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SUMMARY

A solvent system has been developed consisting of the solvent chloroform: methanol: acetic acid: water (85:15:10:3) and silica gel H impregnated in ammonium sulphate (40 gm silica gel H in 100 ml of .15M $(NH_4)_2 SO_4$ in distilled water). With this system phospholipids of _E. coli_ can be separated by single dimension thin layer chromatography. Envelopes from _E. coli_ B/r, its N plasmid bearing derivatives, and a few spontaneous revertants which are fully antibiotic sensitive have been examined for phospholipid composition using this solvent system. Fatty acid analysis was carried out by using gas liquid chromatography technique.

No difference has been found in the percentage of total lipids, phospholipids or neutral lipids of these strains. No qualitative changes in the phospholipid species have been detected. As compared to the plasmidless host, a reduction in the ratio of PG/CL has been observed in most of the plasmid bearing strains. However, the alterations in the PG/CL ratio cannot be correlated to antibiotic resistance as a few of the antibiotic sensitive revertants also showed a reduced ratio of PG/CL.

Fatty acid analysis of N^+ strains revealed a significant increase in the level of cis-vaccenic acid relative to that found in the host strain. Elevated levels

of this fatty acid have also been encountered in a few antibiotic sensitive revertants. This illustrates that the increase in cis-vaccenic acid does not play any role in conferring resistance to antibiotics in N plasmid bearing derivatives.

The altered level of cis-vaccenic acid seen in the strains examined could be fully correlated to the property of cellular clumping shown by these cultures. No clumps were seen in the host. In those N^+ strains (and in one antibiotic sensitive revertant) in which the level of cis-vaccenic acid is comparable to that of the control, no clumping is seen.

The strains forming clumps and having increased level of cis-vaccenic acid showed a decline in absorbance and viability after maximum growth was reached (rRM100$^+$ and its sensitive revertant are exceptional in this respect). The analysis of representative strains harvested after 16 hours growth demonstrated a relatively increased level of cis-vaccenic acid and decreased levels of cyclopropane fatty acids. On the other hand, the strains having fatty acids comparable to those found in the N^- host have high survival ratios in the stationary and in the post-stationary phase. The strains tending to lose viability in the post-stationary phase have an increased ratio of total unsaturated to saturated fatty acids whereas the host and other strains showing a relatively higher viability revealed a decrease in

this ratio. The data suggests that the inefficient conversion of unsaturated fatty acids to cyclopropane fatty acids in *E. coli* strain LEB18 bearing N plasmids is somehow correlated with decreased viability.

It is possible that the fatty acid alterations observed might alter cellular permeability and permit an uptake of toxic products from the growth medium.

INTRODUCTION

Plasmids are small, circular and covalently closed DNA molecules which are stably inherited in an extrachromosomal state and do not require any selective pressure for their maintenance inside the host. They vary in size from one plasmid to another and are generally less than one-twentieth the size of bacterial DNA. They act as replicons inside their hosts. F plasmids, toxinogenic plasmids, antibiotic resistant plasmids and colicinogenic plasmids are a few examples of typical plasmids (Novick et al, 1976).

Plasmid Incompatibility and Entry Exclusion

Plasmids have been classified into a number of incompatibility groups. Incompatibility has been defined as the inability of two different plasmids (belonging to the same incompatibility group) to coexist stably in the same host cell and in the absence of continued selection pressure. The resident plasmid codes for specific alterations at molecular level in its host, which in turn prevent the establishment of a second plasmid belonging to the same incompatibility group. Nevertheless, there is no barrier to a plasmid belonging to another incompatibility group being able to infect and replicate in the same host. The barrier functions which restrict the entry of closely related plasmids are abolished when the cells are grown to stationary

phase of growth with aeration, or when they are starved of amino acids or treated with iodate. This strongly suggests that changes in the membranes of the host are mediated by the resident plasmid. However, the exact mechanism and functions of the barrier responsible for the entry exclusion of a closely related plasmid are not known in precise biochemical terms and are doubtless very complex.

To explain incompatibility many authors have proposed a replicon model which assumes the existence of different attachment sites needed for replication by plasmids of different incompatibility groups, whereas plasmids of the same incompatibility group compete for the same attachment site(s) occupied by a related resident plasmid. This has been well reviewed by Falkow, 1975.

Cell Envelope of E. Coli

The cell envelope of _E. coli_ is thought to consist of three distinct layers - the outer membrane containing lipopolysaccharide (LPS), a few proteins and phospholipids - a rigid peptidoglycan layer composed of N-acetylglucosamine, N-acetyl-muramic acid and polypeptide chains attached to N-acetylmuramic acid and forming cross bridges - the cytoplasmic membrane made up of many proteins and phospholipids. The changes in the composition of the major

macromolecules e.g. lipopolysaccharide, lipoproteins, phospholipids, proteins and peptidoglycan will alter the properties of cell envelope. These changes may occur by spontaneous or induced mutation of the cell genome. Plasmids are also known to change the properties of E. coli cell envelope.

Alterations in Host Envelopes Mediated by F Plasmid

Cells carrying the F plasmid are known to be different from cells lacking it. F^+ cells differ from F^- in respect to their surface charge as well as their motility in semisolid medium. Orskov and Orskov (1960) showed that F^+ cells acquired an additional antigen designated by them as f^+, not found in F^- cells. F^+ cells were also demonstrated to possess long, hairlike structures termed as F-pili (Brinton, c.c., 1971) which are assembled from a single phosphoglycoprotein known as F-pilin. This protein is deficient in amino acids. The F-pilus measures up to 20 μm in length and 8.5 nm in width and has a central axial hole about 3.5 nm in diameter. F-pili are also known as sex pili and are different from the commonly found fimbriae also called common pili or type I pili. Common pili are comparatively very short and are about 1.5 μm in length (Brinton, 1971).

F-Pili and Conjugation

Sex pili are quite fragile and can be removed by simple blending. They, however, resist ordinary pipetting and gentle dilution. Blended F^+ cells are ineffective in the transfer of F factor to female (F^-) cells. The role of sex pili in conjugation is not unequivocally established. One opinion considers sex pili as hollow channels which affect pair formation between male and female cells; the F factor is also transmitted through these channels from the male to the female. The second view hypothesizes that sex pili are the organs which mediate the formation of specific pairs only. Once the specific pair is formed, the sex pilus is thought to retract inside the male, bringing the male and the female cells in close surface to surface contact. The F factor is then passed onto the recipient by some other mechanism(s) independent of the sex pili. Both the models are interesting; however, they lack experimental confirmation because efforts to localise the DNA inside the sex pili as well as the presence of retracted pili inside the donor, did not yield any definitive results.

N Plasmids and their Intergeneric Transfer

As discussed already, sex pili play an important role in conjugation. This has been confirmed at least in the case of F and I plasmids. The picture is surprisingly quite complex in plasmids of the N-incompatibility group. The extensive and exhaustive search by electron microscopic examination of

negatively stained cells carrying rRM98 (N-plasmid) failed to reveal any structures or appendages similar to sex pili (Brodt et al, 1974). Sex pili have been shown in strains carrying F and I plasmids. F pili also specifically adsorb RNA-containing isometric phages as well as DNA-containing filamentous phages (Brinton Jr. et al, 1964). Similarly Khatoon et al (1972) discovered a filamentous bacteriophage named IKe which is specific to E. coli strains carrying N plasmids. This phage has been shown to attach directly to the surface of the E. coli strains harbouring N-plasmids (Brodt et al, 1974). These findings are highly significant in that they demonstrate interbacterial plasmid DNA transfer from N-plasmid-bearing donors to females occurs by a mechanism which does not involve structures like sex pili. These findings also suggest the possibility of the existence in nature of mechanisms for gene transfer which are independent of and different from those in which sex pili are implicated.

Phenotypes of E. coli B/r Harbouring N-Plasmids

N plasmids have been shown to mediate some observable changes in the phenotypic properties of the B/r strain of E. coli (Iyer, 77 and Iyer et al, 78). These include:

a) Sensitivity to bacteriophage IKe.
b) Intergeneric transfer of plasmid.
c) Resistance to various antibiotics.
d) Changes in membrane components.

Alterations in Membranes Mediated by Plasmids

The classical example of an alteration in the membrane protein is the synthesis of sex pili (Brinton Jr..et al, 1964). Sex pili are assemblies of fimbriae-like protein that lack arginine, cystine, histidine and proline. R. Iyer (1977) studied *E. coli* B/r harbouring each of seven N-plasmids with respect to the changes in the membrane proteins evoked by these plasmids. All the plasmids conferred on the host *E. coli* B/r, resistance to various antibiotics; sensitivity to the filamentous phage IKe as well as the ability to donate the plasmid to appropriate recipients. Strains bearing the plasmids rR48, rRM98 and rR269 lacked the 36500 dalton protein which is one of the major proteins found in outer membranes of plasmidless *E. coli* host and the density of the outer membranes in these strains was also reduced. Plasmids rR45, rR199 and rR205 did not mediate any of these alterations. Plasmid rR46 behaved differently from these two groups in that it increased the density of the outer membranes but did not alter the level of 36500 dalton protein. Thus it appears that the 36500 dalton protein is not implicated in the expression of IKe sensitivity or conjugative plasmid transfer.

Freeze Fracture Analysis of N Plasmid Harbouring Strains

Freeze fracture analysis of strains lacking the major 36.5 K protein showed cleavage within the outer membrane,

whereas the strains in which this protein was present at levels equivalent to those in the plasmidless host showed a fracture plane within the cytoplasmic membrane (Iyer, R. 1977). The loss of this major protein was seen in the N+ E. coli B/r but was not seen in N+ E. coli K12 transconjugants. A 31000 dalton envelope protein present in transfer proficient N+ E. coli K12 strains is absent in transferless derivatives. An envelope protein which coincides with the transfer phenotype in the B/r host has a molecular weight of 46,500 (Iyer, R., unpublished data).

Phospholipids and their Localisation in E. coli

The total lipids of E. coli are entirely located in the cell envelope (Lennarz, W., 1967). Phospholipids are the major components and comprise about 90% of the total lipids; neutral lipids account for the rest (Suling and O'Leary, 1977). The total lipid content of E. coli has been reported by many workers to be around 10% of the dry weight of the cells. (Law, J.H. 1961; Bishop and Work, 1965; Kaneshiro and Marr, 1962; Kanemasa et al, 1967; Cronan, Jr. J.E., 1968 and Damoglou and Dawes, 1968).

Phospholipids are located in both the outer and inner membranes (Miura and Mizushima, 1968; Schnaitman, 1970 and White et al, 1972). The distribution of the phospholipid species is reported to be similar in both the membranes. However, lysophosphatidyl ethanolamine is located only in the

outer membrane (White et al, 1972). This may not be the case in vivo because the phospholipids are comparatively protected against the enzyme phospholipase which degrades phosphatidyl ethanolamine to lysophosphatidyl ethanolamine in in vitro studies during the extraction procedure.

Phospholipid Species Reported in E. coli

This aspect has been extensively studied by many workers including Law, 1961; Kanfer and Kennedy, 1963; Tarlov and Kennedy, 1965; Kanemasa et al, 1967; Cronan, 1968; Ames, 1968 and Desiervo, 1969. According to these workers phosphatidyl ethanolamine forms a major part, 70-80% of the phospholipids. The next in order are phosphatidyl glycerol and cardiolipin each of which constitute about 5-15% of the cellular phospholipids. The other minor phospholipids reported are phosphatidyl serine and lysophosphatidyl ethanolamine. The proportion of the phospholipid species has been shown to vary from one growth phase to another (Cronan, Jr. and Vagelos, 1972).

It is of interest that whereas phosphatidyl glycerol and cardiolipin show turnover during the growth period, phosphatidyl ethanolamine does not seem to do so (Kanemasa et al, 1967). The role played by the turnover of the phospholipid species still remains to be determined. The need to have a complex composition of these phospholipids is questioned by the isolation of mutants which are defective in

the synthesis of phosphatidyl ethanolamine or cardiolipin. (Raetz, 1975; Ohta et al, 1974; Hawrot and Kennedy, 1975 and Pluschke et al, 1978). From these results it appears that phosphatidyl ethanolamine and cardiolipin do not play an essential role in the function of cellular membranes of E. coli. Ultimately we may be in a position to acquire mutants which function normally and have membranes with phosphatidyl glycerol as the only phospholipid species. It will be interesting to analyse the behaviour and functions of the membranes in such a host.

Phospholipid Associated Fatty Acids of E. Coli

The fatty acids of the phospholipids of E. coli have been analysed by many workers (Kaneshiro and Marr, 1962; Scheuerbrandt and Bloch, 1962; Hilderbrand and Law, 1964; Bishop and Work, 1965; Karkas et al, 1965; Law et al, 1963; VanGolde and VanDeenen, 1967; Kanemasa et al, 1967; Silbert et al, 1968; Cronan, 1968; Peypoux and Michel, 1970; Silbert, 1970; Hendler et al, 1970; Suling and O'Leary, 1977.) The fatty acids reported by these workers consist of saturated fatty acids comprising lauric, myristic, palmitic and stearic. Palmitoleic and cis-vaccenic were the unsaturated fatty acids encountered in E. coli. Cyclopropane fatty acids were cis - 9, 10 - methylene hexadecanoic acid and lactobacillic. β-hydroxymyristic has been reported by a few of these workers.

Fatty Acids in Major Phospholipid species of E. coli

Phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin are the major phospholipids. Kito et al (1972) analysed the fatty acid composition of these phospholipids in E. coli and found that the proportion of various fatty acids was different. Major differences were observed in the proportion of cis-vaccenic acid which comprised approximately 30, 50 and 70% of the total fatty acids in phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin respectively. As the temperature of growth was lowered, a progressive decrease in palmitic acid and corresponding increase in palmitoleic and cis-vaccenic acid was found in phosphatidyl ethanolamine while only cis-vaccenic acid increased as palmitic acid decreased in phosphatidyl glycerol and cardiolipin.

Fatty Acids in Lipopolysaccharides of E. coli

A small proportion of the total fatty acids is present in the lipopolysaccharides of E. coli. The fatty acids are mainly lauric acid, myristic acid and palmitic acid. (Burton and Carter, 1964; Taylor et al, 1966). Hydroxydecanoic acid, C_{16} unsaturated, cyclopropane fatty acids C17 and C_{19} and β-hydroxymyristic acid have also been reported in small amounts by these workers in the lipopolysaccharides of E. coli.

Effect of Growth Phase on Lipids and Fatty Acid Composition

There are marked changes in the composition of lipids and their fatty acid composition as growth progresses from logarithmic to stationary phase. These changes are reflected in the increase in cardiolipin; decrease of phosphatidyl glycerol; an increase in cyclopropane fatty acids and a simultaneous decrease in unsaturated fatty acids (Kanfer and Kennedy, 1963; Peypoux and Michel, 1970; Randle et al, 1969; Starka and Moravova, 1970). Phosphatidyl glycerol is known to be a precursor of cardiolipin, hence the increase in cardiolipin is accompanied by corresponding decrease of phosphatidyl glycerol. Both phosphatidyl glycerol and cardiolipin have been shown to turn over but the role played by their turnover is not clear so far.

Effect of Temperature on Fatty Acid Composition

To maintain fluidity of the membranes, E. coli has been shown to alter its fatty acid composition. Unsaturated fatty acids which remain fluid at lower temperature are increased with a corresponding decrease of saturated fatty acids during a shift from higher to lower temperature (Marr and Ingraham, 1962; Shaw and Ingraham, 1965; Haest et al, 1969; Hechemy and Goldfine, 1971; Sinesky, 1971). Saturated fatty acids solidify at the lower temperature whereas the unsaturated fatty acids remain in the fluid state at the same temperature, hence the change from saturated to unsaturated

fatty acids takes place as expected by a shift from higher to lower temperature. A shift from lower to higher temperature is accompanied by increase in saturated fatty acids and a relative decrease in unsaturated fatty acids.

Role of Cyclopropane Fatty Acids

The role played by the cyclopropane fatty acids still remains to be resolved. These fatty acids are produced in large proportions in the stationary phase of E. coli. Low pH (Knivett and Cullen, 1965), high temperatures (Marr and Ingraham, 1962; Kito et al, 1972; Armstrong et al, 1967), high sodium chloride concentration (McGarrity and Armstrong, 1975) and anaerobiosis (Knivett and Cullen, 1965) have also been shown to favour the increased production of the cyclopropane fatty acids. It has been suggested that the cyclopropane group protects the double bonds from oxidation (Law et al, 1963) but this theory has been contradicted by the increase of cyclopropane acids by anaerobiosis (Knivett and Cullen, 1965).

Lipids and Antibiotic Resistance

Several workers have related an increased lipid content to bacterial resistance to different anti-microbial drugs. Streptococcus pyogenes resistant to tetracycline showed an increase of up to threefold in extractable lipids (Norrington and James, 1970). Similar findings were reported by Brown and Wood (1972) on examination of Pseudomonas aeruginosa and

Klebsiella aerogenes resistant to polymyxin. The cell wall fractions of these polymyxin resistant strains had lower amounts of phospholipids than the sensitive strains. Rhizobium meliloti resistant to viomycin had increased levels of phospholipids (MacKenzie and Jordan, 1970).

Suling and O'Leary (1977) analysed strains of the family Enterobacteriaceae susceptible and resistant to various antibiotics. They did not observe any qualitative differences in the composition of phospholipids and fatty acids. They did not find any significant correlation between the total lipids, neutral lipids and phospholipids and resistance to antibiotics. They found, however, that the ratio of phosphatidyl glycerol to cardiolipin changed in the strains harbouring the R factor 222 which imparted resistance to tetracycline. These strains showed a higher phosphatidyl glycerol to cardiolipin ratio than the plasmidless host. Strains resistant to polymyxin had a decreased ratio of phosphatidyl glycerol to cardiolipin. These workers also reported differences in the relative amounts of unsaturated and cyclopropane fatty acids between strains sensitive and resistant to antibiotics. The findings of these workers with respect to cyclopropane fatty acids corroborated the results reported by Dunnick and O'Leary (1970).

Legakis et al (1977) examined the lipid composition of E. coli strains in relation to resistance to penicillin. No qualitative or quantitative differences in the composition of

extractable lipids were observed by them. The sensitive strains had a slight increase in the triglyceride fraction. The highly resistant strains showed an increased level of myristate which was accompanied by a decrease in the level of other fatty acids. Bound lipids of resistant cells had a higher level of β-hydroxy decanoic acid than those of sensitive cells. They postulated that the change in the lipid metabolism results in the alteration of the architecture of the cell wall and is responsible for decreased permeability in the uptake of penicillin. No penicillinase activity could be detected in the supernatants after pelleting the cells.

Ultrasonic treatment of the cells released all the penicillinase activity. The strains showing high resistance towards penicillin showed reduced levels in the uptake of this drug. The modification in the permeability of the membranes which reduces the uptake of the drug, in conjunction with the production of the enzyme penicillinase renders these strains highly resistant to penicillin. This illustrates the role played by the permeability barrier built in the cell envelope of the highly resistant strains.

Lipids Versus Proteins in Antibiotic Resistance

As already mentioned, many workers have associated bacterial antibiotic resistance to the total lipid content and composition. Resistant gram negative microorganisms have

been shown to have decreased cyclopropane fatty acids whereas the resistant gram positive bacteria failed to reveal this change (Dunnick and O'Leary, 1970). Chopra and Eccles (1977) studied mutants of E. coli deficient in cyclopropane fatty acids and observed that they were more sensitive to tetracycline than the wild type parent strain. They also speculated that the change in the fatty acid pattern can be mediated by products of genes on the plasmid other than those of the tetracycline resistance controlling regions.

Plasmid mediated resistance to tetracycline in E. coli has been reported to be associated with the synthesis of certain membrane proteins referred to as tetracycline resistance proteins. (Levy and McMurray, 1974; Yang, Zubay and Levy, 1976; Wojdani, Avtalion and Sompolinsky, 1976). Wojdani and coworkers (1976) prepared a specific antiserum by immunizing rabbits with cell evelopes prepared from an E. coli strain carrying an R plasmid which mediated resistance to tetracycline (Tc). The antiserum so obtained was absorbed with cell envelopes of an isogenic E. coli strain which did not harbour an R plasmid and was tetracycline sensitive. The absorbed antiserum contained antibodies which reacted specifically with cell envelopes of E. coli strains resistant to tetracycline but failed to react with tetracycline susceptible strains. These workers were also successful in preparing antiserum which reacted specifically with tetracycline resistant strains of

Staphylococci, by following the same procedure used for *E. coli*.

They also separated and characterised the antigen for tetracycline resistance by passing the cell envelope preparations of Tc-resistant strains through a column of solidified antiserum prepared against the cell envelopes of Tc-susceptible strains. The antigen which passed through the column gave a single protein band when tested by SDS-polyacrylamide gel electrophoresis. This protein had a molecular weight of 50,000 and reacted specifically with antiserum prepared with cell envelopes of Tc-resistant *E. coli* strains harbouring R plasmid but did not react with antiserum prepared against cell envelopes of Tc-susceptible strain. They characterised the protein responsible for Tc-resistance in *Staphylococci* which had a molecular weight of 30,000 daltons. To confirm their claim, these workers did not demonstrate that these proteins were induced in the presence of tetracycline as tetracycline resistance is inducible. Plasmids code for other changes in the host and it is also possible that these proteins are the products of regions of plasmid DNA other than those controlling tetracycline resistance.

Aims of the Present Investigations

Plasmids of the N incompatibility group are known to mediate several alterations in the phenotypes of *E. coli* B/r

including:

i) Resistance to antibiotics.

ii) Sensitivity to phage IKe.

iii) Alteration in envelope (inner and outer membrane) density.

iv) Alterations in integral envelope protein including elimination or modification of a major outer membrane protein of mol. wt. 36,500.

Proteins constitute important envelope components, which are embedded in envelope phospholipids. Together they play an important role in cellular physiology and comprise the permeability barrier of the cell. Phospholipids play an important role in the structural integrity of membranes. They are also needed for the function of certain membrane-associated enzymes.

The aim of the present study was to investigate the phospholipids and fatty acids of N plasmid bearing strains of E. coli B/r and compare them with those of the plasmidless host. Attempts would also be made to correlate any changes in membrane phospholipids, if found, with observeable effects on cellular growth or other recognizable properties.

MATERIALS AND METHODS

Bacterial Strains: All the bacterial strains were maintained on L agar plates (composition given below) with or without ampicillin or tetracycline at 30 µg/ml. These strains were kindly provided by Dr. Rajul Iyer (Microbiology and Immunology Department, Ottawa University). The strains and their phenotypes are listed in Table 1.

Chemicals: The chemicals used were of the best reagent grade. All the solvents were glass-distilled before use. The phospholipid kit (standards) was purchased from Serdary Research Lab., INC., London (ONTARIO). Fatty acid methyl ester standards for G.L.C. were a gift from Dr. M. Kates (Biochemistry Department, Ottawa University). Cyclopropane fatty acid methyl ester standards (C_{17} and C_{19}) were given by Mr. James T. McGarrity (Biology Department, Ottawa University).

Growth Medium: Cells were grown in L broth which was prepared as follows:

Tryptone (Difco)	10 gms
Yeast extract (Difco)	5 gms
Sodium chloride	5 gms
Distilled water	1000 ml
pH	7.0

L agar was prepared by adding Difco agar (1.0%).

Table 1

ESCHERICHIA COLI STRAINS*

Strain NO.	Plasmid	Relevant Markers**	Selection
LEB18(B/r)	–	prototroph	–
LEB508(rR45$^+$)	rR45	Ap Tc Su Tra$^+$	Tc
LEB509(rR46$^+$)	rR46	Ap Sm Sp Tc Su Tra$^+$	Tc
LEB505(rR48$^+$)	rR48	Pen Sm Tc Su Tra$^+$	Tc
LEB505(rR48$^-$)***	–	–	–
LEB500(rRM100$^+$)	rRM100	Ap Tra$^-$ Ike^{poor}	Ap
LEB500(rRM100$^-$)***	–	–	–
LEB502(rRM98$^+$)	rRM98	Ap Sp Sm Tc Tra$^+$ Ike^S	Ap
LEB510(rR199$^+$)	rR199	Tc Tra$^+$	Tc
LEB511(rR205$^+$)	rR205	Pen Tc Su Tra$^+$	Tc
LEB506(rR269$^+$)	rR269	Ap Km Su Tc Tra$^+$	Tc
LEB506(rR269$^-$)***	–	–	–

* Strains were kindly provided by Dr. R. Iyer.

** Abbreviations: Ap, ampicillin; Tc, tetracycline; Sm, streptomycin; Km, kenamycin; Sp, spectomycin; Su, sulphonamide; Pen, penicillin, Tra$^+$, plasmid transfer; Ike^S, sensitivity to phage Ike.

*** Antibiotic sensitive revertants have been designated with a negative superscript. Although no plasmid DNA has been detected in cesium chloride-ethidium bromide gradients (R. Iyer, personal communication), the absence of plasmid DNA has not been rigorously established.

<u>Growth Curves</u>: All strains were streaked on L agar plates with appropriate antibiotic supplements as required and incubated at 37°C for 16-18 hours. Subcultures were made in 10 ml of L broth which were incubated at 37°C for 16-18 hours. A portion of this subculture was used to inoculate 10 ml of L broth in nephlometer flasks. The inoculum was adjusted so as to achieve an initial absorbance of 0.05 units at 610 nm using a Bausch and Lomb spectrometer. The culture flasks were then incubated at 37°C in a water bath shaker at 150 strokes/minute. The absorbance was recorded at intervals during a period of 24 hours. The growth curves were drawn on semilog paper with time (hours) on the x-axis and absorbance on the y-axis (Fig. 1).

<u>Total Viable Counts</u>: 0.5 ml of broth cultures were withdrawn aseptically and diluted 10^{-6} and 10^{-7} times in physiological saline. One-tenth ml of these dilutions was plated on L agar plates with or without an antibiotic (ampicillin or tetracycline at 30 μg/ml). Colonies were scored after 24 hours at 37°C.

For the extraction of total lipids and dry weight determination, the cells were grown under similar conditions except that 2.4 litres of L broth was used. The volume was kept at one-fifth the capacity of flasks. The growth of cells was terminated by immediate transfer of flasks into ice cold water, after an absorbance of 0.25 was reached.

Dry Weight of Cells: Cells were harvested (after they were grown to an absorbance of 0.25) by centrifugation at 12,000 r.p.m. (16,000 x g) using an International centrifuge model PR2. The pellet was thoroughly washed once with chilled distilled water, pelleted again at the same speed and suspended uniformly in 20 ml of distilled water. Four ml of this suspension was transferred into a preweighed, clean and dry weighing bottle. The cells were then dried at 105°C for 16-18 hours and then over KOH pellets in a dessicator under vacuum for 3 hours. Dry weight of the cells was determined using a Mettler balance. The remaining 16 ml of the cell suspension was used for the extraction of lipids.

Dry weight and total lipid determinations were carried out on a few strains by drying the cells in a freeze dryer. The cells so dried were weighed accurately and lipids were extracted from the dried cells.

Extraction of Total Lipids: A modification (Kates, 1972) of the Bligh and Dyer (1959) procedure was followed for the extraction of total lipids. To 16 ml of the cell suspension (see dry weight of cells) was added 40 ml of methanol and 20 ml of chloroform to yield a mixture of methanol-chloroform-water having a ratio of 2:1:0.8. The extraction was carried out at 20°C for 3 hours with constant stirring. The mixture was then centrifuged at 12,000 r.p.m. (16,000 x g) for 10 minutes. The supernatant was transferred to a

separating glass funnel and stored at 4°C. The pellet was used for a second extraction by uniformly suspending in 38 ml of a mixture of methanol-chloroform-water (2:1:0.8). The second extraction was carried out for 18 hours at 20°C and the supernatant recovered after centrifugation at 12,000 r.p.m. (16,000 x g) for 10 minutes. Supernatants from both the extractions were pooled in the separating funnel. Thirty ml each of chloroform and distilled water was added, shaken thoroughly and allowed to separate overnight at 20°C. The lower chloroform layer was collected and the remaining mixture was washed once with 30 ml of chloroform. The two chloroform layers were pooled and the lipids were recovered by adding one-third the volume of benzene and evaporating the solvents at 30°C under reduced pressure in a rotary evaporator. The dried lipids were dissolved in a small amount of chloroform, centrifuged at 5000 r.p.m. (2000 x g) for 5 minutes; the supernatant was dried under vacuum over KOH pellets and weighed. The lipids were then dissolved in 5 ml of chloroform and stored in glass-stoppered flasks at -20°C in an atmosphere of nitrogen. The percentage lipid content was calculated using the recovery of total lipids (dried) and dry weight of cells.

Neutral Lipids: Neutral lipids were characterised by thin layer chromatography on silica gel H plates using a solvent mixture petroleum ether: ethyl ether - acetic acid

(85:15:1). Identification was carried out by co-chromatography of authentic standards. Phospholipids stayed at the origin in this system.

Phospholipids: The phospholipid species were identified by running thin layer chromatography on silica gel H plates of 0.5 mm thickness (20 x 20 cms) in different single phase solvent systems i.e. (i) chloroform-methanol-water (65:25:4) (Wagner et al, 1961); (ii) chloroform-methanol-7N ammonium hydroxide (65:35:5) (Nichols, B.W., 1963) and (iii) chloroform-methanol - acetic acid-water (85:15:10:3) (a system evolved by trial and error during the present investigations. Silica gel H impregnated in 0.15 M ammonium sulphate was used for preparation of TLC plates).

Single Dimensional Solvent System - A Modification

Silica Gel H Plates: 40 grams of silica gel H was thoroughly emulsified in 100 ml of 0.15 M ammonium sulphate, spread uniformly over glass plates (20 x 20 cms) so as to give a thickness of 0.5 mm using a Desega (Germany) spreader. The plates were allowed to dry at 20°C for an hour and activated for 3 hours at 110°C prior to use.

Solvent System: The solvent mixture consisted of chloroform-methanol-acetic acid-water (85:15:10:3). The glass chromatographic chamber was lined with filter paper soaked in the solvent and the lid of the chamber was sealed with adhesive tape so as to achieve saturation of the chamber with solvent vapours.

Reagent Sprays for Detection of Phospholipids: Ninhydrin spray (Wagner et al, 1961) was employed for the detection of free amino group. Phosphate groups were identified by the phosphate stain of Dittmer and lester (1964) modified by Vaskovsky and Kostetsky (1968). Periodate Schiff reagent (Shaw, N., 1968) was employed to spot viccinal glycol containing lipids.

Lipids were visualized by exposure to iodine vapours for 30 seconds. The spots were marked and scraped off the chromatogram for quantitative estimation of phosphorus content.

To acquire permanent records the chromatograms were treated with sprays of 50% sulphuric acid and subsequently heated at 150°C. The chromatograms were then photographed and stored. These chromatograms were not used for any other test.

Estimation of Phospholipids: The phospholipids were estimated by determining the phosphorus content using the

modified procedure of Bartlett (1959). The lipid sample containing 0.5 to 5 µg of P was digested with 0.8 ml of 72% perchloric acid for 5 minutes by heating in a Kjeldahl digestion rack over a gas burner. Distilled water (4.2 ml was added followed by 0.2 ml of 5% ammonium molybdate solution in distilled water and 0.2 ml of amidol solution (freshly prepared by dissolving 0.5 gram of amidol in 50 ml of 20% sodium bisulphite solution and filtered). The tube contents were mixed, heated in a boiling water bath for 7 minutes and cooled for 15 minutes. Silica gel was removed by centrifugation at 2,500 r.p.m. for 10 minutes. Silica gel controls from corresponding areas were also treated similarly. Absorbance was read at 820 nm using a Coleman Junior Spectrophotometer in round 12 mm cuvettes. A standard curve was obtained each time by running 0.5-5 µg P standards. Standard phosphate solution was prepared by dissolving 1.097 g of KH_2PO_4 in 250 ml of distilled water to give a stock solution of 1000 µg P per ml. Samples as well as standards were run in duplicate. Care was taken to wash the glass equipment with strong sulphuric acid and thoroughly rinse with deionised distilled water.

<u>Analysis of Total Fatty Acids of E. coli</u>

Fatty acid methyl esters were prepared from the total lipids following the procedure of Kates (1964). One ml of total lipids containing 1-5 mg of lipid sample was taken in

an erlenmeyer flask (50 ml) with a side arm. The solvent was evaporated in a stream of nitrogen. To this was added 4.5 ml of methanolic-HCL (2.5%). The mixture was heated under reflux (calcium chloride tube on condenser) for 2 hours. Half a ml of distilled water was added to the mixture and the volume was adjusted to fill the side arm by adding methanol-water (9:1) and the fatty acid methyl esters were extracted with four portions (5 ml) of petroleum ether (boiling point: 30-60°C). The solvent was evaporated at 30°C, in a stream of nitrogen and the dried residue was dissolved in 50 µl of chloroform.

Gas Liquid Chromatography: The methylated fatty acids were analysed using a CARLO ERBA Model GV gas chromatograph equipped with flame ionization detectors. Glass columns 180 cms x 2 mm (1D) coated with 10% SP2300 on 100/120 chromosorb WAW 01-1849 (Supelco Inc., Bellefonte, PA) were used. The column temperature was maintained at 190°C. Nitrogen was used as the carrier gas (0.18 kg per square inch). Fatty acids were identified by comparison of retention times relative to palmitate and with those obtained for the standards as well as published ones (Kates, 1974). The percentage of the fatty acids were calculated from the chromatograms using the peak-height times retention time method of Carroll, 1961.

Separation of Outer and Inner Membranes of E. coli

The procedure of Smit et al (1975) was followed with some modifications. The procedure adopted by Smit et al (1975) was a modification of the procedure of Koplow and Goldfine (1974) which in turn was modified from the one followed by Schnaitman (1970). The procedure used in the present studies is as follows:

i) Cells were grown in 2.4 litres of L broth at 37°C with aeration to an absorbance of 0.25 at 610 nm.

ii) Cells were pelleted at 12000 r.p.m. (16,000 x g) for 10 minutes using an International centrifuge model PR2. The pellet was washed once with ice cold 10 m M N-2-hydroxy-ethyl piperazine-N'-2-ethane sulphonic acid (HEPES) buffer pH 7.4 and suspended in 10 ml of the same buffer. Cells were stored at -70°C till further use.

iii) The cell suspension was diluted to 100 ml with HEPES buffer. Ten milligrams each of deoxyribonuclease and ribonuclease were added and the suspension was sonicated with 4 x 30 seconds pulses in a Bronwill Biosonik Disintegrator. The cells were kept ice cold during sonication.

iv) The unbroken cells were removed by pelleting at 12000 r.p.m. (16,000 x g) for 10 minutes.

v) The supernant from (iv) was further centrifuged at 18,000 r.p.m. (35,000 x g) for 2 hours. The pellet

constituted the total membranes.

vi) Separation of the outer and inner membranes was carried out using a discontinuous sucrose gradient comprised of 8 ml of 2.02 M; 24 ml of 1.44 M and 20 ml of 0.77 M sucrose prepared in HEPES buffer pH 7.4. The total membranes were layered on top of the gradient (10-40 milligram proteins). The gradient was then centrifuged at 22,500 r.p.m. (50,000 x g) for 21 hours in a Spinco SW25.1 rotor in a Beckman LT-2 ultracentrifuge. Fractions were collected by piercing the bottom of cellulose nitrate centrifuge tubes with 18-gauge needle. The contents were collected in separate glass tubes (50 drops in each). Absorbance of each fraction was recorded at 280 and 260 nm with a Carl Zeiss Spectrophotometer model M4 Q3. Corrections were made for the presence of nucleic acids and proteins estimated using the formula i.e. (A280/A260) x A280. Fraction numbers (X-axis) were plotted against protein levels (Y-axis).

Peak fractions were pooled, diluted four times with HEPES buffer and the membranes were recovered by pelleting at 40,000 r.p.m. (170,000 x g) for 2 hours in FA50 rotor using Beckman LT-2 ultracentrifuge and polycarbonate tubes. The membrane pellets so obtained were washed with distilled water four times and finally suspended in a small volume of

distilled water (one ml for outer membranes and 0.5 ml for inner membranes). Steps (ii) to (iv) were carried out at 0-4°C.

RESULTS

Growth Curves of Escherichia coli Strains at 37°C

The growth curves of the E. coli strains used in the present investigations all followed almost the same pattern, at least in the exponential phase (Fig. 1). The mid-log phase occurred at 0.25 units absorbance. The maximum growth levels were obtained in about 8-10 hours. The plasmidless host, strains bearing plasmid rR205 and the strain cured of the plasmid rR48 followed an identical pattern of growth over a period of 24 hours. They reached a maximum growth level of 1.15 units absorbance and did not show any decline in O.D. thereafter up to 24 hours. The strains bearing the plasmid rRM100 and the strain cured of this plasmid reached a maximum plateau level of 1.1 absorbance units and maintained this level up to 24 hours. The rest of the strains (bearing N plasmids as well as some sensitive revertants) showed a decline in absorbance after completion of maximum growth levels. Most of the N plasmids affected the growth of the strains in the stationary phase of the host. Plasmids rR45, rR269, rR48 and rR199 proved to be most detrimental to the host strain in its growth pattern. The strains bearing these plasmids failed to attain the same growth level as that of the plasmidless host and also showed a decline in absorbance in the stationary phase. The strain cured of the plasmid rR48 followed the growth pattern of the host whereas the

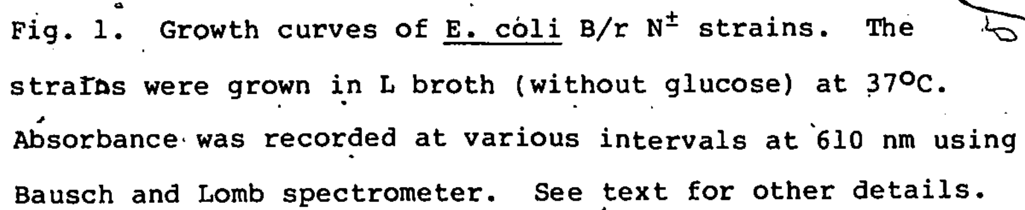


Fig. 1. Growth curves of <u>E. coli</u> B/r $N^{\pm}$ strains. The strains were grown in L broth (without glucose) at 37°C. Absorbance was recorded at various intervals at 610 nm using Bausch and Lomb spectrometer. See text for other details.

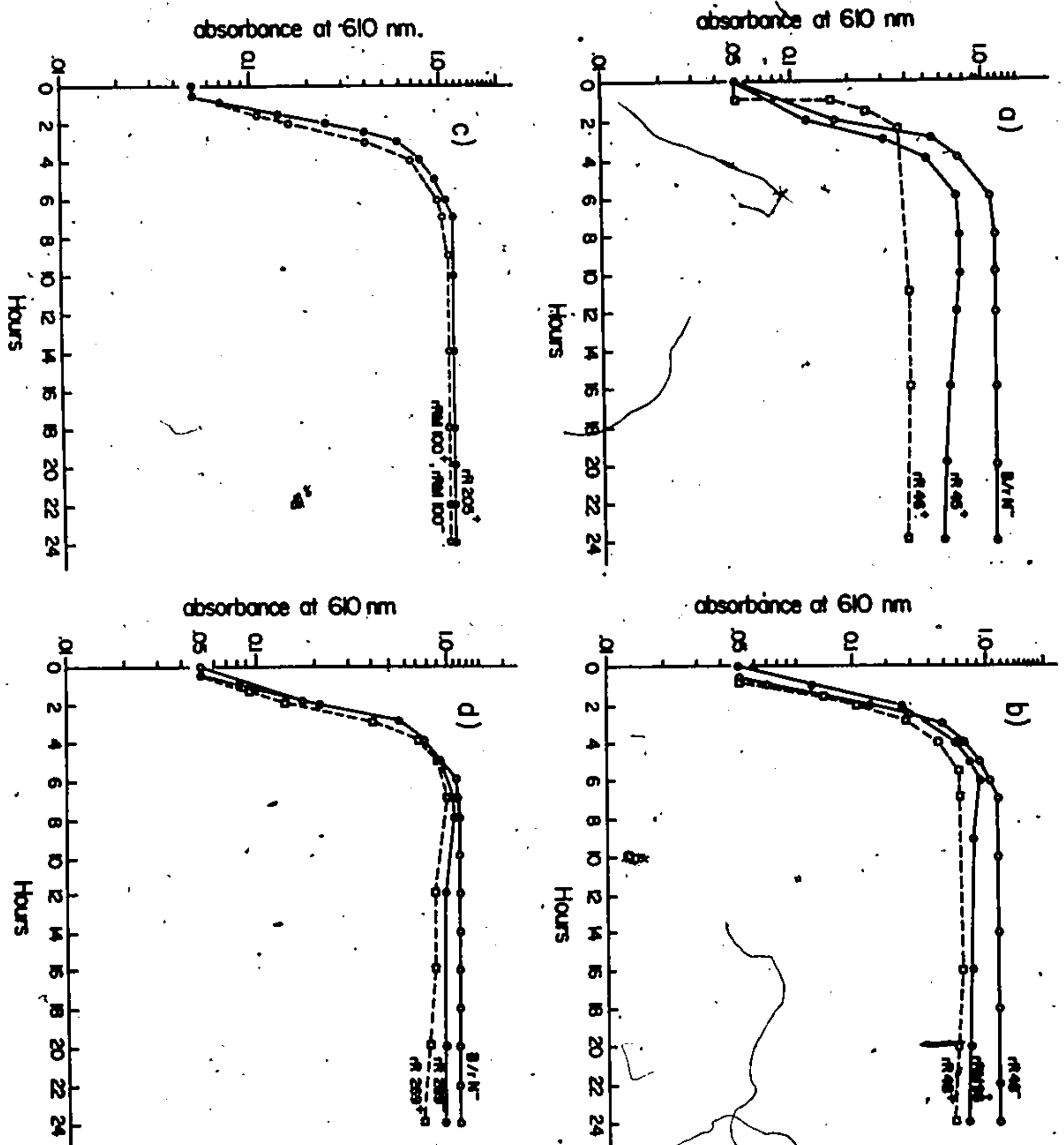


Figure 1

rRM100 and rR269 plasmid cured strains behaved like the plasmid bearing strains. It is quite likely that some of the plasmid functions are retained by the strains cured of the plasmids. The strain bearing the plasmid rR46 reached only about 0.4 units absorbance and maintained this level up to 24 hours when grown on L broth without glucose. It showed normal growth in L broth supplemented with glucose and reached a growth level of 1.2 units absorbance. This growth level was maintained up to 24 hours.

Viability and Clumping of Cells

There was a steady increase in the viable cells of all the strains in the first 8-10 hours of growth at 37°C. After this period, a decline in the total viable cells was observed with most of the N plasmid bearing strains except for the $rR46^+$, $rRM100^+$ and $rR205^+$ strains. Strains bearing plasmids rR46*, rR205 as well as the strain cured of the plasmid rR48 showed viable counts comparable to those of the plasmidless host (Fig. 2).

Microscopic examination of the cultures revealed an interesting phenomenon. Strains which showed a decrease in absorbance as well as in the total number of viable cells

* Viable counts when grown on L broth with 0.1% glucose were comparable to plasmidless host. The $rR46^+$ strain grew poorly (low viable counts) in L broth without glucose.

Fig. 2. Comparison of growth (total viable counts) of *E. coli* B/r $N^{\pm}$ strains. The strains were grown in L broth (without glucose) at 37°C. 0.5 ml of broth cultures were withdrawn and diluted 10^{-6} and 10^{-7} times in physiological saline. One-tenth ml of these dilutions were plated on L agar with or without an antibiotic (tetracycline or ampicillin at 30 μg/ml). Colonies were scored after 24 hours at 37°C. Care was taken to disrupt the clumps by vigorous shaking for one minute before proceeding to the next dilution.

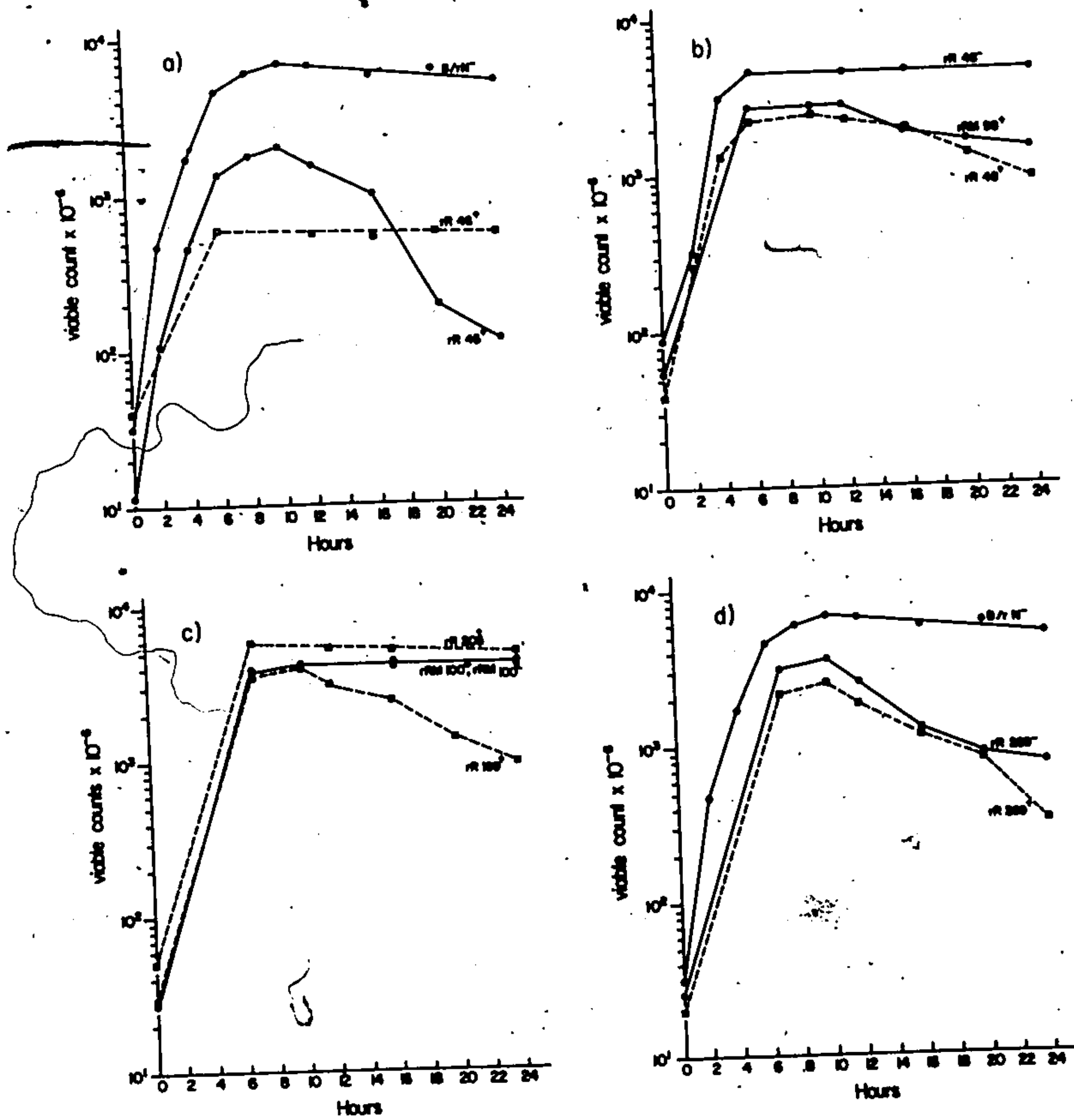


Figure 2

formed clumps. The strain bearing N plasmid rRM100 as well as the one cured of this plasmid also showed clumps. No clumps were seen in LEB18; LEB509($rR46^+$); LEB505($rR48^-$) and LEB511($rR205^+$) in 24 hour old cultures. The strains cured of the plasmids rR100 and rR269 also formed clumps which indicates that some of the functions mediated by these plasmids are retained by these strains. The decline in the total viable counts appears to be because of defective growth and death of the cells.

The strains which formed clumps did so in L broth with or without glucose and also in minimal medium containing glucose as the sole carbon source. There was no change in the clumping behaviour of the strains when grown at (42°C).

Overnight broth cultures of the strains forming clumps became clear after one hour of standing at room temperature as the cells settled to the bottom of tubes. Broth cultures of strains not forming clumps stayed turbid for more than 24 hours at room temperature.

It was observed that the strains bearing plasmids presented problems in their maintenance on L agar at 20°C. The plasmidless host always grew well on repeated subcultures whereas the plasmid bearing strains tended to lose viability when stored at 20°C for intervals longer than 15 days. The plasmidless host presented no problem in recovery from cultures kept up to one month under the same conditions. It is interesting that the plasmid bearing strains show normal

growth and increased viable counts during the exponential growth. The decline in optical density as well in viable counts occurs after reaching the stationary phase which lasts for a shorter duration than that of the host.

The alterations in the composition of fatty acids mediated by N plasmids will be presented in detail at a later stage of this thesis. The strains forming clumps had increased concentrations of cis-vaccenic acid (C18:1) as compared to the level found in the plasmidless host as well as the other strains not forming clumps.

Total Lipids

The percentage of total lipids extracted by the Bligh and Dyer method varied from 6 to 8 (Table 2) irrespective of the method of dry weight determinations of the cells. (The dry weight was determined by the freeze drying method as well as by drying the cells at 105°C. For details see Materials and Methods). No significant difference was observed in the percentage of total lipids among the strains analysed. The percentage of total phospholipids was found to be almost the same in all strains as measured by the estimation of total phosphorus in the lipids extracted by the Bligh and Dyer method. These results imply that the N plasmids did not mediate any significant change in the percentage of phospholipids and the neutral lipids.

Composition of Neutral Lipids

Thin layer chromatography of the total lipids using the solvent system petroleum ether - ethyl ether - acetic acid (85:15:1) did not reveal any qualitative changes in the neutral lipids. All the strains had diglycerides and free fatty acids which were identified by co-chromatography with authentic standards. Phospholipids stayed at the origin in this solvent system (Plates 1 and 2). TG, FAME, sterol ester or hydrocarbons were not found in all the strains.

Composition of Phospholipids

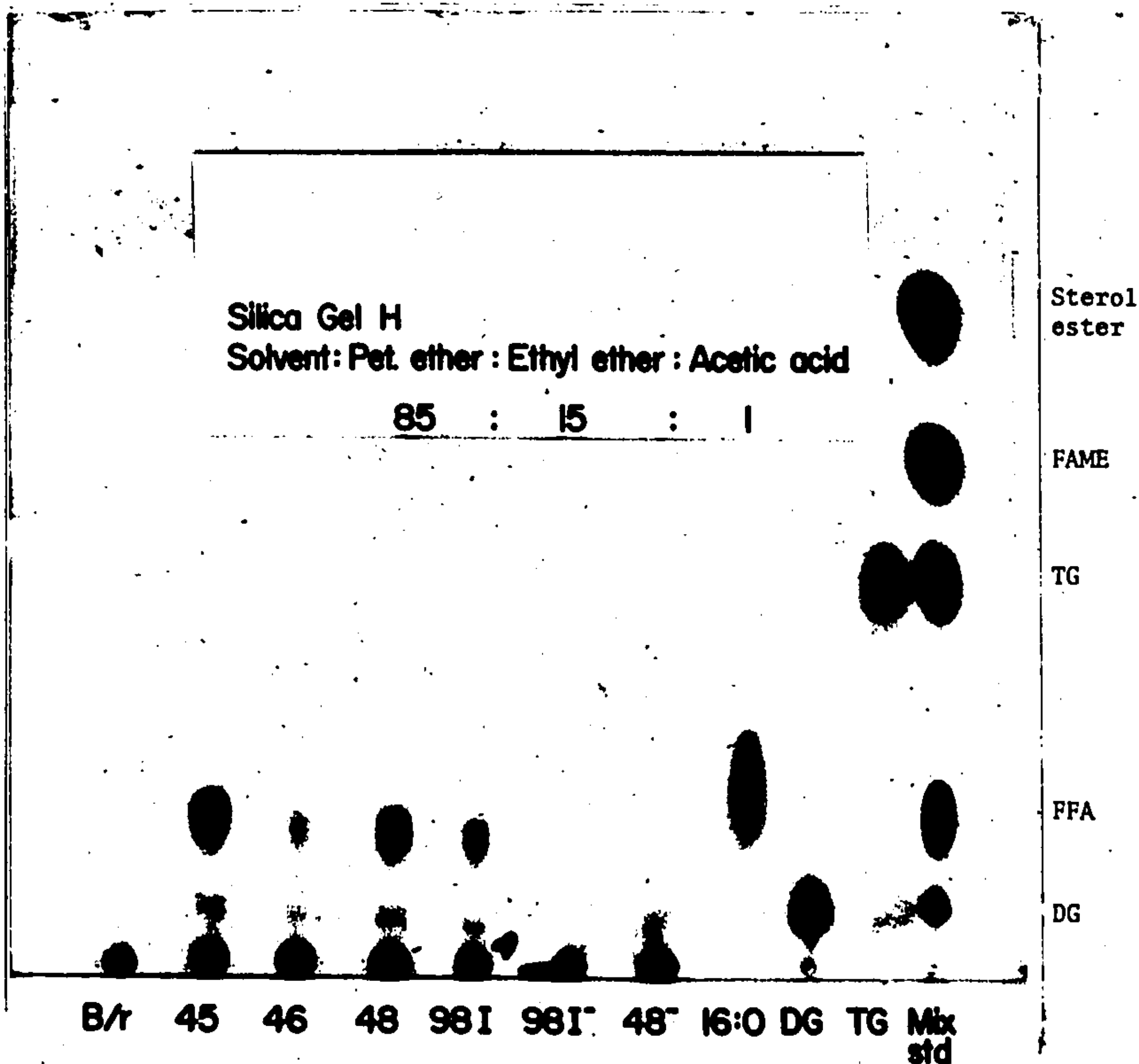
Some of the previously described solvent systems failed to isolate all the phospholipid species in single dimensional chromatography. The solvent systems tried were (i) chloroform-methanol-water (65:25:4) (Wagner et al, 1961), (ii) chloroform-methanol-7N ammonium hydroxide (65:35:5) (Nichols, B.W., 1963). By repeated trial and error, a solvent system was developed for the present investigations (see Material and Methods). This system resolved all the species of phospholipids encountered in the strains under study, in a single dimension. Except for phosphatidyl serine (present only in small proportions), the separation of the remaining phospholipid species was extremely good (Plate 3) and should be useful in the metabolism and turnover studies of bacterial phospholipids.

Five types of phospholipids were detected in all the E.

Plate 1 and 2. Characterisation of neutral lipids.

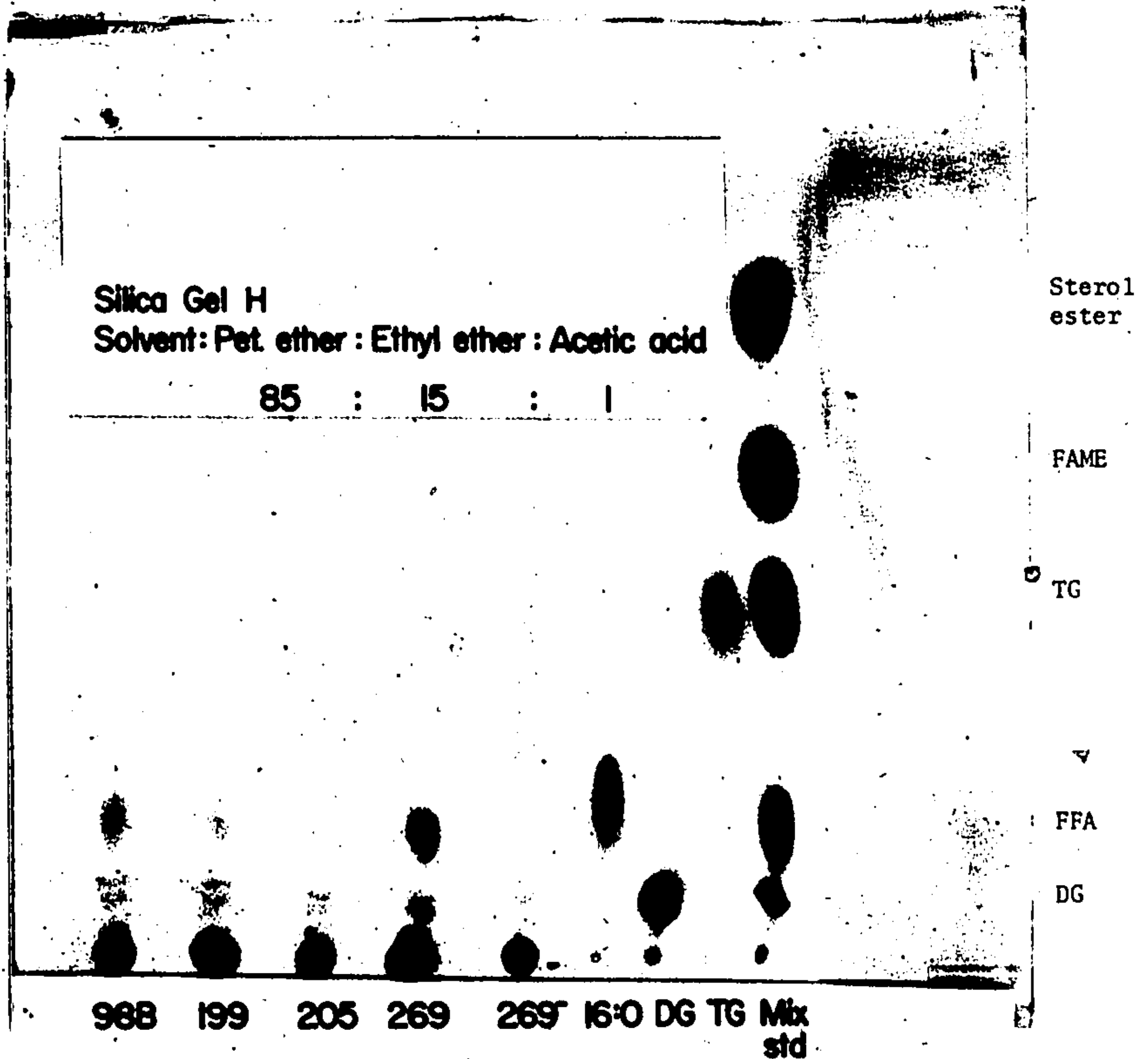
Separation of neutral lipids was carried out by thin layer chromatography on silica gel H plates (40 gms in 100 ml of distilled water). The solvent mixture was petroleum ether: ethyl ether: acetic acid (85:15:1). Neutral lipids were identified by co-chromatography with authentic standards. Phospholipids stayed at the origin. TG, FAME and sterol ester were not detected.

Plate - 1



Key: B/r = LEB18
45 = LEB508(rR45$^+$)
46 = LEB509(rR46$^+$)
48 = LEB505(rR48$^+$)
98I = LEB500(rRM100$^+$)
98I$^-$ = LEB500(rRM100$^-$)
48$^-$ = LEB505(rR48$^-$)
16:0 = Palmitic acid
DG = Diglyceride
TG = Triglyceride
FFA = Free fatty acid
FAME = Fatty acid methyl ester

Plate - 2



Key: 98 B = LEB502(rRM98^{+})
199 = LEB510(rR199^{+}).
205 = LEB511(rR205^{+})
269 = LEB506(rR269^{+})
269^{-} = LEB506(rR269^{-})
16:0 = Palmitic acid
DG = Diglyceride
TG = Triglyceride
FFA = Free fatty acid
FAME = Fatty acid methyl ester

Plate 3. Separation and characterisation of phospholipids of <u>E. coli</u> B/r $N^{\pm}$. Phospholipids were separated by thin layer chromatography using silica gel H plates (40 gms in 0.15 M ammonium sulphate). The solvent mixture was chloroform-methanol-acetic acid-water (85:15:10:3). Identification was carried out by co-chromatography with authentic standards and using various other solvent systems. Spots were visualised by spraying the chromatograms with 50% sulphuric acid and heating at 150°C. For assay of phosphorus the spots were visualised by exposure to iodine vapours for 30 seconds. (For other details see Materials and Methods.)

Key: B/r = LEB 18
45 = LEB 508($rR45^{+}$)
46 = LEB 509($rR46^{+}$)
48 = LEB 505($rR48^{+}$)
$\overline{48}$ = LEB 505($rR48^{-}$)
98I = LEB 500($rRM100^{+}$)
$98\overline{I}$ = LEB 500($rRM100^{-}$)
98B = LEB 502($rRM98^{+}$)
199 = LEB 510($rR199^{+}$)
269 = LEB 506($rR269^{+}$)
$\overline{269}$ = LEB 506($rR269^{-}$)
LPE = Lysophosphatidyl ethanolamine
PS = Phosphatidyl Serine
PE = Phosphatidyl ethanolamine
PG = Phosphatidyl glycerol
CL = Cardiolipin
NL = Neutral lipid

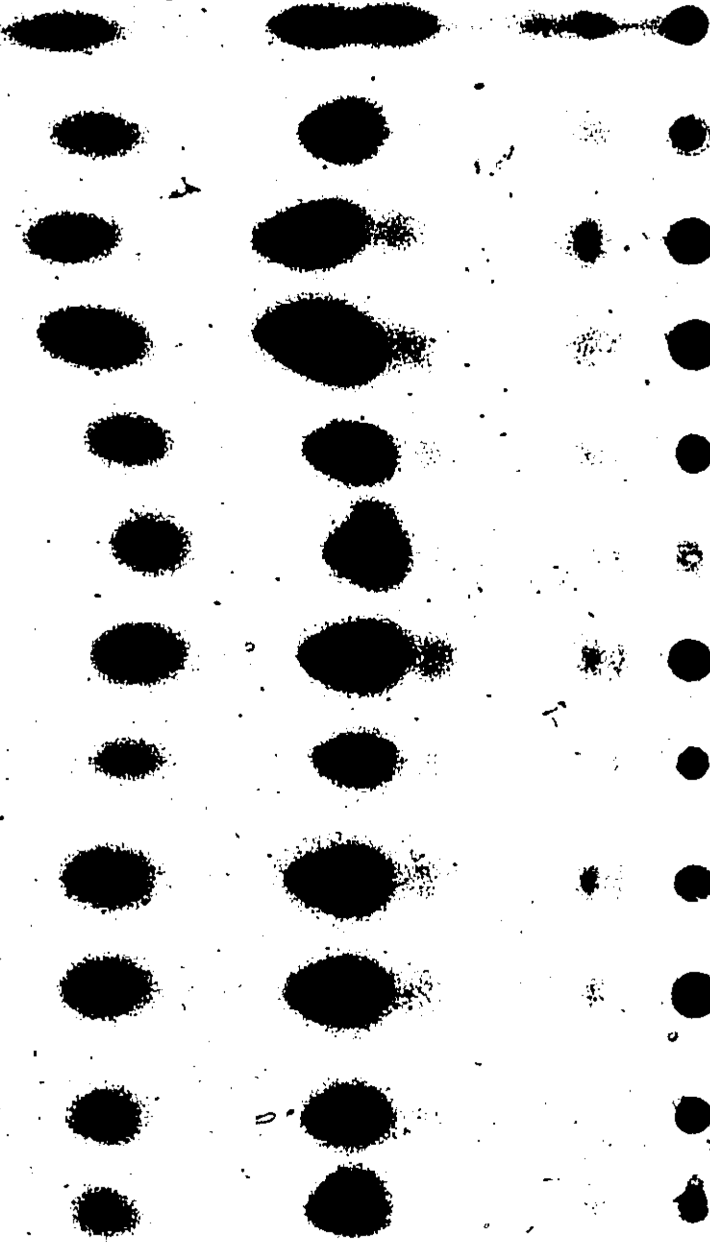


Plate - 3

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coli strains and they were characterised by their behaviour towards various staining reagents (see Material and Methods) and by co-chromatography with authentic standards. These consisted of lyso-phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin. Neutral lipids ran with the solvent front.

Phosphatidyl ethanolamine constituted the bulk of the phospholipids and its proportion varied from 68-75%, followed by phosphatidyl glycerol and cardiolipin which comprised about 14-18% and 5-9% respectively of the total phospholipids. Lyso-phosphatidyl ethanolamine varied from 1-10% and phosphatidyl serine constituted approximately 1.5-4.4% of the total phospholipids. The phasmidless host and the plasmid cured strains LEB505(rR48$^-$) as well as LEB500(rRM100$^-$) had a higher proportion of phosphatidyl serine (about 4%) than the plasmid cured strain LEB506(rR269$^-$) that had 1.5% of the total phospholipids (Table 2).

LEB508(rR45$^+$) showed the highest proportion of lysophosphatidyl ethanolamine (LPE) (10% of the total phsopholipids). The proportion of LPE in the plasmidless host and the plasmid cured strains was between 2-4%. In the other strains harbouring various plasmids, LPE varied from 1-7% approximately. The gross variation observed in the proportion of LPE may be because of the presence of phospholipase enzymes which degrade PE to LPE and free fatty

Table 2

LIPID ANALYSIS OF E. COLI
% of Phospholipids*

	Total Lipids**		Total Phospho-lipids***		PE		PG		CL		PS		LPE		PG/CL
LEB18(B/r)	7.8	0.7	4.6	1.1	69.3	4.9	17.4	2.4	5.3	0.7	4.1	1.0	3.8	2.7	3.3
LEB508(rR45$^+$)	6.7	0.4	4.2	0.1	69.1	1.3	13.9	1.4	5.4	0.9	1.7	0.6	9.8	2.9	2.6
LEB509(rR46$^+$)	6.6	1.9	4.9	0.9	73.9	0.6	15.2	0.4	8.6	0.2	1.6	0.0	1.6	1.0	1.8
LEB505(rR48$^+$)	6.8	2.3	5.1	0.2	68.2	1.8	16.3	1.6	7.3	1.4	2.9	0.2	5.4	0.9	2.2
LEB500(rRM100$^+$)	5.9	0.5	4.7	0.1	68.5	0.1	17.1	3.3	6.0	0.4	1.5	0.5	6.9	2.1	2.9
LEB502(rRM98$^+$)	6.0	1.4	4.7	0.3	74.1	0.0	15.9	1.5	6.4	0.2	1.7	1.0	2.0	0.6	2.5
LEB510(rR199$^+$)	6.7	2.5	4.3	0.9	67.3	5.3	16.7	4.8	7.2	1.5	2.6	0.7	6.0	2.2	2.3
LEB511(rR205$^+$)	7.2	2.3	N.D.		74.7	0.9	15.2	1.4	7.4	1.3	1.4	0.5	1.0	0.3	2.1
LEB506(rR269$^+$)	6.8	1.4	N.D.		69.1	0.2	18.4	1.6	5.2	2.3	2.4	0.8	5.1	1.6	3.5
LEB505(rR48$^-$)	7.6	2.6	N.D.		70.8	2.8	16.1	0.3	7.4	0.6	3.9	1.8	1.9	1.3	2.2
LEB500(rRM100$^-$)	8.0	0.4	N.D.		69.7	2.0	16.5	0.8	5.4	0.1	4.4	0.4	3.1	1.1	3.1
LEB506(rR269$^-$)	7.1	0.1	N.D.		72.6	4.6	16.0	0.4	7.6	0.9	1.6	1.2	2.9	1.3	2.1

* Percent of total phospholipids the standard deviation; N=4.
** Percent of total lipids in dry wt. of cells.
*** Percent of Phosphorus in total lipids.

Abbreviations: PE = Phosphatidyl ethanolamine;
PG = Phosphatidyl glycerol;
CL = Cardiolipin;
PS = Phosphatidyl serine;
LPE = Lysophosphatidyl ethanolamine.
ND = Not done.

acids.

As is apparent from Table 2, the ratio of phosphatidyl glycerol to cardiolipin was reduced in the strains harbouring the plasmids, except for LEB506(rR269^{+}) which showed a slightly higher ratio of these two phospholipids than the plasmidless parent.

Of the plasmid cured strains, LEB500(rRM100^{-}) demonstrated a shift of the ratio of PG to CL close to that found in LEB18. The other two plasmid cured strains LEB505(rR48^{-}) and LEB506(rR269^{-}) had a reduced ratio of PG to CL indicating that some of the functions mediated by the plasmid were not lost.

Separation of Outer and Inner Membranes

The proportion of phospholipids in the total lipids of whole cells did not reveal any major differences which could be attributed to the presence of the plasmids. The only significant difference was in the proportion of LPE which was higher in some of the strains harbouring plasmids. This, in fact, may be the result of the degradation of PE by phospholipase. The inner and outer membranes of these strains were separated and analysed in order to elucidate the alterations, if any, in the composition of the phospholipids of these membranes.

The plasmidless host and the plasmid bearing strains LEB509(rR46^{+}); LEB502(rRM98^{+}) and LEB510(rR199^{+}) were

Fig. 3. Separation of outer and inner membranes of E. coli B/r $N^{\pm}$ strains. The procedure of Smit et al (1975) was followed with some modifications. Cells were grown in L broth (without glucose) to 0.25 absorbance at 610 nm. The outer and inner membranes were separated by using a discontinuous sucrose gradient in 10m M Hepes buffer pH 7.4. (For details see Materials and Methods.)

B/r N^{-} = LEB18

B/r, $rR46^{+}$ = LEB509($rR46^{+}$)

B/r, $rRM98^{+}$ = LEB502($rRM98^{+}$)

B/r, $rR199^{+}$ = LEB510($rR199^{+}$)

0.M = outer membranes

1.M = inner membranes

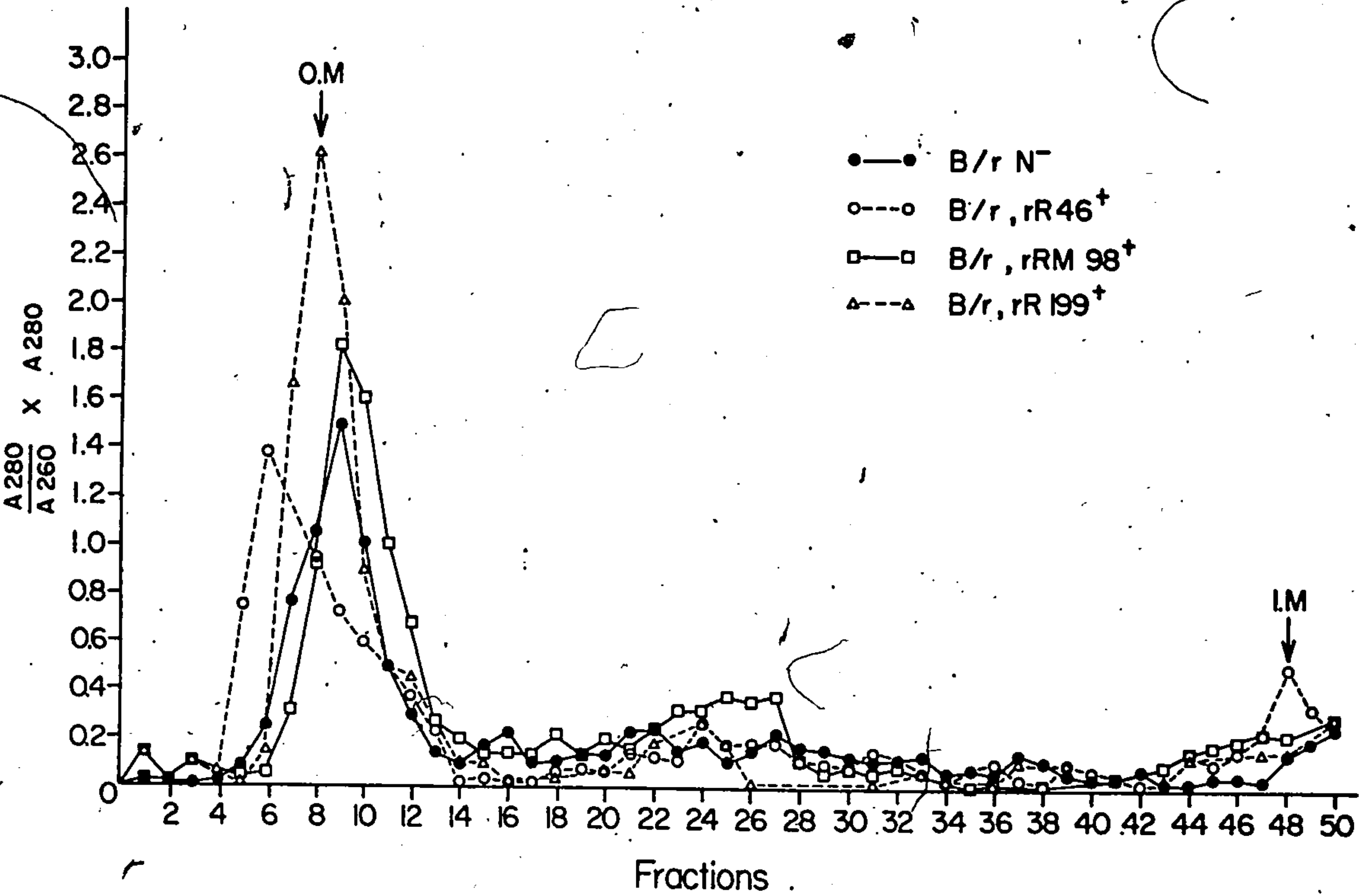

Figure 3

employed for the separation of outer and inner membranes using a modification of the technique of Smit, J. et al (1975) (for details see Materials and Methods). Fig. 3 shows the protein concentration (determined by the absorbance ratio method) of fractions collected after discontinous sucrose gradient ultracentrifugation. The recovery of the inner membranes being poor, further studies were conducted only on the outer membrane fractions. As is evident from Fig. 3, the density of the outer membrane of the strain LEB509(rR46$^+$) and LEB510(rR199$^+$) increased whereas the strains LEB502(rRM98$^+$) did not show any such increase as compared to LEB18.

Phospholipids of Outer Membranes

The outer membranes of the strains examined contained all the phospholipid species encountered in the whole cells. The major components were phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin. Phosphatidyl serine was present in trace amounts only (..5%) (Table 3). The composition of the phospholipids of outer membranes varied from strain to strain. PE and LPE showed a great variation but the sum total of both was found to be the same in all the four strains examined. This suggests that the variation was because of the action of enzyme phospholipase *in vitro*. As in the whole cells (Table 2) the ratio of PG to CL was found

Table 3

PHOSPHOLIPID COMPOSITION OF OUTER MEMBRANES OF E. COLI
% of Phospholipids*

Strain No.	LPE	PE	PG	CL	PG/CL
LEB18(B/r)	27.4	61.7	6.9	3.9	1.8
LEB509(rR46$^+$)	21.3	68.5	5.9	4.4	1.3
LEB502(rRM98$^+$)	35.1	58.3	3.3	3.3	1.0
LEB510(rR199$^+$)	28.8	63.7	3.5	4.4	0.8

* Percent of phosphorus in total phospho lipids.
Abbreviations: See Table 2
Numbers represent mean of two runs on the same batch.

to be lower in the outer membranes of plasmid bearing strains than that of the plasmidless host. PE + LPE was much higher (89-93%) in outer membrane than in whole cells (73-76%) and PG + CL was much lower in outer membrane (7-11%) than in whole cells (19-24%).

Fatty Acid Composition of Bligh and Dyer Extractable Lipids

Gas liquid chromatography of methyl esters derived from lipids of E. coli strains showed gross differences in the pattern of fatty acids. Major fatty acids were C16:0, C16:1 and C18:1 which constituted 86-94% of the total fatty acids in the mid-log phase of strains (Table 4).

The major change was in the content of C18:1 (% of total fatty acids) of the strains analysed. The plasmid bearing strains LEB508(rR45$^+$); LEB505(rR48$^+$); LEB500(rRM100$^+$); LEB502(rRM98$^+$); LEB510(rR199$^+$) and LEB506(rR269$^+$) had relatively higher concentrations of this fatty (38-51%) than those in the plasmidless host which had about 33% thus reflecting an increase of 14-53%. LEB511(rR205$^+$) had the same proportion as that found in the plasmidless host whereas LEB509(rR46$^+$) had about 26% which is lower by 22% than that found in the control strain. LEB508(rR45$^+$) and LEB506(rR269$^+$) had the highest content of C18:1 which amounted to about 51% of the total fatty acids. LEB509(rR46$^+$) had the lowest proportion, only 26%, which is almost half of that found in LEB508(rR45$^+$) as well as

Table 4

Fatty Avids of *E. coli* LEB18 N Plasmids at 37°C
% of total fatty acids**

(Mid-log phase absorbance = 0.25)

Fatty Acid*	LEB 18 (B/r)	LEB508 ($rR45^+$)	LEB509 ($rR46^+$)	LEB505 ($rR48^+$)	LEB500 ($rRM100^+$)	LEB502 ($rRM98^+$)	LEB510 ($rR199^+$)	LEB511 ($rR205^+$)	LEB506 ($rR269^+$)	LEB505 ($rR48^-$)	LEB500 ($rRM100^-$)	LEB506 ($rR269^-$)
12:0	1.1	.4	.2	.4	.3	.4	.6	1.2	.5	1.0	1.1	1.4
13:0	.03	-	-	-	-	-	-	-	-	-	-	-
14:0	3.0	1.7	2.8	2.0	2.1	2.2	3.8	3.6	2.4	3.5	2.2	2.0
14:1****	.5	.2	.2	.4	.3	.3	.3	.4	.2	.3	.3	.3
15:0	1.4	-	-	-	-	.03	.1	.3	-	.04	.6	.2
15:1****	0.1	-	-	.03	-	-	-	-	-	-	-	.2
16:0	26.4	22.9	38.8	23.5	23.2	23.2	26.7	28.0	23.0	27.1	24.8	23.8
16:1	26.7	19.6	29.7	20.7	22.4	22.8	25.6	28.4	19.1	28.0	20.3	20.6
17:0	.7	.1	.1	-	-	.2	.06	.3	-	.1	.6	.3
17:0 cy	4.7	2.5	1.6	2.7	3.0	2.9	3.9	3.4	2.7	3.9	4.3	4.1
18:0	.5	.6	-	1.2	.4	.6	.3	.4	.6	.3	.8	.6
18:1	33.4	51.1	25.9	48.9	47.4	46.4	38.1	33.0	50.8	34.9	43.7	45.4
U1***	.9	.8	.3	.5	.5	.5	.5	1.2	.4	.7	1.2	.6
U2***	.2	.3	-	.4	.4	.5	.2	.05	.3	.06	.2	.3

* Number preceding the colon indicates the length of carbon chain; number following the colon represents the number of unsaturated bonds; cy refers to the presence of cyclopropane; U stands for unidentified.

** Numerical values are the average of duplicate analysis on each strain harvested in two different experiments.

*** The relative retention times of unidentified fatty acids (denoted by U_1 and U_2) to that of palmitic acid were 2.41 and 2.92.

**** Tentatively identified.

LEB506(rR269$^+$). Of the plasmid cured strains, LEB505(rR48$^-$) had a level of this fatty acid which is close to the level found in the host strain whereas LEB500(rRM100$^-$) and LEB506(rR269$^-$) still demonstrated the presence of increased levels of C18:1 and were in fact comparable to those found in some of the plasmid bearing strains.

The other major fatty acids, C16:0 and C16:1, each constituted about 26% of the total fatty acids in the host strain. The remaining strains had variable amounts of these fatty acids except that LEB510(rR199$^+$) had almost the same levels of these two fatty acids as found in the host. LEB509(rR46$^+$) had the highest content of about 39% of C16:0 as compared to the other strains and had 13% more than was found in the host strain. LEB511(rR205$^+$) and LEB505(rR48$^-$) had slightly increased content of C16:0 whereas the remaining strains had reduced content (less by 2-3%) relative to LEB18 (Table 4).

C16:1 was also found in variable amounts in the strains. LEB509(rR46$^+$) demonstrated an increase of 3% whereas LEB505(rR48$^-$) and LEB511(rR205$^+$) had a comparable proportion of this fatty acid to that found in the plasmidless host. The remaining strains had reduced levels which ranged from 4-7% of that in the control strain.

C14:0 constituted about 3% of the total fatty acids in LEB510(rR199$^+$); LEB511(rR205$^+$); LEB505(rR48$^-$) and

LEB509(rR46$^+$). The rest of the strains showed a slight reduction of this fatty acid.

C17:0 cyclopropane formed 5% of the fatty acids of the plasmidless host whereas strains cured of the N plasmids rR48, rRM100, rR269 and the strain bearing the plasmid rR199 had about 4%. The remaining strains had slightly reduced levels of this fatty acid.

C12:0 was present in low levels (1% or less) in the strains. C13:0 was encountered only in the host and in trace amounts (.03%). C14:1, C15:0, C15:1, C18:0 and the other two unidentified fatty acids were detected in trace amounts in all strains.

The plasmidless host had the maximum number of different fatty acids followed by the plasmid cured strains. LEB509(rR46$^+$) had only 9 of the 14 fatty acids encountered in the host.

Fatty Acids and Clumping of Cells

It was observed during the present investigations that most of the strains harbouring plasmids formed clumps during their growth. The phenomenon of clumping was observed with the strains bearing the N plasmids rR45, rR48, rRM100 and rR269. The plasmid cured strains LEB510(rR199$^-$) and LEB506(rR269$^-$) also formed clumps similar to the above mentioned plasmid bearing strains.

The plasmidless host failed to demonstrate such clumps.

Table 5

CORRELATION OF C18:1, 36.5K PROTEIN AND CLUMPING OF E. COLI STRAINS

Strains	LEB18 (B/r)	LEB508 ($rR45^+$)	LEB509 ($rR46^+$)	LEB505 ($rR48^+$)	LEB500 ($rRM100^+$)	LEB502 (rR98)	LEB510 ($rR199^+$)	LEB511 ($rR205^+$)	LEB506 ($rR269^+$)	LEB505 ($rR48^-$)	LEB500 ($rRM100^-$)	LEB506 ($rR269^-$)
36.5K*	+	+	+	-	-	-	+	+	-	+	-	+
Clumping**	-	+	-	+	+	+	+	-	+	-	+	+
C18:1***	33	51	26	49	47	46	38	33	51	35	44	45

* Data on 36.5K dalton protein was provided by Dr. Iyer.
** - No clumping; + clumping.
*** Figures indicate the % of C18:1 in total fatty acids in mid-log phase.

The strains bearing the plasmids rR46 and rR205 along with the plasmid cured strain LEB505(rR48$^-$) also behaved like the plasmidless host and did not reveal any clumps during their growth. As is evident from Table 5, the presence or absence of the major protein 36.5K daltons could not be implicated in this behaviour. The fatty acid analysis of the strains demonstrated the correlation between the amount of cis-vaccenic acid (18:1) and the formation of clumps. The strains which showed clumps had increased levels of C18:1 comprising between 38-51% of the total fatty acid whereas the strains not forming any clumps contained 35% or less of this particular fatty acid.

Fatty Acids of $N^{\pm}B/r$ in the Stationary Phase

The growth curves and viable counts of the strains (Figs. 1 and 2) over a period of 24 hours revealed that the plasmidless host along with some of the other strains (rR46$^+$, rR48$^-$, rRM100, rRM100$^-$ and rR205$^+$) entered a stationary phase that extended up to 24 hours. These strains maintained high viable counts through the stationary phase. The remaining strains (rR45$^+$, rR48$^+$, rR199$^+$, rR269$^+$ and rR269$^-$) had stationary phases of a very short duration and showed decrease in total viable counts thereafter. Hence the fatty acid composition of some selected strains was determined on cultures harvested at 16 hours following growth at 37°C to find out if there was any correlation of

Table 6

FATTY ACIDS** OF E. COLI LEB18 ± N PLASMIDS IN STATIONARY PHASE*

Fatty Acid	LEB18 (B/r)	LEB509 ($rR46^+$)	LEB505 ($rR48^+$)	LEB505 ($rR48^-$)	LEB500 ($rRM100^+$)	LEB500 ($rRM100^-$)	LEB510 ($rR199^+$)	LEB511 ($rR205^+$)
C14:0	5.1	3.9	2.6	5.4	3.0	3.8	2.9	6.0
C15:0	4.2	2.8	1.5	4.9	2.0	1.9	2.1	3.5
C16:0	36.8	42.6	36.8	36.5	36.7	39.4	36.0	39.4
C16:1	2.8	20.7	5.5	2.8	1.6	–	4.2	2.1
C17:0	2.8	4.7	1.9	3.1	1.9	1.6	2.9	2.1
C17:0 cy	21.3	8.2	17.3	22.1	19.8	20.0	19.0	23.4
C18:0	–	0.8	–	–	[illegible]	1.6	–	0.3
C18:1	11.1	16.3	25.6	11.1	15.9	11.4	21.2	8.5
U1***	3.3	–	2.3	2.5	2.4	2.9	3.1	2.4
C19:0 cy	11.3	–	6.7	11.0	13.7	15.3	8.6	12.2
U2***	1.4	–	–	0.47	1.5	2.4	–	0.2

* 16 hours growth at 37°C in L broth (no glucose).

** Numbers represent percentage of total fatty acids and are the mean of two analyses per culture.

*** The relative retention times of unidentified fatty acids (denoted by U_1 and U_2) to that of palmitic acid were 2.38 and 4.24.

Abbreviations: See legend on Table 4.

decreased viability with the composition of fatty acids. The results are presented in Table 6.

The major fatty acids encountered were C16:0, C17:0 cyclopropane, C18:1 and C19:0 cyclopropane. C16:0 formed about 36% of total fatty acids of the host, LEB505(rR48+), LEB500(rRM100$^+$) LEB505(rR48$^-$) and LEB510(rR199$^+$) whereas LEB511(rR205$^+$) and LEB500(rRM100$^-$) contained about 39% of this fatty acid. LEB509(rR46$^+$) had 42%.

The levels of C17:0 cyclopropane and C19:0 cyclopropane varied from 8-23% and 0-15% respectively amongst the strains analysed. Strains forming clumps and having decreased viable counts had decreased levels of cyclopropane fatty acids as compared to the levels found in the host as well as in the other strains (not forming clumps and with no decreased viable counts in the stationary phase).

The major changes were found in the levels of cis-vaccenic acid (C18:1). It is interesting that strains forming clumps and with decreased viable counts had considerable increase in the levels of C18:1 which was about 10 and 15% respectively in LEB510(rR199$^+$) and LEB505(rR48$^+$) as compared to the level found in the host strain. This same correlation between clumping and levels of C18:1 was observed in the mid-log phase (Table 5).

C14:0, C15:0, C16:1 and C17:0 fatty acids were present in all the strains and their levels varied from about 2-21%. Two unidentified fatty acids were also present in most

strains and their levels varied between 0-3% (Table 6).

It is quite interesting that the host strain, the strain bearing plasmid rR205 as well as the strain cured of the plasmid rR48 had very similar patterns of fatty acids. These strains do not form clumps and do not show a decrease in absorbance or in viable counts in the stationary phase. LEB509($rR46^{+}$) also does not form clumps and shows no decline in absorbance but its fatty acid profile was different from the rest of the strains.

DISCUSSION

N-plasmids are known to confer changes in the phenotypes of E. coli B/r (Iyer, 77 and Iyer et al, 78). Some of these phenotypes are:

i) Sensitivity to the filamentous bacteriophage IKe.

ii) Resistance to various antibiotics.

iii) Plasmid transfer to suitable recepients.

iv) Change in the density of outer membranes.

v) Elimination of 36500 dalton protein from outer membrane by some of the N-plasmids.

In the light of the above-mentioned alterations which may be due to the changed lipids of the cell envelope, the present investigations were carried out to find out the changes in the composition of lipids and fatty acids of strains harbouring N-plasmids and compare them with the plasmidless host and with the available plasmid cured strains.

As the composition of lipids changes under various conditions of growth (O'Leary, 1967), the strains used in the present investigations were grown under identical conditions at all times. As is apparent from Fig. 1, the growth of all strains followed an identical pattern only during the exponential phase, hence the cells of all the strains were harvested at an absorbance of 0.25 units which approximately

represents the mid-exponential phase of all the strains.

Total Lipids, Neutral Lipids and Phospholipids

No changes were observed in the chloroform-methanol extractable total lipids, neutral lipids and phospholipid content of the strains (Table 2). This is in agreement with the findings of other workers (Winshell and Neu, 1974; Suling and O'Leary, 1977 and Legakis et al, 1977). Vaczi et al, 1957; Hill et al, 1963; Hugo and Stretton, 1966; Hugo and Franklin, 1966 and Chang and Tsang, 1972, however, correlated the antibiotic resistance of strains studied by them, to the increased lipid content of strains.

Composition of Phospholipids

The phospholipid composition of strains (Table 2) did not reveal any significant difference between them which could be correlated with any change in the resistance phenotype of the strains harbouring the plasmids. Suling and O'Leary (1977) reported a quantitative difference in the phospholipids of antibiotic susceptible and resistant strains of Serratia marcescens. The E. coli strains studied by them did not reveal such changes in phospholipid composition.

In the present studies the strains harbouring plasmids were found to have changed ratios of phosphatidyl glycerol to cardiolipin. The PG/CL ratio was found to be lower in the

strains harbouring plasmids, except for LEB506(rR269^{+}) which had a higher PG/CL ratio than that of the plasmidless host. These findings were similar to those reported by Suling and O'Leary (1977). These workers thought that antibiotic resistance was due to changed lipids of the outer membranes. The present investigations demonstrate that the data on PG/CL ratio could easily be interpreted wrongly and correlated with antibiotic resistance but the results obtained on the plasmid cured strains proved otherwise (Table 2). The ratio of PG/CL was found to be lower in the plasmid cured strains LEB505(rR48^{-}) and LEB506(rR269^{-}) and was close to the PG/CL ratio encountered in most of the plasmid carrying strains (Table 2). This suggests that the ratio of PG/CL is not controlled by the region of plasmid DNA which imparts antibiotic resistance to the host. Presumably the ratio of PG/CL is controlled by gene(s) other than those imparting antibiotic resistance. The genes controlling PG/CL ratio could possibly be transposable and become integral parts of the host genome. Thus, they would not be lost during loss of the plasmid, whether this occured spontaneously or by artificial manipulations. It is also likely that the sensitive revertants still have a smaller plasmid with the PG/CL ratio determinants.

Phospholipids of Outer Membranes

All the phospholipid species found in the intact cells

were encountered in the outer membrane fractions also. However, the relative concentration of the various phospholipids was different in the latter (Table 3). Though the percentage of PE varied from strain to strain, the sum total of PE and LPE remained the same. The increase in the level of LPE observed in the outer membrane fractions may possibly be due to an in vitro effect of a phospholipase which is usually present in the outer membranes. Similar findings were reported by White et al, 1972.

The ratio of PG/CL changed in the outer membrane fractions. The ratio of these two phospholipid species was lower in the outer membrane fractions of strains harbouring plasmids. Similar results were observed in the PG/CL ratio of the intact cells also (Table 2). Results obtained on the ratio of PG/CL in intact cells of plasmid cured strains ruled out the correlation of this alteration to antibiotic resistance. Whether such changes are to the advantage or disadvantage of the cell remains to be investigated. These changes, however, do not seem to have any bearing on any of the known phenotypes of E. coli strains harbouring various N-plasmids investigated in the present studies.

Fatty Acids of E. coli in Mid-log Phase

The analysis of fatty acids of the total lipids revealed marked changes in the composition of various fatty acids in the E. coli strains. The most obvious alterations were found

in the proportion of C18:1 (cis-vaccenic acid) (Table 4). The plasmids rR45, rR48, rRM100, rR199 and rR269 increased the level of this unsaturated fatty acid. The increase in the level ranged from 14-53% of the total fatty acids higher than the level found in the plasmidless parent. This observation corroborates the findings of previous workers (Dunnick and O'Leary, 1970 and Suling and O'Leary, 1977). Plasmids rR46 and rR205 differed from the other plasmids. rR205 did not change the level of C18:1 whereas rR46 reduced the level of cis-vaccenic acid considerably. In fact the plasmid rR205 infected E. coli strain showed the same levels of various fatty acids as encountered in the plasmidless parent (Table 7). Plasmid rR46 on the other hand increased the level of C16:0 by 47% as compared to the level of C16:0 in the host strain. As is apparent from Table 4, the increased level of C18:1 was mostly at the expense of C16:1 and C16:0.

The fatty acid analysis of the plasmid cured strains gave interesting results (Table 8). The pattern of the fatty acids of the plasmid cured strain LEB505(rR48$^-$) showed a close resemblance to that of the plasmidless host whereas the other two plasmid cured strains LEB500(rRM100$^-$) as well as LEB506(rR269$^-$) had fatty acid content which was similar to that of several N$^+$ strains examined (Table 4). This clearly proves that some of the plasmid functions that mediate alterations in the composition of fatty acids, are

Table 7

FATTY ACIDS OF E. COLI B/r*
% of total fatty acids**
(mid-log phase absorbance = 0.25)

Strain	12:0	13:0	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:0 cy	18:0	18:1	U1	U2
LEB 18 (B/r)	1.1	.03	3.0	.5	1.4	.1	26.4	26.7	.7	4.7	.5	33.4	.9	.2
LEB511 (rR205+)	1.2	-	3.6	.4	.3	-	28.0	28.4	.3	3.4	.4	33.0	1.2	.05

* Excerpt from Table 4.

** Numbers represent mean of values obtained on four runs on two different batches.

Table 8

FATTY ACIDS OF *E. COLI* B/r*
% of total fatty acids***
(mid-log phase absorbance = 0.25)

E. Coli Strain	12:0	13:0	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:0 cy	18:0	18:1	U1	U2
LEB18 (B/r)	1.1	.03	3.0	.5	1.4	.1	26.4	26.7	.7	4.7	.5	33.4	.9	.2
LEB505** ($rR48^-$)	1.0	–	3.5	.3	.04	–	27.1	28.0	.1	3.9	.3	34.9	.7	.06
LEB500** ($rRM100^-$)	1.1	–	2.2	.3	.6	–	24.8	20.3	.6	4.3	.8	43.7	1.2	.2
LEB506** ($rR269^-$)	1.4	–	2.0	.3	.2	.2	23.8	20.6	.3	4.1	.6	45.4	.6	.3

* Excerpt from Table 4.

** Plasmid cured strains.

*** Figures represent the average of four separate runs on two different batches.

not lost. This is consistent with the hypothesis that some of the genes on the plasmids can act as transposons which have the capability of translocation to the host genome and become its integral part. Thus these transposable genes could be retained by the bacterial host even after the subsequent spontaneous or induced loss of the plasmid. It is also possible that a smaller plasmid which carries the genes for such functions exists in sensitive revertants.

The fatty acid patterns of the strains LEB509(rR46^{+}), LEB511(rR205^{+}) (plasmid carrying and resistant to antibiotics), LEB500(rRM100^{-}) and LEB506(rR269^{-}) (sensitive to antibiotics) (Table 4) convincingly demonstrate the absence of correlation between resistance and unsaturated fatty acid levels. Strains LEB509(rR46^{+}) and LEB511(rR205^{+}) did not show increased levels of unsaturated fatty acids but were resistant to various antibiotics, whereas LEB500(rRM100^{-}) and LEB500(rR269^{-}) (plasmid cured strains) had increased levels of unsaturated fatty acids but were susceptible to antibiotics. This is contrary to the opinion of other workers (Dunnick and O'Leary, 1970; Ringrose and Higgins, 1974 and Suling and O'Leary, 1977) who correlated the increase of unsaturated fatty acids and decrease in cyclopropane fatty acids with antibiotic resistance. Legakis et al (1977) observed no change in the composition of fatty acids of E. coli strains susceptible and resistant to penicillin. In the present studies,

LEB511(rR205^{+}) showed almost the same composition of fatty acids as found in the host. Chopra and Eccles (1977) also ruled out the association of the content of cyclopropane fatty acids with tetracycline resistance. The present studies revealed no changes in the level of cyclopropane fatty acid amongst various strains susceptible or resistant to tetracycline (Table 4). Plasmid mediated resistance to tetracycline has been associated with the synthesis of new membrane proteins designated as tetracycline resistance proteins (Levy and McMurry, 1974; Yang et al, 1976; Wojdani et al, 1976).

From the analysis of fatty acids of E. coli strains included in the present investigations, it can be concluded that:

i) Some of the N-plasmid genes mediate the alterations in fatty acids. The fact that the fatty acid content of LEB505(rR48^{-}) is similar to that of LEB18 suggests that the determinants of changes of fatty acid content are plasmid based.

ii) The N-plasmid gene(s) controlling the antibiotic resistance are different from the gene(s) controlling the alterations in fatty acid composition.

iii) Plasmid rR205 lacks the gene(s) which mediate the alterations in fatty acids.

Whether the same is true with the rest of the plasmids

needs to be investigated. Plasmid rR48, however, did not produce a similar effect, at least in the plasmid cured rR48⁻ strain tested and its fatty acid picture resembled that of the plasmidless host. It is interesting that strains LEB500(rRM100⁻) and LEB506(rR269⁻) express identical phenotypes with respect to clumping and fatty acid contents (Tables 5 and 7).

Correlation of Fatty Acid C18:1 to Clumping

It was observed during the present investigations that some E. Coli strains harbouring various N plasmids formed clumps during their growth. This was encountered with the strains harbouring the plasmids rR45, rR48, rRM100, rR199 and rR269. Plasmids rR46 and rR205 failed to show this phenotype. Plasmid-cured strains LEB500(rRM100⁻) and LEB506(rR269⁻) also yielded clumps whereas the plasmid cured strain LEB505(rR48⁻) behaved like its plasmidless host in not forming any clumps. Manning et al (1977) reported the phenomenon of clumping in their studies on E. coli K_{12} mutants and correlated this with the loss of one of the major proteins of outer membranes, 3A. OMB mutants of E. coli K_{12} which lacked the major protein I, did not show any clumps in their investigations. Some of the E. coli strains used in the present investigations also lacked 36.5K dalton protein, the omp B product; however, there was no correlation between clumping and the absence of this protein

at least in the B/r N^+ strain of E. coli (Table 5).

Correlation was found between clumping of cells and their fatty acid composition. Strains which have higher levels of cis-vaccenic avid (C18:1) formed clumps (Table 5). The strains which had a concentration of 35% or less of cis-vaccenic acid did not clump whereas an increase of 9% or more of the cis-vaccenic acid resulted in the clumping of cells. The increase in the concentration of cis-vaccenic acid was observed to be mainly at the expense of the saturated and unsaturated fatty acids palmitic (C16:0) and palmitoleic (C16:1). No correlation was observed between clumping and level of palmitic or palmitoleic acid.

Fatty Acids, Clumping and Viability of LEB18 $N^{\pm}$

Fig. 2 shows that strains that form clumps show a decline in viable counts with the exception of LEB500(rRM100$^+$) and LEB500(rRM100$^-$), beyond the stationary phase. This decline may be attributable to the clumps in the cultures. Surko and Iyer (personal communication) found that the LEB18 $N^{\pm}$ strains that form clumps begin to do so only in the exponential phase and at characteristic optical densities. Clumps were not seen in the lag or very early exponential phase of growth. The growth curves and results obtained on the total viable counts (Figs. 1 and 2) demonstrate the steady increase in the absorbance and total viable counts throughout the log phase

of growth although clumps were present during this period. Clumps could also be disrupted by hundredfold dilution and vigorous shaking for one minute, irrespective of the phase of growth. This confirms that the decline in viable counts observed with some of the strains was due to the death of the cells and not because of the clumps.

As discussed earlier there is a correlation between clumping and levels of cis-vaccenic acid (C18:1) determined in the mid-log phase (Table 5). The analysis of fatty acids on some of the selected strains in their post stationary phase showed that the strains that formed clumps still have increased levels of cis-vaccenic acid (C18:1) plus C19 cyclopropane fatty acid (Table 9). Marr and Ingraham (1962) showed that E. coli adjusts its membrane fluidity by changing the proportion of cis-vaccenic acid. In the present investigations, many of the N plasmids have been found to increase the proportion of cis-vaccenic acid (Tables 9 and 10) as compared to the plasmidless host at the same temperature and under similar conditions of growth.

It is obvious from the present investigations that the increased level of unsaturated fatty acids does not affect the viability of the cells in the exponential phase of growth. As growth progresses towards the stationary phase, the cells change their fatty acid composition considerably in order to cope with the changed environment. These alterations, which have been attributed to the resident

Table 9

FATTY ACIDS, CLUMPING AND VIABILITY OF E. COLI B/r N±

Plasmid	Clumping	Expo C18:1*	Stat C18:1*	Stat C18:1 +19 cy*	Stat 17 cy + 19 cy*	Expo** Ratio of FA	Stat Ratio of FA**	Rel. Viabi-lity***
-	-	33.3	11.1	22.4	32.6	1.83	0.87	72
rR45+	+	51.1	ND	ND	ND	2.72	ND	5
rR46+	-	25.9	16.3	16.30	8.2	1.34	0.82	86
rR48+	+	48.9	25.6	32.2	23.9	2.60	1.2	37
rRM100+	+	47.4	15.9	29.7	33.6	2.67	1.04	89
rRM98+	+	46.4	ND	ND	ND	2.58	ND	52
rR199+	+	38.1	21.2	29.8	27.6	2.09	1.13	20
rR205+	-	33	8.5	20.7	35.6	1.84	0.86	73
rR269+	+	50.8	ND	ND	ND	2.65	ND	13
rR48−	-	34.9	11.1	22.1	33.1	2.01	0.89	89
rRM100−	+	43.7	11.4	26.6	35.2	2.15	0.87	83
rR269−	+	45.4	ND	ND	ND	2.35	ND	19

* Numbers represent percentage of total fatty acids.
** Sum of unsaturated and cyclopropane derivatives devided by sum of total saturated plus unknown.
*** Total viable counts 24/10 hrs. x 100.
Abbreviations: Expo, mid-exponential phase;
Stat, stationary phase (16 hours growth);
ND, not done.

Table 10

FATTY ACIDS OF E. COLI B/r*
% of total fatty acids**

Fatty Acid	LEB18 (B/r)	LEB508 (rR45+)	LEB505 (rR48+)	LEB500 (rRM100+)	LEB502 (rRM98+)	LEB510 (rR199+)	LEB506 (rR269+)
12:0	1.1	.4	.4	.3	.4	.6	.5
13:0	.03	–	–	–	–	–	–
14:0	3.0	1.7	2.0	2.1	2.2	3.8	2.4
14:1	.5	.2	.4	.3	.3	.3	.2
15:0	1.4	–	–	–	.03	.1	–
15:1	.1	–	.03	–	–	–	–
16:0	26.4	22.9	23.5	23.2	23.2	26.7	23.0
16:1	26.7	19.6	20.7	22.4	22.8	25.6	19.1
17:0	.7	.1	–	–	.2	.06	–
17:0 cy	4.7	2.5	2.7	3.0	2.9	3.9	2.7
18:0	.5	.6	1.2	.4	.6	.3	.6
18:1	33.4	51.1	48.9	47.4	46.4	38.1	50.8
U1	0.9	.8	.5	.5	.5	.5	.4
U2	.2	.3	.4	.4	.5	.2	.3

* Excerpt from Table 4.
** On 2 batches/culture harvested in mid log phase on 2 different occasions.

plasmids are such that most of the strains tend to lose viability in the stationary phase (Fig. 2). Most of these strains had increased level of cis-vaccenic acid in the mid-log phase. LEB500(rR100^{+}) and its plasmid cured derivative proved to be exceptions whose relative viabilities were comparable to that of the host strain (Table 9).

The fatty acid composition of the strains analysed in the stationary phase demonstrated that the strains showing a dramatic decrease in relative viability (viable counts 24 hrs/10 hrs x 100) contained a high level of cis-vaccenic acid and decreased levels of cyclopropane fatty acids. E. coli mutants defective in the synthesis of cyclopropane fatty acids have been shown to behave like the wild type strain (Taylor and Cronan, 1976).

In the present studies the fatty acid picture of LEB509(rR46^{+}) (Table 9) in the stationary phase, was found to be different from the rest of the strains. In spite of the low levels of cyclopropane fatty acids (8% of total fatty acids), this strain had relative viability comparable to the host strain and others having elevated levels of cyclopropane fatty acids.

The ratios of the unsaturated to saturated fatty acids in the mid-log and stationary phase reveal that the strains could afford to have increased proportions of unsaturated fatty acids in the log phase but as the growth progresses to the stationary phase, the strains adjust the composition of

fatty acids by decreasing unsaturated fatty acids with a concomittant increase of saturated fatty acids (Table 9). The strains that could adjust the composition of fatty acids so as to have half (or more than half) of the saturated fatty acids in their membranes showed high relative viability (viable counts 24 hrs/10 hrs x 100). On the contrary, the strains unable to do so (and had more than half of the unsaturated fatty acids) tended to lose viability. It is likely that the unsaturated fatty acid content and its inefficient conversion to cyclopropane fatty acids in the stationary phase is responsible for the death of the cells in the stationary phase. The accumulation of toxic products and other factors which include changes in pH may be detrimental to the cells with membranes of increased fluidity. The fatty acid alterations observed might alter permeability of cell membranes and permit an uptake of toxic products from the growth medium.

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