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CROSS RESISTANCE AMONGST COLIPHAGES

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CROSS RESISTANCE

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SUMMARY

The general pattern of resistance to bacteriophages in *Escherichia coli* K-12 and the effect of mutations to bacteriophage resistance on the structure of the cell wall has been studied.

A set of 56 virulent bacteriophages lysing *E. coli* K-12 was obtained from various workers or by isolation from raw sewage. Resistant mutants were isolated to 42 of these bacteriophages in one strain of *E. coli* K-12 and tested for resistance or sensitivity to the full set of 56 bacteriophages. Most of the mutants fell into eleven groups with respect to their resistance patterns. The bacteriophages that were isolated from sewage were partially characterized with respect to their electron microscopic morphology and neutralization by various antisera. Similar data about many of the other bacteriophages was obtained from the literature and the taxonomic relationships of various bacteriophages revealed. The pattern of resistant mutants obtained in this study is discussed with reference to bacteriophage taxonomy.

Various cell envelope subfractions and chemically or enzymically modified cell wall preparations were tested in neutralization experiments with many of the 56 bacteriophages. From the results obtained, conclusions could be made as to the nature of the receptor for these bacteriophages.

It was found that many of the bacteriophages to which the Ktw, Ttk, Bar, Wrm and miscellaneous groups were resistant, were neutralized by lipopolysaccharide preparations. Alterations in the response of the above mutants to a series of antibiotics and to bacteriophages C21 and U3 indicated that they had lipopolysaccharide

alterations. This was confirmed by direct sugar analysis of the mutant lipopolysaccharides using gas liquid chromatography which showed six distinct classes. The results are in agreement with the published partial rough core structures of *Salmonella* and *E. coli* O 100. Twelve representative mutants of the Bar and Wrm(2) resistance groups were shown by P1 transduction or conjugation to be between *pyr*E and *mtl* which has been previously shown to be the site of the rfa locus in *E. coli* K-12. Outer membrane protein defects were also demonstrated in some of the above lipopolysaccharide-altered mutants by polyacrylamide gel electrophoresis. The possible involvement of protein in so-called lipopolysaccharide receptors and vica versa is discussed on the basis of these and other results presented in this thesis.

Analysis of the protein composition of Con$^-$ mutants revealed that these mutants were deficient in two major cell wall proteins named 3a and 3b in the nomenclature of Schnaitman. These mutants have been subsequently shown by colleagues to be defective as recipients in conjugation and tolerant to colicins K and L. They have also been partly mapped in this study. Based on the results obtained for this and other mutants, a hypothesis is put forward concerning the first steps of infection of cells by bacteriophages.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

Robert E. W. Hancock

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CHAPTER 1

INTRODUCTION

d'Herelle as early as 1926 divided the infectious process of the bacteriophage into four stages, (1) adsorption of the phage particle, (2) penetration of the phage particle into the host cell, (3) the intracellular multiplication of the bacteriophage, and (4) the lysis of the host cell and release of the phage progeny (d'Herelle, 1926). Although it has been since discovered that stages (2) and (3) involve only the nucleic acid of the phage particle (Herschey and Chase, 1952), this picture remains essentially correct.

A bacteriophage resistant mutant can be altered such that it prevents any of the four stages. Classically described resistant mutants however are often altered at the level of adsorbtion to the receptor, i.e. stage (1).

Bacteriophages have been described for a large number of hosts, and it seems that they are present for most species in which they have been looked for (Adams, 1959; Ackerman, 1969; Tikhonenko, 1970). They differ in morphology (see below), nucleic acid type and content (Ackerman, 1969; Wildy, 1971) and ability to lysogenize a host bacterium. A bacteriophage which is able to infect a cell and lysogenize it is called a temperate bacteriophage (Herschey, 1971; Echols, 1972). The resultant lysogenic strain often has the bacteriophage DNA incorporated into it and replicating under the control of the host replicative systems (Herschey, 1971). If a bacteriophage is unable to lysogenize bacteria, then it is called a virulent bacteriophage

and undergoes a lytic cycle of replication.

The studies involved in this thesis have been mainly limited to double-stranded-DNA-containing, virulent bacteriophages capable of lysing one strain of Escherichia coli K-12. In this introduction there has been no attempt to review the literature about bacteriophages exhaustively, but rather it is concerned with those topics of relevance to the thesis.

CLASSIFICATION AND TAXONOMY OF BACTERIOPHAGES

The first extensive attempt to classify bacteriophages was made by Burnet and McKie (1933) and Burnet (1933, 1934), who distinguished bacteriophages by means of cross resistance, serology and simple biochemical tests. Burnet (1934) found some correlation between the latter two criteria; however many of his bacteriophages were subsequently lost and much of his work cannot be related to modern bacteriophage taxonomy. Other early studies of a similar nature were reviewed in a book by Adams (1959). Although many of the early studies involved serology, which appeared to be a good basis for classification (Adams, 1953), the discovery that there were antigenic differences between wild type and mutant λ bacteriophages (Fry and Waites, 1969), has since demonstrated its limitations.

Examination of gross bacteriophage morphology under the electron microscope revealed that bacteriophages were striking and characteristic in appearance (Ruska, 1941; Luria, Delbruck and Anderson, 1943). Adams (1953) showed that the size and shape of bacteriophages correlated well with other criteria, and using the various criteria, Adams and Wade

(1955) divided a limited number of coli-dysentry phages into four taxonomic groups.

By means of extensive studies on their morphologies, Bradley (1963, 1967) found he could divide all of his bacteriophages into six distinct types; three of which included double-stranded-DNA bacteriophages differentiated on tail morphology (contractile, long non-contractile or short non-contractile). Ackerman (1969) exhaustively reviewed the literature concerning the electron microscopy of bacteriophages and proposed a further differentiation on head morphology (isometric, long and elongated). Of the nine morphological permutations of head and tail structure, described by this system, Ackerman found that he could demonstrate examples of eight of these morphologies. The ninth category has since been described (Ackerman, Petrow and Kasatiya, 1974) but no other morphologies have been noted except in aberrant mutant bacteriophages (Cummings et al., 1967; Cummings, Couse and Forrest, 1970).

In 1966, an International Committee on Nomenclature of Viruses was set up and this group published their first report in 1971 (Wildy, 1971). Their classification system for bacteriophages is still in the preliminary stages; however they have proposed generic names for the T-even bacteriophages (myovirus) and for bacteriophage λ (caudaevirus). Due to the preliminary nature of this classification, laboratory strain names have been used in this thesis.

Limited studies have been done on the DNA homology (Cowie, Avery, and Champe, 1971; Davis and Hyman, 1971; Brunovskis, Hyman and Summers, 1973; Hyman, Brunovskis and

Summers, 1974) and genetic recombination (Luria and Dulbecco, 1949; Adams, 1953; Mizobuchi, Anderson and McCorquodale, 1971) of various strains of bacteriophages. Studies of this nature will have significance not only to the further classification of bacteriophages which are similar in appearance, but also to the study of evolution of bacteriophages.

RECEPTORS FOR BACTERIOPHAGES IN THE CELL WALL

One of the reasons for the study of bacteriophage resistance, is so that we can ultimately discover more about not only the nature of the adsorbtion of bacteriophages to their receptors in the cell wall (by studying the defect in the resistant mutant) but also about the two components involved in the interaction; the bacteriophage tail and its receptor. Thus a discussion of the structure of the cell wall, with emphasis on those components of the cell wall able to act as receptors is relevant to this thesis.

Structure and composition of the cell wall

The cell wall is defined here as that part of the cell external to the cytoplasmic membrane. In gram negative cells it has been shown by electron microscopy (Silva and Sousa, 1973) to consist of a dense rigid layer, and external to this a trilaminar membranous structure, called the outer membrane. The rigid layer or murein sacculus is thought to be the shape maintaining layer of the cell (Weidel, Frank and Martin, 1960; Burman, Nordstrum and Bloom, 1972; Braun *et al.*, 1973), although it does not appear to be such for the membrane of bacterial ghosts (Henning, Hohn and Sonntag, 1973). The structure of the murein sacculus is

known (Braun and Bosch, 1972; Braun et al., 1973). It appears to be attached to the outer membrane by means of a lipoprotein (Braun and Rehn, 1969; Braun and Bosch, 1972, 1973a,b). The lipid portion of the murein lipoprotein is inserted into the outer membrane (Schnaitman, 1971b; Burman, Nordstrum and Bloom, 1972). Inouye (1974) has recently presented an attractive model for the molecular assembly of the murein lipoprotein, in which six molecules of lipoprotein, each with α-helix structures form a 12.5Å pore. These pores are proposed to extend through the membrane and thus provide channels for the passive diffusion of molecules. However, DNA from bacteriophages is too large to travel down these pores.

The outer membrane has three classes of components; proteins, phospholipids and lipopolysaccharide (LPS). There are many theories as to the structure of the outer membrane (de Petris, 1967; Schnaitman, 1971b; Van Gool and Nanninga, 1971; Trauble and Overath, 1973; Costerton, Ingram and Cheng, 1974), however most of these consider the basic structure to be a lipid bilayer, typical of biological membranes. This has been borne out by biophysical studies (Forge and Costerton, 1973; Forge, Costerton and Kerr, 1973).

Assuming the membrane has as its basic structure a lipid bilayer, and the membrane is at least partly fluid (Oldfield, 1971), then it is possible that the integral proteins are of three types (1) traversing the membrane (2) mainly in the outer half of the membrane, and (3) mainly in the inner half of the membrane (Chapman, 1972; Razin, 1972; Singer and Nelson, 1972; Coleman, 1973). Thus since a

bacteriophage attacks from without one would imagine that its receptor, if a protein, would be in one of the first two classes. The integral proteins would be capable of lateral movement within the membrane (Kaback and Barnes, 1971; Harold, 1972). The lipid A portions of the lipopolysaccharide molecules probably interact hydrophobically with the outermost lipid layer of the outer membrane (Rothfield and Romeo, 1971; Schnaitman, 1971b; Benedetto, Shands and Shah, 1973); while the carbohydrate portions of the molecules either extend away from the surface of the cell (de Petris, 1967; Schnaitman, 1971b) or consist of an ordered and cross-linked mass of polysaccharide chains on part of the surface of the cell (Costerton, Ingram and Cheng, 1974). When the components of the outer membrane are separated and then mixed, a membrane essentially identical to the outer membrane, is formed (Bragg and Hou, 1972; Rothfield *et al.*, 1972; Sekizawa and Fukui, 1973). This is evidence for the self assembly of outer membrane components.

Electrophoretic measurements have shown that the cell possesses a large net negative charge in media suitable for bacteriophage binding (Brinton, Buzzell and Lauffer, 1964). Other studies using isoelectric equilibrium analysis (Sherbet and Lakshmi, 1973) have characterized the number of anionogenic and cationogenic groups more thoroughly. This work has shown that the phospholipid is probably at a depth of 60 Å below the cell surface while the proteins and lipopolysaccharide may be on the cell surface.

The study of phospholipids has revealed differences in the distribution of various phospholipids in the cell wall and cytoplasmic membranes of *E. coli* (White, Lennarz, and

Schnaitman, 1972). The various functions of phospholipids have been described (Cronan and Vagelos, 1972) but as yet no purified bacteriophage receptor has been shown to be dependent on a phospholipid. However, the synthesis of proteins, which can act as bacteriophage receptors (see for example Lindberg, 1973), and phospholipids does appear co-ordinated (Crowfoot, Esfahani and Wakil, 1972; Forge, Costerton and Kerr, 1973).

Bayer (1968a) has shown that when *E. coli* is plasmolysed, the cytoplasmic membrane remains attached to the outer membrane at between 200 and 400 locations. These adhesions have been shown to be the site of bacteriophage adsorbtion for the T-group phages (Bayer 1968a), attachment of the F pilus (Bayer 1968b), export of newly synthesized lipopolysaccharide (Muhlradt *et al.*, 1973) and binding of DNA to the envelope (Olsen *et al.*, 1974). The number of such adhesions is also compatible with each of the adhesions being a component of the membrane subunits which segregate on cell division (Green and Schaechter, 1972). However, recent evidence has suggested that there is more than one class of adhesion sites (M. Bayer, manuscript in preparation).

Structure and composition of the lipopolysaccharide and its ability to act as a receptor

The outer membrane of *E. coli* K-12 comprises approximately 16% lipopolysaccharide by weight (Sekizawa and Fukui, 1973). In its purified state it adopts a membranous structure (Katayama *et al.*, 1971), however it is generally thought not to be responsible for the trilaminar structure of the outer membrane. It is thought to be associated with protein in the cell wall (Wober and Alaupovic, 1971; Wu and Heath,

1973), and there is evidence that the lipid A portion of the lipopolysaccharide molecule is covalently bound to the protein.

It has been shown in Salmonella that lipopolysaccharide is synthesized sequentially (Osborn, 1966; Mühlradt, 1971) by a number of enzymes, some of which reside in the cytoplasmic membrane (Osborn, 1966). It is then exported to the outer membrane (Osborn, Gander and Parisi, 1972; Mühlradt et al., 1973) via Bayer adhesion sites (Mühlradt et al., 1973) and spreads out laterally over the bacterial cell surface. There is evidence (Rothfield and Takeshita, 1966; Rothfield and Romeo, 1971) for the role of phospholipid in the biosynthesis of lipopolysaccharide.

The structure of the lipopolysaccharide rough core has been best studied in Salmonella (Holme et al., 1968; Droge et al., 1970; Hellerqvist and Lindberg, 1971; Lehman, Lüderitz and Westphal, 1971; Hämmerling, Lehman and Lüderitz, 1973; Lehman et al., 1973), in which it has been shown that there is only one type of structure for all strains. However, Schmidt and co-workers have demonstrated three different core types, R1, R2 and R3, for the E. coli strains they have studied (Schmidt, Jann and Jann, 1970; Schmidt, Fromme and Mayer, 1970; Schmidt, 1972). The structure of the lipopolysaccharide of E. coli O 100, which has an R2-type core has been largely characterized (Hämmerling et al., 1971), but few other structural studies have been done. In fact, in addition to the above, and studies on the heterogeneous rough core structure of E. coli O111-B_4 (Fuller, Wu, Wilkinson and Heath, 1973; Wu and Heath, 1973), only a few less detailed studies of lipopolysaccharide

composition have been done on strains of *E. coli* (Malchow *et al.*, 1969; Morton and Stewart, 1972).

E. coli K-12 is a rough organism, lacking O-specific sugars (Ørskov and Ørskov, 1962; Rapin and Mayer, 1966). This is probably due to a mutation at the *rfb* locus situated near *his*$^+$ on the genetic map (Ørskov and Ørskov, 1962; Jones, Koltzow and Stocker, 1972). Schmidt has shown that the enzymes produced by the *rfa* genes of *E. coli* K-12 strain 2578 can synthesize a complete core structure (Schmidt, 1973) and he has excluded the possibility of a leaky *rfa* mutant as suggested by Jones, Koltzow and Stocker (1972). He has also shown by serological tests and phage typing that the *E. coli* K-12 core is different from other known core types of *Salmonella* (Ra) and *E. coli* (R1-R3).

The *E. coli* K-12 core contains glucose, galactose, heptose and a small amount of rhamnose (Rapin and Mayer, 1966; Monner, Jonsson and Boman, 1971; Eriksson-Grennberg, Nordstrum and Englund, 1971), although the latter is missing from one subline of *E. coli* K-12 (Nikaido, Nikaido and Rapin, 1965). In those strains which contain it, rhamnose has been shown to exist bound to the KDO-lipid A portion of the molecule (Sugimoto and Okazaki, 1967). The KDO-lipid A portion of the molecule has been chemically but not structurally characterized (Rooney and Goldfine, 1972).

As early as 1934, Gough and Burnet (1934) were able to show that a polysaccharide extract of *E. coli* from an autolysed culture could inhibit the adsorbtion of bacteriophages to the *E. coli* cell. Since then, many bacteriophages have been shown to attach to extracted

lipopolysaccharide (as reviewed by Rapin and Kalckar, 1971; Lindberg, 1973). In addition, electron micrographs showing bacteriophage T4 whole particles and isolated tail fibres attached to purified lipopolysaccharide have been published (Jesaitis and Goebel, 1953; Wilson, Luftig and Wood, 1970). However, for irreversible attachment these bacteriophages require, as a receptor, the aggregated lipopolysaccharide complex (Lindberg, 1973). Furthermore, it has been shown that lipopolysaccharide from bacteriophage sensitive cells can provide functional receptor for spheroplasts of cells normally lacking receptor sites (Watson and Paigen, 1972). The requirements for functional receptor have also been extensively studied (Rapin and Kalckar, 1971; Lindberg, 1973) and this will be discussed more fully later.

Lastly, the genetics of lipopolysaccharide biosynthesis has been studied in Salmonella (Kuo and Stocker, 1972; Sanderson and Saeed, 1972; Stocker and Mäkela, 1971) and E. coli (Schmidt, Jann and Jann, 1970; Eriksson-Grennberg, Nordstrum and Englund, 1971; Schmidt, 1973) and it has been shown that many of the genetic loci responsible for the enzymes involved in the biosynthesis of the rough core (rfa genes) lie between pyrE and mtl on the respective genetic maps.

Cell wall proteins - their composition, functions and ability to act as bacteriophage receptors

The protein composition of the outer membrane of E. coli was first described by Schnaitman (1970a,b) who used the technique of polyacrylamide gel electrophoresis. He was able to show using his solubilization techniques and gel

buffer system one major protein band of 44,000 molecular weight accounting for as much as 40% of the total protein of the cell envelope. By separating cell wall and cytoplasmic membrane using sucrose gradient centrifugation, he showed that there were only six protein bands in the cell wall, one of which, the 44,000 molecular weight protein, accounted for 70% of the total protein of the cell wall. In a later study, Schnaitman (1971a) was able to show that the cytoplasmic membrane proteins were soluble in 2% Triton X-100 and this provided a simple technique for the separation of wall and membrane protein. He was also able to show that by treating the cell wall with Triton X-100 and Ethylenediamine-tetraacetic acid he could reduce the protein content and also the vesicle-like structure (Schnaitman, 1971b).

The first serious challenge to Schnaitman's ideas of one major protein band came from Bragg and Hou (1972) who were able to show that in their buffer system the major band ran as two distinct major proteins and one minor protein which had molecular weights (as estimated using their polyacrylamide gel system) ranging from 44,000 to 33,400 when partially purified (Bragg and Hou, 1971). Moldow, Robertson and Rothfield (1972) showed that the major band of the cell envelope contained several polypeptides while Inouye and Yee (1973) demonstrated the presence of three bands of varying molecular weights. The dilemna was finally solved by Schnaitman (1973a,b, 1974a,b) who showed that the major band consisted of not one, but four proteins (proteins 1, 2, 3a and 3b) of 40,000 molecular weight. Proteins 3a and 3b ran together

in all the gel systems tried by Schnaitman but he managed to separate them by column chromatography and proved they were different by comparing their cyanogen bromide peptides (Schnaitman, 1973b, 1974a). The anomolous results described previously could be explained by the fact that many solubilization techniques did not completely dissociate the sub-units or unravel the polypeptide chains of the four proteins. E. coli K-12 has only proteins 1, 3a and 3b in its outer membrane (Schnaitman, 1974b), as protein 2 is only found in strains of E. coli lysogenic for a certain bacteriophage (C.A. Schnaitman, personal communication). A more thorough discussion of Schnaitman's work is included in the text. The concept that peak 3 contains two proteins has been recently challenged by Reithmeier and Bragg (1974), who have shown that when they partially solubilize the cell wall of E. coli NRC 482 in 0.5% SDS at 100C for 1 hour, a single protein, with the characteristics of the acrylamide gel peak 3 (shown by Schnaitman to consist of proteins 3a and 3b) can be isolated. However, they have not studied the residual cell wall proteins, and it is by no means clear whether or not they have selectively solubilized only one of the proteins 3a and 3b. Inouye and Lee (1973) have shown that all of the membrane proteins produced by stable mRNA are in the outer membrane and there are differences not only in the stability of the mRNA's of the major proteins but also in their assembly mechanisms.

The nature and function of the proteins of the cell wall of E. coli is poorly understood. The murein lipoprotein has been well characterized (Braun and Bosch,

1972, 1973a,b). It will only partly run on polyacrylamide gels, unless the cell wall is treated with lysozyme (Schnaitman, 1971b), due to the fact that it exists in two states; free and bound to the murein sacculus (Hirashima et al., 1973). Phospholipase A1 (M.W. 30,000) has also been shown to exist in the outer membrane of E. coli K-12 (Ohki, Osamu and Nojima, 1972), however none of the other enzymes involved in phospholipid metabolism appear to be associated with the cell wall (Bell et al., 1971) with the possible exception of the phosphatidyl serine synthesizing enzymes (White et al., 1971). Both 3' nucleotidase and 5' nucleotidase are also associated with the outer cell wall layers of E. coli (Nisonson, Tannenbaum and Neu, 1969). Other enzymes and proteins which have not been localized in the cell wall or cytoplasmic membrane of the envelope are summarized by Machtiger and Fox (1973) and Costerton, Ingram and Cheng (1974). Recently, Koplow and Goldfine (1974) have shown that heptose deficient mutants have large alterations to their protein compositions.

Jesaitis and Goebel (1953) and Weidel (1958) first described the receptors of bacteriophages T2 and T6 as lipoprotein. The fact that bacteriophage T2 resistant mutants were sensitive to bacteriophage T6 and vice versa (Demerec and Fano, 1945) suggested they were probably different lipoproteins. Chemical characterization of the receptors showed that the specifity of the receptors of bacteriophages T2 and T6 was determined by different chemical groupings (Weltzein and Jesaitis, 1971). Studies by Michael (1968) and DePamphilis (1971) have confirmed that the receptor for T2 is probably a lipoprotein, while

the receptor for T6 could possibly be associated with protein 1 of the cell wall (C.A. Schnaitman, 1974b).

The receptor for bacteriophage T5 was isolated originally as a lipo-glycoprotein complex by mild alkali extraction of the cell wall of E. coli B (Weidel, Koch and Bobosch, 1954). The properties of this complex have been well studied (Zarybnicky, Zarybnicka and Frank, 1973). Braun and co-workers (Braun, Schaller and Wolff, 1973; Braun and Wolff, 1973) have shown that the actual receptor, which they have isolated from the complex, is a single polypeptide chain of 85,000 molecular weight which resides in the outer membrane.

The receptor for bacteriophage λ has also been shown to be a protein located in the outer membrane (Randall-Hazelbauer and Schwartz, 1973). Sabet and Schnaitman (1973a, 1973b) have isolated a protein of 60,000 molecular weight which is missing or altered in bfe$^-$ mutants. This protein is the receptor for colicins E2 and E3 and although it has not been directly demonstrated, the fact that the bfe$^-$ mutant which is missing it is also resistant to bacteriophage BF23 (Buxton, 1971), suggests that this protein is the receptor for bacteriophage BF23 also.

ADSORBTION OF BACTERIOPHAGES

In order to understand the nature of resistance to bacteriophages, one must consider the process that is deleted in many of these mutants, the adsorbtion of the bacteriophage tail to the cell wall.

Structure of the bacteriophage tail and changes undergone

in adsorbtion

All double-stranded DNA bacteriophages have tails that can be fitted into three main types: (A) contractile, (B) long non-contractile, and (C) short non-contractile (Ackerman, 1969). Within this simple classification there appear to be some minor variations (Tikhonenko, 1970; Krzywy, 1972a,b). This introduction will afford a brief summary of the three classes.

The proteins of the type (A) tail of T-even bacteriophages have been extensively characterized, by molecular weight (Cummings et al., 1970a,b, 1973; King and Laemmli, 1973; King and Mykolajewycz, 1973) and functionally (Mason and Haselkorn, 1971; Poglazov, Rodikova and Sultanova, 1972; Beckendorf, 1973; Beckendorf, Kim and Lielausis, 1973; Dawes and Goldberg, 1973a,b; Kells and Haselkorn, 1974). The genes on the bacteriophage T4 genome which code for these proteins have been largely characterized (Dawes and Goldberg, 1973a,b; King and Laemmli, 1973; King and Mykolajewycz, 1973).

The main structural features of the type (A) tail are a tail core, a contractile sheath (Kellenberger and Arber, 1955), a base plate and six pins, as well as both long and short tail fibres (Kells and Haselkorn, 1974). The adsorbtion process has been shown by electron microscopy to involve attachment of long tail fibres to the cell wall, followed by attachment of the tail pins of the base plate, contraction of the sheath, and injection of the DNA through the tail tube (Simon and Anderson, 1967). This process occurs at sites of adhesion of the cell wall and cytoplasmic membrane (Bayer, 1968a). The major protein of the T-even tail, which itself has contractile properties (Kozloff and Lute, 1959), is the sheath protein responsible for the contraction of the tail. It has been

shown that the contraction process is similar for another bacteriophage with a type (A) tail, but with different head morphology (Donnelli, Guglielini and Paoletti, 1972).

As a result of these and other studies, Benz and Goldberg (1973) have postulated that there are three types of receptors for bacteriophage T4-like particles. Two of these are postulated to be in the outer membrane and are the receptors for long tail fibres, and the subsequent attachment of the tail pins. The third is postulated to be in the cytoplasmic membrane and is the receptor for the tail tube after the bacteriophage tail has contracted.

The nature of the proteins for the type (B) tail of bacteriophage T5 (Zweig and Cummings, 1973a) and the type (C) tails of bacteriophages T7 (Studier, 1973) and P22 (Botstein, Waddell and King, 1973) have been studied, but the actual process of adsorbtion/penetration is not understood. It is known that some bacteriophages with type (C) tails have enzymic activities associated with these tails which have the ability to digest cell wall components (Stirm _et al_., 1971; Kanegasaki and Wright, 1973; Lindberg, 1973; Leske, Wallenfels and Jann, 1973). However, whether these findings are of general significance to these bacteriophages is as yet unknown.

The finding that bacteriophages of all three tail types can adsorb to the adhesions between outer and cytoplasmic membranes that are formed after plasmolysis (Bayer, 1968a) is probably important. It may be that these adhesions are of general significance to all bacteriophages, in allowing the transport of DNA excreted from bacteriophages which attach around them. It has been shown that isolated receptor preparations can trigger DNA ejection from bacteriophage T5

in the absence of such pores (Zarybnicky, Zarybnicka and Frank, 1973).

Kinetics of adsorbtion

The kinetics of adsorbtion has been dealt with thoroughly and critically in a number of reviews (Tolmach, 1957; Weidel, 1957; Garen and Kozloff, 1959; Adams, 1959) and it will be dealt with only briefly in this thesis. It was shown by Krueger (1931) and subsequently confirmed by Schlessinger (1932) that adsorbtion follows the kinetics of a first order reaction, with the rate of disappearance of free phage from the medium being proportional to the instantaneous concentration of free phage and the concentration of host cells.

Adsorbtion probably involves at least two successive steps, the first of which is reversible (Puck, Garen and Cline, 1951; Stent and Wollman, 1952; Gamow, 1969). The first step has been postulated to involve the establishment of electrostatic bonds (salt bridges) between appropriate configurations of ionic charges on the two bodies (Puck, Garen and Cline, 1951). Lindberg (1973) considers that this step is the binding of tail fibres to a cell wall receptor. It is probably non-enzymatic as it is not a temperature dependent step (Tolmach, 1957).

The second step is irreversible attachment. For this to occur with certain bacteriophages which have contractile tails, it requires aggregated lipopolysaccharide complex (Lindberg, 1973). It will not occur with alkali- or acid-hydrolysed lipopolysaccharide (Lindberg, 1973), nor with lipopolysaccharide broken down with polymyxin B (Koike and Iida, 1971), sonication, or treatment with sodium deoxycholate (Lindberg,

1967). This step probably involves the anchoring of the tail pins and is dependent, in bacteriophage T4, on functional gene 12 product (Simon, Swan and Flatgaard, 1970). It might be enzymatic as it is dependent on temperature (Tolmach, 1957).

Certain organic compounds are able to act as adsorbtion co-factors for bacteriophage particles; for example bacteriophage T4 requires free L-tryptophan (T.F. Anderson, 1945, 1946). These co-factors act by virtue of their ability to be electron donor compounds (Kanner and Kozloff, 1964), and by forming a molecular complex with a component of the tail plate, they lower the activation energy (Gamow, 1969).

RESISTANCE TO BACTERIOPHAGES

The resistance of cells to bacteriophages can be of three main types. Firstly, the cells can be lacking some component necessary for adsorbtion, these being referred to below as receptor mutants. Secondly, the cells might be lacking a component that is necessary during some post-adsorbtion step of bacteriophage multiplication, without which viable progeny bacteriophage particles are not produced. These are called "tolerant" mutants below in agreement with the nomenclature for colicins (Reeves, 1972). The third type is a miscellaneous type, where resistance is due to either lysogeny of the cell with a related temperate bacteriophage, restriction by either the host or a resident plasmid restriction system, or by some other mechanism which is not well understood. Resistance to bacteriophages may also give rise to resistance or tolerance to colicins.

Receptor mutants

Early workers (Bail, 1923; Burnet and McKie, 1933) were able

to show that mutation of a cell to bacteriophage resistance involved only some of the bacteriophages capable of lysing the strain. These mutations were shown to occur spontaneously during cell reproduction at a frequency of 10^{-7} to 10^{-10} mutations per bacterial division, and prior to the addition of selecting agent (Luria and Delbruck, 1943; Newcombe, 1949; Lederberg and Lederberg, 1952).

Demerec and Fano (1945) did the first comprehensive study of cross resistance amongst a group of seven bacteriophages which they called the T-bacteriophages. They were able to show that E. coli B could mutate to resistance to one bacteriophage or as many as three different bacteriophages in a single mutational step; and that there were a limited number of resistant mutant types that could be isolated using these bacteriophages. They described five bacterial colony types associated with resistant mutants and were able to correlate one of these types to some extent with resistance to bacteriophage T7. The fact that they were able to demonstrate two different mutants resistant to a given bacteriophage (e.g. T1, T3 or T4), showed that bacteriophage resistance could occur by more than one type of host cell alteration.

Some mutations to bacteriophage resistance were shown to be accompanied by the loss of ability to synthesize growth factors such as tryptothan (E.H. Anderson, 1946; Luria, 1946; Gots, Koh and Hunt, 1954) or proline (Wollman, 1947; Curtiss, 1965; Baich, 1968). A deletion covering the tonB and trp genes was shown to give rise to the two properties, T1 resistance and tryptothan requirement, for one mutant cell line (Franklin, Dove and Yanofsky, 1965); while Curtiss (1965) demonstrated a chromosomal aberration in his bacteriophage resistant pro^- mutants. Baich (1968), however, considered

the relationship of T4 resistance and proline deficiency to be structural rather than genetic for some T4 resistant mutants. Resistance to bacteriophage λ was often accompanied by the inability of the mutant cell to utilize maltose as a sole carbon source (E.M. Lederberg, 1955; Ronen and Raanan-Ashkenazi, 1971). This was because the maltose biosynthesis and lambda receptor genes are both under the positive control of the mal T regulator gene in E. coli K-12 (Thirion and Hofnung, 1972). Only about 20% of the resistant mutants map inside a gene lam and yield the λ^r mal^+ phenotype (80% are mal T λ^r). Wang and Newton (1971) consider that the association of resistance to bacteriophages T1 and Ø80 with deficiencies in iron transport might be due to the role of the iron transport system in an early function of these bacteriophages. In addition to these studies, it has been shown that when sucrose is added to the medium in which a culture of E. coli B is growing, the cells become phenotypically resistant to bacteriophages T2, T3, T4, T6 and T7 (Jackson, Buller and Shankel, 1967); however the significance of this is not, as yet, known.

For many years, bacteriophage resistant mutants were used mainly in genetic studies as they were easily selected and offered reasonable variety. Linkage relationships were established for some bacteriophage resistant mutants including mutants resistant to many of the T-group phages, λ and BF23 (J. Lederberg, 1947; E.M. Lederberg, 1955; Hayes, 1957; Weinberg, 1960; Tamaki, Sato and Matsuhashi, 1971; Curtiss, 1965; Buxton, 1971; Jasper, Whitney and Silver, 1972; see also Taylor and Trotter, 1972).
Other workers used mutation to bacteriophage resistance as a means of testing the capability or specificity

of various mutagenic agents (Novick and Szilard, 1951; Bryson and Davidson, 1951). However, these studies shed little light on the nature of bacteriophage resistance.

Garen and Puck (1951), working on the two step nature of adsorbtion, showed that the two bacteriophage T1 resistant mutants of E. coli B, B/1 (resistant to T1 and now known as ton B^-) and B/1,5 (resistant to T1 and T5 and now known as ton A^-), differed in that the former could adsorb T1 reversibly. This implied that the resistance of strain B/1 to bacteriophage T1 was not due solely to the failure of attachment. The further study of this mutant has been somewhat hindered by the inability of T1 to adsorb reversibly or irreversibly to either killed cells or cell wall fragments (Stent, 1963). The B/1,5 mutant however is unable to adsorb either T1 (Garen and Puck, 1951) or T5 (Weidel, Koch and Bobosch, 1954) reversibly or irreversibly. Weidel and co-workers (Weidel, Koch and Bobosch, 1954) were able to extract a lipoglycoprotein complex from their B/1,5 mutant and demonstrated that it was unable to adsorb bacteriophage T5, while a similar preparation from the sensitive strain did adsorb T5. It was later shown by Braun and co-workers (Braun, Schaller and Wolff, 1973; Braun and Wolff, 1973) that the T5 receptor was a protein present in the outer membrane of sensitive strains. This protein was also present in bacteriophage T5 resistant mutants, but was altered in such a way that it could not adsorb bacteriophage T5.

Mutants of E. coli selected as resistant to bacteriophages T3, T4 or T7 and resistant to all three of these bacteriophages were sometimes also resistant to T2 and T6 (Demerec and Fano, 1945). In these mutants the structure of

the lipopolysaccharide was substantially altered (Weidel, 1955). It was shown in Salmonella (Lindberg, 1967; Rapin and Kalckar, 1971; Wilkinson, Gemski and Stocker, 1972; Lindberg, 1973) and in E. coli 08 (Schmidt, Jann and Jann, 1970) that the pattern of bacteriophages lysing mutants with altered lipopolysaccharide, varied with the composition of the lipopolysaccharide. Although only some of the mutants were generated by selecting for bacteriophage resistance (see for example Wilkinson and Stocker, 1968), one can still make conclusions about the receptor specificities of the various bacteriophages and thus the alterations which give rise to resistance to these bacteriophages. For instance, bacteriophage Br10 (Schmidt, Jann and Jann, 1970) is able to lyse rough core mutants with phosphate attached to the heptose residues, but not those lacking phosphate. It has also been shown that bacteriophage FO in Salmonella can only lyse strains containing the terminal N-acetyl-D-glucosamine of the common core polysaccharide (Lindberg, 1967). Similar work on rough mutants of Salmonella and E. coli 08 has contributed much to an understanding of the nature of the receptors of bacteriophages C21, P22, Br2, 6SR and Br60 (Schmidt, Jann and Jann, 1970; Lindberg and Hellerqvist, 1971; Gemski and Stocker, 1972), although the requirements seem quite complex in some cases (Schmidt, Jann and Jann, 1970; Lindberg, 1973). Mutants failing to meet these receptor requirements are resistant to the bacteriophage.

In E. coli K-12, studies on the basis of resistance to bacteriophages with lipopolysaccharide receptors have been limited; mainly concentrating on a few bacteriophages.

Rapin, Kalckar and Alberico (1966) showed that bacteriophage C21 could lyse E. coli K-12 only if it was mutated such that the amount of galactose in the lipopolysaccharide was reduced. They further demonstrated a heptose requirement for adsorbtion in agreement with the findings in Salmonella (Lindberg and Hellerqvist, 1971) and E. coli 08 (Schmidt, Jann and Jann, 1970). Other authors have also related bacteriophage C21 resistance or sensitivity to alterations in the composition of the lipopolysaccharide of E. coli K-12 (Eriksson-Grennberg, Nordstrum and Englund, 1971; Rapin and Kalckar, 1971).

Mutants with altered lipopolysaccharide compositions, selected as supersensitive to novobiocin were shown by Tamaki and co-workers (Tamaki, Sato and Matsuhashi, 1971) to be also resistant to bacteriophages T4, T7, P1 and Mu1. These workers selected many bacteriophage T4 resistant mutants and fitted them into eight types, which were shown to vary with respect to their sensitivities to novobiocin and bacteriophages T3 and T7, as well as their lipopolysaccharide compositions. From this work, they hypothesized that the receptors for bacteriophages T3, T4 and T7 were not simply residues of lipopolysaccharide but complex structures with other components of the cell surface; which they thought might be lipoproteins. Watson and Paigen (1971) showed that certain mutants of E. coli K-12 lacking galactose in their lipopolysaccharide (e.g. gal U and gal E) were resistant to bacteriophage U3.

Mutants at the bfe genetic locus are resistant to bacteriophage BF23 and colicins E1, E2 and E3 (Buxton, 1971; Jasper, Whitney and Silver, 1972). In these mutants the

60,000 molecular weight protein which is the receptor for colicins E2 and E3, and possibly, as described earlier, bacteriophage BF23, is missing or altered (Sabet and Schnaitman, 1973a). However, this protein is probably only one component of the receptor of colicin E1 (Sabet and Schnaitman, 1971b), and this may also hold true for bacteriophage BF23. Randall-Hazelbauer and Schwartz (1973) have shown that the receptor protein of bacteriophage λ is missing or altered in lam mutants (λ resistant).

Thus, in summary, it appears that in all the cases above, mutation to resistance leads to loss or alteration of the receptor.

Tolerant mutants

Although many colicin tolerant mutants have been described (Hill and Holland, 1967; Nagel de Zwaig and Luria, 1967; Nomura and Whitten, 1967) only one type of bacteriophage resistant mutant has been described as tolerant. This was the so-called tet (mnemonic for T-even tolerant) mutants (Matthews, 1970). Bacteriophages T2, T4 and T6 could adsorb to tet^- mutants and inject their DNA (Matthews and Hewlett, 1971) but infectious centres were not formed. After a thorough study of the properties of tet^- cells, it was found that they lacked uridine diphosphate glucose phosphorylase and produced non glucosylated progeny phage which were sensitive to the host restriction enzyme (Hewlett and Matthews, 1973).

Cronan and Vagelos (1971) were able to demonstrate a similar type of tolerance when bacteriophage T4 infected a mutant, temperature sensitive for membrane lipid biosynthesis, at the restrictive temperature. If the

mutant cell was infected by bacteriophage T4 at the permissive temperature, and at any time during the first 70% of the latent period the temperature was shifted to the restrictive level, abortive infection occurred. Cessation of bacteriophage synthesis occurred within a few minutes of the temperature shift, leading to premature lysis. It was shown that lysozyme was required for premature lysis to occur and thus surmised that phospholipid biosynthesis was necessary for the integrity of the infected cell. Another tolerant mutant associated with the membrane was bfm which was tolerant to bacteriophage BF23 (Shinozawa, 1973). Infection by BF23 was lethal to the bfm$^-$ cell and caused leakage of potassium, but not permeability to ONPG. Thus the mutant probably results in a relatively specific change in the structure or conformation of the cell.

Hausmann (1968) isolated a tolerant mutant sin in Shigella sonnei D2 371-48 which could be complemented by the sin$^+$ gene from E. coli. Infection of this mutant by bacteriophage T7 was abortive, leading to double-stranded scission of the T7 DNA. Other mutants have been found in E. coli K-12 which also affect the DNA of certain infecting bacteriophages. These include gro N785 and gro P which can adsorb but not plaque lamboid bacteriophages (Herschey, 1971) and gro_7 which is tolerant for bacteriophage T7 (Hausmann, 1973). A tab A temperature sensitive mutant of E. coli K-12 produced non-infective T-even progeny bacteriophage when infected at the restrictive temperature (Pulitzer and Yanagida, 1971). This was shown to occur as a result of defective tail fibre production at this temperature. A similar mutant tab B was also able to adsorb bacteriophage T4

but at the restrictive temperature the assembly of phage heads was affected. This mutation was shown to have no major mutational role in the E. coli cell. Two similar mutants, mop (Takano and Kakefuda, 1972) and gro E (Georgopoulos, Hendrix and Kaiser, 1972) could adsorb bacteriophages T4 and λ, but subsequently affected the morphogenesis of the bacteriophage capsid resulting in abortive infection. It has been further shown that gro E (Zweig and Cummings, 1973) prevents cleavage of a minor protein tail component of bacteriophage T5 thus preventing T5 multiplication.

Tamaki, Sato and Matsuhashi (1971) have described bacteriophage T4 tolerant mutants of E. coli K-12 but have not studied the defects involved.

Other types of resistance

It was noted, very early in the study of bacteriophages (Burnet and Lush, 1936), that bacteria could become resistant to a bacteriophage, after adsorbtion of that bacteriophage. This process, known as lysogeny, only occurs with certain types of bacteriophages (i.e. temperate bacteriophages). The immunity to superinfection, which is mediated by a repressor, has been thoroughly studied by many workers (see Hershey, 1971; Echols, 1972; for reviews).

Other types of resistance, not specifically concerned with the receptor or some post adsorbtion event, include the blocking of bacteriophage adsorbtion by slime material (Gratia, 1922; Koransky and Anderson, 1973) or by mutation from roughness to smoothness (Burnet, 1929; Burnet and McKie,

1933). The acquisition of slime material or capsular polysaccharide can be observed after mutation of a cell to lon^- ($capR^-$) or $capS^-$ (Lieberman and Markovitz, 1970). Encapsulation can render a cell sensitive to a series of capsular bacteriophages (Park, 1956; Sutherland and Wilkinson, 1965; Stirm et al., 1971). Conversely E. coli K-12 being a rough strain (Ørskov and Ørskov, 1962), would be resistant to smooth-strain-specific bacteriophages and a female or recipient strain of E. coli K-12, being unable to produce a pilus, would be resistant to the male-specific bacteriophages which adsorb to sex-pili (Curtiss, 1969). Thus from this point of view, E. coli K-12 strains can exhibit certain types of resistance due to their inherent properties.

Every wild type bacterium has a restriction system, which will attack foreign DNA endonucleolytically unless this DNA has been modified by the host-specific modification system (Arber, 1965; Arber and Linn, 1969; Revel and Luria, 1970; Boyer, 1971). When bacteriophage λ is propagated on E. coli B or C, it subsequently forms plagues at an efficiency of plating of 10^{-4} on E. coli K-12 (Arber, Hattman and Dussoix, 1963; S. Lederberg, 1965). This is because the E. coli K-12 restriction mechanism recognizes as foreign the λ DNA which has been modified by the E. coli B or C host modification systems. The extent of this phenomena was shown to be wide and varied when studied for 28 coliphages and four different hosts (Eskridge, Weinfeld and Paigen, 1967). It was also shown that if a cell was made lysogenic for bacteriophages P1 (S. Lederberg, 1957, 1965; Drexler and Christensen, 1961; Glover et al., 1963;

Eskridge Weinfeld and Paigen, 1967) or P2 (S. Lederberg, 1957), or contained either F fertility factors (Schnell *et al.*, 1963) or various R factors (Bannister and Glover, 1968), that the growth of certain bacteriophages previously propagated on the non-lysogenic, F^- or R^- cells respectively was restricted. The bacteriophages that were restricted varied in each system; thus indicating that the restriction was specific, and giving rise to various patterns of "resistance".

The P1 restriction enzyme has been shown to attack two specific areas of the bacteriophage T1 genome (Drexler and Christensen, 1961). The restriction of T7, a female specific bacteriophage, by the F factor has been shown to result from two sex factor genes (Morrison and Malamy, 1971) which prevent late mRNA from being translated (Morrison, Blumberg and Malamy, 1974; Blumberg and Malamy, 1974). The restriction of bacteriophages T5 and BF23 by Col Ib plasmid-containing cells has also been described, and it has been shown that this episome directly interferes with the expression of late genes (Mizobuchi, Anderson and McCorquodale, 1971).

In 1953, Wahl (1953) described semi resistance which involved a lowered efficiency of plating and very turbid plaques when the bacteriophage used was plated on his semi resistant mutant. This phenomena of inhibition of bacterial growth without complete lysis has also been demonstrated by Marsik and Parisi (1971). Wahl (1953) explained it by postulating that the bacteria had a low level of receptor activity, and thus not every bacterium could be infected.

Other authors have described partial resistance (see for example Burnet and McKie, 1933) which might be due to a similar mutation. The lowered level of plaques might also be due to a host range mutant of the bacteriophage lysing a strain resistant to the parent bacteriophage strain (Luria, 1945). Infection of strain B/3,4,7(2,6) by high concentrations of bacteriophages T2 and T6 caused lysis of this strain, however dilution did not yield individual plaques. This phenomena is also caused by a host range mutant (Luria and Human, 1952).

Cross resistance between bacteriophages and colicins

Colicins are produced by bacteria which contain an extrachromosomal plasmid called a colicinogenic factor. They are narrow range antibiotics each capable of killing a few strains of bacteria which are related to the colicin-producing strain (Reeves, 1965; Nomura, 1967; Reeves, 1972). They have been shown to adsorb to a receptor on the cell surface (Konisky and Cowell, 1972; Sabet and Schnaitman, 1973a,b) and exert their effects on the cell either by staying at the cell surface and transmitting a message through the cell envelope to the target (Changeux and Thiery, 1967) or by entering the cell and acting directly on the target (Boon, 1971; Bowman *et al*., 1971). Two types of resistant cells have been shown to be insensitive to the lethal effects of colicins; these being receptor mutants such as *bfe* (Sabet and Schnaitman, 1973a,b) and tolerant mutants (Hill and Holland, 1967; Nagel de Zwaig and Luria, 1967; Nomura and Whitten, 1967); the categories being analogous in part to the bacteriophage resistant mutants discussed in this thesis.

The major difference is that the binding of bacteriophages to tolerant mutants often gives rise to a lethal event, in contrast to the binding of colicins to colicin tolerant cells. Certain colicin resistant mutants have been shown to have alterations in their cell envelopes (Sabet and Schnaitman, 1973a; Braun, Schaller and Wolff, 1973), however not all of these alterations are in the outer membrane (Holland and Tuckett, 1972).

Fredericq and Gratia (1949) first showed that mutants resistant to colicin E and colicin K were cross-resistant to their group II and III bacteriophages respectively. Since then _ton_ A (Fredericq, 1951; Gratia, 1964) _ton_ B (Gratia, 1964), _tsx_ (Weltzein and Jesaitis, 1971) and _bfe_ (Buxton, 1971; Jasper, Whitney and Silver, 1972) mutants have been shown to be cross resistant to specific bacteriophages and colicins. The protein that Braun and co-workers (Braun Schaller and Wolff, 1973; Braun and Wolff, 1973) isolated and described as the receptor for bacteriophage T5 was also shown to be the receptor for colicin M. Neither bacteriophage T5 or colicin M could adsorb to a similar protein isolated from a strain (ton A^-) cross resistant to both. Thus it is clear that they have a common receptor protein.

OBJECTS OF THE STUDY

The aims of this study were:

1. To use a wide range of virulent bacteriophages to select for bacteriophage resistant mutants in one strain of _E. coli_ K-12 and to attempt to group these mutants in a meaningful way.

2. To demonstrate that the resistant mutants are altered in some way and to characterize not only the outer membrane defects of these mutants but also the receptor specificity of the bacteriophages to which these mutants are resistant.

CHAPTER 2

MATERIALS AND METHODS

MEDIA

Nutrient broth (Difco 0003) was prepared double strength plus 0.5% (w/v) sodium chloride. Nutrient agar was blood agar base (Difco 0045) prepared as directed without the addition of blood. Brain heart infusion medium (Difco 0037-01) was prepared as directed. Minimal liquid medium was that of Davis and Mingioli (1950). Minimal agar was prepared by the addition of 2% (w/v) agar (Difco 0140) to minimal liquid medium. Glucose was added as a carbon source at 0.5% (w/v). Growth supplements and other carbon sources were used at the following final concentrations: purines, pyrimidines and amino acids, 20 µg/ml; galactose, lactose, mannitol, xylose and maltose, 1.0% (w/v). Fermentation characteristics of recombinants were determined by plating cultures on eosin-methylene blue (EMB) agar (Difco-0511) with sugars added at the concentrations noted above. 0.7% agar for overlays was prepared by diluting mutrient agar 1:1 with nutrient broth.

BACTERIAL STRAINS

The strains of *E. coli* K-12 and the single strain of *E. coli* B used, together with their source or derivation, are listed in Table 2-1, with the following exceptions: the bacteriophage resistant mutants derived from P400 (Tables 4-1, 4-2, 4-3, 4-4, 4-5 and 4-6); and the colicin

TABLE 2-1

Bacterial strains (i)

Strain	Genotype/mating type	Source/derivation
AB1133	thi argE his proA thr leu mtl xyl ara galK lacY str supE/F$^-$	A.L. Taylor
ES368	lon non leu ade trp strA/F$^-$	E.C. Siegel
P400	thi argE non proA thr leu mtl xyl ara galK lacY str supE/F$^-$	by P1kc cotransduction of non with his$^+$ from ES368 into AB1133
E. coli B	prototroph/F$^-$	J. Eigner
P839	thi/HfrH	K. Brooks Low
AB259	thi rel/HfrH	B. Bachmann CGSC259
RC749	thr leu thi mal lac gal ton λ^r/HfrP10(J4)	R. Clowes
KL209	malB sup53 (unmapped amber suppressor) λ^r/HfrP10(J4)	B. Bachmann CGSC4315
JG20	thr leu thi malB λ^r/Hfr P10(J4)	J. Gross
RC750	met/HfrC	R. Clowes
KL141	thi pyrE argG thyA rbs malA str gltS λ^r/F$^-$	B. Bachmann CGSC4224
Ra2	sfa-4 (sfa-5) sup42 (unmapped amber suppressor/HfrP048)	B. Bachmann CGSC4241

(i) The abbreviations and nomenclature are essentially that of Taylor and Trotter (1972). The mating type is included after the genotype and is separated from it by an oblique line.

resistant and tolerant mutants derived from AB1133 (Table 4-7) kindly provided by J.K. Davies. All strains were stored as freeze-dried cultures and working stocks were maintained on nutrient agar slopes at 4C. All cultures were grown by incubation at 37C. Overnight cultures were prepared by innoculation from a slope or single colony into 10 ml of media and aerated by agitating on a reciprocating shaker. Logarithmic cultures were prepared by diluting an overnight culture 10- to 100-fold in fresh media and aerating as above.

Strain P400 was derived as follows: It was known that non was over 50% cotransducible with his (Radke and Siegel, 1971). Therefore the his^+ marker from strain ES368 was transferred into strain AB1133 using Plkc transduction. Recombinant colonies were tested for their inability to form T7-resistant colonies by plating with bacteriophage T7 and incubating at 37C overnight. One recombinant with non^- properties was freeze dried as P400.

BACTERIOPHAGE STRAINS

The bacteriophage strains used in this study together with their sources are described in Table 2-2. In some cases, bacteriophages have been given laboratory strain names, either for the sake of simplicity or due to the similarity of names in the literature. The previous names where appropriate and a literature reference to the bacteriophages have been listed in Table 2-2. The bacteriophages listed as having been isolated from sewage are discussed more thoroughly in Chapter 3 together with other bacteriophages shown to be similar. In addition to the bacteriophages of Table 2-2 used for the isolation and testing of mutants, bacteriophages C21 and U3

TABLE 2-2

Bacteriophage Strains used

Bacteriophages	Source(i)	Previous(ii) Name	References(iii)
T1,T2,T3,T4,T5,T6,T7	1	–	a
BF23	1	–	b
A,B,C,D,F,G,J,E4,E7,E11, E15,E21,E25,H1,H3,H8	2	–	–
K2	3	IISK = SsII	c
K3	3	VSH = SHV	d
K4	3	VSK = SsV	c
K5	3	VISK = SsVI	c
K6	3	VIISK= SsVII	c
K8	3	IXSK = SsIX	c
K9	3	XSK = SsX	c
K10	3	XISK = SsXI	c
K11	3	XIISKo= SsXII	c
K12	3	XIISHo= SHXII	d
K15	3	G36 = SG36	e
K16	3	G42 = SG42	e
K17	3	T1881	f
K18	3	α	g
K19	3	a	g
K20	3	D8	g
K21	3	D2a	g
K22	3	D2b	g
K25	3	F2	g
K26	3	F4	g
K27	3	F5	g
K29	3	F7	g
K30	3	F9	g

TABLE 2-2 Continued

K31	3	F10	g
Ox1,Ox2,Ox3,Ox4,Ox5	4	-	h
M1	4	phage 3	h
M3	4	C1 = Ø1	i
Ac3	5	3	j
Ac4	5	4	j
H^+,V	6	-	k
Ø1	7 and 10	-	l
H	7	-	m
Ø11-T	8	-	n
F27	9	-	o
W31	11	-	p

(i) Sources were:

1, Lab. stocks; 2, isolated from sewage in this study (see Chapter 3); 3, S. Slopek; D. Kay; 5, M.W. Ackerman; 6, J. Beumer; 7, R.W. Hyman; 8, M.H. Malamy; 9, I.W. Sutherland; 10, R. Dettori; 11, T. Watanake.

The scientists mentioned above are thanked for their kind gifts of bacteriophages.

(ii) Two alternatives have been given where more than one name has been used in the literature.

(iii) References in which the bacteriophages have been previously described are:

a, Adams (1953); b, Buxton (1971); c, Krzywy, Kucharewicz-Krukowska and Slopek (1971); d, Krzywy, Kucharewicz-Krukowaka and Slopek (1972); e, Kryzywy (1972b); f, Slopek _et al._ (1973); g, Slopek _et al._ (1972); h, Kay and Fildes (1962); i, Bradley (1963); j, Ackerman and Berthiaume (1969); k, Beumer _et al._ (1965); l, Dettori, Maccacaro and Piccinin (1961); m, Brunovskis, Hyman and Summers (1973); n, Morrison and Malamy (1971); o, Sutherland and Wilkinson (1965); p, Watanabe and Okada (1964).

(Table 6-1) kindly provided by Dr. Roy Russell, and bacteriophage P1kc from laboratory stocks were used.

Some of the bacteriophages used were originally isolated on other strains of E. coli or Shigella, a closely related genus (Ewing, Hucks and Taylor, 1952; Luria and Burrous, 1957). These included: bacteriophages H^+ and V originally isolated on Shigella dysenteriae PB (Beumer et al., 1965); K2, K3, K4, K5, K6, K8, K9, K10, K11, K12, K15, K16 and K17 originally isolated on Shigella sonnei strains (Krzywy, Kucharewicz-Krukowska and Slopek, 1971, 1972; Krzywy, 1972; Slopek et al., 1973); K18, K19, K20, K21, K22, K25, K26, K27, K28, K29, K30 and K31 originally isolated on Shigella flexneri strains (Slopek et al., 1972); Ox1, Ox2, Ox3, Ox4, Ox5, and M1 originally propagated on E. coli c2 (Bradley, 1963); and Ac3 and Ac4 originally propagated on E. coli 0127:B8 (Ackerman and Berthiaume, 1969). These bacteriophages were all re-isolated three times on strain P400 from single plagues and propagated in liquid media (Adams, 1959).

GENERAL BACTERIOPHAGE METHODS

The method for assaying bacteriophage solutions was the agar overlay technique. 0.1 ml of each of a series of 100-fold dilutions of a bacteriophage solution were added to 0.1 ml of a standing overnight culture (10^9 cells/ml) of indicator bacteria in 3 ml of 0.7% Agar at 45C, mixed by gently swirling and poured onto a nutrient agar plate. After allowing the agar overlay to set, and incubating overnight at 37C, the plate containing the largest number of discrete plaques was selected and the plaques enumerated.

All bacteriophages were propagated by one of two methods on E. coli K-12 strain P400; with the exception of the T group of bacteriophages which are classically grown on E. coli B and bacteriophages H1, H3, H8 and E11 which were also propagated on E. coli B. For the first method, a solution of a given bacteriophage was assayed by the agar overlay technique and after overnight incubation a plate showing semi-confluent lysis selected. The 0.7% agar overlay was scraped with a spreader from two such plates into a sterile bottle, 10 ml of nutrient broth added, and the bottle allowed to stand for 30 min at room temperature after which the agar was removed by centrifugation (3000xg, 10 min). The supernatant was extracted and assayed by the agar overlay technique described above for plaque forming units, (pfu).

The second method involved adding approximately 10^7-10^8 pfu of a given bacteriophage to 10 ml of a culture of strain P400 or AB1133 in logarithmic-phase growth (1-2x10^8 cells/ml). The mixture was then incubated at 37C until lysis occured, or otherwise incubated overnight. Chloroform (0.5 ml) was added and after incubating a further 30 min at 37C the bacteria pelleted by centrifugation (5000xg, 10 min). The supernatant was then assayed as above. All bacteriophage cultures were stored for routine use at 4C with chloroform (5% v/v) and assayed every six months or in some cases more regularly for pfu. Samples of each bacteriophage were also added to double strength milk and freeze dried (Ghitter and Wolfson, 1960) for long term maintenance.

Efficiency of plating studies were done by plating a defined number (10^5-10^6 pfu) of a given bacteriophage, in a 0.7% agar overlay with enough bacteria (5 x 10^7 cells) to

normally give confluent growth.

ISOLATION OF BACTERIOPHAGES FROM SEWAGE

Raw sewage was obtained on three separate occasions, with the kind assistance of Mr. Lloyd Goss, from Bolivar sewage treatment farm near Adelaide. The technique for isolation of bacteriophages was based on that of Brown and Parisi (1966). Raw sewage was allowed to settle and the top layer filtered through a Seitz S6 filter. To 9 ml of this solution 1 ml of 10 times concentrated brain heart infusion broth was added after which it was incubated (37C, 4 hr). Chloroform (0.5 ml) was added and the solution incubated further (37C, 30 min) and then the bacteria removed by centrifugation (10,000 x g, 15 min) and the supernatant plated with strain P400 by the agar overlay technique. In the third isolation the supernatant was treated with anti-serum raised against bacteriophages from the first and second isolations, in order to increase the range of bacteriophages obtained. After overnight incubation of the overlaid plates single bacteriophage plaques were picked off and purified by two single plaque isolations.

SEROLOGICAL TECHNIQUES

One rabbit was used to raise antiserum against each of bacteriophages T3, T6, H3 and F. The technique used was based on the three course immunization schedule of Barry (1954). Bacteriophage lysates (10^9 pfu/ml) were prepared by centrifugation (10,000 x g, 30 min) to remove most of the cell wall fragments. In the first course of immunization, on five

successive days 0.75 ml - 1.0 ml of bacteriophage solution was injected intravenously into the ear after which the rabbit was rested 7 days and a test bleed taken. For the second course, 1 ml was injected intravenously on four successive days and the rabbit again rested 7 days and test bled; while for the third course this was repeated with the exception of the fourth day's injection. At this stage, the anti-phage antibody titres were sufficiently high for neutralization experiments and the rabbits were bled out, either through the ear or by cardiac puncture. The blood from the various bleeds was allowed to clot at room temperature and then ringed with a hot loop, stood in the cold for two hours, and the serum collected and freed from red blood cells by centrifugation (5,000 x g, 10 min). The supernatant was then frozen in 1 ml quantities.

Assay of anti-phage antibodies was done with the homologous bacteriophage by the method of Barry (1954). 0.9 ml of a solution of bacteriophage (5×10^5 pfu/ml) was added to 0.1 ml of each of a series of 10-fold dilutions of anti-phage sera (or 0.1 ml of normal sera as a control), and the solution incubated (37C, 30 min) diluted 100-fold, and 0.1 ml plated with 0.1 ml of a standing overnight culture of strain P400 by the agar overlay technique. After overnight incubation of the plates at 37C they were enumerated, and a plate with a 90% reduction in plaque count used to calculate the K value using the formula (Adams, 1959):

$$K = 2.3 \frac{D}{t} \log \frac{po}{p}$$

where D = dilution of antiserum; t = time of incubation; po = number of plaques on control plate; and p = number of plaques on plate with dilution D.

In order to test the neutralization of other bacteriophages, either a K value was calculated using the method above, or a neutralization assay was performed using a given dilution of antiserum (1/10). In this second test, 90% neutralization (approximately equivalent to K>1) or greater indicated serological relatedness. Two other anti-sera were used in these experiments; anti-phage T4 sera, a gift of Dr. A. Osmond; and anti-phage T2 sera, a gift of Dr. P. Reeves.

ELECTRON MICROSCOPY

The technique of preparation of bacteriophages for electron microscopy, was based on that of Bradley (1967). Bacteriophages were harvested from five overlaid plates (0.7% agar overlay) showing semi-confluent lysis by collecting each overlay into 3 ml of 1% (w/v) ammonium acetate pH 7.0. They were then purified and concentrated by one cycle of differential centrifugation (5,000 x g, 20 min then 50,000 x g, 60 min) and the resultant bacteriophage pellet resuspended in a small volume of ammonium acetate buffer. Uranyl acetate was added at 2% (w/v) and the specimens were then examined with a Siemens Elmiskop 1 electron microscope.

Grid preparation, mounting, staining and electron microscopy were skilfully performed mainly by Miss P. Dyer, The Department of Biochemistry, The University of Adelaide. The electron microscopy of bacteriophage T3 was done by Dr. J. Casely-Smith, The Department of Zoology, The University of Adelaide.

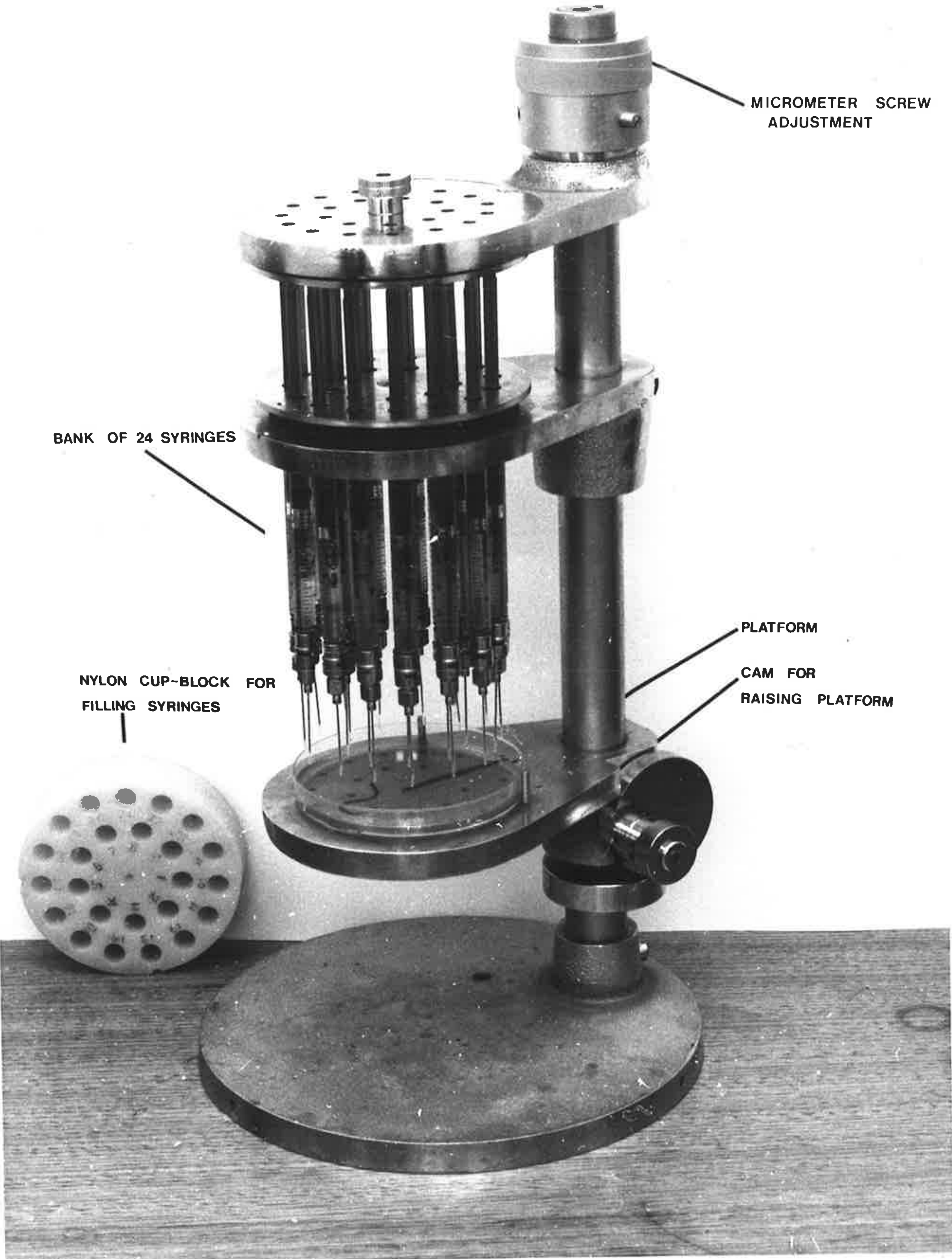
ISOLATION AND SCREENING OF BACTERIOPHAGE RESISTANT MUTANTS

In order to isolate bacteriophage resistant mutants of independant origin, individual colonies of strain P400 were picked off and streaked across nutrient agar plates on which 10^7-10^8 pfu of the appropriate bacteriophage had been spread. From each streak, a single colony was then picked off after overnight incubation, purified by two successive single colony isolations, and screened for sensitivity or resistance to the bacteriophages shown in Table 2-2.

Suspected resistant mutants were screened using a multiple syringe bacteriophage applicator (M.S.B.A.) shown in Fig. 2-1. This machine, skilfully made by Mr. Barry Parker of the Workshop, Medical Building, The University of Adelaide, was based on that of Zierdt, Fox and Norris (1960). The technique of using this machine involved filling each of the syringes from a nylon block with 24 wells, each containing 2 ml of a different bacteriophage solution (2×10^5 pfu/ml). The mutants to be tested were grown as overnight standing cultures at 37C and 0.1 ml of each plated on a nutrient agar plate using the agar overlay technique. The overlaid plates were then dried face downwards (45 min, 37C), and placed in turn on the platform of the M.S.B.A. and the micrometer rotated to produce a hanging drop at the end of each of the syringes. The platform was raised so that each of the drops were touched off onto a discrete area of the plate. After the spots of bacteriophage solution had dried into the surface of the overlay, the plate was incubated overnight at 37C. The parent strain P400, treated similarly, acted as control. A reference line on the plate, served to indicate the placement of the various spots. The M.S.B.A. was

FIG. 2-1

Multiple syringe bacteriophage applicator for testing resistance or sensitivity to 24 different bacteriophages consecutively. The machine was constructed from solid brass and nickel electroplated. It was fitted with 24 Luer-lock 1 ml tuberculin syringes each of which had a 27 gauge needle with a tight fitting teflon tubing jacket. The cup block was machined from nylon. The micrometer screw adjustment has a has a travel of 80 turns which lowers the syringe plungers sufficiently to deliver 1 ml of liquid. One half of a turn produces a hanging drop of sufficient size for the testing of resistance or sensitivity to the 24 bacteriophages contained in the syringes. The applicator, adapted from that of Zierdt, Fox and Norris (1960) was constructed by Mr. Barry Parker of the Workshop, Medical Building, University of Adelaide.


MICROMETER SCREW ADJUSTMENT
BANK OF 24 SYRINGES
PLATFORM
CAM FOR RAISING PLATFORM
NYLON CUP-BLOCK FOR FILLING SYRINGES

sterilized by autoclaving and could be used twice in one day (allowing resistance or sensitivity of the mutants to 48 bacteriophages to be tested in this time period).

One or two isolates of each type of resistant mutant were selected for further study and freeze dried. Further characterization of the resistance pattern of these selected strains was done by e.o.p. studies whenever the mutant was not fully resistant or sensitive to a bacteriophage (see Chapter 4). All mutants isolated arose spontaneously with the exception of strain P479 which was selected after mutagenesis of strain P400 by N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) according to the method of Adelberg, Mandel and Chen (1965).

NOMENCLATURE OF RESISTANT MUTANTS

The relative efficiency of plating (e.o.p.) in this thesis represents the number of plaques formed on a mutant relative to the number formed on the parent strain P400.

The following terms define the patterns of resistance that occur when a defined number (10^4-10^6 pfu) of a given bacteriophage is plated, in a 0.7% agar overlay, with enough mutant bacteria (5×10^7 cells) to normally give confluent growth. The initials or symbols in brackets are the abbreviations used in Tables 4-1, 4-2, 4-3, 4-4, 4-5, 4-6 and 4-7.

Full Resistance (R) The bacteriophage is unable to form plaques on a particular mutant (e.o.p. $<10^{-7}$).

Bacterial Inhibition (I) The bacteriophage plaques are very turbid (i.e., bacterial growth is inhibited where a plaque would normally form; the area of inhibition being equal to the

normal area of a plaque). Plaques occur with an e.o.p. of 1.

Partial Resistance (P) The bacteriophage forms wild type plaques with an e.o.p. of 10^{-2} or less.

Partial Resistance with Inhibition (IP) A combination of the above two effects with very turbid plaques and a lowered e.o.p.

Slight Resistance (SL) The bacteriophage either forms wild type plaques at an e.o.p. of greater than 10^{-2}, or only minute plaques are formed with an e.o.p. of approximately 1. These are very minor alterations in the resistance pattern and thus they have been grouped.

Sensitivity (-) An e.o.p. of approximately 1 with normal plaques.

The terms resistance or resistant, where used in this thesis, cover any of the above terms except for sensitivity. These terms are distinguished from full resistance or fully resistant as defined above.

The designation Wrm (2) for a strain implies that the strain belongs to sub-group 2 of the Wrm resistance group.

PREPARATION OF CELL ENVELOPE AND WALL FRACTIONS

For neutralization studies, whole cell envelope was prepared from cells grown to late logarithmic phase (10^9 cells/ml) in a 10 litre fermentor. The media used was phosphate buffer pH 7.0 containing 1.5% (w/v) yeast extract and 1% (w/v) casamino acids. After the cells had reached the late logarithmic stage of growth, chloroform (0.5% w/v) was added for 10 min at 37C and the bacteria centrifuged down (10,000 x g, 10 min). The pellet was then re-suspended in water and treated by a technique similar to that used by

Weidel, Koch and Bobosch (1954) in the first stage of preparation of the bacteriophage T5 receptor complex. The suspension was stirred vigorously with a glass rod and the pH raised from 6.5 to 9.8 by dropwise addition of 0.1N sodium hydroxide and immediately lowered to pH 7.0 by bubbling carbon dioxide through the suspension, after which it was centrifuged (10,000 x g, 20 min) and the supernatant set aside. The pellet was re-suspended in distilled water and extracted again as above. The two supernatants were combined as the "sodium hydroxide supernatant" and the pellet treated according to the technique of Weltzein and Jesaitis (1971).

The pellet remaining after sodium hydroxide treatment was re-suspended in 70 ml of 0.01M Tris buffer pH 7.5 and 0.4 mg ribonuclease A (Sigma Chemical Co., St. Louis, Mo.), 0.4 mg of crude beef heart pancreas deoxyribonuclease (Sigma Chemical Co.,) and 10 mg of $MgCl_2$ added. This solution was then incubated at 37C for 30 min, and the supernatant removed after centrifugation (10,000 x g, 20 min). The pellet was re-suspended in Tris buffer and sonicated for 30 min at 0C, and collected by centrifugation (10,000 x g, 30 min). After re-suspension of the pellet, phase contrast microscopy revealed that the preparation was not contaminated with whole cells, and no clumping of the membraneous structure was observed. The suspension was then dialysed (12 hr, 4C then 24 hr, 4C) against fresh changes of water. The cell envelope was collected by centrifugation (50,000 x g, 60 min) dissolved by sonication in distilled water and lypholized as the cell envelope preparation. The yield was 620 mg (dried weight) of cell envelopes.

This cell envelope preparation was dissolved at a concentration of 2 mg/ml in 2% Triton X-100 in 10 mM Hepes buffer pH 7.4 and treated by the techniques of Schnaitman (1971a,b). Suspension was aided by 10 sec sonication. The solution was incubated for 10 min at 23C and then centrifuged (150,000 x g, 60 min). The supernatant was set aside as the Triton X-100 soluble supernatant. The pellet was re-suspended in water and washed by centrifugation (15,000 x g, 60 min). Re-suspension of the pellet in the original volume of water yielded the Triton X-100 insoluble "cell wall" preparation. This cell wall preparation was then re-extracted with the same Triton-Hepes buffer, but with 10 mM Ethylene-diamine-tetraacetic acid (Edta) pH 7.4 added (Schnaitman, 1971b). This yielded after centrifugation and washing, the Triton-Edta soluble and insoluble preparations. Triton X-100 was removed from the Triton-Edta soluble supernatant by alcohol precipitation and centrifugation (10,000 x g, 20 min) which left the Triton X-100 in the supernatant (Sabet and Schnaitman, 1971, 1973a). The protein content of all preparations was analysed by the technique of Schacterle and Pollack (1973).

For polyacrylamide gel electrophoresis, whole cell envelope was prepared from 1 litre of cells grown in nutrient broth to logarithmic-phase (7 x 10^8 cells/ml). The cells were harvested by centrifugation (10,000 x g, 20 min) in 0.05 M Tris-buffer pH 7.8, washed in the same buffer, and broken with a French pressure cell, or after freezing with an alcohol-dry ice mixture in an X-press cell disintegrator (L.K.B. Biotec, Stockholm). Cell debris was pelleted by centrifugation (5,000 x g, 15 min) and $MgCl_2$ was added to

2 mM. The supernatant fluid was centrifuged (78,000 x g, 60 min), and the pellet re-suspended in Tris-$MgCl_2$ as above and centrifuged (78,000 x g, 60 min). The resultant pellet was re-suspended in deionized distilled water at a protein concentration of 10 mg/ml (estimated by the Schacterle and Pollack (1973) technique) as the cell envelope preparation. Cell wall was prepared by the method of Schnaitman (1971a) as described above using an initial concentration of 10 mg/ml.

LIPOPOLYSACCHARIDE PREPARATIONS

For neutralization studies, lipopolysaccharide (LPS) was extracted by the hot phenol-water technique (Westphal, Luderitz and Bister (1952). The preparation was treated with deoxyribonuclease and ribonuclease as described above for the cell envelope preparation. For gas liquid chromatography, LPS was isolated by the technique of Galanos, Luderitz and Westphal (1969) using solubilization in phenol-chloroform-petroleum ether.

ADSORBTION AND NEUTRALIZATION STUDIES

Two methods were used for testing the adsorbtion of bacteriophages to whole cells. In the first a given bacteriophage was added to logarithmically growing bacteria (2×10^8 cells/ml) at a multiplicity of infection of 0.5 to 1.0 in the presence of 0.001M KCN. After 30 min at 37C this was diluted 100-fold, 5% (v/v) chloroform added, the solution vortexed for 5 sec on a Whirlymixer (Fissons Scientific Industries Ltd.), shaken for 10 min at 37C, and suitable dilutions plated for infective centres. Nutrient broth

substituted for bacteria in the control. This method tested only irreversible adsorbtion. The second method used tested both reversible and irreversible adsorbtion. Bacteriophages and bacteria were mixed for 7 min at 37C as described above, but without the addition of cyanidc. They were then centrifuged and the supernatant assayed for unattached bacteriophages.

For neutralization studies, 0.5 ml of a given bacteriophage ($5x10^5$ pfu/ml) in nutrient broth was added to 0.5 ml of distilled water containing 1 mg, 100 µg or 10 µg of LPS or of one of the cell envelope or wall preparations. The mixtures were then incubated for 3 hr at 37C, diluted 100-fold to stop further reaction and 0.1 ml plated with 0.1 ml of an overnight standing culture of strain P400 in a 0.7% agar overlay, and incubated overnight at 37C. In the control 0.5 ml of distilled water was substituted for the LPS or cell envelope or wall preparation. A 50% or greater reduction in plaque count relative to the control indicated neutralization.

For enzymatic studies of the receptor, cell wall was treated with sodium periodate according to the technique of Sabet and Schnaitman (1971) or pronase according to the technique of Weltzein and Jesaitis (1971) prior to neutralization studies as above.

ANTIBIOTIC RESISTANCE TESTING

Oxoid multodisks 11-14D and 30-9C were used to test alteration in the pattern of resistance or sensitivity to a range of antibiotics. The discs contained chloramphenicol (50 µg), erythromycin (50 µg), tetracycline (50 µg), colistin methane sulphonate (50 µg), kanamycin (5 µg), neomycin (10 µg)

and ampicillin (2 µg) to which strain P400 is sensitive; novobiocin (30 µg) to which it is slightly sensitive; and sulphafurazole (500 µg), oleanodymycin (10 µg), fusidic acid (10 µg) cloxacillin (5 µg), and novobiocin (5 µg) to which it is resistant. In the case of erythromycin a decrease or increase in zone size of 50% over the control strain P400 was taken to mean resistance or sensitivity respectively. Any lesser change was described as slight (Table 1). All other antibiotics except for ampicillin showed absolute changes (i.e. resistance going to sensitivity). Ampicillin resistance was further tested by plating approximately 1000 cells on nutrient agar plates containing 0.5, 1.0, 2, 3, 4, 5, 15, 30 or 75 µg/ml of ampicillin (Beecham Research Laboratories, Australia). Strain P400 was found to be resistant to 1.0 ug/ml but sensitive to 2.0 µg/ml.

POLYACRYLAMIDE GEL TECHNIQUES

The solubilization of cell envelope proteins and the subsequent analysis of these proteins by gel electrophoresis were carried out by three techniques, the first of which, Method (1) was essentially that of Neville (1971). Samples of whole envelope, cell wall or standard protein preparations, were made 50 mM in sodium carbonate, and sodium dodecyl sulphate (SDS) was added at 4 mg/mg of protein, followed by the addition of 10% by volume 2-mercaptoethanol; the maximum time for this step being 2 min. This preparation was diluted to 4 mg protein/ml in upper gel buffer (Neville, 1971) + sucrose (2%) + bromophenol blue, and samples containing 100-150 µg of protein (25-40 µl) were loaded onto gels

(10 x 0.6 cm) with a Hamilton microsyringe. The gels, in a SDS borate-sulphate discontinuous buffer system (Neville, 1971), were run at 0.5-1.0 mA per tube at 25C until the sample entered the upper gel then the current was increased to 1.5 mA per tube. After electrophoresis for 6 hr the gels were stained for 15 hr in 0.2% coomasie brilliant blue in fixative (Weber and Osborn, 1969) and de-stained with 20% methanol in 7.5% acetic acid.

The solubilization of method (2) was the complete method II of Schnaitman (1973a) and involved preliminary heating of the sample in SDS at 37C for 2 hr, dialysis against SDS-urea and final heating at 100C for 5 min. The solubilization of method (3) again employed method II of Schnaitman (1973a) omitting only the final 100C heating. Samples of cell wall and cell envelope prepared by methods (2) and (3) were run on 7.5% polyacrylamide gels using either the pH 7.2 Maizel buffer system (Maizel, 1966) or the pH 11.4 - pH 4.1 Bragg-Hou gel system (Bragg and Hou, 1972). These were the conditions used by Schnaitman (1973a,b, 1974a,b).

Gel staining and de-staining was by the techniques of Swank and Munkres (1971). Stained gels prepared by all techniques were scanned with either a Joyce Loebel Chromoscan Mk II densitometer, a Gilford gel scanner or a Helena Quick Scan Jr.

GAS LIQUID CHROMATOGRAPHY

To 2 mg of LPS in deionized distilled water, 100 µg of arabinose was added as an internal standard. The LPS was then hydrolized with 0.1N HCl (100C, 64 hr) in a total volume of 2 ml in an ampoule sealed under vacuo. The resultant

hydrolysate was then centrifuged (3000 x g, 15 min) to remove lipid, excess acid neutralized on a column of ion exchange resin AG1-X2 (HCO_3^- form) (100-200 mesh, Bio. Rad Laboratories, Richmond, Calif.) and brought to pH 8.5 with $NaHCO_3$. This mixture of aldoses was reduced by adding 14 mg of sodium borohydride and incubating for 2 hr at room temperature. Excess borohydride was destroyed with glacial acetic acid, and passage through a column of ion exchange resin AG50W-X4 (H^+ form) (50 - 100 mesh, Bio. Rad Laboratories). The aldoses thus obtained were converted to their alditol acetates by the technique of Holme et al. (1968). These were then dried down and dissolved in a small amount of chloroform and injected into a column of 3% ECNSSM (Applied Science Laboratories, State College, Pa.) on Gas ChromQ (100-120 mesh)(Applied Science Laboratories), in a Varian Aerograph series 1700 gas chromatograph connected up to a Hitachi Model QPD 54 chart recorder. The column temperature was 200C as suggested by Eriksson-Grennberg et al. (1971). As a control, arabinose was added to an equal amount of glucose and subjected to the above procedure, including hydrolysis.

GLUCOSAMINE ANALYSIS

"Degraded polysaccharide" was extracted from LPS by treatment with 1% acetic acid (100C, 2 hr) and lipid A was removed by centrifugation (Müller-Seitz, Jann and Jann, 1968). Some samples were further extracted 2 times with an equal volume of chloroform. All samples were then lypholized, after which they were hydrolysed according to the technique of Monner et al. (1971). The glucosamine content of the

hydrolysate was analysed according to the technique of Strominger *et al*. (1959).

GENETIC ANALYSIS

The techniques of conjugation and gradient transfer analysis of mutants were those of de Haan *et al*. (1969). For scoring of growth factors replica plating was used; however, this was found to be inaccurate for bacteriophage resistance/sensitivity testing and another technique was used. Single recombinant colonies were picked off, spread on a nutrient agar plate, grown overnight at 37C, and a single colony then spread with a platinum loop on one ninth of a dried nutrient agar plate. A drop (0.01 ml) of bacteriophage lysate containing 10^6 pfu or more was applied to the spread area and after incubation at 37C overnight, an area of lysis indicated sensitivity. The resistant mutant used in the study and its parent strain P400 acted as controls. The technique of P1kc transduction was that of Lennox (1955).

CHAPTER 3

CHARACTERIZATION OF BACTERIOPHAGES ISOLATED FROM SEWAGE

INTRODUCTION

Bacteriophages capable of lysing *E. coli* have been isolated on many other genera of the order Eubacteriales. These include many members of the family Enterobacteriaceae (of which *E. coli* is a genus): *Shigella* (Beumer *et al.*, 1965; Gromkova, 1968; Dhillon and Dhillon, 1972); *Serratia* and *Salmonella* (Adams and Wade, 1955); *Klebsiella* and *Aerobacter* (Sutherland and Wilkinson, 1965; Souza, Ginoza and Haight, 1972); as well as *Pasteurella pestis* (Hertman, 1964; Brunovskis, Hyman and Summers, 1973) of the family Brucellaceae. Many of these are enteric organisms and hence excreted in the faeces. *E. coli* K-12 too, was originally isolated as an enteric organism from the stool of a diphtheria patient at Stanford University in 1922 (Jones, Koltzow and Stocker, 1972). Therefore, as one might expect, coliphages have been previously isolated from the faeces of various animals and man (see for example, Burnet and McKie, 1933), from sewage (e.g. Dettori, Maccacaro and Piccinin, 1961; Brown and Parisi, 1966), water (Bradley, 1963), and a host of other sources in which one might expect to find faecal contamination (Dhillon and Dhillon, 1972). In this study, it was decided to isolate coliphages from raw sewage which appeared to be a valuable source of such bacteriophages.

ISOLATION OF BACTERIOPHAGES

Three separate isolations of bacteriophages from raw sewage were made, in which seven, eleven and twenty-five bacteriophages respectively were selected originally on the basis of different plaque morphologies. The first and second series of bacteriophages were classified serologically and by electron microscopy, and were also used to isolate bacteriophage resistant mutants (see Chapter 4). It was shown that each series contained a number of isolates of similar or identical bacteriophages. Antisera against some of these bacteriophages (anti F, anti T6 and anti H3) were present during the third isolation to select against these common isolates, thus providing a wider range of bacteriophages. The bacteriophages isolated were: bacteriophages A,B,C,D,F,G and J (from the first isolation); bacteriophages H1 - H11 (second isolation); and bacteriophages E1 - E25 (third isolation).

ELECTRON MICROSCOPY

Morphological studies were performed on bacteriophages A,D,F,G,J,H1,H3,H4,H5,H6,H7,H8,H9,E4,E7,F27,E11,T4 and T3 and examples of the structures observed are shown in Fig. 3-1A, 3-1B, 3-1C and 3-1D.

Bacteriophages A,F,G,E4,E7,F27 and J had similar morphology (Fig. 3-1D which was the A1 type on the classification of Ackerman (1969). The essential features were a head that was not quite truly hexagonal, joined to a contractile tail. The appearance of the tail sheath was altered by contraction of the tail; while the head, which

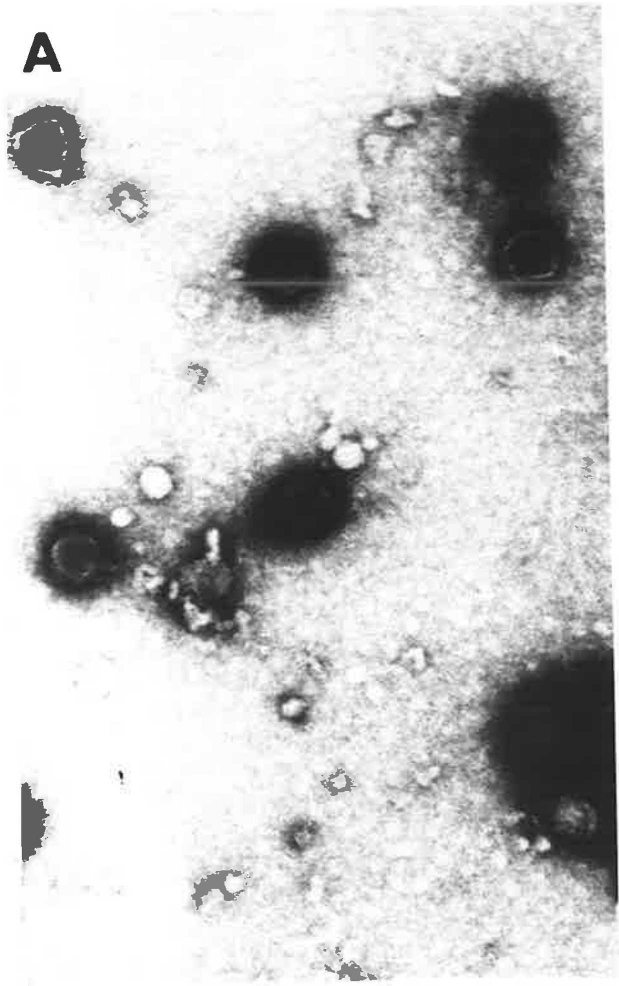
FIG. 3-1

(A) Electron micrograph of bacteriophage T3 (x90,000) negatively stained with Uranyl acetate. The bacteriophage has a C1-type morphology (Ackerman, 1969).

(B) Electron micrograph of bacteriophage D (x200,000), showing B1-type morphology (Ackerman, 1969). A base plate structure and four long tail fibres are visible.

(C) Electron micrograph of bacteriophage H1 (x245,000) showing A2-type morphology (Ackerman, 1969). Tail fibres are lined up along the tail sheath.

(D) Electron micrograph of bacteriophage A (x245,000) showing A1-type morphology (Ackerman, 1969). The tail fibres are shorter than those of bacteriophage H1 (C) and are more numerous (there being 8) than those of T-even (A2) bacteriophages (Simon and Anderson, 1967).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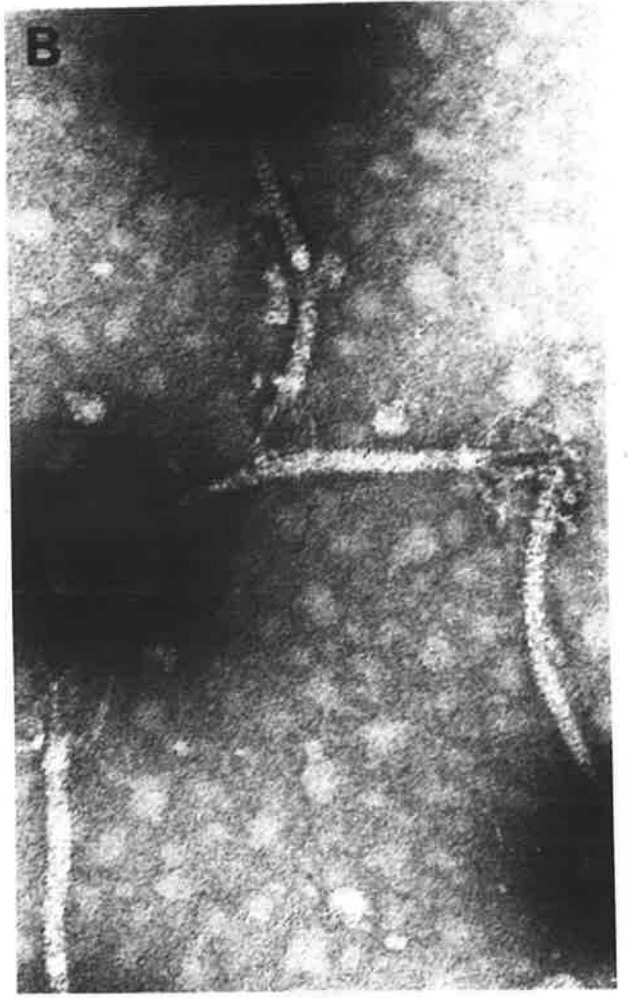
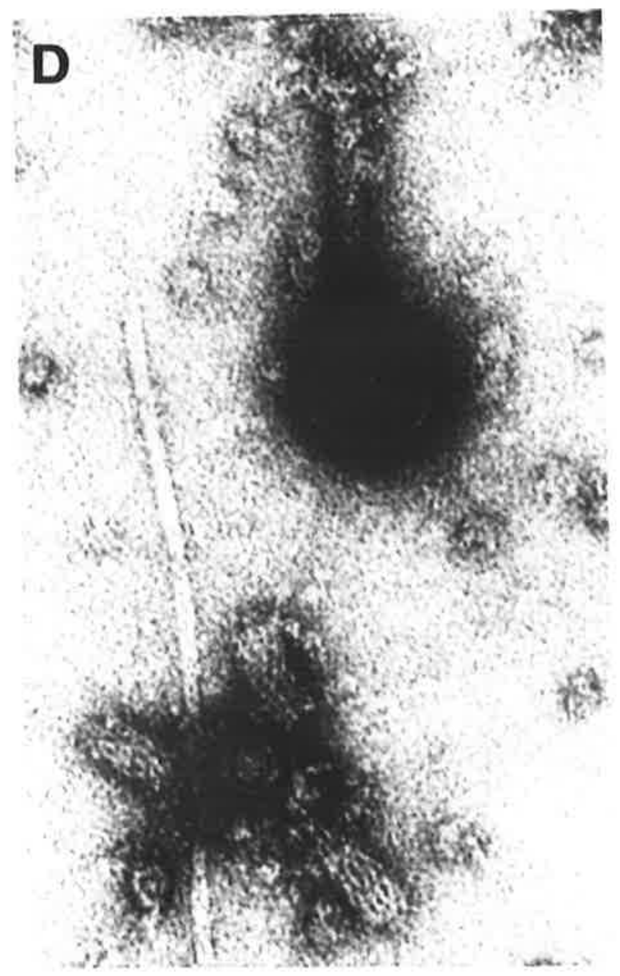

B

C


D

was joined to the tail by a fairly flexible joint, was often lost after contraction. The tail core could be seen to partly extend from the contracted bacteriophage tails and it was often associated with membranous vesicular-like material. There appeared to be a base plate, pins and eight fibres associated with the tail. The sizes of bacteriophages A,J,F and E4 were similar, with the arithmetic means of their measurements (calculated using greater than ten measurements) varying by a maximum of 3%. The measurements of bacteriophage A were: Head, 930±39Å x 852±52Å; Tail, 1159±350Å x 205±7Å; Contracted Tail, 550±18Å x 280±16Å; Fibres, approximately 730Å long. Bacteriophages G, E7 and F27 were of similar morphology but had heads which were 10% smaller: 832±31Å x 759±25Å.

Bacteriophage D (Fig. 3-1B) had a morphology similar to bacteriophage T5 (Lunt and Kay, 1972) being of the B1 type according to the classification of Ackerman (1969). The head appeared to be larger than that of T5, being 849±50Å long and 927±60Å when measured diagonally from vertex to vertex; while the tail length (1984±60Å) was also greater than that reported for T5 (Lunt and Kay, 1972). The length of the tail fibres (606±19Å) and the width of the tail (100±6Å) were very similar to the measurements reported for T5. The size and morphology (Fig. 3-1A) of bacteriophage T3 (of C1 type morphology), were similar to that previously reported (Ackerman, 1969; Tikhonenko, 1970) and to that of bacteriophage E11.

The electron microscopic morphologies of bacteriophages T4, H1, H3, H4, H5, H6, H7, H8 and H9 (Fig. 3-1C), were all T-even in nature (A2 in the Ackerman (1969) classification). The bacteriophages, as well as having this similar morphology,

were of similar size, the measurements being: Head, 1164Å (with the arithmetic mean masurement for any one bacteriophage differing from the arithmetic mean measurement of all bacteriophages by a maximum of ±5.9%) x 864Å (±6.6%); Tail, 1109Å (±7.7%) x 236Å (±8.5%). They all had a contractile tail, a base plate and pins, a tail collar, tail fibres, and a tail core which are the typical features of T-even bacteriophages, (Simon and Anderson, 1967). The measurements noted above, although greater than others reported for T-even bacteriophages in the literature (Bradley, 1967; Ackerman, 1969; Tikhonenko, 1970), were proven by Miss P. Levy, who performed the electron microscopy, to be correct to within 5% by comparison with standard sized styrene beads.

SEROLOGICAL CLASSIFICATION

When the various anti-bacteriophage sera were tested in neutralization experiments with the homologous bacteriophages, the K values obtained were: anti-T6 (K=712), anti-H3 (K=658), anti-T3 (K=230), anti-F (K=410), anti-T4 (K=659), anti-T2 (K=1091) which is equivalent to 90% neutralization by a 10^{-3} to 10^{-4} dilution of the respective antisera. The full set of results obtained by using these antisera is presented in Table 3-1.

All of the bacteriophages from the first isolation, with one exception (bacteriophage D), were neutralized by anti-bacteriophage F sera. The degree of neutralization varied; however all of these bacteriophages were clearly related serologically to each other. It is interesting that bacteriophage G, which appeared to have a different sized

TABLE 3-1

Neutralization of T bacteriophages, and bacteriophages from the first and second isolations by various antisera.

Bacteriophage tested	K Value for Antisera raised using [a,b]					
	T6	H3	T3	F	T2	T4
T1	0	0	0	-	0	0
T2	5.0	-	-	-	1090	6.4
T3	0	0	230,157	0	0	0
T4	6.5	0	0	0	20	659
T6	712,565	-	0	-	12.2	9.5
T7	0	-	0.5,0.23	-	-	-
H1	86.1,91.5	0	0	0	8.9	-
H2	97.6,86.2	0	0	0	-	-
H3	0.8,0.8	661,658	0	0	0	0
H4	0.7,1.5	720	0	0	0	0
H5	1.0,1.4	931	-	0	0	0
H6	0.8,0.9	750,1072	-	0	0	0
H7	0.5,0.8	836	-	0	0	0
H8	932,462	0	0	0	20	8.2
H9	1.0,1.4	495	-	0	0	0
H10	1.2,0.8	490	-	0	0	0
H11	0.9,0.8	767,612	-	0	0	0
A	-	-	0	144,114	-	-
B	-	-	0	180	-	-
C	-	-	-	606	-	-
D	0	-	0	0	0	0
F	0	0	0	410,230	0	-
G	-	-	-	560	-	-
J	-	-	0	49.5,59.7	-	-

[a] - means not done.

[b] Where two figures appear, these are separate determinations.

head to bacteriophages A, F and J, was not significantly serologically different. The reason for this is that antigenicity appears to be associated with tail components (Adams, 1959); therefore the tails of these bacteriophages, which are morphologically similar, must also be antigenically similar.

Bacteriophages H1 - H11 which are morphologically similar to T-even bacteriophages, seemed to fit into three serological groups:

1) bacteriophages H3, H4, H5, H6, H7, H9, H10 and H11, which were neutralized slightly by anti-T6 sera, well by anti-H3 sera but not at all by anti-T2 or anti-T4 sera;
2) bacteriophages H1 and H2 which were more closely serologically related to T6 than T4 and T2 but are not at all related to H3;
3) bacteriophage H8 which was essentially serologically identical to T6.

The serological relatedness of the T-even bacteriophages, and of bacteriophages T3 and T7 (Adams and Wade, 1955; Adams, 1959) were largely borne out by the results shown in Table 3-1. Since bacteriophage T7 had a very low K value when tested with anti-T3 sera (Table 3-1) another isolate of bacteriophage T7 was obtained from Dr. M.H. Malamy, and shown to have a very similar K value. The 500-1000 fold difference in K values obtained after neutralization of bacteriophages T7 and T3 by anti-T3 sera has been previously reported (Adams, 1959). H3-like bacteriophages are as serologically related to bacteriophage T6 as T3 and T7 are

to each other, however they are clearly not related to T4 and T2 and thus they have been placed on a serological group of their own in Table 3-2.

SELECTION AND CHARACTERIZATION OF BACTERIOPHAGES USED IN RESISTANT MUTANT TESTING

Bacteriophages used in resistant mutant testing are summarized in Table 3-2. When resistant mutants were isolated against bacteriophages A, B, C, F, G and J, and tested by cross streaking it was shown that mutants resistant to one of these bacteriophages were resistant to all of them. Thus, since these bacteriophages are similar both morphologically and serologically, and have common resistant mutants (i.e. they are probably different isolates of the same bacteriophage), it was decided to use for future studies only bacteriophage A in addition to bacteriophage D (which was clearly different from the others) from the first isolation. Similar logic was applied to bacteriophages from the second isolation, and bacteriophages H1, H3 and H8 were selected for future use.

In the third isolation, twenty-five bacteriophages were purified. These were selected as different from the above bacteriophages, by the inclusion of antisera during their isolation.

When these bacteriophages were tested against previously isolated resistant mutants, it was found that they were of six main types. Examples of each of these types, which included bacteriophages E4, E7, E11, E15, E21 and E25 were selected for further study and of these only one was

TABLE 3-2

Bacteriophages, their taxonomic criteria and the phenotypic groups that they are commonly resistant to. [a]

Bacteriophages	Electron Microscopy [b]	Serology [c]	Phenotypic Resistance Groups [d]
T5,D,E21	B1	-	TonA
T1,E25,K22,K26,K27,K30	B1	-	TonA,TonB
BF23,E15,K6,K8,K11, K12,M3,Ac4	B1	-	Bfe
K10	B2	-	Ktn,Wrm
H3	A2	anti H3	Tsx
T6,H1,H8,K9,K18,K31,Ox1	A2	anti T-even	Tsx,Wrm
K3,K4,K5,Ox3,Ac3	A2	anti T-even	Con,Wrm
Ox2,Ox4,M1	A2	anti T-even	Con,Wrm,Bar
Ox5	A2	anti T-even	Con,Wrm,Bar, Ttk,Misc
E4	A1	-	Efr,Wrm,Bar, Misc
K2	A2	anti T-even	Ktw,Wrm,Bar
K20,K21	A2	anti T-even [e]	Ktw,Wrm,Bar
K29	A2	-	Ktw,Wrm,Bar
K16	C1	-	Ttk,Wrm,Bar, Misc
F27	A1	-	Ttk,Wrm,Bar, Misc
H+	A1	-	Ttk,Wrm,Bar, Misc
V	-	-	Ttk,Wrm,Bar, Misc
E7	A1	-	Ttk,Wrm,Bar, Misc
K17	C1	-	Wrm,Bar,Misc
A,F,G,J	A1	anti F	Bar,Wrm
T3	C1	anti T3	Bar,Wrm
T4	A2	anti T-even	Ttk,Wrm,Bar, Misc
K19	A1	-	Ttk,Wrm,Bar
T2	A2	anti T-even	Ttk,Wrm
T7,Ø1,H,W31	C1	anti T3	Wrm
E11	C1	anti T3	Wrm
K15	B1	-	Wrm

TABLE 3-2 Continued

K25	B1	-	-
Ø11-T	C1	anti T3	-

[a] Details of minor variations in resistance pattern are included in Tables 4-1, 4-2, 4-3, 4-4, 4-5 and 4-6.

[b] Electron microscopic appearance is as described by Ackerman (1969):-

A1 (P1-like) = long contractile tail, isometric head.

A2 (T-even-like) = long contractile tail, elongated head.

B1 (T5-like) = Long non-contractile tail, isometric head.

B2 = long non-contractile tail, elongated head.

C1 (T3-like) = short non-contractile tail, isometric head.

- means not done.

[c] Rabbit anti-bacteriophage sera tested against each bacteriophage were anti T2, anti T4 or anti T6 (anti T-even), anti T3, Anti H3, and anti F. Antisera with neutralizing activity are included in the table.

- means not neutralized by above antisera.

[d] See Chapter 4 for full details and Chapter 9 for discussion. Mnemonics are the same as the genotypic mnemonics where one member of the group has previously been mapped:-

Con is Conjugational recipient deficiency (Skurray, Hancock and Reeves, 1974); Ktw is K two resistance; Wrm is Wide range mutants; Ktn is K ten resistance; Ttk is T2, T4 or K19 resistance; Bar is bacteriophage A resistance; Misc is miscellaneous group; Efr is E four resistance;

[e] Only shows partial neutralization ($0.8<K<1.2$) with anti T2 serum and anti T6 serum (anti T4 serum not tested).

neutralized by any of the available sera (i.e. bacteriophage E11).

Other bacteriophages used in resistance studies were imported from various laboratories (see Table 2-2). Many of these had been morphologically characterized previously (see Table 2-2 for references and Table 3-2 for summary). They were tested for their neutralization by the various antisera available and it was found that many of the bacteriophages with T-even morphologies were neutralized by anti-T2, anti-T4 or anti-T6 sera. The only exception appeared to be bacteriophage K29, while bacteriophages K20 and K21 from the same resistance group (see Table 4-3) were only poorly neutralized by anti-T2 and anti-T6 sera (anti-T4 sera was not tested). The serological relatedness of T3 and the other so-called female-specific bacteriophages W31, H, ØII-T and Ø1, is in agreement with their other closely related properties (Morrison and Malamy, 1971; Brunovskis, Hyman and Summers, 1973); but it is interesting that bacteriophage E11, which is not female-specific, is more closely related to T3 than is bacteriophage T7, which is female-specific.

SUMMARY AND CONCLUSIONS

Only elementary conclusions that have been made in the text can be made at this point. This work in part bears out the conclusion (Adams, 1953; Ackerman, 1959) that classification by serological and by morphological techniques give similar results. Discussion of this work in relation to resistance studies is included in Chapter 9.

CHAPTER 4

ISOLATION AND TESTING OF RESISTANT MUTANTS

INTRODUCTION

As discussed in Chapterl, studies of bacteriophage resistance in *E. coli* K-12 or *E. coli* B have been restricted to a few limited studies on mutants resistant to the T bacteriophages or bacteriophage λ (Demerec and Fano, 1945; Garen and Puck, 1951; Curtiss, 1965; Jackson, Buller and Shankel, 1967; Radke and Siegel, 1971; Ronen and Raanan-Ashkenazi, 1971; Tamaki, Sato and Matsuhashi, 1971). For this study the range of virulent bacteriophages lysing *E. coli* K-12 was increased by the utilization of typing strains from a closely related genus, *Shigella* (Ewing, Hucks and Taylor, 1952; Luria and Burrows, 1957) and other bacteriophages isolated from sewage (see Chapter 3).

ISOLATION OF MUTANTS

When bacteriophage resistant mutants are selected in *Escherichia coli* K-12 many of the mutants are mucoid, their resistance apparently being due to the physical barrier presented to bacteriophage attachment by a layer of capsular polysaccharide. This layer is formed in the single step mutants lon$^-$ or capS$^-$ (Lieberman and Markovitz, 1970). These mutants were avoided by using a non$^-$ strain P400, as the parent strain for selection of bacteriophage resistant mutants. The non$^-$ mutation is blocked in capsular synthesis, and the lon$^-$ non$^-$ and capS$^-$ non$^-$ double mutants have the

phenotype of the non$^-$ single mutant (Radke and Siegel, 1971). Thus, mucoid mutants are not picked up in this strain. It has been shown that the non$^-$ mutation does not alter the composition of the lipopolysaccharide (Table 6-2).

Over 500 resistant mutants were isolated using 42 different virulent bacteriophages to select the mutants. Between 20 and 200 apparent mutant colonies were selected with each bacteriophage used, although many, when purified, were found to be fully sensitive.

PHENOTYPIC RESISTANCE GROUPS

Each mutant was tested against the full set of bacteriophages in use at the time, so that about 400 were tested against all bacteriophages. Those selected for further study were carefully re-tested against all bacteriophages. The following phenotypic patterns arose.

TonA, TonB, Bfe, Con, Efr and Ktn groups

The mutants in these phenotypic groups were fully resistant to all bacteriophages in the group, as summarized in Table 4-1.

Tsx group

Two sub-groups of mutants were found in this resistance group, which showed common resistance to a set of 8 bacteriophages (see Table 4-2). The most common mutant type (99%) is probably (Table 4-7) the classical _tsx_ mutant. The only mutant of sub-group 2 was fully resistant to bacteriophages H1, H3, H8 and K18, and showed partial or slight resistance to the others.

TABLE 4-1

Fully resistant mutants of the TonA, TonB, Bfe, Con, Ktn and Efr groups of resistant mutants.

Phenotype	Isolated Against	No. of Mutants	Representative Mutant	Resistant to
TonA	T1,D,E21, E25,K22, K26	32	P417	T1,E25,K22, K26,K27, K30,T5,E21, D
TonB	T1,E25,K26	10	P442	T1,E25,K22, K26,K27, K30.
Bfe	E15,K8,K12	21	P445	BF23,E15, K6,K8,K11, K12,M3,Ac4
Con	K3,K5	8	P460	K3,K4,K5, Ox2,Ox3, Ox4,Ox5, Ac3,M1
Ktn	K10	10	P466	K10
Efr	E4	10	P448	E4

TABLE 4-2

Bacteriophage resistance of the Tsx Group of mutants.

Sub-group	Representative Mutant	Isolated against	No. Isolated	Resistances [a]							
				H3	T6	H1	H8	K9	K18	K31	Ox1
1	P407	T6,H1,H3, H8,K18	97	R	R	R	R	R	R	R	R
2	P433	H1	1	R	SL	R	R	P	R	P	SL

[a] All abbreviations as in the Nomenclature section of Chapter 2.

Ktw group

Mutants in this group are resistant to a set of four bacteriophages. Sub-groups 2 and 3 (one mutant each) are distinguished from the other mutants by cross resistance to bacteriophages E4 and H^+ respectively (see Table 4-3). The mutants in sub group 1 are resistant (to a varying extent) only to the four bacteriophages.

Ttk group

This group is less well-defined than the previous groups. Common resistance to bacteriophages Ox5, K16, F27, H^+ and V (see Table 4-4) has been used to justify the grouping of these mutants. They are differentiated into sub-groups according to their pattern of resistance to bacteriophages T2, T4 and K19. Resistance to both T4 and K19 is found (sub-group 3) as well as resistance to one but not the other (sub-groups 2 and 4). The bacteriophage T2 resistant mutant that comprises sub-group 1 of Ttk differs from the others as it is uniquely cross resistant to bacteriophage E4. Some of the mutants are cross resistant to bacteriophage E7 and one is also slightly resistant to bacteriophage K17.

Miscellaneous group

This group is even less well-defined than the Ttk group and includes five mutant types, which do not fit well into other groups. They are resistant to bacteriophages which are common to the Ttk group. Two are resistant to some extent to bacteriophage T4 (sub-groups 1 and 2). Sub-group 3 (containing 3 identical mutants) and sub-group 4 (only 1 mutant) appear to be similar (see Table 4-4).

TABLE 4-3

Bacteriophage resistance of the Ktw Group of mutants.

Sub-Group	Mutants	Isolated against	Resistances [a]					
			K2	K20	K21	K29	E4	H^+
1	P477	K20	IP	IP	IP	IP	-	-
	P458	K2	IP	R	R	SL	-	-
	P456,P457	K2	IP	R	R	R	-	-
2	P476	K2	IP	R	R	R	SL	-
3	P240	K29	SL	IP	IP	IP	-	I

[a] All abbreviations as in Nomenclature section of Chapter 2.

TABLE 4-4

Resistant mutants of the Ttk group and miscellaneous (Misc) mutants resistant to between two and five of the bacteriophages to which Ttk group mutants are resistant.

Group	Sub-Group	Mutants	Iso-lated against	Resistances [a]										
				Ox5	K16	F27	H^+	V	E7	K17	T4	K19	T2	E4
Ttk	1	P429	T2	P	R	R	R	R	-	-	-	R	R	R
	2	P423	T4	SL	R	R	R	SL	R	SL	R	-	-	-
	3	P425	T4	R	R	R	R	R	IP	-	R	R	-	-
		P432	T4	SL	R	R	R	R	IP	-	R	R	-	-
	4	P474	K19	P	R	R	R	R	-	-	-	R	-	-
Misc	1	P491	T4	-	SL	-	R	-	P	SL	P	-	-	-
	2	P443	E7	-	-	-	-	-	P	-	SL	-	-	-
	3	P498, P499, P238	F27, K16	-	SL	R	SL	-	-	-	-	-	-	-
	4	P237	F27	SL	SL	R	SL	-	-	-	-	-	-	-
	5	P493	K17	SL	SL	-	-	-	-	SL	-	-	-	I

[a] All abbreviations as in the Nomenclature section of Chapter 2.

Bar group

Nineteen mutants were sorted into eight sub-groups as shown in Table 4-5. The mutants were resistant to between ten and nineteen bacteriophages. Sub-groups 1 to 3 were sensitive to T3 while sub-groups 4 to 8 were resistant. Sub-group 1 was distinguished by its sensitivity to bacteriophage K16, while sub-group 2 was uniquely sensitive to bacteriophages A and E7. Sub-group 3 contained all the remaining T3 sensitive strains and showed, at least, resistance to a set of twelve bacteriophages. Of the T3 resistant strains, sub-group 4 was uniquely sensitive to bacteriophage K2. Sub-group 5 was found to be sensitive to H^+ and only slightly resistant to T3. Sub-group 6 was sensitive to bacteriophage K17 but resistant to T4 and K2. Sub-group 7 was resistant to all bacteriophages except K19 while sub-group 8 was resistant to this bacteriophage too. While this group is extremely heterogeneous, it nevertheless seems to fit well as a group. It is interesting that nearly all of the bacteriophages to which the Misc, Ktw, and Ttk groups are resistant, are included in the set of bacteriophages to which Bar mutants are resistant.

Wrm group

Only one of these mutants, P479, was selected to a bacteriophage specific to the group, and this after NTG mutagenesis. These mutants appear to be single mutants as they do not have the combined properties of the resistance groups they embrace (see Chapters 5 and 7). They fall clearly into two groups as can be seen from Table 4-6. Wrm mutants are resistant to bacteriophages from eight out of

TABLE 4-5

Mutants of the Bar resistance group.

Sub-group	Mutants[a]	Resistances[b]																		
		M1	Ox2	Ox4	E4	K2	K20	K21	K29	Ox5	K16	F27	H^+	V	E7	K17	A	T3	T4	K19
1	P455	-	P	P	SL	SL	IP	IP	I	P	-	-	-	-	SL	-	SL	-	-	-
2	P492	SL	P	R	R	I	R	R	I	R	IP	R	-	R	-	P	-	-	-	-
3	P409	-	R	R	R	I	IP	IP	SL	R	R	-	-	-	P	SL	P	-	-	-
	P413	P	R	R	R	IP	P	P	IP	R	R	-	-	-	P	SL	R	-	-	-
	P404	P	IP	IP	R	IP	IP	IP	R	IP	IP	R	-	-	IP	P	IP	-	-	-
	P495	SL	R	R	R	R	IP	R	R	R	P	R	-	-	R	P	R	-	-	-
	P497	SL	P	R	R	I	R	R	R	R	R	R	-	-	R	P	R	-	-	-
	P494	P	P	R	R	I	SL	SL	IP	R	P	R	R	-	R	SL	R	-	-	-
	P496	P	P	R	R	I	IP	IP	IP	R	IP	R	R	-	R	SL	R	-	-	-
	P415	P	P	R	R	I	IP	IP	R	R	P	R	I	R	R	P	R	-	-	-
4	P490	-	P	R	R	-	IP	IP	IP	R	SL	R	R	-	R	-	R	R	-	-
	P428	SL	P	R	IP	-	I	-	I	R	P	R	R	-	R	-	R	R	-	-
	P405	SL	R	R	R	-	I	I	IP	R	P	R	R	-	R	-	R	R	-	-
	P436	SL	R	R	P	-	SL	SL	I	R	SL	R	R	-	R	-	R	R	-	-
5	P402	SL	I	R	R	R	IP	IP	I	R	I	R	-	R	R	R	IP	SL	-	-
6	P451	SL	R	R	R	R	R	R	R	R	R	R	R	-	R	-	R	R	SL	-
7	P487	SL	P	R	R	I	IP	IP	SL	R	P	R	R	I	R	SL	R	R	SL	-
	P488	SL	R	R	R	I	R	R	I	R	P	R	R	R	R	SL	R	R	SL	-
8	P489	SL	R	R	R	IP	R	R	R	R	R	R	R	R	P	P	R	R	P	R
		Con[c]			Efr[c]	Ktw[c]				Ttk[c]							Bar[c]		Ttk[c]	

TABLE 4-5 Continued

[a] The mutants were selected using the bacteriophages in brackets:-

P402 (against F), P404(F), P405(F), P409(J), P413(B), P415(G), P428(J), P436(H1), P451(E7), P455(K2), P487(T3), P488(T3), P489(T3), P490(T3), P494(K17), P495(K17), P497(F27), P496(F27).

[b] All abbreviations as in the Nomenclature section of Chapter 2.

[c] Mutant class which is characteristically resistant to the bacteriophages.

TABLE 4-6

Mutants of the Wrm resistance group.

Sub-Group	Mutants[a]	Resistances[b]																					
		K10	H3	H1	H8	K18	K31	Ox1	K9	T6	K3	K4	K5	Ac3	Ox3	M1	Ox2	Ox4	E4	K2	K20	K21	K29
1	P435	–	–	R	SL	R	SL	–	P	–	–	–	–	R	R	R	R	R	R	R	R	R	R
	P479	–	–	R	SL	R	P	SL	–	–	–	–	–	R	R	R	R	R	R	R	R	R	R
2	P416	–	–	R	R	SL	R	SL	R	SL	R	R	I	SL	SL	SL	R	R	R	SL	P	R	–
	P424	–	–	R	R	SL	R	SL	R	SL	P	R	I	SL	SL	SL	R	R	R	SL	R	R	–
	P239	I	–	R	P	SL	R	SL	R	SL	R	I	I	SL	SL	SL	R	R	R	R	P	P	R
		Ktn[c]	Tsx[c]								Con[c]								Efr[c]	Ktw[c]			

Sub-Group	Mutants[a]	Resistances[b]																	
		Ox5	K16	F27	H^+	V	E7	K17	A	T3	T4	K19	T2	T7	E11	Ø1	W31	K15	H
1	P435	R	R	R	R	R	R	P	R	R	SL	R	–	P	–	SL	–	R	SL
	P479	R	R	R	R	R	R	SL	R	R	SL	R	–	SL	SL	R	SL	R	SL
2	P416	R	R	R	R	–	R	SL	R	R	R	–	SL	–	–	–	–	R	P
	P424	R	R	R	R	–	R	SL	R	R	R	–	SL	–	–	–	–	P	–
	P239	R	P	R	R	–	SL	SL	R	R	P	–	SL	–	–	–	–	R	P
		Ttk[c]							Bar[c]		Ttk[c]			Wrm[c]					

[a] The bacteriophages that the mutants were selected against were P416 (against C), P424(T4), P435(H1), P479(T7), P239(K29).

[b] All abbreviations as per Nomenclature section of Chapter 2.

[c] Mutant group which is characteristically resistant to the bacteriophages.

the eleven other sets defined in this study and show resistance to between thirty and thirty-three bacteriophages. These mutants may be similar to other mutants described in the literature (Curtiss, 1965; Radke and Siegel, 1971).

CROSS RESISTANCE TO COLICINS

Surveys on the resistance of *E. coli* K-12 to a wide range of bacteriophages, as presented in this Chapter, and colicins (Davies and Reeves, 1974a,b) have provided the basis for a more extensive study of the phenomenon of cross resistance between bacteriophages and colicins. The testing of the colicin resistance of the bacteriophage resistant mutants in this section of Chapter 4, was done by John Davies and is the subject of a joint publication (Hancock, Davies and Reeves, 1974).

Results shown in Table 4-7 confirm and extend the results of other workers, who found that bacteria mutated in the *ton*B (Fredericq, 1951; Gratia, 1964), *ton*A (Fredericq, 1951), *tsx* (Weltzein and Jesaitis, 1971) and *bfe* (Buxton, 1971) loci were cross resistant to certain colicins and bacteriophages, suggesting a common cellular component for the action of both on the *E. coli* K-12 cell. A similar conclusion can be made from the resistance of the Con, Ktw(1) and Ktw(2) bacteriophage resistant mutants to specific colicins (Table 4-7).

Four of the classes of colicin resistant mutants exhibited the pattern of resistance of Ktw(1) mutants. One of these classes TolIb appeared to be identical to Ktw(1) mutants in that all of the Ktw(1) mutant strains were resistant to colicins K, L, A, S4 and N; however there were

TABLE 4-7

Cross resistance between bacteriophages and colicins in *E. coli* K-12 - mutants shown to be similar.

Phenotypic Colicin Resistance Class (a)	Colicin Resistance Pattern (a)	Phenotypic Bacteriophage Resistance Class (b)
TonB	B,D,G,H,Ia,Ib,M,S1,V	TonB
TonA	M	TonA
Bfe	E1,E2,E3,A	Bfe
Tsx	K	Tsx(1)
Con	K,L	Con
TolIb	K,L,A,S4,N	Ktw(1)
TolIa	K,L,A,S4	Ktw(2)
TolX	pK,L,A,S4,N)	Ktw(1)[c]
TolXIV	E2,E3,K,L,A,N)	
TolXV	E2,E3,K,L,A,S4,N)	

(a) Full details will be published in 1974 by Davies and Reeves - manuscript submitted for publication to J. Bacteriol.

(b) Details of bacteriophage resistance are included in Tables 4-1, 4-2 and 4-3.

(c) The brackets indicate that these three mutants showed a Ktw(1) bacteriophage resistance pattern. However, no Ktw(1) mutant showed a colicin resistance pattern other than that described for TolIb.

no bacteriophage resistant mutants with the colicin resistance pattern of the other 3 classes, suggesting that these mutants are altered in a way that cannot be easily selected using bacteriophages. These mutants might have similar defects since they are commonly resistant to colicins K, L, A and N and sensitive to colicin E1 (Davies and Reeves, 1974a), being the only mutants isolated with these colicin resistance properties.

Many of the colicin resistant mutants are sensitive to all of the bacteriophages (Table 2-2) tested, these being: strain P224 (Rcx phenotypic class), P651 (TolIIb), P555 (TolIIc), P660 (TolIII), P692 (TolIV), P596 (TolIX), P653 (TolXII), P520 (TolXIII), P516 (TolXVI), P625 (Cir), P575 (ExbB), P535 (Exbc), P295 (Cbt), P1209 (Cmt), P645 (Ivt) and P1235 (Cvt). The colicin patterns of these mutants are extremely varied (Davies and Reeves, 1974a,b). In addition, mutants from many of the bacteriophage resistance groups described in this Chapter were sensitive to all colicins, these groups being the Tsx(2), Ktn, Efr, Ktw(3), Ttk(1), (2), (3) and (4), and Misc(1), (2), (3) and (4) groups. Strains P494 (Bar(3)) and P424 (Wrm(2)) were also sensitive to all colicins. These findings suggest that differing requirements for the action of bacteriophages and colicins on bacterial cells, as judged by the lack of cross resistance of many mutants, are quite common.

Other colicin- and bacteriophage-resistant mutants were resistant to a set of bacteriophages or colicins which do not fit well into any of the previously defined classes (see Table 4-7). Mutants from the Bar, Wrm and Misc(5) groups were resistant to one of six combinations of colicins

A, L, S4 and N, with no clear pattern arising from these studies (Hancock, Davies and Reeves, 1974). The combinations to which these mutants were resistant were: (1) colicins A, L, S4 and N; (2) A, L and S4; (3) A and L; (4) L and S4; (5) A or (6) L. However, as pointed out by Davies and Reeves (1974a), mutants with relatively minor changes in the structure or composition of the cell envelope, as judged by their changed sensitivity to a wide range of antibiotics, detergents and surfactants, are often resistant to these four colicins.

There are also two types of colicin resistant mutants which are resistant to many bacteriophages, the first of which includes strains P689 (TolVII), P602 (TolVIII) and P220 (TolXI) which are resistant to between 7 and 13 of the bacteriophages which fail to lyse Bar mutants, and which partially inhibit all other bacteriophages. Although these mutants could be loosely classified as Bar mutants, they were not similar to other Bar mutants (Table 4-5), and the finding that they exhibit slight mucoidicity (J.K. Davies, personal communication) raises the possibility that their bacteriophage resistance properties are at least partly caused by this. Strains P602 and P689 appear to be LPS-altered (Davies and Reeves, 1974a), in common with the Bar mutants (Chapter 6). The other type of colicin resistant mutant with an unusual bacteriophage resistance pattern has only one representative, strain P652 (TolXVII) resistant to colicins E1, E2, K, L, A, S4 and N (Davies and Reeves, 1974a). This mutant shows partial sensitivity to bacteriophages T6, Ox1, Ox2, Ox4, Ox5, Ac3, Ac4, H1, M1, K2, K20, K21, K29, K3, K4, and K5, all of which have T-even morphology (Table 3-2), while it shows

resistance to all other bacteriophages. Although this mutant appears to have a large defect in the cell envelope, the nature of this defect is as yet unknown (J.K. Davies, personal communication).

SUMMARY AND CONCLUSIONS

500 bacteriophage resistant mutants were isolated to 42 virulent bacteriophages in one strain of *Escherichia coli* K-12 and tested for resistance or sensitivity to a set of 56 bacteriophages. It was possible to fit all of these mutants into 11 main groups, and one group of miscellaneous (Misc) mutants which did not fit well into any of the other groups. Each group of resistant mutants was characterized by a particular set of bacteriophages to which most of the mutants showed resistance of one type or another. Some of these groups were shown to be cross-resistant to defined sets of colicins, while others were either sensitive to all colicins or showed resistance to a set of bacteriophages that did not fit well into any of the defined phenotypic resistance groups.

CHAPTER 5

ADSORBTION OF BACTERIOPHAGES TO CELL ENVELOPE SUBFRACTIONS

INTRODUCTION

Beumer *et al* (1965) studied the adsorbtion of various bacteriophages to cell wall and lipopolysaccharide (LPS) preparations. This enabled them to partially characterize the nature of the receptor for these bacteriophages.

Sabet and Schnaitman (1971) examined the adsorbtion of colicin E3 to various subfractions of the cell envelope of *E. coli* K-12 derived by sequential solubilization with Triton X-100 and Triton X-100 + Edta (Schnaitman, 1971a,b). They were able to localize the receptor activity in the Triton-Edta soluble fraction, at a level equivalent to the receptor activity of the cell envelope, despite the fact that this fraction contained only 50% of the LPS and 25% of the protein of the cell envelope (Schnaitman, 1971b). Chemical and enzymatic studies partially characterized the nature of this fraction (Sabet and Schnaitman, 1971) which was later used as the starting preparation for the isolation of the receptor protein (Sabet and Schnaitman, 1973a). Similar but less detailed studies have been done on the colicin I receptor of *E. coli* K-12 (Konisky and Liu, 1974).

An understanding of the nature of the receptor, for a given bacteriophage, facilitates the study of the basis of resistance to that bacteriophage, since one can then study one component or class of components of the outer membrane to see if it is altered in resistant strains.

ADSORBTION TO LIPOPOLYSACCHARIDE

LPS isolated by the phenol-water technique usually has some protein associated with it (Wober and Alaupovic, 1971; Wu and Heath, 1973b). Galanos, Lüderitz and Westphal (1969) described a technique for isolating LPS with less protein attached to it; however, when this phenol-chloroform-petroleum ether extracted LPS was used in neutralization experiments with bacteriophages T3 and K10, it neutralized neither bacteriophage. Therefore, phenol-water extracted LPS was used in these studies.

Twenty-three bacteriophages were tested for neutralization by LPS and cell wall preparations (Table 5-1). Of these, seventeen bacteriophages, to which the Ktn, Ktw, Ttk, Bar and Wrm groups of mutants are resistant, showed adsorbtion to LPS. Four other bacteriophages to which some mutants of these groups are also resistant, namely E4, Ox5, K15 and T2, were not adsorbed; however, of these only T2 showed appreciable neutralization by cell wall preparations. Bacteriophage Ox5 was neutralized by a cell envelope preparation and this is discussed in the following section.

Two other bacteriophages were included in this testing, since receptor activity could not be found in any other subfractions of the cell envelope (Table 5-2). These were bacteriophage H3 which appears atypical both serologically (Table 3-1) and with respect to its resistance patterns (Table 4-6) from the other bacteriophages failing to lyse *tsx* mutants; and K26, a T1-like bacteriophage (Table 4-1). Neither were shown to adsorb to LPS preparations.

TABLE 5-1

Adsorbtion of bacteriophages to lipopolysaccharide (LPS) and cell wall preparations.

Bacteriophage	Resistant Groups [a]	Adsorbtion to [b]	
		Cell Wall	LPS
K26	TonA, TonB	0	0
K10	Ktn, Wrm	0	1
H3	Tsx	0	0
E4	Efr, Bar, Wrm, Misc	0	0
K2	Ktw, Bar, Wrm	0	1
K20	Ktw, Bar, Wrm	1	1
Ox5	Con, Bar, Wrm, Ttk, Misc	0	0
K16	Ttk, Bar, Wrm, Misc	2	3
F27	Ttk, Bar, Wrm, Misc	3	3
H+	Ttk, Bar, Wrm, Misc	0	2 [c]
V	Ttk, Bar, Wrm, Misc	N.D. [d]	3 [c]
E7	Ttk, Bar, Wrm, Misc	0	1
A	Bar, Wrm	0	1
T3	Bar, Wrm	0	3
T4	Ttk, Bar, Wrm, Misc	3	2
K19	Ttk, Bar, Wrm	0	1
T2	Ttk, Wrm	3	0
T7	Wrm	3	3
Ø1	Wrm	2	2
W31	Wrm	1	3
E11	Wrm	1	3
K15	Wrm	0	0
ØII-T	-	1	3

[a] See Tables 4-1, 4-2, 4-3, 4-4, 4-5, 4-6.

[b]
0 means less than 50% neutralization by 1mg/ml LPS or cell wall.
1 means greater than 50% neutralization by 1mg/ml LPS or cell wall.
2 means greater than 50% neutralization by 100µg/ml LPS or cell wall.
3 means greater than 50% neutralization by 10µg/ml LPS or cell wall.

[c] See Beumer *et al.*, 1965.

[d] Not done.

ADSORBTION TO OTHER CELL ENVELOPE SUBFRACTIONS

The results included in Table 5-2 are discussed in the text in terms of sets of bacteriophages failing to lyse certain resistance groups. The preparations tested were those of Schnaitman (1971a,b) and a sodium hydroxide supernatant, prepared by the technique of Weidel, Koch and Bobosch (1954). The Triton X-100 soluble supernatant of the cell envelope which has been described by Schnaitman as a cytoplasmic membrane preparation was also tested and neutralization of bacteriophages (which is analogous with receptor activity) could often be explained by contamination with cell wall proteins. Thus it has not been included in Table 5-2.

Bacteriophages unable to lyse the Con group of resistant mutants

There is some degree of receptor heterogeneity amongst the set of bacteriophages which do not lyse Con$^-$ mutants (Table 4-1). The bacteriophages separate into two classes represented by K3 and Ox5 (Table 5-2). These bacteriophages have different activity spectra on mutants other than Con$^-$ (Table 4-6). The bacteriophage K3 receptor is found in the cell envelope and is not soluble in Triton X-100 (i.e. full activity is found in the cell wall preparation). When the cell wall is treated with Triton X-100 and Edta, all of this receptor activity can be traced to the supernatant (i.e. soluble fraction) after centrifugation and removal of the Triton X-100. The bacteriophage Ox5 receptor, however, is completely destroyed by the first Triton X-100 treatment. This may be because the Ox5 receptor lies in the Triton X-100

TABLE 5-2

Bacterio-phages	Major Resistance Groups(s) [a]	Neutralization by Cell Envelope Subfractions [b,c]					
		Cell Env.	Cell Wall	Triton-Edta Soluble	TES-Triton	Triton-Edta In-soluble	NaOH Super-natant [d]
K3	Con	0.42	0.27		0.13	>130	380
K4	Con	4.2	2.7				
K5	Con	4.2	2.7				
Ac3	Con	0.42	0.27		1.3	>130	
Ox3	Con	4.2	2.7	>130	130	>130	
M1	Con, Bar	4.2	2.7	13	13	130	380
Ox2	Con, Bar	42	>275		>130	>130	>380
Ox4	Con, Bar	21	275		>130	>130	
Ox5	Con, Ttk	21	>275		>130	>130	
BF23	Bfe	42	2.7	1.3		1.3	≤380
E15	Bfe	21	27	13		>130	0.38
K6	Bfe	4.2	27	13			≤38
K8	Bfe	420	27	13			≤38
K11	Bfe	42	27	13			≤38
K12	Bfe	42	27	13			≤380
M3	Bfe	42	27	13			≤38
Ac4	Bfe	42	27	13			≤38
T5	TonA	420	275	130			380
D	TonA	420	275	13			≤38
K28	TonA,TonB	>420	>275	>130		>130	>380
T6	Tsx, Wrm	4.2	2.7	>130	1.3	>130	380
H3	Tsx	>420	>275	>130	>130	>130	>380
T2	Ttk	412	217	>130	13	>130	>380
K2	Ktw, Bar	>420	>275	>130		>130	
E4	Efr, Bar	>420	>275	>130		>130	
T4	Ttk, Wrm	42	2.7	>130		>130	>380

[a] Resistance groups which are characteristically resistant to the various bacteriophages are given.

[b] Neutralization figures are the protein content (in μg/ml as estimated by the technique of Schacterle and Pollack (1973)) of the preparation or one of the serial ten-fold dilutions of it which gives 50% neutralization of bacteriophages in a neutralization assay. The neat solutions were obtained by Schnaitman or Weidel preparation of 1 mg of cell envelope (containing 420μg of protein) per ml. The amount of protein per ml in the various preparations was:-

Triton X-100 insoluble cell envelope: 275μg (CELL WALL)
Triton X-100 + Edta soluble cell wall: 130μg (TRITON-Edta SOLUBLE)
Triton X-100 + Edta soluble cell wall with Triton X-100 removed: 130μg (TES-TRITON)
Triton X-100 + Edta insoluble cell wall: 130μg (TRITON-Edta INSOLUBLE)

TABLE 5-2 Continued

Sodium hydroxide supernatant: 130μg (NaOH supernatant)

Mnemonics used in the table are given in brackets after the amount of protein in the preparation. Preparative techniques etc. are given in Chapter 2.

[c] Blanks in table mean not tested.

[d] Figures for sodium hydroxide supernatant often represent the highest dilution tried in a neutralization assay.

soluble fraction (which is not borne out experimentally), or it may be that it requires the co-operation of a Triton X-100 soluble factor. It has been shown (Schnaitman, 1971a,b) that Triton X-100 will dissolve 50% of the LPS, 80% of the phospholipid and 20% of the protein of the cell envelope. Thus one of these components might represent either the receptor or a requirement for active receptor.

The pattern of neutralization of bacteriophages K4, K5, Ac3 and M1 is similar to that for K3, while Ox3 differs only in that the receptor activity is reduced 50-fold during solubilization with Triton X-100 and Edta. Bacteriophage M1 is the only one of these bacteriophages which is neutralized by the Triton X-100 + Edta insoluble cell wall preparation, however the actual difference may be minor. The pattern of neutralization for Ox2 and Ox4 is similar to that for Ox5, although Ox4 is neutralized slightly by the cell wall preparation.

Comparison of activity spectra on resistant mutants (Tables 4-1, 4-4, 4-5 and 4-6) with neutralization patterns (Table 5-2), divides these bacteriophages into two sets (with one exception, bacteriophage M1). These are (1) bacteriophages K3, K4, K5, Ac3 and Ox3, and (2) Ox2, Ox4 and Ox5. Therefore, these two sets of bacteriophages probably have a common requirement (mutated in the Con^- mutants), and a different requirement (as demonstrated by different resistance and neutralization patterns). Bacteriophage M1 which has a similar neutralization pattern to K3 and a similar activity spectra on resistant mutants to Ox2 (Table 9-1) is, as mentioned above, the exceptional

case. However, it is able to plaque to a limited extent on all Bar mutants (which K3 can plaque on but Ox2 generally can't (Table 4-5)), and this might be evidence that it has receptor requirements intermediate to the above two classes of bacteriophages.

Bacteriophages unable to lyse the Bfe, TonA or Tsx resistance groups

The receptor for the BF23-like bacteriophages is found in the Triton-Edta soluble fraction (Table 5-2). This is in agreement with the results of Sabet and Schnaitman (1971) for colicin E3, which is cross resistant to these bacteriophages (Table 4-7). There is also considerable receptor activity in the sodium hydroxide supernatant. In fact, for bacteriophage E15, this activity is forty times greater than the receptor activity of the Triton-Edta soluble fraction. Both of these preparations probably contain solubilized LPS-lipid-protein complexes (Weidel, Koch and Bobosch, 1954; Schnaitman, 1971b) and therefore the receptor for the BF23-like bacteriophages may be associated with such complexes. Although the sodium hydroxide supernatant was originally isolated as the T5 receptor complex (Weidel, Koch and Bobosch, 1954) the amount of exposed E15 receptor in this fraction is considerably greater. These figures suggest that the sodium hydroxide supernatant would provide a considerably better starting point for the isolation of the bacteriophage E15 receptor than the Triton-Edta soluble supernatant, which was used (Sabet and Schnaitman, 1973a,b) to isolate the colicin E3 receptor.

The receptor of T5-like bacteriophages is also found in the Triton-Edta soluble and sodium hydroxide supernatant subfractions (Table 5-2). There are quantitative differences in the neutralization of bacteriophages T5 and D (both of which have tonA as their only resistant mutants) by these two preparations. Bacteriophage K28, a T1-like bacteriophage, is also unable to lyse tonA (or tonB) mutants, but is not neutralized by any of the preparations tested. This is not surprising as bacteriophage T1 seems to require a component provided only by living cells, but not by dead cells or cell wall fragments (Stent, 1963).

Two bacteriophages which fail to lyse tsx mutants, T6 and H3, differ considerably in their neutralization patterns. They have also been shown to differ serologically (Table 4-6). Bacteriophage H3, like K28, is not neutralized by any of the preparations listed in Table 5-2. The receptor activity for T6 is found in the Triton-Edta soluble fraction, but only after removal of Triton X-100 by alcohol precipitation and washing of the proteins (c.f. bacteriophage M1). This suggests that either bacteriophage T6 is inactivated by Triton X-100 or that the T6 receptor is an aggregation of Triton-Edta soluble components and Triton X-100 by disaggregating (i.e. solubilizing) this, destroys the ability of these components to act as a receptor.

Other Bacteriophages

Bacteriophages K2 and E4, the former of which was shown to be adsorbed by LPS (Table 5-1), were neutralized by none of the preparations tested (Table 5-2). Therefore, while the result for bacteriophage E4 merely mimics the result for K28 (and thus might also indicate a similar

requirement for an active cell function), the result for K2 tends to indicate that the LPS might be at least partly masked by other cell wall components. This is borne out by the 10- to 100-fold differences in the neutralization of many bacteriophages by cell wall and LPS (Table 5-1). As can be seen from the results for bacteriophage T4 (which is also neutralized by LPS), the LPS must be exposed to some extent in the cell wall and envelope preparations.

The receptor for bacteriophage T2, which has been shown to be a lipoprotein (Michael, 1968; DePamphilis, 1971), is found in the same preparations as the receptors of bacteriophage T6 and the K3-like bacteriophages. The presence of Triton X-100 in the Triton-Edta soluble supernatant prevented the neutralization of T2; which is a similar result to that obtained using bacteriophages T6 and Ox3 (Table 5-2).

ENZYMATIC TREATMENT OF THE RECEPTOR

The enzymatic treatment of the receptors (Table 5-3) shows that bacteriophages K3, Ac3, Ox3 and M1 (of the Con resistance group), T6 (Tsx resistance group), T2 (Ttk resistance group) and E15 (Bfe resistance group) have periodate sensitive receptors while the receptor of T5 is insensitive to periodate. These results have been previously demonstrated for T5 (Braun, Schaller and Wolff, 1973), T6 and T2 (Weltzein and Jesaitis, 1971). The colicin E3 receptor which is related to or identical with the bacteriophage E15 receptor has been also shown to be periodate sensitive (Sabet and Schnaitman, 1971). The periodate sensitivity of a receptor, suggests the involve-

TABLE 5-3

Bacteriophages	Major Resistance Groups[a]	50% Neutralization by[b]		
		Cell Wall	Periodate treated	Pronase treated
K3	Con	0.27	>105	≤1.4
Ac3	Con	2.7	>105	≤14
Ox3	Con	2.7	>105	≤14
M1	Con, Bar	2.7	>105	≤1.4
E15	Bfe	27	>105	>140
T5	TonA	105	105	>140
T6	Tsx	27	>105	14
T2	Ttk	2.7	>105	>140

[a] Represents only the major groups of mutants which are resistant to the bacteriophages.

[b] Figure given is the protein concentration leading to 50% neutralization. Some of pronase treated cell wall figures represent the highest dilution attempted in a neutralization experiment.

ment of LPS or some other carbohydrate-associated moeity in the binding of bacteriophages to this receptor (Sabet and Schnaitman, 1971, 1973a).

Pronase treatment of the outer membrane destroys three of the proteins of the cell wall (Bragg and Hou, 1972); as well as modifying one of the major peaks (Bragg and Hou, 1972) which might contain either two proteins 3a and 3b (Schnaitman, 1973b, 1974a) or a single protein (Reithmeier and Bragg, 1974). This treatment destroys the E15, T5 and T2 receptors, but has little effect on the receptors of T6, K3, Ac3, Ox3 and M1 (Table 5-3). The effect of pronase on the receptors of T2 and T6 has been demonstrated previously by Weltzein and Jesaitis (1971).

SUMMARY AND CONCLUSIONS

Many of the bacteriophages to which the Ktw, Ttk, Ktn, Bar, Wrm and miscellaneous groups are resistant, are neutralized by LPS preparations. There are only four exceptions, as described in the text. It is interesting that the T2 receptor which has been described as a lipoprotein probably requires or interacts with LPS, as many of the bacteriophages, to which the T2 resistant mutant shows cross resistance, are neutralized by LPS. Further evidence of this is the periodate sensitivity of the T2 receptor.

A number of bacteriophages were more efficiently neutralized by LPS than cell wall preparations. This is partly accountable for by the fact that the cell wall contains only 20% LPS. However, this does not explain the

100-fold difference in neutralization efficiency that occurs using bacteriophages ØII-T, W31, E11 and H^+. This effect might be due to the fact that bacteriophages have only about 250-300 attachment sites on the cell wall (Bayer, 1968a) and, one would cxpect, many more molecules of LPS per cell than this. Thus, other cell wall components must in some way limit the interaction of bacteriophage and receptor.

The receptor activity of many of the bacteriophages to which the Con and Bfe mutant groups are resistant, is solubilized from the cell wall by Triton X-100 in the presence of Edta, which also solubilizes most of the LPS of the cell wall. This, in addition to the periodate sensitivity of these receptors, suggests the involvement of LPS or some other Triton-Edta soluble, carbohydrate-associated moiety in the adsorbtion of bacteriophages to these receptors. A similar argument applies to the receptor of bacteriophage T6 (and T2 as above). The receptor for bacteriophage T5, however, seems to be insensitive to periodate as previously described (Braun, Schaller and Wolff, 1973), and its receptor probably doesn't involve carbohydrate.

The sodium hydroxide supernatant used as the basis for the preparation of bacteriophage T5 receptor (Weidel, Koch and Bobosch, 1954; Braun, Schaller and Wolff, 1973) seems to be a better receptor preparation for bacteriophage E15 than it does for T5.

CHAPTER 6

THE NATURE OF THE ALTERATIONS IN THE CELL WALL OF RESISTANT MUTANTS - LIPOPOLYSACCHARIDE ALTERATIONS

INTRODUCTION

It is well known in *Salmonella* that there is a correlation between the composition of the lipopolysaccharide (LPS) and the pattern of sensitivity to certain bacteriophages (Wilkinson, Gemski and Stocker, 1972). Tamaki and co-workers (1971) have demonstrated that some mutants selected for resistance to bacteriophage T4 in *E. coli* K-12, also have altered LPS. It has been shown (Chapter 5) that many of the bacteriophages to which the Bar, Ktw, Ttk and Wrm groups of mutants are resistant, were neutralized by LPS preparations. Therefore, in this Chapter, antibiotic sensitivity and lysis by bacteriophages C21 and U3 were used as indicators of LPS deficiency. The LPS alterations suggested by these results were further characterized by direct chemical analysis of the representative strains using gas liquid chromatography.

ANTIBIOTIC RESISTANCE/SENSITIVITY

Tamaki *et al*. (1971) have shown that LPS-deficient strains selected as novobiocin supersensitive can also be resistant to bacteriophages T4 and T7. Eriksson-Grennberg and co-workers (1971) also showed that many of their class II ampicillin resistant mutants were LPS-deficient. Thus it was decided to test these and other antibiotics to determine

TABLE 6-1

Response to Antibiotics, and resistance or sensitivity to bacteriophages C21 and U3.

Phenotype Resistant Group (a)	Sub-Group	Representative Mutants	Response to Antibiotics (b)							Bacteriophages Resistance or Sensitivity (c)	
			(d) NV5	NV30	PN	E	OL	FD	CB	C21	U3
TonA	-	P417	-	-	-	SL	-	-	-	-	-
TonB	-	P442	-	-	-	-	-	-	-	-	-
Bfe	-	P445	-	-	-	-	-	-	-	-	-
Tsx	1	P407	-	-	-	-	-	-	-	-	-
	2	P433	-	-	-	-	-	-	-	-	-
Con	-	P460	-	-	-	-	-	-	-	-	I
Efr	-	P448	-	-	-	-	-	-	-	-	-
Ktn	-	P466	-	-	-	-	-	-	-	-	-
Misc	1	P491	-	-	SL	-	-	-	-	-	R
	2	P443	-	-	-	-	-	-	-	-	-
	3	P498	-	-	-	R	-	-	-	I	P
	4	P237	-	-	-	-	-	-	-	S	R
	5	P493	-	-	-	-	-	-	-	S	R
Ktw	1	P456,P457	-	-	-	R	-	-	-	-	-
	2	P476	-	-	-	R	-	-	-	-	-
	3	P240	-	-	SL	-	-	-	-	S	I
Ttk	1	P429	-	-	-	-	-	-	-	S	R
	2	P423	-	-	-	-	-	-	-	P	I
	3	P425	-	-	-	-	-	-	-	-	R
	4	P474	-	-	SL	-	-	-	-	-	R
Bar	1	P455	-	S	-	-	-	-	-	P	R
	2	P492	S	S	S	SL	-	S	-	S	R
	3	P409,P413	-	S	S	-	-	-	-	S	R
		P404	S	S	-	-	-	-	-	S	R
		P495	-	S	-	S	-	-	-	S	R
		P497	S	S	-	S	-	-	-	S	R
		P494	S	S	-	S	S	S	-	S	R
		P496,P415	S	S	-	S	-	SL	-	S	R
	4	P405	S	S	-	S	S	-	-	S	R
	5	P402	-	S	-	-	-	SL	-	S	R
	6	P451	S	S	-	SL	-	-	SL	S	R
	7	P487	S	S	S	S	-	S	-	S	R
	8	P489	S	S	-	-	-	SL	-	-	R
Wrm	1	P435	S	S	-	S	S	S	-	-	R
		P479	S	S	R	S	-	S	S	-	R
	2	P416	SL	S	SL	SL	SL	SL	-	S	R
		P424	-	S	-	SL	-	SL	-	S	R
		P239	SL	S	-	SL	-	SL	-	S	R

TABLE 6-1 Continued

(a) Abbreviations as per Table 3-2 and Chapter 4.

(b) —— means response same as strain P400.

R means resistant, i.e., producing a smaller zone of inhibition than strain P400.

S means sensitive or supersensitive, i.e., larger zone of inhibition than strain P400.

SL means slight increase in sensitivity; with the exception of strain P417 which shows a slight decrease in sensitivity to erythromycin.

(c) —— means wild type response, i.e., sensitive to bacteriophage U3 or resistant to C21.

R means resistance.

S means sensitivity.

I means inhibited.

P means partial resistance.

All terms are defined in Nomenclature section of Chapter 2.

(d) Symbols, from multodisks, represent:

NV5 - 5μg of novobiocin; NV30 - 30μg of novobiocin; PN - ampicillin; E - erythromycin; OL - oleandomycin; FD - fusidic acid; CB - methicillin.

whether various bacteriophage-resistant mutants were altered in their response to them.

The results obtained using multodisks 30-9C and 11-14D showed that alterations occurred in the response to novobiocin, methicillin, fusidic acid, oleanodymycin, ampicillin and erythromycin (see Table 6-1), of mutants of the Ktw, Ttk, Bar, Wrm and miscellaneous groups.

Novobiocin supersensitivity (to 30μg but not 5μg; or to 5μg and 30μg) occurred amongst all of the mutants in the Bar and Wrm groups. In the Wrm group, two patterns occurred which seemed to correlate with the sub-grouping; however, the pattern for the Bar group was more complex.

Of the other antibiotics, erythromycin sensitivity occurred frequently in the Bar and Wrm groups, while resistance only occurred in two sub-groups of Ktw and one miscellaneous mutant. Fusidic acid sensitivity occurred only amongst sub-groups 2, 3, 5, 7 and 8 of the Bar group and in both sub-groups of Wrm. Ampicillin resistance occurred only with one Wrm mutant which was only partially resistant to 2μg/ml ampicillin, whilst ampicillin supersensitivity was more widespread. Sensitivity to oleandomycin (which is related to erythromycin) and methicillin (which is related to ampicillin) did not seem to follow any clear pattern.

ALTERATIONS IN THE SENSITIVITY TO BACTERIOPHAGE U3 OR TO BACTERIOPHAGE C21

It has been shown that the receptors for bacteriophages C21 (Rapin, Kalckar and Alberico, 1966; Rapin and Kalckar, 1971) and U3 (Watson and Paigen, 1971) are in the LPS rough

core and that mutants in which the composition of the LPS is altered may be changed in their response to these bacteriophages. A large number of bacteriophage resistant mutants were tested by spotting these bacteriophages onto an agar layer containing the mutant bacteria and various resistance patterns were revealed (Table 6-1). Of particular interest were the mutants which were resistant to both bacteriophages C21 and U3, since they differed considerably in their resistance to other bacteriophages (see Tables 6-1 and 9-1). Other mutants were sensitive to C21 and resistant to U3 and these included mutants of the Bar and Wrm groups (which are resistant to many bacteriophages). Thus it was decided to look at the composition of the LPS of selected mutants to see if the alterations suggested by both the antibiotic and bacteriophages C21 and U3 studies, were detectable.

ANALYSIS OF NEUTRAL SUGARS

When the LPS of strain P400 was hydrolysed with 1N H_2SO_4 for 12 hr in a boiling water bath, neutralized, reduced, acetylated and run on a gas liquid chromatograph, the amount of glucose was found to be greater than the amount of heptose (glu: gal: hept=0.38μM/mg LPS: 0.15: 0.30). This was a different result to that found by other workers (Rapin and Mayer, 1966; Eriksson-Grennberg, Nordstrom and Englund, 1971; Monner, Jonsson and Boman, 1971; Tamaki, Sato and Matsuhashi, 1971) for E. coli K-12. Schmidt, Fromme and Mayer (1971) showed that when 0.1N HCl hydrolysis was used the amount of heptose released from LPS increased with time

TABLE 6-2

Analysis of LPS of strain P400, and some of its phage resistant mutants, and of strain AB1133.

Strain	Resistance Group (a)	LPS (b)		
		Glucose	Galactose	Heptose
AB1133	-	0.39	0.24	0.56
P400	-	0.39	0.26	0.58
P460	Con	0.40	0.19	0.55
P456	Ktw(1)	0.32	0.15	0.32
P457	Ktw(1)	0.41	0.14	0.41
P429	Ttk(1)	0.21	0.18	0.38
P425	Ttk(3)	0.23	0.19	0.37
P474	Ttk(4)	0.19	0.18	0.35
P404	Bar(3)	0.23	0	0.35
P415	Bar(3)	0.21	0	0.40
P495	Bar(3)	0.14	0.03	0.31
P405	Bar(4)	0.04	0.03	0.39
P436	Bar(4)	0.02	0	0.33
P416	Wrm(2)	0.02	0.01	0.26
P424	Wrm(2)	0.09	0	0.27
P435	Wrm(1)	0	0	0

(a) Sub-group of each resistance group is included in brackets where appropriate. For full bacteriophage resistance details, see Chapter 4.

(b) Values given as μM sugar/mg LPS.

up to 48 hr at which time it was much greater than the amount released by H_2SO_4 hydrolysis and double the amount released by 0.1N HCl over 12 hr. Jackson and Redman (1971) used an even milder hydrolysis with Dowex 50 (H^+) in 0.01N HCl for 7 days at 105C in their studies on the LPS of Vibrio cholera. It was decided to use a time of 64 hr at 100C using 0.1N HCl as the hydrolysing agent. This caused the amount of heptose, relative to the sulphuric acid liberation, to double when compared to an internal standard although the amount of glucose liberated was fairly constant (Table 6-2).

When heptose from E. coli, which has been shown to be L-glycero-D-manno heptose, is reduced and acetylated, it forms a peak X which runs just after glucose on a gas liquid chromatograph (Schmidt, Fromme and Mayer, 1971). Under our preparation procedures perseitol and L-glycero-D-manno heptose form the same heptitol acetate. Equal amounts of perseitol and glucose were run together in a control experiment, and it was found that the area under the glucose peak was equal to the sum of the areas under the heptose and X peaks. Therefore, for each mutant studied, this sum was used to represent the total amount of heptose (Table 3). In addition to heptose, there was glucose and galactose but no other sugars in the LPS of strains AB1133 and P400 (see Fig. 6-1).

It was interesting to note that the non^- mutation made no difference to the sugar composition as can be seen from the similarity of strain AB1133 and its his^+ non^- derivative P400. The con^- strain P460 was also unchanged.

Strains P456 and P457 from the Ktw resistance group have reduced levels of heptose and galactose. Strains P429,

P425 and P474 from the Ttk group are deficient in glucose and heptose, while the 3 Bar (3) mutants are additionally deficient in galactose. The other strains tested, P436 and P405 (Bar (4)), P416 and P424 (Wrm (2)), and P435 (Wrm (1)) had little or no glucose or galactose in their LPS. The Wrm group mutants also showed lower levels of heptose than other mutants tested, with the Wrm (1) mutant P435 being totally deficient in this and all other neutral sugars. When the other Wrm (1) mutant P479 was tested, it reverted on two separate occasions and showed not only a wild type bacteriophage sensitivity pattern but a sugar composition similar to strain P400. Strain P466, a Ktn mutant, was also tested in an early experiment and found to be unchanged.

GLUCOSAMINE ANALYSIS

The last sugar to be attached to the rough core of the LPS of S. typhimurium is glucosamine (Mäkela and Stocker, 1969). The lipid A portions of the LPS molecules of Salmonella and E. coli K-12 also have glucosamine attached to them (Rooney and Goldfine, 1972). Thus degraded polysaccharides, obtained by removing Lipid A from the LPS of selected strains, were tested for the presence of glucosamine. There was less than 0.01μM of glucosamine per mg of LPS in the polysaccharide portion of the LPS of strains P400, P460 (Con) or P425 (Ttk (3)). The LPS of a smooth strain of S. typhimurium, kindly provided by R. Davies, was subjected to a similar analysis and it was found that there was 0.20μM of glucosamine/mg LPS which represented 1 mole of glucosamine per molecule of LPS (see Chapter 9). This was a similar result to that previously discovered for Salmonella (Lüderitz et al. 1971).

SUMMARY AND CONCLUSIONS

Some bacteriophage resistant mutants of the Ktw, Ttk, Bar, Wrm and miscellaneous groups were shown to be altered in their response to a series of antibiotics and to bacteriophages C21 and U3. The neutral sugar compositions of the LPS's of many of the mutants were investigated, and shown to fit into six classes. The parent strain P400, has glucose, galactose and heptose in its LPS but no other neutral sugars, and no glucosamine in the degraded polysaccharide.

CHAPTER 7

THE NATURE OF THE ALTERATION IN THE CELL WALL OF RESISTANT MUTANTS - PROTEIN ALTERATIONS

INTRODUCTION

Mutants resistant to various bacteriophages have been shown to have a protein band missing or altered when compared to the sensitive strains. These include *bfe* (Sabet and Schnaitman, 1973a,b), *ton*A (Braun, Schaller and Wolff, 1973; Braun and Wolff, 1973), *lam*B (Randall-Hazelbauer and Schwartz, 1973) and a *tfr*A, *tsx* double mutant (Schnaitman, 1974b). In most cases the bacteriophages, to which these mutants are resistant, probably have as their receptor the protein deleted in these mutants, one exception being the bacteriophages to which *tfr*A mutants are resistant (which are thought to have LPS receptors (Beumer *et al*., 1965)).

Other workers have shown that heptose-deficient strains of *E. coli* K-12 have altered protein patterns (Koplow and Goldfine, 1974). Therefore, it was decided to look at the protein composition of the LPS-altered mutants described in Chapter 6 as well as other suspected protein-altered mutants including Con$^-$ mutants.

CON$^-$ MUTANTS - GENERAL PROPERTIES

Various of the bacteriophages, which are unable to lyse Con$^-$ mutants (Table 4-1), were tested for their ability to adsorb to strain P400. Adsorbtion could be readily demonstrated for all bacteriophages tested, these being:

K3 (99.5% adsorbed), M1 (87.5%), Ox3 (81.4%) and Ox4 (77.9%). In contrast, adsorbtion of bacteriophages K3 (to strains P459, P460 and P461), M1 (to strain P459), Ox3 (to strain P459) and Ox4 (to strains P459, P460 and P461) was undetectable. The two methods used (see Chapter 2) tested both reversible and irreversible adsorbtion, and thus resistance of the Con$^-$ strains to these bacteriophages is apparently due to the loss or alteration of receptor such that adsorbtion cannot occur.

It has been shown that these mutant strains are unaltered in their response to a variety of antibiotics (Table 6-1) and to bacteriophage C21, while bacteriophage U3 causes inhibition of Con$^-$ mutants, but not lysis (Table 6-1). This altered response to bacteriophage U3 was not due to a changed LPS composition, as gas liquid chromatographic analysis revealed that strains P460 (Table 6-2) and P459 had identical sugar compositions to strain P400.

The Con$^-$ mutants were found to be uniquely (amongst bacteriophage resistant mutants) resistant to lethal zygosis, due to their 100- to 1000-fold decrease in ability to act as recipients during conjugation with Hfr and F' donors (R. Skurray, Ph.D thesis, University of Adelaide, 1974; Skurray, Hancock and Reeves, 1974). This conjugational recipient deficiency was not due to recombinational deficiency of the Con$^-$ strains, as P1kc transduced the <u>leu</u>$^+$ and <u>pro</u>$^+$ markers from strain P839 at the same frequency into strains P400 and P460 (Con$^-$). It was shown by Dr P. Reeves that the inability of Con$^-$ mutants to act as recipients in conjugation was due to an inability to form mating pairs (Skurray, Hancock and Reeves, 1974).

Con$^-$ mutants have also been shown to be tolerant to

colicins K and L (Table 4-7).

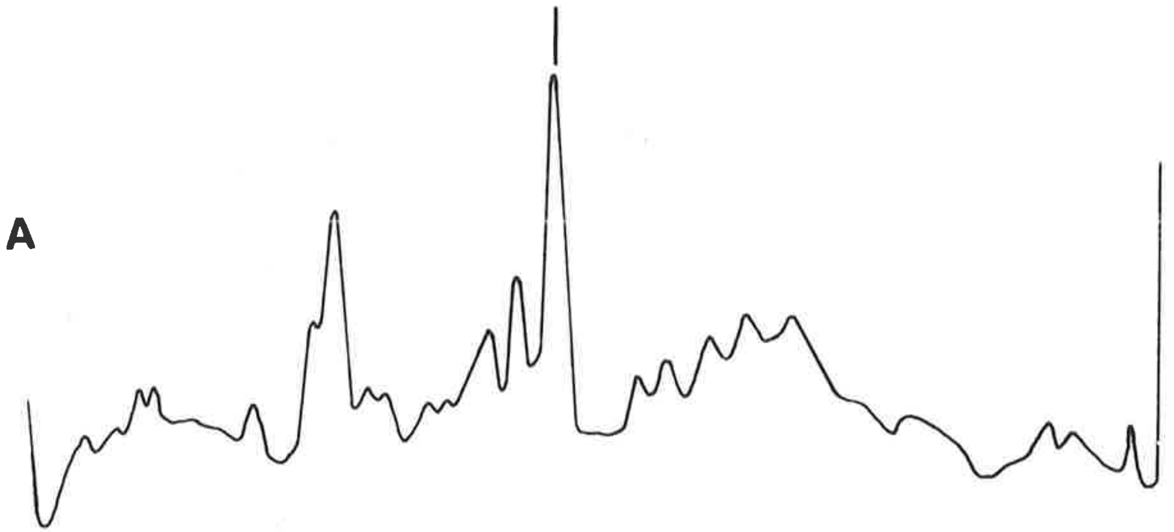
CON$^-$ MUTANTS: ALTERATIONS AS REVEALED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

The polyacrylamide gel electrophoresis system used first was the SDS system developed by Neville (1971) for mammalian membranes (Method 1) and this clearly showed that a major band of the cell envelope was dramatically reduced in amount in strain P460 in comparison to that of the parent strain P400 (Fig. 7-1A and 7-1B). The Triton X-100 treatment described by Schnaitman (1971a) did not solubilize the band (Fig. 7-1C) which was thus identified as an outer membrane or cell wall component. The molecular weight (MW) of the affected band, when compared to standard proteins (serum albumin, ovalbumin, carbonic anhydrase and lysozyme) was estimated at 28,000. The 28,000 MW band is apparently completely absent in the Triton X-100 treated preparation of the mutant (Fig. 7-1D), and it is possible that the small band present at this position in the envelope of the mutant is a different, Triton X-100 soluble protein which runs at the same place in the gel and is not a residual amount of the protein(s) affected by the mutations in strain The assistance of Ron Skurray in the Neville gel analysis of the mutants is acknowledged, as the work above was done in conjunction with him.

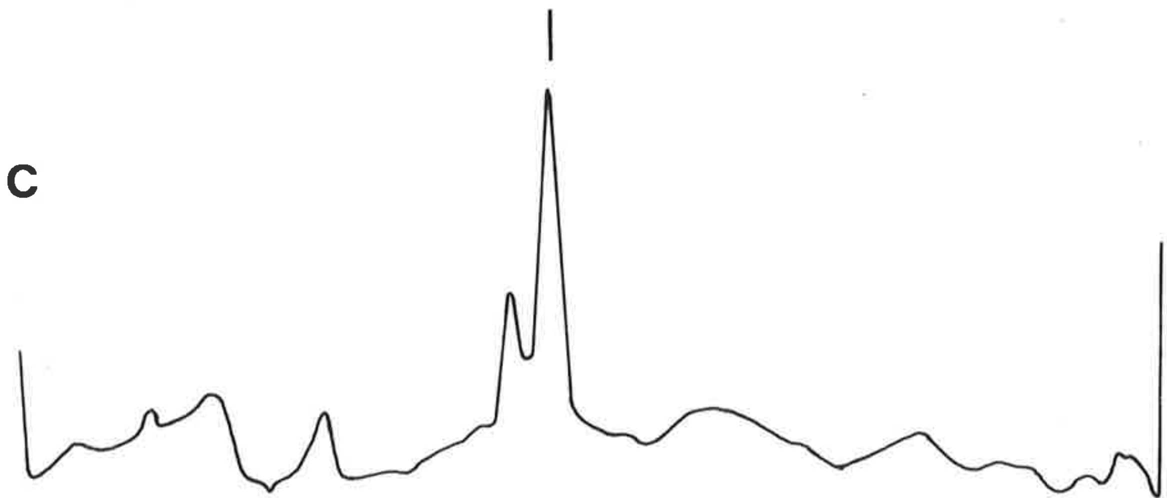
Although the Neville system gave a very good resolution of bands, the pattern was different from that found by Schnaitman (1971a, 1973a,b, 1974a,b), Bragg and Hou (1972) or Inouye and Yee (1973) using various different SDS gel systems. To enable direct comparison with other

FIG. 7-1

Comparison by densitometry, of the whole envelope proteins of P400 (Fig. 7-1A) and P460 (Fig. 7-1B), run using the Neville (1971) gel system. Figures 7-1C and 7-1D show profiles of outer membrane proteins of strains P400 and P460 respectively, in the same gel system. The 28,000 MW major peak is indicated by the vertical line.


A

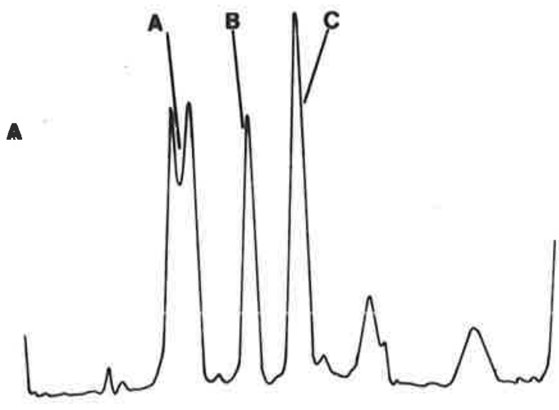
B

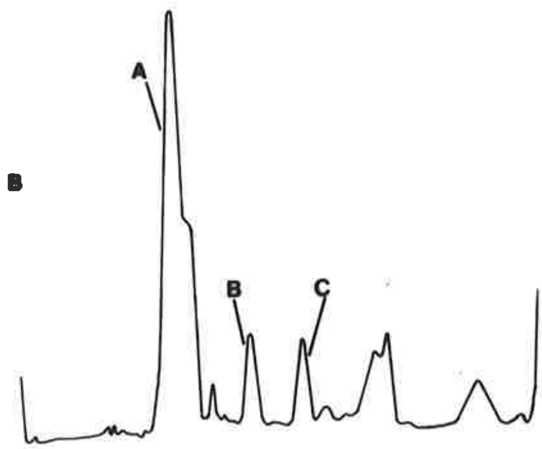

C

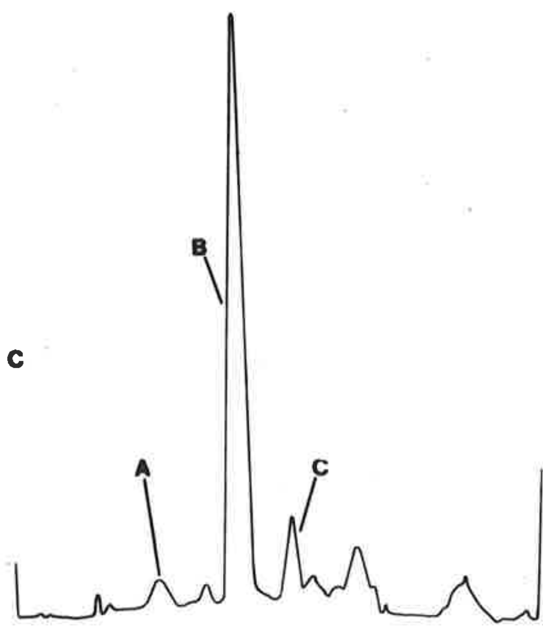
D

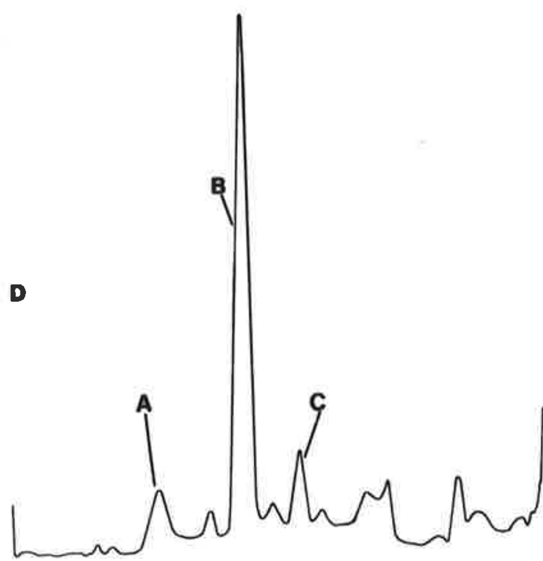
FIG. 7-2

Comparison by densitometry, of stained bands of outer membrane proteins, run using the pH 7.2 Maizel buffer system, and either heating (method 2) or not heating (method 3) the samples at 100C, before loading onto SDS polyacrylamide gels. Figures 7-2A and 7-2B show the outer membrane proteins of strains P400 (Con^+) and P460 (Con^-) respectively, solubilized by method 3. Figures 7-2C and 7-2D show the outer membrane proteins of strains P400 (Con^+) and P460 (Con^-) respectively, solubilized by method 2. The peaks are labelled A, B and C according to Schnaitman (1974a) nomenclature as described in the text.


A
B
C
A


A
B
B
C


B
C
A
C


B
D
A
C

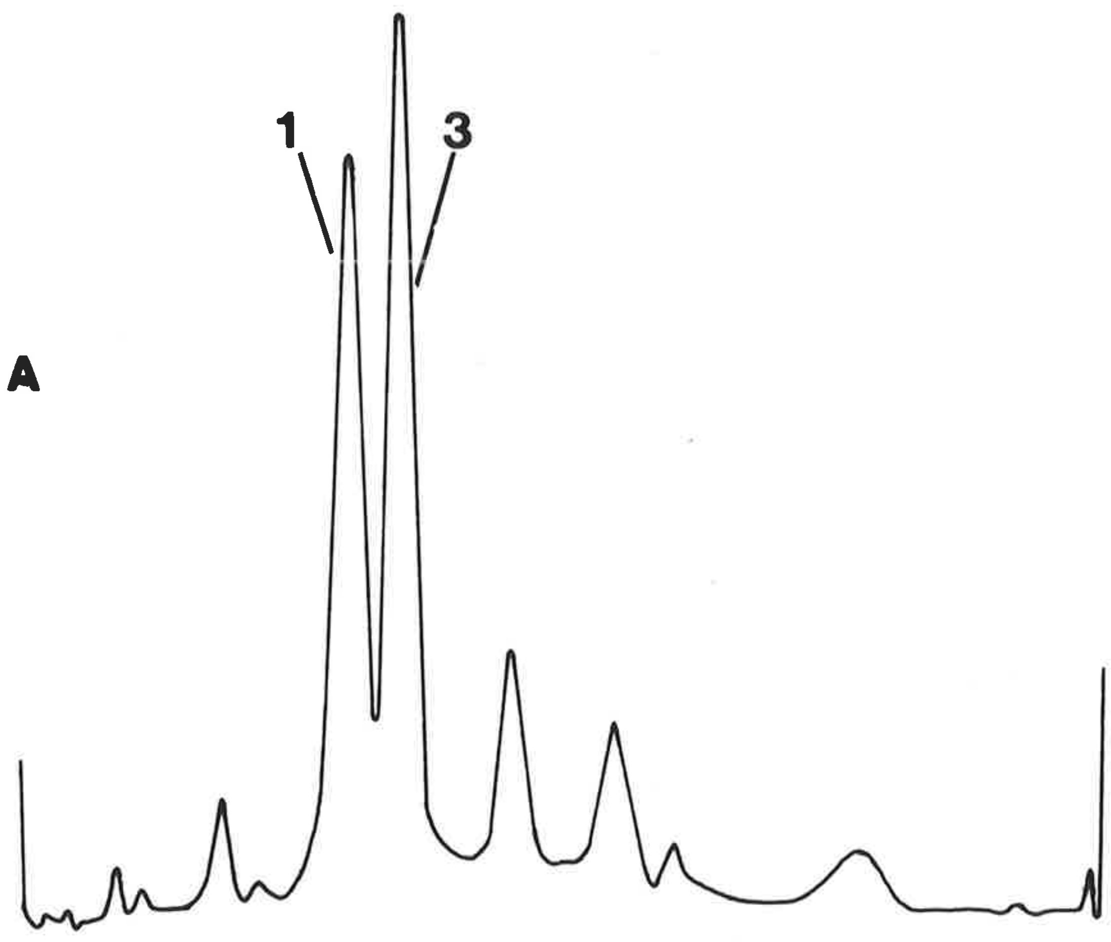
work on E. coli, cell wall preparations were solubilized and run under the conditions of Schnaitman (Fig. 7-2 and 7-3).

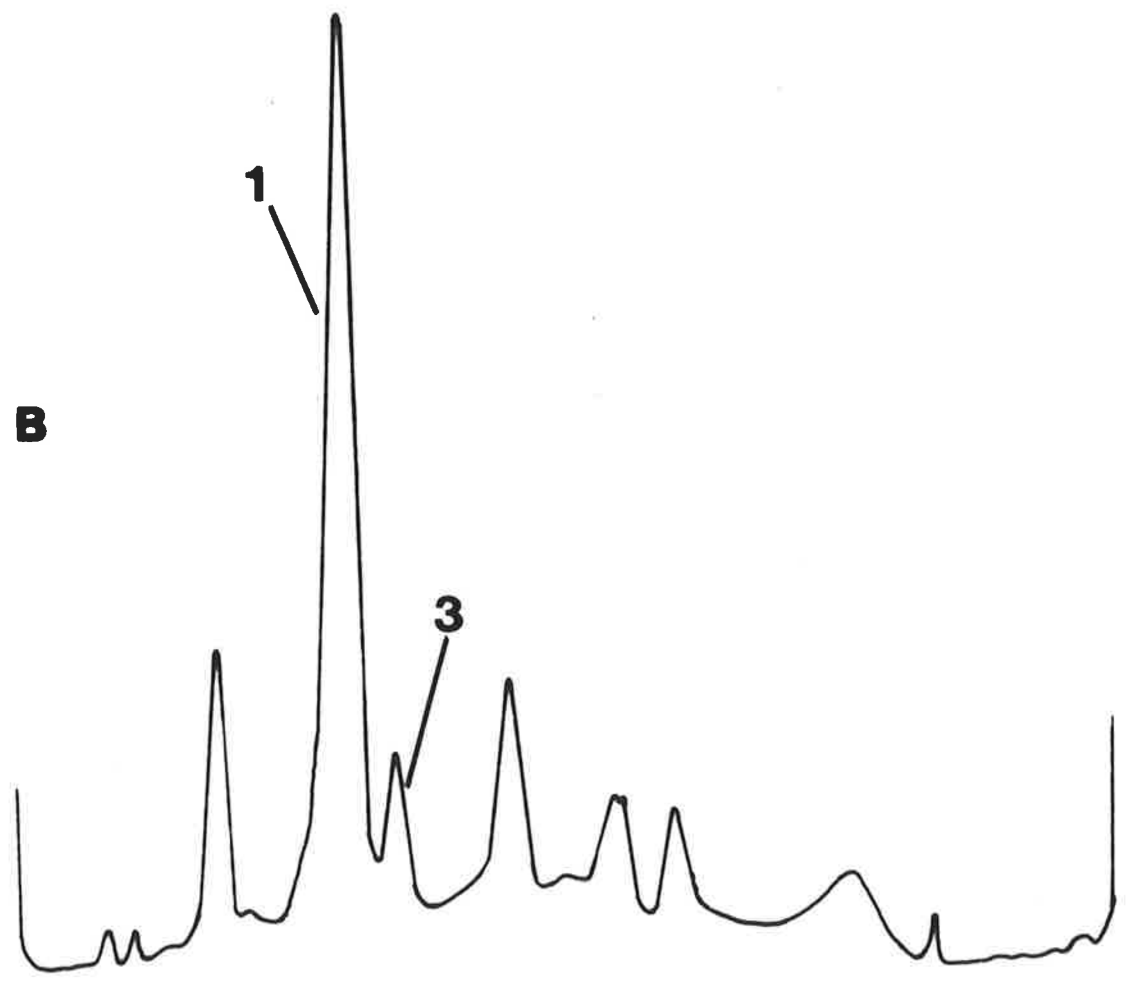
E. coli K-12 has 3 major outer membrane proteins, 1, 3a and 3b (Schnaitman, 1974b). Using method 3 for sample preparation and the pH 7.2 Maizel buffer system, protein 1 runs as a peak A and proteins 3a and 3b run together as peak C (Schnaitman, 1973a, 1974a,b). The Con$^-$ mutant P460, run using these conditions, had peak C very much reduced (Fig. 7-2B in comparison with strain P400 (Fig. 7-2A). The heated samples (method 2) run under the same conditions showed that all the major proteins ran at peak B (Fig. 7-2C,D) as observed by Schnaitman (1973a). However, both with strains P400 and P460, a small amount of material ran at the position of peak C, comparable in amount to that observed with unheated P460 (Fig. 7-2B). It seems likely, therefore, that this residual amount of material in peak C (Figs. 7-2B, C and D), represents a minor protein and is not a residual amount of proteins 3a or 3b; although further analysis may be required, it is provisionally postulated that strain P460 lacks both proteins 3a and 3b.

Unheated preparations (method 3) of strains P400 and P460 also differed in the amount of peak B and possibly also peak A (Fig. 7-2A,B). The material in peak B is presumably due to either proteins 1, 3a or 3b running in this position, even when the heating is omitted, since peak B is a product of these proteins (Fig. 7-2C,D). Because there is so much more peak B in P400 than in P460 (Fig. 7-2A,B), it must be largely derived from proteins 3a and 3b. It should be noted that the material in peaks B and C of P460 (Fig. 7-2B) is the maximum amount of proteins 3a and 3b that could be present.

FIG. 7-3

Comparison, by densitometry, of stained bands of the outer membrane proteins of strains P400 (Fig. 7-3A) and P460 (Fig. 7-3B) run on the Bragg-Hou gel system after solubilization by method 2. The peaks are labelled 1 and 3 in accordance with the Schnaitman (1974a) nomenclature as described in the text.


1
3
A


1
B
3

The reduction in one of the components of peak A in P460 is much less than the reduction in peak C (Fig. 7-2B), and may not be significant.

Under alkaline conditions (Bragg and Hou gels), Schnaitman (1973b, 1974a) showed that both proteins 3a and 3b run together as peak 3, which is again virtually absent in strain P460 (Fig. 7-3B) in comparison to strain P400 (Fig. 7-3A). Thus results with both neutral and alkaline gels support the hypothesis that Con$^-$ mutants lack the two outer membrane proteins 3a and 3b.

LIPOPOLYSACCHARIDE-DEFICIENT MUTANTS - PROTEIN ALTERATIONS

A number of representative bacteriophage resistant mutants were tested for protein alterations in their cell walls and envelopes by the techniques discussed previously in this chapter. Results obtained are summarized in Table 7-1. It was found that strains were sometimes altered in peak 1 (using the Schnaitman (1974a) nomenclature for Bragg-Hou gels), which consisted of a single protein, protein 1 (Schnaitman, 1974a). For demonstrating the alterations, the Bragg-Hou gel system was by far the best, although equivalent alterations were seen in the Neville and Schnaitman gel systems. Representative results are seen in Figs. 7-4, 7-5, 7-6, 7-7 and 7-8. In each case the gel runs of mutants (bold lines) are compared with the parent strain P400 (dashed lines) prepared and run under the same conditions.

It was demonstrated that, with the exception of the Con$^-$ mutants (Figs. 7-1, 7-2 and 7-3), only mutants previously shown to be LPS-altered (Chapter 6) had

TABLE 7-1

Summary of alterations to major polyacrylamide gel peaks caused by mutation to bacteriophage resistance in various strains.

Strain	Resistance Class	Relative amounts of major polyacrylamide gel peaks [a,b]	
		Peak 1	Peak 3
P400	-	1	1
P460	Con	1	0
P448	Efr	1	1
P466	Ktn	1	1
P493	Misc(5)	1	1
P456	Ktw(1)	0.3-0.5	1
P476	Ktw(2)	1	1
P240	Ktw(3)	1	1
P429	Ttk(1)	0.8	1
P423	Ttk(2)	0.8	1
P425	Ttk(3)	1	1
P474	Ttk(4)	0.8	1
P495	Bar(3)	0.5	1
P405	Bar(4)	1	1
P436	Bar(4)	1	1
P489	Bar(8)	0.3-0.5	? [c]
P435	Wrm(1)	1	1
P424	Wrm(2)	0.1	0.1

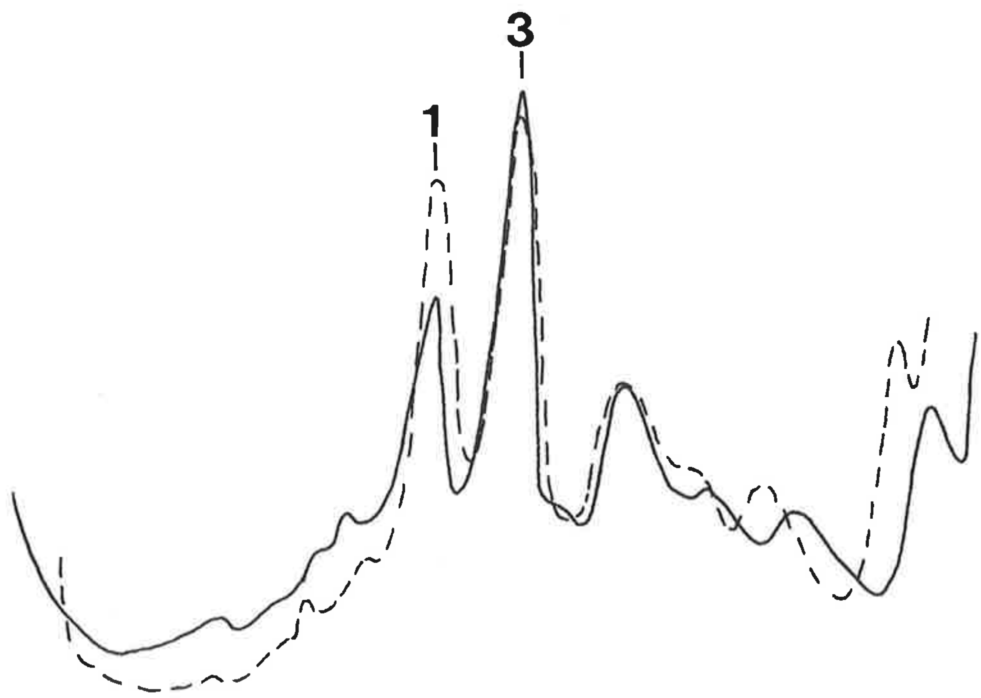
[a] Major polyacrylamide gel peaks are named in the Schnaitman (1973b, 1974a,b) nomenclature for Bragg-Hou gels as described earlier in this Chapter (Fig. 7-3).

[b] Results are expressed as approximate peak areas relative to those of strain P400.

[c] Results of analysis of the cell wall proteins of P489 using methods (2) and (3) (see Chapter 2) for solubilization and the Maizel buffer system (Maizel, 1966) suggest that peak C may be reduced in this strain. However the level of alteration has not, as yet, been quantitated.

FIG. 7-4

Comparison by densitometry of the outer membrane proteins of P400 (- - -) and P456 (——) run using the Bragg-Hou gel system after solubilization by method 2. The peaks are labelled 1 and 3 in accordance with the Schnaitman (1974a) nomenclature as described in the text. The Ktw(1) strain P456 has relatively less of protein 1 than has P400.


3
1

alterations in the protein composition of their cell wall (Table 7-1). The Efr, Ktn and Misc (5) strains had no obvious alterations in their cell wall proteins (Table 7-1). This does not necessarily imply that no proteins are mutated or absent in these mutants, but rather that the changes are too small to be picked up by polyacrylamide gel electrophoresis or are masked by other cell wall proteins. This effect has been demonstrated for bfe$^-$ mutants (Sabet and Schnaitman 1973a,b) and tsx$^-$ mutants (Schnaitman, 1974b) which, although mutated in a protein of the cell wall, have no apparent defects in cell wall composition.

Of the Ktw strains, P456 (Ktw(1)) was alone shown to have an altered protein composition (Fig. 7-4), having only 30% - 50% of protein 1 remaining in its cell wall. This strain has also been shown to have an altered LPS (Table 6-2). The other Ktw strains, P476 (Ktw (2)) and P420 (Ktw (3)) have no such protein deficiency and this might explain in part the slightly different resistance patterns obtained for these strains (Table 4-3).

Three of the four Ttk strains tested had minor changes in peak 1 size (Table 7-1) as shown in Fig. 7-5 for strain P429 (Ttk (1)). However, no correlation was obvious between the resistance pattern and the presence or absence of this small protein 1 alteration.

Of the Bar mutants studied, strain P495 (Bar (3)) had a 50% reduction in the amount of peak 1 (Fig. 7-6) while strain P489 (Bar (8)) which was resistant to a greater number of bacteriophages than strain P495, had a similar alteration in peak 1 and possibly also an alteration in peak C. However,

FIG. 7-5

Comparison by densitometry of the outer membrane proteins of P400 (- - -) and the Ttk(1) mutant P429 (——) run using the Bragg-Hou gel system after solubilization by method 2. The peaks are labelled 1 and 3 in accordance with the Schnaitman (1974a) nomenclature as described in the text.

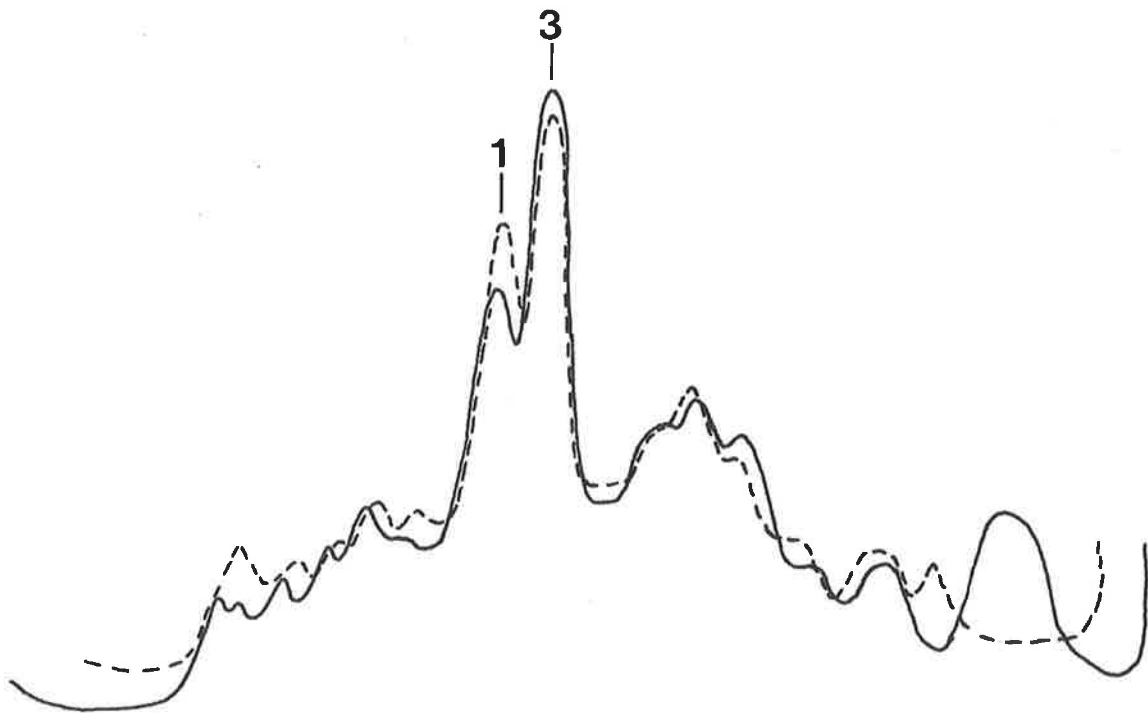

3
1

FIG. 7-6

Comparison by densitometry of the outer membrane proteins of P400 (- - -) and the Bar (3) mutant P495 (——) run using the Bragg-Hou gel system after solubilization by method 2. The peaks are labelled 1 and 3 in accordance with the Schnaitman (1974a) nomenclature as described in the text.

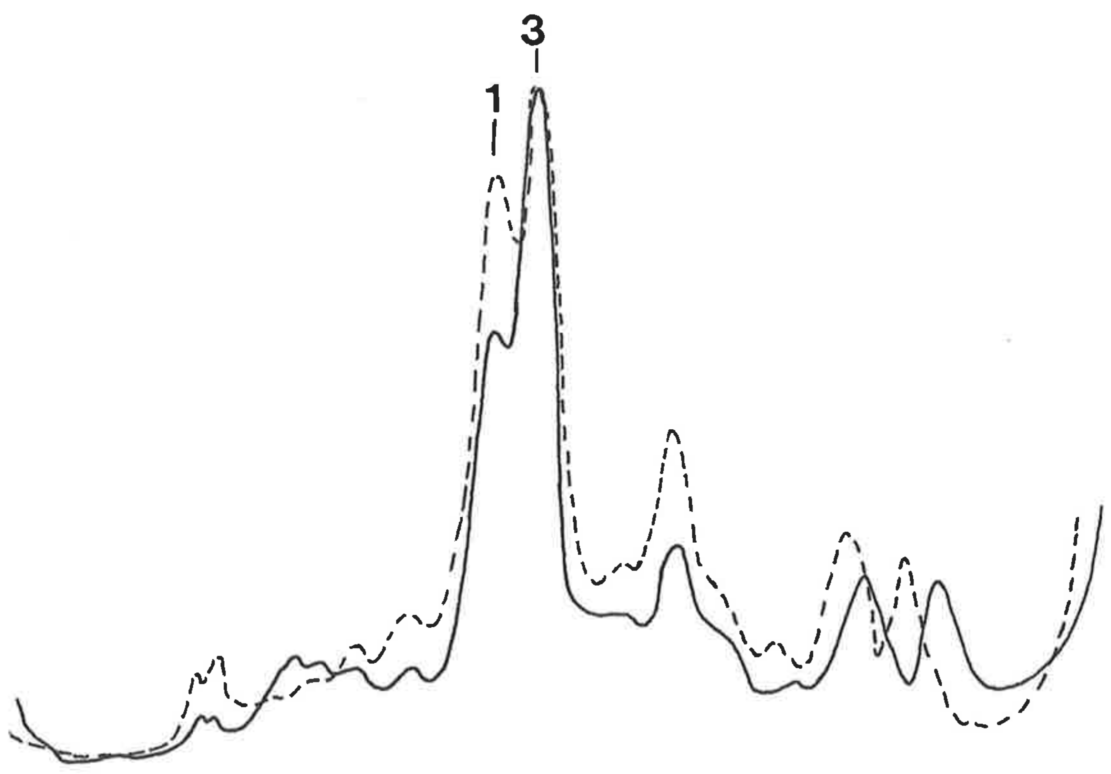

3
1

FIG. 7-7

Comparison by densitometry of the outer membrane proteins of P400 (- - -) and the Wrm (2) mutant P424 (——) run using the Bragg-Hou gel system after solubilization by method 2. The peaks are labelled 1 and 3 in accordance with the Schnaitman (1974a) nomenclature as described in the text. Peaks 1 and 3 of P424 appear drastically reduced. The increase in protein in the lower molecular weight region (right-hand end of the gel) is due to the increased loading necessary to visualize the bands in P424.

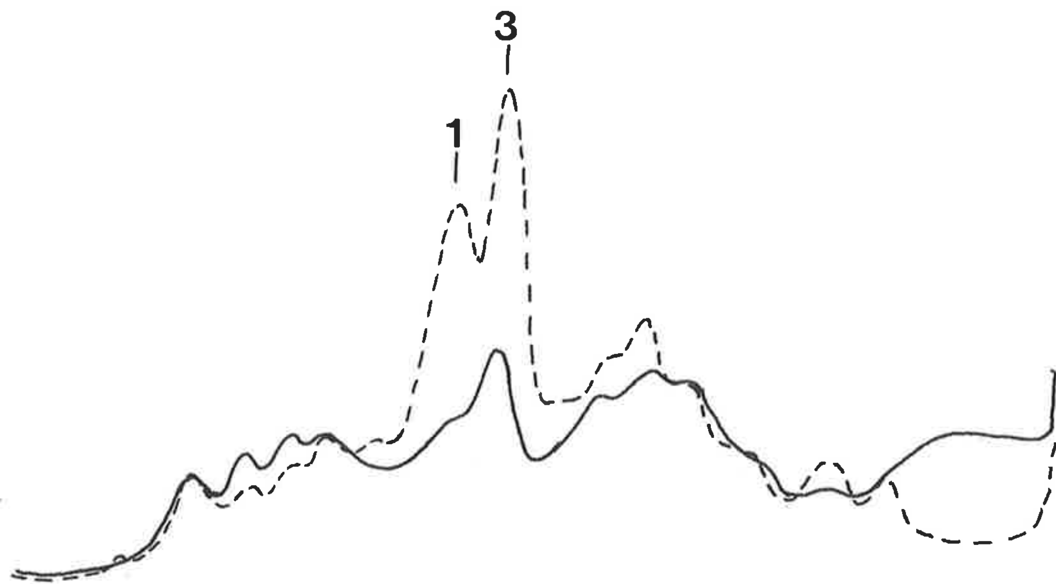
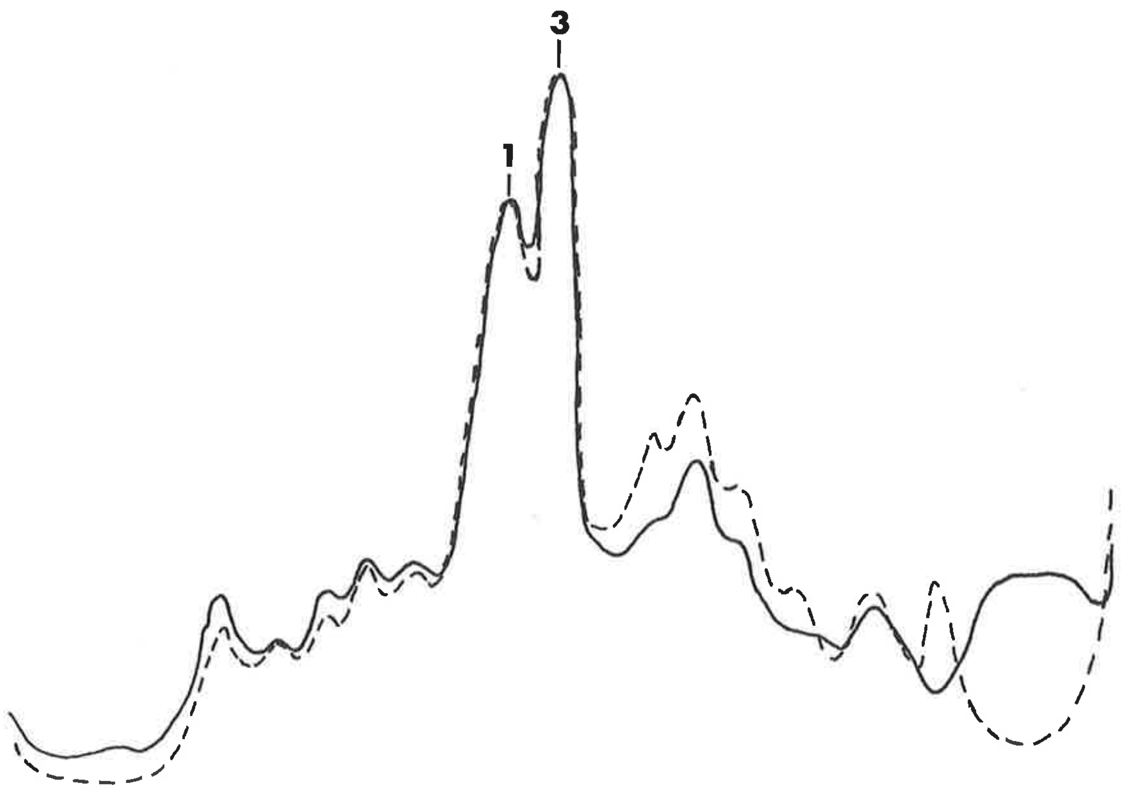

3
1

FIG. 7-8

Comparison by densitometry of the outer membrane proteins of P400 (- - -) and the Wrm (1) strain P435 (——) run on the Bragg-Hou gel system after solubilization by method 2. The peaks are labelled 1 and 3 in accordance with the Schnaitman (1974a) nomenclature as described in the text.


3
1

it was shown that two Bar (4) mutants, P405 and P436, which had intermediate resistance patterns (Table 4-5) to the other Bar mutants studied were unaltered in cell wall protein composition.

The Wrm mutant P424 (Wrm (2)) which has a large LPS alteration (Table 6-2) also had greatly reduced levels of peaks 1 and 3 (Fig. 7-7). Thus it is probably similar to the heptose deficient mutants of Ames, Spudich and Nikaido (1974) and Koplow and Goldfine (1974). On the other hand, strain P435 (Wrm (1)), which has an even larger LPS-defect than strain P424 (Table 6-2) and a similar resistance pattern (Table 4-6), has an unaltered protein composition (Fig. 7-8). This result was repeated for a fresh cell wall preparation of strain P435.

There appears to be some correlation between resistance pattern and protein composition for the Wrm mutants. For instance, bacteriophages K3, K4 and K5 which are unable to lyse Con$^-$ mutants, probably have as a receptor or receptor-component one of the proteins of peak 3 which is deleted in Con$^-$ mutants (Fig. 7-3). Strain P424 which has considerably reduced peak 3 (Fig. 7-7) is resistant to these bacteriophages (Table 4-6) while strain P479 with normal peak 3 (Fig. 7-8) is sensitive to them (Table 4-6). However, the correlation does not hold for the other bacteriophages which fail to lyse Con$^-$ mutants, and in this case one might suggest that these bacteriophages are more sensitive to alterations in the LPS component (see Chapter 5) of the receptor. The resistance or sensitivity of the Wrm strains to bacteriophages T2 and T6 might also be related to the absence or presence of peaks 1 and 3.

SUMMARY AND CONCLUSIONS

Two of the major proteins of the E. coli K-12 cell wall, proteins 3a and 3b, are missing in the cell walls of Con$^-$ mutants. Of the other strains tested, only certain mutants shown in Chapter 6 to be LPS-altered, were shown to have protein alterations and most of these were shown to have alterations in protein 1. Strain P424 (Wrm (2)) had greatly reduced amounts of both of the major peaks, 1 and 3, while of the other peak 1 altered mutants, summarized in Table 7-1, strain P489 had a possible peak 3 alteration also. The protein alterations probably resulted from specific mutations rather than being merely the result of increasing LPS alterations leading to increasing disturbance of the outer membrane. This was shown by the fact that strain P456 (Ktw (1)) which had a minor LPS alteration (Table 6-2) had an altered protein composition (Fig. 7-4), while P435 (Wrm (1)) with a very large LPS alteration (Table 6-2) had no obvious cell wall protein defect (Fig. 7-8).

There appears to be a limited relationship between protein composition and bacteriophage resistance pattern for some mutants.

CHAPTER 8

MAPPING OF THE MUTATIONAL LESIONS

INTRODUCTION

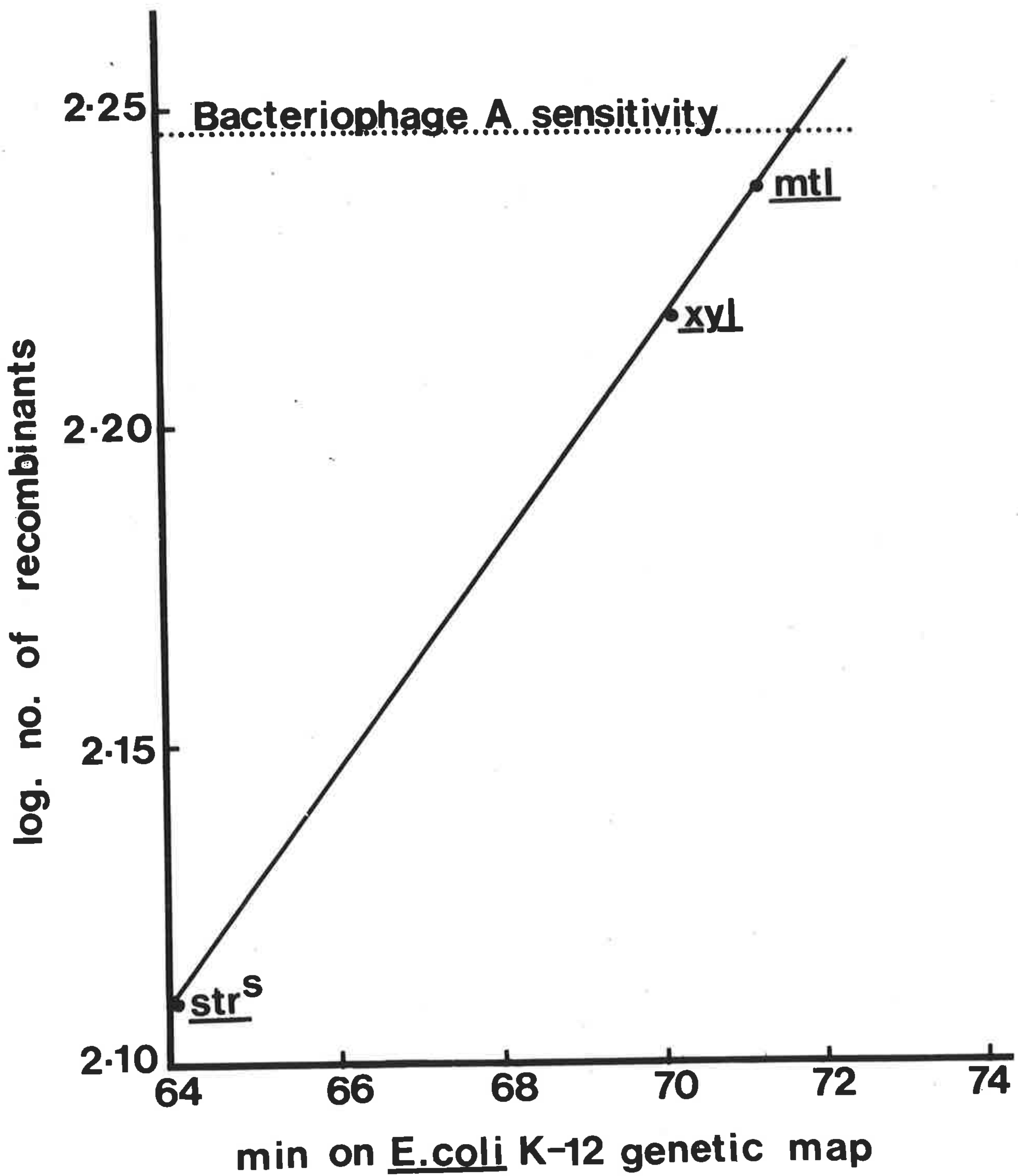
The genetic study of bacteriophage resistant mutants in *E. coli* has been mainly involved with mutants resistant to the T group of bacteriophages, BF23 and λ. Well defined bacteriophage resistance loci include *ton*A (J. Lederberg, 1947), *ton*B (Curtiss, 1965), *tsx* (J. Lederberg, 1947), *lam*B (Thirion and Hofnung, 1972), *tfr*A (Curtiss, 1965) and *bfe* (Buxton, 1971; Jasper, Whitney and Silver, 1972). Apart from these, only a few limited genetic studies of bacteriophage resistance have been done in *E. coli* K-12 (Hayes, 1957; Curtiss, 1965; Tamaki, Sato and Matsuhashi, 1971). The adaptation of the technique of gradient transfer analysis (de Haan *et al.*, 1969) to the mapping of bacteriophage resistant mutants (Chapter 2), has provided a rapid technique for the approximate genetic location of mutational lesions.

MAPPING OF Bar AND Wrm MUTANTS

When strain P404 was mated with various Hfr's, it was found that only strains KL209, RC749 and JG20 (Hfr J4) gave appreciable transfer of sensitivity to bacteriophage A. A de Haan gradient transfer analysis (de Haan *et al.*, 1969) was performed by mating strains KL209 (*mal*B *arg*E$^+$/Hfr) and P404 (*mal*B$^+$ *arg*E/F$^-$) using *arg*E as the selective marker and *mal*B as the counterselection against the donor (Fig. 8-1). Sensitivity to streptomycin, fermentation of mannitol and

FIG. 8-1

de Haan gradient transfer analysis of conjugation between strain P404 (bacteriophage A^r, argE mtl xyl malB$^+$ str^r/F$^-$) and KL209 (bacteriophage A^S, argE$^+$ mtl$^+$ xyl$^+$ malB str^S/Hfr J4): Analysis of malB$^+$ argE$^+$ recombinants. This shows that the bacteriophage resistance gene (Bar (3)) in this strain maps at approximately 72 min.



2·25
2·20
2·15
2·10
Bacteriophage A sensitivity
mtl
xyl
str^s
log. no. of recombinants
64
66
68
70
72
74
min on E.coli K-12 genetic map

xylose, and bacteriophage sensitivity was scored and, by plotting $\log_{10}$ of the number or recombinants against the E. coli K-12 genetic map position, the mutation was mapped at approximately 72 min. By Plkc transduction into strain KL141, it was shown that bacteriophage A resistance was 55% co-transducible with pyrE^{+} which was itself 20% cotransducible with mtl, and that bacteriophage resistance lay between these two markers. Thus, using Taylor and Trotter's (1967) relationship between map distance and percentage cotransduction, this places this bacteriophage resistance gene at approximately 71.8 minutes. Similar conjugation or transduction experiments indicated that strains P455 (Bar (1)), P492 (Bar (2)), P413 (Bar (3)), P490, P405 (Bar (4)), P402 (Bar (5)), P451 (Bar (6)), P487 (Bar (7)), P489 (Bar (8)), P416 and P239 (Wrm (2)) all map between pyrE and mtl (Fig. 8-3).

Many of the rfa genes for biosynthesis of LPS in Salmonella (Stocker and Mäkela, 1971; Wilkinson, Gemski and Stocker, 1972), E. coli 08 (Schmidt, Jann and Jann, 1970) and E. coli K-12 (Eriksson-Grennberg, Nordstrom and Englund, 1971; Schmidt, 1973) have been previously located in this region of the respective genetic maps. It is therefore likely that the LPS-altered, bacteriophage resistant mutants of the Bar and Wrm (2) groups form a rfa gene cluster in E. coli K-12. In support of this hypothesis, it has been shown in E. coli 08 (Schmidt, Jann and Jann, 1971) and Salmonella (Wilkinson, Gemski and Stocker, 1972; Lindberg, 1973) that mutants deficient in the enzymes of LPS core biosynthesis (rfa mutants), often have an altered pattern of resistance or sensitivity to various bacteriophages.

MAPPING OF Ktw MUTANTS

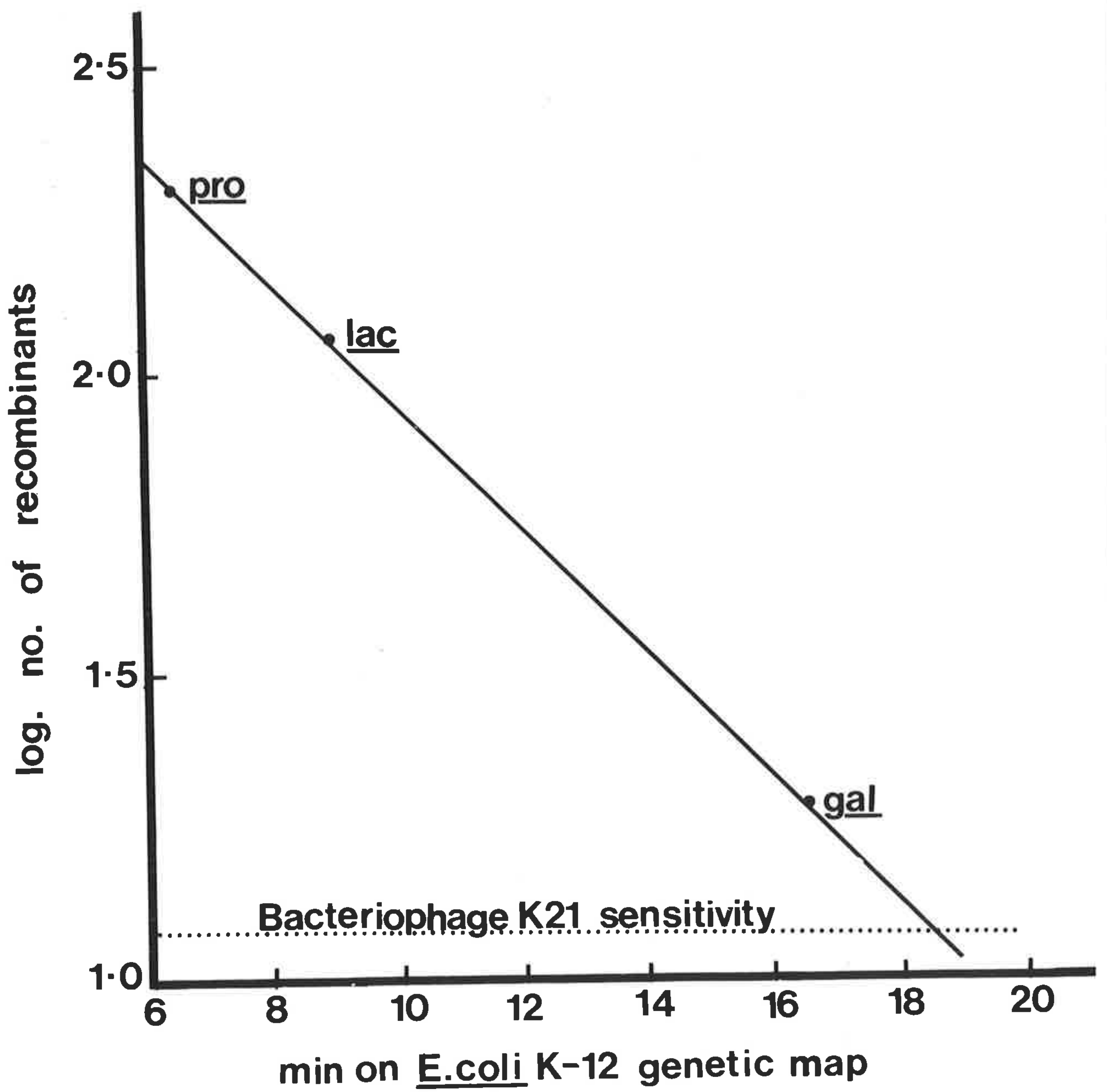
It was shown by mating with strain KL209 and by P1kc transduction that Ktw mutants did not map at or near the rfa locus. Further experiments indicated that these mutants could be mapped using HfrH. Therefore, strain P458 (Ktw (1), thr str/F⁻) was mated with strain P839 ($thr^{+}str^{+}$/HfrH) by the technique of de Haan *et al.* (1969) and 200 recombinants selected. These were scored for *thr*, *leu*, *pro*, *lac*, *gal* and sensitivity to bacteriophage K21. Although the level of bacteriophage sensitive recombinants was low (2%), a de Haan gradient transfer plot (such as illustrated in Fig. 8-1) gave a straight line and indicated that the mutation mapped at 19.2 min on the *E. coli* K-12 genetic map. Similar studies indicated that the defects in strains P476 (Ktw (2)), P477 (Ktw (1)) and P240 (Ktw (3)) mapped at 18.4, 20 and 17.4 minutes respectively. This indicated that these mutants mapped at either the same or linked loci; allowing for the error introduced by the small number of phage sensitive recombinants (due to the distance of the Ktw loci from the HfrH origin). Therefore, the results of the four experiments were collated (Fig. 8-2) and a de Haan plot indicated the genetic map position of the Ktw resistance gene in these mutants to be 18.6 min. It is possible that one or more of these mutants do not map at this locus, and in fact preliminary experiments with strain P456 (Ktw (1)) indicate that this strain does not.

MAPPING OF Con⁻ MUTANTS

Preliminary matings of the Con resistant mutant P460 with

FIG. 8-2

de Haan gradient transfer analysis of results of conjugation of strain P839 (bacteriophage K21^{S}, *pro*$^{+}$ *lac*$^{+}$ *gal*$^{+}$ *str*^{S}/HfrH) with strains P458 (Ktw(1), *pro* *lac* *gal* *str*$^{+}$/F^{-}), P477 (Ktw(1), *pro* *lac* *gal* *str*$^{+}$/F^{-}), P476 (Ktw(2), *pro* *lac* *gal* *str*$^{+}$/F^{-}) and P240 (Ktw(3), *pro* *lac* *gal* *str*$^{+}$/F^{-}): Analysis of *pro*$^{+}$ *str*$^{+}$ recombinants. The Ktw bacteriophage resistance genes in these strains map at approximately 18.6 min.



2·5
2·0
1·5
1·0
log. no. of recombinants
pro
lac
gal
Bacteriophage K21 sensitivity
6
8
10
12
14
16
18
20
min on E.coli K-12 genetic map

strains KL209 (Hfr J4), AB259 (HfrH) and RC750 (HfrC) indicated that the latter two could transfer sensitivity to bacteriophage K3. Results from gradient transfer analyses were very hard to analyse as Con$^-$ mutants or recombinants gave spurious results on EMB media, which was used to score fermentation of galactose and lactose. A number of experiments were done in an attempt to determine the reason for these spurious results. These included plating strains P400 and P460 on various synthetic media or mixtures of EMB agar with nutrient or minimal agar. It was shown that on EMB agar the growth of strain P460 was inhibited, relative to strain P400. Furthermore, after 2-3 days, the relatively small colonies of strain P460 took up the EMB stain giving a pseudo-positive result. Although this appeared to be the reason for the spurious results, it was not possible to demonstrate sensitivity to Eosin Y, methylene blue or to the Eosin Y-methylene blue complex, even at four times the concentrations that existed in EMB agar.

Scoring of fermentation with minimal agar containing growth factors and lactose or galactose as the sole carbon source was difficult (especially for the scoring of _gal_), but, using the two above systems together, the Con$^-$ genetic loci was shown to be between _lac_ and _gal_. Gradient transfer analysis (de Haan _et al_., 1969) of the data indicated the map position to be somewhere between 12 and 15 min. on the _E. coli_ K-12 genetic map. It was shown also that sensitivity to bacteriophage K3 was transferred by HfrC strain RC750, using _pro_$^+$ as a selective marker; but transfer of K3 sensitivity occurred only at a low frequency (3-10%). This was surprising as the HfrC origin lies at about 13.5 min or a maximum of 1.5 min from the map position of Con$^-$ as

indicated by previous experiments. It was further shown that when Con$^-$ was transferred to HfrC by Plkc transduction, this strain would not transfer Con$^-$ into a recipient cell, even if purE$^+$ (12 min) was used as the selective marker in the recipient. Furthermore, it could not be shown that the Con$^-$ marker was cotransducible with purE$^+$, even though 150 transductants were tested.

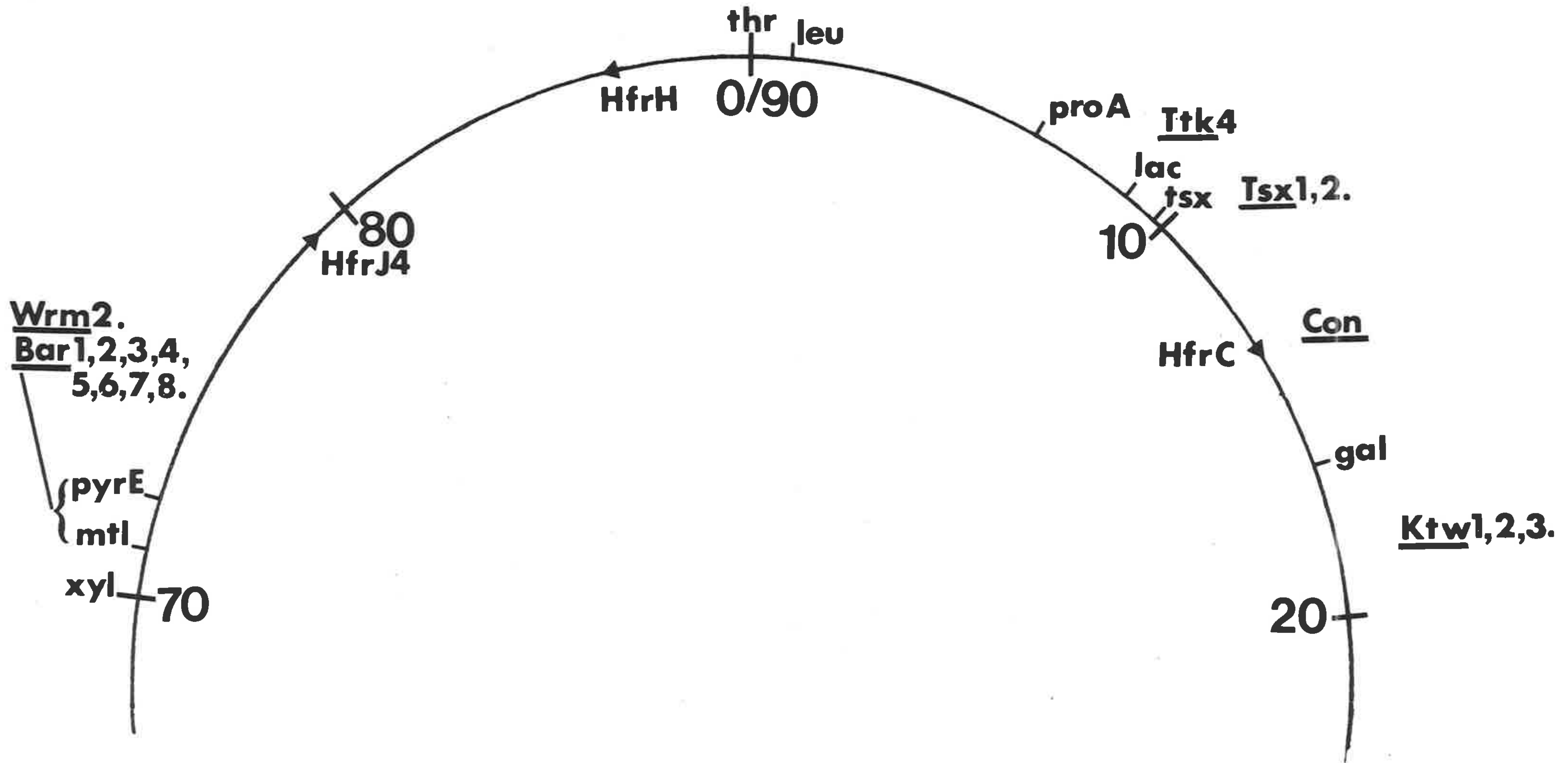
To summarize, a hypothesis to explain these results, would be that the Con$^-$ defect is a deletion or chromosomal aberration, such as has been demonstrated for other bacteriophage resistant mutants (Curtiss, 1965; Franklin, Dove and Yanofsky, 1965). This would help explain why Con$^+$ could be transferred into the Con$^-$ strain P460 by HfrC, but Con$^-$ could not be transferred into a Con$^+$ strain. The mutational lesion probably maps very near to the HfrC origin (Fg. 8-3) based on its inability to be cotransduced with the purE marker.

MAPPING OF OTHER MUTANTS

By Plkc transduction or conjugation with strain KL209 (Hfr J4), it was shown that the following mutants do not map at the rfa locus: strains P423 (Ttk (2)), P474 (Ttk (4)), P448 (Efr), P407 (Tsx (1)), P433 (Tsx (2)), P237 (Misc (4)) and P479 (Wrm (1)). The mapping of mutations of strain P400 by conjugation, is inhibited by the lack of genetic markers for selection purposes between gal (16.5 min) and str (64.2), and thus attempts to locate the above mutations, in addition to those in strains P429 (Ttk (1)) and P443 (Misc (2)) have been largely unsuccessful.

FIG. 8-3

Partial genetic map of *E. coli* K-12 (adapted from Taylor and Trotter, 1972) including genetic loci and origins of various Hfr's mentioned in Chapter 8 and the approximate genetic locations of various bacteriophage resistance loci isolated and mapped in this study.



thr
leu
0/90
HfrH
proA
Ttk4
lac
tsx
Tsx1,2.
10
80
HfrJ4
Wrm2.
Bar1,2,3,4,
5,6,7,8.
Con
HfrC
gal
Ktw1,2,3.
pyrE
mtl
xyl
70
20

The only mutants, of those mentioned above, that have been located on the E. coli K-12 genetic map are strains P407 and P433 of the Tsx resistance group and P474 of the Ttk resistance group (Fig. 8-3). Gradient transfer analyses of these mutants indicated that the Tsx mutants mapped at or close to the previously discovered tsx locus, while P474 mapped close to proA. This is interesting as strain P433 (Tsx (2)) is not fully resistant to bacteriophage T6 after which the tsx locus is named. Further experiments are required to determine whether or not the Tsx (2) mutant allele is in fact linked to the tsx allele. The discovery that the Ttk (4) mutant P474 maps near proA indicates that it may map close to the tfrA locus despite the fact that it is not resistant to bacteriophage T4 (Table 4-4).

SUMMARY AND CONCLUSIONS

All results are summarized in Fig 8-3. The mapping of the Bar and Wrm (2) mutants at a position between pyrE and mtl on the E. coli K-12 genetic map, suggests that these mutants which often have altered LPS (Table 6-2) are mutated in rfa genes which form a cluster at this location. The Con^- mutant probably maps close to the HfrC origin, and genetic experiments suggest that the multiple properties of Con^- mutants (i.e. deficiency in two cell wall proteins, colicin tolerance, bacteriophage resistance, sensitivity to EMB agar, conjugational recipient deficiency) might or might not be due to a deletion or chromosomal aberration, causing more than one genetic locus to be affected. However, this is by no means proven and the properties are consistent with the

defect being a point mutation (see Chapter 9) and thus Con^- mutants have been discussed in this thesis with this surmise. Both sub-groups of the Tsx resistance group map at closely-linked loci or the same locus; while representatives of the three sub-groups of Ktw map together at a distinct locus. Strain P474 (Ttk (4)) maps close to the previously discovered tfrA gene, however neither the Ttk (1) nor the Ttk (3) mutants do.

CHAPTER 9

DISCUSSION

In this work, bacteriophage resistant mutants were selected in *E. coli* K-12 using a wide range of different virulent bacteriophages. The results summarised in Figure 9-1 showed that the mutants fell into 11 main groups, and on group of miscellaneous mutants which did not fit well into any of the other groups. Each group of resistant mutants was characterized by a particular set of bacteriophages to which most of the mutants showed resistance of one type or another. Although the mutants within a group were not always identical, no difficulty was experienced in recognising the relationship between them. A number of the mutant groups have already been described including TonA, TonB, Bfe and Tsx (Demerec and Fano, 1945; Buxton, 1971). A few new mutant types are described, but extending the number of bacteriophages studied did not greatly increase the range of mutant types. It would seem that there are not many undiscovered types resistant to different sets of virulent bacteriophages. It remains possible of course that other types remain undetected, because they occur less frequently than those described previously or in this paper. However, selecting a large number of mutants enables one to try to arrange them in a meaningful way.

THE GENERAL PATTERN OF BACTERIOPHAGE RESISTANT MUTANTS AND ITS RELATIONSHIP TO BACTERIOPHAGE TAXONOMY

Each type of mutation must have its effect on

bacteriophage resistance by preventing some stage of the bacteriophage infectious cycle. The best characterized mechanism is by loss of receptor, although not all mutants act at the level of adsorbtion. Many examples of receptor mutants have been found in these studies; however, the poor adsorbtion, generally, of many of the bacteriophages, using a wide range of techniques, has inhibited the search for tolerant mutants similar to those found in studies of colicin resistant mutants (see Reeves, 1972). Garen and Kozloff (1959) have postulated a class of resistant mutants with subtle surface modifications. They propose that the rate of phage attachment to these mutants would be slow, but still sufficient to prevent colony formation in the presence of bacteriophages. Some of the mutants discussed in Chapter 4 appear to be inhibited by certain bacteriophages and it is possible that they may be of this class. Wahl (1953) has described semi resistance which may be similar to the partial resistance with inhibition (IP) pattern of resistance.

The various mutant groups described serve also to define sets of bacteriophages, most of which are unable to form plaques on more than one of the mutant groups described. Correspondingly, some of the mutant groups confer resistance to more than one set of bacteriophages. In general, the mutants seem to be of two main types. The majority of resistance groups confer resistance to one or two sets of bacteriophages, while the Bar and Wrm mutants are resistant to bacteriophages from many sets. Mutants of the first type are presumably affected in a cell component necessary for only one or two bacteriophage sets during infection, whereas the Bar and Wrm mutants affect a function necessary, either directly

or indirectly, for infection by many different bacteriophage sets. One possibility, expanded later in this discussion, is that Bar and Wrm mutants affect the cell surface in such a way that many different receptors are absent or non-functional.

Bacteriophage taxonomy is based primarily on morphology (Bradley, 1967; Ackerman, 1969; Tikhonenko, 1970; Wildy, 1971), which serves to identify the major natural groupings. Furthermore, some of the well studied bacteriophages are known to be closely related genetically and able to undergo recombination readily. On classical criteria for higher organisms one can say that they are strains of one species. Thus BF23 and T5 (Mizobuchi, Anderson and McCorquodale, 1971) and T2, T4 and T6 (Luria and Dulbecco, 1949; Adams, 1953) recombine readily and constitute two separate species. Unfortunately, information of this type is not available for most bacteriophages.

It is interesting that the bacteriophages with B1 type morphology studied (Table 3-2), resembled either T1 or T5 and BF23 in their dimensions. Most bacteriophages with similar dimensions to T1 required both the *ton*A and *ton*B functions for infection; the others resembled T5 in requiring only *ton*A function, or BF23 in requiring only *bfe* function. Both *ton*A and *bfe* mutants are known to lack a specific surface protein (Braun, Schaller and Wolff, 1973; Sabet and Schnaitman, 1973) which may be the bacteriophage receptor. The nature of *ton*B, however, is not fully understood as T1 adsorbs reversibly to this mutant (Garen and Puck, 1951). It seems that many of the bacteriophages resembling T1 morphologically have similar requirements for infection, while those resembling T5 and BF23, probably all one

species, use either the T5 or BF23 receptor and, like the T1 set, are unaffected by Bar or Wrm mutations. Thus, it seems that resistance patterns might be the simplest method of distinguishing strains of the same species of bacteriophages.

Bacteriophages of A2 morphology (Table 3-2) are far more heterogenous with respect to resistance patterns (Fig. 9-1). Seven of the mutant groups are resistant to bacteriophages of this type. Three groups, Tsx, Con and Ktw, are commonly resistant to a set of between four and nine bacteriophages with A2 morphology. Within the set of bacteriophages affected by the Tsx mutations, H3 has only one requirement, for the tsx function. This function is also required by T6 and six other bacteriophages of A2 morphology; however these bacteriophages, unlike H3, have altered activities on one or both sub-groups of the Wrm resistance group. Heterogeneity also exists amongst those bacteriophages which require the Con function. Only the Con mutants and one sub-group of Wrm mutants have altered sensitivity towards bacteriophage K3; however, in the case of Ox5 there are also mutants in the Ttk, Bar and miscellaneous groups which are resistant to it. The mutational lesions affecting the infection of these strains by bacteriophage Ox5, differ considerably (Chapters 6 and 7) and appear to reside in the outer membrane. Since the mutants, with the exception of Con, appear to affect the same component (LPS) of the outer membrane, one might postulate, as with bacteriophage K3, that there are only two factors influencing the infectious cycle, but that

TABLE 9-1

General pattern of resistance.

Resist.[c] Group (Sub-group)	BACTERIOPHAGES RESISTANT TO[a,b]																													
	T5-like	T1-like	BF23-like	K10	H3	H1-like	T6	K3-like	Ac3-like	M1	Ox2-like	E4	K2	K20-like	K29	Ox5	K16	F27	H^+	V	E7	K17	A	T3	T4	K19	T2	T7-like	K15	H
TonA	R	R																												
TonB		R																												
Bfe			R																											
Ktn				R																										
Tsx(1)					R	R	R																							
(2)					R	V	V																							
Con								R	R	R	R					R														
Efr												R																		
Ktw(1)													V	V	V															
(2)												V	V	R	R															
(3)													V	V	V				V											
Ttk(1)												R				V	R	R	R	V						R	R			
(2)																V	R	R	R	V	R	V			R					
(3)																V	R	R	R	R	V				R	R				
(4)																V	R	R	R	R						R				
Misc(1)																	V		R		V	V			V					
(2)																					V				V					
(3)																	V	R	V											
(4)																V	V	R	V											
(5)												V				V	V					V								
Bar(1)											V	V	V	V	V	V					V		V							
(2)										V	V	R	V	R	V	R	V	R		R		V								
(3)										V	V	R	V	V	V	R	V	V	V	V	V	V	V							
(4)										V	V	V		V	V	R	V	R	R		R		R	R						
(5)										V	V	R	R	V	V	R	V	R		R	R	R	V	V						
(6)										V	R	R	R	R	R	R	R	R	R		R		R	R	V					
(7)										V	V	R	V	V	V	R	V	R	R	V	R	V	R	R	V					
(8)										V	R	V	V	R	R	R	R	R	R	R	V	V	R	R	V	R				
Wrm(1)						V			R	R	R	R	R	R	R	R	R	R	R	R	R	V	R	R	V	R		V	R	V
(2)				V		V	V	V	V	V	R	R	V	V	V	R	R	R	R		V	V	R	R	V		V		V	V
	T5-like	T1-like	BF23-like	K10	H3	H1-like	T6	K3-like	Ac3-like	M1	Ox2-like	E4	K2	K20-like	K29	Ox5	K16	F27	H^+	V	E7	K17	A	T3	T4	K19	T2	T7-like	K15	H

TABLE 9-1 Continued

[a] When two or more bacteriophages have a similar pattern of resistant mutants, they have been grouped, and a representative bacteriophage nominated to describe this set of bacteriophages.

T1-like - T1, E25, K22, K26, K27, K30; T5-like - T5, D, E21; BF23-like - BF23, E15, K6, K8, K11, K12, M3, Ac4; H1-like - H1, H8, K18, K31, Ox1, K9; K3-like - K3, K4, K5; Ac3-like - Ac3, Ox3; Ox2-like - Ox2, Ox4; K20-like - K20, K21; T7-like - T7, E11, ØI, W31.

[b] Resistance is described as either:

R - mutants in the group or sub-group are fully resistant to all the bacteriophages.

V - some or all of the mutants are not fully resistant and/or not all of the bacteriophages involved are able to lyse the mutant.

[c] Sub-groups are included in brackets.

bacteriophage Ox5 is far more sensitive to one of these factors.

T-even (A2 morphology) bacteriophages probably have three types of receptors, for initial tail fibre attachment, for subsequent attachment of the spikes to the baseplate and for the tail tube in order to position it prior to unplugging (Benz and Goldberg, 1973). This last type of receptor might reside on the plasma membrane but the other two receptors are probably in the outer membrane. Alteration of these two receptors could occur independently, causing bacteriophage resistance. This might help to explain the results discussed above.

Bacteriophage E4 of A1 morphology has a complex set of requirements for infection if one considers its resistant mutants. Besides its specific resistant mutant, Efr, mutants from the Ttk, Ktw, Bar, Wrm and miscellaneous groups are resistant to it, although in all except the Bar and Wrm groups, these E4 resistant mutants are limited to one or two examples per group. It is not known whether these mutants each represent specific requirements for infection, or whether they demonstrate the sensitivity of the bacteriophage E4 receptor to outer membrane alterations.

One might ask whether bacteriophages from various parts of the world which select for the same resistant mutants and are similar using electron microscopic or serological techniques, are different isolates of the same bacteriophage. This seems quite likely as most of the differences are minor. For the enterobacteriaceae closely related to E. coli K-12, there are probably a limited number of bacteriophage species which can be found in most parts of the world with some

variation. The 54 bacteriophages studied (Table 2-2) vary considerably in their apparent requirements for infection, from those such as BF23 which requires a receptor (the bfe protein) but is apparently unaffected by a change in the cell which affects most other bacteriophages, through to those which are affected by up to six different types of host mutational changes.

The 54 bacteriophages represented in Figure 9-1 can be reduced to five main resistance types based on their ability to plaque on the resistant mutants shown. These are:-

(1) Those bacteriophages for which there is only one type of fully resistant mutant. This includes bacteriophage H3, the BF23-like bacteriophages and the T5-like bacteriophages.

(2) Those bacteriophages for which there are two types of fully resistant mutants, e.g. the T1-like bacteriophages.

(3) Those bacteriophages for which there are two types of resistant mutants, one of which is a specific fully resistant mutant and another which is cross resistant to many bacteriophages. This second type of mutant is not fully resistant to all members of the resistance group. The bacteriophages included in this category and their fully resistant mutants are T6, H1, H8, K9, K18, K31, Ox1 (Tsx); K3, K4, K5, Ox3, Ac3 (Con); K10 (Ktn); and T2 (Ttk).

(4) Those bacteriophages for which there are a range of mutants, which are resistant to them in any one of the ways described in Chapter 2 (Nomenclature). Most of these bacteriophages are included in the

Bar, Ktw, and Ttk resistance groups (Tables 4-3, 4-4 and 4-5), with the exception of bacteriophage T2 which is designated as a Type 3 bacteriophage above.

(5) This type is restricted to bacteriophages K15 and H, and the T7-like bacteriophages. The only mutants to these bacteriophages found in this survey are those cross resistant to a wide range of bacteriophages implying that in order for resistance to occur a large change in the cell surface must take place such that it interferes with the binding of other bacteriophages to their receptors.

THE STRUCTURE OF THE LIPOPOLYSACCHARIDE OF E. COLI K-12 AND THE ALTERATIONS IN CERTAIN BACTERIOPHAGE RESISTANT MUTANTS

Some of the mutants described in this thesis were defective in their LPS's (Chapter 6). E. coli K-12 is a rough organism (Rapin and Mayer, 1966) with no O-antigen sugars (Schmidt, 1973). Reports in the literature vary considerably as to the exact proportion of glucose, galactose and heptose in the LPS of E. coli K-12 (Rapin and Mayer, 1966; Eriksson-Grennberg, Nordstrom and Englund, 1971; Monner, Jonsson and Boman, 1971; Tamaki, Sato and Matsuhashi, 1971), as well as the presence of rhamnose in the LPS (Rapin and Mayer, 1966; Tamaki, Sato and Matsuhashi, 1971) or glucosamine in the polysaccharide chain (Schmidt, 1973; Koplow and Goldfine, 1974). Thus it appears that there are strain differences in E. coli K-12 LPS extracted in various laboratories. The

results, shown in Table 6-2, suggest that the ratio of the three neutral sugars in the LPS of P400 is approximately 3 moles of heptose: 2 moles of glucose: 1.3 moles galactose.

Nikaido _et al._ (1965) described a strain Y10 which they showed was deficient in the biosynthesis of thymidine diphosphate L-rhamnose and thus had no rhamnose in its LPS; unlike other derivatives of wild type _E. coli_ K-12 (Nikaido _et al._, 1965; Rapin and Mayer, 1966; Eriksson-Grennberg, Nordstrom and Englund, 1971) which had no such enzyme deficiency (Nikaido _et al._, 1965). The fact that strain AB1133 (and hence P400) is directly derived from strain Y10 (Bachmann, 1972) explains the absence of rhamnose in the LPS of our mutants. There is also no glucosamine attached to the polysaccharide portion of the LPS of strains P400, P460 (Con) and P425 (Ttk (3)), despite the previous finding of Schmidt (1973) that his strain of _E. coli_ K-12 has a complete core. Glucosamine appears to be an essential part of the completed rough cores of smooth strains of _Salmonella typhimurium_ (Osborn, 1966; Lüderitz _et al._, 1971; Stocker and Mäkela, 1971; Hämmerling, Lehman and Lüderitz, 1973) and _E. coli_ O 100 (Hämmerling _et al._, 1971) but not _E. coli_ O 71 (Müller-Seitz, Jann and Jann, 1968). Rooney and Goldfine (1972) have shown that their _E. coli_ K-12 strain CR34 has no glucosamine in the polysaccharide portion of its LPS and it is interesting that both this strain and AB1133 were derived from strain Y53 (Bachmann, 1972). However, whether or not other _E. coli_ K-12 strains have glucosamine in their LPS's, other than that attached to the lipid A portion, is uncertain.

Rather than propose a model for the structure of the _E. coli_ LPS in this thesis, it was found on examining the

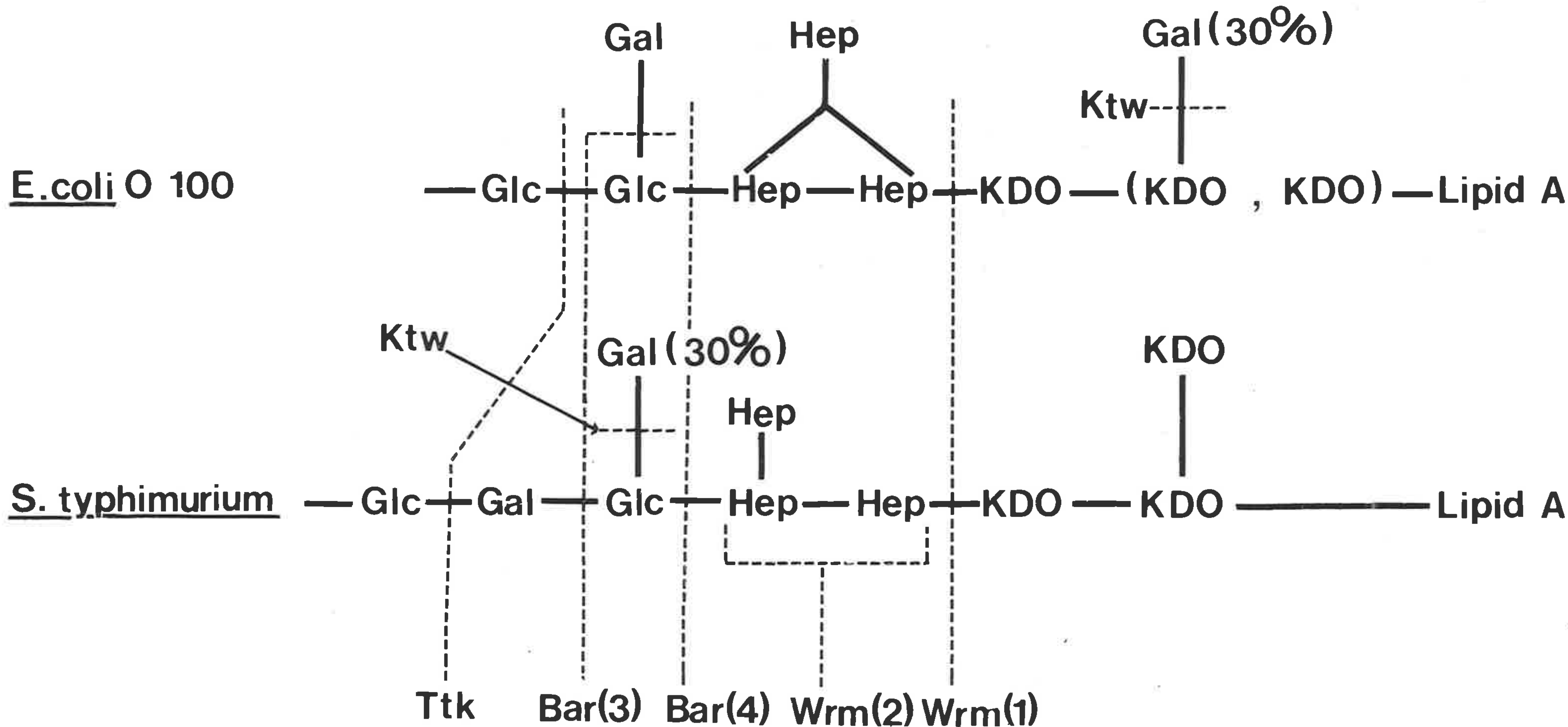
literature that the results obtained (Fig. 6-2) could be explained using partial rough core structures for the LPS of *Salmonella* (Hammerling, Lehman and Luderitz, 1973) or *E. coli* 0 100 (Hammerling et al., 1971). Using these structures (Fig. 9-1) simple derivations for the LPS's of the various resistant mutants could be demonstrated. This does not imply that the core of *E. coli* K-12 has the same linkages or even that the sugar sequence is identical to that of either model. However, there are almost certainly similarities between them. Schmidt (1972, 1973) has shown that *E. coli* K-12 LPS is serologically distinct from the rough cores of *Salmonella* Ra mutants and *E. coli* 0 100, but in this context, it is interesting that *Salmonella* Rb mutants which differ from *Salmonella* Ra mutants by a single glucosamine molecule (which is also lacking in the degraded polysaccharide of P400) are also serologically distinct (Lüderitz *et al*., 1971).

The most significant difference between the molar ratios for the partial structures of *S. typhimurium* and *E. coli* 0 100 shown (Fig. 9-1) and that of P400, is that the ratio of galactose to glucose is lowered in the latter strain (1.3: 2 moles as opposed to 2:2 moles). Rapin and Mayer (1966) have postulated two galactose molecules in the LPS of *E. coli* K-12, although they also get a lower ratio of galactose to glucose than would be expected. This is difficult to explain unless one considers that the galactose molecule, deleted in the Ktw strains (Fig. 9-1) is present in only 30% of the wild type LPS molecules. Hammerling *et al*. (1973) have discussed previously that the substitution of the main chain by side chain sugars, including galactose

FIG. 9-1

Visualization of the stages at which the lipopolysaccharide-deficient, bacteriophage resistant mutants of *E. coli* K-12 are altered. The lipopolysaccharide models used are the partial rough core structures of *E. coli* 0 100 (Hammerling *et al.*, 1971) and *S. typhimurium* (Hammerling, Lehman and Luderitz, 1973). Phosphate and ethanolamine bound to the rough cores of these strains have been omitted.

Abbreviations: KDO, 3-deoxy-D-manooctulosonate; Hep, L-glycero-D-mannoheptose; Glc, glucose; Gal, galactose.



Gal
Hep
Gal (30%)
Ktw
E. coli O 100
Glc
Glc
Hep
Hep
KDO
(KDO , KDO)
Lipid A
Ktw
Gal (30%)
KDO
Hep
S. typhimurium
Glc
Gal
Glc
Hep
Hep
KDO
KDO
Lipid A
Ttk
Bar(3)
Bar(4)
Wrm(2)
Wrm(1)

(Hellerqvist and Lindberg, 1971), can be incomplete.

The other anomally found is that there is one mole of heptose less than predicted by the model (Fig. 9-1), in all the mutants with altered LPS from the Ktw, Bar and Ttk groups (see Table 6-2). The presence of a branched heptose trisaccharide in the LPS core has been documented by Hämmerling *et al.* for R strains of *E. coli* O 100 (Hämmerling *et al.*, 1971) and *S. typhimurium* (Hämmerling *et al.*, 1973). In *Salmonella* only mutants with more complete LPS have been shown to have a heptose III unit and, in these cases, the degree of substitution of the heptose main chain with heptose III can vary from 20 to 90%. It was shown that for a *S. typhimurium* Ra mutant most heptose main chains were substituted by a heptose side chain while for two *gal*E mutants of *S. landau* M and *S. typhimurium* M only 25-50% of molecules had the heptose III unit. It is interesting to note that other authors (Schmidt, Jann and Jann, 1970; Eriksson-Grennberg, Nordstrom and Englund, 1971) have also found reduced heptose levels in mutants of *E. coli* which have other more distal sugars still present in their basal cores. Oneof these mutants (*lpc*A) maps in a similar place to the resistant mutants of the Bar and Wrm (2) groups (Fig. 8-3).

The simplest assumption is that the various mutants are blocked at the stages shown in Figure 9-1. Ktw (1) mutants have reduced levels of galactose and thus might be missing the galactose molecule shown which, as discussed above, is apparently present in only 30% of LPS molecules of strain P400. Rapin and Mayer (1966) have demonstrated that in *gal*E

mutants of E. coli K-12 the amount of galactose is halved. However, the reduced level of galactose in our Ktw strains does not lead to bacteriophage C21 sensitivity as it does in theirs, and genetic experiments (Chapter 8) indicate our mutant is not galE. The exact position of the defect in the Bar (4) mutants is not easily calculated from the results obtained (Table 6-2), but these mutants might be similar to the rfaF mutant of S. typhimurium (Wilkinson, Gemski and Stocker, 1972) i.e., defective in the addition of heptose II. Other classes of mutants might well correspond with other of the Salmonella rfa mutants.

Representative LPS-altered mutants of the Bar resistance group and sub-group 2 of the Wrm resistance group have been found to map between pyrE (72 min) and mtl (71 min) on the E. coli K-12 genetic map (Fig. 8-3). This is similar to results obtained by other authors for Salmonella (Stocker and Mäkela, 1971; Wilkinson, Gemski and Stocker, 1972) and E. coli (Schmidt, Fromme and Mayer, 1970; Eriksson-Grennberg, Nordstrom and Englund, 1971; Schmidt, 1973) rfa mutants. Salmonella has been well studied and a series of mutants affecting LPS core biosynthesis have been shown to map at various positions, with an rfa cluster cotransducible with pyrE or mtl. However, the Salmonella rfa mutants (rfaF, rfaG, rfaH) which correspond with Bar and Wrm (2) mutants do not all map in this region, and in fact, other mutants (rfaJ) with similar LPS compositions to Ttk mutants, unlike Ttk mutants, do map in this region of the Salmonella genetic map. Thus, although anomalies do exist, it is probable that the Bar and Wrm (2) genes form part of an rfa gene cluster, and the mutants in this region are affected in the enzymes of LPS

core biosynthesis.

The increased sensitivity to novobiocin of many mutants of the Bar and Wrm groups (Table 6-1) suggests that this antibiotic might be useful in revealing the larger changes in the structure of the LPS. This is in agreement with the results of Tamaki, Sato and Matsuhashi (1971) who studied novobiocin supersensitive and bacteriophage T4 resistant mutants.

When the ampicillin resistance of various bacteriophage resistant mutants was tested (Table 6-1) none of the classes demonstrated by Eriksson-Grennberg _et al_. (1971) were found. Their Class II mutants were galactose deficient, sensitive to bacteriophage C21 and in one case resistant to bacteriophages T3 and T4. However, the results in Chapter 6 suggest that alterations in the LPS composition brought about by mutation to phage resistance, are not sufficient to cause significant resistance to ampicillin, and only slight changes have been observed. It is probably significant that their ampicillin resistant class II mutants all contained two mutations (AmpAl, AmpII).

Other antibiotic changes are consistent with findings of Davies and Reeves,(1974a) who found alterations in the pattern of novobiocin, ampicillin, oleandomycin, fusidic acid and methicillin resistance or sensitivity as revealed by multodisks in a tol C mutant with an altered response to bacteriophages C21 and U3. Thus, it seems that these antibiotics together with erythromycin (Table 6-1) are useful in revealing differences in the LPS composition of _E. coli_ K-12 strains.

THE RECEPTOR SPECIFICITY OF BACTERIOPHAGES WHICH ADSORB TO LIPOPOLYSACCHARIDE

Alterations in the resistance of *E. coli* K-12 to bacteriophage C21 or sensitivity to U3 have been shown to be brought about by changes in the LPS (Rapin, Kalckar and Alberico, 1966; Rapin and Kalckar, 1971; Watson and Paigen, 1971). Strains of three different LPS compositions were found in these studies to be resistant to bacteriophage C21. These were the parent strain P400, mutants with a major defect in the LPS, and mutants with a relatively minor loss of only one glucose residue. These latter two C21 resistant mutant types correspond to those of Schmidt, Jann and Jann (1970). Thus, the actual nature of the bacteriophage C21 receptor remains obscure, but it probably requires at least two linked heptoses and possibly also a defined configuration of the LPS different to that found in the parent or some Ttk mutant strains which are resistant to C21. The bacteriophage U3 receptor appears to be made non-functional by the loss of galactose as previously described (Watson and Paigen, 1971).

Many of the bacteriophages, to which the LPS deficient mutants described in this thesis are resistant, are neutralised by LPS extracted by hot phenol-water (Table 5-1). These bacteriophages all belong to types 4 and 5 as defined earlier in this discussion. However, the relationship between resistance to a given bacteriophage and the LPS composition is not simple. Often a relatively small change in the LPS of a strain will lead to that strain being resistant to a given bacteriophage while larger changes in other strains leave the strains sensitive to the bacterio-

phage and very large changes lead again to resistance. Examples of this include bacteriophage C21 as discussed above, bacteriophage T4 (Tamaki, Sato and Matsuhashi, 1971; and Table 6-2) and bacteriophages K2, K20, K21 and K29 of the Ktw resistance group, as well as many others. Thus it appears that for many bacteriophages the receptor requirements are quite complex.

Furthermore, mutants with similar but not identical resistance patterns (e.g., strains P456 and P457, P425 and P474, P415 and P495, P405 and P436, P416 and P424 (see Tables 6-2 and 9-1)) often have similar amounts of core sugars. The variation in the resistance patterns of individual mutants is too great to be accounted for simply by the small number of possible defects in LPS biosynthesis indicated in Fig. 9-1, and there must be some method of introducing microheterogeneity. Based on the work of Hellerqvist and Lindberg (1971) and themselves, Hammerling *et al*. (1973) have suggested that there is evidence for considerable heterogeneity of the basal core structure of LPS within a strain and this may result in the heterogeneity noticed in phage resistance patterns.

POSSIBLE INVOLVEMENT OF THE LIPOPOLYSACCHARIDE-PROTEIN AS A RECEPTOR

Wu and Heath (1973) have postulated that essentially all the LPS of the cell envelope of *E. coli* is present as a LPS-protein in its native state. Hot phenol-water extraction of the LPS results in partial but not necessarily complete degradation of the molecule. Thus it may be that the protein part of this molecule is the receptor or a component of the

receptor for bacteriophages attaching to "lipopolysaccharide" extracted by this technique. In this context it is interesting that bacteriophages T3 and K10 attach to phenol-chloroform-petroleum ether extracted LPS, which has relatively less protein attached to it (Galanos, Lüderitz and Westphal, 1969), with a lower efficiency than to hot phenol-water extracted LPS (see Chapter 5). Changes in either the protein or the LPS might affect the conformation of the other component and hence its ability to act as a receptor.

From this study (Chapters 4, 6 and 7) it seems possible that other proteins are affected by alterations in the LPS, in that certain LPS mutants can lose their ability to bind bacteriophages which have been shown to have protein receptors. There are two main examples of this. The Ttk mutant P429 has an alteration in its LPS backbone and has become resistant to bacteriophage T2, which appears to have a lipoprotein receptor (Michael, 1968; De Pamphilis, 1971; Lindberg, 1973). Wrm mutants, which are also deficient in their core sugars, are in some cases resistant to bacteriophage T2 and also to some of the bacteriophages which, by virtue of their inability to lyse con$^-$ and tsx$^-$ mutants, probably have protein receptors. (Schnaitman, 1974b; see also Chapter 7).

The mutations which alter the LPS might directly affect the proteinaceous receptors (since some of the proteins of the cell wall may be carbohydrate associated (Schnaitman, 1974a), and one enzyme may be involved in the synthesis of both). Alternatively, if the LPS or LPS-protein interacts with the protein receptor of these bacteriophages in some

way, then a mutation in the former might affect the ability of the latter to bind bacteriophages. Wu and Heath (1973) have shown that there are proteins, other than the described LPS-protein, which are associated with LPS and thus probably interact with it. The fact that periodate treatment of the cell wall (Table 5-3) destroys the ability of bacteriophages T2, T6, E15 and K3 to bind to their receptors, which have been described as proteins (Michael, 1968; Weltzein and Jesaitis, 1971; Sabet and Schnaitman, 1973a,b; Skurray, Hancock and Reeves, 1974), further suggests the possible involvement of LPS in these receptors.

By examining the protein composition of the cell wall of various mutants, it was found that certain LPS-altered mutants (Chapter 6) were also protein-altered (Table 7-1). The altered protein was often protein 1 as defined by Schnaitman (1973b, 1974a). In addition, strains P489 (Bar (8)) and P424 (Wrm (2)) had peak 3 (proteins 3a and 3b) changes. These major peak alterations were possibly not the only protein changes in the mutants studied, however they were the only obvious ones. In a few cases it was possible to correlate differences in protein composition with resistance or sensitivity to specific bacteriophages; for instance peak 3 differences in Wrm mutants correlated with resistance or sensitivity of the two sub-groups of Wrm mutants to bacteriophages K3, K4 and K5 (which had peak 3 as their receptor (Chapter 7)). However, there was no obvious correlation between LPS alterations and protein defects, and the alteration of the LPS in the various mutants generally seemed to correlate far better with the bacteriophage resistance patterns than did the concomitant

protein alterations.

An alternative hypothesis to explain the observations that bacteriophages thought to have protein receptors are also affected by mutants with altered LPS, is that bacteriophages have two or more receptors of different specificity. It has been postulated that bacteriophage T4 has two different stages of adsorbtion to the outer membrane (Benz and Goldberg, 1973) which appear to correlate with the two thermodynamic stages (Lindberg, 1973). The first stage of adsorbtion, which is the reversible, non-enzymatic attachment of tail fibres (Lindberg, 1973) has been shown to occur using isolated purified LPS (Wilson, Luftig and Wood, 1970). However, it is possible that the second stage, which is the irreversible and possibly enzymatic (Lindberg, 1973) adsorbtion of tail pins, could involve protein in one of the ways discussed above. It is certainly known that aggregated LPS complex is required for this stage (Lindberg, 1973).

It seems likely that mutation to phage resistance might be a useful tool for selecting strains with altered LPS and the further bacteriophage receptor, enzymic and genetic studies of these mutants might be useful in elucidating not only the nature of the host-bacteriophage interaction but also the pathway of LPS biosynthesis in E. coli K-12.

CON$^-$ MUTANTS

Con$^-$ mutants were selected as resistant to bacteriophages K3 or K5 and found to be resistant to nine bacteriophages of T-even (A2) morphology (Table 4-1). These bacteriophages had altered efficiencies of plating on LPS-deficient mutants; some like bacteriophage Ox5 being affected by smaller LPS

defects such as those found in Ttk and Bar mutants, while others like bacteriophage K3 were affected only by the larger LPS alterations of Wrm (2) mutants. The fact that periodate destroyed the receptor activity of the cell wall for these bacteriophages (Table 5-3), further indicated the complicity of LPS in this receptor. Work by Koplow and Goldfine (1974) and Ames, Spudich and Nikaido (1974) in addition to results presented in this thesis (Chapters 6 and 7) has indicated that certain strains had a reduced amount of major proteins in the outer membrane and also an LPS deficiency resulting from a single mutational step. This, however, was not the case for Con$^-$ mutants which were shown to lack the major proteins 3a and 3b (Chapter 7) and yet they had a normal LPS composition (Table 6-2).

It is interesting that while relatively minor LPS changes can alter the pattern of sensitivity to various antibiotics (Table 6-1), and larger LPS changes can cause quite large alterations in this pattern (Tamaki, Sato and Matsuhashi, 1971; Table 6-1), the loss of two of the three major outer membrane proteins of E. coli K-12 in Con$^-$ mutants caused no alteration in the pattern of sensitivity to the antibiotics tested. Furthermore, while large LPS alterations (e.g. in Wrm mutants) caused resistance to up to 34 bacteriophages (Table 4-6), Con$^-$ mutants were resistant to only 9. It is likely that the Con$^-$ mutants, being unable to adsorb these bacteriophages (Chapter 7), are receptor-altered mutants. Thus it is obvious that relatively few bacteriophages can use the major proteins 3a or 3b as a receptor or receptor-component when compared with those using the LPS. In addition, although Con$^-$ mutants

are tolerant to colicins K and L (Table 6-3), they are sensitive to 17 colicins (J.K. Davies, personal communication) which represent nearly all of the known colicins active on E. coli K-12 (Davies and Reeves, 1974a). This implies that none of these 19 colicins have proteins 3a or 3b as their receptor in the outer membrane. It is therefore very interesting that these proteins have been implicated as the F pilus receptor in the recipient cell (Skurray, Hancock and Reeves, 1974).

A HYPOTHESIS CONCERNING THE METHOD BY WHICH DNA AND OTHER MACROMOLECULES ENTER THE CELL

Past work (summarized in Chapter 1) has implicated LPS and protein as the two components of the outer membrane that can act as receptors for bacteriophages. The results described in this thesis indicate that many "protein" receptors require the LPS in some way for activity, while concomitantly "LPS" receptors might involve protein. In bacteriophage resistant mutants, one or other of these receptor components is missing or altered (Chapters 6 and 7). Other studies have shown that changes brought about by mutation to phage resistance can also cause colicin tolerance (Reeves, 1972; Hancock, Davies and Reeves, 1974; Table 4-7), colicin resistance (see Chapter 4 for summary) and conjugational recipient deficiency (Skurray, Hancock and Reeves, 1974).

It is postulated that the receptors for bacteriophages, colicins and the F pilus are spatially arranged around a region which is probably a Bayer adhesion site. Bayer adhesion sites or "pores" have been visualized in plasmolysed

but not normal cells, and are the sites of adsorbtion of the T bacteriophages (Bayer 1968a,b). Evidence for the arrangement of receptor components around a specific site includes the co-operation of LPS and protein in various bacteriophage receptors, and the finding (Chapter 5) that many of the bacteriophage receptors are associated with LPS-protein-phospholipid complexes isolated by the extraction of the cell with dilute sodium hydroxide (Weidel, Koch and Bobosch, 1954) or Triton X-100 + Edta (Schnaitman, 1971b).

The way in which bacteriophages with noncontractile tails (e.g. T3 and T5) inject their DNA into the cell is not known, however it is postulated in this hypothesis that the second irreversible and possibly enzymatic step of adsorbtion (Lindberg, 1973) leads to unblocking of the tail and allows the phage DNA to be released into the Bayer pore from which it is taken up by an unknown mechanism. Adsorbtion of bacteriophage T5 to isolated LPS-protein-phospholipid complexes (Zarybnicky, Zarybnicka and Frank, 1973) leads to release of phage DNA under certain conditions, as does adsorbtion of λ to the partially purified receptor in the presence of chloroform (Randall-Hazelbauer and Schwartz, 1973). The above mechanism might also account for the entry of DNA via the pilus into the recipient cell during conjugation.

Large alterations to the outer membrane such as those caused by Wrm mutants (Table 6-2), Con mutants (Chapter 7) or tfr tsx double mutants (Schnaitman, 1974b) do not lead to the complete breakdown of Bayer pores since no bacteriophage resistant mutant isolated so far is resistant to all bacteriophages (Table 9-1). Thus neither the major

proteins nor the carbohydrate portion of the LPS of the cell wall is an essential component of Bayer pores; that is to say bacteriophage receptors are probably not themselves an essential component of the Bayer pores. The fact that all colicin tolerant mutants permit the entry of some bacteriophages (see Chapter 4) indicates that the proposed colicin transmission or entry mechanisms and their components are also not essential components of the Bayer pores.

It is postulated therefore that the Bayer pores are composed not of outer membrane components (with the possible exception of phospholipids, the KDO-lipid A portion of LPS and certain minor proteins) but of components derived from some more internal layer of the cell envelope.

APPENDIX

PUBLISHED AND SUBMITTED MATERIAL

The material contained in this thesis has been published or submitted for publication, in part, in the following papers:-

1. SKURRAY, R.A., R.E.W. HANCOCK, and P. REEVES. 1974. Con^- mutants : class of mutants in *Escherichia coli* K-12 lacking a major cell wall protein and defective in conjugation and adsorbtion of a bacteriophage. J. Bacteriol. 119 : 726-735.

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3. HANCOCK, R.E.W., J.K. DAVIES, and P. REEVES. 1974. Cross resistance between bacteriophages and colicins. Submitted to J. Bacteriol.

4. HANCOCK, R.E.W., and P. REEVES. 1974. Bacteriophage resistance in *Escherichia coli* K-12: Preliminary characterization of bacteriophage resistant mutants. Submitted to J. Bacteriol.

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