$\frac{\text{no. of injured cells at certain time}}{\text{no. of cells survivingat that time}} \times 100).$$

N.D.: not determined.

to enumerate bacterial cells may be inhibitory to stressed cells, and plating of stressed bacteria on media supplemented with catalase can enhance their survival. This possibility was tested by plating heated cells on nutrient agar containing 150 U/ml catalase (Table 9). After heating at 60°C for 5 min, the percenatge of survival of the ColV$^-$ strain as measured by plating on nutrient agar (0.8/75 =1.1%) is similar to that obtained when nutrient agar + catalase(1.1%) is used as the plating medium. for strain ED1829 ColV,I-K94, the percentage of cells survivingthe heating at 60°C for 5 min is two fold higher when plating on nutrient agar + catalase (0.19%) than when plating on nutrient agar(0.18/173 =0.1). However, the number of unheated ColV$^+$cells plated on NA + catalase is not higher than those plated on NA indicating that unheated ColV$^+$ cells do not show enhanced sensitivity to hydrogen peroxide. Heating may inactivate the enzyme, catalase, inside the cell or increase the cellular permeability to H_2O_2, thus rendering the ColV$^+$ cells slightly more susceptible to the toxic effects of this compound. Consequently, the addition of H_2O_2 degrading substances, such as catalase, slightly increases the survival of these cells. The effect of exposure to high temperature on the integrity of the outer membrane of ColV$^-$ and ColV$^+$ cells is discussed in detail in section 3.2. Thus catalase has a slight effect on the ColV$^+$ cells but

Table 9: Effect of the type of plating medium on enumeration of thermally stressed cells.

Time of heating (min)	ED1829			ED1829ColV,I-K94		
	NA	NA+catalase	NA+DOC	NA	NA+catalase	NA+DOC
		%			%	
0	75	100	33	173	100	12
2	17	16	1.6	45	53	0.7
5	0.8	1.1	0.04	0.18	0.19	0.008

Bacteria were grown to the stationary phase at 37°C, heated to 60°C and plated as described in Materials and Methods. Percentage of enumeration was calculated using the zero-time counts on NA + catalase as 100%.

NA: nutrient agar, NA+catalase: nutrient agar supplemented with catalase (150U/ml), NA+DOC: nutrient agar supplemented with sodium deoxycholate (o.5%).

H_2O_2 is not a major factor in sensitivity.

Heated cells were also plated on nutrient agar containing deoxycholate to calculate the percentage of structurally injured cells. Tables 7 and 8 reveal that heated ColV$^-$ and ColV$^+$ strains show reduced survival on this medium indicating that damage to the outer membrane of both strains has occurred. Gram-negative bacteria are generally not permeable to deoxycholate; heating may alter the outer membrane allowing deoxycholate permeation into the cell, and consequently inability to grow on this medium is observed. ColV$^+$ cells may, however, be somewhat permeable even before heating (Table 9 zero time).

The growth behaviour of the ColV$^-$ and ColV$^+$ cells after heating at 60^{o}C for 2 min was investigated. Figure 11 reveals that heated ColV$^+$and ColV$^-$ strains have lag periods before they are able to re-start cell division and growth. This indicates the presence of injured cells which are repairing heat induced damage. The lag period of the ColV$^+$ strain is longer; and the growth rate after the lag period is slower in the heated ColV$^+$ strain compared with the heated ColV$^-$ strain. This indicates the presence of extensively injured ColV$^+$ cells and reflects the higher percentage of death in the ColV$^+$ strain, respectively.

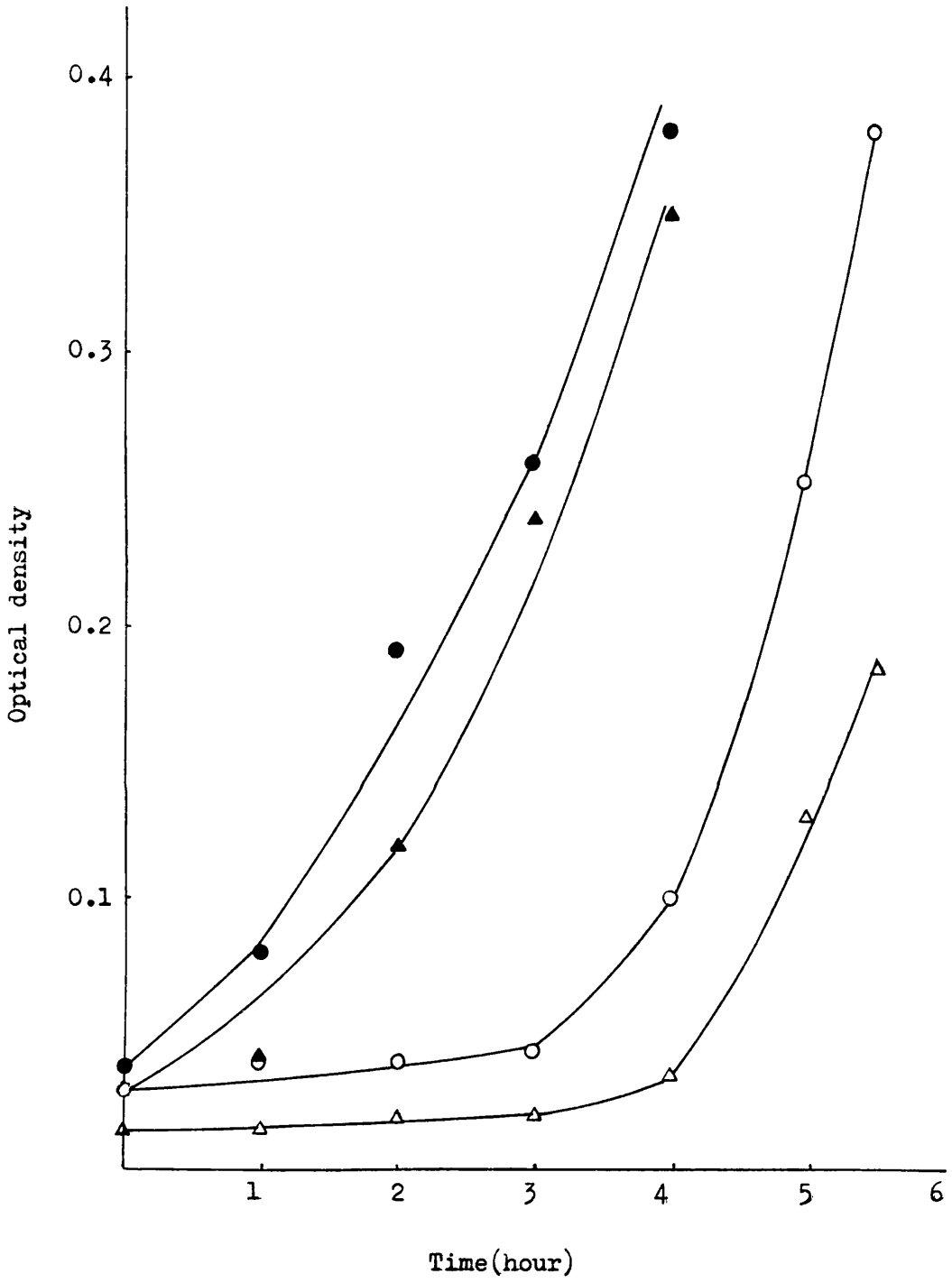


Fig 11: Growth behaviour after heat treatment at 60°C. Organisms were grown to the stationary phase at 37°C. o ED1829 heated at 60°C for 2 min, ● ED1829 unheated, △ ED1829ColV,I-k94 heated for 2 min, ▲ ED1829ColV,I-k94 unheated.

The above data show that the ColV plasmid-bearing cells are more sensitive to heat than ColV$^-$ ones. However, exposure to high temperature is one of the methods used in plasmid elimination. The possibility that heating at 60^{o}C may select for ColV$^-$ cells and that the cells surviving the heat treatment are actually ColV$^-$ cells present at very low numbers in the ColV$^+$ population was tested. Cells of the unheated ColV$^+$ population and of those heated at 60^{o}C for 15 min were tested; for both, 100% produced colicin V, indicating that all survivors retain the plasmid.

Accordingly, the presence of the ColV plasmid sensitizes organisms grown to stationary phase at 37^{o}C to heating. A small part of the increased sensitivity may be due to H_2O_2 present in nutrient agar but most of it is not. None of the survivors from heating ColV$^+$ strains have lost the plasmid.

3.1.2. The role of ColV- encoded components in heat sensitivity.

The ColV,I-K94 plasmid encodes the production of derepressed levels of transfer components and also the production of colicins V and Ia and the corresponding immunity components and an outer membrane protein (VmpA protein); other components may also be encoded. To

determine which component(s) is responsible for the enhanced heat sensitivity of the ColV plasmid- bearing strains, the thermal sensitivity of derivatives deficient in one or more components was studied. Table 7 shows that the heat sensitivity of a strain carrying a mutant plasmid that does not encode transfer components (ED1829 ColV-M50(1)) is similar to that of the strain lacking the ColV plasmid (ED1829). In accord with this, repression of the transfer properties of the $ColV^+$ strain by $\underline{fin}^+$ plasmids results in increase in the thermal resistance of the $ColV^+$ strain. After exposure to heat at 60^oC, the percentages of survival of ED1829 R124 and ED1829 ColV,I-K94, R124 strains are similar (Table 7). These observations suggest that the transfer components are involved in the increased heat sensitivity of the $ColV^+$ strains.

The heat sensitivity of a strain carrying a mutant plasmid that does not encode colicin components (ED1829 ColV-M40(5)) was investigated. Table 7 shows that this strain has similar heat sensitivity to that of the $ColV^-$ cells.

The above findings suggest that presence of both colicin and transfer components is essential for the increased heat sensitivity of the ColV plasmid- bearing cells. In fact, transfer components are reduced in level

in stationary phase. Accordingly, either a transfer component or components is/are not affected in stationary phase or the concentration of the component responsible for heat sensitivity is still high enough in the stationary phase to influence sensitivity.

3.1.3. Effect of growth temperature on bacterial heat sensitivity.

Growing cells at 25°C prior to heat treatment resulted in similar heat sensitivity of the ColV$^-$ and ColV$^+$ strains. After 5 min heating at 60°C, the strain harbouring the ColV plasmid was only 3 times as sensitive as ED1829 (Table 10) compared to a larger difference in the heat sensitivity of the same strains grown at 37°C (Table 7). Heating at 52°C showed similar results; the percentages of survival of the ColV$^-$ and ColV$^+$ cells grown at 25°C are nearly identical (Table 8). Synthesis of transfer components (Tewari et al. 1985), colicin components and VmpA protein (Davies et al. 1986) is reduced in cells grown at 25°C, indicating that one or all of these components are responsible for the increased heat sensitivity of the ColV$^+$ cells.

Comparing the survival of ColV$^-$, ColV$^+$ and derivatives deficient in synthesis of colicin or transfer components grown at 25°C with the survival of those grown at 37°C shows that prior growth at 37°C increases

Table 10: Effect of heat on bacterial strains grown at 25°C.

Strain	Exposure to heat for 2 min		Exposure to heat for 5 min		Exposure to heat for 10 min		Exposure to heat for 15 min	
	%survival	%injury	%survival	%injury	%survival	%injury	%survival	%injury
ED1829	0.13	83	0.03	89	0.004	75	0.002	80
ED1829ColV, I-K94	0.2	76	0.01	75	0.001	62	0.001	65
ED1829ColV-M40(5)	ND	ND	0.1	82	0.05	71	0.02	78
ED1829ColV-M50(1)	ND	ND	0.03	72	0.01	70	0.004	65

Organisms were grown to the stationary phase in broth at 25°C, heated at 60°C and treated as described in Materials and Methods.

the thermal resistance of all the studied bacteria (Tables 7 and 10). A possible explanation for this relative resistance is that there is a correlation between temperature of growth, composition of the cytoplasmic membrane, and sensitivity to heat. Increase in growth temperature from 30 to 43°C was accompanied by an increase in the thermal resistance of E. coli and increase in the saturated: unsaturated fatty acid ratio of the phospholipids of the cytoplasmic membrane (Beuchat 1978). It was observed that, irrespective of the growth temperature, bacteria maintain a constant membrane fluidity to provide the normal membrane functions. Increasing the growth temperature causes increase in the average chain length of the fatty acids or increase in the ratio of saturated: unsaturated fatty acids (Ellar 1978). Consequently, membrane phospholipids of bacterial cells grown at high temperature (e.g. 37°C) are less susceptible to heat, causing an increased cellular resistance to heat. Alternatively, higher levels of heat shock proteins in 37°C cells may be responsible allowing protection of the heat- sensitive target or its better repair.

3.1.4. Effect of growth phase on bacterial heat sensitivity

Tables 11 and 12 show that for exponentially grown cells, the ColV plasmid bearing strain has only slightly higher heat sensitivity than the ColV$^-$ strain.

Table 11: Effect of heating at 60°C on bacterial strains in the exponential phase.

A.

Strain	Exposure for 30 sec		Exposure for 1 min		Exposure for 2 min	
	%survival	%injury	%survival	%injury	%survival	%injury
ED1829	43	97	21	98	3.1	99
ED1829 ColV, I-K94	41	97	13	99	1	99

B.

Strain	Exposure for 5 min		Exposure for 10 min	
	%survival	%injury	%survival	%injury
ED1829	0.04	83	0.01	86
ED1829ColV, I-K94	0.03	86	0.001	93

Organisms were grown to the exponential phase in broth at 37°C (A) or 25°C (B), heated at 60°C and treated as described in Materials and Methods.

Table 12: Effect of heating at 52°C on bacterial strains in the exponential phase.

	Strain	Exposure to heat for 10 min		Exposure to heat for 20 min		Exposure to heat for 30 min	
		%survival	%injury	%survival	%injury	%survival	%injury
A	ED1829	44	99	33	99.9	26	100
	ED1829ColV, I-K94	38	99	28	99	12	99
B	ED1829	27	88	18	98	4	96
	ED1829ColV, I-K94	25	86	12	98	4	94

Organisms were grown to the exponential phase in broth at 37°C (A) or 25°C (B), heated at 52°C and treated as described in Materials and Methods.

Comparing the heat sensitivity of strains in the exponential phase with that of cells in the stationary phase reveals that cells grown to the exponential phase at 37°C are more sensitive to heat than those grown to the stationary phase at the same temperature (compare tables 11 and 7 for cells heated at 60°C and tables 12 and 8 for cells heated at 52°C). The increased heat sensitivity in exponentially grown cultures may occur due to increased susceptibility of DNA during this growth phase.

On the other hand, when the 25°C- grown strains are heated at 60°C, cells in the stationary and exponential phases of growth have similar sensitivity to heat (Tables 11 and 10). This may be observed due to the high heat sensitivity of cells grown at low temperatures (25°C) whether they are in the stationary or exponential phase.

3.2. The basis for the increased heat sensitivity of ColV, I-K94$^+$ strains

Heat can damage several sites in the microbial cell (see section 1.4. of the Introduction); those most affected appear to be nucleic acids, ribosomes, and the cell envelope. This section investigates the nature of the heat - induced injury in the ColV plasmid - bearing strains compared to the Col$^-$ parental strains.

3.2.1. The effect of heat on the outer membrane

a. Effects on the lipopolysaccharide

Since heat is known to affect the outer membrane, studies have been made of whether the lipopolysaccharide (LPS) component, which is important for outer membrane integrity and impenetrability, is altered in ColV$^+$ strains and whether increased heat sensitivity is associated with LPS changes.

Firstly, release of LPS has been studied and, secondly, dye sensitivity which reflects LPS structure and penetrability has been investigated. In each case, ColV$^-$ and ColV$^+$ strains have been compared without heating and then the effects of heating considered.

i. Release of LPS from unheated cells

Both unheated ColV$^-$ and ColV$^+$ cells released some lipopolysaccharide but the unheated ColV$^+$ strain released

greater amounts of lipopolysaccharide; 53% of the LPS of the P^+ strain was released into the medium compared with 38% for the $ColV^-$ strain (Fig 12). However, the strain used in this experiment (LE1) is a mutant that incorporates low concentrations of galactose solely into the LPS (Rothfield & Pearlman-Kothencz 1969), and the lipopolysaccharide release from this strain is probably greater than that in other laboratory strains. The increased release of LPS from the unheated $ColV^+$ cells compared with tne $ColV^-$ cells may explain the enhanced permeability of the unheated ColV plasmid - bearing strains to deoxycholate (Table 9). The compact arrangement of lipopolysaccharide in the outer membrane of Gram-negative bacteria is the primary factor that prevents entry of hydrophobic substances into the cell; if this structure is more disturbed in the $ColV^+$ strain, greater sensitivity to deoxycholate might be expected.

ii. Release of LPS from heated cells

The effect of heat on the lipopolysaccharide was studied by measuring the amount of LPS released into the heating medium after exposure of bacterial cells to 60^oC for 5, 10 and 15 min. Both heated $ColV^-$ and $ColV^+$ cells released considerable amounts of lipopolysaccharide. Figure 12 shows that on heating at 60^oC for 5 min the increase in the amount of LPS released from heated LE1 strain compared with LPS released from LE1 cells after

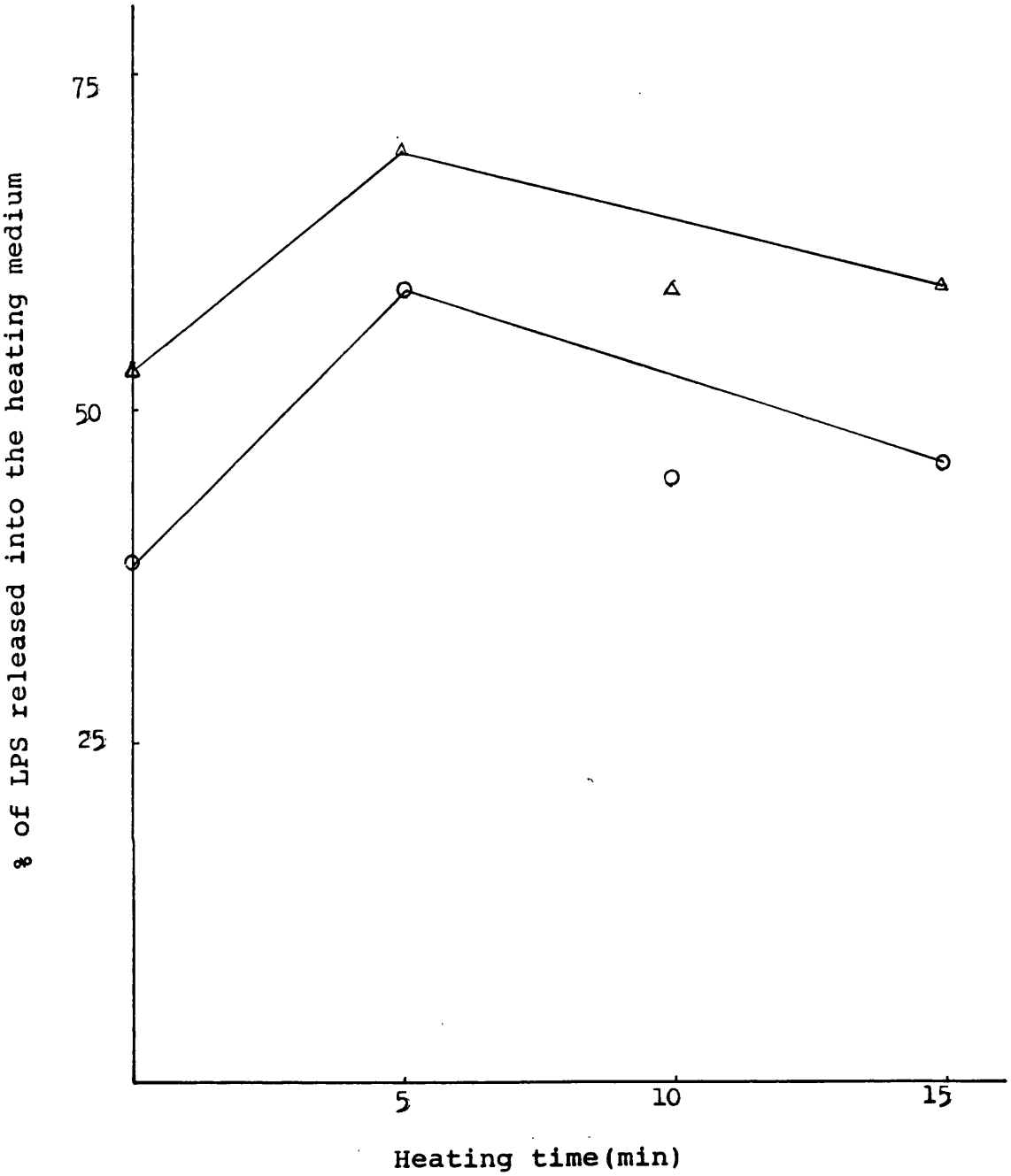


Fig 12: Release of lipopolysaccharide from heated cells. Bacterial strains were heated at 60°C and treated as described in Materials and Methods.
o LE1, Δ LE1ColV,I-k94.

0 min heating is nearly the same as the increase in the amount of LPS released from heated LE1 ColV,I-K94 strain compared with LPS released from these cells after 0 min heating. However, after heating at 60°C for 5 min, the ColV$^+$ strain is releasing 70% of its LPS whereas the ColV$^-$ is releasing only 59% (Fig 12).

To correlate the heat induced release of lipopolysaccharide with the thermal susceptibility of the bacterial strains used in this experiment, the heat sensitivity of LE1 and LE1 ColV,I-K94 cells was tested. After heating at 60°C for 2 min, LE1 ColV,I-K94 cells were 3 times more sensitive than the isogenic ColV$^-$ strain, and after heating for 5 min the ColV$^+$ strain was 1.5 times as sensitive as LE1 strain.

The heat induced release of lipopolysaccharide (Fig 12) may be correlated with the increased heat sensitivity of the ColV$^+$ cells compared with the ColV$^-$ cells, since about $\frac{3}{4}$ of the LPS in the outer membrane of these cells is released (after heating at 60°C for 5 min) making the bacterial envelope weaker and vulnerable to physical stresses.

iii. Sensitivity of heated and unheated cells to gentian violet.

Any alterations in lipopolysaccharide structure

in ColV$^+$ atrains and LPS associated changes on heating may be reflected by changes in sensitivity to hydrophobic agents. Hydrophobic antibiotics and dyes (e.g. gentian violet) can penetrate E. coli and Salmonella mutants with deficient lipopolysaccharide (rough mutants) but are unable to penetrate cells with complete lipopolysaccharide (smooth strains). Therefore, the uptake of gentian violet by heated and unheated cells was examined. Figure 13 shows that unheated ColV$^+$ and ColV$^-$ strains take up gentian violet (5µg/ml) to a similar extent, indicating that the ColV$^+$ strain does not possess a lipopolysaccharide sufficiently altered to affect the permeability to this dye. Uptake of this hydrophobic dye is increased in strains having large LPS defects. Stan-Lotter et al. (1979) reported that 60% of gentian violet (10µg/ml) was absorbed by the heptoseless (Re) strain (rough mutant) of S. typhimurium whereas the smooth strain absorbed ~20% of the added dye.

The effect of heating on the permeability of the outer membrane to gentian violet was also studied. Heating at 60^{o}C for 5, 10, 15 min did not increase the permeability of ED1829 or ED1829 ColV,I-K94 to gentian violet (Fig 13) indicating that exposure to heat does not cause sufficiently drastic changes in the lipopolysaccharide structure to affect uptake of this dye.

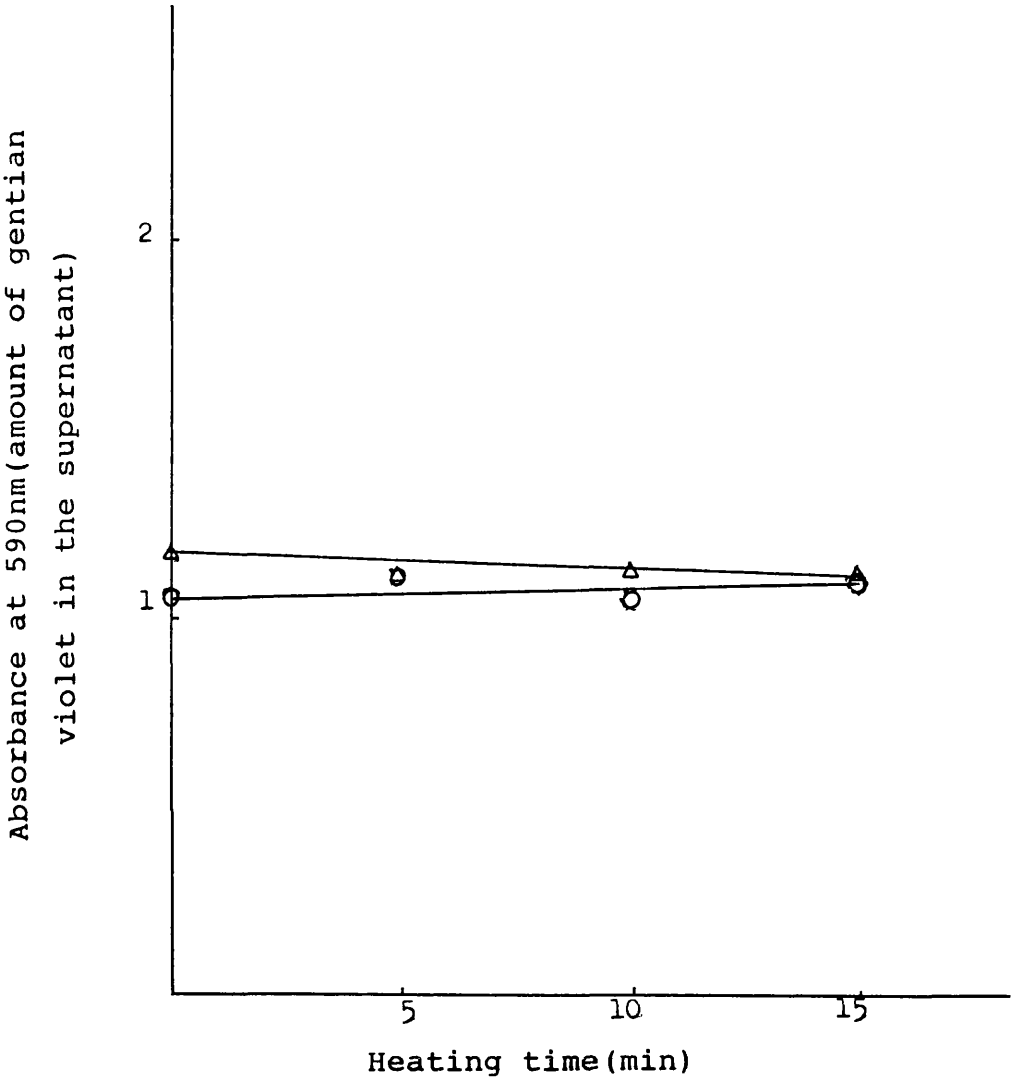


Fig 13: Permeability to gentian violet. Cells were grown to the stationary phase at 37°C, heated at 60°C. o ED1829, Δ ED1829ColV,I-k94.

b. Effects on the proteins of the cell envelope

The cell envelope of the unheated *E. coli* has four major proteins. These include the porins, OmpF (37Kd) and OmpC (36Kd), the OmpA (33Kd) and the lipoprotein (7.2kd). The envelope of the ColV plasmid bearing cells has, in addition to these proteins, plasmid encoded proteins. These include the VmpA (33Kd) and some of the products of the *tra* genes.

The protein content of the cell envelopes of the heated and unheated strains was analysed by SDS-polyacrylamide gel electrophoresis. Figure 14 shows that after heating $ColV^-$ and $ColV^+$ cells at 60^oC, all the major outer membrane proteins (OmpF, OmpC and OmpA) can be observed. The relative amounts of the porins or the OmpA proteins were not changed, however, the quantity of these proteins appeared less in the heated strains.

Cell membranes are usually dissociated in 2% SDS at temperatures above 60 or 70^oC for complete solubilization of the cell envelope proteins. Dissociation of cell envelopes at lower temperatures (e.g. 37^oC) does not allow denaturation of the proteins causing absence of most of the protein bands from the gel. Comparing proteins of the 30^oC - dissociated cell envelopes of heated and unheated cells shows that

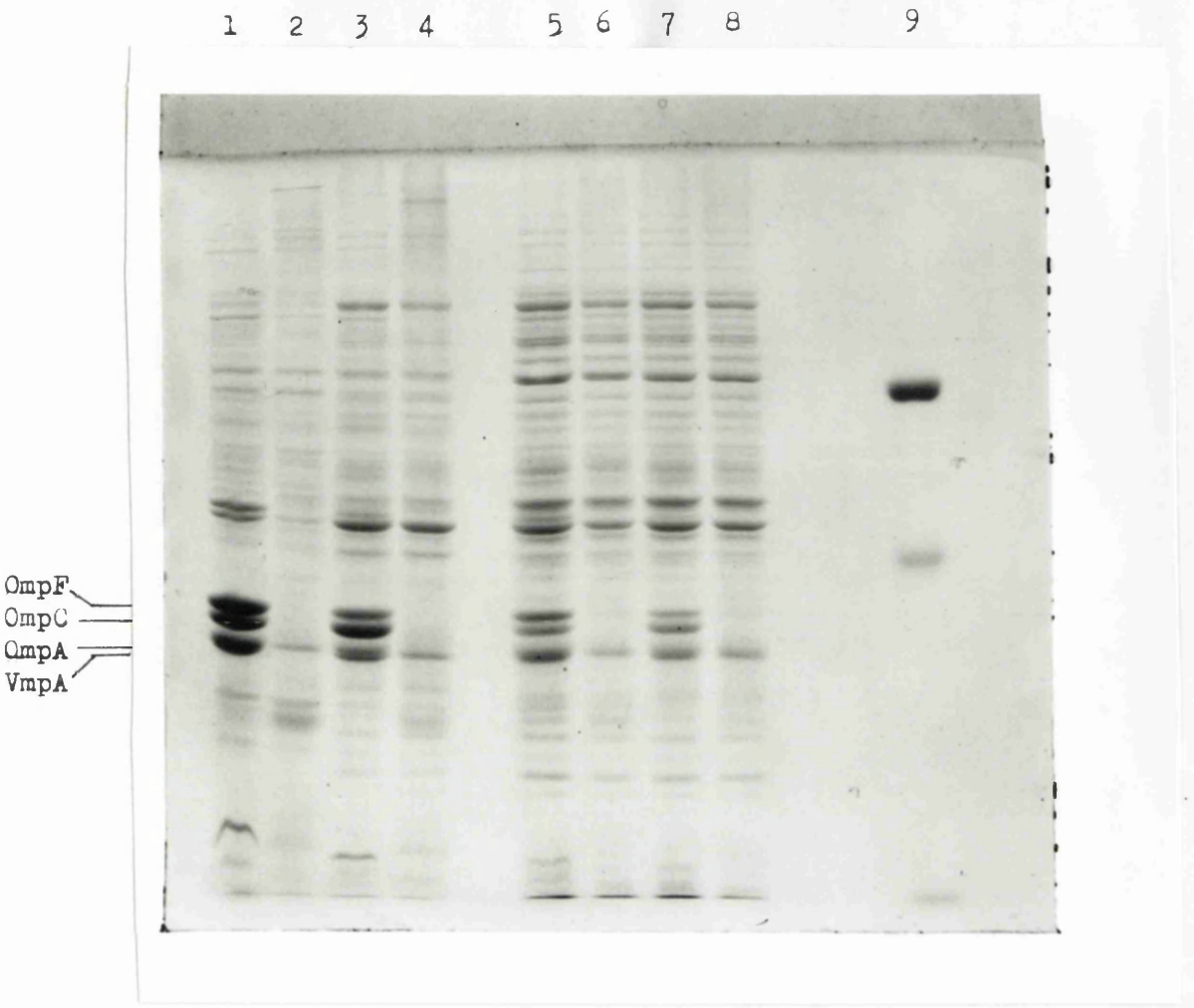


Fig 14: SDS- polyacrylamide gel electrophoresis of envelopes of heated and unheated cells of ED1829 and ED1829 ColV,I-K94. 1: unheated ED1829, dissociated at 100°C; 2: unheated ED1829, dissociated at 30°C; 3: unheated ED1829ColV,I-K94, dissociated at 100°C; 4: unheated ED1829ColV,I-K94, dissociated at 30°C; 5: heated ED1829, dissociated at 100°C; 6: heated ED1829, dissociated at 30°C; 7: heated ED1829ColV,I-K94, dissociated at 100°C; 8: ED1829ColV,I-K94, dissociated at 30°. ; 9: Molecular weight standards, 68Kd and 43Kd.

heating of ColV$^-$ or ColV$^+$ cells at 60^{o}C for 5 min causes accentuation of some of the existing bands or appearance of protein bands that could not be revealed when the cell envelopes of unheated cells were dissociated at 30^{o}C (Fig 14 lanes 2 & 6 and 4 & 8). In fact, the 30^{o}C dissociated heated membranes resembled the 100^{o}C membranes. This is presumably because heating of bacteria denatures some of the proteins of the cell envelope or weakens the interactions between these proteins and the other constituents of the cell envelope.

C. Effects on permeability properties of the outer membrane.

As mentioned in the Introduction, the outer membrane of the Enterobacteriaceae is impermeable to hydrophobic compounds. However, heat may affect the structure of the outer membrane and consequently its permeability barrier properties. To investigate this hypothesis, the permeability of the outer membrane of heated cells to a variety of hydrophobic and high molecular weight substances was studied.

It was observed that heating at 60^{o}C for 2, 5, 10 or 15 min causes reduced survival of ColV$^-$ and ColV$^+$ strains when plated on nutrient agar containing 0.5% deoxycholate (Table 7). Figures 15 and 16 present the

effect of sodium deoxycholate on growth of heated and unheated cells in liquid medium. Presence of deoxycholate at 0.5% in the growth medium of the unheated ED1829 cells does not affect their growth rate. However, the growth rate of heated ($60^{\circ}C$, 2 min) $ColV^-$ cells in media containing DOC is reduced, where the percentage of inhibition is 15% compared with 0% inhibition of the unheated cells.

The unheated ColV plasmid - bearing cells grow at a slower rate in the presence of DOC, where the percentage of growth inhibition is 22%. This indicates that, in contrast to $ColV^-$ cells, the $ColV^+$ cells are partially permeable to this hydrophobic compound suggesting that these cells possess an altered envelope. The heated $ColV^+$ cells grow at a much reduced rate in broth containing deoxycholate, where the percentage of growth inhibition is 47% (Fig 16). These observations indicate that heat affects the outer membrane structure of both $ColV^-$ and $ColV^+$ cells, causing increased permeation of this hydrophobic compound. The larger effect of DOC on the heated $ColV^+$ cells can be explained by the fact that the unheated $ColV^+$ cells have partially defective envelope and heating increases the extent of this damage causing permeation of larger quantities of deoxycholate.

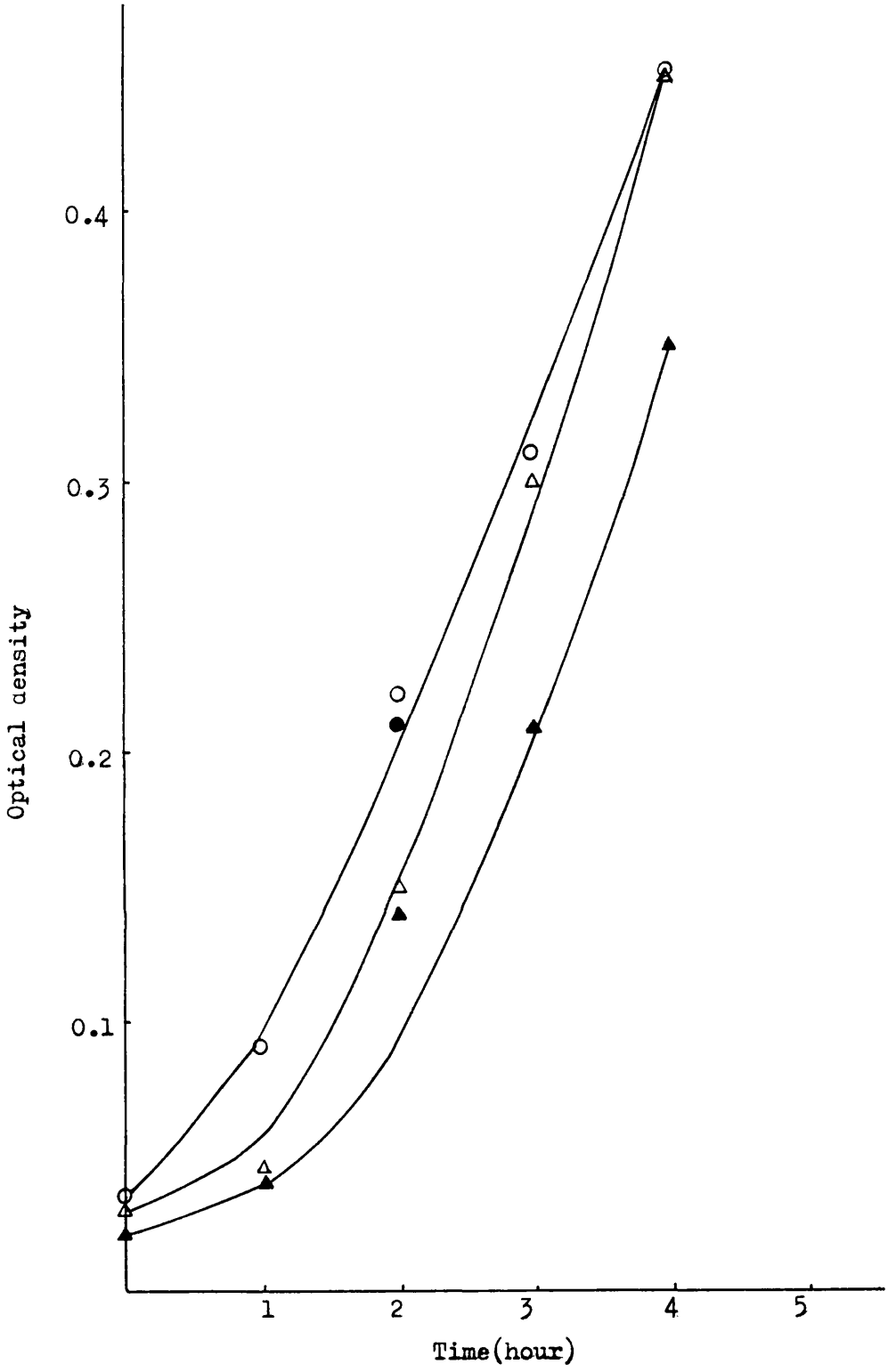


Fig 15: Effect of deoxycholate (0.5%) on unheated ColV$^-$ and ColV$^+$cells. o ED1829, ● ED1829+DOC, △ ED1829ColV,I-k94, ▲ ED1829ColV,I-k94 + DOC.

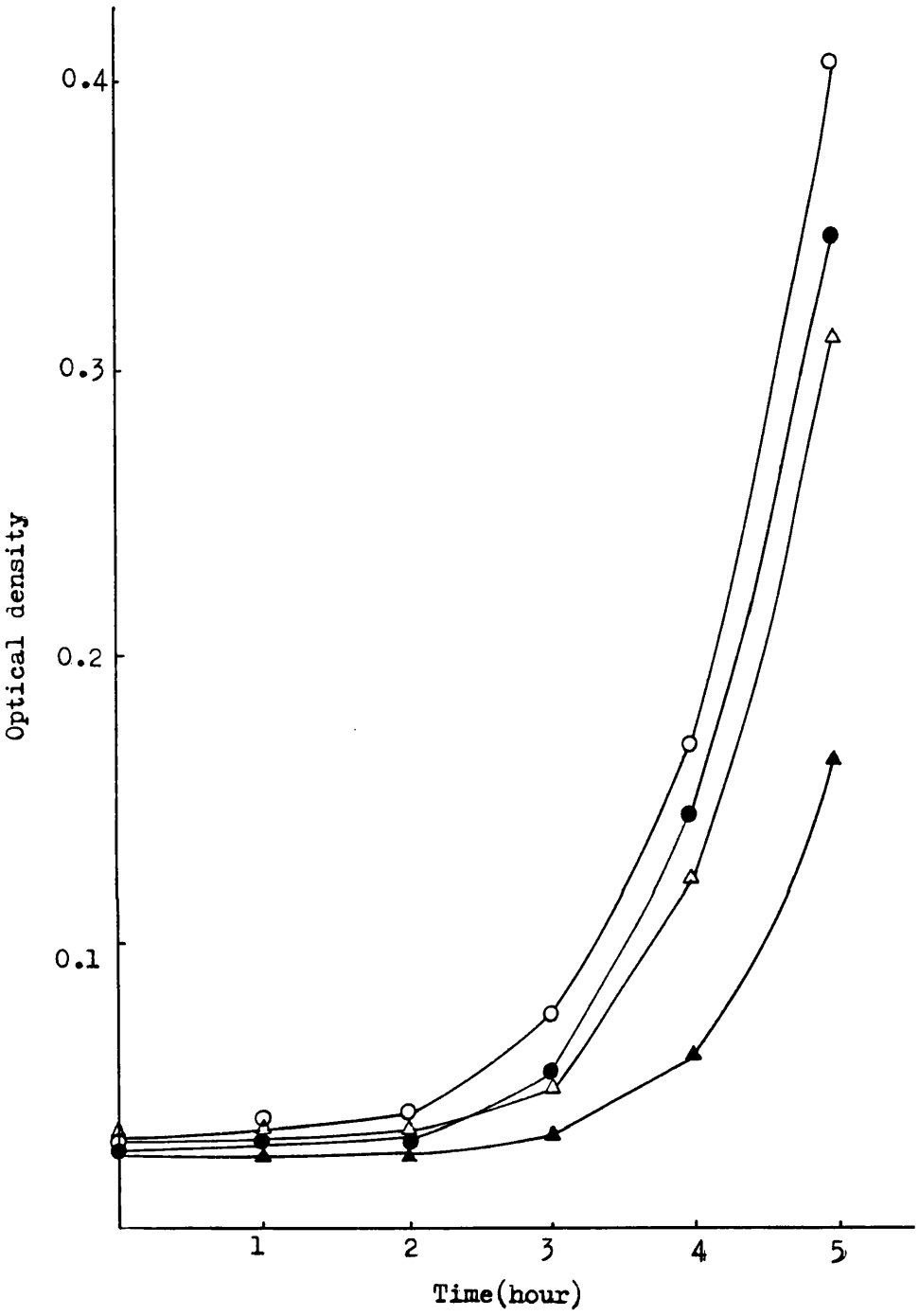


Fig 16: Effect of deoxycholate (0.5%) on heated cells. Cells were grown to the stationary phase at 37°C, and heated at 60°C for 2 min. o ED1829, ● ED1829+DOC, △ ED1829ColV,I-k94, ▲ ED1829ColV,I-k94 + DOC. **The graph shows optical density of broth cultures.**

The effect of EDTA (0.01M) on heated and unheated ED1829 and ED1829 ColV,I-K94 cells was also studied as this agent can permeabilize the outer membrane and release LPS. The unheated $ColV^-$ and $ColV^+$ cells show reduced growth rate when grown in media containing EDTA (0.01M). The percentage of growth inhibition of both strains was 41% after 5 hours incubation in this medium (Fig 17).

Heating at 60^oC for 2 min increased the sensitivity of both $ColV^-$ and $ColV^+$ cells to EDTA but the effect was greater on the p^+ ones (Fig 18).

The permeability of heated and unheated cells to lysozyme is presented in Figures 19 and 20. Lysozyme alone is generally unable to act on Gram-negative bacteria since it cannot penetrate the outer membrane. It was possible however that ColV might affect sensitivity and that heating might also permeabilise cells to lysozyme. Figures 19 and 20 show that the heated and unheated cells of $ColV^-$ and $ColV^+$ strains were not lysed by lysozyme alone, indicating that heating did not cause large alterations in the structure of the outer membrane and that lysozyme permeation into heated cells is still hindered.

Addition of EDTA to the bacterial suspensions

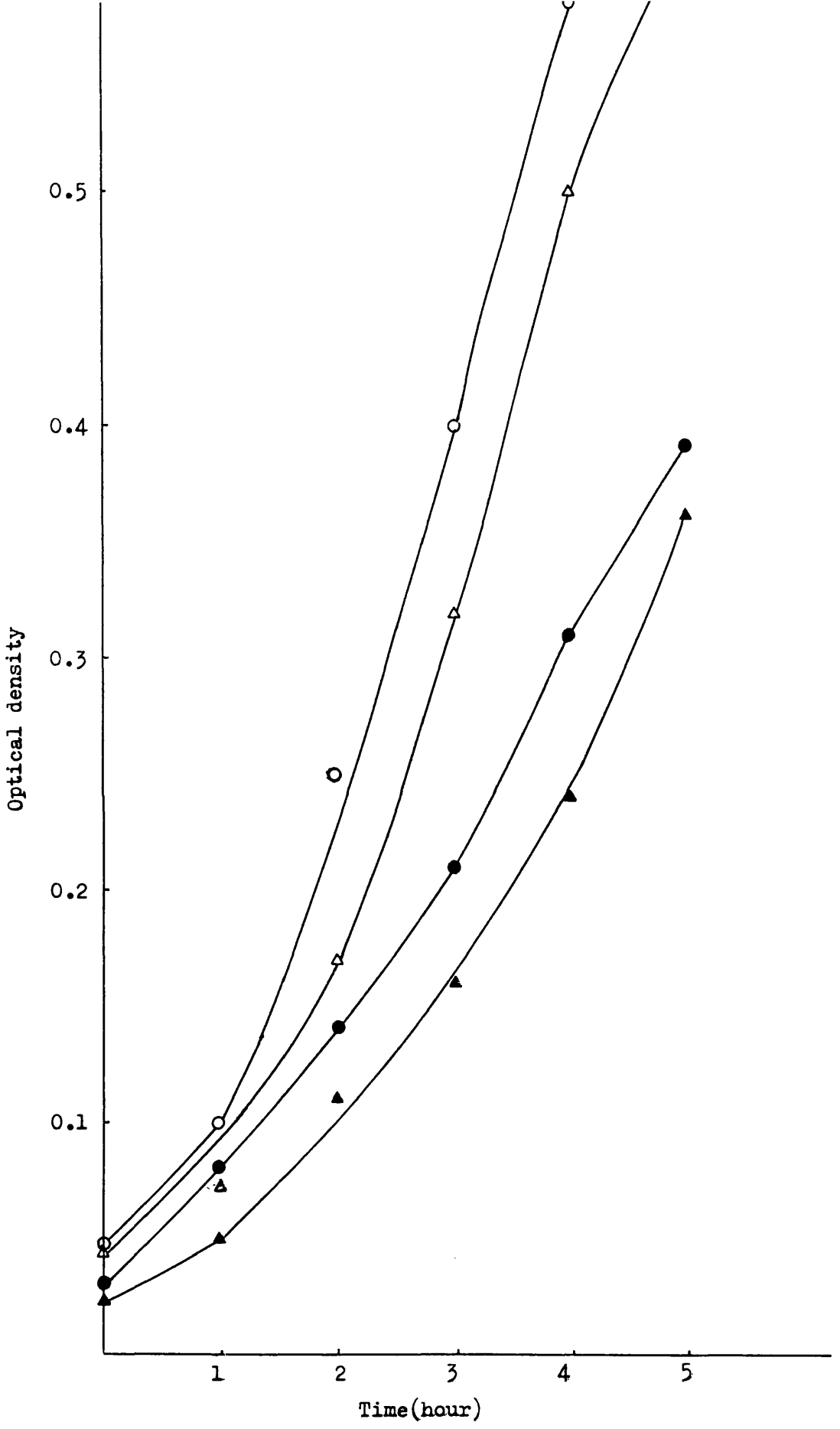


Fig 17: Effect of EDTA (0.01M) on unheated cells.

○ ED1829, ● ED1829+EDTA, △ ED1829ColV,I-k94, ▲ ED1829ColV, k94+EDTA.

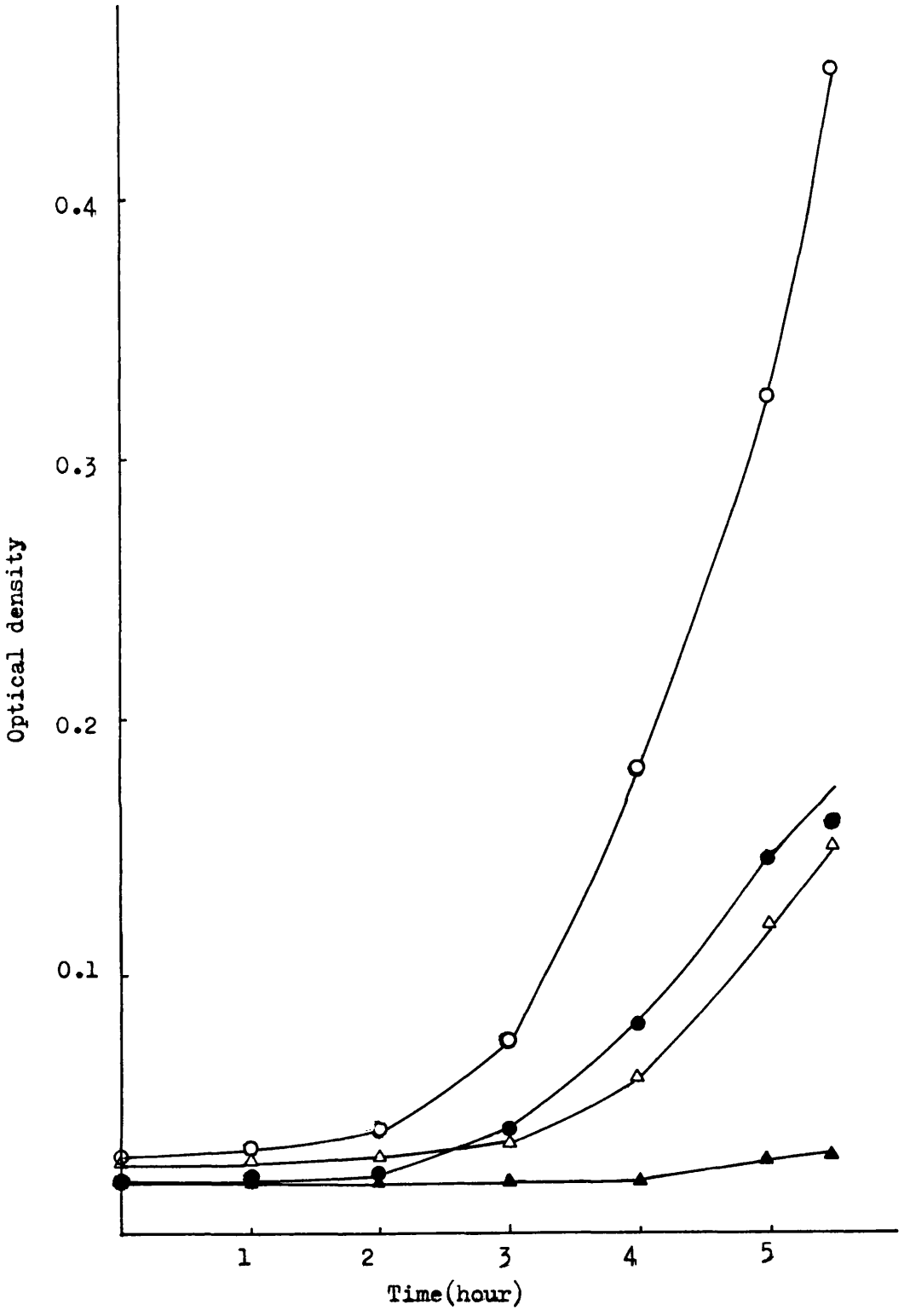


Fig 18: Effect of EDTA (0.01M) on heated cells. Cells were grown to the stationary phase at 37°C, and heated at 60°C for 2 min. o ED1829, ● ED1829+EDTA, △ ED1829 ColV,I-k94, ▲ ED1829ColV,I-k94 + EDTA.

caused lysozyme lysis of cells. Figure 19 shows that lysis of heated (60^0C, 2 min) and unheated ColV$^-$ cells was to a nearly similar extent. After 60 min incubation of bacterial cells in EDTA - lysozyme, a drop in relative optical density from 100 to 65 and 73 is observed in the unheated and heated cells, respectively (Fig 19). For the ColV$^+$ strain, lysis of the unheated cells by lysozyme- EDTA is more than the lysis of the heated cells by the same treatment. After 60 min incubation, a drop in relative optical density from 100 to 69 and 82 was observed in unheated and heated ColV$^+$ cells, respectively (Fig 20).

The sensitivity of heated cells to antibiotics that are usually not effective against members of the Enterobacteriaceae was also tested. The unheated ED1829 cells were resistant to erythromycin (Table 13). However, heating at 60^oC for 2 min sensitized ED1829 cells to erythromycin (Table 13). The unheated ColV plasmid-bearing cells were sensitive to erythromycin, but exposure to high temperature increased the extent of inhibition. This suggests that heating damages the outer membrane of both ColV$^-$ and ColV$^+$ cells, allowing penetration of this antibiotic and consequently its access to its target site.

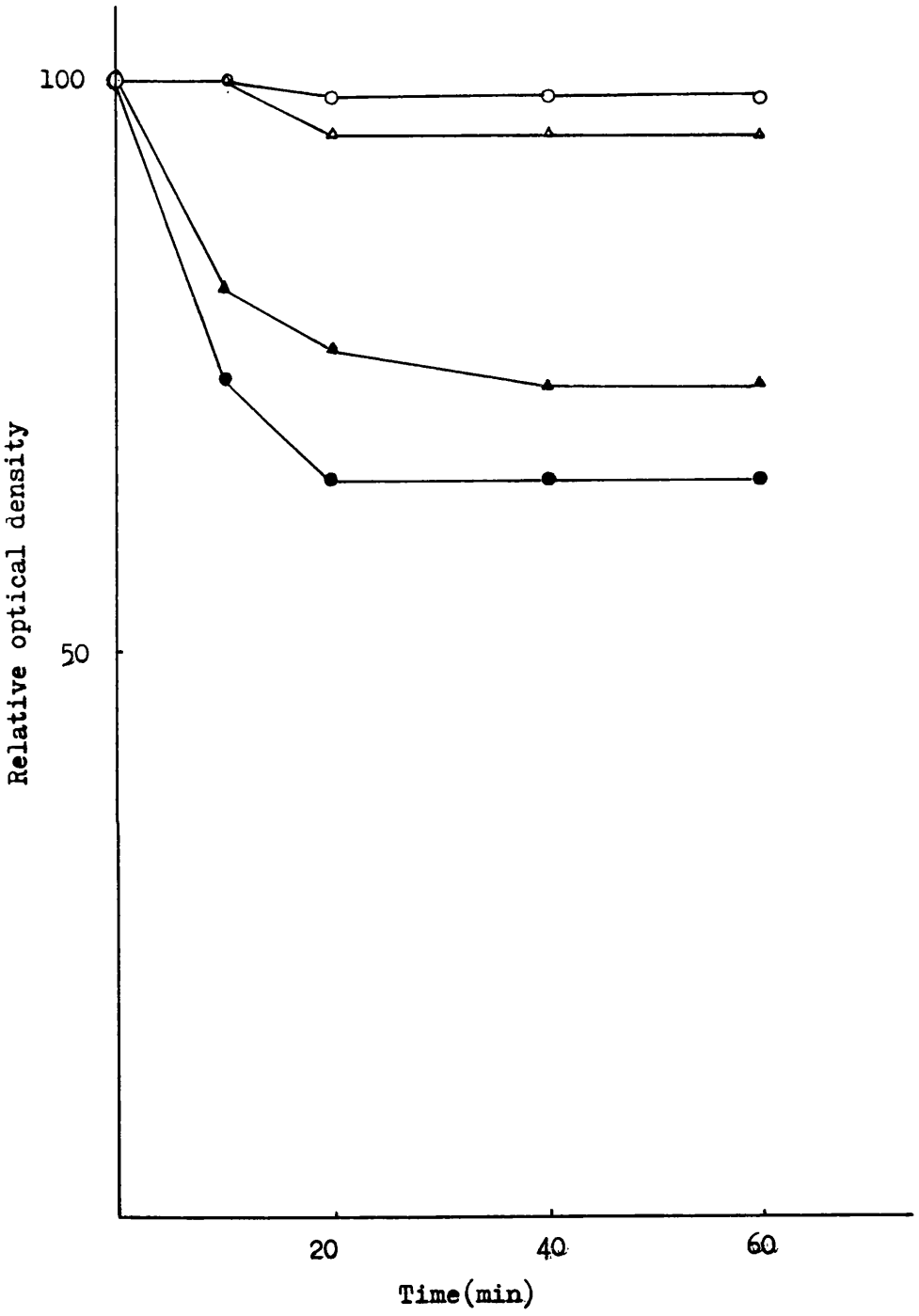


Fig 19: Effect of lysozyme on ColV$^-$ cells. ED1829 cells were grown to the stationary phase at 37^{o}C. o unheated cells+lysozyme, ● unheated cells+lysozyme +EDTA, △ heated cells+lysozyme, ▲ heated cells+ lysozyme+EDTA.

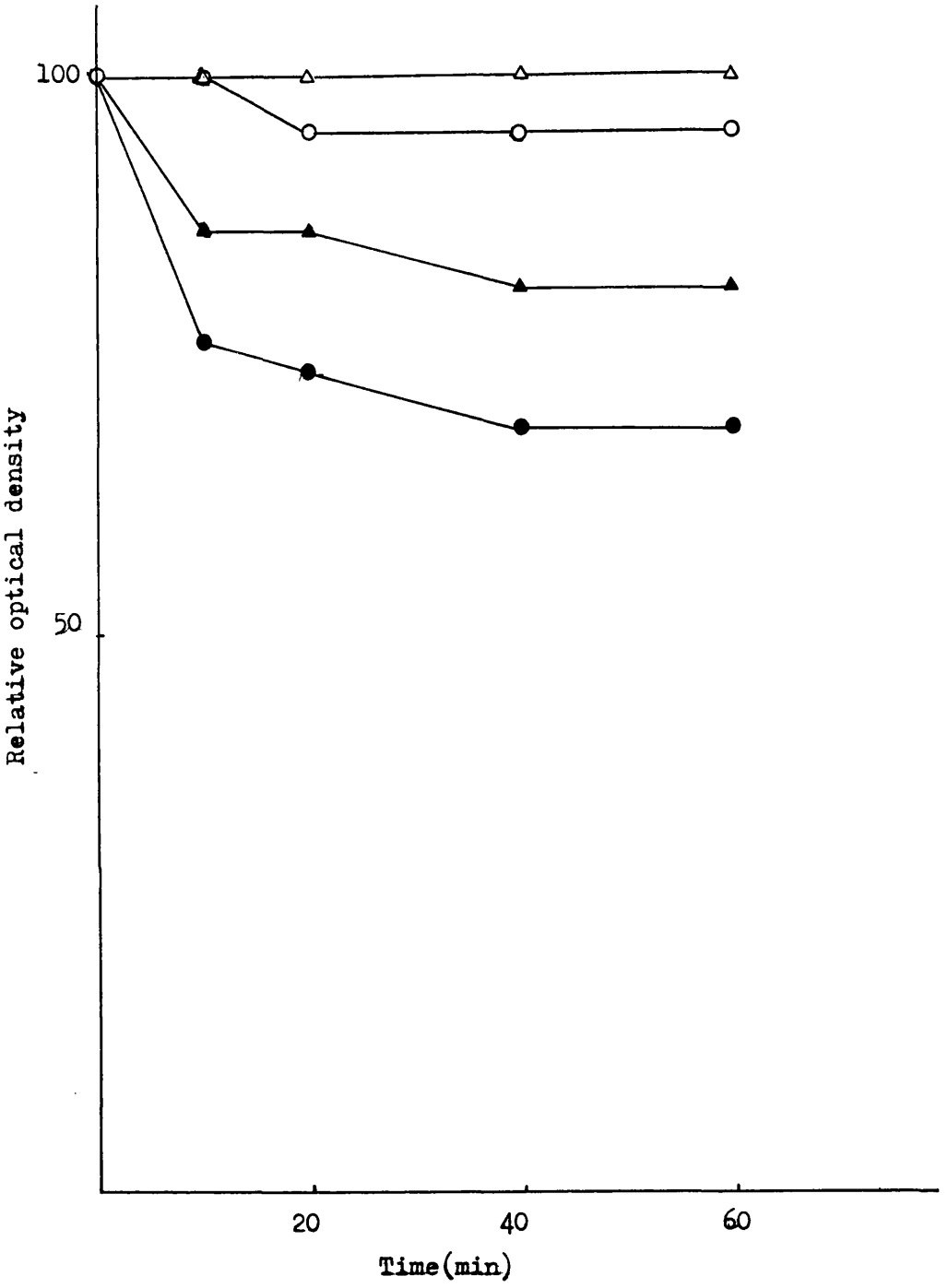


Fig 20: Effect of lysozyme on ColV^{+} cells. ED1829 ColV,I-k94 cells were grownto the stationary phase at 37^{o}C. o unheated cells+lysozyme, ● unheated cells +lysozyme+EDTA, △ heated cells+lysozyme, ▲ heated cells+lysozyme+EDTA.

Table 13: Effect of hydrophobic or bulky antibiotics on heated cells.

Antibiotic	ED1829		ED1829ColV,I-K94	
	Unheated	Heated	Unheated	Heated
Erythromycin (10μg)	0	10mm	11mm	14mm
Sulphafurazole (100μg)	0	0	0	0
Novobiocin (5μg)	0	0	0	0
Penicillin (1.5iu)	0	0	0	0

Strains were tested for antibiotic sensitivity by the disc diffusion method. The values indicate the diameter of the zone of growth inhibition.

Testing the sensitivity of heated ColV$^-$ and ColV$^+$ strains to novobiocin, penicillin or sulphafurazole shows that heating did not change the susceptibility of the cells to these antibiotics. The heated and unheated cells of both strains were resistant to these antibiotics in these tests (Table 13). It is possible that the heat- induced damage to the outer membrane is not severe enough to allow enough permeation of the above mentioned antibiotics to cause growth inhibition. Also, it is possible that the concentration of these antibiotics was lower than the required concentration for inhibition of bacterial growth.

3.2.2. Effect of heat on release of cellular constituents

Damage to the cytoplasmic membrane may be indicated by release of some of the internal constituents of the cell into the heating medium. Figure 21 reveals that for cells grown to the stationary phase at 37^{o}C, both heated ColV$^+$ and ColV$^-$ cells released materials absorbing at 260nm into the heating medium. After 5 min heating at 60^{o}C, ED1829 ColV,I-K94 cells leaked twice the amount released from ED1829 strain. This observation is correlated with the lower percentage of survival of the heated ColV$^+$ cells compared with the heated ColV$^-$ cells (see section 3.1.1.); suggesting that damage to the cytoplasmic membrane may contribute to the loss

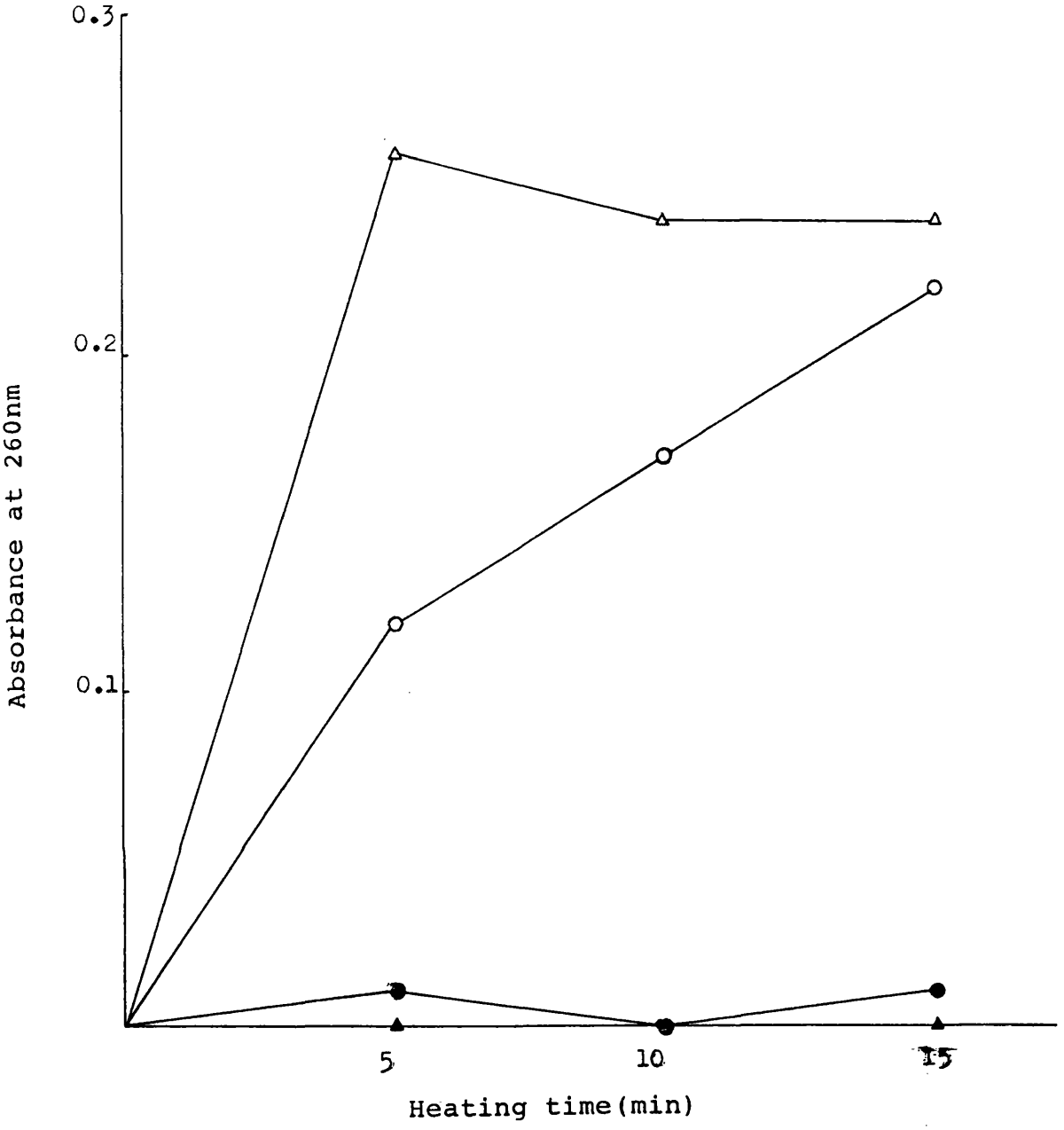


Fig 21: Release of materials absorbing at 260nm from cells grown to the stationary phase at 37°C. o ED1829 heated, △ ED1829ColV,I-k94 heated, ● ED1829 unheated, ▲ ED1829ColV,I-k94 unheated.

of viability of heated strains and that this damage has a greater effect on the ColV plasmid- bearing cells. After 15 min heating at 60°C , the leakage from both strains was nearly the same ... ; the release of internal substances from heated ColV$^+$ cells did not increase whereas leakage from the ColV$^-$ cells increased with increasing the time of heating. Some of the released materials may re-enter the ColV$^+$ cells more readily than the ColV$^-$ cells, resulting in the observation of unchanged leakage from the ColV$^+$ cells after 15 min heating.

When cells are grown to the stationary phase at 25°C, the amount of the leaked compounds from heated ColV$^-$ cells (Fig 22) was similar to that released from the heated ColV$^-$ cells that were grown to the stationary phase at 37°C (Fig 21). This similarity in leakage was observed for cells heated at 60°C for 5, 10 or 15 min, however, after 15 min heating the amount of leaked materials did not increase compared to 10 min. This may suggest that with increasing the time of heating the cytoplasmic membrane may become severely damaged resulting in uncontrolled inward passage of the released substances causing a drop in the amount of these materials in the heating medium. The materials released from heated ColV$^+$ cells that were grown to the

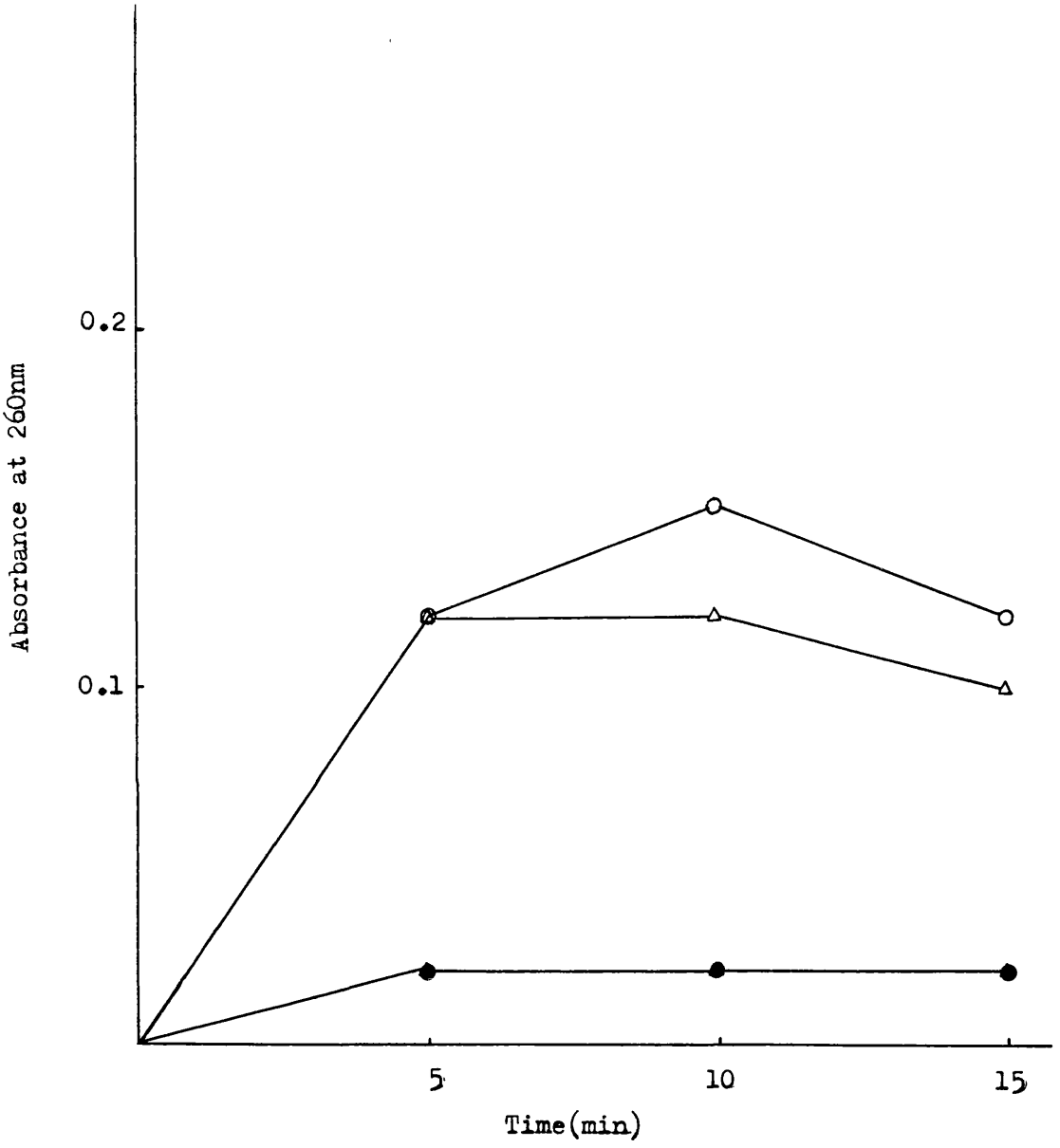


Fig 22: Release of materials absorbing at 260nm from cells grown to the stationary phase at 25°C. o ED1829 heated, △ ED1829ColV,I-k94 heated, ● ED1829 unheated, ▲ ED1829ColV,I-k94 unheated.

stationary phase at 25°C is less than the amount released from the heated ColV$^+$ cells that were grown to the stationary phase at 37°C but similar to the amount released from the ColV$^-$ cells. The synthesis of colicin and transfer components is reduced at 25°C, it is possible that the transport of these components from their site of synthesis (ribosomes) to the outer membrane or outside the cell weakens the cytoplasmic membrane or that some colicin or transfer components are actually embedded in the cytoplasmic membrane altering its properties. This would explain the greater damage to the cell membrane of heated ColV$^+$ cells grown at 37°C compared with the heated ColV$^+$ cells grown at 25°C.

When cells are grown to the exponential phase at 25°C (Fig 23) or 37°C (Fig 24) there was little leakage of the 260nm- absorbing materials from the heated ColV$^-$ or ColV$^+$ cells. The increased heat sensitivity of cells at exponentional phase (see section 3.1.4.) is not correlated with increased leakage from cells, indicating the presence of another mechanism of injury in exponentially growing cells. Inactivation of enzymes or other components needed for bacterial growth or enhanced damage to nucleic acids may be the primary factor that causes heat- induced death of these bacteria, in this growth phase. The release of 260nm- absorbing materials

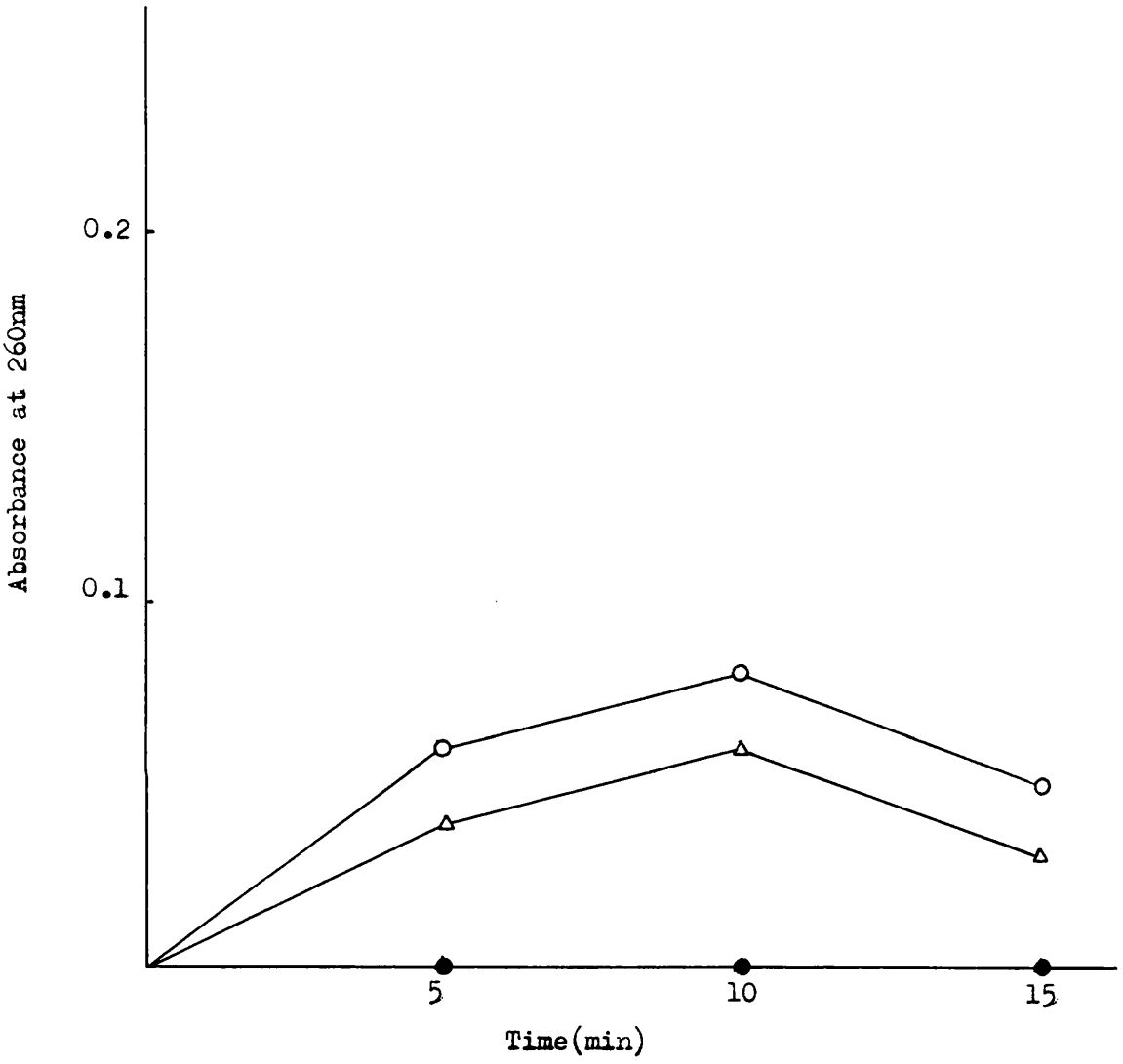


Fig 23: Release of materials absorbing at 260nm from cells grown to the exponential phase at 25°C. o ED1829 heated, Δ ED1829ColV,I-k94 heated, ● ED1829 unheated, ▲ ED1829ColV,I-k94 unheated.

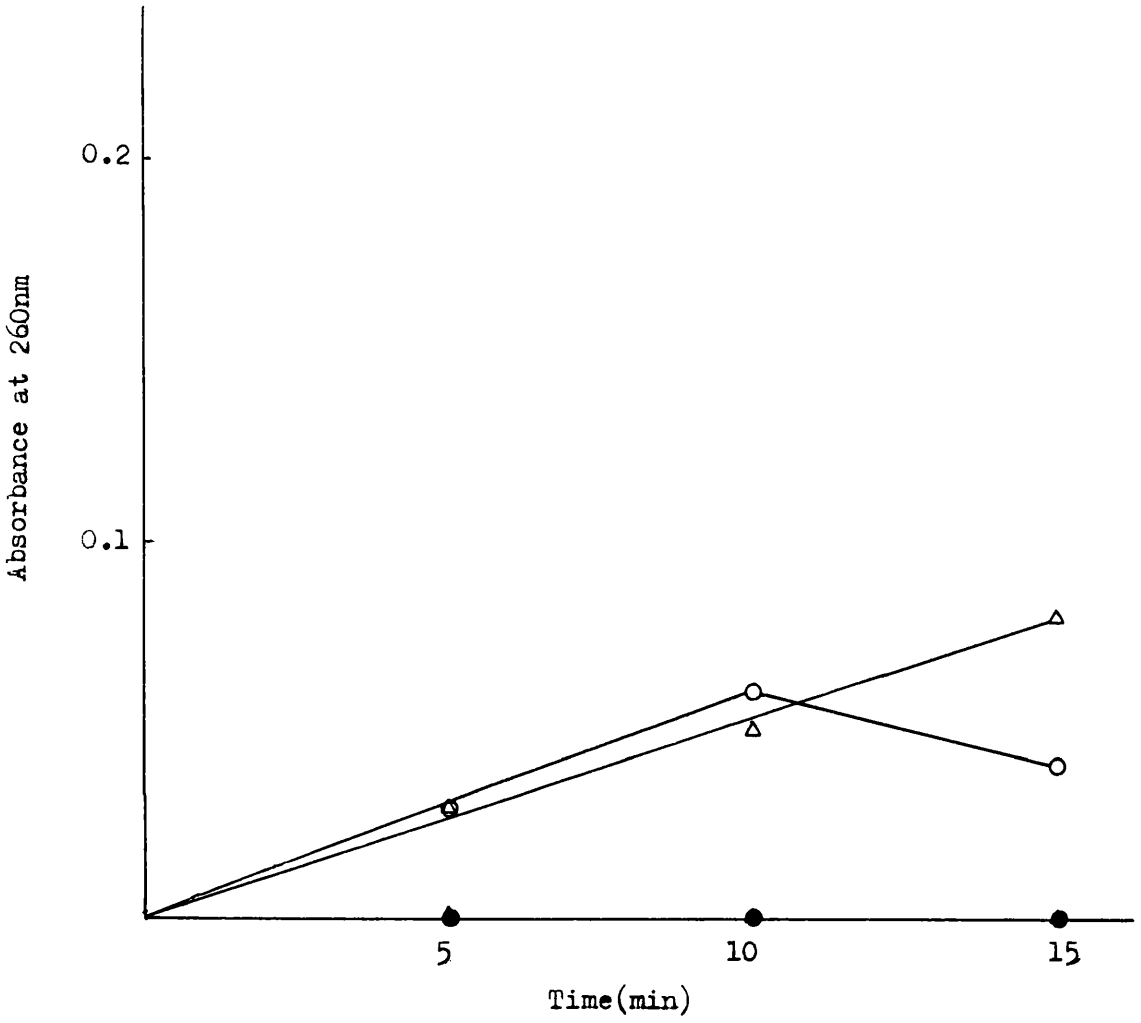


Fig 24: Release of materials absorbing at 260nm from cells grown to the exponential phase at 37°C. o ED1829 heated, △ ED1829ColV,I-k94 heated, ● ED1829 unheated, ▲ ED1829ColV,I-k94 unheated.

from unheated ColV$^-$ and ColV$^+$ cells was examined. The unheated cells that are grown to the stationary or exponential phase at 25°C or 37°C either release negligible amounts or do not leak their internal constituents indicating that leakage from heated cells is a heat-inducible process.

In these experiments, the cells were heated in 0.75% sodium chloride instead of nutrient broth. However, the above findings can be applied to cells heated in nutrient broth since the heat sensitivities of the strains heated in sodium chloride are similar to those observed when cells are heated in nutrient broth.

Damage to the cytoplasmic membrane was also tested by measuring the penetration of o-nitrophenyl β-D-galactoside into the cytoplasm. Cells were induced for production of β-galactosidase by addition of isopropyl β-D-thiogalactoside. Then samples of heated and unheated cells were assayed for transport of ONPG. P678-54 strain and its ColV$^+$ derivative were used in this experiment as P678-54 is deficient in the permease which allows entry and accumulation of ONPG. Therefore, penetration of ONPG and its subsequent hydrolysis (by β-galactosidase) can be observed only if the cytoplasmic membrane is made permeable to ONPG or to

β-galactosidase either by heat or by toluene treatment. The amount of 0-nitrophenol (ONP) (measured as absorbance at 420nm) released from ONPG in heated and unheated cells is presented in Table 14. The unheated P678-54 cells did not hydrolyse ONPG whereas heated cells of the same strain hydrolysed a small amount of the ONPG. For the unheated P678-54ColV,I-K94 strain, a small amount of the ONPG was able to penetrate the cell; however , heating allowed penetration of greater amounts of ONPG into cells. This suggests that heating damages the cytoplasmic membrane of both $ColV^-$ and $ColV^+$ cells, and indicates that this damage is more extensive in the ColV plasmid bearing strains. It also appears that the cytoplasmic membrane of the $ColV^+$ strain may be slightly permeable to ONPG. This may correlate with the slow growth of some *lacY* mutants on lactose when ColV,I-K94 is introduced (Rowbury *et al.* 1985).

To test whether heating inactivates the β-galactosidase, which may result in a lower estimation of ONPG penetration into cells, the activity of this enzyme in heated and unheated cells was examined by measuring the hydrolysis of ONPG after disruption of the cytoplasmic membrane by toluene treatment. The β-galactosidase activity of the heated cells of both $ColV^-$ and $ColV^+$ strains was the same as that of the

Table 14: Hydrolysis of o-nitrophenyl β-D-galactoside

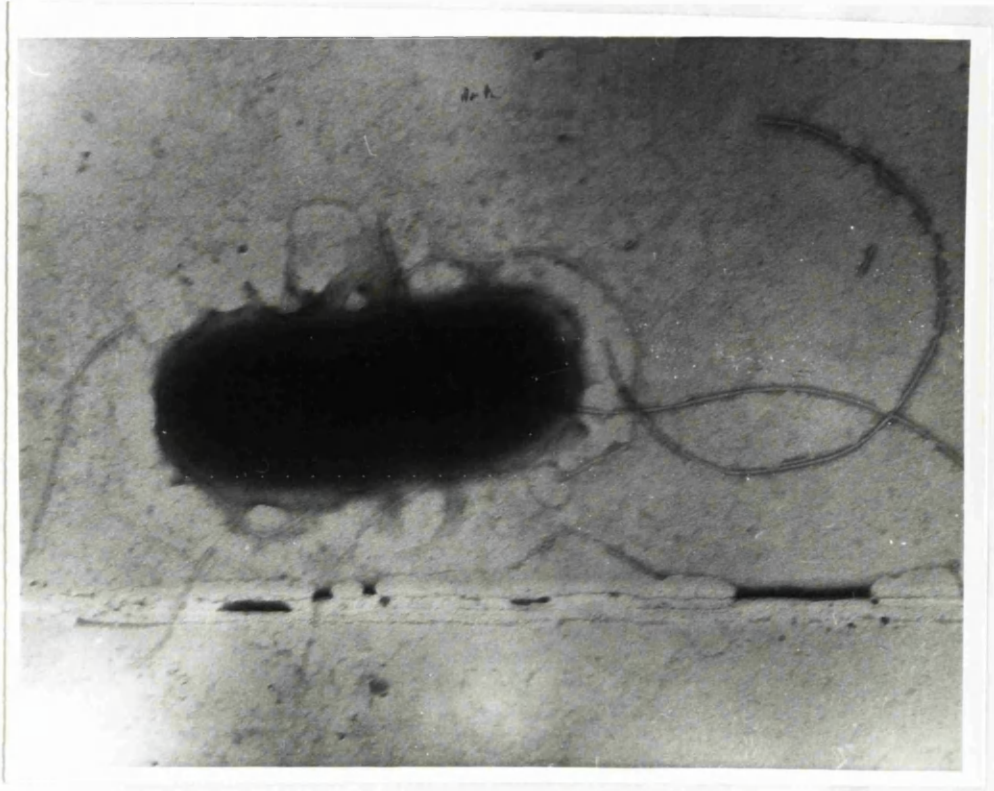
Strain	Unheated cells		Heated cells	
	-Toluene	+Toluene	-Toluene	+Toluene
P678-54	0	0.24	0.02	0.24
P678-54ColV,I-K94	0.02	0.33	0.06	0.33

Cells were grown to the stationary phase at 37°C, induced for production of β-galactosidase and treated as described in Materials and Methods. The values represent absorbance at 420nm. Heating was for 5 min at 60°C.

unheated cells (Table 14). This indicates that this enzyme is not inactivated by exposure of cells to 60°C for 5min.

3.2.3. Heat effects on DNA and other cytoplasmic components

The effect of heat on DNA was measured indirectly by following the division of heated cells under the electron microscope. DNA replication is a pre-requisite to cell septation, therefore failure to observe cell division can be indicative of DNA damage. Stationary phase cells of control (unheated) and of heated (60°C, 5 min) strains were diluted into fresh nutrient broth, allowed to grow until a marked increase in optical density was observed and examined under the electron microscope. Figures 25 and 26 reveal that the heated ColV$^-$ and ColV$^+$ cells show normal cell division where septum formation is complete resulting in production of two similar daughter cells. Formation of long filaments without transverse constrictions or septated filaments (chains) was not observed in these heated cells. This suggests that the processes of cell septation and separation and DNA replication can occur normally in ColV$^-$ and ColV$^+$ cells exposed to high temperatures. However, the possibility that DNA damage occurs in heated cells cannot be ruled out. It is possible that



b.

Fig 25: Electron micrographs of strain ED1829. a.unheated cell. b. heated cells. Mag 15000X.

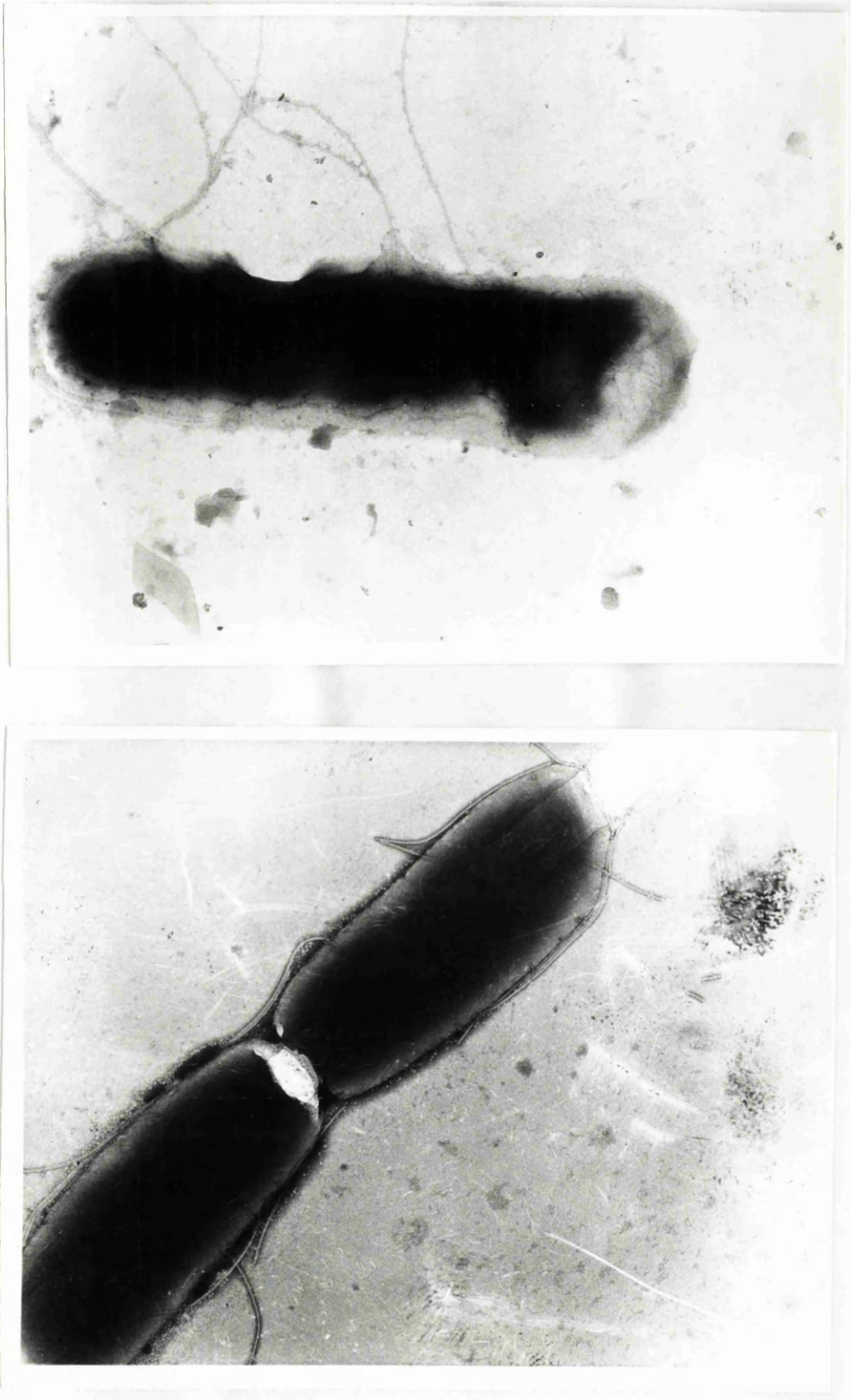
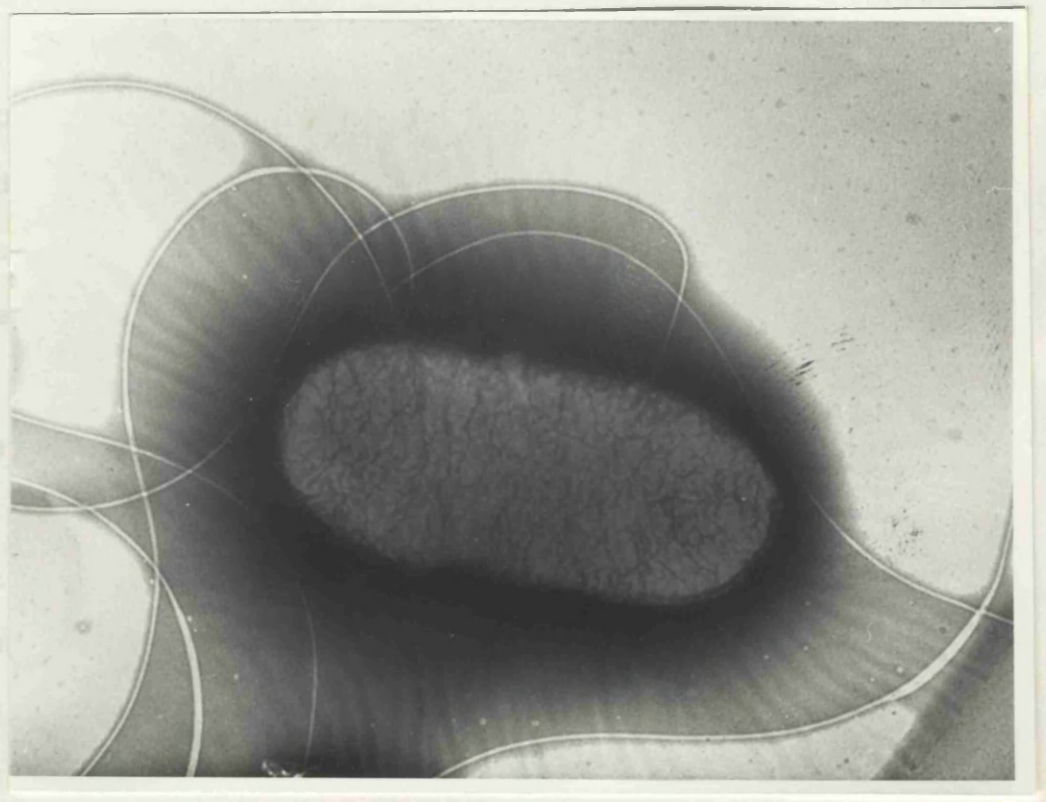


Fig 26: Electron micrographs of ED1829ColV,I-K94 strain. a. unheated cells. Upper micrograph, 15000X; lower micrograph, 12500X. b. heated cells. Upper micrograph, 20000X; lower micrograph, 15000X.

b.



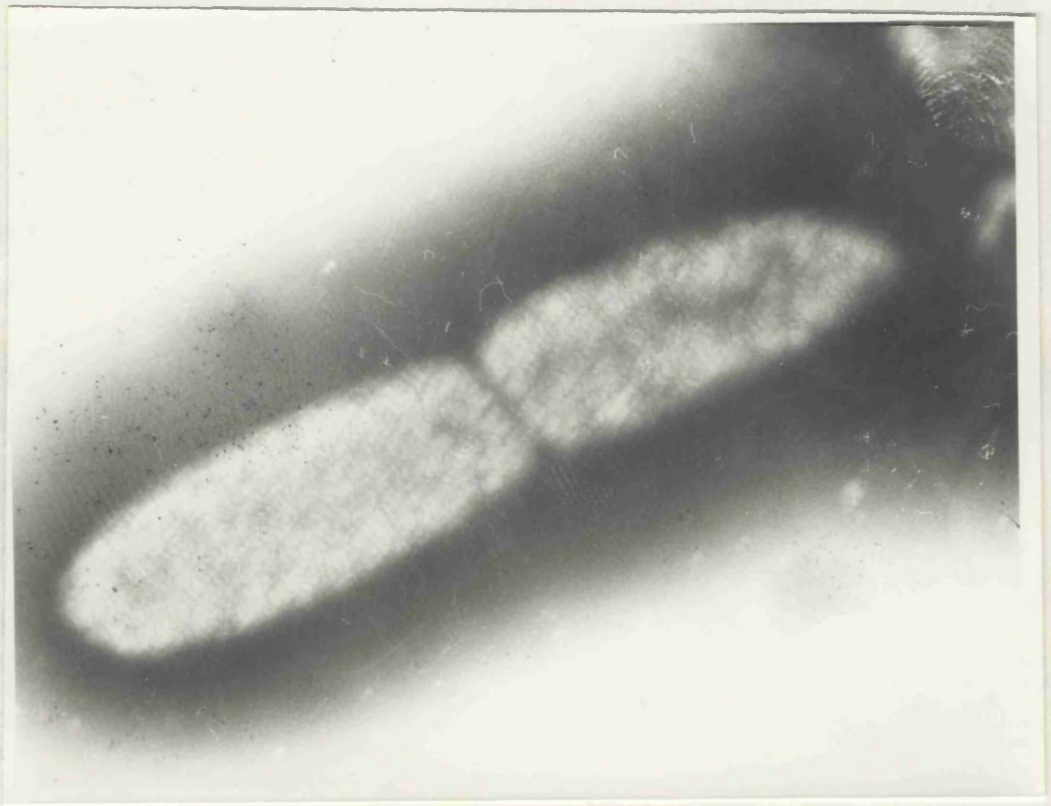


Fig 26 continued.

repair of DNA damage and resynthesis of the enzymes involved in its replication and of the enzymes and proteins required for cell division occurred rapidly after heating.

The effect of heat on DNA was investigated using another method. Heat stressed cells were plated on nutrient agar supplemented with DL-pantoyllactone and their percentages of survival were compared with those of cells plated on nutrient agar alone. DL-pantoyllactone is known to reverse the cell division inhibition observed in uv irradiated cells.

To study whether addition of pantoyllactone can enhance the recovery of cells exposed to high temperature, the heated cells were plated on nutrient agar containing 0.05M pantoyllactone and the percentage of cells forming colonies on this medium was compared with that of cells plated on nutrient agar. Table 15 shows that after heating at 60°C for 2 or 5 min, the percentages of survival of the ColV$^-$ strain as measured by plating on nutrient agar were similar to the percentages obtained when heated cells were plated on nutrient agar + pantoyllactone. Similarly, plating on nutrient agar containing pantoyllactone did not increase the percentage of survival of the heated ColV$^+$ cells (Table 15). These

Table 15: Effect of plating heated cells on media containing DL-pantoyllactone.

Strain	Plating medium	%survival after heating for	
		2 min	5 min
ED1829	NA	91.5	9.6
ED1829ColV, I-K94	NA	31	0.1
ED1829	NA+pantoyllactone	84	12
ED1829ColV, I-K94	NA+pantoyllactone	34	0.1

Organisms were grown to the stationary phase at 37°C, heated to 60°C and treated as described in Materials and Methods.

NA: nutrient agar.

observations may indicate that heat does not damage DNA. However, it is possible that both DNA damage and damage to other cytoplasmic components had occurred. In this case, increase in cell mass cannot occur, which explains the ineffectiveness of pantoyllactone in enhancing the survival of heated cells.

The thermal sensitivity of a polA strain (DP1152) was tested. Table 16 shows that the DNA polymerase I-deficient strain is more heat sensitive than the parent strain (AB1157), the former was 20 times more sensitive than AB1157 cells after heating at 60°C for 2 min. This indicates that polA cells exposed to high temperature have reduced ability to repair the damaged DNA and suggests that heating may induce formation of single strand breaks in DNA.

The heat sensitivity of the ColV$^+$ derivatives of these strains was also studied. Presence of the ColV plasmid in AB1157 cells increased their sensitivity to heat (Table 16). On the other hand, DP1152 ColV,I-K94 cells are 2.5 times more heat resistant than the P$^-$ strain when heated at 60°C for 2 min; and after heating for 5 and 10 min similar percentages of survival were observed in both strains (Table 16). TheColV plasmid may encode the production of DNA polymerase I which can

Table 16: Heat sensitivity of polA cells.

Strain	Exposure to heat for 2 min		Exposure to heat for 5 min		Exposure to heat for 10min	
	%survival	%injury	%survival	%injury	%survival	%injury
AB1157	80	99	16	99	0.2	99.6
AB1157ColV, I-K94	5	99.7	0.2	99	0.01	99
DP1152	4	95	0.24	97	0.002	85
DP1152ColV, I-K94	10	96	0.2	99	0.002	95

Bacteria were grown to the stationary phase at 37°C, heated at 60°C and treated as described in Materials and Methods.

substitute for the missing enzyme in polA strains. Alternatively, the effect of ColV on normal strains may depend on its ability to reduce polA dependent repair of heat damage. If so, ColV would not affect the polA strain.

To investigate the damage to other cytoplasmic components, the effect of storage of heated cells in liquid minimal medium before enumeration of the surviving cells on nutrient agar was tested. Repair of some damaged cellular components may occur during storage in minimal medium. Table 17 shows that the percentages of survival of the heated strains, ED1829 and ED1829 ColV, I-K94, did not increase when cells were stored in minimal medium for 120 min. Similar percentages of survival were obtained whether cells were stored in minimal medium or plated immediately on a complex medium, suggesting that damaged cells are not repaired during incubation in minimal medium. Heated cells may be severely damaged requiring storage for longer time to repair the damage. It is also possible that the damaged cells require complex nutrients (amino acids or sugars) for repairing the damaged sites in the cell. Enzymes necessary for synthesis of these complex substances may be inactivated by the heat treatment.

Table 17: Effect of incubation in liquid minimal medium on recovery of heated cells.

Storage of cells in minimal medium	ED1829		ED1829ColV,I-K94	
	%survival	%injury	%survival	%injury
+	26	84	2	98
−	24	82	2	97

Organisms were grown to the stationary phase at 37°C, heated at 60°C for 5 min and treated as described in Materials and Methods. The percentages of survival and injury are calculated as described in Table 7.

3.3. Growth conditions influencing the thermal resistance of ColV$^+$ cells.

3.3.1. Growth in magnesium - enriched medium.

The heat sensitivity of ED1829 and ED1829 ColV,I-K94 strains grown in broth supplemented with $MgSO_4$ (0.05M) is presented in Table 18. Growth in magnesium - enriched medium did not increase the heat resistance of ColV$^-$ cells. The heat sensitivity of the ColV$^-$ strain grown in nutrient broth containing $MgSO_4$ is similar to that of ColV$^-$ cells grown in nutrient broth alone.

On the other hand, growth of the ColV$^+$ cells in magnesium enriched medium increased the heat resistance of these cells. After 5 min heating at 60^{o}C, the cells grown in medium supplemented with magnesium are 20 fold more resistant than the same cells grown in nutrient broth (Table 18). Also, growth in magnesium - enriched medium greatly reduced the difference between the heat sensitivity of the ColV$^-$ and ColV$^+$ cells. Production of colicin and VmpA protein is reduced in cells grown in Mg - enriched medium, which is in accord with synthesis (or secretion) of colicin being involved in the increased heat sensitivity of the ColV$^+$ cells and these results may also implicate VmpA protein in heat sensitivity.

The heat sensitivity of the ColV$^-$ and ColV$^+$ cells grown in magnesium - depleted broth is presented in

Table 18: A. Heat resistance of cells grown in magnesium - enriched broth.

Time of heating	ED1829		ED1829 ColV,I-K94	
	% survival	% injury	% survival	% injury
2	44	97	43	99
5	10	98	6	97
10	0.4	91	0.3	99

Table 18: B. Heat resistance of cells grown in nutrient broth.

Time of heating	ED1829		ED1829 ColV,I-K94	
	% survival	% injury	% survival	% injury
2	72	80	41	85
5	17	91	0.3	78
10	1.6	95	0.02	88

Organisms were grown in nutrient broth containing 0.05M $MgSO_4$ or in nutrient broth to the stationary phase at 37°C. The bacterial suspensions were heated at 60°C and treated as described in Materials and Methods. The percentages of survival and injury were calculated as described in Table 7.

Table 19. Both ColV$^-$ and ColV$^+$ cells showed increased sensitivity to heat when grown in this medium although the effect was greater for the ColV$^+$ strain. On heating at 60^{o}C for 5 min, growth in Mg - depleted medium resulted in 5 fold increase in the heat sensitivity of the ColV$^-$ strain although the effect was less marked at 10 min. For the ColV$^+$ strain, cells grown in this medium were 15 fold more heat sensitive than the ColV$^+$ cells grown in nutrient broth.

3.3.2. Growth at low pH.

It is important to know whether organisms grown at mild acid pH have altered heat sensitivity because, for example, chicken carcasses are sometimes washed with lactic acid or succinic acid as a final step in their processing and these will have slightly acidic surfaces; altered heat sensitivity of such organisms might have implications for food safety. In this study, the effect of growth of ΔD1829 ColV, I-K94 cells at pH 5 and 7 on their heat sensitivity was examined. Table 20 shows that when heated at 60^{o}C for 5 or 10 min, the cells grown at pH 5 are 2 fold more sensitive to heat than those grown at pH 7; indicating that growth at low pH only slightly increases the heat sensitivity of the ColV$^+$ cells.

Table 19: Heat resistance of cells grown in magnesium - depleted broth.

Time of heating (min)	ED1829		ED1829 ColV,I-K94	
	% survival	%injury	% survival	% injury
5	3	88	0.02	99.7
10	0.8	98	0.002	93
15	0.1	95	0.001	90

Organisms were grown to the stationary phase at 37°C, and treated as described in Materials and Methods. The percentages of survival and injury are calculated as described in Table 7.

Table 20: Heat sensitivity of ColV$^+$ cells grown at low pH.

Time of heating (min)	Growth at pH 5		Growth at pH 7	
	% survival	% injury	% survival	% injury
2	65	92	83	98
5	6	99	11	99
10	0.1	100	0.2	100

Cells were grown at 37^{o}C to the stationary phase in L-broth, and heated in nutrient broth at 60^{o}C.

3.3.3. Exposure to gradual rise in temperature before heating.

To examine the effect of exposure to rise in temperature on the heat resistance of bacteria subsequently exposed to higher temperatures, the cells were subjected to a gradual rise in temperature from 34 to 50°C and the percentage of survival after heating at 60°C for 5 min was determined. Table 21 shows that for the ED1829 strain this treatment increased the heat resistance of the cells 8 fold. For the ED1829 ColV,I-K94 strain, exposure to gradual rise in temperature before heating at 60°C resulted in 50 fold increase in the heat resistance of this strain and almost abolished the difference between the Col^- and $ColV^+$ strains. However, gradual temperature increase (using the same method) on another strain, P678-54 and its $ColV^+$ derivative, did not increase its heat resistance (Table 21). Specific proteins (the heat shock proteins) are synthesised when cells are exposed to a shift to a higher temperature, the synthesis of these proteins is presumably associated with increased cellular heat resistance. The unchanged heat resistance of P678-54 strain after heat shocking may be due to absence of some of the heat shock genes in this strain. It is also possible that some P678-54 cells are highly sensitive to heat and killed during the temperature rise to 50°C, but this can be ruled out since after heating at 50°C the percentage of survival of this

Table 21. A. Resistance of cells exposed to gradual rise in temperature before heating at 60°C.

Strain	% survival	% injury
ED1829	60	92
ED1829 ColV,I-K94	25	87
P678-54	4	97
P678-54 ColV,I-K94	0.4	96

Table 21: B. Resistance of cells heated directly at 60°C.

Strain	% survival	% injury
ED1829	7.4	97
ED1829 ColV,I-K94	0.5	98
P678-54	6	94
P678-54 ColV,I-K94	0.5	96

60°C exposure time was 5 min.

strain was only slightly lower than that of ED1829 (data not shown).

To examine the stability of the heat resistance induced during the rise in temperature, the cells were heat shocked, left at room temperature for 120 min and their survival was measured after exposure to high temperature (60^{o}C for 5 min). The percentage of survival of the cells exposed to gradually increasing temperature (Table 22A) was nearly similar to that of the cells heated directly at 60^{o}C (Table 22B). This suggests that the thermal resistance induced by exposure to a gradual rise in temperature before heating at higher temperatures is transient. The heat shock proteins synthesised after heat shocking cells may be degraded rapidly.

Table 22: Extent of heat shock response.

	Strain	% survival	% injury
A.	ED1829	0.3	98
	ED1829 ColV,I-K94	0.3	100
B.	ED1829	0.2	100
	ED1829 ColV,I-K94	0.1	99

Cells grown to the stationary phase at 37^{o}C were exposed to rise in temperature from 34-50^{0}C (A) or from 34-37^{o}C (B), left at room temperature for 120 min and heated at 60^{o}C for 5 min.

3.4. Studies on wild type ColV$^+$ isolates from the chicken processing plant.

The ColV plasmid bearing *E. coli* cells are more pathogenic than plasmid-free cells in that they have increased ability to survive in body tissues. Contaminated food is a likely source of pathogenic *E. coli* and investigation of the incidence and properties of ColV$^+$ strains in samples from chicken processing plants may play a role in reducing the prevalence of these infections.

3.4.1. Identification and properties of *E. coli* strains

a. Biochemical identification

Specimens were obtained from the scalding tanks of chicken processing plants. Samples from tanks of 52^{o}C and 60^{o}C scalding temperatures were examined. The isolates were streaked on MacConkey agar and identified to the genus or species level by using the API 20E identification system. The source and classification of each isolate is presented in Table 23; 74% of the isolates were *E. coli*.

b. Colicin production.

The strains were tested for colicin production by using ED1829 as the indicator strain. The colicin producing isolates are presented in Table 23; 16 of the 35 *E. coli* strains (46%) produced a colicin or colicins to which ED 1829 was sensitive. Out of 13 Col$^+$ strains

Table 23: Classification and properties of isolates from the chicken processing plant.

Isolate Number	Classification	Source	Growth on MacConkey agar	Colicin production (size of zone of inhibition)
1	Escherichia coli	Scalding tank	+,red colonies	+(11mm)
3	E. coli	Scalding tank	+,red colonies	-
5	Salmonella spp	chicken skin	+,yellow colonies	-
6	E. coli	Scalding tank	+,red colonies	+(7mm)
13	E. coli	Scalding tank	+,red colonies	+(8mm)
14	E. coli	Scalding tank	+,red colonies	-
15	E. coli	Scalding tank (60°C)	+,red colonies	+(11mm)
16	E. coli	Scalding tank(60°C)	+,red colonies	-
17	Acinetobacter calcoaceticus	Scalding tank(60°C)	+,red colonies	-
18	Escherichia hermannii	Scalding tank(60°C)	+,yellow colonies	-
19	E. coli	Scalding tank(60°C)	+,red colonies	+(4mm)
20	Kluyvera spp.	Scalding tank(60°C)	+,red colonies	-
21	Kluyvera spp.	Scalding tank(52°C)	+,red colonies	-
22	Proteus mirabilis	scalding tank(52°C)	+,yellow colonies	-

Table 23 continued.

Isolate Number	Classification	Source	Growth on Macconkey agar	Colicin production (size of zone of inhibition)
23	*E. coli*	Scalding tank(52°C)	+,red colonies	-
25	*E. coli*	Scalding tank(52°C)	+,red colonies	+(11mm)
27	*E. coli*	Scalding tank(52°C)	+,red colonies	-
28	*Escherichia hermannii*	Scalding tank(52°C)	+,yellow colonies	-
29	*E. coli*	Scalding tank(52°C)	+,red colonies	+(12mm)
30	*E. coli*	Scalding tank(52°C)	+,red colonies	-
32	*E. coli*	Scalding tank(52°C)	+,red colonies	-
34	*E. coli*	Scalding tank(52°C)	+,red colonies	+(12mm)
35	*E. coli*	Scalding tank(52°C)	+,red colonies	-
37	*E. coli*	Scalding tank(52°C)	+,red colonies	-
39	*E.coli*	Scalding tank(52°C)	+,red colonies	+(4mm)
40	*E. coli*	Scalding tank(52°C)	+,red colonies	+(10mm)
41	ND	Scalding tank(60°C)	+,yellow colonies	ND
42	*E. coli*	Scalding tank(60°C)	+,red colonies	+(12mm)
43	*E. coli*	Scaldnig tank(60°C)	+,red colonies	+(11mm)

Table 23 continued.

Isolate Number	Classification	Source	Growth on MacConkey agar	Colicin production (size of zone of inhibition)
44	*E. coli*	Scalding tank (60°C)	+, red colonies	+(11mm)
45	ND	Scalding tank (60°C)	+, yellow colonies	ND
46	ND	Scalding tank (60°C)	+, yellow colonies	ND
48	*E. coli*	Scalding tank (60°C)	+, red colonies	-
50	*E. coli*	Scalding tank (60°C)	+, red colonies	-
51	*E. coli*	Scalding tank (60°C)	+, red colonies	-
52	*E. coli*	Scalding tank (60°C)	+, red colonies	-
53	*E. coli*	Scalding tank (60°C)	+, red colonies	+(16mm)
54	*E. coli*	Scalding tank (60°C)	+, red colonies	-
55	*E. coli*	Scalding tank (60°C)	+, red colonies	+(.8mm)
56	*E. coli*	Scalding tank (60°C)	+, red colonies	-
57	*E. coli*	Scalding tank (60°C)	+, red colonies	+(21mm)
58	*E. coli*	Scalding tank (60°C)	+, red colonies	-
59	ND	Scalding tank (60°C)	+, yellow colonies	ND
60	ND	Scalding tank (60°C)	+, yellow colonies	ND

Table 23 continued.

Isolate Number	Classification	Source	Growth on MacConkey agar	Colicin production (size of zone of inhibition)
61	*E. coli*	Scalding tank(60°C)	+,red colonies	-
62	*E. coli*	Scalding tank(60°C)	+,red colonies	-
63	*E. coli*	Scalding tank(60°C)	+,red colonies	-

ED 1829 was used as the indicator strain for colicin testing.

ND: not determined

isolated from scalding tanks of specified temperature, 8 were isolated from 60°C tanks (out of a total of 19 E. coli strains) whilst 5 (out of 11 E. coli) derived from 52°C tanks. To identify the colicinV producing strains, the isolates were overlaid with indicator strains that produce colicinV, colicin Ia or both. If the unknown colicin (from the wild isolate) does not inhibit or only slightly inhibits the growth of ED1829 ColV,I-K94 (as observed in colicins produced by strains 6, 13, 19, 25, 29, 39 and 57) the strains are considered likely to be producing colicins V and Ia or one of them (Table 24A).

To confirm the colicin type, the immunity of these strains to the colicins produced by ED 1829 ColV-K30 and ED 1829 R483 Ia was tested. The strains 19, 39 and 57 produce colicins V and Ia since they are immune to both types (Table 24B). The strains 25 and 29 probably produce colicin Ia plus a colicin V of a subtype different from that encoded by ED 1829 ColV-K30 (Table 24B). Strains 6 and 13 produce colicins V and Ia since they are immune to both (Table 24B); in addition, these strains probably produce another type of colicin(s) because ED 1829 ColV,I-K94 strain is not fully immune to the colicins produced by strains 6 and 13 (Table 24A).

c. Antibiotic sensitivity.

Table 24A: Identification of colicin V-producing strains

Isolate Number	zone sizes with indicator strains			
	ED1829	ED1829ColV,I-K94	ED1829ColV-K30	ED1829R483Ia
1	11mm	12mm	12mm	ND
6	7	4	12	6mm
13	8	4	4	8
15	11	11	11	ND
19	4	0	3	7
25	11	0	6	4
29	12	0	6	4
34	12	10	8	ND
39	4	0	3	9
40	10	6	6	ND
42	12	11	10	9
43	11	11	11	9
44	11	10	11	10
53	16	10	12	17
55	8	7	9	9
57	21	0	4	20
ED1829 ColV, I-k94	5	0	7	23

The values represent the sizes of zones of growth inhibition.

ND: not determined.

Table 24B: Immunity of isolates to colicins V and Ia

Overlay with isolate number (Indicator strain)	Zone sizes with producer strain	
	ED1829ColV-K30	ED1829R483Ia
6	0	0
13	0	0
19	0	0
25	8	0
29	9	0
39	0	0
57	0	0

The values represent the sizes of zones of growth inhibition.

The sensitivity of the isolates from the chicken processing plant to 11 antibiotics was tested. All the strains were sensitive to gentamicin (Table 25). Variable percentages of resistance were obtained for other chemotherapeutically important drugs. Twenty nine percent, 43% and 98% of the isolates were resistant to chloramphenicol, ampicillin and cotrimoxazole, respectively. For tetracycline (50µg), an antimicrobial drug that is used widely in poultry, 67% of the strains were resistant (Table 25). All isolates were resistant to erythromycin except strain 17, this antibiotic is effective against only few Gram negative bacteria due to the permeability barrier properties of the cell envelope. Also all isolates were resistant to sulphafurazole. Table 25 also shows that multiple antibiotic resistance is frequently observed in the isolates. Excluding resistance to erythromycin and sulphafurazole, the mean number of resistances was 2.7 $\pm$0.3 for the Col^- *E. coli* strains, and 3.7$\pm$0.4for the Col^+ *E. coli* strains.

d. Plasmid content

Plasmid DNA of the colicin V producing strains was isolated and subjected to agarose gel electrophoresis to attempt to confirm the presence of ColV plasmids. Also, the plasmid content of other wild type isolates was examined. Fig 27 shows that strain 14 (lane 4) does not have a plasmid. Strain 5 may also be p^-. These 2

Table 25: Antibiotic sensitivity of isolates from the chicken-processing plant.

Isolate number	GN	CR	CT	SF	AMP	CAR	SXT	TE	C	E	S	TE
1	S	S	S	R	S	S	R	R	S	R	R	R
3	S	S	S	R	S	S	S	S	S	R	S	S
5	S	S	S	R	S	S	R	R	R	R	R	R
6	S	R	S	R	R	S	R	R	S	R	S	R
13	S	R	S	R	S	S	R	R	S	R	S	R
14	S	R	S	R	R	S	R	R	S	R	R	R
15	S	S	S	R	S	S	R	S	S	R	S	R
16	S	S	S	R	S	S	R	R	S	R	S	R
17	S	R	S	R	R	S	R	R	R	S	R	R
18	S	S	S	R	S	S	R	R	S	R	S	R
19	S	S	S	R	S	S	R	R	S	R	S	R
20	S	S	S	R	S	S	R	R	R	R	S	R
21	S	S	S	R	S	S	R	S	S	R	S	R
22	S	R	R	R	R	S	R	S	R	R	S	R
23	S	S	S	R	R	S	R	R	R	R	S	R
25	S	R	S	R	R	R	R	R	S	R	R	R
27	S	S	S	R	R	R	R	R	R	R	S	R
28	S	S	S	R	S	S	R	R	S	R	R	R
29	S	R	S	R	R	R	R	R	S	R	S	R
30	S	S	S	R	R	S	R	S	S	R	R	R
32	S	S	S	R	R	S	R	R	R	R	S	R
34	S	S	S	R	R	R	R	R	R	R	R	R

Table 25 continued

Isolate number	GN	CR	CT	SF	AMP	CAR	SXT	TE	C	E	S	TE
35	S	S	S	R	S	S	R	R	R	R	S	R
37	S	S	S	R	R	R	R	R	S	R	S	R
39	S	S	S	R	S	S	R	R	S	R	S	R
40	S	R	S	R	R	R	R	R	S	R	R	R
42	S	S	S	R	R	S	R	R	S	R	S	R
43	S	S	S	R	R	S	R	R	R	R	S	R
44	S	S	S	R	R	S	R	S	R	R	S	R
48	S	S	S	R	S	S	R	R	S	R	S	R
50	S	S	S	R	S	S	R	S	S	R	S	R
51	S	S	S	R	S	S	R	S	S	R	S	R
52	S	S	S	R	S	S	R	S	R	R	S	R
53	S	S	S	R	R	R	R	S	S	R	S	R
54	S	S	S	R	S	S	R	S	S	R	S	R
55	S	R	S	R	R	S	R	R	S	R	R	R
56	S	S	S	R	S	S	R	S	S	R	S	R
57	S	S	S	R	S	S	R	S	S	R	S	R
58	S	S	S	R	S	S	R	S	S	R	S	R
61	S	S	S	R	S	S	R	R	S	R	S	R
62	S	S	S	R	S	S	R	R	S	R	S	R
63	S	S	S	R	S	S	R	R	S	R	S	R

GN: gentamicin (10μg), CR: cephaloridine (25μg),

CT: colistin sulphate (10μg), SF: sulphafurazole (500μg),

AMP: ampicillin (25μg), CAR: carbenicillin (100μg),

SXT: cotrimoxazole (25μg), TE: tetracycline (50μg),

C: chloramphenicol (10μg), E: erythromycin (10μg),

S: streptomycin (10μg).

S: sensitive (zone of growth inhibition is observed),

R: resistant.

strains are Col$^-$. Strikingly both show multiple antibiotic resistance, presumably chromosomally- encoded. Strain 6 (Fig 27 lane 2) probably has two plasmids. The band marked A shows a similar mobility to the ColV, I-K94 plasmid. The band marked B may be the covalently closed circular form of this. This could be established by heating the plasmid at 100^{o}C followed by cooling, the CCC form will renature after this treatment whereas the OC form will not. The band marked C is a smaller second plasmid. Strain 13 (Fig 27 lane 3) has 3 plasmids; the larger one shows the same mobility as the ColV,I-K94 plasmid.

Strains 16, 19 & 30 have several plasmids of variable sizes (Fig 28 lanes 1, 2 & 3); one of those in 16 and 19 resembles ColV,I-K94 in mobility. Strain 39 (Fig 28 lane 4) has a larger plasmid with a similar mobility to the ColV,I-K94 plasmid in addition to several small plasmids.

Fig 29 lane 2 shows that strain 57 has a plasmid of a similar mobility to that of the ColV,I-K94 plasmid. Strain 1 (Fig 30 lane 1) has many small plasmids and strain 3 (Fig 30 lane 2) has plasmids of variable sizes.

3.4.2. Transfer of ColV plasmids

To study the behaviour of wild ColV plasmids

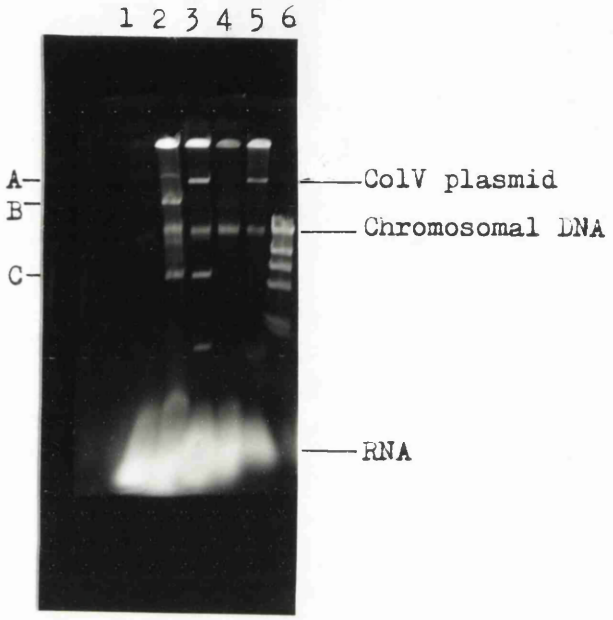


Fig 27: Plasmid content of wild type isolates. Lane 1: strain 5, lane 2: strain 6, lane 3: strain 13, lane 4: strain 14, lane 5: ED1829ColV,I-K94, lane 6: λDNA Hind III digest.

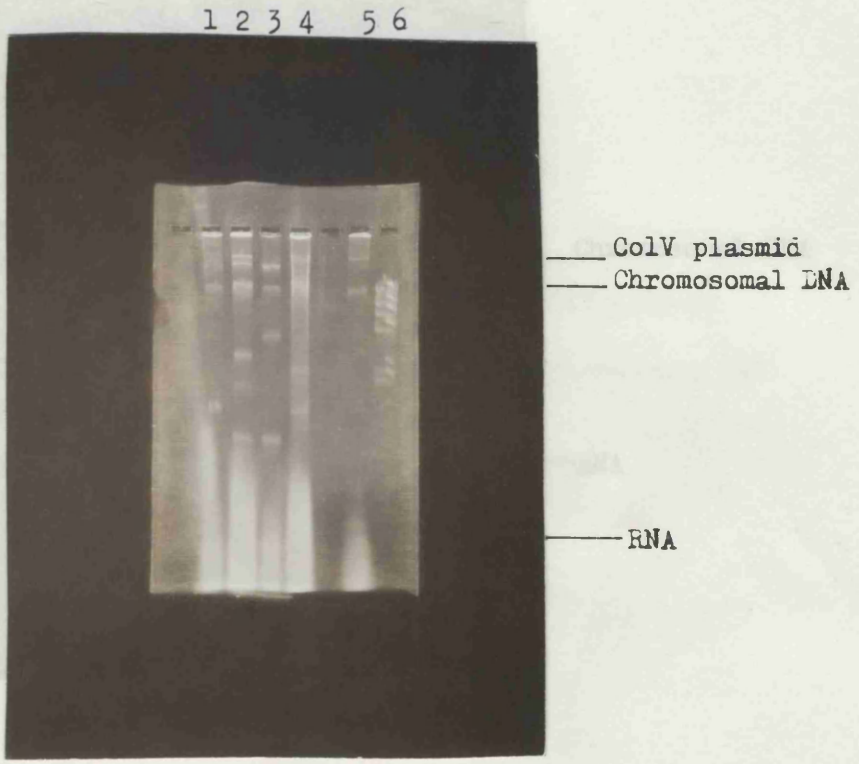


Fig 28: Plasmid content of wild type isolates. Lane 1: strain 16, lane 2: strain 19, lane 3: strain 30, lane 4: strain 39, lane 5: ED1829ColV,I-K94, lane 6: λDNA Hind III digest.

Fig 29: Plasmid content of wild type isolates. Lane 1: strain ED1829ColV,I-k94, lane 2: strain 57, lane 3: strain 55, lane 4: strain 51, lane 5: strain 50.

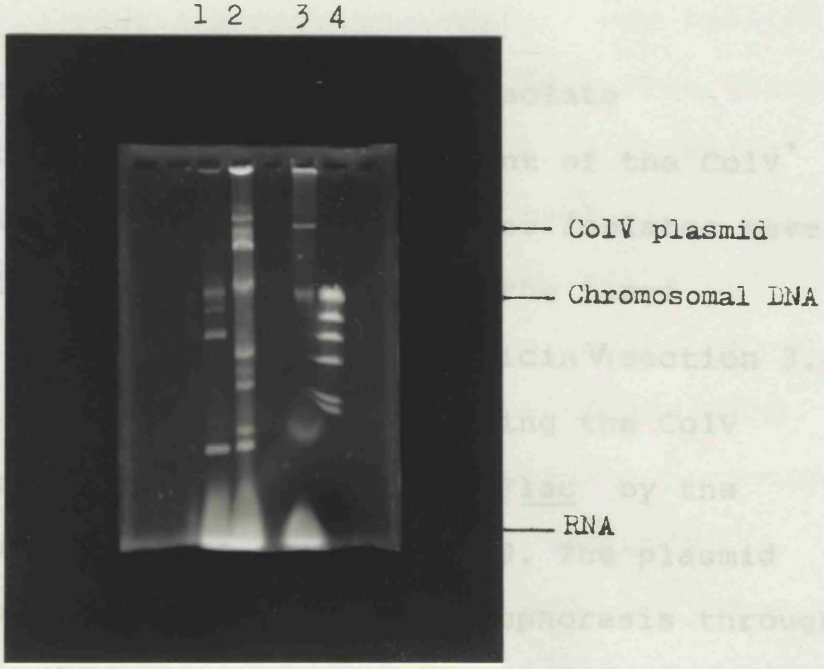


Fig 30: Plasmid content of wild type isolates. lane 1: strain 1, lane 2: strain 3, lane 3: ED1829ColV,I-K94, lane 4: λDNA Hind III digest.

alone in laboratory strains of E. coli (particularly their effects on heat resistance) and to examine how ColV,I-K94 behaves in a naturally p^- chicken isolate (especially effect on heat resistance) transfer of ColV plasmids has been attempted.

a. DNA transfer from a $ColV^+$ chicken isolate

Examination of the plasmid content of the $ColV^+$ wild type isolates showed that all these isolates have one or more plasmid(s) in addition to the large plasmid which is assumed to encode colicin V (section 3.4.1. d). Therefore, to obtain cells harbouring the ColV plasmid only transformation of ED1829 Flac by the ColV plasmid of strain 13 was attempted. The plasmid DNA was extracted, subjected to electrophoresis through agarose gels and the band corresponding to the ColV plasmid was electroeluted and mixed with permeabilised ED1829 Flac cells. Transformants carrying the ColV plasmid were not observed.

b. Transfer to a Col^- chicken isolate of ColV,I-K94

When transfer of ColV,I-K94 plasmid from a K12 strain (ED1829$ColV^+$) into a wild type plasmid free strain (14) was tested by conjugation 14$ColV^+$ cells were isolated.

3.4.3. Curing of ColV plasmids

To study the effect of wild type ColV plasmids on the host's heat sensitivity, comparing these strains with isogenic strains lacking the plasmid is necessary. To eliminate the ColV plasmid, two curing procedures were used. For curing strain 39, exposure to sodium dodecylsulphate (SDS) and high temperature was applied. Colonies from the treated bacterial culture were tested for colicin production and the non-colicin producing ones were examined for the presence of the ColV plasmid. Fig 31 shows that the cells have not lost the plasmid but may be carrying mutant or deletion derivatives of the plasmid. The same method was used to attempt to cure strain 13. The non-colicin producing derivatives were tested for the loss of the ColV plasmid; Fig 32 shows that these cells did not lose the plasmid either. Curing by SDS and high temperature depends on the increased sensitivity, to the selection procedures, of the cells carrying the plasmid compared with the plasmid-free cells. Presence of the plasmid-specified components in the cell envelope (e.g. some of the products of the tra genes) may allow for SDS permeation and consequent lysis of plasmid-bearing cells. Therefore, plasmid-free cells or cells carrying mutated derivatives of the plasmid may be selected.

For curing strain 57, rifampicin at a concentration of 5 or 8μg/ml was used. The treated bacteria were

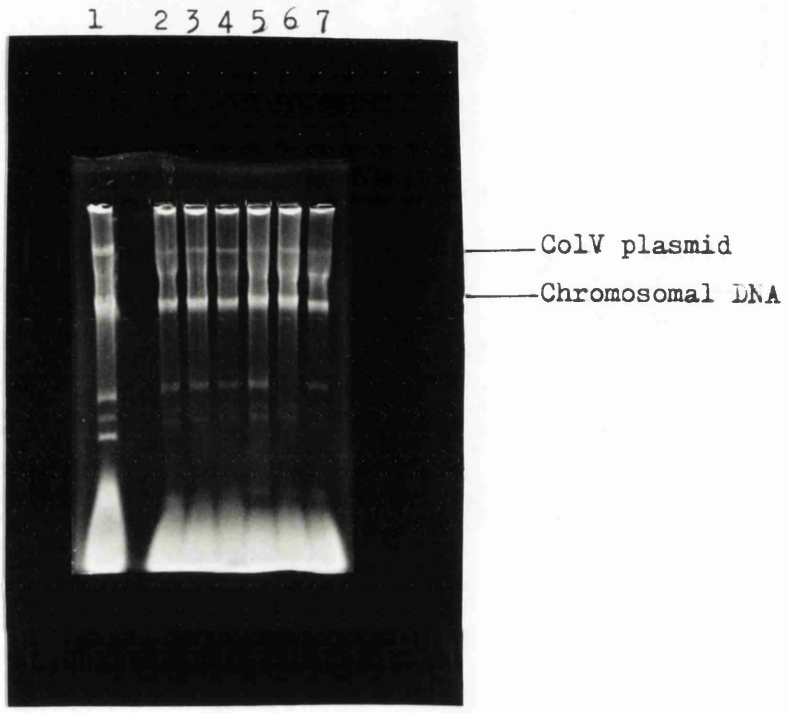


Fig 31: Plasmid content of the non-colicin producing derivatives of strain 39. lane 1: strain 39, lanes 2-7: non-colicin producing derivatives of strain 39.

Fig 32: Plasmid content of the non-colicin producing derivatives of strain 13. Lane 1: strain 13, lane 2: non-colicin producing derivative of strain 13.

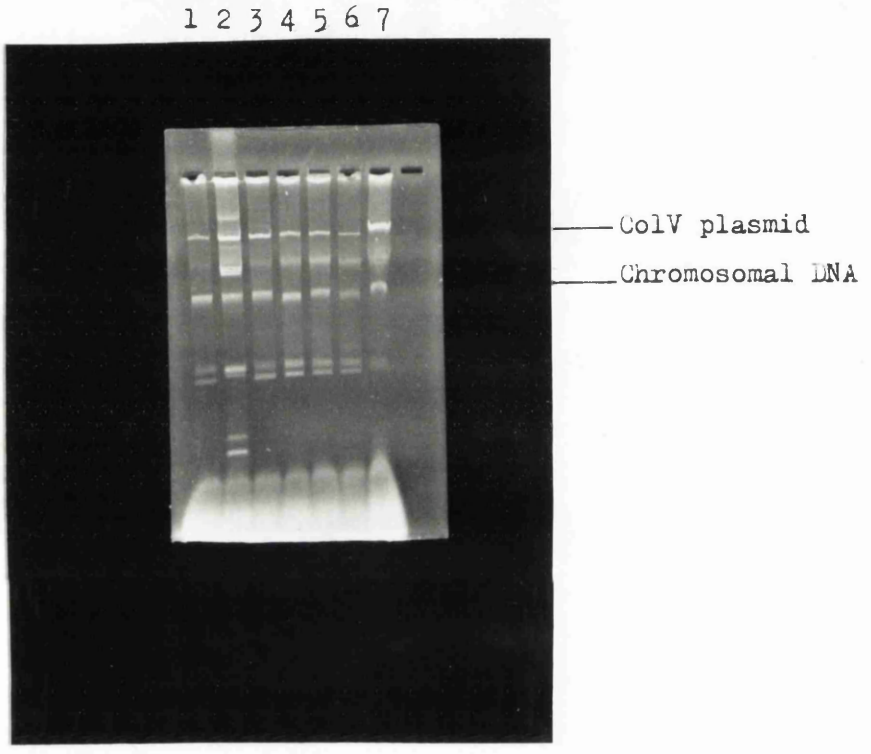


Fig 33: Plasmid content of the non-colicin producing derivatives of strain 57. Lanes 1-6: non-colicin producing derivatives of strain 57, lane 7: strain 57.

tested for colicin production and the non-colicin producing colonies were examined for the presence of the ColV plasmid; fig 33 shows that these derivatives retained the plasmid also.

3.4.4. Properties of the wild ColV$^+$ strains

a. VmpA protein production

Some ColV plasmids encode the production of a 33Kd outer membrane protein, VmpA. It is not possible to compare strains carrying the wild ColVs with isogenic p$^-$ strains because the chicken isolates have not been cured neither was the attempted transfer of ColVs from chicken isolates successful. We have nonetheless scored the ColV$^+$ chicken isolates for a 33K protein resembling VmpA protein. This protein has a similar molecular weight to the OmpA protein, and to test for the presence of VmpA the protein content of the cell envelopes that are dissociated at 100^{o}C is compared with those that are dissociated at 30^{o}C. The OmpA protein is a heat modifiable protein and its apparent molecular weight decreases to ~28Kd when cell membranes are dissociated at low temperatures (30^{o}C) in SDS; VmpA protein is not heat modifiable and can be observed at the 33Kd position. Figure 34 shows that cell envelopes of the wild type ColV$^+$ isolates, strains 57, 13 and 6 (lanes 2, 8 & 10) contain a protein resembling VmpA protein. It is apparently absent from strains 39 and 19.

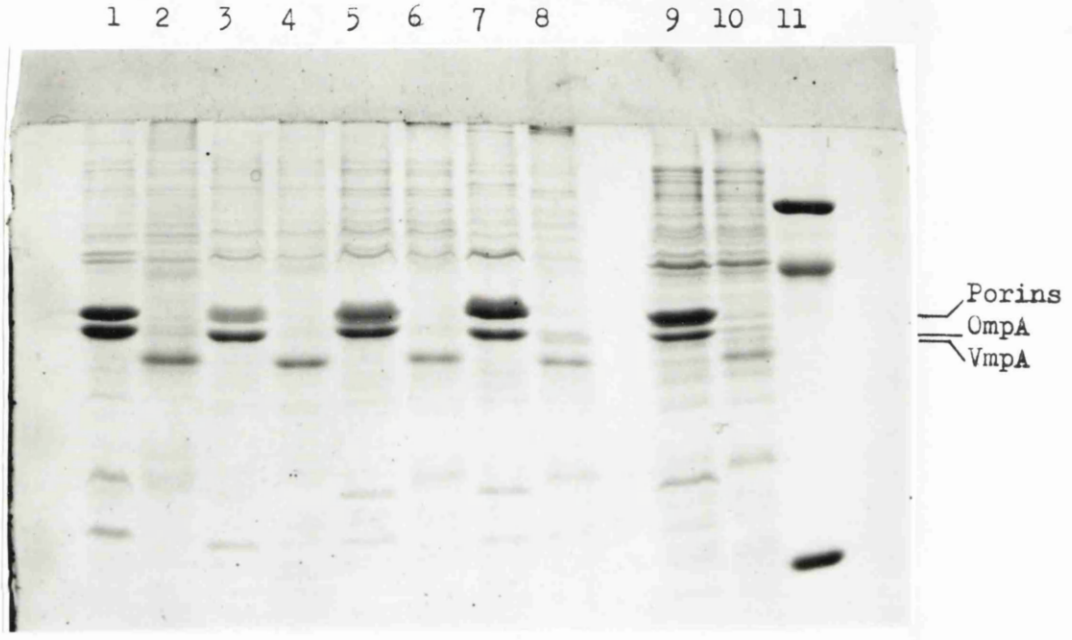


Fig 34: SDS-polyacrylamide gel electrophoresis of cell envelopes of ColV$^+$ strains. 1&2: strain 57, 3&4: strain 39, 5&6: strain 19, 7&8: strain 13, 9&10: strain 6. Lanes 1, 3, 5, 7, and 9: 100^{o}C dissociation, lanes 2, 4, 6, 8 and 10: 30^{o}C dissociation. Lane 11: molecular weight standards, 68, 43&14kd.

b. Heat sensitivity

It has not been possible to examine the effects of the chicken isolate ColV plasmids on heat sensitivity but the effect of ColV,I-K94 on a p^- chicken isolate has been tested. To examine the effect of ColV,I-K94 , strain 14 (isolated from a chicken scalding tank) and its $ColV^+$ derivative (obtained by transfer of the plasmid from ED1829ColV,I-K94 into strain 14) were heated at 60°C for 2, 5 and 10 min. Table 26 shows that the ColV plasmid bearing strain has similar heat sensitivity to the p^- strain. Comparing this observation with the heat sensitivity of E. coli K12 strains and their $ColV^+$ derivatives (Table 7) shows that, in contrast to the wild type strain, presence of this ColV plasmid in K12 strains increases their sensitivity to heat. Since the greater heat sensitivity of K12 $ColV^+$ strains compared to Col^- ones may reflect a greater sensitivity of the $ColV^+$ envelope to damage, the absence of an effect of ColV,I-K94 on strain 14 may be related to a different envelope protein profile in 14 allowing 14ColV,I-K94 envelopes to be more resistant to heat.

To test this, the protein content of the envelopes of the heated and unheated cells of strains 14 and 14 $ColV^+$ was analysed by SDS-polyacrylamide gel electrophoresis. After heating the $ColV^-$ and $ColV^+$ cells at 60°C for 5 min, all the major outer membrane

Table 26: Effect of the ColV,I-K94 plasmid on the heat sensitivity of a wild type isolate

Time of heating (min)	14		14 ColV,I-K94	
	%survival	%injury	%survival	%injury
2	19	92	22	90
5	0.3	99	0.6	99
10	0.02	100	0.03	100

Bacteria were grown to the stationary phase at 37°C, heated at 60°C and treated as described in Materials and Methods.

proteins (porins and OmpA) can be observed (Fig 35). Comparing the 100°C dissociated envelopes with the 30°C dissociated ones gives an indication of the degree of the denaturation of the cell envelope proteins. Dissociation of cell envelopes in 2% SDS at 30°C does not allow solubilization of the proteins causing absence of most of the protein bands from the gel. However, comparing proteins of the 30°C dissociated cell envelopes of heated and unheated cells shows that heating of cells at 60°C for 5 min causes appearance of protein bands that could not be revealed when the cell envelopes of unheated cells were dissociated at 30°C (Fig 35 lanes 2 & 6 and 4 & 8). These observations are similar to those obtained when the heated K12 strains (ED1829 and ED1829 ColV,I-K94) were dissociated at 30°C (Fig 14), suggesting that heating of 14ColV$^+$ cells weakens the interactions between the proteins and other constituents of the cell envelope to a similar degree to that observed in K12ColV$^+$ cells.

Also, the lipopolysaccharide structure in wild type isolates differs from that of K12 strains, the former have usually complete LPS in contrast to K12 strains that do not possess the O-antigen portion of the LPS. The amount of LPS released from heated 14ColV$^+$ cells may be less than that observed in heated K12

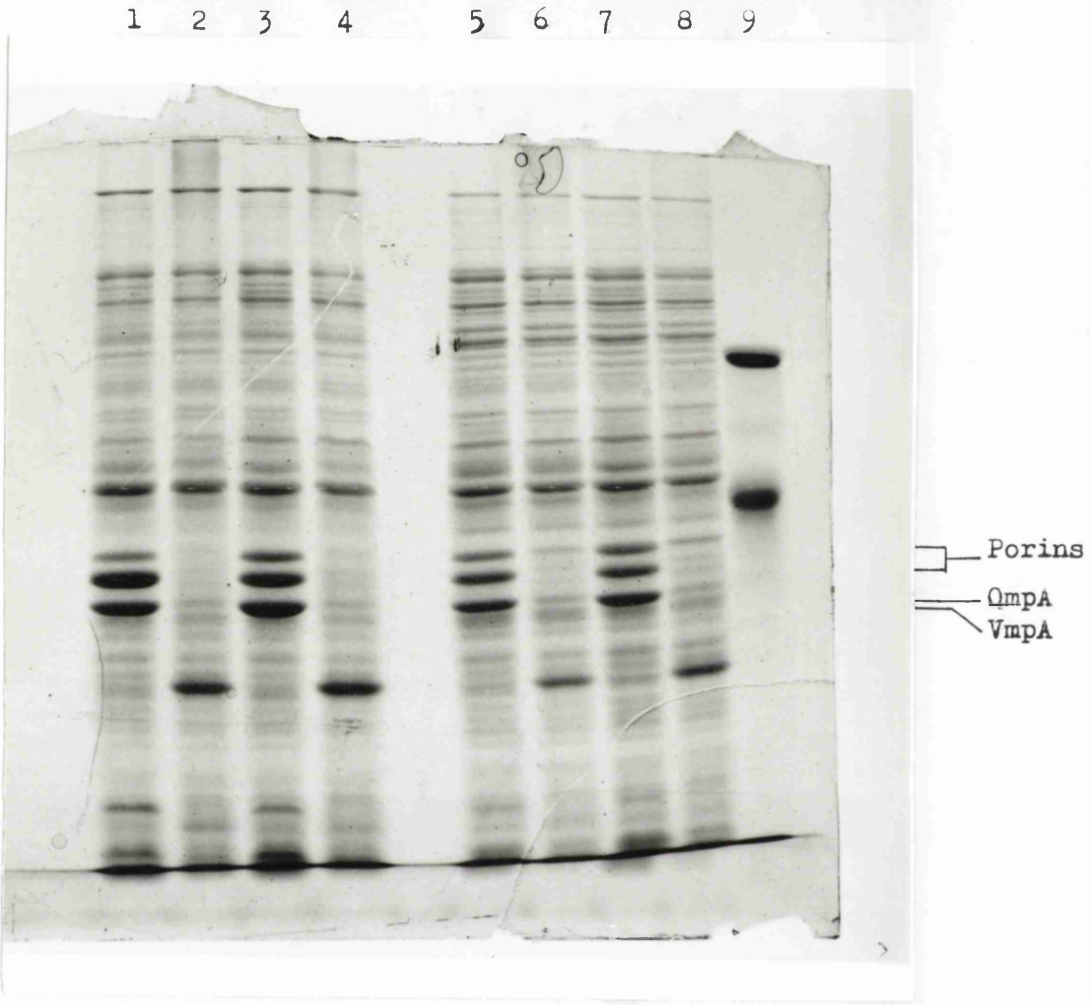


Fig 35: SDS-PAGE of envelopes of heated and unheated cells of strains 14 and 14ColV,I-K94. 1&2: unheated strain 14, 3&4: unheated strain 14ColV,I-K94, 5&6: heated strain 14, 7&8: heated strain 14ColV,I-K94, 9: molecular weight standards, 68 and 43Kd. lanes 1, 3, 5 & 7: dissociated at 100^{o}C, lanes 2, 4, 6, & 8: dissociated at 30^{o}C.

strains resulting in higher thermal resistance of the ColV plasmid - bearing wild type strain.

4. Discussion

4.1. Heat effects on ColV,I-k94$^+$ cells

It was reported that E. coli strains harbouring the ColV plasmid are more pathogenic than plasmid-free parental strains and that they possess increased ability to survive in blood stream and internal organs of infected hosts. Pathogenic E. coli strains are associated with several types of enteric and systemic disease which affect both adults and children. Since contaminated food is a major source of enterobacterial infections proper processing and cooking of food are important factors in providing bacteria-free food to consumers. Because heat plays an important role in cooking and meat processing, the thermal sensitivity of the ColV$^+$ cells and the factors influencing it have been investigated.

4.1.1. The ColV plasmid components involved in heat sensitivity

The presence of the ColV,I-k94 plasmid greatly enhances the heat sensitivity of 37^{o}C grown stationary phase host cells. The production of both colicin and transfer components is responsible for this increased sensitivity. Several observations suggested the involvement of these components. Firstly, the thermal resistance of the strain harbouring a deletion

derivative of the ColV plasmid that does not encode colicin production (ED1829 ColV-M40(5)) is similar to that of the ColV$^-$ cells. Secondly, the thermal resistance of the strain carrying a derivative of the ColV plasmid that does not encode transfer components (ED1829 ColV-M50(1)) is significantly higher than the ColV$^+$ strain. Thirdly, the ColV$^+$ strain that has had its transfer properties repressed by introduction of a fin$^+$ plasmid (as observed in ED1829 R124, ColV$^+$) shows a similar heat sensitivity to the isogenic ColV$^-$ strain. Fourthly, the ColV$^+$ and ColV$^-$ cells that are grown to the stationary phase at 25^{o}C have similar heat sensitivities. Synthesis of the transfer and colicin components is reduced in cells grown at 25^{o}C which confirms that these components, either alone or in combination with other unidentified plasmid encoded components, are responsible for the increased thermal sensitivity of ColV plasmid bearing strains.

Colicin components (including the colicins themselves, immunity proteins, lysis proteins etc), sex pili and some of the proteins encoded by the tra genes are located in the outer and cytoplasmic membranes. The presence of these proteins in the cell envelope may alter the stability of the envelope's structure by weakening the interactions between the various constituents of the envelope. Interactions

that may be affected are LPS-Mg, LPS-porins interactions in the outer membrane and phospholipid-phospholipid, phospholipid-protein interactions in the cytoplasmic membrane. Such interactions can be altered by e.g. loss or gain of a particular protein component; thus ompA mutants appear to have lps lesions presumably due to altered LPS-OmpA protein interactions; such lps lesions in ompA mutants can be reversed by ColV due to VmpA protein insertion (Reakes et al. 1988). Heating damages the outer and cytoplasmic membranes; and since the envelope of ColV$^+$ cells is altered compared with p$^-$ cells, the ColV$^+$ strains may be more vulnerable to heat action than the plasmid-free strains.

4.1.2. Effects on the outer membrane

The outer membrane of the ColV$^-$ and the ColV$^+$ cells is damaged structurally and functionally when bacterial suspensions are exposed to high temperature. Heating at 60^{o}C for 5, 10 or 15 min caused the release of lipopolysaccharide into the heating medium. Also, analysis of the protein content of the cell envelope of heated and unheated cells showed that heating at 60^{o}C for 5 min alters the structure of the outer membrane. Some of the envelope proteins of the heated cells were easily solubilized in SDS at low temperature which indicates that the interactions between the proteins and other components of the outer membrane

(lipopolysaccharide) are weakened by exposure to high temperature.

The outer membrane permeability properties are altered when cells are subjected to high temperature. Most cells that survived the heating at 60°C for 2, 5, 10 or 15 min had damaged outer membranes because only a very small proportion of the cells that formed colonies on nutrient agar were able to grow on nutrient agar + deoxycholate (0.5%). The inhibitory effect of DOC was also observed in heated ColV$^-$ and ColV$^+$ cells grown in liquid medium containing 0.5% DOC. On heating, both strains showed slower growth rate in this medium but the growth of the ColV$^+$ strain was more reduced than the p$^-$ strain. This sensitization to DOC almost certainly indicates a change in outer membrane permeability because DOC is known to damage the cytoplasmic membrane if it can reach it.

Heating at 60°C for 2 min also allowed permeation of erythromycin into ColV$^-$ cells and increased the extent of penetration of erythromycin into ColV$^+$ cells. Erythromycin is usually unable (or only poorly able) to penetrate enteric bacteria and this is due to outer membrane impermeability because Gram positive bacteria are sensitive. Accordingly, heating alters the structure of the outer membrane causing permeation

of these substances. On the other hand, heating to 60°C did not change the permeability of ColV$^-$ and ColV$^+$ cells to gentian violet. The penetration of this dye is significantly increased in Gram-negative bacteria with profound lipopolysaccharide defects (e.g. Rd or heptoseless Re mutants) but is not affected in strains with minor LPS alterations, which may indicate that heating causes outer membrane alterations of moderate degree that do not result in increased penetrability of this dye.

The effect of 0.01M EDTA (a compound that releases lipopolysaccharide from the outer membrane) on the growth of heated cells was studied and compared with growth of unheated cells. Both unheated ColV$^-$ and ColV$^+$ cells were slightly inhibited by EDTA. Exposure to EDTA for a short period removes magnesium ions from the outer membrane causing the release of 33%-50% of the lipopolysaccharide. However, prolonged exposure to EDTA (several hours) may cause degradation of the rRNA and ribosomes (Russell 1971) where EDTA chelates divalent ions required for maintaining the integrity of the ribosome, loss resulting in cell death. Heating increases the sensitivity of these strains to EDTA. Subjecting cells to high temperature releases part of the lipopolysaccharide into the heating medium; and exposure of heated cells to EDTA may result either in

extraction of more LPS causing increased disruption of the outer membrane integrity or in increased penetration of EDTA into cells causing degradation of the ribosomes, consequently inhibition of cell growth occurs.

Testing the effect of lysozyme on heated p^- and ColV plasmid bearing cells shows that heating at 60^oC for 2 min did not cause sufficiently large alterations to allow entry of lysozyme. Addition of EDTA (0.7mM) caused penetration of lysozyme and subsequently lysis of cells. For both $ColV^-$ and $ColV^+$ strains, lysis of heated cells by this treatment was not greater than the lysis of unheated cells. EDTA treatment did not increase the lysozyme permeability of heated cells compared with unheated cells. It is possible that after EDTA treatment the overall amount of lipopolysaccharide released from heated and unheated cells is the same. Although heating releases part of the LPS, addition of EDTA to heated cells may not result in extraction of more LPS. The lipopolysaccharide layer is usually stabilised by two mechanisms, the first involves divalent cation binding of the lipopolysaccharide molecules, and the other involves interactions of LPS molecules with other components of the outer membrane through hydrophobic or ionic interactions. Treatment with EDTA results in release of only the fraction of

the LPS that is linked by divalent cations.

To summarise, the outer membrane of the ColV plasmid bearing and plasmid free cells is damaged on heating. This was indicated by permeation or increased penetration of hydrophobic and bulky compounds that cannot usually permeate enteric bacteria. Although the ColV$^+$ strain was slightly permeable to some of these substances, heating increased the extent of this permeability. This may occur as a consequence of the heat induced lipopolysaccharide release because the presence of tightly packed LPS molecules is essential for maintaining the permeability barrier properties of the outer membrane. For example, S. typhimurium strains with complete LPS are resistant to hydrophobic compounds (e.g. novobiocin, erythromycin, nafcillin) but isogenic strains with rough LPS are sensitive (Roantree et al. 1977). In rough mutants, the interaction between lipopolysaccharide molecules is decreased which may allow penetration of hydrophobic substances. The heated ColV$^+$ strains show increased damage to the outer membrane compared with the p$^-$ ones. Some of the tra gene products, colicin components and VmpA protein are embedded in the outer membrane. Some of these proteins may bind LPS or other constituents of the cell envelope which decreases the LPS-LPS or LPS-protein interactions and causes the weakening of the

outer membrane. This results in enhanced heat induced damage to this membrane in the ColV$^+$ strains.

The damage to the outer membrane may contribute to the loss of cellular viability. Inhibitory substances that are usually excluded may penetrate and cause death if the damage is severe or repair of the damaged outer membrane did not occur due to heat induced damage to other parts of the cell such as DNA or ribosomes.

4.1.3. Effects on release of internal components

Release of 260nm-absorbing materials (such as amino acids and nucleotides) was observed in heated cells. For cells grown to the stationary phase at 37^{o}C, heating at 60^{o}C caused leakage of some of the cellular constituents from both ColV plasmid-bearing and plasmid - free strains but the release was greater from the p$^+$ strain. Also, heating (60^{o}C for 5 min) of a strain deficient in permease (P678-54) allowed penetration of o-nitrophenyl β-D- galactoside (ONPG) which usually cannot enter unheated cells. For the ColV$^+$ derivative of this strain, the unheated cells were slightly permeable to ONPG but heating significantly increased the permeation of this compound. These observations probably indicate that heating induces damage to the cytoplasmic membrane so that

it cannot function as an effective barrier. This damage occurs in ColV$^-$ and ColV$^+$ cells and is greater in the p$^+$ strains.

The presence of colicin and transfer components probably plays a major role in the increased damage to the cytoplasmic membrane of heated ColV$^+$ strains. Growth of the strains under certain conditions that reduce synthesis of these components (e.g. growth to the stationary phase at 25^{o}C) resulted in leakage of similar amounts of 260nm-absorbing materials from the heated ColV$^-$ and ColV$^+$ strains. Some of the proteins involved in the transfer process (e.g. traS and traG gene products) are located in the inner membrane (Willetts and Skurray 1987). In addition, the polypeptide that confers immunity to colicin Ia resides in the inner membrane (Weaver et al. 1981) and that which confers colicin V immunity may be located in this membrane also. The presence of these proteins in the cytoplasmic membrane may weaken the membrane causing its increased vulnerability to heat damage.

The increased leakage of the internal substances and the increased penetration of ONPG into heated ColV$^+$ cells was associated with the higher sensitivity of these cells to heat. Thus damage to the cytoplasmic membrane may contribute to the heat induced death.

The leakage may occur by a combination of loss of the cell membrane permeability barrier and degradation of some of the intracellular compounds (e.g.nucleic acids and proteins). Repair of the damaged cell membrane allows re-entry and accumulation of the necessary precursors from the medium and subsequently re-synthesis of the degraded compounds provided that the DNA is functional. However, if this damage is not repaired either due to extensive cell membrane damage or to DNA damage, death may occur.

4.1.4. Effects on DNA and other cytoplasmic components

Damage to DNA occurs on heating, double and single strand breaks are observed in DNA of heated cells (Gomez 1977). The DNA breakage occurs by the action of heat-activated endonucleases. Most of the endonuclease activity is due to the action of the apurinic/apyrimidinic - endonuclease of exonuclease III. Other endonucleases involved in the DNA breakage after heating include endonucleasesIV and V (Ljungquist 1977). The number of single strand breaks is larger in the wild type strain compared to the strains deficient in the AP endonuclease activity of exonuclease III, and this is associated with increased sensitivity to heat ($52^{o}C$ for 1hr) of the wild type strain in comparison with the endonuclease-deficientones (Williams-Hill and Grecz 1983).

Repair of DNA damage is associated with increased

survival after heating, and recovery from DNA injury involves utilisation of DNA repair processes. E. coli strains deficient in DNA repair (e.g. recA, polA strains) have higher sensitivity to heat (52°C) compared to parental strains with normal DNA repair functions (Mackey and Seymour 1987). In this study, exposing a polA strain to 60°C showed that this strain had increased heat sensitivity compared with the isogenic polA$^+$ strain. DNA polymerase I is involved in addition of deoxynucleotides to DNA with large gaps, suggesting that DNA breaks occur on heating at 60°C.

The presence of the ColV,I-k94 plasmid in polA strain did not increase the heat sensitivity of the cells, whereas the parental polA$^+$ cells that harbour this plasmid showed higher heat sensitivity than the plasmid-free polA$^+$ cells. The ColV plasmid may encode the production of DNA polymerase I. This may repair some of the DNA breaks that are not repaired in polA strains and substitute for the missing function of DNA polymerase I in these strains.

The DNA damage may be accompanied with injuries to other cytoplasmic components because plating of heated cells on nutrient agar containing 0.05M pantoyllactone did not increase the percentage of survival compared with plating on nutrient agar alone. Since chromosome replication

and increase in cell mass are pre-requisites to cell division, damage affecting both or one of these processes prevents cell division.

Damage to DNA and other cytoplasmic components is also indicated by the observation that incubation of heated cells in minimal medium for 120 min before enumeration on nutrient agar did not enhance the recovery of heated cells. Heated cells may have extensive DNA damage and incubation in minimal medium for longer time may be necessary for repairing the injured sites. Also, some of the heat induced lesions may not be repaired in nutritionally deficient media and may require complex nutrients for their repair. The effect of incubation in minimal medium on recovery of heated cells may depend on the severity of the heat induced damage. Wilson and Davies (1976) reported that holding heated cells at 52^{o}C for 15 or 35 min in liquid minimal medium increases the percentage of survival of cells. However, percentages of cells forming colonies on minimal medium or nutrient agar were nearly similar when cells were heated at 52^{o}C for 90 min (Mackey and Derrick 1982).

4.2. Growth conditions enhancing the thermal resistance of ColV,I-k94$^+$ strains

4.2.1. Growth in magnesium enriched medium

Cultures of the ColV$^-$ strain grown in broth supplemented with $MgSO_4$ (0.05M) or in broth alone had nearly similar heat resistance when heated at 60^oC for 2,5 or 10 min. Presumably Mg^{++} will stabilize the outer membrane and will also affect the cellular ribosomes, another component putatively affected by heat. The unaltered heat sensitivity may indicate that in the p$^-$ strain the outer membrane and other components that are stabilized by Mg^{++} (e.g. ribosomes) are not the primary sites of heat action but damage to these components may still contribute along with other changes (e.g. damage to DNA) to the loss of viability.

Growth of the ColV$^+$ strain on magnesium enriched broth increased its heat resistance. Since the outer membrane of the ColV$^+$ cells is weaker compared to the p$^-$ cells, as evidenced by its increased permeability to hydrophobic or large compounds, and heating damages this membrane to a higher degree in the ColV$^+$ cells. (as evidenced by penetration of greater amounts of hydrophobic compounds into heated ColV$^+$ cells compared to heated p$^-$ cells), the presence of high concentrations of magnesium in the growth medium may stabilise the outer membrane of these cells. This may decrease the

amount of the lipopolysaccharide released on heating and consequently results in increase in the percentage of survival of ColV^{+} cells.

Growth in magnesium depleted broth increased the heat sensitivity of the ColV plasmid-bearing and the p^{-} cells, with greater effect on the ColV^{+} strain. Of the major sites of heat damage, namely outer and inner membranes, nucleic acids, ribosomes and proteins; this Mg^{++} depletion is likely to affect the outer membrane and ribosomes most. Magnesium ions cross link the lipopolysaccharide molecules, and growth in magnesium depleted medium may cause weakening of the LPS interactions as a result of the increased electrostatic repulsion between the negatively charged groups of the LPS molecules. Since exposure to high temperature releases part of the LPS into the heating medium, growth in Mg-depleted broth may cause the release of more LPS on heating. This causes the penetration of harmful substances and leakage of essential components into and out of the cells, subsequently decrease in the percentage of survival of these cells is observed on heating.

Also, heating damages the ribosomes and degrades rRNA. Magnesium is involved in stabilisation of ribosomes, and lowering the Mg^{++} concentration of the

growth medium may cause unfolding of the ribosomal subunits and consequently render them more susceptible to heat damage. Subjecting cells grown in magnesium depleted medium to high temperature may cause ribosomal breakdown at a faster rate resulting in the higher heat sensitivity of these cells. This could be tested by examining the degradation of ribosomes from heated and unheated cells that are grown in Mg-depleted broth and comparing it with that of broth grown heated and unheated cells. The cultures are labelled with ^{3}H-uracil, and the ribosomes are extracted, centrifuged through 5-20% sucrose gradients and the sedimentation rate of the ribosomal subunits is compared. Also, the activity of the ribosomes from these cells can be tested by measuring protein synthesis in vitro.

4.2.2. Exposure to gradual increase in temperature before heating

Subjecting cells of ED1829 and its ColV^{+} derivative to a gradual temperature increase from 34 to 50^{o}C before heating at 60^{o}C enhanced their survival. Exposing cells to a gradual rise in temperature to a sublethal level induces synthesis of specific proteins while synthesis of all other types of proteins is decreased or inhibited. The induction of these proteins may be associated with the increased resistance to heat on subsequent exposure to a higher temperature.

Heating affects many cell components including outer and cytoplasmic membranes, proteins, DNA, RNA and ribosomes. The heat shock proteins may protect all or some of these sites and consequently increase the percentage of survival after heating at high temperatures. Some of the heat shock proteins are associated with ribosomes (e.g. GroEL), and they may interact with these components preventing the heat induced unfolding of ribosomal subunits and consequently protect rRNA molecules from degradation by the heat activated ribonucleases. Other heat shock proteins (e.g. GrpE, GroES, DnaK) are involved in nucleic acid synthesis at high temperature because mutants defective in these proteins are unable to synthesise RNA and DNA and cannot grow at high temperature.These proteins may have polymerase activity or protect or activate DNA polymerase or repair activities, and therefore are able to repair the heat induced DNA breaks. Also, some of these proteins may bind or associate with DNA which increases DNA stability and protects it from the action of the heat activated endonucleases.

The DnaJ gene product is located in the cell envelope and is induced by the heat shock treatment. This protein has large hydrophobic regions (Zylicz et al. 1985) and may stabilise the outer membrane structure by substituting for the lipopolysaccharide

released by heating at high temperatures, causing re-creation of hydrophobic bilayers. The presence of these bilayers may prevent entry of harmful substances that penetrate damaged cells or mutants with decreased amounts of LPS or with defective LPS structure (e.g. rough LPS). Other heat shock proteins may be located in the outer or cytoplasmic membrane although this has not been confirmed.

The increase in thermal resistance of cells exposed to a gradual rise in temperature before heating at 60°C was greater in the ColV$^+$ strain compared with the ColV$^-$ one. The cell envelope of ColV$^+$ cells is altered structurally allowing increased permeation of hydrophobic compounds, and the presence of heat shock proteins in the outer and probably in the inner membranes of ColV$^+$ cells may stabilise the cell wall. This may prevent the increased leakage of internal constituents and penetration of inhibitory compounds from and into ColV plasmid-bearing cells on subsequent exposure to a high temperature which enhances survival after heating. It would be of interest to examine LPS loss on heating from cells grown at 37°C compared to those subjected to a gradual rise in temperature.

The effect of subjecting cells to a gradual temperature rise on their heat resistance after heating at high temperatures is not confined to cells heated in broth and may be observed in foods. The heat resistance of

Salmonella thompson exposed to 54 or 60°C in broth, liquid whole egg, minced beef or reconstituted dried milk was increased when cells are exposed to 48°C for 30 min before heating at the higher temperatures (Mackey and Derrick 1987). This situation may be encountered frequently in daily life where bacteria in foods heated up slowly during cooking may acquire thermotolerance, and therefore longer heating time may be required to provide bacteria free food.

The thermal resistance induced after exposure to a gradual rise in temperature occurs rapidly after this treatment but is transient and decreases after ~120 min. The HtpR gene product (sigma - 32) regulates the synthesis of the heat shock proteins. Sigma - 32 is usually present at low amounts in cells which are not exposed to a gradual temperature rise. However, the induction of the synthesis of the HtpR protein is auto-regulatory and factors that denature sigma - 32 or decrease its concentration causes induction of synthesis of greater quantities of this protein and consequently synthesis of heat shock proteins. If the cells are exposed after induction, immediately or within a short time to a high temperature, heat shock proteins protect the susceptible sites in the cells from heat action. If cells are subjected to gradual increase in temperature and left at room temperature for long time (e.g. 2hr

or longer) before exposure to a higher temperature, the heat shock proteins may be degraded and the level of sigma - 32 may fall to its initial amount causing inhibition of further synthesis of the heat shock proteins.

4.3. Studies on wild type colicin V-producing strains

4.3.1. Characteristics of *E. coli* isolates

a. Prevalence of colicin producing strains

The frequency of colicin production, particularly colicin V, is usually higher in pathogenic strains compared with non-pathogenic types. More than 80% of the enterotoxigenic *E. coli* strains isolated from pigs produced colicin whereas only 25% of the non-enterotoxigenic *E. coli* strains were Col^+ (Harnett and Gyles 1984). In this study, 46% of the *E. coli* strains isolated from chicken processing plants encode the production of one type of colicin or several colicins. Thirty one percent (5 of 16) of the colicin producing strains produce colicins V and Ia. This incidence of $ColV^+$ strains is similar to that observed in strains isolated from clinical cases but significantly higher than the percenatge of $ColV^+$ strains in samples from the environment or from non-clinical cases. Testing 1474 *E. coli* strains isolated from humans with meningitis, septicaemia or enteritis showed that 39% of these strains were colicinogenic; 23% of the Col^+ strains encoded the production of colicin V (Milch *et al.* 1984). In contrast, few $ColV^+$ strains appear to be present in healthy animals or in faecally contaminated water. Thus examination of the incidence of colicin producing strains in 231 *E. coli* isolates recovered from healthy pigs in England revealed that 44% were colicinogenic but that only a small proportion of these were colicin V producers (deAlwis and Thomlinson 1973). Also a

similar result was obtained in the Ivory Coast. Twenty nine percent of the 178 coliform strains isolated from water samples and from human and animal faeces produced colicin and only 1% of these produced colicin V (Trudel et al. 1984).

The high percentage of ColV$^+$ strains recovered from chicken processing plants in this study may indicate that some of the apparently healthy chickens may be asymptomatic carriers of pathogenic ColV$^+$ strains. Also, it may indicate that the external surfaces of healthy birds are cross- contaminated by excreta of infected birds either during transport or during rearing, which introduces pathogenic ColV$^+$ E. coli strains into processing plants and consequently results in isolation of high numbers of ColV$^+$ strains.

b. Antibiotic sensitivity of isolates

Gentamicin was the most effective drug against the wild type isolates. This antibiotic is rarely used in poultry and bacteria are not exposed to the selective pressure exerted by the presence of the antibiotic, consequently gentamicin resistant strains are not selected for.

The incidence of drug resistant bacteria is usually high in animals that are frequently exposed

to antimicrobial drugs. Ninety eight percent of enteric bacteria isolated from chickens fed on antibiotic- containing feeds were drug- resistant whereas only 39% of isolates from cattle which had not had antibiotics in their feed were resistant (Dhillon and Dhillon 1981).

In the present study, ~30% of the isolates were resistant to chloramphenicol, more than 40% were resistant to ampicillin and almost all the isolates were resistant to cotrimoxazole. These findings are similar to those observed in other studies. A high percentage of E. coli strains isolated from calves were resistant to antibiotics; 39.5%,49% and 94% of these strains were resistant to ampicillin, chloramphenicol and sulphonamide, respectively (Linton 1984). A similar pattern of antibiotic resistance was also observed in pathogenic E. coli strains isolated from humans. The drug sensitivity of 232 enteropathogenic E. coli strains isolated in the U.K. during 1980 and 1981 from infants under 3 years old was tested (Gross et al. 1982). Susceptibility to ampicillin, chloramphenicol, gentamicin, nalidixic acid, streptomycin, sulphonamide, tetracycline and trimethoprim was tested. Fifty eight percent of the strains were resistant to one or more of these drugs. Thirty seven percent, 46% and 13% were resistant to ampicillin, sulphonamide and chloramphenicol,

respectively; and all isolates were sensitive to gentamicin.

The similarity of antibiotic resistance patterns of isolates from human and animal samples suggest that a high proportion of the drug -resistant enteric bacteria recovered from humans may be of animal origin. The resistant strains may be transmitted to humans by direct handling of animals or carcasses during rearing and processing of animals, or by ingestion of food contaminated with these bacteria. It was reported that the normal gut flora of healthy humans in contact with animals show a higher percentage of antibiotic resistance. Also, handling and eating food contaminated with drug- resistant strains caused the appearance of the same resistant serogroups in the faecal flora of the people handling the food (Hinton et al. 1986).

Seventy percent of the isolates were resistant to tetracycline (50µg). The high incidence of tetracycline resistance in strains from poultry may be, in part, due to its occasional use for prophylaxis, growth promotion and treatment (Linton 1984). In addition, the use of other antibiotics in poultry may facilitate the maintenance of the tetracycline resistant strains in animals, the presence of one type of antibiotics may select for strains carrying other drug resistances

including tetracycline. Also, these strains may spread from faeces of animals and contaminate bedding, feed, and water in poultry farms, which causes spread of drug resistant strains to other birds in the same pen or to other flocks. Also, feed ingredients carrying antibiotic resistant bacteria contribute to the spread of these strains in poultry flocks.

Multiple antibiotic resistance was prevalent in strains recovered from chicken processing plants in this survey. In the Col^- *E. coli* strains, the mean number of resistances was ~ 3, and in the Col^+ *E. coli*, the mean number of resistances was ~ 4 antibiotics. These results are similar to observations reported in other countries. In the Far East, 44% of the enterotoxigenic *E. coli* strains were multiply- resistant (cited in Rowe and Threlfall 1984). Also, the percentage of multiply resistant enterotoxigenic strains isolated in Mexico from sick childern less than 3 years old was high. The sensitivity of the strains to ampicillin, tetracycline, streptomycin, chloramphenicol, gentamicin and kanamycin was tested. Sixty eight percent of the isolates were resistant to four or more antibiotics and all the strains were resistant to one or more drugs (Martinez *et al.*1987).

The use of antibiotics as feed additives may select

for multiply resistant strains. Although one antibiotic is usually used (e.g. tetracycline), the presence of a single antibiotic in animal feed can select for multiply resistant strains in the animals which ingest them. Tetracycline resistance is associated with resistance to a wide range of other antimicrobial agents, and the presence of tetracycline as feed additive would select for the whole pattern of multiple antibiotic resistance, resulting in increase in the pool of multiply resistant enteric bacteria.

The majority of the chicken isolates examined for presence of plasmids had one or more plasmids. These could be encoding enterotoxin production, adhesins, antibiotic resistance or other properties that are usually specified by plasmids.

Some of these phenotypes, such as antibiotic resistance, may be chromosomally encoded too. Two of the multiply resistant wild type strains, strains 14 and 5, did not contain plasmids but were drug resistant. Strain 14 is resistant to cephaloridine, ampicillin, cotrimoxazole, tetracycline, and streptomycin; and strain 5 is resistant to cotrimoxazole, tetracycline, chloramphenicol & streptomycin. Chromosomally determined drug resistance may result by mutations causing alterations in the target site or preventing

the access of antibiotics to this site (Saunders 1984). Resistance to streptomycin may occur by mutations leading to lack of a specific protein receptor on the 30S subunit of the ribosome, which prevents attachment of the antibiotic to ribosomes and consequently its action on protein synthesis and cells. Chromosomally-encoded resistance to chloramphenical may result from permeability changes in the outer or cytoplasmic membrane which hinder penetration of the antibiotic into cells. Also, some chloramphenicol resistant strains may have alterations in the target site (Smith and Burns 1984). Resistance to ampicillin and cephaloridine may result from permeability changes in the cell envelope or from production of chromosomally encoded β- lactamases which hydrolyse and inactivate these drugs (Medeiros 1984). Chromosomally encoded resistance to cotrimoxazole may result from presence of altered form of dihydropteroate synthetase (the target site of sulphonamides) to which sulphonamide has a lower affinity, and from presence of insusceptible form of dihydrofolate reductase (the target enzyme of trimethoprim) (Hamilton-Miller 1984). Resistance to tetracycline may occur due to mutations causing alterations in the target site (ribosomes) or changes in outer membrane permeability which prevent tetracycline penetration (Levy 1984).

4.3.2. Heat sensitivity of wild type ColV,I-K94 plasmid-bearing cells.

It is important to eliminate pathogenic bacteria, including ColV$^+$ strains, during the first step in chicken processing (the scalding process). Some of the chickens may harbour bacteria on their skin and feathers which introduces bacteria into processing plants and contaminates other machines used in chicken processing, such as defeathering machines and eviscerators. This enhances the spread of bacteria to other processed carcasses that are originally free from pathogens.

In this study, the heat sensitivity of a p$^-$ wild type chicken isolate (strain 14) and its ColV,I-K94$^+$ derivative was tested. The presence of this ColV plasmid in strain 14 did not increase its heat sensitivity in contrast to the effect of the plasmid on K 12 strains. However, the presence of the ColV,I-K94 plasmid in other wild type chicken isolates may enhance the heat sensitivity of these strains. Strain 14 has chromosomally encoded multiple resistance to cephaloridine, ampicillin, cotrimoxazole, tetracycline and streptomycin; therefore, this strain probably has altered envelope or cytoplasmic components. Heat damages many cellular sites including the cell envelope, ribosomes and nucleic acids but structure of some of the cellular or envelope components of strain 14 differs from that of other wild type or k12 strains;

and the presence of ColV,I-k94 plasmid in strain 14 did not increase the heat sensitivity of this strain.

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