Characterization of conjugation defective mutants of *Escherichia coli* K-12

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I wish to dedicate this thesis to
my patient, understanding and loving parents.

CONTENTS

STATEMENT

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

Paul A. Manning

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SUMMARY

This thesis demonstrates that *ompA* is the structural gene for the outer membrane protein 3A, and this protein is shown to be the receptor for bacteriophage K3. Of all the genes involved in recipient functions the *ompA* gene has the major effect. It is strongly suggested, although it has not been possible to directly show by *in vitro* studies, that protein 3A is an essential recipient requirement for the F-pilus to function efficiently in conjugation. Protein 3A is also shown to be involved in transmembrane transport of amino acids and may function as a transmembrane pore. Under some conditions it is apparently essential for the integrity of the cell.

A wide range of mutants of *Escherichia coli* K-12, selected as resistant to either bacteriophages or colicin, have been tested for their recipient ability in conjugation with a number of donors. Of all the mutants examined, the *ompA* mutants (previously called *con* or *tolG*, which are shown to be identical) were the most defective. This defect was specific for donors bearing an F-like pilus.

The *ompA* mutants have been previously shown to be resistant to bacteriophage K3, tolerant to colicins K and L, defective as recipients in conjugation with F' and Hfr donors and to have greatly reduced amounts of outer

membrane protein peak 3 on Bragg and Hou gels. These mutants were further studied and shown to have lost only the major outer membrane protein 3A. This protein has been purified and shown to function as the receptor for bacteriophage K3. It has not, however, been possible to block conjugation *in vitro* and thus to implicate protein 3A directly in conjugation.

Using bacteriophage K3, a wide range of *ompA* mutants and also a series of extended host range mutants of the phage have been isolated. These mutants show that it is possible to independently vary the properties of phage resistance, colicin tolerance and recipient ability in conjugation. Protein 3A was also purified from one of these *ompA* mutants and was shown to be an altered protein demonstrating that *ompA* is the structural gene for protein 3A.

In order to determine the primary function of protein 3A, various properties of *ompA* mutants were studied. They were shown to grow less well under a variety of conditions and after completion of growth, to enter a decline phase in which viability is lost and the cells become heavily piliated. They are also shown to be defective in the uptake of amino acids, and to grow poorly at 42°C.

The results of these studies are discussed in terms of the possible functions of the ompA gene product, protein 3A.

During the course of these studies it was shown by examining the cross-resistance pattern of a large number of mutants selected as resistant to a wide range of colicins that the colicin produced by Serratia marcescens strain JF246, which has also been used to select ompA mutants, was a colicin of type L. It was also shown that tsx mutants, resistant to bacteriophage T6 and colicin K, lack a major outer membrane protein which is shown to be the receptor for these lethal agents. Tsx mutants were therefore useful as controls in determining the functions of protein 3A. It was also necessary in these studies to devise means of identifying and differentiating proteins 3A and 3B. This has been accomplished and confirms that the two are distinct protein species.

CHAPTER 1

INTRODUCTION

The three processes by which it is possible to transfer genetic material from one cell to another are transformation, transduction and conjugation. Transformation is the mechanism, whereby free molecules of deoxyribonucleic acid (DNA), derived either by natural or artificial lysis of the donor cells, are taken up by the recipient cells. Transduction is mediated by bacteriophages. In certain strains of bacteriophage, a proportion of the mature phage contain DNA derived from the bacterial cell on which they were propagated. When such a phage injects its DNA into the next host, it injects the bacterial DNA along with any phage DNA. Conjugation is the process by which it is possible to get the transfer of DNA from a donor to a recipient cell by direct cell to cell contact. Lederberg and Tatum (1946a,b) were first to demonstrate this phenomenon of conjugal gene transmission between strains in Escherichia coli and it has subsequently been shown to occur within a number of other genera: ~~Salmonella (Zinder and Lederberg, 1952)~~, Pseudomonas (Holloway, 1955), Serratia (Belser and Bunting, 1956), Vibrio (Bhaskaran, 1958), Yersinia (previously Pasteurella) (Lawton, Morris and Burrows, 1968),

Rhizobium (Heumann, 1968) and Enterobacter (de Graaf, Tieze, Bong and Stouthamer, 1968). Intergeneric conjugation has also been shown to occur between strains of a number of genera (Jones and Sneath, 1970).

In this introduction a full survey of the literature on conjugation itself has not been attempted, but the survey has been restricted to the discovery of the conjugal process, properties of the F sex factor, the role of the parents and the outer membrane in that process and to the ways in which mutants may be defective in conjugation. With regard to the outer membrane the literature has been surveyed with respect to its structure and to the isolation of the receptors for certain colicins and bacteriophages.

CONJUGATION AND THE F FACTOR IN ESCHERICHIA COLI K-12

The work of Lederberg and Tatum (1946a,b) was essential to the discovery of conjugation. They first reported the formation of prototrophic recombinants from mixed cultures of multiple auxotrophic strains of Escherichia coli K-12. At that time the only possible mechanisms which were considered likely were transformation or cell fusion. It was soon shown that sterile filtrates from cultures of either parent could not produce the

observed results (Lederberg and Tatum, 1947). Later, Davis (1950) provided good evidence for a requirement for cell to cell contact, by showing that genetic recombination did not occur between the bacterial strains if they were separated only by a filter, which allowed the free exchange of extracellular fluids, but did not allow cell contact. The studies of Nelson (1951) also further indicated the direct role of the bacterial cells in recombination.

It was first discovered by Hayes (1952) that the roles of the two parents were not equivalent during conjugation and it was subsequently shown (Lederberg, Cavalli and Lederberg, 1952; Hayes, 1953a,b; Cavalli, Lederberg and Lederberg, 1953) that the cells of E. coli K-12 could exist in either of two sexual states, dependent upon the presence of a transmissible fertility factor, F (Lederberg et al., 1952). The cells which are genetic donors or males harbour F and are termed F^+, whereas cells which are capable of acting only as recipients or females, lack F and are termed F^-. F^+ cells were capable of transferring F itself at high frequency (approaching 100%) to F^- cells but were only capable of promoting transfer of chromosomal markers at a much lower frequency (10^{-2} to 10^{-4}%). $F^+ \times F^+$ matings were less fertile than $F^+ \times F^-$ matings and $F^- \times F^-$ matings were infertile. The F factor

also appeared to multiply autonomously and more rapidly than the harbouring cells, since the introduction of a few F^+ cells into an F^- culture causes the F^+ character to spread throughout the entire population (Lederberg, 1958: cited in Adelberg, 1960).

It is possible for cells to be cured of the F factor and become F^-. This can occur either spontaneously or by the use of chemicals such as acridine dyes (Hirota, 1956, 1960; Hirota and Iijima, 1957). It was as a consequence of the spontaneous curing of one of the early derivatives of E. coli K-12 that Lederberg and Tatum (1946a,b) were able to discover the conjugal process and the uni-directional nature of the transfer of DNA. F replicates so that it maintains one to two copies per chromosome (see Clowes, 1972 for review).

In an $F^+ \times F^-$ mating most of the unselected markers are derived from the F^- parent and the recipients invariably become donors, or F^+. However a number of deviant strains derived from F^+ strains were isolated which produced recombinants (at up to 1000 times that of the original F^+ strain), amongst which it was found only a very small proportion inherited the donor state. These strains were termed Hfr (high frequency of recombination) (Cavalli, 1950; Cavalli, Lederberg and Lederberg, 1953;

Hayes, 1953b). When the donor state was inherited from an Hfr, it too was Hfr and not F^+. The mutation from F^+ to Hfr appeared to have resulted in the loss of F as an autonomous independently transmissible agent.

It was the experiments of Wollman and Jacob (1955, 1958) on the kinetics of recombinant formation, which revealed the true nature of Hfr strains and of gene transfer during conjugation. They isolated a large number of Hfr strains from a single F^+ strain (Jacob and Wollman, 1956) and showed that each was able to transfer a different sequence of markers at high frequency. These experiments also showed that the genetic determinant of Hfr character appeared to be linked to those genetic determinants transferred last during conjugation. These findings led Jacob and Wollman (1957) to believe that the different Hfr strains arise by the insertion of the F factor into the chromosome at different points, with a site immediately adjacent to the F factor becoming the leading point or origin of chromosome transfer. Since only recombinants receiving terminally transferred markers became donors, it was also postulated that the F factor in the Hfr was attached distal to the origin so that it was the last marker transferred: during conjugation the circular Hfr chromosome in some way opens at one side of the point of attachment of F and generates a

linear structure with a unique direction and orientation of transfer, with F being on the distal end of this structure.

In Hfr x F^- matings a gradient of recombination frequencies is always observed when different markers from the Hfr parent are selected. This gradient is such that recombination is maximal for markers closest to the origin. Using gradients of transfer of markers and also experiments in which matings are interrupted at different times, it has been possible to show that it takes about 100 minutes at 37°C to transfer the whole chromosome of E. coli K-12 and this has enabled a circular linkage map to be constructed (Bachmann, Low and Taylor, 1976).

The F factor can also exist in another form as the F' (F-prime). Strains bearing F's have been isolated as variants of Hfr strains. They have the ability to produce high yields of particular recombinant classes and all recombinants become donors (Jacob and Adelberg, 1959; Adelberg and Burns, 1960; Hirota and Sneath, 1961). F' strains were discovered as those recombinants in an Hfr x F^- mating which had received a genetic marker much earlier than would be expected with the normal process of genetic transfer. These recombinants only received the F factor and a small part of the chromosome. Unlike the

recombinants normally formed in an Hfr x F^- cross, they are unstable and in the case of sugar fermentations could be readily shown to segregate bacteria with the recipient parent phenotype at a frequency of about 1 in 10^3 cells (Jacob and Wollman, 1961).

It has subsequently been shown that in F' strains the F factor has become detached from the chromosome of the parent Hfr strain by a reciprocal recombination event and has carried with it a segment of chromosomal material (Broda, Beckwith and Scaife, 1964; Scaife, 1966; Berg and Curtiss, 1967; Freifelder, 1968; Low, 1968, 1972, 1974). F' strains exist as two types, primary and secondary. A primary F' strain is derived from the cell in which the F' was formed; the chromosome is deleted for the material carried by the F'. A secondary F' strain is derived by conjugal transfer of an F' into a normal haploid recipient strain and so produces a strain which is diploid for that region of the chromosome carried by the F'.

The F-factor is classed as a plasmid (see Novick, Clowes, Cohen, Curtiss, Datta and Falkow, 1976 for a discussion of plasmids). Plasmids are extrachromosomal genetic elements capable of independent replication within the host cell, and are readily maintained without specific

selection. F also behaves as an episome: it can exist integrated into or independent of the host chromosome.

The nature of the sex factor F

The sex factor has been shown to consist of DNA (Driskell and Adelberg, 1961; Lavalle and Jacob, 1961). By caesium chloride density gradient centrifugation of the DNA extracted from bacterial cultures, an additional small band can be seen in the DNA from F^+ cells which is not present in F^- cells (reviewed in Hayes, 1968). When the DNA of the sex factor F is extracted in this way and analysed, nine tenths is found to contain a G+C content of 50% like the chromosomal DNA, and the remaining tenth has a G+C content of 44%. Also, isolated F DNA is able to form DNA-DNA hybrids with E. coli chromosomal DNA, and Falkow and Citarella (1965) have shown that an F'lac element had approximately 40% nucleotide sequence homology with the chromosome of its host cell.

It has been shown that the sex factor F exists as a small DNA molecule (Hickson, Roth and Helinski, 1967; Freifelder, 1968) and that it can be isolated as a covalently closed circular (CCC) duplex (Freifelder, Folkmanis and Kirschner, 1971). It is thought to exist in that form for at least some of its intracellular existence (Clowes, 1972).

The molecular weight of the F factor has been estimated by a number of techniques, including neutral sucrose sedimentation (Freifelder and Freifelder, 1968; Bazaral and Helinski, 1970) and X-ray inactivation (Freifelder, 1968). These measurements, however, did not give very good agreement and values ranging from 35 to 75 megadaltons were obtained. More recent measurements with the aid of electron microscopy have been based on the contour length of the DNA of the F factor and have given consistent values between 61 and 64 megadaltons (Kline and Helinski, 1971; Palchaudhur, Mazaitis, Maas and Klineschmidt, 1972; Sharp, Hsu, Ohtsubo and Davidson, 1972). This value is taken as 62 megadaltons and corresponds to 94.5 kilobases (94,500 nucleotide base pairs), which is thought to be sufficient DNA to code for about 100 proteins (Sharp et al., 1972).

The F factor codes for the F pilus

Cells harbouring the F factor have a specific surface antigen, f^+ (Ørskov and Ørskov, 1960), and by subsequent studies (Ishibashi, 1967; Knolle and Ørskov, 1967; Lawn, Meynell, Meynell and Datta, 1967) this was shown to correspond to the F-pilus. F-pili can be readily distinguished from the other surface appendages such as common pili and flagella, since they are able to adsorb male specific ribonucleic acid (RNA) phages along their

sides (Crawford and Gesteland, 1964) and male-specific DNA phages adsorb to the tips of the F-pili (Caro and Schnös, 1966).

F pili are 850 to 950 nm wide and up to 20 µm in length (Brinton, 1965, 1971; Lawn, 1966; Hardy, 1975) and are under the control of the F-factor (Brinton, Gemski and Carnahan, 1964). F-pilin, the subunit of which pili are composed, is a phosphoglyco-protein of molecular weight 11,800 daltons (Brinton, 1971). The mean number of pili per donor cell varies from 1.4 to 2.7 with the maximum numbers appearing as growth conditions become more anaerobic (Curtiss, Caro, Allison and Stallions, 1969).

Col- and R- factors

The other classes of plasmids which have been discovered include the Col- and R- factors, which code for colicin production and antibiotic resistance respectively.

Colicins were discovered by Gratia (1925) when he showed that E. coli V produced a substance which was capable of killing E. coli Ø. Fredericq and Betz-Bareau (1953a,b,c) initiated work on the genetic basis of colicinogeny and demonstrated that although the transfer occurred at a low frequency it was possible to transfer the colicinogenic property of certain strains by conjugation.

Subsequently, Fredericq (1954) demonstrated that transfer of colicinogeny was independent of recombination of other markers. From this evidence he inferred the existence of a plasmid which conferred the ability to make a colicin and which could be transferred from one cell to another. These Col- factors are not always self-transmissible and some require a self-transmissible plasmid to coexist in the same cell before they can be transferred (Hardy, 1975).

Resistance-transfer factors or R-factors were discovered from 1957 onwards as the result of the isolation of increasing numbers of strains which were simultaneously resistant to more than one of the commonly used antibiotics. In 1959 it was discovered that several drug resistances could be transferred together to a sensitive bacterium (Akiba, Koyama, Ishiki, Kimura and Fukushima, 1960) and that this transfer was dependent upon contact between live cells (see Hayes, 1968 and Falkow, 1975 for reviews). These initial observations were subsequently extended and the mechanism of transfer was shown to be via conjugation (Mitsuhashi, Harada and Hashimoto, 1960; Nakaya, Nakamura and Murata, 1960; Watanabe and Fukasawa, 1960, 1961). R-factors are also capable of mediating transfer of chromosomal markers with

low frequency in the same way as the F factor (Sugino and Hirota, 1962).

Col- and R- factors also comprise DNA similar in size to F (44 to 113 x 10^6 daltons) compared with the non-self-transmissible or non-conjugative plasmids which are much smaller (3 to 6 x 10^6 daltons) (Helinski, Lovett, Williams, Katz, Kupersztoch-Portnoy, Guiney and Blair, 1974; Hardy, 1975). One complication arises in measuring the size of various R-factors is that in some hosts they dissociate into two smaller replicons (units of replication) (Nisioka, Mitani and Clowes, 1970; Rownd and Mickel, 1971). One of these replicons is thought to be the sex factor itself while the other replicon carries some or all of the markers for antibiotic resistance (Cohen and Miller, 1970a,b; Haapala and Falkow, 1971). In general they exist as only 1 to 2 copies per host cell chromosome as does F, compared with 10 to 15 copies for the non-conjugative plasmids (Helinski et al., 1974.

Most of the well studied conjugative plasmids fall into two groups depending upon whether they produce F-like or I-like pili (Meynell et al., 1968; Hardy, 1975). There are also specific phages which attach to the I-pilus which is usually no more than 2 µm long (Lawn et al., 1967;

Meynell and Lawn, 1967, 1968; Meynell, 1972). The two pilus types can be differentiated serologically and the F-like pili can be subdivided into four types and the I-like pili into two types (Lawn and Meynell, 1970; Harden and Meynell, 1973).

It has also been possible to demonstrate the transfer of R- factors from Escherichia coli to species as remote phylogenetically as Vibrio cholerae, Yersinia pestis, Serratia, ~~Pseudomonas~~, Proteus and Salmonella (see Watanabe, 1963; Meynell, Meynell and Datta, 1968). The demonstration of the transfer of drug resistance from a generally non-pathogenic bacterium to a wide variety of pathogens indicates the reason for clinical concern at the increasing incidence of R- factors (Richmond, 1974).

GENETIC STRUCTURE OF THE F SEX FACTOR

Incompatability

Bacteria which harbour one plasmid cannot normally be stably infected by another isogenic or closely related plasmid: it was originally observed that it was very difficult to stably infect an Hfr with an F' element (Scaife and Gross, 1962; Maas and Maas, 1962; Maas, 1963). It was just as difficult to isolate cells

carrying two autonomous F' elements; they seemed to rapidly segregate pure clones carrying only one or other of the F's (Scaife and Gross, 1962; Echols, 1963). This property of plasmids is known as incompatability (inc) (Novick, 1969) and has been used as a basis for classification of plasmids (Novick et al., 1976). Willetts (1974) has mapped inc on the F factor (see Figure I-1). However, the mechanism by which this phenomenon operates is unknown.

Two major models for the control of replication have been presented and include mechanisms which account for incompatability. In one model (Jacob, Brenner and Cuzin, 1963), replication is initiated at a specific site in the cell, thought to be on the cytoplasmic membrane; incompatible plasmids compete for a limited number of sites. The alternative model is that of Pritchard, Barth and Collins (1969) which is based on the properties of a postulated inhibitor of replication which is produced after initiation of a round of plasmid replication and is diluted during cell growth until a critical level is reached, whereupon a new round is initiated. The commencement of replication of an incoming plasmid will increase the concentration of inhibitor. Since replication is only initiated when the inhibitor concentration again

falls to a critical level due to an increase in cell volume, segregation of the plasmids will occur before a new round of replication could occur. This incompatibility could be explained on a random segregation of sex factors, with compatible plasmids being those which are not producing the same inhibitor. This second model does not contradict the membrane attachment model, since membrane attachment sites could still be involved in segregation of replicated elements. Discussion of these two models is included in a number of reviews (Novick, 1969; Clowes, 1972; Achtman, 1973; Hardy, 1975).

Surface or entry exclusion

Surface exclusion is a property associated with the transfer system itself; exponentially growing cells harbouring plasmids (either autonomous or integrated into the chromosome) are poor recipients when mated with donors harbouring identical or closely related sex factors (Lederberg, Cavalli and Lederberg, 1952). Under a variety of conditions donors can be made to act as good recipients; such cells are termed surface exclusion deficient (sfx^-) phenocopies (Lederberg et al., 1952; Bonhoeffer, 1966; Curtiss et al., 1969). Achtman, Willetts and Clark (1971) have shown that surface exclusion acts at least partly at the level of mating pair formation, leading to a

corresponding reduction in DNA transfer to the recipient cells (Matsubara, 1968; Sheehy, Orr and Curtiss, 1972).

Curtiss *et al.*, (1969) have concluded that the presence of F-pili is not responsible for surface exclusion since F^+ or F' derived cells, which rarely possess pili (Cohen, Fisher, Curtiss and Adler, 1968) and also many transfer defective (Tra^-) mutants (see later) which lack pili, are still surface exclusion proficient (Achtman *et al.*, 1971).

Although the loss of pili by a donor cell does not eliminate exclusion, Meynell and Ewins (1973) have found that the surface exclusion expressed by an F^+ cell, acting as a recipient, did not operate if the donor was an Hfr and was also carrying an F-like R-factor and producing mixed pili. If the Hfr donor carried an I-like R-factor instead and produced discrete F-like and I-like pili, then the F^+ recipient cells were proficient at exclusion. These results suggest that there is an exclusion specificity associated with the type of pili present. The gene for surface exclusion (*traS*) has now been mapped on the F-factor (Figure I-1) within the *tra* operon and is co-controlled with the transfer genes (see later) (Achtman and Helmuth, 1974; Willetts, 1974; Helmuth and Achtman, 1975).

Fertility inhibition

Most plasmids isolated from nature transfer at a rather low frequency of about 0.1 to 1% that observed with F (Willetts, 1972a). This inhibition of transfer is attributed to the formation of a transfer inhibitor by certain plasmid gene products. Derepressed mutants of both F- and I-like plasmids have been isolated which transfer at a frequency close to that of F (Egawa and Hirota, 1961; Meynell et al., 1968; Ohki and Ozeki, 1968). Willetts and Finnegan (1972) proposed a model for the inhibition of F'lac transfer by the F-like R-factor R100. This has subsequently been modified and expanded by Gasson and Willetts (1975). They propose a mechanism whereby two separate proteins (the finO and finP products) act together as the FinOP system to inhibit the synthesis or function of the traJ control gene (Finnegan and Willetts, 1971 and 1973), which is required for the synthesis of other tra gene products. (A discussion of the various transfer (tra) genes occurs later in this introduction).

finP is the F transfer inhibition gene and has been mapped between the origin of transfer (ori) and traJ (Willetts, Maule and McIntire, 1976). The site of action of the transfer inhibitor, traO, has also been mapped and it lies immediately adjacent to traJ, between finP and traJ. The F factor is thought to be a naturally occurring

mutant in *finO*, so that it is derepressed for transfer (Finnegan and Willetts, 1971).

Female specific phage restriction

A number of bacteriophages give lower efficiencies of plating and reduced plaque size on male strains (F^+, F' or Hfr) (Monner and Boman, 1970; Williams and Meynell, 1971), and these results are strain dependent (Linial and Malamy, 1970). These phages can be readily separated into two groups. Most of them, such as ØII (Cuzin, 1965) and T7 (Mäkelä, Mäkelä and Soikkeli, 1964) give large plaques at about 100 fold lower frequency on male strains. Phage *tau* (Hakura, Otsuji and Hirota, 1964) is different in that it is a temperate phage which gives small plaques on F^- strains and does not plaque at all on male strains.

ØII and T7-like phage have been shown to adsorb normally to male cells (Mäkelä *et al.*, 1964; Watanabe and Chada, 1964; Cuzin, 1965) but are not restricted in the classical way of DNA degradation (Arber and Linn, 1969; Boyer, 1971; Meselson, Yuan and Heywood, 1972). Linial and Malamy (1970) have demonstrated that host macromolecular syntheses are inhibited in male cells within a few minutes after infection with ØII. The phage DNA becomes associated with the membrane but is inhibited

from replication. The nature of the restriction has been the subject of a number of studies (Studier and Maizel, 1969; Morrison and Malamy, 1971; Whitaker, Yamada and Nakada, 1975; Yamada and Nakada, 1975).

Morrison and Malamy (1971) have isolated mutants of F which correspond to two genes pifA and pifB. pifA mutants are still somewhat female-specific phage resistant with intermediate plaque size and intermediate efficiency of plating, whereas pifA pifB double mutants give the same plating efficiency as F^- strains. Malamy has positioned the location of the pif region on the F factor (see Helmuth and Achtman, 1975).

The transfer (tra) genes

It has been possible to analyze genetically the transfer function of plasmids. Transfer defective (Tra^-) mutants of both F-like and I-like plasmids have been isolated (see Willetts, 1972), but only the transfer system of F-like plasmids has so far been genetically analysed in detail. There have been two studies on the basis of conjugational transfer.

Ohtsubo, Nishimura and Hirota (1970) devised a system for complementation analysis of the two compatible

F-like plasmids F'gal and R100-1. Using cells carrying both an F'gal Tra$^-$ mutant and an R100-1 Tra$^-$ mutant, they examined for complementation as shown by elevated levels of transfer. Seven complementation groups could be thus identified.

The second set of analyses demonstrated complementation between Tra$^-$ mutants in unstable transient heterozygotes, carrying two different F'lac Tra$^-$ mutants, which had been introduced into the same cell by conjugation (Achtman, Willetts and Clark, 1972) or P1 transduction (Willetts and Achtman, 1972). These analyses yielded complementary results; the first defined nine genes and the second confirmed the existence of eight of these and defined two more for a total of eleven genes, named traA through to traK. Genes traI, traD, traG, traF, traC and traE corresponded to groups A, B, C, D, E and F of Ohtsubo et al., (1970), traH, traB, traK, traA and traJ were additional genes and a twelfth gene now identified as traL corresponds to group H of Ohtsubo et al., (1970) (Willetts, 1973).

By complementation analyses with a series of F'gal deletions (Ohtsubo, 1970) and a series of Hfr deletions (Ippen-Ihler, Achtman and Willetts, 1972; Willetts, 1973) these genes have been ordered in a unique

linear sequence: tra J, A, L, E, K, B, C, F, H, G, D, I.

More recent studies have identified traS coding for surface exclusion, as mapping between traG and traD (Willetts, 1974). Also by means of insertion mutants made with phage Mu-1, tra A, L, E, K, B, C, F, H, G, S, D and I have been shown to constitute one polycistronic operon (Achtman and Helmuth, 1974; Helmuth and Achtman, 1975).

All mutants in tra A, B, C, E, F, H, J, K and L were resistant to all F-specific phages (Ohtsubo et al., 1970; Achtman et al., 1971, 1972; Willetts and Achtman, 1972) and lacked the F-pilus (Ohtsubo et al., 1970; Brinton, 1971). Mutants in tra I, D and some in traG still synthesized the F-pilus and were sensitive to all F-specific phages, except that RNA phages such as f2, MS2 and R17 can adsorb to, but not infect traD mutants (Walker and Pittard, 1969, 1971; Ohtsubo et al., 1970; Achtman et al., 1971, 1972). Since traG mutations can affect both pilus synthesis and DNA transfer (Ohtsubo et al., 1970; Achtman et al., 1972) it is thought that the traG product is bifunctional or the traG mutations may affect two genes, in between which complementation is prevented by polarity interactions (Willetts, 1972a).

It is also now known that traA in fact codes for the pilus subunit (M. Achtman, personal communication).

F-like R-factors are able to complement mutations in most tra genes with the exceptions of traI and J (Willetts, 1971; Alfaro and Willetts, 1972), which are thought to be plasmid specific. The traI product has been implicated in the initiation of transfer replication, and traJ appears to be a positive control gene regulating all the tra genes (Finnegan and Willetts, 1971, 1972: Willetts, 1971; Willetts and Finnegan, 1972; Achtman, 1973a,b), since traJ mutants lack F pili, traI function, pilus specificity (traA) and surface exclusion (traS) all of which lie in the one operon.

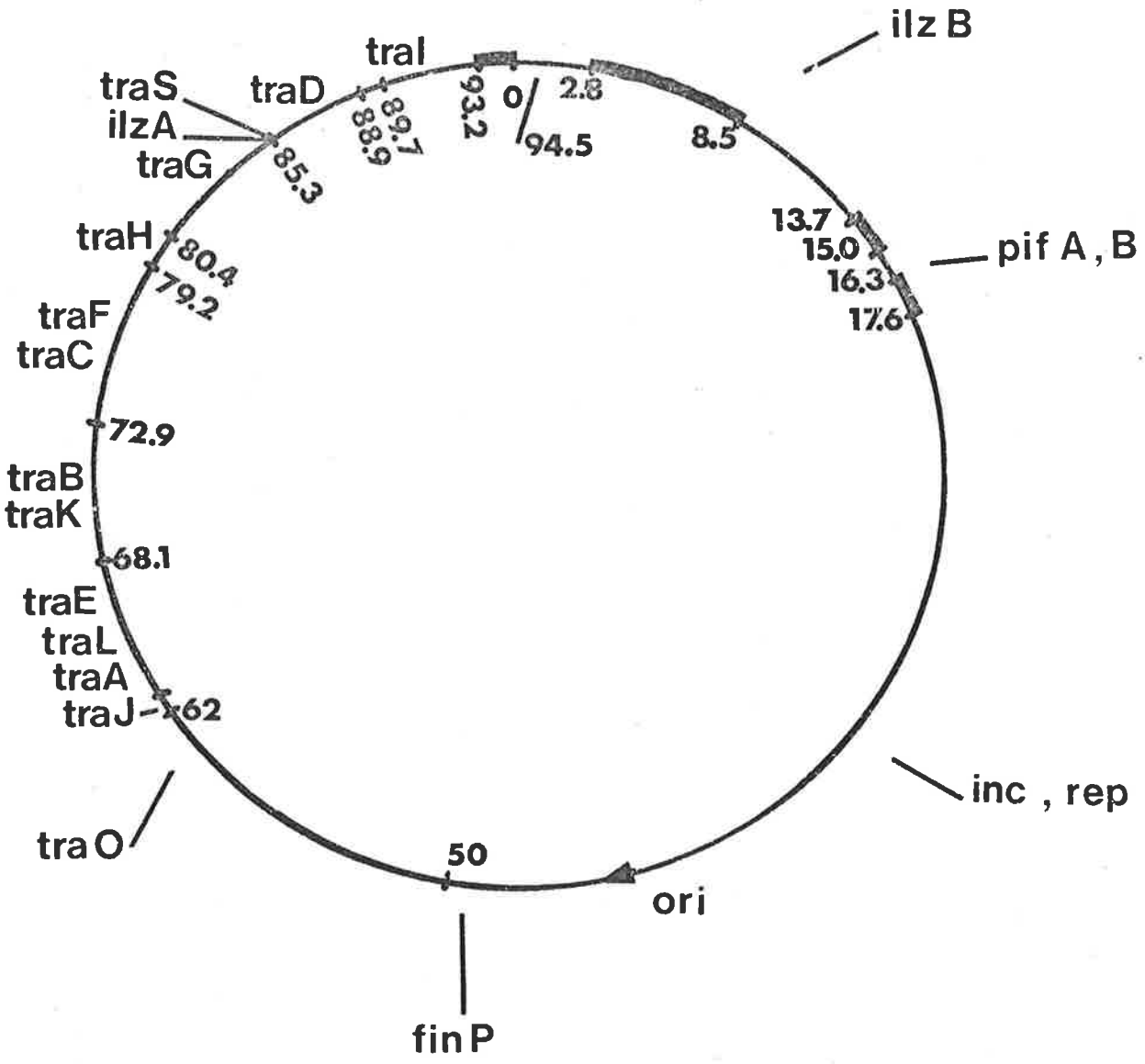
The location of the origin (ori) from which transfer begins has also been mapped (Willetts, 1972b). Reeves and Willetts (1974) have shown that of the F-like plasmids, ColV2 and ColVBtrp could initiate transfer from the F origin, whereas R100-1, R1-19 and R538Fdrd-1 could not. This can be correlated with the plasmid specificities of the traI products of these plasmids and is consistent with the suggestion of Willetts (1972b) that the traI product is an endonuclease which recognizes the origin sequence, and opens the DNA for transfer.

Immunity to lethal zygosis

Skurray and Reeves (1973a,b; 1974) have shown that Hfr, F^+ and F' cells are, unlike F^- cells, insensitive to an excess of Hfr donor cells, indicating that there is an F factor mediated immunity to lethal zygosis (Ilz). By use of a series of Hfr deletion strains and F'*lac* deletion strains it has been possible to demonstrate that Ilz is genetically complex and that it is probably due to the presence of two genes; *ilzA*, lying between *traG* and *traS*, and *ilzB* lying outside the *tra* operon beyond *traI* (Skurray, Willetts and Reeves, 1976). Sensitivity to lethal zygosis would require a strain to be an *ilzA ilzB* double mutant. Recent results using chimeric plasmids incorporating regions of the *tra* operon are consistent with these results (Skurray, Nagaishi and Clark, 1976).

The present genetic and physical structure of F can thus be summarized as shown in Figure I-1. This was accomplished by combining the aforementioned genetic analyses with electron microscopic analysis of DNA heteroduplexes made with Tra^- mutants (Sharp *et al.*, 1972; Davidson, Deonier, Hu and Ohtsubo, 1974) and is a modification of the map presented by Helmuth and Achtman (1975). The map units are in kilobases (Kb).

FIGURE I-1: A combined genetic and physical map of the F-factor of Escherichia coli K-12. Obtained from Helmuth and Achtman (1975) and Skurray et al., (1976).


ilz B
traI
traS
traD
ilzA
traG
traH
traF
traC
traB
traK
traE
traL
traA
traJ
traO
finP
ori
inc , rep
pif A , B
0
2.8
8.5
13.7
15.0
16.3
17.6
50
62
68.1
72.9
79.2
80.4
85.3
88.9
89.7
93.2
94.5

ROLE OF THE PARENTS IN CONJUGATION

Bacterial conjugation has been described in terms of five stages (Curtiss, 1969):

i. specific pair formation, defined as the formation of donor-recipient cell unions which are stable during gentle dilution;

ii. effective pair formation, defined as the establishment of a cellular connection between donor and recipient cells;

iii. chromosome, or sex factor mobilization, defined as the preparation of the circular donor chromosome or sex factor for linear sequential transfer;

iv. chromosome or sex factor transfer;

v. recombination of the transferred parts of the donor chromosome into the chromosome of the recipient cell or circularization of the transferred sex factor.

Specific pair formation

The examination of mating mixtures by light microscopy has revealed the existence of mating pairs (Lederberg, 1956; Anderson and Maze, 1957; Anderson, 1958), and these pairs have also been studied using

electron microscopy (Anderson, Wollman and Jacob, 1957). De Haan and Gross (1962) have shown that these pairs are stable enough to resist gentle dilution and this has enabled the study of specific pair formation to progress. Specific pairs form in the absence of energy metabolism in either parent, and the rate and frequency of pair formation is a function of the population density as well as of the viscosity and temperature of the medium (Falkow, 1975; Novotny and Lavin, 1971; Walmsley, 1976). Thus specific pair formation largely follows the kinetics of a random collision between two particles.

The F-pilus has been shown to be essential for this specific pair formation on the basis of a number of observations. Careful examination of mating mixtures by light microscopy showed the presence of pairs which stream together separated by an invisible thread about the length of a donor pilus (Brinton, 1965). Also, if mating mixtures to which donor specific RNA phages have been added (Marvin and Hoffman-Berling, 1963; Marvin and Hohn, 1969; Zinder, 1965) are examined under electron microscopy, then it can be seen that the donor pili in fact span the distance between the donor and recipient cells (Brinton, 1965, 1967, 1971; Curtiss et al., 1969). Brinton (1971) has also shown that pili can be effectively removed from the donor cells by high

speed blending, without loss of viability to the cells, and along with this loss goes loss of the ability to form specific pairs. The rate of return of specific-pair forming ability is identical to the rate of resynthesis of donor pili. The frequency of specific pair formation is directly proportional to the mean number of donor pili per cell (Curtiss et al., 1969). This result was obtained by using donor cultures grown under a variety of conditions which influence the number of pili per cell. Lederberg, Cavalli and Lederberg (1952) showed that F^- phenocopies could be produced by growing cultures with vigorous aeration into late stationary phase and such cultures have been shown to lack pili (Brinton, 1965). The addition of donor specific RNA- or DNA- phages to donor cultures prior to or at the time of mating interferes with specific pair formation as measured by recombinant production (Ippen and Valentine, 1967; Knolle, 1967; Novotny, Knight and Brinton, 1968). In particular, if male specific DNA-phages which adsorb only to the tip of the pili (Caro and Schnös, 1966) are added to the donor cultures prior to mating, then specific pair formation is effectively reduced. Also the many tra mutants described earlier which lack pili do not appear to be able to form specific mating pairs whereas tra mutants still possess-

-ing pili are capable of forming specific pairs although some later stage of conjugation is defective (Achtman et al., 1973).

Curtiss (1969) has likened pili to grappling hooks necessary for specific pair formation. However, the interaction of the donor pilus with the recipient cell surface is thought to be of a more specific nature with a particular pilus receptor site being present on the recipient cell surface (Brinton, 1971).

Effective pair formation

Even though two cells can form a specific pair this does not mean a successful conjugal event can occur, because specific pairs can even be detected between dead cells (Brinton, 1967). It is proposed that effective pair formation is the process by which a specific pair establishes a cellular connection between donor and recipient so that the transfer of genetic material from the donor to the recipient cell can occur (Curtiss, 1969). However, this stage is poorly understood, mainly because of the lack of knowledge concerning the nature of the bridge used to conduct the DNA from the donor to the recipient cell.

Brinton (1967, 1971) has asserted that the F-pilus, which he has shown to have a central axial hole of 200 to 250 nm serves as both the organelle for specific pair formation and also as a tunnel through which the DNA subsequently enters the recipient. No one, however, has yet been able to isolate DNA present within the pilus channel during conjugation. (Wendt, Ippen and Valentine, 1966; Brinton, 1971).

Curtiss (1969) has expressed doubts about the pilus conduction model, and suggests that wall to wall contact is required for efficient transfer. Ou and Anderson (1970), however, have suggested that DNA can be transferred between members of a mating pair connected only by an F-pilus. These two models are not incompatible. It would be possible for DNA to be transferred via the pilus and have the cells in very close if not wall to wall contact. Such contacts have been observed (Anderson, 1958; Anderson et al., 1957) and might be formed by the retraction of F pili which has been demonstrated in male cells (Jacobson, 1972; Novotny and Fives-Taylor, 1974; Fives-Taylor and Novotny, 1976). However, the model of Jacob et al., (1963) in which there is cytoplasmic fusion between the donor and recipient can be discounted since only DNA is transferred and no cytoplasmic components from

the donor such as β-galactosidase can be detected in the recipient (Curtiss et al., 1968). The steps involved in specific and then effective pair formation are quite rapid since pairs are formed within three to five minutes after mixing of the donor and recipient cells, and DNA appears in the recipient soon after. The cells must undergo some surface interaction during this short period, but also the sex factor or chromosomal DNA must undergo some change in readiness for transfer. Ou (1975) has studied the generation of a mating signal, which also appears to require cell to cell contact, although not necessarily cell wall to cell wall contact. This signal is proposed to start the preparations for DNA transfer. The transmission of such a signal may constitute what converts a specific pair into an effective one.

Chromosome or sex factor mobilization

F and all other sex factors exist as circular molecules within the bacterial cell; even when a sex factor is present in the Hfr state it is still part of one large circular chromosome (Falkow, 1975). It is thought that the mobilization of the sex factor or of a chromosome containing an integrated sex factor most likely involves the same series of events, which are under the control of the sex factor (Curtiss, 1969). Since

mobilization leads directly to DNA transfer, it follows that it would be uneconomical for the donor cell to be carrying out this process all the time in the absence of a suitable recipient. The mating stimulus proposed by Jacob et al., (1963) which is being investigated by Ou (1975) is thought to be a suitable switch to start mobilization. It is proposed that after such a switch an F-coded initiator, a specific endonuclease, makes a single stranded nick in the DNA. The product of traI has been suggested to be such an endonuclease and ori the sequence which it recognizes (Willetts, 1972b; Reeves and Willetts, 1974).

Chromosome or sex factor transfer

Chromosome or sex factor transfer is the transmission of genetic information from the donor to the recipient. By using an Hfr whose DNA was labelled with density isotopes prior to mating, Jacob et al., (1963) found that DNA transferred to recipient cells consisted of one strand synthesized before and the other during the mating. Ptashne (1965) obtained a similar result by following the conjugal transfer of an F' containing a λ prophage. The localization by autoradiography of F (Herman and Forro, 1964) and Hfr chromosomal DNA (Gross and Caro, 1966) transferred to recipient cells indicated

that only one of the two strands of the donor DNA molecule is transferred rather than both strands being transferred. Ohki and Tomizawa (1968) have confirmed this finding by showing that F' DNA recovered from a recipient cell is composed of one strand transferred from the donor and one strand synthesized in the recipient cell. In addition, Cohen et al., (1968a,b) have demonstrated that DNA transferred from F^+, F' and Hfr donors to DNA deficient minicells was single stranded and that F and short F' DNA, but not long F' and Hfr chromosomal DNA, were converted to partially double-stranded DNA within the minicells. When they examined the transfer of λ prophage DNA from F' and Hfr donors, Ohki and Tomizawa (1968) and Rupp and Ihler (1968) found that each donor transferred a specific λ strand that was dependent upon the polarity of F' or Hfr chromosome transfer to the recipient cell. The transferred donor DNA always entered the recipient with its 5' terminus as the leading extremity. Vapnek and co-workers (Vapnek and Rupp, 1970, 1971; Vapnek, Lipman and Rupp, 1971) have confirmed the asymmetric nature of the transfer of F DNA and that this asymmetry applies to both F-like and I-like R factors. These workers have also shown that the non-transferred strand of F is conserved in the donor cell where it is replicated to reform a complete F molecule. This newly synthesized

strand was identical in sequence to the one transferred to the recipient. It has been demonstrated that donor cells carrying dnaB (TS) mutations, which inhibit vegetative replication of both plasmid and chromosomal DNA at 42°C, are still able to carry out conjugal transfer at the restrictive temperature (Bonhoeffer, 1966; Bonhoeffer, Hosselbarth and Leliman, 1967). This was originally thought to be evidence that DNA synthesis in donor cells was not required for transfer, however, dna (TS) mutants do synthesize DNA during conjugation at 42°C (Bresler, Lanzov and Lukjaniec-Blinkova, 1968; Marinus and Adelberg, 1970), and most of the replicated DNA is that synthesized to replace the strand being transferred to the recipient cells (Fenwick and Curtiss, 1973a). Thus, the mechanisms of vegetative and conjugal DNA synthesis are distinguishable from one another, even though they share common elements. Also, Sarathy and Siddiqui (1973) have provided very good evidence that conjugal DNA synthesis is not necessary even to initiate conjugal chromosome transfer by Hfr donors. The many factors involved in conjugal DNA replication and transfer have been combined in a model by Curtiss and Fenwick (1974), which is a modification of the rolling circle model of DNA replication proposed by Gilbert and Dressler (1968).

A more comprehensive review of this stage of conjugation can be found in a number of reviews (Curtiss, 1969; Brinton, 1971; Curtiss and Fenwick, 1974).

Recombination or plasmid circularization

The DNA entering the recipient cell is single stranded and a complementary strand is required to be synthesized. The resulting double-stranded molecule must be refractory to nucleases within the cell, be able to undergo recombination with the bacterial chromosome or alternatively, in the case of a plasmid, to circularize and eventually replicate.

Recombination

Curtiss (1969) has stated that the formation of recombinants following conjugal transfer of genetic material from a donor to a recipient requires synapsis between homologous segments of donor and recipient genome, formation of continuous or discontinuous regions of effective homologous pairing between the synapsed homologues, reassortment of donor and recipient genetic information to yield new combinations of genetic information, and segregation of recombinant chromosomes from non-recombinant chromosomes. A similar definition of

recombination has also been proposed by Clark (1971, 1973) who has comprehensively reviewed the literature with respect to the metabolism of DNA during recombination and to the isolation and characterization of mutants defective in recombination (rec mutants).

The most direct method for detecting mutants that might be blocked in genetic recombination is to screen survivors of mutagenic treatment for the ability to produce recombinants when mated with an Hfr (Clark and Margulies, 1965), or transduced with a generalized transducing phage (Clark, 1973) or transformed with purified DNA (Beattie and Setlow, 1971). In order to confirm the deficiency in recombination, such suspected mutants must be shown to be normal as recipients in relation to DNA transfer. This can be detected directly by using radioactive and density labels, however, biological methods are also available: transfection (transformation with phage DNA), plaque formation by generalized transducing phages or zygotic induction may be used to indicate the occurrence of transformation, transduction, or conjugation respectively. The inheritance of plasmid DNA which occurs by a process termed repliconation (Clark, 1967) also indicates successful DNA transfer to putative recombination defective mutants. It is thought that mutants which show reduced recombinant

frequencies but show normal ability to receive DNA and to inherit it by repliconation are defective in one of the steps of genetic recombination. The genes involved are known as rec genes.

Plasmid Circularization

It can be seen that the processes of inheritance of a plasmid and recombinant formation can be differentiated: plasmid inheritance requires that the transferred DNA circularizes and replicates itself whereas recombinant formation requires the integration of the transferred DNA into the chromosome of the recipient cell.

On the basis of their own experiments and those of Falkow, Tompkins, Silver, Guerry and Le Blanc (1971), Curtiss and Fenwick (1974) have suggested what happens to the linear single-stranded plasmid DNA transferred to the recipient. They propose that the linear DNA transferred from the donor initially attaches to the inner cell membrane where an RNA primer would be synthesized followed by the synthesis of the complementary DNA strand. The formation of the circular plasmid DNA in the recipient could involve the action of exonucleases to digest 3' nucleotides. The plasmid could then become an open circular molecule containing the RNA primer which is then converted to an open circular molecule

with the RNA replaced by DNA and finally to CCC DNA free in the cytoplasm. Alternatively the exonucleases could digest the 5' ends of the DNA molecule.

THE CELL WALL OF ESCHERICHIA COLI

The cell wall is that part of the cell envelope which is external to the cytoplasmic membrane. Electron microscopic sections show this to be composed of an inner, electron dense layer with a typical unit membrane on the outer surface (De Petris, 1967; Silva and Sousa, 1973). The inner electron dense layer is the peptidoglycan or murein sacculus which forms a rigid string-bag-like network and is thought to be the shape maintaining structure in bacteria (Weidel and Pelzer, 1964; Braun, Gnirke, Henning and Rehn, 1973; Braun and Hantke, 1974). Bacterial ghosts, however, do not appear to require the peptidoglycan layer for their shape, but are also not subject to osmotic pressure or surface tension (Henning, Holm and Sonntag, 1973). Henning (1975) has recently reviewed the various factors determining cell shape.

The Murein Lipoprotein

Braun and his coworkers (Braun and Rehn, 1969; Braun and Sieglin, 1970; Braun and Wolff, 1970) have shown that there is a specific lipoprotein, 12 to 14 nm

long, composed of 57 amino acids (Braun and Bosch, 1972) and covalently linked to the peptidoglycan (Inouye, Shaw and Shen, 1972) and which extends outwards towards the outer membrane. Schnaitman (1971) has proposed that the lipid component of this molecule anchors the outer membrane by its hydrophobic interactions. This has been confirmed by the studies of Burman, Nordström and Bloom (1972) which also suggest that the peptidoglycan serves as a foundation for the outer cell wall layers.

There are about 10^5 lipoprotein molecules per cell which can exist as a free form as well as bound (Hirashima, Wu, Venkateswaran and Inouye, 1973). In pulse chase experiments using labelled arginine, the label first appears in the free form, then equilibrates between the two forms and retains this distribution for several generations. The free lipoprotein is about twice as abundant as the bound form, and is found mainly associated with the outer membrane. The primary and secondary structures and biosynthesis of the murein lipoprotein have been extensively reviewed (Braun and Hantke, 1974; Braun, 1975; Inouye, 1975).

Composition of the outer membrane

Schnaitman (1970b) has shown that the cell wall, or outer membrane has a much greater density on sucrose

gradients than the cytoplasmic membrane. However, the cell wall material contains cytoplasmic membrane which is specifically attached to the cell wall (Schnaitman, 1971a). The cytoplasmic membrane and cell wall obtained by sucrose gradient centrifugation, have also been shown to correspond, both in composition and electron microscopic appearance, to those fractions of the cell envelope which are soluble and insoluble, respectively, in the non-ionic detergent Triton X-100 (Schnaitman, 1971a).

The outer membrane is typical of other biological membranes in displaying the double track appearance after fixation for electron microscopy (Dreher, Schulman, Anderson and Roels, 1967). A number of studies have shown that the outer membrane contains phospholipids and proteins (Schnaitman, 1970a,b; White, Lennarz and Schnaitman, 1972) as well as lipopolysaccharide (Schnaitman, 1971; Reske and Jann, 1972). The basic structure of the outer membrane as indicated by biophysical studies is a phospholipid and protein bilayer with the oligosaccharide portion of the LPS associated with the surfaces of the bilayer (Costerton, Ingram and Cheng, 1974). Differences have been demonstrated in the distribution of the phospholipids of the outer and cytoplasmic membranes (White et al., 1972). Freeze

etching studies of Pseudomonas have shown that there is a cleavage plane in the outer membrane of the cell wall (De Voe, Costerton and MacLeod, 1971; Forge, Costerton and Kerr, 1973; Gilleland, Stinnet, Roth and Eagon, 1973), which indicates that the hydrophobic parts of the phospholipid and LPS molecule form a zone in the centre of this layer. Studies on E. coli (Bayer and Remsen, 1970; Bayer, Koplow and Goldfine, 1975; Verkleij, Lugtenberg and Ververgaert, 1976) have shown that the membrane only fractures for short distances within its hydrophobic regions. This suggests that the outer membrane is not a completely typical lipid bilayer, but that the proteins present disturb the freeze-fracturing because of their orientation in the membrane.

The lipopolysaccharide (LPS) is a major component of the outer membrane, and for Salmonella its structure, biosynthesis and genetics have been extensively studied and reviewed (Osborn, 1969, 1971; Weinbaum, Kadis and Ajl, 1971; Nikaido, 1973). Recent studies using antibiotics and bacteriophages to select resistant mutants have also enabled the structure and composition of the LPS of E. coli to be elaborated (Eriksson-Grennberg, Nordström and Englund, 1971; Monner, Jonsson and Boman, 1971; Tomaki, Sato and Matsuhashi, 1971; Tamaki and Matsuhashi, 1973; Boman and Monner, 1975;

Hancock and Reeves, 1976). Boman and Monner (1975) have shown that the LPS of E. coli K12 is composed of a core region, composed of lipid A, ketodeoxyoctonic acid (KDO) and a phosphorous component, and a side chain region containing the sugars, heptose, glucose, galactose and rhamnose. By using an independent set of bacteriophage resistant mutants, Hancock and Reeves (1976) have also arrived at a similar LPS structure which closely resembles that obtained for Salmonella.

Sites of adhesion of the outer and cytoplasmic membranes

In Salmonella the LPS is synthesized sequentially by a number of enzymes (Osborn et al., 1972). The complete O-antigen (or side chain) and the core region are synthesized and attached to each other in the cytoplasmic membrane (Mühlradt, 1971). The LPS is then rapidly and irreversibly translocated into the outer membrane via discrete export sites (Mühlradt, Menzel, Golecki and Speth, 1973). After 2 to 3 min the LPS is randomized over the surface. In ultrathin sections of plasmolyzed cells, the sites of export were localized over adhesion sites between the cytoplasmic and outer membrane. There appeared to be approximately 250 such points.

More recently, Bayer (1974, 1975) has used a

lysogenic phage of Salmonella anatum to convert the host cell, which then produces LPS that differs antigenically and in its phage receptor properties. Using ferritin labelled antibody and phage adsorption he was able to visualize approximately 20 to 30 insertion points for the new LPS in the outer membrane. Kulpa and Leive (1976) have estimated the number of initial insertion points of new LPS by using galactose density labelling and then density gradient fractionation of the membrane. They have suggested that there are between 10 and 50 such sites per cell.

Bayer (1968a,b) first showed that there were about 200 to 400 sites of adhesion of the cytoplasmic and outer membranes visible after cells were plasmolyzed. He showed that bacteriophages T1 to T7, λ, and ØX174 all appeared to be preferentially located over these adhesions following 4 to 6 min of adsorption (Bayer, 1968b,c). The F-pili of E. coli K-12 HfrH have also been shown to originate at a wall membrane fusion (Bayer and Starkey, 1972), so it is possible that these adhesion areas are suitable sites for the transport of infecting nucleic acid. Worcel and Burgi (1974) have also shown that during the cell cycle there is a temporary association between the chromosome and the cell envelope, and it has been shown that the sites of attachment of the DNA

are at adhesions of the cytoplasmic and outer membranes (Olsen, Heidrich, Hannig and Hofschneider, 1974). Thus it would seem that the 200 to 400 adhesion sites observed by Bayer probably fall into several different classes.

The outer membrane proteins

Much of the outer membrane of E. coli is protein and Schnaitman (1970a,b) has described its composition with the aid of polyacrylamide gel electrophoresis. Using different solubilization and running conditions the single band of molecular weight 44,000 daltons which he first observed has been split into several proteins in the range of 35,000 to 45,000 and these comprise about 70% of the total outer membrane proteins (Bragg and Hou, 1972; Moldow, Robertson and Rothfield, 1972; Inouye and Yee, 1973; Schnaitman, 1973a,b; Koplow and Goldfine, 1974). The most effective means of separating these major outer membrane proteins is to analyze proteins which have been boiled in sodium dodecyl sulphate (SDS) solutions, with an alkaline buffer system as described by Bragg and Hou (1972), or with discontinuous gel systems of the types described by Neville (1971) or Laemmli (1970). A combination of these latter two methods using the slab gel apparatus of Studier (1973) at present gives the best method of resolving the different

envelope proteins in E. coli K-12 (Lugtenberg, Meijers, Peters, van der Hoek and van Alphen, 1975). As mentioned earlier Schnaitman (1971a) has also shown that the cytoplasmic membrane proteins are able to be solubilized using the non-ionic detergent Triton X-100, and that the Triton insoluble component corresponds to the outer membrane or cell wall. He was also able to show that treatment of the Triton insoluble component with Triton in the presence of ethylene-diamine-tetraacetic acid (EDTA), solubilized most of the protein in this fraction (Schnaitman, 1971b).

When the outer membrane proteins of E. coli K12 are examined with the gel system of Bragg and Hou (1972), two major protein bands are observed and these have been designated by Schnaitman (1974a) as proteins 1 and 3. No protein 2 is normally present in E. coli K-12 but it is found in E. coli 0111 and a number of enterotoxin producing strains. In at least one case the production of protein 2 is determined by a temperate phage, and cells made lysogenic for this particular phage will also produce protein 2 (Schnaitman, 1974a; Schnaitman, Smith and Forn de Salsas, 1975).

Rosenbusch (1974) has purified and extensively characterized protein 1 from E. coli BE. This protein

has a molecular weight of 36,500 and can be isolated bound to the peptidoglycan in continuous sheets with a regular hexagonal symmetry. This has led Rosenbusch to term this protein the outer membrane matrix protein. Schnaitman (1974a) has shown that when this protein is solubilized in SDS solutions without heating, it migrates on gels as an aggregate with a much higher apparent molecular weight than its true molecular weight. This property was confirmed by Rosenbusch (1974) who showed that SDS does not bind to protein 1 until it is heated strongly.

At the time of commencement of this thesis there was some confusion as to the number of the major outer membrane proteins: the protein described as protein 1 by Schnaitman (1973b) has been given various designations: protein I (Henning, Hohn and Sonntag, 1973), protein A, (Bragg and Hou, 1972), and protein B (Koplow and Goldfine, 1974). Rosenbusch (1974) showed that it was a single polypeptide in E. coli B, however, it has been recently shown that in E. coli K-12 there are two very closely related polypeptide species constituting protein 1 (Schmitges and Henning, 1976; C.A. Schnaitman, personal communication). These have been designated 1a and 1b and can be separated electrophoretically on the system of Lugtenberg et al., (1975), where they are designated

by these authors as bands b and c.

The confusion with the composition of protein peak 3 (nomenclature of Schnaitman, 1973b) is worse. Schnaitman (1974a) has suggested that it consists of two proteins 3A and 3B, which have been confirmed in this thesis, and of which protein 3A is normally the more abundant. From the work in this thesis it is now clear that protein 3A is identical to the proteins designated by Henning et al., (1973) as protein II*, as protein B by Bragg and coworkers (Bragg and Hou, 1972; Reithmeier and Bragg, 1974), as protein C by Koplow and Goldfine (1974), as protein G by Chai and Foulds (1974) and as band d by Lugtenberg et al., (1975). This protein is heat modifiable in its behaviour on SDS gels: when protein 3A is analyzed without heating, it moves with an apparent molecular weight lower than that which is observed after the protein has been boiled in SDS.

The murein lipoprotein is also a major outer membrane protein and has been well characterized, but only the free form will run on acrylamide gels (Braun and Bosch, 1973a,b) unless the cell wall is treated with lysozyme (Schnaitman, 1971,b).

Very few enzymes have been localized in the outer membrane (Costerton et al., 1974). Phospholipase A1 has been shown to be present (Ohki, Osamu and Nojima, 1972), however, none of the other enzymes involved in phospholipid metabolism appear to be associated with the outer membrane (Bell, Mavis, Osborn and Vagelos, 1971), with the possible exception of the enzymes involved in synthesis of phosphatidyl serine (White, Albright, Lennarz and Schnaitman, 1971).

A number of minor proteins have also been shown to exist in the outer membrane. The protein coded for by the tonA gene is the receptor for bacteriophages T1, T5 and Ø80 and for colicin M (Braun, Schaller and Wolff, 1973; Braun and Wolff, 1973; Hantke and Braun, 1975). This protein is a single polypeptide chain of molecular weight 85,000. This protein is now known to be the site for the uptake of ferrichrome complexed iron (Wayne and Neilands, 1975; Luckey, Wayne and Neilands, 1975; Hantke and Braun, 1975).

The receptor for colicins E2 and E3 has been purified by Sabet and Schanitman (1973) and been shown to be a protein of 60,000 molecular weight. This protein is missing or altered in bfe mutants and is the receptor site for the uptake of vitamin B12 (cyano-

cobalamin) (Di Masi, White, Schnaitman and Bradbeer, 1973; Kadner and Liggins, 1973). Bfe mutants are resistant to bacteriophage BF23 as well as colicins A, E1, E2 and E3 (Nagel de Zwaig and Luria, 1967; Buxton, 1971; Davies and Reeves, 1975b; Hancock, Davies and Reeves, 1976) and it has now been shown that they all use the same receptor protein of which there are about 200 to 250 copies per cell (Sabet and Schnaitman, 1973; Bradbeer, Woodrow and Khalifah, 1976).

Bacteriophage λ has also been shown to have an outer membrane protein receptor (Randall-Hazelbauer and Schwartz, 1973). This 55,000 molecular weight protein is the product of the lamB gene which is located in one of the maltose operons (Hofnung, 1974) so that the number of the receptor molecules at the cell surface can be varied by adding inducers of the maltose operons to the growth medium or by changing the degree of catabolite repression (Ryter, Shuman and Schwartz, 1975). It appears to be integrated in the cell envelope during the last quarter of each generation and the integration is initiated in the vicinity of the septum (Ryter et al., 1975). Recently it has been shown that the λ receptor functions like a specific pore for the uptake of maltose and maltotriose (Szmelcman, Schwartz, Silhavy and Boos, 1976).

Pugsley and Reeves (1976a,b,c and 1977a,b) have shown that colicins B and D are very closely related and share a common receptor. This receptor is a 78,000 dalton protein absent in cbr mutants and is also the receptor for enterochelin complexed iron as suggested by other authors (Guterman, 1971, 1973; Davies and Reeves, 1975a). This protein is derepressed under iron starvation (Pugsley and Reeves, 1976a; Hancock, Hantke and Braun, 1976) as is the tonA protein and the colicin I receptor, which has also been identified as an outer membrane protein (Konisky and Lin, 1974).

The receptors for bacteriophages T2 and T6 have been suggested to be in the outer lipoproteic layer of the cell envelope and be protein in nature and different from each other (Beumer, Beumer-Jochmans, Dirkx and Dekegel, 1965, 1966). Fredericq (1953, 1956) has suggested that on the basis of cross-resistance studies bacteriophage T6 and colicin K share a common receptor. Weltzien and Jesaitis (1971) have shown that tsx mutants have lost receptor activity for these agents and also showed that their receptors differed slightly in chemical groupings. Sabet and Schnaitman (1971) have localized the receptor activity for colicin K in the outer membrane.

A number of recent reports have shown the effects that various LPS mutations have on the protein composition of the outer membrane. In particular, some mutants with heptose deficient LPS have been shown to be reduced in the major outer membrane proteins, especially protein 1 (Koplow and Goldfine, 1974; Bayer et al., 1975; Hancock and Reeves, 1976; van Alphen, Lugtenberg and Berendsen, 1976). However, there is not a strict correlation between LPS defects and loss of outer membrane proteins as shown by Hancock and Reeves (1976), who demonstrated that an LPS mutant which had no detectable heptose, glucose or galactose in its LPS core, still had a normal outer membrane protein composition.

CONJUGATION DEFECTIVE MUTANTS

The various stages of conjugation which have been described have been elaborated mainly by biophysical techniques. A wealth of mutants have been identified and characterized in donor strains and these are providing many answers to the role of the donor in the conjugal process. Only in the last stage of genetic recombination have extensive studies been made of recipient function and a series of recombination deficient (rec) mutants have been obtained, which are proving extremely useful in gaining an insight into

the exact mechanism by which recombination occurs (Clark 1971, 1973). The stages of conjugation cannot readily be synchronized and so it is difficult to characterize intermediate steps of gene transmission in a population of cells. However, mutants blocked at specific stages of conjugation should greatly reduce this difficulty. Ideally it would therefore be an advantage to have defective recipients which are blocked at these different stages.

Three methods have been used for measuring the competence of bacteria at forming mating pairs. The method of de Haan and Gross (1962) uses the rate of formation of recombinants as an indicator of the number of mating pairs. Achtman, Willetts and Clark (1971) used a Coulter counter and measured mating pairs by comparison of the size of "particles" in the parent cultures with those in the mating mixture. Skurray, Hancock and Reeves (1974) assayed mating pairs by measuring Lac^+/Lac^- sectored colonies derived from mating mixtures of Lac^+ males and Lac^- females.

At the time of commencement of this study only the conjugation defective mutants of Monner, Jonsson and Boman (1971) and those of Skurray et al., (1974) were known. The mutants of Monner et al., (1971) were

high level ampicillin resistant mutants which were defective in the composition of their LPS. Using the method of de Haan and Gross (1962) these mutants were shown to be greatly reduced in their ability to form mating pairs. Skurray (1974) has also shown these mutants to be resistant to lethal zygosis. The Con^- mutants of Skurray *et al.*, (1974) were resistant to lethal zygosis and defective in pair formation both under their new experimental procedure, and also when measured using a Coulter counter (M. Achtman, personal communication). These Con^- mutants were selected as resistant to bacteriophage K3 (Skurray *et al.*, 1974; Hancock and Reeves, 1975) and were thought to have lost the major outer membrane proteins 3A and 3B. Unlike the mutants of Monner *et al.*, (1971) they had a normal LPS composition (Hancock and Reeves, 1976). These mutants have also been isolated as tolerant to colicins K and L (Davies and Reeves, 1975b,c). The defect in recipient ability was shown to affect a number of F-like plasmid bearing and Hfr donor strains but not the F-like R-factors R100-1 and R136fin^- (Skurray *et al.*, 1974).

During the course of this study several other classes of conjugation defective mutants have been described. Reiner (1974) predicted that, among mutants resistant to infection by single stranded DNA viruses,

there would be some also resistant to infection by single stranded conjugal DNA. Using the single stranded DNA phage ST-1, he was able to isolate two classes of defective recipients. The type A mutants form mating aggregates whereas the type B mutants do not (Achtman, 1975). The type A mutants were grossly defective in mating with F'lac and Hfr donors, and also appeared to be defective as recipients with a donor carrying a plasmid coding for an I-like pilus (Reiner, 1974). This implied that it was defective as a recipient for at least two different pilus types. The type B mutants were not as defective in conjugation but the defect was only observed with an F'lac and Hfr donors. The type B mutants map in the region of the rfa genes: however, the type A defect has not been localized genetically because of the extreme nature of the defect in recipient ability and also its resistance to infection by transducing phages such as P1 (Reiner, 1974).

Havekes, Lugtenberg and Hoekstra (1976) have recently shown that two classes of mutants with heptose-less LPS and also lacking some outer membrane proteins, are conjugation deficient. These mutants were found among strains selected as resistant to bacteriophages T3, T4 and T7. Both mutants were defective in mating pair formation using the method of Skurray et al., (1974),

but the defect in conjugation observed in liquid medium could be greatly alleviated by mating on the surface of a membrane filter (Havekes et al., 1976). One of these mutants maps near the rfa region and the other is a deletion in the region of 6.5 to 8.5 min. Both mutants have only been shown to be defective with F' and Hfr donors.

By means of a zygotic induction enrichment procedure, Havekes and Hoekstra (1976) have isolated a mutant defective as a recipient with an F' and several Hfr donors but competent with R100-1 and R144drd3 bearing donors. This again shows a specificity for a defect with the F-pilus. This mutant is also very similar to the Con$^-$ mutants of Skurray et al., (1974) in the other properties of resistance to lethal zygosis and defect in mating pair formation (Havekes and Hoekstra, 1976). This mutation is cotransducible with pyrD which maps at 21.5 min on the new linkage map. (Bachmann, Low and Taylor, 1976).

Falkinham and Curtiss (1976) have isolated a series of mutants which appear to be defective as recipients at a number of different stages of conjugation. These mutants were isolated by selection for mutations which were thought to bring about defects in the cell wall

and in inner and outer membrane function. These mutants have many pleiotropic properties and it is understood that further work is being carried out to fully characterize the mutations in these strains (J.O. Falkinham III, personal communication).

THE AIMS OF THIS STUDY

This study was undertaken to determine the nature of the components of the cell envelope which are essential for conjugation. Such work then lead to a characterization of a particular class of mutants defective as recipients in conjugation, to a study of the pleiotropic effects of these mutants, and to the determination of the primary function of the gene product affected in these mutants.

CHAPTER 2

MATERIALS AND METHODS

Media

Nutrient broth (Difco 0003) was prepared at double strength with the addition of 0.5% (w/v) sodium chloride. Nutrient agar was Blood Agar Base (Difco 0045) prepared as directed without adding blood. Luria broth was prepared as described by Miller (1972) and LB-agar was made by the subsequent addition of 1.5% (w/v) agar (Difco 0140-01). Tetrazolium agar was made by the addition of 2×10^{-5}% (w/v) 2,3,5-triphenyl tetrazolium chloride to LB-agar. Minimal liquid medium A was that described by Davis and Mingioli (1950) and minimal agar was prepared by the addition of 2% (w/v) agar. Minimal liquid medium B was as described by Pugsley and Reeves (1976b). Growth supplements and carbon sources were used at the following concentrations unless otherwise specifically stated: purines, pyrimidines and amino acids, 20 µg/ml; glucose, 0.5% (w/v); galactose, lactose, glycerol and maltose, 1.0% (w/v); succinate and lactate, 10 mM. 0.7% nutrient overlays were prepared by diluting nutrient agar 1:1 with nutrient broth. 1.0% minimal overlays were

prepared by diluting minimal agar with minimal salts medium A.

Chemicals

All chemicals were of the highest purity available. Enterochelin was purified by Dr. A.P. Pugsley (Pugsley and Reeves, 1976b). Spectinomycin was a gift of the Upjohn Co., Kalamazoo, Michigan, U.S.A. (^{57}Co)-cyanocobalamin (vitamin B12) was a generous gift of Dr. R. Ryell of the Flinders University Medical Centre, South Australia. (U-^{14}C)-glutamine (57.3 mCi/mMol), (G-^{3}H)-Proline (677 mCi/mMol) and $^{55}FeCl_3$ (11.4 mCi/mMol) were obtained from the Radiochemical Centre, Amersham, England. Pure sodium dodecyl sulphate (SDS;BDH30176) was used throughout with the exception of slab gel electrophoresis where in addition impure SDS (BDH 30175) was used as well as a mixture of the two types, as described in the text.

Bacterial strains

The bacterial strains used are described in Table 2-1. The bacteriophage resistant mutants of Hancock and Reeves (1975) are listed in Table 3-1 and are all derivatives of strain P400. The colicin

TABLE 2-1

Bacterial strains[a]

Strain	Mating type/Genotype	Source
AB257	HfrC/*metB*, *rel*, λ^-.	b
AB259	HfrH/*thi*, *rel*, λ^-.	b
AB1133	F^-/*thi*, *argE*, *proA*, *thr*, *leu*, *his*, *mtl*, *xyl*, *ara*, *galK*, *lacY*, *supE*, *rpsL*, λ^-.	
AN366	F^-/*pabA*, *his-4*, *arg-3*, *ilv*, *purE*, *aroE*, *rpsL*, *rpsE*, λ^-.	G. Woodrow
CSH23 (E5014)	F'*lac*$^+$ *proA*$^+$, B^+/ Δ(*lac pro*), *supE*, *thi*, *rpsE*.	Cold Spring Harbor
ED267	F^-/*his*, *trp*, *lac*, *proA*, *gal*, *tsx*, Su^-, *rpsL*.	N. Willetts
JC6256	F^-/*trp*, Δ*lac*.	N. Willetts
JF404	HfrH/*thyA*.	J. Foulds
JF404-2a	*tolG* mutant of JF404.	J. Foulds
KLF11/ JC1553	F'111/*argG*, *metB*, *his*, *leu*, *recA*, *mtl*, *xyl*, *malT*, *gal*, *lacY*, *tonA*, *tsx*, *supE*, *rpsL* λ^R, λ^-.	b
P400	*his*$^+$, *non* transductant of AB1133.	R.E.W. Hancock

(TABLE 2-1 Cont....)

Strain	Mating type/Genotype	Source
P801	K12, F^+/prototroph, λ^+.	-
W620	F^-/*thi*, *pyrD*, *gltA*, *galK*, *rpsL*, *rel*, λ^-.	b
χ342	HfrC/*proC*, *metB*, *relA*, λ^-.	b

a. All the strains listed are derivatives of *Escherichia coli* K-12 and the genotypes are according to the current linkage map of Bachmann, Low and Taylor (1976).

b. These strains were obtained from Dr. Barbara Bachmann of the *Escherichia coli* Genetic Stock Center, Yale University, Connecticut, U.S.A.

TABLE 2-2

Colicinogenic strains

Strain	Colicins produced	Source
Citrobacter freundii CA31	A	a
Escherichia coli T20 (colB-K260)	B	b
E. coli CA23	D	a
E. coli K53	E1	a
E. coli CA42	E2	a
E. coli K-12 W3100 (colE3-CA38, I-CA38)	E3[c]	a
E. coli CA46	G	a
E. coli CA58	H	a
E. coli CA53	Ia	a
E. coli K-12 (ColIb-p9)	Ib	a
E. coli K-235	K, X[d]	a
E. coli 398	L	a
E. coli M32.T19 (colM-K260)	M	b
E. coli 284	N, E3	a
E. coli II	Q, E1, D, I	a
Shigella boydii	S1	a
S. dispar	S4	a

(TABLE 2-2 Cont....)

Strain	Colicins produced	Source
E. coli CA7	V	a
E. coli K-12 185 II N x II S7a (colX-K235)	X	a
Serratia marcescens JF246	JF246	J. Foulds

a. Obtained from cultures maintained in this laboratory and described in Davies (1974) and Davies and Reeves (1975b).

b. Isolated as strong colicin producers by A.P. Pugsley.

c. Produces no detectable colicin I.

d. Produces no detectable colicin X.

TABLE 2-3

R- and Col- factors

R- or Col- factor	Incompat-[a] ability type	Antibiotic Resistances[b]					Colicins Produced
		Tc_{20}	Str_{10}	Str_{100}	CM_{50}	Kan_{50}	
ColV2	FI	S	S	S	S	S	V
ColVBtrp	FI	S	S	S	S	S	V,B
R386	FI	R	S	S	S	S	
R1drd19	FII	R	R	S	S	S	
R538Fdrd1	FII	S	R	S	R	S	
R100-1	FII	R	R	S	R	R	
R136fin$^-$	FII	R	S	S	S	S	
R64-11	Iα	R	R	S	S	S	
R144drd3	Iα	S	S	S	S	R	I
R163drd1	Iα	R	R	S	S	R	I
R538Idrd2	Iα	R	R	S	S	S	

a. Incompatability groups were as described by Novick (1974).

(TABLE 2-3 Cont....)

b. The antibiotic resistances or colicins which have been underlined are those used for selection of transfer. The subscripts indicate the level of resistance used in micrograms per millilitre.

resistant mutants of Davies and Reeves (1975a,b) are listed in Table 3-2 and are all derivatives of AB1133. The colicinogenic strains used are listed in Table 2-2 and have been described elsewhere (Davies and Reeves, 1975b; Foulds, 1972). The R- and Col- factors used are listed in Table 2-3 and were all maintained in strain JC6256.

Bacteriophages

The bacteriophages used in this study were: T1, T2, T3, T4, T5, T6, T7, BF23, A, B, C, D, F, G, J, E4, E7, E11, E15, E21, E25, H1, H3, H8, K2, K3, K4, K5, K6, K8, K9, K10, K11, K12, K15, K16, K17, K18, K19, K20, K21, K22, K25, K26, K27, K29, K30, K31, 0x1, 0x2, 0x3, 0x4, 0x5, M1, M3, Ac3, Ac4, H^+, V, ØI, ØII, H, F27, W31, ST-1, ØXtB and TuII*. All bacteriophages were from laboratory stocks and have been previously described (Hancock and Reeves, 1975) with the exception of ST-1 and ØX-tB which were obtained from A.M. Reiner and TuII* which was obtained from U. Henning. A series of mutants of bacteriophage T4 containing amber, ochre and opal suppressible mutations were kindly supplied by A.J. Clark.

General bacteriophage methods

The method for assaying suspensions of bacteriophage was by the agar overlay technique. 0.1 ml of each of a series of dilutions (usually 100-fold) of a bacteriophage suspension were added to 0.1 ml of a standing overnight culture (10^9 cells/ml) of indicator bacteria in 4 ml of 0.7% nutrient agar at 44°C, mixed by gentle swirling and pouring onto a nutrient agar plate. The plates were then incubated overnight at 37°C.

For measurement of the efficiency of plaquing (E.O.P.) of the bacteriophages on various strains a modification of this procedure was used. Instead of a standing overnight culture, log phase cultures of cells (2×10^8 cells/ml) grown at the appropriate temperature were used and preincubated at that temperature for 10 min prior to the addition of 4 ml of the 0.7% nutrient agar. Overnight incubation was at the same temperature.

The bacteriophages were maintained as lysates and kept at 4°C. They were propagated on the appropriate strain (Hancock and Reeves, 1975) by either harvesting from a 0.7% nutrient agar overlay or by propagating in liquid medium (Adams, 1959).

Bacteriophage adsorption

Bacteriophage adsorption was measured as follows. The bacterial strain being tested was grown to a density of 2×10^8 cells per ml, and bacteriophages were added to a portion at a multiplicity of 1.0. Immediately after the addition of the phage suspension, the culture was returned to incubate, and at 1.5 min intervals samples were taken and diluted 10^{-4} in prewarmed nutrient broth containing 20 µg of chloramphenicol per ml. A 0.1 ml volume was added to 0.1 ml of a culture of strain JC6256/R538Fdrd1 at a density of 2×10^8 cells per ml; the mixture was allowed to stand for 15 min at 37°C and was then poured as an overlay with 4 ml of 0.7% nutrient agar containing chloramphenicol (20 µg/ml) onto a nutrient agar plate. Plates were then incubated overnight and scored for plaque-forming units.

Selection for bacteriophage resistant mutants

All mutants were independently derived and spontaneous. They were obtained by plating 2×10^7 log-phase cells with 2×10^8 plaque forming units of phage in a 4 ml 0.7% nutrient agar overlay. Plates were incubated overnight at 37°C. Each mutant was

derived by using a culture grown from a separate single colony to ensure that the mutations were of independent origin. The mutants were purified by three single-colony isolations.

For mutant selection bacteriophage K3 was used for obtaining ompA mutants, bacteriophage T6 for tsx mutants and bacteriophage T5 for tonA mutants.

Screening of mutants for bacteriophage resistance

Suspected bacteriophage resistant mutants were screened using a multiple syringe bacteriophage applicator of the type described by Zierdt, Fox and Norris (1960). The mutants to be tested were grown as standing overnight cultures in nutrient broth at 37°C. 0.1 ml of each culture was added to 5 ml of 0.7% nutrient agar and poured as an overlay onto a predried (face downwards for 30 min at 37°C) nutrient agar plate. The overlaid plates were then dried (face downwards for 30 min at 37°C), and placed in turn on the platform of the phage applicator. The micrometer on the applicator was then rotated to produce a drop at the end of each of the syringes, the platform raised, and so that each of the drops touched onto a discrete area of the plate. After the spots of

bacteriophage suspension had dried into the surface of the overlay, the plate was incubated overnight at 37°C.

The bacteriophage suspension used in the applicator contained between 10^5 and 10^6 plaque forming units (p.f.u.) per ml.

Further characterization of the bacteriophage resistant mutants was by measurement of the efficiency of plaquing.

Selection of extended host range bacteriophages

To 2 x 10^7 log phase cells of the ompA mutants in 4 ml 0.7% nutrient agar overlays were added 10^9 plaque forming units (in 0.1 ml nutrient broth) of bacteriophage K3 and poured onto nutrient agar plates and incubated overnight at 37°C. Plaques occurring at a frequency of less than 10^{-5} were picked and propagated through three single-plaque isolations on the mutant on which they were isolated. These phage were the extended host range mutants.

Colicin preparations

Colicins K-235, L-398 and L-JF246 were prepared by induction; a culture of the colicinogenic strain

at a density of 4×10^8 cells/ml was induced by the addition of 50 ng of mitomycin C per ml. The culture was allowed to continue growing, with vigorous aeration, for 2.5 h, centrifuged at 5000 x g for 20 min and sterilized by the addition of 1% chloroform (v/v). Samples of this crude colicin were then stored frozen until required. A partially purified colicin K preparation was also provided by A.P. Pugsley.

Colicin adsorption

Colicin adsorption was measured by determining the amount of colicin which had not adsorbed to the strain under test by the ability of the unadsorbed colicin to kill an indicator strain. To 1.0 ml of the bacterial strain being tested (2×10^8 log phase cells/ml) was added 0.1 ml of colicin and 0.1 ml of chloramphenicol (200 µg/ml) and the whole incubated at 37°C. At 1.5 min intervals, 0.1 ml was removed and added to 1.0 ml of strain JC6256/R538Fdrd1 (2×10^8 log phase cells per ml in the presence of 20 µg/ml of chloramphenicol) and incubated for 15 min at 37°C. This mixture was then diluted and plated out on nutrient agar plates containing chloramphenicol (20 µg/ml), incubated overnight at 37°C and scored for colony forming units.

Selection of colicin resistant mutants

As for the bacteriophage resistant mutants all colicin resistant mutants were spontaneous and independently derived.

A fresh overnight of the colicinogenic strain was streaked onto a nutrient agar plate and incubated overnight at 37°C. This streak was then killed by exposure to chloroform for 15 min, when it was then overlayed with 5 ml of normal nutrient agar containing 2×10^7 log phase cells obtained from a culture grown from a separate single colony. Plates were then incubated overnight at 37°C and a colony was picked from the zone of clearing around the streak produced by the colicinogenic strain. Colonies obtained in this way were then put through three single colony isolations.

Screening of mutants for colicin resistance

Suspected colicin-resistant mutants were tested for their resistance to the full set of colicinogenic strains listed in Table 2-2. This was done using the conventional cross-streak plate test as described by Davies and Reeves (1975b).

Further characterization of the colicin resistance involved the titration of colicin preparations on the strain under test, and the titre compared with that on the wild type. This was done by taking log-phase bacteria diluted to a density of 2×10^3 cells per ml. To 0.1 ml of cell suspension was added 0.1 ml of a dilution of colicin. This was then incubated at 37°C with gentle shaking for 30 min, 4 ml of 0.7% nutrient agar was added, and the entire 4.2 ml was poured as an overlay on a nutrient agar plate, prior to overnight incubation and scoring for colony forming units. The titre of the colicin was taken to be the reciprocal of the dilution that gave 50% bacterial survival.

Antibiotic resistance testing

Oxoid "Multodisks" 30-9C and 11-14D were used to test any alteration in the pattern of resistance or sensitivity to a range of antibiotics. A nutrient agar plate was overlayed with 5 ml of soft nutrient agar containing 2×10^7 bacteria. Onto this was layered the "Multodisk", and the plates were then incubated overnight at 37°C, and the zone of inhibition of growth measured. Novobiocin resistance was tested further by plating about 500 cells on nutrient agar plates containing 1, 3, 5, 7.5, 15, 30 and 60 µg/ml of novobiocin.

Mating procedures

Donor strains were grown overnight in nutrient broth at 37°C as standing cultures, diluted 1:10 into fresh broth and incubation continued by standing at 37°C until a density of 2 x 10^8 cells/ml was reached. The recipient strains were grown to a density of 2 x 10^8 cells/ml in nutrient broth with vigorous aeration for at least four generations at the same temperature as used for the mating. For the transfer of colicin-producing plasmids, the donor cultures were preincubated at the appropriate mating temperature for 5 min in the presence of 5 mg of trypsin per ml to destroy any colicin present. A volume of 0.1 ml of donor culture was added to 1.0 ml of recipient culture and incubated for 30 min at the appropriate temperature, except for Hfr matings which were allowed to proceed for 60 min. The mating mixture was then diluted in minimal salts solution A and plated out for transconjugants or recombinants.

Cell envelope preparations

For the preparation of components of the cell envelope, bacteria were grown to two thirds the maximum density obtained in the medium. This corresponded to the logarithmic phase of growth. For culture volumes

of less than 25 litres, cells were harvested by centrifugation at 5000 x g for 20 min in a 6 x 750 ml head in an MSE Mistral 6L centrifuge (M.S.E. Ltd., London, England). For larger volumes, which has been grown in a fermenter, the cells were harvested by centrifugation in a Veronesi KLE160 continuous flow centrifuge (Veronesi Separatori, s.a.s., Bologna, Italy). The harvested cells were then either frozen as a paste or resuspended in about one hundredth the original volume in 10 mM HEPES buffer (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4).

Cells which had been frozen as a paste were broken at 28,000 p.s.i. in an X-Press Cell Disintegrator (LKB Biotec, Bromma, Sweden). The broken cell pellet was then resuspended in 5 volumes of 10 mM HEPES buffer (pH 7.4), containing 2 mM $MgCl_2$ and a small amount of DNase and RNase, and centrifuged for 10 min at 5,000 x g in a Sorvall SS-34 rotor (Du Pont Instruments, Newtown, Conn.) to remove the unbroken cells. Cells which had been resuspended in 10 mM HEPES buffer (pH 7.4) were broken in a French pressure cell (Aminco, Silver Spring, Maryland) at 12,000 p.s.i. The unbroken cells were then removed by centrifugation.

The supernatant obtained after the removal of the unbroken cells was then centrifuged at 78,000 x g for 60 min in a Spinco 30 rotor (Beckman Instruments, Palo Alto, California) to pellet the cell envelope or whole membrane. The supernatant was the cytoplasmic material.

The outer membrane of the cell envelope was then extracted using the method of Schnaitman (1971a). The whole membrane was resuspended to a protein concentration of about 10 mg per ml in 10 mM HEPES buffer (pH 7.4) containing 2% (v/v) Triton X-100, and incubated for 20 min at room temperature. This was then centrifuged for 60 min at 78,000 x g at $4^{o}C$ in a Spinco 30 rotor. This extraction was then repeated on the pellet. The supernatant, the Triton soluble component of the cell envelope, is used as the cytoplasmic membrane. The Triton insoluble component (TIW) is referred to as the outer membrane.

The outer membrane could be subsequently solubilized for chromatography by using ethylenediaminetetraacetic acid (EDTA) in the presence of Triton X-100 (Schnaitman, 1971b). This material, referred to as the TES component of the outer membrane, was obtained by extracting the TIW with 5 mM EDTA and 2% Triton

X-100 in 10 mM HEPES buffer (pH 7.4) and incubating for 10 min at room temperature. This was then centrifuged at 78,000 x g in a Spinco 30 rotor for 60 min at 20°C. This extraction was then repeated on the pellet. This resulted in solubilization of about 60 to 70% of the outer membrane protein.

Proteins in solutions containing Triton X-100 could be freed of this detergent by ethanol precipitation overnight at -15°C with 2 volumes of cold 95% ethanol. The ethanol precipitate could then be collected by centrifugation at 10,000 x g for 20 min in a Sorvall SS-34 rotor. The precipitate was then washed twice with cold ethanol. Prior to ehtanol precipitation it was usually necessary to concentrate the protein solution and this was accomplished using an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) with a PM10 membrane.

Trypsin digestion

Protein at a concentration of 2 mg per ml in distilled water was digested with trypsin (100 µg/ml) for 12 hours at 37°C. The digest was then prepared for SDS-polyacrylamide-gel-electrophoresis as described later.

Lipopolysaccharide extractions

Lipopolysaccharide was extracted using the phenolchloroform-petroleum ether procedure of Galanos, Lüderitz and Westphal, (1969).

Protein estimation

Two methods were employed for estimating the concentration of protein in samples. For protein solutions in water or non-Triton-containing buffers, the method of Schacterle and Pollack (1973) was used. For samples containing Triton X-100, the method of Wang and Smith (1975) was used. Bovine serum albumin was used as standard in both cases.

Ketodeoxyoctonate (KDO) estimation

The amount of KDO present was estimated using the method of Keleti and Lederer (1974). An extinction coefficient at 540 nm of 19 for a 1 mM solution of KDO under the assay conditions was taken as standard.

Carbohydrate estimation

Hexoses and heptoses were determined using the method of Dische, Shettles and Osnos (1949) using

glucose as standard.

Column chromatography

Ethylene diaminetetraacetic acid (EDTA) in the presence of Triton X-100 was used for solubilizing the outer membrane for chromatography (Schnaitman, 1974a). This material is referred to as the Triton-EDTA-soluble (TES) component of the outer membrane and was separated from the insoluble component by centrifugation (78,000 x g, 60 min, 16°C) in a Spinco 30 rotor. The TES material was concentrated in an Amicon pressure dialysis apparatus using a PM10 membrane and was then precipitated overnight at -15°C with two volumes of cold ethanol. The material was collected by centrifugation (20,000 x g, 20 min, 4°C) and was then washed with cold ethanol. This material was then ready to be redissolved in the appropriate buffer for chromatography. Ion-exchange chromatography of the TES component of the outer membrane on DEAE-cellulose (Whatman DE-52) using Tris-Triton-EDTA buffer (TTE) adjusted to pH 7.2 was as previously described (Schnaitman, 1974a). Chromatography on QAE Sephadex was essentially the same except that TTE buffer was adjusted to pH 8.0.

Gel filtration chromatography on Sephadex G-200 in the presence of SDS was carried out as described by Schnaitman (1974a).

Polyacrylamide gel electrophoresis

Samples for disc gel electrophoresis were prepared by the methods of Schnaitman (1973a, 1974a) and electrophoresed using unheated and heated preparations with the pH 7.2 buffer system of Maizel (1966), and heated samples with the pH 11.4 buffer system of Bragg and Hou (1972).

Gels were routeinely loaded with 50 µl of sample at a protein concentration of 1 mg/ml.

Gels were stained in Coomassie brilliant blue (Swank and Munkres, 1971) and densitometer tracings were obtained with a Quick Scan Jnr. gel scanner (Helena Laboratories Corp., Beaumont Texas).

Samples for slab gel electrophoresis were prepared according to Lugtenberg et al., (1975) and were electrophoresed under his conditions. The slabs were stained with Coomassie brilliant blue R according to Fairbanks, Steck and Wallach (1971). Samples

consisted of 12.5 µl containing protein at a concentration of 1 mg/ml. The sample preparations were heated for 5 min at 100°C immediately prior to loading onto the gels.

Amino acid analysis

Amino acid analysis was performed by M. Calder of the Department of Biochemistry, the University of Adelaide.

1 mg of protein in 1 ml distilled water was diluted with 1 ml of 0.1N HCl, transferred to the hydrolysis tube and evaporated to dryness. The sample was then hydrolysed under N_2 at 105°C for 21 hours in 1 ml of 6N HCl plus 1 drop of 5% aqueous phenol. The resulting hydrolysate was then evaporated to dryness and then redissolved in 1.5 ml of the loading solution, of which 0.5 ml was loaded onto the amino acid analyser.

Bacteriophage neutralizations

Bacteriophage neutralization experiments were carried out by incubating 0.1 ml volumes of dilutions of the cell envelope preparations (in 0.1 M sodium phosphate buffer, pH 7.2) with 0.1 ml of nutrient broth

containing 10^3 p.f.u. of bacteriophages for 3 h at 37°C. After this time 0.1 ml of a culture of indicator bacteria (strain P417, 2 x 10^8 log phase cells per ml in nutrient broth) was added and incubation continued for a further 15 min. 4 ml of molten 0.7% nutrient agar was added and the whole poured as an overlay on a nutrient agar plate, incubated overnight at 37°C and scored for plaque forming units.

Colicin neutralizations

Colicin neutralization experiments were carried out by two (similar) methods. In the first method 0.1 ml volumes of the cell envelope preparations (in 0.1 M sodium phosphate buffer, pH 7.2) were incubated with 0.1 ml of colicin for 30 min at 37°C. After this time 0.1 ml of a culture of indicator bacteria (strain P417, 10^9 log phase cells per ml in nutrient broth) was added and incubation continued for a further 30 min. 0.1 ml of this mixture or of a 10^{-2}, 10^{-4}, 10^{-5} dilution were added to 4 ml of molten 0.7% nutrient agar plate, incubated overnight at 37°C and scored for colony forming units. The amount of colicin used in this assay was sufficient to give 400 colonies when plated out neat.

The second method was basically the same as above except that a series of 2 fold dilutions of cell envelope material were used and only 10^3 log phase cells of indicator bacteria were added. The 4 ml of 0.7% nutrient agar was also added directly to the assay tube before pouring as an overlay. The colicin used in this assay was derived from a standing overnight culture of the colicinogenic strain: the culture was centrifuged for 20 min at 5,000 x g and the supernatant sterilized by the addition of 1% $CHCl_3$. This material gave about 10% survival of the indicator bacteria in control experiments.

Inhibition of mating

Experiments to inhibit mating were carried out in two ways: using outer membrane or purified LPS and using whole cells.

In the first method 0.1 ml of sodium phosphate buffer (0.1 M, pH 7.2) containing outer membrane (0, 1, 10, 100 or 1000 µg of contained protein) or similar amounts of LPS was added to the normal mating mixture and the mating allowed to proceed as normal.

In the second method, transfer to strain ED267 was measured.

To 0.1 ml of the donor strain AB259 was added 1.0 ml of F^- cells which consisted of strain ED267 and either P400 or P460 (*ompA-1*) in a ratio of 1:1. After allowing the mating to proceed for 60 min at 37°C the mating mixture was plated out for *pro*$^+$ recombinants. Streptomycin (100 µg/ml) was used for contraselection.

Determination of growth rates

Growth rates were determined either by measuring the absorbance of optical density (OD; 625 nm), or by viable cell counts. Strains grown for 16 hr at 37°C in appropriate media were subcultured into 10 ml of the same, fresh medium to an OD of 0.025 or 0.05 in a 100 ml side arm flask. Cultures were then shaken in an orbital water bath shaker at 200 revolutions per min at the appropriate temperature and the OD measured at intervals. In some experiments 0.1 ml samples were also taken for dilution in nutrient broth and the viable cells per ml determined on nutrient agar (incubated at 37°C). For growth experiments at 30°C and 42°C all inocula were grown at 30°C.

Uptake of transport substrates

Uptake of glutamine, proline or cyanocobalamin (vitamin B12) was measured using cells grown in minimal

medium A with glucose and harvested in the logarithmic phase of growth (OD_{650} = 0.4) by centrifugation (5,000 x g, 20 min). The cells were washed twice and resuspended in fresh medium (final OD = 0.8) containing 100 µg per ml of chloramphenicol to inhibit protein synthesis. The cells were stored at 4°C and used within 2 hr of harvesting. For uptake a 1 ml cell suspension was equilibrated at the appropriate temperature in a gently shaking water bath and uptake initiated by the addition of the labelled transport substrate. Samples (0.1 ml) were removed at intervals and filtered through Gelman GA-6 membrane filters (mean pore diameter 0.45 µm).

The filters were quickly rinsed twice with 10 ml saline, dried and placed in scintillation vials. Toluene containing 0.6% butyl-1,3,4 phenyl-oxidiazole was added and the radioactivity retained by the filters counted in a Packard model 3003 liquid scintillation counter, corrected for non-specific retention of radioactivity by the filters, and uptake determined as weight of transport substrate accumulated per mg cell dry weight.

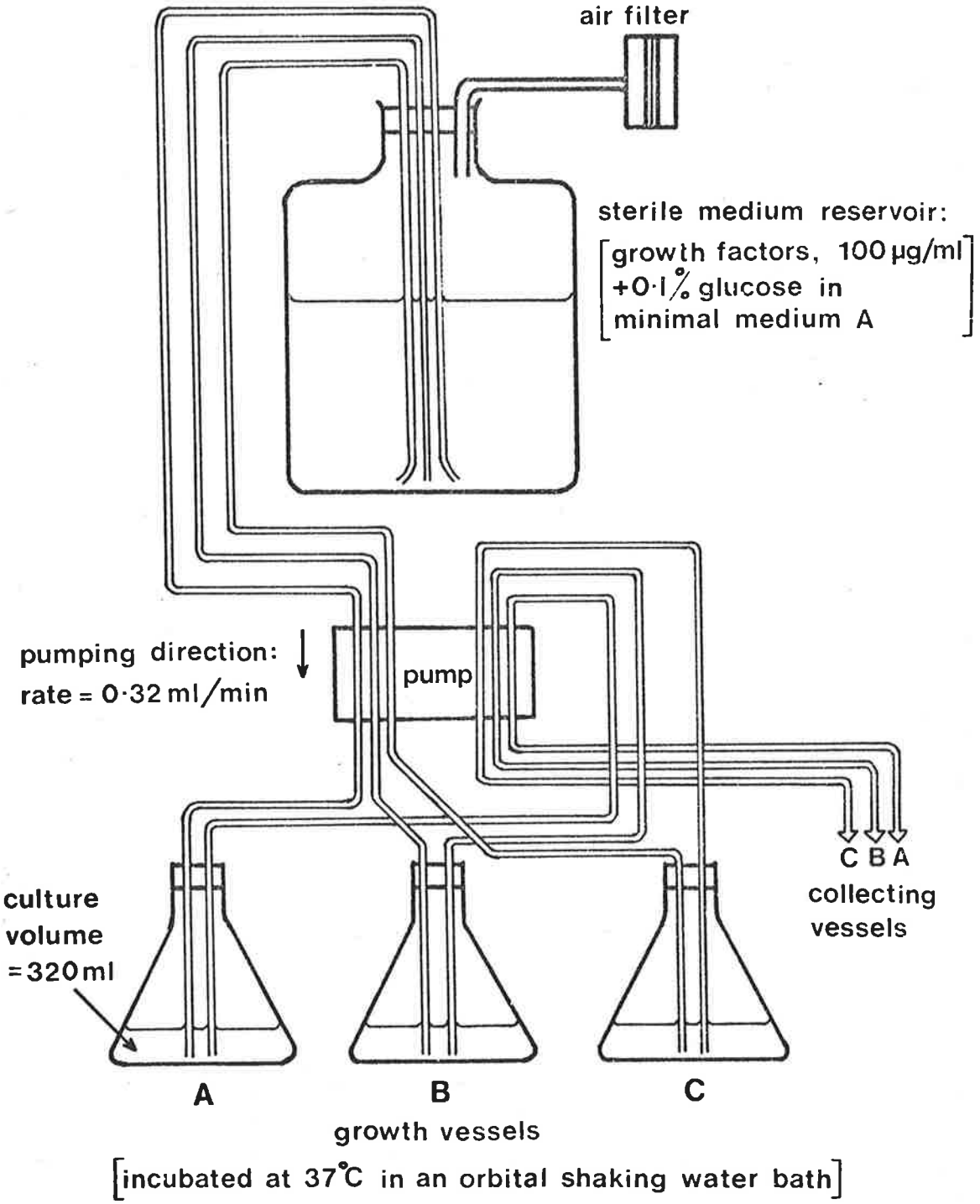
Enterochelin mediated iron uptake was measured at 37°C using $^{55}Fe(III)$ (Pugsley and Reeves, 1976c).

The procedure used was a modification of the method described by Langman, Young, Frost, Rosenberg and Gibson (1972). aroE derivatives of the strains were used in order to eliminate problems caused by the secretion of enterochelin during the assay. Bacteria were grown overnight in minimal medium B (5 μM Fe), washed twice in iron depleted minimal medium B and subcultured into iron depleted minimal medium B. The remainder of the method is essentially similar to that described above for amino acid and cyanocobalamin uptake except that the cells were grown to an OD_{650} = 0.2 washed and ferrienterochelin uptake determined in iron depleted medium B containing 100 μM nitrilotriacetic acid to prevent iron uptake through the low affinity uptake system.

Growth on limiting glucose

To test for growth on a limiting concentration of glucose as sole carbon source, a chemostat was set up as shown in Figure 2-1. All inocula were logarithmic phase cultures (2×10^8 cells/ml) in the same medium and grown at the same temperature as that used in the chemostat.

FIGURE 2-1: Diagram of the chemostat apparatus used for growing cultures on limiting amounts of glucose.


air filter
sterile medium reservoir:
[growth factors, 100 µg/ml
+0·1% glucose in
minimal medium A]
pumping direction:
rate = 0·32 ml/min
pump
C B A
collecting
vessels
culture
volume
=320ml
A
B
C
growth vessels
[incubated at 37°C in an orbital shaking water bath]

ONPG Hydrolysis

The hydrolysis of O-nitrophenyl-β-D-galacto-pyranoside (ONPG) was used as a measure of leakage from intact cells. Cells were grown in the presence of 10^{-3}M IPTG (isopropyl- β -D-thiogalactopyranoside) prior to assaying and the procedure used was that of Pardee, Jacob and Monod (1959).

Transduction

P1 phage stocks were prepared as described by Miller (1972) using P1 cml clr 100, which is a heat inducible P1 carrying an R-factor derived gene for chloramphenicol (50 µg per ml) resistance. The transduction procedure was that of Pittard (1965).

Photomicrography and electron microscopy

Electron microscopy and phase contrast microscopy were expertly performed by Miss Pam Dyer of the Department of Biochemistry, the University of Adelaide.

For electron microscopy of bacteriophages, the phage lysates were concentrated by differential centrifugation: 5,000 x g for 10 min to remove the cell

debris, followed by 35,000 x g for 20 min in Spinco 30 rotor to pellet the phage. The pellets were resuspended in a small volume of ammonium acetate buffer (1% (w/v), pH 7.0). Phage were depicted by negative staining using uranyl acetate (2%, w.v).

CHAPTER 3

RECIPIENT-ABILITY OF BACTERIOPHAGE- AND COLICIN-RESISTANT MUTANTS

INTRODUCTION

The evidence that the early stages of conjugation involve the interaction of the tip of the pilus of the donor cell with the cell wall of the recipient bacterium was discussed in Chapter 1. This interaction is thought to be specific and so, unless mutants affected in the pilus attachment site are lethal, it should be possible to obtain mutants defective in this site. The sex pilus has been likened to a bacteriophage (Brinton, 1971), and bacteriophages and colicins both attach to the outer membrane of the cell envelope as the first step in their infectious and killing processes. It was therefore thought, that, since the receptors for these agents must have a selective advantage to the cells for them to be retained, it may be that this advantage is to enable efficient genetic transfer in a population of cells by means of conjugation. Thus the loss of the receptor for the phage or colicin may make the cell defective as a recipient in the early stages of conjugation

perhaps even by loss of the receptor for the sex pilus. Preliminary studies with the con mutants of Skurray et al., (1974) have shown that these mutants are defective in the early stages of conjugation. These mutants were shown to be altered in the composition of their outer membrane and to be defective in pair formation suggesting that the proposed hypothesis was likely to be fruitful. Because of the availability of the sets of bacteriophage resistant mutants (Hancock and Reeves, 1975) and colicin resistant mutants (Davies and Reeves, 1975a,b) previously isolated in this laboratory, these mutants were screened for their recipient ability in conjugation. Although there is some overlap in these two groups (Hancock, Davies and Reeves, 1976) they have been treated separately because of the difference in their parent strains.

For this study both an Hfr and an F' donor was used, both of which produce an identical F-like pilus. Use of these two donors enables one to recognize mutants defective as recipients because of either a defect in recombination of conjugally transferred Hfr DNA or a defect in plasmid replication (Clark, 1973). Mutants defective with both types of donor and presumably affected in the early steps common to both processes. An R100-1 donor, producing a serologically different

F-like pilus and an R64-11 donor, producing a totally unrelated I-like pilus (Lawn and Meynell, 1970; Hardy, 1975) were also used as donors for this screening.

The recipient ability of the con mutants is also measured with a wide range of F-like pili producing donors and also I-like pili producing donors. A comparison is made with a tolG mutant (Foulds and Barrett, 1973) in this and a number of other properties.

RECIPIENT ABILITY OF BACTERIOPHAGE-RESISTANT MUTANTS

When the bacteriophage resistant mutants were examined for recipient ability in conjugation with a number of donors, the results shown in Table 3-1 were obtained.

If we consider 50% of the number of trans-conjugants or recombinants formed with the parent to be the cut-off point, then Wrm1, Wrm2, Bar2, Bar3, Bar4, Bar5, Bar6, Bar7, Bar8, Ktw3 and Con are defective with the F'lac pro and of these Wrm1, Wrm2, Bar3, Bar5, Bar7, Bar8 and Con are also defective with HfrH. The defects with HfrH are comparable to those observed with the F'lac pro with the exception of Bar2, Bar4 and Ktw3 which do not appear to be as defective with HfrH. This

TABLE 3-1

Recipient ability of bacteriophage resistant mutants.

Mutant class[a]	Mutant	Recipient ability[b] with			
		F'lac pro	HfrH	R100-1	R64-11
Bar 1	P455	1.08	1.35	1.11	1.04
2	P492	0.44	0.95	1.85	0.64
3	P495	0.19	0.17	3.92	0.041
4	P436	0.24	0.63	2.59	0.090
5	P402	0.16	0.31	2.30	0.058
6	P451	0.45	-	1.21	-
7	P487	0.15	0.17	1.63	0.018
8	P489	0.017	0.083	3.11	0.15
Bfe	P445	0.70	0.86	0.58	0.69
Con	P460	0.0005	0.005	1.10	0.77
Efr	P448	0.89	0.91	1.14	1.29
Ktn	P466	1.28	0.82	0.77	1.78
Ktw 1	P456	0.96	1.09	3.30	1.90
2	P476	1.12	0.94	1.65	4.65

(TABLE 3-1 Cont....)

Mutant class[a]	Mutant	Recipient ability[b] with			
		F'lac pro	HfrH	R100-1	R64-11
Ktw 3	P240	0.40	0.85	2.66	0.40
Misc 1	P491	1.33	1.57	0.69	1.55
2	P443	1.12	0.92	1.66	0.94
3	P498	1.11	1.05	0.53	1.91
4	P237	1.00	0.90	0.52	0.84
5	P493	1.13	1.36	0.83	0.64
TonA	P417	0.74	0.93	0.63	0.64
B	P442	1.14	1.09	0.38	0.72
Tsx 1	P407	0.69	1.34	0.68	0.75
2	P433	0.93	0.74	1.68	0.81
Ttk 1	P429	1.05	0.81	0.66	0.42
2	P423	0.88	0.96	1.71	0.56
3	P425	0.86	1.17	1.03	0.23
4	P474	1.15	1.23	3.76	0.35
Wrm 1	P435	0.079	0.17	6.65	0.22
2	P424	0.34	0.45	2.99	0.064

(TABLE 3-1 Cont....)

a. The mutant classes are defined in Hancock and Reeves (1975).

b. Recipient ability was measured as a percentage of input donor cells and given with respect to the parent recipient strain, P400, which is taken as 1. F'*lac pro* gave 25.5% transfer selecting for *lac*$^+$ *pro*$^+$ transconjugants, HfrH gave 19.8% transfer selecting for *thr*$^+$ recombinants, R100-1 gave 9.86% transfer selecting for tetracycline resistance and R64-11 gave 0.38% transfer selecting for tetracycline resistance. All results are the mean of at least three matings. Streptomycin (100 µg per ml for F'*lac pro* and HfrH, and 1000 µg per ml for R100-1 and R64-11) was used for contraselection.

is probably a reflection of the arbitrarily chosen cut off point. All these defective mutants with the exception of the Con mutant have alterations to their LPS (Hancock and Reeves, 1976). For these mutants the correlation is quite good between LPS composition and recipient ability: those mutants with the most defective LPS being the poorest recipients. The fact that those mutants which are defective as recipients with HfrH are also defective with F'lac pro rules out the possibility that these mutants are affected in either recombination of transferred Hfr DNA or plasmid replication, unless of course both are equally affected. However, it is considered much more probable that the early common stages of conjugation are affected. Also, none of the LPS mutants are as defective as the Con mutant.

If one looks at the recipient ability of the mutants with the R100-1 donor then only the TonB mutant is reduced at all. If those mutants which scored as defective with the F'lac pro and HfrH donors are looked at, it can be seen that also closely correlated with the LPS defect is an increase in recipient ability for the R100-1 plasmid. The Con mutant is an exception and is discussed later. However, one significant exception to this correlation is the Ktwl mutant which has only minor LPS alterations but is quite hyper-receptive for

R100-1, and is normal as a recipient with F'lac pro and HfrH donors. The Ttk4 mutant which is also normal for F'lac pro and HfrH is also a much better recipient for R100-1, but it does have a more markedly altered LPS composition.

If one now considers the recipient ability of these mutants for the R64-11 plasmid, a number of mutants can again be classed as defective. The defective mutants are Bar3, Bar4, Bar5, Bar7, Bar8, Ktw3, Ttk1, Ttk3, Ttk4, Wrm1 and Wrm2. All are known to be defective in the structure of their LPS (Hancock and Reeves, 1976).

When recipient ability with the HfrH or F'lac pro donors is compared with the R64-11 donor, two different patterns are observed. Bar8 and Wrm1, the most affected in their LPS structure and, of the LPS mutants, the most affected in their recipient ability with HfrH and F'lac pro donors, are affected to a lesser extent in their recipient ability with R64-11. In contrast the other mutants such as Bar3 and Wrm2 show the converse effect with recipient ability for R64-11 being affected to a greater extent. The effect of recipient ability for the R64-11 donor is thus not correlated with the extent of the LPS alteration; mutants with only lipid A and KDO in the LPS being affected less than mutants with

only minor modifications to their LPS. The Con mutant, shown to be defective with HfrH and F'lac pro donors, is normal as a recipient for R64-11.

RECIPIENT ABILITY OF COLICIN RESISTANT MUTANTS

The colicin resistant mutants of Davies and Reeves (1975a,b) were examined for their recipient ability with the same set of donors and the results of these matings are summarized in Table 3-2.

If, as before, those mutants which form less than 50% the number of recombinants or transconjugants as the parent strain are taken as defective, then it can be seen that there are a number of defective mutants. Those mutants which are defective as recipients with HfrH (Con, TolIIb, TolIIc, TolIII, TolXII and ExbB) are also defective with F'lac pro and the extent of the defect is very similar. However, the Con mutant is by far the most defective. The only mutants which can be classed as defective with the R100-1 donor are TolIIc and TonB, and those defective with the R64-11 donor are TolIIb, TolIIc and TolXI. There does not appear to be a general correlation between those mutants which are defective with one donor being either defective or proficient with another donor.

TABLE 3-2

Recipient ability of colicin resistant mutants.

Mutant class[a]	Mutant	Recipient ability[b] with			
		HfrH	F'lac pro	R100-1	R64-11
Bfe	P525	1.18	1.12	0.78	0.55
Con	P212	0.042	0.023	1.63	0.58
Rcx	P224	0.59	0.73	0.68	0.56
Tsx	P209	0.93	0.83	1.40	0.56
Tol Ia	P218	1.15	0.62	1.41	0.68
Ib	P210	0.90	0.71	1.41	1.11
IIb	P651	0.24	0.28	0.71	0.33
IIc	P555	0.30	0.29	0.42	0.32
III	P660	0.47	0.49	0.60	0.52
IV	P692	0.87	0.70	1.36	0.85
VII	P689	0.88	0.80	1.25	0.64
VIII	P602	0.61	0.59	0.53	0.66
IX	P596	1.10	0.99	1.01	0.72
X	P661	1.04	1.01	0.80	1.23

(TABLE 3-2 Cont....)

Mutant class[a]	Mutant	Recipient ability[b] with			
		HfrH	F'lac pro	R100-1	R64-11
XI	P220	0.71	0.64	1.29	0.43
XIII	P520	0.38	0.46	0.51	0.68
XIV	P530	0.93	0.79	1.45	1.12
XV	P686	0.92	0.71	2.10	1.11
XVII	P652	0.60	0.76	0.65	0.60
Cbr	P295	0.58	0.84	0.51	0.64
Cir	P625	0.82	0.74	0.56	0.74
Cmt	P1209	0.62	0.78	0.50	0.67
Cvt	P1235	0.61	0.61	1.09	0.74
ExbB	P575	0.40	0.49	0.91	0.68
C	P535	0.56	0.71	1.33	0.61
Ivt	P645	0.63	0.88	0.70	0.51
TonA	P1205	0.64	1.20	0.68	0.67
B	P585	0.73	0.83	0.40	1.06

(TABLE 3-2 Cont....)

a. The mutant classes are described in Davies and Reeves (1975a,b).

b. Recipient ability was measured as a percentage of input donor cells and given with respect to the parent strain AB1133 which is taken as 1. F'*lac pro* gave 27.82% transfer selecting for *lac*$^+$ *pro*$^+$ transconjugants, HfrH gave 19.31% transfer selecting for *thr*$^+$ recombinants, R100-1 gave 7.77% transfer selecting for tetracycline resistance and R64-11 gave 0.32% transfer selecting for tetracycline resistance. All results are the mean of at least three matings. Streptomycin (100 µg/ml for F'*lac pro* and HfrH, and 1000 µg per ml for R100-1 and R64-11) was used for contraselection.

The colicin tolerance or resistance mutation in some of these strains is known to result in a defect in the outer membrane. The Bfe, Rcx, Tsx, Cbr, Cir and TonA mutants lack the receptors for colicins A, E1, E2 and E3; X; K; B and D; Ia and Ib; and M respectively (Sabet and Schnaitman, 1973; Davies and Reeves, 1975b; Weltzien and Jesaitis, 1971; Pugsley and Reeves, 1976a, 1977a; Konisky and Liu, 1974; Braun, Schaller and Wolff, 1973). The Tol IV, Tol XIV and Tol XV mutants have no or greatly reduced amounts of proteins 1a and/or 1b (Davies and Reeves, manuscript in preparation) and now known to map together at a locus designated ompB (Sarma and Reeves, 1977). The tol Ia, Tol Ib and Tol X mutants have been shown to be the same as the TolF mutants of Foulds (Foulds and Barrett, 1973; Foulds, 1976) and have reduced amounts of protein 1a under certain growth conditions (J. Foulds, personal communication).

The lack of any effect on recipient ability in these mutants indicates that none of the outer membrane proteins mentioned are required for conjugation. However the importance for conjugation of the product of the gene which is altered in Con mutants is again shown.

FURTHER STUDIES OF THE RECIPIENT ABILITY AND OTHER PROPERTIES OF CON MUTANTS

The properties reported for *tolG* mutants (Foulds and Barrett, 1973) showed considerable overlap to those which had been previously described for the *con* mutants (Skurray *et al.*, 1974; Davies and Reeves, 1975c) although there were some inconsistencies. The *tolG* mutants were also observed to be defective in recombinant formation with an HfrH (J. Foulds, personal communication). The two classes of mutants thus seemed to be very similar and it was therefore decided to study the properties of a *tolG* mutant at the same time as studying those of the *con* mutant. The *con* mutants had been chosen to study further because of their marked affect on recipient ability in conjugation.

Further recipient ability studies of *con* mutants

To further test the specificity of the defect in recipient ability of the *con* mutants, it was decided to use a large number of donors. In order to compare a *tolG* mutant this required to first put the *tolG* mutation into the same F^- strain. Strains P1635 and P1636 (*tolG*) were obtained by mating JF404-2a with P400 for 32 min at 37°C, interrupting the mating, by blending

and selecting for gal$^+$ str^r recombinants. The growth factor requirements of the two strains were identical as was the suppressor status (Su$^-$ or SupE$^+$), measured using a series of amber, ochre or opal suppressible mutants, of bacteriophage T4. The only difference detectable between the two strains was that the tolG locus had not been transferred to strain P1635.

From Table 3-3 it can be seen that the defects for con and tolG are identical. The mutants were competent as recipients with all the donors bearing I-like pili, but of the donors bearing F-like pili, they were only competent with the R100-1 and R136fin$^-$ donors as shown previously (Skurray et al., 1974). If one takes the data for P400 and its con mutant P460, then it is possible to order the F-like pili bearing donors according to their ability to transfer to P460.

In their increasing ability to transfer the order is: (F'111, HfrH, ColV2, HfrC, ColVBtrp), F'lac pro, R386, R538Fdrd1, R1drd19, and (R136fin$^-$ and R100-1). On the basis of pilus serotype (Lawn and Meynell, 1970) and efficiency of plating of various F-specific RNA phages (Dennison and Hedges, 1972; Alfaro and Willetts, 1972), the same order is obtained if the F', Hfr and Col donors are considered as one group. (Thanks to Sheena

TABLE 3-3

Recipient abilities of *con* and *tolG* mutants with the different donors.

Donor	% Transfer[a] to				
	P400	P460 (*con*)	P212 (*con*)	P1635	P1636 (*tolG*)
HfrH	1.8	<0.01	0.13		
HfrC	1.5	<0.01		2.0	<0.01
F'111	12.5	<0.01		13.0	<0.01
F'*lac pro*	25.5	0.013	0.73		
R1drd19	16.9	1.31		12.3	0.74
R100-1	13.8	14.8	9.3	19.4	18.9
R136fin$^-$	14.9	14.0		15.7	18.2
R386	13.0	0.02		14.5	0.04
R538Fdrd1	11.3	0.07	0.30	13.2	0.03
ColV2	5.3	<0.01		5.0	<0.01
ColVB*trp*	1.2	<0.01		1.2	<0.01
R64-11	1.1	1.2	0.72	0.7	0.7
R144drd3	8.0	7.2		6.5	10.5
R163drd1	6.4	5.8		8.7	9.8
R538Idrd2	2.4	2.0	1.8	2.3	1.9

(TABLE 3-3 Cont....)

a. Recombinants (Hfr crosses) or transconjugants (plasmid crosses) were recorded as a percentage of input donor cells. HfrH transfer was measured selecting for *gal*$^+$ recombinants and HfrC and F'111 transfer were measured selecting for *arg*$^+$ recombinants or transconjugants. F'*lac pro* transfer was measured by selecting for *lac*$^+$ *pro*$^+$ transconjugants. Transfer of the R- and Col- factors was using the appropriate antibiotic or colicin resistance (Table 2-3). Streptomycin (at a concentration of 100 µg/ml for Hfr and F' matings and at 1000µg/ml for the remainder) was used for contraselection throughout.

Dennison for noticing this correlation).

Antibiotic sensitivity, bacteriophage resistance and colicin tolerance of *con* mutants

The similarity of the specific nature of the defect of the *con* and *tolG* mutants as recipients in conjugation further suggested that they may be identical. All the previously reported properties for both mutations were therefore examined.

The mutations resemble their parent strains in being resistant to (per millilitre) 500 µg of sulfafurazole, 10 µg of oleandomycin, 5U of penicillin G, 10 µg of methicillin, 10 µg of fusidic acid, 5 µg of novobiocin, and 5 µg of cloxacillin and sensitive to 50 µg of colistin methane sulfonate, 5 µg of kanamycin, 10 µg of neomycin, and 2 µg of ampicillin as shown previously for *con* mutants (Skurray *et al.*, 1974). However, the mutants had become sensitive to 30 µg of novobiocin, whereas both parent strains are resistant, as shown previously for *tolG* mutants (Foulds and Barrett, 1973).

Both mutants were resistant to only a group of nine K3-like phages (K3, K4, K5, 0x2, 0x3, 0x4, 0x5, M1

and Ac3) of the sixty four phages tested, and were unable to adsorb phage K3 (Figure 3-1). *tolG* mutants had been previously reported as being sensitive to colicin K (Foulds and Barrett, 1973), but it has now been shown (Table 3-4) that both they and the *con* mutants are tolerant to only colicins K and L-398 of the 19 colicins tested, as previously shown for *con* mutants (Davies and Reeves, 1975b,c) and to bacteriocin JF246 as shown for *tolG* mutants (Foulds and Barrett, 1973).

Thus, it was not possible to differentiate *con* and *tolG* mutants.

Map position of *con* and *tolG* mutants

Because of the similarity in phenotype of the *con* and *tolG* mutants with respect to conjugation deficiency, resistance to only the set of K3-like bacteriophages and to colicins K, L-398 and JF246, it was decided to see if the two were both altered at the same genetic locus. It was reported that *tolG* mutants are cotransducible with *pyrD* (Foulds, 1975) at 21.5 min on the *E. coli* K-12 linkage map (Bachmann, Low and Taylor, 1976), which contradicted a result obtained by Davies and Reeves (1975c) suggesting that *con* was

FIGURE 3-1: Adsorption of bacteriophage K3 to whole cells of strain P400 (O) and its con mutant P460 (●); JF404 (■) and its tolG mutant, JF404-2a (▲). Cells were at a density of 2 x 10^8/ml, and phage were added to a multiplicity of 1.0.

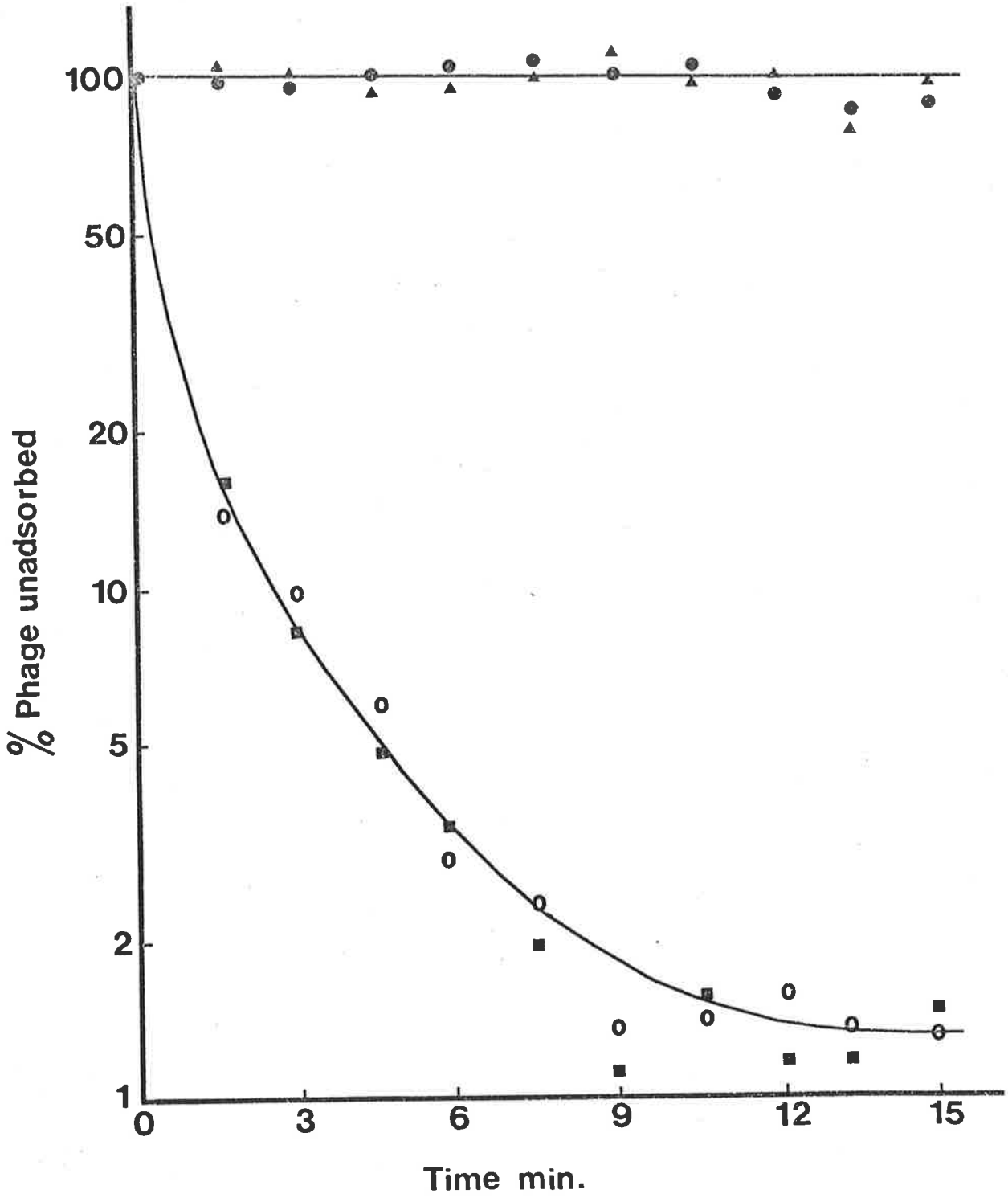

100
50
20
10
5
2
1
% Phage unadsorbed
0
3
6
9
12
15
Time min.

TABLE 3-4

Colicin titres on the mutants.

Strain	Titre of Colicin			Mutation
	L-398	JF246	K-235	
P400	256	512	4028	-
P460	1	1	4	con
AB1133	256	512	4028	-
P212	1	2	8	con
JF404	256	512	256	-
JF404-2a	1	1	2	tolG
P1635	256	512	4028	-
P1636	1	1	2	tolG

The mutants and their parents were fully sensitive to all other colicins tested.

cotransducible with *lip* at 14.5 min. Both transductions were tried, and it was found that both of the *con* mutants P212 and P460 and the *tolG* mutant JF404-2a were cotransducible with *pyrD* (Table 3-5). It has not been possible to repeat the result of Davies and Reeves (1975c). The two mutants therefore appeared to be identical.

IDENTITY OF COLICINS PRODUCED BY *E. COLI* 398 AND *SERRATIA MARCESCENS* JF246

It had been possible to select *con* (*tolG*) mutants using either colicin L-398 (Davies and Reeves, 1975b,c) or bacteriocin JF246 (Foulds and Barrett, 1973). As well as these colicins both mutants were tolerant to only colicin K. It was also known that *tsx* mutants were specifically resistant to only colicin K of a wide range of colicins (Davies and Reeves, 1975b). As it was of interest to know all the pleiotropic effects of the *con* mutants, and since the colicin produced by strain JF246 had not been typed previously, it was decided to test it against all of the type mutants isolated by Davies and Reeves (1975a,b). It was observed that the patterns of resistance to L-398 and JF246 were identical. 80 spontaneous mutants of P400 resistant to bacteriocin JF246 were also isolated, and examined for their pattern

TABLE 3-5

Cotransduction of *con* and *tolG* with *pyrD*[a].

Strain	Mutation	Resistance of *pyrD*$^+$ transductants to phage K3 and colicins K-235, L-398 and JF246	% Cotransduction
P212	*con*	33/152	21.7
P460	*con*	35/152	23.0
JF404-2a	*tolG*	10/56	17.6

a. Resistance to phage K3 and colicins K-235, L-398 and JF246 were all transduced together. All transductants were *glt*, *gal* and *rpsL* and sensitive to colicins E2, E3 and A.

of colicin resistance. All were also resistant to colicin L-398 and could be included in the classification of Davies and Reeves (1975b) for mutants resistant to the A group of colicins. Thus according to the nomenclature principle of Fredericq (1965) bacteriocin JF246 is a colicin of type L. With the agreement of Foulds (personal communication) it was therefore decided to call this colicin L-JF246, and it has been subsequently shown that antibody prepared against each of colicins L-398 and L-JF246 is able to neutralize the other (Foulds, 1976), thus confirming the results of the cross-resistance studies. The con mutants were therefore still only tolerant to colicins K and L.

SUMMARY AND CONCLUSIONS

It has been demonstrated that from a wide range of mutants selected as resistant to either bacteriophages or colicins, the *con* mutants are the most defective and this defect is specific. The maximum defect is observed with donors bearing F-pili, and the defect is less with donors bearing less closely related pili. The wide range of mutants tested has also made it possible to exclude a number of the outer membrane proteins from having a role in recipient functions in conjugation. The *tolG* mutants of Foulds (Foulds and Barrett, 1973; Foulds, 1974) have been shown to be identical to the *con* mutants in all their described properties (the outer membrane defect is the subject of the next Chapter). The bacteriocin produced by *Serratia marcescens* strain JF246 has also been shown to be a colicin of type L.

CHAPTER 4

CHARACTERIZATION OF THE OUTER MEMBRANE DEFECT OF *ompA* (*con* OR *tolG*) MUTANTS AND PURIFICATION AND IN VITRO PROPERTIES OF PROTEIN 3A.

INTRODUCTION

It has been shown in Chapter 3 that *con* and *tolG* mutants appeared to be identical. Both classes of mutants have also been shown to have outer membrane defects (Chai and Foulds, 1974; Skurray *et al.*, 1974). *con* mutants have been suggested to be missing proteins 3A and 3B using the pH 11.4 polyacrylamide gel buffer system of Bragg and Hou (1972) under the conditions of Schnaitman (1973a), whereas *tolG* mutants were shown to be missing a major outer membrane protein using the gel system of Neville (1971). Also, Schnaitman (1974a) has shown that proteins 3A and 3B can be separated by DEAE-cellulose chromatography. The outer membranes of a *con* and a *tolG* mutant were therefore examined on polyacrylamide gels and by DEAE-cellulose chromatography.

A number of authors (Lugtenberg *et al.*, 1975; Henning and Haller, 1975; Garten *et al.*, 1975) have

disputed the existence of protein 3B and so the various DEAE-cellulose column fractions were examined to see if protein 3B exists in *con* and *tolG* mutants and whether it is distinct from protein 3A. The examination of the outer membrane proteins at different growth temperatures has also been undertaken to see if the presence of protein 3B is temperature dependent.

In Chapter 3 it was also shown that both *con* and *tolG* mutants were unable to adsorb bacteriophage K3. It was therefore decided to see what fraction of the cell envelope contained the receptor for bacteriophage K3 and if this corresponded to the protein missing in these mutants.

ompA MUTANTS (*con* OR *tolG*) LACK ONLY PROTEIN 3A

The outer membrane from *con* mutants was shown previously (Skurray *et al.*, 1974) to have a greatly reduced amount of peak C, using the pH 7.2 buffer system of Maizel (1966), and of peak 3 using the pH 11.4 buffer system of Bragg and Hou (1972). This has been confirmed and shown to apply to the *tolG* mutant isolated by Foulds and Barrett (1973) (Figures 4-1, 4-2).

FIGURE 4-1: Comparison, by densitometry, of the outer membrane proteins of strains P400, P460 (*con*) and JF404-2a (*tolG*) run on sodium dodecyl sulphate-polyacrylamide-gels, using the pH 7.2 buffer system of Maizel (1966) with unheated (i, ii and iii) and heated (iv, v and vi) samples. Peaks are labelled by the method of Schnaitman (1974a).

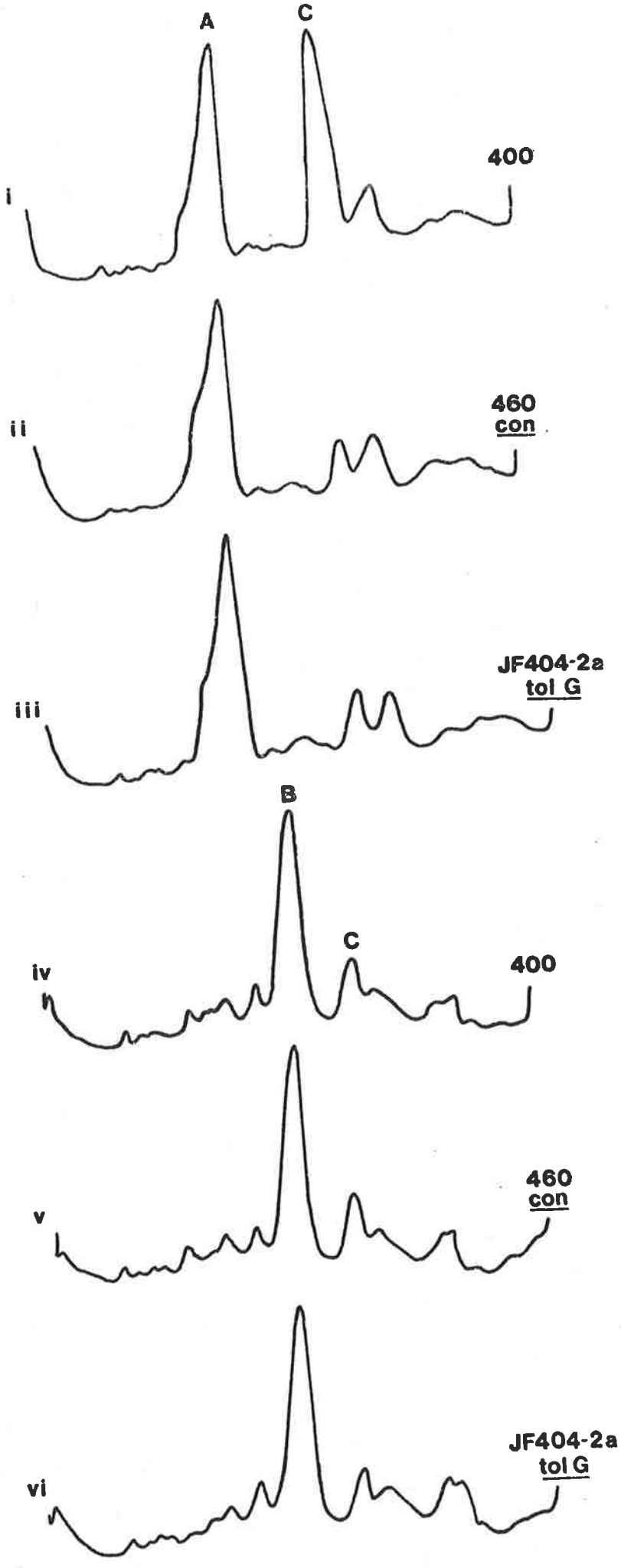
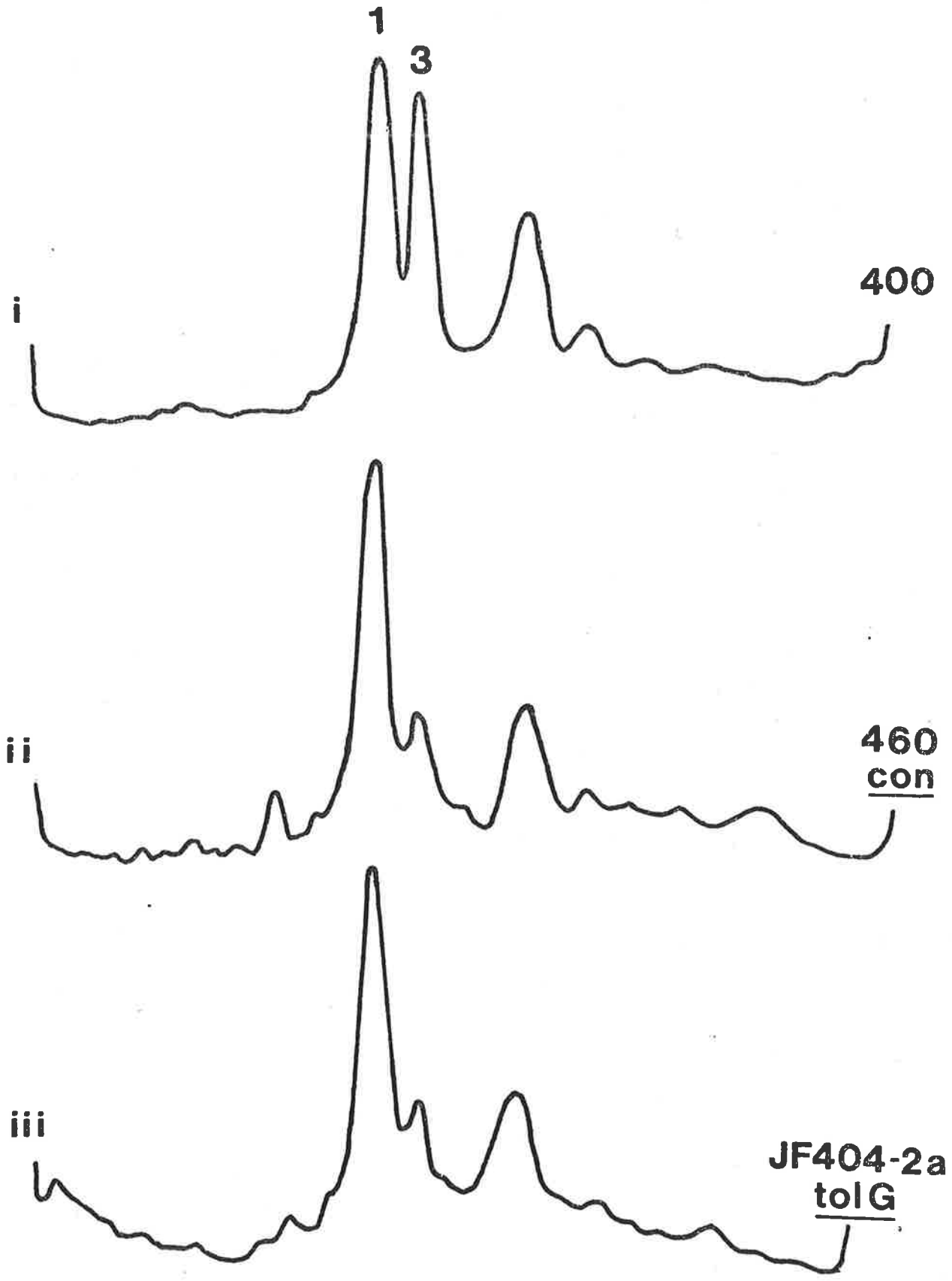

A
C
400
i
460
con
ii
JF404-2a
tol G
iii
B
C
iv
400
460
con
v
JF404-2a
tol G
vi

FIGURE 4-2: Comparison, by densitometry, of the outer membrane proteins of strains P400, P460 (*con*) and JF404-2a (*tolG*) run on sodium dodecyl sulphate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1972) with heated samples. Peaks are labelled by the method of Schnaitman (1974a).


1
3
i
400
ii
460
con
iii
JF404-2a
tolG

Originally, it was thought that both proteins 3A and 3B were absent in *con* mutants (Skurray *et al.*, 1974). However, since Schnaitman (1974a) has shown that these proteins can be separated using ion-exchange chromatography on DEAE-cellulose, the defect in the outer membrane proteins of *con* mutants was further examined. When the outer membrane of the *con* mutant P460 was chromatographed on DEAE-cellulose, it was observed that only protein 3A was absent and that the level of protein 3B was unaltered (Figures 4-3, 4-4). Protein 3A occurs (when present) in fractions I, II, III and perhaps IV, whereas protein 3B is only eluted from the column at high salt concentrations and occurs in fractions IV and V.

The *tolG* mutant, strain JF404-2a, gave a result identical to that for the *con* mutant P460.

The specific loss of only protein 3A in *con* and *tolG* mutants confirms the results of Chapter 3 and these two mutations are therefore identical. This locus which maps at 21.5 min is therefore a strong contender for being the structural gene for protein 3A and it was decided, at the suggestion of Schnaitman (personal communication) and in agreement with Foulds (personal communication), to rename the locus *ompA* (*o*uter *m*embrane *p*rotein).

FIGURE 4-3: DEAE-cellulose chromatography using Whatman DE-52 of the Triton plus-EDTA-soluble (TES) outer membrane proteins of strain P400. The various pooled fractions were run on sodium dodecyl sulphate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1972). Densitometer traces of the stained gels are labelled by the method of Schnaitman (1974a).

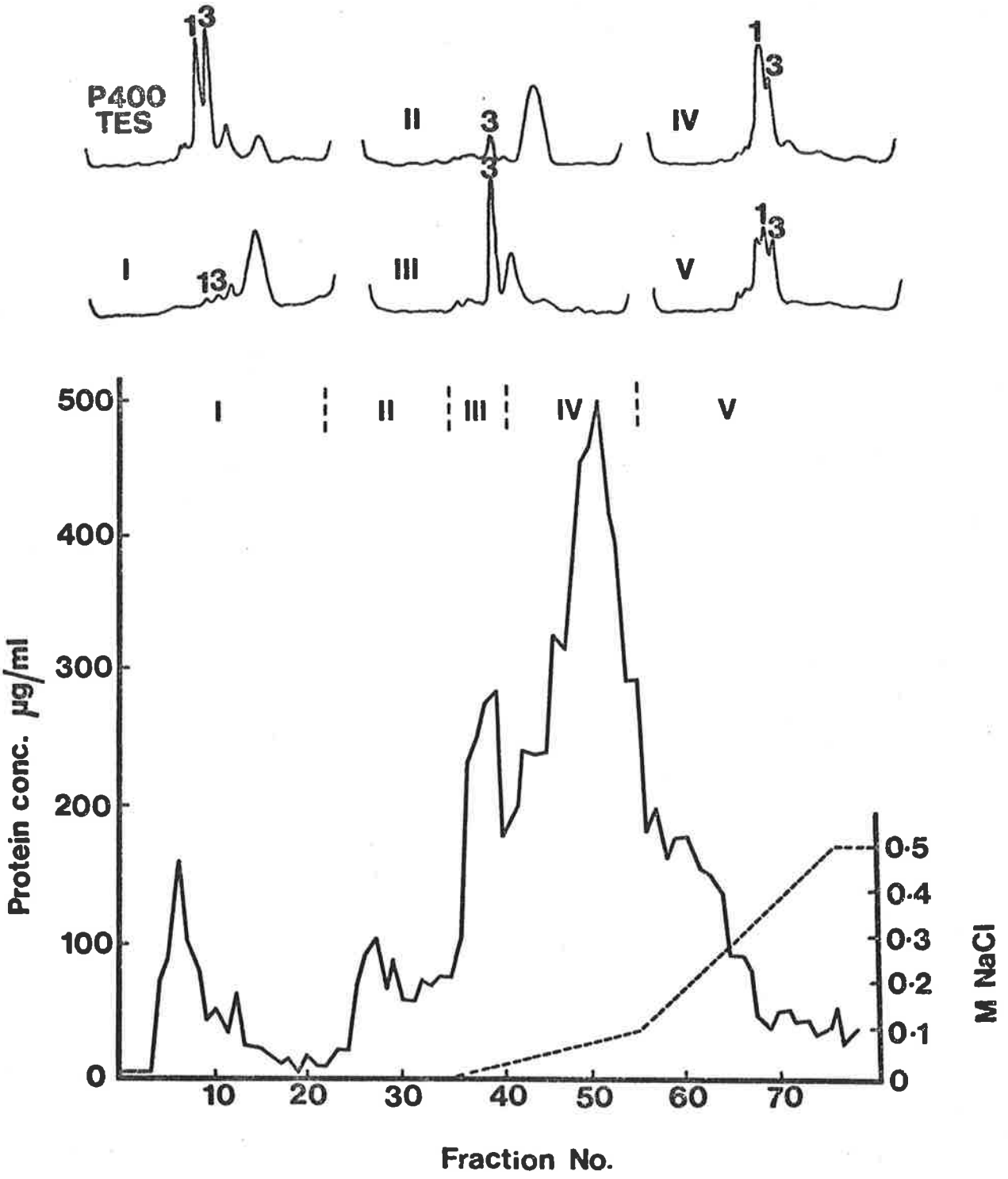
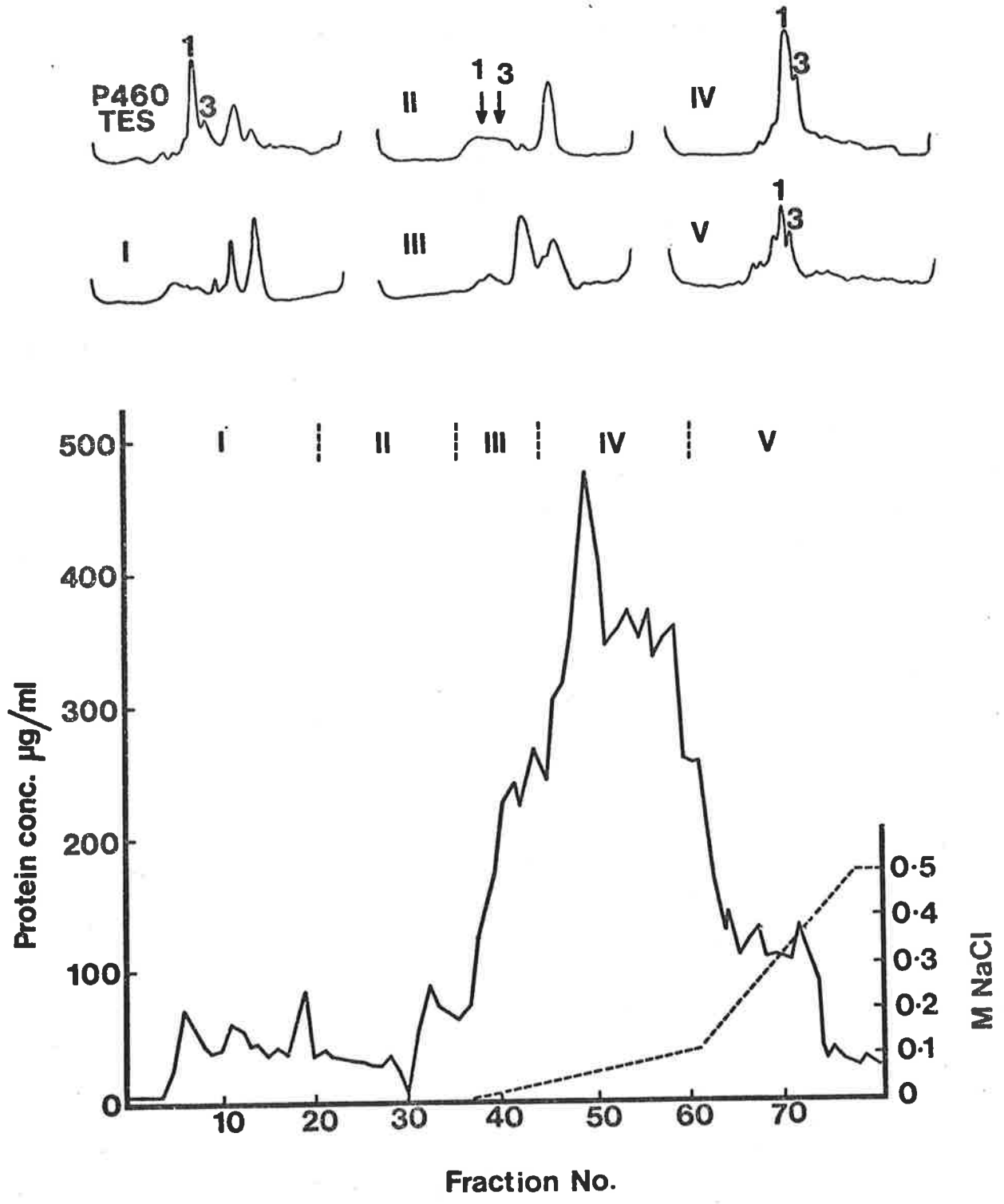

P400
TES
I
II
III
IV
V
1
3
13
Protein conc. µg/ml
M NaCl
Fraction No.
0
100
200
300
400
500
0·1
0·2
0·3
0·4
0·5
10
20
30
40
50
60
70

FIGURE 4-4: DEAE-cellulose chromatography, using Whatman DE-52, of the Triton-plus-EDTA-soluble (TES) outer membrane proteins of strain P460 (con). The various pooled fractions were run on sodium dodecyl sulphate-polyacrylamide gels, using the pH 11.4 system of Bragg and Hou (1972). Densitometer traces of the stained gels are labelled by the method of Schnaitman (1974a).

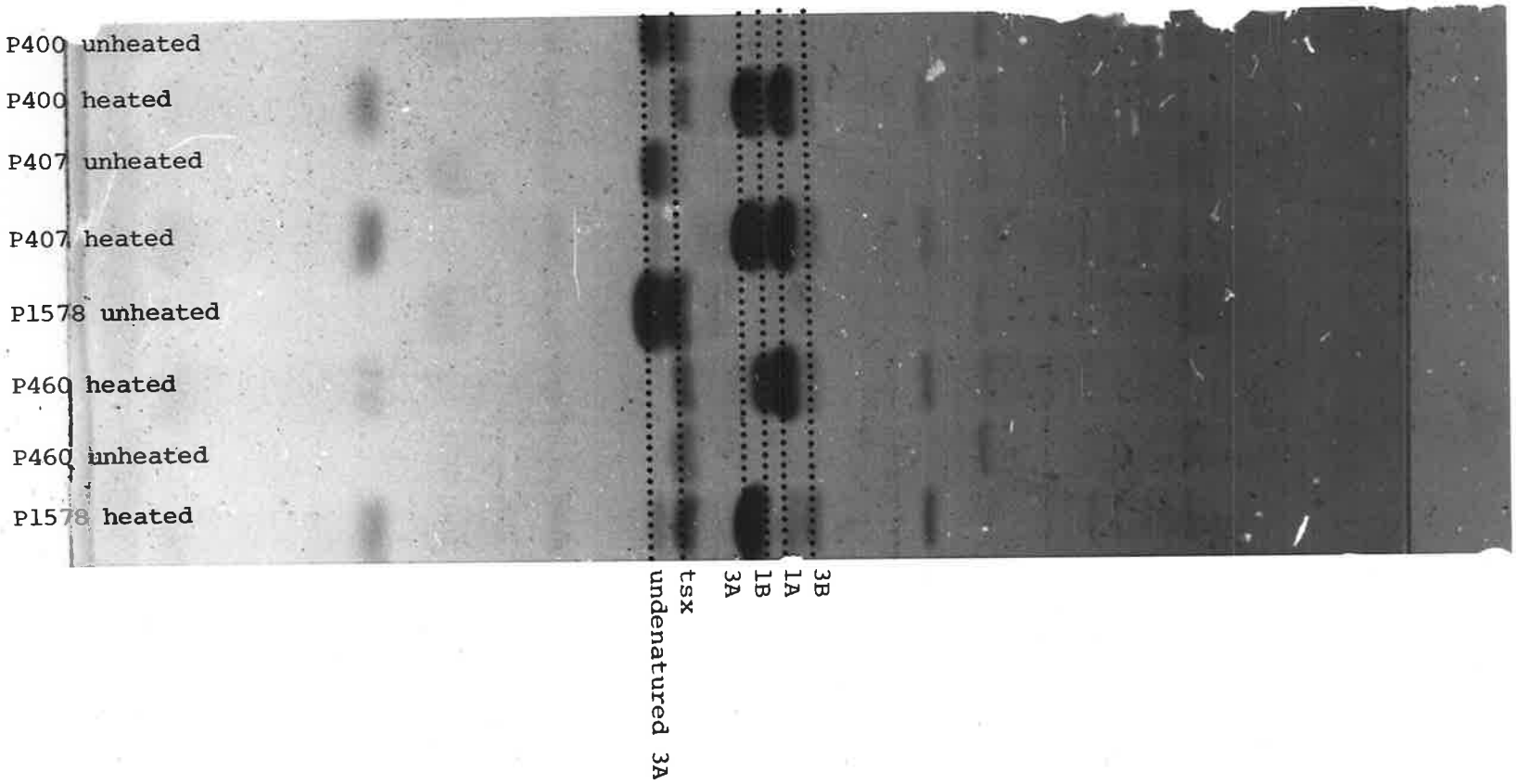

P460
TES
1
3
I
II
III
IV
V
500
400
300
200
100
0
Protein conc. µg/ml
10
20
30
40
50
60
70
Fraction No.
0·5
0·4
0·3
0·2
0·1
0
M NaCl

The absence of protein 3A in ompA mutants enables one to now identify this protein on the polyacrylamide slab gel system of Lugtenberg et al., (1975). It can be seen to correspond to the band designated as d by these authors. The heat modifiability of this protein is also shown on this gel system (Figure 4-5). The samples, which have not been heated, clearly show a faster moving band which is greatly reduced in the ompA mutant P460, and that band d which is slower moving is also absent in this mutant when using heated samples. No bands corresponding to b and c can be detected in the unheated samples, as shown by the lack of any difference between P400 and the ompB mutant, P1578, which lacks bands b and c (Chapter 7). This presumably is because these proteins are aggregated in the unheated samples and the aggregates are too large to enter the main gel. Band a is also not detected in the unheated samples.

IDENTIFICATION OF PROTEIN 3B ON POLYACRYLAMIDE GEL ELECTROPHORESIS

Schnaitman (1974a) showed that, with heated samples of outer membrane, proteins 3A and 3B run together as peak 3 on the pH 11.4 gel system of Bragg and Hou (1972) and as part of peak B on the pH 7.2

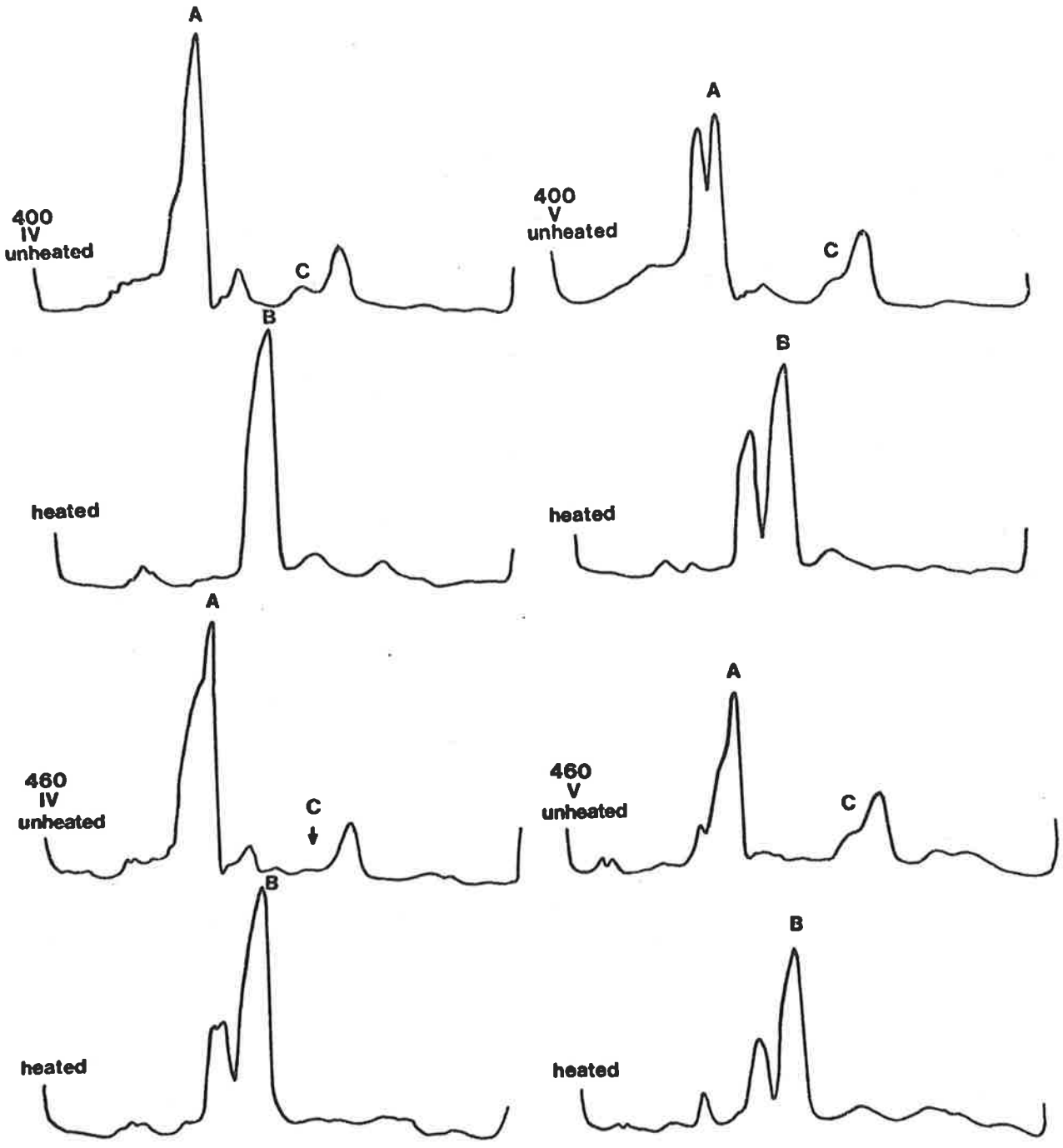
FIGURE 4-5: Sodium dodecyl sulphate polyacrylamide gels of the outer membranes of strains P400, P407 (tsx), P460 (con) and P1578 (ompB) using the slab gel electrophoresis system described by Lugtenberg et al., (1975) with unheated and heated samples. The major bands are labelled according to Schnaitman (1974). Mutants P407 (tsx) and P1578 (ompB) are described in Chapters 6 and 7 respectively.


P400 unheated
P400 heated
P407 unheated
P407 heated
P1578 unheated
P460 heated
P460 unheated
P1578 heated
undenatured 3A
tsx
3A
1B
1A
3B

gel system of Maizel (1966). These observations are confirmed by the results shown in Figures 4-1, 4-2, 4-3 and 404, but it has also been possible to differentiate proteins 3A and 3B by using unheated samples on Maizel gels. In the DEAE-cellulose column fractions which are enriched in protein 3B and contain no 3A (fractions IV and V in Figure 4-6), a small peak running slightly faster than peak C is correspondingly enriched. This peak and peak A, disappears upon heating, and peak B appears in their place.

Henning and coworkers (Garten et al., 1975) have been unable to show the existence of protein 3B in outer membrane preparations. This suggests that either it is not present in their strains or that protein 3B is not produced under their growth conditions. The latter seemed the most likely explanation since Henning (personal communication) could not detect protein 3B even in strains in which it has been shown to exist. It was noted that these workers routinely grew bacteria at 30°C for preparing outer membranes, whereas the routine temperature used in this laboratory and the laboratory of Schnaitman, who first proposed the existence of protein 3B, was 37°C. The outer membranes of a number of strains grown in nutrient broth

FIGURE 4-6: Comparison, by densitometry, of polyacrylamide gels of the protein composition of the pools IV and V from the DEAE-cellulose columns of strain P400 and its con mutant P460. The samples were run on sodium dodecyl sulphate polyacrylamide gels, using the pH 7.2 buffer system of Maizel (1966) with unheated and heated samples. Peaks are labelled by the method of Schnaitman (1974a).


A
400
IV
unheated
C
B
heated
A
400
V
unheated
C
B
heated
A
460
IV
unheated
C
B
heated
A
460
V
unheated
C
B
heated

at different temperatures were therefore examined. In Figures 4-7, 4-8 and 4-9 are shown the results for the strains grown at 30°C and 42°C. P1578 is an ompB mutant lacking proteins 1a and 1b from its outer membrane (Chapter 7), and P407 is a tsx mutant missing the tsx protein (Chapter 6). The tsx protein runs in the same position, of peak C, in Maizel gels with unheated or heated samples and as a distinct peak in the system of Bragg and Hou.

It can be seen that the peak running immediately ahead of peak C (labelled D) increases dramatically when the strains are grown at 42°C instead of at 30°C (Figure 4-7). This peak is present in all strains and so cannot be protein 1 (1a or 1b), the tsx protein or protein 3A. It also moves upon heating to the position of peak B (Figure 4-8), and the increase in the size of this peak corresponds to that observed with peak 3 in Bragg and Hou gels of P460 (Figure 4-9). This protein thus has all the properties previously described for protein 3B (Schnaitman, 1974a) and its presence has been additionally shown to be dependent upon growth temperature.

Thus the positions of the major outer membrane proteins under the different disc gel conditions used

FIGURE 4-7: Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis of the outer membranes of strains P400, P407 (tsx), P460 (ompA) and P1578 (ompB) using unheated samples with the pH 7.2 buffer system of Maizel (1966). Cells were grown in nutrient broth at 30°C and 42°C. Peaks are labelled according to Schnaitman (1974a).

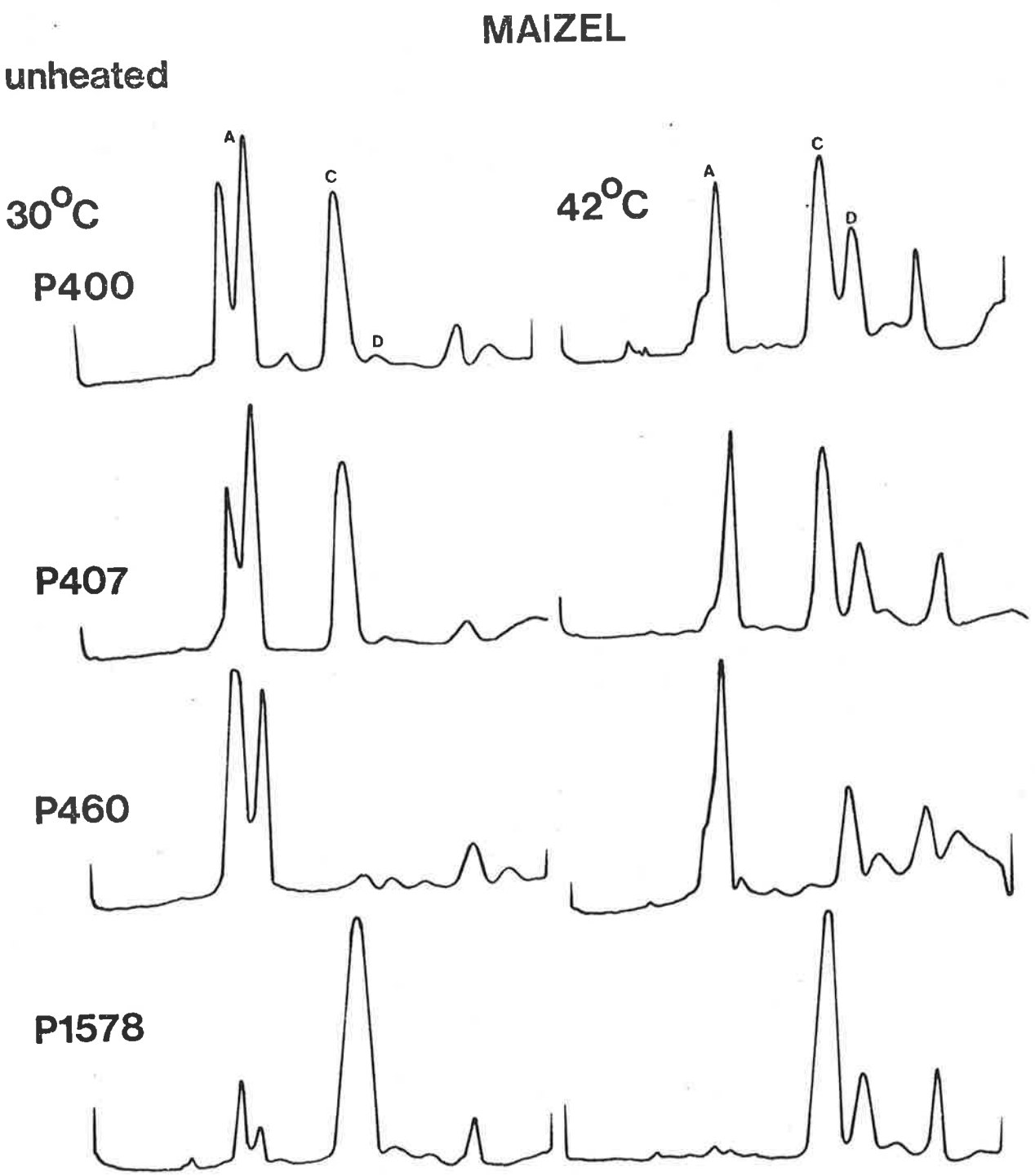

MAIZEL
unheated
30°C
42°C
P400
P407
P460
P1578
A
C
D
A
C
D

FIGURE 4-8: Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis of the outer membranes of strains P400, P407 (tsx), P460 (ompA) and P1578 (ompB) using heated samples with the pH 7.2 buffer system of Maizel (1966). Cells were grown in nutrient broth at 30°C and 42°C. Peaks are labelled according to Schnaitman (1974a).

MAIZEL

heated

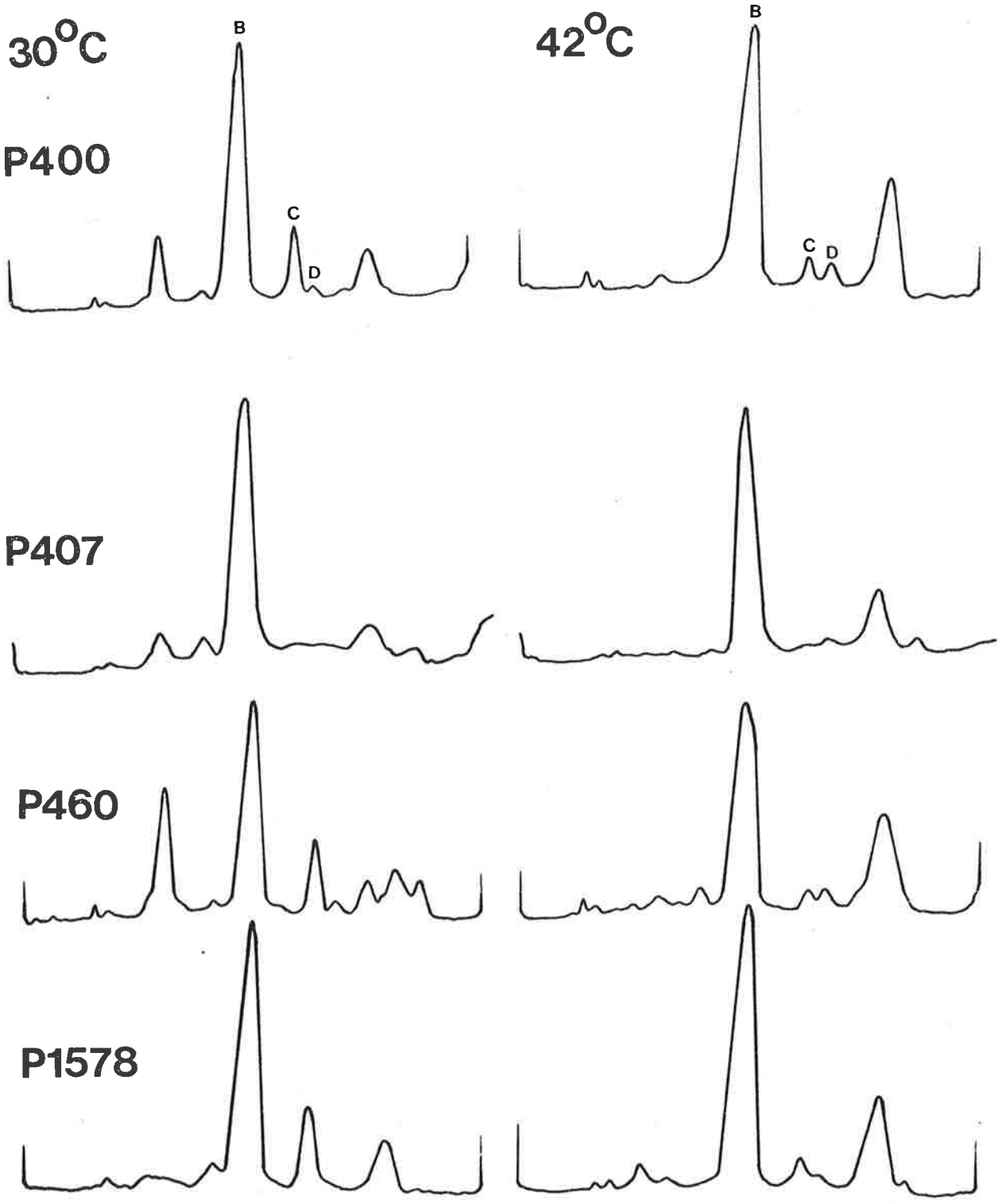
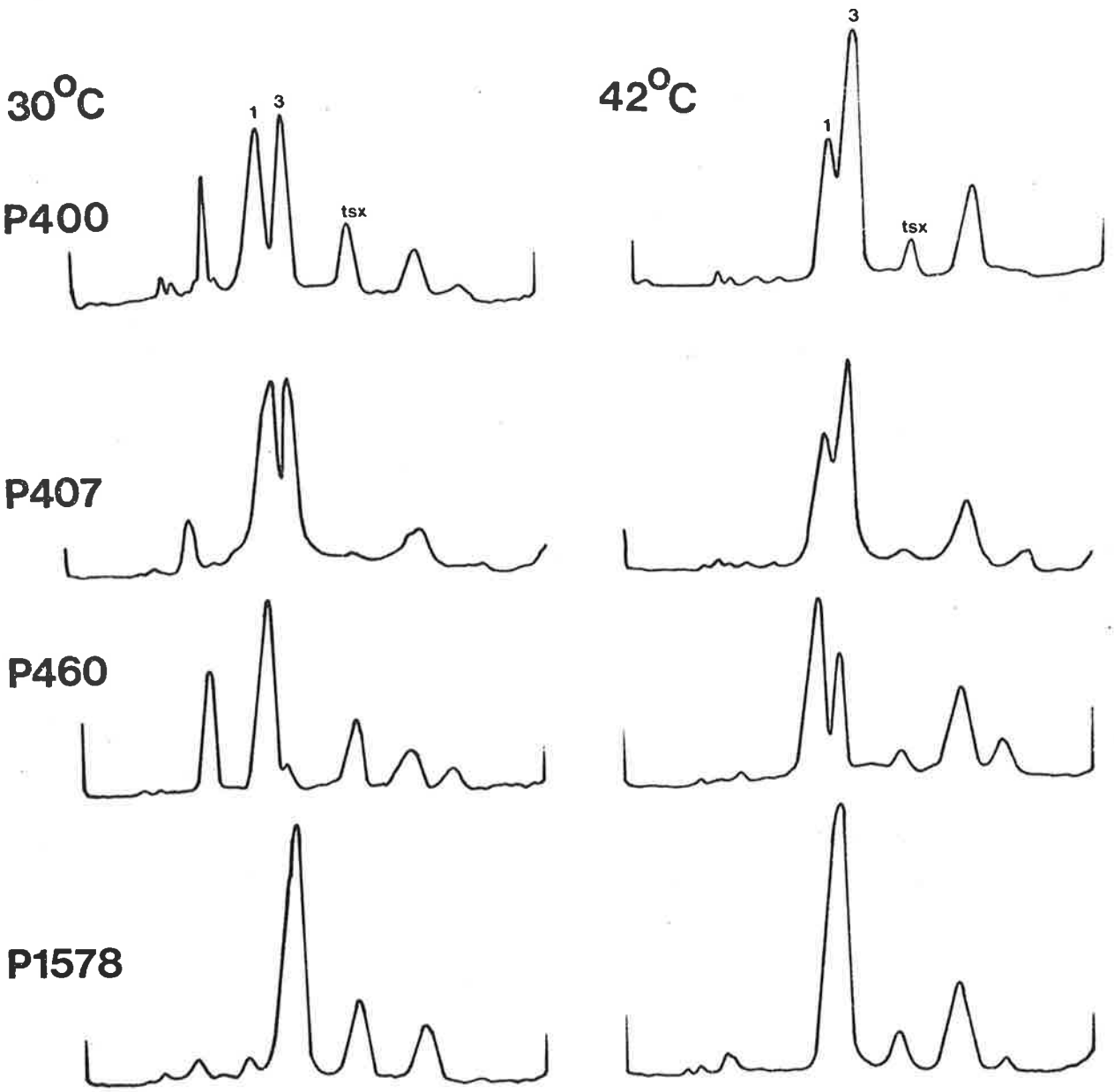


FIGURE 4-9: Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis of the outer membranes of strains P400, P407 (tsx), P460 (ompA) and P1578 (ompB) using the pH 11.4 buffer system of Bragg and Hou (1972). Cells were grown in nutrient broth at 30°C and 42°C. Peaks are labelled basically according to Schnaitman (1974a).

BRAGG & HOU



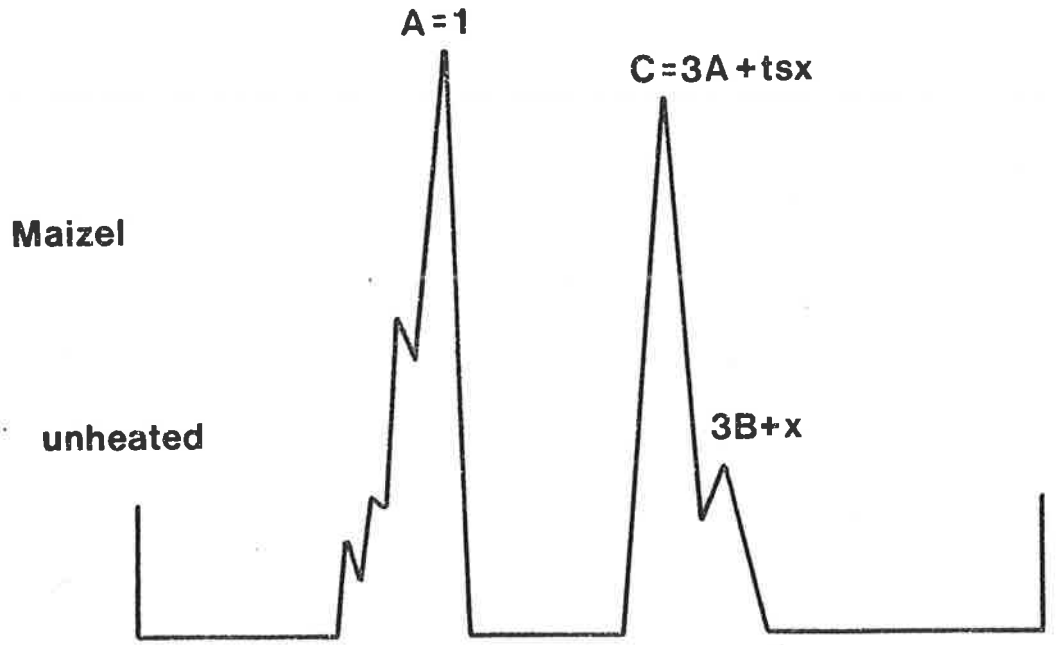
in this thesis can now be represented diagrammatically as shown in Figure 4-10.

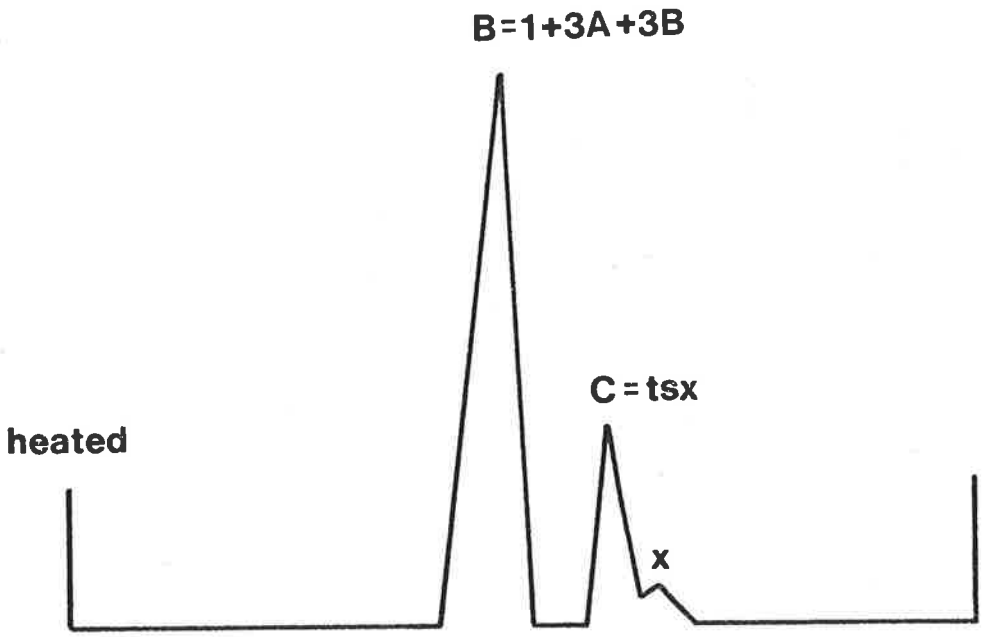
By running the same series of mutants on the SDS-polyacrylamide slab gel system of Lugtenberg et al., (1975) it is now possible to identify the running position of protein 3B. This is best shown using strain P1578 (Figure 4-11), where protein 3B can be seen to run as band a.

From Figures 4-8, 4-9 and 4-11 it can also be seen that the amount of tsx protein decreases and the amount of protein 1b (band c in Figure 4-11) increases with change in growth temperature from 30°C to 42°C. The ratio of the amounts of proteins 1 to 3 also decreases under these conditions.

Thus not only has it been possible to account for the inability of Henning's group to demonstrate the existence of protein 3B, but it can be seen that there appears to be quite complex interactions affecting the different outer membrane proteins depending upon the growth temperature used.

FIGURE 4-10: Diagrammatic representation of the positions into which the major outer membrane proteins of E. coli K12 move upon sodium dodecylsulphate polyacrylamide gel electrophoresis, using the pH 7.2 buffer system of Maizel (1966) and the pH 11.4 buffer system of Bragg and Hou (1972). Peaks are labelled according to Schnaitman (1974a).


A=1
C=3A+tsx
Maizel
unheated
3B+x


B=1+3A+3B
C=tsx
heated
x

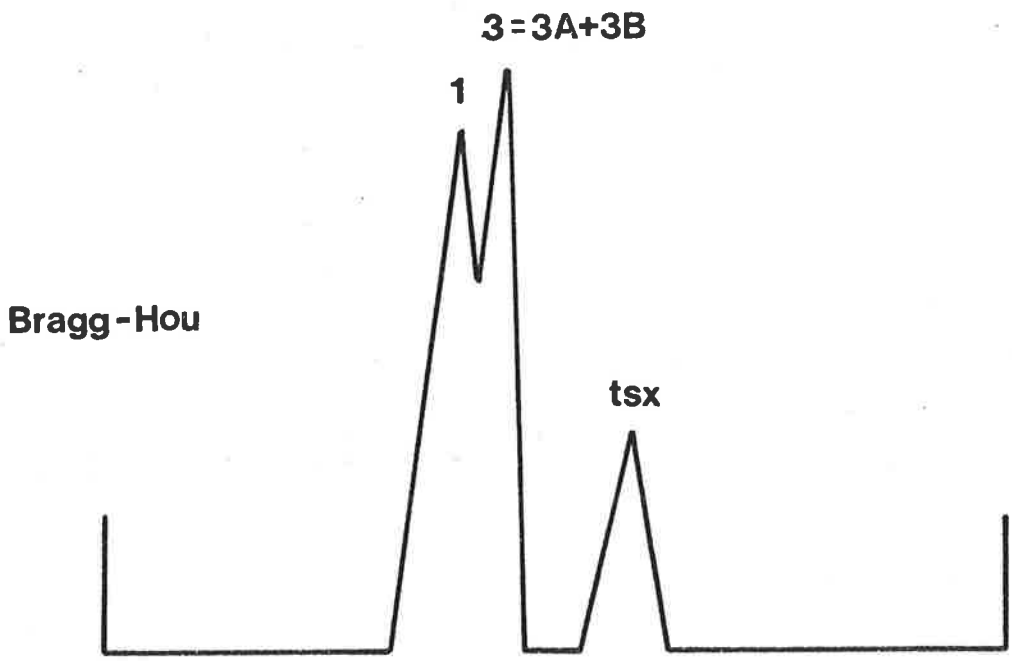
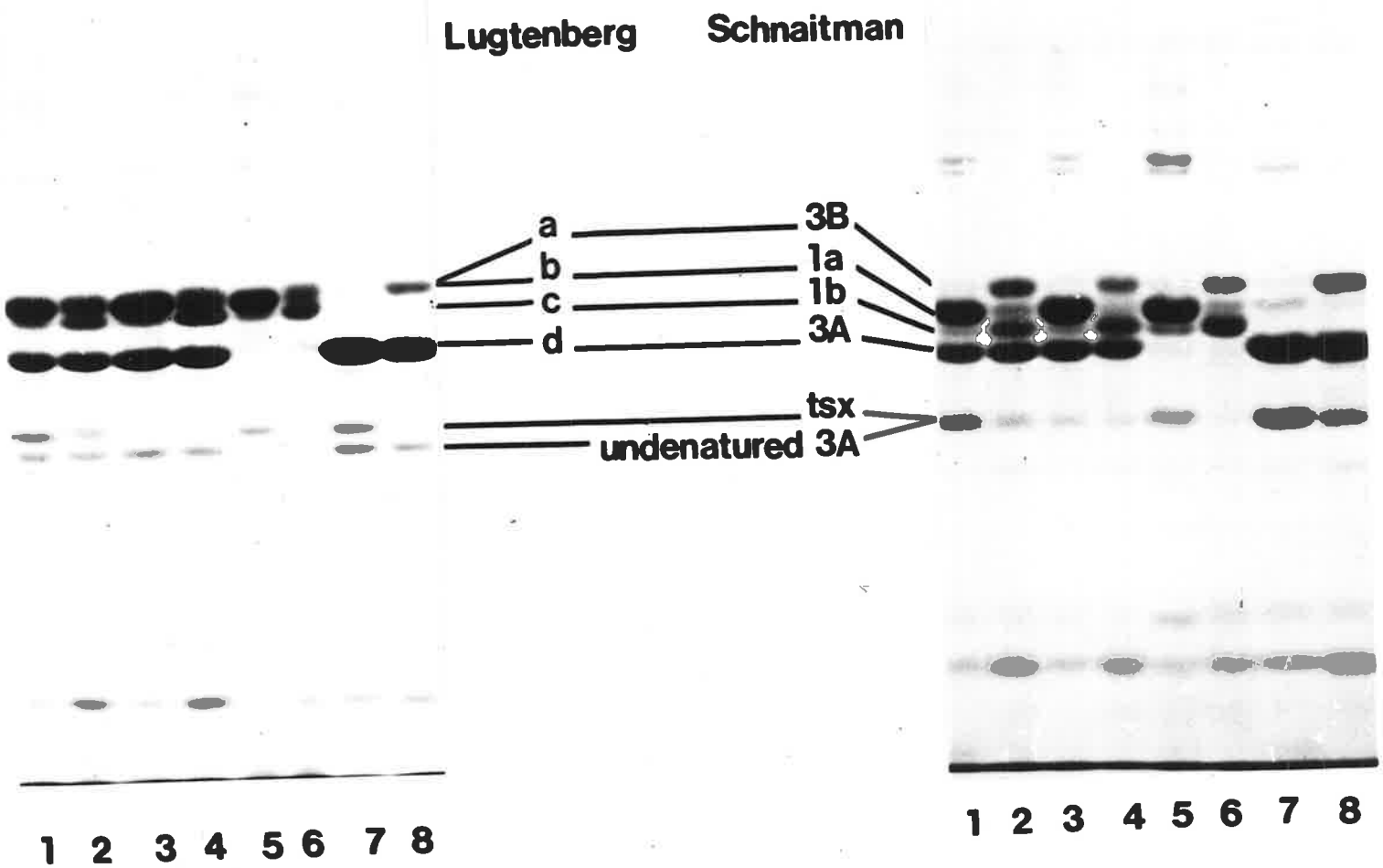

3=3A+3B
1
Bragg-Hou
tsx

FIGURE 4-11: SDS polyacrylamide slab gel electrophoresis run using the system of Lugtenberg et al., (1975) and stained according to Fairbanks et al., (1971). Bands a, b, c and d are labelled according to Lugtenberg et al. with the corresponding designations, 3B, 1a, 1b and 3A, in the nomenclature of Schnaitman (1974a).

a. Using BDH 30176 SDS, tsx is well separated from the residual undenatured protein 3A.

b. Using 50:50 mixture of BDH 30175 and 30176 SDS, bands a, b, c and d are well separated.

The samples correspond to the strains P400, P407 (tsx), P460 (ompA) and P1578 grown at 30°C (1, 3, 5, 7 respectively) and at 42°C (2, 4, 6, 8 respectively).


Lugtenberg
Schnaitman
a
b
c
d
3B
1a
1b
3A
tsx
undenatured 3A
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

NEUTRALIZATION OF BACTERIOPHAGES BY CELL ENVELOPE FRACTIONS

When the various components of the cell envelope are examined for their ability to neutralize bacteriophage K3, the Triton X-100 insoluble component or the outer membrane has the highest specific activity (Table 4-1). This neutralizing ability is absent in the ompA mutant which lacks protein 3A in its outer membrane. When the other K3-like bacteriophages were examined for their ability to be neutralized, they too were able to be neutralized by outer membrane from P400 but not from P460 (Table 4-2).

The neutralizing ability of the outer membrane for bacteriophage K3 can be solubilized by using EDTA in the presence of Triton X-100 (the TES component). This procedure is also known to solubilize most outer membrane proteins (Schnaitman, 1971a).

Henning and Haller (1975) have looked at the outer membrane of tolG mutants on their gel system and have shown that these mutants are missing the protein described in that laboratory as protein II* (Garten and Henning, 1974). Thus protein II* is identical to

TABLE 4-1

Neutralization of bacteriophage K3 by cell envelope fractions

Source of cell envelope	Cell envelope fraction			
	WM[a]	CM[b]	OM[c]	LPS[d]
P400	5[e]	50-100	1	>1000
P460 (*ompA-1*)	>1000	>1000	>1000	>1000

a. WM = whole membrane; 78,000 x g pellet after breaking bacteria in French pressure cell.

b. CM = cytoplasmic membrane; Triton X-100 soluble component of WM.

c. OM = outer membrane; Triton X-100 insoluble component of WM.

d. LPS = lipopolysaccharide.

e. Figures represent the lowest concentration (in µg/ml contained protein or LPS) of the envelope fraction

(TABLE 4-1 Cont....)

which gave 50% neutralization of the 10^3 bacteriophages used in the assay: the concentrations of material used were 1000, 500, 100, 50, 10, 5, 1 and 0.1 µgm/ml. >1000 indicates that the phage were not neutralized by this, the maximum amount of material used.

TABLE 4-2

Neutralization of other K3-like bacteriophages by outer membrane

Bacteriophage	Source of outer membrane	
	P400	P460 (ompA-1)
K4	1[a]	>1000
K5	1	>1000
0x2	5	>1000
0x3	1	>1000
0x4	10	>1000
0x5	10	>1000
M1	5	>1000
Ac3	1	>1000

a. Figures represent the lowest concentration (in µg/ml contained protein) of outer membrane material which gave 50% neutralization of the 10^3 bacteriophages used in the assay: the concentrations of material used were 1000, 500, 100, 50, 10, 5, 1 and 0.1 µg/ml.

protein 3A. These workers have also isolated a bacteriophage TuII* which is unable to plaque on tolG mutants (Henning and Haller, 1975). This phage has been used to select mutants referred to by these authors as tut (Henning, Hindennach and Haller, 1976) which are identical to the ompA mutants in their map position and phenotypic properties as far as it has been tested. When the cross-resistance pattern of this phage was examined with the bacteriophage resistant mutants of Hancock and Reeves (1975), it appeared to be identical to the K3-like bacteriophage 0x3.

INHIBITION OF CONJUGATION

Attempts to inhibit conjugation with components of the cell envelope were unsuccessful. This was attempted to see if protein 3A did in fact have a role in recipient functions. However, the inability to demonstrate activity, could possibly be similar to the situation described by Randall-Hazelbauer and Schwartz (1973). These authors showed that the receptor for bacteriophage λ required modification by such chemical agents as chloroform or ethanol before it could function in vitro. Thus the only biological activity detected in vitro was the neutralization of phage K3.

In preliminary experiments attempting to inhibit conjugation with whole cells, somewhat confusing results were obtained (Table 4-3). It was not possible to show any inhibition of conjugation to strain ED267 but the presence of this recipient appeared to decrease transfer to strain P400 and to increase transfer to strain P460 (ompA).

PURIFICATION OF PROTEIN 3A - THE BACTERIOPHAGE K3 RECEPTOR

Protein 3A has been previously purified (Garten et al., 1975; Reithmeier and Bragg, 1974) but under conditions in which receptor activity for bacteriophage K3 is lost. As it was strongly suspected that protein 3A was the receptor for bacteriophage K3, a T-even like phage (Figure 4-12), it was decided to purify this protein under conditions which retained this biological activity. In this purification strain P1605 was used. This is an ompB tsx tonA mutant which produces neither protein 1 nor the tsx protein and hence produces protein 3A as its most abundant protein (Figure 4-13). Thus for strain P1605, protein peak C, of unheated samples run using the pH 7.2 buffer system of Maizel (1966), consists only of protein 3A.

TABLE 4-3

Inhibition of conjugation using whole cells

Mating (1:10)	% Transfer to[a]		
	P400	P460	ED267
HfrH x P400	28.4	-	-
x P460	-	0.08	-
x E267	-	-	40.3
x (P400 + ED267)	13.6	-	38.8
x (P460 + ED267)	-	2.2	41.8

a. Transfer to P400, P460 and ED267 was measured by selecting for <u>pro</u>$^+$ recombinants and using streptomycin (100 µg/ml) for contraselection. Transfer is expressed as a % of the input donor cell number.

FIGURE 4-12: Electron micrographs of bacteriophage K3 negatively stained with uranyl acetate.

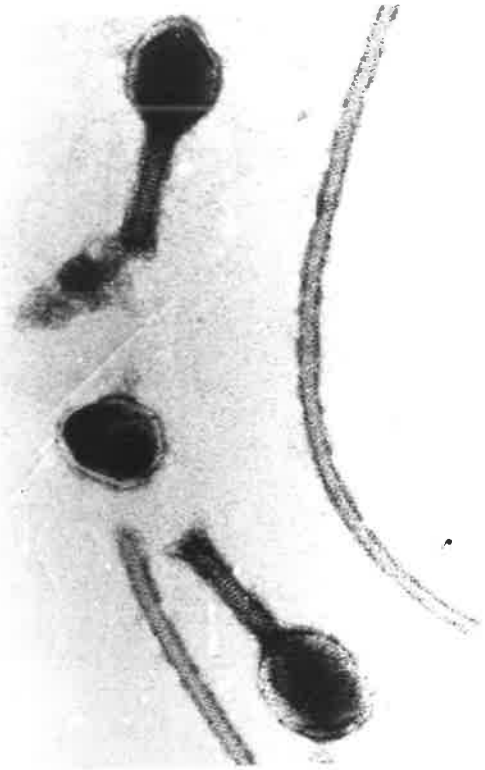
a. Field showing a number of K3 phage (x 44,000).

b. Phage K3 and examples of poly-tail-tubes in the preparation (x 90,000).

c. High magnification of a single phage (x 190,000)

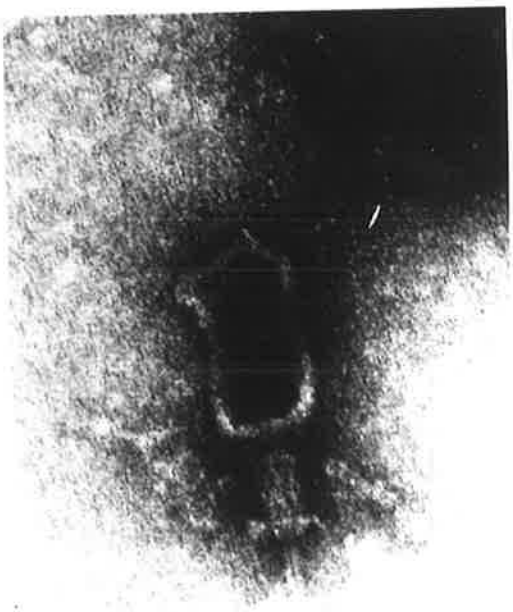
d. Example of a K3 phage which has injected its DNA. Note the empty head, contracted tail sheath and the spikes or tail pins (x 120,000).

a



b

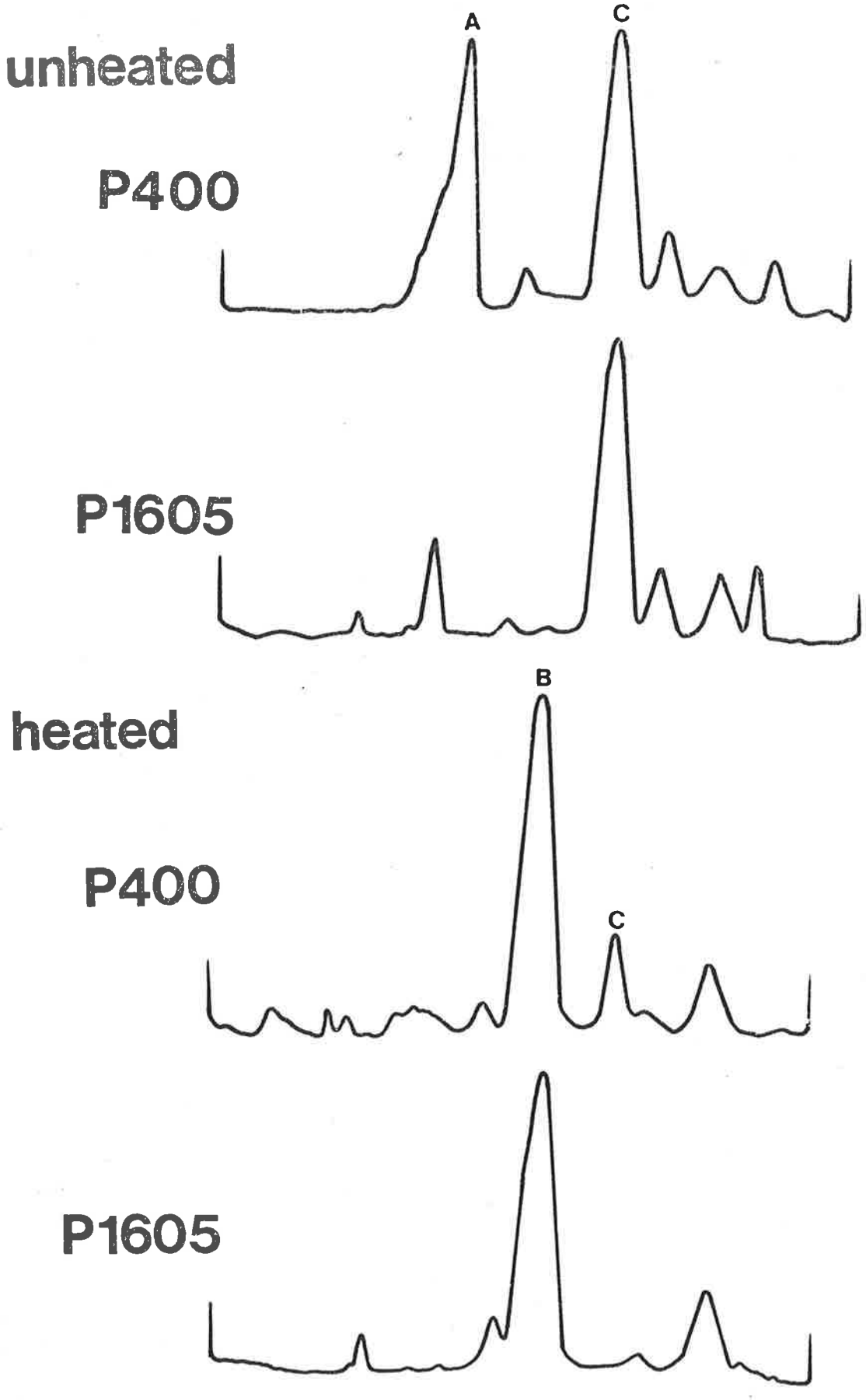
c



d

FIGURE 4-13: Comparison of the outer membranes of P400 and P1605 (*ompB*, *tsx*, *tonA*) by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis using unheated and heated preparations with the pH 7.2 buffer system of Maizel (1966). Peaks are labelled according to Schnaitman (1974a).

MAIZEL



When the TES component of the outer membrane of strain P1605 was chromatographed on DEAE-cellulose and the fractions analyzed, it was observed that the bacteriophage K3 receptor activity could only be correlated with the presence of protein 3A (Figures 4-14, 4-15, 4-16 and 4-17).

Fractions 42-55 which contained the major peak of activity were pooled and concentrated 10-fold, dialyzed overnight against 20 volumes of buffer and rechromatographed on QAE-Sephadex (Figure 4-18). Protein 3A is present in each of the pools (Figure 4-19), possibly due to the column being somewhat overloaded, and each is able to neutralize bacteriophage K3 (Table 4-4). However, pool IV contained negligible amounts of any protein species other than protein 3A and this was concentrated and dialyzed overnight as before and rechromatographed on DEAE-cellulose to give pure protein 3A in pool III (Figures 4-20, 4-21). This material was able to neutralize bacteriophage K3 (Table 4-5) and contained no detectable carbohydrate (<1%) or KDO (<0.1%). It had the amino acid composition as shown in Table 4-6.

FIGURE 4-14: DEAE-cellulose chromatography in TTE buffer pH 7.2 of the Triton X-100 and EDTA soluble (TES) outer membrane of P1605. 500 mg of protein was applied to a 2.5 x 50 cm column. This was obtained from 50 litres of bacteria grown in a fermenter under vigorous aeration at $37^{o}C$ to a density of 7-8 x 10^{8} cells/ml.

25 ml fractions were collected from the column and 2 ml of each taken and ethanol precipitated for phage neutralization and ketodeoxyoctonate (KDO) determination.

Top : Distribution of protein in the fractions.

Centre: KDO concentration of the fractions.

Bottom : Ability of the fractions to neutralize bacteriophage K3, expressed as the negative log of the dilution required to give 50% neutralization.

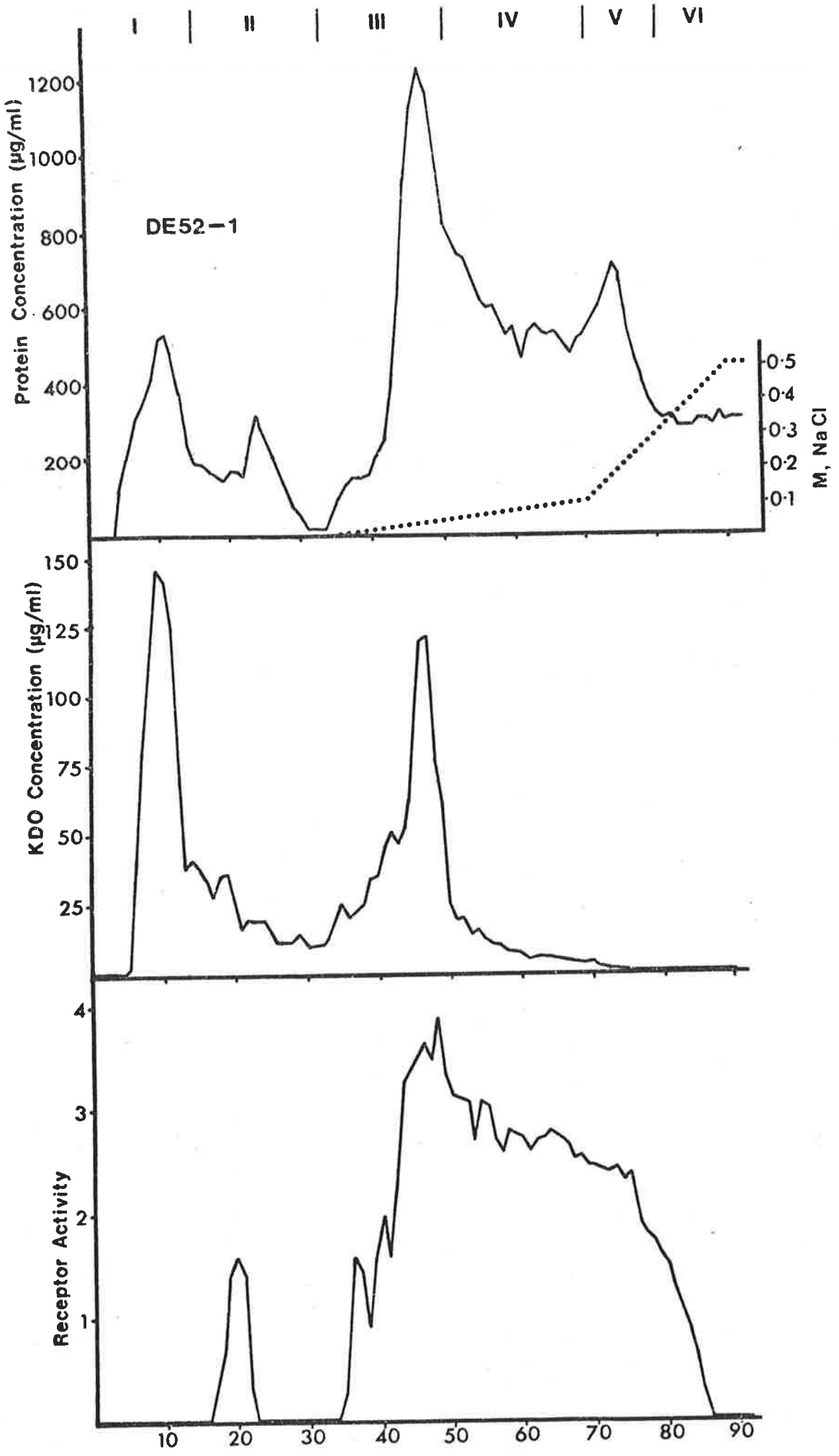

I
II
III
IV
V
VI
DE52–1
Protein Concentration (µg/ml)
1200
1000
800
600
400
200
0·5
0·4
0·3
0·2
0·1
M, NaCl
KDO Concentration (µg/ml)
150
125
100
75
50
25
Receptor Activity
4
3
2
1
10
20
30
40
50
60
70
80
90

FIGURE 4-15: Protein composition of the fractions of the DEAE-cellulose column pooled as shown in Figure 4-14. The proteins were analyzed on sodium-dodecyl-sulphate polyacrylamide gels using unheated samples with the pH 7.2 buffer system of Maizel (1966). Peaks are labelled according to Schnaitman (1974a).

MAIZEL
unheated

DE52 – I

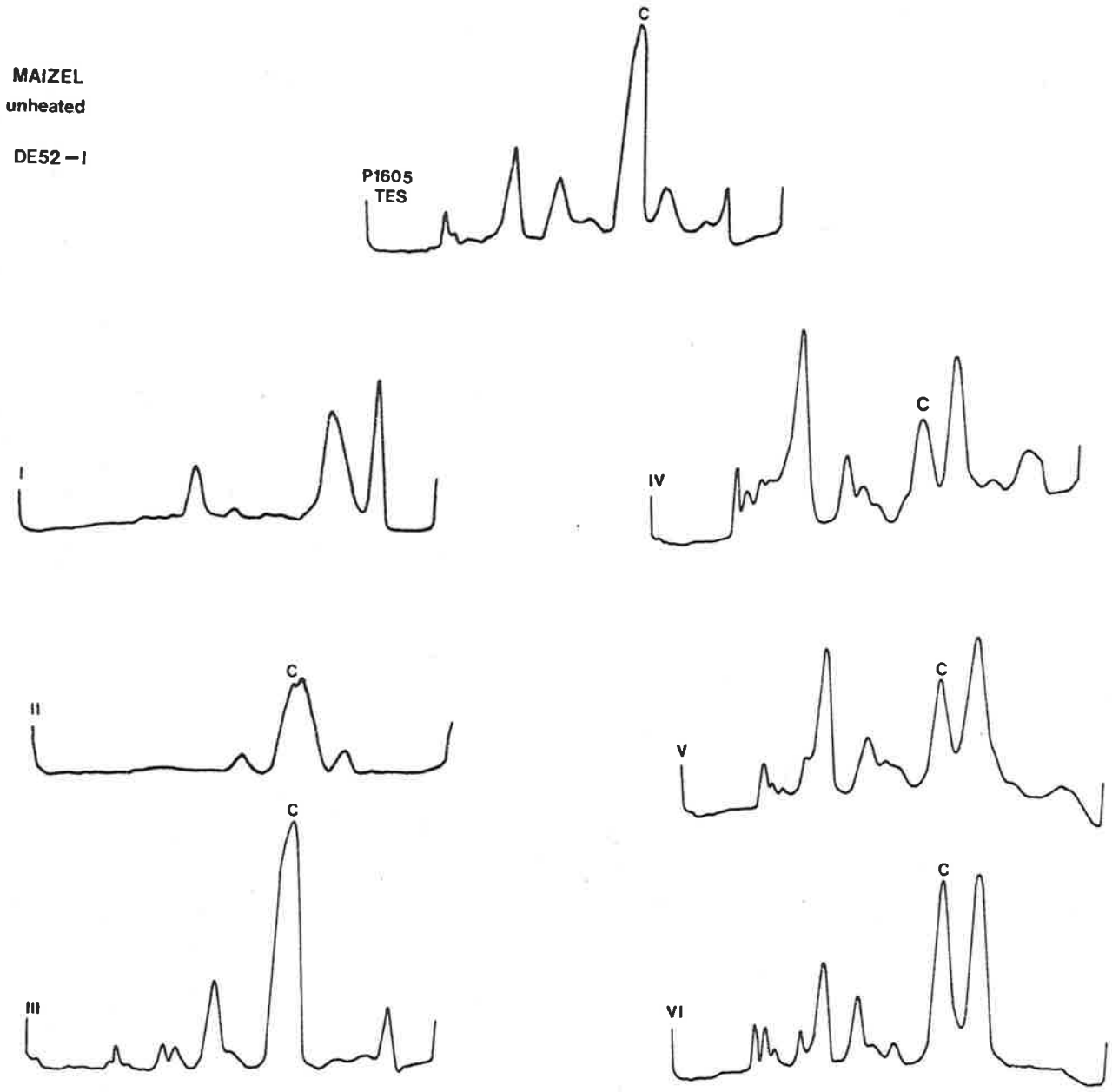
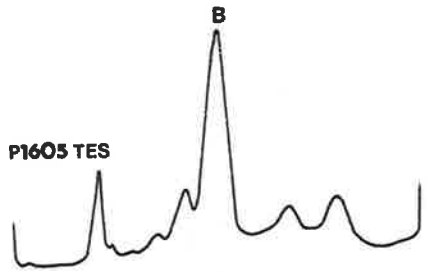
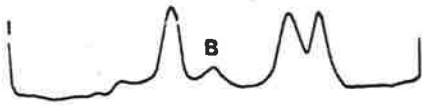


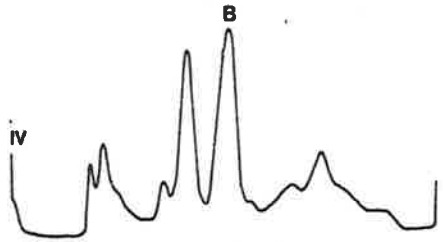
FIGURE 4-16: Protein composition of the pooled fractions of the DEAE-cellulose column shown in Figure 4-14. The proteins were analyzed on sodium dodecyl sulphate polyacrylamide gels using heated samples with the pH 7.2 buffer system of Maizel (1966). Peaks are labelled according to Schnaitman (1974).

MAIZEL
heated

DE 52–1







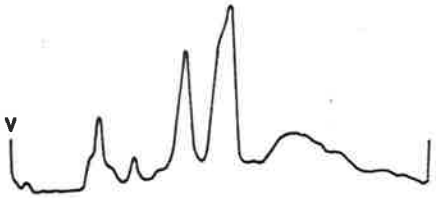


FIGURE 4-17: Protein composition of the pooled fractions of the DEAE-cellulose column shown in Figure 4-14. The proteins were analyzed on sodium dodecyl sulphate polyacrylamide gels using the pH 11.4 buffer system of Bragg and Hou (1972). Peaks are labelled according to Schnaitman (1974a).

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DE52 – I

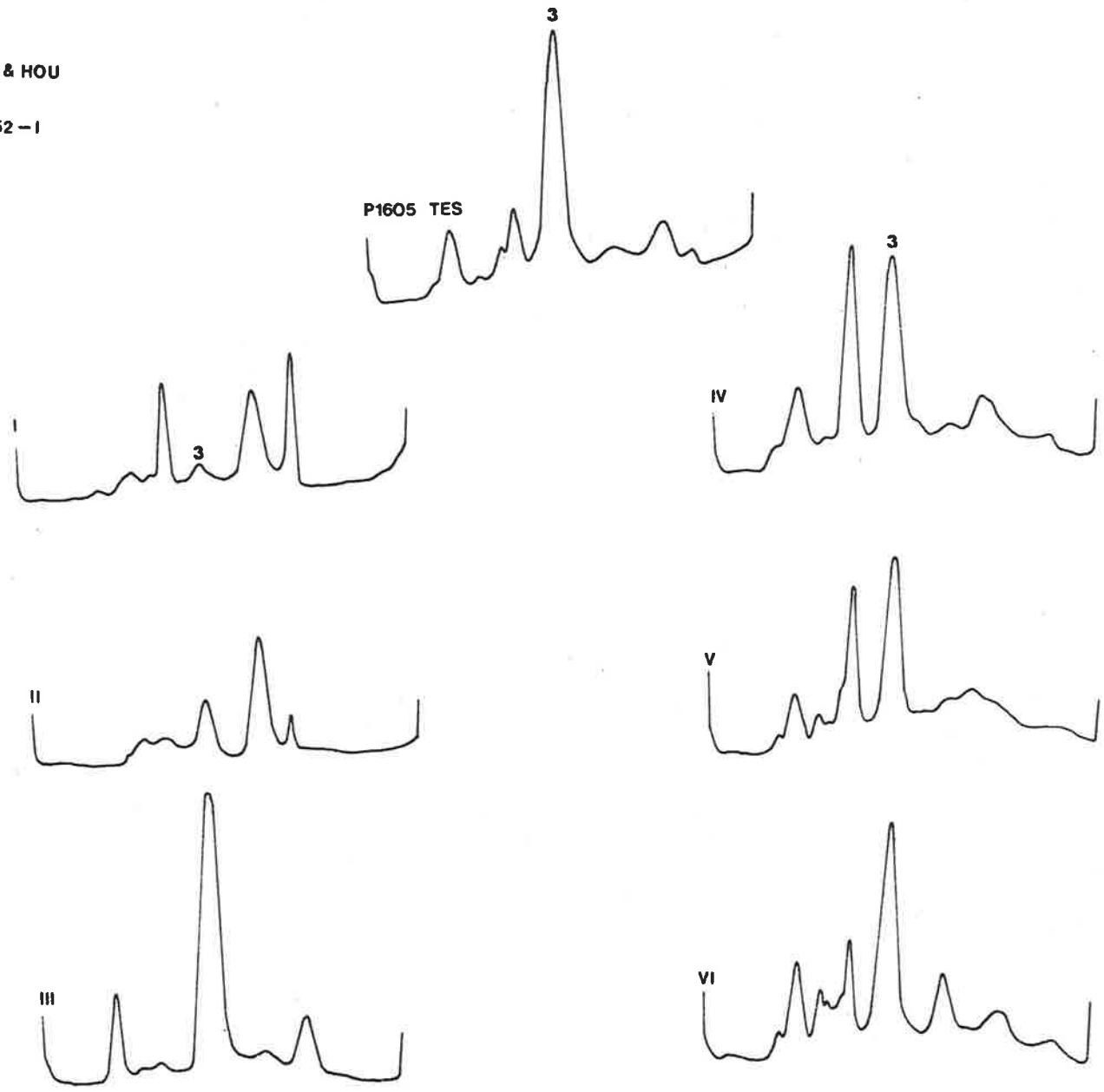
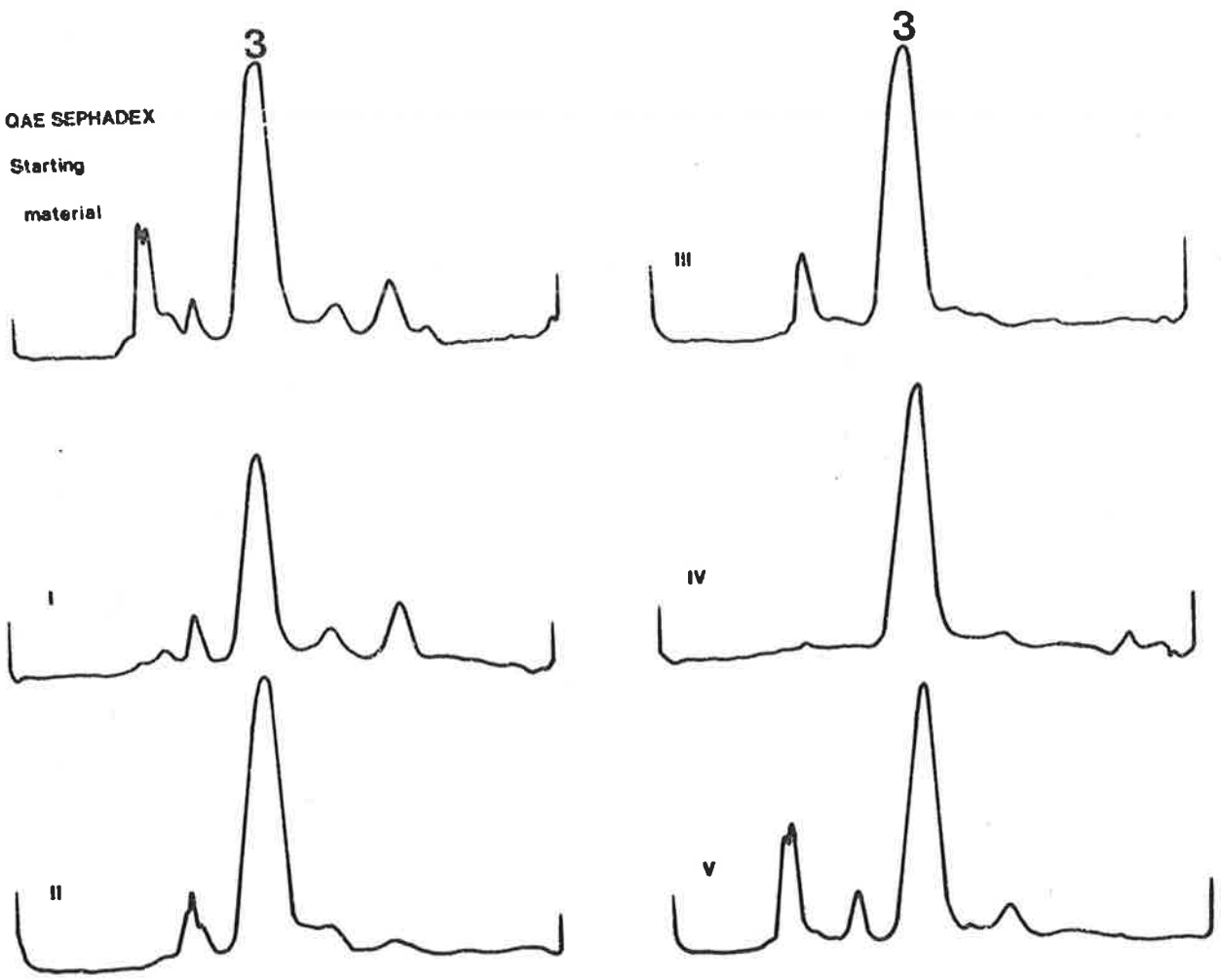


FIGURE 4-18: QAE-Sephadex chromatography in TTE buffer pH 8.0 of fractions 42 to 55 (labelled as the starting material) from the DEAE-cellulose column shown in Figure 4-14. The elution profile of the protein is shown in the lower panel and the protein composition of the pools analyzed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis using the pH 11.4 buffer system of Bragg and Hou (1972) is shown in the upper panel. The peaks are labelled according to Schnaitman (1974a).


QAE SEPHADEX
Starting
material
3
3
I
II
III
IV
V

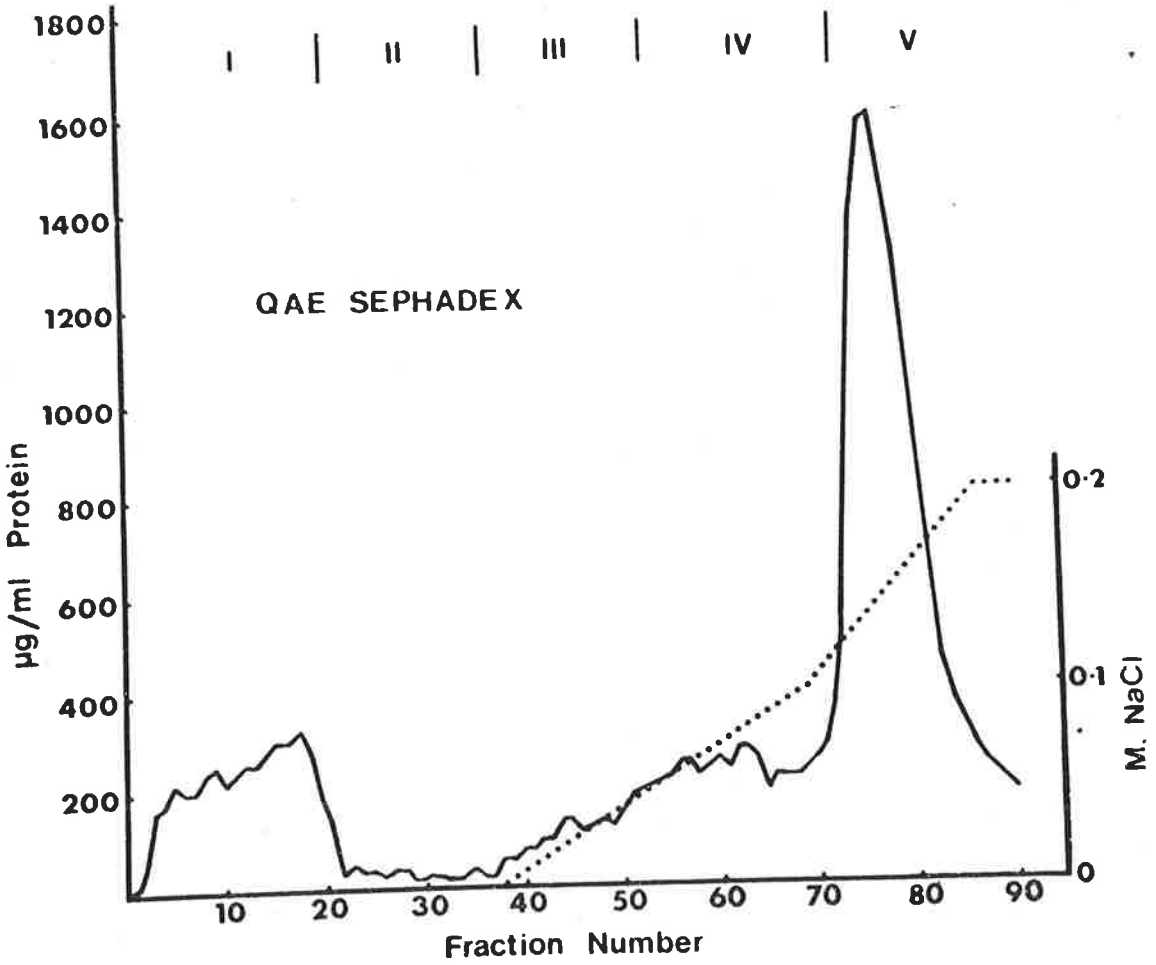
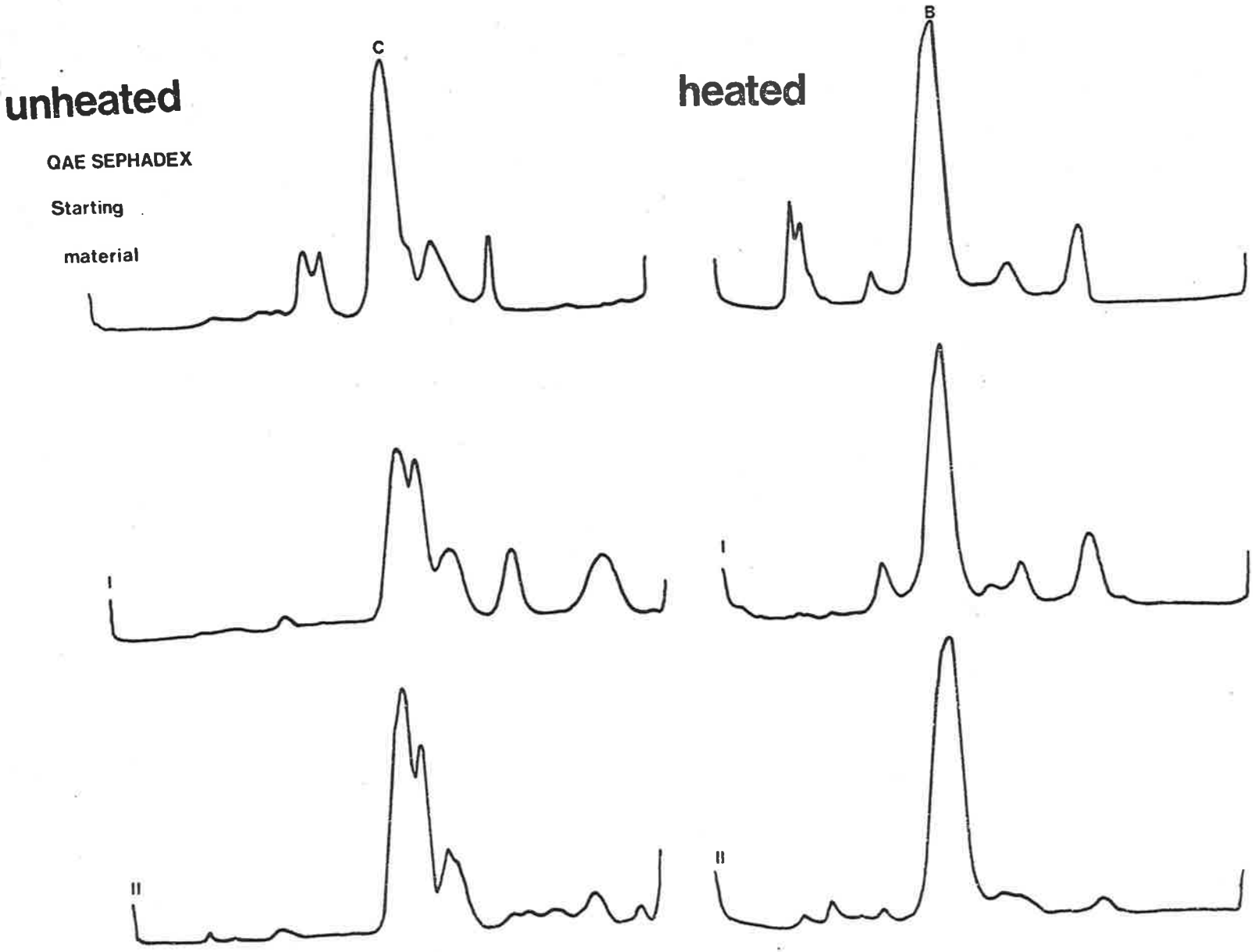

QAE SEPHADEX
I
II
III
IV
V
1800
1600
1400
1200
1000
800
600
400
200
µg/ml Protein
0·2
0·1
0
M. NaCl
10
20
30
40
50
60
70
80
90
Fraction Number

FIGURE 4-19: Analysis of the protein composition of the pooled fractions of the QAE Sephadex column shown in Figure 4-18. Unheated and heated samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis using the pH 7.2 buffer system of Maizel (1966). Peaks are labelled according to Schnaitman (1974a).

MAIZEL



MAIZEL

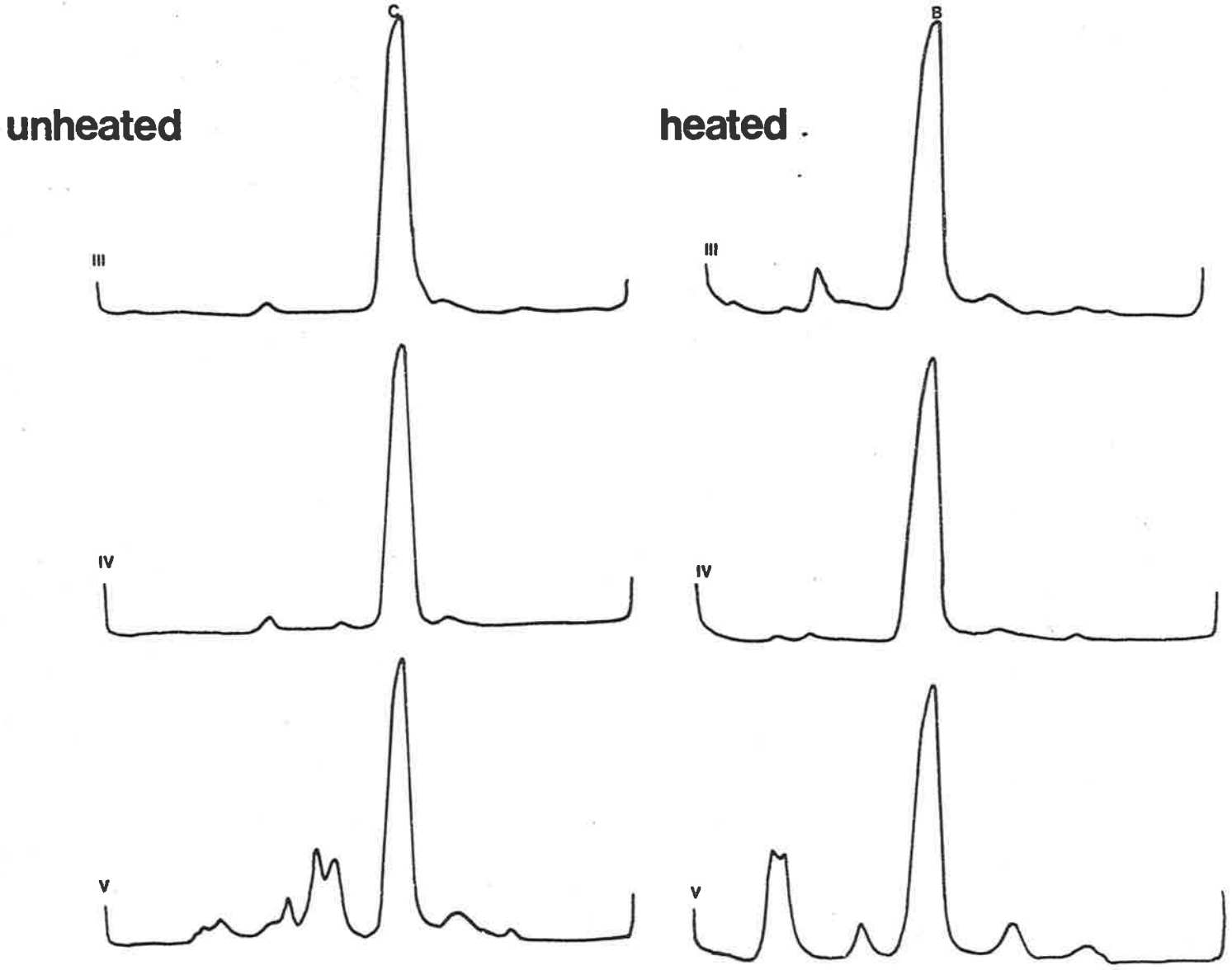


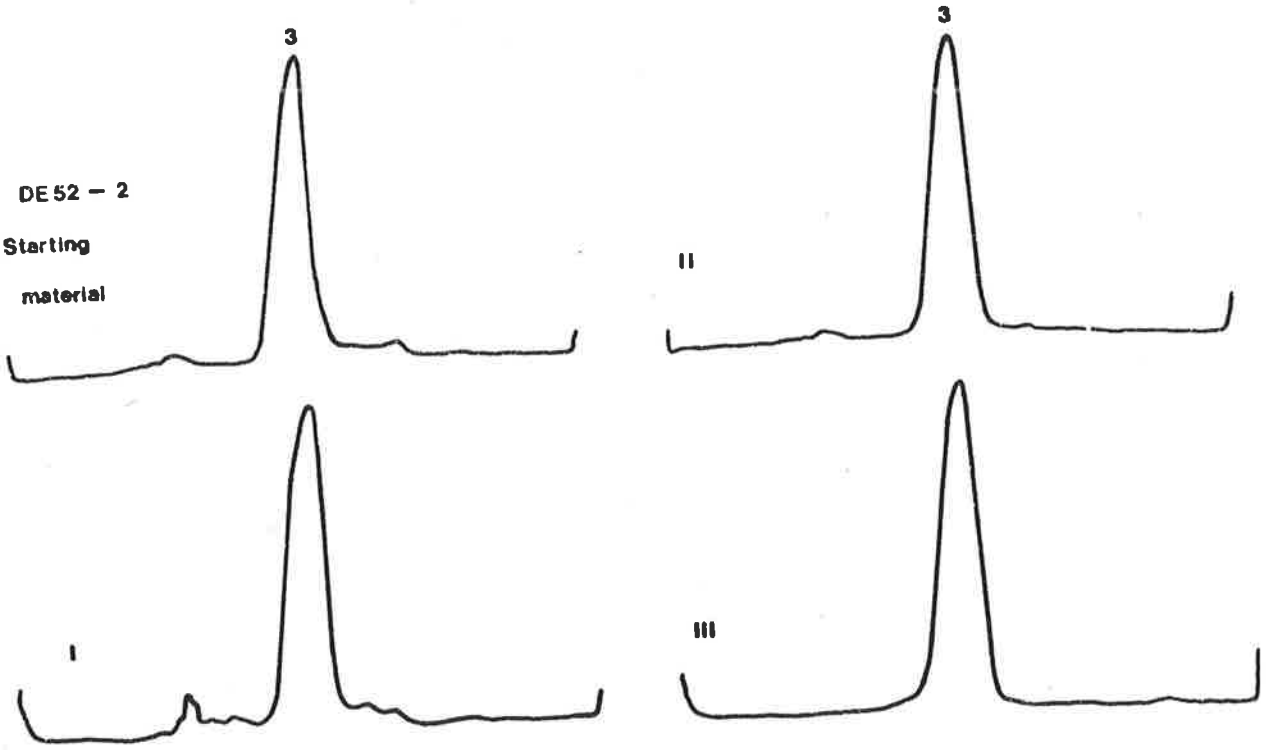
TABLE 4-4

Neutralization of bacteriophage K3 by purified protein obtained by QAE-Sephadex chromatography

Pool	Neutralizing ability[a]
Starting material	1.0
I	1.0
II	1.0
III	0.5
IV	0.5
V	1.0

a. Neutralizing ability is expressed as the lowest concentration (in µg/ml) protein required to give 50% neutralization of bacteriophage K3 under the conditions used (see Materials and Methods). Samples containing 1000, 100, 10, 5, 1, 0.5, 0.1 µgm/ml of protein were used.

FIGURE 4-20: DEAE-cellulose chromatography in TTE buffer pH 7.2 of pool IV from the QAE-Sephadex column shown in Figure 4-17. The protein composition of the pools was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis using the pH 11.4 buffer system of Bragg and Hou (1972) and peaks are labelled according to Schnaitman (1974a).


DE 52 – 2
Starting
material
3
I
3
II
III

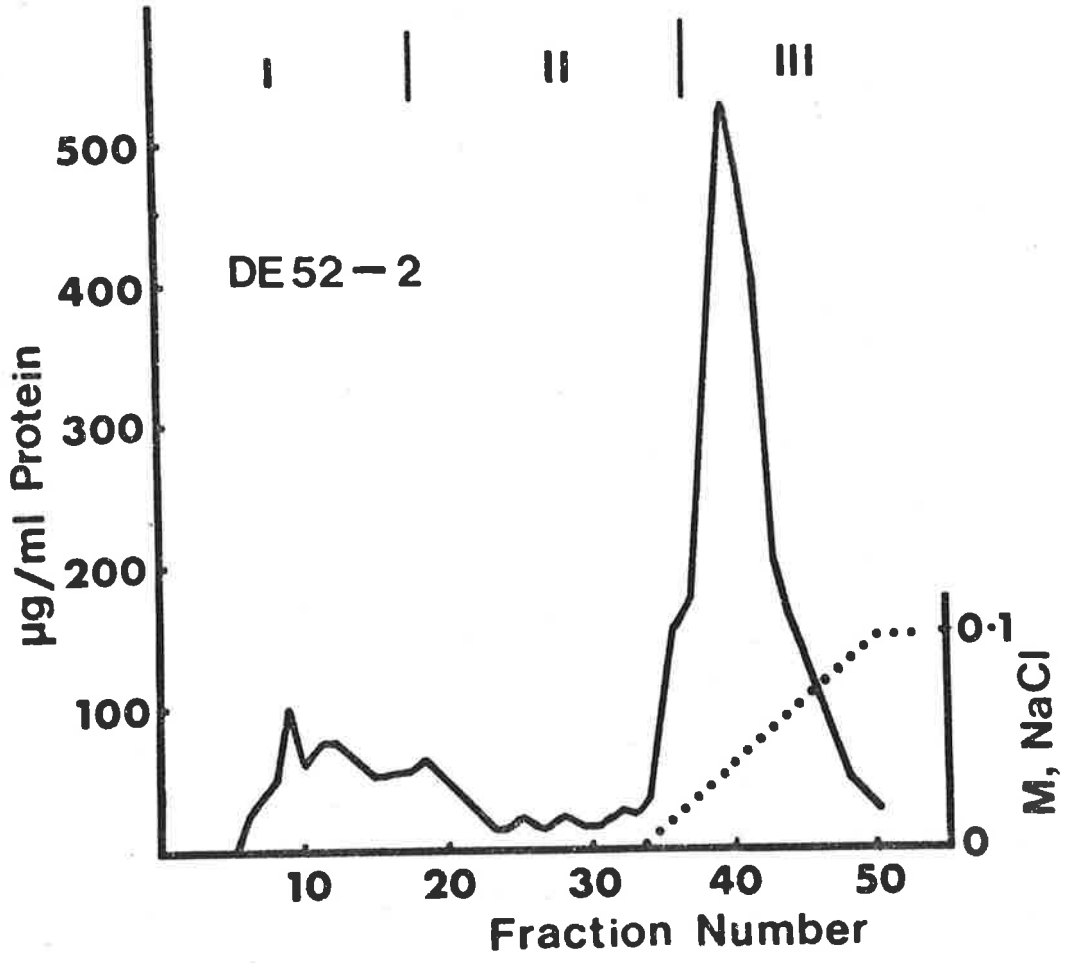

I
II
III
DE 52 – 2
500
400
300
200
100
µg/ml Protein
0·1
0
M, NaCl
10
20
30
40
50
Fraction Number

FIGURE 4-21: Analysis of the protein composition of the pooled fractions of the DEAE-cellulose column shown in Figure 4-20. Unheated and heated samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis using the pH 7.2 buffer system of Maizel (1966). Peaks are labelled according to Schnaitman (1974a).

MAIZEL

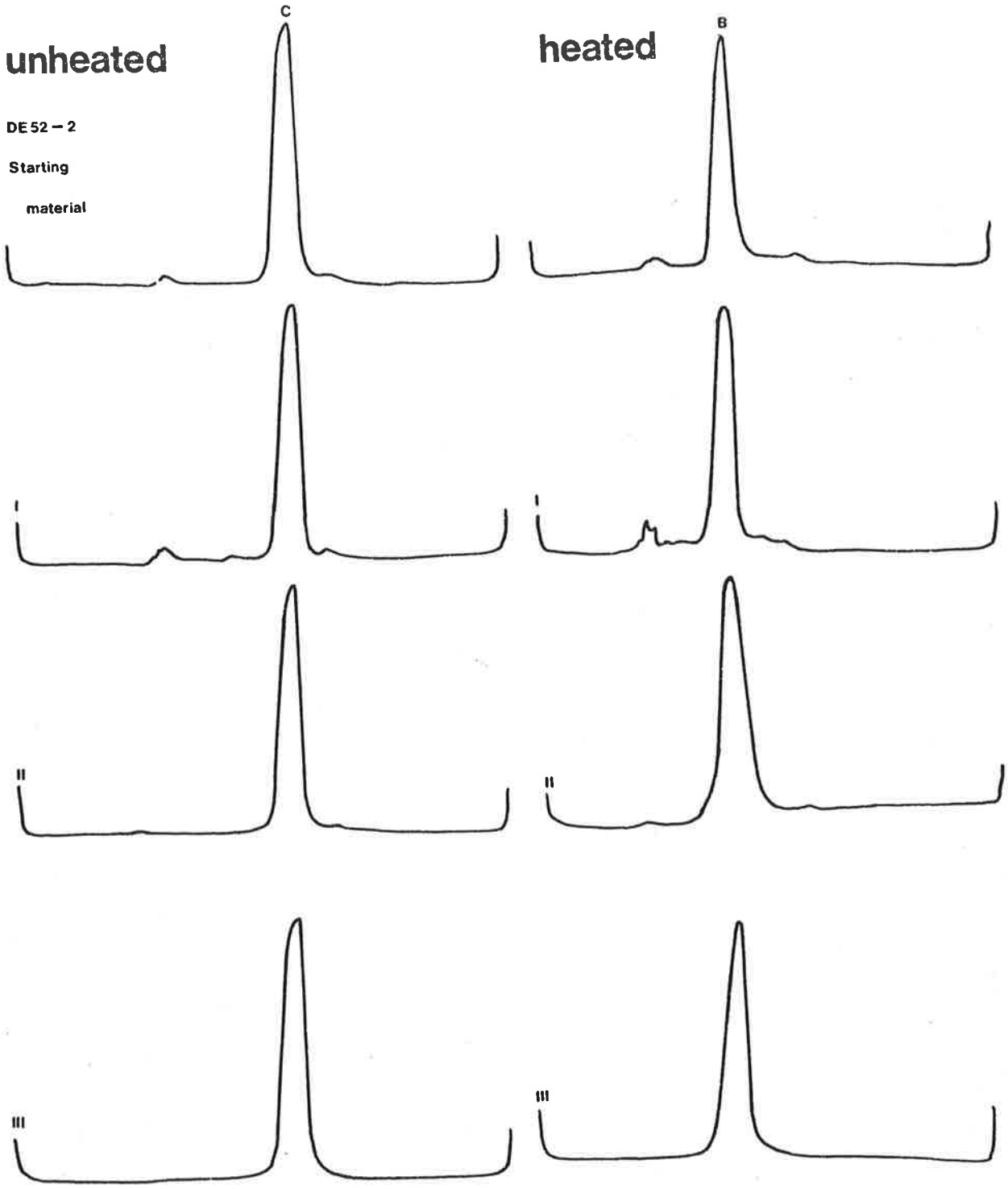


TABLE 4-5

Neutralization of bacteriophage K3 by purified protein obtained by DEAE-cellulose chromatography of pool IV from Figure 4-19

Pool	Neutralizing ability[a]
Starting material	0.5
I	1.0
II	0.5
III	0.5

a. Neutralizing ability is expressed as the lowest concentration (in µg/ml) protein required to give 50% neutralization of bacteriophage K3 under the conditions used (see Materials and Methods). Samples containing 1000, 100, 10, 5, 1, 0.5, 0.1 µgm/ml of protein were used.

TABLE 4-6

Amino acid composition of purified receptor

Amino Acid	μ Moles %
Cysteic acid	-
Methionine*	1.22
SCM-cysteine	-
Aspartic acid	13.3
Threonine	6.3
Serine	5.08
Glutamic acid	9.5
Citrulline	-
Proline	5.93
Glycine	12.1
Alanine	9.31
Half cysteine	-
Valine	6.88
Isoleucine	4.14
Leucine	7.15
Tyrosine	5.27
Phenylalanine	2.45
Lysine	5.46
Histidine	1.79
Arginine	4.23
Tryptophan	n.d.[a]

(TABLE 4-6 Cont....)

* Methionine was measured as methionine sulphone.

a. n.d. = not determined.

TRYPSIN DIGESTION OF PROTEIN 3A

Hancock (1974) has previously shown that trypsin treatment of the outer membrane has little effect on its ability to neutralize bacteriophage K3. When purified protein 3A is digested with trypsin, it yields a fragment of molecular weight of about 25,000 daltons (Figure 4-22). An identical result has also been obtained by Haller and Henning (1974) who showed that trypsin treatment of their protein II* yields protein II. The trypsin treated purified protein 3A was still able to neutralize bacteriophage K3 suggesting that much, if not all, of the receptor specificity of this protein, was present in this larger fragment.

FIGURE 4-22: The degradation of protein 3A by trypsin. A comparison of protein 3A and its tryptic digest on sodium dodecyl sulphate polyacrylamide gels using the pH 11.4 buffer system of Bragg and Hou (1972). Outer membranes of P400 and P460 (ompA) are shown for comparison.

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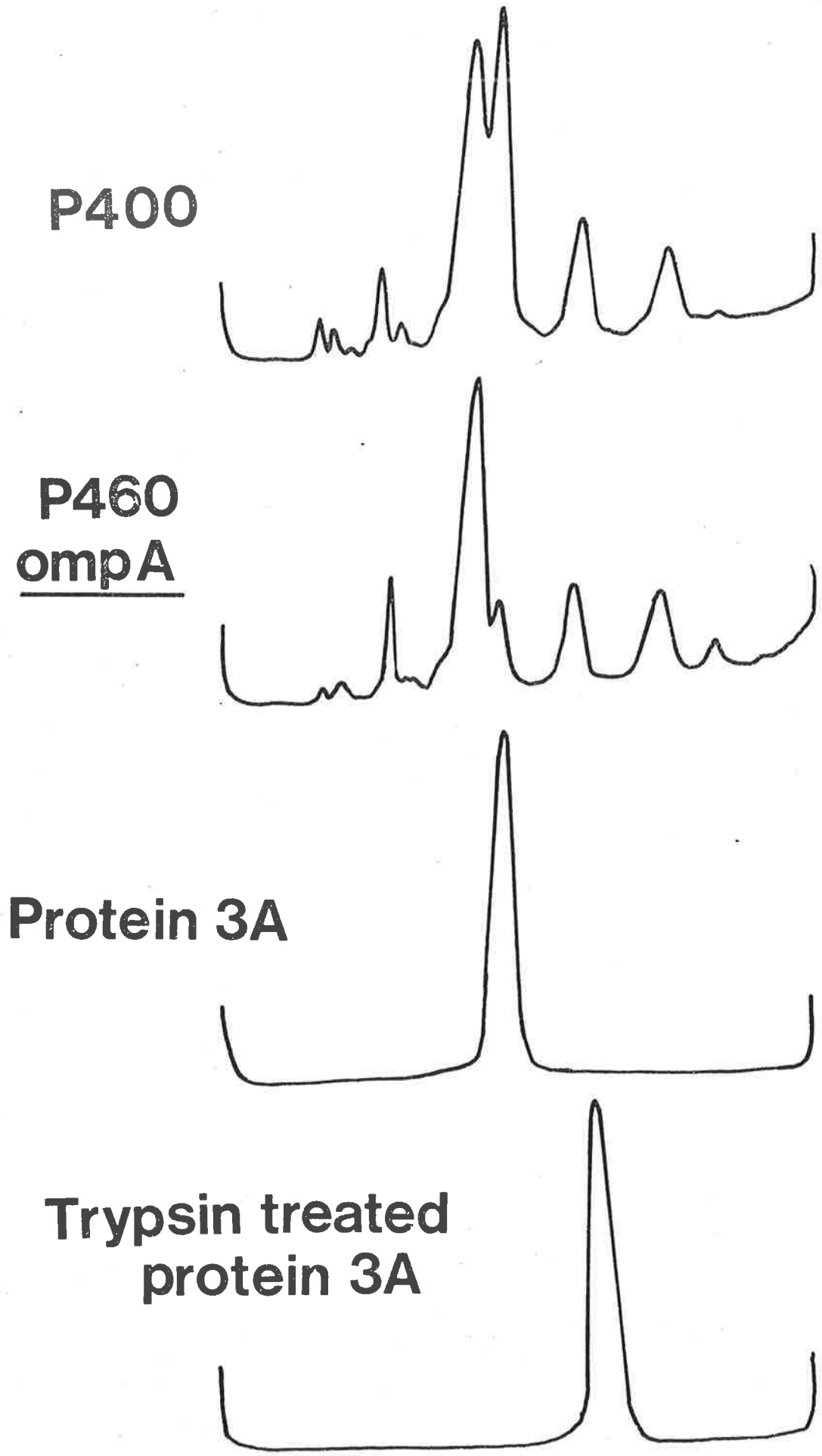


SUMMARY AND CONCLUSIONS

It has been demonstrated that *con* and *tolG* mutants are both lacking the outer membrane protein 3A which together with information from Chapter 3, shows them to be identical. Because of this they have been renamed as the *ompA* locus.

Protein 3A has been purified and shown to account for the bacteriophage K3 neutralizing activity of the outer membrane. Since *ompA* mutants have also been shown to be unable to adsorb bacteriophage K3 (Chapter 3) it is concluded that protein 3A is the receptor for bacteriophage K3. It has not been possible to demonstrate a role in recipient functions for protein 3A *in vitro*.

Protein 3B has also been shown to be a distinct protein species with different electrophoretic behaviour to the other major outer membrane proteins. The amount of this protein that is produced has been shown to be dependent upon the growth temperature.

CHAPTER 5

ISOLATION OF MUTANTS WITH AN ALTERED PROTEIN 3A USING HOST RANGE MUTANTS OF BACTERIOPHAGE K3

INTRODUCTION

In the previous Chapter it has been shown that protein 3A is absent in the *ompA* mutants P460 and JF404-2a, that this protein is able to neutralize bacteriophage K3 and similar phage, and presumably functions *in vivo* as the receptor for the phage. It has been suggested that *ompA* was the structural gene for protein 3A, however, much more convincing evidence would be the demonstration of an altered protein 3A produced by an *ompA* mutant.

The only class of mutants selected using bacteriophage K3 are *ompA* mutants. Since bacteriophage K3 uses protein 3A as its receptor on the cell surface, *ompA* mutants might occur which produce an altered protein 3A, unable to act as receptor for wild type phage K3, but able to act as a receptor for extended host range mutants of the phage. Such *ompA* mutants could also be used to isolate extended host range phage mutants. A series of *ompA* mutants, resistant to bacteriophage K3, have been isolated and it was possible to isolate

extended host range mutants of bacteriophage K3 on some of these.

BACTERIOPHAGE RESISTANCE OF *ompA* MUTANTS

48 independent bacteriophage K3-resistant bacterial mutants were isolated and of these only 10 initially gave plaques of extended host range mutants when plated out in a lawn with 10^9 p.f.u. of bacteriophage K3. A further 9 mutants were shown to be able to plaque the extended host range phage. All of the mutants were resistant to wild type bacteriophage K3 and were also resistant to only K4, K5, 0x2, 0x3, 0x4, 0x5, M1 and Ac3 of the 64 phages tested. That is, they appeared to be typical *ompA* mutants (Skurray *et al.*, 1974; Hancock and Reeves, 1975). However it was found that there was considerable variation in the ability of these mutants to plaque the extended host range phage mutants (Table 5-1). The overall pattern ranges from the ability to plaque all of the extended host range phage down to the typical *ompA* mutants, of which P460 is the type strain, and on which it has not been possible to isolate any extended host range mutants. The data also allow the extended host range mutants to be arranged in an order of increasing potency, with wild type K3 (h^+) being the least potent.

TABLE 5-1

Efficiency of plating of the extended host range mutants of bacteriophage K3 at 37°C.

Type Strain	No. of similar mutants	Genotype[a]	EOP of bacteriophage K3 extended[b] host range mutants							
			h^+	h4	h15 h23 h40	h5	h3 h7	h30	h44	h1
P400		ompA$^+$								
P1668	2	ompA-12	R							
P1675	3	ompA-19	R							
P1676	2	ompA-20	R							
P1658	2	ompA-2	R							
P1673	2	ompA-17	R							
P1667	1	ompA-11	R	<0.01	<0.01	R	<0.1			
P1665	1	ompA-9	R	R	R	<0.01	<0.01		<0.1	<0.1
P1666	2	ompA-10	R	R	R	R	<0.01	<0.1	R	<0.1
P1672	1	ompA-16	R	R	R	R	R	R	<0.1	<0.1
P1662	2	ompA-6	R	R	R	R	R	R	R	<0.1
P1663	1	ompA-7	R	R	R	R	R	R	R	<0.01
P460	29	ompA-1	R	R	R	R	R	R	R	R

(TABLE 5-1 Cont....)

a. All mutants were shown to map at ompA by cotransduction with pyrD (see Table 5-2).

b. EOP, efficiency of plating; the blank spaces indicate an EOP of >0.1 with respect to P400; R, resistant (EOP $<10^{-6}$).

MAP POSITION OF THE MUTANTS

All of the mutations were shown to be linked to *pyrD* at 21.5 min on the linkage map (Bachmann *et al.*, 1976), by cotransduction into strain W620. The co-transduction frequencies were comparable for all the classes of *ompA* mutants as listed in Table 5-2.

COLICIN RESISTANCE OF THE MUTANTS

Of the nineteen colicins tested the *ompA* mutants were only affected by colicins K and L. The variability of the resistance of the mutants to these colicins is shown in Table 5-3.

RECIPIENT-ABILITY IN CONJUGATION OF THE MUTANTS

The recipient ability of the mutants with an F'*lac pro* donor (Table 5-3) shows there is a general reduction, although in most cases it remains at a level greater than that observed in mutants such as P460, an extreme *ompA* mutant.

OUTER MEMBRANE PROTEIN COMPOSITION OF THE MUTANTS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

In order to make it easier to compare the outer membranes of the mutants on polyacrylamide gels, *tsx*

TABLE 5-2

Cotransduction frequencies of the mutants with *pyrD*.

Strain	Transductants[a]		Cotransduction (%)
	ompA *pyrD*$^+$	*pyrD*$^+$	
P1668	4	10	40
P1675	23	42	55
P1676	11	24	46
P1658	19	43	44
P1673	12	25	48
P1667	28	50	56
P1665	10	37	27
P1666	13	38	34
P1672	18	37	49
P1662	17	32	53
P1663	20	43	47
P460	19	43	44

a. Transductants were scored for *glt*, *rpsL* and *gal*, as well as for resistance to bacteriophage K3.

TABLE 5-3

Colicin tolerance and recipient ability of the mutants at 37°C.

Strain	Resistance to colicin[a]		Recipient ability[b] with F'lac pro
	K-235	L-JF246	
P400	S	S	1.0
P1668	S	S	0.066
P1675	P	P	0.050
P1676	S1	P	0.0004
P1658	P	R	ND[c]
P1673	P	R	0.012
P1667	S	S	0.39
P1665	P	R	0.016
P1666	P	S	0.023
P1672	S1	R	0.011
P1662	P	R	0.009
P1663	P	R	0.004
P460	P	R	0.0002

a. S, sensitive; S1, slight resistance; P, partially resistant; R, resistant, S<S1<P<R.

(TABLE 5-3 Cont....)

b. Recipient ability is measured with respect to the number of input donor cells and is expressed as a fraction of the parent strain P400 (taken as 1.0) that gave a mean transfer of 29% for the series. Each result was the mean of at least three independent matings.

c. ND, not detectable.

mutants of all the mutants were obtained by selecting for resistance to bacteriophage T6 (see Chapter 6).

In most cases, except for P1667, no readily detectable protein 3A was found (Figure 5-1: P1668 is used as an example). This was determined by measuring the amount of heat modifiable peak C on Maizel gels using unheated and heated samples. In no case were any extra peaks, which may have corresponded to an altered protein, detected. However, any proteins below about 15,000 daltons run in with the dye front and are not resolved. In the case of P1667, normal amounts of peak C were detected (Figure 5-1) which appeared to run in the position identical to that normally occupied by protein 3A. Samples of outer membranes from a tsx mutant of strain P1667 were run mixed with equal amounts of outer membrane from the parent strain, P400 and its tsx derivative, strain P407. There was no detectable difference in the shape and size of peak C using the mixtures as compared to the strains alone, which indicates that any alteration in protein 3A of P1667 is such as to have no effect on its mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis under these conditions. On Bragg and Hou gels (Figure 5-2) a greatly reduced peak 3 was observed in most mutants (P1668 is again used as an example), with the exception of P1667, which has

FIGURE 5-1: Densitometer tracings of sodium dodecyl sulphate polyacrylamide gels of outer membrane preparations of P407 (a tsx mutant of P400, the parent strain) and tsx mutants of the ompA mutants P1668 and P1667 run with the pH 7.2 buffer system of Maizel (1966) with unheated and heated samples. tsx mutants were used to simplify the interpretation of the gels (see Chapter 6). Peaks are labelled according to Schnaitman (1974a).

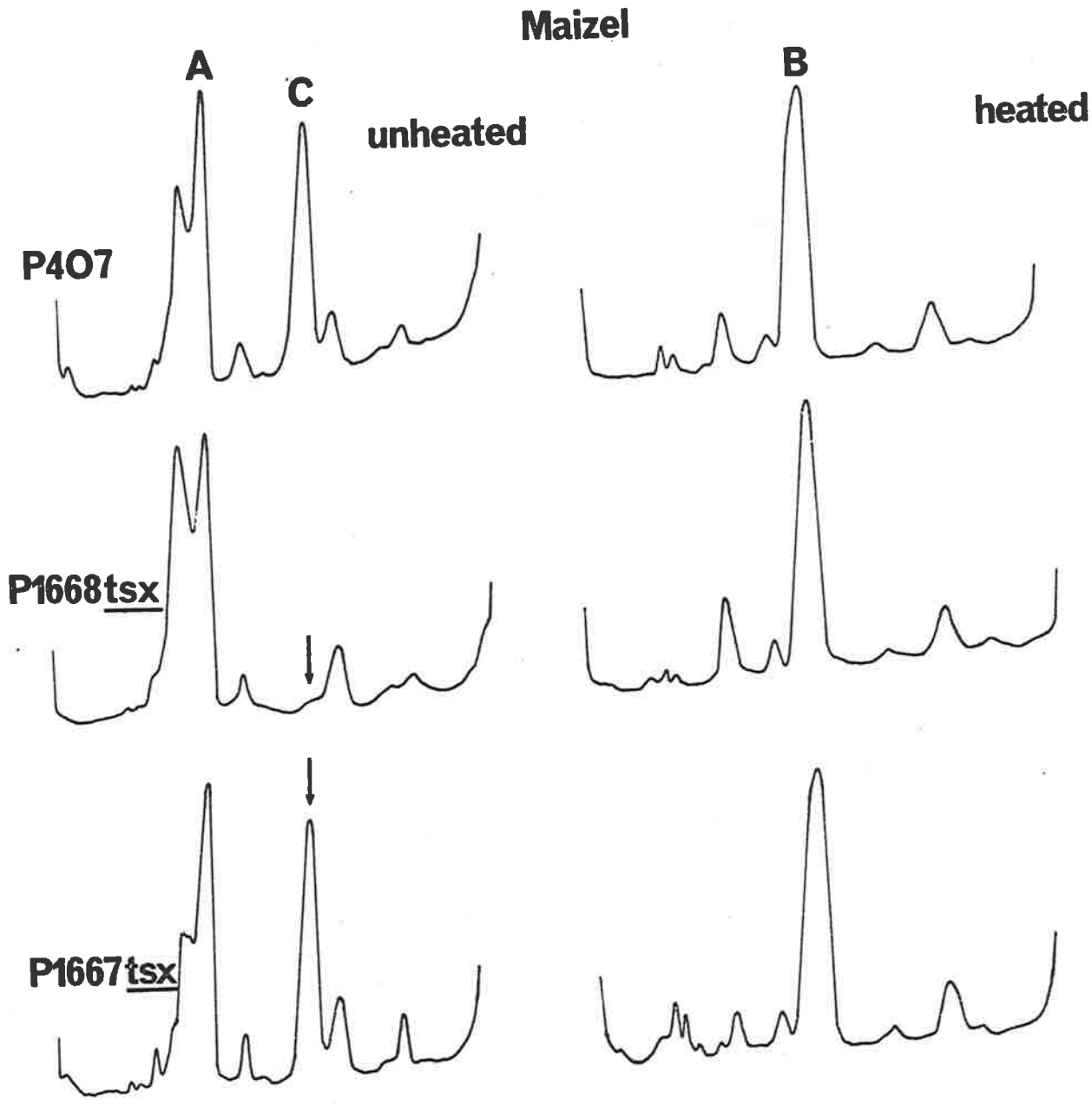
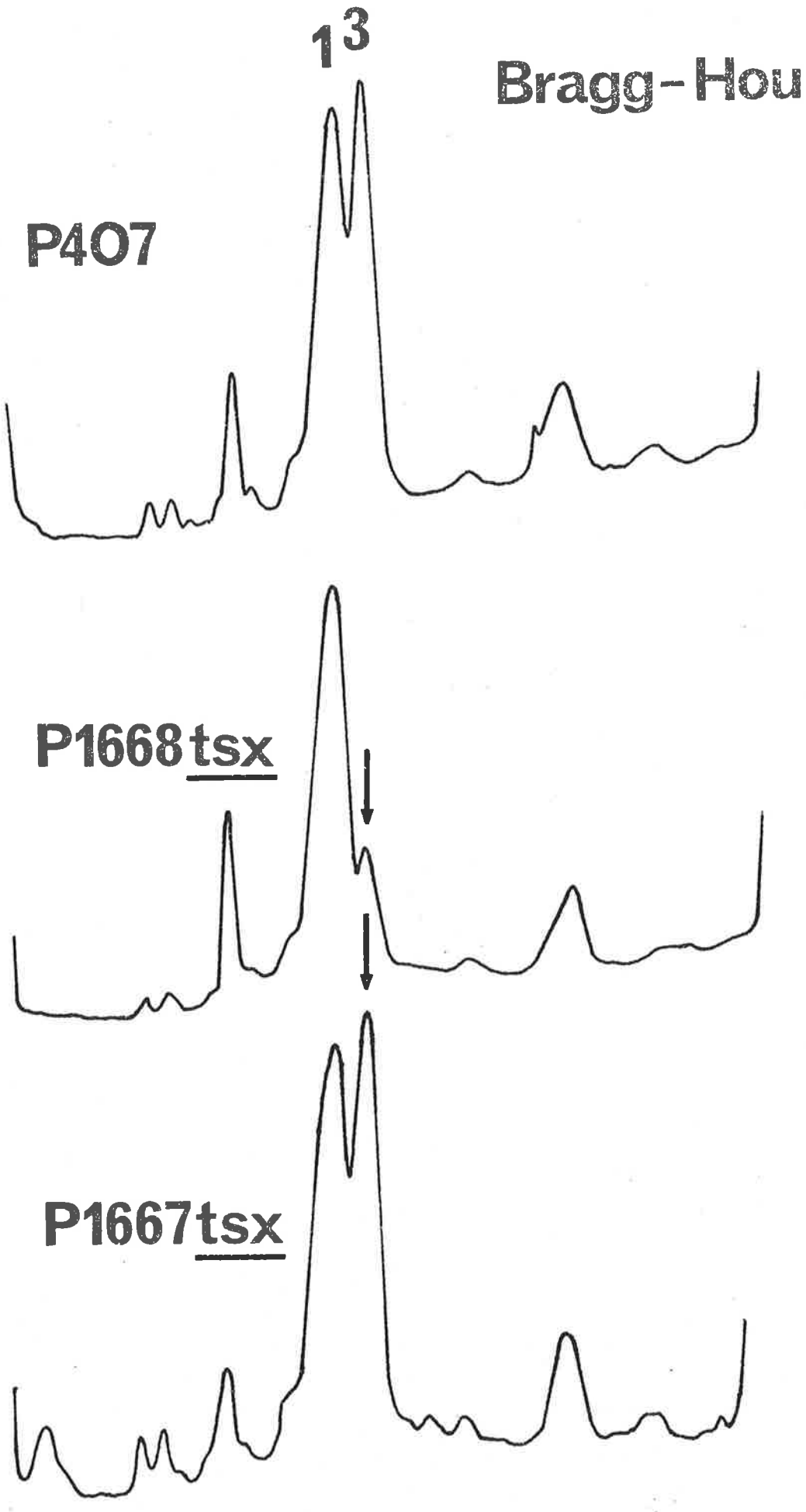

Maizel
A
C
unheated
B
heated
P407
P1668tsx
P1667tsx

FIGURE 5-2: Densitometer tracings of sodium dodecyl sulphate polyacrylamide gels of outer membrane preparations of P407 (a tsx mutant of P400, the parent strain) and tsx mutants of the ompA mutants P1668 and P1667 run with the pH 11.4 buffer system of Bragg and Hou (1972) using heated samples. Peaks are labelled according to Schanitman (1974a).


1 3
Bragg-Hou
P407
P1668 tsx
P1667 tsx

a normal peak 3.

All of the mutants isolated as resistant to bacteriophage K3 were confirmed to map at ompA, at 21.5 min on the E. coli K-12 linkage map as shown by cotransduction with pyrD. Of all the mutant classes studied, about 40% of the ompA mutants are able to plaque at least one of the extended host range phages. Since it is known that the wild type phage can use protein 3A as its receptor (Chapter 4), it is thought that all of these ompA mutants have some protein 3A present, although at undetectable levels except in strain P1667. It can be seen from Table 5-1 that, with some exceptions, the activity spectra of the K3h$^-$ mutants allows the ompA mutants to be arranged in an order of increasing phage resistance and the K3h$^-$ mutants in an order of increasing potency. This suggests that for both the bacterial and phage mutant groups the alterations are of one type but vary in degree.

It is thought that the different groups of ompA mutants differ in the amount of protein 3A present and that the bacteriophages differ in the amount of receptor that must be present for them to be able to form plaques.

NEUTRALIZATION OF EXTENDED HOST RANGE BACTERIOPHAGES AND PURIFICATION OF PROTEIN 3A FROM AN *ompA* MUTANT

Outer membrane from strains P400, P460 (*ompA-1*) and P1667 (*ompA-11*) were tested for neutralization of the extended host range phages (Table 5-4). All the phages resembled the wild type phage K3 in being neutralized by very small amounts of outer membrane from strain P400, but were not neutralized by outer membrane from P460 (*ompA-1*). Neutralization of these phages required high amounts of outer membrane from strain P1667 (*ompA-11*), whereas the wild type phage was not neutralized. Clearly the receptor activity of outer membrane from strain P1667 is altered although it contains wild type levels of protein 3A. The neutralizing ability of the outer membrane of strain P1667 for different extended host range phages did not correlate, however, with the activity spectra of the phages shown in Table 5-1.

Protein 3A from strain P1881 (*ompB*, *tsx*, *tonA*, *ompA-11*) was purified using the same procedure as described in Chapter 4. The purified protein was unable to neutralize bacteriophage K3 (not neutralized by 1000 µg/ml under standard conditions) and hence clearly differs from the protein 3A of an *ompA*$^{+}$ strain.

TABLE 5-4

Neutralization of extended host range mutants of bacteriophage K3 by outer membrane

Bacteriophage K3 extended host range mutant	Source of outer membrane		
	P400	P460	P1667
K3(h^+)	1[a]	>1000	>1000
h1	1	>1000	500
h3	1	>1000	100
h4	1	>1000	500
h5	1	>1000	500-1000
h15	1	>1000	100
h23	1	>1000	>1000
h30	1	>1000	500
h40	5	>1000	100-500
h44	1	>1000	1000
h47	1	>1000	500

a. Figures represent the lowest concentration (in µg/ml contained protein) of outer membrane material which gave 50% neutralization of the 10^3 bacteriophages used in the assay: the concentrations of material used were 1000, 500, 100, 50, 10, 5, 1 and 0.1 µg/ml.

However, although the extended host range phage K3h1 plaques with reasonable efficiency on ompA-11 strains, this phage was not significantly neutralized (<10% neutralization with 1000 µg/ml) by protein 3A from strain P1881.

Thus the protein 3A present in ompA-11 strains is altered in its biological properties confirming that ompA is the structural gene for protein 3A.

TEMPERATURE SENSITIVE ompA MUTANTS

Amongst the ompA mutants described it has been possible to demonstrate the existence of a class which is temperature sensitive.

The efficiencies of plating of the bacteriophages on these mutants is shown in Tables 5-5 and 5-6. The mutant P1670 (ompA-14), not described before, was similar to P1675 (ompA-19) in its properties at 37°C. It can be seen that at 42°C the ompA mutants with the exception to the extreme ompA mutant P460, which has been used as a control, have become sensitive to wild type bacteriophage K3. However, if one also looks at the efficiencies with which they plate the extended host range phage, in general, only minor changes are observed.

TABLE 5-5

Temperature sensitivity to bacteriophage K3.

Strain	EOP of wild type[a] K3 (h^+)	
	30°C	42°C
P400	1.0	1.0
P1658	R	0.44
P1670	R	0.035
P1675	R	0.43
P1676	R	0.32
P460	R	R

a. EOP, efficiency of plating; R, resistant (EOP $<10^{-6}$).

TABLE 5-6

Temperature sensitivity to some of the extended host range mutants of bacteriophage K3.

Strain	EOP of extended host range mutants[a]											
	h1		h3		h30		h40		h44		h47	
	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
P400	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
P1658	0.65	0.29	0.49	0.65	0.65	0.54	0.55	0.41	0.74	0.63	0.58	0.18
P1670	0.67	0.14	0.38	0.45	0.63	0.44	0.29	0.38	0.46	0.38	0.48	0.36
P1675	0.67	0.21	0.52	0.48	0.55	0.58	10^{-4}	0.42	0.18	0.31	0.38	0.31
P1676	0.44	0.11	0.37	0.27	0.37	1.20	0.32	0.31	0.31	0.28	0.42	0.62
P460	R	R	R	R	R	R	R	R	R	R	R	R

a. EOP, efficiency of plating; R, resistant (EOP $<10^{-6}$).

The only exception is for P1675 with K3h40 which has shown the same trend as observed with wild type K3.

When the recipient abilities of these mutants with an F'lac pro donor were examined, strains P1670 (ompA-14) and P1675 (ompA-19) again showed a temperature sensitive effect, becoming better recipients at 42°C (Table 5-7). This was most pronounced with strain P1675. These two mutants also showed the same trend with their colicin tolerance patterns (Table 5-7), becoming more sensitive at 42°C.

The protein composition of the outer membranes of a tsx derivative of one of these mutants, P1675, and P407 (a tsx mutant of P400, the parent strain of P1675) were examined and are shown in Figures 5-3, 5-4.

From the Maizel gels (Figure 5-3) it can be seen that P1675 has no detectable protein 3A at 30°C, but that significant amounts are present at 42°C, although not the full normal amount. The ompA-1 mutant P460 showed no protein 3A at either temperature (not shown) whereas the parent strain P400 has the protein at both temperatures.

TABLE 5-7

Recipient ability in conjugation and colicin tolerance at 30°C and 42°C.

Strain	Sensitivity to colicins[a]				Recipient ability[b] with F'lac pro	
	K-235		L-JF246			
	30°C	42°C	30°C	42°C	30°C	42°C
P400	S	S	S	S	1.0	1.0
P1658	P	P	R	R	nd[c]	nd
P1670	P	P	R	P	0.0074	0.068
P1675	P	S	R	P	0.0062	0.303
P1676	P	P	R	P	nd	nd
P460	P	P	R	R	0.0003	0.0003

a. S, sensitive; S1, slight resistance; P, partial resistance; R, resistant; S<S1<P<R.

b. Recipient ability was measured with respect to the number of input donor cells and is expressed as a fraction of the parent strain P400 that gave a mean transfer of 22.5% at 30°C and 29.0% at 42°C. Each result is the mean of three matings.

(TABLE 5-7 Cont....)

c. nd : not detectable.

FIGURE 5-3: Densitometer traces of sodium dodecyl sulphate polyacrylamide gels run using the pH 7.2 buffer system of Maizel (1966), comparing a tsx mutant of P1675 with P407 (a tsx mutant of P400, the parent strain of P1675). Unheated and heated preparations are compared of outer membranes of the bacteria grown at 30°C and 42°C in nutrient broth.

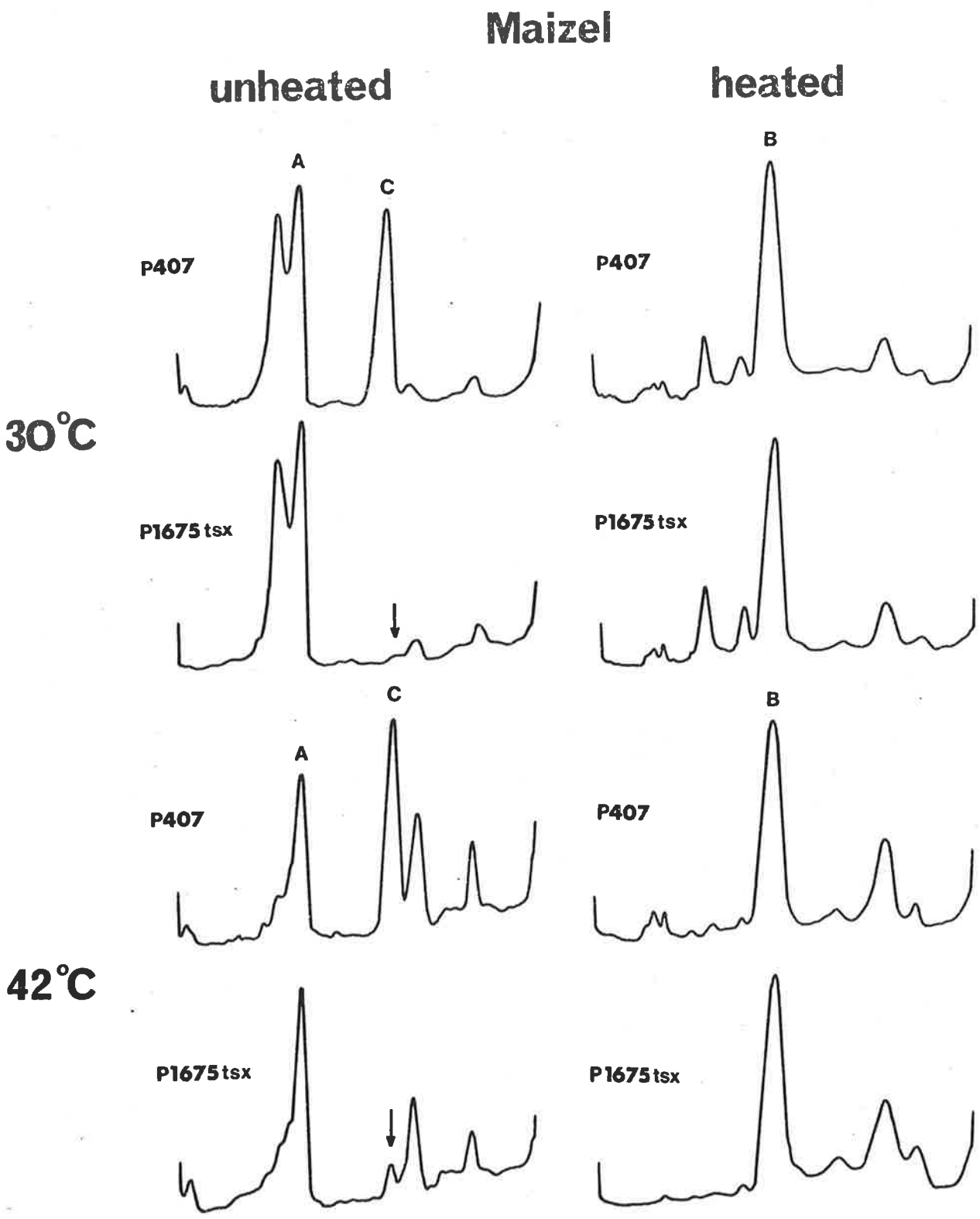
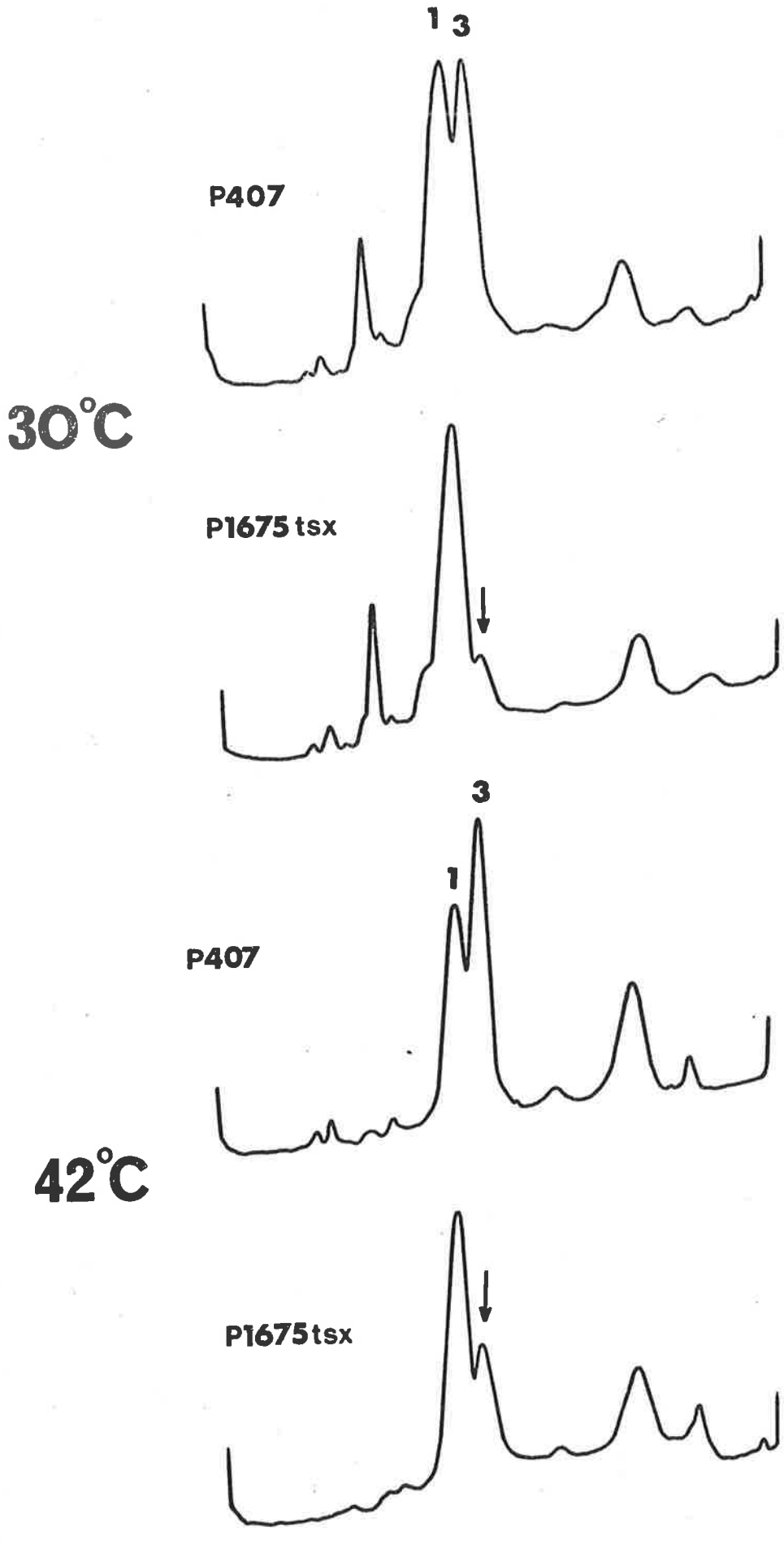

Maizel
unheated
heated
30°C
42°C
A
C
B
P407
P1675 tsx

FIGURE 5-4: Densitometer traces of sodium dodecyl sulphate polyacrylamide gels run using the pH 11.4 buffer system of Bragg and Hou (1972), comparing a tsx mutant of P1675 with P407 (a tsx mutant of P400, the parent strain of P1675). Heated outer membrane preparations are compared of the bacteria grown at 30°C and 42°C in nutrient broth.

Bragg-Hou



SUMMARY AND CONCLUSIONS

It has been demonstrated that by using extended host range mutants of bacteriophage K3, it is possible to obtain ompA mutants with different degrees of alteration in their ability to plate the extended host range phage, in their tolerance to cclicins K and L and in their recipient ability in conjugation. Since protein 3A is the receptor for bacteriophage K3 it is concluded that these mutants have different amounts of an altered protein 3A; in most cases this is undetectable but in the case of strain P1667 it is produced in wild type amounts and has been shown to be altered. The demonstration of an ompA mutant with a protein 3A altered in its biological properties is extremely good evidence that ompA is the locus for the structural gene for protein 3A. The importance of protein 3A in conjugation is further supported by the increase in recipient ability correlated with an increase in the amount of protein 3A as shown in a temperature sensitive ompA mutant.

CHAPTER 6

THE TSX PROTEIN: THE RECEPTOR FOR BACTERIOPHAGE T6 AND COLICIN K

INTRODUCTION

As a result of the cross resistance studies between colicins by Fredericq (1949), the loss of specific surface receptors was postulated to account for mutant bacteria being resistant to some colicins while remaining sensitive to others. Such studies with bacteriophages and colicins suggested that certain bacteriophages and colicins shared these receptors (Fredericq, 1953, 1956). It was observed that bacteria which were sensitive to colicins E, M or K were always sensitive to bacteriophages BF23, T1 or T6 respectively, and also that cells which were resistant to one of the colicins or bacteriophage were always resistant to the corresponding bacteriophage or colicin (Fredericq, 1953, 1956). The conclusions which have been drawn from these cross resistance studies have subsequently been confirmed by the identification and isolation of proteins which function as a receptor for both the E colicins and bacteriophage BF23 (Sabet and Schnaitman, 1973; Bradbeer, Woodrow and Khalifah, 1976) and another which functions for bacteriophage T5 and colicin M

(Braun and Wolff, 1973; Braun, Schaller and Wolff, 1973). However the bacteriophage T6 and colicin K receptor has not been identified.

Sabet and Schnaitman (1971) have shown that the receptor activity for colicin K is found in the Triton X-100 plus EDTA soluble fraction of the outer membrane. It has also been suggested that bacteriophage T6 used a protein containing receptor located in the outer lipid-protein layer of the cell envelope (Beumer, Beumer-Jochmanns, Dirkx and Dekegel, 1965, 1966). However, Weltzien and Jesaitis (1971) have found some differences in the effects of a number of chemical agents on colicin K and bacteriophage T6 receptor activities suggesting that they may differ.

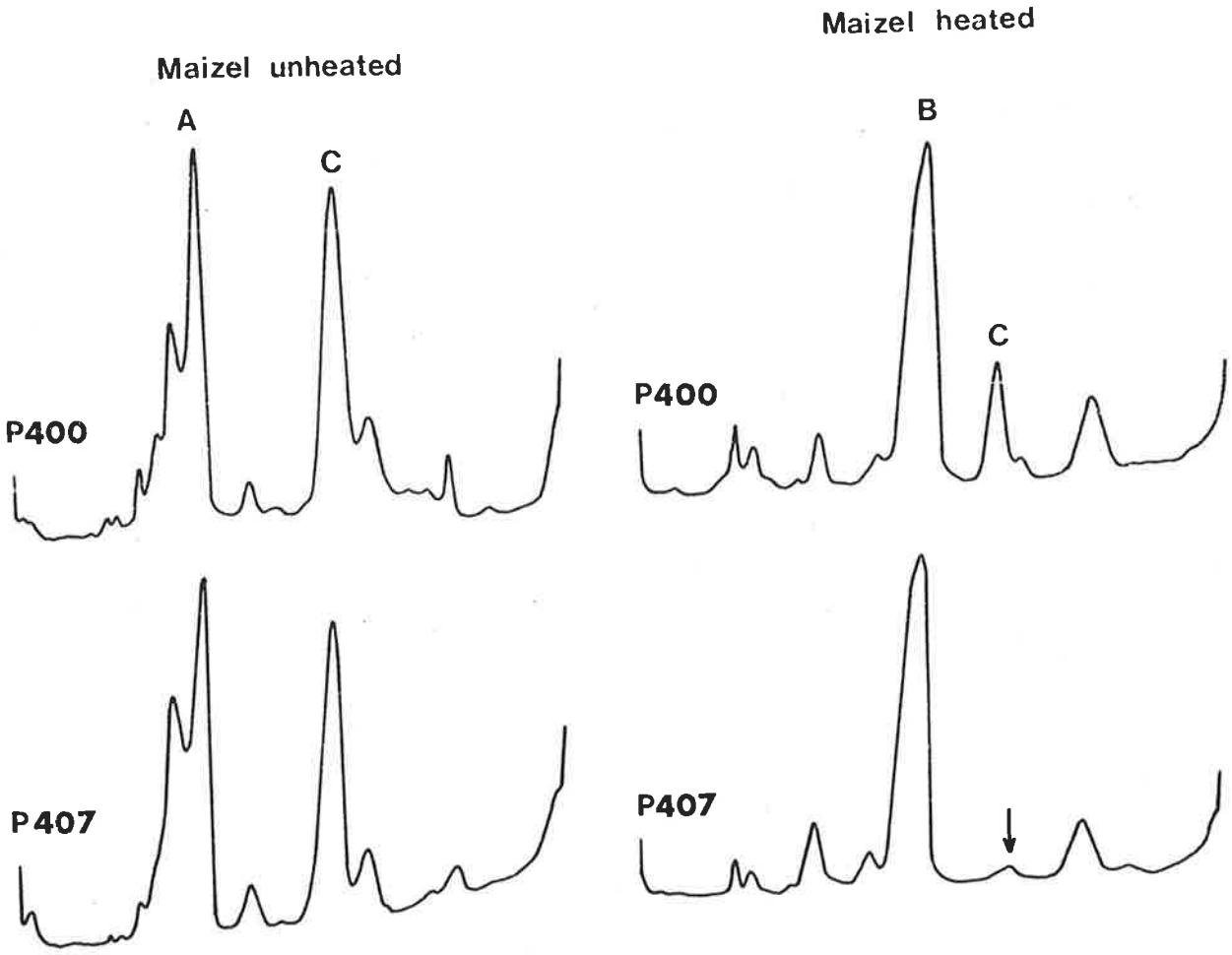
Recent cross resistance studies of bacteriophages (Hancock and Reeves, 1975) and colicins (Davies and Reeves, 1975b) have also demonstrated the highly specific nature of the tsx mutation (simultaneous resistance to colicin K and bacteriophage T6). It was decided to determine the nature of the bacteriophage T6 receptor as it appeared to be an outer membrane protein, and so tsx mutants would be a suitable control for the experiments to be described in Chapter 7.

TSX MUTANTS LACK AN OUTER MEMBRANE PROTEIN

When the outer membranes of a number of tsx mutants in different genetic backgrounds were examined by SDS-polyacrylamide gel electrophoresis, a protein (called the tsx protein) was not detected in these mutants. This protein is not modified upon heating. The loss was only detectable on Maizel gels using heated samples, in which case there is no detectable residual peak C, or on Bragg and Hou gels where a peak running faster than peak 3 was absent (Figure 6-1). However, if an ompA mutant was used (Figure 6-2) it could be seen that on Maizel gels there was again no peak C and this could be seen with both unheated as well as heated samples. The tsx protein therefore corresponded to that portion of peak C which did not move upon heating. This suggests that the protein becomes fully denatured by SDS treatment at 37°C, whereas the major proteins (1 (a and b), 3A and 3B) require boiling to be fully denatured. Under all the conditions in Figures 6-1 and 6-2 the tsx protein moves with an apparent molecular weight of 26,000 daltons (Figure 6-3).

LOSS OF RECEPTOR ACTIVITY IN TSX MUTANTS, ISOLATION OF EXTENDED HOST RANGE MUTANTS OF BACTERIOPHAGE T6 AND

FIGURE 6-1: Comparison by SDS polyacrylamide gel electrophoresis of the outer membranes of strains P400 and its tsx mutant P407 grown at 37°C in nutrient broth. Gels were run using the pH 7.2 buffer system of Maizel (1966) with both unheated and heated samples, and the pH 11.4 buffer system of Bragg and Hou (1972) with heated samples. Peaks are labelled according to Schnaitman (1974a).


Maizel unheated
A
C
P400
P407
Maizel heated
B
C
P400
P407

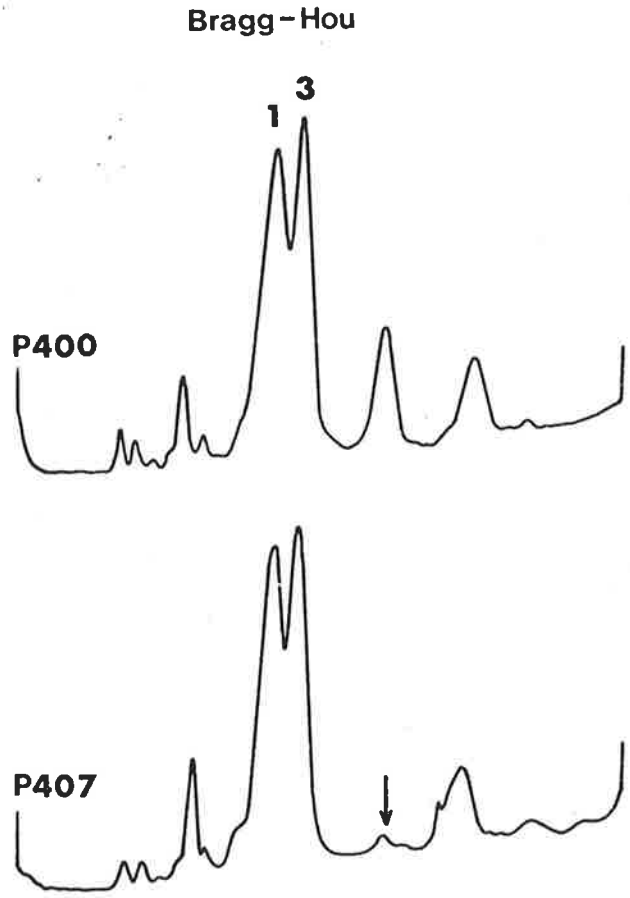
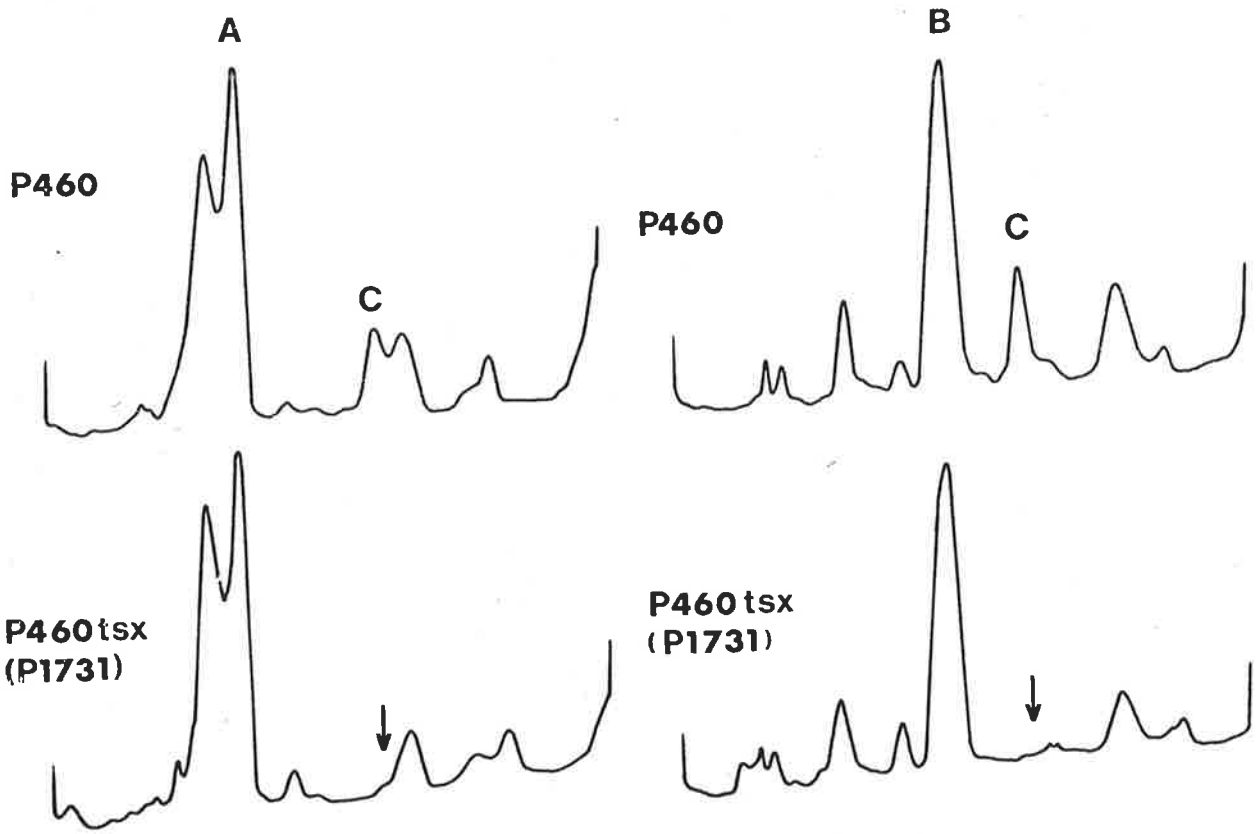

Bragg-Hou
1
3
P400
P407

FIGURE 6-2: Comparison by SDS polyacrylamide gel electrophoresis of the outer membranes of strains P460 (ompA-1) and its tsx derivative P1731 grown at 37°C in nutrient broth. Gels were run using the pH 7.2 buffer system of Maizel (1966) with both unheated and heated samples, and the pH 11.4 buffer system of Bragg and Hou (1972) with heated samples. Peaks are labelled according to Schnaitman (1974a).

Maizel unheated

Maizel heated



Bragg - Hou

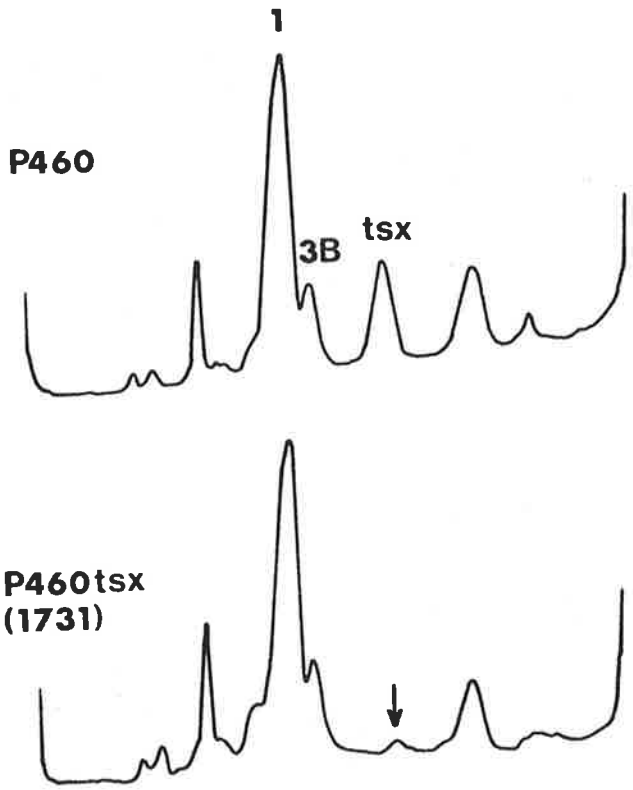
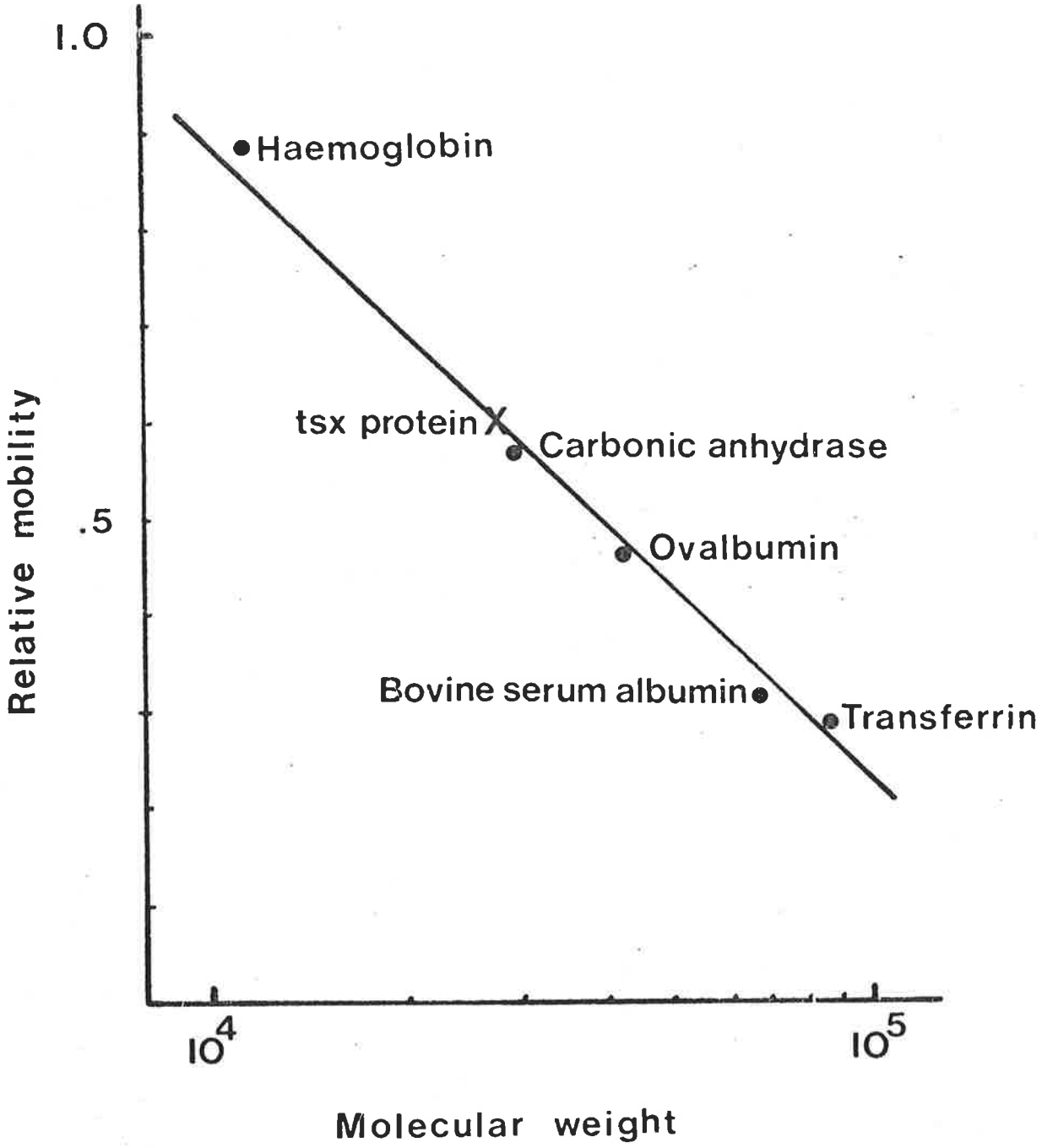


FIGURE 6-3: Molecular weight of the tsx protein as determined by SDS polyacrylamide gel electrophoresis using the pH 11.4 buffer system of Bragg and Hou (1972) with the following standard proteins: haemoglobin (15,500), carbonic anhydrase (29,000), ovalbumin (43,000), bovine serum albumin (68,000) and transferrin (86,000).


1.0
.5
Relative mobility
Haemoglobin
tsx protein
Carbonic anhydrase
Ovalbumin
Bovine serum albumin
Transferrin
10^4
10^5
Molecular weight

MUTANTS OF *E. COLI* K-12 WITH AN ALTERED TSX PROTEIN

In the same way that *ompA* mutants have been shown to have lost the receptor activity for bacteriophage K3, *tsx* mutants have been shown to have lost receptor activity for bacteriophage T6 and colicin K. The outer membrane has the highest specific activity for this receptor(s) and this is where the tsx protein is predominantly found. This activity can also be solubilized using EDTA in the presence of Triton X-100. Similar to the studies in Chapter 5, *tsx* mutants with an altered tsx protein and also extended host range mutants of the phage can be isolated. All *tsx* mutants isolated map at 9.1 min as determined by cotransduction with *proC* into strain χ342.

Full details of all these and other properties of *tsx* mutants have not been included because they are not directly relevant to the body of this thesis. However, in the next section it is shown that it is justified to say that the tsx protein is a common receptor for bacteriophage T6 and colicin K.

PURIFICATION OF THE RECEPTOR ACTIVITY FOR BACTERIOPHAGE T6 AND COLICIN K

From the studies in Chapter 4 it could be seen that the major outer membrane protein 3A co-chromatographed on DEAE-cellulose with the tsx protein. It was therefore decided to use an ompA mutant, lacking protein 3A, in the purification of the bacteriophage T6 and colicin K receptor. It has been previously shown (Hancock and Reeves, 1975; Davies and Reeves, 1975b; Hancock, Davies and Reeves, 1976) that there was no cross resistance between tsx mutants and ompA mutants although ompA mutants were tolerant to colicin K, that is, they still had the receptor on the cell surface.

When the TES component of the outer membrane of strain P460 was chromatographed on DEAE-cellulose the receptor activities for bacteriophage T6 and colicin K copurify (Figure 6-4), and these fractions also contain the tsx protein (Figure 6-5). Rechromatography on DEAE-cellulose or on QAE-Sephadex did not yield significant amounts of electrophoretically pure tsx protein. However, it was possible to detect near pure protein by chromatography of the P460 TES material on QAE-Sephadex with 0.005M NaCl in the buffer (Figures 6-6, 6-7), but this was only a fraction of the total tsx protein and receptor activities. The remainder was bound to the column.

FIGURE 6-4: DEAE-cellulose chromatography in TTE buffer pH 7.2 of the Triton plus EDTA soluble component (TES) of the outer membrane of strain P1677 (ompA-1, tonA-204). 650 mg of protein was applied to a 2.5 x 50 cm column. This material was obtained from 50 litres of bacteria grown in a fermenter under vigorous aeration at 37°C to a density of 7-8 x 10^8 cells/ml. 25 ml fractions were collected from the column and 2 ml of each was taken and ethanol precipitated for phage and colicin neutralization. Phage receptor activity is expressed as the reciprocal of the dilution required to give 50% neutralization of the bacteriophages under the assay conditions. Colicin receptor activity is expressed as the log of the fraction survival of the indicator bacteria added to the neutralization assay.

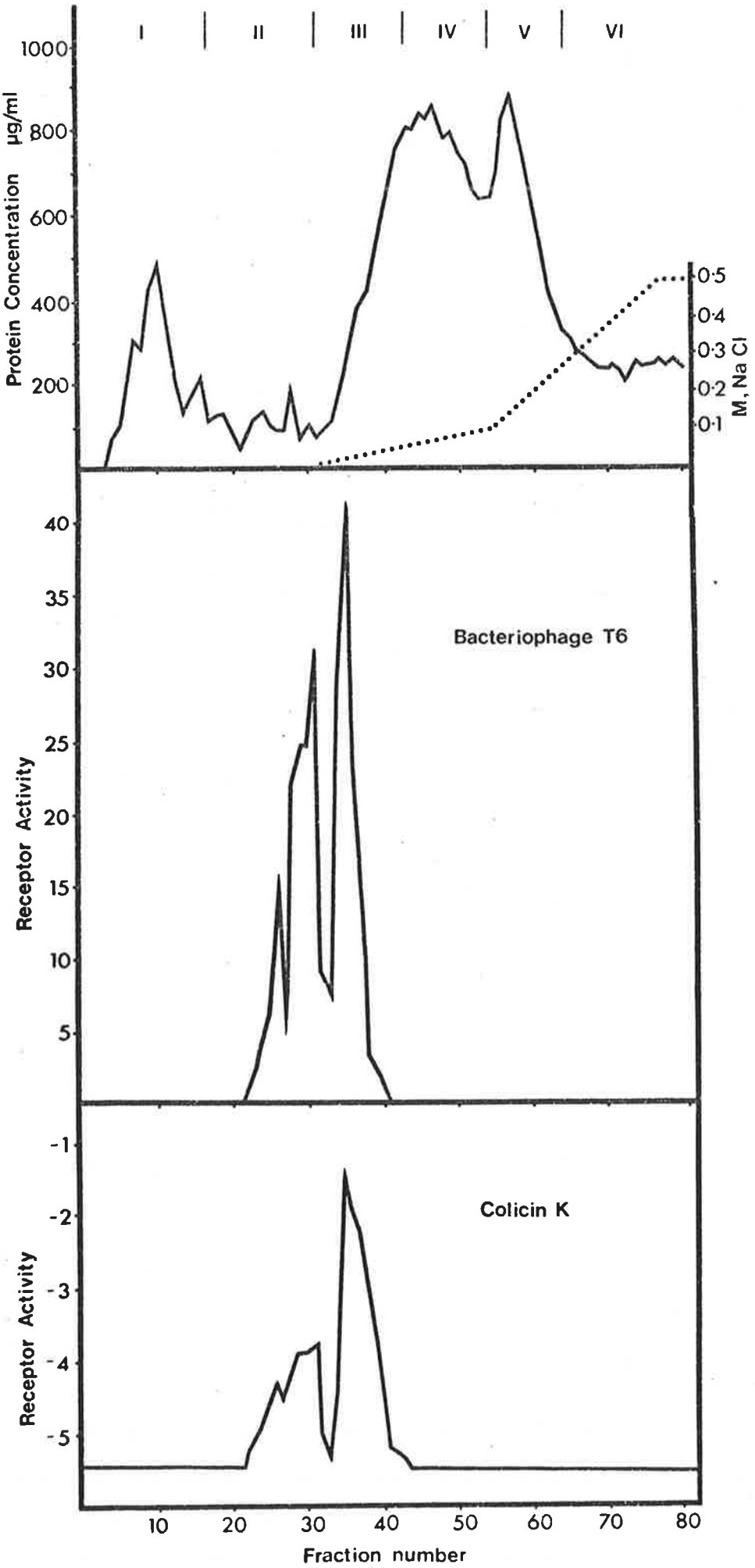

I
II
III
IV
V
VI
Protein Concentration µg/ml
1000
800
600
400
200
0·5
0·4
0·3
0·2
0·1
M, Na Cl
Bacteriophage T6
Receptor Activity
40
35
30
25
20
15
10
5
Colicin K
Receptor Activity
-1
-2
-3
-4
-5
10
20
30
40
50
60
70
80
Fraction number

FIGURE 6-5: Protein composition of the fractions of the DEAE-cellulose column in Figure 6-4 and pooled as shown. The proteins were analyzed on sodium dodecyl sulphate polyacrylamide gels using the pH 11.4 buffer system of Bragg and Hou (1972). Peaks are labelled according to Schnaitman (1974a). The dotted lines indicate the position of the tsx protein.

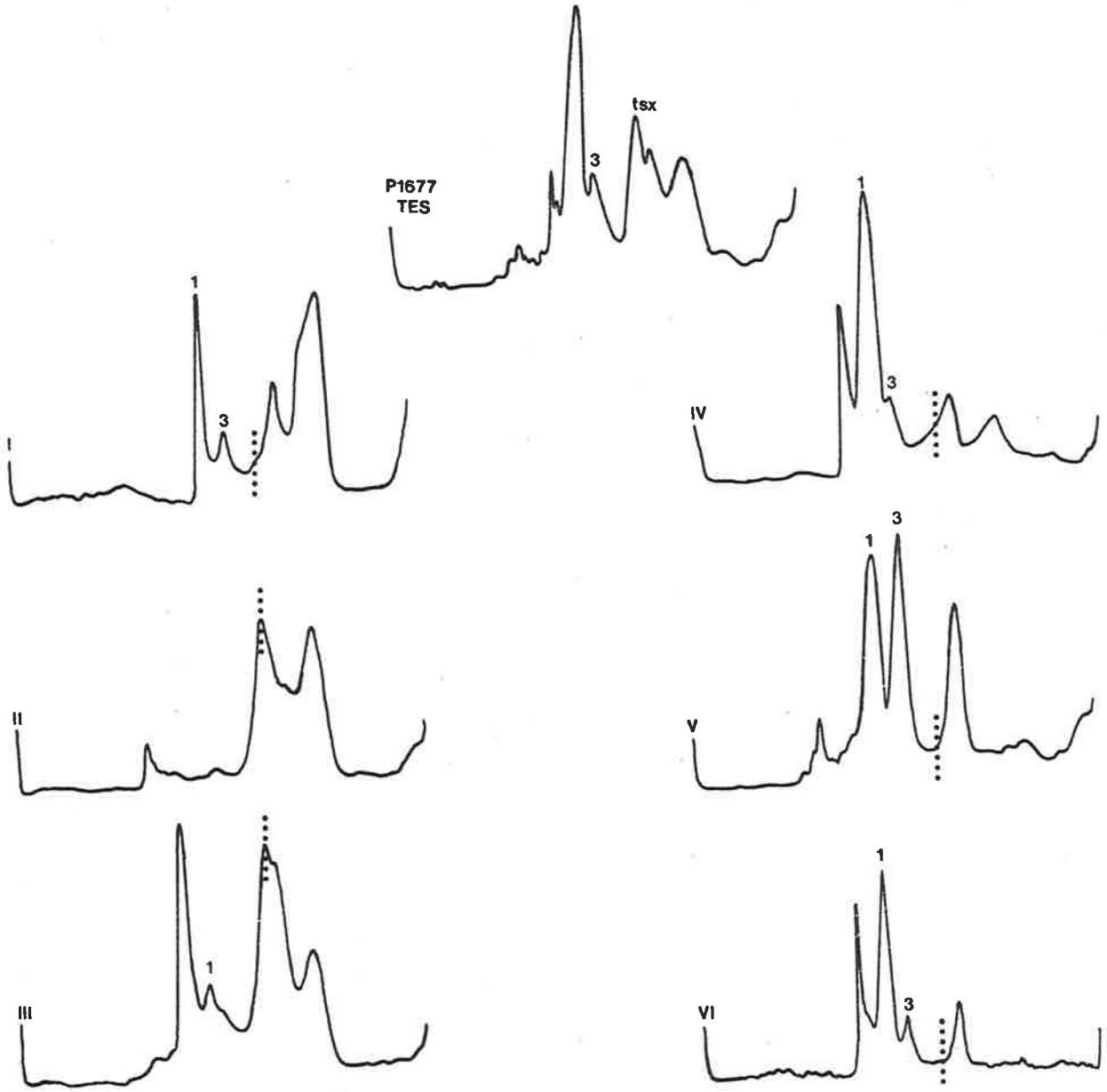

P1677
TES
tsx
3
I
1
3
II
III
1
IV
1
3
V
1
3
VI
1
3

FIGURE 6-6: QAE-Sephadex chromatography in TTE buffer (pH 8.0 containing 0.005M NaCl) of the TES component of the outer membrane of P460 (ompA-1). 105 mg of protein was applied to a 2.0 x 20 cm column. This was obtained from 10 litres of bacteria grown in 1 litre amounts with vigorous aeration at 37°C to a density of 7-8 x 10^8 cells/ml. 5 ml fractions were collected from the column and 1 ml of each was taken and ethanol precipitated for phage and colicin neutralizations. Only that part of the column prior to the addition of the salt gradient is shown. Phage receptor activity is expressed as the reciprocal of the dilution required to give 50% neutralization of bacteriophages under the assay conditions. Colicin receptor activity is expressed as the reciprocal of the dilution required to give 50% survival of the indicator strain in the assay.

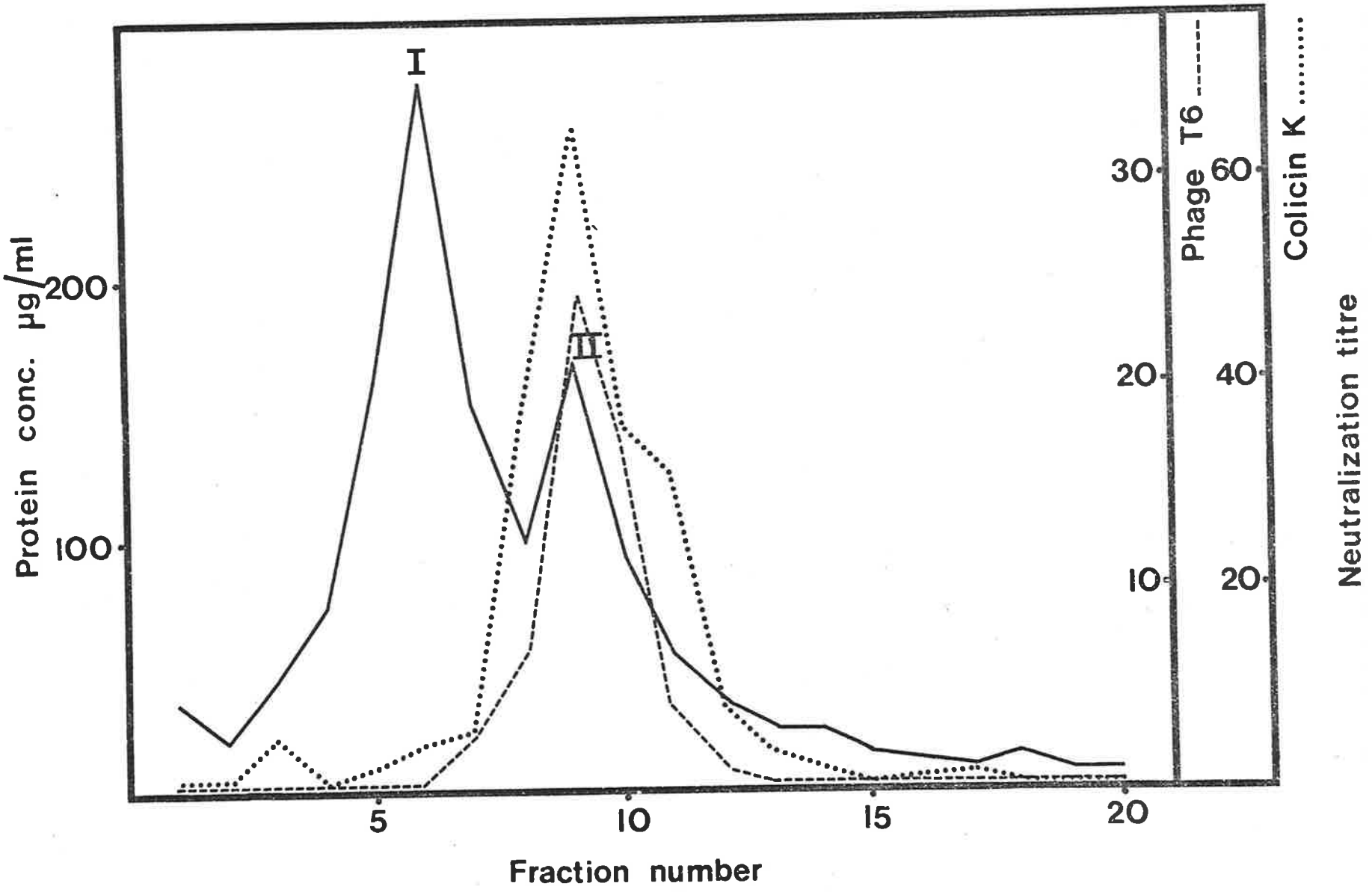
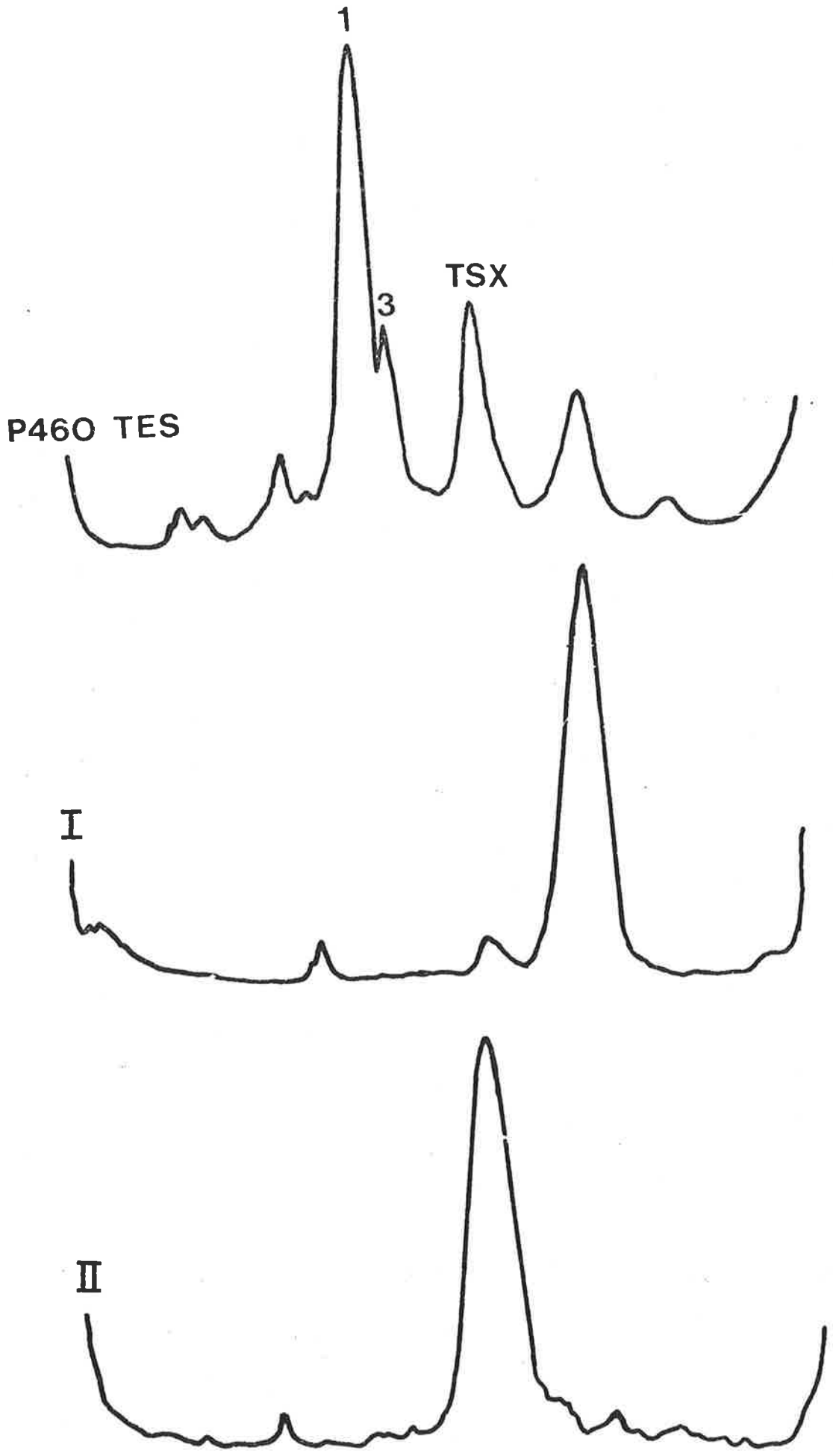

I
II
Protein conc. µg/ml
200
100
Phage T6 ------
Colicin K .....
30
20
10
60
40
20
Neutralization titre
5
10
15
20
Fraction number

FIGURE 6-7: Protein composition of the fractions labelled I and II of the QAE-Sephadex column shown in Figure 6-6. The proteins were analyzed on sodium dodecyl sulphate polyacrylamide gels using the pH 11.4 buffer system of Bragg and Hou (1972). Peaks are labelled according to Schnaitman (1974a).


1
3
TSX
P460 TES
I
II

The tsx protein has only been purified in significant amounts by chromatography in the presence of SDS. However, no receptor activity was detected in this material nor any other material which has been in SDS solutions.

Amongst the tsx mutants, which have been isolated and shown to be able to plaque extended host range mutants of bacteriophage T6, was a mutant with wild type levels of the tsx protein. When the TES component of the outer membrane of this mutant was chromatographed on DEAE-cellulose no in vitro receptor activity was detected.

All these analyses are very similar to the results obtained with ompA mutants, bacteriophage K3 and protein 3A. They all point to the tsx protein being the receptor for both bacteriophage T6 and colicin K and to tsx being the structural gene for this protein.

SUMMARY AND CONCLUSIONS

tsx mutants have been shown to be lacking or altered in an outer membrane protein (designated the tsx protein) of 26,000 daltons molecular weight. This protein is a major protein in the outer membrane of strain P400 which has been used in most of the studies in this thesis. This protein functions as the receptor for bacteriophage T6 and colicin K and is coded for by the *tsx* gene at 9.1 min.

CHAPTER 7

DEFECTIVE GROWTH FUNCTIONS IN *ompA* MUTANTS

INTRODUCTION

The outer membrane of *E. coli* contains a number of proteins and these have properties which may be divided into two types. All of these membrane proteins presumably have a primary function, that is, a function without which the cell would be disadvantaged to a greater or lesser extent. Primary functions can be attributed to only a limited number of the outer membrane proteins, although many also serve as receptors for bacteriophages and for colicins; however, these properties are usually regarded as secondary. Thus, the protein originally identified as a receptor for bacteriophage λ is now known to in some way allow maltose and maltotriose to diffuse through the outer membrane (Szmelcman *et al.*, 1976) and presumably this is its primary function. The receptor protein for bacteriophage BF23 and for colicins E2 and E3 has as its primary function an involvement in the uptake of vitamin B12 (Di Masi *et al.*, 1973; Bradbeer *et al.*, 1976). The receptor protein for bacteriophages T1, T5 and Ø80 and colicin M is involved in the uptake of iron complexed

as ferrichrome (Hantke and Braun, 1975; Luckey et al., 1975; Wayne and Neilands, 1975; Hancock and Braun, 1976) and the receptor protein for colicins B and D functions primarily as a receptor for iron complexed with enterochelin (Pugsley and Reeves, 1976a,b and 1977b).

Protein 3A may have as its primary function a role in recipient functions in conjugation, however, this seems unlikely for a protein present in such large amounts. Although the efficiency with which a cell conjugates with donors bearing F-like pili is related to the amount of protein 3A present (Chapter 5). Therefore ompA mutants, together with tsx mutants and an ompB mutant as controls, have been looked at under a variety of conditions to see if the loss of protein 3A has any specific effects on the physiology of the cell.

For this work several mutants were isolated. The mutant strain P1774 (an ompA-48 derivative of P801) was obtained by selecting for resistance to bacteriophage K3. This mutant was also resistant to K3h1, the most potent of the extended host range mutants of bacteriophage K3 (Chapter 5) and had no detectable protein 3A in its outer membrane. P1774 was defective as a recipient in conjugation with the F-like R-factor R538Fdrd-1 (3% of the level of the control

strain P801) and competent as a recipient with the I-like R-factor R64-11 (91% of control level) and with the F-like R-factor R100-1 (105% of control level). The mutation was 46% cotransducible with the pyrD mutation in strain W620 (37 ompA$^-$ pyrD$^+$/84 pyrD$^+$). Thus P1774 was typical of the extreme ompA mutants.

The tsx mutant P1773 was selected as resistant to bacteriophage T6 and in addition was fully resistant to only colicin K. By polyacrylamide gel electrophoresis it was shown to lack the tsx protein (Chapter 6), and was 56% cotransducible with the proC mutation in strain χ 342 (40 tsx$^-$ proC$^+$/71 proC$^+$). Thus P1773 was a typical tsx mutant.

The ompB mutant P1578 was selected as resistant to colicin L-JF246 and was typical in its colicin and bacteriophage resistance pattern to the Tol XIV type mutants (Davies and Reeves, 1975; Hancock, Davies and Reeves, 1976). It was also typical in having no protein 1 in its outer membrane (Davies, 1974) and has been shown to map at the ompB locus (V. Sarma, personal communication).

GROWTH OF *ompA* MUTANTS UNDER DIFFERENT CONDITIONS

When strain P460 (*ompA-1*) is grown in minimal medium A with glucose as sole carbon source and compared with its parent P400, there is a substantially longer lag period before the absorbance or optical density at 625 nm (OD) begins to rise (Figure 7-1A). The growth rate in logarithmic phase of P460 was also slightly reduced and after completion of growth the OD declined, whereas that of P400 remained steady. The decline in OD was accompanied by a substantial drop in viable count (Figure 7-1B). The rapid onset of this decline can be better seen in the same medium but with a 5 times concentration of the required amino acids (Figure 7-2). In Figure 7-1B, the "resting" phase cells used for the inoculum of strain P460 in fact had about five fold less viable cells than the P400 inoculum and this may account for the larger lag before the OD rises. P1578 (*ompB*) and P407 (*tsx*) had growth curves indistinguishable from that for P400: the lag period was short; they reached a similar plateau level and there was no decline either of OD or viable count. Thus the effect appears to be specific to the *ompA* locus.

A number of other *ompA* mutants, described in Chapter 5, were also studied and in general showed both

FIGURE 7-1: Comparison at 625 nm by optical density (A) and viable cell count (B) of growth of P400 (●) and its ompA-1 mutant P460 (O) at 37°C on minimal medium A containing growth factors at 20 µg/ml and 10 mM glucose as carbon source.

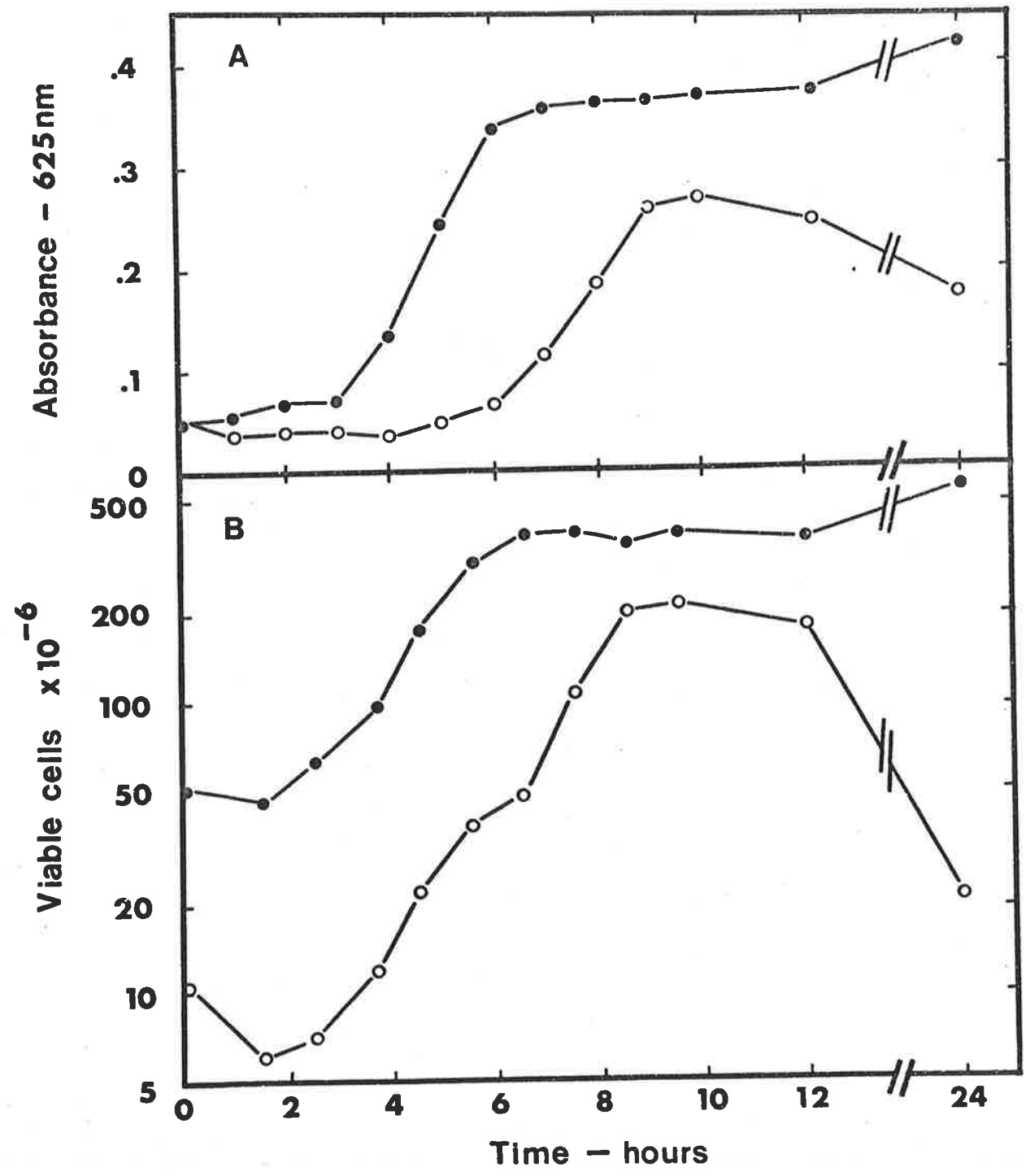
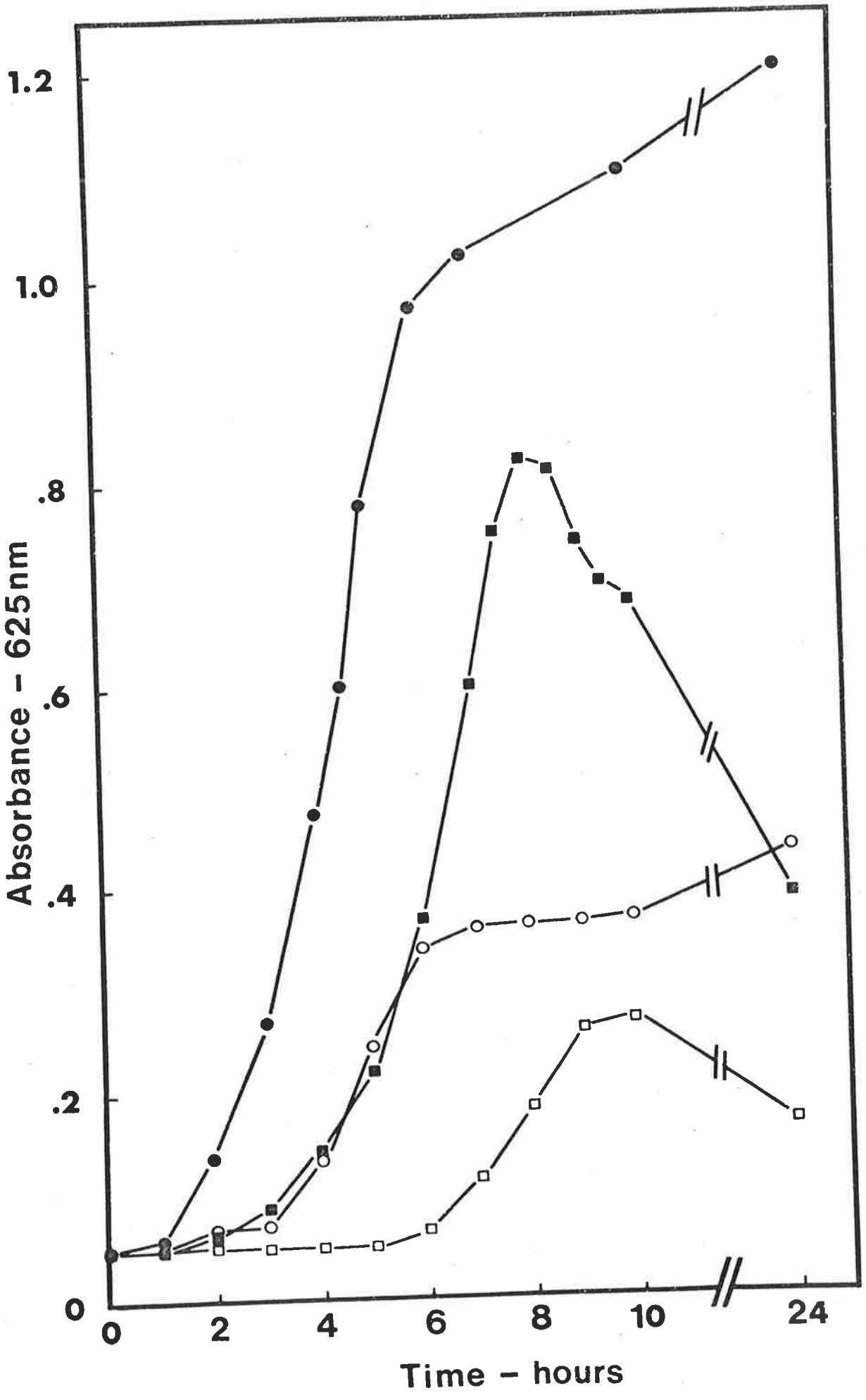

A
B
Absorbance – 625nm
.4
.3
.2
.1
0
500
200
100
50
20
10
5
Viable cells x 10⁻⁶
0
2
4
6
8
10
12
24
Time – hours

FIGURE 7-2: Comparison by optical density at 625 nm of P400 (●, ○) and its ompA-1 mutant P460 (■, □) grown at 37°C in minimal medium A with 10 mM glucose and growth factors at 20 µg/ml (○, □) and at 100 µg/ml (●, ■).


1.2
1.0
.8
.6
.4
.2
0
Absorbance - 625nm
0
2
4
6
8
10
24
Time - hours

an extended lag and a decline phase. The growth rate tended to be lower than that of P400 but with the exception of P1658 (ompA-2) the reduction was barely significant (Table 7-1). The lag in OD rise varied considerably amongst strains but this is most likely due to the proportion of viable cells in the inoculum rather than indicating any specific variation in the strains. Although in strain P1667 (ompA-11) which does not have an uptake defect there is still a small but detectable lag, suggesting that there is an additional factor involved in the growth of the ompA mutants. The major variation observed was with strains P1667 and P1668 which showed the least extension of lag phase and exhibited zero or very slight decline respectively over 24 hr. It is of interest that P1668 is the least affected of the ompA mutants in respect to phage sensitivity and P1667 has wild type amounts of an altered protein 3A.

Data for P1667 and P1666 (which shows an intermediate lag length) and P400 and P460 are shown in Figure 7-3.

The effect of the ompA mutation varies in degree according to the medium used (Table 7-2) but in no case were the ompB or tsx mutants affected.

TABLE 7-1

Rates of growth and uptake of glutamine in *ompA* mutants with different patterns of resistance to extended host range mutants of bacteriophage K3.

Strain No.	*ompA* allele number	Other relevant markers	Mid log[a] doubling time (min)	Rate of uptake of glutamine (% of control)
P400	*ompA*$^+$	-	67	100
P1668	*ompA-12*	-	70	31.3
P1675	*ompA-19*	-	69	15.8
P1658	*ompA-2*	-	102	30.3
P1673	*ompA-17*	-	68	9.4
P1667	*ompA-11*	-	69	106.0
P1665	*ompA-9*	-	69	12.2
P1666	*ompA-10*	-	68	25.2
P1672	*ompA-16*	-	85	22.5
P1662	*ompA-6*	-	70	19.9
P1663	*ompA-7*	-	73	13.8
P460	*ompA-1*	-	77	21.9
P407	-	*tsx-200*	69	94.0
P1578	-	*ompB-105*	65	105.0

(TABLE 7-1 Cont....)

a. Doubling times in the logarithmic phase of growth at 37°C were measured by increase in OD (625 nm), in minimal medium A with growth factors added at 20 g/ml with 10 mM glucose. Uptake of glutamine was measured at 37°C. Four samples were taken within the first 4 minutes of glutamine uptake and the rate of uptake expressed as a % of that in strain P400. Each uptake experiment was performed at least twice, and the results agreed within 5%.

FIGURE 7-3: Comparison by optical density at 625 nm of growth of the ompA mutants P1667 (O; ompA-11), P1666 (□; ompA-10), P460 (Δ; ompA-1) with the parent strain P400 (●) at 37°C in minimal medium A containing growth factors at 20 µg/ml and 10 mM glucose as carbon source.

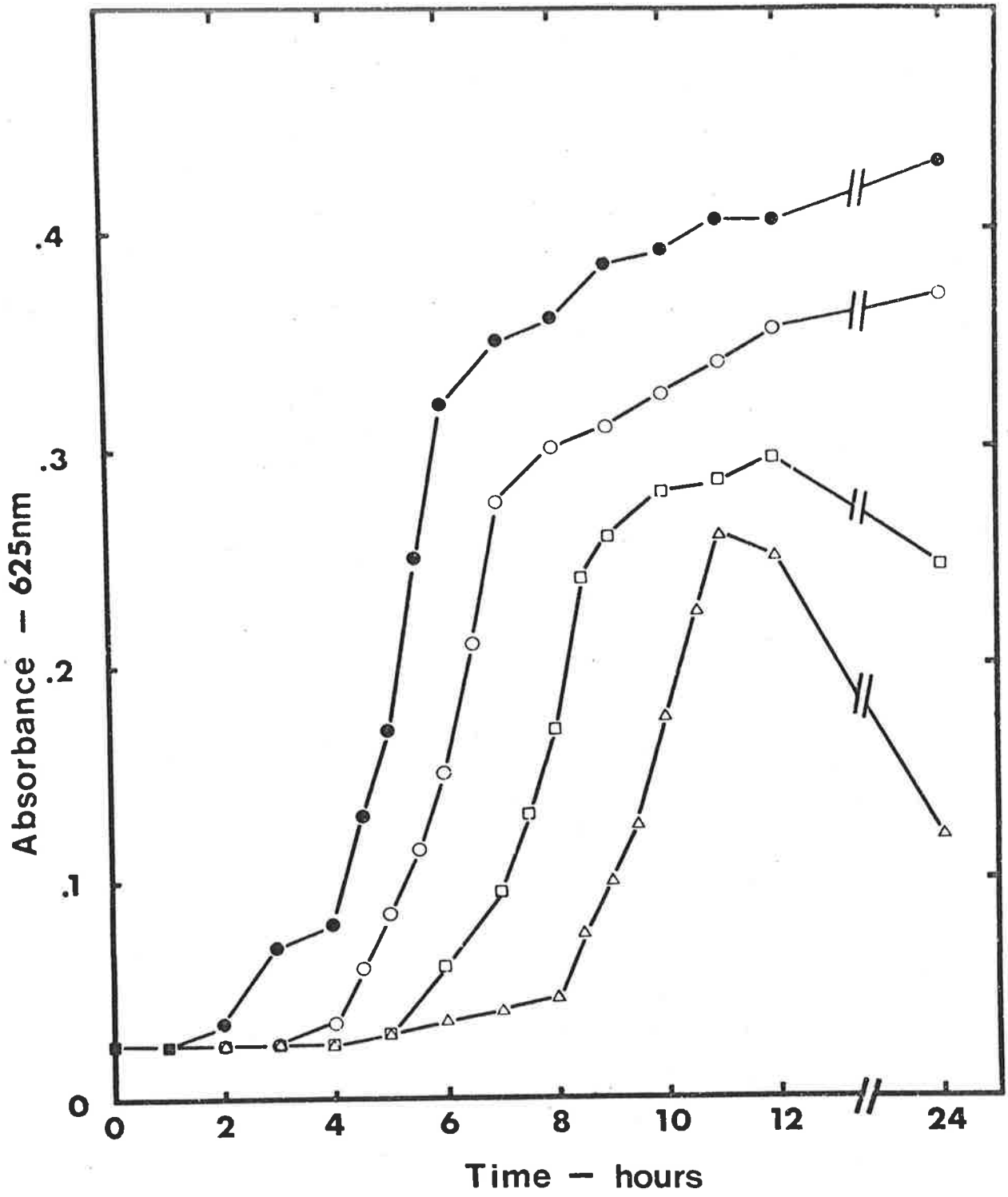

Absorbance — 625nm
.4
.3
.2
.1
0
0
2
4
6
8
10
12
24
Time — hours

TABLE 7-2

Doubling times (min) on various media in logarithmic phase of growth at 37°C based on growth curves followed by OD measurement.

Medium	Strain and relevant mutation						
	P801	P1774	P1773	P400[a]	P460[a]	P407[a]	P1578[a]
	-	ompA-48	tsx-202	-	ompA-1	tsx-200	ompB-105
Nutrient Broth	38	39	38	81	83	82	81
Minimal Medium A + 10mM glucose	63	80	65	72	87	75	81
+ 1% glycerol	107	138	108	126	228	138	132
+ 1% lactose	98	101	98	-	-	-	-
+ 1% maltose	-	-	-	78	87	81	75
+ 10mM lactate	108	115	108	125	145	-	-

(TABLE 7-2 Cont....)

Medium	Strain and relevant mutation						
	P801	P1774	P1773	P400[a]	P460[a]	P407[a]	P1578[a]
	-	ompA-48	tsx-202	-	ompA-1	tsx-200	ompB-105
+ 10mM succinate	95	121	105	126	148	127	102
Luria Broth							
+ 10mM glucose	34	50	36	39	46	40	39

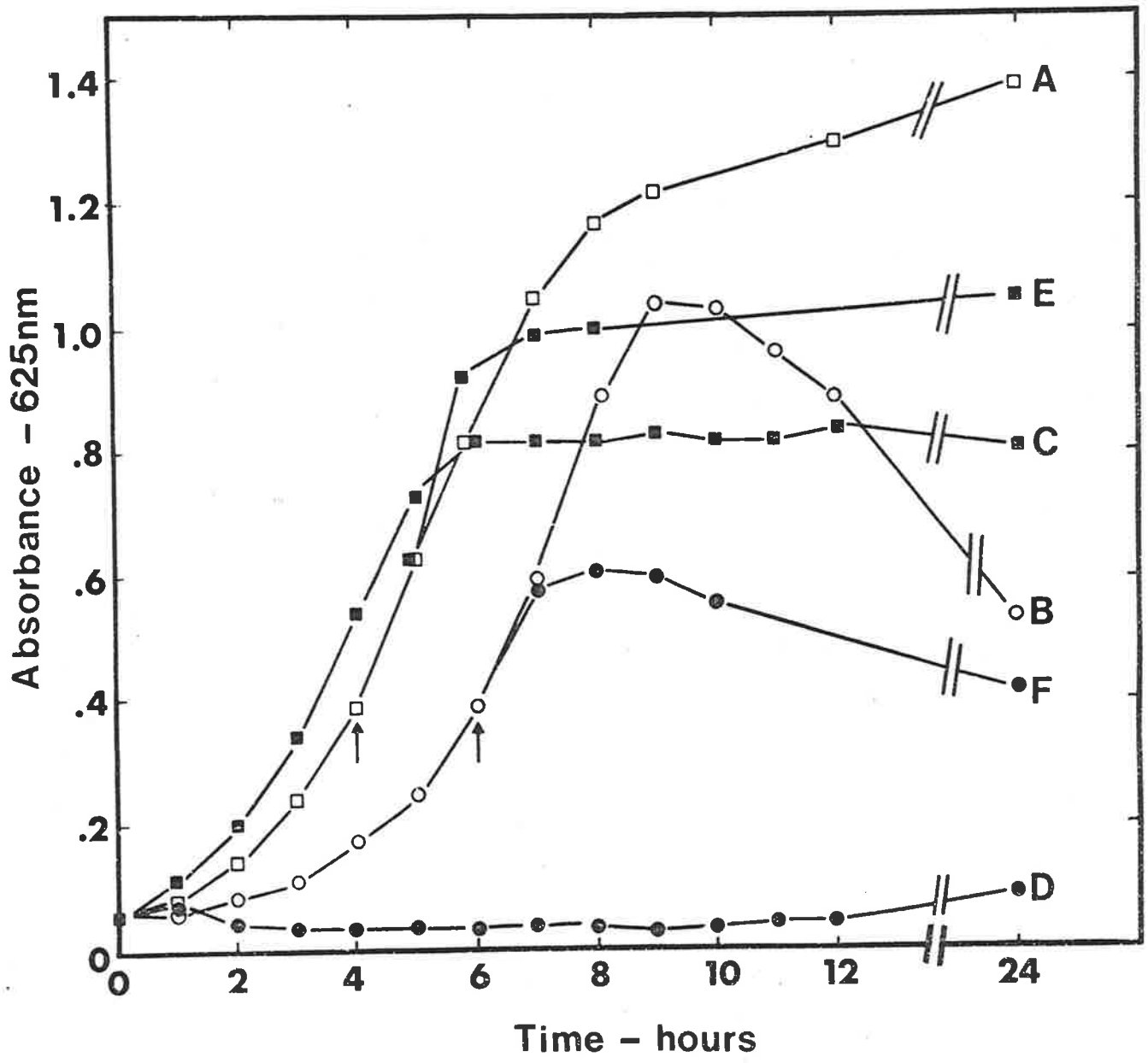
a. The minimal medium A in which P400, P407, P460 and P1578 were grown was supplemented with arginine, proline, threonine, leucine and thiamine each at 100 µg/ml.

The ompA mutants of P400 grow less well at 42°C than at 37°C or 30°C and this is most pronounced in minimal medium A with glucose. In Figure 7-4 curves A and B representing growth at 30°C clearly resemble the 37°C data showing lag and decline phases with P460. Curves C and D are quite different: no significant growth was observed with P460 at 42°C, whereas P400 grew normally although the plateau level was lower. When P400 was first grown at 30°C and then transferred to 42°C (curve E), its growth continued and then plateaued at a level intermediate to that observed when grown at either of the temperatures alone. However, when P460 was transferred to 42°C (curve F) its growth rate soon slowed down and cell density plateaued and then began to decline. Both the tsx mutant, P407, and the ompB mutant, P1578, behaved like the parent strain P400 at both temperatures.

GROWTH OF ompA-1 MUTANTS ON LIMITING GLUCOSE

In preliminary experiments, when two independent cultures of a tonA derivative of the ompA-1 strain P460 were grown under chemostat conditions with a dilution time of 1000 min, it was observed that these cultures declined in optical density and also viable cells and then regained the same steady state optical density

FIGURE 7-4: Effect of temperature on the growth of P400 (■ , □) and its ompA-1 mutant P460 (●, O) in minimal medium A with 10 mM glucose and growth factors added at 100 µg/ml. Solid symbols are for measurements taken at 42°C and open symbols for those at 30°C. Arrows indicate the point at which a temperature shift from 30°C to 42°C was made.


1.4
1.2
1.0
.8
.6
.4
.2
0
Absorbance – 625nm
0
2
4
6
8
10
12
24
Time – hours
A
E
C
B
F
D

observed before the decline. This was accompanied by increasing numbers of cells which had reverted and become sensitive to colicins K and L and bacteriophage K3. No decline in optical density or viable cells was detected in a tonA derivative of the parent strain P400. This suggests that the ompA-1 mutant, P460, is incapable of growth under such limiting conditions.

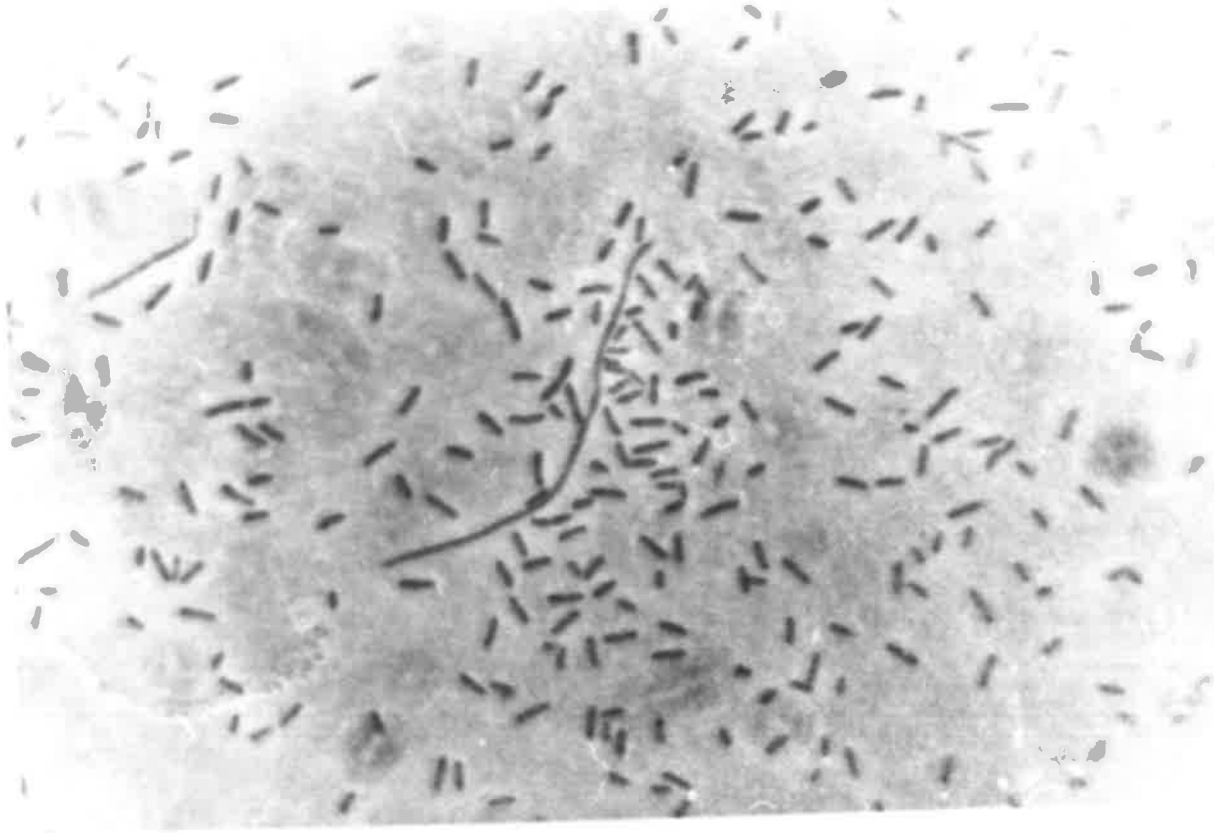
MORPHOLOGY OF CELLS IN THE DECLINE PHASE

When observed, under phase contrast microscopy, after 24 hr growth in minimal medium A plus glucose, the ompA mutant P460 showed many clumps of cells (Figure 7-5), whereas the culture of the parent strain P400 contained no clumps and existed as a typical suspension of single cells. Electron microscopy of the same cells of strain P460, revealed large numbers of very fine fibres, probably pili, attached to their surfaces (Figure 7-6).

OUTER MEMBRANE PROTEINS UNDER DIFFERENT GROWTH CONDITIONS

Bacteria were grown under different conditions and the outer membrane extracted and run on polyacrylamide gels. Considerable variation was observed in the amount of the various major outer membrane proteins present (Table 7-3). These results do not differentiate proteins 1a and 1b but it can be seen that if the slab gel system

FIGURE 7-5: Comparison by phase contrast microscopy of cells of strain P400 (top) and its ompA-1 mutant P460 (bottom) grown for 24 hours in minimal medium A with 10 mM glucose and growth factors each added at 100 µg/ml.



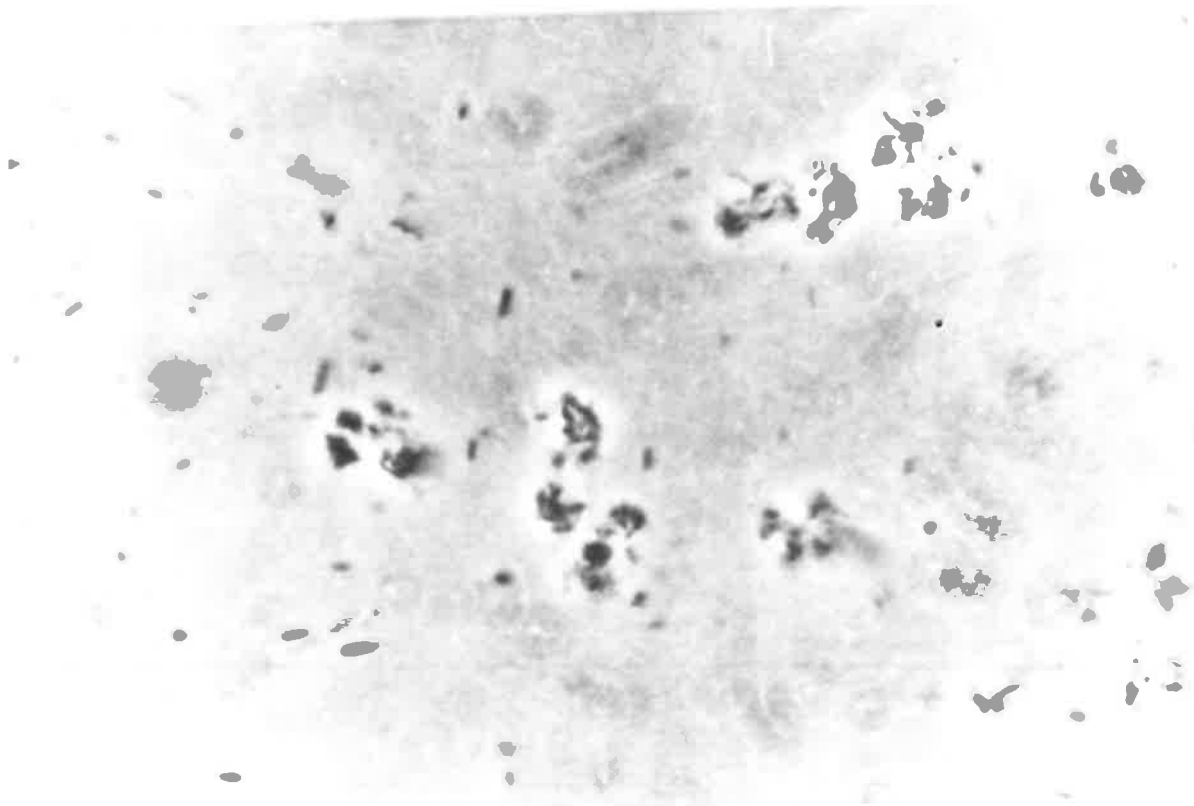


FIGURE 7-6: Cells of P460 (ompA-1) under the same conditions as for Figure 7-5 and examined by electron microscopy.

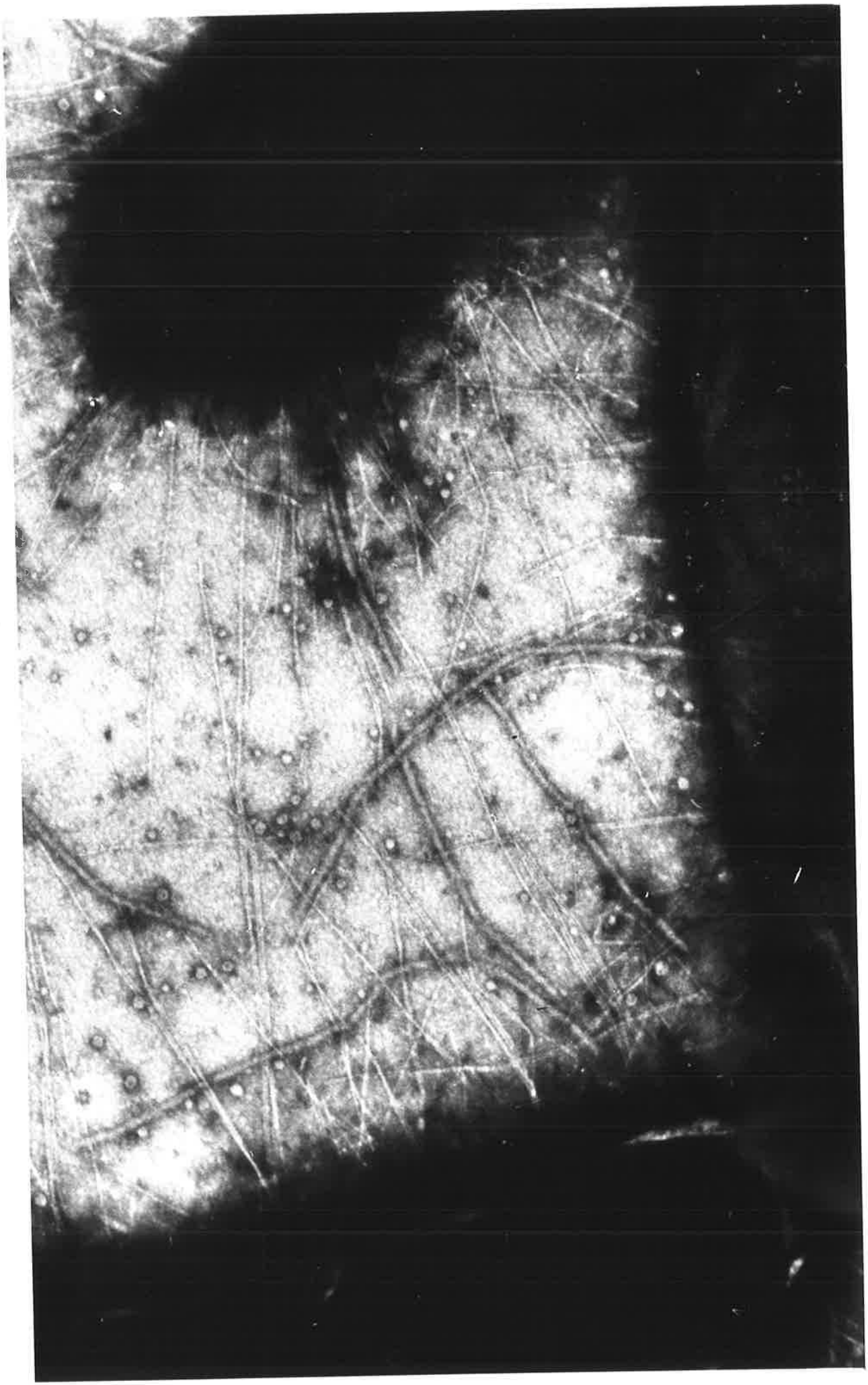


TABLE 7-3

Major outer membrane protein composition on various media.

Growth medium[a]	Strain	Proteins[b] 1	3A	3B	tsx
Minimal medium (MM)	P400	47	43	8	2
+ glucose	P407	47	50	3	0
	P460	88	0	9	3
	P1578	0	86	12	2
MM + succinate	P400	55	32	5	8
	P407	58	37	5	0
	P460	80	0	8	12
	P1578	0	71	12	17
MM + lactate	P400	58	26	4	12
	P407	60	34	6	0
	P460	80	0	8	12
	P1578	0	67	11	22
MM + glycerol	P400	56	28	4	12
	P407	61	36	3	0
	P460	85	0	4	11
	P1578	0	65	15	20

(TABLE 7-3 Cont....)

Growth medium[a]	Strain	Proteins[b] a	3A	3B	tsx
Luria Broth + glucose	P400	44	51	2	3
	P407	45	51	4	0
	P460	88	0	6	6
	P1578	0	92	4	4
Nutrient Broth	P400	35	42	6	17
	P407	38	56	6	0
	P460	70	0	10	20
	P1578	0	71	6	23

a. Growth media are the same as described in Table 7-2.

b. The relative amounts of the proteins are calculated from the areas of the peaks on densitometer tracings of Coomassie Blue R-stained gels (Swank and Munkres, 1971). Use of unheated and heated preparations in the pH 7.2 buffer system of Maizel (1966) under the conditions of Schnaitman (1973), enable us to differentiate these different proteins as shown in Chapter 4.

of Lugtenberg et al., (1975) is used most of the protein 1 is in the 1a form (Figure 7-7).

Table 7-3 also demonstrates that the tsx protein is catabolite repressible, and it has recently been reported by Kumar (1976) that cya and crp mutants have greatly reduced levels of T6 receptor activity.

The presence of glucose in the minimal medium results in the production of more protein 3A than when other carbon sources are used. This may reflect an added requirement for its function in growth on minimal medium plus glucose, and the decline phase observed with ompA strains may be a consequence of the cells being unable to fulfil this requirement.

UPTAKE OF TRANSPORT SUBSTRATES

Uptake of the amino acid glutamine by strains P460 (ompA-1), P407 (tsx-202) and P1578 (ompB-105) was compared with that by the parent strain P400. As shown in Figure 7-8, only the ompA mutant was defective in glutamine uptake. Defective glutamine uptake was found to be a characteristic of a wide range of ompA mutants of P400 tested with the exception of P1667 (ompA-11) (Table 7-1). Although within the group of ompA mutants

FIGURE 7-7: Comparison of strains P400 (a), P407 (*tsx-200*) (b), P460 (*ompA-1*) (c), and P1578 (*ompB-105*) (d) by SDS polyacrylamide slab gel electrophoresis according to Lugtenberg *et al*., (1975). Cells were grown in minimal medium A with growth factors at 100 µg/ml and either glucose, succinate, glycerol or lactate as sole carbon source. Cells grown in nutrient broth are also shown. Gels were stained with Coomassie Blue R according to Fairbanks *et al*., (1971) and bands are labelled according to Schnaitman (1974a).

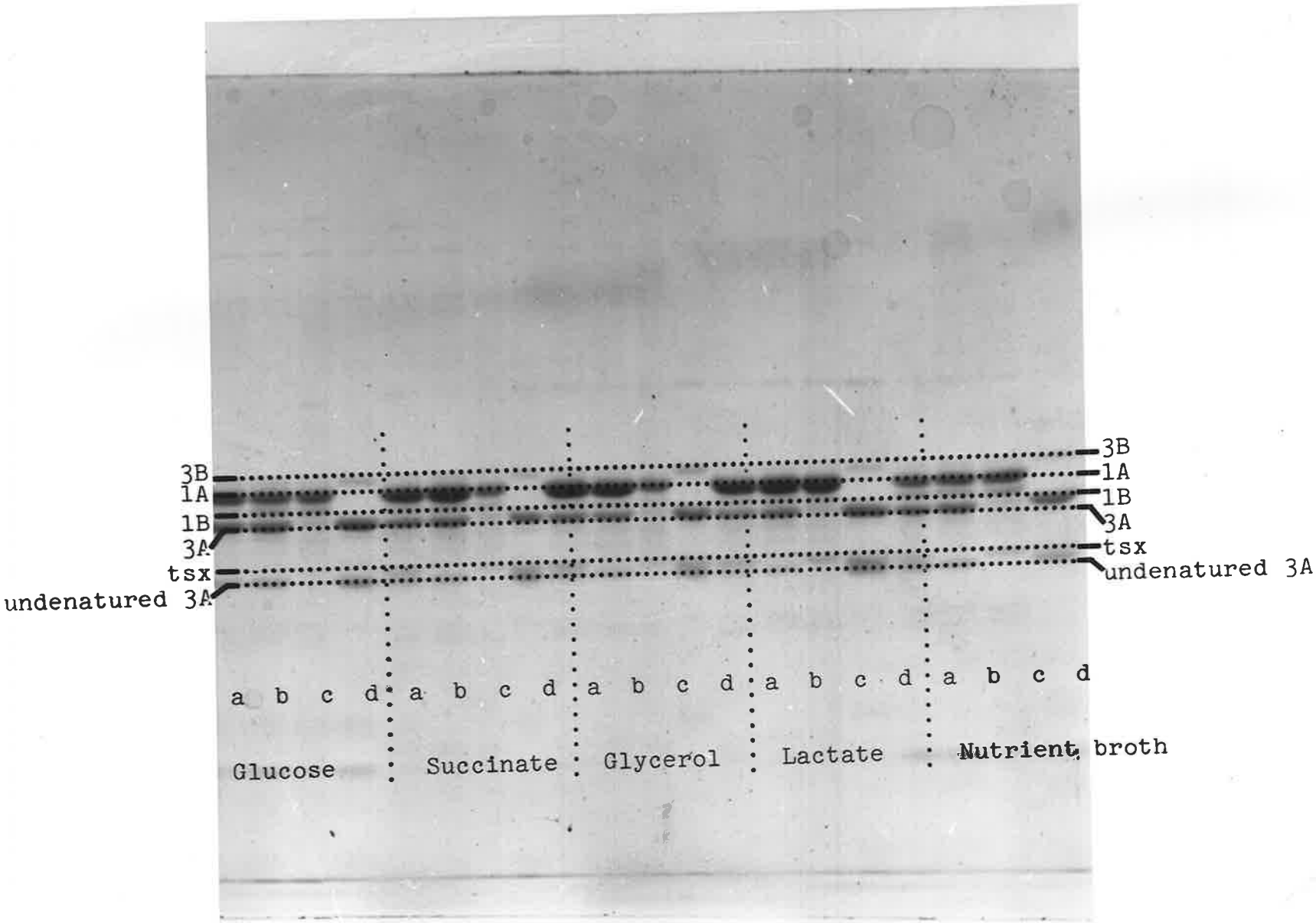
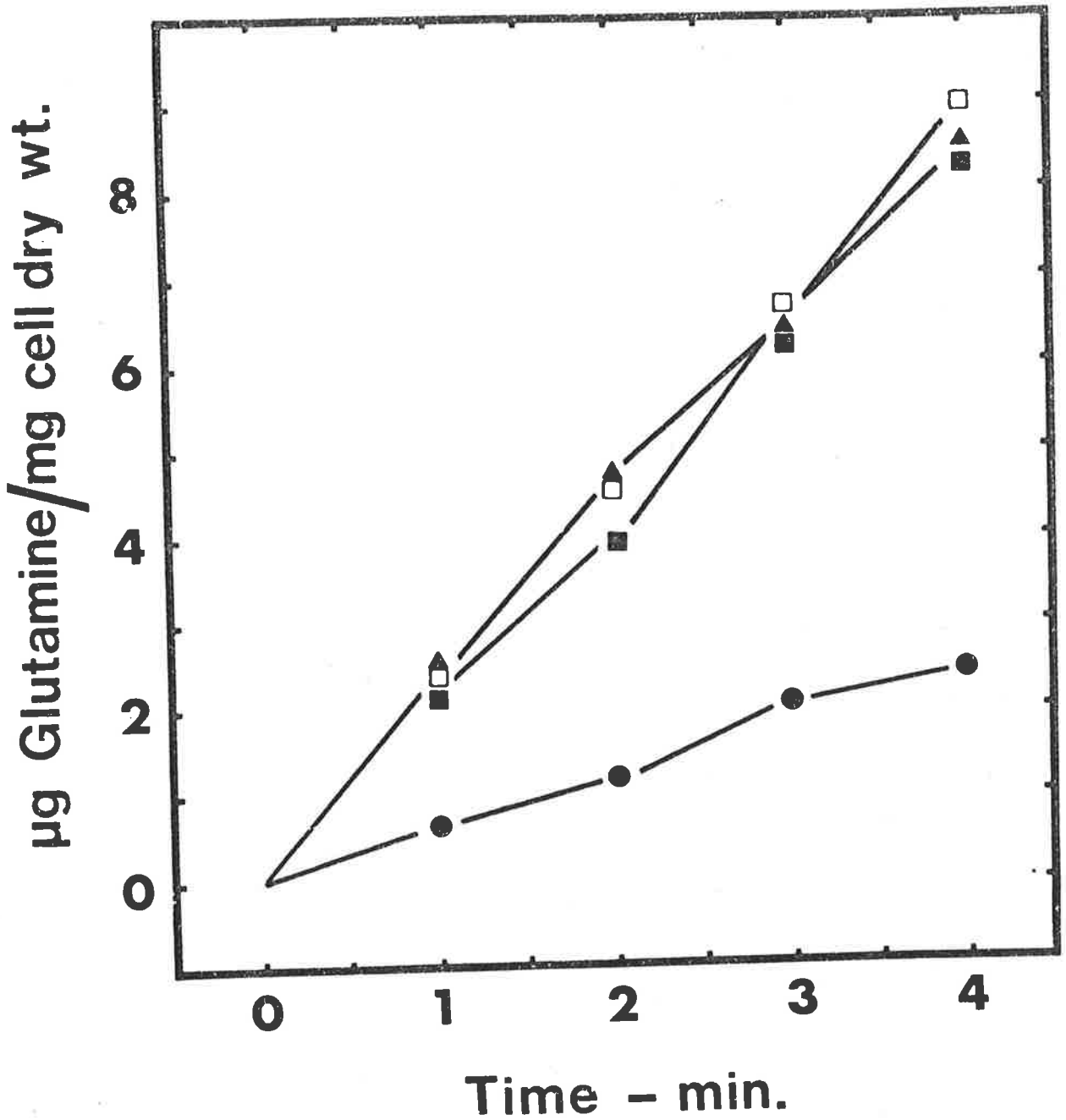

3B
1A
1B
3A
tsx
undenatured 3A
3B
1A
1B
3A
tsx
undenatured 3A
a b c d a b c d a b c d a b c d a b c d
Glucose
Succinate
Glycerol
Lactate
Nutrient broth

FIGURE 7-8: Initial uptake of ^{14}C-glutamine by strains P400 (□), P407 (▲; tsx-200) P1578 (■; ompB-105) and P460 (●; ompA-1) at 37°C. Cells were grown at 37°C prior to uptake, and the uptake mixture contained 500 µM glutamine.


µg Glutamine/mg cell dry wt.
0
2
4
6
8
0
1
2
3
4
Time – min.

the range of glutamine uptake rates was quite wide, there appears to be no obvious correlation between the rate of uptake of glutamine and bacteriophage and colicin resistance patterns or recipient ability in conjugation as described in Tables 5-1 and 5-3.

Pro^+ derivatives of strains P400 and P460 (ompA-1) were also tested for their ability to accumulate the amino acid proline. The ompA-1 strain again accumulated less of the amino acid than the isogenic omp^+ strain (25.6% of the uptake of the P400 derivative over the first 4 minutes). The effects of an ompA mutation on both glutamine and proline in a wild type K-12 background were tested using P801 and its ompA-48 derivative, P1774. Similar results were obtained (Figure 7-9), but the reduction in uptake rate was less than in the P400 genetic background.

The temperature at which the uptake was measured also had a pronounced effect on the rate of glutamine uptake by P400. Over a range of temperatures from 22°C to 42°C the rate of uptake increased with the temperature. In all cases the rate of uptake by the ompA-1 mutant P460 was less, but the difference between the two strains was much more marked at higher temperatures due to the higher level of uptake by P400 (Figure 7-10).

FIGURE 7-9: Initial uptake of (A) ^{3}H-proline and (B) ^{14}C-glutamine by strains P801 (■), P1773 (tsx-202; ▲) and P1774 (ompA-48; ●) at 37°C. Cells were grown at 37°C prior to uptake and the uptake mixture contained 500 µM proline or glutamine.

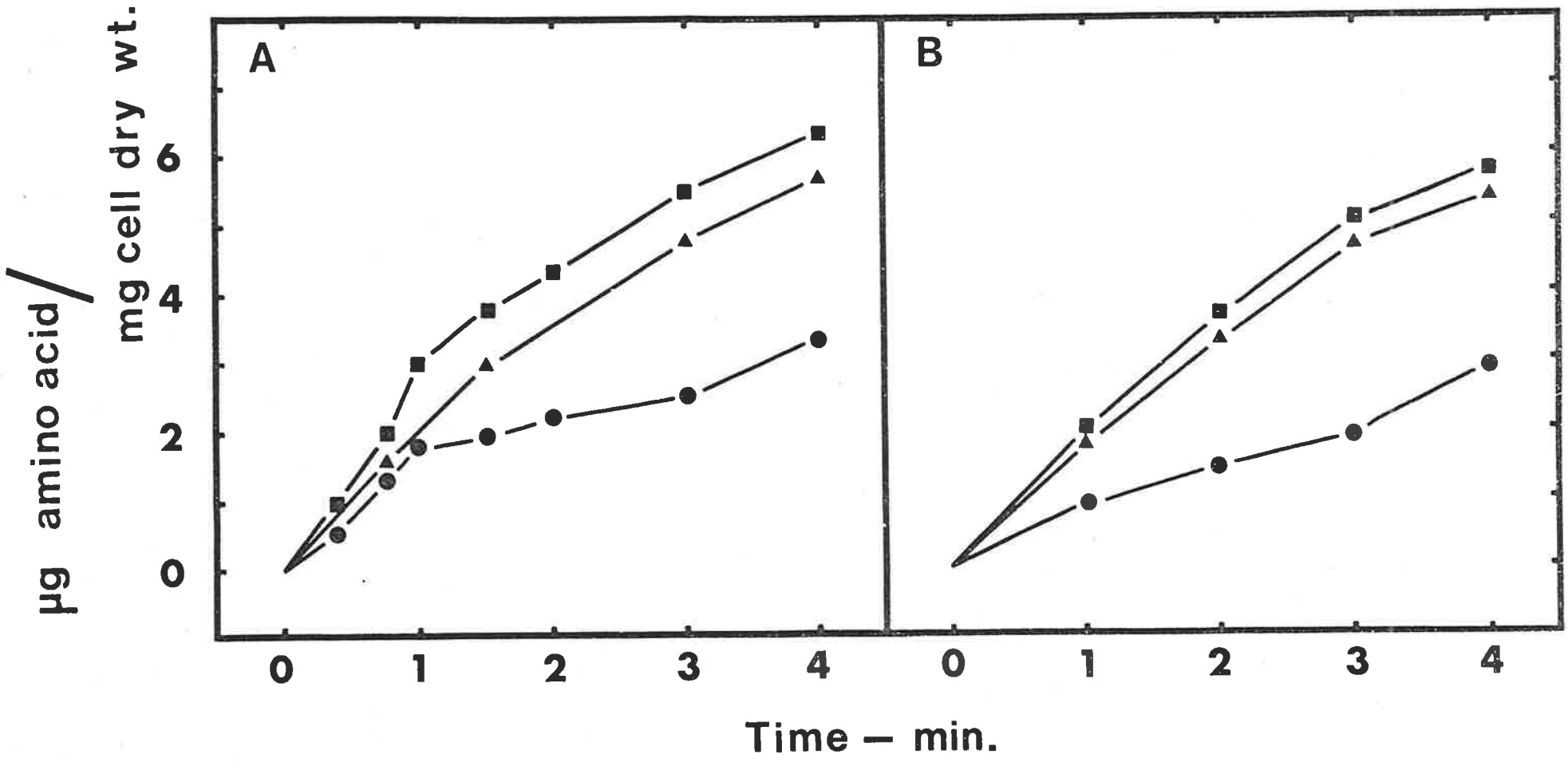
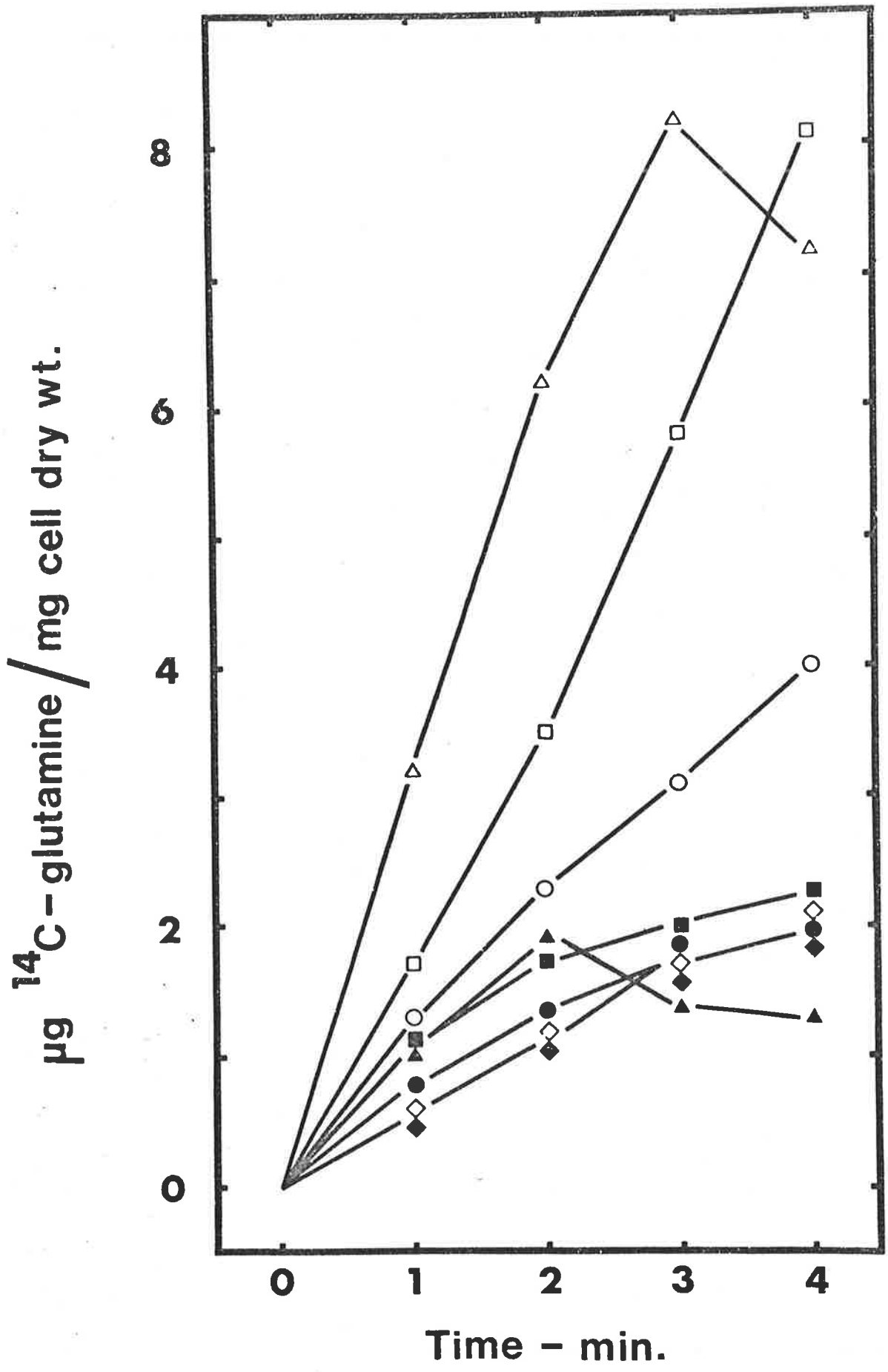

A
B
µg amino acid / mg cell dry wt.
0
2
4
6
0
1
2
3
4
Time — min.

FIGURE 7-10: Initial uptake of ^{14}C-glutamine by strains P400 (open symbols) and P460 (*ompA-1*; closed symbols) at 22°C (◇, ◆), 30°C (○, ●), 37°C (△, ▲) and 42°C (□, ■). Cells were grown at 30°C prior to uptake, and were equilibrated at the appropriate temperature for 15 min before uptake was initiated by the addition of ^{14}C-glutamine.


μg ^{14}C-glutamine / mg cell dry wt.
8
6
4
2
0
0
1
2
3
4
Time – min.

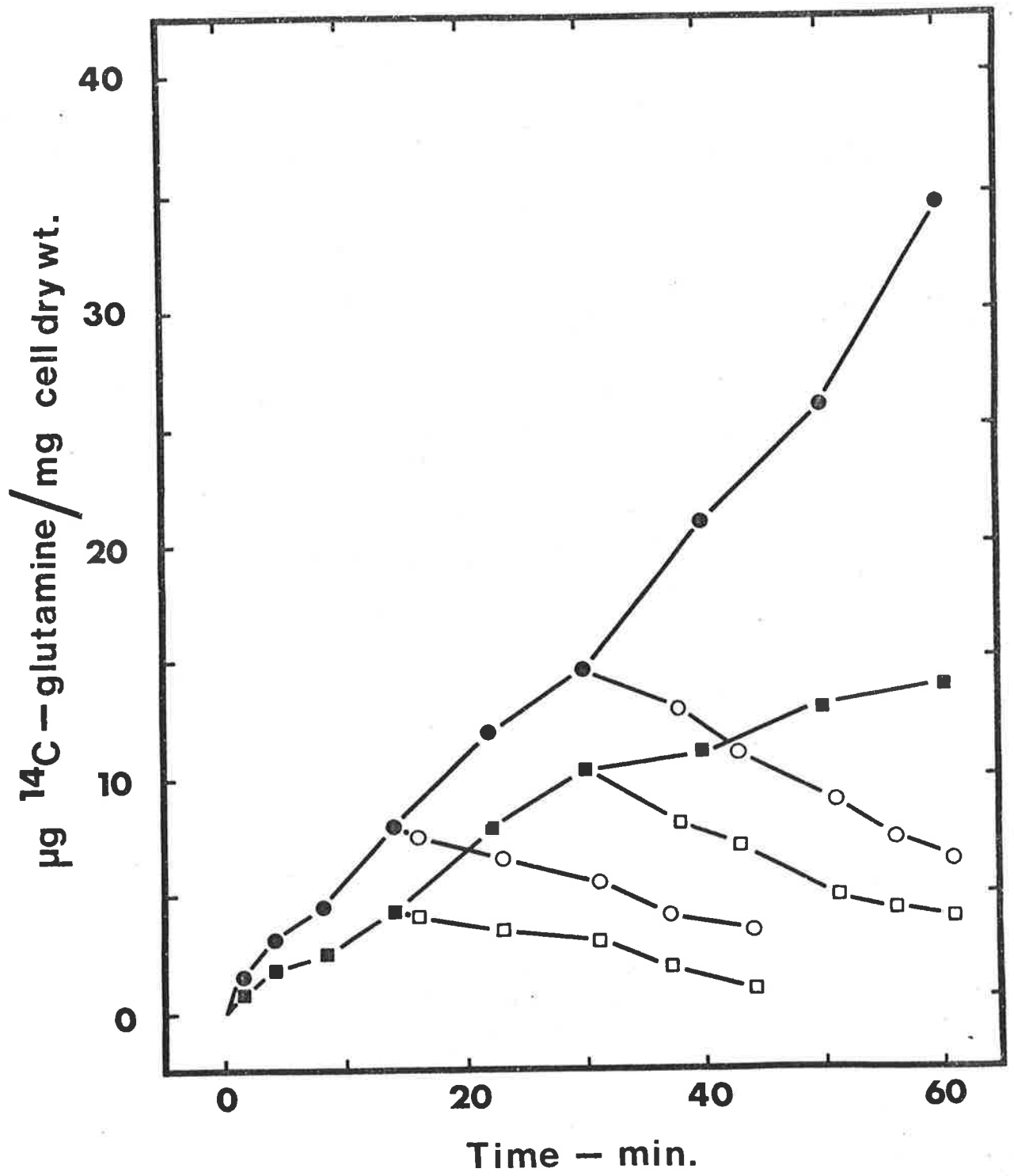
When measured in the presence of lactate as a sole energy source, the rate of active glutamine uptake by P801 was reduced compared with uptake measured in cells grown and tested in the presence of glucose. The glutamine uptake rate of P801 and its ompA derivative, P1774, under these conditions were indistinguishable.

One possible explanation for the reduced ability of ompA mutants to accumulate amino acids is that leakage is more rapid than from omp^+ cells. This possibility was examined by incubating cells in the presence of radioactive glutamine and then washing and resuspending in the absence of glutamine (Figure 7-11). These results demonstrated that there was little or no difference in the rate of glutamine leakage in ompA (P1774) and omp^+ (P801) cells.

Leakiness of the cell wall was also tested by comparing the ability of the mutants to hydrolyse ONPG. No significant difference was detected between the parent strain P400 and its ompA mutant P460, ompB mutant P1578 or tsx mutant P407.

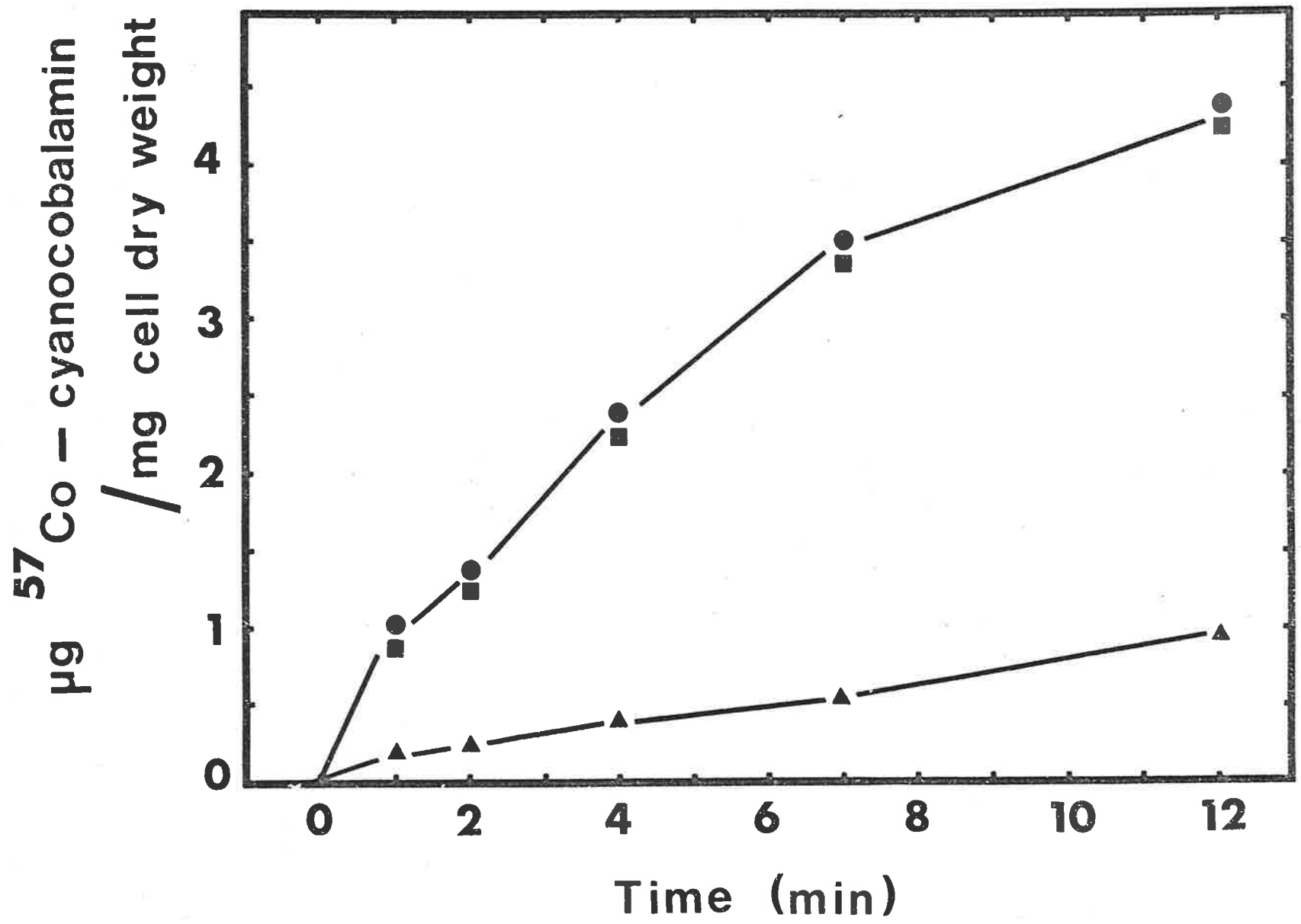
Strains carrying the ompA-1 mutation were assayed for the ability to accumulate ferrienterochelin (an iron-siderophore complex) and cyanocobalamin (vitamin B12),

FIGURE 7-11: Uptake (closed symbols) of ^{14}C-glutamine by strains P801 (●, ○) and P1774 ompA-48; ■, □) in glucose minimal salts solution A at 37°C. Cells were grown at 37°C in the presence of glucose. Samples of the uptake mixture which contained 1mM glutamine were removed after 14 and 30 min, centrifuged, resuspended in fresh uptake medium (containing chloramphenicol) in th e absence of glutamine, and incubation continued at 37°. Samples of the cells were then also assayed for ^{14}C-glutamine content at subsequent time intervals (open symbols).


40
30
20
10
0
μg ^{14}C – glutamine / mg cell dry wt.
0
20
40
60
Time – min.

both of which have specific outer membrane transport systems (Pugsley and Reeves, 1976b,c, 1977b; Di Masi et al., 1973; Bradbeer et al., 1976). Uptake of ^{55}Fe(III)-ferrienterochelin was found to be normal in iron-starved cells of strain P1567 (ompA-1, aroE) compared with the isogenic omp^{+} strain P1711. Uptake of ^{57}Co(III)-cyanocobalamin was shown to be normal in P460 (ompA-1) compared with the parent strain P400 (Figure 7-12): no uptake of cyanocobalamin was detected in control experiments using strain P445 (bfe) as reported previously for other bfe mutants (Di Masi et al., 1973).

FIGURE 7-12: Uptake of ^{57}C-cyanocobalamin (vitamin B12) by strains P400 (●), P445 (bfe; ▲) and P460 (ompA-1; ■) at 37°C. Cells were grown at 30°C prior to uptake.


μg ^{57}Co – cyanocobalamin / mg cell dry weight
0
1
2
3
4
0
2
4
6
8
10
12
Time (min)

SUMMARY AND CONCLUSIONS

The experiments described in this chapter were undertaken in an attempt to discover the primary function of the major outer membrane protein 3A. Recent studies have suggested, on the basis of limited data, that mutants of *E. coli* K-12 lacking one or more of the major outer membrane proteins were not defective in such parameters as cell shape or integrity (Henning and Haller, 1975; Verkleij *et al.*, 1976).

The results presented here show that in general *ompA* mutants are defective in the uptake of both glutamine and proline, and in their growth on various media; furthermore, cells grown on certain media enter a decline phase and lose viability after growth ceases. During this decline phase cells become heavily piliated and aggregated in clumps. These defects are specific in that the techniques used did not reveal any defect in *ompB* mutants lacking both proteins 1a and 1b or *tsx* mutants lacking the tsx protein. These defects presumably reflect the role of protein 3A in normal strains.

CHAPTER 8

DISCUSSION

In order to elucidate the recipient functions involved in the early stages of conjugation, it is highly desirable to have mutants which are blocked at different stages. In this thesis a wide range of bacteriophage- and colicin-resistant mutants have been screened with a number of donors. The mutants with the most profound effect on recipient ability were the ompA mutants, and it was therefore decided to concentrate on characterizing this class of mutants. From previous studies (Skurray et al., 1974; Hancock and Reeves, 1975; Davies and Reeves, 1975c) these mutants had a well defined phenotype and it was of interest to inter-relate the different aspects of the phenotype and to see if they were independent of one another.

DIFFERENTIATION OF PROTEINS 3A AND 3B AND DEMONSTRATION THAT ompA MUTANTS LACK ONLY PROTEIN 3A

Prior to the work described in this thesis it had only been possible to differentiate the major outer membrane proteins 3A and 3B by the way in which they bind to DEAE-cellulose columns (Schnaitman, 1974a). The two

proteins were known to co-electrophorese on polyacrylamide gels using heated samples with both the pH 11.4 buffer system of Bragg and Hou (1972) and the pH 7.2 buffer system of Maizel (1966). However, they had different cyanogen bromide cleavage patterns (Schnaitman, 1974a).

Skurray et al. (1974) had previously suggested that ompA mutants had lost both proteins 3A and 3B. However, by DEAE-cellulose chromatography it has been shown that ompA mutants lack only protein 3A (Chapter 4). This fact was then used in an attempt to identify the position to which protein 3B moves on Maizel gels using unheated samples. The results obtained suggested that protein 3B moved independently to protein 3A and this was confirmed by varying the growth conditions of the bacteria, which also showed that the amount of protein 3B produced was dependent upon the growth temperature. Under such conditions it could also be seen that when samples were run using the slab gel system of Lugtenberg et al. (1975) protein 3B moved as an independent protein and could be identified as the band a described by these authors. A number of authors (Henning and Haller, 1975; Garten et al., 1975; Lugtenberg et al., 1975) had previously disputed the existence of protein 3B. However, the results presented in this thesis clearly show that protein 3B does exist and is distinct from the other major outer membrane proteins in its

electrophoretic behaviour. The ability to readily differentiate proteins 3A and 3B made it possible to easily show that ompA mutants had lost only protein 3A.

The demonstration that ompA mutants were lacking only protein 3A implicated ompA as the structural gene for this protein. The fact that ompA mutants were also unable to adsorb bacteriophage K3, were very poor recipients and were defective in mating pair formation suggested a direct role of protein 3A in the adsorption of the phage, specifically the outer membrane receptor for its tail fibres, and in conjugation with F-like pili.

PROTEIN 3A IS THE RECEPTOR FOR BACTERIOPHAGE K3 AND ompA IS THE STRUCTURAL GENE FOR PROTEIN 3A.

It has been demonstrated that the bacteriophage K3 neutralizing activity of the outer membrane of E.coli K12 is accounted for by protein 3A (Chapter 4). This same protein is missing or altered in the ompA mutants, which are also unable to adsorb bacteriophage K3. It is therefore concluded that protein 3A is the receptor for bacteriophage K3.

Protein 3A has recently been purified by Garten, Hindennach and Henning (1975) and by Reithmeier and Bragg (1974), but they have used SDS which destroys the bacterio-

phage K3 neutralizing capacity of the protein. A comparison of results is shown in Table 8.1. The results obtained in this study are in agreement with the results obtained by Reithmeier and Bragg (1974) who found no cysteine whereas Garten *et al*. (1975) find 2 moles/polypeptide of cysteine. The reason for this discrepancy is not known.

It has also been shown that the *ompA-11* mutation leads to the production of wild type amounts of an altered protein 3A. This altered protein differs from the wild type protein in that it is unable to neutralize either wild type phage K3 or the extended host range phage mutant K3h1.

The production by an *ompA* mutant of a protein 3A altered in its biological properties strongly suggests that the *ompA* gene is the structural gene for protein 3A, supporting the evidence of Henning *et al*. (1976) who have isolated mutants, resistant to a K3-like phage, TuII*, and which had a protein 3A with an altered electrophoretic mobility. Recently, Datta, Krämer and Henning (1976) have confirmed *ompA* to be the structural gene for protein 3A by use of homogenotes, heterogenotes and intergeneric hybrids.

RECIPIENT ABILITY OF *ompA* MUTANTS AND ROLE OF PROTEIN 3A IN CONJUGATION

When examined for their recipient ability in con-

TABLE 8-1

Amino acid composition of purified receptor

Amino Acid	Residues/polypeptide (mole/mole)		
	Purified K3[a] receptor	Protein II*[b]	Protein B*[c]
Methionine*	3	5	1
Aspartic acid	35	33	36
Threonine	17	17	16
Serine	13	13	13
Glutamic acid	25	25	27
Citrulline	-		
Proline	16	16	17
Glycine	32	30	34
Alanine	25	24	31
Valine	18	20	22
Isoleucine	11	12	15
Leucine	19	20	23
Tyrosine	14	14	14
Phenylalanine	6	7	8
Lysine	14	15	18
Histidine	5	5	5
Arginine	11	11	15

(TABLE 8-1 Cont....)

Amino Acid	Residues/polypeptide (mole/mole)		
	Purified K3[a] receptor	Protein II*[b]	Protein B*[c]
Tryptophan	n.d.[d]	6	n.d.
Half cysteine	-	2	0
Cysteic acid	-		
SCM-cysteine	-		

a. Expressed as nearest integer, based on a molecular weight of 33,000.

b. From Garten, Hindennach and Henning (1975).

c. From Reithmeier and Bragg (1974).

d. n.d. = not determined.

* Methionine was measured as methionine sulphone.

jugation, the ompA mutants were not affected for any of the donors bearing I-like pili or for R100-1 and R136fin⁻ donors (Table 3.3).

Although the latter two R-factors encode F-like pili, these pili are the least closely related to the E. coli K12 F-pilus both antigenically (Lawn and Meynell, 1970) and in their sensitivity to male specific phage (Alfaro and Willetts, 1972); Dennison and Hedges, 1972). The recipient ability of the ompA mutants in fact corresponds to the degree of divergence in similarity to the F-pilus: the less similar the pilus of the donor is to that of F, the better the ompA mutant is as a recipient with that donor. Thus the defect with Hfrs, F's, ColV2 and ColVBtrp donors is greater than with R386 and R538-Fdrd-1, which is greater than with R1drd19 which is in turn greater than with R100-1 and R136fin⁻. No defect exists with any of the I-like R-factors tested.

Although F and R100-1 are at the two extremes of F-like pilus variation, the pilus subunits are sufficiently closely related that a mixed pilus can be formed (Lawn, Meynell and Cooke, 1971), which does not occur with an F and an I-like R-factor. This may be an indication that ompA is not affecting the F-pilus receptor, since donors with these mixed pili conjugate as well as donors with a

"pure" pilus, and so presumably the interaction of the pilus and its receptor is not affected.

Attempts to block conjugation with both outer membrane preparations and whole cells of *ompA*$^{+}$ and *ompA* strains were unsuccessful. This may have been due to an inability to obtain the optimal assay conditions so that non-significant results were obtained. Perhaps the interaction of the F-pilus with the recipient cell is reversible and so blocking would not be detected.

Skurray *et al*., (1974) previously showed that for Hfr matings the defect in the *ompA* mutants was at the stage of mating pair or aggregate formation. A series of the *ompA* mutants isolated in Chapter 5 were sent to Dr. Mark Achtman for further studies. It was shown using a Coulter counter that those mutants defective in recipient ability were defective in mating aggregate formation. It was also shown that if matings were allowed to proceed on the surface of a membrane filter (Havekes and Hoekstra, 1976), prior to selecting for recombinants then the defect in conjugation is greatly alleviated. The mutants isolated by Havekes and Hoekstra (1976) by a zygotic induction enrichment procedure are extremely similar (and are probably

identical) to the ompA mutants. If protein 3A, which is lacking in ompA mutants, was the primary receptor for the F-pilus then it would be expected that holding the cells close together would not greatly increase the level of mating, in the same way that a bacteriophage resistant (receptorless) mutant is not infected even in the presence of a vast excess of phage. However, if the interaction of the F-pilus with its receptor is reversible and requires stabilization, then a defect in this stabilization stage would be expected to have a much greater effect in liquid medium matings than matings carried out on a membrane filter. Thus the ompA mutants may be blocked at some stage required for mating aggreagate formation. This would also imply that the ompA gene product is not the only requirement for stabilization and that donors with the more distantly related F-like pili have a greater requirement for some other factor(s) and or have a lesser requirement for protein 3A.

OmpA mutants as they have been examined are so markedly affected in their recipient ability that it seems that the outer membrane protein 3A plays an essential role in recipient functions for at least some F-like donors.

THE ROLE OF OTHER CELL ENVELOPE COMPONENTS IN CONJUGATION

The wide range of mutants, examined for their recipient ability in conjugation, included strains which are defective in a variety of components of the cell envelope. Of the strains bearing mutations in different outer membrane proteins, only the ompA mutants already discussed were affected in their recipient ability and these were the most dramatically altered mutants. However, a number of other mutants showed altered recipient ability with the different donors. These mutants all had an altered LPS structure. In Table ~~8-1~~ 8-2 the results with the defective mutants obtained in Chapter 3 are again shown with the inclusion of their LPS composition as described previously by Hancock and Reeves (1976). The general increase in defect in the LPS composition corresponds well with a decrease in recipient ability for F'lac pro and HfrH and to an increase in recipient ability for R100-1. The mutants were also defective for R64-11 but this defect is less for mutants with a more defective LPS composition.

The ompA mutant P460 has a normal LPS composition (Hancock and Reeves, 1976) but it has been shown to have lost only the major outer membrane protein 3A. This does not affect recipient ability for any of

TABLE 8-2

Mutant Class	Mutant	Recipient ability with				LPS[a]		
		F'*lac pro*	HfrH	R100-1	R64-11	Glu	Gal	Hep
-	P400	1	1	1	1	.39	.26	.58
OmpA	P460	0.0005	0.005	1.10	0.77	.40	.19	.55
Bar7	P487	0.15	0.17	1.63	0.018	-	-	-
Bar5	P402	0.16	0.31	2.30	0.058	-	-	-
Bar3	P495	0.19	0.17	3.92	0.041	.14	.03	.31
Bar4	P436	0.24	0.63	2.59	0.09	.02	.00	.33
Wrm2	P424	0.34	0.45	2.99	0.064	.02	.01	.26
Bar8	P489	0.017	0.083	3.11	0.15	.02	.04	.12
Wrm1	P435	0.079	0.17	6.65	0.22	.00	.00	.00

a. The composition of the LPS is that obtained by Hancock and Reeves (1976). No data are shown for Bar5 and Bar7 but these are thought to have an altered LPS composition less altered than Bar3 on the basis of a comparison of a number of other properties (Hancock, 1974).

the donors bearing I-like pili or for the R100-1 and R136fin$^-$ donors (Tables 3-1, 3-2 and 3-3). However, LPS clearly plays a role in recipient functions but the role is apparently more complex, and the limited magnitude of the effect on recipient ability observed in the studies described in this thesis provides no evidence that LPS acts as the major determinant for specificity for either F-like or I-like pilus attachment, although a number of authors (Monner et al., 1971; Reiner, 1974; Havekes et al., 1976) have observed a greater reduction in recipient ability in similar mutants. The reason for this discrepancy is not apparent, although the mutants of Monner et al., (1971) are quite complex and another of the mutants had a large chromosomal deletion and so it is possible that the phenotype is the result of mutation in several closely linked genes (Havekes et al., 1976).

By selecting for mutants resistant to lethal conjugation with an I-like R-factor, it has recently been shown (L. Havekes and W.P.M. Hoekstra, personal communication) that such mutants were all defective in their LPS structure. It is also now believed that the I-like pilus recognizes specific regions of the LPS as its receptor.

It is known that mutations affecting primarily the synthesis of LPS can lead to the loss of outer membrane proteins (Koplow and Goldfine, 1974; Lugtenberg *et al.*, 1976) and it is possible that LPS mutations affect recipient function by altering the local environment of the protein 3A or other molecule in the outer membrane, and thereby modifying its function.

EXTENDED HOST RANGE MUTANTS OF BACTERIOPHAGE K3 AND *ompA* MUTANTS WITH AN ALTERED PROTEIN 3A

It has been proposed that the different groups of *ompA* mutants, described in Chapter 5, differ in the amount of protein 3A present and that the extended host range bacteriophages differ in the amount of receptor that must be present for them to be able to infect the *ompA* mutant and give rise to plaques.

The T-even bacteriophages are very similar genetically (Russell, 1974) and structurally (Cummings, Chapman, DeLong, Kusy and Stone, 1970; Cummings, Kusy, Chapman, DeLong and Stone, 1970). The observations of Bayer (1968) have suggested that the process by which the different T-even bacteriophages infect the cell is

also very similar. Goldberg and coworkers (Wais and Goldberg, 1969; Bauman, Benz, Wright and Goldberg, 1970; Dawes and Goldberg, 1973a,b) have postulated that, for bacteriophage T4, the six tail fibres each interact with a receptor molecule and that only when all have interacted can the tail pins on the base plate go on to interact with their receptor. The nucleic acid of the infecting phage can then be injected. Bacteriophage K3 is morphologically similar to the other T-even phages (Chapter 3; Krzywy, Kucharewicz-Krukowska and Slopek, 1972), and it has been possible to perform crosses between bacteriophage K3 and T2, T4 and T6 (A. Puspurs, personal communication).

It has been proposed that the bacteriophage K3 extended host range mutants are able to plaque on bacteria with greatly reduced levels of the protein 3A receptor, perhaps because the tail pins can be activated after less than six tail fibre-receptor interactions. It is not difficult to believe there is some protein 3A present, although not detected on polyacrylamide gels, in the ompA mutants, since the receptors for other phages, such as λ, T5 and BF23 are not normally detected by polyacrylamide gel electrophoresis.

During the course of this study a similar pattern

to that obtained with extended host range mutants of bacteriophage K3 and ompA mutants, was obtained by Hofnung et al., (1976) using host range mutants of bacteriophage λ and lamB mutants. Schwartz (1975) has shown that the reaction of bacteriophage λ with its receptor is a two step process. The initial specific interaction of its single tail fibre with the receptor is reversible, but this complex normally proceeds to an irreversible stage. Mutations in the lamB gene affecting the receptor may reduce the probability that the complex becomes irreversible, whereas host range mutations of the phage may increase this probability (Randall-Hazelbauer and Schwartz, 1973; Hofnung, Jezierska and Braun-Breton; 1976). Further, in some cases the irreversible step can occur in vivo but not in vitro (Schwartz, 1975) with purified receptor.

The fact that purified protein 3A can neutralize bacteriophage K3, a T-even like phage, suggests that as for λ, no other host receptor is required for stable phage-host complex formation and the interaction between bacteriophage K3 and protein 3A can become irreversible. The altered protein 3A of P1667 (ompA-11) is apparently able to act as a functional receptor in vivo for bacteriophage K3h1, but is unable to neutralize this phage in vitro. It may be that, by analogy with the λ

phage situation, the protein behaves differently *in vitro* and *in vivo*, and that the altered protein - extended host range phage complex is able to proceed to the irreversible stage only *in vivo*.

The hypothesis presented at the beginning of this section does not allow any simple explanation of the resistance of P1667 to K3h5 or P1666 to K3h44 (Table 5-1). The other properties however can be explained by the fact that the protein 3A of P1667 is altered in its receptor activity. Perhaps these phage carry more than just a mutation affecting host range.

If the hypothesis is correct that the different groups of *ompA* mutants differ in the amount of protein 3A present and that the bacteriophages differ in the amount of receptor that must be present for them to be able to form plaques, then the *ompA* mutants in Table 5-1 are arranged in approximate order of decreasing amounts of protein 3A (with the exception of strain P1667). It can be seen that the sensitivity to colicins K and L and reduction in recipient ability do not decrease in the same order. It appears that reducing the level of protein 3A does in itself reduce recipient ability well below that expected from their sensitivity to extended host range phage. Perhaps, the

sensitivity of the ompA mutants to K3h$^-$ mutants is determined largely by the amount of protein 3A present, whereas the recipient ability and colicin L sensitivity are also affected by alterations to the structure of the protein.

It thus appears possible to independently vary all three of these properties of the ompA mutants - the ability to plaque the host range phage, sensitivity to colicins K-235 and L-JF246, and recipient ability in conjugation.

For example, strains P1658 and P1668 have similar abilities to plaque the host range mutants, but P1668 is sensitive to L-JF246 and permits conjugation at 6.6% of normal, whereas P1658 is resistant to the colicin and no detectable conjugation occurs.

Similarly, P1675 and P1676 can be compared. Both have similar efficiencies of plating of the extended host range phage and are partially resistant

to colicin L, but P1675 is a much better recipient in conjugation.

If one compares P1672 and P1673, both of which are resistant to colicins and have similar abilities as recipients in conjugation, it can be seen that they are quite different in their ability to plaque the extended host range phage.

OmpA mutants have also been shown to be defective in the uptake of glutamine (discussed later). If the glutamine uptake by the altered protein 3A mutants is considered, in addition to the other properties, mutants P1673 and P1665 appear very similar as do their colicin tolerances and recipient ability in conjugation, but they differ markedly in their ability to plate the extended host range phage. Also, strains P460 and P1672 which have similar glutamine uptakes and colicin tolerances are different in their recipient ability and extended host range phage sensitivity.

It can therefore be concluded, that in general those mutants able to plaque K3h$^-$ mutants contain

residual amounts of protein 3A, and that in some at least of those mutants, it is an altered protein which is present. In the case of P1667, the alteration is such as to reduce the receptor activity but not the amount of protein in the outer membrane.

It appears that mutations in the structural gene for protein 3A are able to affect both the nature of the protein and the amount which is synthesized, or inserted in the outer membrane. This is presumably by alterations to different regions of the protein which may affect its tertiary structure or alter particular regions which are necessary for anchoring the protein in the membrane.

DEFECTIVE GROWTH FUNCTIONS OF *ompA* MUTANTS

The experiments described in Chapter 7, were undertaken to discover the primary functions of the major outer membrane proteins of *E. coli* K-12. The results demonstrated that only the *ompA* mutants were defective under the conditions used. That is, the defects were specific for alterations to or loss of the outer membrane protein 3A. These defects presumably reflect the role of protein 3A in normal strains but are unable to be accounted for on any simple hypothesis.

The defect in uptake affects both proline and glutamine, which use the energized membrane and high energy phosphate energy sources respectively, making a specific effect on energy metabolism unlikely. Furthermore there was no effect on the uptake of either ferrienterochelin or cyanocobalamin, again suggesting that the effect was not on energy provision but on some other aspect of uptake.

Outer membrane proteins do not appear to have a structural role (Henning and Haller, 1975) and the only primary functions postulated for outer membrane proteins are as mediators for specific transport of substrates such as maltose (Szmelcman et al., 1976) ferrichrome (Hantke and Braun, 1975; Luckey et al., (1975; Wayne and Neilands, 1975) cyanocobalamin (Bradbeer et al., 1976; Di Masi et al., 1973) or ferrienterochelin (Pugsley and Reeves, 1976a,b; 1977a) or for formation of hydrophilic pores through the membrane. It is well documented (Nakae and Nikaido, 1975; Decad and Nikaido, 1976) that outer membranes have pores which allow free entry of water soluble molecules up to a size limit of about 700 molecular weight. It seems highly improbable that the phospholipid and lipopolysaccharide components could form such

hydrophilic pores and this function is most likely served by proteins. Indeed Nakae (1976a,b) has shown that protein 1 in E. coli B and a similar protein in Salmonella typhimurium can be incorporated in vitro into membranes to form transmembrane pores or channels.

The variety of effects we observe in ompA mutants suggests that protein 3A is not involved in any specific transport, which leaves either an involvement in pore formation or an as yet unexpected role for outer membrane proteins. The possibility that protein 3A is involved in pore formation will be discussed first.

The observed defects have some, but not all, of the properties expected of a defect in transmembrane diffusion. As expected, the effect is non-specific, affecting uptake of glutamine and proline but not cyanocobalamin or ferrienterochelin for which specific outer membrane transport systems exist. There are also defects in growth in minimal medium when only salts and a given carbon compound are taken up, but the magnitude of these effects on growth rate in minimal medium depends on the carbon source, whereas one might expect a reduction in diffusion across the outer membrane to affect growth equally in any medium.

However, it is clear that the mutants are not completely defective in diffusion across the outer membrane, as in no case is the effect enough to halve the growth rate. The magnitude of an effect due to an incomplete block in diffusion across the outer membrane would depend on the extent to which diffusion was limiting in the mutant and this would depend on other parameters, in addition to the number and perhaps size of the pores. If the uptake from the periplasm across the cytoplasmic membrane were highly effective for a given substrate, a low periplasmic concentration might provide sufficient gradient to allow diffusion across the pore depleted outer membrane and not limit growth rate. Likewise, the anomalous behaviour of proline, which is taken up by an ompA mutant at only 25 to 50% of the wild type rate, but is taken up adequately under growth conditions, may be accounted for if only under the former conditions is the diffusion step rate limiting.

Recently, Hantke (1976) has shown that the defect in nucleoside uptake observed by McKeown, Kahn and Hanawalt (1976) in certain strains of E. coli K-12 was due to the tsx mutation. He has also suggested that the tsx protein forms a pore required for nucleoside uptake. The defect in uptake observed is of the same order as that described in Chapter 7 for proline and

glutamine in ompA mutants. It is thought that the various outer membrane pores are not the only means of uptake of these particular substrates but their specificity (lamB protein for maltose and maltodextrins, tsx protein for nucleosides and protein 3A for amino acids) allows rapid diffusion or facilitated transport of the appropriate substrate into the cell.

There remain, however, some difficulties in accepting the hypothesis that protein 3A is involved in outer membrane transmembrane diffusion. Firstly, Nakae (1976b) found in his experiments that protein 1 accounted for all of the ability of a crude protein extract to ass pores to a membrane in vitro. However, these experiments did not exclude the possibility that other proteins form pores in E. coli (Nakae, 1976b). Protein 1 is extracted from membranes as aggregates whereas protein 3A is extracted as a monomer which is not fully denatured (Schnaitman, 1973a) and the tsx protein and probably most others are fully denatured by the detergent (SDS) extraction used. It may be that only the aggregated protein 1, which is also known to bind very little SDS at the temperatures used. (Rosenbusch, 1974), can be reintegrated in functional form into membranes in vitro, but that other proteins can form pores in vivo. The disaggregation of protein 1

has in fact been shown to result in the loss of this ability to form pores (Nakae, 1976b). Also, the trypsin treatment Nakae uses is known to degrade the 33,000 dalton protein 3A molecule to a 25,000 dalton component (Garten and Henning, 1974). Nakae's findings are therefore not inconsistent with an *in vivo* role for protein 3A as a transmembrane pore.

What is most difficult to understand is how the loss of protein 3A could affect the uptake of several substrates, whilst protein 1 is still present, as protein 1 has clearly been shown by Nakae (1976b) to be able *in vitro* to form a pore in the absence of any other protein. If protein 3A does indeed form a transmembrane pore, then perhaps it has a different specificity to that of a protein 1 pore, which could occur if the various cytoplasmic membrane transport systems were specifically localized beneath either protein 1 or protein 3A pores. The observation that the defects in uptake or growth are not absolute, would, under this hypothesis, be accounted for by diffusion through other pores being adequate for the reduced uptake levels observed, even in the complete absence of protein 3A. However an *ompA ompB* double mutant did not have a further reduced rate of glutamine uptake,

indicating that protein 1 pores are not critical in this case.

The possibility of a more complex role for protein 3A than pore formation is also indicated by a number of lines of evidence. OmpA mutants are tolerant to colicins K and L (Davies and Reeves, 1975b,c), that is, even though the colicins can bind to their receptors they are unable to mediate their usual killing effect. These colicins are far larger than the size limit suggested for outer membrane pores, unless perhaps only a fragment of these colicins normally enters the cell. The decline phase phenomenon is also suggestive of a more complex role for protein 3A, as is the role of protein 3A in mating pair formation in conjugation with most F-like pili bearing donors.

CONCLUDING REMARKS

This thesis has concentrated on the study of a particular class of conjugation defective mutants, *ompA*, of *Escherichia coli* K-12. These mutants have been previously shown to be blocked at the stage of mating pair or aggregate formation and consequently DNA transfer has not been looked at because this occurs at a later stage of conjugation. However, it may be possible that DNA transfer is required for the stabilization of mating pairs or aggregates and that the primary role of protein 3A is in DNA transfer which then stabilizes pair formation.

It has been clearly demonstrated that the *ompA* locus at 21.5 min is the structural gene for the major outer membrane protein 3A. The *in vitro* studies which were carried out in order to demonstrate the role of protein 3A in conjugation were unsuccessful. However, the use of *ompA* mutants with different levels of protein 3A and with different degrees of alteration to protein 3A shows that this protein is essential for conjugation, in liquid medium matings, with donors bearing most F-like pili. Protein 3A is also shown to be the receptor for bacteriophage K3 and a number of similar phages. All these properties are thought to

be involved with the uptake of amino acids for growth and for maintenance of cell integrity under starvation conditions. It is suggested that colicin tolerance may be a reflection of a block in the primary function of the protein.

APPENDIX

Publications

Material contained in this thesis has been published, accepted, or submitted for publication, in the following papers:

1. Manning, P.A. and P. Reeves. 1975.
   Recipient ability of bacteriophage-resistant mutants of *Escherichia coli* K-12.
   J. Bacteriol. 124: 576-577. (Reprint enclosed).

2. Manning, P.A. and P. Reeves. 1976.
   Outer membrane of *Escherichia coli* K-12: *tsx* mutants (resistant to bacteriophage T6 and colicin K) lack an outer membrane protein.
   Biochem. Biophys. Res. Commun. 71: 466-471.
   (Reprint enclosed).

3. Manning, P.A. and P. Reeves. 1976.
   Outer membrane of *Escherichia coli* K-12: differentiation of proteins 3A and 3B on acrylamide gels and further characterization of *con* (*tolG*) mutants.
   J. Bacteriol. 127: 1070-1079. (Reprint enclosed).

4. Manning, P.A., A. Puspurs and P. Reeves. 1976.
   Outer membrane of *Escherichia coli* K-12: isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3.
   J. Bacteriol. 127: 1080-1084. (Reprint enclosed).

5. Manning, P.A. and P. Reeves. 1976.
   Outer membrane of *Escherichia coli* K-12: demonstration of the temperature sensitivity of a mutant in one of the major outer membrane proteins.
   Biochem. Biophys. Res. Commun. 72: 694-700.
   (Reprint enclosed).

6. Manning, P.A. and P. Reeves. 1977.
Further studies on the recipient ability of Escherichia coli K-12 bacteriophage resistant mutants.
J. Bacteriol. in press (April).

7. Manning, P.A. and P. Reeves. 1977.
Outer membrane of Escherichia coli K-12: effect of growth temperature on the production of protein 3B and further characterization on acrylamide gels.
FEMS Microbiology Letters, in press.

8. Manning, P.A., A.P. Pugsley and P. Reeves. 1977.
Defective growth functions of mutants of Escherichia coli K-12 lacking a major outer membrane protein.
J. Mol. Biol. in press.

9. Manning, P.A. and P. Reeves. 1977.
The major outer membrane protein 3A of Escherichia coli K-12 is the receptor for bacteriophage K3.
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OUTER MEMBRANE OF ESCHERICHIA COLI K-12: DEMONSTRATION OF THE TEMPERATURE SENSITIVITY OF A MUTANT IN ONE OF THE MAJOR OUTER MEMBRANE PROTEINS.

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Summary

A con mutant of E. coli K-12 previously described as having an altered protein 3A present at a level not detectable on polyacrylomide gel electrophoresis, is shown to have a temperature dependent phenotype. It has no detectable protein 3A at 30°C, and is defective as a recipient in conjugation and is resistant to bacteriophage K3 and colicins K and L. At 42°C the protein is detectable and the strain is sensitive to phage K3 and shows a marked increase in recipient-ability. However, full sensitivity to the colicins is not regained.

Introduction

The outer membrane of Escherichia coli K-12 consists of a number of major outer membrane proteins (1,2), and of these protein 3A (using the nomenclature of Schnaitman, ref. 1) is the second most abundant. This protein has been shown to be missing in con mutants (2), which are resistant to bacteriophage K3 (3) and tolerant to colicins K and L (5,6). The loss of protein 3A also results in the loss of receptor activity for bacteriophage K3 (2,3) and protein 3A has now been shown to be the receptor (Manning and Reeves, manuscript in preparation). Con mutants are also defective as recipients for most F-like plasmids (2,3).

We have recently isolated a series of con mutants unable to plaque wild type

bacteriophage K3, but on which we were able to isolate host range mutants (Manning, Puspurs and Reeves, submitted to J. Bacteriol.). In this communication we demonstrate that amongst these bacterial mutants are a type which is cold sensitive, in that no detectable protein 3A is observed at 30°C but in which the protein can be identified at 42°C. The temperature dependence of a number of properties of these mutants is also described.

Materials and Methods

The bacterial strains used are all derivatives of Escherichia coli K-12 and are listed in Table 1. Bacteriophage and colicinogenic strains are those we have used previously (2). Nutrient media and minimal media supplemented with the appropriate growth factors and carbon source were as previously described (7,8).

Recipient-ability in conjugation was measured as previously described (9), with the exception that the bacteria were grown and mated at the test temperature and after plating out for recombinants the plates were incubated at 37°C.

Bacteriophage and colicin sensitivity was measured as previously described (2,4,5), using the conventional cross-streak plate test.

Table 1.

Bacterial strains

Strain	Characteristics	Source
P400	F^-/thi, argE, proA, thr, leu, mtl, xyl, ara, galK, lacY, supE, non, λ^-	3
P460	con-1 mutant of P400	3
P1675	con-19 mutant of P400	a.
CSH23 (E5014)	F'lac^+ $proA^+$, B^+ / Δ(lac pro)	Cold Spring Harbor

a. This is one of a series of mutants selected as resistant to bacteriophage K3 and on which host range mutants could be isolated. They will be described elsewhere (Manning, Puspurs and Reeves, submitted to J. Bacteriol.)

Outer membrane preparations were obtained and prepared for electrophoresis on SDS-polyacrylamide gels with the pH7.2 buffer system of Maizel (11) and the pH11.4 buffer system of Bragg and Hou (12) using the methods of Schnaitman (1,10).

Results and Discussion

We have previously shown that under our conditions protein 3A always runs

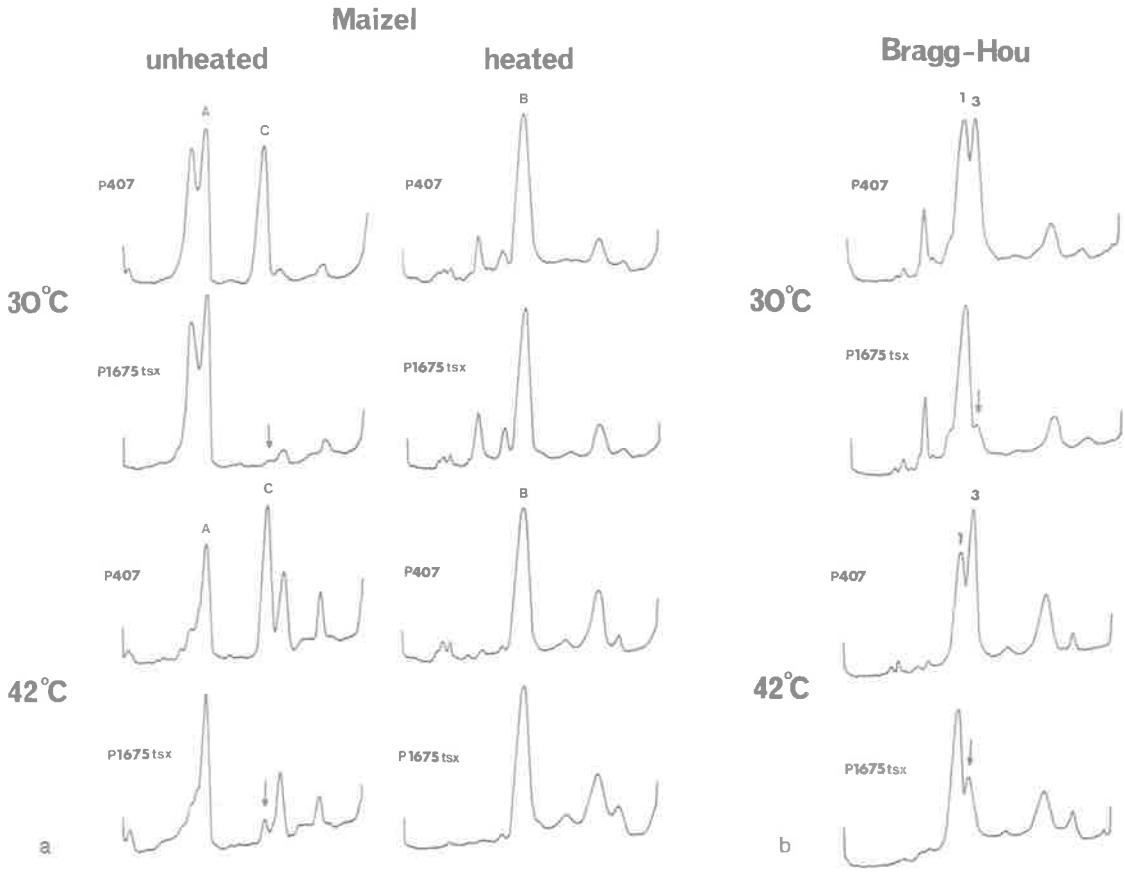

Figure 1a : Densitometer traces of SDS-polyacrylamide gels run using the pH7.2 buffer system of Maizel (11), comparing a tsx mutant of P1675 with P407 (a tsx mutant of P400, the parent strain of P1675).

Figure 1b : Densitometer traces of SDS-polyacrylamide gels run using the pH11.4 buffer system of Bragg and Hou (12), comparing a tsx mutant of P1675 with P407 (a tsx mutant of P400, the parent strain of P1675).

on SDS-polyacrylamide gel electrophoresis with other major outer membrane proteins : with the tsx-protein in unheated samples on Maizel gels, with 1 and 3B in heated samples on Maizel gels and with 3B on Bragg-Hou gels (2). We therefore made tsx mutants from our mutant and the parent strain by selecting for resistance to bacteriophage T6, and it is the outer membranes from these strains which we present in figure 1. These strains have no tsx-protein as shown by the absence of any residual peak C using heated samples on Maizel gels and also the tsx-protein peak is absent in the Bragg-Hou gels. We can therefore say that the peak C observed in the strains represents only

Table 2.

Sensitivity to bacteriophage K3

Strain	E.O.P. of wild type K3(h^+)	
	30°C	42°C
P400	1	1
P460	nd*	nd
P1675	nd	0.43

Strain	E.O.P. of K3 host range mutants											
	h1		h3		h30		h40		h44		h47	
	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°
P400	1	1	1	1	1	1	1	1	1	1	1	1
P460	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
P1675	0.6	0.2	0.5	0.5	0.6	0.6	10^{-4}	0.4	0.2	0.3	0.4	0.3

* nd = not detectable; eop of less than 10^{-6}.

protein 3A. Thus, from the Maizel gels with the unheated samples, it can be seen that strain P1675 has no protein 3A at 30°C but that significant amounts are present at 42°C, although not the full normal amounts. The con mutant P460 shows no protein 3A at either temperature (Manning and Reeves, unpublished results), whereas its parent strain P400 has the protein at both temperatures. From figure 1. it can also be seen that protein 3B, which constitutes the majority of the protein in the peak running immediately faster than peak C, increases significantly in proportion to the other proteins at 42°C, as shown by comparing this peak using unheated and heated samples on the Maizel gels (fig. 1), since protein 3B is known to move into peak B upon heating (1,2).

From table 2 it can be seen that P1675 becomes sensitive to wild type bacteriophage K3 at 42°C, and that a similar effect is observed with one of the host range phages tested, namely K3h40.

Table 3.

Colicin Resistance Patterns

Strain	Colicins			
	K-235		L-JF246	
	30°	42°	30°	42°
P400	S	S	S	S
P460	P	P	R	R
P1675	P	Sl	R	P

Resistance was determined using the convential cross-streak plate test (5). S = sensitive, Sl = slight resistance, P = partial resistance and R = full resistance (S<Sl<P<R)

The fact that full colicin sensitivity is not regained at 42°C (table 3) suggests that the protein may be altered so as to affect its role in sensitivity to colicins K and L, as some mutants with undetectable amounts of protein 3A have previously been shown to be sensitive to colicins K and L (Manning, Puspurs and Reeves, submitted to J. Bacteriol.)

The increase in recipient ability in conjugation at 42°C (table 4) correlating to the return of protein 3A, is further evidence supporting our hypothesis (2, 3) that protein 3A is required for efficient conjugation with most F-like plasmid bearing donors.

Table 4.

Recipient-ability with F' lac pro

Recipient	% Transfer with respect to donor*	
	30°C	42°C
P400	22.5	29.0
P460	0.006	0.009
P1675	0.14	8.8

* Each is the mean of at least three experiments.

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Outer Membrane of *Escherichia coli* K-12: Differentiation of Proteins 3A and 3B on Acrylamide Gels and Further Characterization of *con (tolG)* Mutants

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Two classes of mutants, *con* and *tolG*, that appeared to be very similar in a number of respects have been shown to be identical and cotransducible with *pyrD*. By diethylaminoethyl-cellulose chromatography of the outer membranes, we have shown that the mutants are missing only protein 3A and retain protein 3B. Using *con* mutants, we were thus able to identify protein 3B on the pH 7.2 gel system of Maizel where it runs separately from protein 3A if unheated samples are used. *tolG* mutants were shown to be identical to *con* mutants in being conjugation defective with most F-like plasmid donors but not with I-like plasmid donors, and in their resistance pattern to bacteriophages and colicins. During the course of this study, it was observed that the bacteriocin produced by *Serratia marcescens* JF246 was identical in its activity spectrum to colicin L-398 and is now considered to be a colicin of type L.

Recent studies of bacteriophage- and colicin-resistant mutants of *Eschericha coli* have led to the identification of outer membrane proteins able to be used as receptors for these bacteriophages and colicins, and also to characterization of the biological role of the proteins.

Sabet and Schnaitman (30) showed that *bfe* mutants, resistant to colicins E1, E2, E3, and A and to bacteriophage BF23, have lost the receptor for at least some of these lethal agents and that this receptor is a protein involved in the transport of vitamin B12 (7). Bacteriophages T1, T5, and ϕ80 and colicin M also share a receptor, which in this case has been shown to be the binding site for the uptake of ferrichrome-bound iron (2, 16, 22, 41). The receptor for bacteriophage λ has been identified as an outer membrane protein involved in the chemotaxis and high-affinity binding of maltose (18, 19, 28, 38). Each of these receptor proteins is only a relatively minor component of the outer membrane.

con mutants have lost the receptor for bacteriophage K3 (34; P. A. Manning and P. Reeves, manuscript in preparation), and analysis of the proteins of the outer membrane by polyacrylamide gel electrophoresis has shown them to be virtually lacking peak 3 (nomenclature of Schnaitman [33]), using the pH 11.4 buffer system of Bragg and Hou (1), and peak C, using unheated samples on the pH 7.2 buffer system of Maizel (24). *con* mutants have been isolated as resistant to phage K3 (15, 34) or as tolerant to colicins K and L (4, 6).

tolG mutants isolated by Foulds and Barrett (10) as tolerant to bacteriocin JF246 have also been shown to be missing a major outer membrane protein (3).

In this study we show that the previously described defect in the outer membranes of both *con* and *tolG* mutants is identical and consists of the absence of protein 3A only. We are able to distinguish proteins 3A and 3B by their behavior upon polyacrylamide gel electrophoresis.

We also extend the known phenotypes of both *con* and *tolG* mutants to show that *tolG* mutants exhibit properties previously described for *con* mutants and vice versa, indicating that the two are identical.

We also present evidence that bacteriocin JF246 is a colicin of type L.

MATERIALS AND METHODS

Media and culture conditions. Nutrient broth, nutrient agar, and minimal agar, supplemented with the appropriate growth factors and carbon source, were as described previously (35, 36). All cultures were incubated at 37°C unless stated otherwise.

Bacterial strains. The colicinogenic strains were described elsewhere (4, 8, 11, 17). All other strains were derivatives of *E. coli* K-12 and are listed in Table 1. The Col-factors and R-factors used, the colicins and antibiotic resistances for which they code, and those used for selection in the matings are

summarized in Table 2. All Col-factors and R-factors were maintained in strain JC6256.

Bacteriophages. All bacteriophages are from stocks maintained in this laboratory and described previously (15).

The 64 bacteriophages used were: T1, T2, T3, T4, T5, T6, T7, BF23, A, B, C, D, F, G, J, E4, E7, E11, E15, E21, E25, H1, H3, H8, K2, K3, K4, K5, K6, K8, K9, K10, K11, K12, K15, K16, K17, K18, K19, K20, K21, K22, K25, K26, K27, K29, K30, K31, Ox1, Ox2, Ox4, Ox5, M1, M3, Ac3, Ac4, H^+, V, ØI, ØII-T, H, F27, and W31.

Colicin sensitivity. Sensitivity was measured in two ways. Initially this was done by the conventional cross-streaking plate test, as described previously (4), using colicins A, B, D, E1, E2, E3, G, H, Ia, Ib, K, L, M, N, Q, S1, S4, V, and X.

In the second method, preparations of colicins K-235, L-398, and L-JF246 were titrated on the strain being tested, and the titer was compared with that on the wild type. This was done by taking log-phase bacteria diluted to a density of 2×10^3 cells per ml. To 0.1 ml of cell suspension was added 0.1 ml of a dilution of colicin. This was then incubated at 37°C with gentle shaking for 30 min, 4 ml of 0.7% nutrient agar was added, and the entire 4.2 ml was poured as

TABLE 1. *Bacterial strains*

Strain	Characteristics	Source/reference
AB1133	F⁻/*thi argE proA thr leu his mtl xyl ara galK lacY strA supE* λ⁻	A. L. Taylor
P212	*con* mutant of AB1133	5
P400	*non his*⁺ transductant of AB1133	34
P460	*con* mutant of P400	34
JF404	HfrH/*thyA*	J. Foulds (10)
JF404-2a	*tolG* mutant of JF404	J. Foulds (10)
P1635	F⁻/*thi argE mtl xyl ara str non* λ⁻	[a]
P1636	F⁻/*thi argE mtl xyl ara str non* λ⁻ *tolG*	[a]
AB259	HfrH/*thi rel* λ⁻	B. Bachmann
AB257	HfrC/*metB rel* λ⁻	B. Bachmann
KLF11/JC1553	F′ 111/*argG metB his leu recA mtl xyl malA gal lacY str tonA tsx supE* λʳ λ⁻	B. Bachmann
CSH23(E5014)	F′ *lac*⁺ *proA*⁺,*B*⁺/Δ(*lac pro*)	Cold Spring Harbor
JC6256	F⁻/*trp lac* Δ	N. Willetts
W620	F⁻/*thi pyrD gltA galK str rel* λ⁻	B. Bachmann

[a] Strains P1635 and P1636 were obtained by mating JF404-2a with P400 for 32 min at 37°C, interrupting the mating, and selecting for *gal*⁺ *str*ʳ recombinants. The growth factor requirements of the two strains were identical as was the suppressor status (Su⁻ or *supE*⁺), measured by using a series of T4 amber, ochre, and opal phage kindly supplied by A. J. Clark.

TABLE 2. *List of plasmids*[a]

Plasmid	Incompatibility type[b]	Antibiotic resistance[c]					Colicin
		Tc_{20}	Str_{10}	Str_{100}	Cm_{50}	Kan_{50}	
F′ *lac pro*	FI	S	S	S	S	S	
F′ 111	FI	S	S	S	S	S	
R1*drd*19	FII	*R*	R	S	S	S	
R100-1	FII	*R*	R	S	R	R	
R136*fin*⁻	FII	*R*	S	S	S	S	
R386	FI	*R*	S	S	S	S	
R538F*drd*1	FII	S	R	S	R	S	
ColV2	FI	S	S	S	S	S	*V*
ColVB*trp*	FI	S	S	S	S	S	*V,B*
R64-11	Iα	*R*	R	S	S	S	
R144*drd*3	Iα	S	S	S	S	*R*	
R163*drd*1	Iα	*R*	R	S	S	R	I
R538I*drd*2	Iα	*R*	R	S	S	S	

[a] Strains bearing the plasmids were kindly supplied by N. Willetts and E. Meynell.

[b] Incompatibility types were obtained from reference 27.

[c] R, Resistant; S, sensitive. Tc, Tetracycline; Str, streptomycin; Cm, chloramphenicol; Kan, kanamycin. The subscripts indicate the level of resistance used in micrograms per milliliter. The antibiotic resistances and colicins italicized are those used for selection of transfer, and 1,000 μg of streptomycin per ml was used as contraselection in all cases.

an overlay on a nutrient agar plate, prior to overnight incubation and scoring for colony-forming units. The titer of the colicin was taken to be the reciprocal of the dilution that gave 50% bacterial survival.

The conventional plate test was used for the initial screening of mutants, and the second method of titrating the colicins on the mutants was used to quantitate the extent of the resistance detected.

Bacteriophage sensitivity and adsorption. Bacteriophage sensitivity was scored by using the multiple-syringe phage applicator used previously (15), with phage at a density of 10^5 to 10^6 plaque-forming units per ml.

Bacteriophage adsorption was measured as follows. The bacterial strain being tested was grown to a density of 2×10^8 cells per ml, and bacteriophages were added to a portion at a multiplicity of 1.0. Immediately after the addition of the phage suspension, the culture was returned to incubate, and at 1.5-min intervals samples were taken and diluted 10^{-4} in prewarmed nutrient broth containing 20 μg of chloramphenicol per ml. A 0.1-ml volume was added to 0.1 ml of a culture of strain JC6256/R538F*drd*1 at a density of 2×10^8 cells per ml; the mixture was allowed to stand for 15 min at 37°C and then was poured as an overlay with 4 ml of 0.7% nutrient agar containing chloramphenicol (20 μg/ml) onto a nutrient agar plate. Plates were then incubated overnight and scored for plaque-forming units.

Mating procedures. Matings were performed as described previously (25) with the exception of Hfr matings, which were incubated for 60 min. For the transfer of colicin-producing plasmids, the donor cultures were preincubated at 37°C for 5 min in the presence of 5 mg of trypsin per ml to destroy any colicin present. Transfer was then detected as described previously (34).

Preparation of outer membranes, column chromatography, and polyacrylamide gel electrophoresis. Outer membranes were prepared by using Triton X-100 as described by Schnaitman (33), although cells were broken in an X-Press (LKB Instruments, Bromma, Sweden). For chromatography on diethylaminoethyl (DEAE)-cellulose (Whatman DE-52), the outer membranes were solubilized in Triton X-100 plus ethylenediaminetetraacetate (EDTA) and chromatographed as described previously (33).

Samples for electrophoresis were prepared by the method of Schnaitman (32, 33) and run under his conditions using both the pH 7.2 buffer system of Maizel (24) and the pH 11.4 buffer system of Bragg and Hou (1).

Gels were stained in Coomassie brilliant blue (37), and densitometer tracings were obtained with a Quick Scan Jr. gel scanner (Helena Laboratories Corp., Beaumont, Tex.).

Protein estimation. Two methods were employed for estimating the concentration of protein in samples. For protein solutions in water or non-Triton-containing buffers, the method of Schacterle and Pollack (31) was used. For samples containing Triton X-100, the method of Wang and Smith (40) was used. Bovine serum albumin was used as a standard in both cases.

Transduction. P1 phage stocks were prepared as described by Miller (26), using heat-inducible P1 carrying chloramphenicol resistance derived from the R-factor R100.

Colicin preparations. Colicins K-235, L-398, and L-JF246 were prepared by induction; a culture of the colicinogenic strain at a density of 4×10^8 cells per ml was induced by the addition of 50 ng of mitomycin C per ml. The culture was allowed to continue growing, with vigorous aeration, for 2.5 h, centrifuged at $5,000 \times g$ for 20 min, and sterilized by the addition of 1% chloroform. Samples of this crude colicin were then stored frozen until required.

RESULTS

***con* mutants lack only protein 3A.** The outer membrane from *con* mutants was shown previously to have a greatly reduced amount of peak C, using the pH 7.2 gel buffer system of Maizel (24), and of peak 3, using the pH 11.4 buffer system of Bragg and Hou (1). This has been confirmed and shown also to apply to the *tolG* mutants isolated by Foulds and Barrett (10) (Fig. 1 and 2). Originally, it was thought that both proteins 3A and 3B were absent in *con* mutants. Schnaitman has since shown (33) that these proteins can be separated by ion-exchange chromatography using DEAE-cellulose (Whatman DE52), and this technique has enabled us to reexamine the defect in outer membrane proteins of *con* mutants. It was shown previously (33) that proteins 3A and 3B have quite different cyanogen bromide peptide profiles, indicating that these two proteins are distinct polypeptides. When the outer membrane of strain P460 was examined by DE-52 chromatography, it was observed that only protein 3A was absent and that the level of protein 3B was unaltered (Fig. 3 and 4). Protein 3A occurs (if present) in fractions I, II, III, and perhaps IV, whereas protein 3B occurs in fractions IV and V.

A *tolG* mutant, strain JF404-2a, gave a result identical to that for the *con* mutant P460 (unpublished data).

Differentiating proteins 3A and 3B upon polyacrylamide gel electrophoresis. Schnaitman (33) showed that, with heated samples of outer membrane, proteins 3A and 3B run together as peak 3 on Bragg-Hou pH 11.4 gels (1) and as part of peak B on Maizel pH 7.2 gels (24).

Our results confirm these observations but show that the two proteins can be differentiated by using unheated samples on Maizel gels. If we look at the fractions from the DE-52 columns that are enriched in protein 3B and contain no 3A, we find that the small peak which runs slightly faster than peak C is correspondingly enriched, that this peak (and peak A) disappears upon heating, and that peak B appears in their place (Fig. 5).

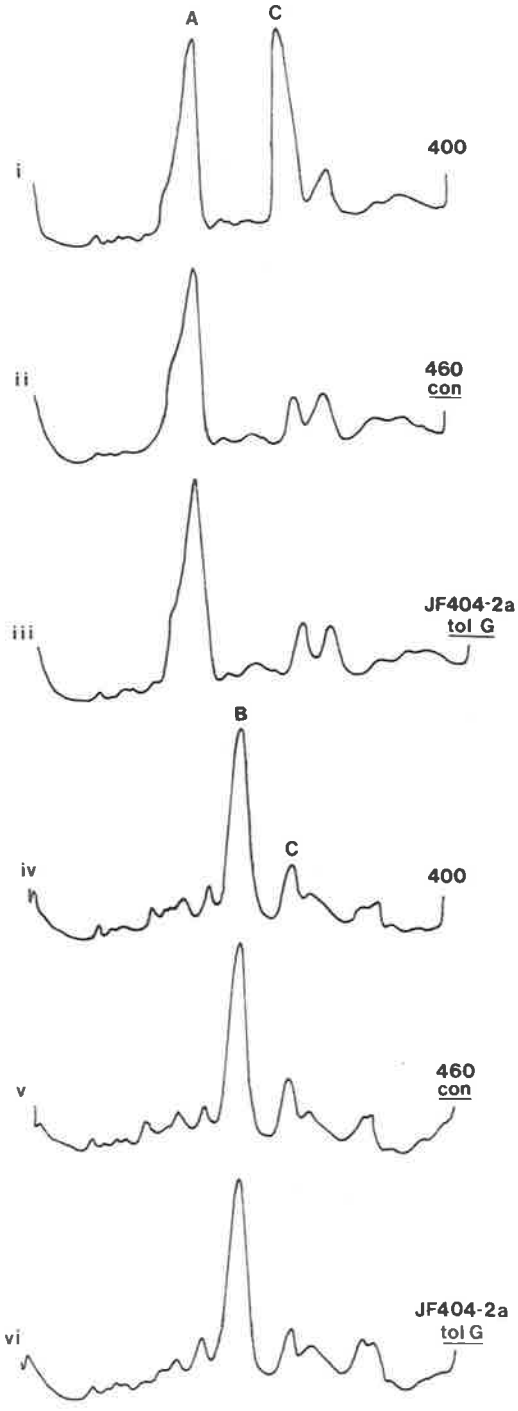

FIG. 1. *Comparison, by densitometry, of the outer membrane proteins of strains P400, P460 (con), and JF404-2a (tolG) run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 7.2 buffer system of Maizel (24) with unheated (i, ii, and iii) and heated (iv, v, and vi) samples. Peaks are labeled by the method of Schnaitman (33).*

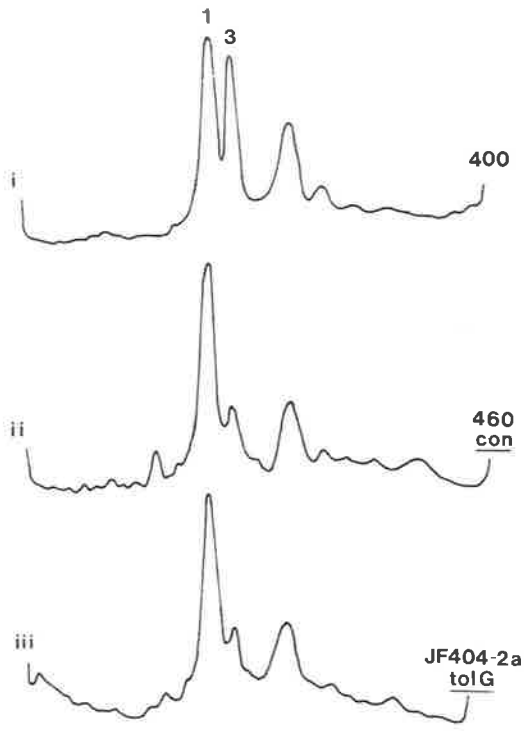

FIG. 2. *Comparison, by densitometry, of the outer membrane proteins of strains P400, P460 (con), and JF404-2a (tolG) run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1) with heated samples. Peaks are labeled by the method of Schnaitman (33).*

In Fig. 4 the amount of protein 3B relative to protein 1 in the Bragg-Hou gels of fractions IV and V seems higher than expected since this is also the only fractions that contain protein 1. This may be due to some of the protein 1 selectively remaining bound to the column.

Other properties of *con* and *tolG* mutants. We examined both *con* and *tolG* mutants for properties previously reported as being affected by one or both of the mutations. We found that both *con* and *tolG* mutants were identical as far as can be determined.

The mutants resemble the parent strains in being resistant to (per milliliter) 500 μg of sulfafurazole, 10 μg of oleandomycin, 5 U of penicillin G, 10 μg of methicillin, 10 μg of fusidic acid, 5 μg of novobiocin, and 5 μg of cloxacillin and sensitive to 50 μg of colistin methane sulfonate, 5 μg of kanamycin, 10 μg of neomycin, and 2 μg of ampicillin as shown previously for *con* mutants (34). However, the mutants had become sensitive to 30 μg of novobiocin, whereas both parent strains are resistant, as shown previously for *tolG* mutants (10).

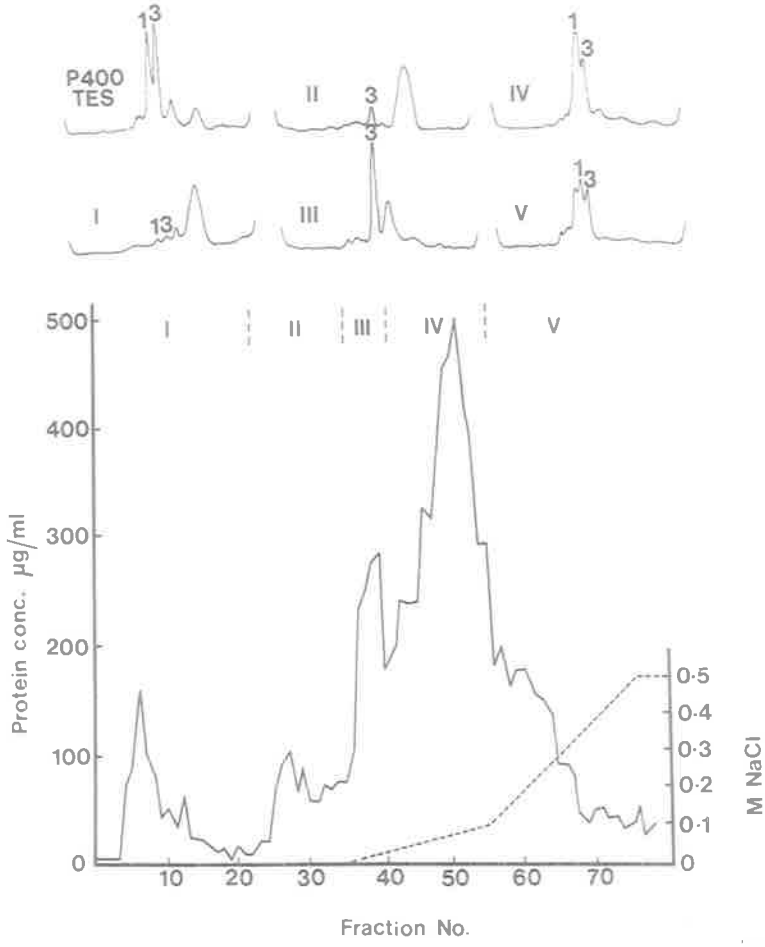

FIG. 3. *DEAE-cellulose chromatography of the Triton-plus-EDTA-soluble (TES) outer membrane proteins of strain P400. The various pooled fractions were run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1). Densitometer traces of the stained gels are labeled by the method of Schnaitman (33).*

The mutants were resistant to a group of nine K3-like phage, were unable to adsorb K3 (Fig. 6), were tolerant only to colicins K and L (Table 3), and were defective as recipients in conjugation with Hfr strains and most F-like plasmid donors (Table 4), as described previously for *con* mutants (10, 15, 34). *tolG* mutants had been reported as being sensitive to colicin K (10), but it has now been shown that, like *con* mutants, they are partially resistant (Table 3).

Map position of *con* and *tolG* mutants. It was reported previously that *tolG* mutants are cotransducible with *pyrD* (9) at about 21.5 min on the *E. coli* K-12 linkage map (39) and that *con* mutants are cotransducible with *lip* at 14.5 min (6). We therefore repeated both transductions because of the similarity of the phenotypes of the mutants. We showed that both of the *con* mutants, P460 and P212, as well as the *tolG* mutant, JF404-2a, are cotransducible with *pyrD* (Table 5). We have been unable to repeat the result previously obtained in our laboratory (6) demonstrating cotransduction with *lip* and suggest that the earlier result may be due, in part, to the difficulty we encountered using the strain HfrH *lip-22*.

Identity of colicins produced by *E. coli* 398 and *Serratia marcescens* JF246. Because it had been possible to select *con* (*tolG*) mutants using either colicin L-398 or bacteriocin JF246, we suspected they may be similar colicins.

The colicin produced by strain JF246 had not been typed previously, and, when it was tested against all of the type mutants isolated by Dav-

ies and Reeves (4, 5) (except for strains P516 and P653, which have been lost), it was observed that the patterns of resistance to L-398 and JF246 were identical. We also isolated 80 spontaneous mutants of strain P400 resistant to bacteriocin JF246 and examined their pattern of colicin resistance; all were also resistant to colicin L-398 and could be included in the classification of Davies and Reeves for mutants resistant to the A group of colicins (4). We suggest, therefore, that according to the nomenclature of Fredericq (12) and with the agreement of Foulds (J. Foulds, personal communication) bacteriocin JF246 is a colicin of type L and that it be called L-JF246.

DISCUSSION

We have confirmed that the properties previously ascribed to either *con* or *tolG* mutants apply to both. Thus, *tolG* mutants resemble *con* mutants in being resistant to only one group of 9 phages out of the 64 phages tested, in being tolerant to only colicins K and L of the set of 19 colicins used in this laboratory, and in being defective as recipients in conjugation with most F-like plasmid donors. Conversely, *con* mutants resemble *tolG* mutants in tolerance to the colicin of strain JF246; indeed, we showed that this colicin activity is indistinguishable from colicin L and it has been renamed as colicin L-

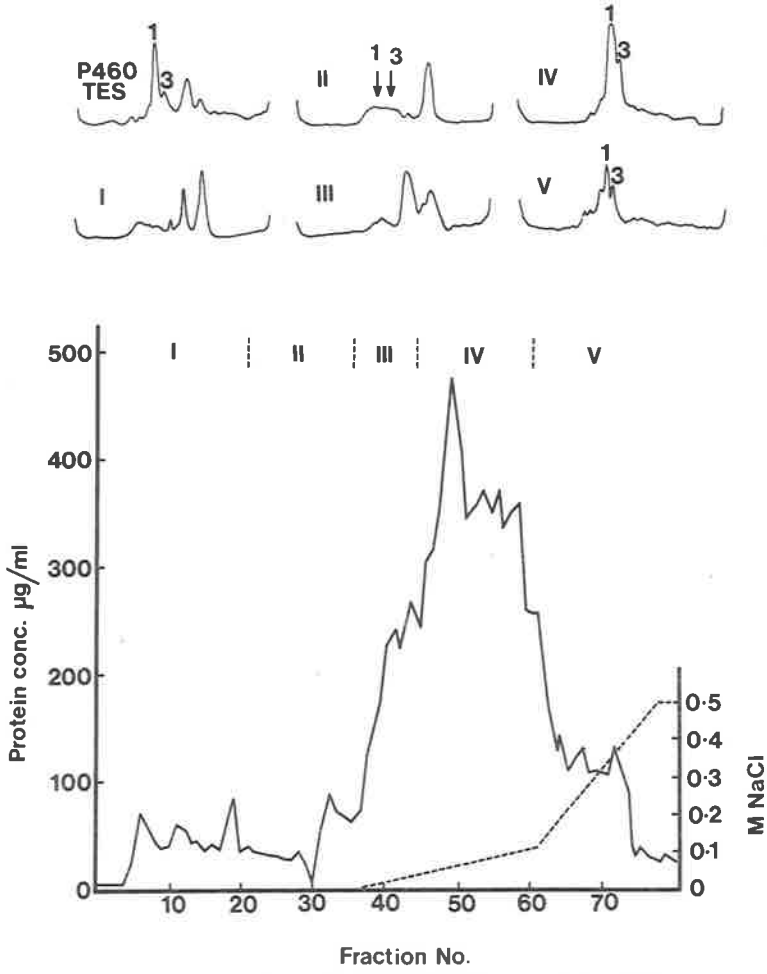

FIG. 4. *DEAE-cellulose chromatography of the Triton-plus-EDTA-soluble (TES) outer membrane proteins of strain P460 (con). The various pooled fractions were run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1). Densitometer traces of the stained gels are labeled by the method of Schnaitman (33).*

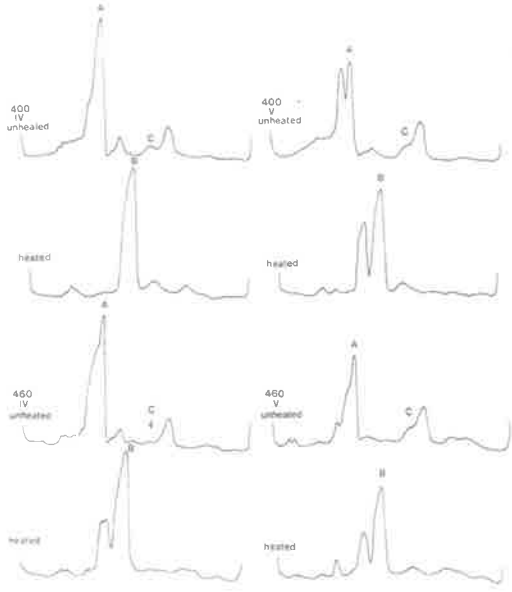

FIG. 5. *Comparison, by densitometry, of polyacrylamide gels of the protein composition of pools IV and V from the DEAE-cellulose columns of strains P400 and P460. The samples were run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 7.2 buffer system of Maizel (24) with unheated and heated samples. The peaks are labeled by the method of Schnaitman (33).*

JF246. Both types of mutation are cotransducible with *pyrD* and have a defect in their outer membrane composition, which is shown in both cases to be the loss of protein 3A only.

From Fig. 1 it can be seen that peak C is reduced in the *con* (and *tolG*) mutant; if samples are heated, this peak remains the same size in the *con* (and *tolG*) mutant but in P400 is reduced to the smaller size observed in the mutant. This suggests that it is only the portion of peak C that moves into peak B upon heating which is missing in *con* (and *tolG*) mutants. The residual peak C in P460 corresponds to another protein (the *tsx* protein), which has been shown to be absent in *tsx* mutants and functions as the receptor for phage T6 and colicin K (P. A. Manning, M. Lavoie, and P. Reeves, manuscript in preparation).

The small peak of protein running slightly faster than peak C also appears to decrease upon heating (Fig. 1), and this position corresponds to that of the heat-modifiable protein enriched in the column fractions containing protein 3B (Fig. 5). We believe that this peak, in fact, contains protein 3B. Thus, the positions of the various outer membrane proteins can be summarized diagrammatically as in Fig. 7.

The heat-modifiable protein B* of Reithmeier and Bragg (29), which Henning has shown to be identical with his protein II* (13, 14, 20, 21) and to be missing in *tolG* mutants (21), must therefore be protein 3A. This confirms the recent suggestion of Lugtenberg and his co-workers (23).

Phage TuII*, isolated by Henning as being unable to plaque on *tolG* mutants (20), thus fits into the group of K3-like phage as defined by Hancock and Reeves (15).

The loss of the ability of *con* mutants to plaque phage K3 had been assumed to be due to

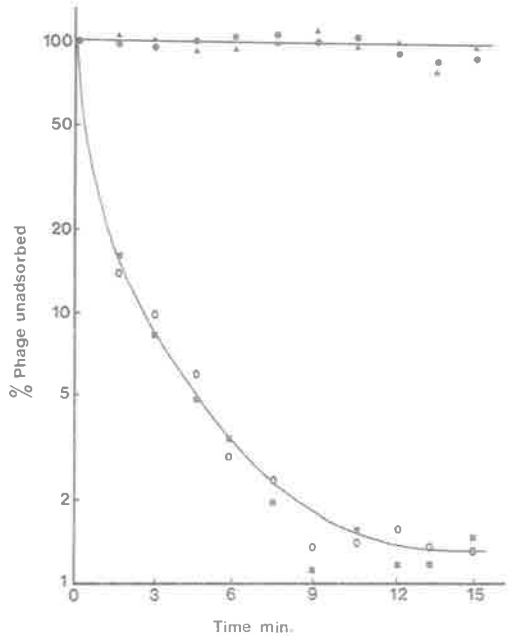

FIG. 6. *Adsorption of bacteriophage K3 to whole cells of strain P400 (○); its con mutants, P460 (●) and JF404 (■); and its tolG mutant, JF404-2a (▲). Cells were at a density of 2 × 10⁸/ml, and phage were added to a multiplicity of 1.0.*

TABLE 3. *Colicin titers on the mutants*[a]

Strain	Titer of colicin			Mutation
	L-398	L-JF246	K-235	
P400	256	512	4,028	
P460	1	1	4	*con*
AB1133	256	512	4,028	
P212	1	2	8	*con*
JF404	256	512	256	
JF404-2a	1	1	2	*tolG*
P1635	256	512	4,028	
P1636	1	1	2	*tolG*

[a] The titers of the colicins were measured by using method two as described in the text. The mutants and their parents were fully sensitive to all other colicins.

TABLE 4. *Recipient abilities with the different donors*

Donor	% Transfer[a] to:				
	P400	P460 (*con*)	P212 (*con*)	P1635	P1636 (*tolG*)
HfrH	1.8	<0.01	0.13		
HfrC	1.5	<0.01		2.0	<0.01
F′ 111	12.5	<0.01		13.0	<0.01
F′ *lac pro*	25.5	0.013	0.73		
R1*drd*19	16.9	1.31		12.3	0.74
R100-1	13.8	14.8	9.3	19.4	18.9
R136*fin*	14.9	14.0		15.7	18.2
R386	13.0	0.02		14.5	0.04
R538F*drd*1	11.2	0.07	0.30	13.2	0.03
ColV2	5.3	<0.01		5.0	<0.01
ColVB*trp*	1.2	<0.01		1.2	<0.01
R64-11	1.1	1.2	0.72	0.7	0.7
R144*drd*3	8.0	7.2		6.5	10.5
R163*drd*1	6.4	5.8		8.7	9.8
R538I*drd*2	2.4	2.0	1.8	2.3	1.9

[a] Transfer was measured as a percentage of input donor cells. Each result is the mean of at least three experiments.

TABLE 5. *Cotransduction of con and tolG with pyrD*[a]

Strain	Mutation	Resistance of *pyrD*$^+$ transductants to phage K3 and colicins K-235, L-398, and L-JF246	% Cotransduction
P212	*con*	33/152	21.7
P460	*con*	35/152	23
JF404-2a	*tolG*	10/56	17.6

[a] Resistances to phage K3 and colicins K-235, L-398, and L-JF246 were all transduced together. All transductants were *glt*, *gal*, and *str* and sensitive to colicins E2, E3, and A.

the loss of the receptor (34). Protein 3A can be shown to be the receptor for phage K3 and also for some of the other K3-like phage able to be neutralized by cell fractions (Manning and Reeves, manuscript in preparation).

Our studies with *con* mutants are continuing. We are investigating the role of protein 3A in conjugation and also in phage K3 sensitivity. We understand that its role in sensitivity to colicin L-JF246 is also being investigated (Foulds, personal communication).

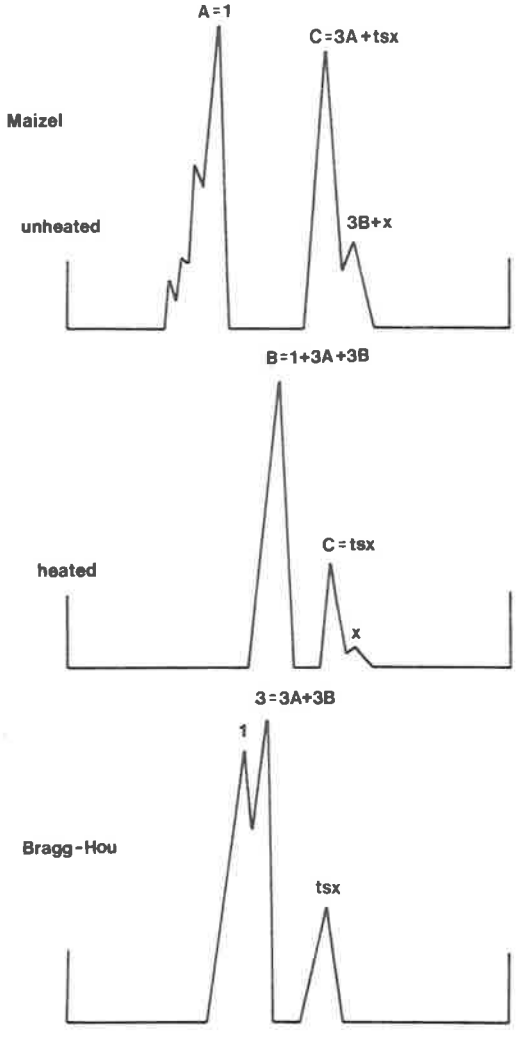

FIG. 7. *Diagrammatic representation of the positions into which the major outer membrane proteins of E. coli K-12 move upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using the pH 7.2 buffer system of Maizel (24) and the pH 11.4 buffer system of Bragg and Hou (1). Peaks are labeled by the method of Schnaitman (33) with the exception of tsx, which represents the protein now shown to be the receptor for bacteriophage T6 and colicin K (Manning, Lavoie, and Reeves, manuscript in preparation).*

ACKNOWLEDGMENTS

We wish to thank John Foulds for bacterial strains and for providing results prior to publication. We would also like to thank Carl Schnaitman for his many helpful discussions with regard to column chromatography and polyacrylamide gel electrophoresis of the outer membrane proteins of *E. coli*.

ADDENDUM IN PROOF

We have shown in this paper that our *con* mutants and the *tolG* mutants described by Foulds and Barrett (J. Bacteriol. **116**:885–892, 1973) are identical. These mutants are also identical to the *tut* mutants recently described by Henning et al. (FEBS Lett. **61**:46–48, 1976). It has now been resolved that these three designations, *con*, *tolG*, and *tut*, be renamed *ompA* (outer membrane protein) since this is the site of the structural gene for protein 3A (Manning et al., J. Bacteriol. **127**:1080–1084, 1976; Henning et al., FEBS Lett. **61**:46–48, 1976). Any reference to this mutation should now use the *ompA* designation.

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OUTER MEMBRANE OF ESCHERICHIA COLI K-12: TSX MUTANTS (RESISTANT TO BACTERIOPHAGE T6 AND COLICIN K) LACK AN OUTER MEMBRANE PROTEIN.

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Summary

Tsx mutants of Escherichia coli are fully resistant to a set of T6-like bacteriophage and are resistant to colicin K. We demonstrate that these mutants are missing an outer membrane protein (the tsx-protein) of molecular weight 32,000 as measured by SDS-polyacrylamide gel electrophoresis. Tsx mutants are receptor mutants which are unable to absorb either the bacteriophages or the colicin and the loss of receptor function can be demonstrated using outer membrane preparations.

We suggest that the tsx-protein is the receptor for both the bacteriophage and colicin.

Introduction

Cross resistance studies between bacteriophage T6 and colicin K have suggested that these two agents share a common receptor (1). In a recent study on bacteriophage and colicin resistance, it was found that the classical tsx mutants, which map at 11 min. on the chromosome of E.coli K-12 (2), were resistant only to a group of 8 T6-like bacteriophages (3) and to colicin K (4).

Sabet and Schnaitman (5) have shown that colicin K receptor lies in the outer membrane and Weltzien and Jesaitis (6) have shown that it is protein in nature

and that receptor activity is unable to be detected in membrane extracts of *tsx* mutants.

In this communication we show that *tsx* mutants are missing an outer membrane protein which we can readily detect on SDS-polyacrylamide gels.

Materials and Methods

The bacterial strains used are listed in table 1 and all cultures were grown in nutrient broth at 37°C.

TABLE 1.

Bacterial strains

(all derivatives of E.coli K-12)

Strain	Characteristics	Reference
P400	F⁻/*thr argE proA thi leu mtl xyl ara galK lacY str supE* λ	12.
P407	*tsx* mutant of P400	3.
P460	*con* mutant of P400	12.
P1731	*tsx* mutant of P460	This paper

Outer membranes were prepared as previously described (7) using the methods of Schnaitman (8) to obtain the Triton X-100 insoluble component of the cell envelope. Sample preparation and acrylamide gel electrophoresis methods have been described before (8, 9) and are essentially the pH7.2 system of Maizel (10) and the pH 11.4 system of Braggand Hou (11).

Bacteriophage T6 was from stocks maintained in this laboratory (2) and colicin K was prepared by induction as described elsewhere (7).

Phage neutralization experiments were carried out by incubating 0.1 ml volumes of dilutions of the outer membrane preparations (in phosphate

buffer 0.1M, pH7.2) with 0.1 ml of nutrient broth containing 10^3 pfu of bacteriophage T6 for 3 hours, after which 0.1 ml of a culture of indicator bacteria (strain P400, 2 x 10^8 cells/ml in nutrient broth) was added and incubation continued for a further 15 minutes. 4 ml of molten 0.7% nutrient agar was added and the whole poured as an overlay on a nutrient agar plate, incubated overnight and scored for plaque forming units.

The amount of colicin added was such that 20% survival of indicator bacteria was obtained under the assay conditions with 0.1 ml buffer substituting for the membrane preparations.

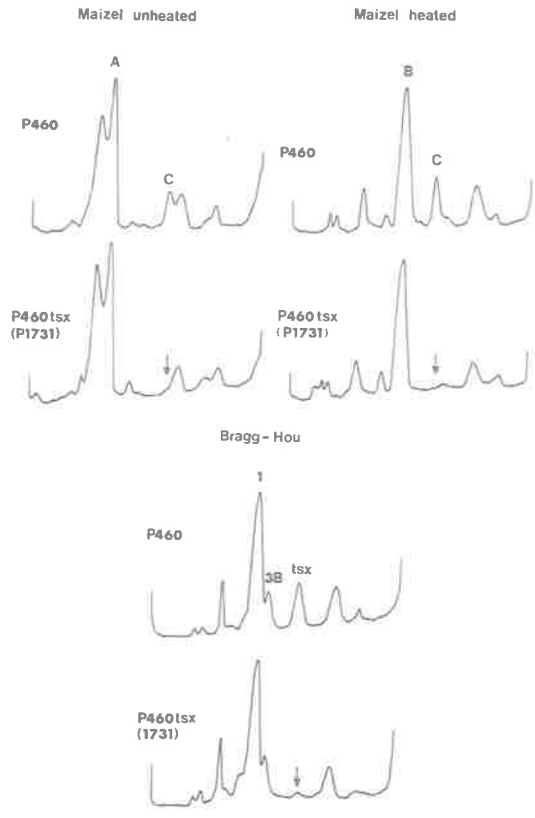

FIGURE 1.

Densitometer tracings of outer membranes of strains P460 (con) and its tsx mutant, P1731, run on SDS-polyacrylamide gels using the pH7.2 buffer system of Maizel (10) with both unheated and heated samples, and using the pH 11.4 system of Bragg and Hou (11) with heated samples. Peaks are labelled according to Schnaitman (8).

Results and Discussion

In our analysis of the outer membrane proteins of *tsx* mutants we have used *tsx*, *con* and *tsx con* double mutants of the parent strain P400. We present results obtained by using a *con* mutant (7, 12) as this enables an easier interpretation of the acrylamide gels in the absence of protein 3A (7, 8).

Thus in Figure 1 it can be seen that there is no peak C detectable when using unheated or heated samples on Maizel gels of strain P1731, a *tsx con* mutant, when compared with the *con* mutant, P460. If the heated samples are run on Bragg-Hou gels, then again there is a peak missing in the *tsx con* mutant when compared with a *con* mutant. In all three cases the protein peak missing in the *tsx* mutants is in a position corresponding to a protein of molecular weight 32000. (The standard proteins used were phosphorylase A, transferrin, bovine-serum-albumin, ovalbumin and carbonic anhydrase.)

If wild type (P400) and a *tsx* mutant is compared, then the protein peak can be shown to be present in P400 and absent in *tsx* mutants, using heated samples on Maizel or Bragg-Hou gels. However, protein 3A masks the protein if unheated samples are used.

We have called the protein missing in *tsx* mutants, the tsx-protein: it accounts in P400 for about 8% of the outer membrane proteins (as measured by comparing the areas of the peaks on the densitometer trace of the polyacrylamide gels). The tsx-protein is thus present in much greater amounts than the receptors for bacteriophages T5 (13), λ(14) and BF23 (15) which are normally undetectable on SDS-polyacrylamide gels of the whole outer membrane. However, under our normal laboratory growth conditions, no defect has so far been detected in *tsx* mutants other than the bacteriophage and colicin resistance. The function of this relatively major protein is not known.

The absence of the tsx-protein corresponds with the loss of neutralizing

TABLE 2.

Neutralization of bacteriophage T6 and colicin K by outer membrane

Strain	Amount required under the test conditions for 50% neutralization	
	Bacteriophage T6	Colicin K
P400	1 µg	5 µg
P407	>100	>100

(a) 50% of the 10^3 pfu of bacteriophage were neutralized in the test as described in the materials and methods.

(b) Only 50% of the indicator bacteria were killed by the residual colicin in the test as described.

activity for bacteriophage T6 and colicin K as previously shown by Weltzien and Jesaitis (6) and confirmed for our mutants as shown in table 2.

We are presently purifying the tsx-protein which we believe to be the receptor for both the bacteriophage T6 and colicin K.

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Recipient Ability of Bacteriophage-Resistant Mutants of *Escherichia coli* K-12

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The ability of a wide range of bacteriophage-resistant mutants to act as recipients in conjugation with F'*lac pro* and R100-1 donors has been studied. A number of mutant types defective in recipient ability with F'*lac pro*, as well as mutants which were hyperreceptive with R100-1, have been detected.

It has been recently shown that a number of mutants isolated as resistant to certain bacteriophages are reduced in their ability to act as recipients in conjugation with F' and Hfr strains (3, 4, 7). The Con mutants have been shown to lack protein 3a (7; Manning and Reeves, manuscript in preparation), using the nomenclature of Schnaitman (6). The type A and B conjugation-defective mutants of Reiner (4), selected as resistant to single-stranded deoxyribonucleic acid phage, probably have alterations in their lipopolysaccharide (LPS), as do the conjugation-defective mutants of Monner and co-workers (3), which were selected as ϕW-resistant mutants of an ampicillin-resistant strain. Lugtenberg and associates (personal communication) have isolated conjugation-defective mutants by selecting for resistance to phages T3, T4, and T7. These mutants have altered cell walls, their LPS being defective in heptose and there being reduced amounts of outer membrane proteins. Thus we have a precedent for a number of mutants, selected as phage resistant and having altered cell walls, being defective in conjugation. In this study we tested the type strains of all the bacteriophage-resistant mutant classes isolated in a recent study (2). Their recipient ability was assessed using both F'*lac pro* and R100-1-bearing donor strains. We were interested in F' and R100-1 transfer, since the Con mutants were not defective with respect to the R100-1 factor, but were with F' and Hfr strains (7).

The strains used were all derivatives of *Escherichia coli* K-12 and are listed in Table 1. The donor strains (CSH23 and JC6256/R100-1) were used as standing overnight cultures grown in nutrient broth at 37 C and diluted to 2×10^8 cells/ml. The recipient strains were grown in nutrient broth with vigorous aeration at 37 C, for at least four generations, to 2×10^8 cells/ml. A volume of 0.1 ml of donor culture was added to 1.0 ml of recipient culture and incubated for 30 min at 37 C, after which the mating mixture was diluted and plated out. F'*lac pro* transfer was measured on minimal plates selecting for proline synthesis and utilization of lactose (1%) as carbon source and with streptomycin (100 μg/ml) for contraselection. R100-1 transfer was measured on nutrient plates (Difco, blood base agar) containing 20 μg of tetracycline and 1,000 μg of streptomycin per ml.

The results of the various matings, each of which is the average of three or more experiments, are summarized in Table 2.

If we consider 50% of the number of recombinants formed with the parent to be the cut-off point, then both the Wrm mutants and all Bar

TABLE 1. *Bacterial strains used*[a]

Strain	Characteristics	Source
AB1133	F⁻/*thi,argE,his,proA,thr,leu, mtl,xyl,ara,galK,lacY,str, supE*,λ⁻	B. Bachmann
P400	*his*⁺*non*9 transductant of AB1133	R. E. W. Hancock (2)
CSH23(E5014)	F'*lac*⁺ *proA*⁺,*B*⁺/Δ(*lac pro*), *supE,spc,thi*	Cold Spring Harbor
JC6256/R100-1	R100-1/*trp,lac*Δ	N. Willetts

[a] All mutations for bacteriophage resistance were selected in P400 and are described in Hancock and Reeves (2).

TABLE 2. *Recipient ability of the bacteriophage-resistant mutants for transfer of F'lac pro and R100-1 plasmids*

Mutant classes[a]	Mutant	F'*lac pro* transfer[b]	R100-1 transfer[b]
Bar 1	P455	1.08	1.11
2	P492	0.44	1.85
3	P495	0.19	3.92
4	P436	0.24	2.59
5	P402	0.16	2.30
6	P451	0.45	1.21
7	P487	0.15	1.63
8	P489	0.017	3.11
Bfe	P445	0.70	0.58
Con	P460	0.0005	1.10
Efr	P448	0.89	1.14
Ktn[c]	P466	1.28	0.77
Ktw 1	P456	0.96	3.30
2	P476	1.12	1.65
3	P240	0.40	2.66
Misc 1	P491	1.33	0.69
2	P443	1 12	1.66
3	P498	1.11	0.53
4	P237	1.00	0.52
5	P493	1.13	0.83
Ton A	P417	0.74	0.63
B	P442	1.14	0.38
Tsx 1	P407	0.69	0.68
2	P433	0.93	1.68
Ttk 1	P429	1.05	0.66
2	P423	0.88	1.71
3	P425	0.86	1.03
4	P474	1.15	3.76
Wrm 1	P435	0.079	6.65
2	P424	0.34	2.99

[a] The mutant classes are defined in reference 2.

[b] Transfer was measured as a percentage of input donor cells and given with respect to P400, the parent recipient strain, which is taken as 1. F'*lac pro* gave 25.5% transfer and R100-1 gave 9.86% transfer to P400. All matings were carried out at least three times.

[c] Ktn is now believed to be a typical λ-resistant mutant.

mutants other than Bar (1), as well as the Con and Ktw (3) mutants, are conjugation defective with respect to F'*lac pro*. All these mutants, with the exception of Con mutants, have LPS alterations, and are thought to be receptor mutants defective in the adsorption of phage which use regions of the LPS as their receptor (R. E. W. Hancock, Ph.D. thesis, Univ. of Adelaide, Australia, 1974; Hancock and Reeves, manuscript in preparation). The only apparent structural defect in the Con mutants is the lack of protein 3a (7; Manning and Reeves, unpublished data) and these mutants have been shown to be defective in the adsorption of phage K3 (7).

The defect in recipient ability of these two classes of mutants differs in that the LPS mutants are not as defective with respect to F' transfer as are the Con mutants, while their recipient ability with respect to R100-1 is increased and that of the Con mutants is normal.

The results suggest that there are two classes of cell wall-defective mutants, altered in either LPS or protein composition, which differ in the nature of their defect in recipient ability. In the case of the Con mutants, the evidence suggests that mating pair formation is the stage affected (7). It is not shown at what stage of conjugation the defect occurs in conjugation-defective mutants with LPS alterations, but, since the LPS is a cell wall constituent, we suggest they may also be affected in the early stages of conjugation, specifically the formation of either preliminary or effective mating pairs.

The results also confirm the difference in specificity of recipient ability for F' and R100-1 factors.

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Outer Membrane of *Escherichia coli* K-12: Isolation of Mutants with Altered Protein 3A by Using Host Range Mutants of Bacteriophage K3

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A series of mutants has been isolated with alterations to protein 3A of the outer membrane. These mutations map at the previously described *con* locus as shown by cotransduction with *pyrD*. Most of them do not have detectable levels of protein 3A but are thought to have low levels of altered protein. These mutants have been detected by screening *con* mutants, isolated as resistant to bacteriophage K3, for their ability to plaque host range mutants of this bacteriophage. These host range phage mutants have activity spectra on the various *con* mutants that enable the bacterial mutants to be arranged in an order of increasing resistance to the host range phage mutants, from mutants sensitive to all host range phage to those sensitive to only one class. Likewise, the phage can be arranged in an order of increasing ability to plaque on the *con* mutants. Some of the mutants resemble the previously described *con* mutants in being tolerant to colicins K and L, and others resemble them in being highly defective as recipients with the F factor. These properties vary independently, suggesting that protein 3A can be modified to independently affect the three properties of bacteriophage receptor function, involvement in colicin sensitivity, and involvement in conjugation.

There are a number of major proteins in the outer membrane of the cell envelope of *Escherichia coli* K-12. Protein 1 (using the nomenclature of Schnaitman [22]) is normally the most abundant of these proteins. The next most abundant protein is protein 3A, which has been shown to be missing in *con* (*tolG*) mutants (4, 17, 23). Protein 3A has now been shown to function as the receptor for bacteriophage K3 and a number of similar phages (P. A. Manning and P. Reeves, manuscript in preparation). The *con* mutants are also of particular interest because of their defect in recipient ability in conjugation with most F-like plasmid donors (16, 17, 23). Proteins 1 and 3A are followed in abundance by protein 3B and the *tsx* protein. Of these, the *tsx* protein has been shown to function as the receptor for bacteriophage T6 and for colicin K (P. A. Manning, M. Lavoie, and P. Reeves, manuscript in preparation), whereas no function has as yet been found for protein 3B. All of the outer membrane proteins are normally produced in much lower amounts and so cannot really be considered as major proteins.

In this paper we report on the properties of a series of mutants that are resistant to bacteriophage K3 and, in general, have undetectable levels of protein 3A. However, the properties of these mutants suggest that they have an altered protein 3A, which is present in greatly reduced amounts. The mutants are altered in some or all of the properties attributed previously to protein 3A.

MATERIALS AND METHODS

Bacterial strains. All strains were derivatives of *E. coli* K-12 and are listed in Table 1.

Media and culture conditions. Nutrient broth, nutrient agar, and minimal agar, supplemented with the appropriate growth factors and carbon source, were as described previously (24, 25). All cultures were incubated at 37°C.

Bacteriophages. All bacteriophages were from stocks maintained in this laboratory and were described previously (11) with the exception of the host range mutants of bacteriophage K3, isolated in this study as described below.

Bacteriophage sensitivity. The sensitivity of the mutants to the set of 64 bacteriophages described previously (11, 17) was determined by using a multiple-syringe phage applicator (11).

Sensitivity to the host range bacteriophage mutants was measured by efficiency of plating using 2×10^7 log-phase bacteria and varying amounts of phage in a 4-ml 0.7% nutrient agar overlay.

Mutant selection. Independently derived, spontaneous bacterial mutants resistant to bacteriophage

TABLE 1. *Bacterial strains*[a]

Strain	Characteristics	Source/reference
P400	F⁻/*thi argE proA thr leu mtl xyl ara galK lacY str supE non* λ⁻	23
P460	*con-1* mutant of P400	23
P407	*tsx* mutant of P400	11
CSH23 (E5014)	F′ *lac*⁺ *proA*⁺,*B*⁺/Δ(*lac pro*)	Cold Spring Harbor
W620	F⁻/*thi pyrD gltA galK str rel* λ⁻	B. Bachmann

[a] All *con* mutants isolated in this study are derivatives of strain P400.

K3 were obtained by plating 2×10^7 log-phase cells with 2×10^8 plaque-forming units of phage in a 4-ml 0.7% nutrient agar overlay. Each mutant was derived by using a culture grown from a separate single colony to ensure that the mutations were of independent origin. The mutants were purified by three single-colony isolations, and 2×10^7 cells were plated with 10^9 plaque-forming units of bacteriophage K3. Plaques occurring at a frequency of less than 10^{-5} were picked and propagated through three single-plaque isolations on the mutant on which they were isolated. These phages are the host range mutants.

Mating procedures. Matings were performed as described previously (16).

Preparation of outer membranes and polyacrylamide gel electrophoresis. Outer membranes were the Triton X-100-insoluble components of the cell envelope prepared by the methods of Schnaitman (22).

Samples for electrophoresis were prepared by the method of Schnaitman (21, 22) and were run under his conditions using both the pH 7.2 buffer system of Maizel (15) and the pH 11.4 buffer system of Bragg and Hou (3). Gels were stained with Coomassie brilliant blue (26), and densitometer tracings were obtained with a Quick Scan Jr. gel scanner (Helena Laboratories Corp.)

Protein estimation. Protein concentrations were determined by using bovine serum albumin as a standard and the method of Schacterle and Pollack (20).

Transduction. P1 phage stocks were prepared as described by Miller (18), using heat-inducible P1 carrying chloramphenicol resistance derived from the R-factor R100. The transduction procedure was that of Pittard (19).

Colicin sensitivity. Colicin sensitivity was determined by the conventional cross-streak plate test (7).

RESULTS

Bacteriophage resistance. Of the 48 independent bacterial mutants isolated, 10 initially gave plaques of host range mutants, and of the others, 9 were shown to be able to plaque the host range phage isolated. All of the mutants were resistant to wild-type bacteriophage K3 and were also resistant to only bacteriophages K4, K5, Ox2, Ox3, Ox4, Ox5, M1, and Ac3 of the 64 phages tested (11). That is, they appeared to be typical *con* mutants (17, 23). However, it was found that there was considerable variation in the ability of the *con* mutants to plaque the host range phage mutants (Table 2). The overall pattern ranges from the ability to plaque all of the host range phage down to the typical *con* mutants (e.g., P460), which are unable to plaque any and on which we have been unable to isolate any host range phage mutants. The data also allow the host range mutants to be arranged in an order of increasing potency, with the wild-type K3 (h^+) being the least potent.

Map position of the mutants. All of the mutations were shown to be linked to *pyrD* by cotransduction into strain W620. The cotransduction frequencies were comparable for all classes of *con* mutation as listed in Table 3.

Colicin resistance. Table 2 also shows variation in the resistance of the mutants to colicin L-JF246.

Recipient ability in conjugation. From Table 2 it can be seen that in the bacterial mutants under study there is a general reduction in recipient ability, although in most cases it remains at a level greater than that observed in mutants such as P460, thought to be totally lacking the protein 3A.

Outer membrane proteins. We obtained *tsx* mutants of all the bacterial mutants, by selecting for resistance to bacteriophage T6, so that it would be easier to analyze the protein 3A content of the mutants (17).

In all cases, except for P1667, no readily detectable protein 3A was found (Fig. 1; P1668 is used as an example). This was determined by measuring the amount of heat-modifiable peak C on Maizel gels using unheated and heated samples. In no case were any extra peaks, which may have corresponded to an altered protein, detected. In the case of P1667, normal amounts of peak C were detected (Fig. 1) which appeared to run in the position identical to that normally occupied by protein 3A. Samples of outer membrane from a *tsx* mutant of strain P1667 were run mixed with equal amounts of outer membrane from the parent strain P400 and its *tsx* derivative, strain P407. There was

TABLE 2. *Properties of the mutants*

Type strain	No. of similar mutants	Genotype[a]	EOP of bacteriophage K3 host range mutants[b]							Resistance to colicin L-JF246[c]	Recipient ability with F′ *lac pro*[d]
			h+	h4	h15 h23 h40	h5	h3 h47	h30	h44	h1	
P400		*con*+									S	1.0
P1668	2	*con-12*	R								S	0.066
P1675	3	*con-19*	R								P	0.050
P1676	2	*con-20*	R								P	0.0004
P1658	2	*con-2*	R								R	ND[e]
P1673	2	*con-17*	R								R	0.012
P1667	1	*con-11*	R	<0.01	<0.1	R	<0.1				S	0.39
P1665	1	*con-9*	R	R	R	<0.01	<0.01		<0.1	<0.1	R	0.016
P1666	2	*con-10*	R	R	R	R	<0.01	<0.1	R	<0.1	S	0.023
P1672	1	*con-16*	R	R	R	R	R	R	<0.1	<0.1	R	0.011
P1662	2	*con-6*	R	R	R	R	R	R	R	<0.1	R	0.009
P1663	1	*con-7*	R	R	R	R	R	R	R	<0.01	R	0.004
P460	29	*con-1*	R	R	R	R	R	R	R	R	R	0.0002

[a] All mutants were shown to map at *con* by cotransduction with *pyrD* (see Table 3).
[b] EOP, Efficiency of plating; R, resistant (EOP $<10^{-6}$).
[c] S, Sensitive; P, partially resistant; R, resistant.
[d] Recipient ability was measured with respect to the number of input donor cells and is expressed as a fraction of the parent strain P400 that gave a mean transfer of 29% for the series. Each result was the mean of at least three matings.
[e] ND, Not detectable.

TABLE 3. *Cotransduction frequencies of the mutants with pyrD*

Strain	Transductants[a]		Cotransduction (%)
	con⁻ *pyrD*+	*pyrD*+	
P1668	4	10	40
P1675	23	42	55
P1676	11	24	46
P1658	19	43	44
P1673	12	25	48
P1667	28	50	56
P1665	10	37	27
P1666	13	38	34
P1672	18	37	49
P1662	17	32	53
P1663	20	43	47
P460	19	43	44

[a] Transductants were scored for *glt*, *str*, and *gal*, as well as for resistance to bacteriophage K3.

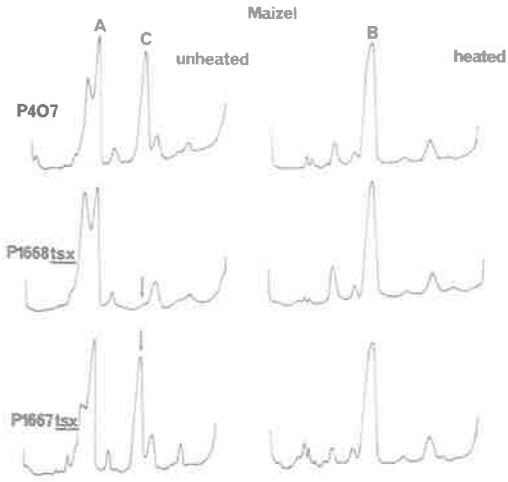

FIG. 1. *Densitometer tracings of sodium dodecyl sulfate-polyacrylamide gels of outer membrane preparations run with the pH 7.2 buffer system of Maizel (15) with unheated and heated samples. tsx mutants were used to simplify the interpretation of the gels (17). Peaks are labeled according to Schnaitman (22).*

no detectable difference in the shape and size of peak C using the mixtures as compared to the strains alone, which indicates that any alteration in protein 3A of P1667 is such as to have no effect on its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. On Bragg-Hou gels (Fig. 2) a greatly reduced peak 3 was observed in most mutants (P1668 is again used as an example), with the exception of P1667, which has a normal peak 3.

DISCUSSION

In this study we isolated a range of mutants at the *con* locus that are able to plaque a series of host range mutants of bacteriophage K3 to different degrees. All of the mutants map at *con* (*tolG*) at 21.5 min on the *E. coli* K-12 linkage map (27) as shown by cotransduction with *pyrD*, and Henning et al. (12, 13) recently showed that this gene is likely to be the structural gene for protein 3A.

All of the mutant classes studied, representing about 40% of the *con* mutants, are able to plaque at least one of the host range phages. Since it is known that the wild-type phage K3 uses protein 3A as its receptor (17, 23; Manning

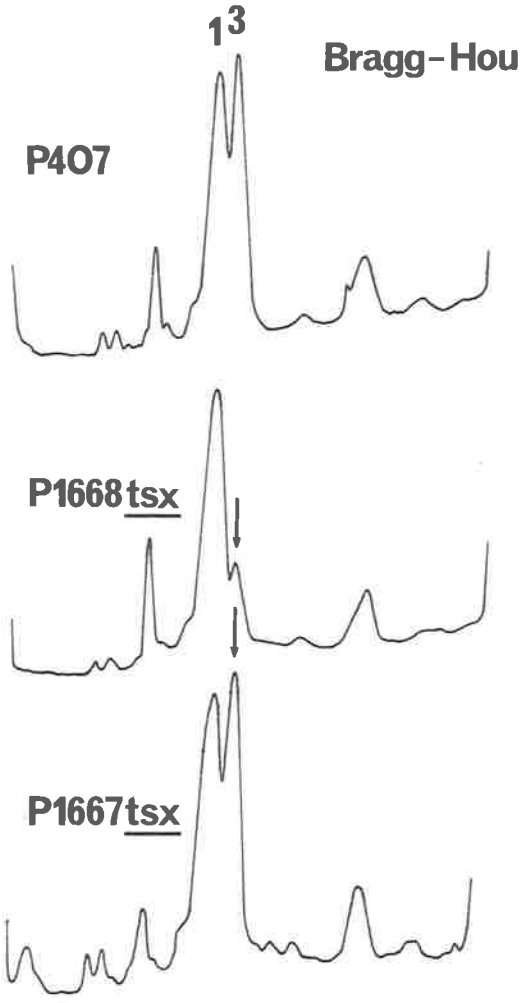

FIG. 2. *Densitometer tracings of sodium dodecyl sulfate-polyacrylamide gels of outer membrane preparations run with the pH 11.4 buffer system of Bragg and Hou (3) with heated samples. Peaks are labeled according to Schnaitman (22).*

and Reeves, manuscript in preparation), we suggest that all of these *con* mutants have some protein 3A present, although at undetectable levels except in strain P1667. It can be seen from Table 2 that, with some exceptions, the activity spectra of the K3h$^-$ mutants allow the *con* mutants to be arranged in an order of increasing phage resistance and the K3h$^-$ mutants in an order of increasing potency. This suggests that for both the bacterial and phage mutant groups the alterations are of one type but vary in degree.

We suggest that the different groups of *con* mutants differ in the amount of protein 3A present and that the bacteriophages differ in the amount of receptor that must be present for them to be able to form plaques.

The structure of all the T-even bacteriophages is similar (5, 6) and so presumably is the process by which they infect the cell (1). Goldberg and co-workers (2, 8, 9) have postulated that, for bacteriophage T4, the six tail fibers each interact with a receptor molecule and that only when all have interacted can the tail pins on the base plate of the phage go on to interact with their receptor. The nucleic acid of the infecting phage can then be injected. Bacteriophage K3 is morphologically similar to other T-even phages (14; Manning and Reeves, unpublished data). We propose that bacteriophage K3h$^-$ mutants are able to plaque on bacteria with greatly reduced levels of the protein 3A receptor, perhaps because the tail pins can be activated after less than six tail fiber-receptor interactions.

However, this hypothesis does not allow any simple explanation of the resistance of P1667 to K3h5 or P1666 to K3h44. The other properties of P1667 could be explained if the protein 3A of this strain, present in normal amounts, were altered so as to reduce its receptor activity.

If our hypothesis to explain the K3h$^-$ activity spectra is correct, then the *con*$^-$ mutants in Table 2 are arranged in approximate order of decreasing amounts of protein 3A (with the exception of strain P1667). It can be seen that sensitivity to colicin L and reduction in recipient ability do not decrease in the same order. It appears that reducing the level of protein 3A does in itself reduce recipient ability, but some mutants have a level of recipient ability well below that expected from their sensitivity to host range phage. We suggest that, whereas sensitivity of our *con*$^-$ mutants to K3h$^-$ mutants is determined largely by the amount of protein 3A present, the recipient ability and colicin L sensitivity are also affected by alterations to the structure of the proteins.

It thus appears to be possible to independently vary all three properties of the *con* mutants—the ability to plaque the host range phage, sensitivity to colicin L-JF246, and recipient ability in conjugation.

For example, strains P1658 and P1668 have similar abilities to plaque the host range mutants, but P1668 is sensitive to L-JF246 and permits conjugation at 6.6% of normal, whereas P1658 is resistant to the colicin and no detectable conjugation occurs.

Similarly, we can compare P1675 and P1676. Both have similar efficiencies of plating of the host range phage and are partially resistant to the colicin, but P1675 is a much better recipient in conjugation.

If one compares P1672 and P1673, both of

which are resistant to colicin L-JF246 and have similar abilities as recipients in conjugation, it can be seen that they are quite different in their ability to plaque the host range phage.

We conclude, then, that in general those mutants able to plaque K3h$^-$ mutants contain residual amounts of protein 3A and that in some, at least of those mutants, it is an altered protein which is present. In the case of P1667, the alteration is such as to reduce the receptor activity but not the amount of protein in the outer membrane.

It appears that mutations in the structural gene for protein 3A are able to affect both the nature of the protein and the amount which is synthesized, or inserted, in the outer membrane.

ADDENDUM IN PROOF

Since submitting this paper, we have learned of the recent work of Hofnung et al. (Mol. Gen. Genet. 145:207–213, 1976), who reported similar findings with bacteriophage λ and *lamB* mutants of *E. coli* K-12.

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