STUDIES ON ANTIBIOTIC-RESISTANT ENTEROBACTERIA ISOLATED FROM POLLUTED RIVER WATER

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by

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CONTENTS

Detailed 'contents' are given at the beginning of each results chapter.

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PUBLICATIONS

Part of the work described in this thesis has been published as follows:

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Hughes, C. and G.G. Meynell (1977) Rapid screening for plasmid DNA. Molec. Gen. Genet. 151, 175-179.

Hughes, V., S. Le Grice, C. Hughes and G.G. Meynell (1978) Two major groups of colicin factors: their molecular weight. Molec. Gen. Genet. 159, 219.

Hughes, C. and P.W. Taylor (1978) Effect of plasmid carriage on the serum sensitivity of enterobacteria. Proc. XII Internat. Cong. Microbiol. p. 180.

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Reprints of these articles are included at the back of the thesis.

SUMMARY

Two methods were devised to simplify the examination of antibiotic-resistant enterobacteria from natural sources.

The efficient isolation of enterobacteria from polluted river water is allowed by supplementing bile salts medium with a critically small amount of nalidixic acid. This medium allows direct counting of coliforms by eliminating all other lactose fermenters.

A further procedure is described for demonstrating plasmid DNA and it's molecular weight, based on rate zonal centrifugation of unlabelled DNA in neutral sucrose gradients containing a low concentration of ethidium bromide. Each plasmid species, nicked during preparation of lysates, forms only a single band, thus simplifying the examination of plasmids from naturally occuring strains.

These methods were used to investigate standard colicinogenic strains and wild-type enterobacteria isolated from polluted river water. The effect of plasmid-carriage on the survival of enterobacteria was examined in river water and also in serum.

ABBREVIATIONS

R factor / R plasmid	Plasmid determining resistance to antibiotics
Col factor / Col plasmid	plasmid determining colicin production
R^+, Col^+	bacterial strains carrying an R plasmid / Col plasmid
$R6K^+$, RPI^+, Col $E1^+$ etc.	bacterial strains carrying the plasmids R6K, RPI, Col E1 etc.
r-determinant, r-det.	Gene(s) specifying resistance to an antibiotic
Ent	enterotoxin production
Hcf	human colonization factor
Hly	haemolysin production
Tra	conjugation ability
recA	chromosomal locus of host recombination system
Nal^R	resistant to nalidixic acid
SDS	sodium dodecyl sulphate
EDTA	ethylenediaminetetra-acetic acid
Tris	2-amino-2-(hydroxy-methyl)-propane-13,-diol
Butyl-BPD	2-(4-t-Butylphenyl)-5-(4-biphenylal)-1,3,4,-oxadiazole
DEAE cellulose	diethylaminoethyl cellulose
TSA	trypticase soya agar
YE	yeast extract
O.N.C.	overnight culture
PEG	polyethylene glycol

METHODS: NOTES AND INDEX

Because of the diverse nature of the work presented in this thesis, a 'Materials and Methods' section is presented in each results chapter. To allow rapid location of individual methods I have included an index.

All commercial bacteriological culture media were obtained from Oxoid Ltd., and prepared as recommended. Media and buffers were sterilized by autoclaving (121^{o} C for 20 minutes). The addition of antibiotics was performed aseptically after cooling of the media to about 50^{o} C. Antibiotic solutions were sterilized either by passage through sterile Millipore filters (pore size 0.45 μm) or by steaming for 30 minutes in a pressure cooker. They were stored, as were antibiotic sensitivity discs, at 4^{o} C.

Bacterial strains were kept on Dorsets' egg slopes at room temperature. Subcultures were made by streaking for single colonies on Blood Agar Base; sweeps of 10 - 30 colonies were then taken for culture and examination. To obtain pure cultures of river bacteria, single colonies were taken from the selection medium and streaked onto Blood Agar Base. Single colonies formed on this medium were then taken for culture.

INDEX OF METHODS

1. INTRODUCTION

1. INTRODUCTION

1.1. INCIDENCE AND SPREAD OF INFECTIOUS DRUG RESISTANCE

1.1.1. Discovery

Bacteria resistant to the action of sulphonamides and antibiotics have been observed since the introduction of these agents, but until the early 1950s, this could be explained only by spontaneous mutation in the bacterial chromosome followed by selection of the resistant clone by chemotherapy (Luria and Delbrück, 1943). In 1952 the use of sulphonamides in treating dysentery in Japan became impossible due to widespread resistance to the drug among *Shigella* (Cavalli Sforza and Lederberg, 1953) and following the introduction of chloramphenicol, tetracycline, and streptomycin individual resistance to these drugs also developed. In 1956 a strain of *Shigella flexneri* resistant to all four drugs was isolated (Kitamoto *et al.*, 1956). This new phenomenon, multiple drug resistance, became commonplace in the following years and could not be accounted for by mutation and selection. When, during a further outbreak of dysentery, commensal *E. coli* were found to possess the same drug resistance pattern as the pathogen, Akiba *et al.* (1960*) and Ochiai *et al.* (1959*) suggested that the genetic material mediating such resistance could be transfered between different bacteria. Further clinical reports (Kagiwada *et al.*, 1960*)

* In Japanese; reviewed by Watanabe (1963)

and experiments by other Japanese workers (Mitsuhashi, Harada and Hashimoto, 1960; Watanabe and Fukasawa, 1961) confirmed the infectivity of drug resistance, and demonstrated that contact between bacteria was required for transfer, as in the case of the F factor (Lederberg and Tatum, 1946). The generic term "plasmid" has been adopted for such autonomous extrachromosomal genetic elements and those determining drug resistance are known as "R factors" or "R plasmids".

Following their discovery in Japan (Watanabe, 1963) R-factors were observed in Salmonella typhimurium isolates in the U.K. (Datta, 1962), and since that time they have been found with increasing frequency among many other species of bacteria.

1.1.2. In human disease

As Falkow (1975) has pointed out, changes in the character of infections caused by Shigella and Salmonella are due more to improvements in sanitation than the introduction of antibacterial agents. In technologically advanced countries widespread epidemics have been largely replaced by sporadic outbreaks. Nevertheless, surveys in Europe, the U.S.A. and Japan show that these two genera frequently possess varied combinations of drug resistance determinants. Not only do different resistance spectra seem to have evolved on R factors found in, for example, Japan and the U.K., but as Anderson (1977) has observed in S. typhimurium different compatability

groups of R plasmids predominate in different parts of the world. In underdeveloped countries, of course, diseases caused by Shigella and Salmonella retain their danger and the acquisition of R factors, combined with antibiotic usage, have led to extensive epidemics, such as those observed recently in Central America with both dysentery and typhoid. In addition to these two genera, enteropathogenic E. coli responsible for epidemic diarrhoe of the newborn are also becoming more difficult to treat as they acquire R factors.

The organisms mentioned above are pathogens which cause severe illness in man by virtue of virulence factors, sometimes plasmid determined, which allow them to invade, colonize and disrupt host tissue. R factors exacerbate problems in treatment. Organisms which have no such ability to cause disease, commensal enterobacteria, have been mentioned earlier (1.1.1.) as providing reservoirs of R factors which can be drawn upon by pathogens. A third group of Gram negative organisms, including Pseudomonas, Escherichia, Proteus, Citrobacter and Enterobacter, may be regarded as opportunist pathogens. These organisms can only infect weakened hosts and their cause is greatly aided by possession of R factors and antibiotic treatment. In recent times hospital populations have comprised a greater proportion of the elderly, the chronic sick, those treated with immunosuppressive and cytotoxic drugs, and those recovering from surgery.

This weakened population, in the presence of intensive antibiotic usage, affords a highly suitable environment for opportunist pathogens, and the high incidence of multiply resistant strains in such nosocomial (hospital-acquired) infections is well documented (Salzman and Klemm, 1966; Finland, 1971; Isenberg and Berkman, 1971; Rose and Schreier, 1968).

1.1.3. In animals

Selective pressure for R^+ enterobacteria in domestic animals is exerted not only by prophylactic and remedial application of antibiotics, but also by the use of antibiotics as growth promoting feed additives. There are now many reports of resistance among specific members of the Enterobacteriaceae obtained from farm animals fed various antibacterial drugs (Smith, 1967; Guinée, 1971; Walton, 1966; Siegel, Huber and Enloe, 1974). As in human medicine, the situation appears to have deteriorated. Anderson (1968) showed that, in the U.K., the proportion of strains of S. typhimurium resistant to antibiotics rose from 3.3 % in 1962 to 61.5 % in 1965, with multiple resistance becoming commonplace. This trend is mirrored by the coliform population. R^+ coliforms have been reported in meat and milk (Linton et al., 1977 a; Kelly et al., 1972; Cooke et al., 1971; Jones, 1971), and Linton et al. (1977 b) found that such contaminants are sometimes able to colonize human consumers. In addition, Fein et al. (1974) and Levy, Fitz-

gerald and Macrone (1976 a) have demonstrated the development of multiply-resistant coliform populations, in both workers and their families, on farms where the animals receive antibiotic-supplemented food.

In the U.K., and more recently the U.S.A., government reports (Report 1969 a; 1969 b) have suggested denial of drugs used in human medicine for use as feed additives and also limitation of their use in veterinary medicine. In the U.K., tetracycline resistance persists after the legislation restricting its use (Smith, 1975) and it is widely believed that antibiotics continue to be administered illicitely. An alternative view is that these resistant clones, in the absence of antibiotic pressure, have been selected on the basis of other characteristics, such as ability to colonize the gut. If this is so (and also because multiple resistance can be selected by the use of a single drug, even one which is not in medical use), withdrawal of selected antibiotics may not produce a rapid reduction in the incidence of R^+ coliforms.

1.1.4. In the community

The normal human community can not be viewed in isolation from the highly selective environments already mentioned. Levy et al. (1976 b) and Linton et al. (1977 b) demonstrated the flow of R-factors from animals to man, and similar spread to the community, via hospitals, was found by Domato et al. (1974). They showed that 45 % of

infants who harboured R^+ coliforms in the gut during hospitalization, were still excreting them a year later; and after this time, a third of the infant's families also carried the R-factor. That R^+ E. coli may spread from individuals under antibiotic treatment to close relatives, not under treatment, has also been demonstrated by Petrocheilou, Richmond and Bennett (1977) during a study of the intestinal flora of two people living together over a 17 month period.

Early surveys in Britain and Ireland by Smith and Halls (1966), Datta (1969) and Moorhouse (1969) showed that R^+ enterobacteria could be isolated from a majority of the normal community, although these organisms constituted only a small proportion of each person's gut flora. In addition, analyses of enterobacteria in sewage and polluted natural waters by Sturtevant and Feary (1969) and Feary et al. (1972) in the U.S.A., and by Smith (1971 a; 1971 b) in the U.K., confirmed the establishment of R^+ strains in the intestinal flora of human communities. Linton et al. (1974) have shown that although hospital effluent exhibits a much higher frequency of R^+ coliforms than other effluents, these still constitute less than 5 % of the total of these organisms in urban sewage. The normal human population appears to be the largest reservoir of such organisms.
Later surveys, including those of polluted river water by Hughes and Meynell (England, 1974) and Cooke (New

Zealand, 1976), and of the gut flora of normal people (Widh and Sköld, Sweden, 1977), indicate that the situation has not improved and has probably worsened. In particular, one notes an increasing abundance of multiply resistant organisms which are a substantial threat to the clinical use of antibiotics.

1.2. RELATIONSHIPS BETWEEN PLASMIDS

1.2.1. Phenotype

The episome concept of Jacob and Wollman(1958) led to F and phage λ being regarded as representatives of a collection of elements behaving genetically in fundamentally the same way; an episome was viewed as a dispensable genome capable of replicating either autonomously, or as an integral part of the chromosome. Difficulties in using the term in the original sense resulted in all extrachromosomal elements capable of autonomous existance being defined as 'plasmids' (Lederberg, 1952). The idea that F, λ, other temperate phages, R and Col factors are, because of their different phenotypes, distinct biological entities, has been further eroded by an understanding of the underlying similarities between them. While replication and distribution are common requirements of such molecules, the phenotypic markers are a reflection of the environmental niche, albeit transient, of the particular plasmid. Thus, the properties of medically important Gram-negative bacteria now associated with plasmids are resistance to antibacterial agents and heavy metals, fermentation of sugars,

and the production of α-haemolysin, colicins, K88 surface antigens and enterotoxins (Falkow, 1975; Hardy, 1975). Recent work has also indicated plasmid involvement in adhesion to human epithelial cells, and resistance to killing by serum (Chapter 7).

1.2.2. Sex factor activity

Conjugation and plasmid transfer are mediated by a sex factor, be it repressed as is normally the case with R and Col factors, or derepressed as in F, ColV and derepressed mutant R factors. After Meynell and Lawn (1967) established that sex pili formed by ColI$^+$ cells were morphologically and antigenically distinct from those determined by F, it became clear that most self-transmissable R and Col factors could be alloted to two groups on the basis of their reaction to F and I specific phages. These plasmids were designated F-like and I-like.

1.2.3. Incompatability

R factors were divided into *fi*$^+$ (fertility inhibition$^+$) and *fi*$^-$ by whether they inhibited the expression of F. Two *fi*$^+$ or two *fi*$^-$ R factors were incompatable (could not coexist stably in a cell line), but one *fi*$^+$ and *fi*$^-$ were stably maintained together (Egawa and Hirota, 1962; Meynell and Datta, 1965; Watanabe *et al*., 1964).

Further work has shown, by means of male-specific phages and superinfection immunity, that both the *fi*$^+$ and *fi*$^-$

groups of R-factors are heterogenous. Over 20 compatability groups now exist and are extremely useful in epidemiological studies. In addition to being correlated with sex pilus type, incompatability also reflects gross similarity in DNA sequence (Grindley, Humphreys and Anderson, 1973; Falkow et al., 1974) and therefore depicts true phylogenetic relationships. The phenomenon appears to be determined by replication control (Uhlin and Nordstrom, 1975; Timmis, Cabello and Cohen, 1975) although this is probably not the only governing mechanism (Timmis, Andrés and Slocombe, 1978).

Despite the high degree of DNA homology shown by incompatable R factors, they display a considerable variation in the resistance determinants which they carry (e.g. Datta, 1975). Watanabe (1963) proposed that R factors consist of a resistance transfer factor (RTF, sex factor) and a group of resistance genes. One may postulate that classification according to incompatability brings together plasmids with related RTF units, and that the variety of resistance spectra carried within an incompatability group is due to a mechanism which allows the interchange of r-determinants between independent genetic elements, or replicons. It has been shown that a piece of DNA carrying an ampicillin resistance determinant occurs on different plasmids belonging to unrelated incompatability groups (Heffron et al., 1975). While reciprocal recA-dependent recombination

can result in the interchange of different r-dets between replicons, fairly extensive regions of homology were thought necessary. To account for the carriage of similar resistance genes on plasmids known to have little homology, it is necessary to invoke a mechanism which does not involve reciprocal recombination.

1.2.4. Transposition

In 1974 Hedges and Jacob described the movement of an ampicillin resistance determinant between plasmids which had no significant homology. This recA-independent (Bennett and Richmond, 1976) insertion of a discrete piece of DNA into one of numerous sites on a recipient replicon is termed 'transposition'. Many such pieces of DNA (transposons) have now been reported, a number of which carry drug resistance genes. Transposition offers the opportunity for an RTF unit to acquire varied combinations of resistance genes and explains the rapid evolution and diversity of R factors. Many aspects of transposition in both prokaryotic and eukaryotic systems have been extensively reviewed by Starlinger and Saedler (1976), Cohen (1976), and Kleckner (1977).

1.3. SURVIVAL OF PLASMID-CARRYING BACTERIA

1.3.1. In vitro

E. coli strains carrying F, whose sex factor is naturally derepressed, die more rapidly in stock cultures than do their F^- counterparts (Hayes, 1953). Derepressed sex factors increase susceptibility to lysis by tris, EDTA (Goldschmidt, Goldschmidt and Wyss, 1967), detergent (Dowman and Meynell, 1970; Salisbury, Hedges and Datta, 1972) and male specific phages. Dowman and Meynell (1970) reported that carriage of derepressed sex factors lengthens the doubling time of strains grown in broth culture, and this effect has also been observed with R plasmids in Staphylococcus aureus (Lacey and Chopra, 1975). Novick (1969) pointed out that even a growth rate difference of 0.1 % between R^+ and R^- cells could give the R^- cell an effective advantage in vivo, though such differences would be difficult to detect in vitro.

Although the increased susceptibility of R^+ cells to SDS and other compounds such as Macarbomycin (Iyobe, Mitsuhashi and Umezawa, 1971) seems to reflect derepression of fertility, this is not the case with all such selective agents e.g. E. coli K12 is made more susceptible to Kasugamycin by the carriage of a range of conjugative plasmids, irrespective of their degree of repression (Danbara and Yoshikawa, 1977). Current studies with both conjugative and non-conjugative plasmids suggest that they might induce increased sensitivity to a significant number of drugs.

Continous culture studies may supply clearer information. Those of Melling, Ellwood and Robinson (1977) indicate that both environment and relative inoculum size are important in R^+/R^- competition studies. While R^- *E. coli* K12s were unable to displace R^+ (RPI^+) progeny under carbon-limited and magnesium-limited conditions, they could do so to a large extent under phosphate limitation, an effect accentuated by increased dilution rate. The authors suggest that the R^+ strain may be less efficient at phosphate uptake.

1.3.2. *In vivo*

In certain environments, many plasmids confer a decisive selective advantage upon their hosts (section 1.1.). Drug resistance determinants clearly enable their hosts to survive in hospitals, farms, and individual infections in both man and animals. Whether they have any effect on host survival where antibiotic pressure is transient or absent, as in the normal human population, is less clear. Apart from resistance determinants, carriage of factors such as K88, Ent, Hly, HCF (human colonization factor) and ColV confer specific advantage on potential pathogens. Evidence for advantage obtained from Col factors other than ColV is less clear (see Hardy, 1975), but indicates that *Col* genes are more often carried by *E. coli* serotypes normally resident in the gut than by serotypes which are only transient members of the gut population. Work by Anderson (1974)

showed that R factor carriage decreases the ability of non-pathogenic E. coli to survive, both in vitro and in the human intestine. However, as Richmond pointed out (1977), when antibiotic pressure is released, competition is often not between R^+/R^- isogenic pairs. Withdrawal of antibiotics tends to result in the displacement of resident R^+ E. coli by sensitive strains, probably because R plasmids are not in general carried by those E. coli that are effective at becoming majority components of the fecal flora (Hartley and Richmond, 1975; Hartley et al., 1975). Certain E. coli O-antigen types are known to be good colonizers of the human gastro-intestinal tract; types O1, O2, O8, O118, O25 and O75 are notable in this respect (Wiedemann and Knothe, 1969). Yet a survey of antibiotic resistant coliforms in man suggest that these E. coli are less commonly carriers of R plasmids than are those less able to survive (Hartley et al., 1975). Nevertheless, when R plasmids appear in O-types with good survival ability, the resistance traits also survive (Petrocheilou, Richmond and Grinsted, 1977; Jonsson, 1974).

Carriage of plasmids has been found to produce varied effects on bacterial virulence in experimental animals. Sometimes virulence appears unchanged (Watanabe, 1971; Smith, 1972), sometimes there are varied degrees of avirulence (Thiele, 1970; Smith and Lingwood, 1971; Smith, 1972). However, the interpretation is confused

by other considerations. Rough (avirulent) cultures appear superior to smooth (virulent) cultures as recipients for R factors (Jarolmen and Kemp, 1969), i.e. by constructing R^+ cells one could select for avirulence, also in such constructions one is manipulating cultures *in vitro* and possibly losing unknown virulence factors. Thiele (1970) noted that while R^+ *Salmonella typhimurium* recombinants from a cross of virulent Salmonella recipients and avirulent R^+ *E. coli* donors were generally avirulent, prolonged subculturing could result in the recovery of virulence without loss of plasmid-mediated drug resistance. She therefore admitted that such a diminution in virulence might not be a simple reflection of plasmid carriage. That such studies on plasmid effect are inconclusive is a reflection of the lack of understanding of the nature and interaction of virulence determinants.

1.4. THE SCOPE OF THIS THESIS

The high incidence of plasmid-carrying antibiotic resistant enterobacteria in both human and animal populations is reflected in their frequency of isolation from sewage and river water. These environments therefore provide convenient points for the collection and subsequent study of such organisms. However, at the beginning of the work described in this thesis, though many medically-important characteristics of the enterobacteria had been attributed to extrachromosomal DNA, investigations into their incidence and nature were clearly hindered by the cumbersome methods available for the isolation and identification of both the bacteria and their plasmids.

Enterobacteria, including coliform bacteria which are used as indicators of fecal pollution in public health surveys, were normally isolated on bile-salts media which even in the most selective of cases failed to prevent growth of some unwanted organisms, particularly pseudomonads. Identification of the required bacteria, which often formed a minority of colonies on such media, required subsequent subculturing and biochemical testing. The accurate counting of enterobacteria in epidemiological studies was therefore laborious.

In order to show that an isolated bacterial strain posessed a plasmid it was necessary to demonstrate the presence of extrachromosomal DNA in cell lysates. The standard procedure for the isolation of plasmid DNA usually involved the growth

of a large volume of bacterial culture and, following lysis of the cells, removal of the chromosomal DNA by differential centrifugation. The presence of plasmid DNA could then be demonstrated by density gradient centrifugation of the supernatant in caesium chloride gradients containing more than 200 μg/ml ethidium bromide. This concentration of the intercalating dye enables plasmid (closed circular) and chromosomal (linear) DNA to be distinguished by inducing a density difference between them. Although this method demonstrated the presence of plasmid DNA, determination of the number and molecular weights of the plasmid species present required further examination, either by electron microscopy or by sedimentation of isotopically-labelled DNA through sucrose gradients.

Clearly the methods available for both the selection of the desired organisms and description of their plasmid complement were unsuitable for epidemiological studies of plasmid-carrying enterobacteria. In an attempt to alleviate these problems the first part of this thesis describes two procedures which together allow such investigations to be economically performed.

Following the initial observation that the enterobacteria have a lower sensitivity to nalidixic acid than do most of the pseudomonads, supplementation of bile-salts medium with low levels of this antibacterial agent was shown, in chapter two, to increase greatly the selection of enterobacteria from the mixed populations present in polluted river water.

Subsequent demonstration of the presence of plasmid DNA and determination of its molecular weight was then simplified by the rapid screening method described in chapter three. This procedure exploits improved lysis of host cells and optimal visualization of DNA-acridine complexes. After removal of RNA and most of the chromosomal DNA, species of plasmid DNA were separated by rate-zonal centrifugation in neutral sucrose gradients. Bands of open-circular DNA-ethidium bromide complexes could then be detected visually under ultra-violet illumination. The sedimentation rates of several known plasmids were determined, thus calibrating the method for a wide range of molecular weights.

Prior to the use of the above method to examine natural isolates, it was applied to a study of plasmids harboured by standard colicinogenic strains (chapter four). This project was undertaken for two reasons; firstly, it confirmed the value of the screening method for studies requiring molecular weight determinations on a large number of plasmids, the parallel use of electron microscopy allowing verification of the rapid estimates obtained. Secondly, the data which were obtained on the size of the Col factors were useful in confirming and extending previous work on the similarities and possible evolutionary relationships existing between these plasmids.

The development of the nalidixic acid medium, described in chapter two, facilitated an investigation into the incidence of antibiotic-resistant enterobacteria in the River Stour,

Kent. This is a small river of generally good water quality but receiving substantial volumes of sewage and industrial effluent from the towns Ashford and Canterbury. The sources and distribution of antibiotic (ampicillin) resistant and sensitive organisms in the river were determined and compared with the variation of chemical and biological indicators which had been measured during surveys performed by the River Authority (chapter five). Because of the particular medical interest in the incidence of multiple antibiotic resistance the coliforms selected were tested for resistance to other clinically useful antibiotics. The incidence with which such isolates posessed simultaneously a number of plasmid mediated characteristics was further indicated by screening for the ability to produce colicins and to transfer antibiotic resistance determinants to a sensitive strain of E. coli K12. The number and molecular weights of plasmid species carried by some of these isolates were determined. Chapter six describes how antibiotic-sensitive and resistant strains were compared with respect to their ability to persist in river water, as this was considered to be of additional public health importance.

The application of the two methods devised in the early part of the thesis to the study of standard colicinogenic strains and antibiotic-resistant river isolates made available a considerable number of characterised strains. These proved to be well-suited to an investigation into the recent suggestion that plasmids could mediate resistance of Gram-negative bacteria to the bactericidal action of serum, a characteristic of clinical importance previously attributed to posess-

ion of chromosomally-determined surface antigens. As described in chapter seven, the use of both laboratory and wild-type enterobacteria enabled an assessment to be made of the importance of factors determined by both plasmids and the chromosome in mediating this phenomenon.

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2. NALIDIXIC ACID AS A SELECTIVE AGENT FOR THE ISOLATION OF ENTEROBACTERIA FROM RIVER WATER

2. NALIDIXIC ACID AS A SELECTIVE AGENT FOR THE ISOLATION OF ENTEROBACTERIA FROM RIVER WATER

2.1. INTRODUCTION

2.1.1. As the river conditions of autumn 1974 (heavy rain and flooding) differed from those of the previous sampling in the spring of 1973, the bacterial population of the River Stour at Canterbury was reexamined. The total counts (lactose fermenting and non-lactose-fermenting) on Mac-Conkey agar Nr. 3 were found to have increased four-fold, and oxidase tests showed that this increase was due largely to organisms other than enterobacteria e.g. Aeromonas and Pseudomonas. A medium was therefore needed which would eliminate these organisms, and this was developed by exploiting their natural greater susceptibility to nalidixic acid.

2.1.2. Susceptibility to antimicrobial drugs as a taxonomic characteristic

Sensitivity to antibiotics and other antimicrobial agents has often been used to characterize and identify groups of bacteria e.g. pseudomonads (Tunstall and Gowland, 1974; Blazevic, Koepcke and Matsen, 1973), micrococci (Lacey and Boswell, 1968) and the Gram-negative anaerobic bacilli (Finegold, Harada and Miller, 1967; Rodriguez et al, 1976).

This limited identification within genera or families has been extended by the investigation of correlation existing

between antibiotic sensitivity and classical taxonomy. This has led to the use of computer identification of clinical isolates solely on their sensitivities to a wide range of antibiotics (Friedman and Maclowry, 1973).

2.1.3. Use of antimicrobial drugs in isolation media

As susceptibility to antibiotics is a useful taxonomic characteristic, antibiotics are correspondingly useful in the primary isolation of organisms from mixed populations. An early example is Fleming's use of penicillin to improve detection of Haemophilus influenza in 'a great variety of pathological conditions' (Fleming, 1929).

While more recent applications have included isolation of moulds (Mossel et al, 1970) and Chromobacter (Ryall and Moss, 1975) most studies are concerned with potential pathogens such as Staphylococcus (Finegold and Sweeney, 1961; Smith and Baird-Parker, 1964; Williams, 1972), Streptococcus (Vincent, Gibbons and Gaafor, 1971; Black and Van Buskirk, 1973; Baker, Clark and Barrott, 1973) and Pseudomonas aeruginosa (Guto and Enomoto, 1970; Thom et al, 1971).

The isolation of coliforms, which as indicators of fecal pollution are of great public health importance, usually depends on the use of a bile salts medium, but, as observed earlier, this does not exclude some related organisms. The basic medium used in this study, MacConkey agar Nr. 3, is one of the more selective media as it contains bile salts and crystal violet.

Most other media used for primary isolation of coliforms are even less selective.

I therefore investigated the possibility of improving selection of coliforms by supplementing MacConkey agar No. 3 with an antibacterial drug.

2.2. MATERIALS AND METHODS

Culture media. Strains were isolated on Oxoid MacConkey agar No. 3 (CM115). Subcultures were made to Oxoid Blood Agar Base (CM55) and to Oxoid Nutrient Broth No. 2 (CM67). Buffer pH 7.2, contained (g/l), gelatin (0.01), KH_2PO_4 (3), $Na_2HPO_4.12H_2O$ (7), NaCl (5).

Bacterial Strains. "River" strains were isolated between January 1974 and January 1975 from six different river sites in East Kent (National Grid references TR138677, TR157676, TR143577, TR174599, TR324588 and TR338618), and were isolated on MacConkey agar either as supplied or containing sodium ampicillin, 20 µg/ml. "Standard" strains were obtained from the National Collection of Type Cultures, National Collection of Industrial Bacteria, National Collection of Plant Pathogenic Bacteria and the American Type Culture Collection.

Examination of strains. The following tests were used. River strains were incubated at 37°; standard strains at their appropriate temperature (30° or 37°).

(i) lactose fermentation, assessed on MacConkey agar or by subculture to lactose-deoxycholate agar (Meynell and Meynell, 1970);

(ii) catalase formation, assessed both immediately and 5 minutes after adding 3% (v/v) H_2O_2 to cultures grown overnight on Blood Agar Base;

(iii) oxidase reaction (Kovacs, 1956);

(iv) oxidation or fermentation of glucose (Hugh and Leifson, 1953);

(v) sensitivity to the vibriostatic agent O/129 (2,4-diamino-6, 7-di-isopropyl pteridine; Bain and Shewan, 1968);

(vi) Flagellar morphology, as determined by electron microscopy. Strains were grown overnight in broth or in sucrose peptone broth to increase flagella formation (Fuerst and Haywood, 1969). They were then fixed in glutaraldehyde, negatively stained with uranyl acetate and examined with an AEI transmission microscope.

Antibiotic sensitivity tests. (a) Preliminary screening of 96 river isolates was carried out using impregnated discs. Plates of Blood Agar Base were spread with 0.1 - 0.2 ml of growing broth cultures containing ca. 10^5 colony-forming units (c.f.u.)/ml and overlaid with Oxoid Multodisks containing chloramphenicol (10 μg), tetracycline (10 μg), nalidixic acid (30 μg), streptomycin (10 μg) and kanamycin (10 μg). Plates were incubated for 15 hrs.

precisely at 37°. Diameters of inhibition zones were measured using a viewing box and calipers, the results being recorded to the nearest mm.

(b) Minimum inhibitory concentrations of nalidixic acid were determined for 47 river strains and 28 standard strains by plating on nalidixic acid agar. Initially, overnight broth cultures were diluted in buffer to contain 2 - 5 x 10^3 c.f.u./ml and 0.02 ml samples inoculated onto MacConkey agar containing 0, 0.5, 1.25, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, or 25 µg nalidixic/ml agar. The tests were then repeated using lower concentrations: 0, 0.06, 0.13, 0.25, 05, 0.75, 1.25 and 2.5 µg/ml. Plates were incubated for 20 hrs. precisely at 37°, and the presence or absence of colonies on each plate then recorded.

Colony counts on river waters. Samples of about 200 ml river water were collected in sterile bottles opened about 6 in. below the surface and about 2 ft. from the bank, using standard precautions (Report, 1969).

Counts were made within 3 hrs. of collecting samples from the River Stour, Site I, downstream of Canterbury sewage works (TR174599). Dilutions in buffer were spread on MacConkey agar as supplied or supplemented with nalidixic acid, 0.13, 0.25, or 0.5 µg/ml. Colonies were counted after 20 hrs. incubation at 37°.

A subsequent series of counts, using 0.4 µg/ml nalidixic acid, were made on samples taken from site II (upstream

of the sewage works at TR164598) in addition to site I. These plates were incubated both aerobically for 20 hrs. at 37° and anaerobically for 48 hrs. at 37° in jars fitted with cold catalysts.

Efficiency of plating. Overnight broth cultures of 5 strains of enterobacteria, E. coli K-12 (2 strains), E. coli B1, Klebsiella aerogenes type II (No. B 5938) and Citrobacter freundii (NCTC 9750) were diluted in buffer.

These were then spread onto these media: Blood Agar Base, MacConkey agar No.3, MacConkey agar No. 3 supplemented with nalidixic acid (0.4 µg/ml), and colony counts were compared.

2.3. RESULTS

Classification of strains

Since the purpose of this investigation was to select enterobacteria, rather than to identify all the species isolated from river water, the following empirical classification was used, based on the phenotypes of 47 river strains summarized in Table 2.1.

(a) Oxidase-negative (Ox^-), lactose-fermenting or non-fermenting ($Lac^{+/-}$), with peritrichous or no flagella. All fermented glucose (G^f). These are presumed to be enterobacteria.

(b) Ox^+ Lac^- with polar or no flagella. Some oxidised glucose (G^o) and presumably included Pseudomonas. Others fermented glucose and presumably included genera such as Aeromonas;

(c) $Ox^{+}Lac^{+}G^{f}$ with polar or no flagella. These presumably included other Aeromonas since this genus may be either Lac^{+} or Lac^{-}.

Antibiotic sensitivities of river strains

All 47 river strains were Gram negative catalase-positive asporogenous rods which utilized glucose. They were not susceptible to the compound, O/129, and were therefore not vibrios (Shewan, Hodgkiss and Liston, 1954).

Fig. 2.1. shows the distribution of inhibition zone diameters for 96 river strains, 69 of which were isolated on ampicillin agar, tested against Oxoid Multodisks. In the case of kanamycin, streptomycin, tetracycline and chloramphenicol, the sensitivities of the three phenotypic classes of organisms overlapped to varying degrees and with each of these drugs, a proportion of strains was not inhibited. However, the distribution for nalidixic acid showed two interesting features. First, the $Ox^{-}Lac^{+/-}$ class (presumptive enterobacteria) were all more resistant than the $Ox^{+}Lac^{+}$ class. Second, the distribution for the Ox^{+} Lac^{-} class was bi-modal, some strains being as sensitive as the $Ox^{+}Lac^{+}$ class but others being almost wholly resistant.

Minimum inhibitory concentrations of nalidixic acid

a) river strains

Table 2.1. shows the M.I.C. for 47 river strains. They showed first, that the $Ox^{-}Lac^{+/-}$ class could grow on MacConkey agar containing nalidixic acid at concentrations

Fig. 2.1.

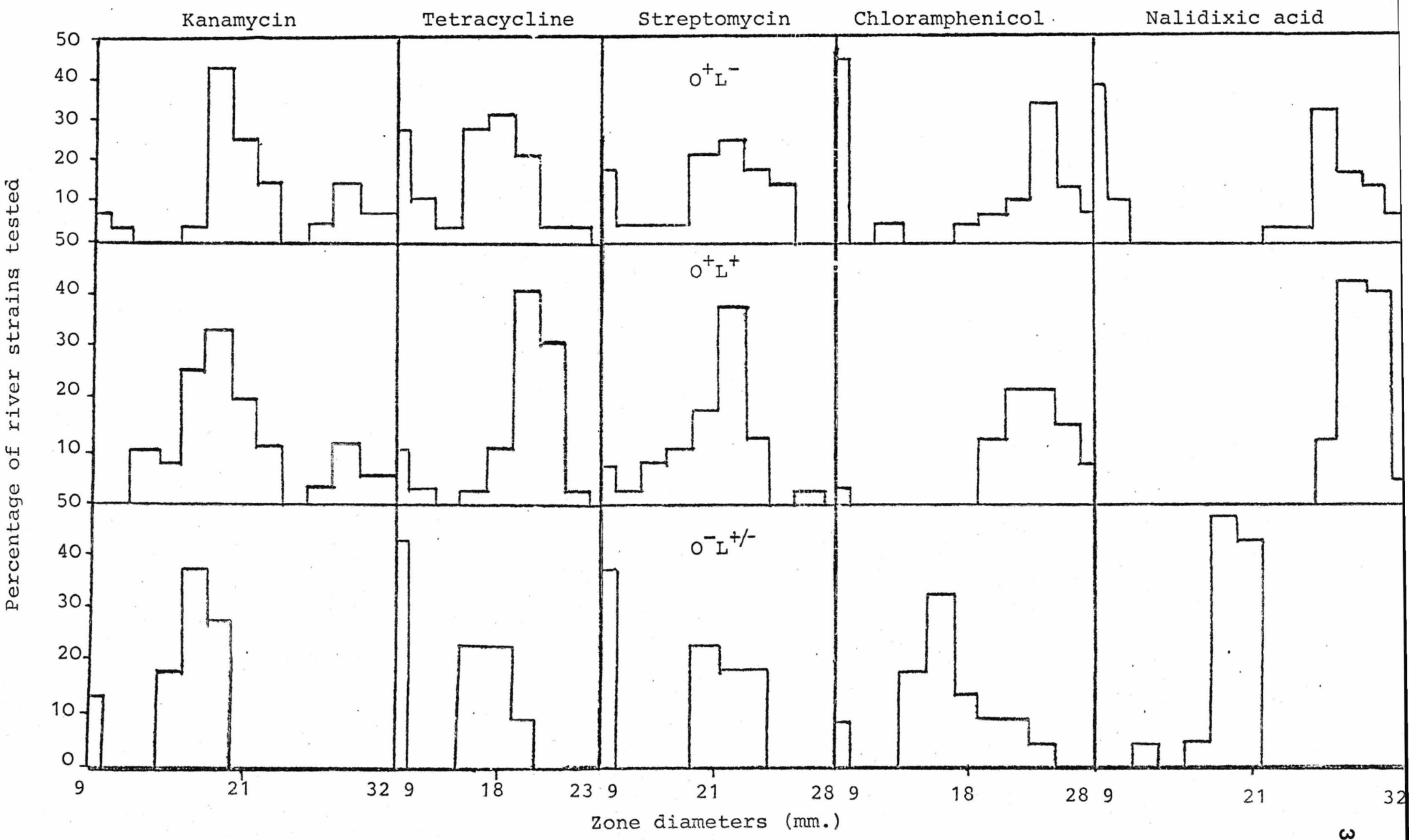
Kanamycin
Tetracycline
Streptomycin
Chloramphenicol
Nalidixic acid
O^+L^-
O^+L^+
$O^-L^{+/-}$
Percentage of river strains tested
50
40
30
20
10
0
9
21
32
18
23
28
Zone diameters (mm.)

Fig. 2.1. Susceptibility of 96 river isolates to five anti-bacterial agents, as measured by the diameters of inhibition zones surrounding Oxoid Multodisks. The diameter of each disc was 9 mm.

The isolates were divided into three phenotypic classes, according to their oxidase reaction (O) and their ability to hydrolyse lactose (L), as described in the text.

TABLE 2.1.

Minimum inhibitory concentrations of nalidixic acid for 47 river strains

Phenotype					Nalidixic acid µg/ml MacConkey agar No.3							
Oxidase	Lactose	Glucose	Flagella	No. tested	0.06	0.13	0.25	0.50	0.75	1.25	2.5	>2.5
-	+/-	F	Peri.	15 (5)					1	8(4)	6(1)	
+	-	O	Polar	8 (3)								8(3)
+	-	F	Polar	12 (5)	5(3)	7(2)						
+	+	F	Polar	12 (5)	6(3)	6(3)						

M.I.C. were determined by inoculating 50 - 100 c.f.u., followed by overnight incubation at 37°C.

Lactose, +/-; fermented / non-fermented.

Glucose, F/O; fermented / oxidized in Hugh & Leifson's test (1953)

Flagella, Peri / Polar; Peritrichous / polar, when present, determined by electron microscopy.

The numbers of strains isolated on ampicillin agar are shown in parentheses.

between 0.13 - 0.5 µg/ml, whereas the $Ox^{+}Lac^{+}$ class was inhibited; and second, that as in the disc tests, the $Ox^{+}Lac^{-}$ class contained two types of strain, one being at least as resistant as the $Ox^{-}Lac^{+/-}$ group and the other as sensitive as the $Ox^{+}Lac^{+}$ group.

Biochemical tests on a total of 20 such river strains, summarized in Table 2.1., showed that of the 8 strains resistant to nalidixic acid, >2.5 µg/ml, none could ferment glucose. These were therefore presumed to be oxidative Pseudomonads. Of the 12 strains sensitive to nalidixic acid, ≤0.13 µg/ml, all fermented glucose. These were therefore thought likely to be Lac^{-} Aeromonas.

b) Standard strains

Tests on 28 standard strains confirmed the previous conclusions, within the limits of the number of species available. Some saprophytic species which might be expected to occur in river water were unable to grow on MacConkey agar No. 3 as supplied. These were the Pseudomonas strains P. phaseolicolor (ATCC 11365), P. fluorescens (NCIB 9494), P. putida (NCIB 9034), P. sp, (NCIB 8858) and Xanthomonas compestris (NCPPB 528), Zymomonas mobilis (NCIB 8938), Achromobacter lwoffi (NCIB 9020), Erwinia caratavora (NCPPB 312).

The following all grew and had M.I.C.s of nalidixic acid within the range 0.75 - 2.5 µg/ml: the coliform organisms Escherichia coli B1, E. coli K12 (2 strains), Klebsiella aerogenes type I, K. aerogenes type II (NCIB 5938), Enterobacter aerogenes (NCIB 10102 and NCTC 10006), Citrobacter

freundii (NCTC 9750), and the non-lactose-fermenting enterobacteria Proteus vulgaris (NCTC 4175), Serratia marcescens (NCTC 1377 and NCIB 2847).

Strains of Pseudomonas aeruginosa (NCTC 10332, NCIB 8295 and NCIB 0950), Pseudomonas alcaligines (NCIB 9398) and Xanthomonas phaseocolor all had M.I.C.s greater than 2.5 µg/ml. Alcaligines faecalis (NCIB 8156) had an M.I.C. of 1.25 µg/ml while of three non-lactose-fermenting strains of Aeromonas (A. caviae NCIB 9671, A. formicans NCIB 9232 and A. liquefaciens NCIB 9233), two were inhibited by 0.13 µg/ml and one by a level of between 0.25 µg/ml and 0.5 µg/ml.

Colony counts on river water

The preceding results suggested that nalidixic acid at 0.13 - 0.5 µg/ml MacConkey agar would selectively inhibit the growth of the $Ox^{+}Lac^{+}$ class and the G^{f} sub-class of $Ox^{+}Lac^{-}$ strains. Colony counts were therefore made on water from site I, downstream from a sewage works, using concentrations of 0, 0.13, 0.25 or 0.5 µg/ml (Table 2.2.).

The selective power of the medium was indicated by the increasing % Ox^{-} colonies obtained on nalidixic agar. Moreover, the colony count/ml of Ox^{-} organisms remained constant, showing that they were not inhibited by this range of concentrations. Results of the 'efficiency of plating' comparisons (Table 2.4.) confirm that there was no significant inhibition of coliforms. Inhibition of $Ox^{+}Lac^{+}$

TABLE 2.2.

Colony counts of river water on 0 - 0.50 µg/ml nalidixic acid agar

Phenotype	Nalidixic acid µg/ml MacConkey agar No. 3											
	0			0.13			0.25			0.50		
	c.f.u./ml	%Ox^-	Ox^-/ml	c.f.u./ml	%Ox^-	Ox^-/ml	c.f.u./ml	%Ox^-	Ox^-/ml	c.f.u./ml	%Ox^-	Ox^-/ml
Lac^+	6.6	26	1.7	2.0	56	1.1	2.3	97	2.2	1.4	100	1.4
Lac^-	4.0	8	0.3	1.3	31	0.4	1.0	35	0.4	1.1	39	0.4
Total	10.6	19	2.0	3.3	46	1.5	3.3	79	2.6	2.5	73	1.8

c.f.u./ml colony forming units, expressed as thousands/ml. river water, as determined after aerobic incubation at 37°C

%Ox^- %Oxidase-negative colonies of a total of 60 - 80 colonies tested at each concentration.

Ox^-/ml Oxidase-negative colonies/ml (= c.f.u./ml x %Ox^-/100)

organisms was complete at 0.5 μg/ml, since all Lac^+ colonies tested proved to be Ox^-. At this concentration, 61 % of Lac^- colonies tested were still Ox^+, as expected from the sensitivity tests of Fig. 2.1. and Table 2.1. and of 20 tested, all were G^o. This remaining fraction of Ox^+ Lac^+ G^o organisms would therefore be expected to be obligate aerobes and anaerobic culture was therefore tested as an additional means of contraselection.

Colony counts were made from sites I and II using aerobic and anaerobic culture on MacConkey agar containing either no nalidixic acid or 0.4 μg/ml (Tables 2.3.a and 2.3.b). The counts are shown in Table 2.3.a and their details are given in Table 2.3.b. The results were consistent with those obtained previously. In both aerobic and anaerobic culture using nalidixic acid, 0.4 μg/ml, 100 % of Lac^+ colonies were Ox^- compared to 21 % (aerobic) and 42 % (anaerobic) without nalidixic acid selection. Of the Lac^- colonies on nalidixic acid agar, only 34 % were Ox^- in aerobic culture whereas the value rose to 88 % in anaerobic culture. These values compare with only 9 % and 22 % Ox^-, respectively, in the absence of nalidixic acid. Thus, nalidixic acid and anaerobiasis together has almost eliminated the unwanted $Ox^+Lac^{+/-}$ group.

TABLE 2.3. a

River water plated on MacConkey agar No. 3 containing nalidixic acid (0.4 µg/ml): Colony counts

		Aerobic			Anaerobic		
Site	Phenotype	-Nal c.f.u./ml	+Nal c.f.u./ml	%	-Nal c.f.u./ml	%	+Nal c.f.u./ml	%
I	Lac^+	$2.1 \cdot 10^3$	$6.3 \cdot 10^2$	30	$1.7 \cdot 10^3$	81	$5.6 \cdot 10^2$	27
	Lac^-	$1.1 \cdot 10^3$	$2.1 \cdot 10^2$	19	$5.8 \cdot 10^2$	53	$6.6 \cdot 10^1$	6
	Total	$3.2 \cdot 10^3$	$8.4 \cdot 10^2$	26	$2.3 \cdot 10^3$	71	$6.3 \cdot 10^2$	20
II	Lac^+	$1.6 \cdot 10^3$	$2.9 \cdot 10^2$	19	$9.6 \cdot 10^2$	60	$2.8 \cdot 10^2$	18
	Lac^-	$1.3 \cdot 10^3$	$2.4 \cdot 10^2$	19	$4.1 \cdot 10^2$	32	$6.2 \cdot 10^1$	5
	Total	$2.9 \cdot 10^3$	$5.3 \cdot 10^2$	18	$1.4 \cdot 10^3$	48	$3.4 \cdot 10^2$	12

% = Colony count expressed as % of aerobic count without nalidixic acid

TABLE 2.3.b

Efficiency of selection of oxidase-negative organisms

Phenotype	Aerobic				Anaerobic			
	-Nal		+Nal		-Nal		+Nal	
	%Ox^-	Ox^-/ml	%Ox^-	Ox^-/ml	%Ox^-	Ox^-/ml	%Ox^-	Ox^-/ml
Lac^+	21	3.4	100	2.9	42	4.0	100	2.8
Lac^-	9	1.2	34	0.8	22	0.9	88	0.6
Total	16	4.6	70	3.7	36	4.9	95	3.3

Ox^-/ml Oxidase-negative colonies, expressed as hundreds/ml, calculated as in table 2.2.

Values calculated as in table 2.2., from counts on site II (in table 2.3.a)

TABLE 2.4.

Efficiency of plating of coliforms

Strain	Medium		
	B.A.B.	M3	M3Nal.
K.aerogenes	1.0	0.87	0.78
C.freundii	1.0	0.88	0.91
E.coli K12(1)	1.0	1.22	1.39
E.coli K12(2)	1.0	1.02	1.02
E.coli B1	1.0	0.78	0.83

Comparisons of spread plate counts of overnight broth cultures made on 3 media (B.A.B. = 1.0).

B.A.B. Oxoid Blood Agar Base (CM 55)

M3 Oxoid MacConkey agar No. 3 (CM 115)

M3Nal M3 supplemented with 0.4 µg/ml nalidixic acid

2.4. DISCUSSION

MacConkey agar No. 3 containing nalidixic acid, 0.4 μg/ml, when incubated aerobically, will inhibit all oxidase-positive lactose-fermenters and a high proportion of oxidase-positive non-lactose-fermenters. It therefore allows the direct counting of typical coliforms in river water contaminated with other organisms from sewage effluent, soil and vegetation. This is sufficient for most studies because the majority of oxidase-negative strains found in such samples are lactose-fermenters. However, if non-lactose-fermenting enterobacteria are sought then the persisting oxidase-positive non-lactose-fermenters may be further inhibited by anaerobic incubation (Table 2.3.b)

Other possible applications of these findings are to the culture media used for colony counts on membrane filters, and also to the liquid media used for counts by the dilution method where organisms such as Aeromonas may produce false positive results in the presumptive coliform test (Holden, 1970).

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3. RAPID SCREENING FOR PLASMID DNA

3. RAPID SCREENING FOR PLASMID DNA

3.1. INTRODUCTION

3.1.1. Having devised a medium for isolating antibiotic-resistant coliforms, I wished to examine the plasmids harboured by some of these strains. In this kind of screening investigation the potentially large number of strains requires an economical method of separating and demonstrating plasmid DNA.
The various alternatives will now be discussed.

3.1.2. Existing methods for the isolation and demonstration of plasmid DNA

The initial step in the isolation of plasmid DNA normally involves enzymatic digestion of the bacterial cell wall followed by lysis of the cells with a non-ionic detergent such as Brij-58 (e.g. Bazaral and Helinski, 1968). Tris (Repaske, 1958) and EDTA (Leive, 1965) are essential for lysis which is often accelerated by dodecylamine (Meynell, 1971) and sodium deoxycholate (Godson and Sinsheimer, 1967).
With few exceptions plasmid DNA comprises a maximum of 3 % of the total cellular DNA so in order to examine this fraction, a primary enrichment, removing most of the chromosomal DNA, is usually performed (Freifelder, 1970).
Short high speed centrifugation deposits the chromosomal DNA, often associated with membrenous debris, leaving

the plasmid DNA and chromosomal fragments in the supernatant, or cleared lysate (Clewell and Helinski, 1969).

An alternative method of preparing cleared lysates is to lyse the cells with a strong anionic detergent such as sodium dodecyl sulphate (SDS), or sodium dodecyl sarcosinate (sarkosyl).

The plasmid DNA can again be partially purified by differential centrifugation. Alternatively lysates prepared with SDS, after addition of high concentration of NaCl, may be cooled so that the detergent precipitates taking with it most of the chromosomal DNA. A short centrifugation again leaves the plasmid DNA in the supernatant (Lee and Davidson, 1970; Guerry, Le Blanc and Falkow, 1973).

Further separation of plasmid DNA is then achieved by exploiting the differences existing between it's usual covalently closed circular configuration and the configurations of nicked circular and linear DNA.

Isopycnic density gradient centrifugation is unable to distinguish between plasmid and chromosomal DNA of E. coli as the (G+C) contents, and therefore the buoyant densities in caesium chloride, are the same. However, separation may be achieved by introducing intercalating dyes such as ethidium bromide (Bauer and Vinograd, 1968) or propidium iodide (Hudson, Upholt, Devinny and Vinograd, 1969) into the caesium chloride gradients.

The binding of intercalative dyes causes partial un-

winding of the duplex structure and in the case of closed circular DNA, a critically small amount of dye-binding (when the mole ratio of bound dye to nucleotide reaches approximately 0.05) reduces the number of helical terms to zero. A relaxed circle is thus formed, though both strands remain intact.

However, further binding results in the formation of superhelices in the opposite direction as can be seen by electron microscopy (Thomas, Kelly and Rhoades, 1969). The restraints inherent in a closed circular duplex, limiting the amount of dye wich can be intercalated, are not present in the relaxed circles produced by nicking or the linear frequents arising through shear. Covalently closed molecules are therefore prevented from binding as much dye as may be bound by relaxed or linear duplexes, and as dye-binding reduces the buoyant density of DNA, there is a correspondingly smaller reduction in the density of closed circular DNA (Radloff, Bauer and Vinograd, 1967). Many supercoiled plasmid DNAs have a density of ca. 1.60 g/ml in caesium chloride-ethidium bromide, whereas nicked or linear E. coli DNA has a density of 1.56 g/ml. These compare with their common density of ca. 1.70 g/ml in the absence of dye.

A further application of the lesser dye-binding of plasmid DNA has been hydroxyapatite chromatography on columns equilibrated with ethidium bromide (Pakroppa, Goebel and Muller, 1975). This followed the discovery that the affinity of DNA-acridine complexes for hydroxy-

apatite is less than that of DNA alone (Pakroppa and Muller, 1974).

Because of their compact supercoiled configuration, closed circular molecules have a lower intrinsic viscosity and lower sensitivity to shear than their linear or relaxed circular counterparts. They therefore have a larger sedimentation coefficient which allows their separation from these other forms of the same plasmid by rate zonal centrifugation in neutral sucrose gradients (e.g. Clewell and Helinski, 1969).

The differences between the rates of sedimentation may be magnified by the use of alkaline sucrose gradients, as closed circular duplexes sediment much more rapidly in strand-separating solvents such as alkali than they do at neutral pH. While a pH in the range 11.5 - 12.0 is enough to separate completely the strands of relaxed and linear DNA, the strands in the closed circular duplex are topologically constrained and at pH 12.5 the denatured molecule forms a tightly compact structure. Thus, if a lysate is centrifuged through a sucrose gradient at pH 12.5, collapsed but intact closed circular DNA sediments approximately 4 times faster than denatured linear fragments (Jaenisch, Hofschneider and Preuss, 1969).

Alkaline denaturation may also be exploited in other ways.

If the pH of a lysate is brought to 12.5 then rapidly reduced to neutrality, the strands of linear and relaxed molecules remain separated but the strands of closed

circular molecules, not having completely separated, are able to reassociate to form the original duplex molecules. Subsequent passage through nitrocellulose filters (Wohlheiter, Falkow, Citarella and Baron, 1966), nitrocellulose columns (Boezi and Armstrong, 1967) or benzoylated naptholated DEAE cellulose columns (Komano and Sinsheimer, 1968) allows separation of the closed circular native DNA by retaining the denatured single stranded material. Closed circular molecules are also resistant to irreversible denaturation by heat.

The situations dealt with so far have been concerned largely with the plasmid in its natural environment i.e. the host cell. However, its isolation may be accomplished by transfer to a foreign environment;

a) Many naturally occuring plasmids of E. coli, Salmonella or Shigella can be transferred to Serratia marcescens or to Proteus mirabilis (Falkow, Wohlheiter, Citarella and Baron, 1964; Marmur et al., 1961). By virtue of the natural difference in base compositions (E. coli plasmid ~ 50 % (G+C); P. mirabilis chromosome 39 % (G+C), separation can be achieved by

(i) chromatography on methylated albumin Kieselguhr (Falkow, Citarella, Wohlheiter and Watanabe, 1966)

(ii) susceptibility to heat denaturation (Wohlheiter, Falkow and Citarella, 1966), or

(iii) isopycnic density centrifugation in caesium chloride gradients (Rownd, Nakaya and Nakamura, 1966).

b) Another method for plasmid isolation is via its segregation into minicells (Inselburg, 1970), which are small, non-growing cells produced by abberant division at the polar ends of rod shaped bacteria. These cells only occasionally contain chromosomal DNA (Kass and Yarmolinsky, 1970) and have the same plasmid DNA as the parents (Roozen, Fenwick, Levy and Curtiss, 1970; Levy and Norman, 1970).

Furthermore, physical characterization of this DNA indicates that a large percentage exists in the closed circular form. This method has been the source of DNA for the study of replicative intermediates (Cohen, Sharp, Silver and McCoubrey, 1971), transformation (Cohen, Chang and Hsu, 1972) and heteroduplex formation (Sharp, Cohen and Davidson, 1973).

3.1.3. Current procedures; requirement for a rapid screening method

Most of the separation methods described above are used only for specialized purposes. The current standard procedure for primary demonstration and isolation of plasmid DNA involves lysis of the host cells and a clearing spin to remove chromosomal DNA, followed by an isopycnic density gradient centrifugation of the cleared lysate in caesium chloride gradients containing 200 - 300 μg/ml ethidium bromide.

This requires a long period (≥ 48 hrs.) of centrifugation at high speed and although the plasmid DNA may be visualized directly by the use of long wave ultra-violet light

(which avoids isotopic labelling and counting), this non the less demands its presence in substantial quantities and thus a large volume of culture, usually a litre. To ensure sufficient DNA, a concentration step in which the DNA is precipitated from the cleared lysate by polyethylene glycol (Humphreys, Willshaw and Anderson, 1975) is often included. However, the use of high concentrations of DNA increases the likelyhood of overloading the gradients, particularly if the chromosomal DNA is not sufficiently sheared (Steensgaard, Møller and Funding, 1975).

The major alternative method for preparative purification of supercoiled DNA, using hydroxyapatite-ethidium bromide chromatography, does not require extended ultracentrifugation. However, it does involve initial passage of a cleared lysate through a Sepharose column to remove protein, RNA and low molecular weight material (Pakroppa et al, 1975).

Initial characterization - i.e. the determination of the number and molecular weights of species present in the isolated plasmid DNA, requires further examination. This involves either sedimentation of isotopically labelled DNA through sucrose gradients, or nicking the supercoils and examining the resulting open circles under the electron microscope (Davis, Simon and Davidson, 1971). Both these methods usually require inclusion of a DNA of known molecular weight, which also applies to the alternative procedure of sedimenting crude (uncleared)

lysates through alkaline sucrose gradients.
The use of isotopic labelling results in a large number of radioactive samples which need to be analysed in a scintillation counter and which eventually require careful disposal.
These procedures are time-consuming and costly and are not suited to surveys of potential plasmid-carrying isolates. This is particularly so as recently a growing number of bacterial functions, notably in the clinical and fermentation fields, are tentatively being attributed to plasmids (e.g. So, Crandall, Crosa and Falkow, 1975; Schrempf, Bujard, Hopwood and Goebel, 1975).
I have therefore developed a method for the rapid demonstration of plasmid DNA and its molecular weight based on rate zonal centrifugation of unlabelled DNA in neutral, isokinetic sucrose gradients containing a low concentration of ethidium bromide.

3.2. PRELIMINARY EXPERIMENTS; EFFICIENCY OF CLEARING SPINS AND POLYETHYLENE GLYCOL CONCENTRATION

3.2.1. Introduction

The procedure depends primarily on
a) the extraction of plasmid DNA, and
b) visualization of the DNA when complexed with acridine.

Many preliminary tests were therefore performed to assess different methods of lysis and visualization of different DNA-acridine complexes. During the early part of the work

with gradients, sufficient plasmid DNA was obtained by using an R6K$^+$ strain grown to stationary phase (Kontomichalou, Mitani and Clowes, 1970) or a ColE1$^+$ strain grown in the presence of 180 µg/ml chloramphenicol (Clewell, 1972) as both these systems produce cells containing an unusually high proportion of plasmid DNA.

However, to obtain sufficient plasmid DNA from normal strains, it was necessary to establish (i) how much of the cleared lysate could be used (i.e. was not contaminated with chromosomal DNA), and (ii) the efficiency of the polyethylene glycol precipitation at various concentrations of DNA.
Radioactive labelling experiments were therefore carried out.

3.2.2. Materials and Methods

Cultures. 28.0 ml Y.E. broth, 0.1 ml 20 % Glucose, 1.5 ml Uridine (5 x 10^3 µg/ml), 0.3 ml ^{3}H Thymidine (10^3 µCi/ml), 0.1 ml unlabelled thymidine (0.2 %), 0.15 ml ONC of E. coli M671 (plasmid free strain C600) shaken O.N. at 37°C.

Procedure

Clearing spin

Cells were harvested, washed in 10 ml saline, deposited again and resuspended in 1.6 ml saline. After sampling and transfer to a Spinco tube, 2.5 ml of a saturated solution of dodecylamine (DDA) in TES_4 and 0.5 ml of

lysozyme (10 mg/ml TES_4) were added, followed by incubation at 37°C for 10 minutes. Addition of 0.5 ml sarkosyl was then followed by a further incubation of 10 minutes x 37°C and a clearing spin of 30,000 r.p.m. x 30 minutes. Samples were then removed from the length of the centrifuge tube by puncture with Yale Microlances attached to 1 ml tuberculin syringes.

PEG treatment. (Humphreys et al. 1975)
The gelatinous pellet of DNA deposited in the clearing spin was resuspended in saline (4 ml), sonicated for 10 minutes and clarified by centrifugation in a bench centrifuge for 15 minutes. A series of 3-fold dilutions in TES_4 was then made, and each dilution, along with a pooled supernatant, comprising all samples taken from the cleared lysate, were treated with NaCl and PEG, cooled over night (4°C) and centrifuged for 30 minutes at 3,000 r.p.m. Samples were then taken from the upper quartile of the supernatant, the remainder of the supernatant, and from the pellet resuspended to original volume in TES_4.

Measurement of radioactivity

Triplicate 0.05 ml samples were absorbed by Whatman 3 mm filter disks and the DNA precipitated by 5 % trichloroacetic acid (2 treatments of ca. 5 ml per disc; 0°). They were then washed in ethyl alcohol for 10 minutes, acetone for 5 minutes (both at room temperature), dried and placed

in vials containing 9 ml of scintillation fluid (6 g Butyl B.P.D./litre toluene). Radioactive counts per minute (CPM) were then measured in either a Packard Tri-Carb spectrometer or a Beckman LS-200B Liquid scintillation system.

3.2.3. RESULTS

Clearing spin

The results (Table 3.1.) show that only 1 - 2 % of the total lysate radioactivity was present in the cleared lysate. Although there was a slight increase in the lower half, it seemed that almost all the cleared lysate could be removed without contamination by bulk chromosomal DNA. Indeed, it was felt that the force used might be too great so a further series of clearing spins were tested, incorporating ethidium bromide so that the unlabelled DNA could be seen. These suggested that forces 25 % as great would give equally good separation but that still smaller forces would not sediment the chromosomal fraction with a defined upper boundary.

PEG treatment

The results for the PEG concentration are shown in Table 3.2. and indicate that the efficiency of precipitation is dependent on DNA concentration. The procedure is effective with the more concentrated solutions (sample 1 ratio of upper supernatant : pellet is 1 : 22.4) but this is not the case when the starting solution is dilute (sample 3 ratio is 1 : 1.4).

TABLE 3.1.

Efficiency of clearing spin (30K x 30')

Sample	Source	C.P.M.
1	Resuspended cells (before lysis)	$1.4 \cdot 10^1$
2	Cleared lysate (top of tube)	$2.2 \cdot 10^5$
3	"	$2.2 \cdot 10^5$
4	"	$2.2 \cdot 10^5$
5	"	$2.2 \cdot 10^5$
6	"	$2.4 \cdot 10^5$
7	"	$2.4 \cdot 10^5$
8	"	$2.4 \cdot 10^5$
9	"	$2.8 \cdot 10^5$
10	"	$2.8 \cdot 10^5$
11	"	$2.8 \cdot 10^5$
12	Cleared lysate (bottom of tube)	$3.2 \cdot 10^5$
13	Clarification supernatant	$1.2 \cdot 10^7$

C.P.M.; Counts per minute per ml (corrected for volume) throughout length of centrifuge tube, following lysis and clearing spin (30,000 r.p.m. x 30')

Clarification supernatant: supernatant from clearing spin pellet, after resuspension, sonication and low speed centrifugation.

TABLE 3.2.

Precipitation of DNA by polyethylene glycol

Dilution of c.s.[b]	Before precipitation	After Precipitation		Pellet	Ratio[d]
		Upper 1/4 Snt.	Remainder Snt.		
0	$4.0 \cdot 10^5$ [a]	$1.7 \cdot 10^4$	$2.2 \cdot 10^4$	$3.8 \cdot 10^5$	22.4
1/3	$1.4 \cdot 10^5$	$2.6 \cdot 10^4$	$3.6 \cdot 10^4$	$1.0 \cdot 10^5$	3.9
1/9	$5.0 \cdot 10^4$	$1.9 \cdot 10^4$	$2.2 \cdot 10^4$	$2.6 \cdot 10^4$	1.4
1/21	$1.7 \cdot 10^4$	$1.1 \cdot 10^4$	$1.2 \cdot 10^4$	$6.6 \cdot 10^4$	0.6
1/81	$7.2 \cdot 10^3$	$3.4 \cdot 10^3$	$3.8 \cdot 10^3$	$3.4 \cdot 10^3$	1.0
1/243	$3.4 \cdot 10^3$	$2.0 \cdot 10^3$	$1.8 \cdot 10^3$	$1.5 \cdot 10^3$	0.8
P.C.L.[c]	$4.0 \cdot 10^4$	$7.2 \cdot 10^3$	$7.0 \cdot 10^3$	$6.8 \cdot 10^3$	0.4

[a] Counts per minute/ml (corrected to original volume)

[b] Dilutions of the clarification supernatant (in TES_4, from clearing spin pellet)

[c] Pooled cleared lysate samples

[d] Ratio of pellet to upper 1/4 snt.

Particularly relevant was the result obtained by treatment of the pooled cleared lysates as the chromosomal DNA concentration in this sample would be similar to that of plasmid DNA encountered in a normal clearing spin (plasmids comprise 1 - 2 % of total cellular DNA. From the previous experiment one can see that this is the concentration of chromosomal DNA left in the cleared lysate). In this case, and in those of samples 3 and 4 which have similar DNA concentrations there was no significant difference between radioactivity levels observed in the pellet and the supernatant.

While, as shown in the original work (Humphreys et al., 1975) the method is valuable when dealing with litre cultures destined for CsCl gradients, it is innapplicable to the low DNA concentrations used in this work.

3.3. RAPID SCREENING FOR PLASMID DNA

3.3.1. Materials and Methods

Details of the procedure are first described, followed by notes in the succeeding section.

Cultures These were incubated at 37° on a reciprocating shaker (50 mm stroke; 100 strokes/min.) in 250 ml capped conical flasks containing 50 ml YE2 broth ($NaH_2PO_4 . 2H_2O$, 3 g; K_2HPO_4, 14 g; NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 2 g; Oxoid acid-hydrolysed casein, 5 g; Oxoid yeast extract, 5 g; L-cysteine HCl, 50 mg; DL-tryptophan, 50 mg per 1 distilled water). After 18 - 22 hrs. incubation, the

optical density at 650 nm was 2.5 - 2.9. The organisms were deposited in a bench angle centrifuge (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England; model 50, type 6/50) and resuspended as evenly as possible by a pasteur pipette in 1.1 ml 0.85 % (w/v) NaCl to give ca. 1.4 ml suspension (Note 1).

Lysis. One ml 5 % (w/v) sodium dodecyl sulphate (SDS) and 3 ml TES_4 (0.1 M-tris, 0.07 M di-Na EDTA, 0.05 M-NaCl, pH 8.0) were placed in a 5 ml polycarbonate centrifuge tube (MSE 34411-111). One ml bacterial suspension was squirted into this lysing mixture from a calibrated pasteur pipette and the tube immediately inverted several times to mix its contents. Lysis often began immediately and was completed by transferring the tube to a 60° waterbath for 8 min., by which time the dense milky bacterial suspension had become uniformly semi-translucent (Note 2).

Enrichment for plasmid DNA. The lysate was centrifuged in the same polycarbonate tube for 15 min. at 30,000 r.p.m. at 20° in the SW50.1 rotor of a Spinco L3-40 centrifuge (Beckman-Riic Ltd., Eastfield Industrial Estate, Glenrothes KY7 4 NG, Scotland). This deposited most of the chromosomal DNA (Note 3), leaving as the supernatant a 'cleared lysate' containing most of the plasmid DNA (Clewell and Helinski, 1969). The upper 3.0 - 3.5 ml supernatant was retained, the remainder being discarded with the deposit (Note 4).

RNase. The cleared lysate was placed either at 4° overnight or at 0° for 2 - 4 hrs. until most of the SDS had precipitated (Becker, Helenius and Simons, 1975). The supernatant was removed and mixed with 0.1 vol RNase solution, 250 µg/ml (E.C. 3.7.4.56. Bovine pancreas ribonuclease, British Drug Houses, Poole, Dorset, England, ref. 39039. This was dissolved in distilled water and heated in a bath of boiling water for 10 min. to inactivate contaminating DNase). The mixture was incubated at 37° for 30 min. (Note 5). Finally, 0.1 vol. ethidium bromide (40 µg/ml) was added (Note 6).

Sucrose gradients. Linear sucrose gradients were prepared in 5 ml cellulose nitrate centrifuge tubes (Spinco 305050) by diffusion overnight at room temperature with the tubes capped to prevent evaporation. Each tube received 0.6 ml 21 % (w/v) sucrose in TES_4, which filled the concave base of the tube, followed by 1.1 ml each of 17 %, 13 %, 9 % and 5 % (w/v) sucrose. All sucrose solutions were prepared in TES_4 and were autoclaved at 121° for 15 min. to inactivate nucleases. Ethidium bromide was added to 4 µg/ml (Note 6).

Centrifugation. A sample of 0.2 - 0.3 ml cleared lysate containing ethidium bromide was placed on the gradient (Note 7) which was immediately centrifuged at 35,000 r.p.m. in the SW50.1 rotor at 20°. The period of centrifugation is specified in Fig. 3.1. and does not include either the run-up or the unbraked run-down times (ca.

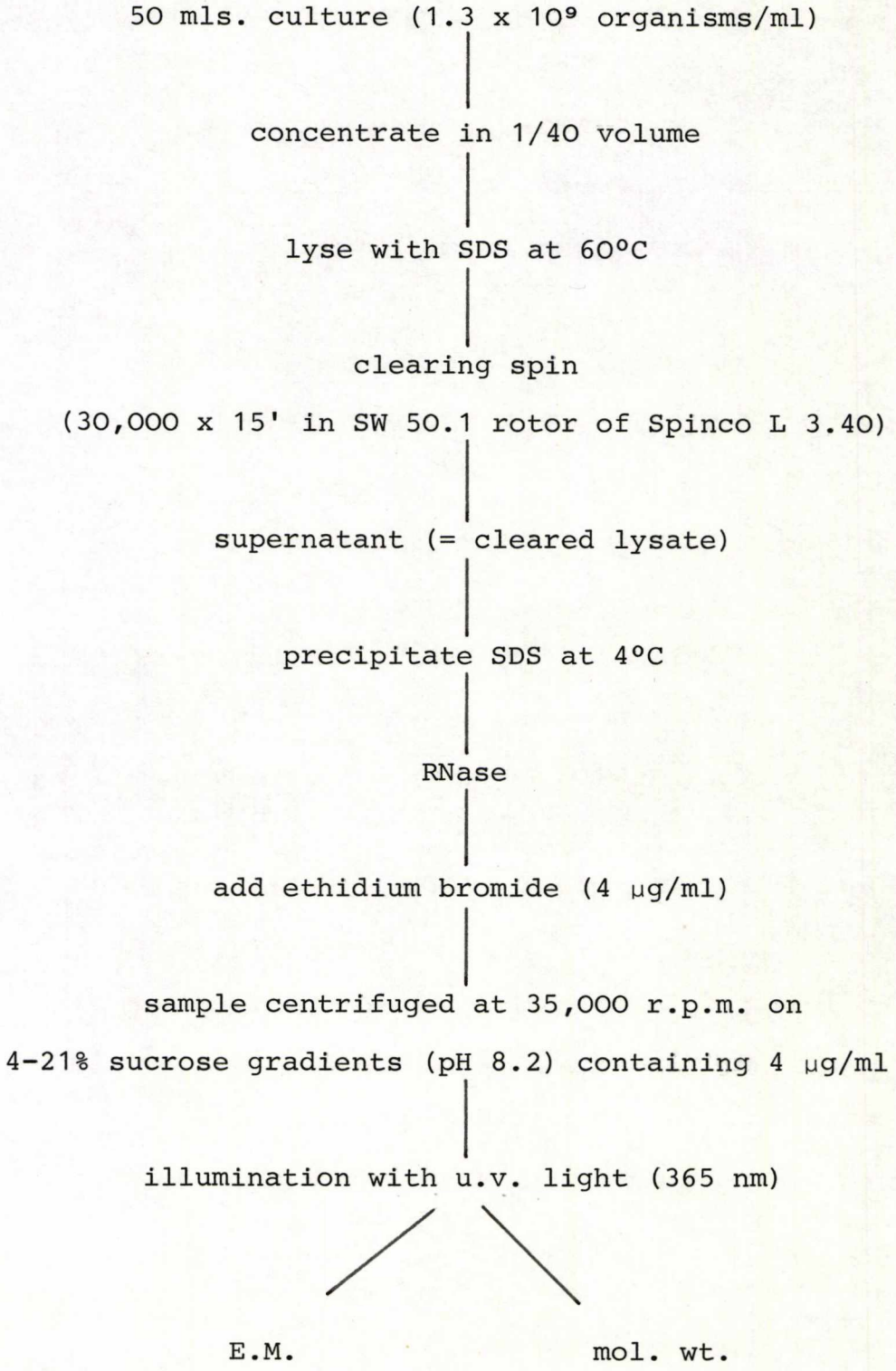
1.8 and 12.7 min., respectively). The radius of rotation to the interface between sample and gradient was ca. 60 mm and to the beginning of the curved base of the tube, ca. 112 mm, giving a usable gradient of ca. 40 mm.

Visualization of bands. The tubes were illuminated in a dark room with an 80 W mercury discharge lamp fitted with a Woods filter transmitting predominantly at 365 nm (Hanovia Ltd., 480 Bath Road, Slough, Bucks SL1 6BJ, England, ref. 16744/1). Plasmid DNA appeared as a thin red fluorescent band within the gradient (Note 6) and the distance of migration was measured in mm from the interface between the sample layer and the top of the gradient.

Electron microscopy of DNA. Plasmid DNA was taken from bands observed in the sucrose gradients using a syringe and was then dialysed against a mixture of 0.01 M TRIS and 0.001 M EDTA (pH 8.5). 50 µl of DNA hyperphase (0.1 mg/ml cytochrome C, 0.5 M NH_4acetate, 1 mM EDTA, ≈0.5 mg/ml DNA) were run onto the hypophase (0.25 NH_4 acetate, pH 7.5) as described in the aqueous technique of Davis, Simon and Davidson (1971) except that a stainless steel ramp and constriction pipettes were used.

DNA was then picked up on Parlodion coated electron microscope grids, stained with Uranyl acetate and shadowed with Platinum-Palladium at a seven degree angle. The complete method is summarized in Fig. 3.1.

Fig. 3.1. Summary of method



3.3.2. Notes

1. Tests with calf thymus DNA in different concentrations of ethidium bromide suggested that as little as 0.5 µg double-stranded DNA could be seen as a band in these gradients, in confirmation of Radloff, Bauer and Vinograd (1967). Since the minimum volume of sample applied to the gradient was 0.2 ml, the minimum concentration of plasmid DNA required was 12.5 µg/5 ml lysate. This fixed the minimum number of bacteria required. If, in round figures, plasmids comprise 1.5 % of total bacterial DNA and if 10^9 Escherichia coli contain 8 µg DNA, then 12.5 µg plasmid will be found in ca. 10^{11} E. coli. 50 ml culture of O.D. = 2.5 contains ca. 1.25×10^{11} E. coli, which is about 1.25 times the calculated minimum. Certain plasmids comprise far more than 1.5 % of total DNA. An example is R6K which contributes ca. 20 % in overnight culture (Kontomichalou, Mitani and Clowes, 1970) and in this case, sufficient plasmid DNA for this technique can be isolated from as little as 7.5 ml overnight culture.

2. Successful lysis depends on good mixing of the organisms with the lysing mixture before lysis begins. Unless this is done, partial lysis causes these dense suspensions to become so viscous that further mixing is impossible and large skeins of unlysed organisms persist in the tube. It was for this reason that lysates were prepared by SDS alone at 60° (Marmur, 1963), rather than by the

now more usual method of preliminary exposure to lysozyme and EDTA followed by detergent (e.g. Bazaral and Helinski, 1968) which never gave proper mixing with these suspensions. Heating at 60° has the further advantages, first, of producing a homogeneous population of relaxed plasmid molecules (Results) and second, taken with the presence of SDS, of being likely to render most Gram negative pathogens harmless. This lysis procedure was found satisfactory with Alcaligenes faecalis (NCIB 8156), Citrobacter freundii (NCTC 9750), Enterobacter aerogenes (NCTC 10006), Proteus vulgaris (NCTC 4175), Pseudomonas aeruginosa (NCTC 10332), Ps. fluorescens (KH 597) and Serratia marcescens (NCTC 1377); but not with Bacillus subtilis (KH 468) or Staphylococcus aureus (UB4008).

3. The conditions for the clearing spin (30 min. at 30,000 r.p.m.) were calculated on the assumption that, under these conditions, the chromosome of Escherichia coli would be unfolded but intact with a sedimentation coefficient (S) of ca. 250 (Stonington and Pettijohn, 1971). However, in our lysates, the effective S value appeared considerably greater, presumably due to the chromosome remaining folded or being associated with membrane fragments. Centrifugation for 15 min. at 30,000 r.p.m. was finally used. It is probably important not to use excessive force at this stage for, although the molecular weight of the chromosome is some 20 times greater than that of a large plasmid like ColV-K94 (mol. wt. 94 x 10^6), its S value, as predicted from the re-

lationships used in Table 3.3., may be only about twice as great.

4. An alternative method for enriching plasmid DNA was also attempted in which an uncentrifuged lysate made with SDS is cooled to 4° so that most of the chromosomal DNA precipitates with the SDS, leaving predominantly plasmid DNA in the supernatant (Lee and Davidson, 1970; Guerry, LeBlanc and Falkow, 1973). However, the lysates were so viscous with the present high concentrations of DNA that the SDS had to be removed by ultra centrifugation so that the procedure lost any advantage over a clearing spin.

5. When cleared lysates were sedimented in sucrose-ethidium bromide without being treated with RNase, two very prominent fluorescent bands were obtained which sedimented at 3.2 and at 4.8 mm / h, respectively. These bands disappeared after treatment with RNase but not DNase and were therefore presumed to be 16s and 23s ribosomal DNA.

6. When irradiated at 365 nm, double-stranded DNA complexed with ethidium bromide fluoresces 80 times more brilliantly than the free dye (Le Pecq, Yot and Paoletti, 1964). The concentration used here (4 μg/ml) is of the same order as that used in the fluorimetric assay of DNA and RNA (Boer, 1975) and is far less than the 100 - 200 μg/ml generally used to introduce density differences

between closed circular and other species of DNA (Radloff et al., 1967; Bauer and Vinograd, 1968). Bands can be seen with these higher concentrations but the visual contrast between the band and the surrounding gradient is far greater at low concentrations.
Ethidium bromide also renders DNA susceptible to nicking by visible light (Smith and Vinograd, 1972) and, although we found no evidence that this occurred in these experiments, the dye-DNA mixtures were kept from strong light as a precaution.

7. If more concentrated lysates were used, gradients generally failed due to migration of the sample as a diffuse band down the gradient, presumably following a density inversion (Britten and Roberts, 1960).

3.3.3. Results

Behaviour of plasmids in sucrose gradients

After centrifugation, the tubes showed one of three appearances. The most usual was that expected: a sharply defined fluorescent band of plasmid DNA perhaps 0.5 mm deep in the main part of the gradient, with a more brilliant band just below the interface between the sample and the gradient, presumed to consist of small DNA and RNA fragments. Occasionally, the main part of the gradient showed either a diffuse band perhaps 10 mm deep (Note 7) or no band whatsoever which, with known plasmid-carrying strains, was taken to follow loss of

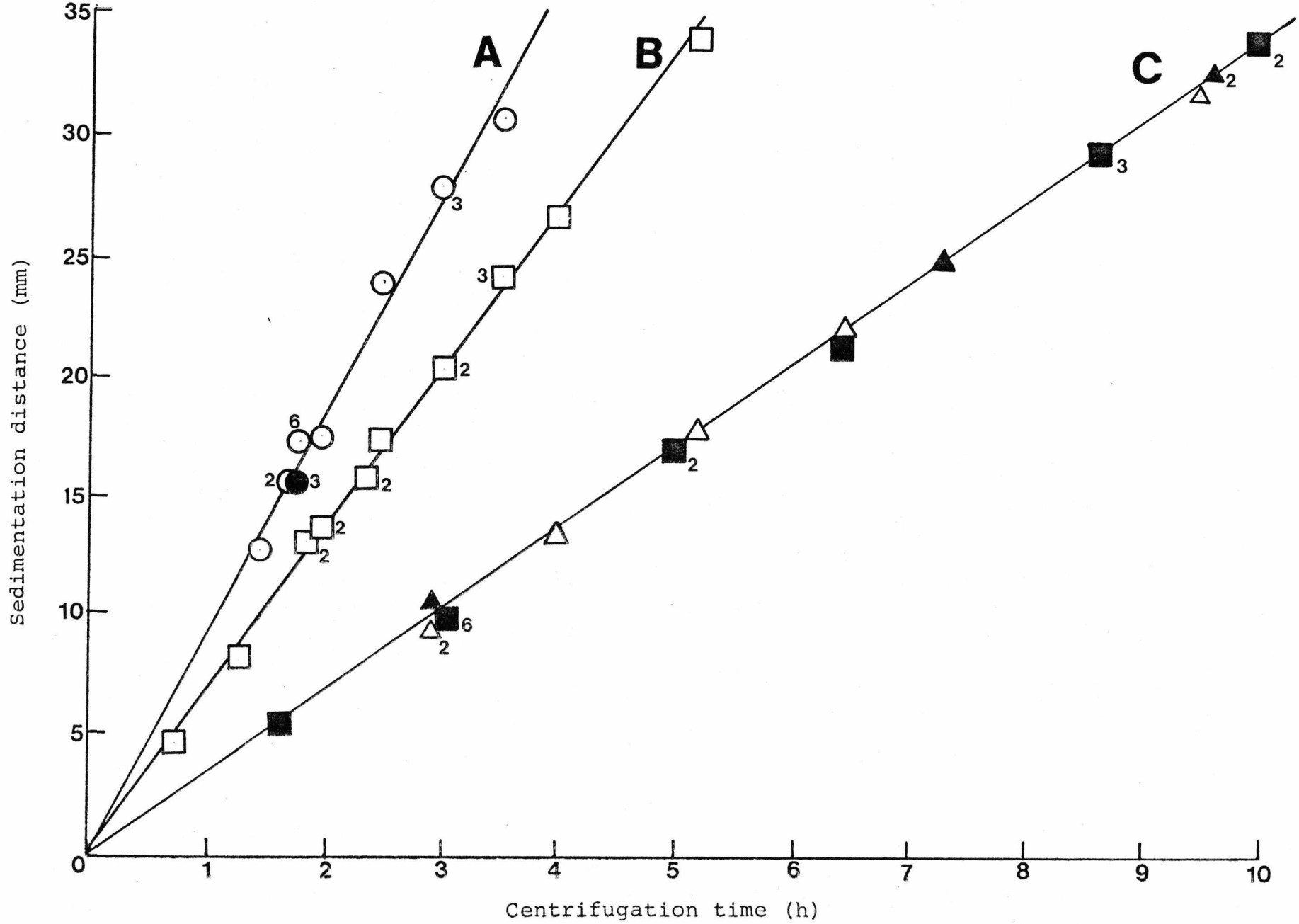
plasmid during the clearing spin or too small a sample. When either of the latter occurred, the expected result was usually obtained on running another sample or by preparing another lysate. No sharp band was ever seen with known plasmid-free strains.

The behaviour of plasmids whose molecular weights ranged from 4.8×10^6 - 61×10^6 is shown in Fig. 3.2. These fell into three groups, depicted by curves A, B and C, each of whose members sedimented at the same rate. In each group, the distance sedimented was proportional to the period of centrifugation. That is, in these linear gradients as in some others (Burgi and Hershey, 1963; Britten and Roberts, 1960) the increased centrifugal force on passing down the gradient cancelled out the increasing viscosity and density of sucrose to produce isokinetic conditions in which the velocity of sedimentation ($\underline{v}$) was constant (Martin and Ames, 1961; Noll, 1967). The observed sedimentation coefficient was therefore proportional to $\underline{v}$.

Conformation of sedimenting plasmids

Comparison of the relative $\underline{s}$ value thus obtained from Fig. 3.2. with the s^o values ($\underline{s}$ at infinite dilution) calculated from the molecular weights, M, reported for these plasmids suggested that the plasmid DNA was sedimenting as relaxed circular molecules. This follows from the relation between sedimentation coefficient and molecular weight, generally expressed as $s^o + c = aM^b$, where $\underline{a}$, $\underline{b}$ and $\underline{c}$ are constants whose values depend on

Fig. 3.2. Sedimentation of plasmid DNA in sucrose gradients

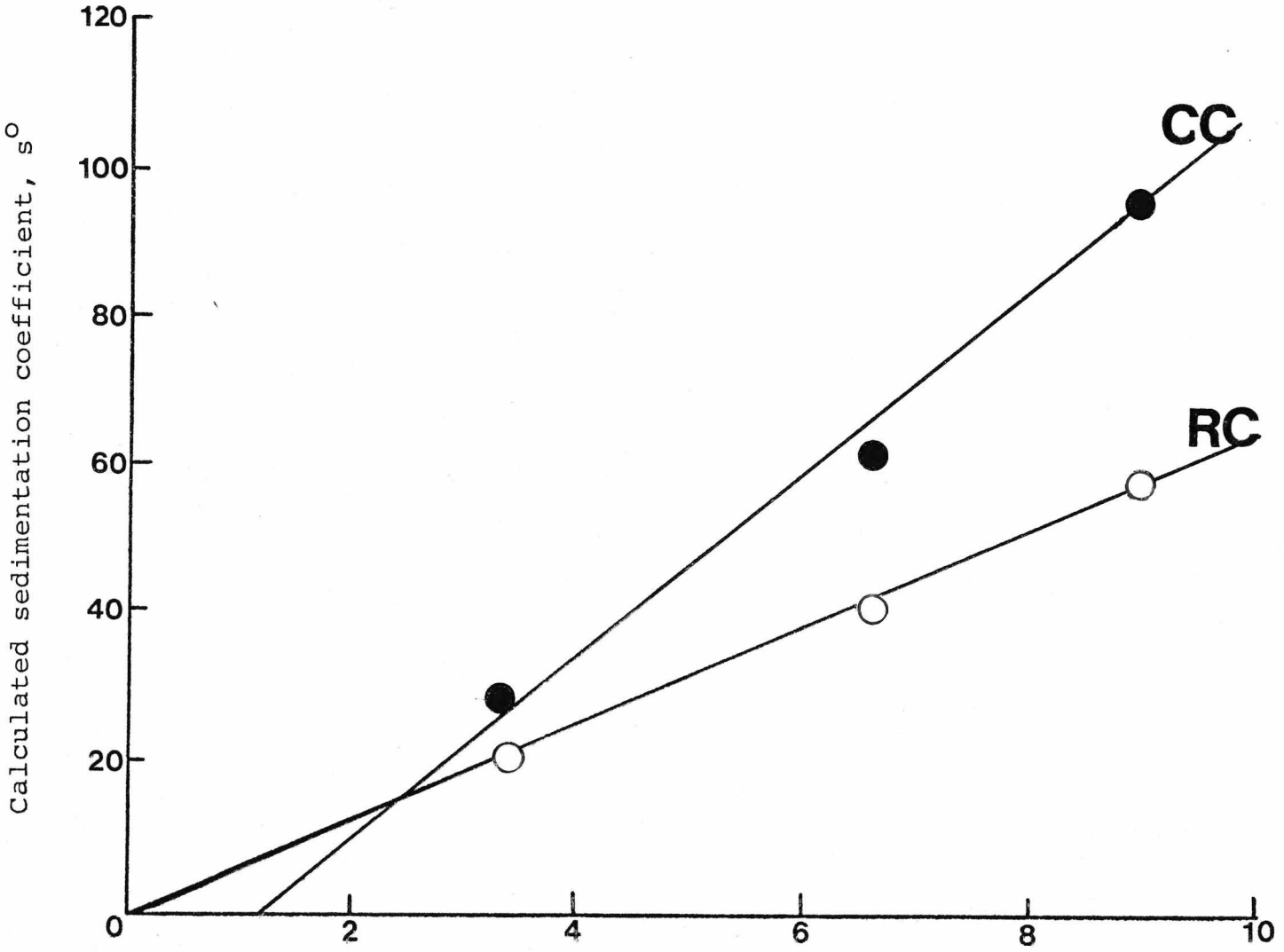


Legends to Figures 3.2. and 3.3.

Fig. 3.2. Sedimentation distance of plasmid DNA in linear 4 - 21 % (w/v) sucrose gradients, pH 8.0, plotted against centrifugation time (SW50.1 rotor, 20°, 35,000 r.p.m.). Many points are averages of several readings, as indicated by the accompanying numbers. The plasmids fell into three groups:
Curve A ($\underline{v}$ = 9.1 mm/h): ColIb-P9 (o),
P1 prophage (●).
Curve B ($\underline{v}$ = 6.7 mm/h): R6K (□).
Curve C ($\underline{v}$ = 3.4 mm/h): ColE1-K30 (■),
ColE2-P9 (△), ColK-235 (▲).
All plasmids were in Escherichia coli K12 except for the P1 prophage which was in E. coli B.

Fig. 3.3. Values of $\underline{s}^{o}$ calculated from reported molecular weights plotted against observed values of $\underline{v}$ (mm/h) from curves A, B and C of Fig 3.2). Closed symbols: closed circular DNA; open symbols: relaxed circles.

Fig. 3.3. Conformation of sedimenting plasmid DNA



whether the DNA is closed circular, relaxed circular or linear (see Bottger, Bierwolf, Wunderlich and Graffi, 1971; and footnote to Table 3.3.). A special case assumes $\underline{c}$ = 0 (Burgi and Hershey, 1963; Bazaral and Helinski, 1968 b). In Table 3.3. relative values of $\underline{s}^{o}$ were calculated from reported values of M. The ratio of these $\underline{s}^{o}$ values were then compared with the ratio of the observed values of $\underline{v}$, taking the values for the smallest plasmids as unity. Table 3.3. shows that the observed ratios of 1.97 and 2.68 did not agree with those predicted for closed circles (2.25 and 3.49) but were consistent with those expected for relaxed circles (1.95 and 2.76). This is expressed in another form in Fig. 3.3. where calculated values of $\underline{s}^{o}$ from Table 3.3. are plotted against observed values of $\underline{v}$ from Fig. 3.2. The expectation from $\underline{s} \propto \underline{v}$ is that the plot will extrapolate to the origin (i.e. when $\underline{s}$ = 0, $\underline{v}$ must be 0). However, this was found only for the plot for relaxed circles. Applying the alternative relationship, $\underline{s} = aM^{b}$, led to the same conclusion. The values of $\underline{b}$ reported for closed and for relaxed circular molecules are 0.43 and 0.36, repectively (Bazaral and Helinski, 1968 b; Barth and Grinter, 1974); our observed value, taken from log $\underline{v}$ plotted against log M, was 0.38.

Cause of nicking

Since at least part of the plasmid DNA <u>in vivo</u> was likely to consist of closed circular molecules (Clewell and Helinski, 1969), these results suggested that nicking

TABLE 3.3.

Comparison of calculated and observed values

Plasmid	Reported value of M[a]	Calculated values of s^o[b] (1)	(2)	Ratio of s^o values (1)	(2)	Observed ratio of v[c]
ColE1-K30 ColE2-P9 ColK-235	4.8×10^6	26.9	20.2	1	1	1
R6K	26×10^6	60.5	39.4	2.25	1.95	1.97
ColIb-P9 P1 prophage	61×10^6	93.8	55.8	3.49	2.76	2.68

M mol. wt. of plasmid DNA. s^o : sedimentation coefficient at infinite dilution
v observed velocity of sedimentation

a From Clowes (1972), Hardy, Meynell, Dowman and Spratt (1973), and Walker and Anderson (1970).

b Calculated from $s^o - c = aM^b$ for closed circular (1) and for relaxed molecules (2). Values for the constants come from Böttger et al. (1971): (1) a = 0.00439, b = 0.553, c = 5.16; (2) a = 0.0219, b = 0.435, c = 2.5.

c Calculated from observed values of v in Fig. 3.2.: curve A, 9.1; burve B, 6.7; curve C, 3.4 mm /h.

occurred during preparation of the lysates. Two causes were considered: DNase contaminating the RNase preparation, despite heating at 100° (McCormick, Larson and Maher, 1974) or thermal hydrolysis during incubation of the lysate at 60° as opposed to the more usual 37°. Tests on lysates containing R6K, prepared with RNase, showed that a 60° lysate produced only a single band ($\underline{v}$ = 6.5 mm/h), presumed to be relaxed circles (Table 3.3.), whereas a 37° lysate produced two bands, one again sedimenting at 6.5 mm/h and a second faster, more conspicous band sedimenting at 10 mm/h. The ratio of these rates was 10/6.5 = 1.54 which is the ratio expected from Table 3.3. for closed circular and relaxed molecules (60.6/39.4 or 1.54). This conclusion was confirmed by electron microscopy of the DNA within each band. Other lysates of R6K were prepared with and without treatment with RNase (this is feasible with R6K because of its high concentration (Note 1) and because it sediments faster than ribosomal RNA), both at 60° and at 37°, with concordant results. Heating at 60°, with or without RNase, produced one band ($\underline{v}$ = 6.5 mm/h); exposure to RNase at 37° yielded two bands ($\underline{v}$ = 6.5 and 10 mm/h, respectively) which were presumed to be relaxed molecules, derived in part from relaxation complexes, and closed circular molecules (Clewell and Helinski, 1969). Hydrolysis at 60° was therefore taken to be the main cause of nicking.

3.3.4. Discussion

This procedure simplifies the screening of strains for plasmid DNA as it does not require either large volumes of cells, radioisotopes or long periods of centrifugation (Fig. 3.1.).

As well as its suitability for detection of plasmid DNA it also gives a rapid estimation of molecular weight.

This is so because of the reproducibility of results obtained, even though no internal standard DNA is employed. This arises in part from the sharpness of the bands which lessens error in determining their position and also because the bands were examined *in situ*, so avoiding artefacts during removal of the gradient for sampling (Morten and Hirsch, 1970).

The system has been calibrated for a wide range of plasmids (Fig. 3.2.), giving the equation

$$\text{m.w.} = \text{antilog.} \left[\frac{\log v + 1.9995}{0.38} \right]$$

for estimating molecular weight in further work. This was determined by fitting values of m.w. and v, for curves A, B and C (Fig. 3.2., Table 3.3.) to $b = 0.38$ (Results) in order to obtain the best fit of a in $s = a.M^b$.

The advantages of nicking the duplexes during lysis are as follows: a) It is in effect producing a concentration of plasmid DNA as unlike in methods such as caesium chloride - ethidium bromide equilibrium cen-

trifugation, the open circular molecules present in the cell are not selected against.

b) Only one band is produced by each plasmid, which would otherwise be expected to produce 2 bands i.e. closed circles as well as open circles. This obviously simplifies the detection of 2 different plasmids in the same host, as confirmed with E. coli K12 carrying both ColIb-P9 (m.w. 61 x 10^6) and ColE1-K30 (4.8 x 10^6)

c) Because the sedimentation rates of plasmid molecules differ less when in the relaxed form (Table 3.3.; Fig. 3.3.) it is possible to adopt a centrifugation time (ca. 3 hrs.) which will allow plasmids of all sizes to band within the gradient. If plasmids were in the supercoiled form this would not be possible.

d) The s^o values of relaxed circles, unlike those of closed circles, are almost unaffected by the ratio of ethidium bromide : nucleotide (Bauer and Vinograd, 1968). This follows from the virtually unaltered configuration after intercalation into relaxed circles.

e) DNA may be taken from the gradient and after dialysis, examined under the electron microscope (Lang and Mitani, 1970; Inman and Schnös, 1970). The usual nicking of the plasmid DNA before examination is eliminated.

Concurrently with this work, an alternative method was described by Meyers, Sanchez, Elwell and Falkow (1976). This technique utilizes agarose gel electrophoresis of plasmid DNA, using ethidium bromide as a stain. This technique is also more economical than those normally

used but its method of DNA isolation is not so rapid or simple as that described here. Also, both closed and relaxed circles may form bands in the gels so that strains possessing more than one plasmid are not as easily examined. These methods can no doubt be improved. The method described here would probably gain in resolution if steeper gradients, e.g. 10 - 40 % sucrose, were used and the speed of detection could be increased by use of a different rotor e.g. a Spinco SW 65 Ti rotor at 65,000 r.p.m. should produce the results shown in Fig. 3.2. in a third of the time.

The method was further tested and applied as described in chapters 4 and 5. It has also been used successfully to isolate and measure the molecular weights of (i) Staphylococcus aureus plasmids. In this case the lysis procedure is altered to include Lysostaphin (W.G. Grubb, personal communication). (ii) derivatives of the plasmid ColV in E. coli K12, the method giving sufficient sensitivity to permit assessment of deletion size (K.G. Hardy, personal communication).

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4. TWO MAJOR GROUPS OF COLICIN FACTORS: THEIR MOLECULAR WEIGHTS

4. APPLICATION OF RAPID SCREENING METHOD TWO MAJOR GROUPS OF COLICIN FACTORS: THEIR MOLECULAR WEIGHT

4.1. INTRODUCTION

Bacterial plasmids can be allotted to taxonomic groups by a variety of criteria (Chapter 1.2.). In the case of colicin (Col) factors, Hardy, Meynell, Dowman and Spratt (1973) suggested that there existed only two major groups, I and II, defined by criteria like the effect on colicin titres of the host recA allele, the proportion of colicin bound to the cells, the molecular weight of the plasmid DNA and the specificity of the colicin protein as reflected in the bacterial alleles determining colicin insensitivity (Table 4.1.). They therefore suggested that Col factors of Group I had evolved from a small 'EK-like' ancestor of molecular weight 5 x 10^6 whereas Group II was descended from a larger 'BIV-like' ancestor (m.w. ca. 60 x 10^6). This interpretation was subsequently supported by Davies and Reeves (1975 a,b) from an extensive study of the host range of colicins on colicin-insensitive mutants. They found that a colicin of their Group A (colicins A, E, K, L, N, S4 and X) was never active on a bacterial mutant selected by a colicin of their Group B (colicins B, D, G. H, I, M, Q, S1 and V), and vice versa. Hence, Col factors of Groups I and II of Hardy et al. (1973) appear to specify colicin proteins of Groups A and B of Davies and Reeves (1975 a,b).

TABLE 4.1.

Characteristics distinguishing two groups of colicin factors

(From Hardy, Meynell, Dowman and Spratt, 1973)

Character	Group I	Group II
Members	E1, E1a, E2, K	B, I, V
Colicin	Mostly free	Mostly bound
Colicin titres in:		
recA host	Decreased markedly	Decreased little, if at all
$recA^+$ host	$\sim$ 512	$\sim$ 16
Lacunae	Unambiguous	Faint or absent
Self-transmissible	No	Yes
Plasmid mol. wt.	$\sim 5 \times 10^6$	$\sim 70 \times 10^6$
Maximum number of copies per chromosome	$\gg 1$	~ 1
Plasmid DNA binding to Cd-sarkosyl crystals	30 %	70 %

The present work describes the molecular weights of plasmids found in standard colicinogenic strains, many kindly provided by Dr. Reeves. The results generally agree with expectation, although some discrepancies occur which may throw further light on the evolutionary relationships between naturally-occurring Col factors.

4.2. MATERIALS AND METHODS

Bacteria Colicinogenic strains are shown in Table 4.2. with their colicins and cross resistance groups according to Davies and Reeves (1975 a,b). Colicin-sensitive indicator strains were Escherichia coli K-12 strain C600 (our M468), and the mutants tonB (Nomura and Witten, 1968, obtained as Fredericq's G43, i.e. M1200) and tolII (Gratia, 1964; obtained as Nomura's KR21, i.e. M1199). Antibiotic resistances were determined by flooding nutrient agar plates (Oxoid CM55) with overnight cultures and adding Oxoid Multodisks containing ampicillin (10 μg), cephaloridine (15 μg), chloramphenicol (10 μg), kanamycin (5 μg), streptomycin (10 μg) and tetracycline (10 μg). Chromosomally-determined streptomycin resistance was detected with discs containing 200 μg streptomycin.

Molecular weights of Col factors Two methods were used: (a) rate zonal sedimentation in sucrose-ethidium bromide gradients, the distance of migration and hence the molecular weight being determined visually under ultra-violet light (Hughes and Meynell, 1977; this thesis Chapter 3).

(b) electron microscopy. Colicinogenic strains were grown in 1 l nutrient broth (Oxoid CN67) in 2 l flasks on an orbital shaker (100 r.p.m.) at 37°C. Bacteria were collected by low speed centrifugation and lysed according to Guerry, LeBlanc and Falkow (1973), with three exceptions: volumes were twenty times greater; lysozyme and sarkosyl were made up in TES (0.05 M Tris, 0.005 M di-sodium EDTA, 0.05 M NaCl, pH 8); and NaCl was not added after lysis. Cleared lysates were obtained after centrifugation at 15°C for 60 min. at 40,000 r.p.m. in a MSE 6 x 5.5 ml swing-out rotor. Caesium chloride (4.6 g) and ethidium bromide (to 500 μg/ml) were added to 4.8 ml cleared lysate. Plasmid DNA was then isolated by dye-buoyant density gradient centrifugation in the same rotor at 15°C for 40 hrs. at 40,000 r.p.m. Supercoiled DNA, the lower band seen under u.v. illumination, was obtained by syringe.

Large plasmids (m.w. $>40 \times 10^6$) were generally found to relax spontaneously. Smaller plasmids were nicked, as the DNA-ethidium bromide complex in polycarbonate tubes, by exposure to visible light (100 W bulb at 40 mm for 7 - 12 hrs. at 4°C). Ethidium bromide was removed by three extractions with propan-2-ol saturated with CsCl in TES, and the CsCl removed by dialysis for ca. 44 hrs. at 4°C against 1 l volumes of TES-EDTA buffer (0.01M tris, 0.001 M EDTA, pH 8.5).

Molecules were prepared for microscopy by the aqueous technique of Davis, Simon and Davidson (1971). Grids were shadowed with platinum-palladium at an angle of 6° in a

vacuum coating unit (Edwards type 306) and examined in an A.E.I. 801 A transmission microscope. Plasmid contour lengths were measured from projected micrograph negatives using a map measurer. Molecular weights were then estimated by using either a diffraction grating or an internal standard such as ColE1-K30.

4.3. RESULTS

Table 4.2 summarizes the results which are of three kinds:
(i) *Grouping by host range of the colicin.* Inspection of Davies and Reeves results suggested that their Group A, presumed by us to be specified by small Col factors of our Group I, were active on tonB but never on tolII indicators; and that the reverse held for their Group B colicins (Davies and Reeves, 1975a, Table 2; Davies and Reeves, 1975b, Table 5). All the strains used here were therefore tested against these indicators and found to behave as expected.
(ii) *Antibiotic-resistance.* This does not appear to have been reported previously although it is clearly material since a strain may carry R factors as well as Col factors. In fact, strain 398 appeared to have both types of determinant on the same plasmid. Some strains showed high level streptomycin resistance, denoted S(h) in Table 4.2., presumed to be due to mutation on the chromosomal strA locus.
(iii) *Molecular weight determinations.* All but three strains were allotted to groups I or II initially by the

TABLE 4.2.

Properties of colicinogenic strains and their plasmids

Strain[1]	Colicins[2]	Group[2]	Antibiotic resistance[3]	Plasmid molecular weights by: sedimentation[4]	microscopy[5]
23	A	A	-	I	<5 (h)
CA31	A	A	Cr	I	<5 (h)
K53	E1	A	-	I+II	Complex (h)
K30	E1	A	-	n.d.	4.2[6]
AB1157	E2	A	S(h)	n.d.	4.4 (0.25)
CA42	E2	A	-	I	3.8 (0.27)
12-317	E2	A	S(h)	I	n.d.
W3110	E3	A	S(h)	I	4.6 (0.19)
CA38	E3	A	-	n.d.	4.6 (0.16)
K216	K	A	-	I	n.d.
235	K+X	A	-	I	4.5 (0.09)
284	N+E3	A	T	I	3.8 (0.49)

Table 4.2. - continued

Strain[1]	Colicins[2]	Group[2]	Antibiotic[3] resistance	Plasmid molecular weights by: sedimentation[4]	microscopy[5]
285	N+E3	A	T	I	n.d.
CA23	D+X	A+B	S(h)	I	3.7 (0.23)
398	L	A	S(l),C,Cr,A,T	(I)	19.1 (1.13)
CA53	Ia	B	-	II	60.3 (1.51)
ST4	Ib-P9	B	S(h)	II	58.8 (1.72)
J5-3	Ib-P9	B	S(h)	II	n.d.
K89	B+M	B	-	II	42.8 (2.7)
CA46	G	B	-	I	3.9 (0.2)
CA58	H	B	-	I+II	Complex (h)
CA7	V	B	-	II	56.6 (3.1)
KH500	VIa-K94	B	S(h)	II	81.2 (2.8)[7]
II	I,Q,E1,D	A+B	-	I+II	n.d.
CA62	E1,I	A+B	-	I+II	n.d.

Table 4.2. - continued

[1]Escherichia coli except for Citrobacter freundii CA31, Salmonella typhimurium ST4 and paracolon CA62.

[2]From Davies and Reeves (1975a,b) except for K30 (= KH573 from K.G. Hardy), AB1157 (= KH293, Hardy et al. 1973), CA38 (= KH28), J5-3 (= KH596) and KH500 (=AB1157 ColV$^+$)

[3]From tests against antibiotic discs (Materials and Methods). A, ampicillin; C, chloramphenicol; Cr, cephaloridin, S(h) and S(l), high and low level streptomycin resistance; T, tetracycline.

[4]From Hughes and Meynell (1977) using visual inspection of plasmid bands in situ. I: mol.wt. ca. 5 x 10^6. II: mol.wt. 40 - 70 x 10^6 . (I) ca. 20 x 10^6; see text.

[5]From electron microscopy (Materials and Methods). Brackets: standard deviation. h: histogram in Fig. 1: mol. wt. x 10^6.

[6]Tomizawa (1974).

[7]K.G. Hardy, personal communication

Fig. 4.1. Mixed populations of plasmids

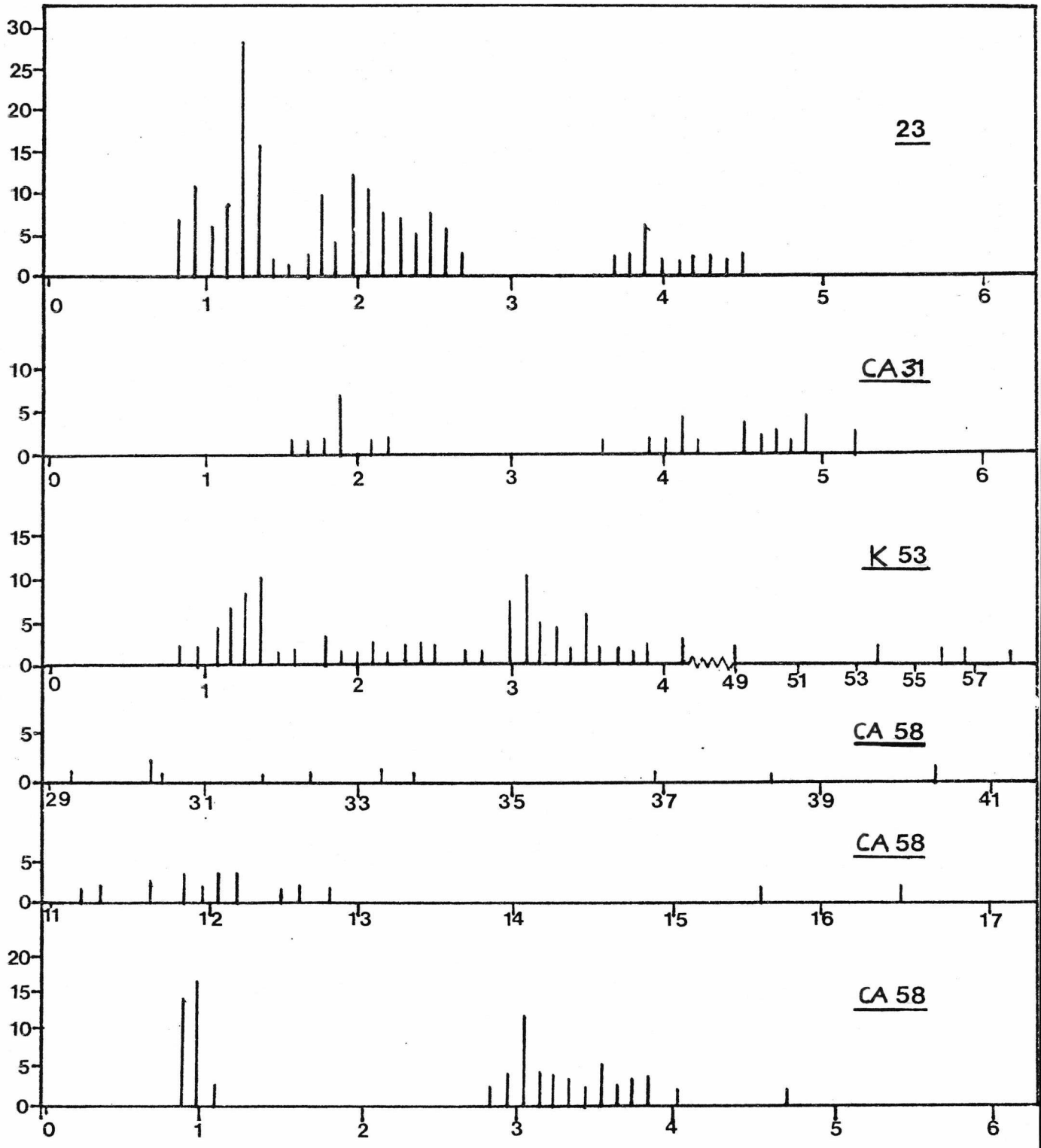

Fig. 4.1. Histograms of the mixed populations of strains 23 (ColA), CA31 (ColA), K53 (ColE1) and CA58 (ColH, 3 histograms). The horizontal axis shows the molecular weight x 10^6, plotted to the nearest 0.1; the vertical axis is the number of plasmids found at a given molecular weight.

rapid screening method of Hughes and Meynell (1977) based on rate zonal sedimentation in sucrose-ethidium bromide gradients. The majority of strains were then examined by electron microscopy. When the contour lengths were distributed unimodally, the mean and its standard deviation were shown in Table 4.2. However, four strains yielded mixed populations of plasmids and these distributions are shown as histograms in Fig.4.1.Thus, strain 23 (ColA) showed three modes, at 1.2, 2.0 and ca. 4.0 x 10^6, of which the last may be a dimer of the second. Strain CA58 (ColH) yielded molecules ranging from 0.8 - 40.5 x 10^6. This heterogeneity may perhaps have arisen in the bacterial stocks from which cultures were inoculated.

4.4. DISCUSSION

Table 4.2. supports the general hypothesis that the small Group I Col factors specify colicins of Group A specificity and that the large Group II factors specify colicins of Group B specificity. This conclusion is further supported by previous observations not included here on other members of Group I (ColEla-16, ColE2-P9, ColK-235; Hardy and Meynell, 1972; Hardy et al. 1973) and Group II (ColB-K77, ColVB-K260; Hardy, 1975, Table 4.1.). Some strains, like 235, 284 and 285, each produced two Group B colicins but only one class of plasmid DNA was detected: that is, the data do not distinguish between two different colicins specified by one plasmid and two plasmids of about the same size, each specifying a different colicin.

Some discrepancies would be expected, since a plasmid might be either larger or smaller than expected from the host range of its colicin. Both types of discrepancy were found here. Strain 398 formed colicin L which belongs to Group A and its plasmid was therefore expected to be Group I but the molecular weight, instead of being ca. 5 x 10^6 , was 19.1 x 10^6. However, this strain was multiply antibiotic-resistant (including low-level streptomycin resistance, presumed to be plasmid-determined) and may therefore carry a single plasmid with linked colicin and antibiotic-resistance determinants. The alternative type of discrepancy was shown by strains CA23 and CA46. Strain CA23 formed colicin D which belongs to Group B but its plasmid has a molecular weight of only 3.1 x 3.7 x 10^6 (Table 4.2.: Timmis, Cabello and Cohen, 1974). Strain CA46 formed colicin G, also belonging to Group B, but its plasmid was only 3.9 x 10^6. It is striking that colicin D is very similar to colicin B (Davies and Reeves, 1975a; Pugley and Reeves, 1977) and that colicin G is likewise very similar to colicin H (indeed they were once grouped together as colicin P: Frédéricq, 1953). Colicin factors D and G may therefore have arisen from extensive deletions of ColB and ColH, respectively, a possibility than can clearly be tested by their degree of homology.

4.5. REFERENCES

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5. ISOLATION OF ANTIBIOTIC-RESISTANT BACTERIA FROM THE RIVER STOUR

5. ISOLATION OF ANTIBIOTIC-RESISTANT BACTERIA FROM THE RIVER STOUR

5.1. INTRODUCTION

With the aid of the Nalidixic acid medium described earlier (Chapter 2), a bacteriological study was made of the River Stour catchment area (Fig. 5.1.). The principal river is the Great Stour which rises at Lenham and flows to Ashford where it is joined by the East Stour, which rises at Westenhanger within a short distance of the sea at Hythe. The Great Stour then cuts through the North Downs to Canterbury and continues to flow in a north-easterly direction to a point just short of the village of Sarre, where it turns eastwards to its outfall at Pegwell Bay near Sandwich (See Fig. 5.1.). The Great Stour is subject to tidal influence as far upstream as Fordwich, below Canterbury, but saline penetration does not reach this point.

The Stour provides a convenient subject for study as the water quality is generally good but the river receives two substantial sewage and industrial effluent discharges, at Ashford and Canterbury, which in the past have sometimes resulted in unacceptable levels of pollution. These two towns are not large (populations about 50,000), but the relatively small volume of the river results in any discharge being sometimes diluted by only a small factor. Typical figures for effluent vo-

Fig. 5.1. River Stour; Catchment area and bacteriological sampling points

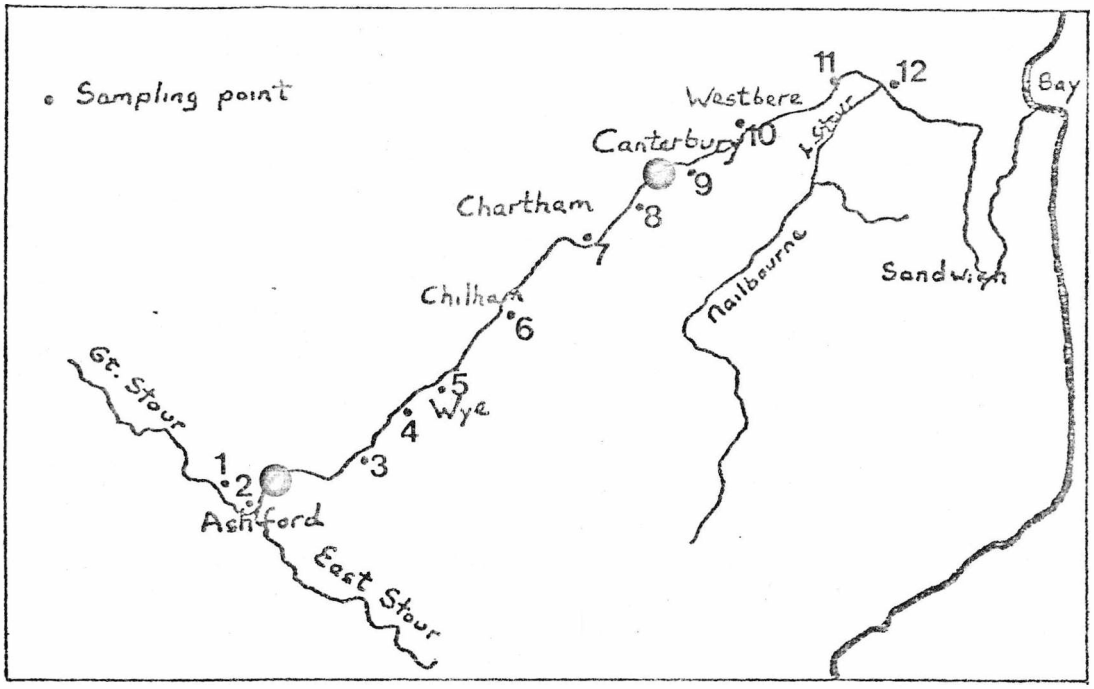


Table 5.1. Main effluent discharges into the Stour

Town	Effluent volume[+]
Ashford ●	3,600
Wye	75
Chilham	1
Chartham	380
Canterbury ●	3,400
Westbere	260

[+] thousands of gallons per day

lumes emitted into the Stour are given in Table 5.1. When the effluent is of acceptable quality, normally between 10 and 20 mg/l biological oxygen demand (B.O. D.; see note in materials and methods) and 15 to 20 mg/l suspended solids (depending on the time of year) a notional acceptable dilution at Ashford is 8 to 1 (i.e. 8 volumes of water to 1 volume of effluent). However, this is not always achieved, e.g. while in November 1976 both Ashford and Canterbury achieved dilutions of approximately 8 to 1, in July of that year, during a drought, effluent was diluted at 1 to 1 at Ashford and 2 to 1 at Canterbury. The river therefore reflects many of the problems experienced by rivers serving the larger cities.

While the river is monitored several times during the year for chemical changes, there has been little or no bacteriological examination. This study is concerned not only with Escherichia coli, the routine indicator of fecal pollution, but also with other coliforms and members of the Pseudomonadaceae. Of particular interest is the presence of organisms which are resistant to antibiotics as these have additional public health importance.

5.2. DISTRIBUTION OF GRAM-NEGATIVE HETEROTROPHS, PARTICULARLY COLIFORMS

5.2.1. Materials and Methods

Samples were taken, as described previously (Chapter 2), from sites (Fig. 5.1.) along the Great Stour and its tributary, the Little Stour/Nailbourne, during the summer of 1975.

Four types of media were used:

(a) Oxoid MacConkey agar No. 3 (CM115); this medium selects for Enterobacteriaceae and some Pseudomonads e.g. Pseudomonas and Aeromonas.

(b) CM115 supplemented with 20 µg/ml ampicillin, indicating the level of ampicillin resistance present in this population.

(c) CM115 supplemented with 0.4 µg/ml nalidixic acid. This medium eliminates the fermentative Pseudomonads, so allowing direct counting of the coliform group (Chapter 2).

(d) Nalidixic acid CM115 further supplemented with 20 µg/ml ampicillin, indicating resistance to this antibiotic within the coliform group.

Lactose-positive and lactose-negative colonies were counted after 18 hrs. incubation at 37°C.

Note:

Biological or biochemical oxygen demand (B.O.D.) is the number of units (p.p.m., mg.) of oxygen taken up per

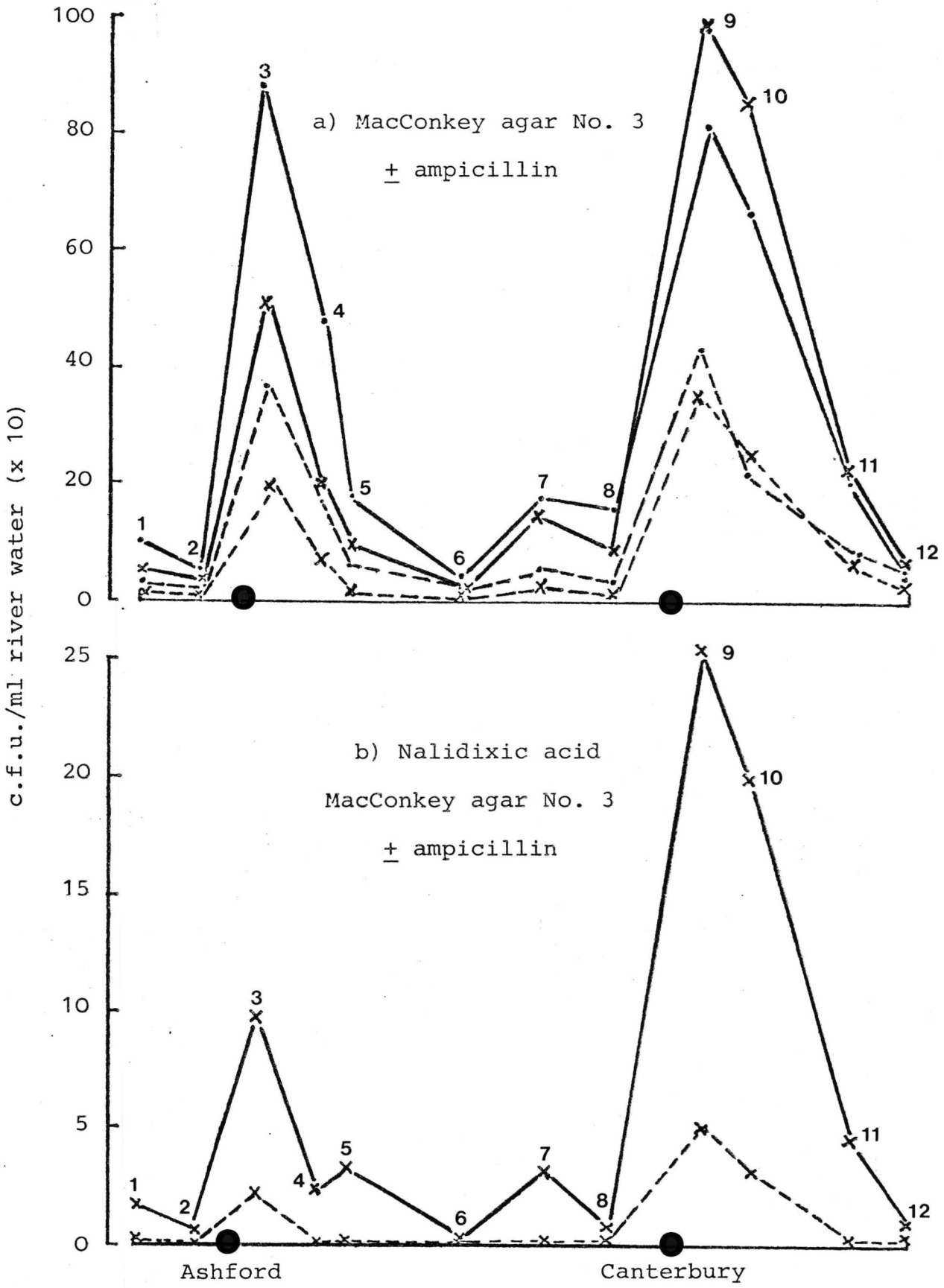
volume liquid sample after 5 days incubation at 20°C. It reflects the amount of oxidizable organic matter present. Oxygen is also taken up by oxidation of ammonia, Fe^{++}, sulphide and sulphite.

5.2.2. Results and discussion

Colony counts obtained from River Stour samples are shown in Figures 5.2.a and 5.2.b. The plots are similar, all six groups of organisms having the same distribution and showing that practically all the bacteria selected originate from effluent entering the river at Ashford and Canterbury. That background levels are very low is confirmed by the counts made on the Little Stour tributary, which passes only through rural land; no organisms were detected at the source and no ampicillin-resistant lactose-fermenters were found at any of the ten sampling points. The mean concentration of lactose-fermenters at these sites was only 13/ml (10 coliforms), the corresponding figure for non-lactose-fermenter being 150/ml. Resistance to ampicillin in the Great Stour isolates was found to be about 40 % among all lactose-fermenters, and about 20 % among coliforms.

In Fig. 5.3. the bacterial distributions depicted in Fig. 5.2.a are compared with data collected by the Kent River Board in its two surveys of the 1975 summer (April/May and August/September). The bacterial counts correspond closely with the chemical indicators, particularly B.O.D.

Fig. 5.2. Colony counts along the course of the River Stour



Course of the River Stour (1 cm. approx. 2 1/2 mile)

Fig. 5.2. Colony counts along the course of the River Stour

5.2.a* c.f.u/ml on MacConkey agar No. 3 ± 20 µg/ml ampicillin

x——— lactose-fermenters

x----- ampicillin resistant lactose-fermenters

•——— non-lactose-fermenters

•----- ampicillin-resistant non-lactose-fermenters

5.2.b* c.f.u/ml on Nalidixic acid MacConkey agar No.3 ± 20 µg/ml ampicillin

x——— coliforms

x----- ampicillin-resistant coliforms

Fig. 5.3. River Stour; biological and chemical analyses, 1975.

3.a,b,c,d ——— samples taken 19th August - 26th August

----- samples taken 22nd April - 1st May

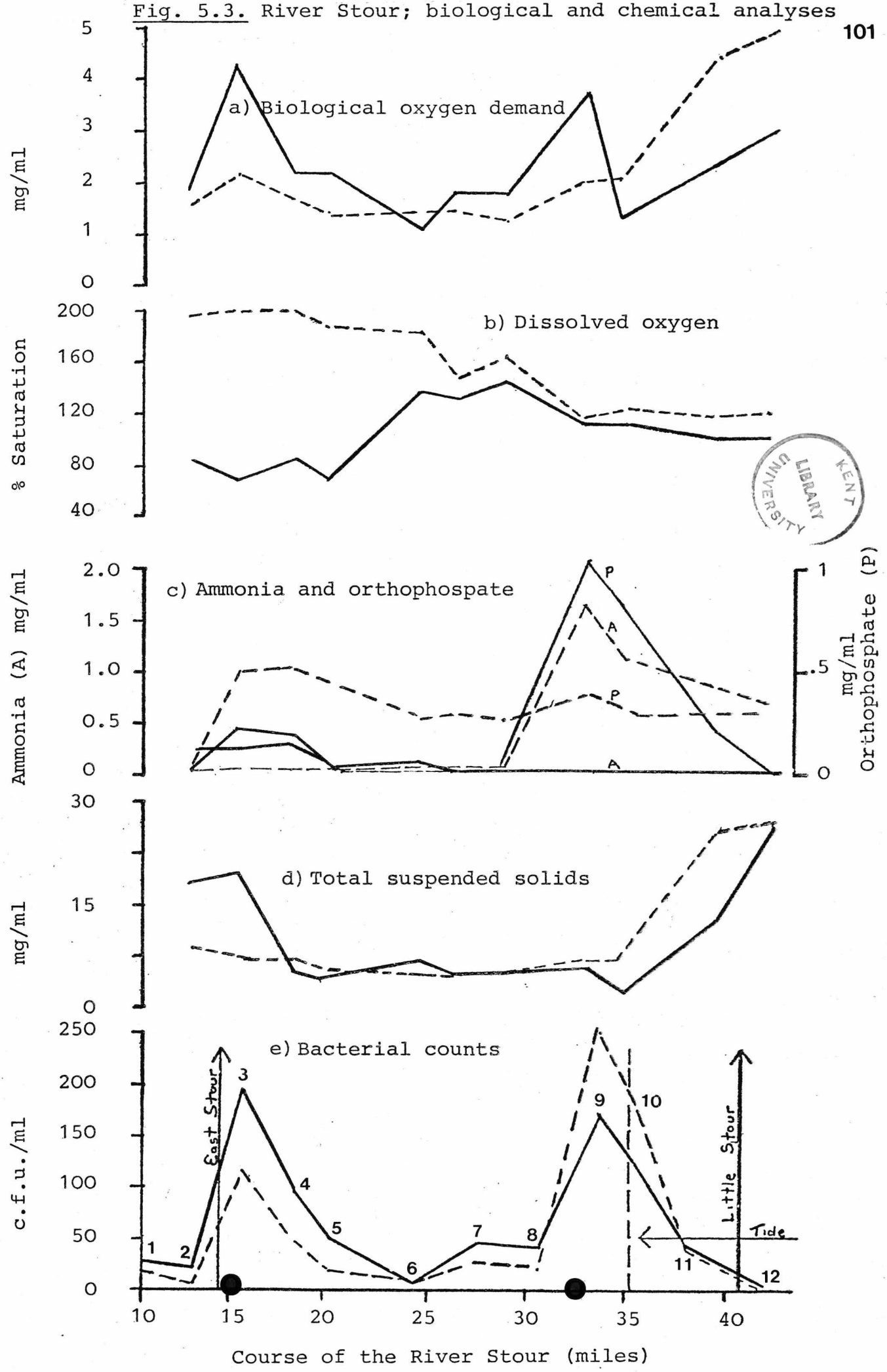
3.e* ----- lactose-fermenters (MacConkey agar No. 3)

——— non-lactose-fermenters (MacConkey agar No. 3)

Samples taken during the summer of 1975.

*** Numbers 1–12 indicate the sampling points shown on p.95.**

Fig. 5.3. River Stour; biological and chemical analyses



and phosphate and ammonia, although there is clearly variation between the two surveys made by the Board.

Although Ritter (1975) observed good correlation between counts of heterotrophic, total coliform and fecal coliform bacteria and determinations of B.O.D. and ammonia in the Neckar river (Germany), such agreement is not universal e.g. attempts by Lin and Evans (Illinois, U.S.A., 1974) to correlate bacterial counts with such chemical parameters proved unsuccessful. This is not surprising as the B.O.D. load industrial wastewaters possess may be either low (i.e. from chemical industry) or high (i.e. from slaughter houses, pulp and paper mills, canning companies) while not reflecting bacterial content. In the present study one observes, below Fordwich, a lack of agreement between counts and determinations of B.O.D. and suspended solid concentration. This may be due to removal of bacteria from the surface water or input of non-bacterial mining effluent into this stretch of the river.

The data illustrates the 'self-cleansing' ability of rivers which are not grossly polluted, as B.O.D., bacterial counts and phosphate and ammonia concentrations return to basal levels within a few miles of the entry points. This contrasts with the situation observed by Coleman et al. (1974), in the Saskatchewan river, Canada, where the effluent from the state capital of Edmonton altered the microbial composition to such an

extent that the effect was still discernable 300 miles downstream of the city. Clearly, a rapid recovery of water quality, as exemplified by the Stour, is desirable from a number of standpoints, not least that of public health.

5.3. EXAMINATION OF ANTIBIOTIC-RESISTANT COLIFORMS

5.3.1. Materials and Methods

(i) The level of ampicillin resistance was determined by colony counts on MacConkey agar No. 3 ± 0.4µg/ml nalidixic acid (Chapter 2.2.) supplemented with ampicillin. Samples were taken from downstream of the Canterbury sewage works (Chapter 2.2.).

(ii) Minimum inhibitory concentrations (M.I.C.) of ampicillin were determined as in Chapter 2.2.

(iii) Resistance spectra of coliforms isolated on ampicillin and chloramphenicol (both 20 µg/ml nalidixic acid agar) were demonstrated by use of multodisks (Chapter 2.2.).

(iv) Colicinogeny was detected as in Chapter 4.2. and the colicins identified by their action on mutants, checked by their response to standard colicins, shown in Table 5.2.

(v) Identification: 'typical' *E. coli* were differentiated from other coliforms by the indole and citrate tests (Cowan and Steel, 1965).

(vi) Self-transfer of resistance determinants was detected by overnight mating with *E. coli* K12 M560 (Chapter 6.2.).

(vii) Examination of plasmid DNA was performed by
(a) the rapid screening method (Fig. 3.1.), and
(b) electron microscopy, following dye-buoyant density centrifugation (Chapter 4.2., except that 500 ml cultures and 15 minute clearing spins were used).

TABLE 5.2.

Insensitivities of colicin indicators

Indicator	Insensitive* to colicins
CL142	-
P585	Q,B,D,G,H,M,I,S,V
M1200	B,I,V
M1199	E,K
CL145	E
CL146	K
M872	K
P295	B,D
P535	B,D,G,H,M
P1391	I
P625	I
P1205	M
P1235	M/Q,V
CL144	B,V (partially)

* Resistant or tolerant.

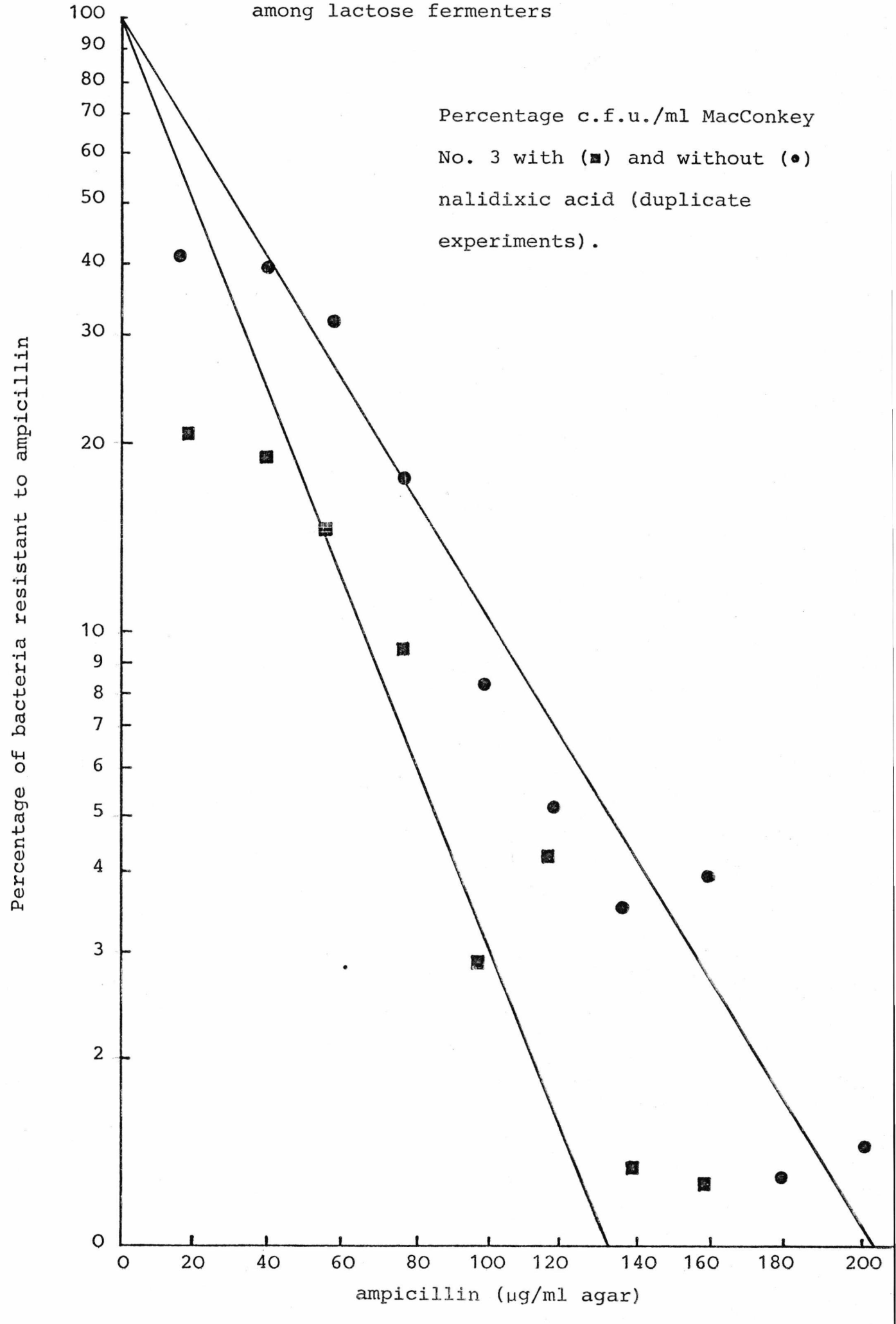
M1200, M1199; this laboratory (Chapter 4.2.). All others supplied by Dr. P. Reeves and described in Davies and Reeves (1975).

5.3.2. Results and discussion

Level of ampicillin resistance. Fig. 5.4. indicates the level of ampicillin resistance among the Enterobacteriaceae and Pseudomonadaceae and also among the coliform bacteria. Both groups display a continuum of resistance levels, approximately logarithmically distributed, up to 250 μg/ml. In the coliform group, this might result from a combination of factors: (i) possession of single amp genes determining different resistance levels, (ii) different levels of gene-expression in different hosts (Smith, 1969), or (iii) the presence of multiple amp genes in certain strains.

This possibility includes the carriage of amp genes on the chromosome, and also as multiple copies carried by a single plasmid 'species', either because of the presence of multiple copies of that plasmid (Nordström, Ingram and Lundbäck, 1972), or because of multiple transposition into a single plasmid molecule, although the latter may be a limited phenomenon, due to transposon immunity (Bennett, Robinson and Richmond, 1977). A more likely possibility, that carriage of multiple amp genes results from possession of more than one 'species' of R-plasmid (Romero and Meynell, 1968; Pinney and Smith, 1974), would be indicated if strains having high level ampicillin resistance possess a greater number of plasmid determined characters and more 'species' of plasmid DNA.

Fig. 5.4. Level of ampicillin resistance among lactose fermenters



Multiple resistance

To examine these possibilities further, and to investigate the incidence of multiple resistance, 50 coliform colonies were selected from both ampicillin and chloramphenicol selection plates. Earlier work had shown that these two antibiotics selected different frequencies of multiple resistance (Hughes and Meynell, 1974). Table 5.3. presents the various resistance spectra of these strains. Multiple resistance was clearly very common and, as observed earlier, the two antibiotic selections yield very different populations (Fig. 5.5.). While half the strains isolated on ampicillin carried no other resistances, no strains were isolated resistant only to chloramphenicol. This is clearly of importance as it dictates that chloramphenicol resistance is under selective pressure from a wide range of antibiotic usage, even if the drug itself is reserved for treatment of human infections, notably typhoid.

Association of high level ampicillin resistance with carriage of multiple plasmid determinants. The 100 strains were further examined for carriage of col and tra genes and their individual resistance to ampicillin was determined. The strains were then grouped according to their M.I.C. of ampicillin: ≤40 μg/ml, low resistance; 60 - 140 μg/ml, intermediate; ≥160 μg/ml, high; and the frequency of multiple resistance, colicinogeny and fertility was determined for each group (Table 5.4.). The re-

TABLE 5.3.

Resistance spectra of coliforms isolated on ampicillin or chloramphenicol

Spectrum	Percentage of isolates Chlor.	Amp.
X	0	48
XY	8	4
XS	0	8
XC	0	6
XT	6	4
XN	2	0
XYS	2	2
XYC	2	0
XYT	22	4
XSC	0	2
XCT	0	6
XYCT	4	2
XYST	26	8
XYTK	2	2
XYTN	2	0
XYSK	2	0
XYSTK	20	0
XYSCT	2	0
XYSCTK	0	2
XSCTKN	0	2

X selective agent

Y amp/chlor (other than X)

S streptomycin

C cephaloridine

T tetracycline

N nalidixic acid

K kanamycin

50 strains isolated on each drug. (i.e. 50 = 100 %)

Fig. 5.5. Incidence of multiple resistance

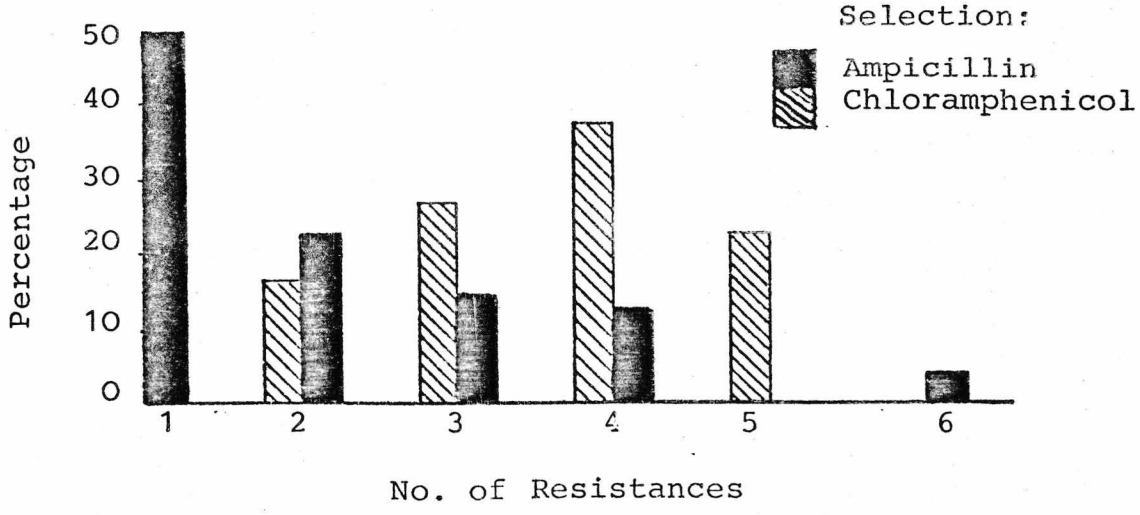


TABLE 5.4.

Percentage of strains exhibiting multiple drug resistance, colicinogeny and fertility

Phenotype	M.I.C. (ampicillin) Low (25[1])	Intermediate (40)	high (35)
col[2]	8	15	45
tra[3]	20	63	83
1-2 r-dets[4]	72	25	43
3-4 r-dets	30	60	37
5-6 r-dets	0	15	20

[1] (25); number of strains in this group

[2] colicinogeny; the percentage of colicinogenic strains in populations not selected by antibiotics was 13 % (100 colonies tested)

[3] transfer of resistance determinants

[4] resistance determinants

sults indicate an association between high ampicillin resistance and carriage of several plasmid determinants, suggesting that high ampicillin resistance may indeed reflect carriage of multiple amp genes. Identification of the genera showed that the relationships did not result from selecting different hosts (only 17 of the 100 strains were not typical E. coli and these were not outstanding in any of the characters studied).

The co-incidence of plasmid determinants in certain strains also means that those resistant to chloramphenicol, in addition to carrying more resistance determinants, proved to be tra$^+$ in a high proportion of cases, 76%, compared to 27% of strains resistant to ampicillin but not chloramphenicol.

Indentification of colicins. The indicator range of the colicins produced by 23 Col$^+$ strains showed that 10 were colicin I, 2 colicin E, 2 colicin V, 2 colicin B or D, and 7 colicin A or X or L. As would be expected, all the strains which were ColI$^+$ or ColV$^+$ were tra$^+$. Both the strains which produced either colicin B or D were tra$^+$, which therefore did not distinguish between them.

Examination of plasmid DNA. Examination of the plasmid DNA of 13 strains, by both rapid screening and electron microscopy, demonstrated the applicability of the rapid method to wild-type strains, although some difficulties

were encountered when (from electron microscopy) a strain was known to possess 3 plasmids.

The results revealed no clear relationship between the number of plasmid species and range of plasmid determinants carried. Only four strains possessed a single plasmid, two of these carrying 4 or 5 resistance determinants, tra and col genes. Results from other strains confirmed that col genes were sometimes linked to r-determinants, as observed with ColL (Chapter 4.3.); two strains with determinants for colicin (A/X/L) had two plasmids, both above 25 . 10^6 molecular weight, which is much larger than the independent Col factors determining these colicins ($\sim$ 5 . 10^6). Clearly simultaneous carriage of a number of plasmid species is common among coliforms and, studies on the physical association of plasmid determinants is confused by the presence of cryptic plasmids, carrying as yet no identifyable markers.

5.4. REFERENCES

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6. DISAPPEARANCE OF BACTERIA FROM POLLUTED SURFACE WATER

6. DISAPPEARANCE OF BACTERIA FROM POLLUTED SURFACE WATER

6.1. INTRODUCTION

The rapid decline in bacterial numbers observed downstream of the main effluent discharges may be due, at least in part, to drainage of unpolluted water into the river. Two additional factors which might be expected to produce such an effect are

a) bacterial death, and

b) deposition of bacteria from the surface water, aided by adherance to sewage particles.

Again of particular interest in this study is whether R-factor carriage affects the persistance of bacteria in river water.

6.1.1. Bacterial death

One of the main purposes of treating sewage and some industrial wastes is to reduce the bacterial load, and reports indicate that both biological filtration and activated sludge treatment eliminate 90 - 99 % of coliforms and pathogenic bacteria such as Salmonella (Kampelmacher and Van Noorle-Jansen, 1970; Smith, 1970).

One would expect the residual enterobacterial population to be susceptable to further injury by the inhospitable river environment, and indeed there has been much work devoted to improving the detection of injured indicator organisms by the use of special recovery media (e.g. Bissonette et al., 1976). In this study survival was monitored as the ability to form colonies on the select-

ive media described. This is not synonomous with irreversible loss of ability to reproduce which is often quoted as the criterion for bacterial death.

6.1.2. Deposition

Organic nutrients absorb to surfaces, and as many aquatic bacteria are adapted to grow on surfaces (Zobell, 1943; Jannasch and Pritchard, 1972), they are often found attached to solids in aquatic environments (Jannasch and Pritchard, 1972; Pearl, 1975). As mentioned earlier, the accepted concentration of suspended solids in sewage effluent destined for the River Stour is between 15 mg (winter) and 30 mg (summer) per litre, but the amount of suspended matter in the river downstream of discharge is normally considerably less (Fig. 5.3.). A proportion of the bacterial population entering the river is likely to be susceptible to rapid deposition in association with this organic matter.

6.2. MATERIALS AND METHODS

Bacterial death

(i) River strains

The strains tested were isolated from the Stour about 300 yd. downstream of the effluent discharge from Canterbury sewage works. They were grouped as either fecal *E. coli*, other coliforms, or non-coliform lactose ferment-

ers (presumptive Aeromonas), on the basis of the following biochemical tests (for references and details see Cowan and Steel, 1965):

Oxidase reaction

Oxidation or fermentation of glucose

Citrate utilization, assessed on Simmon's citrate agar (Oxoid CM155) and confirmed on Koser's citrate.

Acid and gas production from lactose peptone water at 44°C.

Methyl red and Voges-Proskauer tests.

Urease production

Detection of indole by Ehrlich's reagent.

Presence of lysine decarboxylase (Oxoid CM308)

Phenylalanine deamination (Oxoid CM277)

The antibiotic sensitivities of the strains were determined with impregnated discs (Chapter 2.2.), so that possible effects of R-plasmid - carriage could be detected.

(ii) Laboratory strains

To evaluate further the effect of R-factor carriage, such plasmids were transferred from river E. coli (S1, S2, S3; Table 6.2.) to the following E. coli K12 recipients, all of which are resistant to 50 µg/ml nalidixic acid: KH 439 (711), KH 569 (W3110), KH 548 (AB1157), M 560 (Arber's non-restricting mutant 803; Wood, 1966).

Mating procedure

Overnight broth cultures of the donors were diluted 1 in 10 in fresh broth, and incubated for a further 90 min. 5 ml of donor were then added to 1.5 ml of recipient overnight culture. The mixture was then incubated, with gentle shaking, for 25 - 35 minutes after which time it was diluted 100-fold in buffer (Chapter 2.2.), vortexed, and cooled to 4°C. Counts of progeny were made on blood agar base containing 50 µg/ml nalidixic acid and 20 µg/ml ampicillin. Counts of donor and recipient cells were made on ampicillin agar and nalidixic acid agar, respectively. Single colonies of the progeny were purified and tested to confirm that they had received the R-factor. Survival of such progeny (R^+) was then compared with that of the parental (R^-) strains.

(iii) Survival in river water

Overnight broth cultures of test organisms were diluted in river water (taken from the isolation point and filtered through 0.22 µm Millipore filters) to a final concentration of $9 \pm 3 \cdot 10^4$ colony forming units (C.F.U.)/ml. The final suspension (25 ml) was shaken in 50 ml flasks on a reciprocating shaker (100 strokes/minute), at 13 ± 0.5°C (the temperature of the water at time of isolation), for 70 hrs. 5 or 6 samples were taken throughout this time and plated, using the drop method of Miles and Misra (1938), on MacConkey Nº 3 agar.

Sedimentation of bacteria

(i) Estimation of particle size

Samples of river water were collected (Chapter 2.2.) and small volumes (<4ml) were then passed through the following Millipore filters:

BSWP02500 (2.5), SMWP02500 (6.2), SCWP02500 (9.4), LCWP02500 (12) and NCWP02500 (17), the figures in parentheses being the sizes of the largest rigid particles (μm) able to penetrate.

The filtrates were shaken and spread counts were made on the 4 media previously described (Chapter 5.2.1.).

(ii) Electron microscopy

Samples of river water were applied to grids and negatively stained with uranyl acetate, before examination with a transmission electron microscope.

Statistical analyses

The significance of the difference of the means was assessed using the Student's t test.

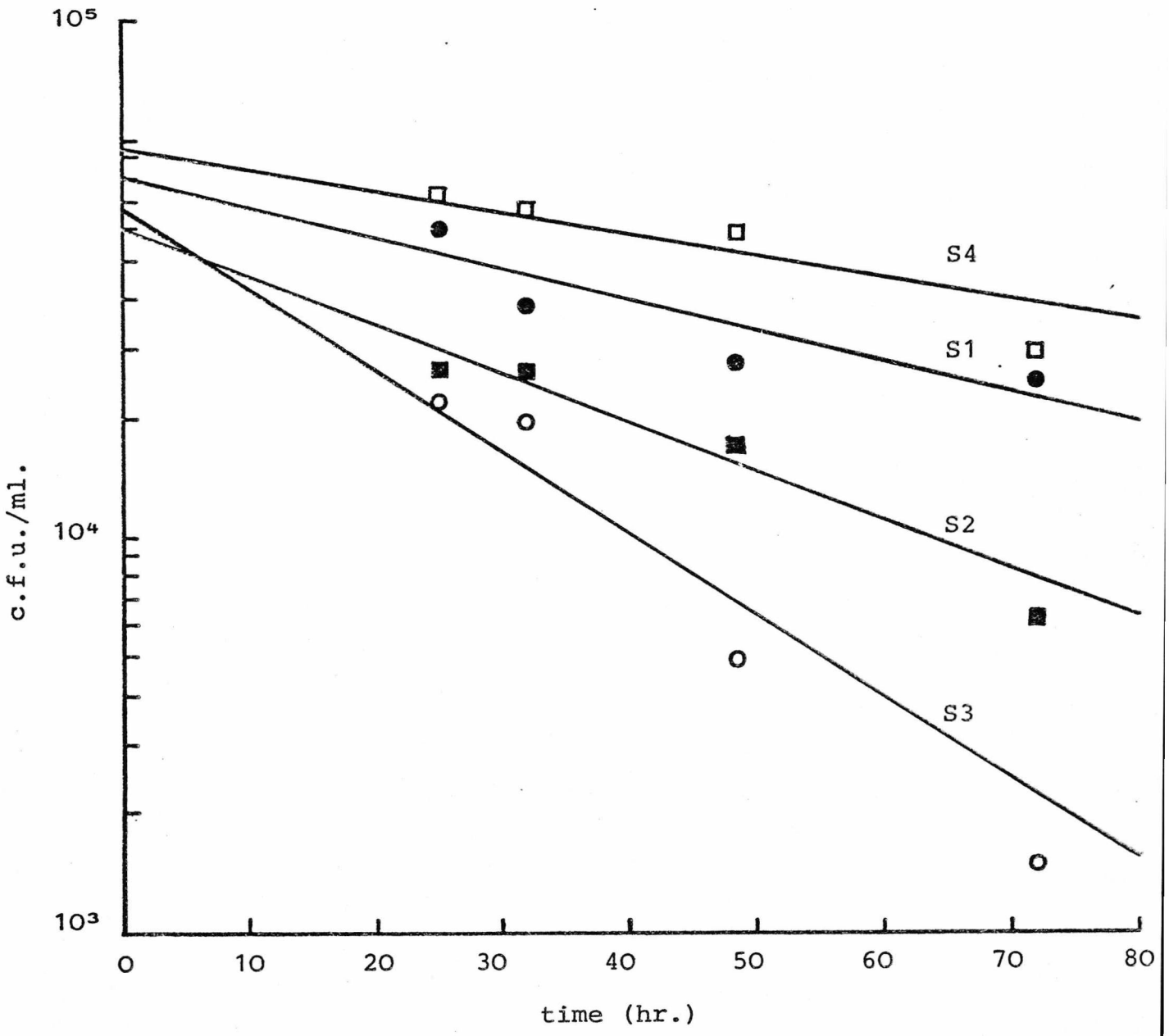
6.3. RESULTS

Survival of E. coli

Survival curves of some E. coli river strains in flasks of filtered river water are shown in Fig. 6.1. As with all other strains tested, the plots appear to show no lag effect and a constant rate of decrease, approximating to logarithmic decay. This agrees with observations made by McFeters et al. (well water; 1974) and Faust, Autaky and Hargadon (Rhode River Estuary, U.S.A.; 1975), and allows a more convenient expression of results, as half-lives, determined from survival curves.

Table 6.1. shows the antibiotic resistance spectra and half-lives of E. coli river strains, including those depicted in Fig. 6.1. There is considerable variation within the group and the mean 1/2 life (t1/2), 31 hrs., concurs with observations made by Geldreich et al. (storm water pollution; 1968), Vasconcelos and Swartz (sea water; 1976), McFeters et al. (well water; 1974), and Faust et al. (River estuary; 1975). In addition to these reports where cultures of natural isolates were reintroduced into aquatic environments, Davenport, Sparrow and Gordon (1976) measured the survival of coliforms by comparing the decay of bacterial numbers with the flow time and dilution of an ice-covered Alaskan river, downstream of a major sewage outfall. Although these results are also in agreement with those of the

Fig. 6.1. Survival of E. coli isolates in filtered river water



c.f.u./ml filtered river water of 4 river isolates. Duplicate samples were plated on MacConkey No. 3 incubated at 37°C. Points represent means of two experiments.

TABLE 6.1.

Survival of antibiotic-resistant and sensitive E. coli isolated from river water

Strain	Antibiotic resistance	half life (t 1/2 hrs.)
S1	C, Cr, P. S, T	40
S2	C, Cr, P, S	25
S3	C, P, S	14
S4	C, S, T	56
S5	C, P	20
S6	P, T	22
S7	P, T	21
S8	Cr, P, S, T	38
S9	C, Cr, P, S, T	16
S10	-	30
S11	-	38
S12	-	33
S13	-	25
S14	-	56

mean half life (t 1/2) of sensitive isolates = 36.4 hrs.

mean half life (t 1/2) of resistant isolates = 28.0 hrs.

mean half life (t 1/2) of all isolates = 31,0 hrs.

present study, the assumption made in the analyses, that all viable bacteria remain in the body of the river, is not supported by results obtained from the Stour (see later discussion).

The survival of the antibiotic resistant and sensitive subgroups (mean values of t1/2, 28 hrs. and 36.4 hrs., respectively) do not show a significant difference at the 5 % level, suggesting that the R-factor carriage does not apply further pressure against E. coli in the river environment. This agrees with Grabow et al. who showed that R^+ coliforms suspended, in dialysis bags, in the River Apies (S. Africa) exhibited similar survival rates to their R^- counterparts. The same conclusion was reached by Smith et al. who performed statistical analyses of R^+/R^- fecal coliform ratios in the sea water at increasing distance from a sewage outfall, arguing that a constant ratio indicated an equal ability to survive.

To test this further, R factors from E. coli river strains S1, S2 and S3 (Table 6.1.) were introduced into four laboratory strains of E. coli K12. The mating frequencies (progeny/donor x %) of the 12 crosses and also the half-lives of the progeny and R^- parents are shown in Table 6.2.

One notes that the mating frequencies are higher for transfer to the non-restricting mutant M560 than for transfer to the other strains having normal restriction

TABLE 6.2.

Mating of R^+ E. coli river isolates with R^- E. coli K12 strains

a) mating frequencies

b) survival of R^- and R^+ hosts

Host	R^+ Donor	Mating Frequencies	Half-life (t 1/2; hrs.)
KH439	-	-	19
	S1	0.6	16
	S2	2.4	19
	S3	2.2	12
KH569	-	-	27
	S1	0.2	19
	S2	1.8	4
	S3	1.1	25
M560	-	-	18
	S1	1.6	17
	S2	5.0	24
	S3	3.2	16
KH548	-	-	19
	S1	0.4	16
	S2	1.2	13
	S3	1.2	16

t 1/2 (R^-) = 20.8 hrs.

t 1/2 (R^+) = 16.4 hrs.

systems. However, the differences although significant (at the 2 % level) are not great, indicating that this system is not a major barrier to such transfer.
The half-lives of the R^- parents and R^+ progeny show firstly that E. coli K12 laboratory strains are less able to survive in river water than are E. coli isolated from the river (mean values (t 1/2 (K12)= 17.5 hrs., t 1/2 (E. coli isolates) = 31 hrs., difference significant at the 1 % level). Secondly although, as in the case of isolated E. coli, the survival times of R^+ organisms are less than their R^- counterparts, the difference is not significant at the 5 % level.

Survival of bacteria other than E. coli

The 1/2 lives of these organisms (Table 6.3.) show that while other river coliforms have similar survival times to E. coli isolates, the oxidase-positive lactose-fermenters (presumptive Aeromonas) show a greater ability to survive in this environment. The mean t 1/2 for the ox^+ group, 62 hrs., is twice that of the E. coli isolates (a difference significant at the 0.1 % level) and this is not surprising as these organisms are often saprophytic on soil and vegetation. Again, laboratory cultures appear to survive for a shorter time than recent natural isolates.

Particle size and deposition

The sizes of particles with which organisms were associated were estimated by measuring the percentage of organisms

TABLE 6.3.

Survival of organisms other than E. coli

(a) Oxidase-negative lactose fermenters

(presumptive coliforms)

Strain	Antibiotic resistances	t 1/2 (hrs.)
S15	P, T	64
S16	C, S, T	22
S17	P, S	42
S18	C, K, S	30
S19	P, T	25
S20	-	35
S21	C, P	28
S22	C, P, T	43

t 1/2 = 36 hrs.

(b) Oxidase-positive lactose fermenters

(presumptive Aeromonas)

Strain	Antibiotic resistances	t 1/2 (hrs.)
S23	P, T	>100
S24	C, S, T	55
S25	-	>100
S26	K, S, T	40
S27	P, T	44
S28	-	75

t 1/2 $\geq$ 62 hrs.

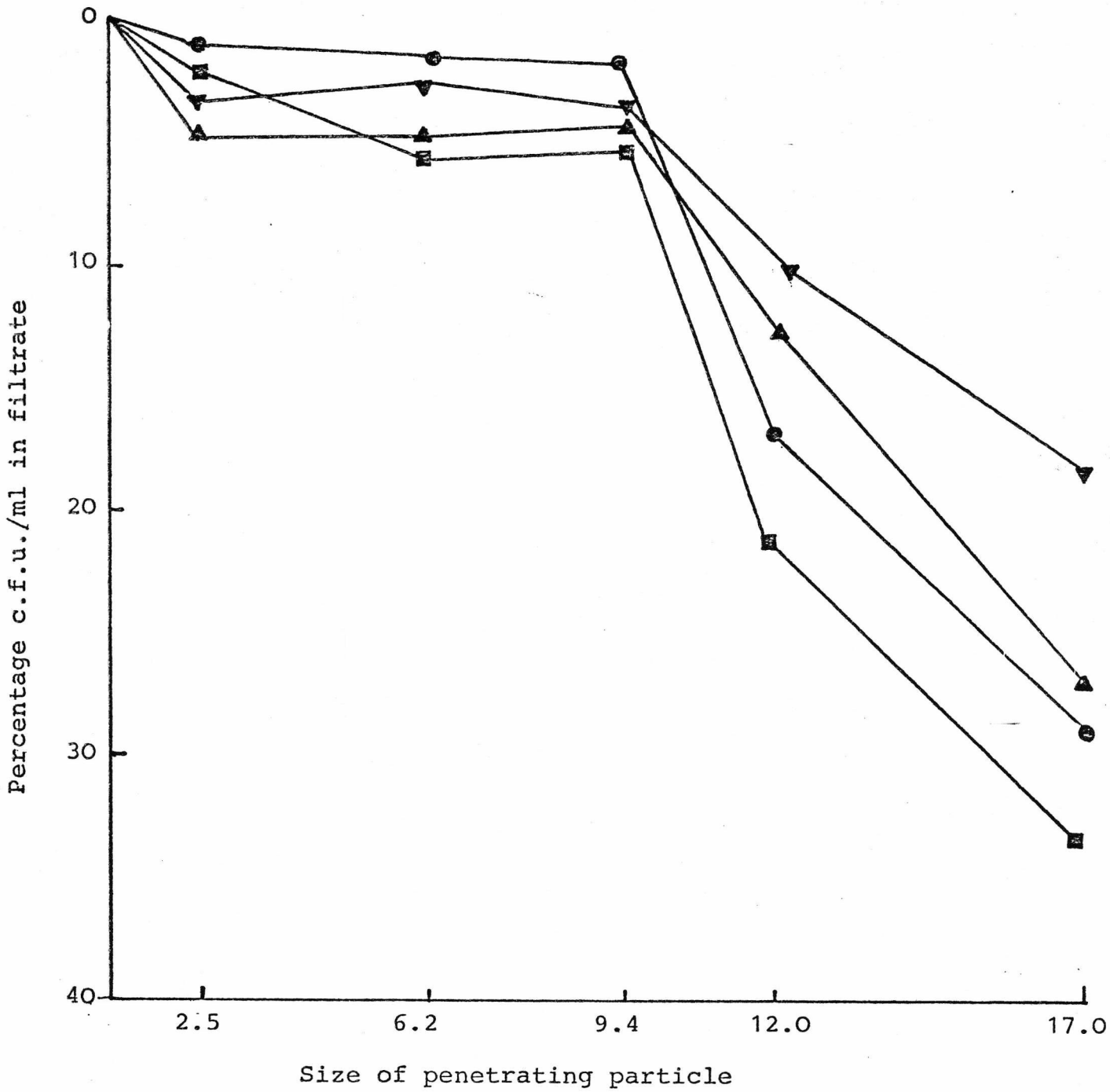
retained by various filters. The results (Fig. 6.2.) show that approximately three quarters of the organisms selected were retained by filters allowing passage of rigid particles 17 μm in diameter. Although bacterial pili might cause adherence to filter pore walls, the results indicate a significant amount of association. This agrees with Goulder (1977) who observed that in the rivers Tyne and Humber (U.K.), concentrations of attached bacteria were usually considerably greater than those of free bacteria, and that the percentage of attached bacteria appeared to rise with the ratio

$$\frac{\text{suspended solid concentration}}{\text{bacterial numbers}}$$

Additional evidence for aggregation of bacteria came from electron microscopy of river water which revealed that over 30 % of organisms sampled from below the Canterbury sewage works bore common pili, a factor which would be expected to aid association.

Deposition of bacteria was indicated by counts made on the surface of the river bed (Table 6.4.), which showed considerable numbers of bacteria to be present in the upper few centimeters.

Fig. 6.2. Estimation of particle size by filtration



c.f.u./ml river water after millipore filtration. Filtrates were vortexed and plated on MacConkey No. 3 (▼ lac^+; • lac^-) and nalidixic acid MacConkey No. 3 with (▲) and without (■) ampicillin. Points represent means of two samplings.

TABLE 6.4.

Estimates of bacterial numbers

in the surface of the river bed

Medium	c.f.u./gm. dry weight sediment	
	lac^+	lac^-
M3	27,000	53,000
M3Amp.	4,800	8,600
M3Nal.	8,600	13,000
M3Nal.Amp.	1,100	5,300

This agrees with Koditschek and Guyre (1974) who, investigating the bacterial population of the sewage disposal bed of the New York Bight (U.S.A.), found that while 'deep water' samples gave coliform counts of <0.1 - 0.5/ml, sediment samples contained between 50 and 60 coliforms per gram (wet weight). Similar results had been recorded earlier by Rittenberg, Mittver and Iuler (1958) with samples from Californian sewage outfalls, and by Van Donsel and Geldreich (U.S.A.; 1971) who, looking at a wider range of aquatic environments, isolated Salmonellae with far higher frequency from bottom sediments than from overlying water.

6.4. DISCUSSION

Bacterial death in aquatic environments is governed by a number of factors and, in different instances, individual factors will assume differing degrees of importance. Survival of coliform bacteria depends on interactions between, for example, concentrations of (and competition for) dissolved nutrients (Mitchell, 1968) and organic matter (Orlob, 1956), dissolved CO_2 (Gray, 1975), the presence of clays (Faust *et al.*, 1975), inhibition by heavy metals (Jones, 1963), and predation (Mitchell and Morris, 1969; Enzinger and Cooper, 1976). There also appears to be a constant relationship between temperature and survival, t 1/2 being extended at lower temperatures (Vasconcelos and Swartz, 1976) and survival therefore improving in winter months.

The results of this study however, suggest that bacterial death in the body of the river is not responsible for the rapid decline in population immediately downstream of effluent discharges. The survival times determined for river isolates are necessarily estimations of *in situ* rates of decline. In this case filtration of the river water removes particulate organic matter and possible predators (protozoa, lytic bacteria and phage). However, it seems from other reports that these determined rates are a reasonable reflection of survival in the river. Bacterial death is then argued against by compar-

isons of t 1/2 measurements with the river flow rate, and also by the equal rates of decline, along the river course, shown by the ox^- and ox^+ groups which have widely different t 1/2 values.

The results suggest that primary removal of bacteria from river water is due to deposition, aided by aggregation to particulate matter. Such aggregation is likely to by due not only to sewage flocculation and bacterial piliation, but also to charged clay particles and microbiol polysaccharides which are known to be of importance in soil association (Brock, 1966).

Enteric pathogens and indicators of fecal pollution have been shown to persist in bottom sediments that have received fecal contamination (Hendrick, 1971). Faust et al. (1975) noted that addition of Montmarillanite clay to river water extends the survival of enterobacteria in river water, and studies by Gerba and McLeod (1976) indicate a similar effect with the addition of sediment to sea water.

In certain polluted sediments selection for R^+ bacteria may also occur as the concentration of heavy metals is above the normal inhibitory level (Koditschek and Guyre, 1974). In the presence of such selection these authors note a further potential hazard when, as in the present study, considerable numbers of Pseudomonas and Aeromonas are present in the same environment.

Watanabe and coworkers (e.g. 1971) point out that certain species of these genera are both potential fish pathogens and carriers of R-factors. Taken with their greater survival ability (results) and the likelyhood that reintroduction of bacteria into the overlying water occurs at an undefined rate, they might conceivably propagate these plasmids along the food chain of acquatic environments.

Survival in sediments will depend on the factors mentioned earlier but the rate of predatory grazing probably attains major importance, as it does in sewage treatment. Fry and Staples (1976) concluded that although the lytic bacterium Bdellovibrio was found in both polluted water and sediment, it was unimportant in reducing coliform populations in these environments. This is supported by Enzinger and Cooper (1976) who found, while investigating an estuarine environment, that removal of E. coli was dependent on the presence of protozoal predators but not on that of lytic bacteria. Further work by Drift et al. (1977) showed such removal in wastewater treated with activated sludge to be a twostep process, rapid absorbtion of bacteria to sludge flocs being followed by their elimination.

6.5. REFERENCES

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7. THE EFFECT OF PLASMID CARRIAGE ON THE SERUM SENSITIVITY OF ENTEROBACTERIA

7. THE EFFECT OF PLASMID CARRIAGE ON THE SERUM SENSITIVITY OF ENTEROBACTERIA

7.1. INTRODUCTION

7.1.1. Normal serum is bactericidal for a wide range of both smooth and rough Gram-negative bacteria by virtue of a system involving antibody, the classical (Inoue *et al.*, 1968), or alternative (Frank, May and Kane, 1973; Traub and Kleber, 1976) pathway of complement activation and, possibly, other serum proteins (Donaldson, 1973). However, some smooth strains are insensitive to this system and there is evidence suggesting that serum resistance may contribute towards the pathogenicity of enterobacterial strains. For example, it has been repeatedly observed that strains isolated from cases of bacteraemia tend to be more serum-resistant than strains isolated from other sources (Roantree and Rantz, 1960; Vost and Randall, 1970; Simberkoff, Ricupero and Rahal, 1976), and bacteraemia caused by sensitive *Escherichia coli* strains is less often associated with shock and death than bacteraemia due to resistant strains (McCabe *et al.*, 1978). In urinary tract infections an increased incidence of serum-resistant strains is associated with both kidney involvement (Gower *et al.*, 1972) and the ability to produce symptomatic infection (Olling *et al.*, 1973). Experiments have shown that only serum-resistant strains of *E. coli* can consistently

produce endocarditis in rabbits (Durack and Beeson, 1977) and that serum-sensitive strains are able to cause kidney infection in rats only after the animals have been depleted of complement (Miller et al., 1978).

Although the nature of serum resistance has been extensively investigated, it's basis has still not been defined with any degree of certainty. Experiments with clinical isolates (Björksten et al., 1976; Olling, 1977; Taylor, 1976) and mutants (Nelson and Roantree, 1967; Taylor, 1976; Taylor and Parton, 1977) have not substantiated earlier suggestions that a full complement of lipopolysaccharide (LPS) O-side chains or the presence of large amounts of acidic polysaccharide K antigens are able to protect completely the bacterial cell from the bactericidal action of serum (Feingold; 1969; Glynn and Howard, 1970; Howard and Glynn, 1971 a; 1971 c). Furthermore, it seems likely that the resistance of Gram-negative bacteria to serum is not due to any one factor, but is determined by several distinct components at or near the cell surface (Taylor and Parton, 1977).

Recently, Reynard and Beck (1976) found that Escherichia coli K12 carrying the plasmids R1 and R100 (NR1) were more resistant to the bactericidal action of dilute rabbit serum than these strains without plasmids. As it has been reported that certain plasmids are able to modify the LPS (Derylo et al., 1975) and protein (Iyer, 1977; Iyer, Darby

and Holland, 1978) components of the cell envelopes of enterobacteria, investigation of the role of plasmid-determined factors is likely to lead to greater understandind of the nature of serum resistance determinants.

This chapter reports on the effect of plasmid carriage on the serum sensitivity of both laboratory and wild-type enterobacterial strains.

7.2. MATERIALS AND METHODS

Bacteria. Laboratory strains and their plasmids are shown, with their source, in Table 7.1. Many of the colicinogenic strains have been described in chapter 4 of this thesis.

The wild-type coliforms, described in Table 7.2., were isolated from the River Stour, Kent, England using Oxoid MacConkey agar CM115 containing 0,4 µg/ml nalidixic acid (this thesis, chapter 2) and supplemented with either chloramphenicol (strain prefix C) or ampicillin (prefix P) at 20 µg/ml to select for antibiotic resistance. These isolates, discussed earlier on pages 108 - 112, were then further examined for plasmid-determined characters:
(i) antibiotic resistance spectra were determined by flooding Oxoid DST agar with a diluted overnight culture and adding Oxoid Multodisks containing sulphonamide (500 µg), ampicillin (100 µg), cephaloradine (15 µg), chloramphenicol (10 µg), kanamycin (5 µg), streptomycin (10 µg) and tetracycline (10 µg). Minimum inhibitory con-

centrations (MIC) of ampicillin were assessed on Oxoid MacConkey agar CM115 containing 0 - 200 μg/ml of the antibiotic.

(ii) colicinogeny was detected by the overlay method (Meynell and Meynell, 1970) using the colicin sensitive indicator E. coli K12 C600. Allotment to group A or group B was determined by action on the mutant indicators tolII and tonB (this thesis, chapter 4).

(iii) the presence of a transfer factor was detected by successful transfer of drug resistance determinants, in overnight culture, to E. coli K12 M560, a Nal^R non restricting mutant described by Wood (1966).

Serum bactericidal assay. Normal human serum was obtained from healthy volunteers and stored in small aliquots at -20°C until required. Normal rabbit serum was also used in some experiments. Bacterial sensitivity to serum was estimated by the technique of Taylor, Roberts and Gower (1972). An early log phase Trypticase Soy Broth (TSB) culture was washed in 0.06 M NaCl and resuspended in 0.05 M Tris-HCl buffer pH 8.4 to a concentration of 10^6 organisms/ml.

A sample (0.5 ml) of this suspension was added to 1.5 ml of serum and viable counts obtained at the beginning of the test and after 1, 2 and 3 hrs. incubation at 37°C.

Laboratory strains were also examined in the system of Reynard and Beck (1976). Log phase cells were washed with 0.054 M NaCl and resuspended in 0.054 M NaCl to a concentration of 9 x 10^6 organisms/ml. A mixture contain-

ing 0.25 ml of this suspension, 0.25 ml of 0.24 M NaCl, 0.20 ml of serum, 0.50 ml of 0.05 M N, N-bis-(2-hydroxyethyl)-2-aminoethane sulphonic acid (BES) (pH 8.4) and 0.30 ml of 0.054 M NaCl was incubated at 37°C. Viable counts were prepared at 0 and 2 hrs.

Curing of plasmid determinants was attempted by overnight growth in TSB containing either a) acridine orange over a range of 5 µg to 200 µg/ml, pH 7.6 (Hirota, 1960), or b) 10 % sodium dodecyl sulphate, pH 7.4 (Tomoeda et al., 1968). Surviving cultures were streaked onto TSA and single colonies inoculated onto TSA and overlaid with indicator culture (to detect loss of colicinogeny) or onto TSA supplemented with ampicillin (to detect loss of R-determinants). That the plasmids were not retained in a non-functional form was indicated by the absence of superinfection inhibition when reintroducing markers from "lost" plasmids into the presumptive cured strain. No physical analyses were made of the DNA content of any strains used in this study. It is therefore not possible to comment upon the presence, or effect, of cryptic plasmids.

Matings. Spontaneous Nal^R mutants of the cured isolates were selected and then mated with prospective donors in overnight culture. Progeny were selected on appropriately supplemented media and colonies purified on TSA and checked for inheritance of other markers.

Statistical analyses. The significance of the difference of the means was assessed using the Wilcoxon sum of ranks (T) test (Wilcoxon, 1945).

7.3. RESULTS

(i) Laboratory strains. The effect of carriage of a range of drug resistance (R) and colicin (Col) factors on the sensitivity to normal human serum of 6 E. coli K12 strains was examined by the methods of Taylor et al. (1972) and Reynard and Beck (1976). With both methods, all K12 strains examined were rapidly killed by serum regardless of plasmid carriage (Table 7.1.).

There was also no evidence to suggest that carriage of prophage λ could confer any degree of serum resistance on these rough strains. In their original study, Reynard and Beck used normal rabbit serum so 15 of the 27 K12 strains listed in Table 7.1. were randomly selected and tested as indicated by these authors; in all cases less than 0.01 % of cells survived 2 hrs. exposure to rabbit serum.

In a separate series of experiments, a number of K12 strains were examined by the technique of Taylor et al. but using BES pH 8.4 as the suspending buffer; the results obtained were identical to those with Tris-HCl pH 8.4. In addition, a tenfold increase in inoculum size had no effect on the percentage survival of K12 strains in this system.

The effect of Col factor carriage by laboratory strains

TABLE 7.1.

Response to normal human serum of laboratory enterobacterial strains as determined by the techniques of Taylor et al. (method A) and Reynard and Beck (method B)

Strain	Host	Plasmids	% survival after Method A 1 hr.	Method A 2 hrs.	Method A 3 hrs.	Method B 2 hrs.
M 671	*E. coli* K12 C600	-	<0.1	<0.1	<0.1	0.06
M 672	"	λ	<0.1	<0.1	<0.1	0.03
M 975	"	R56	<0.1	<0.1	<0.1	0.19
M 976	"	R64	<0.1	0.3	<0.1	<0.01
M 977	"	R56, λ	<0.1	<0.1	<0.1	<0.01
M 978	"	R64, λ	<0.1	<0.1	<0.1	<0.01
M 786	*E. coli* K12 AB1157	-	<0.1	<0.1	<0.1	<0.01
M 830	"	λ	<0.1	<0.1	<0.1	0.04
M 866	"	ColE2-P9	<0.1	<0.1	<0.1	<0.01

Table 7.1. - continued

Strain	Host	Plasmids	% survival after Method A 1 hr.	Method A 2 hrs.	Method A 3 hrs.	Method B 2 hrs.
M1080	*E. coli* K12 J5-3	-	<0.1	<0.1	<0.1	<0.01
M1081	"	R56	<0.1	<0.1	<0.1	<0.01
M1082	"	R64	<0.1	<0.1	<0.1	<0.01
M 941	"	R-Utrecht; F; λ	<0.1	<0.1	<0.1	<0.01
SFI585	*E. coli* K12 J5	NR1	<0.1	<0.1	<0.1	<0.01
SFI587	"	R1	<0.1	<0.1	<0.1	<0.01
SFI609	"	R12	<0.1	<0.1	<0.1	<0.01
M1151	*E. coli* K12 W2637	-	<0.1	<0.1	<0.1	<0.01
M1157	"	R56	<0.1	<0.1	<0.1	<0.01

Table 7.1. - continued

Strain	Host	Plasmids	% survival after Method A 1 hr.	Method A 2 hrs.	Method A 3 hrs.	Method B 2 hrs.
KH462	E. coli K12 W3110	-	<0.1	<0.1	<0.1	<0.01
KH472	"	ColV-K94	<0.1	<0.1	<0.1	0.03
KH517	"	ColV-K94,R1drd19	<0.1	<0.1	0.1	<0.01
KH531	"	R1drd16	<0.1	<0.1	<0.1	0.05
KH573	"	ColE1-K30	<0.1	<0.1	<0.1	<0.01
KH679	"	R1drd16,ColE1-K30	<0.1	<0.1	<0.1	<0.01
M1246	"	ColE3	0.3	<0.1	<0.1	<0.01
M1231	E. coli CA53	ColIa	<0.1	<0.1	<0.1	<0.01
M1232	S.typhimurium ST4	ColIb-P9	45.9	44.8	0.7	7.7
M1235	E. coli K89	ColB, ColM	<0.1	<0.1	<0.1	0.14
M1240	E. coli CA7	ColV1	<0.1	<0.1	<0.1	0.02
M1244	E. coli CA42	ColE2	2.0	0.9	<0.1	3.97

Table 7.1. - continued

Strain	Host	Plasmids	% survival after Method A 1 hr.	2 hrs.	3 hrs.	Method B 2 hrs.
M1247	E. coli K216	ColK	<0.1	<0.1	<0.1	<0.01
M1251	C. freundii CA31	ColA	0.4	<0.1	<0.1	0.02
M1252	E. coli K53	ColE1	3.1	0.2	<0.1	0.09
M1241	E. coli CA46	ColG	158.0	208.0	45.8	75.3
M1245	E. coli 12-316	ColE2	<0.1	<0.1	<0.1	<0.01

Strains obtained from University of Kent (KH; Dr. K.G. Hardy: M; Prof. G.G. Meynell) and Sandoz Forschungsinstitut (SFI). Colicinogenic strains are discussed in chapter 4.

was also examined (Table 7.1.). All were extremely sensitive to human serum with the exception of Salmonella typhimurium ST4, carrying ColIb-P9, and E. coli CA46, carrying ColG. All attempts to cure CA46 were unsuccessful.

(ii) River strains; serum sensitivity. It was found that the river isolates in Table 7.2. could be assigned to one of three groups on the basis of their interaction with normal human serum in the system of Taylor et al. A number of strains were rapidly killed by serum with less than 10 %, and usually less than 1 %, of the inoculum surviving after 1 hr. incubation (promptly sensitive). Others were killed by serum but only after a lag of 1 hr. (delayed sensitive). The remainder grew in serum and were classed as serum resistant as previously defined (Taylor, 1974). An example of each type of response is shown in Fig. 7.1. All 17 strains listed in Table 7.2. carried antibiotic resistance determinants and in 8 cases these were shown to be self-transmissible. In addition, 8 of the isolates carried determinants for colicin production. Only 4 were resistant to human serum and 6 were promptly sensitive. There was no simple relationship between specific plasmid determinants and serum reactivity. However, serum resistant E. coli strains did possess both a larger number of plasmid determinants (antibiotic resistances, colicin production and self-transmissibility), and a higher M.I.C. for ampicillin, than the 11 serum sensitive E. coli.

TABLE 7.2.

Response to normal human serum of smooth enterobacterial strains isolated from river water

Strain	Host[a]	R spectrum[b]	Col[c]	tra[d]	Serum sensitivity
C 5	*E. coli* NT	C,S,Su,P (>200)	-	+	delayed sensitive
C 8	*E. coli* NT	C,P (>200)	A	-	delayed sensitive
C11	*E. coli* NT	C,K,S,Su,T,P (>200)	B	+	resistant
C12	*E. coli* O9:K(A)	C,S,Su,T,P (60)	-	-	delayed sensitive
C14	*Citrobacter sp*	C,Cr,S,Su,T,P (200)	-	-	promptly sensitive
C15	*E. coli* NT	C,K,S,Su,T,P (100)	-	+	delayed sensitive
C22	*E. coli* NT	C,Su,T,P (60)	-	-	delayed sensitive
C25	*E. coli* NT	C,K,S,Su,P (>200)	B	+	resistant
C37	*E. coli* O9:K(A)	C,Su,T,P (60)	-	+	delayed sensitive
C40	*E. coli* NT	C,Su,T,P (60)	A	-	promptly sensitive
C42	*E. coli* NT	C,S,Su,T,P (100)	A	+	resistant
P 5	*E. coli* NT	C,Su,P (40)	A	-	promptly sensitive
P11	*Klebsiella sp*	P (>200)	B	-	promptly sensitive
P21	*E. coli* NT	C,K,Su,T,P (100)	-	+	promptly sensitive
P35	*E. coli* NT	Cr,P 200)	A	-	resistant
P37	*E. coli* NT	C,S,Su,P (20)	-	-	delayed sensitive
P43	*E. coli* NT	P (180)	-	+	promptly sensitive

Legend to Table 7.2.

a Identified according to Cowan and Steel (1965) and serotyped by Dr. A.P. Roberts, London. NT indicates lack of agglutination of heated (100°C; 1 hr.) cells in O1, O2, O4, O6, O7, O9, O11, O18, O39 and O75 antisera.

b Resistance to antibacterial agents determined with impregnated disks C, chloramphenicol; Cr, cephaloridine; K, kanamycin; S, streptomycin; Su, sulphonamide; T, tetracycline; P ( ), ampicillin (minimum inhibitory concentration μg/ml).

c Colicin detected and grouped according to action against indicator strains (this thesis, chapter 4).

d Ability to transfer antibiotic resistance determinants to non-restricting recipient *E. coli* K12 M560.

Fig. 7.1. Response of river isolates to human serum

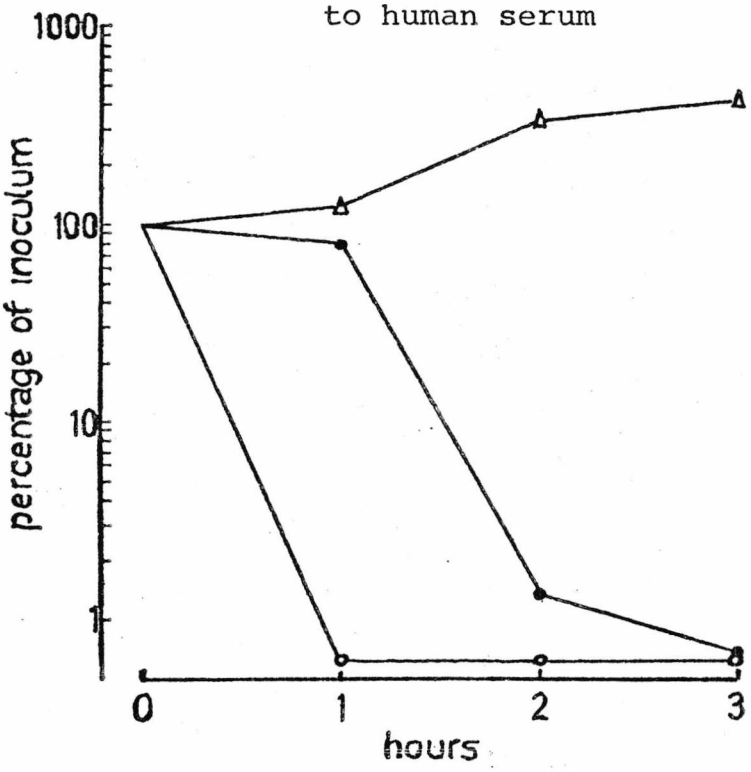
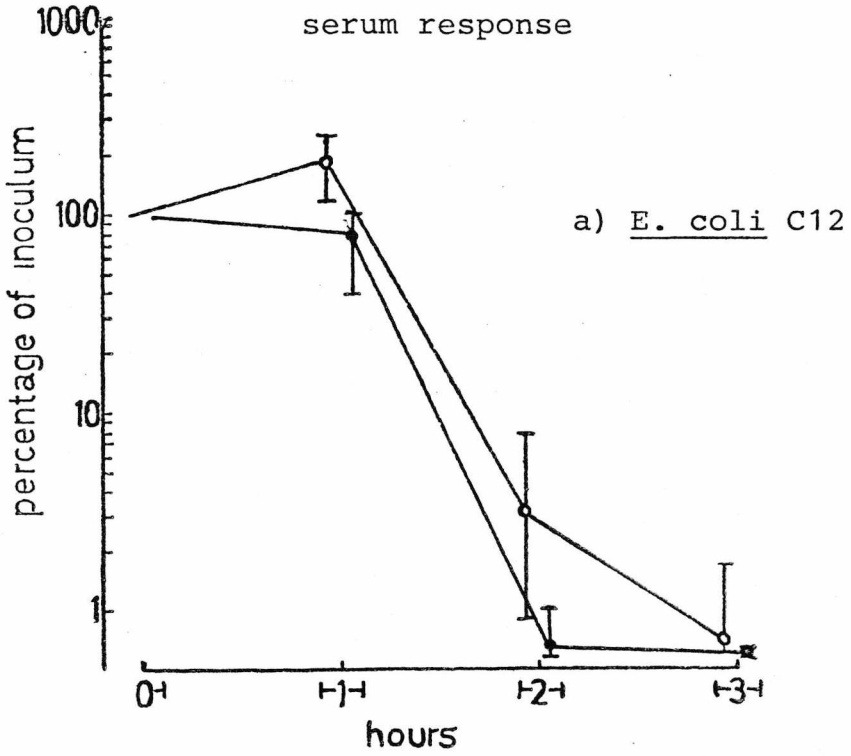
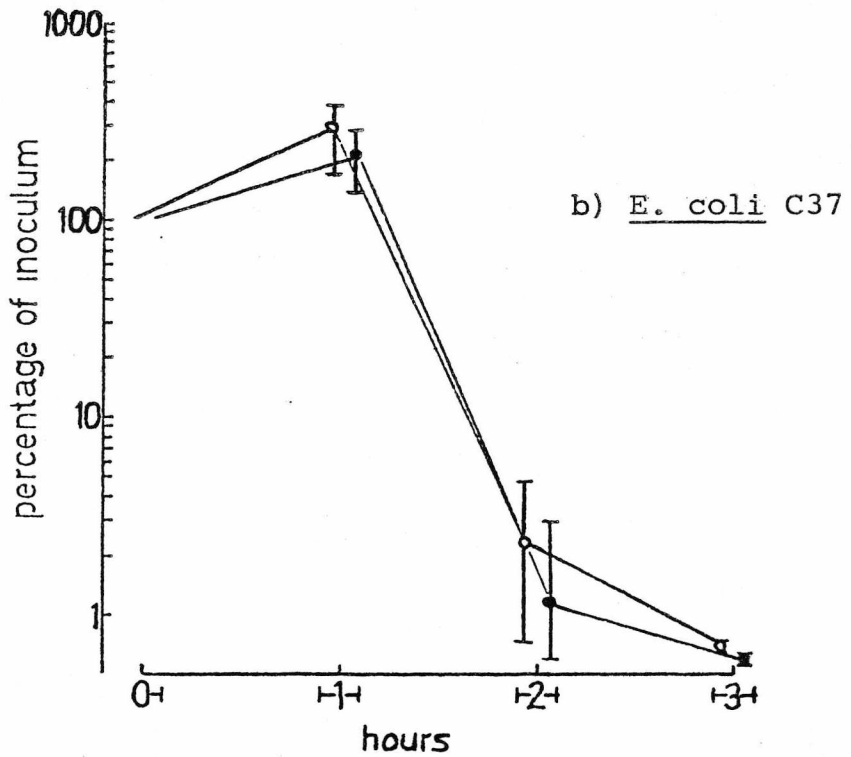


Fig. 7.1. Examples of promptly serum sensitive, delayed serum sensitive and serum resistant enterobacterial strains. Serum bactericidal assays were performed according to Taylor et al. (1972). Symbols: Citrobacter C14 (o); E. coli C5 (•); E. coli C42 (Δ).

Fig. 7.2. The response of $K(A)^+$ (o) and $K(A)^-$ (•) forms of E. coli C12 (a) and E. coli C37 (b) to normal human serum, determined according to Taylor et al. (1972). The means and range of results from 5 determinations are presented for each strain. The difference between E. coli C12 $K(A)^+$ and $K(A)^-$ forms was significant (1h, T = 15, p = 0.01; 2h, T = 17, p = 0.05 ; 3h, invalid); the difference between E. coli C37 forms was not (1h, T = 30, p >0.05; 2h, T = 29, p >0.05; 3h, invalid).

Fig. 7.2. Effect of K(A) antigen loss on serum response





Effect of K antigen loss

Two of the strains were found to belong to E. coli serogroup O9, an O-type associated with R-plasmids (Hartley et al., 1975) and with the production of the A type of K antigen (Ørskov et al., 1977). Both strains were of the delayed serum sensitive type and both reacted with O9 antiserum but only after they had been autoclaved at 121°C for 2 hrs. indicating the presence of a K(A) antigen. Cultures of both E. coli C37 and C12 segregated small numbers of colonies that had lost the ability to produce the K(A) antigen; these variants reacted in O9 antiserum after heating at 100°C for 1 hr. As it has been suggested that the serum resistance of smooth E. coli strains is determined by the presence of large amounts of K antigen (Glynn and Howard, 1970), the reactivity in human serum of the $K(A)^-$ variants was compared to that of the $K(A)^+$ parents (Figs. 7.2.a and 7.2.b). Although both the $K(A)^+$ and $K(A)^-$ forms of E. coli C12 displayed the delayed sensitive response (Fig. 7.2.a), the variant was significantly more serum sensitive than the parent. No significant differences were found, however, between the $K(A)^+$ and $K(A)^-$ forms of E. coli C37, although the means obtained with the variant at 1, 2 and 3 hrs. were slightly lower than those obtained with the parent (Fig. 7.2.b).

Effect of plasmid loss

Attempts were made to cure all strains listed in Table 7.2. of R and Col determinants. Strains C8 and P21 were cured with acridine orange and strains C5 and C42 with sodium dodecyl sulphate.

Loss of R determinants by the promptly sensitive strain P21 was not accompanied by any change in serum reactivity.

Colonies derived from strain C5 and cured of antibiotic resistance determinants gave serum responses identical to that of the parent strain.

E. coli $C42^-$, a cured derivative of C42 that had lost all antibiotic resistance determinants, was resistant to serum, but $C42^-$ cells increased in number less rapidly than did C42 parents when incubated in serum.

The serum sensitivities of 9 colonies derived from E. coli C8, cured of R determinants with 40 μg/ml acridine orange, were measured. All showed the delayed serum killing response but were slightly more serum sensitive than the parent strain. The response to serum of clones that had been treated with acridine orange but not cured of plasmid determinants was identical to that of E. coli C8. One cured colonial form ($C8^-$) was selected for statistical evaluation and a spontaneous Nal^R mutant obtained. The serum responses of $C8^-$ and $C8^-Nal^R$ were identical. Replicate experiments confirmed that the loss of plasmid determinants from E. coli C8 led to a small increase in serum sensitivity (Table 7.3.).

Introduction of R factors into cured river isolates

In order to assess the extent to which plasmids could modify the response of $C8^{-}Nal^{R}$ to human serum, this strain was mated with 5 river and 4 laboratory strains and progeny inheriting appropriate antibiotic resistance markers were selected (Table 7.3.).

Inheritance by $C8^{-}Nal^{R}$ of R-Utrecht from E. coli M941 and of the R and Col determinants from serum resistant E. coli C25 resulted in no significant change in serum response. Progeny from the remaining matings were all significantly more serum resistant than $C8^{-}Nal^{R}$. Inheritance of markers from E. coli C11, C15, C42 and P43 led to comparable, small increases in the serum resistance of progeny, although one of the donor strains was promptly sensitive, one was delayed sensitive and two were serum resistant.

Although plasmids R1 and NR1 were unable to influence the serum sensitivity of K12 strains (Table 7.1.), $C8^{-}Nal^{R}$ progeny inheriting these plasmids were considerably more resistant than the R^{-} parent (Table 7.3.). The level of serum resistance attained by $C8^{-}Nal^{R}$ NR1 (23 % survival after 3 hrs.) prompted one to introduce R12, a round of replication mutant of NR1. Progeny carrying this copy mutant, confirmed by MICs of chloramphenicol, showed a significantly greater increase in serum resistance than progeny carrying the normal NR1 plasmid ($n = 6$; $T = 21$, $p < 0.01$ at 1, 2 and 3 hrs.).

TABLE 7.3.

Effect of plasmid receipt on the response of cured E. coli isolates to normal human serum

Host	Donor	Inherited Markers	% Survival[a] in serum after 1 hr.	2 hrs.	3 hrs.
C8	-	-	162.0 ± 69.5 *T = 21; p > 0.05*	23.6 ± 7.0 *T = 15; p = 0.01*	6.6 ± 4.3 *T = 15; p = 0.01*
C8$^-$Nal^R	-	-	107.6 ± 36.9 -	3.7 ± 2.7 -	0.9 ± 0.8 -
C8$^-$Nal^R	C11	C,P,S,col.	187.5 ± 97.2 *T = 32; p > 0.05*	40.8 ± 38.7 *T = 22; p < 0.01*	13.0 ± 18.1 *T = 25; p < 0.05*
C8$^-$Nal^R	C15	C,K,P,T,S	168.5 ± 30.7 *T = 30; p > 0.05*	46.0 ± 18.2 *T = 21; p < 0.01*	18.5 ± 7.6 *T = 21; p < 0.01*
C8$^-$Nal^R	C25	C,P,S,col.	119.6 ± 38.5 *T = 36; p > 0.05*	7.0 ± 3.5 *T = 35; p > 0.05*	1.7 ± 1.2 *T = 28; p > 0.05*
C8$^-$Nal^R	C42	P,S,col.	127.3 ± 36.4 *T = 21; p < 0.01*	6.8 ± 1.2 *T = 21; p < 0.01*	1.4 ± 0.3 *T = 21; p < 0.01*
C8$^-$Nal^R	P43	P	136.3 ± 31.4 *T = 21; p < 0.01*	13.1 ± 6.0 *T = 21; p < 0.01*	3.1 ± 1.3 *T = 21; p < 0.01*
C8$^-$Nal^R	M941 R-Utrecht	P,T	104.8 ± 36.7 *T = 27; p > 0.05*	2.0 ± 0.9 *T = 36.5; p > 0.05*	0.3 ± 0.2 *T = 34; p > 0.05*

Table 7.3. - continued

Host	Donor	Inherited Markers	% Survival[a] in serum after 1 hr.	2 hrs.	3 hrs.
$C8^{-}Nal^{R}$	SFI585 (NRI)	C,S,Su,T	208.0 ± 30.6 *T = 21; p < 0.01*	47.5 ± 17.0 *T = 21; p < 0.01*	23.3 ± 7.8 *T = 21; p < 0.01*
$C8^{-}Nal^{R}$	SFI587 (RI)	C,K,P,S,Su	153.3 ± 23.4 *T = 21; p < 0.01*	83.2 ± 12.1 *T = 21; p < 0.01*	73.2 ± 21.4 *T = 21; p < 0.01*
$C8^{-}Nal^{R}$	SFI609 (NRI r.o.r.)	C,S,Su,T	276.7 ± 18.3 *T = 21; p < 0.01*	101.2 ± 12.0 *T = 21; p< 0.01*	80.7 ± 10.9 *T " 21; p < 0.01*
P21	-	-	<0.1	<0.1	<0.1
$P21^{-}Nal^{R}$	-	-	<0.1	<0.1	<0.1
$P21^{-}Nal^{R}$	SFI585	C,S,Su,T	<0.1	<0.1	<0.1
$P21^{-}Nal^{R}$	SFI587	C,K,P,S,Su	<0.1	<0.1	<0.1

Legend to Table 7.3.

[a] Estimated according to Taylor et al. (1972). The value given is the mean ± SD of 6 estimations except in the case of C8 (5 estimations) and C8$^-$Nal^R (18 estimations). Each replicate of a strain was prepared on a separate occasion and the appropriate Nal^R cured host included with each set of tests. T and p values are comparisons of replicates of the test strain and equal number of replicates of the Nal^R cured host prepared on the same occasions.

r.o.r.: round of replication mutant

Inheritance of plasmids R1 and NR1 by the cured derivative of promptly sensitive P21 ($P21^{-}Nal^{R}$) had no effect on the serum sensitivity of this strain.

7.4. DISCUSSION

Reynard and Beck (1976) have reported that the plasmids R1 and R100 (NR1) confer high levels of serum resistance on *E. coli* K12 strains. The present work does not confirm this observation; all the K12 strains examined (Table 7.1.), including strains carrying R1, NR1 and other plasmids, were extremely sensitive to normal human and rabbit sera in the serum bactericidal systems of both Reynard and Beck and Taylor *et al.* Fietta, Romero and Siccardi (1977) have also recently found that the R1 plasmid is unable to confer any degree of serum resistance on K12 strains in a system employing highly diluted human serum. These authors did report, however, that 8 out of 26 plasmids examined conferred relative serum resistance to *E. coli* K12 strains. A plasmid was said to have conferred relative resistance if the amount of serum needed to reduce the viable count to 1 % after 30 minutes was greater for R^{+} progeny than for R^{-} parents. The amounts of serum needed to effect this degree of serum killing were extremely small (< 3 % of the total reaction mixture). As many strains are able to grow rapidly in virtually undiluted serum (Taylor, 1974; and this study), one feels there can be little justification in the use of the term "serum resistant" in a situation

where only 1 % of cells survive short exposure to highly diluted serum.

E. coli K12 is morphologically rough due to a lesion in the chromosomal rfb locus determining synthesis of LPS O-side chains (Schmidt, 1973). Although a full complement of O-side chains does not directly determine serum resistance, it appears to be essential for its expression and to be responsible for the delayed killing effect (Taylor, 1975); it therefore seems unlikely that plasmid-determined surface modifications could, in the absence of O-side chains, lead to significant levels of serum resistance. Direct support for this view is provided in the present study. Plasmids R1 and NR1 had no effect on the serum sensitivity of E. coli K12 or of E. coli P21^{-}Nal^{R}, strains promptly killed by serum. However, when either of these plasmids was introduced into C8^{-}Nal^{R}, a cured strain derived from E. coli C8 and showing the delayed serum killing effect, the progeny became significantly less sensitive to the serum bactericidal system. Resistance was particularly marked with plasmid R1, over 70 % of cells surviving 3 hrs. exposure to serum, in contrast to a survival rate of under 1 % for the cured host strain. A round of replication mutant of plasmid NR1 conferred much higher levels of serum resistance on C8^{-}Nal^{R} than the normal NR1, suggesting that the plasmid effect might be related to the number of copies per cell. Acquisition of plasmids by C8^{-}Nal^{R} from 4 of the 6 other donors led to significantly enhanced survival in serum, suggesting

that the ability to modify serum responses in smooth hosts might be a common characteristic of plasmids. In addition, loss by curing of R and Col determinants carried by E. coli C8 led to an increase in serum sensitivity.

It is likely that complete serum resistance has a multifactorial basis. Some plasmids are clearly able to confer a degree of resistance on suitable host bacteria that synthesise LPS with a high degree of core substitution by O-side chains. However, plasmids from resistant strains C25 and C42 had, respectively, no effect and a small but significant effect on the survival of $C8^{-}Nal^{R}$ in serum. The ability of a plasmid to modify the serum response of $C8^{-}Nal^{R}$ was not, therefore, a reflection of the properties of the original host strain. Loss of the K(A) antigen by delayed sensitive strain C12 led to a small increase in sensitivity; this antigen is therefore able to influence the outcome of the cell: serum interaction but obviously does not afford complete protection.

Three of the 4 smooth, serum resistant river isolates were resistant to at least 5 antimicrobial agents and all carried determinants for transfer and colicinogeny. The remaining serum sensitive strains did not exhibit so many extrachromosomally determined characters, suggesting that some strains may be particularly suited for a progressive accumulation of a wide range of determinants enabling them to achieve a high selective advantage in potentially hostile environments.

7.5. REFERENCES

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8. CONCLUSIONS

The nalidixic acid medium and the rapid screening method, described in this thesis, improved greatly on the techniques formerly available for the isolation and identification of enterobacteria and their plasmids.

Supplementation of the Oxoid medium, MacConkey agar No. 3, with 0.4 µg/ml nalidixic acid eliminated all oxidase-positive lactose-fermenting organisms but did not inhibit the growth of enterobacteria. It therefore allowed direct counting and easy isolation of coliforms present in polluted river water. Although it is likely that the supplemented medium could be of benefit in routine public health surveys, it is more probable that it will be used in research studies. Previous investigations into the incidence of antibiotic resistance among coliforms found in sewage and river water have indentified these organisms primarily on the basis of their colonial morphology, ability to ferment lactose on MacConkey agar and Gram reaction (Smith, 1970; 1971; Hughes and Meynell, 1974; Linton *et al.*, 1974). The data presented in chapter two suggest that a proportion of the organisms examined may not have been coliforms but pseudomonads such as *Aeromonas*. The use of the nalidixic acid medium eliminates this possible source of error and is also more economical than the use of presumptive media and biochemical tests (Cooke, 1976).

The detection of plasmids carried by Gram-negative isolates was simplified by the development of a method wich allowed direct observation of plasmid DNA following rate-zonal

centrifugation of cleared cell-lysates through neutral sucrose gradients containing 4 μg/ml ethidium bromide. This concentration was used because it provided, under ultra-violet illumination, the maximum visual contrast between free dye and the dye which was complexed with DNA. Optimal cell lysis was achieved by the use of SDS at 60°C, the high temperature having the additional effect of producing single strand breaks in the supercoiled DNA. The main advantage of this nicking was that each plasmid species was present in the cell lysate as a homogenous population of relaxed molecules. Following centrifugation of the cleared lysate through the gradient each plasmid could then be seen in situ as a single band of DNA-ethidium bromide complexes, a result which made the technique especially useful for the examination of natural isolates carrying an unknown number of plasmids. Subsequent measurement of the migration rate of the observed bands in the gradient afforded an immediate estimate of the molecular weight of the plasmid species present.

The efficacy of this method for rapid demonstration of plasmid DNA was confirmed by its use, in parallel with electron microscopy, in examining plasmid DNA harboured by standard colicinogenic strains. This investigation provided data relating to the evolution of Col factors.

An earlier study on a small number of these plasmids had indicated that they fall into two groups, I and II, determined by various criteria (p. 82), the most obvious being size. Group I Col factors have a molecular weight

of about 5 . 10^6 whereas those of group II are over 40 . 10^6. Subsequent work suggested that the plasmids belonging to these two groups determine colicin proteins which, on the basis of their action on colicin-insensitive mutants, can be assigned to two corresponding, mutually exclusive groups, A and B.

In chapter four, data presented on a wide range of Col factors confirmed and extended these earlier investigations and thus strengthened the hypothesis that these plasmids may have evolved from two ancestors of low and high molecular weight. Some observations did however indicate departures from this evolutionary path. Col factors D and G, while determining colicins of group B, were found to be the same size as the plasmids of group I. In view of earlier evidence that the colicins determined by these plasmids were very similar to those specified by the Col factors B and H of group II, it was proposed that D and G had arisen from B and H by massive deletions, the small plasmids retaining the genes determining colicin synthesis but losing others including those coding for transferability.

A further deviation from the main findings appeared to be the size of Col factor L, a plasmid expected to have a molecular weight of ca. 5 . 10^6 but observed to be approximately 20 . 10^6. Examination of the strain producing colicin L revealed that it was also resistant to several antibiotics, including a low level of streptomycin typical of plasmid-encoded resistance. It was therefore suggested that the size of Col L reflected an association of this

group I plasmid with a number of antibiotic resistance determinants.

The data presented form a firm basis for further study which could determine the precise degree of homology existing between the various Col plasmids and thereby provide a concise example of molecular evolution.

The rapid screening method clearly simplifies examination of well-documented plasmids mediating colicinogeny and antibiotic resistance. In addition, it should encourage future work on plasmids lacking such easily-identifyable characteristics. The study of these plasmids has, in the past, been severely hindered by both the lack of readily-selectable markers and the previous absence of a method suitable for examining large numbers of strains for the presence of plasmid DNA. The concurrent development of rapid screening methods (this thesis and Meyers et al., 1977) and techniques allowing the selection of strains carrying only the desired 'markerless' plasmid (Kretschmer, Chong and Cohen, 1975; So, Heffron and Falkow, 1978) alleviate these problems and facilitate study of these plasmids, many of which determine pathogenic characters such as enterotoxigenicity and adhesion ability.

Examination of antibiotic-resistant coliforms isolated from the River Stour confirmed that both the selective medium and the rapid screening method are suitable for the examination of wild-type enterobacteria. Viable counts obtained from samples taken at various points along the Stour showed that virtually all the enterobacteria and pseudomonads present in the river entered at the sewage outfalls of the

towns Ashford and Canterbury. The bacterial distribution along the course of the river was compared with the variation in biological and chemical indicators which had been measured in two River Board surveys performed in the same year. This comparison showed that although close similarities existed, none of the chemical parameters measured in the River Board surveys can be used routinely to assess accurately the bacterial content of this river. The measurements of biological oxygen demand (BOD) made in the August survey correlated closely with the input of bacteria at the two towns and also with the decline in bacterial numbers observed between them. However, both the August and April figures for both BOD and suspended solid concentration indicated a source of pollution, downstream of Canterbury, which was not revealed by the bacterial survey. Measurements of orthophosphate and ammonia concentrations showed that although these parameters may sometimes reflect bacterial numbers, they do not invariably do so. Levels of dissolved oxygen seemed to be independent of the bacterial content of the river.

The bacteria which entered the river did not persist in the surface water. Viable counts of both enterobacteria and pseudomonads fell to background levels within a few miles of the sewage outfalls. Observation of bacterial half-lives in filtered samples of river water indicated that this rapid decline was not caused by a reduction in viability but was probably due to dispersal of the bacteria from the surface water. This might have been caused by sedimentation of the bacteria, associated with organic matter, onto the

river bed and also by dilution with unpolluted water draining into the river.

The incidence of antibiotic resistance among the isolated bacteria was high. Forty per cent of all lactose-fermenters and twenty per cent of coliforms were resistant to ampicillin. Multiple antibiotic resistance was common among coliforms and examination of the plasmid DNA from a number of such multiply-resistant strains suggested that they usually possess more than one plasmid species. However, carriage of these plasmids did not influence significantly the survival, in filtered river water, of either their original coliform hosts or laboratory E. coli K12 strains to which they were transferred. This is perhaps not surprising as previous observations (section 1.3.) have indicated that only plasmids carrying derepressed sex-factors cause increased susceptibility to chemicals such as detergents. A possible reduction in growth rate caused by plasmid carriage would be relatively unimportant in such a spartan environment.

The frequency with which enterobacteria are able to accumulate several plasmid-mediated traits is of particular medical importance. All chloramphenicol resistant coliforms examined here and in a previous study (Hughes and Meynell, 1974) were resistant to other antibiotics. A similar observation was made by Mitsuhashi et al. (1977) who found no single resistance to chloramphenicol among 15,000 clinical isolates (12,000 Shigella, 3,000 E. coli). Such data indicate that even if chloramphenicol is witheld for the treatment of specific human diseases such as typhoid,

chloramphenicol-resistant strains are likely to persist due to their selection by other antibiotics.

Examination of multiply-resistant coliforms isolated from the River Stour showed that these strains more frequently carried the genes for colicin production and plasmid transfer than did coliforms which were resistant to only one or two antibiotics. This observation is also in agreement with the survey of Mitsuhashi et al. (1977) which showed that resistance to 3 or 4 antibiotics was most often due to the carriage of self-transferable plasmids whereas resistance to one or two antibiotics was generally determined by non-self-transferable plasmids. Widespread antibiotic use might therefore be expected to select strains carrying genes determining transferability, colicinogeny and resistance to unrelated antibiotics.

In addition, and perhaps of more concern, is the possibility that antibiotic use may also increase the incidence of strains possessing plasmid-determined pathogenic characters such as the ability to synthesize toxin.

Echeverria (1978) found that in a survey of enterotoxigenic E. coli isolated in the Far East 70 % were antibiotic resistant, two thirds of these being resistant to four or more antibiotics. Eighty per cent of all antibiotic-resistant strains transferred their resistance determinants to sensitive E. coli by conjugation and 35 % of the successful matings resulted in cotransfer of the genes for enterotoxin production. A high incidence of association between antibiotic resistance and enterotoxin synthesis in patho-

genic strains has also been reported by Scotland et al.(1978). They demonstrated that such association may reflect either the carriage of more than one plasmid species or the carriage of a single plasmid determining both antibiotic resistance and toxin synthesis.

A survey performed by Gyles, So and Falkow (1974) showed that strains producing enterotoxin carried a transfer factor with much greater frequency (90 %) than did those isolated from healthy individuals (35 %). The association of transfer factors with both pathogenic and antibiotic resistance determinants led Smith and Lingwood (1970) to speculate that new pathogenic strains might emerge rapidly under antibiotic pressure. Outbreaks of severe infection caused by Salmonella carrying a number of antibiotic resistance determinants (Gangarosa et al., 1972; Le Minor, 1972) and the association of antibiotic resistance and toxigenity cited above indicate this may be the case.

In future investigations into the incidence and association of such plasmid-determined characters among the enterobacterial flora of the urban community, polluted river water can be used as a convenient source of organisms. For example, river water isolates are currently being examined with strains from urinary tract infections, in order to investigate the possibility (p. 157) that strains resistant to a number of antibiotics are more likely to be resistant to the bactericidal action of serum.

Routine indications of the incidence of antibiotic-resistant enterobacteria among the urban population might pro-

vide useful information for clinicians and at least one pharmaceutical company (Sandoz) is adopting analysis of river bacteria to assess the extent to which resistance has developed against recently-marketed antibiotics.

Of additional value might be an extension of the observations made by Linton et al. (1974) who examined coliform populations from both treated and untreated urban sewage. They observed that although most of the R-factor-carrying coliforms found in the sewage outfall at Bristol came from the normal urban population, the highest incidence of R^+ organisms was found in hospital effluent. The hospital isolates were also more often resistant to several antibiotics. The implication that a large proportion of the multiply antibiotic-resistant strains isolated from river water are hospital strains is supported by Acar et al. (1977) who found that enterobacteria from individuals of the normal urban community were rarely resistant to more than one antibiotic. In contrast, more than half of the hospital isolates examined were multiply-resistant.

Further investigations into the origin and characteristics of enterobacteria entering the River Stour at Canterbury might provide interesting comparisons between strains coming from, for example, the hospital and the abbatoir, in terms of both their plasmid compliment and their O-type. Similarly, the coliforms found in the river at Ashford and Canterbury could be compared, relating any findings to the different components of the waste emenating from these towns. Studies of this kind, relating data on

the incidence and association of bacterial characters to the original bacterial environment could yield useful information on the factors influencing the emergence and persistence of enterobacterial populations possessing various combinations of plasmid genes.

Strains isolated from polluted river water may also form the basis of studies into individual plasmid-mediated effects. Enterobacteria isolated from the River Stour by nalidixic acid selection were used with laboratory E. coli, including those examined by the rapid screening method, to investigate the role of plasmids in determining resistance to the bactericidal action of serum. Such resistance, which appears to contribute towards the pathogenicity of enterobacteria (p. 134), had been previously attributed to chromosomally-specified factors.

Smooth plasmid-carrying enterobacteria gave one of three responses to fresh human serum; prompt sensitivity, delayed sensitivity or resistance. A delayed-sensitive isolate which had been cured of its plasmid markers retained the delayed sensitive response but became more sensitive to serum. Subsequent inheritance of a number of plasmids by this cured derivative resulted in a significant increase in resistance to serum, the effect being particularly marked with R-factors R1 and NR1. However, inheritance of plasmids by a cured derivative of a promptly-sensitive river isolate and a number of E. coli K12 strains had no effect on the serum response of these strains.

These findings suggested that lipopolysaccharide O-side

chains, the cell surface components which are considered to be responsible for the delay in serum killing, are essential for the expression of plasmid-mediated factors that can modify serum response.

The nature of such factors is currently being investigated. Timmis, Moll and Danbara, having found that serum resistance could be conferred on E. coli by the R-factor R6-5, determined the serum sensitivity of strains carrying cloned fragments, generated by restriction endonucleases, of this plasmid (personal communication). This investigation revealed that the gene specifying serum resistance is located on a fragment (EcoR1 E-7) containing the genes traS, traT and traD which determine proteins involved in plasmid transfer and surface exclusion. The probable association of these proteins with the cell-envelope is of particular interest following recent reports of specific alterations in cell envelope protein composition associated with conversion of both E. coli (Taylor and Parton, 1978) and Neisseria gonorrhoeae (Hildebrandt et al., 1978) to serum resistance.

In view of the data on R6-5 and the enhanced level of serum resistance determined by a multicopy mutant of the related R factor NR1 (p. 153) it would be interesting to examine the effect of changes in the control of tra gene expression on the serum resistance conferred by plasmids. It would also be valuable to use the hybrid plasmid containing the R6-5 serum resistance gene in hybridisation experiments with other serum resistance plasmids carried by both laboratory and wild-type strains. This might

provide information on the epidemiology of this particular gene and indicate whether more than one plasmid-encoded protein can, in combination with chromosomally-determined factors, confer serum resistance on enterobacteria.

The recent discovery of many plasmid mediated enterobacterial pathogenic characters such as serum resistance in addition to observations, made here and else where, of the associat-tion of such characters, reemphasize the view discussed earlier (p. 7) that classification of plasmids as R-factors or Col factors is now inappropriate. Populations of both plasmids and their hosts evolve rapidly in response to the bacterial environment, this being particularly so with the medically-important genera of the Enterobacteria-ceae.

The methods described here provide a suitable basis for future isolation and examination of these bacteria and their plasmids. Their application to the study of isolates from the River Stour indicates that polluted river water presents a convenient focus for such epidemiological studies.

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HIGH FREQUENCY OF ANTIBIOTIC-RESISTANT ENTEROBACTERIA IN THE RIVER STOUR, KENT

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Summary The proportion of antibiotic-resistant enterobacteria in natural waters seems to be increasing. For example, in one river in 1974 73% of the enterobacteria isolated were ampicillin resistant, whereas in another study in 1970 27% of organisms isolated from other rivers were ampicillin resistant. The number of antibiotic-resistant enterobacteria is underestimated in colony-counts using pour plates.

INTRODUCTION

IT is to be expected that, as antibiotic resistance increases in the enterobacteria of man and animals, resistant organisms will increasingly be found in natural waters. By 1970-71 a substantial proportion of organisms from various rivers and coastal waters of the U.K. were already resistant, and many strains could transfer their resistance in mixed culture.[1,2] We have determined the frequency of antibiotic-resistant enterobacteria in the River Stour, Kent, this year.

METHODS

Samples.—Six sites were examined. Samples I and II were collected on Jan. 23, 1974: I was taken at the northern edge of Canterbury (national grid reference, TR 134578) and II (TR 175599) about 3 miles downstream and about 1 mile downstream from a sewage works. Samples III–VI were collected on Feb. 8, 1974: III came 2·5 miles upstream from Sandwich (TR 322596); IV and V came 2 and 2·5 miles, respectively, downstream from Sandwich (TR 335612, TR 337618); and VI came from the shoreline of Pegwell Bay where the Stour enters the sea (TR 350641).

Colony-counts.—These were made within 3 hours of collecting the samples, using Oxoid MacConkey No. 3 as supplied or with streptomycin, tetracycline, ampicillin, or chloramphenicol at 20 μg. per ml. agar each. Counts were made either by a drop method [3] or by pour plates in which a 1 ml. sample was added to 20 ml. molten agar at 48°C which was poured immediately and allowed to set at room-temperature. Colonies were counted after overnight incubation at 37°C.

Sensitivity tests. — Antibiotic-resistant strains were purified by subculture to antibiotic-free MacConkey agar and then grown overnight in broth. Plates of Oxoid 'Blood-Agar Base' were spread with 0·1–0·2 ml. of overnight culture and overlaid with sensitivity discs, usually Oxoid 'Multodisks', containing chloramphenicol, tetracycline, ampicillin, or streptomycin, 10 μg. each; kanamycin, 5 μg; cephaloridine, 15 μg; and nalidixic acid, 30 μg.

Transferable resistance.—Strains were grown overnight in broth with *Escherichia coli*, M560, a nalidixic-acid-resistant derivative of the non-restricting mutant, 803, of *E. coli* K-12 described by Wood.[4] Since only one of the strains tested in detail was nalidixic-acid-resistant, transfer of a given resistance determinant could be tested by streaking the mixed overnight culture on nutrient agar containing nalidixic acid (50 μg. per ml.) and the appropriate antibiotic.

RESULTS

The colony-counts are shown in table 1. All the organisms isolated were enterobacteria, judging from their colonial morphology and from gram stains. 71% of the colonies obtained were lactose fermenting.

There were three main findings. First, in the fourteen instances in which drop and pour-plate counts could be compared, the drop method gave the higher value, presumably because this method avoids the rapid changes in temperature inherent in preparing pour plates.[5] The counts on chloramphenicol agar are not included since they were derived from only 1, 4, and 4 colonies, respectively. Second, the colony-counts on antibiotic-free MacConkey agar ranged from $2{\cdot}8 \times 10^2$ to $2{\cdot}3 \times 10^8$, which is comparable to reported values.[1] Third, the percentage of resistant organisms as measured by drop counts was 4–17% with selection for tetracycline resistance, 7–21% for streptomycin, and 50–88% for ampicillin.

The resistance patterns of 71 strains isolated on plates containing individual antibiotics were determined. Every strain proved to be resistant to the antibiotic by which it had been selected. Multiple

TABLE I—COUNTS OF ANTIBIOTIC-RESISTANT ENTEROBACTERIA IN THE RIVER STOUR

Antibiotic	Counting method	Sample						Mean % resistant
		I	II	III	IV	V	VI	
None	Drop	$2{\cdot}8\times10^{3}$	$1{\cdot}9\times10^{3}$	$2{\cdot}3\times10^{3}$	$1{\cdot}4\times10^{3}$	$4{\cdot}8\times10^{3}$	$2{\cdot}2\times10^{3}$	..
Tetracycline ..	Drop	N.C.O.	$1{\cdot}0\times10^{3}$ (5%)	$3{\cdot}0\times10^{3}$ (13%)	$5{\cdot}0\times10^{1}$ (4%)	$5{\cdot}0\times10^{1}$ (10%)	$3{\cdot}8\times10^{2}$ (17%)	10
	Pour	N.C.O.	N.C.O.	$2{\cdot}9\times10^{1}$	$3{\cdot}1\times10^{1}$	$3{\cdot}2\times10^{1}$	$3{\cdot}9\times10^{1}$	
Streptomycin ..	Drop	N.C.O.	$2{\cdot}5\times10^{2}$ (13%)	$2{\cdot}5\times10^{3}$ (11%)	$1{\cdot}0\times10^{3}$ (7%)	$1{\cdot}0\times10^{3}$ (21%)	$1{\cdot}0\times10^{2}$ (7%)	12
	Pour	N.C.O.	N.C.O.	$7{\cdot}5\times10^{1}$	$6{\cdot}0\times10^{0}$	$8{\cdot}0\times10^{0}$	$3{\cdot}6\times10^{1}$	
Ampicillin ..	Drop	Not done	Not done	$2{\cdot}0\times10^{3}$ (88%)	$7{\cdot}0\times10^{2}$ (50%)	$3{\cdot}5\times10^{3}$ (73%)	$1{\cdot}8\times10^{3}$ (80%)	73
	Pour	Not done	Not done	$2{\cdot}0\times10^{3}$	$6{\cdot}5\times10^{1}$	$3{\cdot}0\times10^{0}$	$5{\cdot}4\times10^{1}$	
Chloramphenicol	Drop	N.C.O.	N.C.O.	N.C.O.	N.C.O.	N.C.O.	N.C.O.	..
	Pour	N.C.O.	$1{\cdot}0\times10^{0}$	$4{\cdot}0\times10^{0}$	N.C.O.	N.C.O.	$4{\cdot}0\times10^{0}$	

Percentages of antibiotic-resistant enterobacteria (based on the drop method) are shown in parentheses. N.C.O. = no colonies obtained.

resistance was extremely common. Thus, of the total 71 strains, the numbers resistant to individual antibiotics were: ampicillin, 56 (79%); streptomycin, 40 (56%); tetracycline, 38 (54%); chloramphenicol, 16 (23%); cephaloridine, 23 (32%); kanamycin, 8 (11%); and nalidixic acid, 1 (1%). As an index of the extent of multiple resistance, the mean total resistances possessed by each class of resistant strain were calculated as follows: for strains resistant to ampicillin, 2·8; to streptomycin, 3·2; to tetracycline, 3·1; and to chloramphenicol, 4·1. In particular, of the 8 chloramphenicol-resistant strains tested, all were resistant to streptomycin, 7 to ampicillin, 5 to tetracycline, and 3 to cephaloridine. Some of the resistant strains may represent repeated isolations of the same clone, but the patterns of multiple resistance showed that there was more than one clone amongst the strains selected from each sample by a given antibiotic. When grown overnight in mixed broth culture, 21 of 70 strains tested transferred all their resistance to *E. coli* K–12.

DISCUSSION

The prominent feature of our results is not the absolute concentration of enterobacteria, which was of the same order as that reported by Smith,[1] but the fact that a far higher percentage of enterobacteria were resistant to one or more of the commonly used antibiotics. Thus, our colony-counts on antibiotic-free agar ranged from $2{\cdot}8\times10^2$ to $2{\cdot}3\times10^3$ per ml., whereas Smith's ranged from 0 per ml. to $7{\cdot}5\times10^4$ per ml., with a pronounced skew towards low values because 14 of his 38 counts on rivers were 2×10^2 per ml. or less, while 4 of his counts exceeded 5×10^3 per ml. But in our samples the mean frequency of antibiotic-resistant organisms was about twice that found by Smith in 1970 (table II), and our individual measurements of the percentage of resistant organisms

TABLE II—MEAN AND RANGE OF ANTIBIOTIC RESISTANCE IN ENTEROBACTERIA FROM RIVERS

Antibiotic	River Stour	Smith [1]	
		Table 1*	Table 2†
Ampicillin ..	73% (50–88%)	27% (0–25%)	25% (0–80%)
Streptomycin ..	12% (7–21%)	6% (0–25%)	5% (0–10%)
Tetracycline ..	10% (4–17%)	4% (0–25%)	4% (0–16%)

* Nineteen different rivers.
† Nineteen sites along the River Taff.
Range shown in parentheses.

exceeded his values for resistance to ampicillin, streptomycin, and tetracycline in 37/38, 33/38, and 34/38 instances, respectively. The Stour has a relatively short course, rising just west of Ashford, and is not likely to be atypical of rivers passing through semi-rural country. The implication of our findings is that the frequency of antibiotic resistance may have increased markedly in rivers since 1970.

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Nalidixic acid as a selective agent for the isolation of enterobacteria from river water

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SUMMARY

Enterobacteria are more resistant to nalidixic acid than the majority of other Gram-negative organisms isolated from river water, so allowing their selection on MacConkey agar containing nalidixic acid. Selection is further improved by anaerobic incubation which, with nalidixic acid, virtually eliminates oxidase-positive strains such as *Pseudomonas* or *Aeromonas*.

INTRODUCTION

Following studies on the incidence of antibiotic-resistant Gram-negative rods in the River Stour, Kent (Hughes & Meynell, 1974), heavy rain and flooding resulted in enterobacteria like *Escherichia coli* becoming outnumbered by other organisms of less obvious public health importance like *Pseudomonas* and *Aeromonas*. A selective medium has therefore been devised for the isolation of enterobacteria which depends on their relatively greater resistance to low concentrations of nalidixic acid.

MATERIALS AND METHODS

Culture media

Strains were isolated on Oxoid MacConkey Agar No. 3 (CM115). Subcultures were made to Oxoid Blood Agar Base (CM55) and to Oxoid Nutrient Broth No. 2 (CM67). Buffer pH 7·2, contained (g./l.), gelatin (0·01), KH_2PO_4 (3), $Na_2HPO_4.12H_2O$ (7), NaCl (5).

Bacterial strains

'River' strains were isolated between January 1974 and January 1975 from six different river sites in East Kent (National Grid references TR138677, TR157676, TR143577, TR174599, TR324588 and TR338618), and were isolated on MacConkey agar either as supplied or containing sodium ampicillin, 20 μg./ml. 'Standard' strains were obtained from the National Collection of Type Cultures, National Collection of Industrial Bacteria, National Collection of Plant Pathogenic Bacteria and the American Type Culture Collection.

Examination of strains

The following tests were used. River strains were incubated at 37° C.; standard strains at their appropriate temperature (30 or 37° C.).

(i) Lactose fermentation, assessed on MacConkey agar or by subculture to lactose-deoxycholate agar (Meynell & Meynell, 1970).

(ii) Catalase formation, assessed both immediately and 5 min. after adding 3% (v/v) H_2O_2 to cultures grown overnight on blood agar base.

(iii) Oxidase reaction (Kovacs, 1956).

(iv) Oxidation or fermentation of glucose (Hugh & Leifson, 1953).

(v) Sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-di-isopropyl pteridine; Bain & Shewan, 1968).

(vi) Flagellar morphology, as determined by electron microscopy. Strains were grown overnight in broth or in sucrose peptone broth to increase flagella formation (Fuerst & Haywood, 1969). They were then fixed in glutaraldehyde and negatively stained with uranyl acetate.

Antibiotic sensitivity tests

(*a*) Preliminary screening of 96 river strains was carried out using impregnated disks. Plates of blood agar base were spread with 0·1–0·2 ml. of growing broth cultures containing *ca.* 10^5 colony-forming units (c.f.u.)/ml. and overlaid with Oxoid Multodisks containing chloramphenicol (10 μg.), tetracycline (10 μg.), nalidixic acid (30 μg.), streptomycin (10 μg.) and kanamycin (10 μg.). Plates were incubated for 15 hr. precisely at 37° C. Diameters of inhibition zones were measured using a viewing box and callipers, the results being recorded to the nearest mm.

(*b*) Minimum inhibitory concentrations of nalidixic acid were determined for 47 river strains and 28 standard strains by plating on nalidixic acid agar. Initially, overnight broth cultures were diluted in buffer to contain $2–5 \times 10^3$ c.f.u./ml. and 0·02 ml. samples inoculated on MacConkey agar containing 0, 0·5, 1·25, 2·5, 5·0, 7·5, 10·0, 12·5, 15·0, 20·0 or 25 μg. nalidixic acid/ml. The tests were then repeated using lower concentrations: 0, 0·06, 0·13, 0·25, 0·5, 0·75, 1·25 and 2·5 μg./ml. Plates were incubated for 20 hr. precisely at 37° C. and the presence or absence of colonies on each plate then recorded.

Colony counts on river waters

Samples of about 200 ml. river water were collected in sterile bottles opened about 6 in. below the surface and about 2 ft. from the bank, using standard precautions (Report, 1969). Counts were made within 3 hr. of collecting samples from the River Stour, Site I, downstream of Canterbury sewage works (TR174599). Dilutions in buffer were spread on MacConkey agar as supplied or supplemented with nalidixic acid, 0·13, 0·25, or 0·5 μg./ml. Colonies were counted after 20 hr. incubation at 37° C.

A subsequent series of counts, using 0·4 μg./ml. nalidixic acid, were made on samples taken from site II (upstream of the sewage works at TR164598) in addition to site I. These plates were incubated both aerobically for 20 hr. at 37° C. and anaerobically for 48 hr. at 37° C. in jars fitted with cold catalysts.

RESULTS

Since the purpose of this investigation was to select enterobacteria, rather than to identify all the species isolated from river water, the following empirical classification was used, based on the phenotypes of 47 river strains summarized in Table 1.

(*a*) Oxidase-negative (Ox^-), lactose-fermenting or non-fermenting ($Lac^{+/-}$), with peritrichous or no flagella. All fermented glucose (G^f). These are presumed to be enterobacteria.

(*b*) Ox^+Lac^- with polar or no flagella. Some oxidized glucose (G^o) and presumably included *Pseudomonas*. Others fermented glucose and presumably included genera such as *Aeromonas*.

(*c*) Ox^+Lac^+ G^f with polar or no flagella. These presumably included other *Aeromonas* since this genus may be either Lac^+ or Lac^-.

River strains

All 47 river strains were Gram-negative catalase-positive asporogenous rods which utilized glucose. None were susceptible to the compound 0/129, and were therefore not vibrios (Shewan, Hodgkiss & Liston, 1954).

Preliminary sensitivity tests with disks

Fig. 1 shows the distribution of inhibition zone diameters for 96 river strains, 69 of which were isolated on ampicillin agar, tested against Oxoid Multodisks. In the case of kanamycin, streptomycin, tetracycline and chloramphenicol, the sensitivities of the three phenotypic classes of organisms overlapped to varying degrees and with each of these drugs, a proportion of strains were not inhibited. However, the distribution for nalidixic acid showed two interesting features. First, the $Ox^-Lac^{+/-}$ class (presumptive enterobacteria) were all more resistant than the Ox^+Lac^+ class. Secondly, the distribution for the Ox^+Lac^- class was bi-modal, some strains being as sensitive as the Ox^+Lac^+ class but others being almost wholly resistant.

Minimum inhibitory concentrations of nalidixic acid

Table 1 shows the M.I.C. for 47 river strains. They showed first, that the $Ox^-Lac^{+/-}$ class could grow on MacConkey agar containing nalidixic acid at concentrations between 0·13–0·5 μg./ml.,whereas the Ox^+Lac^+ class was inhibited; and second that, as in the disk tests, the Ox^+Lac^- class contained two types of strain, one being at least as resistant as the $Ox^-Lac^{+/-}$ group and the other as sensitive as the Ox^+Lac^+ group.

Table 1. *Minimum inhibitory concentrations of nalidixic acid for 47 river strains*

Phenotype				Nalidixic acid μg./ml. MacConkey agar No. 3						
Oxidase	Lactose*	Glucose†	Flagella‡	No. tested	0·06	0·13	0·25	0·50	0·75	1·25	2·5	> 2·5
−	+/−	F	Peri	15 (5)	—	—	—	—	1	8 (4)	6 (1)	—
		O	Polar	8 (3)	—	—	—	—	—	—	—	8 (3)
+	−	F	Polar	12 (5)	5 (3)	7 (2)	—	—	—	—	—	—
+	+	F	Polar	12 (5)	6 (3)	6 (3)	—	—	—	—	—	—

M.I.C. were determined by inoculating 50–100 c.f.u., followed by overnight incubation at 37° C.
* Fermented/non-fermented.
† Fermented/oxidized in Hugh & Leifson's test (1953).
‡ Peri/polar; peritrichous/polar, when present, determined by electron microscopy.
The numbers of strains isolated on ampicillin agar are shown in parentheses.

Table 2. *Colony counts of river water on MacConkey agar containing nalidixic acid, 0–0·5 μg./ml.*

Phenotype	Nalidixic acid μg./ml. MacConkey agar No. 3.											
	0			0·13			0·25			0·5		
	c.f.u./ml.	% Ox⁻	Ox⁻/ml.	c.f.u./ml.	% Ox⁻	Ox⁻/ml.	c.f.u./ml.	% Ox⁻	Ox⁻/ml.	c.f.u./ml.	% Ox⁻	Ox⁻/ml.
Lac⁺	6·6	26	1·7	2·0	56	1·1	2·3	97	2·2	1·4	100	1·4
Lac⁻	4·0	8	0·3	1·3	31	0·4	1·0	35	0·4	1·1	39	0·4
Total	10·6	19	2·0	3·3	46	1·5	3·3	79	2·6	2·5	73	1·8

c.f.u./ml.: colony-forming units expressed as thousands/ml. river water as determined after aerobic incubation at 37° C.
% Ox⁻: percentage of oxidase-negative colonies of a total of 60–80 colonies tested at each concentration.
Ox⁻/ml.: oxidase-negative colonies/ml. (= c.f.u./ml. × % Ox⁻/100).

Table 3(*a*). *Colony counts from river water plated on MacConkey agar No. 3 containing nalidixic acid (0·4 μg./ml.)*

Site	Phenotype	Aerobic count c.f.u./ml. −Nal	Aerobic count c.f.u./ml. +Nal	Aerobic count c.f.u./ml. %	Anaerobic count c.f.u./ml. −Nal	Anaerobic count c.f.u./ml. %	Anaerobic count c.f.u./ml. +Nal	Anaerobic count c.f.u./ml. %
I	Lac⁺	2100	630	(30)	1700	(81)	560	(27)
	Lac⁻	1100	210	(19)	580	(53)	66	(6)
	Total	3200	840	(26)	2280	(71)	626	(20)
II	Lac⁺	1600	290	(19)	960	(60)	280	(18)
	Lac⁻	1300	240	(19)	410	(32)	62	(5)
	Total	2900	530	(18)	1400	(48)	340	(12)

% = colony count expressed as percentage of aerobic count without nalidixic acid.

Table 3(*b*). *Efficiency of selection of oxidase-negative organisms*

Phenotype	Aerobic −Nal % Ox⁻	Aerobic −Nal Ox⁻/ml.	Aerobic +Nal % Ox⁻	Aerobic +Nal Ox⁻/ml.	Anaerobic −Nal % Ox⁻	Anaerobic −Nal Ox⁻/ml.	Anaerobic +Nal % Ox⁻	Anaerobic +Nal Ox⁻/ml.
Lac⁺	21	3·4	100	2·9	42	4·0	100	2·8
Lac⁻	9	1·2	34	0·8	22	0·9	88	0·6
Total	16	4·6	70	3·7	36	4·9	95	3·3

Ox⁻/ml. = Oxidase-negative colonies expressed as hundreds/ml., calculated as in Table 2. Values calculated as in Table 2, from counts on site II (in Table 3*a*).

Subclasses of the Ox⁺Lac⁻ class

Biochemical tests on a total of 20 such river strains, summarized in Table 1, showed that of the eight strains resistant to nalidixic acid, $\geqslant 2{\cdot}5$ μg./ml., none could ferment glucose. These were therefore presumed to be oxidative Pseudomonads. Of the 12 strains sensitive to nalidixic acid, $\leqslant 0{\cdot}13$ μg./ml., all fermented glucose. These were therefore thought likely to be Lac⁻ *Aeromonas*.

Standard strains

Tests on 28 standard strains confirmed the previous conclusions, within the limits of the number of species available. Some species which might be expected to occur in river water were unable to grow on MacConkey agar No. 3 as supplied. These were the *Pseudomonas* strains *P. phaseolicolor* (ATCC 11365), *P. fluorescens* (NCIB 9494), *P. putida* (NCIB 9034), *P. sp.* (NCIB 8858) and *Xanthomonas compestris* (NCPPB 528), *Zymomonas mobilis* (NCIB 8938), *Achromobacter lwoffi* (NCIB 9020), *Erwinia caratavora* (NCPPB 312).

The following all grew and had M.I.C.s of nalidixic acid within the range 0·75–2·5 μg./ml.: the coliform organisms *Escherichia coli* B1, *E. coli* K12 (2 strains), *Klebsiella aerogenes* type I, *K. aerogenes* type II (NCIB 5938), *Enterobacter aerogenes* (NCIB 10102 and NCTC 10006), *Citrobacter freundii* (NCTC 9750), and the non-lactose fermenting enterobacteria *Proteus vulgaris* (NCTC 4175), *Serratia marcescens* (NCTC 1377 and NCIB 2847).

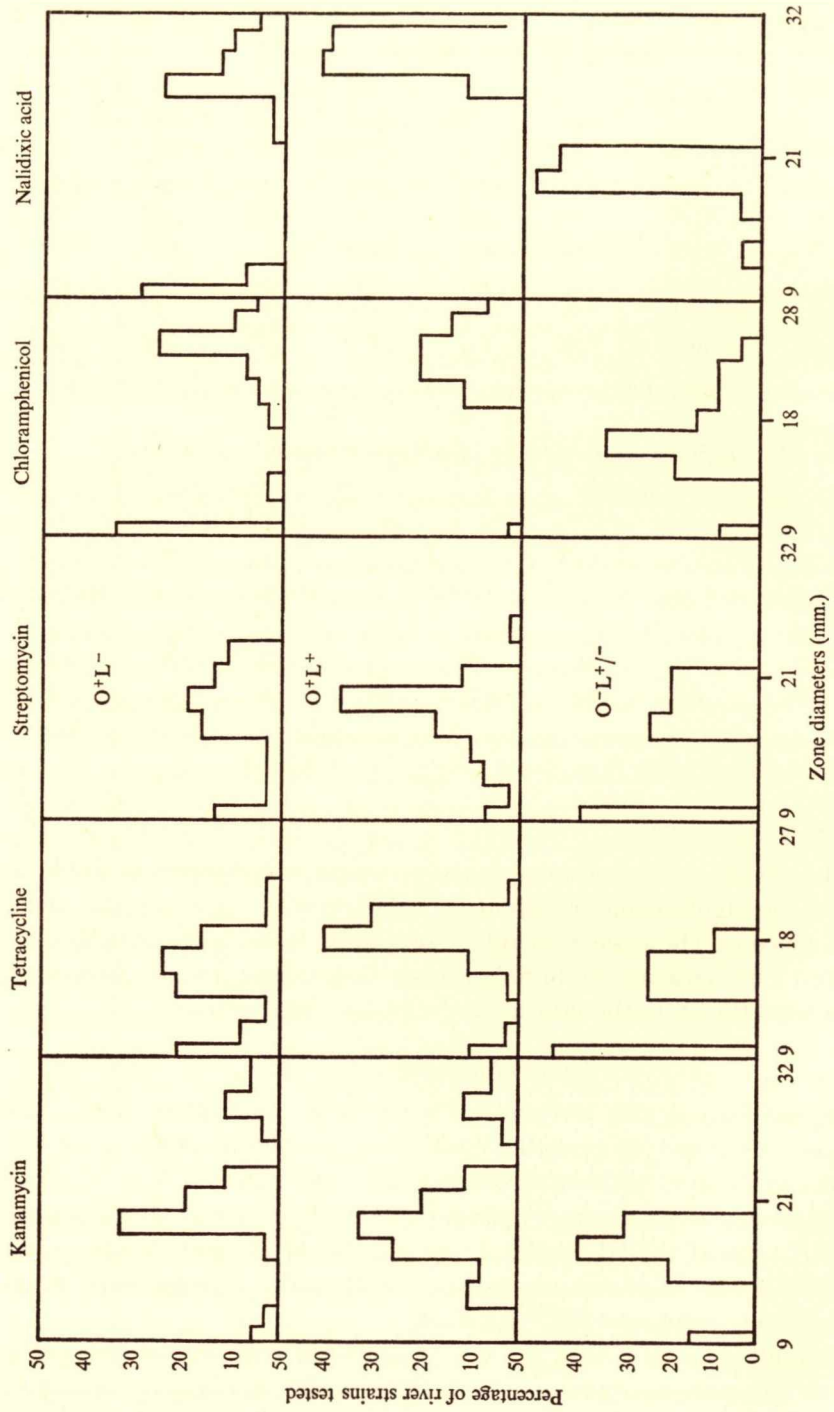

Fig. 1. Susceptibility of 96 river strains to five anti-bacterial agents, as measured by the diameters of inhibition zones surrounding Oxoid Multodisks. The diameter of each disk was 9 mm. The strains were divided into three phenotypic classes, according to their oxidase reaction (O) and their ability to hydrolyse lactose (L), as described in the text.

Strains of *Pseudomonas aeruginosa* (NCTC 10332, NCIB 8295 and NCIB 0950), *Pseudomonas alcaligines* (NCIB 9398) and *Xanthomonas phaseoli* all had M.I.C.s greater than 2·5 μg./ml. *Alcaligines faecalis* (NCIB 8156) had an M.I.C. of 1·25 μg./ml. while of three non-lactose fermenting strains of *Aeromonas* (*A. caviae* NCIB 9671, *A. formicans* NCIB 9232 and *A. liquefaciens* NCIB 9233), two were inhibited by 0·13 μg./ml. and one by a concentration between 0·25 and 0·5 μg./ml.

Colony counts on river water

The preceding results suggested that nalidixic acid at 0·13–0·5 μg./ml. MacConkey agar would selectively inhibit the growth of the Ox^+Lac^+ class and the G^f subclass of Ox^+Lac^- strains. Colony counts were therefore made on water from site I, downstream from a sewage works, using concentrations of either 0, 0·13, 0·25 or 0·5 μg./ml. (Table 2).

The selective power of the medium was indicated by the increasing percentage Ox^- colonies obtained on nalidixic agar. Moreover, the colony count/ml. of Ox^- organisms remained constant, showing that they were not inhibited by this range of concentrations. Inhibition of Ox^+Lac^+ organisms was complete at 0·5 μg./ml., since all Lac^+ colonies tested proved to be Ox^-. At this concentration, 61% of Lac^- colonies tested were still Ox^+, as expected from the sensitivity tests of Fig. 1 and Table 1, and of 20 tested, all were G^o. This remaining fraction of Ox^+Lac^- G^o would therefore be expected to be obligate aerobes and anaerobic culture was therefore tested as an additional means of contraselection.

Colony counts were made from sites I and II using aerobic and anaerobic culture on MacConkey agar containing either no nalidixic acid or 0·4 μg./ml. (Tables 3*a*, *b*). The counts are shown in Table 3(*a*) and their details are given in Table 3(*b*). The results were consistent with those obtained previously. In both aerobic and anaerobic culture using nalidixic acid, 0·4 μg./ml., 100% of Lac^+ colonies were Ox^- compared to 21% (aerobic) and 42% (anaerobic) without nalidixic acid selection. Of the Lac^- colonies on nalidixic acid agar, only 34% were Ox^- in aerobic culture whereas the value rose to 88% in anaerobic culture. These values compare with only 9% and 22% Ox^-, respectively, in the absence of nalidixic acid. Thus, nalidixic acid and anaerobiosis together had almost eliminated the unwanted $Ox^+Lac^{+/-}$ group.

DISCUSSION

MacConkey agar No. 3 containing nalidixic acid, 0·4 μg./ml. when incubated aerobically, will inhibit all oxidase-positive lactose-fermenters and a high proportion of oxidase-positive non-lactose-fermenters. It therefore allows the direct counting of typical coliforms in river water contaminated with other organisms from sewage effluent, soil and vegetation. This is sufficient for most studies because the majority of oxidase-negative strains found in such samples are lactose-fermenters. However, if non-lactose-fermenting enterobacteria are sought then the persisting oxidase-positive non-lactose-fermenters may be further inhibited by anaerobic incubation (Table 3).

Other possible applications of these findings are to the culture media used for

colony counts on membrane filters, and also to the liquid media used for counts by the dilution method where organisms such as *Aeromonas* may produce false positive results in the presumptive coliform test (Holden, 1970).

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Plasmid Carriage and the Serum Sensitivity of Enterobacteria

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The carriage of a range of plasmids by rough, serum-sensitive laboratory strains of *Escherichia coli* made no difference to their reactivity in human serum as determined by two methods. Plasmid-carrying enterobacteria isolated from polluted river water gave a variety of responses to serum. Smooth *E. coli* river isolate C8 was killed by serum but only after a delay of 1 h, and curing of antibiotic resistance and colicin determinants from this strain led to a small but significant increase in serum sensitivity. Plasmids from eight strains were transferred by conjugation to a cured derivative of C8 ($C8^{-}Nal^{R}$), and in six cases a significant increase in the serum resistance of the progeny was observed. Plasmid-mediated enhancement of resistance was particularly marked with plasmids R1 and NR1, and a round of replication mutant of NR1 conferred greater resistance than did the normal R factor. However, R1 and NR1 were unable to modify the serum response of a cured strain ($P21^{-}Nal^{R}$) derived from promptly serum-sensitive isolate P21. These findings suggest that lipopolysaccharide O-side chains, the cell surface components responsible for the delay in serum killing, are essential for the expression of plasmid factors that modify sensitivity to serum. Examination of $K(A)^{-}$ variants of two isolates indicated that the K(A) antigen has only a marginal effect on the serum response.

Normal serum is bactericidal for a wide range of both smooth and rough gram-negative bacteria by virtue of a system involving antibody, the classical (19) or alternative (9, 38) pathway of complement activation and, possibly, other serum proteins (5). However, some smooth strains are insensitive to this system, and there is evidence to suggest that serum resistance may contribute toward the pathogenicity of enterobacterial strains (6, 15, 27, 31). Although the nature of serum resistance has been extensively investigated, the basis of the phenomenom has still not been defined with any degree of certainty. Experiments with clinical isolates (1, 27, 34) and mutants (25, 33, 35) have not substantiated earlier suggestions that a full complement of lipopolysaccharide O-side chains or the presence of large amounts of acidic polysaccharide K antigens are able to protect completely the bacterial cell from the bactericidal action of serum (7, 10, 14, 16). Furthermore, it seems likely that the resistance of gram-negative bacteria to serum is not due to any one factor, but results from the accumulation of a number of distinct components at or near the cell surface (35).

Recently, Reynard and Beck (29) found that *Escherichia coli* K-12 strains carrying the plasmids R1 and R100 (NR1) were more resistant to the bactericidal action of diluted rabbit serum than these strains without plasmids. Because it has been reported that certain plasmids are able to modify the lipopolysaccharide (4) and protein (20, 21) components of the cell envelopes of enterobacteria, investigation of the role of plasmid-determined factors is likely to lead to a greater understanding of the nature of serum resistance determinants. In the present study we report on the effect of plasmid carriage on the serum sensitivity of both laboratory and wild-type enterobacterial strains.

MATERIALS AND METHODS

Bacteria. Laboratory strains and their plasmids are shown, with their source, in Table 1. The wild-type coliforms (Table 2) were isolated from the River Stour, Kent, England, by using MacConkey agar CM115 (Oxoid Ltd., London), containing 0.4 μg of nalidixic acid per ml (17) and supplemented with either chloramphenicol (strain prefix C) or ampicillin (prefix P) at 20 μg/ml to select for antibiotic resistance. All river isolates were colonially and serologically smooth. Isolates were then further investigated in the following manner for plasmid-determined characters.

(i) Antibiotic resistance spectra were determined by flooding Oxoid DST agar with a diluted overnight culture and adding Oxoid Multodisks containing sulfonamide (500 μg), ampicillin (10 μg), cephaloradine (15 μg), chloramphenicol (10 μg), kanamycin (5 μg), streptomycin (10 μg), and tetracycline (10 μg). Minimal inhibitory concentrations of ampicillin were assessed

TABLE 1. *Response to normal human serum of laboratory enterobacterial strains*

Strain[a]	Host[b]	Plasmid(s)[c]	% Survival after:			
			Method A[d]			Method B[e]
			1 h	2 h	3 h	2 h
M671	*E. coli* K-12 C600	—	<0.1	<0.1	<0.1	0.06
M672	*E. coli* K-12 C600	λ	<0.1	<0.1	<0.1	0.03
M975 (22)	*E. coli* K-12 C600	R56	<0.1	<0.1	<0.1	0.19
M976 (22)	*E. coli* K-12 C600	R64	<0.1	0.3	<0.1	<0.01
M977	*E. coli* K-12 C600	R56, λ	<0.1	<0.1	<0.1	<0.01
M978	*E. coli* K-12 C600	R64, λ	<0.1	<0.1	<0.1	<0.01
M786	*E. coli* K-12 AB1157	—	<0.1	<0.1	<0.1	<0.01
M830	*E. coli* K-12 AB1157	λ	<0.1	<0.1	<0.1	0.04
M866	*E. coli* K-12 AB1157	ColE2-P9	<0.1	<0.1	<0.1	<0.01
M1080	*E. coli* K-12 J5-3	—	<0.1	<0.1	<0.1	<0.01
M1081	*E. coli* K-12 J5-3	R56	<0.1	<0.1	<0.1	<0.01
M1082	*E. coli* K-12 J5-3	R64	<0.1	<0.1	<0.1	<0.01
M941	*E. coli* K-12 J5-3	R-Utrecht, F, λ	<0.1	<0.1	<0.1	<0.01
SFI585	*E. coli* K-12 J5	NR1	<0.1	<0.1	<0.1	<0.01
SFI587	*E. coli* K-12 J5	R1	<0.1	<0.1	<0.1	<0.01
SFI609 (24)	*E. coli* K-12 J5	R12	<0.1	<0.1	<0.1	<0.01
M1151	*E. coli* K-12 W2637	—	<0.1	<0.1	<0.1	<0.01
M1157	*E. coli* K-12 W2637	R56	<0.1	<0.1	<0.1	<0.01
KH462	*E. coli* K-12 W3110	—	<0.1	<0.1	<0.1	<0.01
KH472	*E. coli* K-12 W3110	ColV2-K94	<0.1	<0.1	<0.1	0.03
KH517	*E. coli* K-12 W3110	ColV2-K94, R1drd19	<0.1	<0.1	0.1	<0.01
KH531	*E. coli* K-12 W3110	R1drd16	<0.1	<0.1	<0.1	0.05
KH573	*E. coli* K-12 W3110	ColE1-K30	<0.1	<0.1	<0.1	<0.01
KH679	*E. coli* K-12 W3110	R1drd16, ColE1-K30	<0.1	<0.1	<0.1	<0.01
M1246 (3)	*E. coli* K-12 W3110	ColE3	0.3	<0.1	<0.1	<0.01
M1231 (3, 18)	*E. coli* CA53	ColIa	<0.1	<0.1	<0.1	<0.01
M1232 (3, 18)	*S. typhimurium* ST4	ColIb-P9	45.9	44.8	0.7	7.7
M1235 (3, 18)	*E. coli* K89	ColB, ColM	<0.1	<0.1	<0.1	0.14
M1240 (3, 18)	*E. coli* CA7	ColV1	<0.1	<0.1	<0.1	0.02
M1244 (3, 18)	*E. coli* CA42	ColE2	2.0	0.9	<0.1	3.97
M1247 (3, 18)	*E. coli* K216	ColK	<0.1	<0.1	<0.1	<0.01
M1251 (3, 18)	*C. freundii* CA31	ColA	0.4	<0.1	<0.1	0.02
M1252 (3, 18)	*E. coli* K53	ColE1	3.1	0.2	<0.1	0.09
M1241 (3, 18)	*E. coli* CA46	ColG	158.0	208.0	45.8	75.3
M1245 (3, 18)	*E. coli* 12-317	ColE2	<0.1	<0.1	<0.1	<0.01

[a] *E. coli* strains, obtained from G. G. Meynell (prefix M), K. G. Hardy (prefix KH) and Sandoz Forschungsinstitut (SFI). Numbers in parentheses indicate, where appropriate, original sources.

[b] K-12 strains are morphologically rough due to a lesion in the chromosomal *rfb* cluster; cell surface characteristics of other strains used are not known.

[c] —, No known plasmid.

[d] Results obtained by method of Taylor et al. (36).

[e] Results obtained by method of Reynard and Beck (29).

on Oxoid MacConkey agar CM115 containing 0 to 200 μg of the antibiotic per ml.

(ii) Colicinogeny was detected by the overlay method (23), using the colicin-sensitive indicator *E. coli* K-12 C600. Allotment to group A or group B (18) was determined by action on the mutant indicators *tol- II* (11) and *tonB* (26).

(iii) The presence of a transfer factor was detected by successful transfer of drug resistance determinants, in overnight culture, to *E. coli* K-12 M560, an Nal^R nonrestricting mutant described by Wood (40).

Serum bactericidal assay. Normal human serum was obtained from healthy volunteers and stored in small portions at −20°C until required. Normal rabbit serum was also used in some experiments. Bacterial sensitivity to serum was estimated by the technique of Taylor et al. (36). An early log phase Trypticase soy broth culture was washed in 0.06 M NaCl and suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.4) to a concentration of 10^6 organisms per ml.

A sample (0.5 ml) of this suspension was added to 1.5 ml of serum, and viable counts were obtained at the beginning of the test and after 1, 2, and 3 h of incubation at 37°C.

Laboratory strains were also examined by the method of Reynard and Beck (29). Log phase cells were washed with 0.054 M NaCl and suspended in

0.054 M NaCl to a concentration of 9×10^6 organisms per ml. A mixture containing 0.25 ml of this suspension, 0.25 ml of 0.24 M NaCl, 0.20 ml of serum, 0.50 ml of 0.05 M *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 8.4), and 0.30 ml of 0.054 M NaCl was incubated at 37°C. Viable counts were prepared at 0 and 2 h.

Curing of plasmid determinants. Curing of plasmid determinants was attempted by overnight growth in Trypticase soy broth containing either (i) acridine orange over a range of 5 to 200 μg/ml, pH 7.6 (13), or (ii) 10% sodium dodecyl sulfate, pH 7.4 (37). Surviving cultures were streaked onto Trypticase soy agar, and single colonies were inoculated onto Trypticase soy agar and overlaid with indicator culture (to detect loss of colicinogeny) or onto Trypticase soy agar supplemented with ampicillin (to detect loss of R determinants). That the plasmids were not retained in a nonfunctional form was indicated by the absence of superinfection inhibition when reintroducing markers from "lost" plasmids into the presumptive cured strain. No physical analyses were made of the DNA content of any strains used in this study. It is therefore not possible to comment upon the presence or the effect of cryptic plasmids.

Matings. Spontaneous Nal^R mutants of the cured isolates were selected and then mated with prospective donors in overnight culture. Progeny were selected on appropriately supplemented media, and colonies were purified on Trypticase soy agar and checked for inheritance of other markers.

Statistical analyses. The significance of the difference of the means was assessed by using the Wilcoxon sum of ranks (t) test (39).

RESULTS

Laboratory strains. The effect of carriage of a range of drug (R) and colicin (Col) factors on the sensitivity to normal human serum of six *E. coli* K-12 strains was examined by the methods of Taylor et al. (36) and Reynard and Beck (29). With both methods, all K-12 strains examined were rapidly killed by serum, regardless of plasmid carriage (Table 1). There was also no evidence to suggest that carriage of prophage λ could confer any degree of serum resistance on these rough strains. In their original study, Reynard and Beck used normal rabbit serum, so 15 of the 27 K-12 strains listed in Table 1 were randomly selected and tested as indicated by these authors; in all cases, less than 0.01% of cells survived 2 h of exposure to rabbit serum.

In a separate series of experiments, a number of K-12 strains were examined by the technique of Taylor et al., except that *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 8.4) was used as the suspending buffer; the results obtained were identical to those with tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.4). In addition, a 10-fold increase in inoculum size had no effect on the percent survival of K-12 strains in this system.

The effect of Col factor carriage by laboratory strains was also examined (Table 1). All were extremely sensitive to human serum, with the exception of *Salmonella typhimurium* ST4, carrying ColIb-P9, and *E. coli* CA46, carrying ColG. All attempts to cure CA46 were unsuccessful.

River strains. It was found that the smooth river isolates (Table 2) could be assigned to one of three groups on the basis of their interaction with normal human serum by the method of Taylor et al. A number of strains were rapidly killed by serum, with less than 10%, and usually less than 1%, of the inoculum surviving after 1 h of incubation (promptly sensitive). Others were killed by serum but only after a lag of 1 h (delayed sensitive). The remainder grew in serum and were classed as serum resistant (as previously defined; 32). An example of each type of response is shown in Fig. 1. All 17 strains listed in Table 2 carried antibiotic resistance determinants, and in 8 cases these were shown to be self-transmissible. In addition, eight of the isolates carried determinants for colicin production. Only four were resistant to human serum, and six were promptly sensitive. There was no simple relationship between specific plasmid determinants and serum reactivity. However, serum-resistant *E. coli* strains did possess a larger number of plasmid determinants (antibiotic resistances, colicin production, and self-transmissibility), and the minimal inhibitory concentrations of ampicillin were higher for them than for the 11 serum-sensitive *E. coli* strains (Table 2).

Two of the strains were found to belong to *E. coli* serogroup O9, an O-type associated with R plasmids (12) and with the production of the A-type of K antigen (28). Both strains were of the delayed serum-sensitive type, and both reacted with O9 antiserum but only after they had been autoclaved at 121°C for 2 h, indicating the presence of a K(A) antigen. Cultures of both *E. coli* C37 and C12 segregated small numbers of colonies that had lost the ability to produce the K(A) antigen; these variants reacted in O9 antiserum after being heated at 100°C for 1 h. Because it has been suggested that the serum resistance of smooth *E. coli* strains is determined by the presence of large amounts of K antigen (10), the reactivity in human serum of the $K(A)^-$ variants was compared with that of the $K(A)^+$ parents (Fig. 2a and b). Although both the $K(A)^+$ and $K(A)^-$ forms of *E. coli* C12 displayed the delayed sensitive response (Fig. 2a), the variant was significantly more serum sensitive than the parent. No significant differences were found, however, between the $K(A)^+$ and $K(A)^-$ forms

TABLE 2. *Response to normal human serum of smooth enterobacterial strains isolated from river water*

Strain	Host[a]	R spectrum[b]	Col[c]	tra[d]	Serum sensitivity
C5	*E. coli* NT	Ap (>200), Cm, Sm, Su	−	+	Delayed
C8	*E. coli* NT	Ap (>200), Cm	A	−	Delayed
C11	*E. coli* NT	Ap (>200), Cm, Km, Sm, Su, Tc	B	+	Resistant
C12	*E. coli* O9:K(A)	Ap (60), Cm, Sm, Su, Tc	−	−	Delayed
C14	*Citrobacter* sp.	Ap (200), Cm, Cr, Sm, Su, Tc	−	−	Prompt
C15	*E. coli* NT	Ap (100), Cm, Km, Sm, Su, Tc	−	+	Delayed
C22	*E. coli* NT	Ap (60), Cm, Su, Tc	−	−	Delayed
C25	*E. coli* NT	Ap (>200), Cm, Km, Sm, Su	B	+	Resistant
C37	*E. coli* O9:K(A)	Ap (60), Cm, Su, Tc	−	+	Delayed
C40	*E. coli* NT	Ap (60), Cm, Su, Tc	A	−	Prompt
C42	*E. coli* NT	Ap (100), Cm, Sm, Su, Tc	A	+	Resistant
P5	*E. coli* NT	Ap (40), Cm, Su	A	−	Prompt
P11	*Klebsiella* sp.	Ap (>200)	B	−	Prompt
P21	*E. coli* NT	Ap (100), Cm, Km, Su, Tc	−	+	Prompt
P35	*E. coli* NT	Ap (200), Cr	A	−	Resistant
P37	*E. coli* NT	Ap (20), Cm, Sm, Su	−	−	Delayed
P43	*E. coli* NT	Ap (180)	−	+	Prompt

[a] Identified by the method of Cowan and Steel (2) and serotyped by A. P. Roberts, London. NT, Lack of agglutination of heated (100°C; 1 h) cells in O1, O2, O4, O6, O7, O9, O11, O18, O39, and O75 antisera.

[b] Resistance to antibacterial agents determined with impregnated disks. Cm, Chloramphenicol; Cr, cephaloridine; Km, kanamycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Ap, ampicillin. Values in parentheses indicate minimal inhibitory concentrations in micrograms per milliliter of ampicillin.

[c] Colicin detected by action against indicators and grouped by the method of Hughes et al. (18).

[d] tra, Ability to transfer antibiotic resistance determinants to nonrestricting recipient *E. coli* K-12 M560.

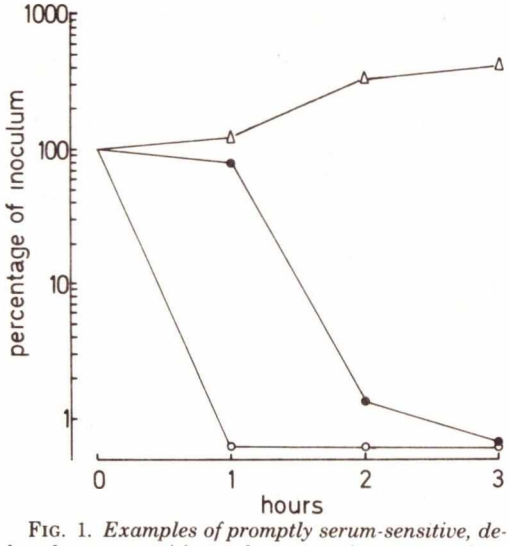

FIG. 1. *Examples of promptly serum-sensitive, delayed serum-sensitive and serum-resistant enterobacterial strains. Serum bactericidal assays were performed by the method of Taylor et al. (36). Symbols: ○, Citrobacter C14; ●, E. coli C5; △, E. coli C42.*

of *E. coli* C37, although the means obtained with the variant at 1, 2, and 3 h were slightly lower than those obtained with the parent (Fig. 2b).

Attempts were made to cure all strains listed in Table 2 of R and Col determinants. Strains C8 and P21 were cured of all resistance and colicin markers with acridine orange, and strains C5 and C42 were cured with sodium dodecyl sulfate.

The serum sensitivities of nine colonies derived from *E. coli* C8, cured of R determinants with 40 μg of acridine orange per ml, were measured. All showed the delayed serum killing response but were slightly more serum sensitive than the parent strain. The response to serum of clones that had been treated with acridine orange but not cured of plasmid determinants was identical to that of *E. coli* C8. One cured colonial form ($C8^-$) was selected for statistical evaluation, and a spontaneous Nal^R mutant was obtained. The serum responses of $C8^-$ and $C8^-Nal^R$ were identical. Replicate experiments confirmed that the loss of plasmid determinants from *E. coli* C8 led to a small increase in serum sensitivity (Table 3).

To assess the extent to which plasmids could modify the response of $C8^-Nal^R$ to human serum, this strain was mated with five river and four laboratory strains, and progeny inheriting appropriate antibiotic resistance markers were selected (Table 3).

Inheritance by $C8^-Nal^R$ of R-Utrecht from *E. coli* M941 and of the R and Col determinants from serum-resistant *E. coli* C25 resulted in no significant change in serum response. Progeny from the remaining matings were all significantly more serum resistant than $C8^-Nal^R$. In-

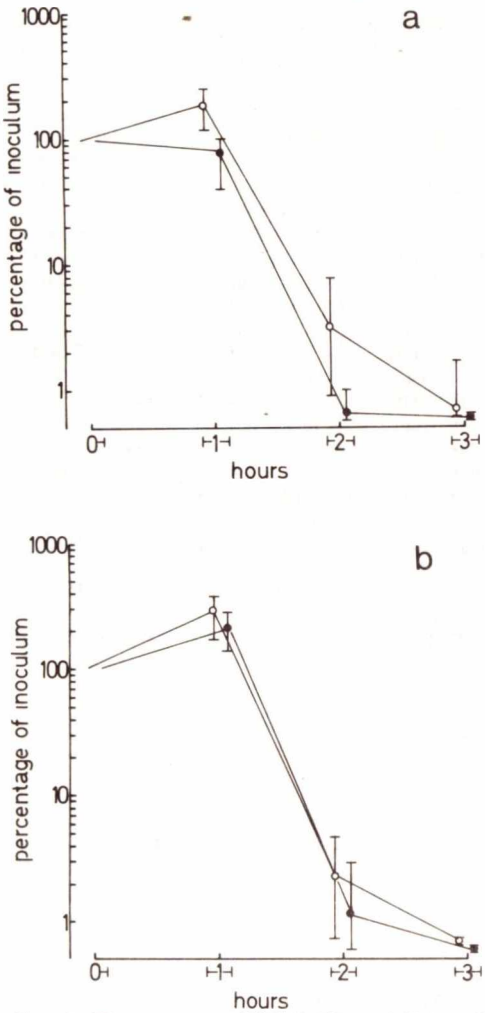

FIG. 2. *The response of K(A)$^+$ (○) and K(A)$^-$ (●) forms of E. coli C12 (a) and E. coli C37 (b) to normal human serum, determined by the method of Taylor et al. (36). The means and range of results from five determinations are presented for each strain. The difference between E. coli C12 K(A)$^+$ and K(A)$^-$ forms was significant (1 h, t = 15, P = 0.01; 2 h, t = 17, P = 0.05; 3 h, invalid); the difference between E. coli C37 forms was not (1 h, t = 30, P > 0.05; 2 h, t = 29, P > 0.05; 3 h, invalid).*

heritance of markers from *E. coli* C11, C15, C42, and P43 led to comparable, small increases in the serum resistance of progeny, although one of the donor strains was promptly sensitive, one was delayed sensitive, and two were serum resistant.

Although plasmids R1 and NR1, both belonging to incompatability group F II, were unable to influence the serum sensitivity of K-12 strains (Table 1), C8$^-$Nal^R progeny inheriting these plasmids were considerably more resistant than the R$^-$ parent (Table 3). The level of serum resistance attained by C8$^-$Nal^R NR1 (23% survival after 3 h) prompted us to introduce R12, a round of replication mutant of NR1. Progeny carrying this copy mutant, confirmed by the minimal inhibitory concentrations of chloramphenicol, showed a significantly greater increase in serum resistance than progeny carrying the normal NR1 plasmid ($n = 6$; $t = 21$, $P < 0.01$ at 1, 2, and 3 h).

Loss of R determinants by the promptly sensitive strain P21 was not accompanied by any change in serum reactivity, and inheritance of plasmids R1 and NR1 had no effect on the serum sensitivity of P21$^-$Nal^R (Table 3).

Colonies derived from strain C5 and cured of antibiotic resistance determinants gave serum responses identical to that of the parent strain.

E. coli C42$^-$, a cured derivative of C42 that had lost all antibiotic resistance determinants, was resistant to serum, but C42$^-$ cells increased in number less rapidly than did C42 parents when incubated in serum.

DISCUSSION

Reynard and Beck (29) have reported that the plasmids R1 and R100 (NR1) confer high levels of serum resistance on *E. coli* K-12 strains. We have been unable to confirm this observation; all the K-12 strains that we have examined (Table 1), including strains carrying R1, NR1, and other plasmids, were extremely sensitive to normal human and rabbit sera in the serum bactericidal systems of both Reynard and Beck (29) and Taylor et al. (36). Fietta et al. (8) have also recently found that the R1 plasmid is unable to confer any degree of serum resistance on K-12 strains in a system employing highly diluted human serum. These authors did report, however, that 8 of 26 plasmids examined conferred relative serum resistance to *E. coli* K-12 strains. A plasmid was said to have conferred relative resistance if the amount of serum needed to reduce the viable count to 1% after 30 min was greater for R$^+$ progeny than for R$^-$ parents. The amounts of serum needed to effect this degree of serum killing were extremely small (<3% of the total reaction mixture). Because many strains are able to grow rapidly in virtually undiluted serum (32), as shown in this study, we feel there can be little justification in the use of the term "serum resistant" in a situation in which only 1% of cells survive short exposure to highly diluted serum.

E. coli K-12 is morphologically rough due to a lesion in the chromosomal *rfb* locus determining synthesis of lipopolysaccharide O-side chains

TABLE 3. *Effect of plasmid receipt on the response of cured E. coli isolates to normal human serum*[a]

Host	Donor	Inherited marker(s)	% Survival[b] in serum after:		
			1 h	2 h	3 h
C8	—	—	162.0 ± 69.5 $t = 21$; $P > 0.05$	23.6 ± 7.0 $t = 15$; $P = 0.01$	6.6 ± 4.3 $t = 15$; $P = 0.01$
C8$^-$Nal^R	—	—	107.6 ± 36.9 —	3.7 ± 2.7 —	0.9 ± 0.8 —
C8$^-$Nal^R	C11	Ap, Cm, Sm, Col	187.5 ± 97.2 $t = 32$; $P > 0.05$	40.8 ± 38.7 $t = 22$; $P < 0.01$	13.0 ± 18.1 $t = 25$; $P < 0.05$
C8$^-$Nal^R	C15	Ap, Cm, Km, Tc, Sm	168.5 ± 30.7 $t = 30$; $P > 0.05$	46.0 ± 18.2 $t = 21$; $P < 0.01$	18.5 ± 7.6 $t = 21$; $P < 0.01$
C8$^-$Nal^R	C25	Ap, Cm, Sm, Col	119.6 ± 38.5 $t = 36$; $P > 0.05$	7.0 ± 3.5 $t = 35$; $P > 0.05$	1.7 ± 1.2 $t = 28$; $P > 0.05$
C8$^-$Nal^R	C42	Ap, Sm, Col	127.3 ± 36.4 $t = 21$; $P < 0.01$	6.8 ± 1.2 $t = 21$; $P < 0.01$	1.4 ± 0.3 $t = 21$; $P < 0.01$
C8$^-$Nal^R	P43	Ap	136.3 ± 31.4 $t = 21$; $P < 0.01$	13.1 ± 6.0 $t = 21$; $P < 0.01$	3.1 ± 1.3 $t = 21$; $P < 0.01$
C8$^-$Nal^R	M941 (R-Utretcht)	Ap, Tc	104.8 ± 36.7 $t = 27$; $P > 0.05$	2.0 ± 0.9 $t = 36.5$; $P > 0.05$	0.3 ± 0.2 $t = 34$; $P > 0.05$
C8$^-$Nal^R	SFI585 (NRl)	Cm, Sm, Su, Tc	208.8 ± 30.6 $t = 21$; $P < 0.01$	47.5 ± 17.0 $t = 21$; $P < 0.01$	23.2 ± 7.8 $t = 21$; $P < 0.01$
C8$^-$Nal^R	SFI587 (Rl)	Ap, Cm, Km, Sm, Su	153.3 ± 23.4 $t = 21$; $P < 0.01$	83.2 ± 12.1 $t = 21$; $P < 0.01$	73.2 ± 21.4 $t = 21$; $P < 0.01$
C8$^-$Nal^R	SFI609 (NRl)[c]	Cm, Sm, Su, Tc	276.7 ± 18.3 $t = 21$; $P < 0.01$	101.2 ± 12.0 $t = 21$; $P < 0.01$	80.7 ± 10.9 $t = 21$; $P < 0.01$
P21	—	—	<0.1	<0.1	<0.1
P21$^-$Nal^R	—	—	<0.1	<0.1	<0.1
P21$^-$Nal^R	SFI585	Cm, Sm, Su, Tc	<0.1	<0.1	<0.1
P21$^-$Nal^R	SFI587	Ap, Cm, Km, Sm, Su	<0.1	<0.1	<0.1

[a] Abbreviations as in Table 2.

[b] Estimated by the method of Taylor et al. (36). The value given is the mean ± standard deviation of six estimations, except in the case of C8 (5 estimations) and C8$^-$Nal^R (18 estimations). Each replicate of a strain was prepared on a separate occasion, and the appropriate Nal^R cured host was included with each set of tests. t and P values are comparisons of replicates of the test strain and equal number of replicates of the Nal^R cured host prepared on the same occasions.

[c] Round of replication mutant R12.

(30). Although a full complement of O-side chains does not directly determine serum resistance, it appears to be essential for its expression and to be responsible for the delayed killing effect (33); it therefore seems unlikely that plasmid-determined surface modifications could, in the absence of O-side chains, lead to significant levels of serum resistance. Direct support for this view is provided in the present study. Plasmids R1 and NR1 had no effect on the serum sensitivity of *E. coli* K-12 or of *E. coli* P21$^-$Nal^R, strains that were promptly killed by serum. However, when either of these plasmids was introduced into C8$^-$Nal^R, a cured strain derived from *E. coli* C8 and showing the delayed serum killing effect, the progeny became significantly less sensitive to the serum bactericidal system. Resistance was particularly marked with plasmid R1; over 70% of cells survived 3 h of exposure to serum, in contrast to a survival rate of under 1% for the cured host strain. A round of replication mutant of plasmid NR1 conferred much higher levels of serum resistance on C8$^-$Nal^R than the normal NR1, suggesting that the plasmid effect might be related to the number of copies per cell. Acquisition of plasmids by C8$^-$Nal^R from four of the six other donors led to significantly enhanced survival in serum, suggesting that the ability to modify serum responses in smooth hosts might be a common characteristic of plasmids. In addition, loss by curing of R and Col determinants carried by *E. coli* C8 led to an increase in serum sensitivity.

It is likely that complete serum resistance has a multifactorial basis. Some plasmids are clearly able to confer a degree of resistance on suitable host bacteria that synthesize lipopolysaccharide with a high degree of core substitution by O-side chains. However, plasmids from resistant strains C25 and C42 had, respectively, no effect and a small but significant effect on the survival of C8$^-$Nal^R in serum. The ability of a plasmid to modify the serum response of C8$^-$Nal^R was not, therefore, a reflection of the properties of the

original host strain. Loss of the K(A) antigen by delayed sensitive strain C12 led to a small increase in sensitivity; this antigen is therefore able to influence the outcome of the cell-serum interaction, but obviously does not afford complete protection.

Three of the four smooth, serum-resistant river isolates were resistant to at least five antimicrobial agents, and all carried determinants for transfer and colicinogeny. The remaining serum-sensitive strains did not exhibit so many extrachromosomally determined characters, suggesting that some strains may be particularly suited for a progressive accumulation of a wide range of determinants, enabling them to achieve a high selective advantage in potentially hostile environments.

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Rapid Screening for Plasmid DNA

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Summary. A procedure is described for demonstrating plasmid DNA and its molecular weight, based on rate zonal centrifugation of unlabelled DNA in neutral sucrose gradients containing a low concentration of ethidium bromide. Each DNA species is then visualized as a discrete fluorescent band when the centrifuge tube is illuminated with ultra-violet light. Plasmids exist as closed circular and as relaxed circular molecules, which sediment separately, but during preparation of lysates, closed circular molecules are nicked so that each plasmid forms only a single band of relaxed circles within the gradient.

1. Introduction

As a growing number of bacterial functions are tentatively being attributed to plasmids, there is a place for a simple and economical method for screening bacterial strains for the presence of plasmid DNA. The generally used methods depend on isotopic labelling of the bacterial DNA followed by either dye-buoyant isopycnic density gradient centrifugation or rate zonal centrifugation and measurement of the distribution of isotope within the gradient. Performed on any scale, these methods become extremely time-consuming whereas the procedure described here using unlabelled bacteria and visual identification of plasmid DNA achieves the same result far more rapidly.

In principle, unlabelled bacterial DNA is separated into predominantly plasmid and chromosomal fractions by differential centrifugation, and the plasmid fraction, after treatment with RNase, is analysed by rate zonal centrifugation through neutral sucrose gradients containing ethidium bromide. The subsequent position of plasmid DNA within the gradient, and hence its molecular weight, is determined by its fluorescence when the centrifuge tube is illuminated with ultra-violet light.

2. Materials and Methods

Details of the procedure are first described, followed by notes in the succeeding section.

Cultures. These were incubated at 37° on a reciprocating shaker (50 mm stroke; 100 strokes/min) in 250 ml capped conical flasks containing 50 ml YE2 broth ($NaH_2PO_4 \cdot 2H_2O$, 3 g; K_2HPO_4, 14 g; NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 2 g; Oxoid acid-hydrolysed casein, 5 g; Oxoid yeast extract, 5 g; L-cysteine HCl, 50 mg; DL-tryptophan, 50 mg; and glucose, 2 g per l distilled water). After 18–22 h incubation, the optical density at 650 nm was 2.5–2.9. The organisms were deposited by low speed centrifugation and resuspended as evenly as possible by a pasteur pipette in 1.1 ml 0.85% (w/v) NaCl to give ca. 1.3 ml suspension (Note 1).

Lysis. One ml 5% (w/v) sodium dodecyl sulphate (SDS) and 3 ml TES4 (0.1 M-Tris, 0.07 M-di-Na EDTA, 0.05 M-NaCl, pH 8.0) were placed in a 5 ml polycarbonate centrifuge tube (MSE 34411-111). One ml bacterial suspension was squirted into this lysing mixture from a calibrated pasteur pipette and the tube immediately inverted several times to mix its contents. Lysis often began immediately and was completed by transferring the tube to a 60° water-bath for 8 min, by which time the dense milky bacterial suspension had become uniformly semi-translucent (Note 2).

Enrichment for Plasmid DNA. The lysate was centrifuged in the same polycarbonate tube for 15 min at 30,000 rp.m. at 20° in the SW50.1 rotor of a Spinco L3-40 centrifuge. This deposited most of the chromosomal DNA (Note 3), leaving as the supernatant a 'cleared lysate' containing most of the plasmid DNA (Clewell and Helinski, 1969). The upper 3.0–3.5 ml supernatant was retained, the remainder being discarded with the deposit (Note 4).

RNase. The cleared lysate was placed either at 4° overnight or at 0° for 2–4 h until most of the SDS precipitated. The supernatant was removed and mixed with 0.1 vol RNase solution, 250 μg/ml (E.C.2.7.7.16. Bovine pancreatic ribonuclease, British Drug Houses, Poole, Dorset, England, ref. 39039. This was dissolved in distilled water and heated in a bath of boiling water for 10 min to inactivate contaminating DNase). The mixture was incubated at 37° for 30 min (Note 5). Finally, 0.1 vol ethidium bromide (40 μg/ml) was added (Note 6).

Sucrose Gradients. All sucrose solutions were prepared in TES4 and were autoclaved at 121° for 15 min to inactivate nucleases. Ethidium bromide was added to 4 μg/ml (Note 6). Linear sucrose gradients were prepared in 5 ml cellulose nitrate centrifuge tubes (Spinco 305050) by diffusion overnight at room temperature with the tubes capped to prevent evaporation. Each tube received 0.6 ml 21% (w/v) sucrose followed by 1.1 ml each of 17%, 13%, 9% and 5% (w/v) sucrose.

Centrifugation. A sample of 0.2–0.3 ml cleared lysate containing ethidium bromide was placed on the gradient (Note 7) which was immediately centrifuged at 35,000 r.p.m. in the SW50.1 rotor at 20°. The period of centrifugation is specified in Figure 1 and does not include either the run-up or the unbraked run-down times (ca. 1.8 and 12.7 min, respectively).

Visualization of Bands. The tubes were illuminated in a dark room with a hand-held 80 W mercury discharge lamp fitted with a Woods filter transmitting predominantly at 365 nm (Hanovia Ltd., 480 Bath Road, Slough, Bucks SL1 6BJ, England, ref. 16744/1). Plasmid DNA appeared as a thin fluorescent red band within the gradient (Note 6). The distance of migration was measured in mm from the interface between the sample layer and the top of the gradient.

3. Notes

1. Tests with calf thymus DNA in different concentrations of ethidium bromide suggested that as little as 0.5 μg double-stranded DNA could be seen in these gradients (Radloff, Bauer and Vinograd, 1967). Since the minimum volume of sample applied to the gradient was 0.2 ml, the minimum concentration of plasmid DNA required was 12.5 μg/5 ml lysate. Assuming plasmids comprise 1.5% of total bacterial DNA and that 10^9 *Escherichia coli* contain 8 μg DNA, then 12.5 μg plasmid should be found in ca. 10^{11} *E. coli* (e.g. 50 ml culture of O.D. 2.5). Certain plasmids comprise far more than 1.5% of total DNA. One is R6K which contributes ca. 20% in overnight culture (Kontomichalou, Mitani and Clowes, 1970) and, in that case, sufficient plasmid DNA can be isolated from as little as 7.5 ml overnight culture.

2. Successful lysis depends on prompt mixing of the organisms with the lysing mixture before lysis begins. Otherwise, partial lysis makes the dense suspensions so viscous that further mixing is impossible and large skeins of unlysed organisms persist in the tube. It was for this reason that lysates were prepared by SDS alone at 60° (Marmur, 1963), rather than with lysozyme and EDTA followed by detergent which never gave adequate mixing with these suspensions. Heating at 60° has the further advantages, first, of producing a homogenous collection of relaxed plasmid molecules (see Results) and second, taken with SDS, of being likely to render most Gram-negative pathogens harmless. In addition to *Escherichia coli,* this lysis procedure was found satisfactory with *Alcaligenes faecalis* (NCIB 8156), *Citrobacter freundii* (NCTC 9750), *Enterobacter aerogenes* (NCTC 10006), *Proteus vulgaris* (NCTC 4175), *Pseudomonas aeruginosa* (NCTC 10332), *Ps. fluorescens* (KH 597) and *Serratia marcescens* (NCTC 1377); but not with *Bacillus subtilis* (KH 468) or *Staphylococcus pyogenes* (UB 4008).

3. The conditions for the clearing spin were calculated initially on the assumption that, in these lysates, the chromosome of *Escherichia coli* would be unfolded but intact with a sedimentation coefficient (s) of ca. 250 (Stonington and Pettijohn, 1971). However, its observed s value appeared appreciably greater and centrifugation for 15 min at 30,000 r.p.m. was finally used. Excessive forces are undesirable at this stage for, although the chromosome is some 20 times bigger than a large plasmid like ColV-K94 (mol. wt. 94×10^6), its predicted s value is only about twice as great.

4. An alternative method for enriching plasmid DNA was also attempted in which an uncentrifuged lysate made with SDS is cooled to 4° so that most of the chromosomal DNA precipitates with the SDS, leaving predominantly plasmid DNA in the supernatant (Guerry, LeBlanc and Falkow, 1973). However, at the present DNA concentrations, the lysates were so viscous that the SDS had to be removed by ultracentrifugation so that this procedure lost any advantage over a clearing spin.

5. When cleared lysates were sedimented in sucrose-ethidium bromide gradients without being treated with RNase, two very prominent fluorescent bands were obtained which sedimented at 3.2 and at 4.8 mm/h, respectively. These bands disappeared after treatment with RNase but not DNase and were therefore presumed to be 16s and 23s ribosomal RNA.

6. When irradiated at 365 nm, double-stranded DNA complexed with ethidium bromide fluoresces 80 times more brilliantly than the free dye (Le Pecq, Yot and Paoletti, 1964). The concentration of ethidium bromide used here (4 μg/ml) is far less than the 100–200 μg/ml generally used to separate closed circular DNA. Bands can be seen at these higher concentrations but the visual contrast between the band and the surrounding gradient is far less than at low concentrations.

Ethidium bromide also renders DNA susceptible to nicking by visible light (Smith and Vinograd, 1972) and, although we found no evidence that this occurred in our experiments, the dye-DNA mixtures were kept from strong light as a precaution.

7. If more concentrated lysates were used, gradients generally failed due to migration of the sample as a diffuse band down the gradient, presumably following a density inversion.

4. Results

After centrifugation, the tubes showed one of three appearances. The most usual was that expected: a sharply defined fluorescent band of plasmid DNA perhaps 0.5 mm deep in the main part of the gradient, with a more brilliant band just below the interface between the sample and the gradient, presumed to consist of small DNA and RNA fragments. Occasionally, the main part of the gradient showed either a diffuse band perhaps 10 mm deep (Note 7) or no band whatsoever which, with known plasmid-carrying strains, was taken to follow loss of plasmid during the clearing spin or too small a sample. When either of the latter occurred, the expected result was usually obtained on running another sample or by preparing another lysate. No sharp band was ever seen with known plasmid-free strains.

The behaviour of plasmids whose molecular weights ranged from 4.8×10^6–61×10^6 is shown in Figure 1. These fell into three groups, depicted by curves A, B and C, each of whose members sedimented at the same rate. In each group, the distance sedimented was proportional to the period of centrifugation. That is, in these linear gradients the increased centrifugal force on passing down the gradient cancelled out the increasing viscosity and density of sucrose to produce isokinetic conditions in which the velocity of sedimentation (v) was constant. The

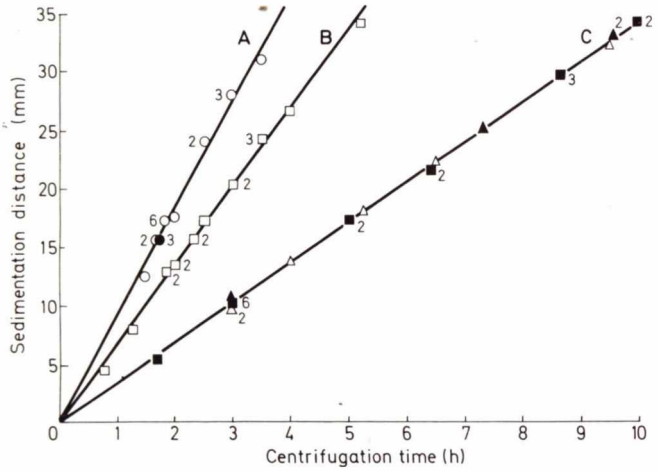

Fig. 1. Sedimentation distance of plasmid DNA in linear 4–21% (w/v) sucrose gradients, pH 8.0, plotted against centrifugation time (SW50.1 rotor, 20°, 35,000 r.p.m.). Many points are averages of several readings, as indicated by the accompanying numbers. The plasmids fell into three groups: Curve A ($v=9.1$ mm/h): ColIb-P9 (○), P1 prophage (●). Curve B ($v=6.7$ mm/h): R6K (□). Curve C ($v=3.4$ mm/h): ColE1-K30 (■), ColE2-P9 (△), colK-235 (▲). All plasmids were in *Escherichia coli* K-12 except for the P1 prophage which was in *E. coli* B

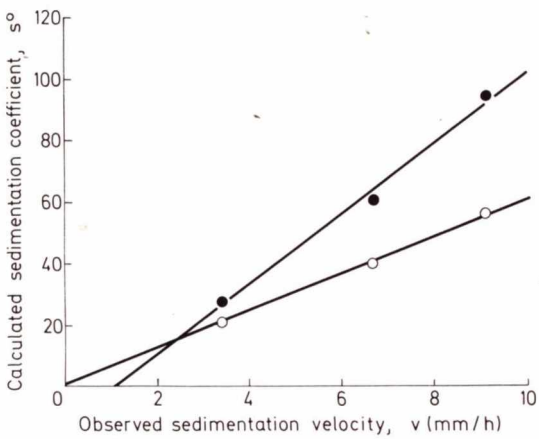

Fig. 2. Values of $s°$ calculated from reported molecular weights plotted against observed values of v (mm/h from curves A, B and C of Fig. 1). Closed symbols: closed circular DNA; open symbols: relaxed circles

observed sedimentation coefficient (s) was therefore proportional to v.

The relative s values thus obtained from Figure 1 for each group of plasmids suggested that the plasmid DNA was sedimenting as relaxed circular molecules. This follows from the relation between sedimentation coefficient and molecular weight: $s° - c = aM^b$, where $s°$ is s at infinite dilution, M is plasmid molecular weight, and a, b and c are constants whose values depend on whether the DNA is closed circular, relaxed circular or linear (see Böttger et al., 1971, and footnote to Table 1. A special case assumes $c=0$: Burgi and Hershey, 1963; Bazaral and Helinski, 1968). In Table 1, reported values of M were used with published values for a, b and c to predict $s°$. The ratios of these predicted $s°$ values were then compared with the observed relative values of v, taking the values of the smallest plasmids as unity. Table 1 shows that the observed ratios of 1.97 and 2.68 did not agree with those predicted for closed circles (2.25 and 3.49) but were consistent with those expected for relaxed circles (1.95 and 2.76). This is expressed

Table 1. Comparison of calculated and observed values

Plasmid	Reported value of M[a]	Calculated of $s°$[b]		Ratio of $s°$ values		Observed ratio of v†
		(1)	(2)	(1)	(2)	
ColE1-K30 ColE2-P9 ColK-235	4.8×10^6	26.9	20.2	1	1	1
R6K	26×10^6	60.5	39.4	2.25	1.95	1.97
ColIb-P9 P1 prophage	61×10^6	93.8	55.8	3.49	2.76	2.68

M: mol. wt. of plasmid DNA. $s°$: sedimentation coefficient at infinite dilution. v: observed velocity of sedimentation

[a] From Clowes (1972), Hardy, Meynell, Dowman and Spratt (1973), and Walker and Anderson (1970)

[b] Calculated from $s° - c = aM^b$ for closed circular (1) and for relaxed molecules (2). Values for the constants come from Böttger et al. (1971): (1) $a=0.00439$, $b=0.553$, $c=5.16$; (2) $a=0.0219$, $b=0.435$, $c=2.5$

† Calculated from observed values of v in Figure 1: curve A, 9.1; curve A, 9.1; curve B, 6.7; curve C, 3.4 mm/h

in another form in Figure 2 where calculated values of $s°$ from Table 1 are plotted against observed values of v from Figure 1. The expectation from $s \propto v$ is that the plot will extrapolate to the origin (i.e. when s is 0, v must be 0). However, this was found only for the plot for relaxed circles. Applying the alternative relationship, $s = aM^b$, led to the same conclusion. The values of b reported for closed and relaxed circular molecules are 0.43 and 0.36, respectively (Bazaral and Helinski, 1968; Barth and Grinter, 1974); our observed value, taken from log v plotted against log M, was 0.38.

Since at least part of the plasmid DNA in vivo was likely to consist of closed circular molecules, these results suggested that nicking occurred during preparation of the lysates. Two causes were considered: DNase contaminating the RNase preparation, despite heating at 100° (McCormick, Larson and Maher, 1974) or thermal hydrolysis during incubation of the lysate at 60° as opposed to the more usual 37°. Tests on lysates containing R6K, prepared with RNase, showed that a 60° lysate produced only a single band ($v = 6.5$ mm/h), presumed to be relaxed circles (Table 1), whereas a 37° lysate produced two bands, one again sedimenting at 6.5 mm/h and a second faster, more conspicuous, band sedimenting at 10 mm/h. The ratio of these rates was $10/6.5 = 1.54$ which is the ratio expected from Table 1 for closed circular and relaxed molecules (60.5/39.4 or 1.54). This conclusion was confirmed by electron microscopy of the DNA within each band. Other lysates of R6K were prepared with and without treatment with RNase (this is possible with R6K because of its high concentration (Note 1) and because it sediments faster than 16s and 23s rRNA), both at 60° and at 37°. Heating at 60°, with and without RNase, produced one band ($v =$ 6.5 mm/h); heating at 37°, with or without RNase, yielded two bands ($v = 6.5$ and 10 mm/h) which were respectively taken to be relaxed molecules, derived in part from relaxation complexes (Clewell and Helinski, 1969), and closed circular molecules. Hydrolysis at 60° was therefore presumed to be the main cause of nicking.

5. Discussion

This procedure should simplify the screening of strains for plasmid DNA, by substituting visual detection of DNA within the centrifuge tube for isotopic labelling (Hughes and Meynell, 1976). An alternative based on visual detection of plasmid DNA in ethidium bromide-agarose gels has been described by Meyers, Sanchez, Elwell and Falkow (1976). The present method uses a simpler method for preparing the DNA and provides sufficient material for electron microscopy (Lang and Mitani, 1970; Inman and Schnös, 1970). Its success depends on isolating sufficient plasmid DNA for immediate centrifugation which depends in turn on achieving uniform lysis of dense bacterial suspensions. What was not forseen was the excellent reproducibility of the results, considering that internal molecular weight standards are usually thought necessary to calibrate such gradients. This reproducibility presumably arose in part from the extreme sharpness of the fluorescent plasmid bands, so that there was virtually no error in determining their position, and partly because the bands were examined in situ, so avoiding artefacts during removal of the gradient for sampling. The advantage of nicking during lysis is in producing a single band for each plasmid which would otherwise be expected to produce two bands: i.e. closed circles as well as relaxed circles. This obviously simplifies the detection of two different plasmids in the same host, as we have confirmed with *Escherichia coli* K-12 carrying both ColIb-P9 (mol. wt. 61×10^6) and ColE1-K30 (mol. wt. 4.8×10^6). Furthermore, the $s°$ value of relaxed circles, as distinct from closed circles, is almost unaffected by the ratio of ethidium bromide: nucleotide (Bauer and Vingrad, 1968).

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Two Major Groups of Colicin Factors: Their Molecular Weights

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Summary. Colicin factors are thought to fall into two taxonomic groups which differ in, amongst other properties, the molecular weight of the plasmid DNA and the host range of the colicin protein. This hypothesis is supported by the plasmids found in 26 colicinogenic strains. Two small Col factors may have arisen from larger factors, judging from similarities between their colicins.

1. Introduction

Bacterial plasmids can be allotted to taxonomic groups by a variety of criteria. In the case of colicin (Col) factors, Hardy, Meynell, Dowman and Spratt (1973) suggested that there existed only two major groups. I and II, defined by criteria like the effect on colicin titres of the host *recA* allele, the proportion of colicin bound to the cells, the molecular weight of the plasmid DNA and the specificity of the colicin protein as reflected in the bacterial alleles determining colicin insensitivity. They therefore suggested that Col factors of Group I had evolved from a small "EK-like" ancestor of molecular weight ca. 5×10^6 whereas Group II was descended from a larger "BIV-like" ancestor (m.w. ca. 60 $\times 10^6$). This interpretation was subsequently supported by Davies and Reeves (1975a, b) from an extensive study of the host range of colicins on colicin-insensitive mutants. They found that a colicin of their Group A (colicins A, E, K, L, N, S4 and X) was never active on a bacterial mutant selected by a colicin of their Group B (colicins B, D, G, H, I, M, Q, S1 and V), and vice versa. Hence, Col factors of Groups I and II of Hardy et al. (1973) appear to specify colicin proteins of Groups A and B of Davies and Reeves (1975a, b).

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The present paper describes the molecular weights of plasmids found in standard colicinogenic strains, many kindly provided by Dr. Reeves. The results generally agree with expectation, although some discrepancies occur which may throw further light on the evolutionary relationships between naturally-occurring Col factors.

2. Materials and Methods

Bacteria. Colicinogenic strains are shown in Table 1 with their colicins and cross resistance groups according to Davies and Reeves (1975a, b). Colicin-sensitive indicator strains were *Escherichia coli* K-12 strain C600 (our M468), and the mutants *tonB* (Nomura and Witten, 1968, obtained as Frédéricq's G43, i.e. M1200) and *tolII* (Gratia, 1964; obtained as Nomura's KR21, i.e. M1199). Antibiotic resistances were determined by flooding nutrient agar plates (Oxoid CM55) with overnight cultures and adding Oxoid Multodisks containing ampicillin (10 μg), cephaloridine (15 μg), chloramphenicol (10 μg), kanamycin (5 μg), streptomycin (10 μg) and tetracycline (10 μg). Chromosomally-determined streptomycin resistance was detected with discs containing 200 μg streptomycin.

Molecular Weights of Col Factors. Two methods were used:

a) Rate zonal sedimentation in sucrose-ethidium bromide gradients, the distance of migration and hence the molecular weight being determined visually under ultra-violet light (Hughes and Meynell, 1977).

b) Electron microscopy. Colicinogenic strains were grown in 1 l nutrient broth (Oxoid CM67) in 2 l flasks on an orbital shaker (100 r.p.m.) at 37° C. Bacteria were collected by low speed centrifugation and lysed according to Guerry, LeBlanc and Falkow (1973), with three exceptions: volumes were twenty times greater; lysozyme and sarkosyl were made up in TES (0.05 M Tris, 0.005 M di-sodium EDTA, 0.05 M NaCl, pH 8); and NaCl was not added after lysis. Cleared lysates were obtained after centrifugation at 15° C for 60 min at 40,000 r.p.m. in a MSE 6×5.5 ml swing-out rotor. Caesium chloride (4.6 g) and ethidium bromide (to 500 μg/ml) were added to 4.8 ml cleared lysate. Plasmid DNA was then isolated by dye-buoyant density gradient centrifugation in the same rotor at 15° C for 40 h at 40,000 r.p.m. Supercoiled DNA, the lower band seen under u.v. illumination, was obtained by syringe.

Large plasmids (m.w. $>40 \times 10^6$) were generally found to relax spontaneously. Smaller plasmids were nicked, as the DNA-ethidium

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bromide complex in polycarbonate tubes, by exposure to visible light (100 W bulb at 40 mm for 7–12 h at 4° C). Ethidium bromide was removed by three extractions with propan-2-ol saturated with CsCl in TES, and the CsCl removed by dialysis for ca. 44 h at 4° C against 1 l volumes of TES-EDTA buffer (0.01 M tris, 0.001 M EDTA, pH 8.5).

Molecules were prepared for microscopy by the aqueous technique of Davis, Simon and Davidson (1971). Grids were shadowed with platinum-palladium at an angle of 6° on a vacuum coating unit (Edwards type 306) and examined in an A.E.I. 801 A transmission microscope. Plasmid contour lengths were measured from projected micrograph negatives using a map measurer. Molecular weights were then estimated by using either a diffraction grating or an internal standard such as ColEl-K30.

3. Results and Discussion

Table 1 summarizes the results which are of three kinds:

(i) Grouping by host range of the colicin. Inspection of Davies and Reeves results suggested that their Group A, presumed by us to be specified by small Col factors of our Group I, were active on *tonB* but never on *tolII* indicators; and that the reverse held for their Group B colicins (Davies and Reeves, 1975a, Table 1; Davies and Reeves, 1975b, Table 5). All the strains used here were therefore tested against these indicators and found to behave as expected.

(ii) Antibiotic-resistance. This does not appear to have been reported previously although it is clearly material since a strain may carry R factors as well as Col factors. In fact, strain 398 appeared to have both types of determinant on the same plasmid. Some strains showed high level streptomycin resistance, denoted S(h) in Table 1, presumed to be due to mutation on the chromosomal *strA* locus.

(iii) Molecular weight determinations. All but three strains were allotted to Groups I or II initially by the rapid screening method of Hughes and Meynell (1977) based on rate zonal sedimentation in sucrose-ethidium bromide gradients. The majority of strains were then examined by electron microscopy. When the contour lengths were distributed unimodally, the mean and its standard deviation were shown in Table 1. However, four strains yielded mixed populations of plasmids and these distributions are shown as histograms in Figure 1. Thus, strain 23 (ColA) showed three modes, at 1.2, 2.0 and ca. 4.0×10^6, of which the last may be a dimer of the second. Strain CA58 (ColH) yielded molecules ranging from $0.8–40.5 \times 10^6$. This heterogeneity may perhaps have arisen in the bacterial stocks from which cultures were inoculated.

Table 1 supports the general hypothesis that the small Group I Col factors specify colicins of Group A specificity and that the large Group II factors specify colicins of Group B specificity. This conclusion is further supported by previous observations not included here on other members of Group I (ColEla-16, ColE2-P9, ColK-235; Hardy and Meynell, 1972; Hardy et al., 1973) and Group II (ColB-K77, ColVB-K260; Hardy, 1975, Table 1). Some strains, like 235, 284 and 285, each produced two Group B colicins but only one class of plasmid DNA was detected: that is, the data do not distinguish between two different colicins specified by one plasmid and two plasmids of about the same size, each specifying a different colicin.

Some discrepancies would be expected, since a plasmid might be either larger or smaller than expected

Table 1. Properties of colicinogenic strains and their plasmids

Strain[a]	Colicins[b]	Group[b]	Antibiotic resistances[c]	Plasmid molecular weights by:	
				sedimentation[d]	microscopy[e]
23	A	A	–	I	<5 (*h*)
CA31	A	A	Cr	I	<5 (*h*)
K53	E1	A	–	I+II	Complex (*h*)
K30	E1	A	–	n.d.	4.2[f]
AB1157	E2	A	S(h)	n.d.	4.4 (0.25)
CA42	E2	A	–	I	3.8 (0.27)
12-317	E2	A	S(h)	I	n.d.
W3110	E3	A	S(h)	I	4.6 (0.19)
CA38	E3	A	–	n.d.	4.6 (0.16)
K216	K	A	–	I	n.d.
235	K+X	A	–	I	4.5 (0.09)
284	N+E3	A	T	I	3.8 (0.49)
285	N+E3	A	T	I	n.d.
CA23	D+X	A+B	S(h)	I	3.7 (0.23)
398	L	A	S(l), C, Cr, A, T	(I)	19.1 (1.13)
CA53	Ia	B	–	II	60.3 (1.51)
ST4	Ib-P9	B	S(h)	II	58.8 (1.72)
J5-3	Ib-P9	B	S(h)	II	n.d.
K89	B+M	B	–	II	42.8 (2.7)
CA46	G	B	–	I	3.9 (0.2)
CA58	H	B	–	I+II	Complex (*h*)
CA7	V	B	–	II	56.6 (3.1)
KH500	VIa-K94	B	S(h)	II	81.2 (2.8)[g]
II	I, Q, E1, D	A+B	–	I+II	n.d.
CA62	E1, I	A+B	–	I+II	n.d.

[a] *Escherichia coli* except for *Citrobacter freundii* CA31, *Salmonella typhimurium* ST4 and paracolon CA62

[b] From Davies and Reeves (1975a, b) except for K30 (=KH573 from K.G. Hardy), AB1157 (=KH293, Hardy et al. 1973), CA38 (=KH28), J5-3 (=KH596) and KH500 (=AB1157 ColV$^+$)

[c] From tests against antibiotic discs (Materials and Methods). A, ampicillin; C, chloramphenicol; Cr, cephaloridin; S(h) and S(l), high and low level streptomycin resistance; T, tetracycline

[d] From Hughes and Meynell (1977) using visual inspection of plasmid bands *in situ*. I: mol. wt. ca. 5×10^6. II: mol. wt. $40–70 \times 10^6$. (I) ca. 20×10^6; see text

[e] From electron microscopy (Materials and Methods). Brackets: standard deviation. *h*: histogram in Figure 1

[f] Tomizawa (1974)

[g] K.G. Hardy, personal communication

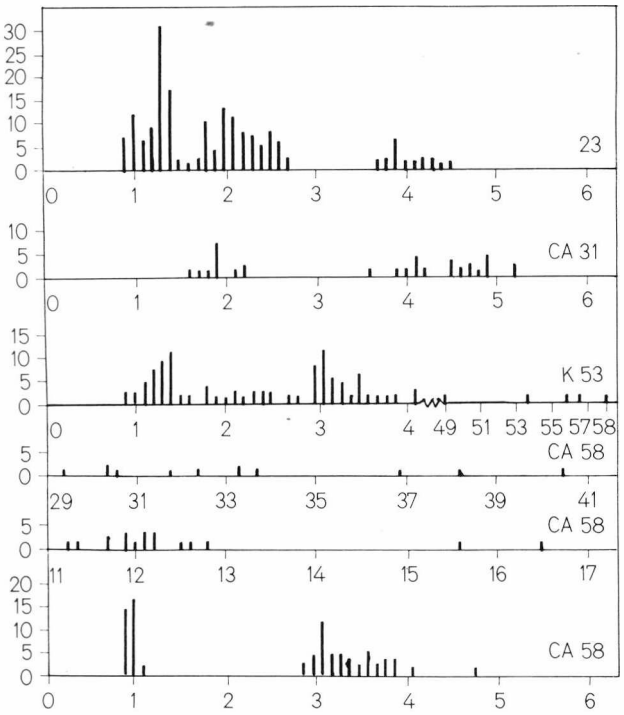

Fig. 1. Histograms of the mixed populations of strains 23 (ColA), CA31 (ColA), K53 (ColEI) and CA58 (ColH, 3 histograms). The horizontal axis shows the molecular weight $\times 10^6$, plotted to the nearest 0.1; the vertical axis is the number of plasmids found at a given molecular weight

from the host range of its colicin. Both types of discrepancy were found here. Strain 398 formed colicin L which belongs to Group A and its plasmid was therefore expected to be Group I but the molecular weight, instead of being ca. 5×10^6, was 19.1×10^6. However, this strain was multiply antibiotic-resistant (including low-level streptomycin resistance, presumed to be plasmid-determined) and may therefore carry a single plasmid with linked colicin and antibiotic-resistance determinants. The alternative type of discrepancy was shown by strains CA23 and CA46. Strain CA23 formed colicin D which belongs to Group B but its plasmid has a molecular weight of only $3.1–3.7 \times 10^6$ (Table 1: Timmis, Cabello and Cohen, 1974). Strain CA46 formed colicin G, also belonging to Group B, but its plasmid was only 3.9×10^6. It is striking that colicin D is very similar to colicin B (Davies and Reeves, 1975a; Pugsley and Reeves, 1977) and that colicin G is likewise very similar to colicin H (indeed they were once grouped together as colicin P: Frédéricq, 1953). Colicin factors D and G may therefore have arisen from extensive deletions of ColB and ColH, respectively, a possibility that can clearly be tested by their degree of homology.

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