Resistance to colicins in

Escherichia coli K12

John K. Davies, B.Sc. (Hons).

Microbiology Department,

University of Adelaide.

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ESCHERICHIA COLI K-12

SUMMARY

This thesis describes an attempt to more fully characterise the alterations in a wide range of colicin resistant mutants (both receptor and tolerant mutants), in an effort to elucidate the mode of action of colicins, particularly in the steps subsequent to adsorption to the colicin receptor.

A collection of colicinogenic strains was obtained from different sources, and the colicins each strain produced were identified and cross-checked. With the aid of techniques that enabled separation of the different colicins produced by multi-colicinogenic strains, and the differentiation of receptor and tolerant mutants, resistant mutants were selected against each of the colicins, and checked for cross-resistance to each of the other colicins.

On the basis of their colicin resistance patterns, it was possible to place the mutants into 30 phenotypic groups. These groups included most of the receptor and tolerant mutant classes previously isolated, as well as many new classes.

Using these results, it was possible to divide colicins into two groups. Group A contains colicins A, E1, E2, E3, K, L, N, S4 and X, while group B consists of colicins B, D, G, H, Ia, Ib, M, S1, Q and V. Mutants selected as resistant to a colicin of group A may or may not be resistant to any other colicin of group A, but are never resistant to a colicin of group B. The reverse also applies.

Each of the phenotypic classes of mutants have been characterised in terms of their sensitivity to a range of antibiotics, detergents and surfactants, their resistance to a wide range of bacteriophages, and the

protein composition of their cell membranes. The various colicin resistance loci have been mapped.

Amongst the 21 phenotypic groups resistant to colicins of group A were the well characterised bfe and tsx receptor mutants, and the con mutants, which, in addition to being tolerant to colicins K and L, are defective in conjugation, resistant to a set of bacteriophages, and appear to lack a major protein in the outer membrane. Many of the mutants tolerate to colicins of group A showed substantial alterations in their sensitivity to a group of antibiotics, detergents and surfactants. Several of the mutant classes showed resistance to a group of bacteriophages and exhibited substantial alterations to another major protein species in the outer membrane.

The 9 phenotypic mutant classes resistant to colicins of group B include the previously described tonA, tonB and exbB mutants. The tonB, exbB and the newly described exbC mutants all appear to excrete colicin inhibitors. Both the tonB and exbB mutants show substantial alterations to the protein composition of the outer membrane.

The mode of action of colicins, in the steps subsequent to receptor adsorption, have been discussed, with the emphasis on the possibility that some colicin molecules may enter the cell. The possible mode of entry of these, and other macromolecules, is also discussed.

STATEMENT

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

John K. Davies.
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FOREWORD

It has become the custom to confine the use of the term colicin resistant to receptor mutants. Here I have used the term resistant to describe all forms of resistance to colicins, as in some cases it has become apparent that no clear distinction can be made between colicin receptor and colicin tolerant mutants.

CONTENTS

CHAPTER 1

INTRODUCTION

Colicins are bactericidal macromolecules produced by some members of the Enterobacteriaceae. Colicin production is associated with the presence in the producing cell of a Col factor, an extra-chromosomal plasmid. The discovery that the genes concerned with colicin production were located on a plasmid was first made by Fredericq (1954).

Classification of colicins.

The colicin which later came to be called V, was originally described by Gratia (1925). Later, Fredericq (1948) described a series of colicins he called A, B, C, D, E, F, G, H, I, J, K, S1, S2, S3, S4 and S5. Since this time, many of the colicins have been re-classified, and several new ones described.

The type strain for colicin C, E.coli CA57, has been lost (Fredericq, personal communication). Colicin E has been subdivided into E1, E2, and E3 (Fredericq (1956). Hamon and Peron (1964a) described a colicin E4, produced by E.coli H, but this colicin was not completely characterized, and has not been studied further.

Colicin F has been re-classified as colicin E2 (Fredericq (1965)). Stocker (1966) has divided colicin I into Ia and Ib. Colicin J, originally described as the colicin produced by E.coli CA62, has been shown to consist of colicins E1 and I (Fredericq (1965)).

The designation L has been used to describe two different colicins. The strain producing the colicin L described by Fredericq (1953) has been lost (Fredericq, personal communication). The colicin L in use today, that

produced by E.coli 398, is apparently the one originally described by Hauduroy and Papavassiliou (1962).

Colicin M (Fredericq (1951a)) was described as the colicin produced by bacteriophage T1 and T5 resistant mutants of E.coli CA7, in addition to V. Colicin N was first described by Hamon and Peron (1964b). Colicin O has not been described, although occassionally it is referred to (Hamon and Peron (1964a)). The name P has been used twice - once by Fredericq (1953) when he suggested that colicins G and H should be amalgamated into a single group called P. A different colicin, called P, was described by Hamon and Peron (1964b).

Colicin Q was described by Smarda and Obdrzalek (1966). Colicin S2 has been lost, and Shigella sonnei P9, the type strain for colicin S3, has been shown to be producing a mixture of colicins E2 and Ib (Fredericq (1965)). Shigella dispar P14, the type strain for colicin S5, has been shown to be producing colicin E1 (Fredericq (1965)).

Again, the designation X has been used twice. A colicin X has been described by Papavassiliou (1961), and the term has also been used by Miyami, Ozaki and Amano (1961) to describe the colicin produced by E.coli K235, in addition to colicin K.

A colicin S8 has been described by Nagel de Zwaig and Vitelli-Flores (1973), although no mention could be found in the literature of a colicin S6 or S7. A bacteriocin called JF246, produced by Serratia marcescens, but active on Escherichia coli, has been described by Foulds (1971).

A summary of all the colicins in use, and their type strains is shown in Table 1-1.

Table 1-1.
General characteristics of colicins.

Colicin	Type strain	Molecular weight	Target
A	Citrobacter freundii CA31	-	energy metabolism
B	Escherichia coli CA18	-	energy metabolism
D	Escherichia coli CA23	92,000	protein synthesis
E1	Escherichia coli K30	56,000	energy metabolism
E2	Escherichia coli K317	62,000	DNA synthesis
E3	Escherichia coli CA38	60,000	protein synthesis
G	Escherichia coli CA46	-	-
H	Escherichia coli CA58	-	-
Ia	Escherichia coli CA53	80,000	energy metabolism
Ib	Shigella sonnei P9	80,000	energy metabolism
K	Escherichia coli K235	45,000	energy metabolism
L	Escherichia coli 398	-	-
M	Escherichia coli CA7/T1,T5	18,000-27,000	-
N	Escherichia coli 284	-	-
Q	Escherichia coli II	-	-
S1	Shigella boydii P1	-	-
S4	Shigella dispar P15	-	-
S8	Shigella AD03-8	-	energy metabolism
V	Escherichia coli CA7	-	-
X	Escherichia coli K235	-	-
JF246	Serratia marcescens JF246	-	energy metabolism

Purification of colicins.

Several of the colicins have been purified and characterized. Timmis (1972) has purified colicin D and shown it to be a protein consisting of a single polypeptide chain with a molecular weight of 92,000. Schwartz and Helinski (1971) have purified colicin E1 and shown it to be a single homogeneous protein with a molecular weight of 56,000.

Colicin E2 has been purified by Reeves (1963), and also by Herschman and Helinski (1967), who again showed it to be a simple protein of 62,000 molecular weight. The same paper describes the isolation and purification of colicin E3 - a homogeneous protein with a molecular weight of 60,000. However, a more recent study (Glick et al. (1972)) has shown that colicin E3 can exist in a series of chromatographically distinguishable forms.

Colicins Ia and Ib are both single polypeptide chains with a molecular weight of approximately 80,000 (Konisky and Richards (1970); Konisky (1972)). Colicin K has been purified by Kunugita and Matsuhashi (1970) and Goebel (1973). It appears to be a protein with a molecular weight of 45,000. Like colicin E3, it can occur in multiple forms and it exhibits slight differences in mobility upon electrophoresis in a polyacrylamide gel (Goebel (1973)).

Colicin M has been isolated (Braun, et al., (1974)) and shown to be a protein complexed with phosphatidylethanolamine. This complex has a molecular weight of 27,000, but if the protein portion is removed it gives a molecular weight in sodium dodecyl sulphate of 18,000.

Thus, in each case where a colicin has been purified it has been shown to be protein in nature, and to consist of a single polypeptide chain. The

size of the different molecules, however, can vary from that of colicin M

(MW 18,000) to the largest colicin so far purified, colicin D (MW 92,000).

The mode of action of colicins.

Although the exact nature of the process which allows colicins to kill

cells is not understood, we do know the nature of the final intracellular

targets. Colicins ultimately kill a sensitive cell by interfering with

one of three basic intracellular functions - protein synthesis, DNA meta-

bolism, and what can be called energy metabolism. Colicins D and E3 inhibit

protein synthesis (Timmis and Hedges (1972); Nomura (1963,1964); Nomura and

Maeda (1965); Reeves (1968)), while colicin E2 inhibits DNA metabolism

(Nomura (1963,1964); Nomura and Maeda (1965); Reynolds and Reeves (1963);

Reeves (1968)).

Several other colicins appear to cause a general inhibition of energy

metabolism. Colicins A (Nagel de Zwaig (1969)), B (Arima, et al., (1968),

Guterman (1973)), E1 (Luria (1964); Reeves (1968); Fields and Luria (1969)),

Ia and Ib (Levisohn, et al., (1968)), K (Nomura (1963); Reeves (1968);

Fields and Luria (1969)), S8 (Nagel de Zwaig and Vitelli-Flores (1973))

and bacteriocin JF246 (Foulds (1971)) all appear to act in this manner.

The cell envelope of Escherichia coli.

Before discussing what is known of the way colicins ultimately come

to inhibit these various processes, it is necessary to examine their target,

the sensitive Escherichia coli cell - and more specifically its cell envelope.

Fig. 1-1 shows a schematic diagram of the Escherichia coli cell envelope,

adapted from those of Costerton, Ingram and Cheng (1974) and Inouye (1974).

The innermost layer, the cytoplasmic membrane, is thought to consist

of a continuous phospholipid bilayer into which various structural proteins are inserted. Fox (1972) has suggested that the various permease molecules, utilized in the active transport of small molecules to the interior of the cell, actually traverse the cytoplasmic membrane. It is suggested (Costerton, et al. (1974)) that the various binding proteins that assist in these transport processes are located on the outside of the cytoplasmic membrane, possibly in close association with the relevant permease molecules.

The cytoplasmic membrane is important as the site of synthesis of the peptidoglycan (Rogers (1970)), lipopolysaccharide (Osborn (1969)) and phospholipid (White et al. (1971)). Presumably the cytoplasmic membrane can also serve as a point of localization for enzymes concerned with cytoplasmic functions. The murein or peptidoglycan layer is immediately external to the cytoplasmic membrane, and Rogers (1970) has suggested that the two may be joined by nascent peptidoglycan, which is synthesized in the cytoplasmic membrane. Other suggestions (Costerton et al. (1974)) of a combination of the turgor pressure on the cytoplasmic membrane, and ionic bonding at the specific sites described by Bayer (1968a) may also explain why the cytoplasmic membrane and peptidoglycan seem to be in close association.

The fact that lysozyme or penicillin can cause the degradation of the peptidoglycan and lead to the formation of spheroplasts, has led to the suggestion that the peptidoglycan is essential for the maintenance of the shape of the cell (e.g. Costerton et al. (1974)), although this assumption has been challenged by Braun, et al. (1973). In the same paper, Braun and coworkers have calculated that the polysaccharide chains of the peptidoglycan are parallel, 1.25 nm apart, and constitute a single layer. Because of this,

and the fact that the peptide cross-linkages are calculated to be at least 1.03 nm apart, one would expect the peptidoglycan to be relatively porous to small molecules, and not to constitute an effective permeability barrier.

Braun and Rehn (1969) have isolated a specific lipoprotein which is covalently linked to the peptidoglycan in such a way that it extends outwards towards the outer membrane (Braun and Sieglin (1970)). Schnaitman (1971) had suggested that the covalently linked lipid portion of this molecule may serve to anchor the outer membrane at a fixed distance from the peptidoglycan, by hydrophobic interactions with the phospholipids in the outer membrane.

Recently, Inouye (1974) described a three dimensional model of the murein-lipoprotein constructed from the amino acid sequence of the protein portion of the molecule. He suggests that the protein consists of an ∝-helix, and that between 6 and 12 of the molecules can arrange themselves into a super-helical array that forms a passive diffusion pore in the outer membrane. The super-helical structure is anchored in the outer membrane by the lipid portions of the molecule, which fold back down the length of the protein molecule, and are inserted into the phospholipid bilayer (see Fig.1-1).

The region between the cytoplasmic and outer membranes (including the peptidoglycan) has been called the periplasmic space. It has been shown to contain free lipopolysaccharide units and murein-lipoprotein (Halegoua, et al. (1974)), as well as certain enzymes - a recent summary is contained in the review by Costerton, et al. (1974).

The outer membrane consists of a phospholipid bilayer complexed with structural protein and lipopolysaccharide. White, et al. (1972) have shown

Figure 1-1 : Schematic diagram of Escherichia coli envelope after Costerton, Ingram and Cheng (1974) and Inouye (1974). ⊕= bound anion, ⊖= bound cation, ⊖⊕ = adhesion point produced by ionic bonding, ⊥ = covalent bond, ✦= peptidoglycan, ⬭ = phospholipid, ⬚ = lipopolysaccharide, BP = binding protein, EC = cytoplasmic orientated enzyme, EM = cell wall synthesizing enzymes, EP = periplasmic enzymes, LP = lipid portion of Braun's lipoprotein, PL = protein portion of Braun's lipoprotein, PS = permease, S = structural proteins.

Mg

OUTER MEMBRANE

LP

PL

EP

LP

PL

EP

PERIPLASMIC SPACE

PL

S S PS BP S S EM

S EM

CYTOPLASMIC MEMBRANE EM

S EM

S EC S EC EM

DETAIL

TURGOR PRESSURE
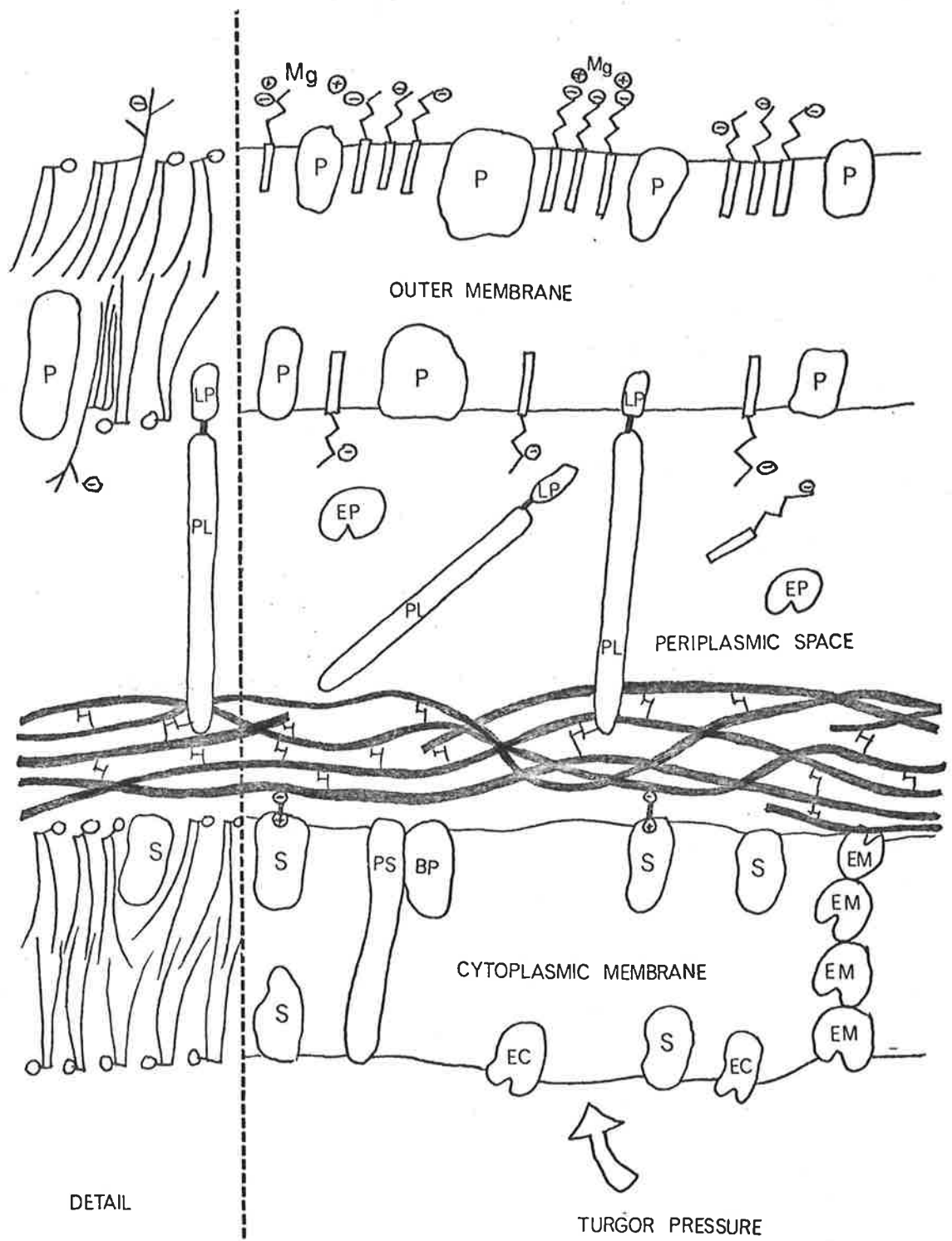
that the lipid content of the outer membrane is quantitatively similar to that of the cytoplasmic membrane, but that the outer membrane contains a greater proportion of lysophosphatidylethanolamine and palmitic acid.

The protein composition of the outer membrane appears to differ markedly from that of the cytoplasmic membrane. Although the results of various experiments with SDS-polyacrylamide gel electrophoresis on the protein composition of the outer membrane conflict in detail, it seems clear that three major protein peaks are observed after electrophoresis of protein preparations from the outer membrane, with approximate molecular weights of 60,000, 40,000, and 30,000 (Wu (1972); Henning, et al. (1973); Inouye and Yee (1973); Schnaitman (1973)). When the solubilization of the proteins is carried out at 100 C, however, all these proteins appear to run as one protein peak, with a molecular weight between 40,000 and 48,000.

Schnaitman (1973, 1974a, 1974b) has shown that E.coli K12 contains three protein species in its outer membrane. When the solubilization is carried out at 37 C, protein 1 runs on gel electrophoresis with an apparent molecular weight of 60,000 (peak A), while proteins 3a and 3b appear as peak C (MW approx. 30,000). When the solubilization is carried out at 100 C, all the proteins unravel and run in a position that is more indicative of their true molecular weight - peak B (MW approx. 40,000). Rosenbusch (1974) has recently purified protein 1, and shown it to be a homogeneous polypeptide, with a molecular weight of 36,500.

Hindennach and Henning (1975) have also purified proteins 1 and 3 (which they call I and II*), and another protein (called III), and shown them to have molecular weights of 38,000, 33,000, and 17,000 respectively.

The nature of the association between the phospholipid bilayer, and
its structural proteins, and the lipopolysaccharide (LPS) is unknown. It
has been suggested (Costerton, et al. (1974)) that there is a considerable
degree of ionic bonding, since chelating agents such as ethylenediamine-
tetraacetic acid (EDTA) cause disruption of the LPS. Schnaitman (1971a),
for example, envisages the site of action of EDTA (and therefore the site
of divalent cation stabilization) to be the site of insertion of the LPS
in the outer membrane.

A considerable amount of evidence has accumulated to suggest that the
outer membrane acts as a penetration barrier, or "molecular sieve", allow-
ing free passage of small molecules, but retarding larger ones. Payne and
Gilvarg (1968) have shown that tetra-lysine is allowed free passage, while
penta-lysine is restricted, which gives some indication of the effective pore
size. It is generally conceded that gram-negative bacteria are less suscep-
tible to a wide range of antibiotics than gram positives, and this tolerance
has been attributed to this penetration barrier. The barrier can be ef-
fectively broken down by treatment with EDTA (Leive, 1965), or in LPS-
defective mutants (Bowman et al. (1971), Monner et al. (1971), Gustafsson
et al. (1973), Tamaki et al. (1971)). The exact nature of these alterations
is uncertain, however, as changes in LPS structure may well be reflected in
other alterations in the outer membrane. Koplow and Goldfine (1974), for
instance, have recently described heptose-deficient mutants which have con-
siderable changes in the protein composition of the outer membrane.

Inouye (1974) has suggested that the pores formed by the murein-lipo-
protein super-helical array could form the "molecular sieve". The channel

size is sufficient to allow free passage of small molecules, while retarding larger ones. He suggests that the polysaccharide portion of the lipopolysaccharide might cover the entrance to the pore without restricting passive diffusion of small molecules. EDTA treatment is envisaged as causing the removal of part of the LPS, thus allowing larger molecules to diffuse through the outer membrane, and resulting in the breakdown of the penetration barrier.

In summary, the cell wall of Escherichia coli can be seen to consist of a cytoplasmic membrane (an effective cell boundary involved in active transport and various synthetic processes) which is firmly attached to a rigid peptidoglycan layer. The peptidoglycan is bound, via the murein-lipoprotein to the outer membrane, which operates as a molecular sieve, allowing passage of small molecules, but retarding larger ones, and therefore affording the cell some protection against antibacterial agents.

Colicin Receptors.

The first step in colicin action appears to be the adsorption of the colicin molecule to a receptor on the cell surface - a suggestion originally put forward by Fredericq (1946) to explain why colicin killing of sensitive cells was so rapidly irreversible. Evidence in support of this hypothesis came in two main forms. Firstly, it was shown that sensitive cells could be rescued from the effects of low concentrations of colicin with trypsin (Nomura and Nakamura (1962), Reynolds and Reeves (1963); Nomura and Maeda (1965)) - clearly suggesting that the colicin molecule is on the surface of the cell and exposed to the action of this proteolytic enzyme.

Secondly, Maeda and Nomura (1966) showed that nearly all of the

radioactive colicin added to a series of cell fractions stayed with the cell envelope fraction. On the basis of this evidence, and also the fact that it had been impossible to demonstrate colicin action in vitro, a general mode of action of colicins was proposed (Nomura (1963, 1964, 1967); Nomura and Maeda (1965); Reeves (1965)).

It was suggested that the colicin molecule adsorbed to a receptor on the surface of the sensitive cell, and stayed there, exerting its effect on the relevant target by transmitting a "message", perhaps via the cytoplasmic membrane. (Changeux and Thiery (1967)).

Since this hypothesis was put forward, several of the colicin receptors have been isolated and characterized. Konisky and Liu (1974) have solubilized the receptor for colicins Ia and Ib with Triton X-100, and shown this trypsin sensitive receptor complex to have a molecular weight of approximately 300,000.

Braun and Wolff (1973) have isolated the receptor for colicin M (and bacteriophage T5), and shown it to be a single polypeptide of molecular weight 85,000. This protein is also essential for ferrichrome-dependent iron uptake (Hantke and Braun (1975); Wayne and Neilands (1975)). Weltzien and Jesaitis (1971) have investigated the nature of the colicin K receptor, and shown it to be sensitive to proteolytic enzymes.

Sabet and Schnaitman (1971, 1973) have isolated the receptor for colicin E3 and shown it to have a molecular weight of 60,000. It appears to contain both protein and carbohydrate, and acts also as the receptor for vitamin B12 (Di Masi, et al. (1973)) and bacteriophage BF23 (Fredericq (1951b)).

Mutants altered in these receptors have been isolated, and the loci mapped on the genetic map of Escherichia coli K12 (Taylor and Trotter (1972)).

The cir locus, conferring resistance to colicins Ia and Ib, maps at 41 minutes (Cardelli and Konisky (1974)), and tonA, giving resistance to colicin M and bacteriophage T5, is located at 3 minutes (Curtiss III (1965)). The tsx locus, the locus for the receptor for colicin K and bacteriophage T6, maps at 9.8 minutes (Curtiss III (1965)), while bfe, which codes for essential parts of the receptors for colicins E1, E2, E3 and A, bacteriophage BF23 and vitamin B12, maps at 78.8 minutes (Buxton (1971); Jasper, et al. (1972); Di Masi, et al. (1973)).

In each of the above cases, it may be that the particular locus codes for a component that is shared by two or more receptors - thus enabling a mutation in the locus to simultaneously confer resistance to more than one colicin, or a colicin and a bacteriophage.

In each of the cases that has been investigated, two points seem clear - the colicin receptor is located in the outer membrane, and protein forms an integral part of the receptor complex.

Tolerance to colicins.

If, as had been suggested, colicin molecules stayed on the receptor on the cell surface, and killed the cell from there, it should be possible to isolate a second class of colicin resistant mutants. Mutants blocked in the transmission of a "message" from the receptor to the target would still be able to adsorb colicin molecules to the intact receptors on their cell surface, but would nevertheless be resistant to the colicin. Mutants with these characteristics - termed colicin tolerant mutants - have been isolated many times, and the various phenotypic classes are summarized in Table 1-2. A genetic map, showing the map location of the various colicin

Table 1-2.

Phenotypic classification of colicin tolerant mutants.

Phenotypic Class	Alternative Class name	Colicin Resistance Pattern (a)	Other colicins tested (b)	Locus	Reference (c)
Tol I	-	K	E1 E2 E3	-	1.
	-	K	E2 E3	-	2.
Tol II	-	E1 E2 E3 K	-	-	1.
	Cim	E1 E2 E3 A K	B D H Ia V	-	3.
	-	E1 E2 E3 A K	-	-	4,5.
	-	E1 E2 E3 A K	-	tol A	6.
	-	E1 E2 E3 A K JF246	Ib	tol A	7.
Tol IIa	-	pE1 pE2 pE3 pA pK	-	-	4.
	-	pE1 pE2 pE3	-	tol P	6.
Tol III	-	E2 E3 K	E1	-	1.
	-	E2 E3 A K	E1	-	4.
	Ref V	E2 E3 K	E1	-	9.
	-	E2 E3 A K	E1	tol B	6.
	-	E2 E3 A K JF246	E1 Ib	tol B	7.
Tol IIIa	-	E2 E3 A pK	E1	-	4.
	-	pE2 pE3 pA pK.	E1	tol B	6.
Tol IV	-	E2 E3	E1 K	-	1.

Table 1-2 continued.

Phenotypic Class	Alternative Class name	Colicin Resistance Pattern (a)	Other colicins tested (b)	Locus	Reference (c)
	Ref IV	E2 E3	E1 K	-	9.
	Cim	E2 E3 A	B D E1 H Ia K V	-	3.
Tol IVt	-	$E2^{40}$ $E3^{40}$	E1 K	-	1,10,11.
Tol IVl	-	$E2^{40}$ $E3^{40}$	E1 K	tol ? (d)	1,12.
Tol V	-	E1 E2 E3	K	-	1.
Tol VI	-	E1 E2	E3 K	-	1.
Tol VII	-	E2	E1 E3 K	-	1.
	Ref II	$E2^{30}$	E1 E3 K	cet B,C	9,13.
Tol VIII	E1-i	E1	E2 E3	-	14.
	-	E1	E2 E3 A K	-	4,5.
	Ref I	E1	E2 E3 K	-	9.
	Tol C	E1	E2 E3 K	tol C	15.
Ton B	bivtl	B I V	-	ton B	16.
	Exb A	B I V	-	ton B	17.
Exb B	biv	B I V	-	-	16.
	-	B I V	-	exb B	17.
Unclassified	-	E2 E3	E1	-	14.
	Cim	E2-K317 A	B E1 E2 E3 D H I K V	-	3.
	Cim	E2-K317	A B E1 E2 E3 D H I K V	-	3.
	A-r	A	E1 E2 E3 K	-	4.
	Ref III	$E3^{40}$	E1 E2	-	9.

Table 1-2 continued.

Phenotypic Class	Alternative Class name	Colicin Resistance Pattern (a)	Other colicins tested (b)	Locus	Reference (c)
	Ref VI	$E1^{40}E2\ E3$	K	–	9.
	Ref VI	$E1^{40}E2\ E3\ K$	–	–	9.
	Ref VII	$E2\ E3^{40}$	E1 K	–	9
	Ref VII	$E2\ E3^{40}K$	E1	–	9.
	Ref VIII	$E1^{40}E2\ E3^{40}$ K	–	–	9.
	Tol D	E2 E3	E1	<u>tol</u> D	18.
	Tol E	pE2 E3	–	<u>tol</u> E	19.
	Tol F	A K JF246	E1 E2 E3	<u>tol</u> F	7.
	Tol G	JF246	A E1 E2 E3 K Ib	<u>tol</u> G	7,8.
	Tol I	I	B E1 E2 E3 K	<u>tol</u> I	20.
bi		B I	V	–	16.
–		B I	V	–	17.
iv		I V	B	–	16.
–		I V	B	–	17.
b		B	I V	–	16.
–		B	I V	–	17.
i		I	B V	–	16.
–		I	B V	–	17.
v		V	B I	–	16.
–		V	B I	–	17.
–		B V	I	–	17.

(a) p = partial resistance, $E1^{40}$ = resistance to E1 at 40 C, $E2^{30}$ =

Table 1-2 continued.

resistant to E2 at 30 C, $E2^{40}$ = resistant to E2 at 40 C, $E3^{40}$ = resistant to E3 at 40 C.

(b) The mutants concerned were all sensitive to these colicins.

(c) References:

1. Numura and Witten (1967).

2. Wendt (1971).

3. Reeves (1966).

4. Nagel de Zwaig and Luria (1967).

5. Nagel de Zwaig and Luria (1969).

6. Bernstein, et al. (1972).

7. Foulds and Barrett (1973).

8. Foulds (1974).

9. Hill and Holland (1967).

10. Rolfe, et al. (1967).

11. Rolfe, et al. (1969).

12. Rolfe, et al. (1971).

13. Buxton and Holland (1973).

14. Clowes (1965).

15. Whitney (1971).

16. Gratia (1964).

17. Guterman and Dann (1973).

18. Burman and Nordstrom (1971).

19. Eriksson-Grennberg and Nordstrom (1973).

20. Cardelli and Konisky (1974).

(d) Described as tol D, but the term tol D has also been used for the locus described by Burman and Nordstrom (1971).

tolerant and receptor loci, is shown in Fig. 1-2. Many of the different mutant classes are not well characterized in terms of their colicin resistance pattern, which makes comparisons of mutants from different isolations difficult at times.

Tol I mutants (tolerant to colicin K, and sensitive to colicins E1, E2, and E3) were isolated by Nomura and Witten (1967). Wendt (1971) isolated mutants that were tolerant to colicin K, and sensitive to colicins E2 and E3, but unfortunately their resistance to other colicins was not tested. These mutants showed a dependence on potassium ions, and could have been Tol I mutants. Nomura and Witten (1967) showed the locus conferring tolerance to colicin K in their mutants mapped near gal, but the exact map position has never been determined.

Tol II mutants (tolerant to colicins E1, E2, E3, A and K) have been isolated by Nomura and Witten (1967), Reeves (1966), Nagel de Zwaig and Luria (1967, 1969), Foulds and Barrett (1973) and Bernstein et al. (1972). In all cases the colicin resistance locus has been shown to map near gal, and Bernstein et al. (1972) showed them to map at tol A, in a cluster of genes at 16.5 minutes (see Fig. 1-2). Nagel de Zwaig and Luria (1967) showed the Tol II mutants released increased levels of β-galactosidase, and also demonstrated a sensitivity to sodium deoxycholate (DOC), and EDTA (also Foulds and Barrett (1973)). Bernstein, et al. (1972) confirmed these sensitivities, and also demonstrated increased susceptibility to sodium dodecyl sulphate (SDS), vancomycin, bacitracin, and a slight sensitivity to zinc ions. Furthermore, they demonstrated an increased plaquing efficiency for λS bacteriophage, and observed that Tol II mutants tend to be

Fig 2: Genetic map of _Escherichia coli_ K12 showing the location

of the various colicin receptor and tolerance loci.

Tol ? = locus for Tol IVl mutants, mapped by Rolfe et al.,

(1971), ★ = region containing loci for some Tol I, Tol IV

and Tol V mutants.
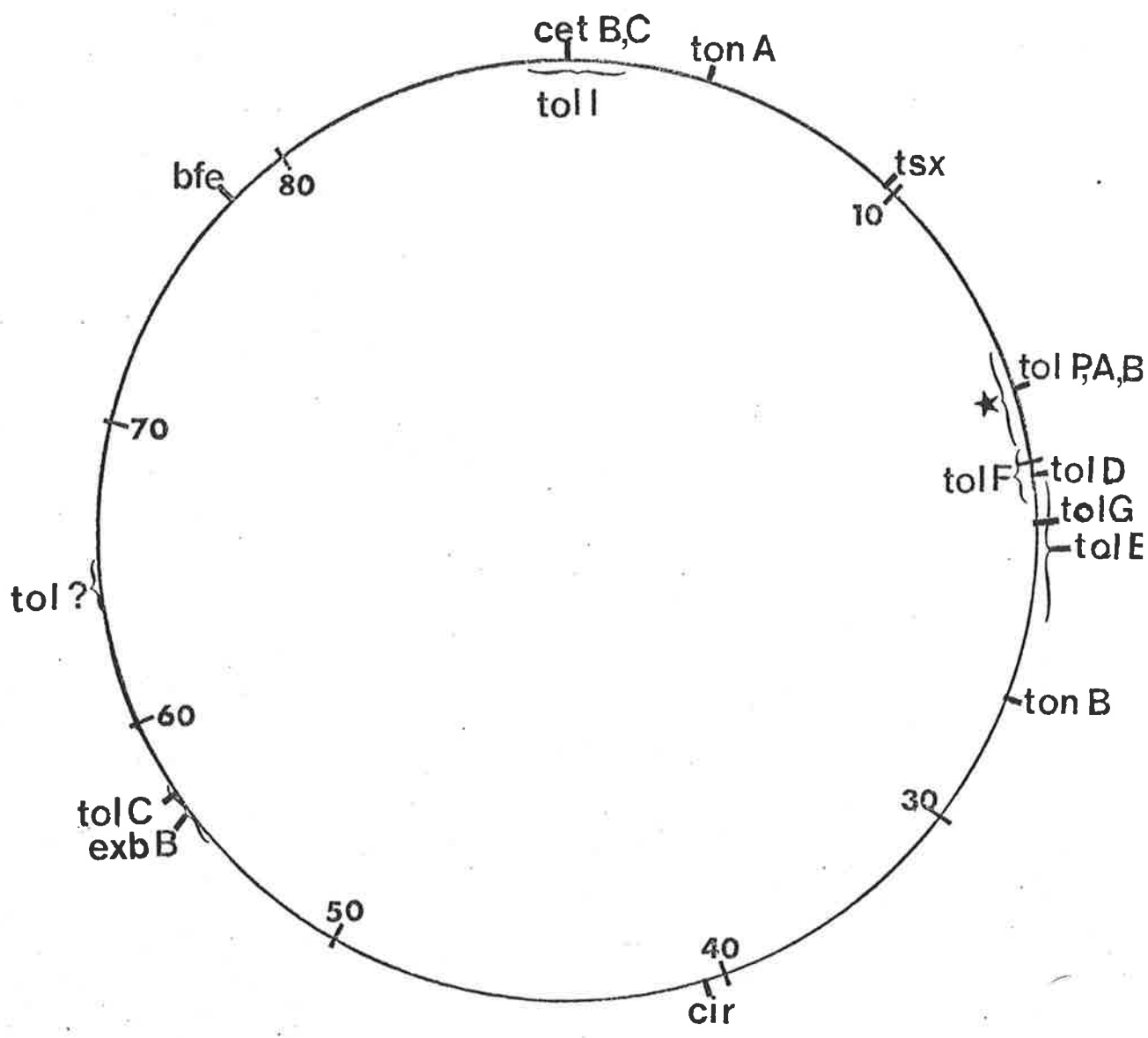
mucoid on minimal agar.

Tol IIa mutants (partially tolerant to colicins E1, E2, E3, A and K) were originally isolated by Nagel de Zwaig and Luria (1967), who showed them to be sensitive to DOC and EDTA, and to map close to gal. This was confirmed by Bernstein, et al. (1972) who placed the resistance locus at tol P at 16.5 minutes. In addition, they also demonstrated, in Tol IIa mutants, the sensitivity to SDS, vancomycin, bacitracin and λS bacteriophage, and the mucoid growth, seen with Tol II mutants.

Tol III mutants are tolerant to colicins E2, E3, A and K, but sensitive to colicin E1, and have been isolated many times (Nomura and Witten (1967), Nagel de Zwaig and Luria (1967), Hill and Holland (1967), Foulds and Barrett (1973), Bernstein et al. (1972)). Again, these mutants have been shown to be sensitive to DOC and EDTA (Nagel de Zwaig and Luria (1967), Foulds and Barrett (1973)), and SDS, vancomycin and bacitracin (Bernstein, et al. (1972)). The same paper shows them to be mucoid, exhibit an increased plaquing efficiency for λS bacteriophage, and to map at tol B (16.5 minutes).

Tol IIIa mutants also map at tol B, and exhibit the same pattern of sensitivity to DOC, SDS, EDTA, vancomycin and bacitracin (Nagel de Zwaig and Luria (1967), Bernstein, et al. (1972)). Nagel de Zwaig and Luria (1967) describe them as being tolerant to colicins E2, E3 and A, and partially tolerant to K, but Bernstein, et al. (1972) state that they are partially tolerant to all four colicins. They exhibit the same reduced plaquing efficiency for λS and mucoid growth on minimal agar as the Tol III mutants.

Tol IV mutants (tolerant to colicins E2 and E3, and sensitive to colicins E1 and K) have been described by Nomura and Witten (1967), Reeves

(1966) and Hill and Holland (1967). The two mutants isolated by Reeves (1966) both mapped near gal, as did 12 of the 15 mutants isolated by Nomura and Witten (1967). Neither the locus near gal, or the other(s) in Nomura and Witten's 3 other mutants has been mapped precisely.

Temperature sensitive mutants, displaying the Tol IV phenotype at 40 C, but not at 30 C, and therefore termed Tol IVt, have been isolated by Nomura and Witten (1967). Rolfe, et al. (1973,1974) have shown this mutant to plate bacteriophages λ and λC190c17 with a reduced efficiency, to plate ∅80 bacteriophage with an abnormal plaque morphology at 40 C, and to have a strong bias towards lysogeny. It has a decreased level of intracellular cyclic AMP, and SDS polyacrylamide gel electrophoresis has shown that it is missing a high molecular weight protein from the cell envelope. In addition to showing an increased tendency to form filaments, and a salt requirement, the Tol IVt mutant exhibited increased sensitivity to EDTA, SDS, DOC, vio-mycin, novobiocin, bacitracin, vancomycin, rifampin and actinomycin D.

A conditionally lethal mutant (Tol IVl), which displays the Tol IV phenotype at 40 C, but will not grow, was also isolated by Nomura and Witten (1967). The mutant is sensitive to colicins E2 and E3 at 30 C, and will grow normally at this temperature. The colicin resistance locus appeared to map in the vicinity of 65 minutes, and this was confirmed by Rolfe, et al. (1971). They also suggest that it has an increased tendency to form fila-ments, and is sensitive to certain antibiotics and detergents.

Tol V mutants are tolerant to colicins E1, E2 and E3, and sensitive to colicin K, and have been described by Nomura and Witten (1967). There is obviously some heterogeneity within this group because, of the two mutants

studied, one has a colicin resistance locus that mapped near gal, and the other mapped elsewhere.

A Tol VI mutant (tolerant to colicins E1, and E2, and sensitive to E3 and K) were isolated by Nomura and Witten (1967). The colicin resistance locus in this rare mutant remains unmapped.

Tol VII mutants (tolerant to colicin E2, and sensitive to E1, E3 and K) have been isolated by Nomura and Witten (1967), and a temperature dependent mutant (tolerant to colicin E2 at 30 C, but sensitive at 40 C, and sensitive to E1, E3 and K at both temperatures) was isolated by Hill and Holland (1967). Some of these mutants are sensitive to ultraviolet light (Holland 1967,1968), Holland and Threlfall (1969)). SDS - polyacrylamide gel electrophoresis has revealed that these Tol VII mutants have a greatly increased amount of a 44,000 molecular weight protein that seems to be loosely attached to the outer surface of the cytoplasmic membrane (Samson and Holland (1970), Holland and Tuckett (1972)). Buxton and Holland (1973) have shown that ultraviolet sensitive mutants displaying the Tol VII phenotype map at a locus called cet C, with other Tol VII mutants mapping at cet B. Both cet B and cet C are located, very close together, near thr on the genetic map.

Tol VIII mutants, which are tolerant to colicin E1, but sensitive to colicins E2, E3 and K, have been isolated many times (Clowes (1965), Nagel de Zwaig and Luria (1967,1969), Hill and Holland (1967)). The colicin re-sistance locus, tol C, has been mapped by Whitney (1971) at 59 minutes. The tol C mutants are sensitive to a wide range of dyes and also to DOC (Clowes (1965), Nagel de Zwaig and Luria (1967), Whitney (1971)). Rolfe and Onodera (1971) have shown that a mutant with a deletion that includes the tolC locus

is missing a membrane protein.

Ton B mutants are resistant to colicins B, I and V, and bacteriophages T1 and ∅80 (Gratia (1964), Guterman and Dann (1973)). Ton B mutants are sensitive to chromium, and are defective in iron transport (Wang and Newton (1971)), requiring iron for growth in minimal media (Guterman and Dann (1973)). They excrete a chelator of iron, enterochelin (Guterman and Dann (1973)), and the locus maps close to trp (Gratia (1964)).

Most of the mutations mapping near gal seem to be recessive to the wild type (Nomura and Witten (1967), Nagel de Zwaig and Luria (1967), Bernstein, et al. (1972)), while the cetB and cetC mutations (Buxton and Holland (1973)) are dominant.

Many other colicin tolerant mutants have been isolated, but because of incomplete data on their colicin resistance patterns, or for other reasons, they cannot be positively assigned to one of the phenotypic classifications used above. Some of the available data on these mutants is summarized in Table 1-2.

Clowes (1965) described the isolation of a mutant that was tolerant to colicins E2 and E3, but sensitive to colicin E1. It could be a Tol III or Tol IV mutant, as the sensitivity to colicin K was not checked. Reeves (1966) describes the isolation of two mutants which were tolerant to colicin E2-K317, but sensitive to several other colicins of type E2. They resembled the Tol VII mutants in that they were sensitive to colicins E1, E3 and K, but both loci map near gal, and not at, or near, cet.

Nagel de Zwaig and Luria (1967) mention a mutant (called A-r) that was resistant to colicin A, but sensitive to colicins E1, E2, E3 and K. They

were not able to determine whether it was a receptor or a tolerant mutant.

Hill and Holland (1967) isolated several temperature dependent tolerant mutants that do not fit easily into any of the classes. Ref III mutants are tolerant to colicin E3 at 40 C, but sensitive at 30 C, and tolerant to colicin K. Ref VI mutants were tolerant to colicin E1 at 40 C, but sensitive at 30 C, and tolerant to colicins E2 and E3. Of the mutants studied, 9 were also tolerant to colicin K, and all the resistance loci mapped close to gal. Thus, at 30 C, the Ref VI mutants were either Tol III or Tol IV mutants, while at 40 C they resembled Tol II or Tol V mutants. The Ref VII mutants were tolerant to colicin E2, and to colicin E3 at 40 C, but sensitive to colicin E3 at 30 C. Of the 19 mutants isolated, 18 were tolerant to colicin K. Thus, at 30 C, they resembled either the Tol VII mutants (E2 tolerant) or a previously undescribed class (tolerant to E2 and K), while at 40 C they appeared to be either Tol III or Tol IV mutants. Both the Ref VIII mutants isolated were tolerant to colicins E2 and K. In addition, at 40 C, as compared with 30 C, they became tolerant to colicins E1 and E3. Thus at 30 C they resembled the majority of the Ref VII mutants, while at 40 C they appear to be Tol II mutants. Even within this class there is evidence of heterogeneity, as only one of the two mutants maps near gal.

Burman and Nordstrom (1971) described an ampicillin resistant mutant that is tolerant to colicins E2 and E3, but sensitive to E1. We do not know whether it is resistant to colicin K, so it could be a Tol III or Tol IV mutant. The mutant is sensitive to DOC and EDTA, and shows changed sensitivity to a range of dyes and antibiotics. The colicin tolerance could be reversed in the presence of high magnesium ion concentrations, and

interestingly, removal of 50% of the lipopolysaccharide by treatment with EDTA did not affect either the tolerance of the mutant, or the sensitivity of its parent strain. No difference could be detected between the lipopolysaccharide of the mutant and its parent. The mutation, call tolD, is dominant over wild type and maps at approximately 20.5 minutes on the genetic map of Taylor and Trotter (1972), between pyr D and bio. It should be noted that tol D has also been used by Rolfe, et al. (1971) to describe the colicin resistance locus in the Tol IVt mutants.

Eriksson-Grennberg and Nordstrom (1973) have described a mutant tolerant to colicin E3, and partially tolerant to colicin E2. The mutants are susceptible to the lipopolysaccharide-specific bacteriophage C21, and analysis of their lipopolysaccharide has shown them to have lowered concentrations of galactose, glucose and rhamnose, when compared with the parent strain. The mutation, which can be suppressed by galactose, maps at tol E, between tol D (20.5 minutes) and pur B (25 minutes).

Foulds and Barrett (1973) have selected two different classes of mutants tolerant to bacteriocin JF246. One class, mapping at tol F were tolerant to colicins A and K as well as JF246, while the other, tol G, are tolerant to JF246 alone. The tol G mutants are sensitive to DOC and EDTA. The tol F locus was located at approximately 21 minutes, while the tol G locus appears to map near 23 minutes. This was confirmed by Foulds (1974), who showed tol G to map at approximately 21.8 minutes, between pyr D and fab A. Chai and Foulds (1974) have demonstrated that tol G mutants lack one of the major outer membrane proteins.

Cardelli and Konisky (1974) have isolated a mutant that is tolerant to

colicins Ia and Ib, but sensitive to colicin B. The relevant locus, which they have called <u>tol</u> I, maps between 89 and 1 minutes. The tol I mutant is resistant to sodium azide and phenethyl alcohol (PEA), and cannot utilize succinate, acetate or malate as sole carbon source.

Both Gratia (1964), and Guterman and Dann (1973) have isolated a series of mutants tolerant to different combinations of colicins B, I and V. All of those isolated by Gratia (1964), appear to have loci mapping between <u>his</u> and <u>str</u>, with the exception of the <u>ton</u> B mutants, and those tolerant to B, but sensitive to colicins I and V, which have loci mapping near <u>trp</u>. Some of Gratia's mutants (those tolerant to colicins B, I and V, or to B and I) had a methionine requirement. This was also noted by Guterman and Dann (1973), who also showed that some of the mutants isolated, like <u>ton</u> B mutants, excreted enterochelin. This second class of enterochelin excreting mutants, called <u>exb</u> B, appear to often be deletion mutants, and map between 56 and 58 minutes on the <u>Escherichia</u> <u>coli</u> K12 genetic map of Taylor and Trotter (1972).

This confusing array of data on tolerant mutants, although incomplete in many areas, did lend some support to the theory that colicins transmitted a "message" to their targets. The fact that colicin action could not be demonstrated <u>in</u> <u>vitro</u>, and that colicin action could be negated with proteolytic enzymes, combined with the evidence for membrane alterations in tolerant mutants, supported the assertion that the colicin molecule absorbed to its receptor, and acted from there.

Direct action of colicin.

Senior and Holland (1971) and Bowman, et al. (1971a) were able to show that colicin E3 acted to inhibit protein synthesis by causing a specific

cleavage of the 16S RNA in the 30S subunit of the ribosome. This led to the dramatic discovery by Boon (1971) and Bowman et al. (1971b) that colicin E3 could act in vitro. The fact that colicin E3 could act directly on its target, the ribosome, to cause the same specific damage it did in the intact cell, meant a drastic reappraisal of the hypotheses on the general mode of action of colicins. This evidence, when combined with the evidence of Mayhack, et al. (1973) that it is the colicin molecule itself that acts as the nuclease, clearly suggests that the colicin E3 molecule, or part of it, must enter the cell.

How a macromolecule like colicin E3 is able to penetrate the outer membrane, let alone the cytoplasmic membrane, is difficult to understand. Yet macromolecules quite clearly are capable of penetrating the cell envelope without causing immediate death of the cell, as the entry of DNA during con- jugation and bacteriophage action ably demonstrate. An answer may lie in the observation by Bayer (1968a) that in plasmolysed cells a series of sites become obvious at which the cytoplasmic membrane remains adhered to the cell wall. Bayer demonstrated that a wide range of bacteriophage attach to the surface of the cell at sites adjacent to these adhesions. Evidence has been put forward that these sites represent the site of export of lipopoly- saccharide to the cell surface (Muhlradt, et al. (1974)), and also the site at which there is an association between the DNA and the cell membrane (Olson et al., (1974)). It is suggested that these sites are areas where the cytoplasmic and outer membranes fuse to form a single structure, and that they represent the site of entry of bacteriophage DNA (Bayer (1968b)).

The frequent cross resistance of colicin receptor mutants to

bacteriophages suggests that they may have receptor components in common. If colicin molecules were also using these sites to gain entry to the cell, it would explain one of the anomalies evident in the observations of colicin action.

Although in all cases studied, the colicin receptors appear to be located in the outer membranes (Konisky and Liu (1974), Braun and Wolff (1973), Weltzien and Jesaitis (1971), Sabet and Schnaitman (1971,1973), there is a considerable amount of evidence that some colicins can act directly on the cytoplasmic membrane. Smarda and Vrba (1962) and Smarda (1965) have shown that colicin Q can act on spheroplasts, as will E1 (Obdrzalek, et al. (1971)). In addition, colicins E2 and G will act on stable L forms of Proteus mirabilis (Smarda and Taubeneck (1968)). Bhattacharyya et a. (1970) have demonstrated that colicin E1 blocks proline accumulation in membrane vesicles prepared from sensitive strains and receptor mutants, but not in vesicles prepared from tolerant mutants.

In addition, Takagaki, et al. (1973) have suggested that colicin K acts directly on the cytoplasmic membrane. If they made tsx mutants (receptor mutants for colicin K and bacteriophage T6) resistant to bacteriophage T4, they appeared to induce a specific change in the outer membrane. Although the double mutant was still tsx, as its resistance to bacteriophage T6 demonstrated, it had become sensitive to colicin K.

Finally, Phillips and Cramer (1973) and Cramer, et al. (1973) have shown a change in the fluorescent probe response of colicin E1 treated cells that they suggest is indicative of the movement of a colicin molecule (or a colicin-induced molecule) through the cell envelope to the cytoplasmic membrane.

These results could be explained in two ways. Firstly, the colicin receptors could be located on the cytoplasmic membrane. In this case, tolerant mutants would have alterations to the cell wall such that the colicin molecule could no longer bind to the receptor. Yet, in the cases so far investigated, the receptors are quite clearly located on, or in, the outer membrane.

An alternative hypothesis is that the colicin receptors are located on the surface of the cell, near regions where the cytoplasmic membrane is either fused with the outer membrane, or exposed in some way. The colicin molecules are then transmitted to the cytoplasmic membrane, and in the case of E3, to the interior of the cell. In this case, tolerant mutants would be mutants blocked in the transmission of the colicin molecule from the receptor to the exposed cytoplasmic membrane.

It should be emphasized that colicin E3 is the only colicin for which there is direct evidence that the colicin acts on an intracellular target. Indeed, some recent evidence has been interpreted as meaning that some other colicin molecules may not enter the cell (Phillips and Cramer (1973); Cramer, et al., (1973); Almendinger and Hager (1972)). These results have, however, been disputed (Cramer and Keenan (1974); Buxton and Holland (1974)).

It may be that colicins inhibiting DNA or energy metabolism have no need to enter the cell, but can act directly on their targets (which are located in, or near, the cytoplasmic membrane itself).

It seemed, therefore, that the isolation of mutants tolerant to a wider range of colicins, in a common genetic background, and the compilation of more complete data on their pleiotropy might well yield a better understanding

of the still poorly understood area of colicin action - that between adsorption and the final action on the target.

Aims.

The aims of this study were:

(1) The collection, cross-checking, and partial characterization of a set of colicinogenic strains producing all of the available colicins, in order to establish an updated classification of the colicins now known.

(2) The selection, in a common genetic background, of a set of resistant mutants, using each of the available colicins. This should allow a study of the relationship of the modes of action of all these different colicins (studies to date have concentrated on only a few colicins).

(3) As part of this study of the relationship between colicins to determine the extent, in these mutants, of cross-resistance to colicins other than the one with which they were selected.

(4) The determination of the map position of as many of the colicin resistance loci as possible.

(5) A more complete characterization than had previously been performed, of the various pleiotropic properties associated with mutation to colicin resistance (at the various known loci, as well as any newly described loci).

(6) The characterization of any changes in the protein composition of the cell membranes of the various mutants.

CHAPTER II

MATERIALS AND METHODS.

Media.

Nutrient broth (Difco 0003) was prepared double strength plus 5 mg/ml

sodium chloride. Nutrient agar was blood agar base (Difco 0045), prepared

as directed, without the addition of blood. Soft agar was prepared by mixing

equal volumes of nutrient broth and nutrient agar. Minimal liquid medium

is that described by Davis and Mingioli (1950). Minimal agar was prepared

by the addition of 20 mg/ml agar (Difco 0140) to minimal liquid medium.

Glucose was added as a carbon source at a final concentration of 5 mg/ml,

while growth supplements were at a concentration of 20 µg/ml. Eosin

Methylene Blue (EMB) agar was Difco 0511, prepared as directed. Tetrazolium

agar was made according to the formula of Achtman, Willetts and Clark (1972).

The relevant sugars were added at a concentration of 10 mg/ml, streptomycin

was used at a concentration of 200 µg/ml, and lipoic acid, where needed, was

at a final concentration of 0.005 µg/ml in all media used.

Bacterial strains.

The non-colicinogenic strains, other than the mutants isolated, are

shown in Table 2-1. The standard indicator strain used throughout was

Escherichia coli K12, strain AB1133, and all colicin receptor and tolerant

mutants were derived from this strain. Table 2-2 lists all the genetic

symbols used in this thesis. The colicinogenic strains used are shown in

Table 2-3. An effort was made to obtain, from different sources, several

stocks of the same or different colicinogenic strains producing the one type

of colicin.

Table 2-1.

Non-colicinogenic bacterial strains.

Strain	Relevant characteristics (a)	Source(b)
E.coli K12		
AB1133	thi argE his proA thr leu ara mtl xyl lacY galK supE strR	4.
P118	Tol II mutant of AB1133	4.
P117	Tol III mutant of AB1133	4.
A837	Tol II mutant of C600. thr leu thi supE lacY tonA	1.
A586	Tol VIII mutant of C600	1.
A597	Colicin A resistant mutant of C600	1.
A845	Tol III mutant of C600	1.
A9	Tol II mutant of AB1133	2.
B1	Tol III mutant of AB1133	2.
ASH120	Ref IV mutant of Hfr H.thyA thi lac Also tonB from selection for colicin I resistance	3.
AB259	thi λ^- rel HfrH	4.
RC740	met HfrC	4.
P601	met λ^- F1gal	4.
W3101 F2gal	galT λ^- F2gal	4.
JC3272	tsx str^R his lys trp lac gal mal	4.
P801	E.coli K12 wild type	4.
P1194	HfrH lip	4.
KL253	thi pyrD his trp tyrA recA mtl xyl malA galK strR F-	4.
Bacteriophage resistant mutants :-		
P400	non mutant of AB1133	(c)

Table 2-1 continued

P417	Ton A	(c)
P442	Ton B	
P445	Bfe	
P466	Ktn	
P407	Tsx I	
P433	Tsx II	
P460	Con	
P448	Efr	
P456	Ktw I	
P476	Ktw II	
P240	Ktw III	
P429	Kts III	
P429	Ttk I	
P423	Ttk II	
P425	Ttk III	
P474	Ttk IV	
P491	Miscellaneous : class I	
P443	Miscellaneous : class II	
P498	Miscellaneous : class III	
P237	Miscellaneous : class IV	
P493	Miscellaneous : class V	
P455	Bar I	
P492	Bar II	
P494	Bar III	

Table 2-1 continued

		(c)
P409	Bar III	
P404	Bar III	
P413	Bar III	
P415	Bar III	
P495	Bar III	
P496	Bar III	
P497	Bar III	
P405	Bar IV	
P428	Bar IV	
P436	Bar IV	
P490	Bar IV	
P402	Bar V	
P451	Bar VI	
P487	Bar VII	
P488	Bar VII	
P489	Bar VII	
P435	Wrm I	
P479	Wrm I	
P416	Wrm II	
P424	Wrm II	
P235	Wrm II	

(a) The abbreviations and nomenclature are essentially those of Demerec, et al. (1966), with the exceptions noted by Curtiss (1968).

(b) Strains kindly provided by
1. S.E. Luria   2.   B. Rolfe   3.   I.B. Holland   4. Stocks of this laboratory.

(c) The bacteriophage resistant mutants were kindly provided by R.E.W. Hancock. Full details of their isolation and characterization are described by Hancock and Reeves (1974a,1974b). They are all derivatives of P400.

Table 2-2.

Genetic symbols used in this thesis.

Locus	Mnemonic	Phenotypic trait affected	Map Location (a)	Reference (b)
ara	Arabinose	fermentation	1.2	1.
arg	Arginine	requirement	78.7	1.
bar	bacterio-phage A resistance	resistance to 10-19 differ-ent bacteriophages.	unknown	2.
bfe	BF23, colic-in E	receptor for colicins A,E1, E2,E3,phage BF23,vitamin B12	78.8	3,4,5.
bio	biotin	synthesis	17.5	1.
cbt	colicin B tolerant	tolerance to colicins B and D.	13.2	17.
cet	colicin E-two	tolerance to colicin E2	89.9	6,7.
chlA	chlorate	nitrate-chlorate reductase	17.6	1.
cim	colicin immune	identical with tolA	-	8.
cir	colicin I receptor	receptor for colicins Ia Ib and S1.	41	9,17.
cmt	colicin M tolerant	tolerance to colicin M	40-80	17.
con	conjugation	conjugation deficient	14.5	17.
cvt	colicin V tolerant	tolerance to colicins Q and V.	40-80	17.
efr	E4 resist-ant	resistance to bacteriophage E4	Unknown	2.
exbA	excretor	see tonB	-	-
exbB	excretor	excretes an inhibitor of colicin B.	56-58	10.
exbC	excretor	excretes an inhibitor of colicin B.	40-65	17.

Table 2-2 continued.

fabA	fatty acid biosynthesis	fatty acid biosynthesis	21.9	1.
gal	galactose	fermentation	16.8	1.
his	histidine	requirement	38.5	1.
ivt	IV tolerant	tolerance to colicins I and V.	38-45	17.
ktn	K-ten	resistance to bacteriophage K10.	unknown	2.
ktw	K-two	resistance to bacteriophage K2 and three others.	unknown	2.
lac	lactose	fermentation	9	1.
leu	leucine	requirement	1.5	1.
lip	lipoic acid	requirement	14.6	1.
lysA	lysine	requirement	54.7	1.
mal	maltose	fermentation	65.7	1.
metC	methionine	requirement	57.2	1.
mis	miscellaneous	resistance to miscellaneous bacteriophages.	unknown	2.
mtl	mannitol	fermentation	71	1.
non	non-mucoid	inability to form mucoid colonies.	39.5	1.
proA	proline	requirement	6.5	1.
purB	purine	requirement	24.9	1.
pyrD	pyrimidine	requirement	21.5	1.
rcx	resistant to colicin X	resistance to colicin X	8-13	17.
rel	relaxed	regulation of RNA synthesis	53	1.
serA	serine	requirement	56	1.
strA	streptomycin	resistance	64	1.

Table 2-2 continued

supE	suppressor	amber suppressor	14.9	1.
thi	thiamine	requirement	57.2	1.
thr	threonine	requirement	0	1.
thyA	thymine	requirement	54.3	1.
tolA	tolerance	tolerance to colicins E1, E2 and E3.	16.5	11.
tolB	tolerance	tolerance to colicins E2 and E3.	16.5	11.
tolC	tolerance	tolerance to colicin E1	59	12.
tolD	tolerance	tolerance to colicins E2 and E3	20.5	13.
tolE	tolerance	tolerance to colicin E3, partial tolerance to E2.	20.5-25	14.
tolF	tolerance	tolerance to colicins A,K and JF246.	21	15.
tolG	tolerance	tolerance to bacteriocin JF246.	21.8	15,16.
tolI	tolerance	tolerance to colicin I.	89-1	9.
tolJ	tolerance	tolerance (partial) to colicin E2.	0.1	17.
tolP	tolerance	partial tolerance to colicins E1,E2 and E3.	16.5	11.
tonA	T-one	resistance to colicin M, and bacteriophages T1,T5 and Ø80.	3	1.
tonB	T-one	resistance to colicins B,I and V, and bacteriophages T1 and Ø80.	26.8	1,10.
trp	tryptophane	requirement	27	1.
tsx	T-six	receptor for colicin K and bacteriophage T6.	9.8	1.
ttk	T2,T4,K19	resistance to bacteriophages T2,T4,K19 and others.	unknown	2.
wrm	wide-range mutant	resistance to a wide range of bacteriophages.	unknown	2.

Table 2-2 continued.

xyl	xylose	fermentation	70.1	1.

(a) Map location (in minutes) on the genetic map of Taylor and Trotter (1972).

(b) References :-

1. Taylor and Trotter (1972).

2. Hancock and Reeves (1975a).

3. Buxton (1971).

4. Jasper, et al. (1972).

5. Di Masi, et al. (1973).

6. Hill and Holland (1967).

7. Buxton and Holland (1973).

8. Reeves (1966).

9. Cardelli and Konisky (1974).

10. Guterman and Dann (1973).

11. Bernstein, et al. (1972).

12. Whitney (1971).

13. Burman and Nordstrom (1971).

14. Eriksson-Grennberg and Nordstrom (1973).

15. Foulds and Barrett (1973).

16. Foulds (1974).

17. This thesis.

Table 2-3.

Colicinogenic strains.

Strain	Source (a)
Citrobacter freundii CA31	1,2,8.
E.coli 23	2.
E.coli CA18	8.
E.coli K89	8.
E.coli AG097	7.
E.coli CA23	8.
E.coli K12 W3110 str^R (Col CA23)	6.
E.coli K53	8.
Shigella dispar P14	8.
E.coli CA42	8.
Shigella sonnei P9	8.
E.coli 12-317	1.
E.coli K317	8.
E.coli K12 W3110 (Col CA38)	3.
E.coli CA46	4,5,8.
E.coli CA58	5.
Paracolon CA62	4,5,8.
E.coli CA53	5,8.
Salmonella typhimurium ST4 (Col P9)	4.
E.coli K12 (Col P9)	8.
E.coli K216	8.
E.coli K235	8.

Table 2-3 continued.

E.coli 398	2.
E.coli 206 Md33 TH106 F'lac (Col K260)	4.
E.coli K260	8.
E.coli 284	2,4.
E.coli 285	2.
E.coli II	2,4.
Shigella boydii P1	4,5.
Shigella dispar P15	4,5.
E.coli CA7	8.
E.coli Ar19a	5,8.
E.coli K12 185II NxII S7a (Col K235)	2,4.

(a) Immediate source : -

    1. N. Atkinson.

    2. Y. Hamon.

    3. D. Helinski.

    4. J. Smarda.

    5. P. Fredericq.

    6. K. Timmis.

    7. B. Stocker.

    8. Stocks of this laboratory.

All strains were stored as freeze-dried cultures, and working stocks were maintained on nutrient agar slopes, which were stored at 4 C.

The set of bacteriophage resistant mutants were kindly provided by R.E.W. Hancock.

Bacteriophages.

The bacteriophages employed in this study are shown in Table 2-4. A large set of virulent bacteriophages of Escherichia coli K12, not used in the initial classification of the mutants, was provided by R.E.W. Hancock.

Cultural Conditions.

Overnight cultures were obtained by innoculating a 10 ml culture from a single colony or nutrient agar slope. The culture was shaken overnight at 37 C. Overnight cultures normally achieved an O.D.650 of 2.0, logarithmic (log) phase cultures were obtained by diluting an overnight culture 10 to 50-fold, and shaking the culture at 37 C. Log phase cultures were taken as those with an O.D.650 of 0.3 to 0.4. Late log phase cultures were those with an O.D.650 of 0.7 to 0.9.

Typing of colicinogenic strains.

The media, biochemical tests, and methods employed in the biochemical typing of several of the colicinogenic strains were those described by Edwards and Ewing (1966).

Isolation of colicin resistant mutants.

Two main methods were used to isolate mutants resistant to the various colicins. Early experiments were done by streaking an overnight culture of the colicinogenic strain across a nutrient agar plate, and then incubating overnight at 37 C. The bacterial growth was killed by exposure to chloroform

Table 2-4.

Bacteriophages.

Bacteriophage	Comments	Source (a)
BF23		1.
Ø80vir	virulent mutant of Ø80	2.
T1		1.
T5		1.
T6		1.
λcI90cI7		2.
C21	lipopolysaccharide specific	1.
U3	lipopolysaccharide specific	1.

Virulent bacteriophage set : (b)

Ac3,Ac4.

B

D

E4,E7,E11,E15,E21,E25.

F27.

H,H$^+$,H1,H3,H8.

K2,K3,K4,K5,K6,K8,K9,K10,K11,K12,K15,K16,K17,K18,K19,K20,K21,K22,K25,K26,
K27,K28,K29,K30,K31.

M1.

Ox1,Ox2,Ox3,Ox4,Ox5.

T2,T3,T4,T7.

W31.

V.

(a) Source :

    1. Stocks of this laboratory.

    2. Kindly supplied by B. Rolfe.

(b) Provided by R.E.W. Hancock.

vapour, and the plate overlayed with soft agar seeded with an overnight culture of AB1133 at a concentration of approximately 10^7 cells/ml. All the resistant colonies appearing in the zone of inhibition were then picked off, and streaked on nutrient agar plates so as to give single colonies.

So as to reduce the possibility of isolating resistant mutants derived from a single clone of cells, an alternative procedure was used in later experiments. After the colicinogenic strain was killed with chloroform, the plates were overlayed with nutrient agar. Individual overnight cultures of AB1133 were then streaked over the surface of a series of plates so as to give single colonies. A few colonies were picked from above the colicinogenic strain on each plate, and subjected to a further single colony isolation.

Except when otherwise stated, all mutants were either selected against the colicin from a colicinogenic strain that had been shown to be producing only one colicin, or in the case of a polycolicinogenic strain, from a colicin zone which had previously been separated by electrophoresis (see below).

In many cases, it was impossible to directly select for revertants, so no analysis of the reversion frequency of the various mutants was done. In every case, however, the mutations seemed to be stable, and no instances of apparent reversion were observed.

Triple layer plate test to distinguish receptor and tolerant mutants

Because of the large number of mutants being screened, and the fact that many colicins are difficult to obtain in liquid media, a plate test was used to distinguish colicin sensitive, tolerant and receptor strains. The method

employed is an adaption and amalgamation of those of Fredericq (1957) and
Hill and Holland (1967). The colicinogenic strain was streaked across a
nutrient agar plate and grown overnight at 37 C. The plate was then ex-
posed to chloroform and overlayed with nutrient agar. The strain to be
tested was then streaked across the plate at a right angle to the original
colicinogenic streak and grown overnight at 37 C. It, too, was then killed
by exposure to chloroform vapour, the plate overlayed with soft agar seeded
with an overnight culture of the indicator strain, AB1133, at a concentration
of approximately 10^7 cells/ml, and incubated overnight at 37 C. A similar
plate test has been developed by Fredericq (personal communication).

Receptor plate test.

To test the ability of the triple layer plate test to distinguish
receptor and tolerant mutants for some of the colicins, AB1133 was streaked
on a sterile "Millipore" membrane lying on the surface of a nutrient agar
plate. After overnight growth at 37 C, the bacteria were killed with
chloroform vapour, and the membrane removed and placed on the surface of a
nutrient agar plate that had previously been streaked with the colicinogenic
strains, grown overnight at 37 C, killed with chloroform, and overlayed with
nutrient agar. The plate was then overlayed with soft agar seeded with AB1133,
and incubated overnight at 37 C. The seeded soft agar layer above the cross-
streak on the membrane was removed, and the shape of the inhibition zone
inspected.

Quantitation of colicin resistance.

Crude colicin preparations were obtained by centrifuging overnight
culture of the colicinogenic strain, and sterilizing the supernatant with

chloroform. Partial resistance, undetectable on the triple layer plate test, can be detected by spotting serial two-fold dilutions of the colicin preparation in nutrient broth, onto the surface of a soft agar layer seeded with the strain to be tested. By comparing the dilution needed to inhibit the growth of a particular mutant with that of its parent, slight differences in sensitivity to a particular colicin can be detected.

Electrophoretic techniques.

In cases where a colicinogenic strain was producing more than one colicin, electrophoresis was used to achieve separation of the colicins. Large flat dishes, approximately 30cm by 30cm by 3cm deep were used to grow the colicinogenic strains. These dishes were made from plate glass, held together with autoclave tape, and with aluminium lids. The colicinogenic strain was streaked across the dish, containing nutrient agar, and grown overnight. The bacterial growth was scraped off, and the dish sterilized by exposure to chloroform. The plate was then subject to electrophoresis for 16 h at 4 C, with a constant voltage of 200 V. Nutrient broth, diluted two-fold in distilled water, was used as an electrolyte. During electrophoresis, the current rose from approximately 30 mA per plate to approximately 150 mA per plate. For the isolation of resistant mutants, the plate was then over-layed with soft agar plus streptomycin, seeded with an overnight culture of AB1133 at a concentration of approximately 10^7 cells/ml, and grown overnight at 37 C. Resistant colonies were then picked from the zone required, and streaked on nutrient agar plates so as to give single colonies.

To test a series of mutants for colicin resistance, the plate was exposed to chloroform vapour, overlayed with nutrient agar, and the strains

to be tested streaked at right angles to the original colicinogenic streak. After incubation overnight at 37 C, the plate was exposed to chloroform vapour once more, and overlayed with a soft agar layer seeded with the indicator strain. A similar electrophoretic technique has been used by Smith (1965).

Plate test for strains excreting colicin inhibitors.

The plate test used by Guterman and Luria (1969) was used to test the strains excreting substances that inhibited colicin action.

Inhibition of colicins by enterochelin.

The ability of enterochelin to interfere with the action of a particular colicin was tested by streaking the relevant colicinogenic strain across a nutrient agar plate, and growing it overnight at 37 C. The colicinogenic strain was killed by exposure to chloroform, and the plate overlayed with nutrient agar. Varying dilutions of enterochelin were streaked across the plate at right angles to the original colicinogenic streak, and allowed to dry in. The plate was then overlayed with soft agar seeded with approximately 10^7 cells/ml of AB1133, the indicator strain. The ability of the indicator strain to grow across the colicin inhibition zone, at the spot where the enterochelin was cross streaked, was interpreted as meaning that enterochelin interferred with the action of the particular colicin. I.G. Young kindly supplied a gift of some enterochelin.

Bacterial Crosses.

In bacterial crosses in which F'gal strains were used as donors, 1ml of a log phase culture was mixed with 5 ml of an overnight culture of the F⁻ strain. After 1 h incubation at 37 C, the culture was centrifuged, and the

bacterial pellet resuspended in minimal liquid medium and plated on the appropriate media. Other matings were done by plating 0.1 ml samples of the Hfr and F⁻ strains directly onto the selective plate.

For analysis of genetic linkage by gradient of transmission studies, the method of de Haan, et al. (1969) was employed. Recombinants were picked off and streaked for single colonies on nutrient agar plages, before being innoculated onto master plates for replica plating on the appropriate media.

Transduction with bacteriophage P1.

Phage lysates were prepared by plating various dilutions of phage P1 in a soft agar layer plus 2mM Ca^{++}, seeded with the donor strain. A plate in which lysis was just complete was selected, the soft agar layer removed, and an equal volume of nutrient broth added. This was shaken, chloroform added, and allowed to stand before centrifuging. The supernatants were collected, and the phage assayed on AB1133. The phage and the recipient bacteria were mixed in the ratio 1:10 in nutrient broth plus 2mM Ca^{++}. The mixture was incubated at 37 C for 30 min. before resuspending the bacteria in minimal liquid medium and plating on the appropriate selective medium. Transductants were picked off, streaked for single colonies on nutrient agar plates, and then stabbed into nutrient agar master plates with sterile toothpicks. They were then replica plated onto the appropriate media.

The formula of Wu (Taylor and Trotter (1972)) was used to convert cotransduction frequencies to map distances.

Antibiotic sensitivity tests.

The sensitivity of various mutants to a range of antibiotics was tested

by overlaying a nutrient agar plate with 5 ml of soft agar seeded with an overnight culture of the strain to be tested, at a concentration of approximately 10^7 cells/ml. A "Multodisk" (Oxoid Ltd., codes S-1 and 30-9C) was then layered onto the surface of the plate, which was then incubated overnight at 37 C. The sensitivity of the various mutants to ampicillin was also determined by plating aliquots of an overnight culture on a series of nutrient agar plates, containing varying concentrations of ampicillin. This gave a more accurate indication of the minimal inhibitory concentration of ampicillin for individual mutants.

Bacteriophage sensitivity tests.

The sensitivity of the mutants to various phages was tested by streaking the mutants against each phage on nutrient agar plates, and incubating the plates overnight at 37 C.

A much wider survey of the bacteriophage resistance patterns of the various mutants was performed by R.E.W. Hancock, using a multiple syringe bacteriophage innoculator (Hancock and Reeves (1974a)), and the virulent bacteriophage set shown in table 2-4, as well as most of the phage used in the initial tests. Resistance to both high (10^7 pfu) and low (10^3 pfu) titres of phage were checked.

Testing sensitivity to detergents and surfactants.

The method employed is essentially that of Bernstein, et al. (1972). An overnight culture of the strain to be tested was diluted 20-fold in 10 ml of nutrient broth. The reagent was then added at the concentration stated, and the culture shaken at 37 C for 4 h, at which time a viable count was performed. The log of the percentage survival (related to the viable count

of the control after 4 h growth), was used as an index of sensitivity.
Thus 2.00 indicates that the agent had no effect on growth over the 4 h
period. It should be noted that only Triton X-100 had no effect on the
parental strain AB1133.

Membrane preparations.

The methods used for the preparation and solubilization of cell membranes
were basically those of Schnaitman (1971b, 1973). These methods were employed
because Schnaitman has more satisfactorily than others identified the major
outer membrane proteins of Escherichia coli, and developed procedures for
separating them on SDS-polyacrylamide gels.

Whole cell envelope protein preparations were prepared from cells
grown in nutrient broth to late log phase. The parental strain, AB1133,
was observed to grow in nutrient broth with mean generation times varying
between 44 and 57 minutes, but under these conditions had a mean generation
time of approximately 50 minutes. When harvested at an O.D.650 of 0.8
(9 x 10^8 cells/ml), the cells had not yet entered stationary phase, which
occurred at an O.D.650 of between 0.9 and 1.1. The various mutants all
had mean generation times between 43 and 59 minutes, with the exceptions of
P535 and P295 (see Chapter IV), which had mean generation times between 37
and 38 minutes. None of the mutants had shown any indications of having
entered stationary phase growth at the time they were harvested.

The cells were harvested by centrifugation at 5000 x g for 20 min,
washed in 0.01M Tris-HCl buffer, pH 7.4, and centrifuged again. The pellet
was "snap-frozen" at -20 C, and broken in an X-press (LKB-Biotec).

The broken cells were resuspended in Tris-HCl buffer, a speck of

deoxyribonuclease (Sigma Chemical Company) added, and left on ice for 30 min. Cell debris was removed by centrifugation at 5000 x g for 20 min. $MgCl_2$ was added to 2 mM, and the supernatant centrifuged (78,000 x g, 60 min) in a Spinco 30 rotor. The resultant pellet was resuspended in Tris-HCl buffer, $MgCl_2$ added to 2 mM, and centrifuged again at 78,000 x g for 60 min. The pellet was resuspended in distilled water, and the protein concentration estimated by the method of Schacterele and Pollack (1973).

Outer membrane preparations were prepared from the whole cell envelope protein preparations by using Triton X-100 to solubilize the cytoplasmic membrane. The method used was basically that of Schnaitman (1971b). Hepes buffer was added to the whole cell envelope protein preparation to 0.01M, and an equal volume of 4% (v/v) Triton X-100 in 0.01M Hepes buffer added for 15 min at 23 C. The preparation was centrifuged in either a Spinco 65 (161,000 x g, 60 min) or Spinco 40 (68,000 x g, 120 min) rotor, the pellet resuspended in distilled water, and the centrifugation repeated. The resultant pellet was resuspended again in distilled water.

The methods employed for solubilization of the above preparations in SDS prior to polyacrylamide gel electrophoresis are basically those described by Schnaitman (1973). The sample was suspended at a cencentration of about 10 mg protein/ml in a solution containing 3% (w/v) SDS, 0.15% (v/v) β-mercaptoethanol, and 7.5 mM EDTA in 0.1 M sodium phosphate buffer, pH7.2. The sample was then incubated for 2 h at 37 C under nitrogen. This sample was used as an "unheated" preparation. "Heated" samples were then dialysed overnight against a solution containing 48% (w/v) urea, 0.1% (w/v) SDS, 0.1% (v/v) β-mercaptoethanol and 0.5 mM EDTA in 0.1 M sodium phosphate buffer,

pH 7.2, and heated at 100 C for 4 min. All samples were stored frozen until used.

Polyacrylamide gel electrophoresis.

Approximately 80 µg of protein (total volume of 80 µl) was loaded onto each gel. The gels contained 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide and 0.5 M urea in 0.1% (w/v) SDS in 0.1 M sodium phosphate buffer, pH 7.2, and were run under the conditions described by Schnaitman (1973). Some "heated" samples were run on the alkaline gel system described by Bragg and Hou (1972).

Gels were stained with coomassie brilliant blue (Neville (1971), Swank and Munkres (1971)), and after destaining by the method of Schnaitman (1973), scanned with a Quick Scan Jr. (Helena Laboratories Corp.).

CHAPTER III

CHARACTERIZATION OF COLICINOGENIC STRAINS

Introduction.

Before undertaking a survey of the extent of cross-resistance to coli-
cin, it was obviously important to determine how many colicins each colicino-
genic strain was producing, and which colicins they were. It was also
necessary to be able to separate the various colicins produced by a multi-
colicinogenic strain, in order to test their ability to kill the various
mutants.

It was found that it was possible to achieve reasonable separation of
the various colicins by electrophoresis in nutrient agar. Fig. 3-1 shows
the results of such an experiment. The difference in electrophoretic mobility
and inhibition zone morphology of the various colicins was sufficient, in most
cases, to enable a determination of the number of colicins a strain was pro-
ducing, and also an identification of each of those colicins.

Singly colicinogenic strains.

Most of the colicinogenic strains produced only one inhibition zone
upon electrophoresis of the colicin they were producing. In each case, this
inhibition zone was subsequently shown to be due to a single colicin.

Fredericq (1948) observed that colicinogenic Citrobacter strains produced
only one colicin, which he called A. Citrobacter freundii CA31 is the type
strain for colicin A (Fredericq (1965)). We have confirmed, by a series of
biochemical tests shown in Table 3-1, that the stocks of CA31 used here are
in fact Citrobacter species. In addition, the single colicin produced by
each of the strains failed to kill Tol II, Tol III or Bfe mutants (Table 3-2).
The invariable resistance of Bfe mutants to colicin A has been noted before

Fig. 3-1 : Use of electrophoresis to separate colicins.
Colicin was allowed to diffuse into the nutrient agar before
being subjected to electrophoresis, and overlayed with a
sensitive indicator strain to visualise the zones of inhibition.

Site of application of original colicinogenic cultures

D-CA23

X-CA23

$\oplus$

E1·CA62   I-CA62

$\ominus$

N-284   E3-284

?-CA7

V-CA7

Table 3-1.

TYPING OF VARIOUS COLICINOGENIC STRAINS BY BIOCHEMICAL TESTS (a)

Biochemical Test	Strain (b)						
	CA31	23	CA46	CA58	398	CA57	P14
Indol	-	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-
Simmon's Citrate	+	-	-	-	-	-	-
H_2S	+	-	±	-	-	+	+
Gelatin	-	-	-	-	-	-	-
Lysine Decarboxylase	-	+	+	+	+	+	+
Arginine Dihydrolase	-	-	-	-	-	-	-
Orthithine Decarboxylase	-	+	-	-	+	+	+
Lactose	+	+	+	+	±	-	-
Sucrose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	±	-	-
Arabinose	+	±	+	±	+	+	+
Glucose	+	+	+	+	+	+	+
Suggested Type	Citrobacter	E.coli	E.coli	E.coli	E.coli	Shigella dispar	Shigella dispar

(a) Biochemical tests and interpretation of results used are described by Edwards and Ewing (1966).

(b) + = positive, - = negative reaction, ± = weak positive.

Table 3-2.

Ability of colicins from certain singly colicinogenic strains to kill various
colicin resistant mutants (a)

| Colicinogenic strain | Colicin resistant mutant (b) | | | | | | | Suggested colicin type |
	Bfe P525	Tol II P651	Tol III P660	Tsx P209	Rcx P224	TonA P1205	TonB P585	
CA31	R	R	R	S	S	S	S	A
23	R	R	R	S	S	S	S	A
K53	R	R	S	S	S	S	S	E1
P14	R	R	S	S	S	S	S	E1
CA42	R	R	R	S	S	S	S	E2
K317	R	R	R	S.	S	S	S	E2
P9	R	R	R	S	S	S	S	E2
K12 (CA38)	R	R	R	S	S	S	S	E3
CA46	S	S	S	S	S	S	R	G
CA58	S	S	S	S	S	S	R	H
CA53	S	S	S	S	S	S	R	I
ST4	S	S	S	S	S	S	R	I
K12 (P9)	S	S	S	S	S	S	R	I
K216	S	R	R	R	S	S	S	K
K235	S	R	R	R	S	S	S	K
398	S	R	R	S	S	S	S	L
P1	S	S	S	S	S	S	R	S1
P15	S	R	R	S	S	S	S	S4
K12 (K235)	S	S	S	S	R	S	S	X
CA57	R	R	S	S	S	S	S	E1

(a) R = resistant, S = sensitivity.

(b) The isolation and characterization of the various mutants is described
   in Chapter IV.

(Nagel de Zwaig and Luria (1967), Fredericq, personal communication). Escherichia coli 23, also said to produce colicin A (Y. Hamon, personal communication), occasionally shows indications of producing small amounts of a second colicin. The main colicin produced, however, has an actively spectrum on the mutants shown in Table 3-2 that is identical with the colicin produced by CA31. Thus it is concluded that both Citrobacter freundii CA31 and Escherichia coli 23 are producing colicin A.

Both Shigella dispar P14 and E.coli K53 are said to produce colicin E1 (Fredericq (1965), Fredericq, personal communication). Both strains appear to produce only one colicin, and as expected for E1, fail to kill a Bfe or Tol II mutant, but are active on Tol III mutants.

Escherichia coli CA42, Escherichia coli K317 and Shigella sonnei P9 have been shown to be producing colicin E2 (Fredericq (1965)). As expected, the single colicin produced by all these strains fails to kill Bfe, Tol II and Tol III mutants. Shigella sonnei P9 is also said to produce colicin Ib (Fredericq (1965)), but only occasionally could small amounts of a second colicin be detected with the particular stock of the strain used in this study.

E.coli CA38 produces both colicin E3 and colicin I (Fredericq (1965)), and because of their similar electrophoretic mobilities under the conditions used here, it was found to be impossible to separate them by electrophoresis. A strain of Escherichia coli K12 harbouring both the Col I-CA38 and Col E3-CA38 factors was obtained. This strain was, however, said to be producing only colicin E3 (D. Helinski, personal communication). It produced only one colicin inhibition zone after electrophoresis (as did CA38). The colicin, however, failed to kill Bfe, Tol II or Tol III mutants - thus confirming an

absence of colicin I production. Colicins E2 and E3 were finally differentiated on their activity spectra on the full range of mutants isolated (see Chapter IV).

E.coli CA46 and E.coli CA58 are the type strains for colicins G and H respectively (Fredericq (1965)). The series of biochemical tests employed (Table 3-1) confirmed that both strains were Escherichia coli. As expected from the results of Fredericq (1953), both strains produced single colicins with a similar, weak inhibition zone. The results shown in Table 3-2, as would be expected, showed that both colicins were active against Bfe, Tol II, Tol III and Ton A strains. Both colicins, however, failed to kill a Ton B mutant.

Escherichia coli CA53, the type strain for colicin Ia (Fredericq (1965)), produced a single colicin which, as would be expected, killed a Ton A, but not a Ton B mutant (Table 3-2).

As the stock of Shigella sonnei P9 used here failed to produce any colicin Ib, strains of Salmonella typhimurium ST4 and Escherichia coli K12 carrying the Col Ib-P9 factor were obtained. Both strains produced a single colicin that was active on a Ton A mutant but failed to kill a ton B mutant (Table 3-2).

Both Escherichia coli K216 and Escherichia coli K235 produce colicin K (Fredericq, personal communication, Goebel (1973)). E.coli K235 is also said to produce colicin X (Miyami, et al. (1961)), but no second inhibition zone could be observed. Strain K216, however, did occasionally show evidence of the production of a second, unidentified colicin. The colicins from both strains failed to kill a Tsx mutant.

Colicin L is defined as the colicin produced by E.coli 398 (Hauduroy and Papavassiliou (1962)). This strain, which the results in Table 3-1 confirm is Escherichia coli, produces a single colicin that is cross resistant to the E group colicins (Table 3-2). This cross-resistance has been observed before by Hauduroy and Papavassiliou (1962).

Shigella boydii Pl and Shigella dispar P15 produce colicins S1 and S4 respectively (Fredericq (1965)). Both strains appeared to produce a single colicin. Colicin S1 failed to kill a Ton B mutant, but was active against all the other mutants tested in Table 3-2. Colicin S4 has a very weak inhibition zone which contains many resistant mutants, and failed to kill either a Tol II or Tol III mutant.

As E.coli K235 could not be shown to be producing colicin X, two different stocks of the same Escherichia coli K12 strain, which carries the Col X-K235 factor, were obtained. This strain produces a single colicin which kills both Ton B and Tol II mutants. It is possible to isolate mutants (called Rcx - see Chapter IV) specifically resistant to colicin X.

The type strain for colicin C, Escherichia coli CA57, has been lost (Fredericq, personal communication). Yet several stocks of what was apparently CA57 were obtained from this laboratory, and elsewhere. When these strains were typed by a series of biochemical tests, however, they all appeared to be Shigella dispar, and not Escherichia coli (Table 3-1). It therefore appears that the CA57 in circulation at the present time may in fact be Shigella dispar P14, which gives identical results to the various biochemical tests in Table 3-1. In addition, the colicin produced by CA57 appears to be identical to colicin E1, the colicin produced by P14.

Multicolicinogenic strains.

E.coli CA23, the type strain for colicin D, is known to produce two colicins (Timmis (1972)). Two inhibition zones were, in fact, found when the colicins produced by CA23 were subjected to electrophoresis. One had an electrophoretic mobility and zone morphology identical to the colicin D produced by an Escherichia coli K12 strain carrying the Col D-CA23 factor, and producing colicin D alone (Timmis, personal communication). The other zone appeared to be identical to the colicin X originally from K235, and, in fact, was active against all the mutants listed in Table 3-3, with the exception of the Rcx mutant, which is specifically resistant to colicin X-K235 (see Chapter IV).

Fredericq (1965) has stated that E.coli CA62 produces a mixture of colicins E1 and I. The two colicins are separable by electrophoresis, and one zone is active on Tol II, Tol III and Ton A strains, but not Ton B mutants - which is what would be expected. In addition the zone of inhibition is of a similar morphology and position to that of the other colicins of type I tested. The other colicin produced by CA62 kills tol III but not tol II strains, and therefore appears to be E1.

E.coli 284 and E.coli 285 are both said to produce colicin N and one of the E group colicins (Hamon, personal communication). Both strains do appear to be producing two colicins. One colicin fails to kill Bfe, Tol II and Tol III mutants (Table 3-3). It must therefore be either colicin E2, E3 or A. Later experiments showed it to be colicin E3 (see Chapter IV). The other colicin produced by both strains is presumably colicin N. It was active on a Bfe mutant, but as can be seen from Table 3-3, failed to

Table 3-3.

ACTIVITY SPECTRA OF COLICINS FROM VARIOUS MULTICOLICINOGENIC STRAINS

| Colicin Zone (a) | Colicin Resistant Mutant (b) (c) | | | | | | | Suggested Colicin |
	TonA	TonB	Bfe	Rcx	Tsx	Tol II	Tol III	
CA23/1	S	R	S	S	S	S	S	D
CA23/2	S	S	S	R	S	S	S	X
K12 (CA23)	S	R	S	S	S	S	S	D
K12 (K235)	S	S	S	R	S	S	S	X
CA62/1	S	S	R	S	S	R	S	E1
CA62/2	S	R	S	S	S	S	S	I
284/1	S	S	S	S	S	R	R	N
284/2	S	S	R	S	S	R	R	E3
285/1	S	S	S	S	S	R	R	N
285/2	S	S	R	S	S	R	R	E3
AG097	S	R	S	S	S	S	S	B
CA18/1	S	R	S	S	S	S	S	B
CA18/2	R	R	S	S	S	S	S	M
K89/1	S	R	S	S	S	S	S	B
K89/2	R	R	S	S	S	S	S	M
206/1	S	R	S	S	S	S	S	B
206/2	R	R	S	S	S	S	S	M
206/3	S	R	S	S	S	S	S	V
K260/1	S	R	S	S	S	S	S	B
K260/2	R	R	S	S	S	S	S	M
K260/3	S	R	S	S	S	S	S	V
CA7/1	S	R	S	S	S	S	S	?

Table 3-3 continued.

CA7/2	S	R	S	S	S	S	S		V
Ar19a/1	S	R	S	S	S	S	S		?
Ar19a/2	S	R	S	S	S	S	S		V
II/1	S	R	S	S	S	S	S		D
II/2	S	S	R	S	S	R	S		E1
II/3	S	R	S	S	S	S	S		I
II/4	S	R	S	S	S	S	S		Q

(a) The various zones are numbered according to the electrophoretic mobility of the colicin. Zone 1 moved the least distance, zone 2 was further from the original point of application, etc.

(b) The individual mutants are those listed in Table 3-2. Their isolation and characterization is described in Chapter IV.

(c) R = resistant, S = sensitive.

kill either a Tol II or a Tol III mutant.

Several of the colicinogenic strains were eventually shown to be producing various combinations of colicins B, I or V. After electrophoresis, E.coli strains K89, CA18, CA7 and Ar19a were all shown to be producing two colicins, while K260 and 206 appear to be producing three (Fig. 3-2). The colicin V zone was immediately evident, because of its unique morphology. The zone of inhibition, although its edge is sharp, is larger at the surface of the plate than at the bottom of the seeded soft agar layer - this characteristic is quite distinctive for colicin V. Thus it was readily apparent that CA7 (the type strain for colicin V), Ar19a (a CA7 derivative), K260 and 206 (a K260 derivative) were all producing colicin V. Several of these strains are known to produce colicins B and M (Fredericq and Smarda (1970)). Therefore, AG097, a strain known to be producing colicin B alone (B. Stocker, personal communication), was obtained. By comparing the zone morphology and electrophoretic mobility of this colicin with those produced by the multi-colicinogenic strains, it appears that strains CA18, K89, K260 and 206 all produce colicin B. As the results in Table 3-3 show, both these colicins, as would be expected for colicin B and V, kill Ton A, but not Ton B strains. Colicin M can be easily identified because it should not be active on Ton B or Ton A mutants (see Chapter I). As can be seen from Table 3-3, CA18, K89, 206 and K260 all appear to produce colicin M. The other colicins produced by CA7 and Ar19a are identical, but remain unidentified. Fredericq (1951a) has shown that under certain conditions CA7 will produce colicin M, but this unidentified colicin kills Ton A mutants, which are specifically resistant to colicin M (Braun and Wolff (1973)). The colicin has the same activity

Figure 3-2 : Zones of inhibition observed after electrophoresis of the colicins produced by various colicinogenic strains producing combinations of colicins B, M and V.
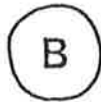
Original application points

AGO97   B

CA18   B   M

K89   B   M

2O6   B   M V
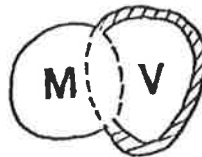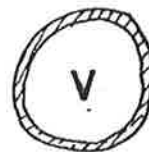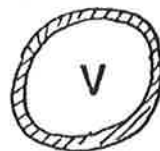
K26O   B   M V

CA7 ?   V
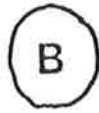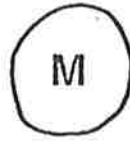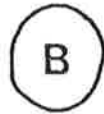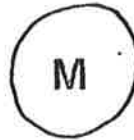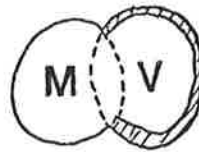
Ar19a ?   V

spectrum as colicin V (see Chapter IV), but differs from this colicin in its zone morphology and electrophoretic mobility (Fig. 3-1).

Escherichia coli II is known to produce colicins, Q, E1, D and I (Smarda and Obdrzalek (1966)). It was not possible to completely separate all these colicins by electrophoresis, as their electrophoretic mobilities are sufficiently similar to cause substantial overlap of their zones of inhibition. Smarda and Obdrzalek (1966) state that colicin Q has an electrophoretic mobility greater than that of colicin V, which must make it the fastest migrating of this mixture of colicins. Later experiments showed this colicin to have an activity spectrum identical to that of colicin V (see Chapter IV), as had been reported previously for colicin Q (Smarda and Obdrzalek (1966)). The results in Table 3-3 suggest that one of the other colicins is in fact E1 (it kills Tol III, but not Bfe and Tol II mutants). The other two zones have a morphology and position similar to colicin D and I, and kill Ton A, but not Ton B mutants, as would be expected. In addition, E.coli II occasionally appeared to produce, in small amounts, an additional colicin. This colicin, which remains unidentified, had a weak inhibition zone, and was the only colicin used that appeared to migrate towards the anode upon electrophoresis.

Summary

A summary of the different colicins produced by each of the colicinogenic strains is shown in Table 3-4. In cases where only one colicinogenic strain is known to produce a particular colicin, an effort has been made to obtain, from different sources, several stocks of the one strain. In such cases, each of the different stocks of the one strain were always used. Thus

colicin L is the only colicin for which subsequent results depend on the ability of a single colicinogenic strain (E.coli 398) to kill the various mutants.

The only colicins, mentioned in the literature, that have not been used, are colicins O, E4 (Hamon and Peron (1964a)), P (Hamon and Peron (1964b)), S8 (Nagel de Zwaig and Vitelli-Flores (1973)), JF246 (Foulds (1971)) and the colicin X described by Papavassiliou (1961).

Table 3-4.

COLICINS PRODUCED BY THE VARIOUS COLICINOGENIC STRAINS

Strain	Colicins Produced
Citrobacter freundii CA31	A
E.coli 23	A (a)
E.coli CA18	B,M
E.coli K89	B,M
E.coli K12 AG097	B
E.coli CA23	D,X
E.coli K12 (Col D-CA23)	D
E.coli K53	E1
Shigella dispar P14	E1
E.coli K317	E2
E.coli CA42	E2
Shigella sonnei P9	E2 (b)
E.coli 12-317	E2
E.coli K12 (Col E3-CA38, I-CA38)	E3
E.coli CA46	G
E.coli CA58	H
Paracolon CA62	E1, I
E.coli CA53	Ia
Salmonella typhimurium ST4 (Col Ib-P9)	Ib
E.coli K12 (Col Ib-P9)	Ib
E.coli K216	K

Table 3-4 continued.

E.coli K235	K (c)
E.coli 398	L
E.coli 206 (Col B-K260, M-K260, V-K260)	B,M,V.
E.coli K260	B,M,V.
E.coli 284	N,E3.
E.coli 285	N,E3.
E.coli II	Q,E1,D,I (d)
Shigella Boydii P1	S1
Shigella dispar P15	S4
E.coli CA7	V (e)
E.coli Ar19a	V (e)
E.coli K12 (Col X-K235)	X

(a) Occasionally produces small amounts of a second unidentified colicin.

(b) Producing no detectable colicin I.

(c) Producing no detectable colicin X.

(d) Some evidence of a fifth unidentified colicin.

(e) Produce a second colicin with an activity spectrum identical to colicin V.

CHAPTER IV

ISOLATION OF COLICIN RESISTANT MUTANTS

Use of the triple layer plate test to distinguish receptor and
tolerant mutants.

The ability of the triple layer plate test to distinguish between
receptor and tolerant mutants was tested by cross-streaking against two
colicinogenic strains, colicin E2 receptor and tolerant mutants. As the
colicin diffuses out and up from the original colicinogenic streak, it
should kill any sensitive bacteria, and they should fail to grow. Any
receptor or tolerant mutant should be unaffected by the colicin, and the
cross-streak should continue to grow. As the colicin diffuses into the
top layer, it should kill the indicator strain, producing what is termed
a zone of inhibition. If the cross-streak is a receptor mutant, it should
not absorb the colicin diffusing through the medium, whereas a tolerant
mutant should absorb colicin from the medium, and stop it from reaching
the top layer and killing the indicator strain. Thus receptor mutants
should have no effect on the shape of the zone of inhibition, but a cross-
streak of a tolerant mutant should be covered by a "cap" of indicator
bacteria.

The result, shown in Fig 4-1, confirms that these two types of mutants
can be distinguished for colicins E2 and E3. The "cap" sometimes fails to
cover the central part of a streak, indicating that the receptors can become
saturated in this area of high colicin concentration. It was found that
tolerant and receptor mutants could be easily distinguished with colicins
E1, E2, E3, K, Ia, Ib and S1.

All mutants resistant to colicins A, D and X, however, appear to give

Figure 4-1 : Triple layer plate test for distinguishing receptor and tolerant mutants. A sensitive strain (AB1133), and a receptor (P525) and a tolerant mutant (P651) were cross-streaked against two colicinogenic strains, and the plate overlayed with the indicator strain. A tolerant mutant can easily be distinguished from a receptor mutant by its effect on the zone of inhibition.
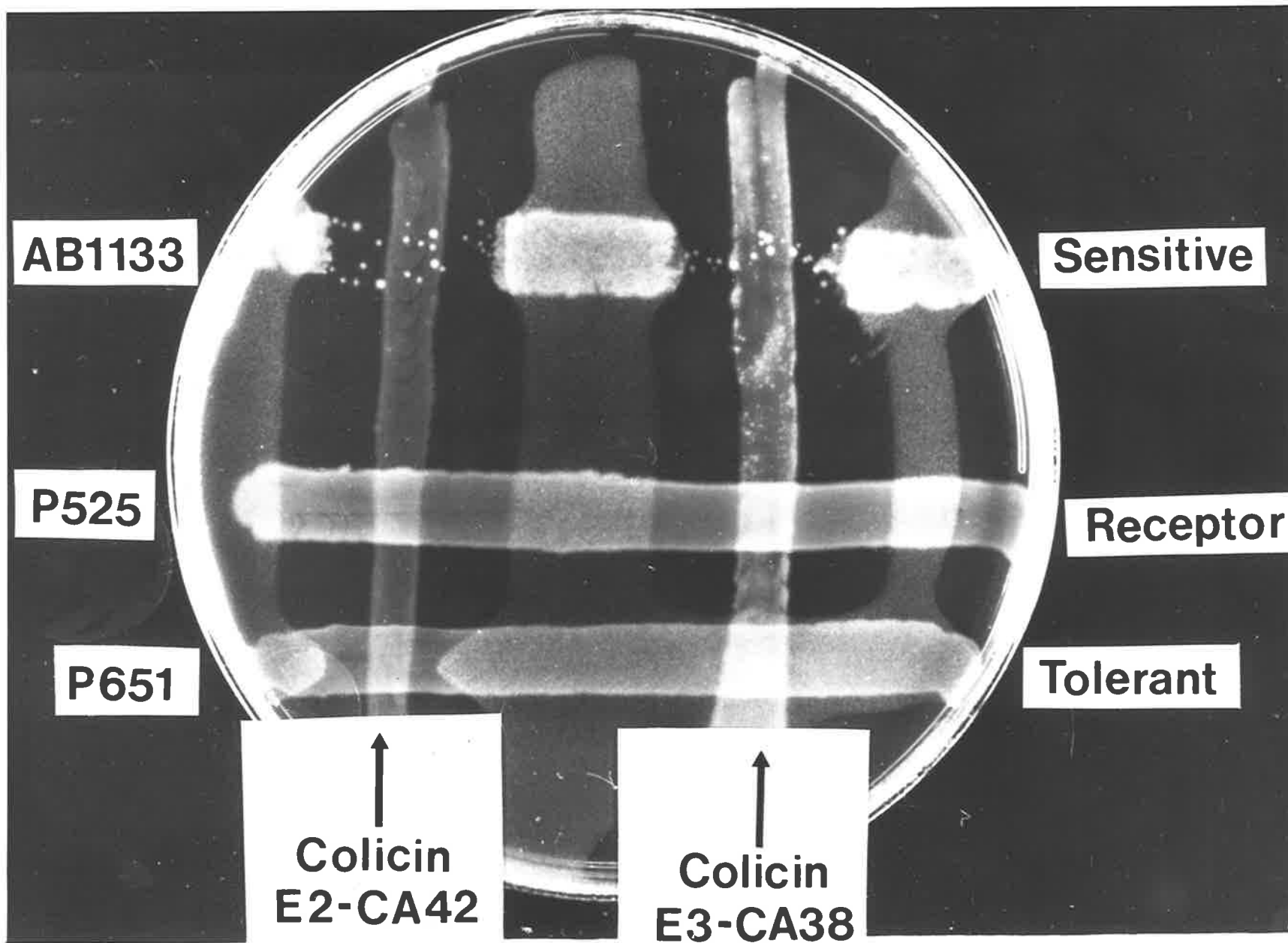
the result expected for a receptor mutant, when cross-streaked against these colicins. The receptor plate test described in Chapter II was used to test the ability to distinguish receptor and tolerant mutants for these colicins. As the parent strain has the receptors for these colicins intact it should elicit the maximum "tolerant" result possible for mutants of this strain. However, when the seeded soft agar layer above the cross-streak was removed and the shape of the inhibition zone inspected, in each case it was unaltered, indicating that the triple layer plate test was unable to distinguish receptor and tolerant mutants for colicins A, D and X. This finding is supported by the evidence of Timmis (personal communication) and Cavard and Barbu (1970) that colicins D and A have fewer receptors on the cell surface than some other colicins.

All mutants resistant to colicins B,G,H,L,M,N,Q,S4 and V appeared to be tolerant to these colicins. Yet one class of these mutants, Ton A, are said to be receptor mutants for colicin M (Braun and Wolff (1973)). There-fore, it appears that for colicin M at least, the triple layer plate test cannot detect receptor mutants.

Some of the mutants that appeared to be tolerant to colicins B and D were excreting inhibitors of these colicins (see chapter VI). These inhi-bitors, by blocking colicin action, mimic the effects of receptor on a tolerant strain, and make it impossible to distinguish with certainty between tolerance and receptor loss.

It was also found that colicin G was chloroform sensitive, and to retain maximum colicin activity, heating at 56 C for 30 min was substituted for chloroform treatment, to kill the bacterial growth at various stages

needed during the plate test.

Colicin S4 exhibited a very weak zone of inhibition, and gave rise to large numbers of resistant mutants, making it difficult at times to distinguish the edge of the zone of inhibition.

The triple layer plate test was also used after electrophoresis (Fig 4-2) to distinguish receptor and tolerant mutants to the various colicins produced by the multicolicinogenic strains.

Isolation of resistant mutants.

Many hundreds of colicin resistant mutants were isolated, using each of the available colicins for selection. These initial isolates were screened for their colicin resistance pattern using the triple layer plate test and a restricted range of colicinogenic strains. In cases where a predominance of one phenotype occurred, only a few were retained for further study.

Approximately 250 mutants were then tested against all the colicinogenic strains listed in Table 3-4. It immediately became obvious that one could divide the colicins into the two groups (group A and group B) shown in Table 4-1. Mutants selected as resistant to a group A colicin may or may not be resistant to other group A colicins, but are never resistant to any colicin of group B. The reverse also applies.

Resistance to colicins of group A.

The mutants resistant to group A colicins can be divided into 21 phenotypic classes on the basis of their colicin resistance patterns. These include both receptor and tolerant mutants.

Figure 4-2 : A combination of the triple layer plate test and the electrophoresis technique, designed to test whether a strain is a receptor or tolerant mutant after separation of the colicin zones of a multi-colicinogenic strain by electro-phoresis.

Original plane of application of colicinogenic culture

Receptor Mutant
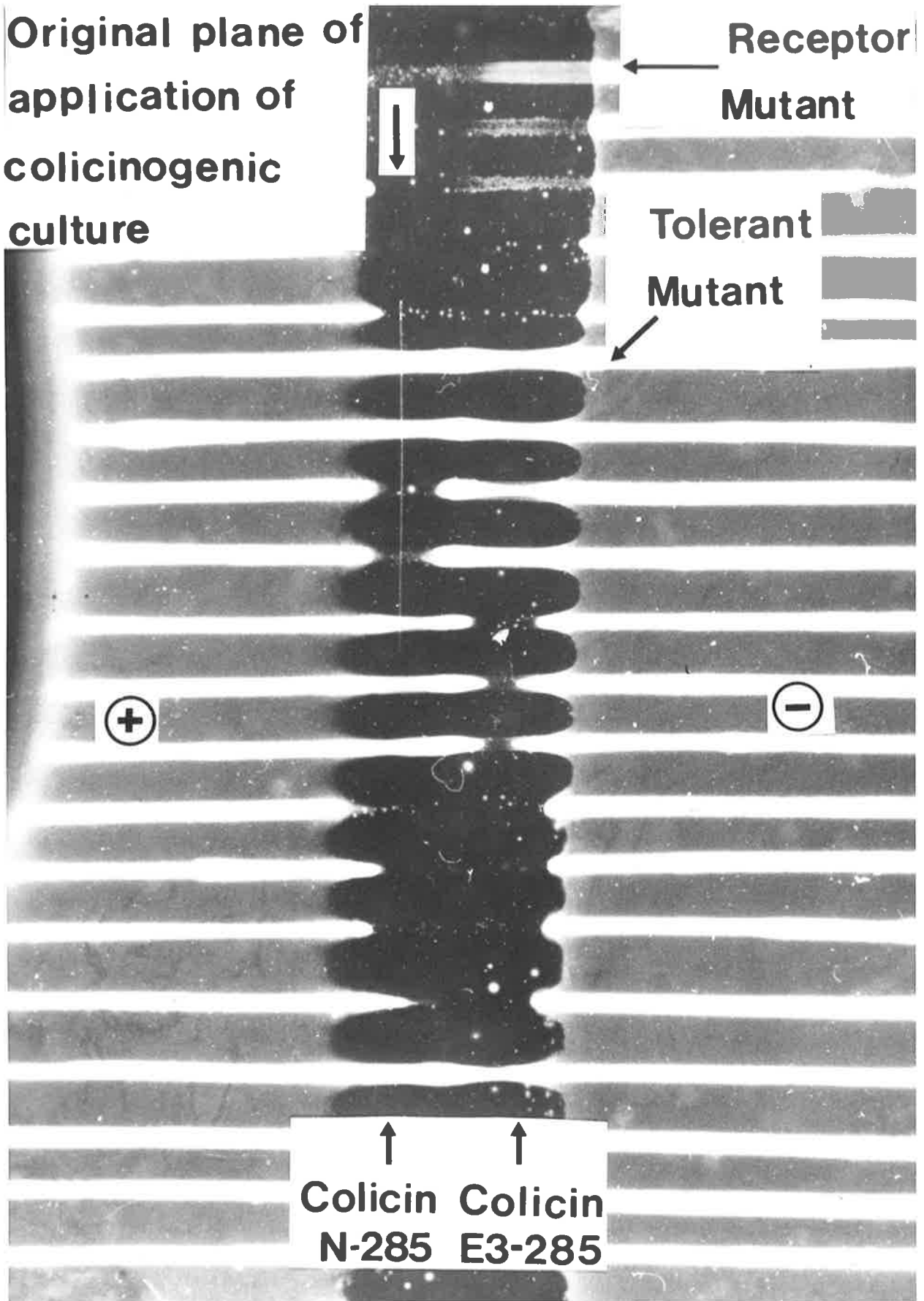
Tolerant Mutant

⊕

⊖

Colicin N-285   Colicin E3-285

Table 4-1.

GROUPING OF COLICINS BY THEIR RESISTANCE PATTERN.

Colicin Group	Colicin	Specific colicins used (a)
A	A	A-CA31, A-23
	E1	E1-K53, E1-P14, E1-CA62, E1-II
	E2	E2-CA42, E2-P9, E2-12-317, E2-K317
	E3	E3-CA38, E3-284, E3-285
	K	K-K216, K-K235
	L	L-398
	N	N-284, N-285
	S4	S4-P15
	X	X-185II, X-CA23
B	B	B-CA18, B-206, B-K260, B-K89
	D	D-CA23, D-II
	G	G-CA46
	H	H-CA58
	I	Ia-CA53, Ib-ST4, Ib-P9, I-CA62, I-II
	M	M-206, M-CA18, M-K89, M-K260.
	Q	Q-II
	S1	S1-P1
	V	V-CA7, V-Ar19a, V-206, V-K260

(a) The nomenclature is that adopted by Nomura (1967). In each case the colicin produced (e.g. E2), is followed by the name of the strain producing it (e.g. CA42), to give, for example E2-CA42.

In order to check for partial resistance to colicins, two fold serial dilutions of colicins A, E1, E2, E3 and K were spotted onto soft agar overlays seeded with the mutant to be tested. The results are shown in Table 4-2. Liquid preparations of colicin, with a sufficient colicin concentration, could not be obtained for colicins L, S4 and X. Colicin N could not be obtained free of colicin E3, as both E.coli 284 and 285 produce both colicins N and E3. The results for the complete cross-resistance tests are shown in Table 4-3.

The Bfe and Tsx mutants isolated are the classical receptor mutants isolated many times previously (Buxton (1971), Curtiss III (1965), Jasper, et al. (1972), Jenkin and Rowley (1955)). The Rcx mutants have not been described before, and are specifically resistant to colicin X. It is impossible to tell from the triple layer plate test whether they are receptor or tolerant mutants, and colicin X cannot be obtained in liquid media at a concentration sufficient to do adsorption studies.

The Con mutants, so called because of their conjugation deficiency (see Chapter VI), are tolerant to colicins K and L. The Tol I mutants (tolerant to colicin K, but sensitive to E1, E2 and E3) described previously (Nomura and Witten (1967)) may be the same as one of the different types described here.

The Tol IIb and Tol III mutants are of the types isolated previously (see Table 4-5), and shown to map at tol A and tol B respectively (Bernstein, et al. (1972)). The Tol IIc mutants, despite the fact that they are resistant to exactly the same colicins as the Tol IIb mutants, were differentiated on their antibiotic, detergent and surfactant sensitivity patterns (see Chapter

Table 4-2.

QUANTITATION OF RESISTANCE TO GROUP A COLICINS.

Phenotypic Class (a)	Type Strain	End-point dilution of colicin preparation(b)				
		A	E1	E2	E3	K
Tol$^+$, Bfe$^+$, Tsx$^+$ Rcx$^+$, Con$^+$	AB1133	9	7	9	7	4
Bfe	P525	0	0	0	0	4
Tsx	P209	8	7	9	7	0
Rcx	P224	6	7	7	7	3
Con	P212	9	7	9	7	0
Tol Ia	P218	0	5	9	7	0
Tol Ib	P210	0	6	8	6	0
Tol IIb	P651	0	0	0	0	0
Tol IIc	P555	0	0	0	0	0
Tol III	P660	0	5	0	0	0
Tol IV	P692	0	5	0	0	4
Tol VII	P689	0	5	2	6	2
Tol VIII	P602	0	0	9	7	2
Tol IX	P596	0	7	9	7	2
Tol X	P661	0	5	7	6	1
Tol XI	P220	1	7	9	7	2
Tol XII	P653	0	7	9	2	4
Tol XIII	P520	0	7	0	0	0
Tol XIV	P530	0	7	0	0	0
Tol XV	P686	0	6	0	0	0

Table 4-2 continued.

Tol XVI	P516	0	6	2	4	4
Tol XVII	P652	0	0	4	1	0

(a) The complete colicin resistance patterns and descriptions of the various phenotypic classes is given in Table 5.

(b) The figures given in the table are n, where $\frac{1}{2}$ n is the last dilution of the colicin preparation to give a complete inhibition zone on a "lawn" of a particular mutant. Where n = o, no or incomplete clearing of the bacterial growth could be seen with an undiluted colicin preparation.

Table 4-3.

PHENOTYPIC CLASSIFICATION OF MUTANTS RESISTANT TO COLICINS OF GROUP A

Phenotypic Class (a)	Type Strain	Number isolated	Colicin resistance pattern (b)									Type of mutant
			E1	E2	E3	K	L	A	S4	N	X	
Bfe (c)	P525	21	E1	E2	E3			A				Receptor
Tsx (d)	P209	3				K						
Rcx (e)	P224	3									X	?
Con	P212	2				K	L					
Tol Ia	P218	3				K	L	A	S4			
Tol Ib	P210	3				K	L	A	S4	N		
Tol IIb (f,g)	P651	10	E1	E2	E3	K	L	A	S4	N		
Tol IIc (g)	P555	9	E1	E2	E3	K	L	A	S4	N		
Tol III	P550	3		E2	E3	K	L	A	S4	N		
Tol IV	P692	1		E2	E3		L	A		N		
Tol VII	P689	1		pE2		pK	L	A	S4			Tolerant
Tol VIII	P602	2	E1			pK		A				
Tol IX	P596	4				pK	L	A		N		
Tol X	P661	3				pK	L	A	S4	N		
Tol XI	P220	1				pK	L	pA	pS4			
Tol XII	P653	1			pE3		L	A	pS4			
Tol XIII	P520	12		E2	E3	K	L	A	S4			
Tol XIV	P530	1		E2	E3	K	L	A		N	X	
Tol XV	P686	2		E2	E3	K	L	A	S4	N	X	
Tol XVI	P516	2		pE2	pE3		L	A				
Tol XVII	P652	1	E1	pE2	pE3	K	L	A	S4	N		

Table 4-3 continued.

(a) The phenotypic classification was adopted so as to conform with that used previously by Nomura and Witten (1967) and Nagel de Zwaig and Luria (1967).

(b) The colicins listed are those to which a particular class of mutants is resistant. They have been tested and found to be sensitive to all the other colicins listed in Table 4-1. p denotes partial resistance, as described in Table 4-2, except for colicin S4, where pS4 denotes partial resistance to colicin S4 on the triple layer plate test.

(c) Also resistant to phage BF23.

(d) Also resistant to phage T6.

(e) This class of mutants has been called Rcx (resistance to colicin X), rather than giving it a more specific Tol classification. As explained in the text, it is impossible to distinguish receptor and tolerant mutants for colicin X.

(f) The classification Tol IIa has been used previously (Nagel de Zwaig and Luria (1967), Bernstein, et al. (1972)), to denote mutants with partial resistance to colicin, and of the Tol II phenotype.

(g) Groups IIb and IIc were differentiated on their sensitivity to detergents and antibiotics (see Chapter VI).

VI).

Mutants tolerant to colicin E2, and sensitive to colicins A, E1 and E3 (Tol VII or Ref II), have been described previously (Hill and Holland (1967, Nomura and Witten (1967)). P689, however, was only partially tolerant to colicin E2. The Tol VIII class appears to be similar to those mutants shown to map at tol C, near met C (Whitney (1971)). In all cases, the phenotype of these mutant classes has been extended considerably.

The Tol IX, XI and XII classes have not been described before, but Tol X has, and been described as a colicin A resistant mutant (see Table 4-5). The Tol XIII, XIV and XV mutants may have been isolated before, but would have been classified as Tol III mutants, from which they differ only in their resistance to colicins N, S4 and X. The single Tol XVII mutant, P652, differs from the Tol II mutants only in that it is partially sensitive to colicins E2 and E3. It does not show the partial sensitivity to colicin E1 exhibited by Tol IIa mutants (Bernstein et al. (1972)).

It has been noted several times before that certain colicin resistant mutants show cross resistance to bacteriophages BF23, Ø80, T1, T5 and T6 (Fredericq and Gratia (1949), Fredericq (1951b) Fredericq and Smarda (1970)). All the mutants resistant to colicins of group A were therefore tested for their sensitivity to these bacteriophages. None of them were resistant to bacteriophages Ø80, T1 or T5. Only the Bfe mutants were resistant to phage BF23, and only the Tsx mutants were resistant to phage T6.

Resistance to colicins of group B.

Each of the 145 mutants resistant to colicins of group B can be placed into one of 7 phenotypic classes, based on their colicin resistance pattern.

These include mutants with the same colicin resistance pattern as the well known Ton A (Curtiss III (1965)) and Ton B mutants (Gratia (1964), Guterman and Dann (1973)). Ton A mutants are resistant to bacteriophages Ø80, T1 and T5, and Ton B mutants are resistant to bacteriophages Ø80 and T1 (Gratia (1964)). Therefore, all the mutants were checked for their resistance to these bacteriophages. This allowed the differentiation of a further two phenotypic classes of mutants. The colicin cross-resistance patterns for the full 9 classes is shown in Table 4-4.

The Cir and Ivt mutants are differentiated by the triple layer plate test, as Cir mutants are receptor mutants for colicins Ia, Ib and S1, while Ivt mutants are tolerant to these three colicins. Both classes of mutants are tolerant to colicins Q and V. The mutants isolated include most of those previously described (Guterman and Dann (1973), Gratia (1964), Cardelli and Konisky (1974)), but again their phenotype has been extended considerably.

Comparison with previously isolated mutants.

As mutants of similar, but less completely characterized colicin resistance patterns to those shown in Tables 4-3 and 4-4 had been isolated previously, it was necessary to compare, as far as was possible, these mutants with the previously isolated strains. Examples of the previously isolated mutant classes were therefore checked for their resistance to all the colicins used in this study. The results are shown in Table 4-5.

Although a large number of mutants were selected as resistant to all the available colicins, a few previously described mutant classes were not isolated. These are summarized in Table 4-6. The Tol IIa and Tol IIIa mutants are partially sensitive to colicin, and any isolated may have been discarded during the initial screening procedure as being fully sensitive.

Table 4-4.

Phenotypic classification of mutants resistant to colicins of group B.

Phenotypic class	Type strain	Number isolated	Colicin resistance pattern (a,b)
Ton A	P1205	5	M (∅80,T1,T5)
Ton B	P585	17	B D G H M I S1 Q V (∅80,T1)
Exb B	P575	70	B D G H M I S1 Q V
Exb C	P535	1	B D G H M
Cbt	P295	16	B D
Cmt	P1209	4	M
Cir	P625	10	$I^R S1^R Q V$
Ivt	P645	12	I S1 Q V
Cvt	P1235	10	Q V

(a) The colicins listed are those to which the particular mutants are
  resistant. They have been tested against, and are sensitive to, all
  the other colicins used.

(b) R = receptor mutant. Otherwise, all the mutants appeared to be
  tolerant to all the colicins listed, although P585, P575 and P535
  were excreting colicin inhibitors that interfere with the ability of
  the plate test to differentiate receptor and tolerant mutants for
  colicins B and D.

Table 4-5.

Comparison of Previously Isolated Mutants with New Phenotypic Classes

Strain	Phenotypic class		Colicin resistance pattern (a)		Reference
	Present class	Previous class	Present pattern	Previous pattern	
A837					1.
A9	Tol IIb(c)	Tol II	E1 E2 E3 K L A S4 N	E1 E2 E3 K A	2.
P118					3.
A845					1.
B1	Tol III	Tol III	E2 E3 K L A S4 N	E2 E3 K A	2.
P117					3.
ASH120	Tol IV	Ref IV	E2 E3 L A N	E2 E3	4.
A586	Tol VIII	Tol VIII	E1 pK A	E1 pA pK	1.
A597	Tol X	A-r	pK L A S4 N	A	1.

(a) pK is partial resistance to colicin A, pA partial resistance to A.

(b) References
1. Nagel de Zwaig and Luria (1967).
2. Bernstein, et al. (1972).
3. Reeves (1966).
4. Hill and Holland (1967).

(c) These mutants were classified as Tol IIb, rather than Tol IIc, on the basis of their antibiotic and detergent sensitivity patterns.

Table 4-6.

Previously Described Mutant Classes not Isolated in this Study (a).

Phenotypic class	Colicin resistance pattern (b)	Reference (f)
Tol IIa	pE1 E2 E3 A K	1,2.
Tol IIIa	pE2 E3 A K	1,2.
Ref III	E3 K	3.
Tol V	E1 E2 E3 K^S (c,d)	4.
Tol VI	E1 E2 K^S (c)	4.
Cim (P116)	E2 (e)	5.
Cim (P137)	E2 (e)	5.
Tol I	I	6,7,8.
–	B I	7,8.
–	B V	8.

(a) In addition, various different receptor mutants, similar to the Bfe group, have been described by Reeves (1966), and Hill and Holland (1967).

(b) p denotes partial resistance.

(c) K^S denotes sensitivity to colicin K.

(d) Also probably included in this group is the single Ref VI mutant, that is sensitive to colicin K. (Hill and Holland (1967)).

(e) These mutants were sensitive to some colicin E2 species (E2-CA42, E2-P9) but were tolerant to others (e.g. E2-K317).

(f) References:
1. Nagel de Zwaig and Luria (1967).
2. Bernstein, et al. (1972).
3. Hill and Holland (1967).
4. Nomura and Witten (1967).
5. Reeves (1966).
6. Cardelli and Konisky (1974).
7. Gratia (1964).
8. Guterman and Dann (1973)

CHAPTER V

GENETIC MAPPING

Loci conferring resistance to colicins of group A.

It has been confirmed by P1 transduction that the bfe locus maps in the region reported previously (Buxton (1971), Jasper, et al. (1972)) - the colicin resistance locus in P525 is 56% cotransducible with argE (see Table 5-1).

Mutants resistant to colicin K and Bacteriophage T6 (tsx) have been shown to map at 9.8 minutes on the genetic map (Curtiss III (1965)) which should make tsx cotransducible with lac. This could not be confirmed, due to the inability to use lac (or gal) as a selective marker in the mutants. The parent strain, AB1133, grows sufficiently well on either lactose or galactose as sole carbon source, to make it impossible to select lac^+ or gal^+ recombinants or transductants.

The rcx locus is transferred by HfrH and HfrC (see Table 5-2), and appears from an examination of the recombinant classes to be between proA and the HfrC origin (i.e. between 6.5 and 13-14 minutes). It is not, however, cotransducible with proA or lip at a frequency greater than 4% (Table 5-1), and so presumably maps in the region 7.8 - 13.3 minutes.

A bacterial cross, using the method of de Haan, et al., (1969), was done using HfrH and the Con mutant, P212, and selecting for thr^+ recombinants. From the gradient of transfer shown in Figure 5-1, the con locus appears to be near 14 minutes on the genetic map. As can be seen in Table 5-1, the con locus appears to be 68% cotransducible with lip, which suggests that it maps at either 14.3 or 14.9 minutes on the genetic map.

Table 5-1.

Mapping of several loci conferring resistance to colicins of group A by P1 transduction

Relevant donor characteristics	Donor strain	Recipient strain	Relevant recipient characteristics	Selection	Number of transductants.	Marker co-transduced	Number of co-transductants.	% co-transduction.
$\underline{argE}^{+}$ $\underline{bfe}^{+}$	P801	P525	$\underline{argE}$ $\underline{bfe}$	arg^{+}	102	$\underline{bfe}$	57	56%
$\underline{proA}^{+}$ $\underline{rcx}^{+}$	P801	P224	$\underline{proA}$ $\underline{rcx}$	pro^{+}	24	–	0	<4%
$\underline{lip}^{+}$ $\underline{rcx}$	P224	P1194	$\underline{lip}$ $\underline{rcx}^{+}$	lip^{+}	37	–	0	<3%
$\underline{lip}^{+}$ $\underline{con}$	P212	P1194	$\underline{lip}$ $\underline{con}^{+}$	lip^{+}	38	$\underline{con}$	26	68%
$\underline{thr}^{+}$ $\underline{leu}^{+}$ $\underline{tolJ}^{+}$	P801	P689	$\underline{thr}$ $\underline{leu}$ $\underline{tolJ}$	thr^{+}	43	$\underline{tolJ}$	37	86%
						$\underline{leu}$	0	<2%
				leu^{+}	4	$\underline{tolJ}$	4	–
						$\underline{thr}$	0	<25%

Table 5-2.

Mapping of several loci conferring resistance to colicins of group A by Hfr crosses

Strain	Colicin resistance locus	Hfr	Marker selected.	Recombinant class						Number of recombinants	Number colicin sensitive.
P224	rcx	H	thr$^+$	thr$^+$	leu$^+$					50	0
				thr$^+$	leu$^+$	pro$^+$				58	3
				thr$^+$	leu$^+$	pro$^+$	lac$^+$			63	15
				thr$^+$	leu$^+$	pro$^+$	lac$^+$	gal$^+$		21	9
				thr$^+$	leu$^+$	pro$^+$		gal$^+$		5	0
				thr$^+$	leu$^+$	$^+$	lac$^+$			4	0
		C	pro$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	arg$^+$		1	0
				lac$^+$	pro$^+$	leu$^+$	thr$^+$			8	7
				lac$^+$	pro$^+$	leu$^+$				9	8
					pro$^+$	leu$^+$				2	0
P218	-	H	his$^+$	his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	16	12
				his$^+$		lac$^+$	pro$^+$	leu$^+$	thr$^+$	2	1
				his$^+$	gal$^+$		pro$^+$	leu$^+$	thr$^+$	1	1
P210	-	H	his$^+$	his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	15	12
				his$^+$		lac$^+$	pro$^+$	leu$^+$	thr$^+$	1	0
				his$^+$	gal$^+$		pro$^+$	leu$^+$	thr$^+$	1	0
				his$^+$			pro$^+$	leu$^+$	thr$^+$	4	1
P651	-	H	his$^+$	his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	7	3
				his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$		1	0
				his$^+$	gal$^+$	lac$^+$		leu$^+$	thr$^+$	1	0
				his$^+$	gal$^+$	lac$^+$	pro$^+$			7	1
				his$^+$			pro$^+$			1	0
				his$^+$	gal$^+$		pro$^+$			2	1

Table 5-2 continued. 93

Strain		H	Sel	Genotype						n₁	n₂
P651	−	H	thr$^+$	thr$^+$						9	0
				thr$^+$	leu$^+$					9	0
				thr$^+$	leu$^+$	pro$^+$				55	0
				thr$^+$	leu$^+$	pro$^+$	lac$^+$			53	0
				thr$^+$	leu$^+$	pro$^+$	lac$^+$	gal$^+$		3	1
				thr$^+$				gal$^+$		2	1
				thr$^+$	leu$^+$			gal$^+$		1	1
P555	−	H	his$^+$	his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	5	4
				his$^+$		lac$^+$	pro$^+$	leu$^+$	thr$^+$	6	0
				his$^+$	gal$^+$		pro$^+$	leu$^+$	thr$^+$	3	3
				his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$		2	2
				his$^+$		lac$^+$	pro$^+$	leu$^+$		3	0
P660	−	H	his$^+$	his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	18	5
				his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$		1	0
		H	thr$^+$	thr$^+$						16	0
				thr$^+$	leu$^+$					14	0
				thr$^+$	leu$^+$	pro$^+$				56	0
				thr$^+$	leu$^+$	pro$^+$	lac$^+$			70	0
				thr$^+$	leu$^+$	pro$^+$	lac$^+$	gal$^+$		22	9
				thr$^+$	leu$^+$	pro$^+$		gal$^+$		3	2
				thr$^+$		pro$^+$	lac$^+$	gal$^+$		3	2
				thr$^+$	leu$^+$		lac$^+$	gal$^+$		2	2
				thr$^+$	leu$^+$			gal$^+$		1	1
				thr$^+$		pro$^+$				7	0
				thr$^+$	leu$^+$		lac$^+$			6	0
P596	−	H	his$^+$	his$^+$	gal$^+$	lac$^+$	pro$^+$	leu$^+$	thr$^+$	15	10
				his$^+$		lac$^+$	pro$^+$	leu$^+$	thr$^+$	4	0
				his$^+$	gal$^+$		pro$^+$	leu$^+$	thr$^+$	1	0

Table 5-2 continued.   94

Strain		H	Sel	his	gal	lac	pro	leu	thr		
P661	-	H	his+	his+	gal+	lac+	pro+	leu+	thr+	16	8
				his+		lac+	pro+	leu+	thr+	4	0
				his+			pro+	leu+	thr+	2	0
				his+	gal+	lac+	pro+	leu+		1	1
				his+			pro+	leu+		2	1
P653	-	H	his+	his+	gal+	lac+	pro+	leu+	thr+	18	10
		H	thr+	thr+						25	0
				thr+				leu+		32	0
				thr+			pro+	leu+		27	0
				thr+		lac+	pro+	leu+		32	0
				thr+	gal+	lac+	pro+	leu+		4	2
				thr+	gal+		pro+	leu+		1	1
				thr+		lac+		leu+		3	0
				thr+			pro+			2	0
				thr+		lac+	pro+			1	0
				thr+	gal+	lac+				1	1
P520	-	H	his+	his+	gal+	lac+	pro+	leu+	thr+	8	6
				his+		lac+	pro+	leu+	thr+	3	3
				his+			pro+	leu+	thr+	2	1
				his+	gal+					1	1
				his+						3	0
P516	-	H	thr+	thr+						9	0
				thr+				leu+		16	0
				thr+			pro+	leu+		52	0
				thr+		lac+	pro+	leu+		104	0
				thr+	gal+	lac+	pro+	leu+		1	0
				thr+	gal+		pro+	leu+		1	1

Table 5-2 continued. 95

				thr	leu	pro	lac	gal	his		
P652	–	H	thr+	thr+	leu+		lac+	gal+		2	1
				thr+		pro+				10	0
				thr+		pro+	lac+			4	1
				thr+						17	0
				thr+	leu+					14	0
				thr+	leu+	pro+				46	0
				thr+	leu+	pro+	lac+			104	0
				thr+	leu+	pro+	lac+	gal+		9	6
				thr+	leu+	pro+		gal+		1	1
				thr+	leu+		lac+			4	0
				thr+	leu+		lac+	gal+		1	1
				thr+		pro+				4	0
				thr+		pro+	lac+			2	0
				thr+		pro+	lac+	gal+		1	0
				thr+			lac+			8	1
				thr+				gal+		4	3
P689	tol J	H	thr+	thr+						47	7
				thr+	leu+					39	10
				thr+	leu+	pro+				40	8
				thr+	leu+	pro+	lac+			46	9
				thr+	leu+	pro+	lac+	gal+		7	1
				thr+	leu+	pro+	lac+	gal+	his+	1	0
				thr+	leu+	pro+	lac+		his+	1	0
				thr+		pro+				3	1
				thr+		pro+	lac+			5	2
				thr+				gal+		1	0

Fig 5-1 : Gradient of transfer in a bacterial mating using the method of de Haan, et al. (1969), between AB259 (HfrH) and the Con mutant P212, selecting for $\underline{thr}^+$ $\underline{str}^R$ recombinants.
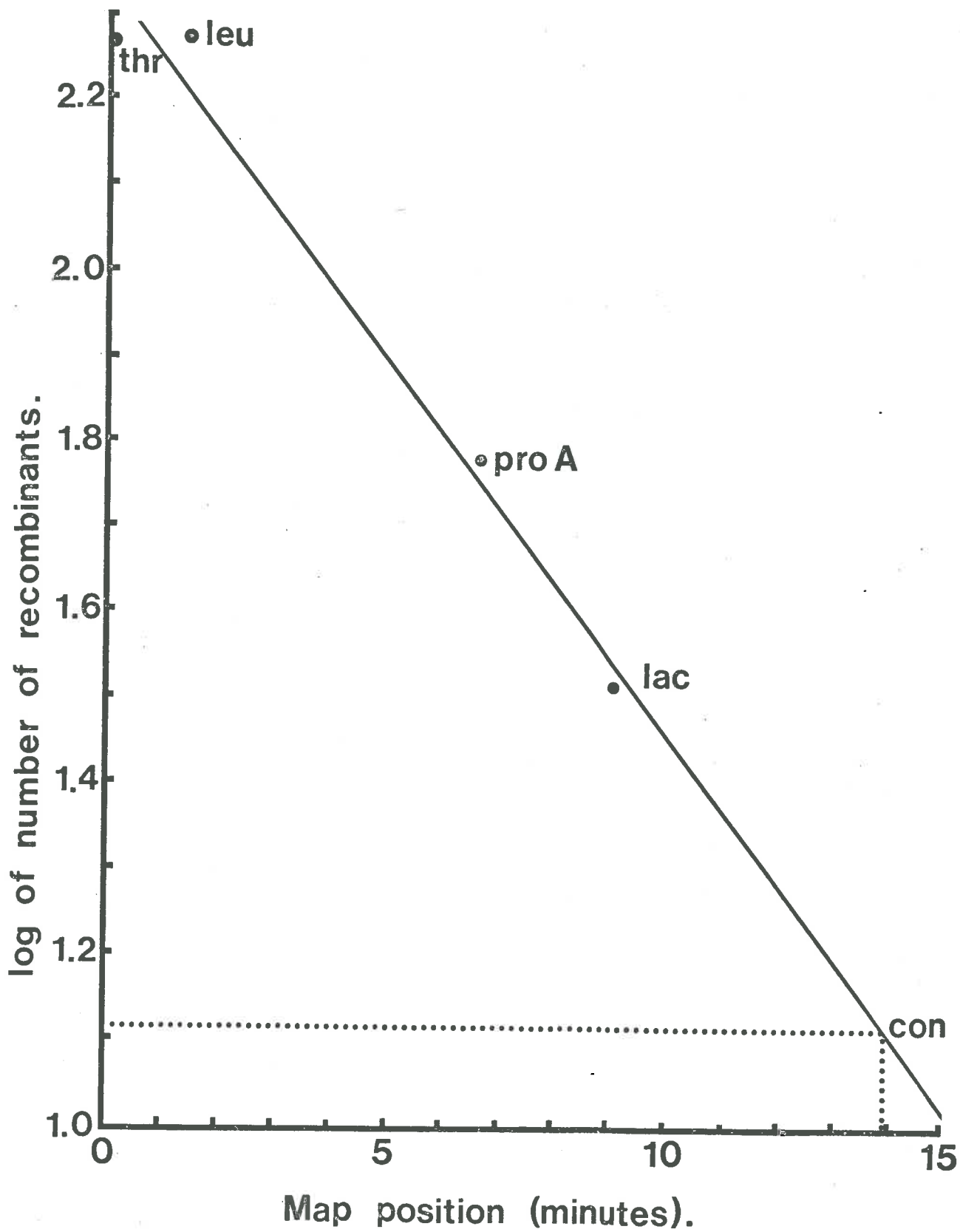
The colicin resistance loci in the Tol Ia (P218), Tol Ib (P210), Tol IIb (P651), Tol IIc (P555), Tol III (P660), Tol IX (P596), Tol X (P661), Tol XII (P653), Tol XIII (P520), Tol XVI (P516) and Tol XVII (P652) mutants are all transferred by HfrH, and many appear to be near gal (see Table 5-2). Each of these mutants was also crossed with HfrC, with a selection for Pro$^+$ recombinants. At least 20 recombinants from each cross were tested and in no case did it appear that the locus could be transferred by HfrC. Because of the inability to use gal as a selective market, a detailed genetic analysis of these mutants was not done. It was possible, however, to more precisely locate the position of some of the loci, by using them as recipients in crosses using donors P601 and W3101, which carry the F1gal and F2gal plasmids, respectively.

Two different sets of experiments were done. In the first set of crosses, gal$^+$ recipients were selected for on minimal media plates containing all the AB1133 growth requirements and streptomycin, with galactose as the sole carbon source. It was possible to isolate what appeared to be gal$^+$, bacteriophage MS2 sensitive, recipients by picking off the largest colonies on the plate. Although these colonies appeared to grow slightly better than the parental strain AB1133 on minimal media plates, they gave only a weak indication of being gal$^+$ on EMB and tetrazolium plates, when galactose was used as the sole carbon source.

In the second series of experiments, all colonies were picked from the initial selection plate (minimal media + AB1133 growth factors, streptomycin, and galactose as sole carbon source), and grown overnight in liquid medium. They were then cross-streaked against strain KL253 (gal recA str) on a

minimal media plate containing KL253 growth factors, streptomycin, and galactose as sole carbon source. Only those cultures which derived from a gal^+ recipient colony on the original plate should be able to provide an F'gal to enable the KL253 streak to grow at the cross-streak. Using this procedure, it was possible to isolate derivatives of all the above mutants, which, although they carried the F1gal or F2gal plasmids (as demonstrated by their ability to transfer them to KL253), were nevertheless phenotypically Gal⁻. It seems that the ability to express a Gal^+ phenotype in AB1133 is not solely dependent on the presence of a functional gal operon.

When these gal^+ recipients were examined for their colicin resistance patterns, it became apparent that the loci in the Tol IIb (P651), Tol IIc (P555), Tol III (P660), Tol XII (P653), Tol XIII (P520), Tol XVI (P516), and Tol XVII (P652) mutants are all located between lip (14.6 minutes) and chlA (17.6 minutes), the region of the chromosome carried by the F1gal and F2gal plasmids (Low (1972)). In each case, the gal^+ recipient became sensitive to the relevant colicins, showing that not only is the locus in the region indicated, but that the wild type is dominant in each case. It seems, therefore, that all these mutations map at or near the tolP, A, B loci (Bernstein, et al. (1972)). A more detailed genetic analysis would be necessary to determine if any new loci are located in this area.

In the Tol Ia (P218), Tol Ib (P210), Tol IX (P596) and Tol X (P661) mutants, neither the presence of an F1gal nor an F2gal plasmid had any effect on the colicin resistance pattern of the recipient. It appears therefore, that the colicin resistance loci in these mutants cannot positively be placed in the region covered by the F1gal and F2gal plasmids. It should be noted,

however, that Nomura and Witten (1967) have shown Tol I mutants, at least, to map close to gal.

The resistance locus in P689, the Tol VII mutant, appears to be transferred early by HfrH (Table 5-2), and although P689 appears to be relatively P1 resistant, it has been shown to be 86% cotransducible with thr (see Table 5-1). None of these transductants were leu$^+$. It was also possible to isolate 4 leu$^+$ transductants that cotransduced this locus, without thr. (i.e. thr$^-$ tolJ$^+$ leu$^+$). Although the number of transductants is too low to obtain a meaningful co-transduction frequency, the fact that the locus will cotransduce with thr without leu, and with leu but without thr, strongly suggests the existence of a new locus (distinct from cet), which we have called tolJ. This locus seems to be located between thr and leu, and probably near 0.1 minutes on the genetic map.

Although the colicin resistance locus in P602 has not been mapped, this Tol VIII mutant has a phenotype similar (tolerant to colicin E1, but sensitive to E2 and E3) to those mutants shown to map at tolC, at 59 minutes on the genetic map (Whitney (1971)).

The resistance loci in the Tol IV, Tol XI, Tol XIV and Tol XV mutants (P692, P220, P530 and P686) have not been mapped. They are not, however, transferred by HfrH when selecting for either thr$^+$ or his$^+$ recombinants, or by HfrC, when selecting for proA$^+$ or argE$^+$ recombinants (at least 20 recombinants were examined in each case). They must all map in the region between his (38.5 min) and argE (78.5 min).

Loci conferring resistance to colicins of group B.

Although the map position of its colicin resistance locus has not been

confirmed, the Ton A mutant, P1205, shows the characteristics of those mutants whose colicin resistance loci have been shown to map at tonA at 3 minutes on the genetic map - resistance to colicin M and bacteriophages T1 and T5 (Fredericq (1951a), Gratia (1964), Braun and Wolff (1973)). The Ton B mutant, P585, has a colicin resistance locus cotransducible with trp (see Table 5-3), as expected from Gratia (1964). We find tonB to be 62% cotransducible with trp.

The colicin resistance locus of the Cbt mutant, P295, was transferred by both HfrH and HfrC (Table 5-4), and appears, from an examination of the recombinant classes, to be between lac (9 min) and the HfrC origin (12-14.5 min). It is cotransducible with lip (14.6 minutes) at a low frequency (see Table 5-3), which means that it maps near either 13 minutes or 16 minutes on the genetic map of Taylor and Trotter (1972). Since a locus mapping at 16 minutes would not be transferred by HfrC, cbt must be located near the ent cluster of loci at 13.2 minutes.

The type strains of the Exb B, Exb C, Cir and Ivt mutant classes (P575, P625, and P645) all have colicin resistance loci that are transferred by HfrH, when his$^+$ recombinants are selected (see Table 5-4). The loci in P625 and P645 appears to be linked to his.

The resistance loci in the Cmt mutant P1209, and the Cvt mutant P1235 are not transferred by HfrH when selecting for thr$^+$ or his$^+$ recombinants, or by HfrC when selecting for proA$^+$ or argE$^+$ recombinants. At least 20 recombinants were examined in each case. The cmt and cvt loci therefore presumably map somewhere between his (38.5 min) and argE (78.8 min).

Table 5-3.

Mapping of several loci conferring resistance to colicins of group B by
P1 transduction

Donor strain	Relevant donor characteristics	Recipient strain	Relevant recipient characteristics	Selection	Number of transductants	Marker cotransduced.	Number of cotransductants.	% cotransduction.
P585	trp^+ $tonB$	JC3272	trp $tonB^+$	trp^+	24	$tonB$	15	62%
P1194	lip^+ cbt^+	P295	lip cbt	lip^+	39	cbt	1	2.6%

Table 5-4

Mapping of several loci conferring resistance to colicins of group B by
Hfr crosses

Strain	Colicin resistance locus	Hfr	Marker selected.	Recombinant class						Number of recombinants	Number colicin sensitive
P295	cbt	H	thr+	thr+	leu+					21	0
				thr+	leu+	pro+				82	1
				thr+	leu+	pro+	lac+			72	23
				thr+	leu+	pro+	lac+	gal+		26	20
				thr+	leu+		lac+	gal+		1	1
				thr+	leu+			gal+		1	0
				thr+		pro+	lac+	gal+		4	1
		C	pro+	lac+	pro+					332	281
				lac+	pro+	leu+				32	29
				lac+	pro+	leu+	thr+			164	153
				lac+	pro+		thr+			14	12
					pro+					90	35
					pro+	leu+				12	8
					pro+	leu+	thr+			33	9
					pro+		thr+			4	2
P575	exbB	H	his+	his+	gal+	lac+	pro+	leu+	thr+	12	2
				his+	gal+		pro+	leu+	thr+	2	0
				his+		lac+	pro+	leu+	thr+	1	0
				his+	gal+	lac+	pro+			1	0
				his+	gal+	lac+				1	1
				his+				leu+	thr+	1	0
				his+						4	1

Table 5-4 continued

				his+	gal+	lac+	pro+	leu+	thr+		
P535	exbC	H	his+	his+	gal+	lac+	pro+	leu+	thr+	6	0
				his+		lac+	pro+	leu+	thr+	10	4
				his+			pro+	leu+	thr+	2	0
P625	cir	H	his+	his+	gal+	lac+	pro+	leu+	thr+	61	53
				his+		lac+	pro+	leu+	thr+	1	0
				his+			pro+	leu+	thr+	3	2
				his+	gal+	lac+				28	24
				his+	gal+					14	13
				his+						21	20
				his+		lac+	pro+			6	6
P645	ivt	H	his+	his+	gal+	lac+	pro+	leu+	thr+	20	20
				his+		lac+	pro+	leu+	thr+	5	1
				his+			pro+	leu+	thr+	10	10
				his+		lac+	pro+		thr+	1	0
				his+		lac+	pro+			20	19
				his+	gal+	lac+				41	29
				his+	gal+					27	25
				his+						21	19

Summary.

The map location of the colicin resistance loci for the various pheno-typic classes is shown in Fig 5-2. Three new colicin resistance loci have been described (tolJ, cbt and con), and it is obvious in some cases that the loci conferring colicin resistance to some of the mutants do not map at any previously described locus.

The Cir mutants described here are almost certainly the same as those shown by Cardelli and Konisky (1974) to map at 41 minutes. Similarly the Exb B mutants display similar properties to the mutants shown by Guterman and Dann (1973) to be cotransducible with serA (56 min).

Fig 5-2 : Genetic map of <u>Escherichia coli</u> K12, after Taylor and
Trotter (1972). The location of the various known colicin re-
sistance loci is shown on the inside of the circle. The regions
in which the loci of the various mutants map is shown on the
outside. The arrows indicate the origin and direction of transfer
of the Hfr strains used. Parentheses around a phenotypic
classification means that the map position of its colicin resistance
locus has not been confirmed.
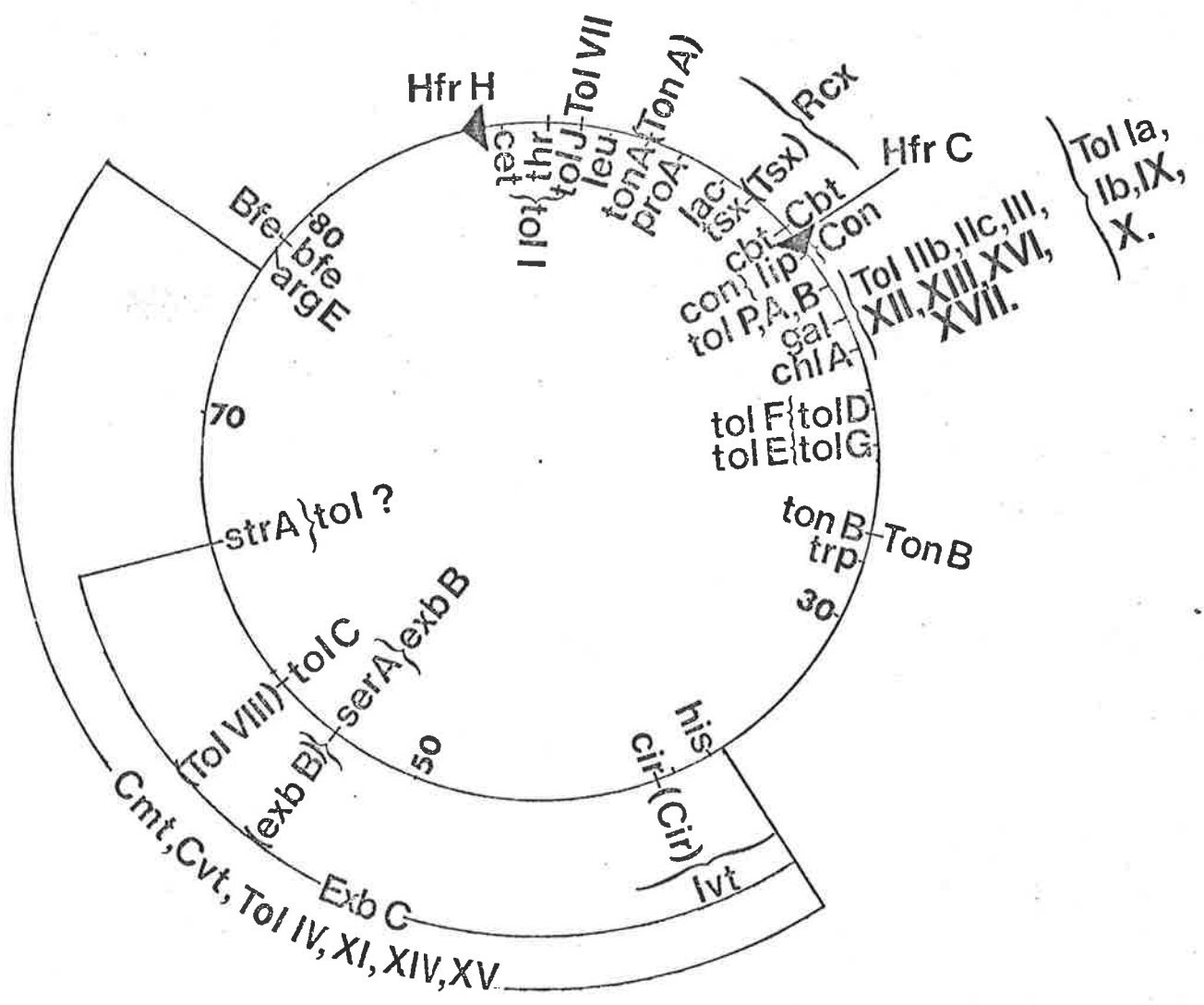
CHAPTER VI

CHARACTERIZATION OF COLICIN RESISTANT MUTANTS

Introduction

Mutations conferring tolerance to colicins have frequently been shown to simultaneously create an increased sensitivity to a range of antibiotics, detergents and surfactants (see Chapter I). For many of the mutants, however, this data was incomplete, and an attempt was made to determine the extent of these various pleiotropic characteristics in the newly isolated mutants.

Sensitivity to antibiotics, detergents and surfactants.

Type strains from each of the 30 phenotypic classes of mutants listed in Chapter IV were tested for their sensitivity to a variety of antibiotics, using "Multodisks". The only mutant to show substantial alteration to its pattern of sensitivity was the Tol VIII strain, P602, which had become sensitive to erythromycin, methicillin, fusidic acid and novobiocin. Other mutants resistant to colicins of group A, however, showed increased resistance or increased sensitivity to ampicillin, as judged by the size of the zone of inhibition of growth. The type mutants of the classes resistant to colicins of group A were therefore tested for their ability to grow on nutrient agar plates containing varying concentrations of ampicillin. The results, shown in Table 6-1, demonstrate that the mutants with a decreased zone size nevertheless have a similar sensitivity to ampicillin, when compared with the parent strain, AB1133. There is however, a quite distinct group of ampicillin "super-sensitive" mutants amongst those resistant to colicins of group A.

The type mutants from each of the mutant classes isolated were also

Table 6-1.

The sensitivity to ampicillin of mutants
resistant to colicins of group A.

Phenotypic class	Type strain	"Multodisk" zone diam. (cm) (a)	Minimal inhibitory concentration of ampicillin (µg/ml) (b)
Tol$^+$, Bfe$^+$, Tsx$^+$, Rcx$^+$, Con$^+$	AB1133	1.2	2
Bfe	P525	1.2	2
Tsx	P209	1.1	2
Rcx	P224	1.2	2
Con	P212	1.3	2
Tol Ia	P218	1.0	2
Tol Ib	P210	1.0	2
Tol IIb	P651	2.0	0.1 - 1.0
Tol IIc	P555	1.3	2
Tol III	P660	1.7	0.1 - 1.0
Tol IV	P692	1.0	2
Tol VII	P689	1.1	2
Tol VIII	P602	1.7	0.1 - 1.0
Tol IX	P596	1.1	2
Tol X	P661	1.0	2
Tol XI	P220	1.2	2
Tol XII	P653	1.1	2
Tol XIII	P520	1.9	0.1 - 1.0
Tol XIV	P530	1.0	2
Tol XV	P686	1.2	2
Tol XVI	P516	1.0	2
Tol XVII	P652	2.0	0.1 - 1.0

(a) The diameter of the disk itself is 0.9 cm.
(b) The lowest concentration in µg/ml of ampicillin at which a noticeable inhibition of growth occurs on nutrient agar plates.

tested for sensitivity to a range of detergents and surfactants that included all those previously used (see Chapter I). The results for mutants resistant to colicins of group A are shown in Table 6-2. None of the mutants resistant to colicins of group B showed any change in sensitivity.

Other properties of mutants resistant to colicins of group A.

Recently other changes have been demonstrated in colicin tolerant mutants. Bernstein, et al. (1972) and Rolfe, et al. (1973) have shown that their Tol II and Tol III mutants were mucoid at 30 C on nutrient agar, and have demonstrated a change in the efficiency of plaquing with $\lambda c190c17$ on a Tol IVt mutant.

Accordingly, all the type mutants resistant to colicins of group A were screened for changes in sensitivity to $\lambda c190c17$. The only mutants to show any resistance were the Tol VII mutants, P689, and the Tol XI mutant, P220. The only mutant to show mucoid growth at 30 C was P220, and it was partially mucoid at 37 C.

Sensitivity to lipopolysaccharide-specific bacteriophages.

The changes in ampicillin sensitivity of some strains suggested that some of the mutants may be altered in their lipopolysaccharide, as some types of ampicillin resistance are accompanied by lipopolysaccharide changes (Monner, et al. (1971)). Therefore, all the type mutants were tested for their sensitivity to two lipopolysaccharide-specific bacteriophages, C21 and U3. The parent strain, AB1133, is resistant to phage C21 and sensitive to phage U3. Any alteration to this pattern has been shown to be accompanied by a change in the lipopolysaccharide (Schmidt, et al. (1970), Watson and Paigen (1971)).

Table 6-2.

Sensitivity to detergents and surfactants of mutants
resistant to colicins of group A.

Phenotypic class	Type strain	Sensitivity index in (a)				
		DOC	SDS	EDTA	PEA	Triton
Tol$^+$,Bfe$^+$ Tsx$^+$,Rcx$^+$,Con$^+$.	AB1133	0.5	0.7	1.3	0.1	2.0
Bfe	P525	0.5	0.6	1.1	0.6	1.9
Tsx	P209	0.5	0.5	1.3	0.4	2.0
Rcx	P224	0.6	0.4	1.1	0.2	2.2
Con	P212	0.6	0.7	0.2	-0.3	1.8
Tol Ia	P218	0.3	0.3	1.3	0.5	2.0
Tol Ib	P210	0.0	0.3	1.1	0.1	1.9
Tol IIb	P651	-1.5	0.4	1.0	-0.4	2.0
Tol IIc	P555	0.1	0.2	0.9	-0.5	1.7
Tol III	P660	-1.5	0.6	0.5	-0.6	1.6
Tol IV	P692	-0.1	0.6	1.0	0.3	0.95
Tol VII	P689	0.6	0.6	1.3	-0.6	1.8
Tol VIII	P602	-2.0	-3.3	0.7	-2.1	-1.3
Tol IX	P596	0.6	0.6	1.2	0.1	2.0
Tol X	P661	0.5	0.8	1.1	0.2	2.0
Tol XI	P220	0.7	0.5	1.3	0.0	1.9
Tol XII	P653	0.7	0.5	1.3	0.1	2.0
Tol XIII	P520	-1.7	0.2	0.3	-0.6	1.7
Tol XIV	P530	0.0	0.2	0.9	0.2	2.0
Tol XV	P686	0.4	0.8	0.9	0.4	2.0
Tol XVI	P516	-1.4	0.3	1.0	0.1	1.8
Tol XVII	P652	-1.1	0.8	0.9	0.1	1.3

(a) DOC is 5mg/ml sodium deoxycholate, SDS is 5mg/ml sodium dedecyl sulphate,
EDTA is 5mg/ml ethylenediaminotetraacetic acid, PEA is 5μl/ml phenethyl
alcohol, Triton is 10 μg/ml Triton X-100.

The only mutants to show a change in the sensitivity pattern were the
Tol VII mutant, P689, which had become resistant to phage U3; the Tol VIII
mutant, P602, which had become partially sensitive to phage C21; and the Tol
XI mutant, P220, which had become resistant to U3, and fully sensitive to
phage C21.

Excretion of colicin inhibitors.

Guterman et al. (1969, 1971, 1973) have shown that ton B (exb A) and
exb B mutants excrete enterochelin, an iron chelator that has the ability to
interfere with the action of colicins B and I (Guterman (1973)).

Using the plate test described in Chapter II, the type and several
other mutants from each of the phenotypic classes resistant to colicins of
group B were tested for the excretion of colicin inhibitors.

None of the mutants produced a substance that could be seen to inhibit
colicins G,H,M,S1, Q or V. Only occasionally could we detect inhibition of
colicins Ia and Ib, and the results were not consistent. Several of the
strains, however, excreted inhibitors of colicins B and D. The results are
summarized in Table 6-3. Ton B mutants excreted inhibitors of both colicins
B and D, as did what are presumably the Exb B mutants, previously isolated
by Guterman and Dann (1973). The Exb C mutant, P535, produced a substance
that interferred with colicin B action, but did not inhibit colicin D action.
The Cbt mutants did not appear to produce any colicin inhibitory substances.
The remaining mutants were all sensitive to colicins B and D, and did not
produce any detectable inhibitor.

In order to check that enterochelin itself would inhibit these colicins,
the plate test described in Chapter II was utilized. Both colicin B and

Table 6-3.

Ability of mutants resistant to colicins of group B to
excrete colicin inhibitors.

Phenotypic Class	Strain	Colicin Inhibitory Excretion (a)	
		Exb (b)	Exd (c)
Ton A	P1205	S	S
Ton B	P585	+	+
Exb B	P575	+	+
Exb C	P535	+	-
Cbt	P295	-	-
Cmt	P1209	S	S
Cir	P625	S	S
Ivt	P645	S	S
Cvt	P1235	S	S

(a) S = sensitive to colicin, + = inhibitor excreted, - = inhibitor not excreted.

(b) Exb = excretes an inhibitor of colicin B.

(c) Exd = excretes an inhibitor of colicin D.

colicin D were inhibited, and both at approximately the same level of enterochelin (100-1000 μg/ml).

Chromium sensitivity of Ton B mutants.

The Ton B mutant, P585, did not appear to have the greatly increased sensitivity to $CrCl_3$, reported previously (Guterman and Dann (1973)), when compared with AB1133, its parent strain. As can be seen from Figure 6-1, the viable count of both strains falls dramatically on the addition of 400 μM $CrCl_3$. Although P585 does appear to be slightly more sensitive to $CrCl_3$, the difference in survival is not great. The difference in optical density observed by Guterman and Dann (1973), could not be demonstrated.

Bacteriophage resistance.

The frequent cross-resistance of colicin resistant mutants to bacterio-phages was discussed in Chapter I. The type mutants from each of the phenotypic classes were tested for resistance to a wide range of bacteriophages by R.E.W. Hancock. The results for mutants resistant to colicins of group A are shown in Table 6-4, and for those resistant to group B colicins in Table 6-5.

As expected the Bfe mutant, P525, is resistant to bacteriophage BF23 and several other similar bacteriophages. Similarly the Tsx mutant, P209, is resistant to T6 and a set of closely related bacteriophages. The Ton A mutant, P1205, was resistant to bacteriophages T1 and T5, and the Ton B mutant, P585, to T1, as expected.

The mutant P212, had a phage resistance pattern identical to that of the Con mutants (see below), a set of mutants that have been shown to be defective in conjugation, and missing a major outer membrane protein

Figure 6-1 : Sensitivity of Ton B mutant P585 to chromium chloride.

$\bigcirc$= AB1133 control, $\triangle$= P585 control, $\bullet$= AB1133 + 400 µM $CrCl_3$,

$\blacktriangle$= P585 + 400 µM $CrCl_3$ in nutrient broth.
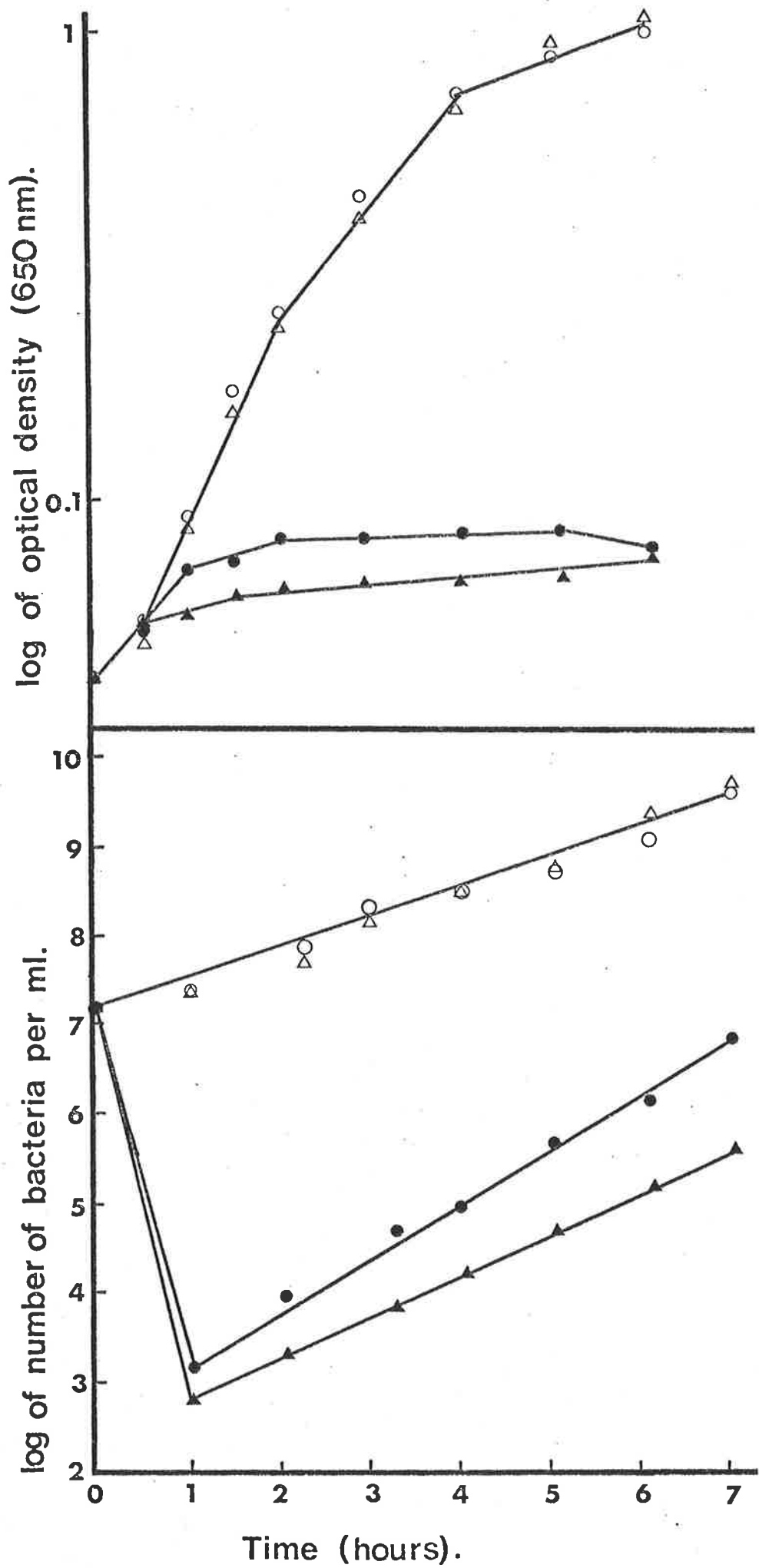
Table 6-4.

Bacteriophage resistance patterns of mutants resistant
to colicins of group A.

Phenotypic Class	Type mutant	Bacteriophage resistance pattern (a)
Bfe	P525	BF23,E15,K6,K8,K11,K12,Ac4.
Tsx	P209	T6,H1,H3,M8,K9,K18,K31,Ox1.
Rcx	P224	-
Con	P212	K3,K4,K5,Ox2,Ox3,Ox4,Ox5,M1,Ac3.
Tol Ia	P218	$E4_{SL}$,$K2_I$,$K20_I$,$K21_I$,K29.
Tol Ib	P210	$K2_I$,$K20_I$,$K21_{IP}$,K29.
Tol IIb	P651	-
Tol IIc	P555	-
Tol III	P660	-
Tol IV	P692	-
Tol VII	P689 (b)	$K2,K20,K21,K29,H^+,Ox5_{SL},E4,V,K16_{SL},K19_{SL},M_P$.
Tol VIII	P602 (b)	$K2_{IP},K20,K21,K29,H^+,Ox5_{SL},A_{SL}$.
Tol IX	P596	$K2_I$,$K20_I$,$K21_I$,$K29_I$.
Tol X	P661	$K2_I$,$K20_I$,$K21_I$,$K29_I$.
Tol XI	P220 (b)	$K2_I$,$K20_P$,$K21_P$,$K29_P$,$Ox2_{IP}$,$Ox4_{IP}$,$Ox5,H^+,E4,V,$ $K16_{SL},K9_P,K31_P$.
Tol XII	P653	-
Tol XIII	P520	-

Table 6-5 continued.

Tol XIV	P530	$K2_I$,K20,K21,K29.
Tol XV	P686	$K2_I$,$K20_I$,K21,K29.
Tol XVII	P652	Partially sensitive to T6,Ox1,Ox2,Ox4,Ox5,Ac3 Ac4,H1,M1,K2,K20,K21,K29,K3,K4,K5. Resistant to all others.

(a) I = bacterial inhibition, IP = partial resistance with inhibition, SL = slight resistance, P = partial resistance, as defined by Hancock and Reeves (1974a). All others are full resistance.

(b) All other bacteriophages, when spotted onto lawns of these mutants, produced hazy areas of lysis after overnight incubation at 37 C. i.e. the bacteriophages were partially inhibited.

Table 6-5.

Bacteriophage resistance pattern of mutants resistant

to colicins of group B.

Phenotypic Class	Type Strain	Bacteriophage resistance pattern
Ton A	P1205	T1,E25,K22,K26,K27,K30,T5,D,E21.
Ton B	P585	T1,E25,K22,K26,K27,K30.
Exb B	P575	-
Exb C	P535	-
Cbt	P295	-
Cmt	P1209	-
Cir	P625	-
Ivt	P645	-
Cvt	P1235	-

(Skurray, Hancock and Reeves (1974)).

The Tol Ia, Tol Ib, Tol X, Tol XIV and Tol XV mutants (P218, P210, P661, P530 and P686) all showed varying degrees of resistance to a specific set of bacteriophages (K2, K20, K21, K29 and occasionally E4), showing a great similarity to the Ktw bacteriophage resistant mutants (see below).

In addition to the Tol VII, Tol VIII and Tol XI mutants (P689, P602 and P220) show a superficial similarity to the Bar mutants (see below). They show resistance (although only partial in some cases) to a wide range of bacteriophage.

The Tol XVII mutant (P652) is remarkable in that it appears to be resistant to almost all of the bacteriophages, including T1, T5 and T6, resistance to which has only been previously demonstrated in ton A, ton B and tsx mutants.

The remaining mutants, including all the mutants resistant to colicins of group B (except for the ton A and ton B mutants) were sensitive to all of the bacteriophages.

Colicin resistance in bacteriophage resistant mutants.

In order to cross check the cross-resistance patterns, a series of mutants, isolated as bacteriophage resistant (Hancock and Reeves (1974a, 1974b)), were checked for their colicin resistance pattern. The results are shown in Table 6-6.

The Ton A, Ton B, Bfe and Tsx I mutants showed the same colicin resistance pattern as P1209, P585, P525 and P209, respectively. Thus the mutants isolated by the two procedures appear to be identical. As expected the Con mutant was tolerant to colicins K and L.

Table 6-6.

Colicin resistance pattern of a set of bacteriophage resistant mutants.

Phenotypic class (a)	Strain	Bacteriophage resistance pattern (b)	Colicin resistance pattern
Ton A	P417	T1,E25,K22,K26, K27,K30,T5,D,E21	M
Ton B	P442	T1,E25,K22,K26, K27,K30	B D G H Ia Ib M S1 Q V
Bfe	P445	BF23,E15,K6,K8, K11,K12,AC4	E1 E2 E3 A
Ktn	P446	K10	-
Tsx	P407	T6,H1,H3,M8,K9, K18,K31,OX1	K
Tsx	P433	$T6_P$,H1,H3,H8, $K9_P$,K18,$K31_P$, $OX1_{SL}$.	-
Con	P460	K3,K4,K5,OX2,OX3, OX4,OX5,M1,AC3	K L
Efr	P448	E4	-
Ktw I	P456	$K2_{IP}$,K20,K21,K29	K L A S4 N
Ktw II	P476	$E4_{SL}$,$K2_{IP}$,K20, K21,K29	K L A S4
Ktw III	P240	$K2_{SL}$,$K20_{IP}$,$K21_{IP}$, $K29_{IP}$,H^+_I	-
Ttk I	P429	E4,T2,K16,K19, $OX5_P$,F27,H^+,V	-
Ttk II	P423	T4,K16,E7,$OX5_{SL}$, F27,H^+,$K17_{SL}$,V_{SL}	-

Table 6-6 continued

Ttk III	P425	$T4,K16,K19,E7_{IP}$, $OX5,F27,H^+,V$	-
Ttk IV	P474	$K16,K19,OX5_P$, $F27,H^+,V$	-
Miscellaneous I	P491	$T4_P,K16_{SL},E7_P$, $H^+,K17_{SL}$	-
Miscellaneous II	P443	$E7_P,T6_{SL}$	-
Miscellaneous III	P498	$K16_{SL},F27,H^+_{SL}$	-
Miscellaneous IV	P237	$OX5_{SL},K16_{SL}$, $F27,H^+_{SL}$	-
Miscellaneous V	P493	$OX5_{SL},K16_{SL}$, $K17_{SL},E4_I$	L
Bar I	P455	(c)	L
Bar II	P492	(c)	Not done
Bar III	P494	(c)	-
Bar III	P409	(c)	L
Bar III	P404	(c)	A L S4
Bar III	P413	(c)	A L S4
Bar III	P415	(c)	A L S4
Bar III	P495	(c)	A L S4
Bar III	P496	(c)	A L S4
Bar III	P497	(c)	A L S4
Bar IV	P405	(c)	L
Bar IV	P428	(c)	L
Bar IV	P436	(c)	L S4

Table 6-6 continued.

Bar IV	P490	(c)	L S4
Bar V	P402	(c)	A L S4 N
Bar VI	P451	(c)	A L S4
Bar VII	P487	(c)	A L
Bar VII	P488	(c)	A L S4
Bar VII	P489	(c)	A L
Wrm I	P435	(d)	L S4
Wrm I	P479	(d)	A L
Wrm II	P416	(d)	L
Wrm II	P424	(d)	-
Wrm II	P235	(d)	A

(a) The isolation and characterization of the various phenotypic groups has been described by Hancock and Reeves (1974a, 1974b).

(b) I = bacterial inhibition, IP = partial resistance with inhibition, SL = slight resistance, P = partial resistance. All others are full resistance. For basis of differentiation see Hancock and Reeves (1974a).

(c) Resistant to between 10 and 19 specific bacteriophages as described in Table 7 of Hancock and Reeves (1974a).

(d) Resistant to between 30 and 33 specific bacteriophages as described in Table 8 of Hancock and Reeves (1974a).

The similarity in bacteriophage resistance between some of the mutants resistant to colicins of group A, and Ktw mutants (Hancock and Reeves (1974a, 1974b)), was confirmed when the colicin resistance pattern of the Ktw mutants was determined. The Ktw I mutant had a colicin resistance pattern identical to that of the Tol Ib mutant, and the Ktw II and Tol Ia mutants also appeared to be identical. However, the Ktw III mutant, P240, did not appear to be resistant to any of the colicins.

In addition, many of the Bar and Wrm mutants showed cross-resistance to various combinations of colicins A, L, S4 and N. The remaining mutants all appeared to be sensitive to the full range of colicins used in this study.

Summary.

Resistance to colicins of group A seems to be accompanied, in some cases, with resistance to bacteriophages and increased sensitivity to detergents, surfactants and antibiotics. The various changes observed in these mutants are summarized in Table 6-7.

Mutants resistant to colicins of group B do not appear to have increased antibiotic, detergent or surfactant sensitivity, and apart from the tonA and tonB mutants, are not cross-resistant to bacteriophages. Several of these mutants, however, excrete colicin inhibitors.

Some of the pleiotropy observed (particularly in the mutants resistant to colicins of group A) could possibly be due to the simultaneous selection of mutations at different genetic loci. The use of spontaneous mutants, however, makes this possibility less likely.

Table 6-7.

Summary of the various properties associated with mutation to resistance to colicins of group A.

Pheno-typic class	Type strain	Colicin resistance pattern	Sensitivity to antibiotics, detergents, surfactants. (a,b)	Mucoid-icity (c)	λc190c17 (d)	C21/U3. (e)	Bacterio-phage resistance (f)
Bfe	P525	E1 E2 E3 A (g)	-	-	-	-	Bfe
Tsx	P209	K (g)	-	-	-	-	Tsx
Rcx	P224	X	-	-	-	-	-
Con	P212	K L	EDTA,PEA	-	-	-	Con
Tol Ia	P218	K L A S4	-	-	-	-	Ktw
Tol Ib	P210	K L A S4 N	-	-	-	-	Ktw
Tol IIB	P651	E1 E2 E3 K	AMP,DOC, pEDTA,PEA	-	-	-	-
Tol IIc	P555	E1 E2 E3 K L A D4 N	pEDTA,PEA	-	-	-	-
Tol III	P660	E2 E3 K L A S4 N	AMP,DOC,EDTA, PEA,pTriton.	-	-	-	-
Tol IV	P692	E2 E3 L A N	pEDTA,pTriton	-	-	-	-
Tol VII	P689	pE2 pK L A S4	PEA	-	+	+	Bar?
Tol VIII	P602	E1 pK A	AMP,DOC,SDS, EDTA,PEA,Triton	-	-	+	Bar?
Tol IX	P596	pK L A N	-	-	-	-	Ktw
Tol X	P661	pK L A S4 N	-	-	-	-	Ktw
Tol XI	P220	pK L pA pS4	PEA	+	+	+	Bar?
Tol XII	P653	pE3 L A pS4	-	-	-	-	-

Table 6-7 continued

Tol XIII	P520	E2 E3 K L A S4	AMP,DOC,EDTA PEA	-	-	-	-
Tol XIV	P530	E2 E3 K L A N X	pEDTA	-	-	-	Ktw
Tol XV	P686	E2 E3 K L A S4 N X	pEDTA	-	-	-	Ktw
Tol XVI	P516	pE2 pE3 L A	pEDTA,DOC	-	-	-	-
Tol XVII	P652	E1 pE2 pE3 K L A S4 N	AMP,DOC,EDTA, pTriton	-	-	-	wide resistance

(a) p = partially sensitive.

(b) Sensitivity to the various agents was defined as when the sensitivity index of a particular mutant was less than -1.0 for SDS, and DOC, 0.8 for EDTA, 0 for PEA, or 0.9 for Triton. AMP means the particular mutant was sensitive to less than 1 µg/ml of ampicillin.

(c) + = mucoid.

(d) + = resistant to bacteriophage λcI90c17.

(e) + = altered sensitivity pattern to the two lipopolysaccharide bacterio-phages C21 and U3.

(f) Bacteriophage resistance phenotype that the mutant class resembled.

(g) Receptor mutant.

CHAPTER VII

SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF MEMBRANE PREPARATIONS

Introduction.

The suggestion that colicin tolerant mutants may have alterations to their membranes was discussed in Chapter I, and changes have previously been demonstrated in certain mutants (e.g. Holland and Tuckett (1972)). The type strains from each of the phenotypic classes were therefore screened for membrane alterations using SDS-polyacrylamide gel electrophoresis.

Whole membrane preparations from each of the mutants (both "heated" and "unheated") were run on the pH 7.2 gel system described by Schnaitman (1973). Outer membrane preparations ("heated" and "unheated") were run on either the pH 7.2 gel system (Schnaitman (1973)), or in the case of some heated preparations, on the alkaline gel system (pH 11.4) described by Bragg and Hou (1972).

"Unheated" outer membrane preparations showed the two major peaks (A and C) described by Schnaitman (1973,1974a,1974b), when run on neutral pH gels (see Fig 7-1). When outer membrane preparations are heated, the peak A (protein 1 in K12) and peak C (proteins 3a and 3b) described by Schnaitman (1973) run as a single peak, called B (Schnaitman (1973,1974a,1974b)), on neutral pH gels (see Fig 7-1). Many of the "unheated" preparations, however, show some evidence of peak B (e.g. Fig 7-1), indicating that despite the fact that the solubilization was carried out at 37 C, some denaturation of proteins 1 and 3 was occurring, and some of the protein was running at a position more indicative of samples heated at 100 C.

When outer membrane samples are run on the alkaline gel system described by Bragg and Hou (1972) (Fig 7-2), the single peak B observed on neutral pH gels is differentiated into two peaks - one peak contains protein 1, the other

Figure 7-1 : Comparison, by densitometry, of stained bands of outer

membrane proteins, run using the SDS - polyacrylamide gel system of

Schnaitman (1973), using neutral pH buffer systems. Figures 7-1(a)

and 7-1(b) show the "unheated" and "heated" outer membrane prepar-

ations from AB1133, respectively. A, B and C refer to the peaks

described by Schnaitman (1973,1974a,1974b). The arrow marks the

position of the dye front. The top of the gel is on the left hand

side of the page.
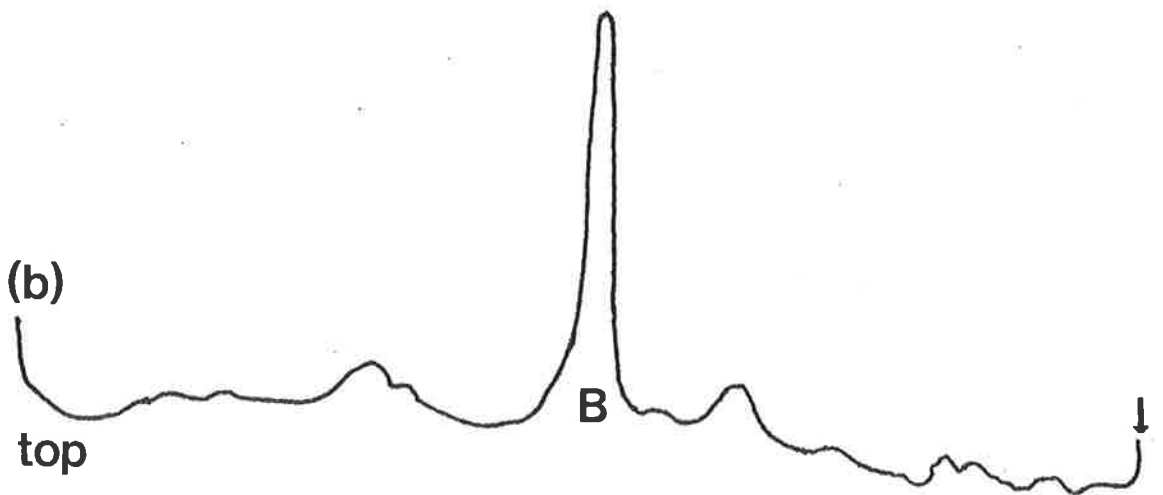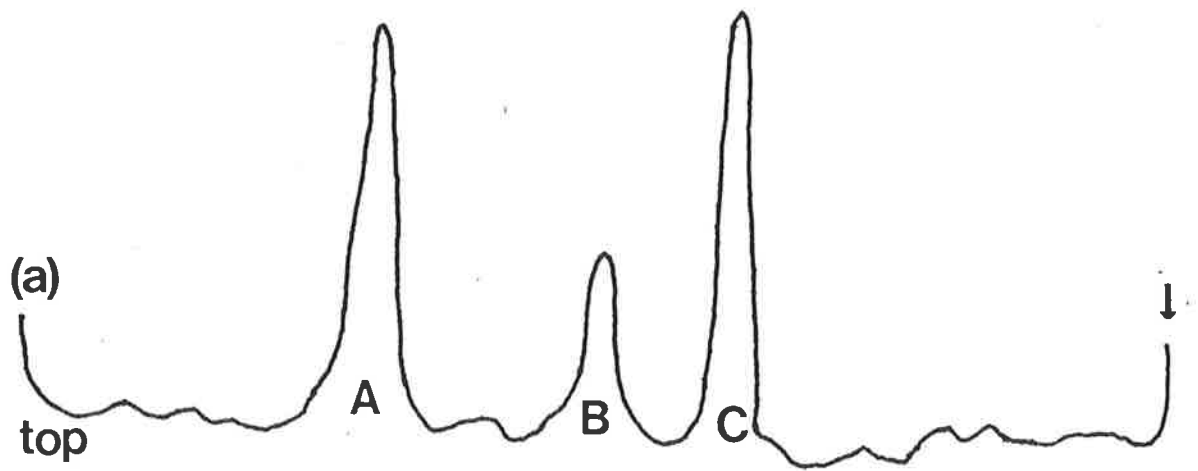
Figure 7-2 : Comparison, by densitometry, of stained bands of outer membrane proteins, run using the SDS-polyacrylamide gel systems of Schnaitman (1973), using either neutral or alkaline pH buffer systems. Figures 7-2(a) and 7-2(b) show "heated" outer membrane preparations from AB1133 run on neutral and alkaline pH gel systems, respectively. B refers to the peak B described by Schnaitman (1973,1974a,1974b). 1 and 3 refer to proteins 1 and 3 (i.e. 3a and 3b) described by Schnaitman (1973,1974a,1974b). The arrow marks the position of the dye front. The top of the gel is on the left hand side of the page.

(a)

top

B

(b)

top

1

3
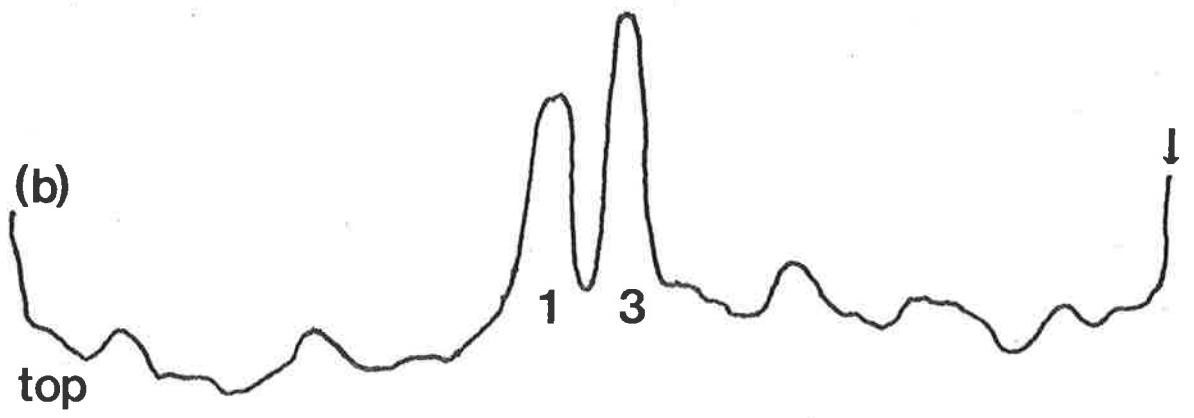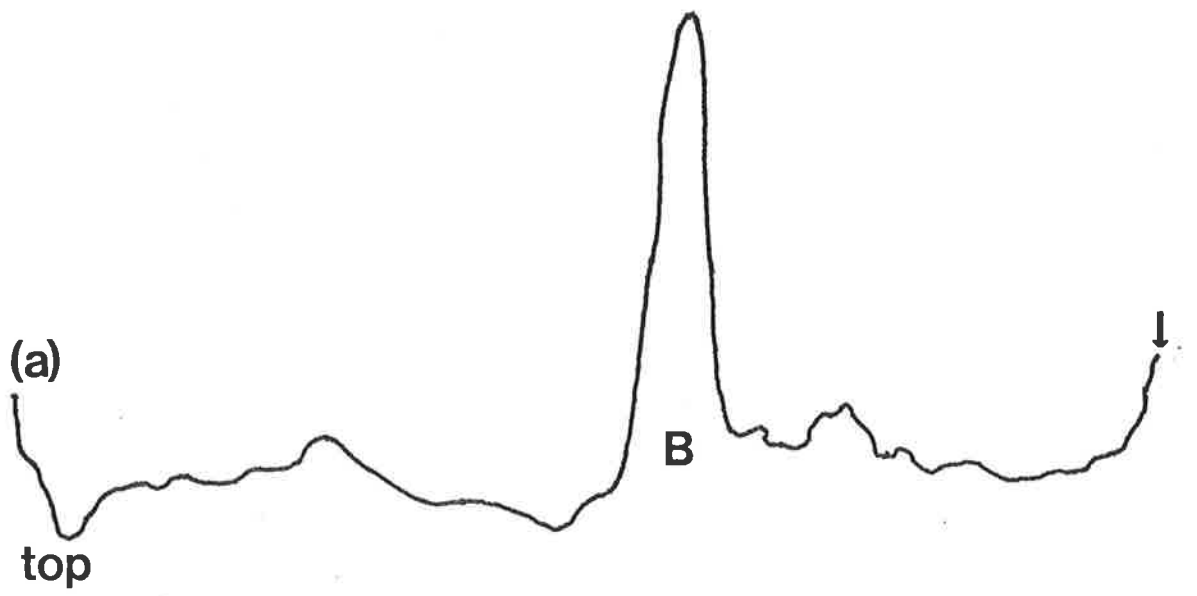
proteins 3a and 3b (Schnaitman (1973,1974a,1974b). It was also observed that peak B could sometimes be resolved into two on neutral gels - apparently due to alkaline conditions developing in the upper resevoir buffer, the partial separation of proteins 1 and 3 was achieved in a manner similar to that shown by Bragg and Hou (1972).

Alterations to the outer membrane in mutants resistant to colicins of group A.

Many of the mutants resistant to colicins of group A showed alterations in their membrane protein composition, as determined by polyacrylamide gel electrophoresis of whole membrane preparations. In each case the same differences could be detected in outer membrane preparations - in no case was it possible to detect changes in what would presumably be the cytoplasmic membrane (i.e. differences detected in whole membrane preparations that were not detectable in outer membrane preparations).

A Con mutant, selected as bacteriophage resistant, has been shown (Skurray, et al. (1974)) to be missing the peak C (containing the proteins 3a and 3b) described by Schnaitman (1973,1974a,1974b). "Unheated" outer membrane preparations from P212 did appear to be deficient in peak C on pH 7.2 gels, and, as expected, in protein 3 on the alkaline gel system (Fig 7-3), confirming the results obtained with the Con mutant P460 (Skurray, et al. (1974)).

"Unheated" outer membrane preparations from several other mutants showed substantial differences when compared to the parent strain, AB1133, on neutral pH gels. The Tol Ia (P218), Tol Ib (P210), Tol VII (P689), Tol IX (P596), Tol X (P661), Tol XI (P220) and Tol XV (P686) mutants all appear to have alterations in the peak A described by Schnaitman (1973), which has been shown

Figure 7-3 : Comparison, by densitometry, of stained banks of outer membrane proteins, run using the SDS-polyacrylamide gel systems of Schnaitman (1973), using either neutral or alkaline pH buffer systems. Figures 7-3(a) and 7-3(b) show the "unheated" outer membrane proteins of AB1133 and P212 (Con) respectively, run on neutral pH gels. Figures 7-3(c) and 7-3(d) show the "heated" outer membrane proteins of AB1133 and P212 respectively, run on alkaline gels. A, B and C refer to the peaks A, B and C described by Schnaitman (1973,1974a,1974b). 1 and 3 refer to proteins 1 and 3 (i.e. 3a and 3b) described by Schnaitman (1973,1974a,1974b). The arrow marks the position of the dye front. The top of the gel is on the left hand side of the page.
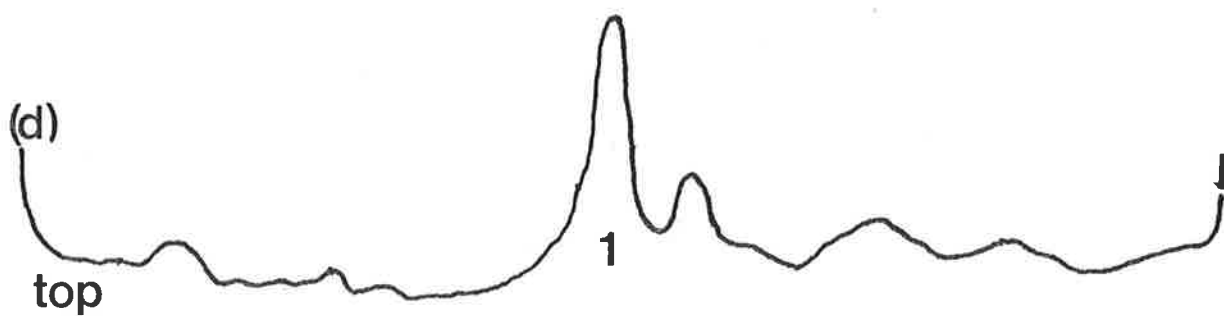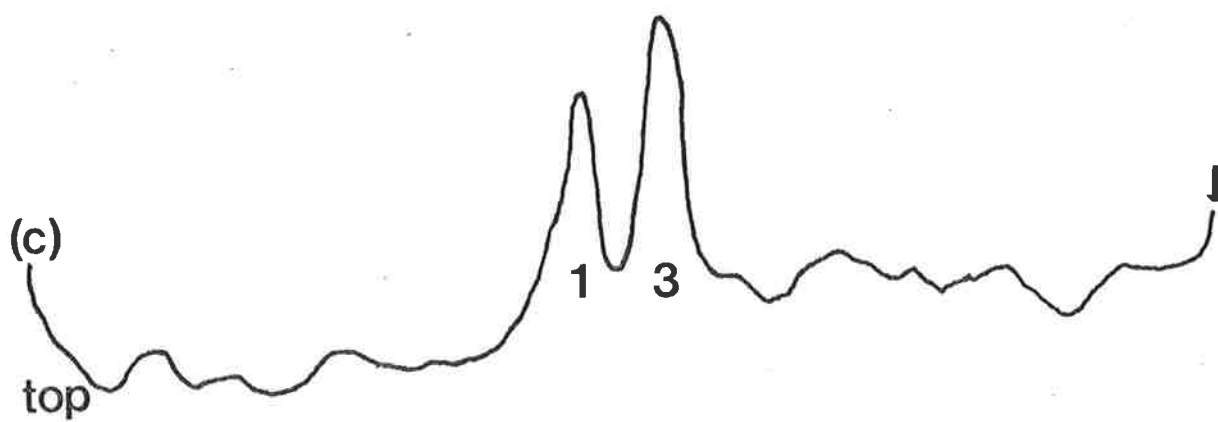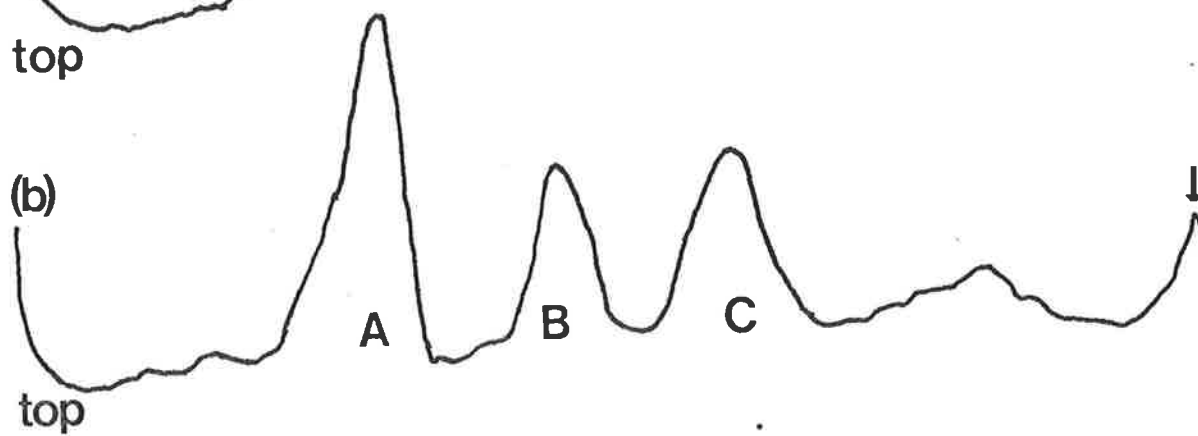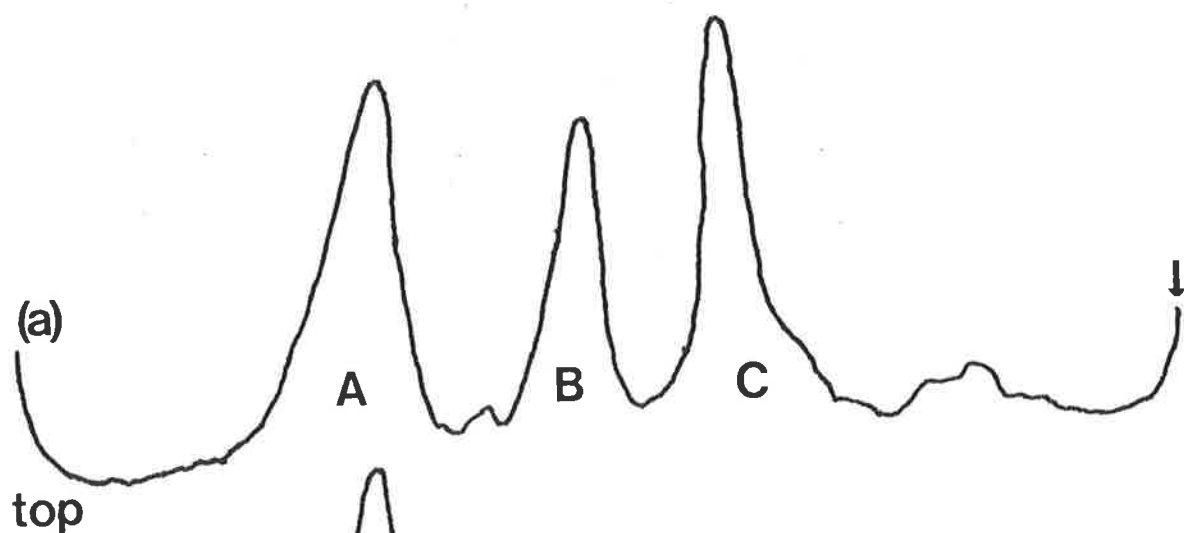
to consist of protein 1 in <u>Escherichia coli</u> K12 (Schnaitman (1973,1974a,
1974b). Densitometry scans of the gels are shown in Figs 7-4, 7-5 and 7-6.

In addition to the mutants with a diminished peak A, the Tol IV mutant
P692, and the Tol XIV mutant P530 appeared to completely lack peak A, when
"unheated" outer membrane preparations are run on neutral pH gels. Densi-
tometry scans of the gels are shown in Fig 7-7 and Fig 7-8.

In each case where a mutant has been shown to have a lowered peak A,
the peak corresponding to protein 1 on alkaline gels, and/or the peak corres-
ponding to protein 1 on neutral gels in which peak B was differentiated into
two, was also lowered, when compared to AB1133. With P692 and P530, these
peaks were completely absent. Both mutants apparently lack protein 1
completely.

None of the other mutants resistant to colicins of group A showed any
major changes in the protein composition of either whole membrane or outer
membrane preparations, on the gel systems used. The scans for the "unheated"
whole membrane preparations are shown in Fig 7-9.

<u>Outer membrane alterations in mutants resistant to colicins of group B.</u>

Whole membrane preparations of the Ton B mutant, P585, and the Exb B
mutant, P575, were compared with that of the parent strain, AB1133, on neutral
pH gels. It was immediately apparent that a new high molecular weight peak
had appeared on scans of the gels loaded with "unheated" whole membrane pre-
parations from the two mutants. It has been suggested that proteins in heated
membrane preparations run in a position that is more indicative of their true
molecular weight, since they are fully unfolded or denatured (Schnaitman
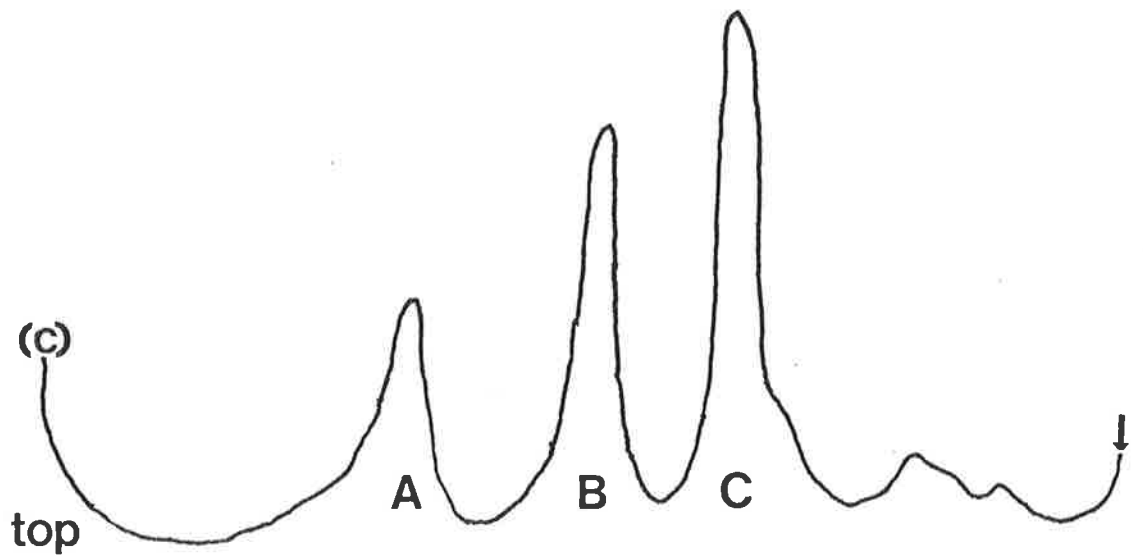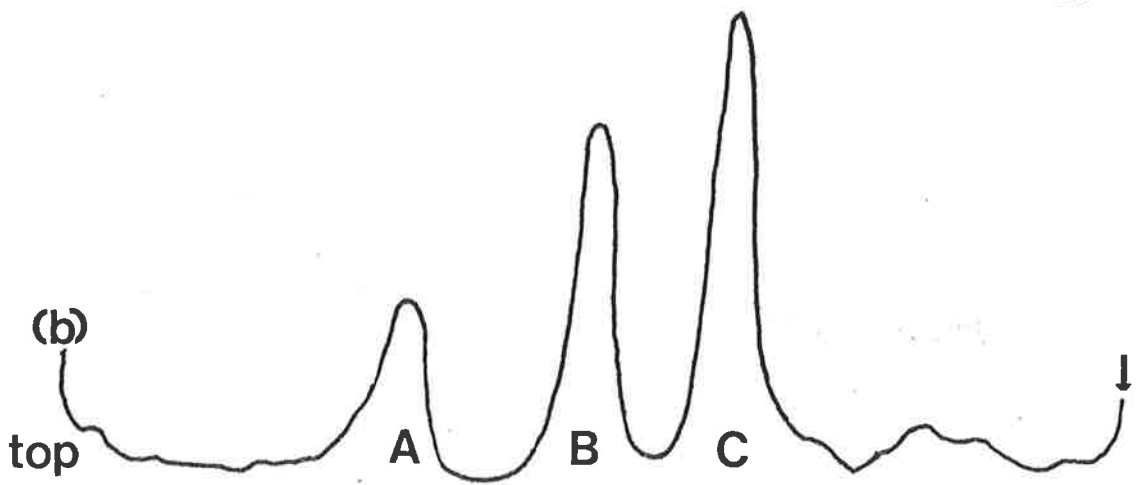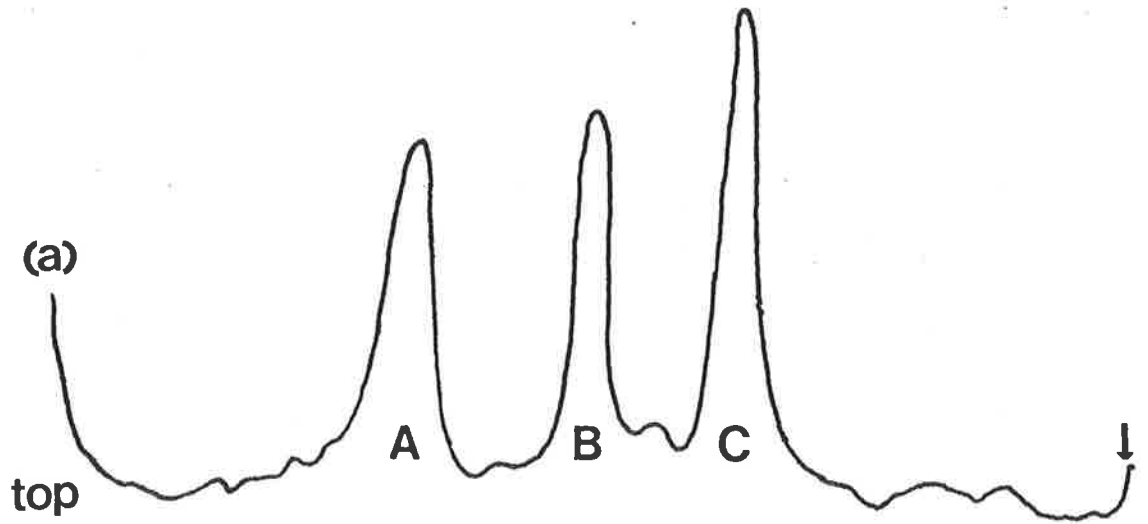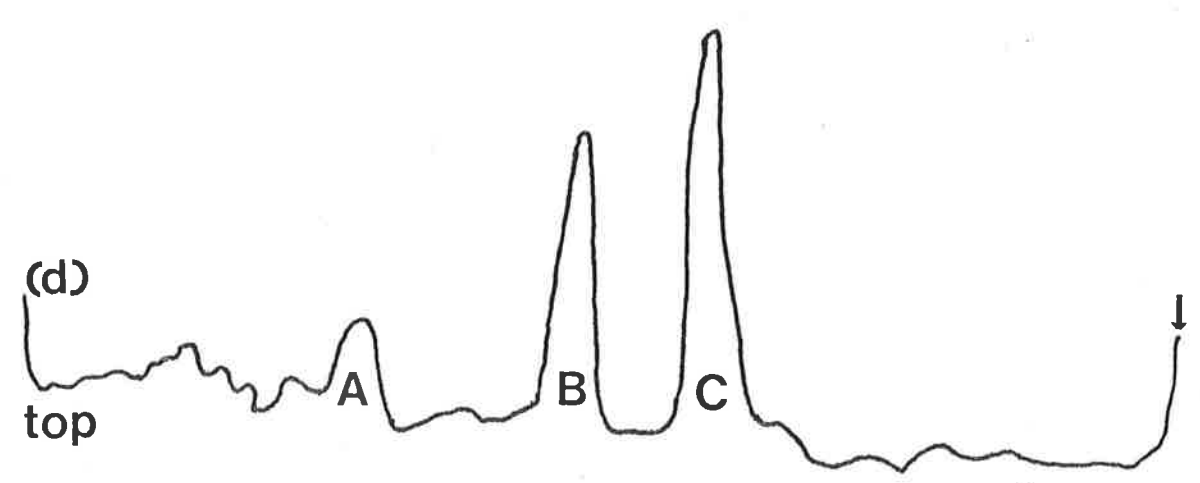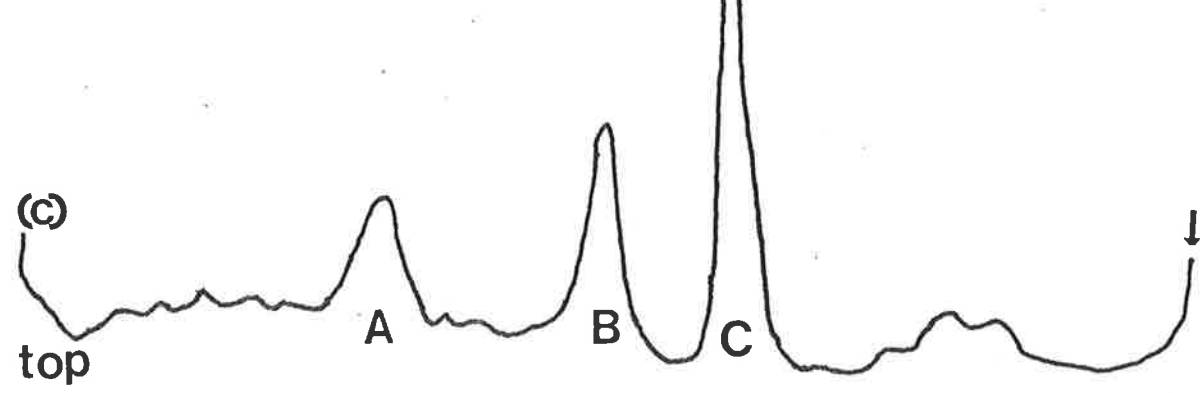(1973)). "Heated" whole membrane preparations of P585 and P575 showed two

Figure 7-4 : Comparison, by densitometry, of stained bands of outer membrane proteins, run using the SDS-polyacrylamide gel system of Schnaitman (1973), using a neutral pH buffer system. Figure 7-4(a), 7-4(b) and 7-4(c) show the "unheated" outer membrane preparations of AB1133, the Tol Ib mutant P210, and the Tol Ia mutant P218 respectively, run on neutral pH gels. A, B and C refer to the peaks A, B and C described by Schnaitman (1973,1974a,1974b). The arrows mark the positions of the dye fronts. The top of the gel is on the left hand side of the page.
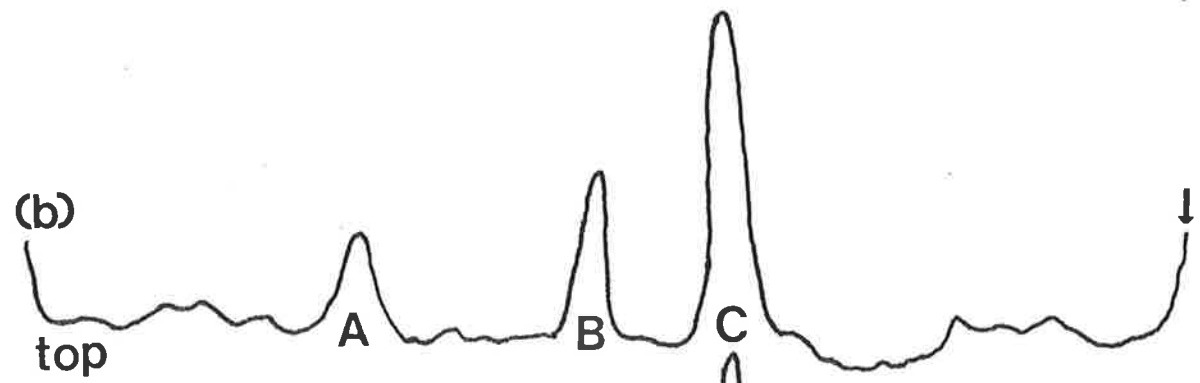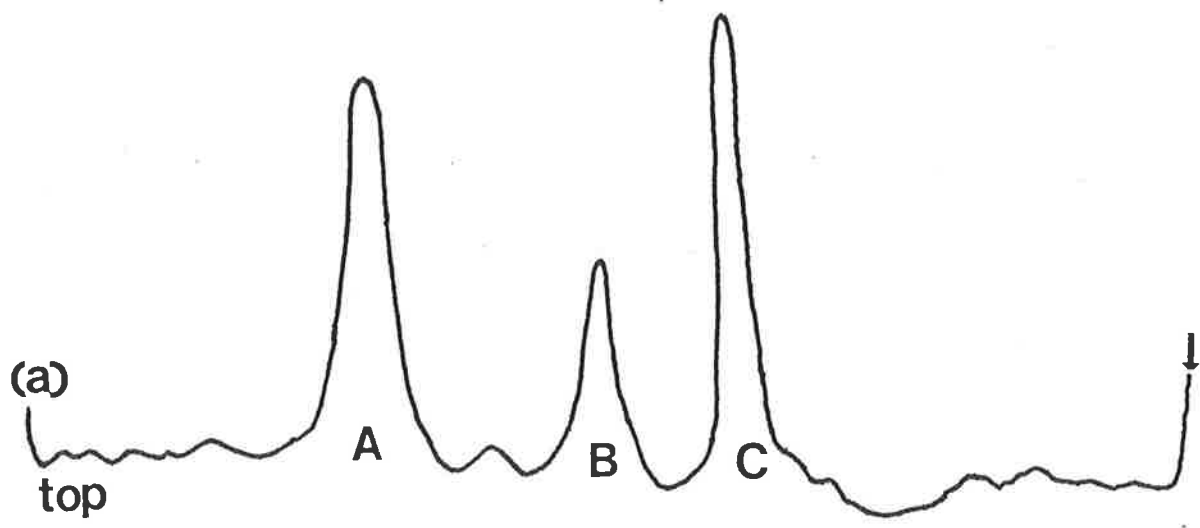
Figure 7-5 : Comparison, by densitometry, of stained bands of outer

membrane proteins, run using the SDS-polyacrylamide gel system of

Schnaitman (1973), using a neutral pH buffer system. Figures

7-5(a), 7-5(b), 7-5(c) and 7-5(d) show the "unheated" outer mem-

brane preparations of AB1133, the Tol VII mutant P689, the Tol

IX mutant P596, and the Tol X mutant P661, respectively, run on

neutral pH gels. A, B and C refer to the peaks A, B and C

described by Schnaitman (1973, 1974a,1974b). The arrows mark

the positions of the dye fronts. The top of the gel is on the
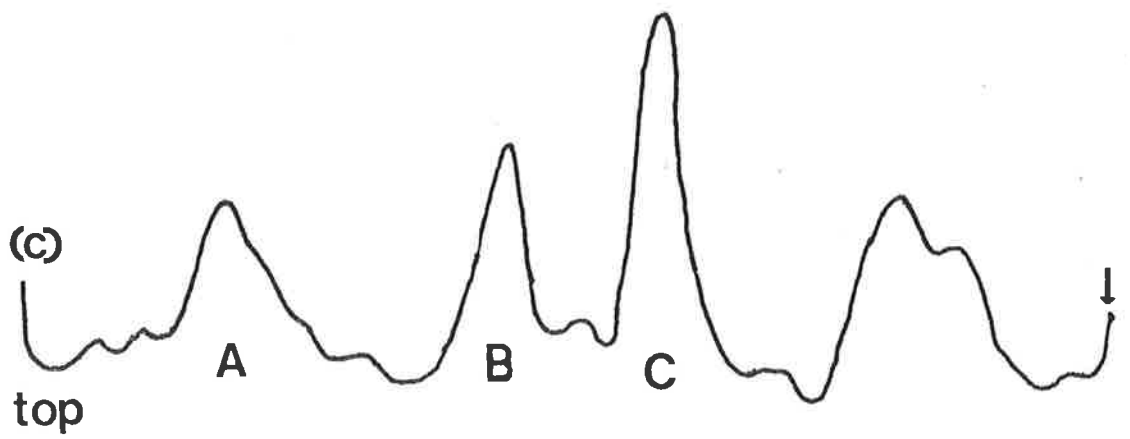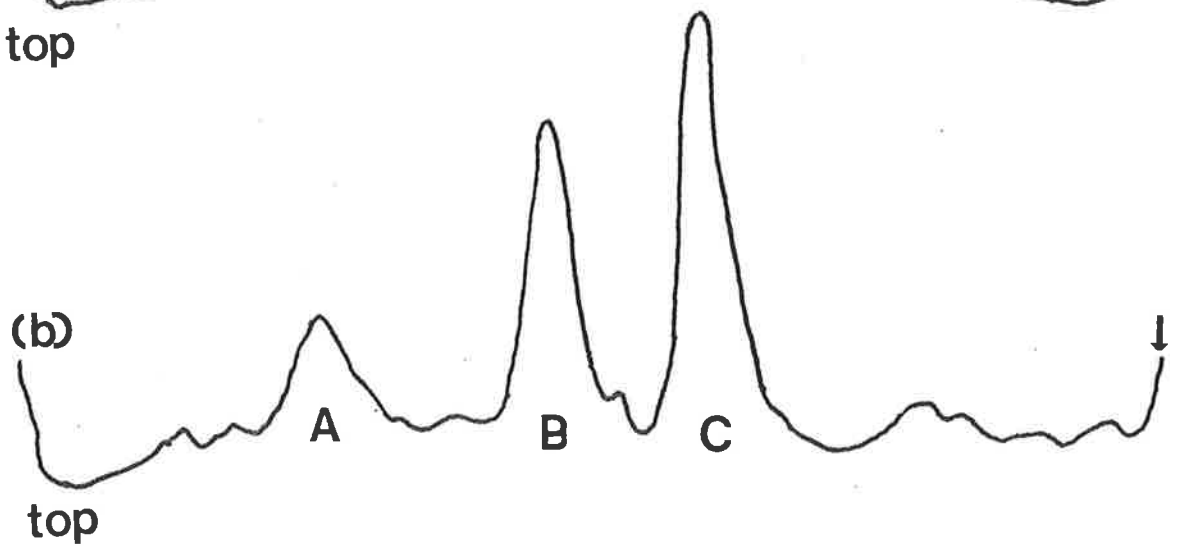
left hand side of the page.

Figure 7-6 : Comparison, by densitometry, of stained bands of outer membrane proteins, run using the SDS-polyacrylamide gel system of Schnaitman (1973), using a neutral pH buffer system. Figures 7-6(a), 7-6(b) and 7-6(c) show the "unheated" outer membrane preparations of AB1133, the Tol XI mutant P220, and the Tol XV mutant P686, respectively, run on neutral pH gels. A, B and C refer to the peaks A, B and C described by Schnaitman (1973, 1974a,1974b). The arrows mark the positions of the dye fronts. The top of the gel is on the left hand side of the page.
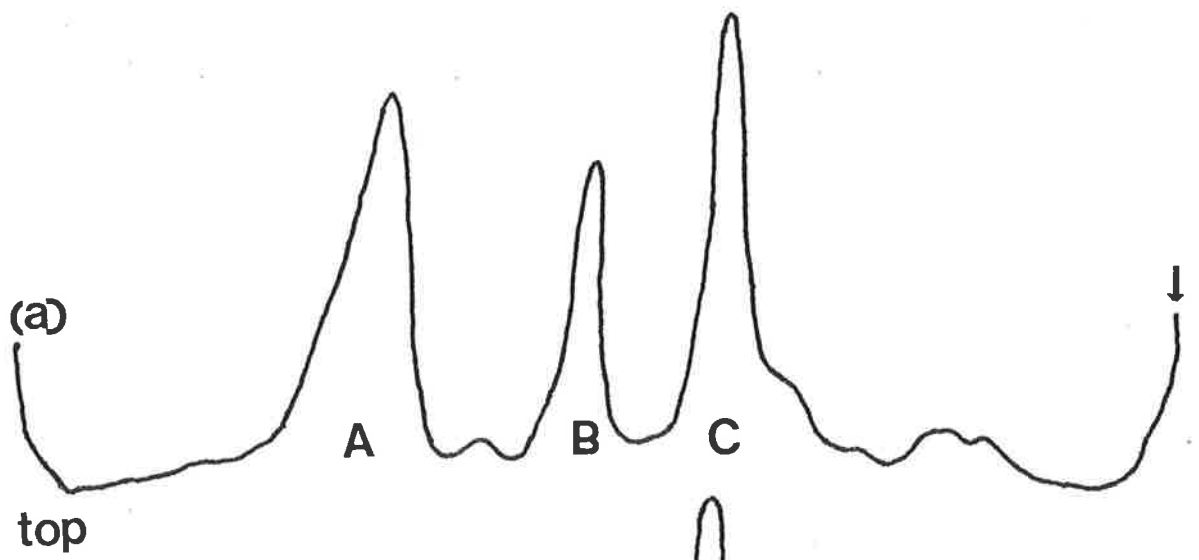
Figure 7-7 : Comparison, by densitometry, of stained bands of outer
membrane proteins, run using the SDS-polyacrylamide gel system of
Schnaitman (1973), and either neutral or alkaline pH buffer systems.
Figures 7-7(a) and 7-7(b) show the "unheated" outer membrane pro-
teins of AB1133 and the Tol IV mutant P692, respectively, run on
neutral pH gels. Figures 7-7(c) and 7-7(d) show the "heated"
outer membrane proteins of AB1133 and P692, respectively, run on
alkaline gels. A, B and C refer to the peaks A, B and C described
by Schnaitman (1973,1974a,1974b). 1 and 3 refer to proteins 1 and
3 (i.e. 3a and 3b) described by Schnaitman (1973,1974a,1974b). The
arrows mark the positions of the dye fronts. The top of the gel
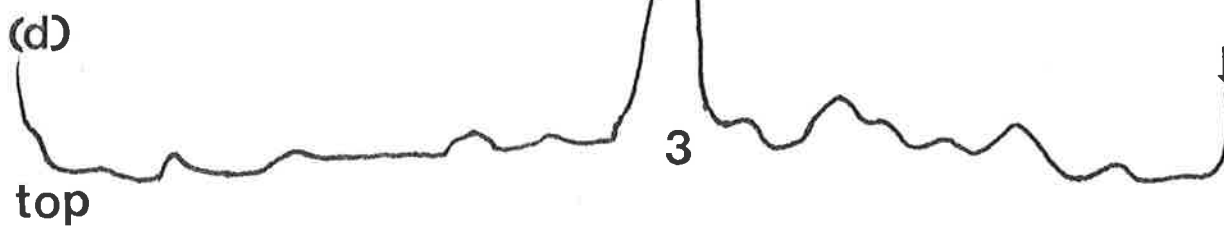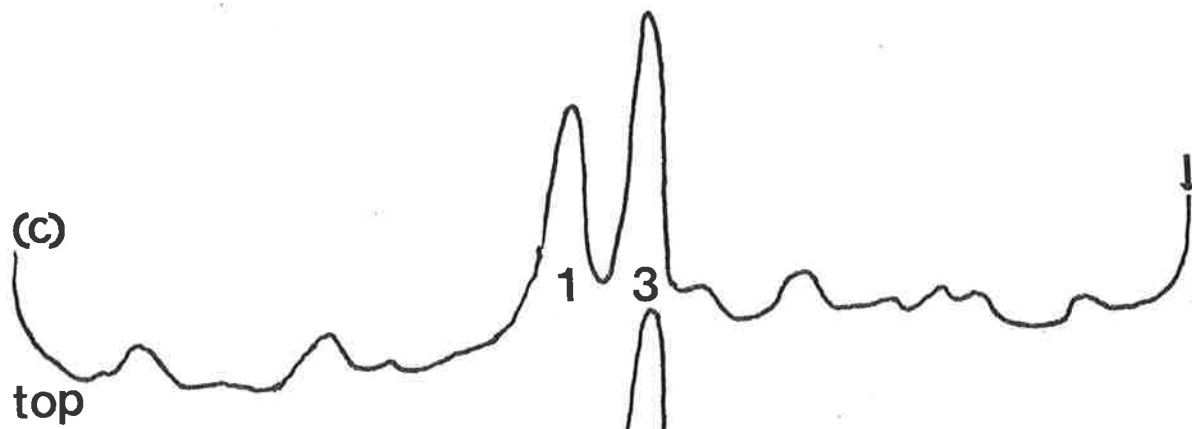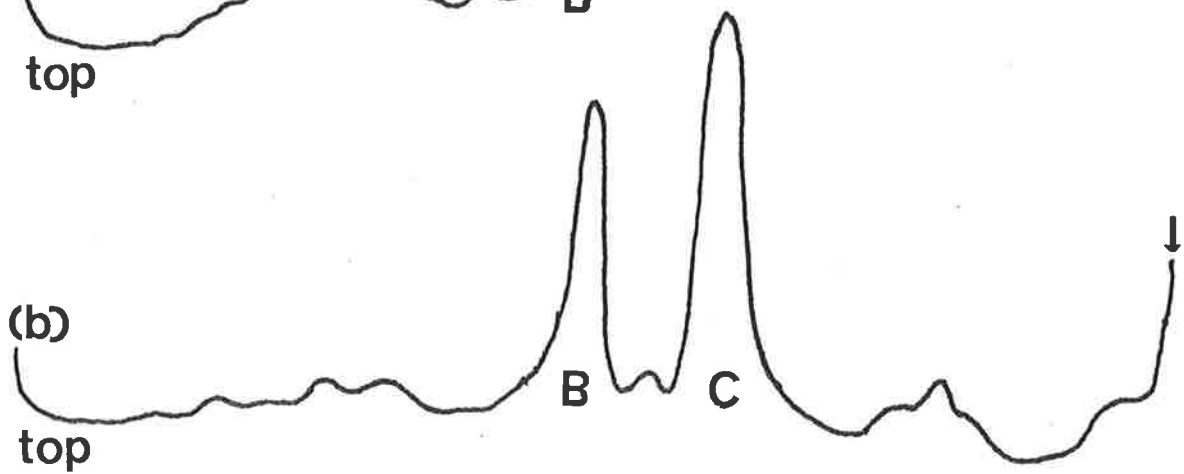is on the left hand side of the page.

Figure 7-8 : Comparison, by densitometry, of stained bands öf outer membrane proteins, run on the SDS-polyacrylamide gel system of Schnaitman (1973), using a neutral pH buffer system. Figures 7-8(a) and 7-8(b) show the "unheated" outer membrane proteins of AB1133 and the Tol XIV mutant P530, respectively, run on neutral pH gels. A, B and C refer to the peaks A, B and C described by Schnaitman (1973, 1974a,1974b). The arrows mark the positions of the dye fronts. The top of the gel is on the left hand side of the page.
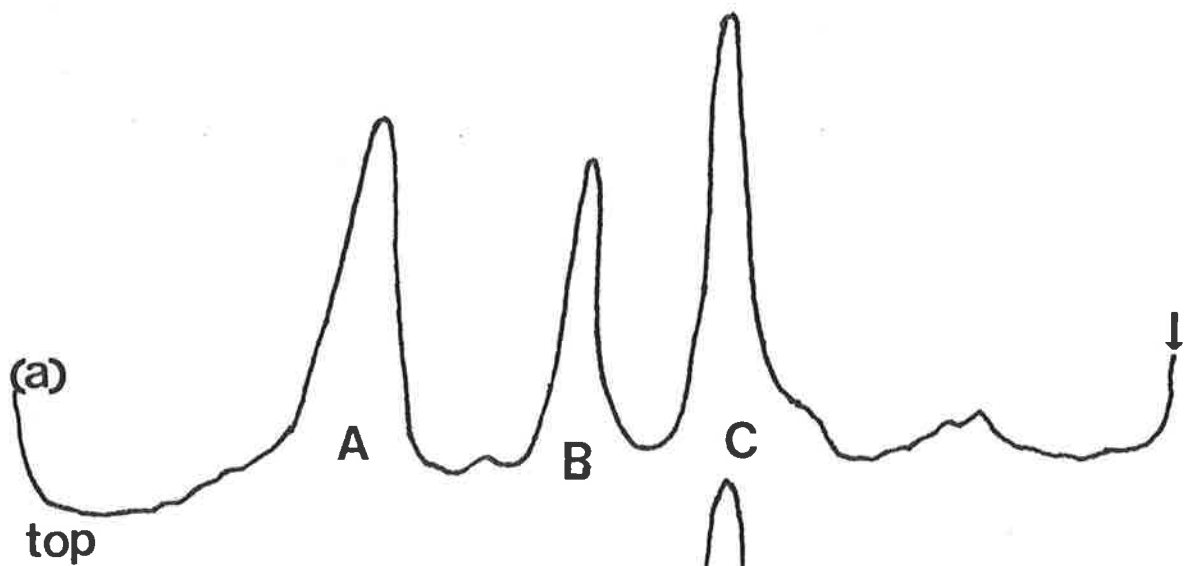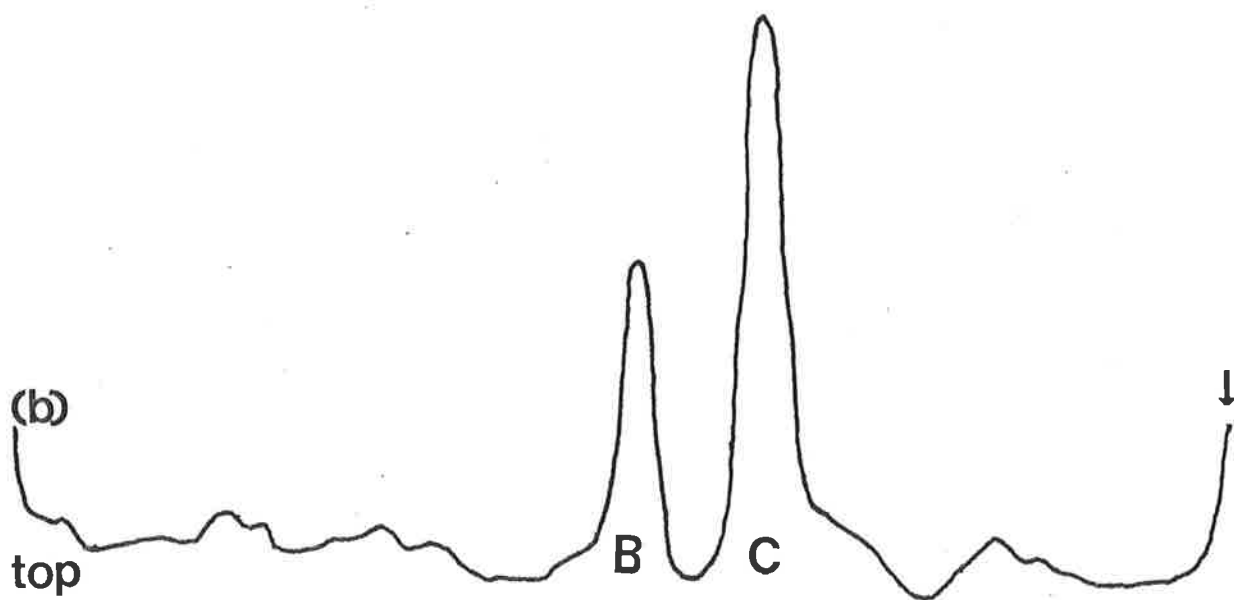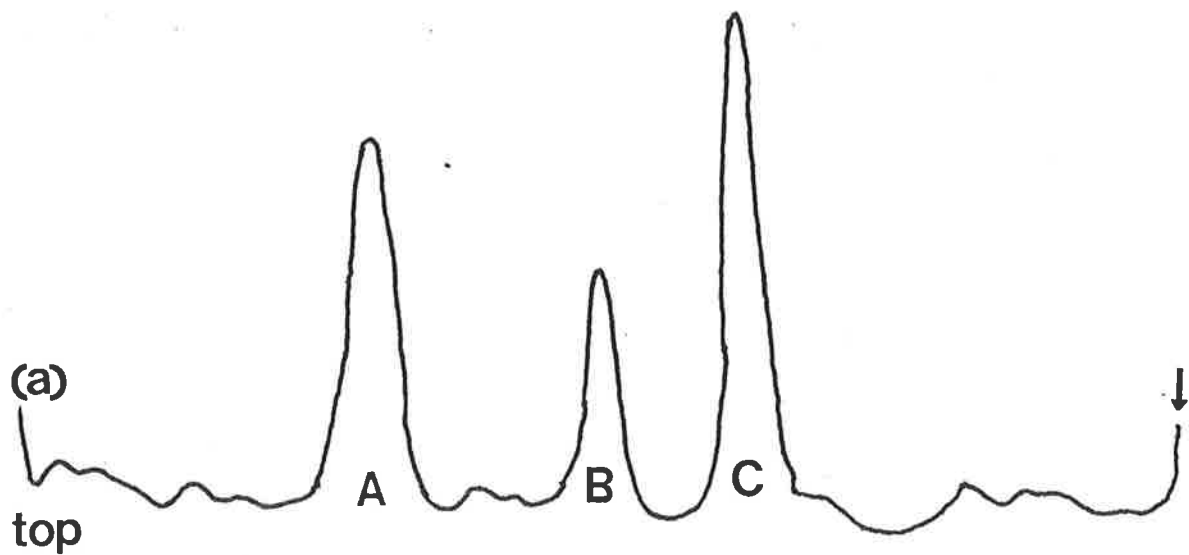
Figure 7-9 : Comparison, by densitometry, of stained bands of whole
membrane proteins, run on the SDS-polyacrylamide gel system described
by Schnaitman (1973). Figures 7-9(a), 7-9(b), 7-9(c) and 7-9(d) show
the "unheated" whole membrane proteins of AB1133, the Bfe mutant
P525, the Tsx mutant P209 and the Rcx mutant P224, respectively, run
on neutral pH gels. Figures 7-9(e), 7-9(f), 7-9(g) and 7-9(h) show
the "unheated" whole membrane proteins of the Tol IIb mutant P651,
the Tol IIc mutant P555, the Tol III mutant P660 and the Tol VIII
mutant P602, respectively, run on neutral pH gels. Figures 7-9(i),
7-9(j), 7-9(k) and 7-9(l) show the "unheated" whole membrane
proteins of the Tol XII mutant P653, the Tol XIII mutant P520, the
Tol XVI mutant P516, and the Tol XVII mutant P652, respectively,
run on neutral pH gels. A, B and C refer to the peaks A, B and
C described by Schnaitman (1973,1974a,1974b). The arrows mark
the positions of the dye fronts. The top of the gel is on the left
hand side of the page.
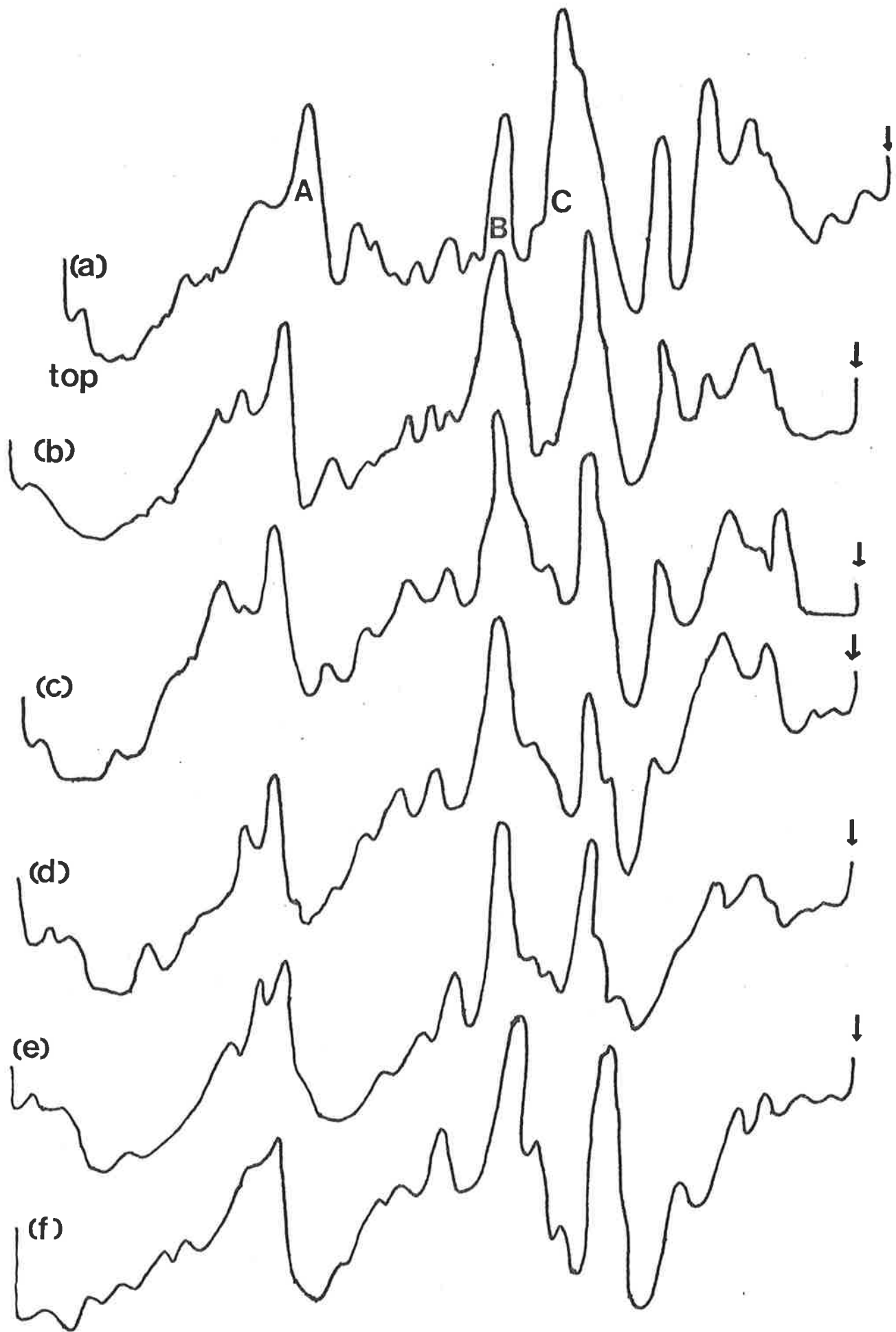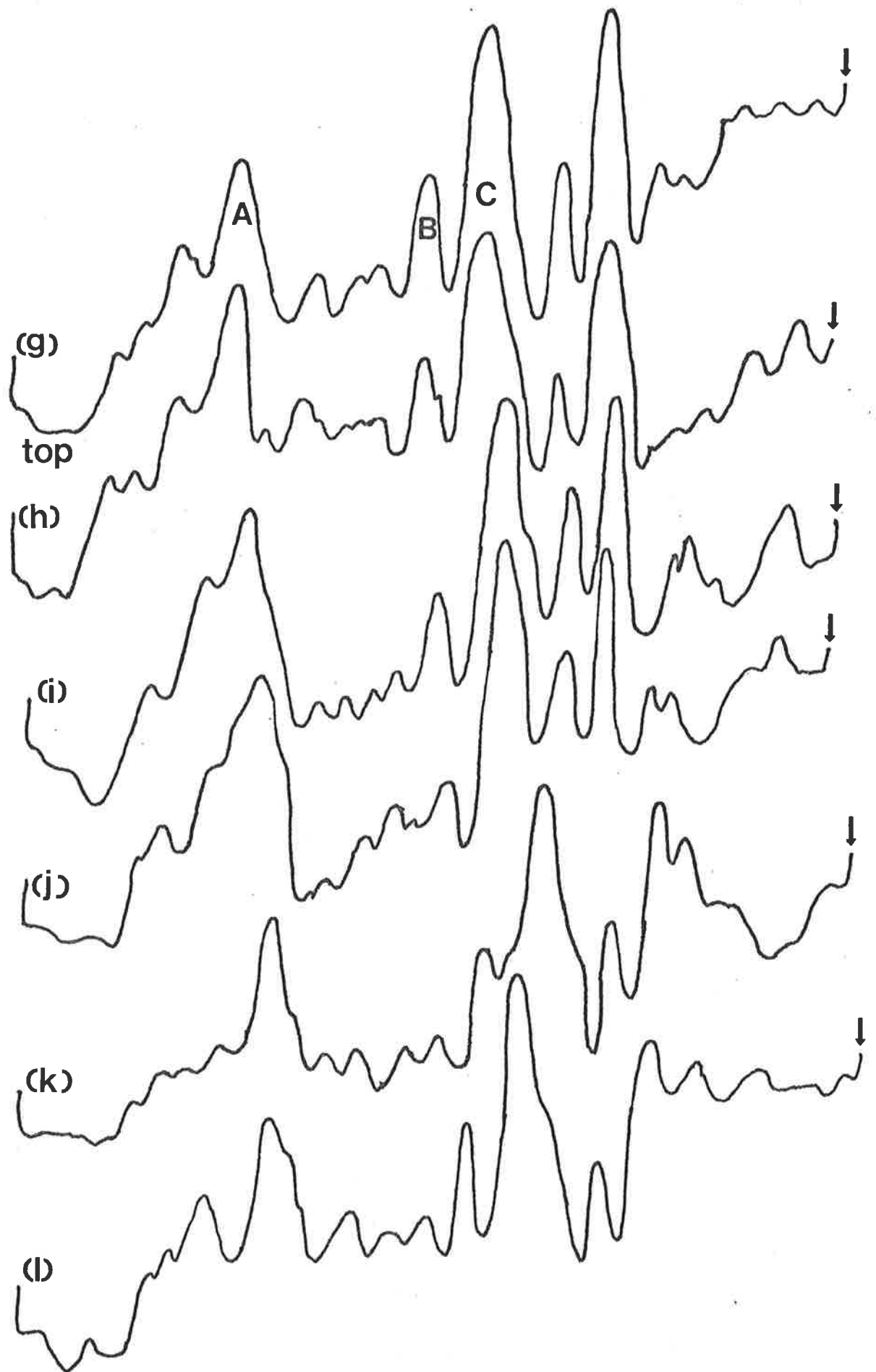
distinct additional peaks running in approximately the same position as the single peak seen in "unheated" samples.

These protein species do not appear to be part of the cytoplasmic membrane, as they are also evident in the outer membrane preparations. The scans of gels loaded with outer membrane preparations from AB1133 and P575 are shown in Fig 7-10. The results for P585, the Ton B mutant, were identical to those of P575.

None of the other mutants resistant to colicins of group B showed any major differences in the protein composition of either the whole or outer membrane preparations, on any of the gel systems used. The scans of the "unheated" whole membrane preparations from these mutants are shown in Figure 7-11.

Summary.

None of the mutants isolated appear to have substantial alterations to the cytoplasmic membrane. These colicin tolerant mutants, however, include examples lacking each of the major protein species in the outer membrane (i.e. proteins 1 and 3). In addition, several of the mutants appear to have a diminished amount of protein 1, and others appear to have two additional proteins.

Figure 7-10 : Comparison, by densitometry, of stained bands of
outer membrane proteins, run using the SDS-polyacrylamide gel
system of Schnaitman (1973), and a neutral pH buffer system.
Figures 7-10(a) and 7-10(b) show the "unheated" outer membrane
proteins of AB1133 and the Exb B mutant P575, respectively,
run on neutral pH gels. Figures 7-10(c) and 7-10(d) show the
"heated" outer membrane proteins of AB1133 and P575, respectively,
run on neutral pH gels. A, B and C refer to the peaks A, B and
C described by Schnaitman (1973,1974a,1974b). The arrows indi-
cate the positions of the dye fronts. The top of the gel is
on the left hand side of the page.
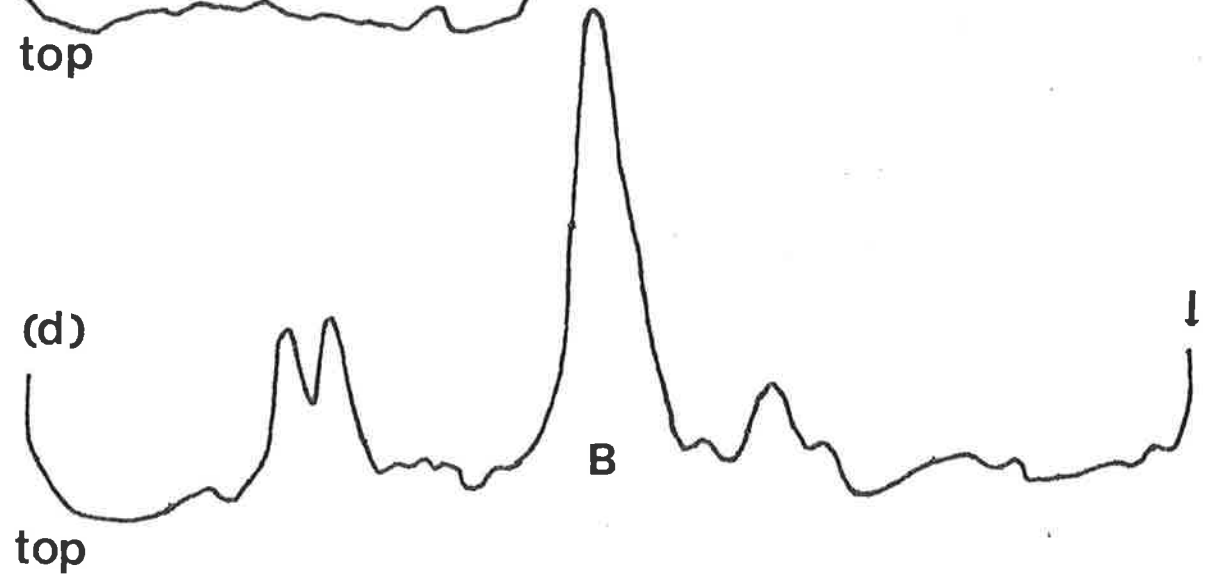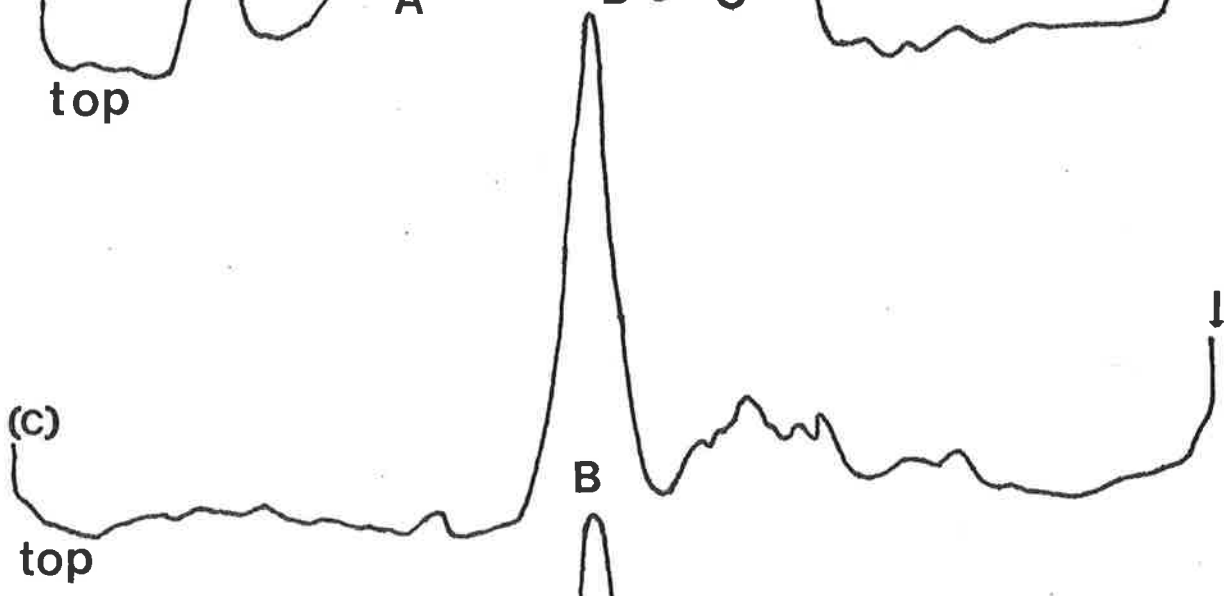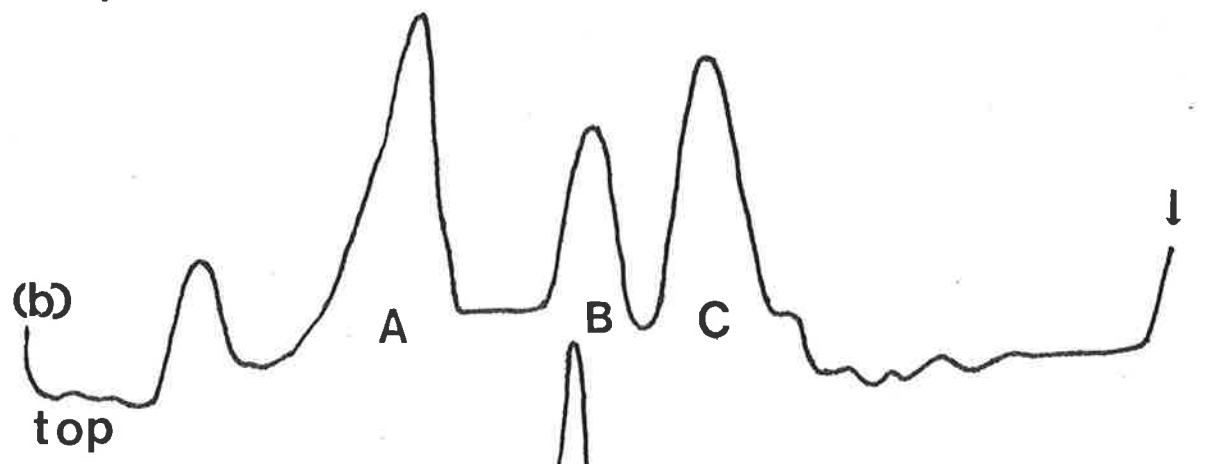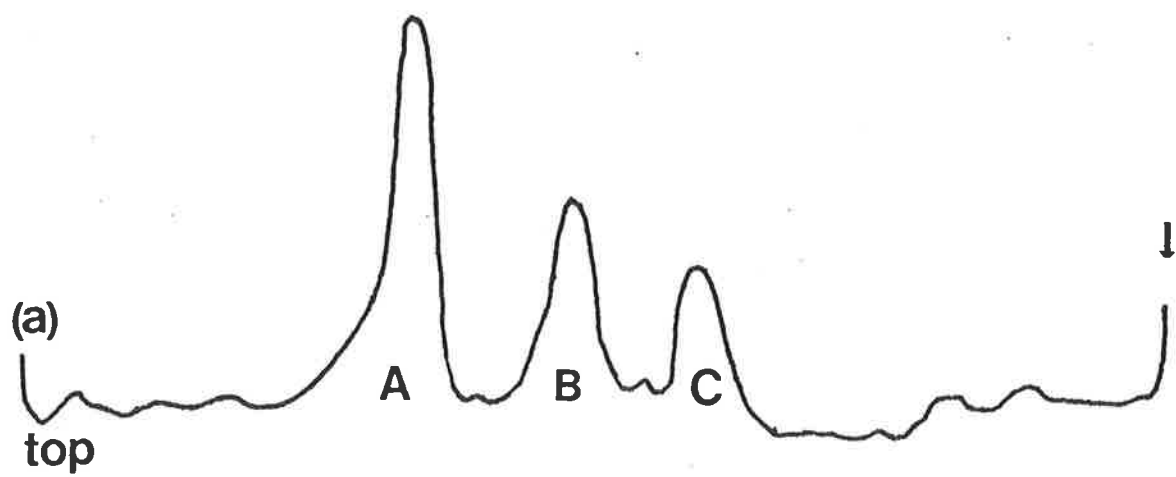
Figure 7-11 : Comparison, by densitometry, of stained bands of whole membrane proteins, run using the SDS-polyacrylamide gel system of Schnaitman (1973), and a neutral pH buffer system. Figures 7-11(a), 7-11(b), 7-11(c) and 7-11(d) show the "unheated" whole membrane proteins of AB1133, the Ton A mutant P1205, the Exb C mutant P535 and the Cbt mutant P295, respectively, run on neutral pH gels. Figures 7-11(e), 7-11(f), 7-11(g) and 7-11(h) show the "unheated" whole membrane proteins of the Cmt mutant P1209, the Cir mutant P625, the Ivt mutant P645, and the Cvt mutant P1235, respectively, run on neutral pH gels. A, B and C refer to the peaks A, B and C described by Schnaitman (1973, 1974a,1974b). The arrows indicate the positions of the dye fronts. The top of the gel is on the left hand side of the page.
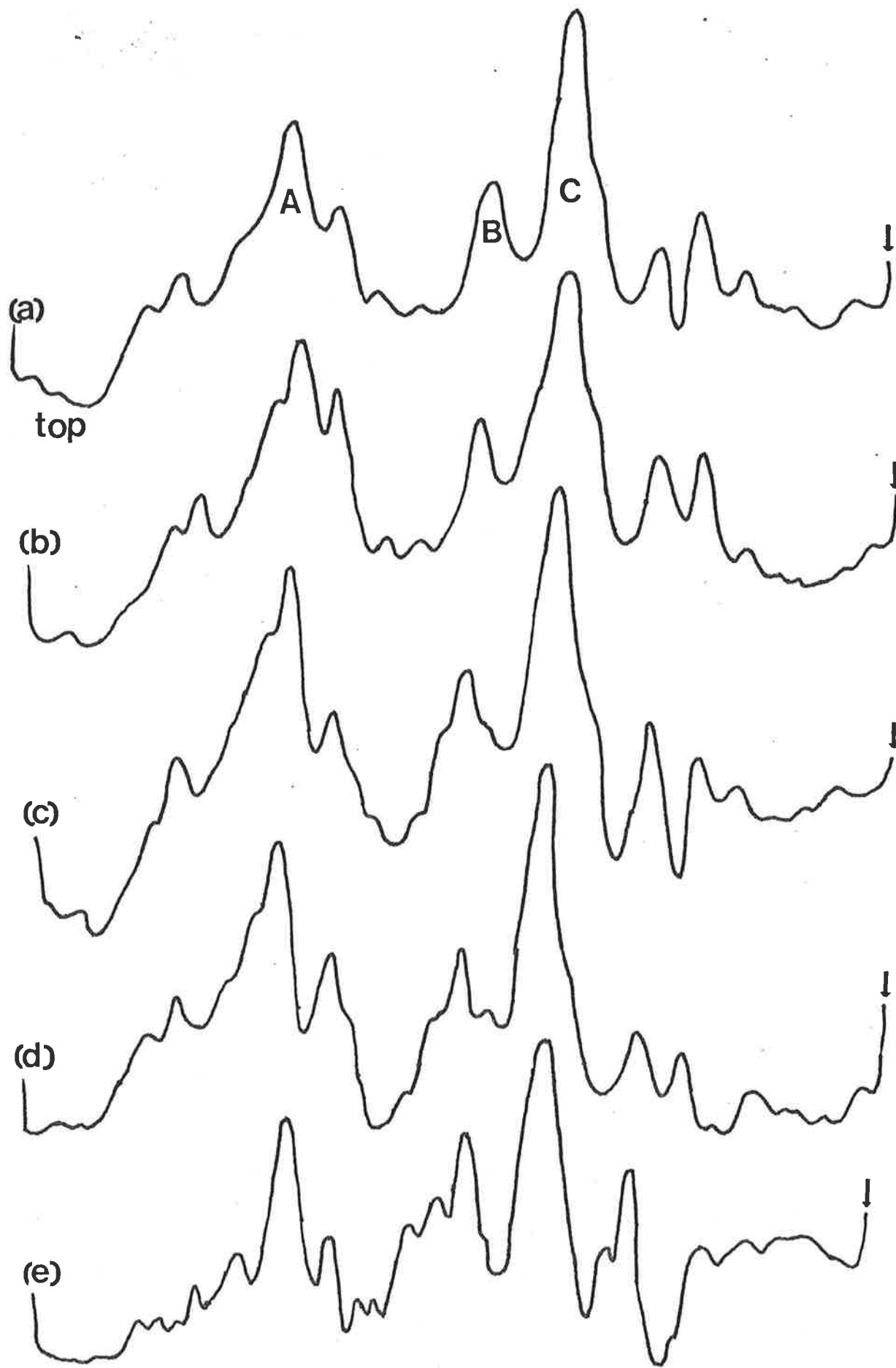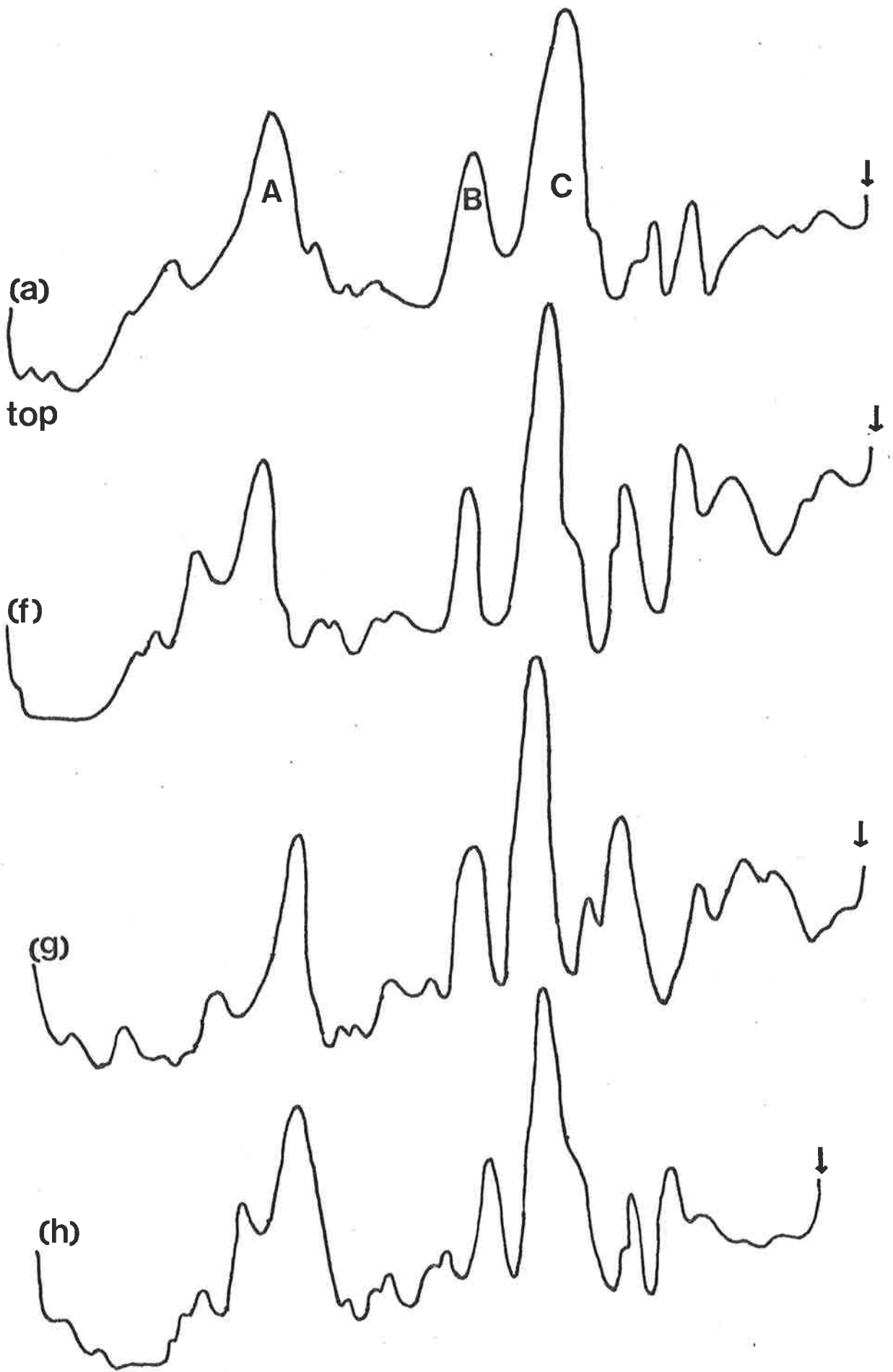
CHAPTER VIII

DISCUSSION

The colicins.

An attempt has been made to collect all the major colicinogenic strains together, and to differentiate the colicins they produce. After the sorting out of various anomalies, this has resulted in a collection of well characterised colicin producing strains, based on the original Fredericq (1948) collection, but including colicins described later, and whose relationship to the Fredericq classification had not always been clear. We have, however, been unable to differentiate (either on the colicin resistance patterns of the mutants isolated, or the zone morphology or electrophoretic mobility of the colicins) between several of the colicins.

Colicins Q and V are almost indistinguishable, having only a very slight difference in electrophoretic mobility, as reported previously (Smarda and Obdrzalek (1966)). It was not possible to differentiate colicins Ia and Ib from S1, and colicins G and H also appear to be very similar. This last point has been commented on before by Fredericq, who at one time classified them both as a single colicin called P (Fredericq (1953)).

In addition, we have been unable to identify the second colicin produced by E.coli CA7. It has an electrophoretic mobility quite different from colicin V (or Q), but cannot be differentiated on the basis of the colicin resistance patterns of the mutants isolated.

The similarity between colicins B and D has not been commented on before. They have similar electrophoretic mobilities and zone morphologies, and cannot be differentiated on the colicin resistance patterns of the mutants, yet they

are reported to have completely different modes of action. Colicin D inhibits

protein synthesis (Timmis (1972)), while colicin B is said to affect energy

metabolism, as simultaneous inhibition of DNA, RNA and protein synthesis is

observed (Guterman (1973)). However, the colicin B preparation used in that

study was contaminated with colicin M, and it is possible, though unlikely,

that this affected the result obtained with colicin B.

Colicin D has also been reported to have a very wide activity spectrum,

and colicin D resistant mutants are reported to be very rare (Fredericq (1953)).

Both of these observations may have been due to the use of E.coli CA23, which

has been shown to produce colicin X, in addition to colicin D. Colicins B

and D were finally differentiated, however, by the substance excreted by the

Exb C strain, P535, which will inhibit colicin B, but not colicin D.

A major finding in this study is the observation that colicins fall into

two well-defined groups. The absence of mutants resistant to colicins from

both groups indicates that there must be two distinct pathways of colicin

action, with little, if any, interaction between them.

Since cross-resistance within groups A and B is common, and between

groups so rare that we have found none by selecting against one colicin at

a time, it is surprising that this division into two groups has not been noted

before. Hardy, et al. (1973), however, have divided a group of 11 Col factors

into two groups on several grounds - the fundamental difference being that

one group, the "E-K like" plasmids, have a molecular weight of 5×10^{6}, while

the "BIV-like" plasmids all have molecular weights of around 7×10^{7}. The

suggestion was made that the two groups of Col factors arose from different

evolutionary origins. Although they did not use as many Col factors as has

been used here, the two groups correspond exactly to groups A and B, support-
ing the suggestion that there are these two very different groups of colicins
active on Escherichia coli K12.

The lack of cross-resistance between the two groups is even more sur-
prising when one considers that both groups contain colicins that have similar
modes of action. Colicins D and E3, for example, act on protein synthesis
(Timmis and Hedges (1972), Senior and Holland (1971), Bowman et al. (1971)),
and colicins A and Ia affect energy metabolism (Nagel de Zwaig (1969),
Levisohn, et al. (1968)), yet mutants resistant to colicins E3 and D, or to
A and Ia, have not been isolated in this study.

Fredericq's original study (1948) showed several examples of apparent
cross-resistance between colicins of the two groups, and there is no obvious
explanation for this discrepancy with the findings presented in this thesis.
It is possible that in some instances selection of double mutants occurred
due to the use of colicinogenic strains producing more than one colicin.
E.coli CA62, for example, which produces what Fredericq called colicin J, has
since been shown to produce a mixture of colicins E1 and I (Fredericq (1965)).
Resistance to colicin J could have occurred by the selection of, for example,
a mutant that simultaneously became ton B and bfe, or by the selection of a
bfe mutant in a strain that already carried a ton B mutation. In both cases,
mutants apparently selected as resistant to a single colicin would show
apparent cross-resistance to colicins from both group A and group B.

The invariable resistance of bfe mutants to colicin A, also commented on
previously by Nagel de Zwaig and Luria (1967), was not observed by Fredericq
(1948). Indeed, mutants specifically resistant to colicins E1, E2 and E3

(presumably bfe mutants) provided the basis for combining colicins E1, E2, E3, F and S5 as colicin E (Fredericq (1956, 1965)). This discrepancy is probably due to the parent strain used to select the mutants, as Fredericq (1948) initially used 6 Shigella sonnei and Escherichia coli strains, but did not include Escherichia coli K12. A later study (Fredericq, personal communication) included strain K12, and substantial cross-resistance between colicins E1, E2, E3 and A was observed. Had Fredericq originally used only strain K12, colicin A would now be considered a subtype of colicin E, but since its cross resistance is strain-dependent, it is probably better to leave the classification as it is.

Mutation to colicin resistance.

The selection of colicin resistant mutants using each of the colicins available has resulted in a very comprehensive collection of mutants in the same genetic background. This, in turn, has enabled a more meaningful comparison of the various mutant classes than has been possible in the past.

Although a large number of colicin resistant mutants were selected, several previously described phenotypes were not isolated. The frequency of mutation to resistance at any particular locus may well be strain-dependent, and many still undescribed mutant classes may exist (indeed there may well be other undescribed colicins which are active on Escherichia coli K12).

It should be remembered, also, that certain mutations to colicin resistance may be lethal - indeed conditionally lethal colicin tolerant mutants have already been described (e.g. Nomura and Witten (1967)). No attempt has been made to isolate conditional lethals in this study, and this may have restricted the number of phenotypic classes of mutants that it was possible to

detect.

Nevertheless, a wide range of mutants have been isolated and characterized, and they are of several distinct types.

Resistance to colicins of group A.

For the first time, it has become apparent that mutants resistant to colicins of group A seem to be of three basic types. The first of these types is the receptor mutant. Both bfe and tsx mutants have been shown to be defective in receptors for specific colicins and bacteriophage. In both cases it has been shown that a protein forms an integral part of the receptor complex (Weltzien and Jesaitis (1971), Sabet and Schnaitman (1973)), but no discernable differences could be detected in this study in outer membrane preparations, using SDS-polyacrylamide gel electrophoresis - suggesting that either the receptor proteins are not major constituents of the outer membrane, or that the changes to the proteins are such that they migrate in an identical fashion to the parent strain on the gel systems used in this study.

Also probably included in this group of receptor mutants is the rcx mutant, P224. This mutant, which is specifically resistant to colicin X, has several characteristics in common with the bfe and tsx mutants. It is insensitive to all the detergents, surfactants and antibiotics tested, yet appears to have an unaltered membrane protein composition (as judged by SDS-polyacrylamide gel electrophoresis) and an unaltered lipopolysaccharide (as judged by the resistance pattern of lipopolysaccharide specific bacteriophages). Most mutants tolerate to colicins of group A are altered in one of the above characters.

The reason why receptor mutants for some of the colicins have never been described remains unanswered. Indeed, it has yet to be shown that the rcx and tsx genes code for the actual receptor proteins for colicins X and K respectively, and it has been shown that the bfe gene codes for a protein that binds colicin E2 and E3 only (Sabet and Schnaitman (1973)). Despite the fact that bfe mutants are receptor mutants for colicin E1, the effect seems to be indirect, and this may also be the case for colicin A, as the cell has been shown to have fewer receptors for colicin A than for some of the E colicins (Cavard and Barbu (1970)). Receptor mutants, resistant to colicins L, N and S4, have still to be described.

The second major group of mutants resistant to colicins of group A are those in which a change to the protein composition of the outer membrane has been demonstrated. Mutations causing simultaneous changes to the outer membrane proteins and colicin tolerance seem to occur at several loci.

The Tol Ia (P218), Tol Ib (P210), Tol IX (P596) and Tol X (P661) mutants all map at a locus (or loci) that is transferred by HfrH, but is not in the area covered by the F1gal and F2gal plasmids. They are resistant to a specific set of bacteriophages (Ktw) and colicins (K,L,A,S4,N), and have a lowered peak A when compared to AB1133 on neutral pH SDS-polyacrylamide gels. None of them show an increased sensitivity to any of the full range of antibiotics, detergents and surfactants tested.

The Tol XIV and Tol XV mutants, P530 and P686, are resistant to the same set of bacteriophages, but appear to map between his and argE, are partially sensitive to EDTA, and are resistant to colicins E2, E3 and X, as well as K,L,A,S4 and N. They have a lowered peak A, and, in fact, in P530 peak A

(i.e. protein 1) appears to be virtually absent.

The Tol VII mutant, P689, and the Tol XI mutant P220, appear to have lowered amounts of protein 1, but in addition appear to be defective in their lipopolysaccharide, as judged by their changed sensitivity to the two lipopolysaccharide-specific bacteriophages, C21 and U3. Both mutants show an increased resistance to bacteriophage λcI90c17, increased sensitivity to PEA, and bear a superficial resemblance to the Bar class of bacteriophage resistant mutants (Hancock and Reeves (1974a)). The mutations in these mutants, however, appear to be at distinct loci - the colicin resistance locus in P689 maps at tolJ, between thr and leu, while the locus in P220 appears to map somewhere between his and argE.

Finally, there are two classes of mutants which appear to totally lack a protein species in the outer membrane. The Tol IV mutant, P692, lacks peak A (protein 1), is insensitive to all detergents, surfactants and antibiotics tested, and has a colicin resistance locus mapping between his and argE. The Con mutant, P212, lacks peak C (proteins 3a and 3b), is sensitive to EDTA and PEA, and resistant to a specific set of bacteriophages. These mutants have been shown to be defective in conjugation (Skurray, et al. (1974)). The con locus appears to map between 14 and 15 minutes on the genetic map of Taylor and Trotter (1972).

The third major group of mutants are those which show an increased sensitivity to ampicillin and DOC, together with an unchanged outer membrane protein composition. The Tol IIb (P651), Tol III (P660) and Tol XIII (P520) mutants all have colicin resistance loci that map near gal, are sensitive to all the bacteriophages tested, and appear to have unaltered outer membrane

proteins and lipolysaccharide.

Several of the mutant classes do not fit exactly into any of the three basic types of mutants resistant to colicins of group A. The Tol IIc mutant, P520, differs from the third group only in its insensitivity to ampicillin and deoxycholate. It has an unaltered outer membrane and lipopolysaccharide, is sensitive to all the bacteriophages tested, and has a colicin resistance locus mapping near gal.

The Tol XII mutant, P653, resembles the receptor mutants in that it is insensitive to the full range of detergents, surfactants and antibiotics tested, and has no detectable changes in the lipopolysaccharide and outer membrane. It is sensitive to the full range of bacteriophages tested. Despite the superficial resemblance to the receptor mutants, P653 appears to be tolerant to colicin L on the triple layer plate test. It is also resistant to colicin A, and partially resistant to colicins E3 and S4.

The Tol XVI mutant, P516, differs from the third main group of mutants only in that it is not "super-sensitive" to ampicillin. Like this group of mutants it is sensitive to DOC, has no detectable changes to the outer membrane or lipopolysaccharide, is sensitive to the full range of bacterio-phages tested, and maps near gal.

The Tol VIII mutant, P602, and the Tol XVII mutant, P652, are unusual in that while they are sensitive to ampicillin and DOC, and have no detectable changes in their outer membrane (like the third main group of mutants), they are both resistant to a wide range of bacteriophages. In addition, P602 has an altered susceptibility to bacteriophages C21 and U3, indicating an altered lipopolysaccharide. The colicin resistance locus in P652 maps near gal, while

that in P602 presumably maps at <u>tol</u> C.

Thus, although there are some variations to the pattern, there are two basic types of mutants tolerant to colicins of group A. One group has an altered outer membrane, may be sensitive to EDTA and PEA, and could have alterations to the lipopolysaccharide. They show cross resistance to bacteriophage and may be tolerant to colicin X, but they are never tolerant to colicin El, and never show "super-sensitivity" to ampicillin and DOC.

The other group have no detectable changes in their outer membrane or lipopolysaccharide, are never resistant to bacteriophages or colicin X, but may be resistant to colicin El. They all have colicin resistance loci near <u>gal</u>, and are "super-sensitive" to ampicillin, DOC, PEA and EDTA.

<u>Resistance to colicins of group B.</u>

Again there seem to be three basic types of mutants resistant to colicins of group B.

The Ton A and Cir mutants, P1205 and P625, are both apparently receptor mutants. The remaining mutants, which are all colicin-tolerant, can be divided into two groups, depending on whether or not they excrete colicin inhibitors. The Ton B (Exb A) mutant, P585, the Exb B mutant, P575, and the Exb C mutant, P535, all appear to excrete colicin inhibitors. Both P585 and P575 appear to be excreting enterochelin, which will inhibit the actions of both colicin B and D at approximately the same concentration.

The inhibitor excreted by the Exb C mutant, P535, appears to be different. It will inhibit the killing action of colicin B, but not colicin D, and is therefore presumably not enterochelin. Two new protein species appear in the outer membrane of the Ton B (P585) and Exb B (P575) mutants

when they are compared to the parent strain AB1133, using SDS-polyacrylamide gel electrophoresis. This alteration to the outer membrane appears to be characteristic of strains excreting enterochelin.

It has been suggested that ton B mutants cannot accumulate enough iron to repress enterochelin synthesis, and are therefore acting as constitutive (Guterman (1973)). If this were due to the observed change in the composition of the outer membrane proteins, one would expect a similar situation to occur in Exb B mutants, which show identical changes. Yet Exb B strains can grow on media in the absence of additional iron (Guterman (1973)), and are therefore not defective in iron transport. It seems, therefore, that the outer membrane changes are characteristic of strains that can excrete enterochelin, rather than mutants defective in iron transport.

The other mutants tolerant to colicins of group B are those whose colicin tolerance does not depend (even in part) on their ability to excrete inhibitors. Of interest here is the fact that the cbt locus appears to map very close to the ent cluster of loci, which are involved in enterochelin synthesis. So once again, resistance to colicins B and D may be linked to enterochelin, and the iron transport system.

It should be pointed out that although the excretion of enterochelin may well afford the cell some protection against colicins B and D, it does not appear to be the sole cause of resistance to colicin B in ton B strains (Guterman and Dann (1973)), and does not appear to cause the resistance to colicin V, for example, that is observed in ton B mutants (colicin V appears to be insensitive to the action of enterochelin).

None of the mutants resistant to colicins of group B appeared to show

any increased sensitivity to any of the antibiotics, detergents and surfac-
tants tested. In addition, none of them showed detectable lipopolysaccharide
alterations, and none (apart from the ton A and ton B mutants) showed resis-
tance to any of the bacteriophages tested, pointing out once again the dis-
tinction between the colicins of the two groups.

Mode of action of colicins of group A.

The lack of cross-resistance between colicins of group A and those in
group B suggests that the two groups of colicins have completely separate
modes of action. As was discussed in Chapter I, certain evidence suggests
that colicins of both groups are capable of acting directly on the cytoplasmic
membrane, given adequate access to it. It may be that access to the exterior
of the cytoplasmic membrane may be all that is needed for many of the colicins
to act on their target - they may not have to enter the cell in a fashion
similar to colicin E3. If colicins from both groups are, in fact, capable of
acting directly on the cytoplasmic membrane, it suggests that there are two
completely different processes for allowing passage of colicin molecules
through the outer membrane.

One possible indication of the difference in these two processes lies
in the observation by Konisky and Cowell (1972) that the cell may have as many
as 5000 receptors for colicin I, as compared to approximately 200 receptor
molecules for colicins E2 and E3 (Sabet and Schnaitman (1971, 1973)). It
was pointed out by Sabet and Schnaitman (1973) that the number of E3 receptor
sites corresponded to the number of sites of adhesion between the cytoplasmic
membrane and cell wall described by Bayer (1968a, 1968b). As was discussed
in Chapter I, the suggestion was made (Schnaitman (1971)) that these sites,

which it is suggested serve as an entry point for bacteriophage DNA (Bayer (1968b)), might also act as a site at which colicins could be given access to the cytoplasmic membrane.

There are quite clearly many more colicin I receptors than adhesion points. This fact together with the lack of cross-resistance between bacteriophages and mutants resistant to colicins of group B suggests that colicin I (and therefore probably all the other colicins of group B) do not utilize the adhesion points to gain access to the cytoplasmic membrane. The mutants resistant to colicins of group A, however, do show substantial cross-resistance to bacteriophages. In addition, there is evidence that the adhesion points are also the site at which the DNA is attached to the cyto-plasmic membrane (Olson, et al. (1974)). Colicin E2 (a colicin of group A) is the only colicin known to act on DNA synthesis, and it appears that this function might well occur at an adhesion point. Therefore, if any of the colicins utilize these sites of adhesion between the cytoplasmic membrane and cell wall as a site to gain access to the cytoplasmic membrane, the colicins most likely to do so would seem to be the colicins of group A.

It seems clear that the first step in the action of colicins of group A is adsorption of the colicin molecule to one of several hundred receptors on the cell surface. The general characteristics of the various tolerant mutants isolated provide some clues as to subsequent events. One group of mutants had substantial alterations to the protein composition of the outer membrane, and in some cases alterations to the lipopolysaccharide were detected, and cross-resistance to bacteriophage observed. It might be that substantial structural alteration of the outer membrane had occurred in these mutants, such

that colicins K, L, A, N and S4 in particular, were no longer able to penetrate it. If the changes observed in these mutants were of a general structural nature, one might expect that the penetration of the outer membrane by other macromolecules would also be affected. This appears to be the case - these mutants are the only mutants besides the receptor mutants to show cross-resistance to bacteriophage, and in one specific case, Con, the ability to act as a recipient for donor DNA during conjugation is also affected.

The changes observed in the other major group of mutants tolerant to colicins of group A seem to be much more specific. It appears that the ability of the outer membrane to act as a penetration barrier has been broken down. The mutants all show an increased susceptibility to ampicillin. This could mean that the cell wall synthesizing enzymes, located on the external surface of the cytoplasmic membrane, have become more accessible to ampicillin, a molecule whose penetration is retarded by the outer membrane. Yet there are no detectable changes to either the protein composition of the outer membrane, or the lipopolysaccharide, in these mutants. This argues against any general structural breakdown of the penetration barrier of the outer membrane, and suggests that the lesions occur at specific sites.

In addition, it would seem to be unlikely that these sites of breakdown of the penetration barrier correspond to the Bayer adhesion points, as none of these mutants are cross-resistant to bacteriophages. The sites, or "pores", appear to be utilized by colicins of group A, and no other macromolecules.

It may be that the outer membrane contains a series of "pores", some of which can be utilized by colicins of group A (and some of which can be utilized by bacteriophages or donor DNA) as a means of penetrating the outer

membrane. If these "pores" could only be successfully utilized if they were then associated with a Bayer adhesion point, several observations could be explained. Firstly it may explain the presence of a step in colicin action in which the components utilized were not also utilized by bacterio-phages - the colicin molecules and bacteriophage DNA use different types of "pores", although both may be connected with Bayer adhesion sites. Secondly it might explain the suggestion that of all the colicin receptor sites on the cell, only a few may actually be sites at which killing occurs (e.g. Luria, et al. (1973)) - these are the sites at which the "pores" are associated with the Bayer adhesion sites.

Mode of action of colicins of group B.

If one accepts the suggestion that colicins of group B do not penetrate the cell envelope at the Bayer adhesion sites, the only other known structures in the cell envelope that occur with a frequency at least as great as the colicin I receptor are the passive diffusion pores described by Inouye (1974). Inouye suggests that there are between 6×10^4 and 1.2×10^5 of these pores located in the outer membrane, depending on the size of the pore (i.e. how many murien-lipoprotein molecules make up the structure).

Konisky (1973) has suggested that colicin I molecules (as well as other colicin molecules) have a high axial ratio, and would therefore be either prolate or oblate shaped molecules. If a prolate shape is considered, the colicin I molecule might well be able to penetrate the larger passive dif-fusion pores, and thus gain access to the cytoplasmic membrane. If one assumes an equal distribution of different sized pores, and suggests that colicin molecules can only penetrate the largest of these pores, the number

of sites at which the colicin molecule could gain access to the cytoplasmic membrane would be of the same order of magnitude as the number of receptor sites.

The first step in the action of colicins of group B also seems to be adsorption of the colicin molecule to a receptor on the cell surface. For both of the receptor mutants isolated in this study, the relevant receptor protein has been isolated and characterized (Braun and Wolff (1973), Konisky and Liu (1974)). If the colicins of group B are, in fact, using the passive diffusion pores to gain access to the cytoplasmic membrane, some of them may also use a portion of the pore, exposed at the surface, as a receptor. This may explain why no receptor mutants for the other colicins of group B were isolated. It seems reasonable to expect that mutations which cause gross structural changes to a structure as important in cell wall organization as the murien-lipoprotein might well be lethal.

If it is assumed that the colicin molecules can only enter the larger pores, this could explain the observation of Konisky and Cowell (1972) that the cell appears to have two different types of receptors for colicin I (one having a higher binding efficiency and leading to cell death, while the other, with a lower binding efficiency, being incapable of leading to cell death). If receptor molecules, capable of weakly binding the colicin molecules, were associated with all the pores, but if they could only bind the molecule strongly and lead to cell death when the pore had attained a size that allowed the colicin molecule to enter, this would explain the effects observed.

Two different types of mutants tolerant to colicins of group B have been isolated. The mutants which hyper-excrete enterochelin suggest that

colicins B and D may utilize some components of the iron transport system. The presence of additional proteins in the outer membranes of these mutants is difficult to explain. It might be that in addition to hyper-excreting enterochelin, both the exb B and ton B mutants overproduce colicin B receptor, and these are the additional proteins one observes. If the mutants were producing large amounts of receptor, many of these molecules would be incapable of assisting in colicin action. This could explain why ton B mutants which can no longer produce enterochelin (ton B aro C mutants) are still partially resistant to colicin B.

The nature of the defect in the Exb C mutant is unknown, but the mutant appears to be excreting an inhibitor other than enterochelin. The nature of the changes in the other mutants tolerant to colicins of group B are also unknown, but the fact that the cbt locus maps very close to the genes concerned with enterochelin synthesis, again suggests a connection between colicin B action and the iron transport system.

Several criticisms can be made of these two general hypotheses for colicin action. For example, it is hard to understand why mutants said to cause general structural re-arrangement in the outer membrane should retard the movement of colicins K, L, A, S4 and N through specific pores in the outer membrane, and yet have no effect on colicin E1 (and only in a few cases on E2 and E3).

Similarly, it is difficult to see why mutants which are said, because of an apparent breakdown in the penetration barrier, to have defective pores in the outer membrane, should not be resistant to colicin X.

In addition, if one speculates that colicins of group A penetrate the

cell wall by entering through pores in the outer membrane, and can only kill the cell when these pores are associated with Bayer adhesion sites, another objection can be raised. In the system described, one would imagine that the number of pores would be much larger than the number of adhesion sites - yet the number of receptors for colicins of group A is approximately the same as the number of Bayer adhesion sites.

Finally, the colicins of group B could only enter the passive diffusion pores described by Inouye (1974) if they were prolate molecules. Yet for colicin I, at least, there is evidence that the molecules are oblate rather than prolate (Konisky (1973)).

Conclusions.

The collection and characterisation of a wide range of colicinogenic strains has enabled mutants to be selected against all of the available colicins. The colicin resistance patterns of these mutants suggest that there are two major groups of colicins active on Escherichia coli K12, each utilizing separate mechanisms to kill the cell.

Colicins of group A act by adsorbing to a receptor on the cell wall. Subsequently two separate steps appear to be involved in the action of these colicins. Components in one of these steps are also utilized by bacterio-phage, and mutants in this step have a structurally altered outer membrane. Defects in the other step can lead to a breakdown in the penetration barrier of the cell.

Colicins of group B also seem to attach to a receptor on the cell surface. Tolerance to colicins of group B can occur by two different means, one of which involves the excretion of colicin inhibitors. Colicin B seems to

utilize several components of the iron transport system.

The evidence presented in this thesis, together with data available on a few of the colicins from the literature, allows a hypothesis to be proposed to account for the two unconnected pathways of colicin action.

APPENDIX.

Published material.

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

(1) "Colicin tolerance and map location of conjugation deficient mutants." John K. Davies and Peter Reeves.

J. Bacteriol. 123:372-373. (1975).

(2) "Genetics of resistance to colicins in Escherichia coli K12 : cross-resistance amongst colicins of group A."

John K. Davies and Peter Reeves. J. Bacteriol. 123:102-117 (1975).

(3) "Genetics of resistance to colicins in Escherichia coli K12 : cross-resistance amongst colicins of group B."

John K. Davies and Peter Reeves. J. Bacteriol.123:96-101 (1975).

(4) "Cross-resistance amongst bacteriophages and colicins in Escherichia coli K12." Robert E.W. Hancock, John K. Davies and Peter Reeves. J. Bacteriol. accepted for publication June, 1976.

(5) "Alterations to the outer membrane of mutants of Escherichia coli K12 resistant to colicins of group A." J. Bacteriol. (submitted).

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