STUDIES ON ANAEROBIC R FACTOR TRANSFER IN FACULTATIVE AND ANAEROBIC ENTERIC BACTERIA

Thesis

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HILDEGARD LAURA MOODIE

Department of Botany and Microbiology

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CONTENTS

Page

PART I

STUDIES ON THE TRANSFER OF R FACTORS AND OTHER SEX FACTORS IN ESCHERICHIA COLI UNDER ANAEROBIC CONDITIONS.

Introduction 1
The Transfer of R factors and other sex factors in E. coli under anaerobic conditions.
Summary 12
(A) Materials and Methods 13
(B) Results 18
(C) Discussion 38

PART II

STUDIES ON R FACTOR TRANSFER IN OBLIGATE ANAEROBIC BACTERIA FROM THE GASTROINTESTINAL TRACT.

Introduction 41
Chapter 1: The Isolation of Obligate Anaerobic Faecal Bacteria.
Summary 54
(A) Materials and Methods 55
(B) Results 64
Chapter 2: Determination of Drug Resistance Spectra of Anaerobic Strains.
Summary 73
(A) Materials and Methods 74
(B) Results and Discussion 76
Chapter 3: Tests for Transferable Drug Resistance in Anaerobic Strains.
Summary 88
(A) Introduction 89
(B) The Isolation of Mutant Strains 90
(C) Transfer Experiments 93

Page

Chapter 4: Identification of Anaerobic Strains.

Summary 98

(A) Methods and Results 99

Chapter 5: Discussion 105

Appendix A 110

Appendix B 117

References 119

ABBREVIATIONS

amp	ampicillin
cpd	cephaloridine
cml	chloramphenicol
ery	erythromycin
kan	kanamycin
lin	lincomycin
nal	naladixic acid
neo	neomycin
nft	nitrofurantoin
pen	penicillin
rif	rifampicin
str	streptomycin
tet	tetracycline
van	vancomycin
O_2	oxygen
CO_2	carbon dioxide
H_2	hydrogen
N_2	nitrogen
P_{O_2}	partial pressure of oxygen
UV	ultraviolet
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
Incubation	incubation was at $37^{o}C$ unless otherwise specified.
Drugs	the terms drugs, antibiotics and chemotherapeutic agents are used synonymously.

PART 1

STUDIES ON THE TRANSFER OF R FACTORS AND OTHER SEX FACTORS IN *ESCHERICHIA COLI* UNDER ANAEROBIC CONDITIONS.

Introduction

R factor mediated transfer of antibiotic resistance between *Enterobacteriaceae* has been reported to occur in the mammalian gastrointestinal tract (Farrar *et al*, 1972; Guinée, 1970; Kasuya, 1964; Reed *et al*, 1969; Wiedemann *et al*, 1970). *In vivo* conjugal transfer of genetic material has also been demonstrated with F^1, F^+ and Hfr *Escherichia coli* strains (Jones & Curtiss, 1970). The environment in the lower gastrointestinal tract, where bacteria are abundant, is mainly anaerobic. This is demonstrated by the dominance of obligately anaerobic bacteria such as *Bacteroides* species (Finegold, 1969; Moore *et al*, 1969) and direct studies of intestinal gas composition (Askevold, 1956). However, most laboratory investigations of the incidence of R factors and their transfer frequencies have been performed under aerobic conditions using faecal facultative strains. The only investigation of resistance transfer under anaerobic conditions *in vitro* is that of Mitsuhashi (1965), who reported complete inhibition of transfer of an R factor from a *Shigella flexneri* donor to an *Escherichia coli* recipient. In addition, Fisher (1957) reported restriction of chromosomal transfer by an *E. coli* Hfr strain under anaerobic conditions in various media.

On the basis of these results, it could be questioned whether *in vivo* R factor transfer is in fact possible (Chabbert *et al*, 1969). The contradictory situation prompted a re-examination of conjugation in facultative strains under anaerobic conditions. Both Fisher (1957) and Mitsuhashi (1965) obtained anaerobic conditions by evacuation. In this investigation, both mating and selection of recombinants were performed under stringent anaerobic conditions using methods de-

veloped for the isolation of obligate anaerobes (Hungate, 1969) to obtain a degree of anaerobiosis similar to that found *in vivo*.

The Mechanism of Bacterial Conjugation

In view of the controversy about anaerobic R factor transfer, it is interesting to note that Curtiss *et al* (1969) reported increased production of sex pili by *E. coli* cells under anaerobic conditions. The process of bacterial conjugation is not yet fully understood and the role of pili is also not clear. However, it is now generally accepted that they are essential, at least for the formation of specific pairs prior to the actual transfer of genetic material (Curtiss, 1969).

Since the discovery of conjugation by Lederberg and Tatum (1946), a great deal of experimental evidence has accumulated regarding the genetic and phenotypic differences between mating types. Actual unions between donors and recipients were first observed by phase contrast microscopy (Lederberg & Tatum, 1946). Anderson *et al* (1957) took electron micrographs and described cellular bridges with the same internal appearances as adjacent cytoplasmic areas. They proposed the "conjugation bridge" theory which suggests that genetic material passes through such a cellular structure. Brinton and his associates (Brinton *et al*, 1964; Brinton, 1965) were the first to question this model and suggested that the bridges were artefacts. They proposed that DNA passes from donor to recipient through the pili. This is now known as the conjugation tube or pilus conduction model. The presence or absence of intercellular bridges and the extent to which pili are involved in the actual transfer of DNA are still controversial issues.

Pili were first so named by Brinton *et al* (1964). Previous work had shown the presence of a periodate-sensitive surface antigen on F^+ cells and the existence of phages specific for donors and recipients (Curtiss, 1969). Brinton demonstrated the control of sex pili on an F^+ cell by the F factor, by showing the simultaneous elimination of male phage susceptibility, F pili and the sex factor by treatment with acridine

orange. The existence of different kinds of pili controlled by a variety of sex factors other than F has now been demonstrated. R factors specify at least two types of pili, F-type and I-type. (For discussion see Marcos, 1972.)

Investigation of the chemical nature of pili and their assay depend on successful purification. This is often hampered as sex pili tend to clump to common pili (Beard et al, 1972). One method of assay is electron microscopy, where numbers of pili are simply counted (Brinton, 1965). Another technique is to measure the total number of phage binding sites in a sample of pili by allowing adsorption of radioactively labelled male specific phages along the length of the pili. However, this method assumes that all adsorption sites bind phage equally (Novotny & Lavin, 1971), whereas purification procedures often alter binding properties (Beard et al, 1972). A third method measures the binding of pilus antibody along the whole length of the pilus (Novotny & Lavin, 1971). Monospecific sera which can only react with a single serotype of sex pilus can be prepared. This method of differentiation is more specific than phage typing, where an F-specific phage will infect all cells producing F-like pili, although with different efficiencies of plating and plaque morphologies (Harden & Meynell, 1972; Nishimura et al, 1967). Recently Beard et al (1972) succeeded in obtaining 5 mg of intact pili of 98% purity from 100 gm of cells from a culture of an E. coli strain carrying a derepressed R factor. Pili are composed mainly of the protein pilin. Although reportedly sensitive to lipid solvents, their buoyant density of 1,309 gm/cm^3 makes a high lipid content improbable. Brinton has found evidence for protein subunits of molecular weight 11,800 with two phosphate groups and one D-glucose residue per molecule. These subunits are probably arranged in a helical manner to form the tubule (Beard et al, 1972).

The sex pili specified by different types of sex factor vary considerably (see Marcos, 1972). The length of F pili as determined by Brinton (1965) and Lawn (1966) ranges from a fraction of a micron to over 20 μ, with an average diameter of

85 - 95 A^{o}. They appear to have an axial hole of 20 - 25 A^{o} diameter and a central dense line of 15 A^{o} maximum diameter on the surface when prepared for electron microscopy by the negative contrast technique. F-like R factor and col factor determined pili are very similar, but show antigenic differences (Lawn et al, 1967). In contrast, I-pili (determined by some fi^{-} type R factors and col factors) are usually shorter, with a maximal length of 2 μ and the axial canal is rarely seen. Terminal knobs of various shapes are often present in both F and I pili (Lawn, 1966; Lawn et al, 1967). These were thought to represent a membranal base or root of the pilus, but since they are present when the pili first emerge, this seems unlikely. The knobs may be an extrusion of part of the cell wall - plasma membrane complex with the emerging tip. It is also possible that the pilus tip has a special affinity for the cell wall material and that a stable association can occur with particles of cell wall complex present in the culture used for microscopy (Lawn, 1966). Phages specific for either F or I pili have been found (see Marcos, 1972). A l-DNA phage, lKe, specific for bacteria carrying some fi^{-} R factors which are insensitive to I-phages (If_1 and If_2) has recently been discovered. This suggests a third type of pilus is present on these bacteria (Khatoon et al, 1972).

The number of pili on a repressed cell is usually one to three, corresponding to the number of sex factors present. Derepressed mutants have more pili; for example Beard et al (1972) obtained up to 9 pili per cell in the R1drd mutant by using optimal culture conditions. However, it seems there are fifty or more potential sites per cell for pilus extrusion. This was demonstrated by Lawn and Meynell (1972), who exposed metabolically active bacteria to pilus antiserum, which caused a great increase in the number of pili per cell. This effect could be due to the prevention of pilus retraction by antibody binding, or a disruption in the equilibrium between free pilin molecules and polymerised pili at the assembly site. Alternatively, antibody may stimulate pilus production in an indirect way.

The synthesis of pili has been studied by using metabolic inhibitors (e.g. NaCN) and antibiotics with a known site of action. Among these are mitomycin C and nalidixic acid, which inhibit DNA synthesis and chloramphenicol, which inhibits protein synthesis. Thymine starvation and exposure to ultraviolet light have also been used to disrupt DNA structure (Brinton, 1965; Novotny et al, 1972). Such studies have revealed that cells contain pools of pilin and that the actual assembly of these molecules is independent of protein or DNA synthesis. Energy is required for assembly, possibly for phosphorylation of pilin and probably also for retention of pili on the cell surface. Exposure to high doses of ultraviolet light results in the production of fewer and shorter pili. The single hit kinetics obtained suggest that DNA is present at the site where pili are produced and may be involved in assembly. This data supports the theory that sex factor DNA is located near the cell surface on a mesosome and that the pilus is extruded at the point of attachment. Since pili have never been seen in the cytoplasm of a cell and blended cell extracts do not show pilus-serum blocking power, it appears that assembly must occur at or near the surface. The site of assembly is probably the membrane, since pili have been seen on spheroplasts, which lack a cell wall (Novotny & Lavin, 1971).

Various procedures which inactivate pili also cause a loss of donor ability. Examples of such procedures are blending, starvation of cells, the formation of F^- phenocopies and the addition of donor specific phages, especially those adsorbing to the tips of pili. The frequency of specific pair formation between donor and recipient is directly proportional to the mean number of pili per donor (Curtiss et al, 1969). Variations in cultural conditions can also affect piliation and therefore fertility. For instance, more pili are produced if donor cultures are grown in a rich medium (Curtiss et al, 1969). As mentioned earlier, Curtiss et al also found maximal piliation when donors were grown under mild anaerobic conditions.

It is interesting to note that Beard et al (1972) found vigorous aeration with minimal foaming to be optimal.

Microscopic examination of mating bacteria revealed pairs which streamed together in liquid (Curtiss et al 1969; Brinton, 1965). The bacteria were not in wall to wall contact, but appeared to be connected by an invisible thread approximately the length of a pilus. The addition of male specific phages to label donor pili showed that these gaps between pairs were in fact spanned by pili. Early attempts to demonstrate transfer between separated pairs on an agar slide were unsuccessful (Curtiss, 1969), but this has more recently been achieved with conjugating cells isolated with a micromanipulator (Ou & Anderson, 1970). These workers found two types of mating pairs resistant to separation by pipetting; intimately and loosely connected pairs. The latter never came into physical contact, but were separated by a distance of up to 3 μm. Motile male cells were seen towing non-motile F^- cells through the medium as though connected by an invisible thread, which was probably the pilus. By observing individual pairs from the time of mixing, it was concluded that transfer in some cases must have occurred while the pair was loosely connected. This suggests that DNA transfer may well occur via the pili, as originally proposed by Brinton et al. This theory is also based on physical considerations, since the dimensions of the axial hole are consistent with conduction of a molecule the size of DNA. Other experimental support for the model is provided by the work of Novotny et al (1968), who found that the presence of another nucleic acid molecule in a pilus prevented conjugation. They added RNA phage to a mating mixture and found that a step in infection prior to penetration prevented the formation of mating pairs. In addition, phage could prevent the completion of mating if added to existing mating pairs. They suggested that the conformation of the pilus had been altered by phage adsorption, thus altering its functioning. Release of phage RNA would also block conduction through the pilus. Similarly, Salzman

(1971) found a specific decrease in mating efficiency if R^+ cells with F or I type pili were pretreated with F or I specific phage respectively. The non-specific effect on the heterologous R factor was very slight, indicating that each pilus functions in co-ordination with its sex factor. The presence of pilus antibody in a concentration too low to completely coat the pili can also block conjugation (Harden & Meynell, 1972). The use of monospecific sera demonstrated that this interaction was also very specific for each pilus type.

In one version of this model, the pilus is compared with a rodlike virus. The pilin coat is assembled around the F factor DNA by polymerisation of presynthesised subunits. Contact of the tip of the pilus with a receptor site releases the DNA in a manner similar to viral infection. It has also been suggested that the pilus itself moves across and is disassembled in the membrane of the recipient (Brinton, 1965; Novotny et al, 1968; Meynell & Ewins, 1973). One objection to the model is that, in order to allow for infections by different types of virus and for conjugation, different kinds of nucleic acid must be actively transported in two different directions inside the pilus. It is difficult to conceive of a molecular mechanism whereby a simple protein polymer like a pilus could achieve this (Marvin & Hohn, 1969). The other major objection is that pili containing nucleic acid have never been isolated. However Curtiss (1969) suggested that nucleic acid would slip out of the pili if they became separated from the donor.

The other classical theory of conjugation bridges was initially based on electron micrographs of conjugating bacteria, (Anderson et al, 1957; Gross & Caro, 1966). The model proposed that close contact is required between mating partners, leading to partial rupture of the cell walls and then connection between the cell membranes. Genetic transfer would then occur through these connecting bridges. Similarly, Schreil and Christensen (1968) found "bubbles" connecting pairs of cells in electron micrographs of conjugation mixtures. The

cytoplasm of one cell often appeared less dense, with the DNA in the nuclear region less closely packed and the cytoplasm distinctly separated from the cell wall. These "leaky" cells were identified as the Hfr parent by labelling with phage T_6. In addition, simple mesosomes were sometimes seen connecting the cell membrane to the nucleoplasm near the contact point between cells and the DNA fibrils in both cells were orientated perpendicularly to the walls at this point. Although both Gross and Caro (1966) and Schreil and Christensen (1968) reported absence of bridges in control cultures of F^- or Hfr cells alone, the work of Lancaster and Skvarla (1970) suggested that these bridges were artefacts caused by non-specific agglutination of cells. Using exponential phase F^- cells only, they demonstrated apparent protrusions of the outer cell wall layers, connections by "bubbles" of cell wall material and apparent fusion of cell wall layers. In addition, they found no correlation between the point of surface contact and nucleoid displacement as suggested by Schreil and Christensen.

More recently the pilus retraction model for conjugation has been proposed (Marvin & Hohn, 1969; Bradley, 1972), based largely on observations of sex specific phage adsorption. According to this theory, the attachment of a pilus to a specific receptor on the recipient wall and the adsorption of an RNA phage to the side of the pilus or a DNA filamentous phage to the pilus tip all trigger retraction of the pilus. A signal, for example a slight change in the pilin conformation, is transmitted from one subunit to the next down the pilus to a mechanism at its base, whereby the equilibrium between polymerised and non-polymerised subunits is reversed. The resulting retraction then brings the two bacteria into close contact, or in the case of phage, the nucleic acid is injected at the base of the pilus. Bradley (1972) observed that RNA phage adsorption to the side of a pilus could only be prevented by actual penetration of an Ff phage or the formation of recombinant pairs and not by mere adsorption of the filamentous phage or F^- cell. This evidence provides support for the model, as

adsorption sites for phages would only disappear once the pilus had been retracted.

Shortening of pili after phage adsorption has been observed in Pseudomonas aeruginosa (Bradley, 1972). In addition, empty phage capsids were located on the outside surface of the bacterium, even though adsorption occurred onto the pili. Bradley's model for phage infection involves ejection of a short length of RNA on the surface of the pilus. Following this stimulus, the pilus retracts and this length of RNA penetrates, followed by the rest of the nucleic acid. Certain chemicals can also cause shortening of pili, which are apparently totally pulled inside at a rapid rate estimated at 100 nm per second. Using the electron microscope, Bradley observed small dark spots at the poles of the bacteria which he suggested were holes in the cell envelope through which pili had protruded before retraction.

As described previously, Ou and Anderson (1970) found that genetic transfer could occur while mating pairs were loosely connected. However, the pairs which were intimately connected were approximately twice as fertile. They proposed that pili play three possible roles in conjugation; attachment organelles for the formation of mating pairs, a means of transfer for DNA and that of bringing mating cells into close contact after which more efficient transfer occurs through some sort of additional conjugation bridge.

Bacteria carrying sex factors are generally poor recipients. This phenomenon of exclusion has significance with regard to the mechanism of conjugation. It appears that exclusion only occurs when the sex factors in donor and recipient determine identical pili (Meynell & Ewins, 1973). It has been demonstrated that sex pili formed by a cell containing two F-like sex factors (e.g. R^{+}Hfr cells) contain a mixture of F and R pilin (Lawn et al, 1971). However, no mixed pili are produced if the donor carries a mixed infection with F-like and I-like sex factors. In this case, it is possible that the two types of

pili are assembled separately from one another or that the pilin subunits are so different that they are incapable of copolymerisation. If now an F^+R^+ (I-like) x F^+R^- mating is performed, only F is excluded. In contrast, exclusion is largely absent if an F^+R^+ (F-like) donor is used. Similar results are obtained with Hfr R^+ donors of different types (Meynell & Ewins, 1973). In addition, exclusion is absent if genes are transferred by transduction instead of by conjugation (Watanabe, Sakaizumi & Furuse, 1968). This suggests that exclusion is eliminated if DNA transfer is involved with a protein coat (phage or pilus) distinct from that determined by the recipient's sex factor. Two theories have been suggested to explain the mechanism of exclusion, both involving DNA transfer via the pilus (Meynell & Ewins, 1973). If disassembly of the pilus occurs in the recipient membrane (page 7), the necessary equilibrium between polymerised and depolymerised pilin might be upset when the same type of pilin is already present in the recipient. Alternatively, if the pilus is only a channel for donor DNA, the recipient's sex factor may recognise the donor pilus and physically block it by attempting to reach the donor in the reverse direction.

Until recently, little attention has been paid to the role of the female in conjugation and most workers have assumed that females are competent for mating. For example Curtiss et al (1969) regarded the ability to form specific mating pairs to be "solely dependent on the presence of F pili". However, Walmsley (1973) has shown that the ability of recipients to produce recombinants varies considerably during their cell division cycle. By counting mating pairs in a liquid culture, he estimated that the proportion of competent cells in two F^- recipient cultures was only 33% and 29%. In addition, killing of female cells by the process of lethal zygosis can occur when there is an excess of donors (Skurray & Reeves, 1973a & 1973b).

Altered recipient ability in cell wall polysaccharide mutants has been interpreted as evidence for a female structural

receptor site (Watanabe et al, 1970; Wiedemann & Schmidt, 1971). Both groups of workers found that removal of the O-side chains (Abequose-Mannose-Rhamnose-Galactose) from the polysaccharide molecules on the surface of recipients increased recipient ability, suggesting that receptor sites had been unmasked. Monner et al (1971) obtained a class of F^- mutants which showed large variations in ability to form mating pairs with a standard Hfr strain. Amongst these mutants there was a correlation between sensitivity to the female specific phage ØW, the lipopolysaccharide composition of the cell wall and a disturbance of resistance to ampicillin. The latter function is related to the biosynthesis of murein, a cell wall constituent (Franklin & Snow, 1971). This correlation suggests the presence of genes which simultaneously mediate a ØW receptor, a female-specific mating site and ampicillin resistance.

THE TRANSFER OF R FACTORS AND OTHER SEX FACTORS IN *E. COLI* UNDER ANAEROBIC CONDITIONS

Summary

Conjugation by three *E. coli* strains containing R factors, an *E. coli* Hfr strain and an *E. coli* F^{1} lac strain was demonstrated under stringent anaerobic conditions. The characteristics of transfer by these strains under aerobic and anaerobic conditions were also investigated. The results indicate that the anaerobic environment of the lower gastrointestinal tract is not a limiting factor for *in vivo* R factor transfer.

(A) MATERIALS AND METHODS

(a) Bacterial Strains

Three E. coli strains were isolated from faecal specimens: two R factor containing strains A_1 (amp^r, cml^r, str^r), A_2 (amp^r, cml^r, str^r, kan^r) and a recipient strain B (nal^r). An E. coli Ps-R (amp^r) strain contained an R factor originally derived from a faecal Pseudomonas aeruginosa strain. This strain was received from Prof. A.J. van Rensburg in Bloemfontein and was also used as a donor in R factor transfer experiments. In addition, three E. coli K-12 strains; an Hfr H wild type strain, an F^1lac wild type strain and an F^- recipient strain (thr^-, leu^-, lac^-, str^r) were used.

(b) Media

The medium used for selection of drug resistant recombinants was nutrient agar containing amp (25 µg/ml), cml (30 µg/ml), str (25 µg/ml), kan (30 µg/ml) or nal (30 µg/ml). Anaerobic nutrient agar was prepared by the methods of Hungate (1969) and Kistner (1960). The medium contained 2 µg/ml methylene blue. This redox indicator has an Eh of 11 mV at pH 7.0 (Smith & Holdeman, 1968). The reducing agent sodium thioglycollate (1.1 mg/ml) and an antifoam agent, Silicolapse 5001 (10 µg/ml) (Woods et al, 1971), were also added. The medium was steamed until homogeneous and in the reduced state. Samples (4 ml) were dispensed into modified Astell roll tubes (Toerien & Siebert, 1967) and perfused for 7 min with O_2-free gas containing 98% N_2 and 2% H_2. After autoclaving, the tubes were maintained at 50°C and drug solutions previously perfused with the N_2-H_2 gas mixture were added anaerobically to give the above concentrations. The anaerobic liquid medium, nutrient broth, was similarly prepared, but the Silicolapse was omitted.

The preparation of the minimal medium and eosin methylene blue-lactose agar (EMB-lac agar) are described in Appendix A. Streptomycin was added to these media at a final concentration of 250 µg/ml. Nutrient broth (anaerobic or aerobic) was used

as a diluent.

(c) Aerobic Mating Experiments

Exponential phase cultures were obtained by diluting .1 ml of a 12-hr resting phase culture in 9 ml nutrient broth and reincubating at 37°C for 90 min. Samples of donor (4 ml) and recipient (0.2 ml) cultures, grown statically or with aeration and in exponential or resting phase, were mixed in 2 ml double strength nutrient broth. As methylene blue and sodium thioglycollate were present in the anaerobic mating mixture, these substances were also added to the aerobic medium. However, this medium was maintained in the oxidised state by aeration and the methylene blue remained unreduced. After static incubation at 37°C for different time intervals, samples were withdrawn, diluted and plated on selective media. Control cultures of donor and recipient strains were similarly treated and plated to check both their drug resistance spectra and the activity of the drugs added to the medium.

(d) Anaerobic Mating Experiments

After aerobic inoculation of strains into anaerobically prepared broth, anaerobiosis was again rapidly achieved by flushing the tube with the N_2-H_2 gas through a sterile needle. The techniques of Hungate (1969) and Kistner (1960) were used for all manipulations involving mating of donor and recipient strains under stringent anaerobic conditions. Samples were added to or withdrawn from Astell roll tubes by injecting through the rubber stoppers with 1 ml or 10 ml all glass Luer Lock syringes fitted with 1 inch 21-gauge needles.

In order to determine the period of exponential growth in an anaerobically growing culture, an anaerobic growth curve was plotted. At time 0, 1 ml of a 24-hr anaerobic culture of strain A_1 was inoculated in 9 ml of fresh broth and reincubated. Samples were removed anaerobically at different time intervals for the determination of viable counts. The results showed that a lag phase of approximately five hours was followed by an exponential phase between 6 to 9 hours. The culture was in

resting phase after 24 hours (Fig. 1). Thus for subsequent work, anaerobic exponential phase cultures were obtained by diluting 1 ml of a 24-hr resting phase culture in 9 ml fresh broth and reincubating for 7 hours.

Samples of donor (4 ml) and recipient (0.2 ml) cultures in log or resting phase were mixed in 2 ml double strength reduced nutrient broth containing methylene blue and sodium thioglycollate. After incubation at 37°C for different time intervals, samples were withdrawn and recombinants were selected anaerobically in Astell roll tubes. Tubes were discarded if the methylene blue was in the oxidised state.

(e) Evacuated Mating Experiments

As a second method of obtaining anaerobic conditions, aerobic resting phase donor and recipient cultures were mixed in loosely capped test tubes. These were placed in anaerobic jars, which were immediately evacuated. After incubation for 6, 12 or 24 hr the jars were opened and the cultures were immediately diluted and plated on selective media.

(f) Donor Cell Counts

Since transfer frequencies were expressed as the number of recombinants per donor cell, donor counts were required for the calculations. These were determined from turbidity readings on a colorimeter for which a calibration curve had been plotted (Fig. 2).

FIG. 1. Growth Curve for Escherichia coli under Anaerobic Conditions.

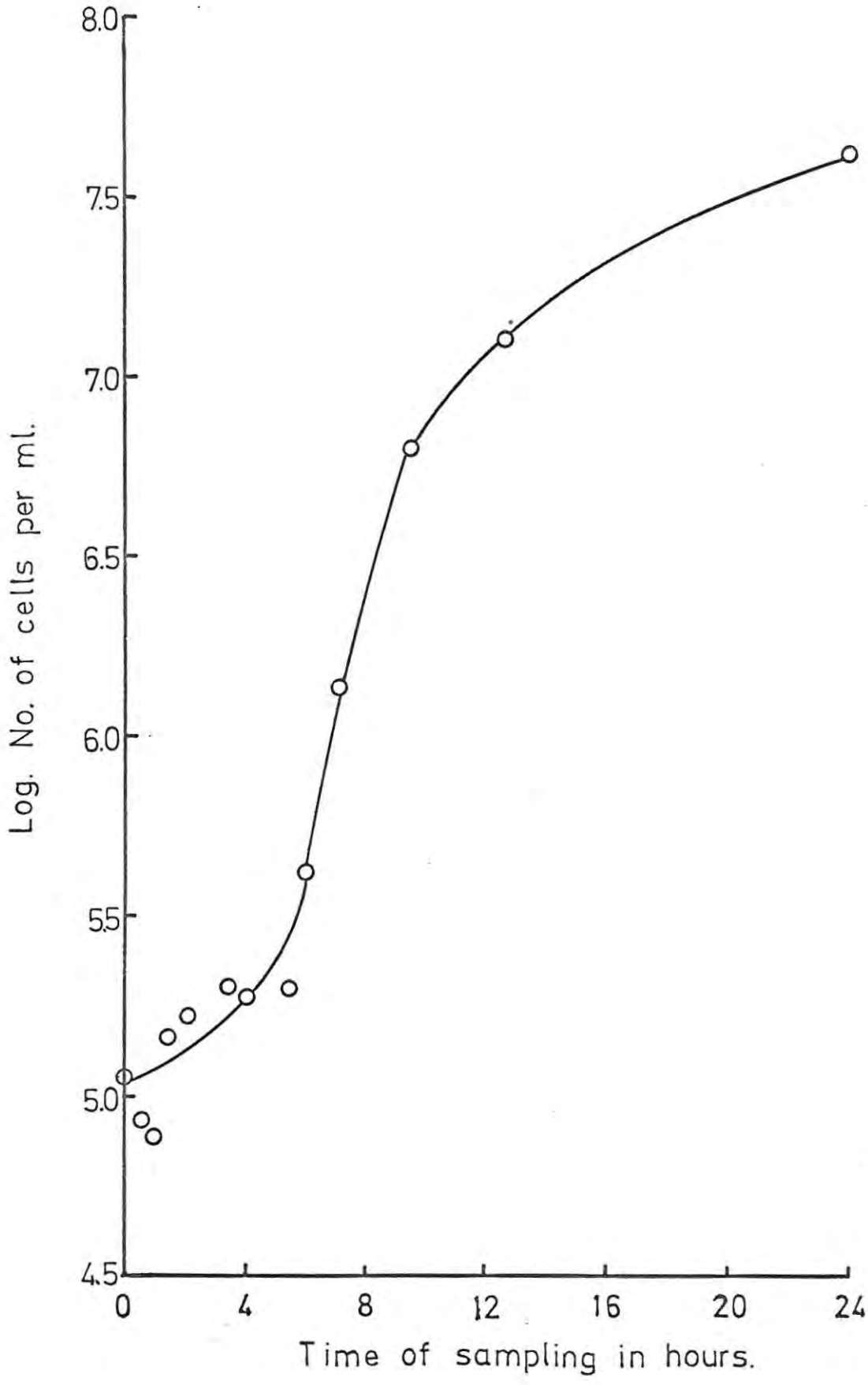
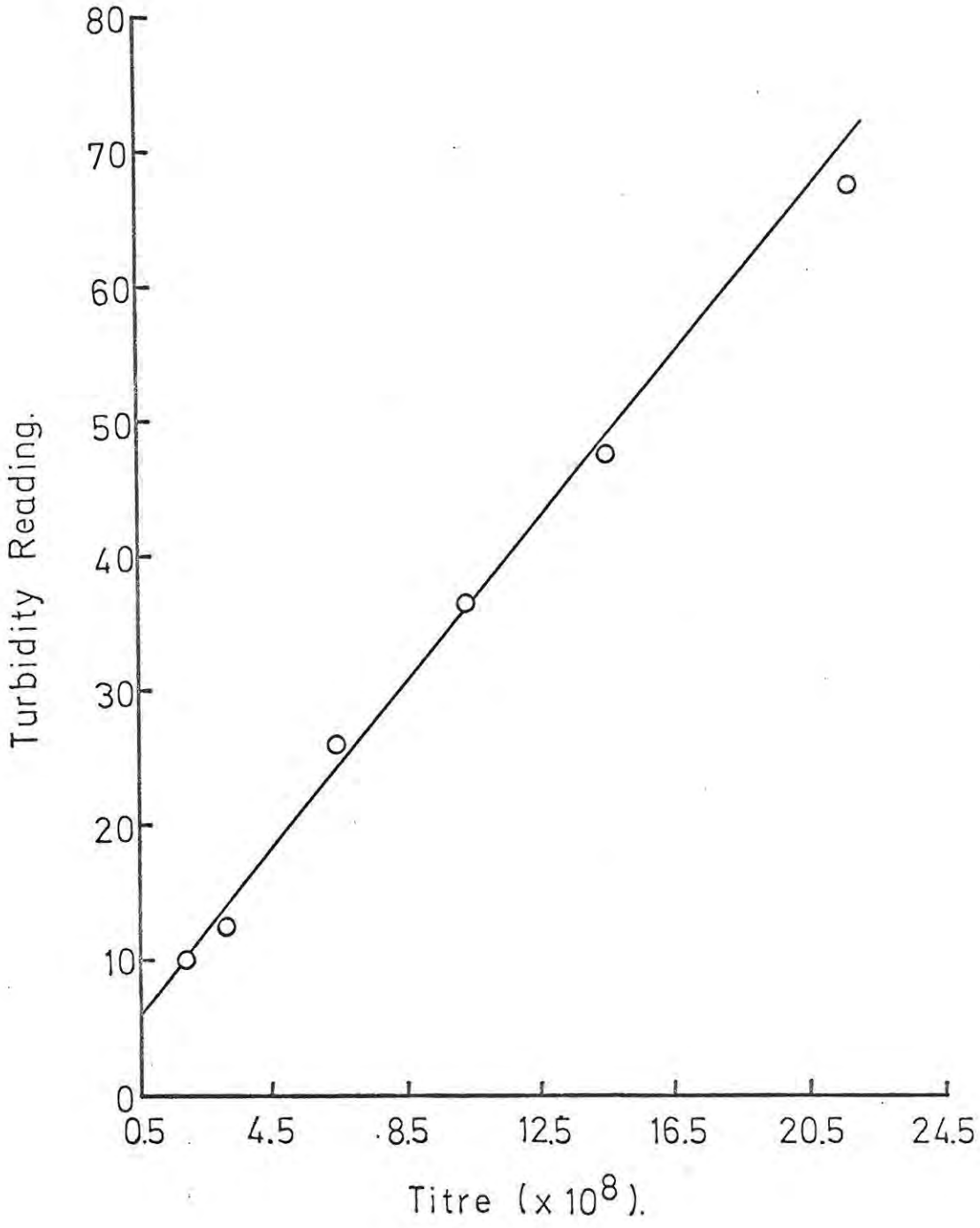


FIG.2. Calibration Curve for Colorimetric Determination of *E.coli* Titre.



(B) RESULTS

(a) Characterisation of Aerobic R Factor Transfer by Strains A_1 and A_2.

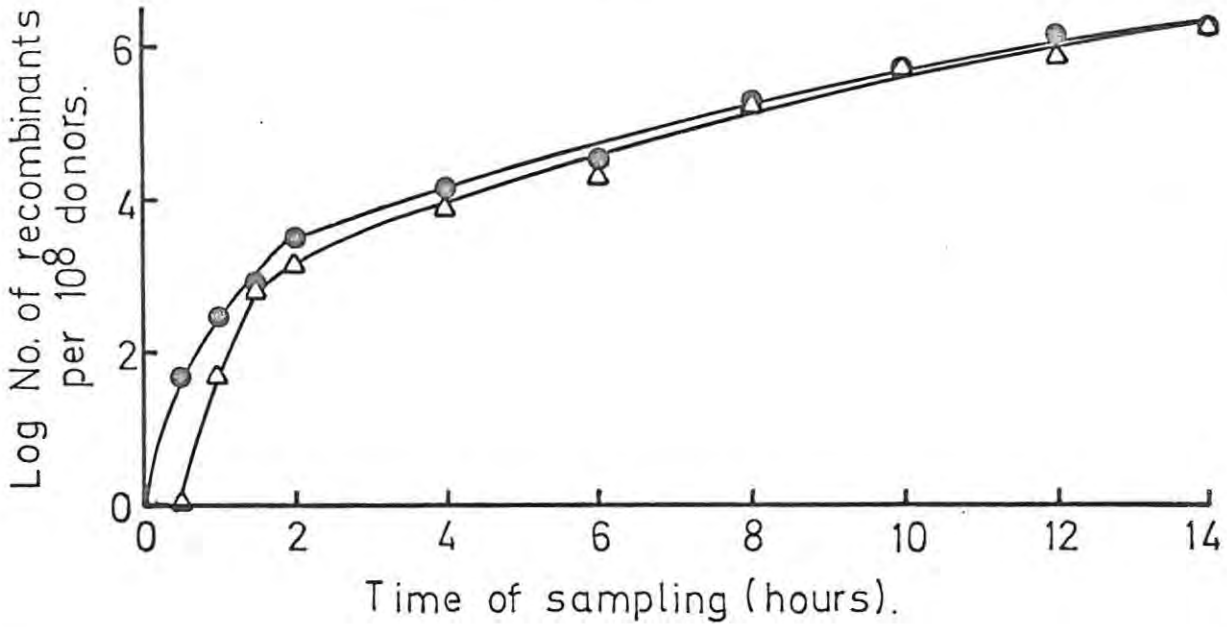
Before investigating anaerobic transfer, the characteristics of transfer between donor strains A_1 and A_2 and recipient strain B were determined under aerobic conditions. Transfer of all drug resistance markers was examined after static or aerated growth of cultures to exponential phase prior to mating (Figs. 3 and 4).

In cross A_1 x B, transfer frequencies of both the amp^r and cml^r markers after static growth were greater than those after aerated growth (compare Figs. 3a and 3b). In contrast, aeration of pre-mating cultures had little effect on the transfer frequencies of the three markers by donor strain A_2 (compare Figs. 4a and 4b). Secondly, recombinants for either marker were always isolated after a shorter time interval in cross A_1 x B than in cross A_2 x B (Figs. 3 and 4). The first - isolation times for amp^r and cml^r recombinants were 30 min and 60 min respectively in cross A_1 x B, but 60 min and 90 min respectively in cross A_2 x B (aerated cultures, see Fig. 3a and 4a). However, transfer of the cml^r marker rapidly reached a similar level to the amp^r marker in both crosses. Recombinants for kan^r in cross A_2 x B appeared between 2 and 4 hours after mating (Figs. 4a and 4b). The level of transfer was lower than for amp^r and cml^r for the duration of mating. No str^r recombinants were isolated in either cross.

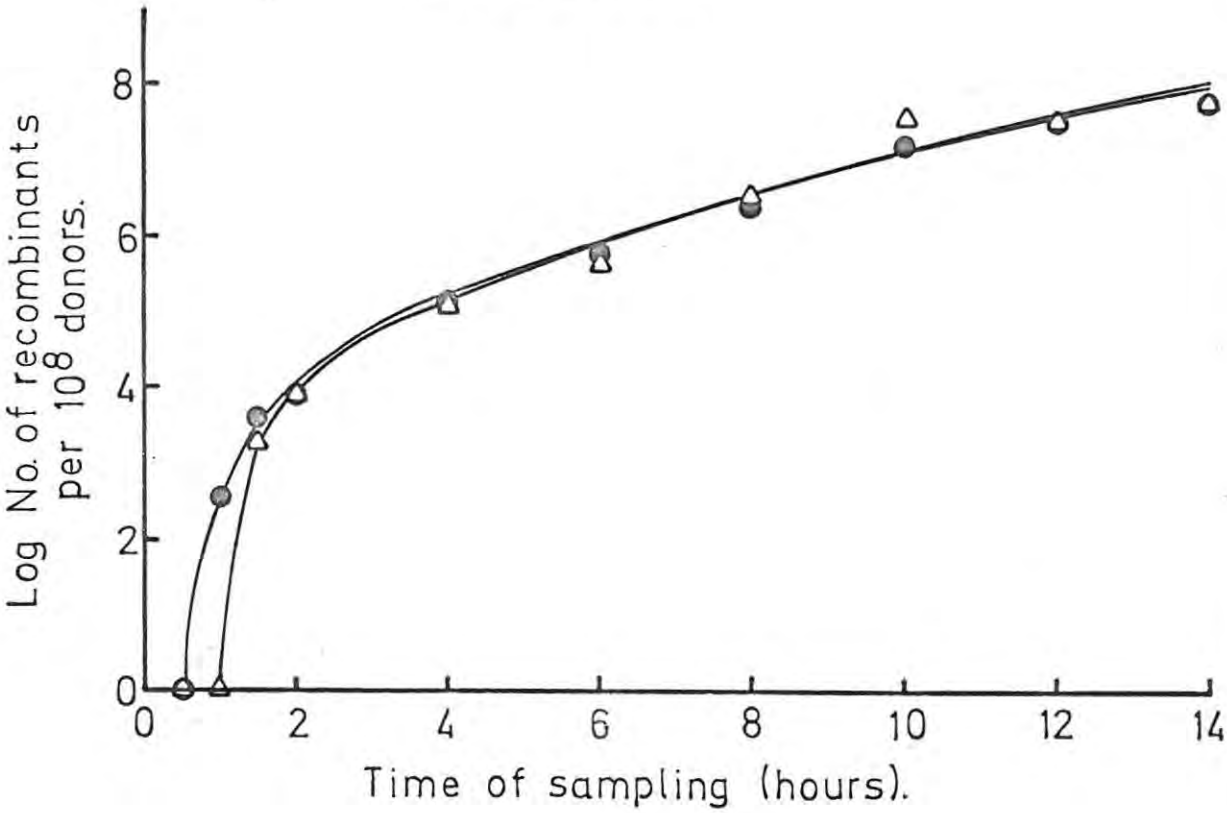
These results demonstrated that the determinants for amp^r and cml^r in donor strains A_1 and A_2 and for kan^r in strain A_2 were situated on R factors. However str^r was probably chromosomally determined in both strains. In addition, amp^r and cml^r appeared to be closely linked in both donors, whereas the kan^r marker was not associated with either amp^r or cml^r in strain A_2. In order to test this hypothesis, recombinant colonies from each type of selective medium were tested for resistance to the other drugs by replica plating or cross

FIG. 3. R Factor Transfer Frequencies in Cross A_1 x B under Aerobic Conditions.

a) Donor and recipient strains mated after aerated growth.



b) Donor and recipient strains mated after static growth.



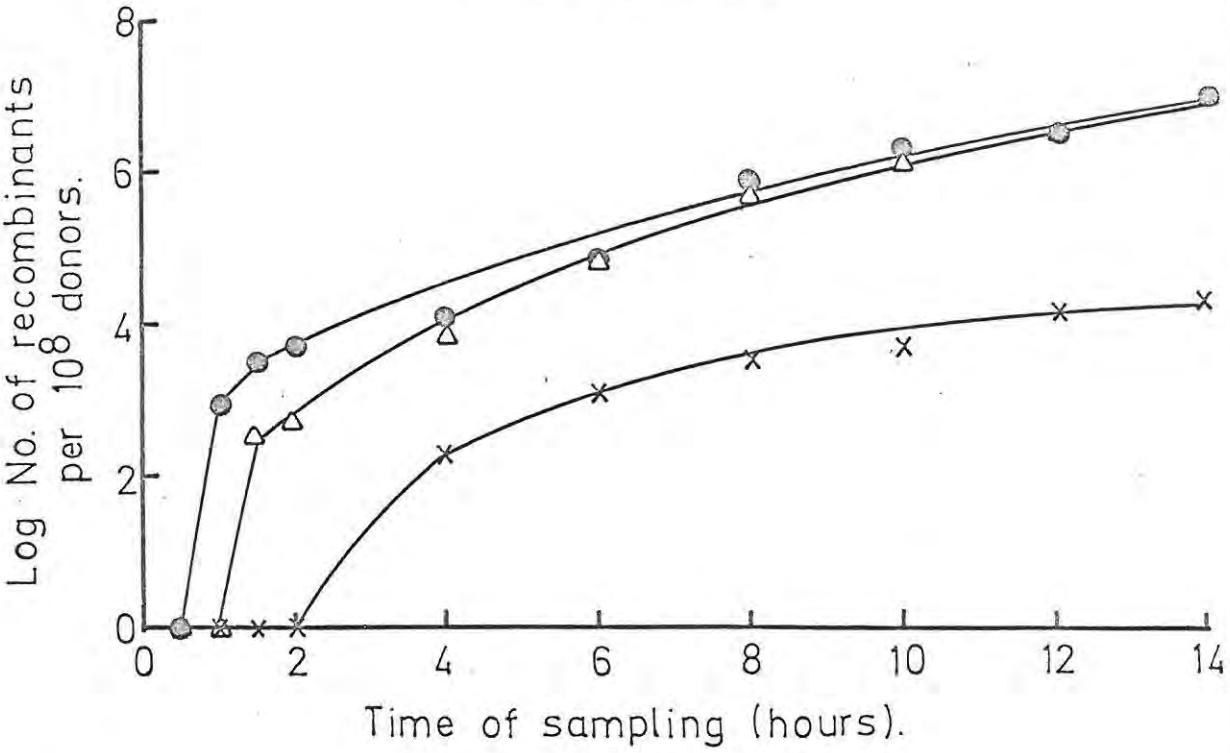
Recombinants selected:

● $amp^r\ nal^r$

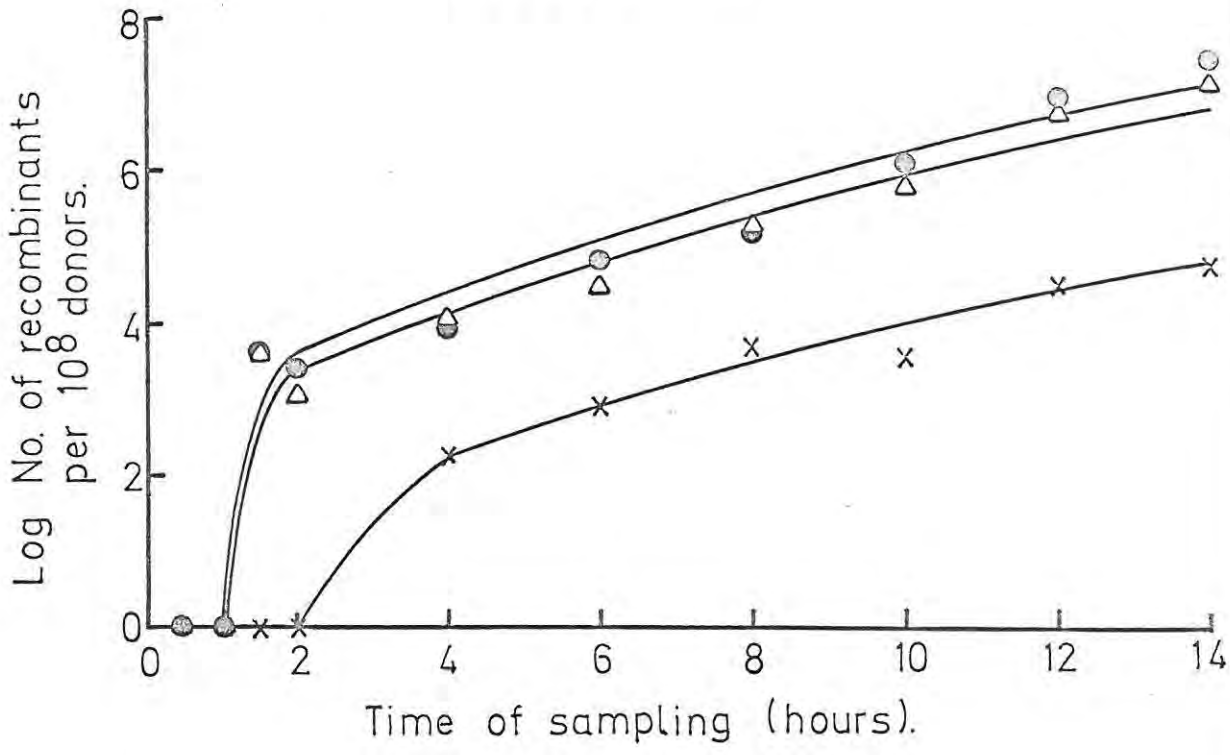
△ $cml^r\ nal^r$

FIG. 4. R Factor Transfer Frequencies in Cross A_2 x B under Aerobic Conditions.

a) Donor and recipient strains mated after aerated growth.



b) Donor and recipient strains mated after static growth.



Recombinants selected:

- ● $amp^r nal^r$
- △ $cml^r nal^r$
- × $kan^r nal^r$

streaking. The results showed that in both donor strains, amp^r and cml^r were closely linked but that the amp^r marker was transferred before the cml^r marker (Tables I and II). In contrast, it appeared that the kan^r marker in A_2 was transferred independently and at a lower frequency than the other two markers. No str^r colonies were isolated from the cross-testing, again indicating that the str^r marker was chromosomally situated in both strains A_1 and A_2.

(b) Effects of Aeration Conditions on R Factor Transfer

R factor transfer frequencies in crosses A_1 x B and A_2 x B were examined under aerobic and anaerobic conditions with donor and recipient cultures in exponential phase pior to mixing. In both matings, transfer of both the amp^r and cml^r markers occurred under stringent anaerobic conditions, but at a lower level than under aerobic conditions (Figs. 5 and 6 and Table III). The initial rate of anaerobic transfer of both markers in cross A_1 x B approached that of the aerobic matings. Anaerobic transfer was more rapid and efficient in cross A_1 x B than in cross A_2 x B. Transfer of the kan^r marker by donor A_2 was not investigated under anaerobic conditions as transfer occurred at such a low level even under aerobic conditions.

Transfer was also demonstrated under anaerobic conditions obtained by evacuation of the mating mixture after aerobic static growth of donor and recipient strains to resting phase. The indicator in the side arm of the anaerobic jar showed that anaerobic conditions were obtained by evacuation. Table IV shows that, for both crosses, the two different anaerobic methods yielded similar results.

(c) Effect of Growth Phase on Anaerobic Transfer

Anaerobic R factor transfer frequencies in crosses A_1 x B and A_2 x B were investigated with donor and recipient strains in exponential phase (7-hr anaerobic culture) and resting phase (24-hr anaerobic culture) prior to mixing. The results indicated that the growth phase did not markedly affect the

TABLE I

CROSS RESISTANCE IN MATING $A_1 \times B$

Time after mixing (hrs)	Cross resistance between amp^r and cml^r				Cross resistance between str^r, amp^r and cml^r			
	$\frac{N^o\ cml^r}{N^o\ amp^r}$ recomb.	% double recomb.	$\frac{N^o\ amp^r}{N^o\ cml^r}$ recomb.	% double recomb.	$\frac{N^o\ str^r}{N^o\ amp^r}$ recomb.	% double recomb.	$\frac{N^o\ str^r}{N^o\ cml^r}$ recomb.	% double recomb.
$\frac{1}{2}$	1/14	7.1	-	-	-	-	-	-
1	57/60	95.0	13/13	100.0	-	-	-	-
$1\frac{1}{2}$	79/81	97.5	28/28	100.0	-	-	-	-
2	30/30	100.0	56/56	100.0	0/30	0	0/28	0
4	366/366	100.0	-	-	0/366	0	0/35	0
6	115/115	100.0	122/122	100.0	0/115	0	0/122	0
8	81/81	100.0	80/80	100.0	0/81	0	0/80	0
10	42/42	100.0	50/50	100.0	0/42	0	0/50	0
12	75/75	100.0	86/86	100.0	0/75	0	0/86	0
14	236/236	100.0	-	-	-	-	-	-
16	620/620	100.0	-	-	-	-	-	-

TABLE II

CROSS RESISTANCE IN MATING A_2 x B

(A) Colonies from Nal + Amp Plates

Time after mixing (hrs)	$\frac{N^o\ cml^r}{N^o\ amp^r}$ recomb.	% double recomb.	$\frac{N^o\ kan^r}{N^o\ amp^r}$ recomb.	% double recomb.	$\frac{N^o\ str^r}{N^o\ amp^r}$ recomb.	% double recomb.
1	12/12	100.0	1/37	2.7	-	-
1½	48/50	96.0	2/17	10.9	0/25	0
2	24/24	100.0	6/45	13.3	0/5	0
4	64/64	100.0	13/75	17.3	0/64	0
6	28/28	100.0	14/123	11.4	-	-
8	91/91	100.0	1/84	11.9	-	-
10	64/64	100.0	-	-	-	-

TABLE II (Cont'd.)

(B) Colonies from Nal + Cml Plates

Time after mixing (hrs)	$\frac{N^o\ amp^r}{N^o\ cml^r}$ recomb.	% double recomb.	$\frac{N^o\ kan^r}{N^o\ cml^r}$ recomb	% double recomb.	$\frac{N^o\ str^r}{N^o\ cml^r}$ recomb.	% double recomb.
1	-	-	-	-	-	-
$1\frac{1}{2}$	18/18	100.0	2/9	22.2	-	-
2	17/17	100.0	3/23	13.0	-	-
4	78/78	100.0	14/83	16.8	0/63	0
6	163/163	100.0	33/225	14.6	0/17	0
8	83/83	100.0	8/126	6.4	-	-

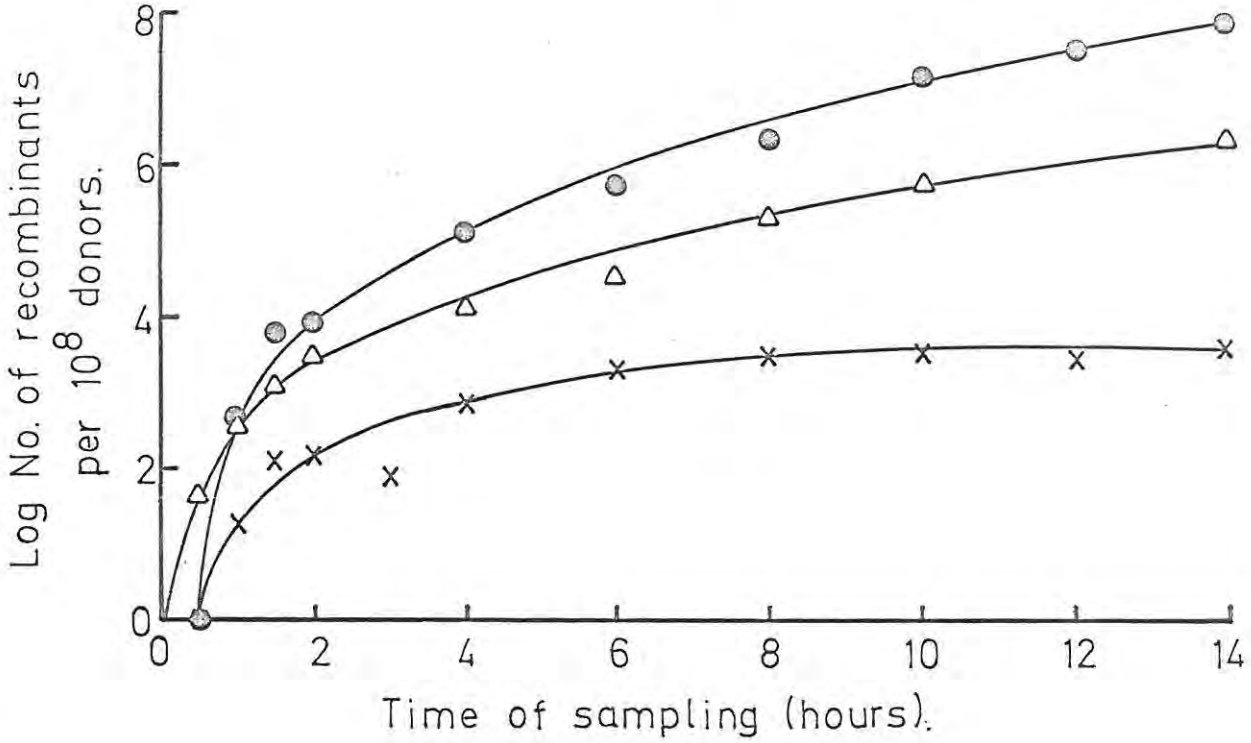
TABLE II (Cont'd.)

(C) Colonies from Nal + Kan Plates

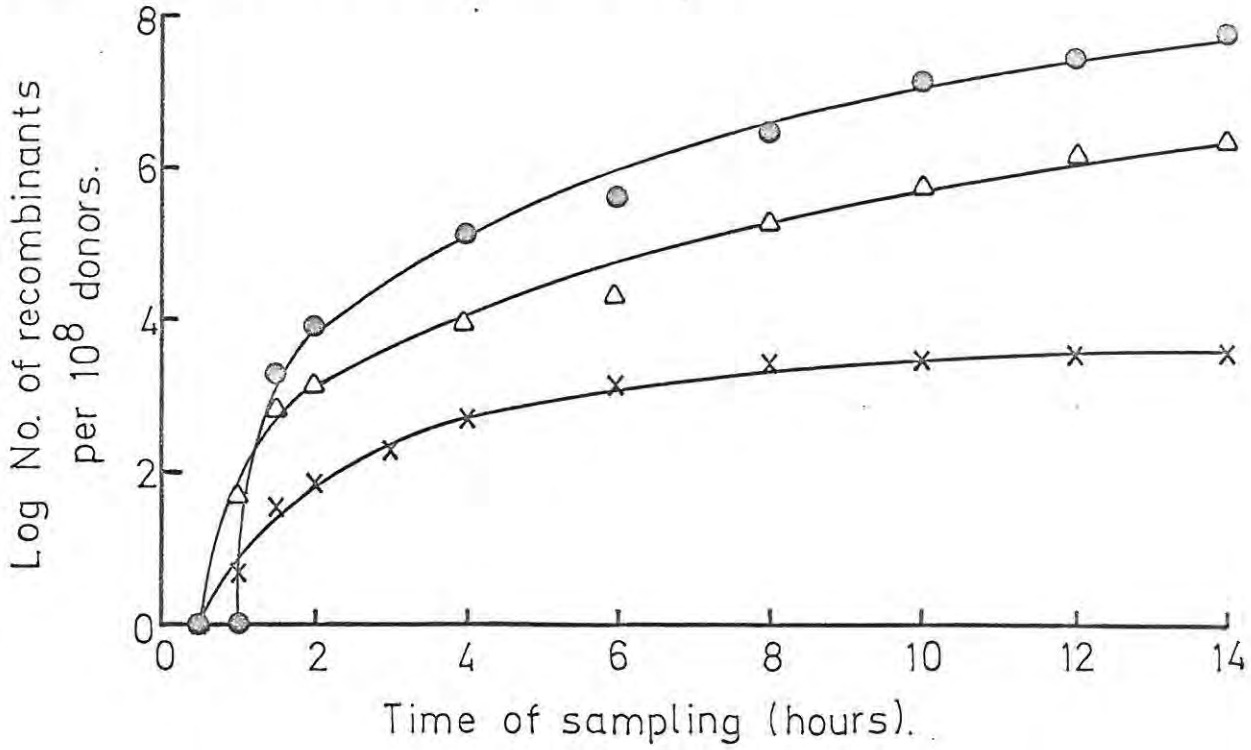
Time after mixing (hrs)	$\frac{N^o\ amp^r}{N^o\ kan^r}$ recomb.	% double recomb.	$\frac{N^o\ cml^r}{N^o\ kan^r}$ recomb.	% double recomb.
4	1/5	20.0	1/5	20.0
6	20/35	57.1	17/33	51.5
8	8/18	44.5	8/8	100.0
10	14/21	66.7	17/26	65.4

FIG. 5. R Factor Transfer Frequencies in Cross A_1 x B under Different Conditions of Aeration.

a). $nal^r amp^r$ recombinants selected.



b). $nal^r cml^r$ recombinants selected.



Mating conditions:

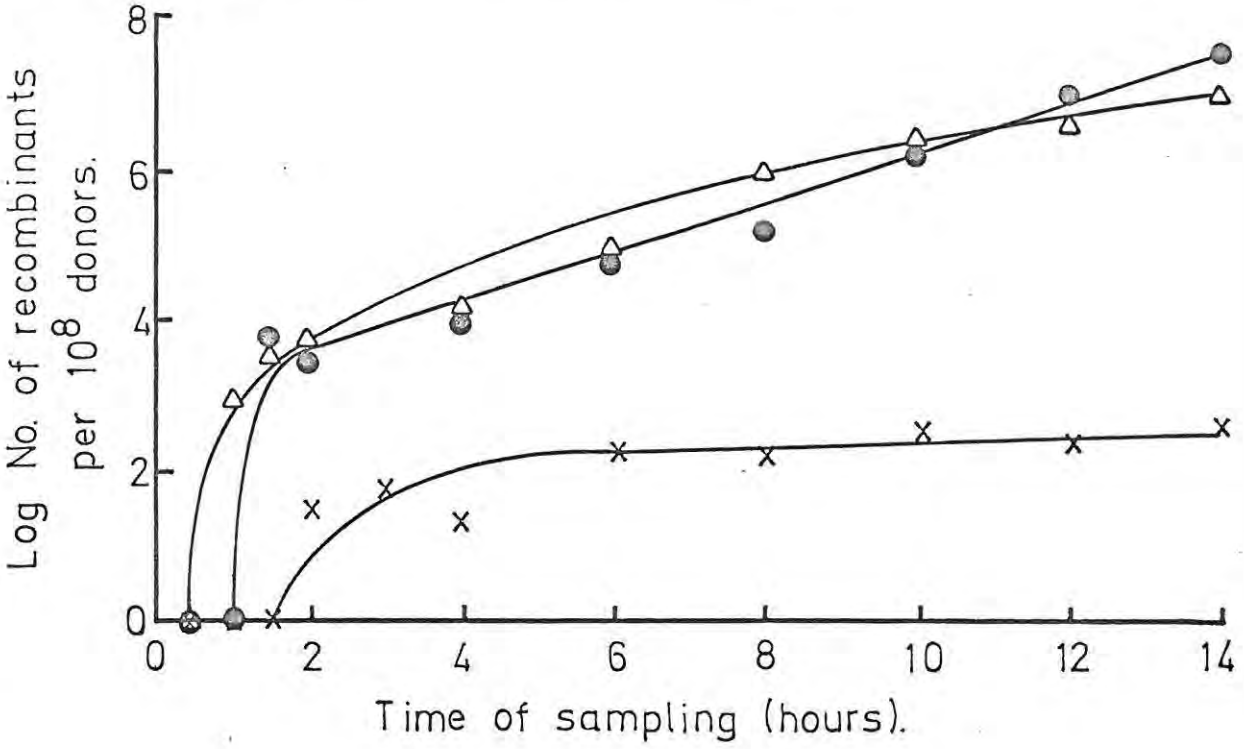
△ aerobic (after aerated growth).

● aerobic (after static growth).

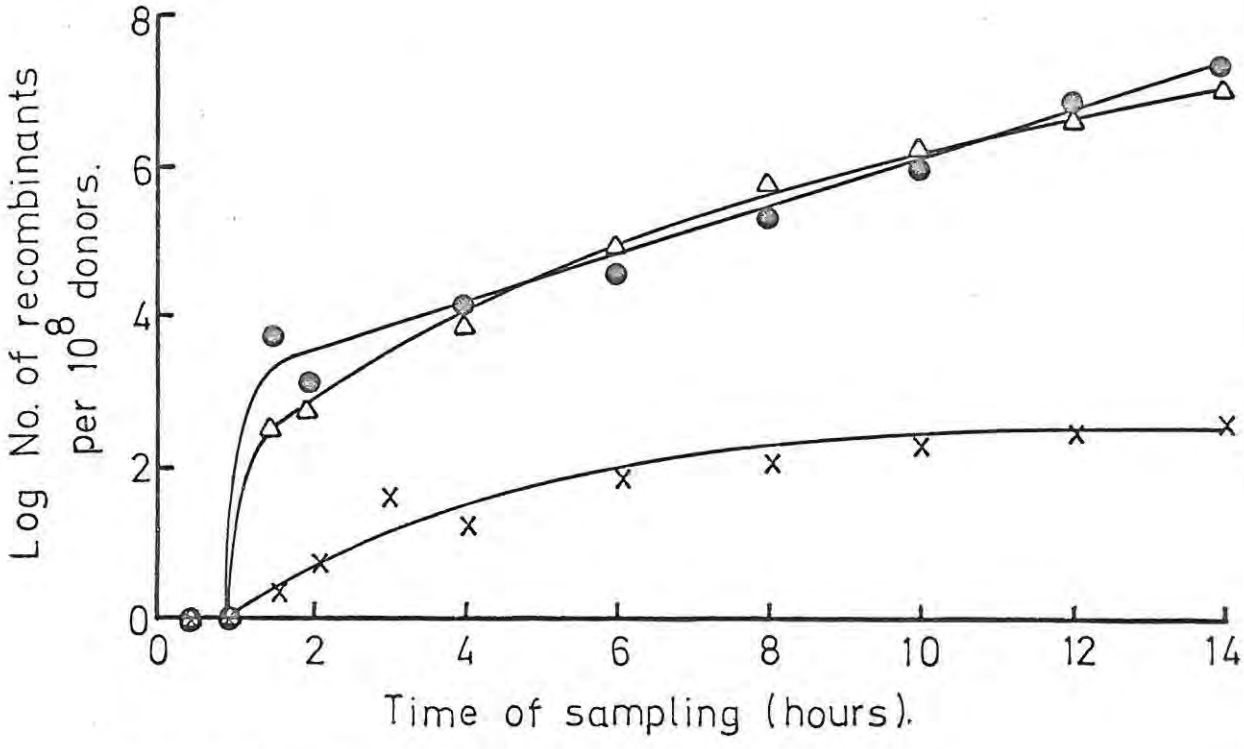
× anaerobic.

FIG.6. R Factor Transfer Frequencies in Cross A_2 x B under Different Conditions of Aeration.

a). $nal^r amp^r$ recombinants selected.



b). $nal^r cml^r$ recombinants selected.



Mating conditions:

△ aerobic (after aerated growth).

● aerobic (after static growth).

× anaerobic.

TABLE III

R FACTOR TRANSFER FREQUENCIES IN CROSSES A_1 x B AND A_2 x B 6 HRS AFTER MIXING DONOR AND RECIPIENT STRAINS

Cross	Transfer frequency			
	Aerobic mating		Stringent anaerobic mating	
	nal^r amp^r recombinants	nal^r cml^r recombinants	nal^r amp^r recombinants	nal^r cml^r recombinants
A_1 x B	3.5×10^{-4}	2.3×10^{-4}	2.4×10^{-5}	1.4×10^{-5}
A_2 x B	7.0×10^{-4}	6.4×10^{-4}	1.1×10^{-6}	7.3×10^{-7}

Frequencies were calculated as the number of recombinants per donor cell. Donor and recipient cultures were in exponential phase prior to mating.

TABLE IV

TRANSFER FREQUENCIES IN CROSSES A_1 x B AND A_2 x B UNDER DIFFERENT ANAEROBIC CONDITIONS

Cross	Transfer frequency			
	nal^r amp^r recombinants selected		nal^r cml^r recombinants selected	
	Stringent anaerobic	Evacuated	Stringent anaerobic	Evacuated
A_1 x B	7.8×10^{-5}	1.7×10^{-4}	7.8×10^{-5}	1.4×10^{-5}
A_2 x B	2.5×10^{-6}	2.8×10^{-6}	7.0×10^{-7}	1.9×10^{-7}

Anaerobic resting phase cultures of donor and recipient strains were mated for 12 hours. Recombinants were selected aerobically.

anaerobic transfer frequency of the cml^r marker in either cross (Fig. 7a). Transfer in cross A_1 x B was again more rapid and efficient than that in cross A_2 x B. Similar results were obtained when nal^r amp^r recombinants were selected in cross A_1 x B (Fig. 7b).

(d) Method of Recombinant Selection after Anaerobic Mating

Experiments involving both mating and recombinant selection under anaerobic conditions were difficult and laborious. In order to facilitate anaerobic work, the effect of selecting recombinants aerobically after anaerobic mating and sampling was investigated in cross A_1 x B. The results indicated that the method of selection had little effect on the anaerobic recombinant yield for either marker. (Fig. 8a and 8b). Similarly, the transfer frequency of the cml^r marker in cross A_2 x B 24 hr after mixing was 1.4×10^{-5} when recombinants were selected anaerobically and 9.6×10^{-6} when selected aerobically.

(e) R Factor Transfer by Ps-R

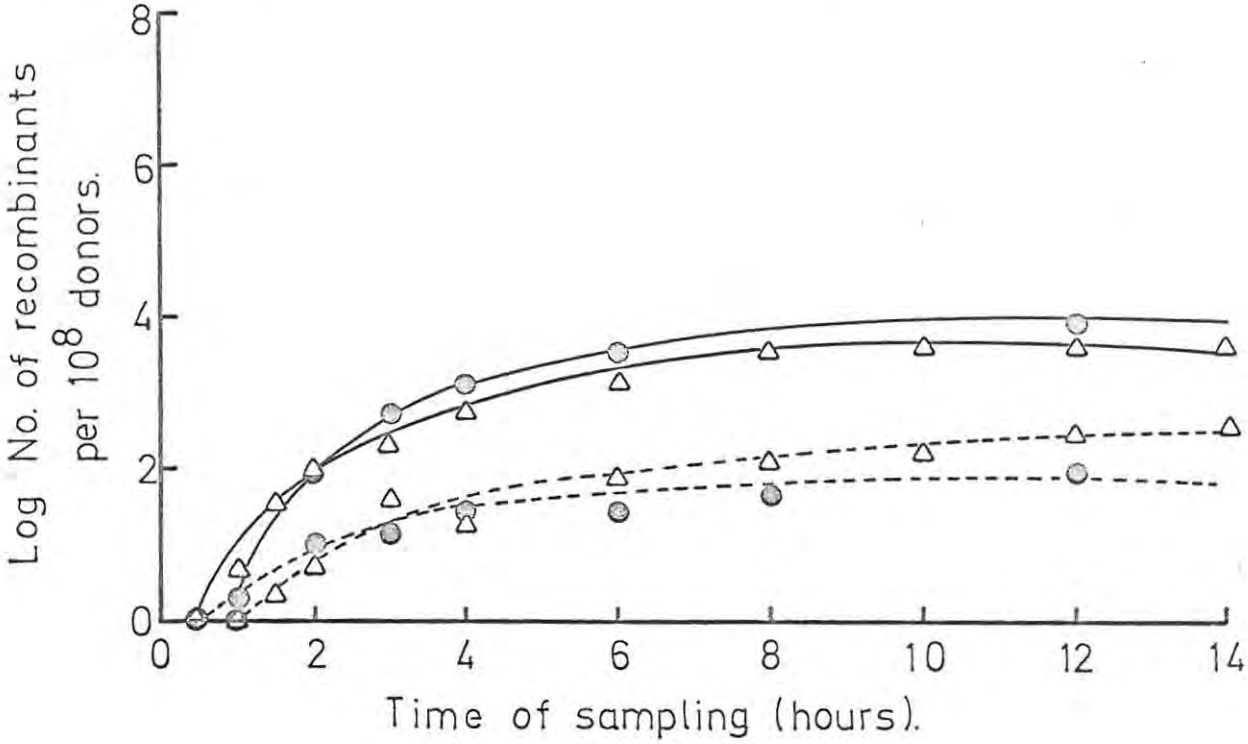
The transfer frequency of a Ps. aeruginosa R factor in an E. coli strain was investigated under aerobic and anaerobic conditions. Results showed that transfer was possible under both stringent anaerobic conditions and after evacuation of the mating mixture (Table V). Aerobic transfer after static growth of donor and recipient strains was again most efficient. Transfer frequencies under both stringent anaerobic and evacuated conditions were similar to the aerobic transfer frequency after aerated growth.

(f) Chromosomal Transfer

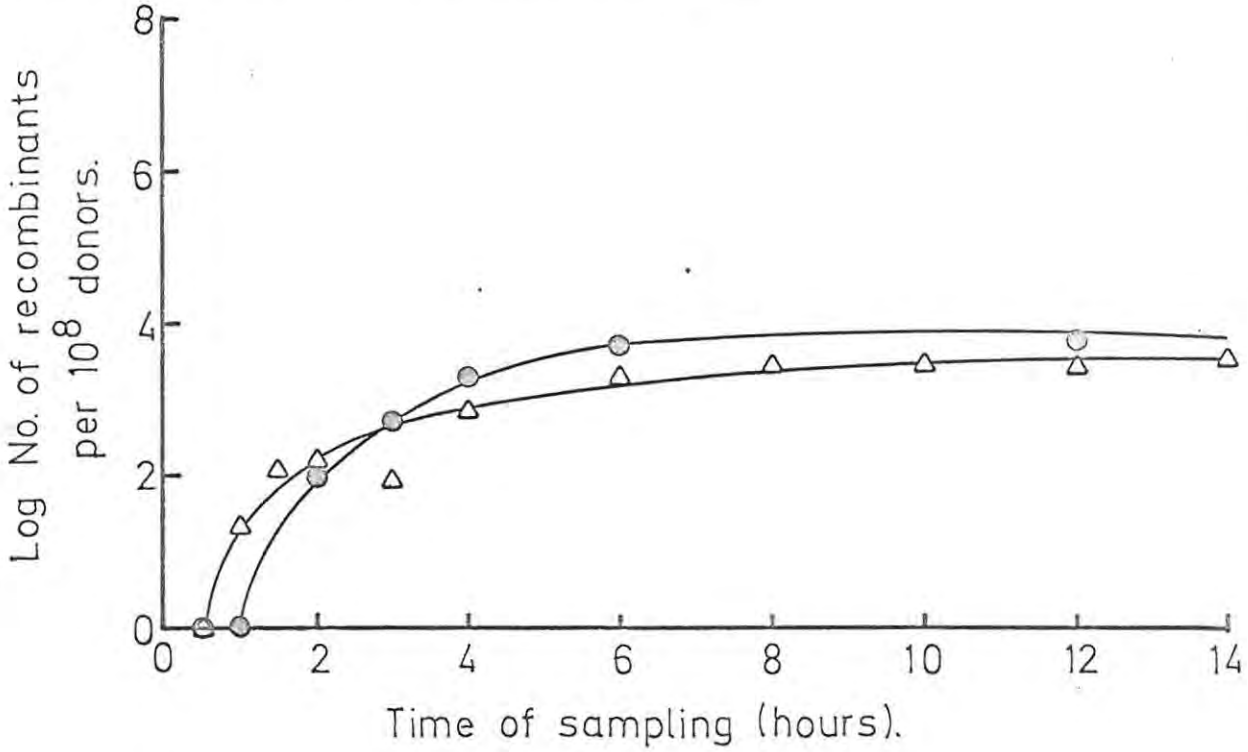
E. coli Hfr H and F^- cultures in resting and exponential phase were mated under aerobic and anaerobic conditions. Minimal medium containing streptomycin was used to select thr^+ leu^+ str^r recombinants. Chromosomal transfer by the Hfr strain was possible under stringent anaerobic conditions (Fig. 9). The transfer frequency was greatest after static aerobic growth

FIG. 7 The Effect of Growth Phase on Anaerobic R Factor Transfer Frequencies in Crosses A_1 x B (○—○, △—△) and A_2 x B (○---○, △---△).

a). $nal^r\ cml^r$ recombinants selected.



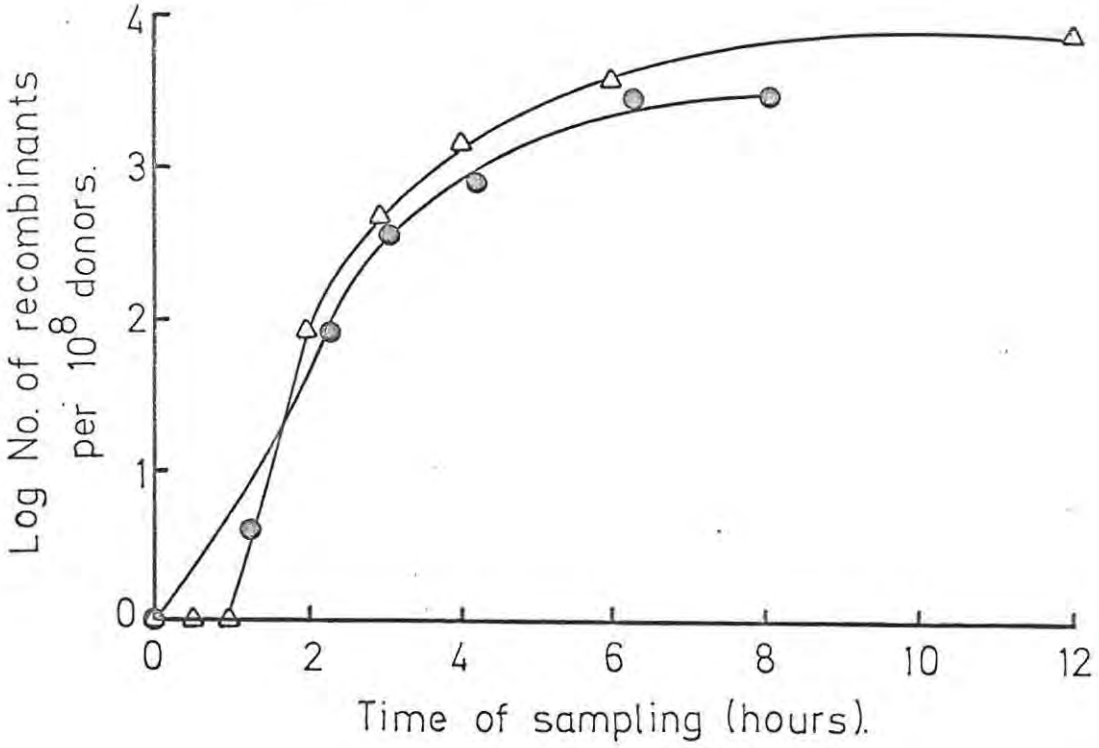
b). $nal^r\ amp^r$ recombinants selected.



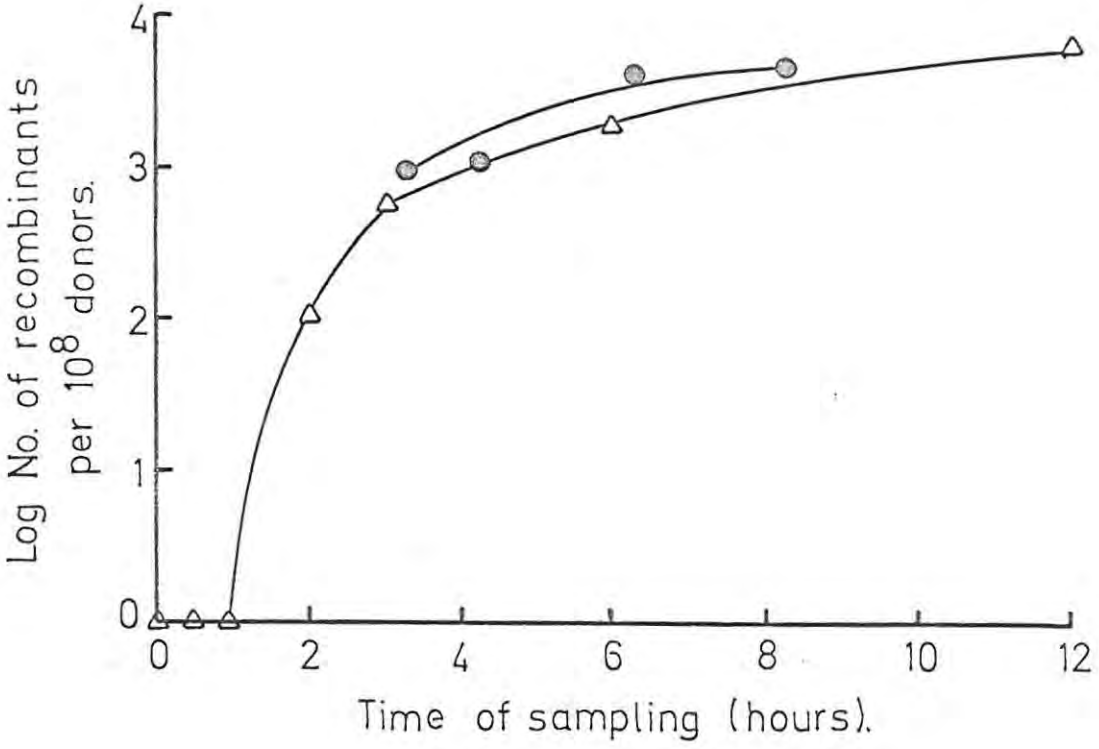
Growth phase of cultures at mating:
△-exponential ●-resting.

FIG. 8. The Effect of Recombinant Selection Method on Recombinant Yields after Anaerobic Mating in Cross A_1 x B.

a) $nal^r amp^r$ recombinants selected.



b) $nal^r cml^r$ recombinants selected.



Recombinant selection:

● anaerobic.

△ aerobic.

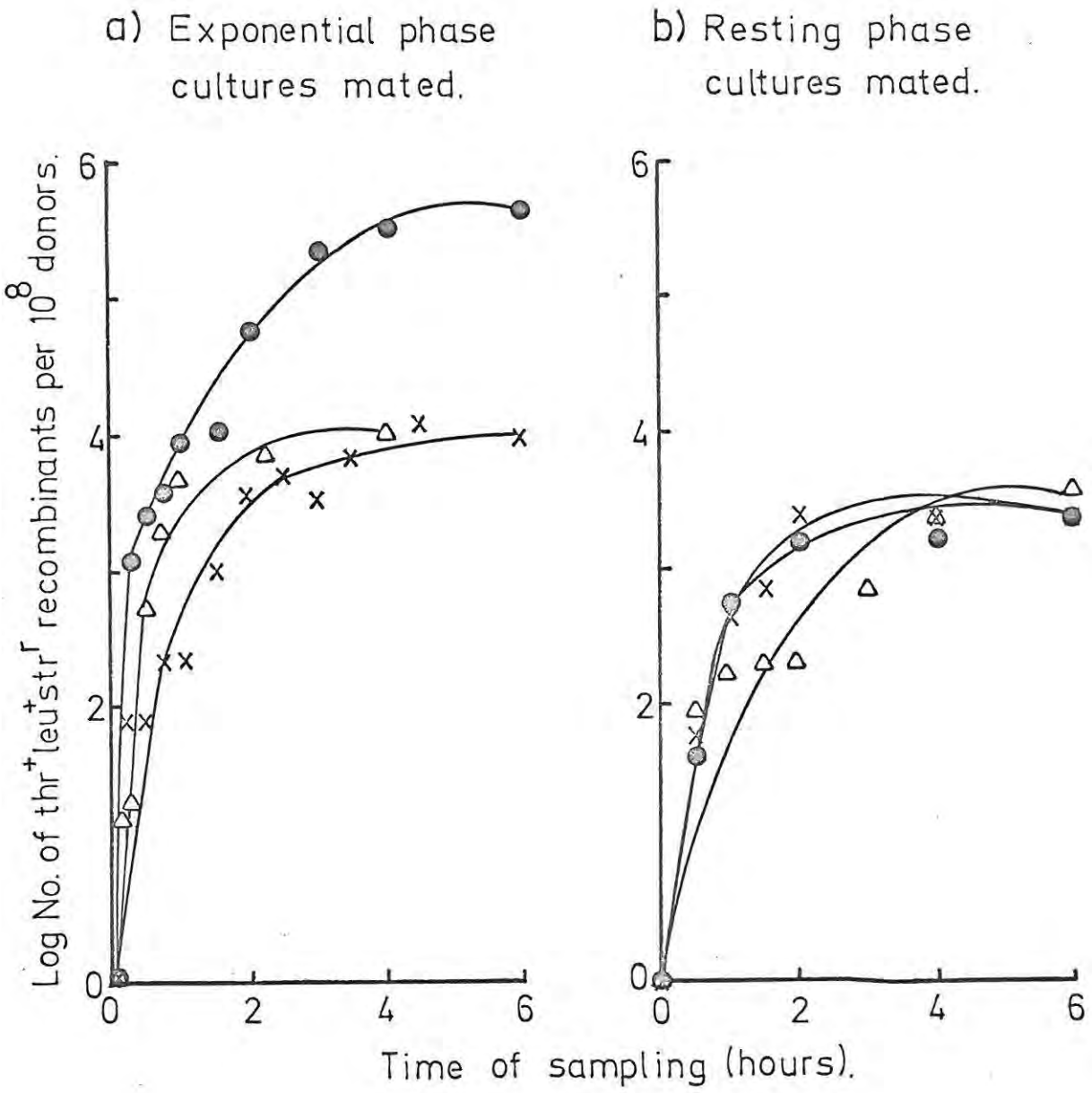
TABLE V

R FACTOR TRANSFER FREQUENCIES IN CROSS Ps-R x B UNDER AEROBIC AND ANAEROBIC CONDITIONS

Time of sampling after mixing (hr)	Transfer frequency			
	Aerobic mating		Anaerobic mating	
	Aerated growth prior to mating	Static growth prior to mating	Anaerobic growth prior to anaerobic mating	Static aerobic growth prior to mating under evacuated conditions
6	3.9×10^{-5}	1.9×10^{-4}	3.6×10^{-5}	8.2 x 10-5
12	3.9×10^{-5}	2.8×10^{-3}	3.0×10^{-5}	1.8×10^{-5}
24	2.4×10^{-3}	5.4×10^{-2}	4.2×10^{-3}	-

All cultures were in resting phase prior to mixing. Transfer frequencies were calculated as the number of nal^r amp^r recombinants per donor cell.

FIG. 9. Chomosomal Transfer Frequency in Cross HfrH x F^- under Different Conditions of Aeration.



Mating conditions:

- △ aerobic (after aerated growth).
- ● aerobic (after static growth).
- x anaerobic.

of donor and recipient strains to log phase prior to mixing. Similar transfer frequencies were shown by exponential phase aerated and exponential phase anaerobic cultures (Fig. 9a). There were no marked differences between the frequencies of chromosomal transfer when resting phase cultures were mated under aerobic and anaerobic conditions (Fig. 9b).

Chromosomal transfer was also demonstrated under anaerobic conditions obtained by evacuation. Donor and recipient strains were grown to resting phase under static aerobic conditions prior to mating. Samples were removed at 6, 12 or 24 hours. The transfer frequencies obtained were similar to those from anaerobic tubes (Table VI).

(g) Transfer by F^1 lac.

E. coli F^1 lac and F^- cultures in resting phase were mated under aerobic and anaerobic conditions. EMB-lac agar was used to select lac^+ str^r recombinants. Transfer frequencies under anaerobic conditions were similar to the aerobic frequencies (Table VII).

TABLE VI

COMPARISON OF TRANSFER FREQUENCIES IN CROSS Hfr H x F^- UNDER DIFFERENT ANAEROBIC CONDITIONS

Time of sampling after mixing (hr)	Transfer frequency	
	Stringent anaerobic conditions	Evacuated conditions
6	2.3×10^{-5}	1.5×10^{-6}
12	1.3×10^{-5}	7.5×10^{-6}
24	1.7×10^{-5}	2.1×10^{-5}

Resting phase donor and recipient cultures were mated. Transfer frequencies were calculated as the number of leu^+ thr^+ str^r recombinants per donor.

TABLE VII

TRANSFER FREQUENCIES IN CROSS $F^{1}lac \times F^{-}$ UNDER DIFFERENT CONDITIONS OF AERATION

Time of sampling after mixing (hr)	Transfer frequency	
	Aerobic mating	Stringent anaerobic mating
8	2.6×10^{-4}	5.2×10^{-4}
12	4.0×10^{-4}	7.8×10^{-4}
24	3.2×10^{-4}	2.6×10^{-4}

Donor and recipient cultures were in resting phase prior to mating. Transfer frequencies were calculated as the number of lac^{+} str^{r} recombinants per donor.

(C) DISCUSSION

The results demonstrated that a variety of donor strains could transfer genetic entities such as R factors, an F^1 factor and the bacterial chromosome to recipient strains by conjugation under stringent anaerobic conditions. The finding that anaerobic R factor transfer is possible differs from that of Mitsuhashi (1965) but agrees with *in vivo* studies (Guinée, 1970; Kasuya, 1964; Reed *et al*, 1969). The anaerobic environment of the lower gastrointestinal tract is therefore not a limiting factor for *in vivo* R factor transfer.

Fisher (1957) reported restriction of zygote formation in an Hfr x F^- mating under anaerobic conditions. However (after completion of this present study), Stallions and Curtiss (1972) pointed out that Fisher used minimal media and also demonstrated that anaerobic chromosomal transfer could occur at high frequency in an Hfr x F^- cross, provided that the mating medium was nutritionally rich. Since the matings were carried out in nutrient broth, the results for chromosomal transfer in this study confirm those of Stallions and Curtiss. It should however be noted that Mitsuhashi (1965) used brain heart infusion broth as the mating medium. This is a nutritionally rich medium, yet he found inhibition of R factor transfer. The present study therefore resolves the controversial situation and demonstrates clearly that anaerobic R factor transfer is possible (Moodie & Woods, 1973a).

Both Fisher (1957) and Mitsuhashi (1965) obtained anaerobic conditions by evacuation of the mating mixture after initial aerobic growth of donor and recipient strains. Stallions and Curtiss (1972) flushed the mating mixture with N_2 for the duration of mating, but also used aerobically grown strains. In this study, R factor and chromosomal transfer were demonstrated under evacuated anaerobic conditions. In addition, far more stringent anaerobic conditions were applied. Exposure to O_2 was avoided during initial growth of donors and recipients, mating and during selection of recombinants.

However, it was also shown that similar recombinant yields were obtained from both the aerobic and anaerobic selection techniques after anaerobic mating. For working with facultative strains, the aerobic selection techniques have the advantage of being far simpler and quicker than anaerobic selection methods.

The results obtained from cross Ps-R x B showed that an R factor could be transferred at a similar frequency under aerobic and stringently anaerobic conditions. Anaerobiosis resulted in reduced transfer frequencies in crosses A_1 x B and A_2 x B. The reason for these differences in response to anaerobic conditions is not known, but is probably dependent in some way on the nature of the donor strains, since the same recipient was used in all mating experiments. The frequency of anaerobic transfer was consistently greater in cross A_1 x B than in cross A_2 x B, again reflecting a strain difference.

In this study the ratio of donors to recipients in the mating mixture was 20 : 1. Skurray and Reeves (1973a and 1973b) have reported that such an excess of donors in an Hfr x F^- mating results in lethal zygosis, which causes a reduction in the number of F^- survivors and recombinant cells. The physiology of the recipient is affected, resulting in inhibition of DNA synthesis, leakage of β-galactosidase from the cell and other changes. Skurray and Reeves noted that only Hfr donors could cause death of the recipients and that lethal zygosis did not occur if an excess of F^+ or F^1 donors was added. They therefore suggested that the death of F^- cells was associated with an event unique to mating with Hfr donors. Lethal zygosis would thus be absent in the present study when the F^1 and R factor containing strains were used as donors, but it is interesting to note its apparent absence in the Hfr x F^- mating system as well. However, Skurray and Reeves reported that carefully controlled growth and mating conditions were necessary for maximum killing. These conditions were probably not favourable for efficient lethal zygosis to occur in the present study.

The highest values for transfer frequencies were obtained when donor and recipient strains were grown to exponential phase under static aerobic conditions prior to aerobic mating. Curtiss et al (1969) found a similar phenomenon in aerobic E. coli Hfr x F^- matings and related this to piliation of the donor cells. The mean number of F pili per cell increased as pre-mating growth conditions were made more anaerobic, firstly by growth in still culture and then by flushing with N_2. There was a direct correlation between the number of pili per donor cell and the recombinant yield from aerobic Hfr x F^- matings. The values obtained for R factor transfer frequencies in the three aerobic crosses are similar to those for wild type repressed R factors in previous reports (Meynell & Datta, 1967; Watson, 1967; Yokota et al, 1972). Similarly, Harada and Mitsuhashi (1971) reported that the transmission frequencies of R factors they had isolated varied considerably, but were generally lower than those of the F factor in E. coli.

PART II

STUDIES ON R FACTOR TRANSFER IN OBLIGATE ANAEROBIC BACTERIA FROM THE GASTROINTESTINAL TRACT.

Introduction

The existence of anaerobic bacteria was first recognised by Pasteur, who noticed that some organisms were motile only in the absence of air (Smith & Holdeman, 1968). Although anaerobes are extremely common and can be isolated from many habitats, including the skin, mouth and intestine of humans (Finegold et al, 1972), they have been largely neglected because of the technical difficulties involved in working with them. However, anaerobes have recently been investigated more thoroughly and their importance in the normal and pathogenic states is becoming increasingly evident.

Anaerobes form a continuous series from organisms just able to grow on agar exposed to air, to those unable to withstand even a very low oxygen level. According to Smith and Holdeman (1968) there are three factors involved in anaerobiosis. Lack of the enzyme catalase in anaerobes, resulting in the formation of lethal concentrations of hydrogen peroxide in the presence of oxygen, was once thought to be of major importance. However it has recently been shown that, although hydrogen peroxide may have detrimental effects, the ability to form catalase is not vital for growth in air. Secondly, inhibiting organic substances are formed rapidly in media containing active reducing agents which become oxidised, for example if anaerobically prepared media are exposed to oxygen. The nature of these inhibitory substances is not known, but they are probably formed by the oxidation of peptone, lipids or agar itself. Although peroxides may again be involved (Barry et al, 1956), the inhibitory action of such media can be demonstrated in the presence of catalase. The strictly anaerobic non-sporing rods in the human intestine are apparently highly sensitive to these

substances (Smith & Holdeman, 1968).

The third factor is the oxidation-reduction (redox) potential of the medium. This is usually expressed as the Eh in mV, being the potential which would exist between the system under study and a H_2 half cell at pH 0. (Jacob, 1970; Morris, 1968; Smith & Holdeman, 1968). Categories often used in describing anaerobes, such as micro-aerophilic, moderately anaerobic and strictly anaerobic, lack precision in defining the degree of anaerobiosis. The redox potential is a much more precise index (Hungate, 1969). The growth of some organisms, for example Bacteroides vulgatus and Clostridium sporogenes, is apparently governed largely by the Eh of the medium and is not much affected by peroxide formation. The Eh values marking the upper limit at which organisms will grow has been measured, for example B. vulgatis 140 mV and C. histolyticum 90 mV (Smith & Holdeman, 1968). Some anaerobes require a far more reduced system, such as the rumen anaerobe Methanobacterium ruminantium, which cannot initiate growth at a potential above -359 mV (Smith & Hungate, 1958).

The Eh is affected by the pH of the medium, becoming more negative as the pH increases. In general, the Eh will become 60 mV more negative for an increase of one pH unit. A buffer system such as CO_2-bicarbonate is therefore usually added to anaerobic media (Hungate, 1969). In nature, oxygen is the most common source of a high redox potential. Hungate has calculated that the O_2 concentration at a potential of -330 mV is 10^{-75} of the concentration in the atmosphere, representing 1.48×10^{-56} molecules of O_2 per litre. Reducing agents such as thioglycollate, cysteine and sodium sulphide can be added to lower the Eh of the medium. The greater the concentration of oxidised materials in the system, the greater the concentration of reducing agents needed to return it to a fixed low potential. High concentrations of reducing agents can be toxic, especially for non-sporing anaerobes, so exposure to oxygen during medium preparation should be avoided (Smith & Holdeman, 1968; Hungate, 1969). Ideally, addition of reducing agent should be the last step in preparation and the medium should already be

partly reduced by boiling (Holdeman & Moore, 1972). Other less active substances which have been used as reducing agents are metallic iron, fragments of chopped meat or steel wool. Suspensions of pasteurised or even unpasteurised aerobes have also been used to reduce the concentration of dissolved oxygen and inactivate any peroxides formed in the medium (Smith & Hungate, 1958).

Anaerobic bacteria are now recognised as the predominant flora of the intestinal tract of adult man (Finegold, 1969; Finegold, 1970; Haenel, 1970; Moore et al, 1969) and many animals (Barnes & Impey, 1972; Bryant & Burkey, 1953; Lee et al, 1968; Syed, 1972; Vervaeke & van Nevel, 1972). Direct studies of intestinal gas composition have demonstrated that the environment of the human lower gastrointestinal tract is anaerobic (Askevold, 1956). The redox potential in humans has been measured at approximately -250 mV (Moore et al, 1969). This reduced state is apparently a direct consequence of the metabolic action of the bacteria present. It has been demonstrated that the potential in germfree animals is approximately 300 mV more positive than in conventional animals. Treatment of germfree rats with the cecal contents of conventional rats resulted in a change to low redox potentials similar to those of normal animals. It has been suggested that in germfree animals, the intestinal contents come into equilibrium with tissues in which the Eh has a positive value, for example +126 mV to +246 mV (venous blood) or +120 mV (subcutaneous tissue). The intestine is therefore probably not intrinsically anaerobic, but becomes anaerobic because of the reducing activity of its flora, the predominance of anaerobes presumably being due to selection (Meynell, 1963).

In man, the gastrointestinal flora is usually established in a logical sequence as one progresses from the relatively aerobic environment of the stomach to the anaerobic bowel. Facultative lactobacilli predominate in the stomach, but the count is usually low and variable, depending on food intake and pH. In the upper duodenum, facultative enterococci and

lactobacilli are found at a count of 10^5 to 10^8 organisms per ml and anaerobes are rare. The terminal ileum contains approximately equal numbers of aerobes (mainly coliforms) and anaerobes (Bacteroides species and bifidobacteria). In the large intestine aerobes are far outnumbered by anaerobes. Estimates of the proportion of facultative bacteria, such as coliforms and streptococci, vary from 0.1% to 10%. The anaerobes include many species of bifidobacteria, proprionibacteria, peptostreptococci and clostridia. However the most prevalent anaerobe is Bacteroides fragilis, with an average count of 10^{10} to 10^{11} per gm. Other gram-negative rods such as Sphaerophorus species, Bacteroides melaninogenicus and Fusobacterium are also abundant (Drasar, 1967; Finegold, 1969; Finegold, 1970; Moore et al, 1969). Little work has been done on the actual sites in which these bacteria are found in vivo in humans, but Savage and Dubos (1967) have demonstrated that bacteria and yeasts occupy specific microenvironments in the mouse intestine. These layers of organisms were formed early in life and persisted for long periods.

Not much is known about the role of these organisms in the normal intestine. It has been suggested that anaerobes play an essential role in maintaining the integrity of the water transport mechanism in the intestinal epithelium in mice (Savage & Dubos, 1968). In humans they appear to be involved in defence against infection and various aspects of host nutrition, such as supplying vitamin K and deconjugating bile acids (Gibbons & Engle, 1964; Finegold, 1969; Finegold, 1970). It is becoming increasingly evident that the normal bowel flora may exert pathogenic effects under certain conditions of stress or physiological disturbance. They have been implicated in a variety of infections such as appendiceal abcess, peritonitis, diverticulitis and post-operative infections following surgery of the bowel or elsewhere in the abdomen. Gram-negative anaerobic bacilli, particularly Bacteroides species, are often associated with such infections and can be isolated in pure culture or together with other anaerobes or facultative organisms (Dubos et al, 1963; Felner & Dowell, 1971; Finegold, 1969;

Finegold, 1970; Finegold et al, 1972; Martin, 1971; Smith & Holdeman, 1968). B. fragilis is among the most common of these opportunist pathogens and has been found to resist many of the commonly used antibiotics, such as kanamycin, gentamicin, streptomycin and penicillin (Finegold & Sutter, 1971; Ingham et al, 1968; Kislak, 1972; Martin et al, 1972; Nastro & Finegold, 1972; Thornton & Cramer, 1970).

Many bowel anaerobes are extremely fastidious and require stringent anaerobic conditions for isolation and culture. The roll tube method developed by Hungate (1969) for obligate rumen anaerobes made it possible to eliminate exposure to oxygen during the entire isolation and cultivation procedure. To avoid the formation of peroxides and other organic inhibitors, methods of preparing pre-reduced and anaerobically sterilised (PRAS) media were also developed. Most intestinal anaerobes will grow at the Eh of -150 mV or lower which can be achieved by this method (Holdeman & Moore, 1972). Such techniques have been used to isolate obligate anaerobes from the intestinal contents of various animals (Holdeman & Moore, 1972; Vervaeke & van Nevel, 1972) and the mouth (Gordon et al, 1971) and intestine (Holdeman & Moore, 1972; McMinn & Crawford, 1970) of humans. Some workers also claim to have achieved improved isolation of anaerobes from clinical specimens using these methods (McMinn & Crawford, 1970). Others dispute this (Rosenblatt et al, 1973) and claim that the conventional jar method is adequate for clinical studies.

This roll tube method is difficult and requires special techniques and complex equipment. As an alternative simpler method for obtaining anaerobic conditions throughout isolation and cultivation procedures, anaerobic glove cabinets have been developed. The aim is to provide an oxygen-free space in which conventional bacteriological techniques may be used in conjunction with PRAS media. Such cabinets have been used to isolate anaerobes from various sources, such as animal intestines (Aranki et al, 1969; Leach et al, 1971; Lee et al, 1968), clinical specimens (Rosenblatt et al, 1973) and the human mouth

(Aranki et al, 1969) and bowel (Drasar, 1967).

Methods of varying complexity were used to remove oxygen from these cabinets. One technique (Lee et al, 1968) involved repeated inflation with nitrogen of a balloon inside the box. The displaced air was then replaced with a 95% N_2 - 5% CO_2 gas mixture. This method was not very satisfactory, since the methylene blue indicator (Eh = 11 mV at pH 7.0, Smith & Holdeman, 1968) did not always become completely reduced. Aranki et al (1969) constructed a collapsible plastic chamber which was evacuated and filled with 10% H_2, 5% CO_2 and N_2. Palladium catalyst in the chamber removed any remaining traces of oxygen and samples were admitted into the cabinet through an air lock. Drasar (1967) used a perspex box from which oxygen was initially removed by burning a spirit lamp and then by pumping the cabinet atmosphere through alkaline pyrogallol or a column of heated copper. The cabinet was then continuously flushed with a 95% N_2 - 5% CO_2 gas mixture. In an attempt to develop a more simple system, Leach et al (1971) constructed a rigid steel cabinet without an air lock. The system worked on the principle of displacement of air by a continuous stream of oxygen-free carbon dioxide. Residual traces of oxygen were removed from the influent gas by passing it over heated copper, but no catalyst was present inside the cabinet and the effluent gas was not recycled. Prior to use, the cabinet was perfused with carbon dioxide for three hours at a flow rate of 6 litres per minute. This was sufficient to reduce the P_{O_2} to less than 1.0 mm Hg and the flow rate was reduced to 3 litres per minute during subsequent procedures. Samples were introduced through two access ports on top of the cabinet. This brief opening did not greatly disturb the internal anaerobic conditions and the resazurin indicator remained colourless. The P_{O_2} in the effluent gas did rise to 8 mm Hg, but this could be restored to zero by flushing with carbon dioxide at 6 litres per minute for 20 minutes.

The methods of media preparation, measurement of the degree of anaerobiosis and other factors in the various glove

cabinets differed considerably. For example, Drasar (1967) poured and dried PRAS media inside the cabinet, thereby avoiding exposure to oxygen throughout. In contrast, Aranki et al, (1969) poured plates outside the cabinet but then placed them under anaerobic conditions for two days before inoculation. Most workers (Aranki et al, 1969; Drasar, 1967; Leach et al, 1971) also placed inoculated plates in anaerobic jars prior to removal from the cabinet. Oxygen levels have been measured with an oxygen electrode (Leach et al, 1971) or various indicators such as phenosafranine, methylene blue and resazurin.

One of the major factors limiting the efficacy of recovery of anaerobic bacteria is the method of collection and transport (Martin, 1971). The sampling method in different studies has varied widely and can be critical. Exposure to oxygen for any prolonged period at this stage will obviously affect the more fastidious anaerobes. According to Holdeman and Moore (1972) and Smith and Holdeman (1968), the isolation of anaerobes requires more exacting conditions than subsequent culture of the pure isolates. Optimum anaerobic conditions should therefore be used for isolation. Some workers recommend that transport media should not be used, as these can allow overgrowth of facultative organisms and loss of anaerobes if conditions are not as reduced as the site of origin (Holdeman & Moore, 1972; Fulghum, 1971). In contrast, Barry et al (1972) found that the recovery of anaerobic bacteria from clinical material on swabs was much more efficient if placed in liquid reduced transport media than if transported in empty dry tubes. It is well known that dessication is an important factor leading to death of bacteria. However in the present study, the organisms would be protected from dessication in the faecal sample, making such transport media unnecessary. A miniature anaerobic jar for tissue transport or the cultivation of anaerobes has been developed (Attebery & Finegold, 1970). Acidified copper sulphate and steel wool were used to obtain anaerobic conditions and "Alka-Seltzer" provided an effective source of carbon dioxide. Another aspect of anaerobic technique is the development of a mobile anaerobic unit for use in hospitals (Fulghum,

1971). This unit facilitates the inoculation of specimens directly into prereduced media so that no transport tubes or media are required.

Little work has been published on non-selective media for the isolation of total intestinal or faecal flora from humans. Various workers have used media such as Reinforced Clostridia agar (Barnes, 1969), brain heart infusion agar, or a complex and very rich medium (Drasar, 1967). Some authors recommend the use of rich media similar to those developed for rumen bacteria, for example rumen - fluid glucose cellobiose agar (RGCA) (Holdeman & Moore, 1972). However, Eller et al, (1971) suggested that such complex media containing large amounts of rich organic substances were unnecessary for bacteria of human origin, which are apparently not particularly nutritionally fastidious. They recommended a medium based on medium 10 (developed by Caldwell and Bryant (1966) for rumen anaerobes) but with deletions of various constituents. The new medium had a low energy content, resulting in small colonies which allowed accurate counting and picking in roll tubes.

Selective media are useful in facilitating the recovery of specific Gram-negative anaerobes from the mixture of facultative and anaerobic organisms found in faeces. Substances such as brilliant green and crystal violet have been used in media for this purpose (Shapton & Board, 1971). However, Finegold et al (1971) recommended the use of antibiotics for the isolation of anaerobes of human origin and this method is becoming widely adopted. The growth of certain organisms on these selective media also provides clues for rapid presumptive identification. For example, blood or laked blood agar containing kanamycin and vancomycin are both selective for Bacteroides species and virtually eliminate the growth of facultative organisms. Strains of Fusobacterium and Sphaerophorus will also grow well, whereas clostridia will not survive. Growth on these media can be regarded as good presumptive evidence of an obligate anaerobe and together with colonial morphology provides reliable tentative identification of Gram-negative non-sporing anaerobes (Finegold et al, 1971).

In this study, it was decided to select for the Bacteroides group of anaerobes, since these organisms predominate in the lower gastrointestinal tract and are amongst the non-sporing anaerobes most frequently associated with clinical material (see page 44). Taxonomy among this group is still in a state flux (Finegold, 1970; Finegold et al, 1967; Moore & Holdeman, 1972; Sutter & Finegold, 1971; Smith & Holdeman, 1968). Some workers even use the term "bacteroides" loosely to describe all obligately anaerobic non-sporing Gram-negative bacilli (Felner & Dowell, 1971). In a commonly used system of classification, the genera Sphaerophorus, Fusobacterium, Dialister and Bacteroides are grouped into the family Bacteroidaceae on the basis of morphological characteristics (Smith & Holdeman, 1968). However, in a later publication, the genera Sphaerophorus and Dialister are incorporated into the genera Bacteroides and Fusobacterium (Moore & Holdeman, 1972). Within the genus Bacteroides the species B. fragilis is most commonly isolated from the intestine (see page 44). Presumptive identification of this organism is often based on the characteristics of colonial growth, cellular morphology and Gram reaction. The ability to grow in desoxycholate broth or broth containing both bile and desoxycholate is also a useful criterion for rapid distinction between some Gram-negative anaerobes (Shimada et al, 1970). Definitive identification is based on physiological characteristics, including the fatty acids produced from glucose. These are identified by gas chromatography (Finegold, 1970; Finegold et al, 1967, 1972; Finegold & Hewitt, 1955, Gibbs & Skinner, 1966; Holdeman & Moore, 1972; Smith & Holdeman, 1968). It has also recently become evident that sensitivity to various antibiotics is a very useful means of identification. Sutter and Finegold (1971) have suggested a scheme for the presumptive identification of Gram-negative anaerobes based solely on antibiotic sensitivities.

Investigators have used a variety of broth or agar dilution and paper disc techniques for testing antibiotic susceptibility of anaerobes and results have differed considerably. For

example, minimum inhibitory concentrations (MIC's) reported for tetracylcine in B. fragilis have varied from 0.1 - 50 µg/ml (Nastro & Finegold, 1972) 1.56 µg/ml (Finegold & Hewitt, 1955) and 0.41 - 0.82 µg/ml (Ingham et al, 1968). Similarly, the reported MIC's for lincomycin in the same organism have varied from 1.1 - 4.4 µg/ml (Ingham et al, 1970), 0.2 - 10 µg/ml (Thornton & Cramer, 1970) and 0.1 - 12.5 µg/ml (Martin et al, 1972). A major drawback when comparing results is the lack of a standard method. Some investigators have recommended standard procedures for disc and broth dilution tests (Felner & Dowell, 1971; Sutter et al, 1972; Wilkins et al, 1972), but even these differ widely with regard to factors such as media and their preparation, age and size of the inoculum and method and length of incubation.

There are numerous problems associated with sensitivity testing under anaerobic conditions (Sapico et al, 1972; Thornton & Cramer, 1970). The standard disc method for aerobes has been developed for rapidly growing strains, whereas anaerobes are often found to grow slowly and erroneous interpretations can result. Another factor to be considered is the anaerobic environment. It has been shown that the MIC's of erythromycin and lincomycin for B. fragilis were 4 to 32 times higher when grown in a H_2 + 10% CO_2 atmosphere than values obtained if grown in a pure hydrogen atmosphere (Ingham et al, 1970). This effect could be due to the acidity of the culture medium produced by incubation under carbon dioxide. In agreement with this hypothesis, Ingham et al demonstrated that lowering the pH of the culture medium from 7.0 to 6.0 reduced the activity of the two drugs to a similar degree. This effect was also demonstrated for erythromycin by Thornton and Cramer (1970). In contrast, carbon dioxide had no effect on the MIC's of rifampin or clindamycin. Another example of drug inactivation is the inhibitory effect of reducing agents on benzylpenicillin (Todd, 1967). When the activity of this drug is tested under anaerobic conditions using a reducing agent in the medium, the results must therefore be interpreted with caution. Other

factors, such as the addition of blood to the medium, variations in the humidity when incubating and the aeration conditions under which plates are stored prior to inoculation may also affect the results of susceptibility tests (Sapico et al, 1972).

It had been demonstrated in Part I that R factor transfer could occur under anaerobic conditions. The aim of the next part of the project was to isolate obligate anaerobes, particularly strains of Bacteroides fragilis, from human faeces and to determine their drug resistance spectra with a view to investigating R factor transfer in this group of bacteria. As discussed previously (page 45), B. fragilis is resistant to many of the commonly used antibiotics. Being the dominant organism in the normal gastrointestinal tract, this organism thereby provides a large reservoir of drug resistance genes. If these are transferable, there could be a spread of resistance genes through the resident B. fragilis population. As Richmond (1972) has pointed out, such transfer even in healthy people would tend to restore resistance to a bacterial cell as soon as it had disappeared through gene loss. Since these endogenous organisms are also potential pathogens, antibiotic therapy could be undermined by resistance transfer. In addition the possibility exists that transfer could occur to other organisms in the gastrointestinal tract, for example to an infecting pathogen. It has been pointed out that probably one of the most important effects of R factors is to extend the range of organisms resistant to drugs. This has been observed with carbenicillin resistance transfer from enteric bacteria to Pseudomonas aeruginosa (Richmond, 1972). Such a phenomenon would also cause a failure of antibiotic therapy. Even if the recipient organism was not pathogenic, transfer would tend to spread resistance genes through an ever increasing range of organisms as the pattern of antibiotic therapy changed. Resistance transfer has not yet been demonstrated in Bacteroides. In order to cover the possible directions of transfer discussed above, it was planned to investigate transfer from one strain of B. fragilis to another and also from B. fragilis to E. coli and vice versa. B. fragilis is a Gram-negative non-sporing rod. This was another important reason for selecting this organism for investigation, as it presumably has various cell wall properties in common with the Gram-negative strains in which R factor transfer has previously been demonstrated.

The conflicting results regarding the efficiency of the different anaerobic techniques make it difficult to adopt a standard method for isolating intestinal anaerobes. The cabinet method seemed simpler and less time consuming than the roll tube method used in Part I, yet equally successful. In addition bacteriological techniques such as the disc method of antibiotic sensitivity testing, "triple" streaking of an agar plate to obtain pure clones from a mixed culture and multiple streaks onto a single plate cannot be used in roll tubes, but are possible in a cabinet. Transferable drug resistance experiments are also difficult to perform by the roll tube method. In South Africa, the cost if importing a cabinet would be exorbitant; £1000 has been described as "inexpensive" in the U.S.A. (Aranki et al, 1969). An inexpensive cabinet was therefore constructed using local materials.

CHAPTER 1

THE ISOLATION OF OBLIGATE ANAEROBIC FAECAL BACTERIA

Summary

A glove cabinet was constructed in which anaerobic conditions were achieved by flushing with a CO_2 + H_2 gas mixture and removing any contaminating O_2 with palladium catalyst. The cabinet was used in conjunction with anaerobic jars and specially constructed perspex containers. The redox dye resazurin was used as an indicator of anaerobic conditions. Liquid and solid media were anaerobically prepared and freshly voided faecal specimens were transported to the laboratory anaerobically. Although a medium selective for Gram-negative obligate anaerobes was used, many of the originally strictly anaerobic strains isolated became facultative after several subcultures in the laboratory. These strains simultaneously developed resistance to ampicillin. The viability of the strains under different conditions of aeration was also investigated.

(A) MATERIALS AND METHODS

(a) Cabinet Construction

The design incorporated various features of the cabinets constructed by Aranki et al, (1969) and Leach et al, (1971). The cabinet (Fig. 10), with dimensions 97 cm x 59 cm x 64 cm, was made of fibre glass moulded on a wooden frame. The sloping viewing window (81 cm x 33 cm) consisted of $\frac{1}{4}$" perspex sheet fixed to the cabinet by an aluminium frame. The 3/8" perspex air lock (22 cm x 26 cm x 21 cm) was designed to accommodate an anaerobic jar. Two air-tight doors provided access to the air lock. A hinged brass frame was made for each door and springs attached to this frame ensured even pressure of the closed door onto a rubber seal. Rapid operation was facilitated by a single screw fastening action.

Three aluminium glove ports projected 2.9 cm from the front of the cabinet. Shoulder length rubber gloves were held in position with rubber rings which fitted over these projections. Each port could be closed with a wooden plug edged with sponge rubber. When closed, each plug was held in place with a hinged bracket and swivel clip to avoid ejection by the positive pressure inside the cabinet. An emergency access port on top (diameter 24 cm), a perspex shelf along the back wall and a glass manometer in one side wall were incorporated.

An ultraviolet light for sterilisation, a 5 amp socket and a 60 watt fluorescent light were fitted inside, but the control panel for these was situated outside the cabinet. To avoid air leakage through electric cables, electrical connections were made by fastening 2 BA $\frac{3}{4}$" threaded rods through the cabinet wall with nuts and bolts. Leads were then taken from either side of these rods to the appropriate fittings.

(b) Additional Equipment

Since a gas flame could not be used in the cabinet, an electric inoculating loop was constructed (Aranki et al, 1969).

Fig. 10: Anaerobic glove cabinet and related equipment.

Heavy gauge platinum wire was used for the loop as nichrome oxidises anaerobically prepared media (Moore, 1966). Four conventional anaerobic jars were used during the initial isolation procedures. This limited the number of plates to 48, which was insufficient and resulted in frequent brief exposure of anaerobes to air.

Two anaerobic containers (32 cm x 30 cm x 14 cm) made of 3/8" perspex were therefore constructed for storage and incubation of plates. The hinged lids were similar to the doors of the air lock. Each box could accommodate 57 plastic petri dishes, bags of silica gel and catalyst pellets (Fig. 11).

Resazurin has a lower redox potential than methylene blue (Smith & Holdeman, 1968). After initial trials using both dyes, resazurin was chosen as the indicator of anaerobic conditions in all containers. The method of preparation was similar to that for Lucas semi-solid indicator, (Willis, 1969), but phenol red was omitted and methylene blue was replaced by 0.0003% resazurin. (Eh = -42 mV at pH 7.0)

(c) Gases

It has been shown that carbon dioxide is required by some anaerobes (Barnes, 1969; Finegold, 1970; Smith & Holdeman, 1968) and that pure nitrogen cannot be used to obtain anaerobic conditions rapidly by flushing a cabinet (Leach et al, 1971). Various gas mixtures were initially tested in this study. Expensive gases such as exact mixtures of 95% CO_2 - 5% H_2, 95% N_2 - 5% CO_2 and highly purified CO_2 were inadequate to produce sufficiently anaerobic conditions to reduce the redox indicator. In order to achieve more stringent anaerobiosis, a Deoxo catalyst cartridge was used to remove any contaminating O_2 in the influent gases from the cylinders (Fig. 12). In addition, 0.5% palladium coated alumina pellets were placed in a perforated metal tray in the cabinet to remove any O_2 entering through possible leaks. Both catalysts depend on the presence of at least a trace of H_2 to react with O_2. An

Fig. 11: Anaerobic incubating container.

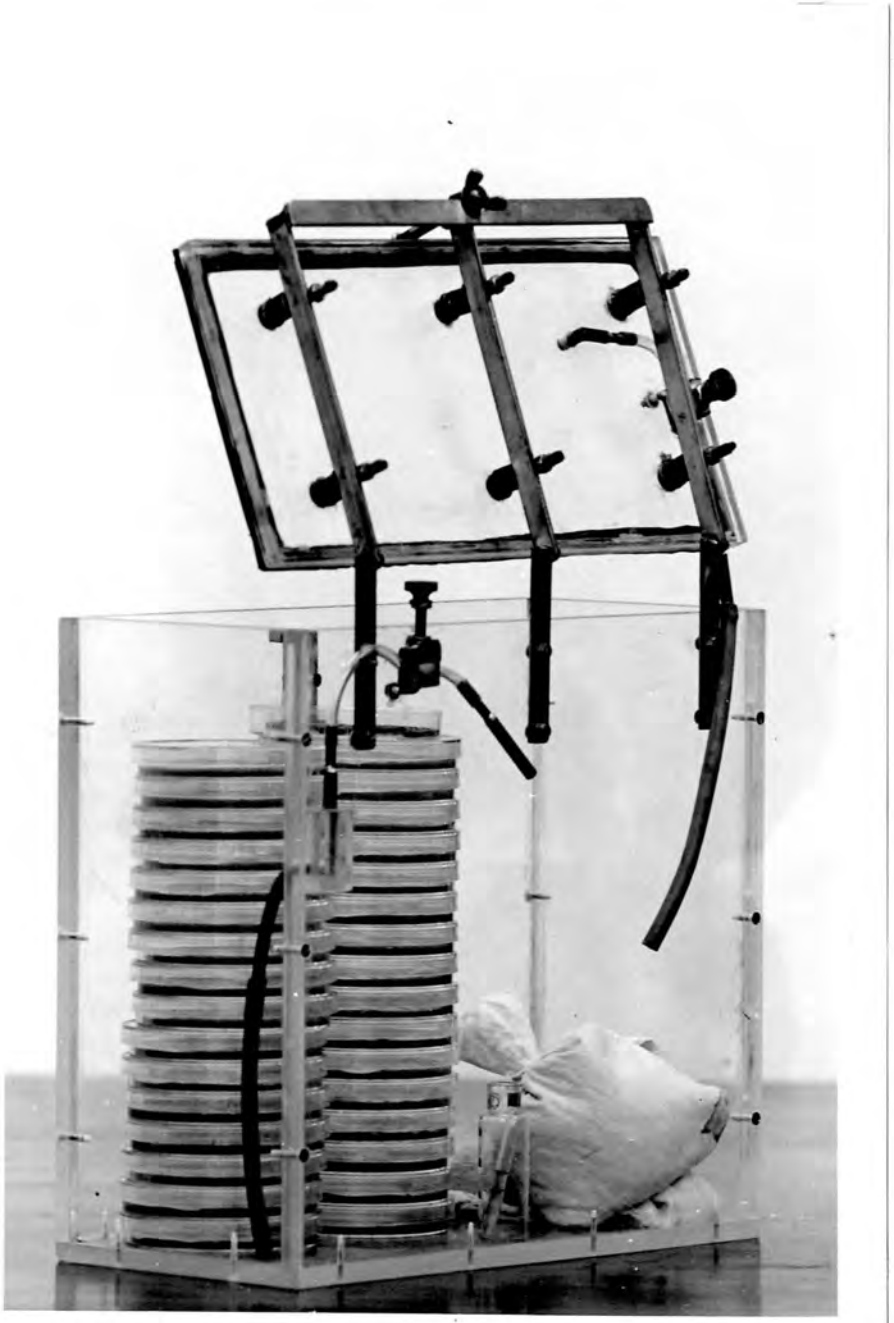
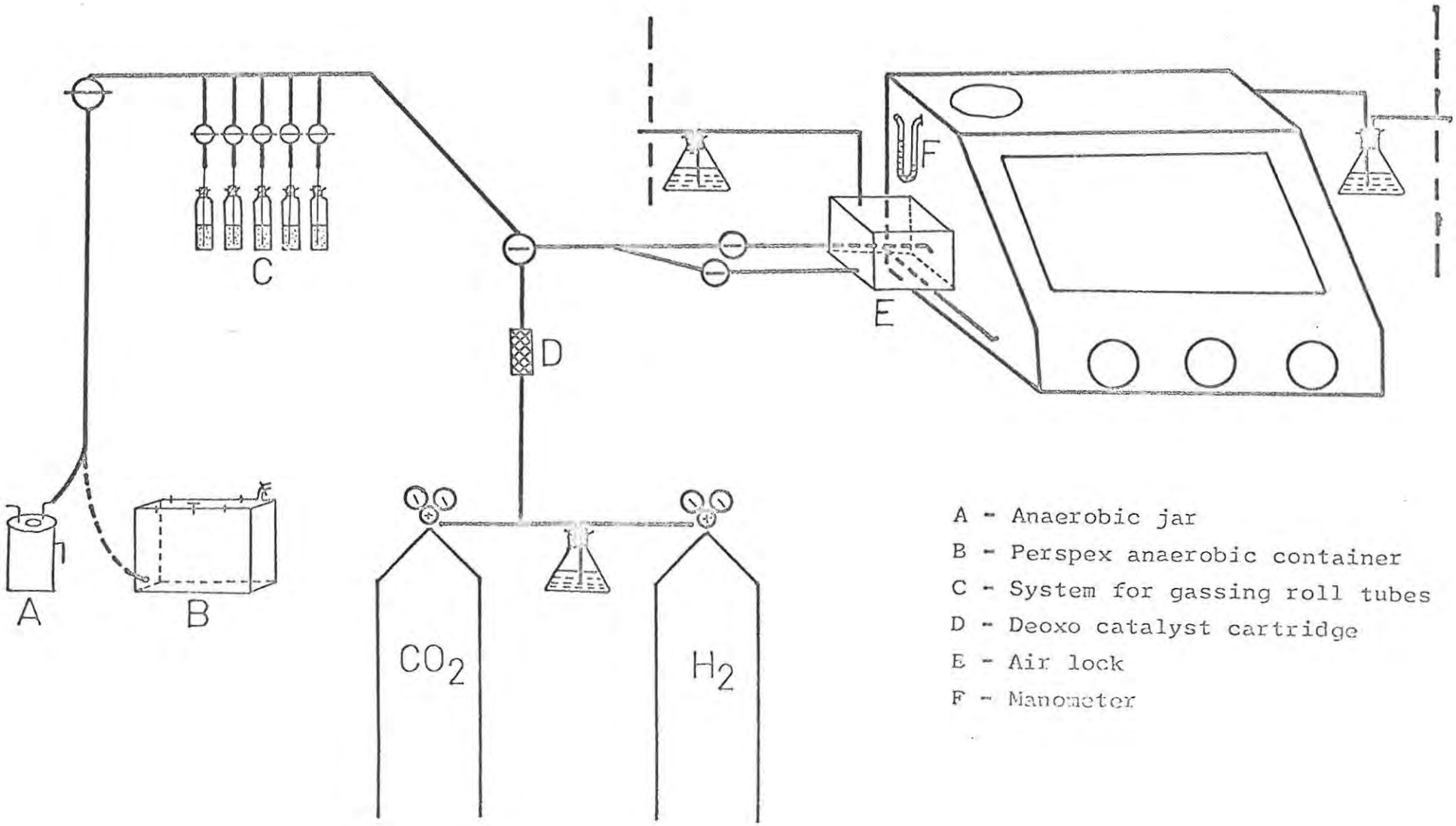


Fig. 12: Diagram of gas flow through anaerobic system.



A - Anaerobic jar
B - Perspex anaerobic container
C - System for gassing roll tubes
D - Deoxo catalyst cartridge
E - Air lock
F - Manometer

efficient and cheap means of achieving this was to purchase separate cylinders of commercial grade H_2 and CO_2. A slow stream of H_2 was "bled" via a bubbler into the CO_2 inlet tube so that the gases merged before entering the Deoxo cartridge. The rate of appearance of bubbles indicated the H_2 flow rate. A 25 kg CO_2 cylinder (price approx. R6) was sufficient for about a month while a 0.5 kg H_2 cylinder (approx. R8) lasted many months. Influent and effluent gas flowed through pressure tubing. Gas entered through 2 inlets at the bottom of the cabinet and one inlet to the air lock. From three outlets at the opposite top corner of the cabinet and one outlet from the air lock, the effluent gas flowed to the outside of the building via wash bottles containing paraffin (Leach et al, 1971). These acted as traps to prevent entry of air into the cabinet when the gas flow was turned off. They also constituted safety devices, as the bubbling of effluent gas could be seen, thus ensuring that the CO_2 was passing to the outside atmosphere.

The loose catalyst pellets require periodic reactivation by heating. Two lots of catalyst were used in rotation so that freshly activated pellets could replace inactive catalyst in the cabinet twice a week (Aranki et al, 1969).

(d) Operation

Initial trials were undertaken using alkaline pyrogallol to absorb some O_2 from the interior of the cabinet before flushing with gas, but this method proved unsatisfactory. In addition the carbon monoxide formed is toxic to some anaerobes (Smith & Holdeman, 1968). For routine use, some oxygen was therefore removed with a spirit lamp (Drasar, 1967) and the cabinet was flushed with the CO_2 + H_2 mixture for 3 hours at 4 litres per minute. Together with approximately 90 gm of catalyst pellets (Aranki et al, 1969) this was sufficient to maintain the indicator in the reduced state for at least 24 hours. While in use, the cabinet was flushed through at 0,5 litres per minute. This maintained the positive pressure on

the gloves necessary for easy manipulations. The air lock was flushed for 20 minutes at 3 litres per minute. Initial trials showed that the resazurin remained colourless for at least 12 hours after this procedure. Approximately 5 gm of freshly activated catalyst was routinely placed in each of the perspex containers before flushing for 30 minutes at 3 litres per minute. Under these conditions, the resazurin remained reduced for at least 48 hours.

Initially the anaerobic jars were routinely introduced into the cabinet via the air lock (Aranki et al, 1969). However, subsequent trials showed that growth of the obligate anaerobes was not affected if uninoculated or inoculated plates were transferred rapidly between air lock and anaerobic jar or perspex box. Anaerobic roll tubes with liquid or slope cultures were sealed inside the cabinet after inoculation. If necessary (for example for introducing pipettes which did not fit through the air lock), the emergency port was opened briefly during operation of the cabinet. The indicator did not become oxidised during this procedure, but as a precaution the cabinet was flushed for 15 minutes at 4 litres per minute before resuming experimental procedures. The flow rate was then returned to the normal low level used during operation.

(e) Media

Detailed methods for preparation of media are described in Appendix A.

PRAS media were prepared according to the principles of Hungate (1969) and Moore (1966). All anaerobic media contained 0.05% cysteine hydrochloride or 0.11% sodium thioglycollate as a reducing agent and 0.4% Na_2CO_3 as a buffer. Brain heart infusion broth (BHIB) was used as a liquid medium and diluting fluid.

The mineral solution (MD) used as an alternative diluent and as a transport medium for the first isolation procedure was based on that of Bryant and Burkey (1953), but cysteine was

replaced by thioglycollate. Both liquid media contained 0.0002% resazurin. After adding all ingredients, the solution was boiled until the colour of the resazurin had changed from blue to pink. Prior to autoclaving, further reduction to the colourless state was achieved by dispensing the medium into Astell roll tubes fitted with rubber stoppers and gassing with CO_2 + H_2 according to the method of Kistner (1960).

A nonselective medium (CM) based on modified medium 10 (Eller et al, 1971) was used to isolate total anaerobes from faeces. Brain heart infusion agar (BHIA), blood agar (BA) or laked blood agar (LBA) were used as the basis for selective and maintenance media. The addition of 0.5 µg/ml menadione, 5 µg/ml hemin and 5 mg/ml yeast extract (Holdeman & Moore, 1972) was found to stimulate the growth of the anaerobic strains isolated on BHIA,which was routinely supplemented with these constituents (BHIA-S). The menadione and hemin solutions were prepared according to the methods of Holdeman and Moore (1972). Kanamycin (1000 µg/ml) and vancomycin (7.5 µg/ml) were added to these media to select for Gram-negative non-sporing anaerobes and particularly Bacteroides fragilis (Finegold et al, 1971). Ampicillin (25 µg/ml) was also added to some selective plates.

During initial trial isolation procedures, media were introduced into the cabinet immediately after autoclaving. All constituents such as cysteine and Na_2CO_3 solutions, blood and drug solutions were prepared anaerobically and added to the stock medium inside the cabinet prior to pouring under anaerobic conditions. The plates were then dried in the cabinet, which had to be kept anaerobic for several days. An electric hot plate was installed in the cabinet to facilitate pouring. This technique proved unsatisfactory for several reasons. The floor of the cabinet sloped slightly unevenly, so that plates of an even depth could not be poured. In addition the limited space made it difficult to pour a sufficient number of plates. The time required for drying was often more than 48 hours, which was inconvenient. For subsequent isolations, media were therefore poured on the bench but when solidified, the

plates were immediately placed under anaerobic conditions and stored for at least 24 hours before use. This prevents the formation of the inhibiting organic peroxides formed when media containing reducing agents become oxidised (see page 41). Silica gel was used to dry plates in the anaerobic containers. To avoid sucking up of the agar during evacuation of anaerobic jars, the plates were not inverted.

(f) Sampling and Isolation

All faecal samples were obtained from Bantu children in the Grahamstown Settlers Hospital. For the first isolation anaerobic MD (mineral diluent) was used as a transport medium. However such transport fluids are not recommended (see page 47) so a dry CO_2 - filled tube was substituted. Using a sterile spatula, a sample of freshly voided faeces was quickly placed in a pre-weighed transport tube. A stream of sterile CO_2 from a CO_2-filled football bladder was immediately flushed through the tube via a long needle. During this procedure the stopper was held in place gently. It was then firmly pressed down as the needle was withdrawn. Samples were immediately taken to the laboratory, weighed and introduced into the cabinet. The time elapsed after sampling was always less than 30 minutes. A 10 ml volume of MD was added to each sample and the faeces were thoroughly dispersed and mixed using a whirlimix. Serial 10-fold dilutions were carried out using a pumpette. The resazurin in the diluent remained colourless throughout this procedure. Appropriate dilutions were spread on CM and the selective medium. Duplicate plates were incubated anaerobically at 37° for 48 hours or more. Plates were then examined inside the cabinet to avoid exposure to O_2 and isolated colonies on the selective medium were streaked on BHIA-S or BA to obtain pure clones. Each pure strain isolated in this way was tested at regular intervals for aerobic growth on BHIA-S. Strains were maintained on BHIA-S slopes in roll tubes and subcultured every 2 weeks.

(B) RESULTS

(a) Isolation of Anaerobes

The plate counts, colony types and number of obligately anaerobic pure strains isolated from six subjects are shown in Tables VIII, IX and X. Those clones from KV plates in isolations 2 and 3 with colonial morphology similar to that of *B. fragilis* were selected for further study (Finegold *et al*, 1971).

(b) Development of Facultative Characteristics in Obligate Anaerobes.

Thirty-four strains from the second isolation, originally all obligate anaerobes (group 1 : strains 1-55) were repeatedly tested for the ability to grow aerobically. Owing to a lack of anaerobic containers (see page 57), the stock cultures of these strains were initially briefly exposed to air on several occasions. Thereafter they were maintained in an anaerobic environment throughout. It was found that the strains became facultative one by one over a period of two months, during which they were subcultured several times.

The obligate anaerobes from the third isolation (group 2 : strains 56-98) were similarly tested. However the stock cultures were maintained under strict anaerobiosis throughout and received absolutely no exposure to O_2. Of the 42 strains, four (strains 58, 59, 64 and 70) were spontaneously able to grow aerobically when subcultured from the stock culture onto aerobic medium (Table XI). In order to simulate the exposure to O_2 of the group 1 strains, broth cultures of the group 2 anaerobes were grown under slightly oxidised conditions (resazurin faintly pink for approximately 1 hour after inoculation) and then tested for facultative ability. This treatment was repeated in several different experiments and a total of 14 more strains became oxygen tolerant (Table XI). All except one of these 14 strains grew vigorously under aerobic conditions. The strains which became facultative in each experiment were not always the same, suggesting that this

TABLE VIII RESULT OF FIRST ISOLATION

Subject	Medium	Count per gm faeces	Remarks
A	CM	8×10^9	Foul smell from plates. Most colonies small, white.
	BA + KV	1×10^9	Large areas of total haemolysis on some plates.
B	CM	1×10^9	Foul smell from plates. Most colonies small, white.
	LBA + KV	1×10^5	Some plates showed slight haemolysis.

74/78 clones tested from subjects A and B were obligately anaerobic. These strains were not studied further.

KV = kanamycin + vancomycin

TABLE IX RESULT OF SECOND ISOLATION

Subject	Medium	Count per gm faeces	Remarks
C	BA + KV	2.9×10^{10}	Foul smell from plates. Some plates showed areas of haemolysis. After 5 days' incubation, one plate had 50 colonies with dark centres. (characteristic of B. melaninogenicus) (Finegold et al, 1971)
D	BA + KV	1.3×10^{9}	Most colonies small and white; convex with entire edges. Some colonies large, white, convex mucoid with undulate edge.

47/80 clones tested from subjects C and D were obligately anaerobic; 34 of these strains selected for further study.
(Group 1 anaerobic strains).

TABLE X RESULT OF THIRD ISOLATION

Subject	Medium	Count per gm faeces	Remarks
F	BA + KV	6.1×10^6	The following colony types were noted for colonies from both subjects F and G:
G	BA + KV or KVA	1×10^6	(a) White to yellowish mucoid raised colony with entire edge (characteristic of B. fragilis)
			(b) Dry matt large colony with undulate edge and wavy surface.
			(c) Creamy white small raised colony with denser centre and undulate edge.

43/78 strains tested from subjects F and G were obligate anaerobes. Most of these were of type (a) colonial morphology. All 43 obligate anaerobes were selected for further study (Group 2 anaerobic strains).

KVA = kanamycin + vancomycin + ampicillin.

KEY FOR TABLE XI

TEST NO: Tests Nos. 1 to 11 were performed sequentially at intervals of 2 to 3 weeks. Where 2 or more tests are grouped together, the results were identical.

TREATMENT: This records the aerobic or anaerobic treatment to which each stock strain (test a) or subculture (tests a, b & c) was subjected prior to the test for facultative growth.

Note: Where subcultures were made, these were taken directly from the stock strains in the cabinet.

(a) Broth culture; no exposure to O_2 at any stage.

(b) Broth culture; anaerobically inoculated and incubated, but aerobically streaked for facultative test.

(c) Deliberate brief exposure of broth culture to O_2 after anaerobic inoculation. Aerobically streaked for facultative test.

(d) Test streaks taken directly from stock cultures in cabinet.

TEST: FAC: test for ability to grow on aerobically prepared medium (BHIA-S without cysteine or $Na_2 CO_3$) under aerobic conditions of incubation.

AMP: test for ability to grow on BHIA-S containing 25 µg/ml ampicillin.

\+ = growth
\- = no growth
N = not tested.

TABLE XI: DEVELOPMENT OF FACULTATIVE ABILITY IN GROUP 2 ANAEROBIC STRAINS

TEST NO.	1 & 2	3	4, 5 & 6	7		8	9	10		11	
TREATMENT	a	b	a	c		a	b	c		d	
TEST	FAC	FAC	FAC	FAC	AMP	FAC	FAC	FAC	AMP	FAC	AMP
Strain 56	-	-	-	N	N	-	-	N	N	N	N
57	-	-	-	N	N	-	-	N	N	N	N
58	-	+	+	N	N	+	+	+	+	+	+
59	-	-	-	N	N	+	+	N	N	+	+
60	-	-	-	-	-	-	-	-	-	-	-
61	-	-	-	-	-	-	-	+	+	-	-
62	-	-	-	+	+	-	-	-	-	+	+
63	-	-	-	-	-	-	-	-	-	-	-
64	-	-	-	N	N	-	+	N	N	+	+
65	-	-	-	-	-	-	-	+	+	+	+
66	-	-	-	-	-	-	-	+	+	+	+
67	-	-	-	-	-	-	-	-	-	N	N
68	-	-	-	-	-	-	-	+	+	-	-
69	-	-	-	-	-	-	-	+	+	+	+
70	-	-	-	-	-	-	+	-	-	-	-
71	-	-	-	-	-	-	-	+	+	-	-
72	-	-	-	-	-	-	-	-	-	N	N
73	-	-	-	-	-	-	-	-	-	-	-
74	-	-	-	-	-	-	-	-	-	N	N
75	-	-	-	-	-	-	-	+	+	-	-
76	-	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	+	+	-	-
78	-	-	-	-	-	-	-	-	-	N	N
79	-	-	-	-	-	-	-	-	-	N	N
80	-	-	-	-	-	-	-	-	-	N	N
81	-	-	-	-	-	-	-	-	-	N	N
82	-	-	-	-	-	-	-	+	+	-	-
83	-	-	-	+	+	-	-	+	+	+	+
84	-	-	-	-	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-	-	-	N	N
86	-	-	-	+	+	-	-	-	-	-	-
87	-	-	-	+	+	-	-	-	-	-	-
88	-	-	-	N	N	-	-	N	N	N	N
89	-	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	-
91	-	-	-	-	-	-	-	-	-	-	-
92	-	-	-	-	-	-	-	-	-	-	-
93	-	-	-	-	-	-	-	-	-	N	N
94	-	-	-	-	-	-	-	-	-	N	N
95	-	-	-	-	-	-	-	-	-	N	N
96	-	-	-	-	-	-	-	+	+	+	+
97	-	-	-	-	-	-	-	-	-	N	N
98	-	-	-	-	-	-	-	-	-	-	-

tendency was present in many of the strains, but was not always immediately expressed. It was also observed that when the strains became facultative, they simultaneously became resistant to ampicillin, whereas the obligate anaerobes remained sensitive. The stock cultures of the 14 strains were also tested by streaking directly onto aerobic and ampicillin containing plates inside the cabinet. Of these, 8 strains were still obligate anaerobes and amp^s, whereas the other 6 strains were now facultative and amp^r (Table XI).

(c) Studies on the viability of anaerobes after exposure to oxygen

Mating, diluting and plating procedures were extremely laborious and time consuming when carried out in the cabinet. Various experiments were therefore performed on the group 2 anaerobes to determine the extent to which cultures could be exposed to O_2 without losing viability.

(i) Growth on Oxidised Anaerobic Plates: The effect of exposing anaerobically prepared medium to oxygen prior to inoculation was investigated. Anaerobic broth cultures were streaked onto BHIA-S plates (containing cysteine) which had been left on the open bench for six hours. The plates were then immediately incubated under anaerobic conditions. Growth of all eleven strains tested in this way was very weak. This inhibitory effect of oxidised medium was probably due to the formation of "organic peroxides" (see page 41).

(ii) Growth of Anaerobes in Oxidised Broth: BHIB was anaerobically prepared, but tubes were opened on the bench and allowed to become oxidised so that the resazurin became pink. These broths were inoculated with 0.1 ml aliquots of 24-hour anaerobic broth cultures. None of the 34 strains tested could grow in this medium. The resazurin did not become reduced again as occurred with tubes which were very briefly opened (see tests (iii) and (v)). The inhibition was probably due to both the organic peroxides formed and the high redox potential.

(iii) Viability of Broth Cultures after Exposure to Air: Twentyfour-hour anaerobic broth cultures were opened on the bench, closed quickly and allowed to stand for various time intervals of up to 90 minutes. The resazurin became oxidised, but gradually turned colourless again. Streaks from these tubes onto anaerobic BHIA-S were anaerobically incubated. All 4 strains tested showed no loss of viability.

(iv) Anaerobic versus Aerobic Dilution and Plating: Twentyfour-hour anaerobic broth cultures were serially diluted in BHIB in the cabinet. Appropriate dilutions were plated on BHIA-S in the cabinet and incubated anaerobically. The same 24-hour cultures were then removed from the cabinet through the air lock and identical diluting and plating procedures were carried out on the bench. The following precautions were observed to ensure minimal exposure to O_2:

(1) Cultures were streaked within 20 minutes of opening the tube or loosening the stopper.

(2) Anaerobic plates were exposed to O_2 for a maximum of 20 minutes whether inoculated or not.

(3) The CO_2 + H_2 gas was flushed through the anaerobic jar or incubator throughout the plating procedures. Unused and inoculated plates were thereby maintained under fairly anaerobic conditions until the container was full and ready for final gassing and incubation.

The results showed that there was no loss of viability after this treatment. For example, viable counts obtained by the two methods were as follows:

	Aerobic method	Anaerobic method
Strain 61	2.6×10^8	2.8×10^8
Strain 67	3.8×10^8	3.5×10^8

All diluting and plating procedures were subsequently performed on the open bench, but the above precautions were always observed.

(v) Anaerobic versus Aerobic Inoculation: In order to simplify mating procedures, an experiment was carried out to determine the effect of inoculating an anaerobic broth culture on the open bench. Duplicate tubes of anaerobic BHIB were inoculated in the cabinet and on the bench from 24-hour anaerobic broth cultures. The aerobically inoculated tubes were opened very briefly and were not shaken. The resazurin turned slightly pink but became reduced again after two to three hours' incubation. The viable counts performed anaerobically and aerobically after different time intervals were as follows:

	Duration of incubation (hours)	Aerobic Method	Anaerobic Method
Strain 66	6	7.7×10^7	5.1×10^7
	12	1.2×10^8	8.9×10^7
Strain 73	6	2.3×10^7	3.5×10^7
	12	1.7×10^8	6.9×10^7

These results showed that the growth rate of the anaerobes was similar for both methods. All anaerobic mating procedures were subsequently performed on the open bench, but exposure to O_2 was always kept to a minimum.

CHAPTER 2

DETERMINATION OF DRUG RESISTANCE SPECTRA OF ANAEROBIC STRAINS

Summary

The resistance spectra of the anaerobic strains were determined for 12 drugs under anaerobic conditions. The paper disc, streak and plate methods were used for sensitivity testing. The results for some drugs were satisfactory, whereas others were difficult to interpret or inconsistent. It was found that cysteine inactivated benzylpenicillin if the drug was incorporated in the medium.

(A) MATERIALS AND METHODS

(a) Disc Tests

Each strain was inoculated into BHIB in the cabinet and incubated for 24 hours. A 0.1 or 0.2 ml sample of the culture was spread onto BA (group 1 strains) or BHIA-S (group 2 strains) with a glass spreader. Sensitivity discs were then placed on the surface. Discs were arranged in such a manner as to avoid possible interactions between drugs. For initial tests, the above procedures were carried out in the cabinet and pre-sterilised spreaders were used. Later tests were performed on the bench (see section on viability). The plates were immediately placed under anaerobic conditions and incubated for 48 hours. They were then examined on the bench for zones of inhibition. The recommendations of Waterworth (1971) regarding the application of discs were taken into account.

(b) Streak Tests

Streak or plate sensitivity tests were required to select for recombinants in transfer experiments. In order to correlate disc tests with streak sensitivities and also to test additional drugs for which discs were not available, drugs were incorporated into the medium (BA or BHIA-S). Streaks from 24-hour anaerobic broth cultures of the test strains were then made onto the medium. These tests were also initially performed inside the cabinet, but later on the open bench. For some drugs the streak tests gave unsatisfactory results when undiluted broth cultures were used; in the case of penicillin, ampicillin, tetracycline and erythromycin, there were often resistant colonies only at the beginning of the streak where the inoculum was heaviest (see Table XIII). This could have been false resistance, since "shielding" could occur due to the high density of cells inoculated onto a small area. To avoid this, a hundredfold dilution of each culture was streaked onto the relevant drug containing plates.

(c) Plate Tests

For some transfer experiments, 0.1 ml inocula of undiluted mating mixtures were spread onto drug plates to ensure maximal possible recovery of recombinants from possible low frequency transfer. Sensitivity tests were therefore also performed by spreading 0.1 ml inocula onto drug plates with a glass spreader to investigate whether "shielding" would occur.

(B) RESULTS AND DISCUSSION

The results for sensitivity testing of group 1 and group 2 strains are shown in Tables XII and XIII respectively. The following observations were made:

(i) Inocula of 0.2 ml gave far better results for disc tests as growth was heavier and the zones of inhibition were easier to measure.

(ii) The results for penicillin, ampicillin, tetracycline and rifampicin were inconsistent. Some strains which on first testing appeared to be sensitive to pen and amp were resistant in subsequent tests (Table XIII). The simultaneous development to amp^r by some strains which became facultative has been discussed previously (see page 70) and the inconsistency for amp^r was probably often due to this phenomenon. The variation in sensitivity to penicillin was possibly due to the effect of cysteine in the medium - see section (vi). Sensitivity to tetracycline and rifampicin varied considerably in different tests (Table XIII). Sensitivities to lincomycin and erythromycin were fairly consistent, with only a few strains giving variable results. Results for the other 8 drugs (including kanamycin and vancomycin) were the most consistent (Table XIII).

(iii) Results on BHIA-S were easier to interpret than those on BA, on which growth was weaker. The presence of blood in the medium also seemed to interfere with the drugs.

(iv) Results were easier to interpret after an incubation period of 36 hours or longer, particularly for the disc tests (result of initial trials). A standard incubation period of 48 hours was chosen.

(v) Strains 56 and 57 were originally isolated on plates containing kanamycin, vancomycin and ampicillin. However they appeared to be amp^s in later tests. These strains and 6 others were therefore tested on combinations of the drugs. Table XIV shows that 5 of the 8 strains gave inconsistent sensitivity results. These strains were sensitive to amp alone, but resistant

to amp in combination with kan and van. This suggested that there was some interaction between the three drugs when present together in the medium which could inactivate the ampicillin.

(vi) As discussed previously (see page 50), benzylpenicillin is reportedly inactivated by cysteine. This effect was investigated on both the disc and plate methods of sensitivity testing as results for this drug had been inconsistent. The results (Table XV) showed that penicillin inactivation occurred when the drug was present in the medium containing cysteine,but not when discs were used on cysteine containing plates. Where cysteine was omitted from the medium, the penicillin streak sensitivities correlated well with disc sensitivities for those strains which remained viable. Discs must therefore be used for benzylpenicillin sensitivity testing unless cysteine (or presumably other reducing agents) can be omitted from the medium. This finding demonstrates that the results of sensitivity testing under anaerobic conditions will remain variable and difficult to interpret until a generally accepted standard technique is defined.

(vii) The results from the plate sensitivity tests were satisfactory and no "shielding" occurred. This method of sensitivity testing could therefore be used in resistance transfer experiments.

KEY FOR TABLE XII

+ = resistant
- = sensitive
+/- = results inconsistent
w = weakly resistant

This table represents the best interpretation of 2 disc sensitivity tests (where discs were available) and 3 streak sensitivity tests.

D + S: disc and streak tests
S: streak tests only (all at 10^{o} dilution of culture)
D: disc tests only.

All sensitivity testing done on Blood Agar.

Drug concentrations (µg/ml):

kan	1000
van	7.5
amp	25
str	25
nal	30
tet	50
cpd	15
cml	30
rif	50
nft	200

TABLE XIIa: DRUG RESISTANCE SPECTRA OF GROUP 1 STRAINS

Drug	kan	van	amp	str	nal	tet	cpd	cml	rif	nft
Test	S	S	D + S	D	D	D + S	D	D + S	S	D + S
Strain										
1	+	+	w	+	+	+	-	-	-	-
2	+	+	+	+	+	+	+/-	+	-	-
4	+	+	+	+	+	w	+	-	-	-
5	+	+	+	+	+	+	+	+	+/-	-
6	+	+	+	+	+	+	-	-	-	-
7	+	+	+	w	+	+	+	+	+/-	-
8	+	+	+	+	+	+/-	-	-	-	-
9	+	+	+	+	+	+	+	-	-	-
10	+	+	+	+	+	+	+	-	-	-
11	+	+	+	+	+	+	+	w	-	-
12	+	+	+	+	+	+	-	+	-	-
13	+	+	+	+	w	+	+/-	-	-	-
15	+	+	+	+	+	+	+/-	+	+/-	-
17	+	+	+	+	+	+	+/-	w	+/-	-
20	+	+	+	+	+	w	+/-	-	-	-
21	+	+	+	+	+	+	+	-	-	-
23	+	+	+	+	+	+	+	-	-	-
24	+	+	+	+	+	+	-	+	+/-	-
25	+	+	w	+	w	+	-	-	-	-
26	+	+	+	+	+	+	+/-	+	-	-
27	+	+	+	+	+	w	+	-	-	-
29	+	+	w	+	+	w	+/-	-	-	-
31	+	+	w	+	+	w	w	-	-	-
35	+	+	+	+	+	+	-	+	w	-
36	+	+	+	+	+	+	+	+	w	-
37	+	+	+	+	+	+	+	+	-	-
39	+	+	+	+	+	+	+	+	w	-
40	+	+	+	+	+	+	+/-	+	w	-
41	+	+	+	+	+	+	+	+	+/-	-
42	+	+	+	+	+	+	+	+	w	-
51	+	+	+	+	+	w	+	w	w	-
52	+	+	+	+	+	+/-	+	-	-	-
54	+	+	+	+	+	+	+	+	+/-	-
55	+	+	+	+	+	+	+	-	-	-

TABLE XIIb: SUMMARY OF DRUG RESISTANCE SPECTRA OF GROUP 1 STRAINS

Drug	No. of strains fully sensitive	No. of strains weakly resistant or +/-	No. of strains fully resistant
kan	0	0	34
van	0	0	34
amp	0	4	30
str	0	1	33
nal	0	2	32
tet	0	8	26
cpd	7	9	18
cml	16	3	15
rif	21	13	0
nft	34	0	0

KEY FOR TABLE XIII

+ = resistant

- = sensitive

w = weakly resistant

b = resistant colonies only at beginning of streak where inoculum was heaviest.

S = streak test

D = disc test

P = plate test

All sensitivity testing done on BHIA-S.

Drug concentrations (µg/ml):

kan	1000
van	7.5
neo	1000
nft	200
str	25
nal	30
cpd	15
cml	30
lin	4
ery	10
pen	1.5 (units/ml)
tet	50
rif	50
amp	25

TABLE XIIIa: DRUG RESISTANCE SPECTRA OF GROUP 2 STRAINS

DRUG	kan		van		neo		nft		str		nal	
TEST	P	P	P	P	S	S	S	S	D	S	S	S
DIL'N	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{0}	10^{-2}
Strain 56	+	+	+	+	+	+	+	+	+	+	+	+
57	+	+	+	+	+	+	+	+	+	+	+	+
58	+	+	+	+	+	+	+	+	+	+	+	+
59	+	+	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	w	+	+	+	+	+
61	+	+	+	+	+	+	+	+	+	+	+	+
62	+	+	+	+	+	+	+	+	+	+	+	+
63	+	+	+	+	+	+	+	+	+	+	+	+
64	+	+	+	+	+	+	+	+	+	+	+	+
65	+	+	+	+	+	+	+	+	+	+	+	+
66	+	+	+	+	+	+	+	+	+	+	+	+
67	+	+	+	+	+	+	+	+	+	+	+	+
68	+	+	+	+	+	+	+	+	+	+	+	+
69	+	+	+	+	+	+	+	+	+	+	+	+
70	+	+	+	+	+	+	+	+	+	+	+	+
71	+	+	+	+	+	+	+	+	+	+	+	+
72	+	+	+	+	+	+	+	+	+	+	+	+
73	+	+	+	+	+	+	+	+	+	+	+	+
74	+	+	+	+	+	+	+	+	+	+	+	+
75	+	+	+	+	+	+	+	+	+	+	+	+
76	+	+	+	+	+	+	+	+	+	+	+	+
77	+	+	+	+	+	+	+	+	+	+	+	+
78	+	+	+	+	+	+	+	+	+	+	+	+
79	+	+	+	+	+	+	+	+	+	+	+	+
80	+	+	+	+	+	+	+	w	+	+	+	+
81	+	+	+	+	+	+	+	+	+	+	+	+
82	+	+	+	+	+	+	+	+	+	+	+	+
83	+	+	+	+	+	+	+	+	+	+	+	+
84	+	+	+	+	+	+	+	+	+	+	+	+
85	+	+	+	+	+	+	+	+	+	+	+	+
86	+	+	+	+	+	+	+	+	+	+	+	+
87	+	+	+	+	+	+	+	+	+	+	+	+
88	+	+	+	+	+	+	+	+	+	+	+	+
89	+	+	+	+	+	+	+	+	+	+	+	+
90	+	+	+	+	+	+	+	+	+	+	+	+
91	+	+	+	+	+	+	+	+	+	+	+	+
92	+	+	+	+	+	+	+	+	+	+	+	+
93	+	+	+	+	+	+	+	+	+	+	+	+
94	+	+	+	+	+	+	+	+	+	+	+	+
95	+	+	+	+	+	+	+	+	+	+	+	+
96	+	+	+	+	+	+	+	+	+	+	+	+
97	+	+	+	+	+	+	+	+	+	+	+	+
98	+	+	+	+	+	+	+	+	+	+	+	+

TABLE XIIIa (Cont'd.)

DRUG	cpd		cml				lin				ery			
TEST	S	S	D	S	S	S	S	S	S	S	S	S	S	S
DIL'N	10^{0}	10^{-2}	10^{0}	10^{0}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{-2}	10^{-2}
Strain 56	-	-	+	-	w	+	+	+	+	+	+	+	+	+
57	-	-	+	-	-	+	+	+	+	+	+	+	+	+
58	+	+	-	-	-	-	+	+	+	+	+	+	+	+
59	-	+	-	-	-	-	-	-	-	-	+	b	-	-
60	-	-	-	-	-	-	-	+	-	-	b	+	-	-
61	-	-	-	-	-	-	-	+	-	-	-	b	-	-
62	-	-	-	-	-	-	-	-	-	-	-	b	-	-
63	-	-	-	-	-	-	-	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-	-	-	-	-	-	-	-
65	-	+	-	-	-	-	-	-	-	-	-	-	-	-
66	-	-	-	-	-	-	-	-	-	-	-	-	-	-
67	-	-	-	-	-	-	-	-	-	-	-	-	-	-
68	-	-	-	-	-	-	-	-	-	-	-	-	-	-
69	-	+	-	-	-	-	-	-	-	-	-	-	-	-
70	-	-	-	-	-	-	-	-	-	-	-	-	-	-
71	-	-	-	-	-	-	-	-	-	-	-	-	-	-
72	-	-	-	-	-	-	-	-	-	-	-	-	-	-
73	-	-	-	-	-	-	-	-	-	-	-	-	-	-
74	-	-	-	-	-	-	-	-	-	-	-	-	-	-
75	-	-	-	-	-	-	-	-	-	-	-	-	-	-
76	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-	+	+	+	+
79	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-	-	-	-	-	-	-
83	-	-	-	-	-	-	-	-	-	-	+	+	w	w
84	-	-	-	-	-	-	-	-	-	-	+	+	w	w
85	-	-	-	-	-	-	-	-	-	-	-	-	-	-
86	-	-	-	-	-	-	-	-	-	-	+	+	w	w
87	-	-	-	-	-	-	-	-	-	-	+	+	w	w
88	-	-	+	-	-	+	+	+	+	+	+	+	+	+
89	-	-	-	-	-	-	-	-	-	-	b	b	-	-
90	-	-	-	-	-	-	-	-	-	-	-	-	-	-
91	-	-	-	-	-	-	-	-	-	-	w	+	-	-
92	-	-	-	-	-	-	-	-	-	-	-	-	-	-
93	-	-	-	-	-	-	-	-	-	-	+	+	+	+
94	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-	+	+	w	w
96	-	+	-	-	-	-	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-	-	-	+	+	+	+
98	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE XIIIa (Cont'd.)

DRUG	Pen				Tet				Rif			Amp			
TEST	D	S	S	S	D	S	S	S	S	S	S	D	S	S	S
DIL'N	10^{0}	10^{0}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{-2}	10^{-2}	10^{0}	10^{-2}	10^{-2}	10^{0}	10^{0}	10^{-2}	10^{-2}
Strain															
56	-	-	+	+	+	+	+	+	-	+	+	-	-	-	-
57	-	b	+	+	+	+	+	+	-	+	+	-	b	-	-
58	+	+	+	+	+	+	-	-	b	-	-	+	+	+	+
59	-	b	+	+	+	+	+	+	-	+	+	-	b	-	+
60	-	b	w	+	+	+	+	+	-	+	+	-	+	-	-
61	-	b	+	+	w	b	+	+	-	+	+	-	+	-	-
62	-	-	+	+	+	+	+	+	-	+	+	-	w	-	+
63	-	-	-	+	+	b	+	+	-	+	+	-	w	-	-
64	-	-	w	+	w	+	+	+	-	+	+	-	-	w	+
65	-	-	+	+	w	+	+	+	-	w	+	-	-	-	+
66	-	-	+	+	w	+	+	+	-	w	+	-	-	-	+
67	-	-	+	+	w	+	+	w	-	w	w	-	-	-	-
68	-	-	+	+	w	+	+	+	-	w	+	-	-	-	-
69	-	-	+	+	w	+	+	+	-	+	w	-	-	-	+
70	-	-	+	+	w	+	+	+	-	+	+	-	-	-	-
71	-	-	+	+	w	b	+	+	-	w	+	-	-	-	-
72	-	-	+	+	-	-	w	w	-	w	+	-	-	-	-
73	-	-	+	+	w	+	+	+	-	+	w	-	-	-	-
74	-	-	+	w	w	+	w	w	-	w	-	-	-	-	-
75	-	-	+	w	w	+	-	w	-	-	-	-	-	-	-
76	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
77	-	-	+	w	w	+	+	w	-	+	+	-	-	-	-
78	-	-	+	w	w	w	+	+	-	+	+	-	-	-	-
79	-	-	+	w	w	-	w	-	-	-	-	-	-	-	-
80	-	-	+	+	w	+	w	w	-	+	-	-	-	-	-
81	-	-	+	w	w	+	+	+	-	+	+	-	-	-	-
82	-	-	+	w	w	+	w	w	-	+	w	-	-	-	-
83	-	-	+	w	w	+	w	w	-	w	w	-	-	-	+
84	-	-	+	-	w	b	+	-	-	+	-	-	-	-	-
85	-	-	+	w	w	b	+	w	-	+	-	-	-	-	-
86	-	-	+	-	w	b	+	w	-	+	+	-	-	-	-
87	-	-	+	-	w	b	+	w	-	+	+	-	-	-	-
88	-	-	+	-	w	+	+	+	-	+	+	-	-	-	-
89	-	-	+	-	w	+	+	+	b	+	+	-	-	-	-
90	-	-	+	-	w	+	+	+	-	+	+	-	-	-	-
91	-	-	+	-	w	+	-	-	-	+	w	-	-	-	-
92	-	-	+	-	w	+	+	w	b	+	+	-	-	-	-
93	-	-	+	-	w	+	+	w	-	+	w	-	-	-	-
94	-	-	+	+	w	+	+	+	-	+	+	-	-	-	-
95	-	-	w	+	w	+	w	w	-	+	+	-	-	-	-
96	-	-	+	+	w	+	+	+	-	+	+	-	-	-	-
97	-	-	+	+	w	+	+	+	-	+	+	-	-	-	-
98	-	-	+	+	w	+	+	+	-	+	+	-	-	-	-

TABLE XIIIb: SUMMARY OF DRUG RESISTANCE SPECTRA OF GROUP 2 STRAINS

Drug	No. of strains fully sensitive	No. of strains weakly resistant or +/-	No. of strains fully resistant
kan	0	0	43
van	0	0	43
neo	0	0	43
nft	0	0	43
str	0	0	43
nal	0	1	42
ery	28	3	12
lin	37	2	4
cpd	38	4	1
cml	40	3	0
pen	0	42	1
tet	1	19	23
rif	4	37	2 (?)
amp	32	10	1

TABLE XIV: SENSITIVITY OF ANAEROBIC STRAINS ON COMBINATIONS OF kan, van AND amp

Strain	amp	kan + van	kan + van + amp
56	-	+	+
57	-	+	+
59	-	+	-
60	-	+	-
61	-	+	-
62	-	+	+
63	-	+	+
64	-	+	+

\+ = resistant

\- = sensitive

TABLE XV: THE EFFECT OF CYSTEINE ON SENSITIVITY TESTING USING BENZYLPENICILLIN (1.5 units/ml)

		MEDIUM USED (basic medium : BHIA-S)		
Strain	Viability without cys	cys + pen (streak)	cys + pen (disc)	no cys, pen (streak)
56	-	+	-	-
57	-	+	-	-
58	+	+	+	+
59	+	+	+	+
60	+	+	-	-
61	+	+	+	+
62	+	+	+	+
63	+	+	+	+
64	+	+	+	+
65	+	+	+	+
66	+	+	+	+
67	+	+	-	-
68	+	+	+	+
69	+	+	+	+
70	+	-	-	-
71	+	+	-	-
72	+	-	-	-
73	+	+	-	-
74	+	+	-	-
75	+	-	-	-
76	+	-	-	-
77	+	+	+	+
78	+	+	+	+
79	+	-	-	-
80	+	-	-	-
81	+	+	-	-
82	+	+	-	-
83	+	+	+	+
84	+	+	-	-
85	+	+	-	-
86	+	+	-	-
87	+	+	-	-
88	+	+	-	-
89	+	+	-	-
90	+	-	-	-
91	+	-	-	-
92	+	-	-	-
93	+	-	-	-
94	+	+	-	-
95	+	-	-	-
96	+	+	+	+
97	+	+	-	-
98	+	+	-	-

\+ = growth

\- = no growth

CHAPTER 3

TESTS FOR TRANSFERABLE DRUG RESISTANCE IN ANAEROBIC STRAINS

Summary

Attempts were made to isolate mutant drug resistant strains in order to obtain a combination of drug resistance markers suitable for transfer experiments between anaerobic strains and also from these strains to *E. coli* and vice versa. However only one mutant proved satisfactory for use in mating experiments. Attempts were then made to demonstrate transfer between various combinations of anaerobic strains and facultative *E. coli* strains. Although some mating experiments were successful, the results were considered invalid as the strains were no longer obligate anaerobes, but had become facultative.

(A) INTRODUCTION

The initial plan was to attempt to demonstrate genetic transfer of drug resistance genes from anaerobe to *E. coli* and vice versa and from anaerobe to anaerobe. However several of these crosses were not possible with all the strains available as the combination of resistance markers was unsuitable. The following *E. coli* strains were available:

(i) R factor containing strains:

E. coli A_1 (amp^r, cml^r, str^r)

E. coli A_2 (amp^r, cml^r, str^r, kan^r)

(ii) Recipient strains:

E. coli B (nal^r)

E. coli MD42 (nft^r)

These recipients were used in attempts to isolate mutant strains carrying different drug resistance markers.

Attempts were also made to isolate mutants of the anaerobic strains in order to obtain a suitable combination of resistance markers for transfer experiments.

The following is a summary of the possible transfer experiments:

(a) Group 1 anaerobes:

(i) Anaerobe x Anaerobe (could not be done).

(ii) Anaerobe (cml^r, rif^s) x *E. Coli* B (cml^s, rif^r) (mutant)

(iii) *E. coli* A_1 (cml^r, nal^s) x anaerobe (cml^s, nal^r)
E. coli A_2 (cml^r, nal^s) x anaerobe (cml^s, nal^r)

(b) Group 2 anaerobes:

(i) Anaerobe x Anaerobe (could not be done).

(ii) Anaerobe x *E. coli* B (nal^r) or *E. coli* B (rif^r) (mutant) (could not be done)

(iii) *E. coli* A_1 or A_2 (amp^r, cml^r, nal^s) x anaerobe (amp^s, cml^s, nal^r)

(B) THE ISOLATION OF MUTANT STRAINS

(a) The isolation of mutants for use as recipients in transfer experiments with group 1 anaerobes.

An E. coli B (nal^r) strain had been successfully used as a recipient in anaerobic R factor transfer experiments with facultative donor strains (Part I). However this strain was not suitable as a recipient in transfer experiments using group 1 anaerobes as donors, as these strains were all nal^r (Table XIIb). The group 1 anaerobes were all nft^s and most were rif^s and attempts were therefore made to isolate nft^r and rif^r strains of the E. coli B strain by various methods.

(i) E. coli B nft^r mutant

1. Gradient plate method: Samples (0.5 ml) of an overnight E. coli B culture were spread onto gradient plates (nutrient agar) containing a maximum of 200 µg/ml of nitrofurantoin and incubated overnight. Out of 30 plates, one had a single resistant colony in the region of high drug concentration. This colony was inoculated into nutrient broth and after overnight incubation, the culture was streaked onto a nft plate (200 µg/ml). All streaks grew well. Three clones of the mutant strain were shown to be stable for nft^r under aerobic conditions. However, when tested under anaerobic conditions, the mutants repeatedly appeared to be unstable and gave inconsistent results. They were therefore unsuitable for use in transfer experiments with anaerobes, and attempts were made to isolate stable mutants by two other methods.

2. Ultraviolet light: A 7 ml sample of an overnight culture of E. coli B was pipetted into a sterile petri dish and irradiated with a UV lamp at a height of 30 cm for 60 seconds. A 0.1 ml sample of the irradiated suspension was added to each of 20 tubes containing 10 ml of fresh nutrient broth and incubated overnight. Samples of 0.5 ml were spread onto 40 nutrient agar plates containing nft (200 µg/ml) and incubated. No nft^r mutants were obtained by this method.

3. NTG (N-methyl-N^1-nitro-N-nitrosoguanidine): A 0.5 ml sample of an overnight broth culture of E. coli B was

added to 4.5 ml prewarmed nutrient broth and incubated for 2 hours so that the culture would be in the exponential growth phase. After centrifuging for 5 minutes at 2000 g, the pellet was resuspended in 5 ml fresh prewarmed broth. NTG was added to give a final concentration of 30 µg/ml. After 15 minutes' incubation at 37°C, the culture was centrifuged and washed twice in T_2 buffer (see Appendix A). A 0.1 ml sample of the washed suspension was added to 10 ml fresh broth. After overnight incubation, 0.5 ml samples were plated directly onto 40 nutrient agar plates containing 200 µg/ml nft. No nft^r mutants were obtained by this method.

(ii) *E. coli* B rif^r mutant

1. Gradient plate method: Thirty gradient plates with a maximum rifampicin concentration of 20 µg/ml were prepared and inoculated as above. Several large isolated colonies grew at the region of highest drug concentration. These were streaked onto rif plates (20 µg/ml) with a control strain (*E. coli* rif^s) on each plate to check the stability of the drug. Several clones of the mutant strains were shown to be stable for rif^r during aerobic and anaerobic growth. One of these, *E. coli* B (rif^r) was used as a recipient in resistance transfer experiments with group 1 anaerobes.

(iii) *E. coli* MD 42 nft^r strain

Marcos (1972) had isolated an *E. coli* strain MD 42 (nft^r, amp^s, cml^s, tet^s). The nft^r gene was non-transferable. It was planned to use this strain as a recipient in transfer experiments as follows: anaerobic strains (nft^s, amp^r, cml^r, tet^r) x *E. coli* MD 42 (nft^r). However, when this strain was tested using both disc and streak plate methods, it was found to be only very weakly resistant to nft (200 µg/ml). Three mutant strains fully resistant to nitrofurantoin were obtained using the gradient plate method. These all proved to be very unstable and lost their resistance when subcultured onto drug-free medium. In addition, resistance was lost when the strains were streaked onto anaerobic medium containing nft and incubated anaerobically,

even without intermediate subculture onto drug-free medium. These strains were therefore unsuitable for use in transfer experiments involving anaerobes.

(b) Attempt to isolate nft^r mutants of anaerobic strains.

An attempt was made to isolate nft^r strains of 5 anaerobic strains (group 1) so that anaerobe x anaerobe transfer experiments could be performed. Anaerobic cultures of the 5 strains were spread onto anaerobically prepared blood agar gradient plates containing nft at a maximum concentration of 200 µg/ml. No nft^r mutants were isolated by this method, so the above transfer could not be attempted.

(C) TRANSFER EXPERIMENTS

(a) Transfer experiments with group 1 anaerobes

(i) Cross 1: Transfer experiments were performed using anaerobic strains (cml^r, rif^s) as donors and *E. coli* B (cml^s, rif^r) as recipient. Prior to each mating experiment, all strains were tested to verify their drug resistance spectra. Anaerobic 24-hr cultures of the donor and recipient strains were mixed (1 : 1 or 1 : 10) into 1 ml double strength BHIB in the cabinet. Control cultures of individual strains were simultaneously inoculated from the same 24-hour broth cultures. After 24 hours' incubation, the mixed cultures and controls were streaked onto selective and control media on the open bench. After 30 hours' anaerobic incubation, the plates were examined aerobically. Since these group 1 anaerobes were already oxygen-tolerant (see page 64), similar transfer experiments were performed under aerobic conditions in nutrient broth.

Of the 17 strains tested (all originally obligate anaerobes), 4 strains were able to transfer the cml^r drug resistance marker to the recipient strain under anaerobic conditions (Table XVI). One of these, strain 26 subsequently lost this ability, but the other 3 strains were repeatedly able to act as donors. Strain 26 was also not able to transfer resistance under aerobic conditions although strains 5, 40 and 54 could do so. All control plates were negative.

The aim of the project was to investigate transferable drug resistance in obligate anaerobes. Since the strains had all developed facultative characteristics and three could transfer under aerobic mating conditions, these positive results were not considered relevant and further attempts were made to demonstrate resistance transfer in group 1 and group 2 anaerobes.

(ii) Cross 2: *E. coli* strains A_1 and A_2 (both cml^r, nal^s) used as donor strains in transfer experiments in Part I, were tested for the ability to transfer their R factors to 16 suitable group 1 anaerobic strains (cml^s, nal^r). The procedure was

TABLE XVI: TRANSFER OF DRUG RESISTANCE FROM GROUP 1 STRAINS (cml^r, rif^s) TO *E. coli* B (cml^s, rif^r)

Cross	TRANSFER (rif^r, cml^r recombinants selected)				
	Anaerobic conditions			Aerobic conditions	
	TEST 1	TEST 2	TEST 3	TEST 1	TEST 2
5 x *E. coli*	++	+	+++	+++	+++
26 x *E. coli*	++	-	-	-	-
40 x *E. coli*	+	+	++	-	++
54 x *E. coli*	+	+	+++	+++	++

- : no growth of streak from mating mixture on selective plates.

+ to +++ : amount of growth on selective plates.

CONTROL PLATES : (a) Individual anaerobic strains on plates containing rif or rif + cml showed no growth.

(b) Streaks of *E. coli* B (rif^r) on plates containing cml or rif + cml showed no growth.

similar to that in cross 1, but matings were performed only under anaerobic conditions. As a control, both donor strains were also mated with recipient strain E. coli B (nal^r,cml^s) under the same anaerobic conditions (i.e. a repetition of transfer experiments performed in Part I).

The cml^r marker was transferred to the E. coli B strain by both donor strains at frequencies similar to those found in previous experiments, indicating that both donors were functional. However no recombinants were obtained from those matings where the group 1 anaerobes were tested as recipients.

(b) Transfer experiments with group 2 anaerobes

(i) Strains and methods: Since the group 2 anaerobic strains were all nal^r (Table XIIIb), this was an unsuitable donor marker for transfer experiments using the facultative E. coli B (nal^r) strain as a recipient. Similarly, the E. coli B (rif^r) mutant recipient strain which had been isolated could not be used as the rifampicin sensitivity results for the group 2 anaerobes were inconsistent (Table XIIIb). There were also no suitable naturally occurring combinations of resistance markers for transfer experiments within the group 2 strains. The group 2 anaerobes could therefore not be tested for donor ability.

Most group 2 anaerobes were amp^s, cml^s, nal^r. These could therefore be tested as recipients using R factor containing strains E. coli A_1 and A_2 (amp^r, cml^r, nal^s) as donors. (Note: At this stage all group 2 strains except strain 58 were still behaving as obligate anaerobes.) The E. coli A_1 strain was chosen as the donor since it had consistently given higher transfer frequencies in mating experiments in Part I. In order to ensure that strain E. coli A_1 would be an efficient potential donor, transfer levels in the cross between facultative strains E. coli A_1 x E. coli B were investigated with donors and recipients mixed in different proportions as follows:

Donor	Recipient	Volume double strength BHIB
0.1 ml	0.1 ml	2.0 ml
0.1 ml	1.0 ml	5.0 ml
0.1 ml	2.0 ml	10.0 ml

The transfer frequencies in the mixtures where recipient cells were in excess were approximately ten to a hundredfold higher than in the 1 : 1 mixture. The effect of resazurin on R factor transfer was also investigated. Transfer frequencies were similar whether resazurin was present or not.

For mating experiments using the group 2 anaerobes as potential recipient strains, it was therefore decided to mix 0.1 ml donor strain *E. coli* A_1 + 1.0 ml recipient (24-hour anaerobic BHIB cultures) into 5 ml double strength BHIB. The mixtures were incubated for 24 hours. Samples (0.1 ml) of the undiluted mixture and a 10^{-2} dilution were then plated onto anaerobic selective media. Control cultures of donor and recipient strains were also inoculated and treated similarly to the mating mixtures.

(ii) Results: There was no growth on plates containing nal + cml, indicating that transfer of the cml^r marker had not occurred. However, the plates containing nal + amp showed semi-confluent growth at the 10^o dilutions and isolated colonies at the 10^{-2} dilutions of all the mating mixtures (approx. 50 - 1000 colonies per 10^{-2} plate). All nal + amp control plates were clear, suggesting that transfer of the amp^r marker from *E. coli* A_1 to the anaerobic strains had occurred at high frequency. Approximately 100 colonies from the 10^{-2} test plates were streaked onto anaerobic nal + amp plates to test for stability of resistance. They were all found to be sensitive, demonstrating that R factor transfer had not occurred. As an explanation for the growth of the mating mixture on the selective nal + amp plates, it could be postulated that "shielding" was occurring due to the high

density of cells. However this argument was not valid, since the possibility of "shielding" had already been eliminated (page 77) and since isolated colonies grew well on the 10^{-2} plates. Since the phenomenon was not found on nal + cml plates, nor when each strain was plated individually on nal + amp at a high cell density (10^{0} of a 24-hour broth culture), some interaction resulting in ampicillin resistance was presumably occurring between the E. coli A_1 and anaerobic strains when plated together. It is interesting to speculate that a similar interaction could occur in vivo in a mixed culture if ampicillin was administered, thereby inactivating the drug.

CHAPTER 4

IDENTIFICATION OF ANAEROBIC STRAINS

Summary

Despite the negative results of tests for the transfer of drug resistance in the anaerobic strains, attempts were made to identify these organisms. Although the strains had been isolated on a medium recommended as selective for Gram-negative non-sporing anaerobes, on which clostridia would reportedly not survive, it appeared that many of the organisms were strains of Clostridium. Another group of organisms consisted of Gram-negative non-sporing rods, but since these were all able to grow under aerobic conditions, they were not considered to be members of the bacteroides group.

(A) METHODS AND RESULTS

Although the results of transfer experiments with group 2 strains were negative, identification tests were performed on all these strains. The 4 strains from group 1 which showed transferable resistance and strain 42 were included in these tests. The isolation of all these strains on KV agar and their colonial morphology on blood agar indicated that the strains were Gram-negative non-sporing anaerobes.

Initial Gram strains on old cultures indicated that all strains were Gram-negative, but subsequent staining of young cultures demonstrated that 35 of the 43 group 2 strains were Gram-positive. Other tests carried out were a spore stain, growth in 0.1% desoxycholate broth and growth in broth containing both 20% bile and desoxycholate. *B. fragilis* is inhibited by desoxycholate alone, but grows well if bile is added. There is apparently a component of bile which overcomes the inhibitory effect of desoxycholate, but the nature of this substance or its action are not known. The combination of these two media is also useful in distinguishing between other Gram-negative non-sporing anaerobes (Shimada *et al*, 1970). A colistin disc sensitivity test was also performed at this stage as the discs had previously been unavailable.

The results are shown in Table XVII correlated with the development to facultative ability discussed previously. The strains could clearly be divided into 2 distinct groups:

Group P: All 35 Gram-positive strains were spore-forming rods. Members of this group displayed similar colonial morphology on blood agar (type p colonies). Of these 35 strains, 10 developed the ability to grow under aerobic conditions. In all strains the spores were usually terminally located, although occasional subterminal spores were observed (Fig. 13). These results indicated that the bacteria were probably strains of *Clostridium*, which are often isolated from the gastrointestinal tract (see page 44).

KEY FOR TABLE XVII

GROUP: P: Gram-positive spore forming long rods.
N: Gram-negative non-sporing short rods.

GRAM STAIN (22 hour culture)
+ : Gram-positive
- : Gram-negative

SPORE STAIN (84 hour culture)
+ : spore-forming; spores oval and wider than vegatative cell; usually terminal.
- : non-sporing

CELL MORPHOLOGY (22 hour culture)
l : medium to long rods of constant width
s : short rods usually broader in the centre; often showing bipolar staining.

COLONIAL MORPHOLOGY (Blood agar + kan + van)
p : grey to white colonies with entire or undulate edge. Sometimes slightly mucoid. 1-2 mm diameter. No haemolysis.
n : very mucoid smooth shiny white to yellowish colonies with entire edge. 1-2 mm diameter. No haemolysis. Sometimes had raised centre (umbonate).

DEVELOPMENT TO FACULTATIVE ABILITY (from Table XI)
+ : able to grow under aerobic conditions
- : obligate anaerobe.

DESOXYCHOLATE INHIBITION: GROWTH IN BILE + DESOXYCHOLATE (incubation up to 5 days)
+ : strain inhibited by desoxycholate alone but growth present in bile + desoxycholate.
- : growth did not follow above pattern.

GROWTH IN DESOXYCHOLATE (incubation up to 5 days)

+ : growth in desoxycholate broth

- : no growth in desoxycholate broth.

COLISTIN RESISTANCE (anaerobic disc test on BHIA-S, 200 µg/ml)

+ : resistant

- : sensitive

x : not tested.

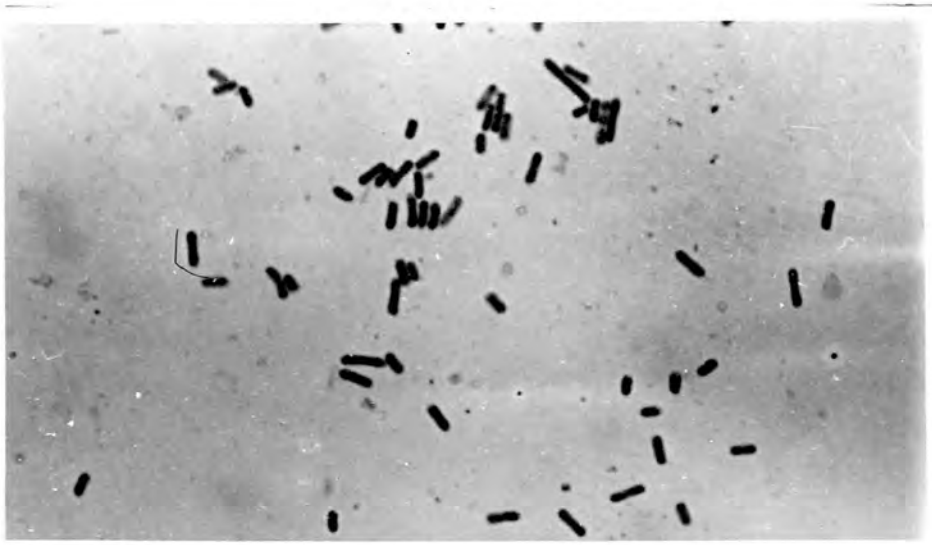
TABLE XVII: IDENTIFICATION OF "ANAEROBIC" STRAINS

Strain	Group	Gram stain	Spore stain	Cell morph.	Colonial morph.	Fac growth	Desox inhib, Bile + Des stim.	Desox growth	Colistin resist
5	N	-	-	s	n	+	-	+	x
26	N	-	-	s	n	+	-	+	x
40	N	-	-	s	n	+	-	+	x
42	N	-	-	s	n	+	-	+	x
54	N	-	-	s	n	+	-	+	x
56	P	+	+	l	p	-	-	-	+
57	P	+	+	l	p	-	-	-	+
58	N	-	-	s	n	+	-	+	-
59	N	-	-	s	n	+	-	+	-
60	P	+	+	l	p	-	+	-	+
61	P	+	+	l	p	+	+	-	+
62	P	+	+	l	p	+	-	+	+
63	P	+	+	l	p	-	-	+	+
64	N	-	-	s	n	+	-	+	-
65	N	-	-	s	n	+	-	+	-
66	N	-	-	s	n	+	-	+	-
67	P	+	+	l	p	-	-	-	+
68	P	+	+	l	p	+	-	+	-
69	N	-	-	s	n	+	-	+	-
70	P	+	+	l	p	+	-	-	+
71	P	+	+	l	p	+	-	-	+
72	P	+	+	l	p	-	-	-	+
73	P	+	+	l	p	-	+	-	+
74	P	+	+	l	p	-	-	-	+
75	P	+	+	l	p	+	+	-	+
76	P	+	+	l	p	-	-	-	+
77	P	+	+	l	p	+	+	-	+
78	P	+	+	l	p	-	+	-	+
79	P	+	+	l	p	-	+	-	+
80	P	+	+	l	p	-	+	-	+
81	P	+	+	l	p	-	+	-	+
82	P	+	+	l	p	+	+	-	+
83	N	-	-	s	n	+	-	+	-
84	P	+	+	l	p	-	-	-	+
85	P	+	+	l	p	-	+	-	+
86	P	+	+	l	p	+	-	-	+
87	P	+	+	l	p	+	-	-	+
88	P	+	+	l	p	-	+	-	+
89	P	+	+	l	p	-	+	-	+
90	P	+	+	l	p	-	-	-	+
91	P	+	+	l	p	-	+	-	+
92	P	+	+	l	p	-	-	-	+
93	P	+	+	l	p	-	+	-	+
94	P	+	+	l	p	-	+	-	+
95	P	+	+	l	p	-	+	-	+
96	N	-	-	s	n	+	-	+	-
97	P	+	+	l	s	-	-	-	+
98	P	+	+	l	s	-	+	-	+

Fig 13: Photographs of anaerobic strains.
Magnification x 800.

(a) Strain 78 (Group P) showing long rods with oval terminal spores wider than vegetative cells.

(b) Strain 66 (Group N) showing shorter rods, some with bipolar staining.



In addition, all strains in this group except No. 68 were colistin resistant and all except Nos. 62, 63 and 68 were inhibited by desoxycholate. Several strains were able to grow in bile + desoxycholate, indicating a stimulatory effect of bile, but this phenomenon could not be correlated with the other data.

Group N: All the Gram-negative short rods from the group 1 (5 strains) and group 2 (8 strains) anaerobes were non-sporing and displayed similar colonial morphology (type n colonies) on blood agar (Fig. 13). All strains developed facultative ability and were sensitive to colistin. They were all able to grow rapidly in desoxycholate broth but were inhibited by a combination of bile and desoxycholate. Together with the fact that they were able to grow under aerobic conditions, this indicates that these strains were not B. fragilis.

No further identification tests were performed as the results of transfer experiments had been negative for the group 2 anaerobes and since all group 1 strains which had shown positive transfer results had become facultative.

As pointed out previously (see page 48), the combination of kanamycin and vancomycin in the selective medium is regarded as inhibitory for strains of Clostridium. In order to rule out the possibility of inactive drugs, the kanamycin and vancomycin were tested for activity against strains of Staphylococcus aureus and Bacillus subtilus. At the same time, all the anaerobic strains were re-tested for sensitivity to the drugs singly and in combination. The S. aureus and B. subtilis strains were fully sensitive, whereas the anaerobic strains were fully resistant to kan, van and kan + van. This indicated that both drugs were active and verified the previous results for the anaerobic strains.

CHAPTER 5

DISCUSSION

Using the anaerobic cabinet and related equipment, the exposure of specimens to oxygen could be avoided throughout the isolation of anaerobes and subsequent procedures. The environment in the cabinet, incubating chambers and media was sufficiently reduced to permit the growth of obligate anaerobes. Thus the two advantages of the roll tube method; the ability to transfer and culture anaerobes without exposure to oxygen and the low redox potential of media, could also be achieved using a cabinet.

Compared to some of the cabinets described in the introduction, the method of achieving anaerobiosis in this system was relatively simple. Only a supply of CO_2 + H_2 and palladium catalyst were required. The equipment was relatively inexpensive, the cabinet construction cost being about R120. Running costs were also low, as the cheap carbon dioxide and hydrogen cylinders were used instead of expensive gas mixtures. Another advantage of the cabinet was that normal bacteriological techniques could be used; for example the triple streaking method for obtaining isolated colonies cannot be used in roll tubes, but was possible in the cabinet. Drying of media is a problem in roll tubes, whereas silica gel could be used to facilitate drying in anaerobic containers. Once it had been established that it was unnecessary to pour media inside the cabinet, the preparation of plates was simplified considerably (Moodie & Woods, 1973b).

The bacterial counts obtained on the non-selective medium (10^9 - 10^{10} per gm faeces) compare fairly well with previous reports. For example, Moore *et al* (1969) and Eller *et al* (1971) found a total count of 10^{11} per gm whereas Finegold *et al* (1971) recovered approximately 10^{10} organisms per gm. The aim of the project was not to isolate total faecal flora, but it was encouraging to note that such high counts could be obtained using the cabinet system.

The gradual development to oxygen tolerance of both the group 1 and some group 2 anaerobes was an interesting phenomenon. As explained previously, the lack of anaerobic containers for storage and incubation at the outset of the project resulted in repeated brief exposure of the group 1 strains to oxygen. The subsequent development to facultativeness suggested that adaptation was occurring. The strains from the next isolation (group 2) were therefore maintained under strict anaerobiosis. The fact that subcultures of many strains, when exposed to oxygen, also developed facultative characteristics, lent support to the theory of adaptation. However there was a spontaneous change from obligate anaerobiosis to facultativeness in some cases where exposure of the strains to oxygen had been avoided throughout. The explanation for the definite correlation between development to facultativeness and ampicillin resistance which was observed amongst the group 2 strains is not known. Possibly there was some alteration of a metabolic pathway which enabled the organism to tolerate exposure to oxygen and simultaneously affected the biochemical mechanism concerned with resistance to this drug.

There are several reports in the literature of similar adaptation phenomena occurring in organisms originally isolated as obligate anaerobes. Varying oxygen sensitivity within a species has also been reported. For example, Drasar (1967) found that only one in 40 of the "bacteroides" strains isolated in his cabinet could be subcultured on the open bench, whereas anaerobes isolated by conventional means were not as sensitive and could be subcultured on the bench. According to Smith and Holdeman (1968), some strains of Bacteroides grow well in the "usual" anaerobic media whereas others are "very fastidious". Barry et al (1972) compared the survival of different anaerobes after brief exposures to oxygen and found B. fragilis to survive this treatment, whereas Peptostreptococcus was very sensitive. In contrast, Moore & Holdeman (1972) isolated aerotolerant strains of Peptostreptococcus, which is usually defined as an obligate anaerobe. Among the clostridia, most organisms are obligate anaerobes but some (e.g. C. histolyticum, C. carnis and C. tertium) are aerotolerant and will grow in broth and on

the surface of agar plates incubated aerobically (Smith & Holdeman, 1968).

Development to facultativeness has been demonstrated in several organisms. For example Sutter (personal communication) observed that some anaerobic cocci and proprionibacteria could become facultative. Similarly, Hill et al (1970) isolated strains of Bacteroides corrodens and found that although the organisms preferred anaerobic conditions on first isolation, this preference was readily lost on subculture. Holdeman & Moore (1972) exclude such organisms from the species B. corrodens, but the fact remains that adaptation was occurring in the strains isolated by Hill et al. A related phenomenon has been demonstrated in strains defined as facultative. For example, strains of streptococci and Enterobacteriaceae (including E. coli) which on first isolation behaved as strict anaerobes, became facultative after several subcultures (Moore & Holdeman, 1972; Moore, personal communication).

Related to this question of growth under aerobic conditions is the extent to which an obligate anaerobe can tolerate exposure to oxygen (or an oxidised environment) and remain viable. For example a type culture of B. fragilis has been shown to survive 24 hours' exposure to oxygen (Drasar, 1967), although this organism is defined as an obligate anaerobe. The precautions recommended to avoid a lethal effect of oxygen on obligate anaerobes vary among different authors. For example Barnes (1969) stated that plates could be inoculated on the open bench but were placed under anaerobic conditions within ten minutes, whereas an interval of twenty minutes was allowed by Holdeman and Moore (1972). The results of tests performed to investigate the viability of the anaerobic strains isolated in this project indicated that they could tolerate a fair amount of oxygen exposure. Dilution, plating and mating procedures could be carried out on the bench if PRAS media were used and precautions were taken to limit the exposure to air to a minimum. An arbitrary limit of twenty minutes' exposure of plates or opened broth cultures was within the tolerance level and allowed sufficient working time.

Other precautions taken such as the flushing of anaerobic containers with $CO_2 + H_2$ while they were being filled with inoculated plates, and the rapid opening and closing of tubes during mating and diluting procedures ensured that the strains remained viable despite their brief exposure to oxygen.

When considering all this data concerning oxygen tolerance and development to facultativeness, a question arises: "what is an anaerobe?" Obviously rigid limits for oxygen exposure cannot always be defined. It seems that the initial oxygen tolerance level of a particular strain can depend on the source of the specimen and that this level can often change, depending on the type of environment to which the organism is subsequently exposed.

A generally accepted standard method for antibiotic sensitivity testing of obligate anaerobes has yet to be developed. As discussed earlier, there are many variables unavoidably introduced when using anaerobic techniques and the effect of these has not yet been adequately studied. The results in this study demonstrating the inactivation by cysteine of benzylpenicillin present in the medium serve to illustrate this point. In addition, nitrofurantoin was apparently somehow affected by anaerobic conditions, since the results of aerobic and anaerobic sensitivity tests on facultative organisms did not correlate (see page 90 and 91).

Most specimens for studies on the composition of the faecal flora and the isolation of B. fragilis have originated from adult Europeans, whereas the subjects in this study were all Bantu children. Organisms tentatively identified as clostridia and other unidentified Gram-negative rods which all became facultative were isolated from these subjects on a selective medium specifically recommended for the isolation of B. fragilis. In addition, no strains of B. fragilis were isolated despite the use of stringent anaerobic techniques. These factors lead one to speculate that the faecal flora of these children might differ considerably in composition and physiology to those of

adult Europeans. It is well known that the average diet of the Bantu is completely different to that of Western Europeans, consisting predominantly of starches and presumably this factor could drastically affect the intestinal flora. However this question requires further investigation before any definite conclusions can be drawn.

Transferable drug resistance was demonstrated in four of the group 1 strains. The aim of the project was to investigate transferable drug resistance in obligate anaerobic organisms. These results were therefore regarded as invalid; the strains had all become facultative and three of the four strains could in fact transfer resistance under aerobic mating conditions. The negative results of transfer experiments with the group two strains were also inconclusive, since many of these had also become facultative and since only one direction of transfer could be attempted. In addition some of the group 2 strains were probably clostridia, which are unlikely to harbour R factors since they are Gram-positive organisms (see page 52).

The goal of this part of the project was therefore only partially achieved. Further studies will require more stringent selection techniques to ensure that the organisms are "stable" obligate anaerobes before proceeding to the investigation of transferable drug resistance.

A paper has recently appeared reporting attempts to transfer antibiotic resistance genes from B. fragilis to E. coli and vice versa and from one strain of B. fragilis to another (Anderson & Sykes, 1973). The fact that these attempts were unsuccessful does not rule out the possibility that such transfer may yet be demonstrated. Only ten strains of B. fragilis were used in this study and it is possible that the physiological conditions were not appropriate for transfer. The significance of such transfer, should it be discovered, has already been discussed.

APPENDIX A

Selective Media

Both aerobic and anaerobic selective media containing one or more drugs were prepared by adding solutions of the drugs to molten agar (50°C) immediately prior to pouring the plates. The nutrient medium base used in each case is mentioned in the text. Each drug powder was assumed to be self sterilising and was dissolved in sterile distilled water (see exceptions below). Fresh drug solutions were prepared for each batch of medium and were used immediately after preparation. The drugs were dissolved in a volume of water such that either 1 or 2 ml of solution was added per 100 ml medium.

Drug	Concentration (µg/ml)	Amount to weigh for 100 ml medium (mg)
amp	25	3.0 (activity: 25 mg per 30 mg)
cpd	15	1.5
cml	30	3.0
ery[1]	10	2.0 (activity: 250 mg per 500 mg)
kan[2]	1000	100
lin	4	0.52 (activity 500 mg per 650 mg)
nal[3]	30	3.0
neo	1000	152.0 (activity 655 µg per mg)
nft[2]	200	20.0
pen	1.5 (units/ml)	0.09 (1670 IU per mg)
rif[1]	50	5.0
str	25	2.5
tet	50	5.0
van	7.5	0.75

(1) Erythromycin and rifampicin were dissolved in 95% ethanol (1 ml per mg).

(2) Kanamycin and nitrofurantoin were added directly to the medium before autoclaving (Todd, 1967).

(3) Naladixic acid was dissolved in 0.1N NaOH.

General Media

Media and solutions not described in the text were prepared as described below. Sterilisation by autoclaving was done at 15 lb/sq in. for 15 min or longer for volumes greater than 250 ml.

Note: distilled water was used in all media.

Nutrient broth:	Bacto Nutrient Broth (Difco)	8 g
	H_2O	1000 ml
Nutrient agar:	Bacto Nutrient Agar (Difco)	23 g
	H_2O	1000 ml

Media for Part I

Minimal medium for E. coli

Minimal salts:

	per 500 ml
NH_4Cl	20 g
NH_4NO_3	4 g
Na_2SO_4 (anhydrous)	8 g
K_2HPO_4	12 g
KH_2PO_4	4 g
$MgSO_4.7H_2O$ (add cold)	0.4 g
H_2O	500 ml

Dissolve each salt in cold water in above order. Wait for complete solution before adding the next. Store over chloroform in fridge.

To make up minimal medium

Minimal Salts Solution	12.5 ml
Ionagar No.2(Oxoid)	2 g
10% Glucose	2 ml
H_2O	85.5 ml

Autoclave.

To make complete medium: L-threonine 0.2 g in 50 ml H_2O
L-leucine 0.2 g in 50 ml H_2O

Autoclave these solutions. Add 0.5 ml of each per 100 ml minimal medium.

Eosin Methylene Blue Lactose Agar (EMB - Lac agar)

	per litre
Nutrient Agar (Difco)	23 g
Lactose	10 g
NaCl	5 g
Methylene blue	0.06 g
Eosin	0.4 g

Adjust pH to 7.3. Autoclave.

Media for Part II

Brain Heart Infusion Broth (BHIB)

Brain Heart Infusion Broth	3.7 g
H_2O	100 ml

Anaerobic BHIB: Resazurin (0.0002%) cysteine and Na_2CO_3 added before dispensing, gassing and autoclaving.

Brain Heart Infusion Agar (supplemented) (BHIA-S)

Brain Heart Infusion Broth	3.7 g
Agar (Oxoid No. 3)	1.5 g
Yeast	0.5 g
H_2O	100 ml

Add cysteine, Na_2CO_3 and 1.0 ml hemin-menadione solution (H-M) to autoclaved, cooled medium.

Hemin-Menadione Solution (H-M)

Menadione stock solution: Add 100 mg menadione to 20 ml 95% ethanol. Filter sterilise and store in fridge.

Hemin stock solution: Add 50 mg hemin to 1 ml 1N NaOH. Make up to 100 ml with distilled water. Autoclave.

H-M Solution: Add 1 ml sterile menadione stock solution to 100 ml hemin stock solution. Use 1 ml of this H-M solution per 100 ml medium. Store H-M solution in fridge.

Cysteine solution: Added to anaerobic media at a final concentration of 0.05 g per 100 ml. For solid media, a solution was prepared and autoclaved so that 1 ml was added per 100 ml cooled medium prior to pouring. Cysteine was added to liquid media prior to dispensing into roll tubes, gassing and autoclaving.

Na_2CO_3 solution: Added Na_2CO_3 to anaerobic media at a final concentration of 0.4 g per 100 ml. The procedure was similar to that for cysteine except that the stock solution for solid media was prepared so that 2 ml solution was added per 100 ml medium (otherwise the Na_2CO_3 precipitated out).

Sodium thioglycollate: added to anaerobic media at a final concentration of 0.11 g per 100 ml. The procedure was the same as that for cysteine.

Blood agar (BA)

Blood agar base (Oxoid)	4 g
Distilled water	90 ml

Autoclave.

7% human blood ("media" blood from Eastern Province Blood Transfusion Service), cysteine and Na_2CO_3 added to cooled medium.

Laked blood agar (LBA): Lake blood by freezing and thawing three times. Prepare as for blood agar.

Selective medium (BHIA-S or BA + KV)

Add kanamycin	1000 µg/ml
and vancomycin	7.5 µg/ml

to BA or BHIA-S.

Non-selective medium for anaerobes (CM)

	per 100 ml medium
Glucose	0.05 g
Soluble starch	0.05 g
Yeast extract	0.05 g
Trypticase	0.20 g
Hemin	1.0 ml stock solution
NaCl	0.26 g

Mineral Solution I	7.5 ml
Mineral Solution II	7.5 ml

Dissolve ingredients except hemin and adjust to pH 6.8. Add 2% Oxoid Agar (No. 3). Make up to required volume (less vol. to be added) with distilled water.

Autoclave. Add cysteine, Na_2CO_3 and hemin to cooled medium.

Mineral Solution I

K_2HPO_4	0.6 g
H_2O	100 ml

Store over chloroform in fridge.

Mineral Solution II

NaCl	1.2 g
$(NH_4)_2\ SO_4$	1.2 g
KH_2PO_4	0.6 g
$CaCl_2$ (anhydrous)	0.12 g
$MgSO_4 . 7H_2O$	0.25 g
H_2O	100 ml

Dissolve constituents one at a time in above order.
Store over chloroform in fridge.

Anaerobic Dilution Fluid (MD)

Mineral Solution I	7.5 ml
Mineral Solution II	7.5 ml
Sodium Thioglycollate	0.11 g
Na_2CO_3	0.4 g
H_2O	85 ml
Resazurin	0.0002%

Add constituents, boil till indicator reduced, while hot dispense in Astell roll tubes (indicator becomes partially oxidised) and gas until indicator becomes colourless again. Seal and autoclave. (Note: if the medium is not dispensed while hot, the gassing takes much longer.)

Method for autoclaving Astell roll tubes

To minimise the popping out of stoppers during autoclaving, gassed roll tubes were allowed to stand for 2 to 3 hours to allow drying between the stopper and the glass neck of the tube. With the water in the autoclave boiling vigorously, the tubes were placed inside and with minimum delay both lid and pressure cap were fitted. This ensured a build up of pressure outside the tubes before they were hot enough to have a raised internal pressure.

Desoxycholate Broth

Brewer thioglycollate medium	4.05 g
Sodium desoxycholate	0.1 g
H_2O	100 ml

Autoclave.

Desoxycholate - Bile Broth

Add 20% Bacto - oxgall (Difco) to desoxycholate broth.

The above two media are prepared by adding all constituents plus cysteine and Na_2CO_3 (The thioglycollate medium contains methylene blue, so no resazurin was added). Heat to boiling (indicator reduced) dispense and gas with pure H_2. If these media are gassed with CO_2, the pH is lowered resulting in precipitation (Shimada et al, 1970).

A heavy inoculum (2 drops of a 24-hr anaerobic culture) was used for these media (Shimada et al, 1970).

T_2 buffer:

Stock solution A:	KH_2PO_4	7.5 g
	NaCl	20.0 g
	Na_2HPO_4 (anhydrous)	15.0 g
	K_2SO_4	25.0 g
	$CaCl_2$ (anhydrous)	0.055 g (add cold)
	Deionised H_2O	1 litre

Store over chloroform at room temp.

Stock solution B:	$MgSO_4$	4.8 g
	Deionised H_2O	100 ml
	Autoclave	
Stock solution C:	Gelatin	0.2 g
	Deionised H_2O	100 ml
	Autoclave	

To make up 200 ml of T_2 buffer

Solution A	40 ml
Deionised H_2O	158 ml
Under sterile conditions add:	
Solution B	1.0 ml
Solution C	1.0 ml

Mix and dispense. Autoclave at 10 lbs. for 13 minutes.

APPENDIX B

Sources of Drugs, Chemicals and Sensitivity Discs

Ampicillin	:	ampicillin trihydrate and Pentrex discs; a gift from Bristol Laboratories.
Cephaloridine	:	"Ceporan" vials (1g); a gift from Glaxo Allenburys (S.A.) (Pty.) Ltd. Discs (15 µg); Oxoid.
Chloramphenicol	:	Chloromycetin powder and discs (Dispens-o-disc) (Difco); a gift from Parke-Davis (Pty.) Ltd.
Erythromycin	:	"Ilosone" - Erythromycin Estolate, 250 mg pulvules; a gift from Lilly Laboratories (S.A.) (Pty.) Ltd.
Kanamycin	:	Kanamycin sulphate and Kantrex discs; a gift from Bristol Laboratories.
Lincomycin	:	Lincomycin Capsules, Upjohn (Pty.) Ltd.
Naladixic acid	:	Naladixic acid powder and discs; a gift from Winthrop, Sterling Drug (S.A.) (Pty.) Ltd.
Neomycin	:	Neomycin sulphate; a gift from Upjohn (Pty.) Ltd.
Nitrofurantoin	:	"Furadantin" tablets or nitrofurantoin powder and discs; gifts from S.K.F. Laboratories (Pty.) Ltd.
Palladium on alumina pellets	:	Engelhard Industries.
Penicillin	:	"Cystapen" - Sodium benzylpenicillin and discs; a gift from Glaxo-Allenburys (S.A.) (Pty.) Ltd.
Resazurin	:	B.D.H. Laboratories.
Rifampicin	:	A gift from Lepetit Pharmaceuticals Ltd.
Silicolapse	:	I.C.I. (S.A.) Ltd.

Streptomycin:	Streptomycin sulphate B.P. and Septrin discs; a gift from Glaxo-Allenburys (S.A.) (Pty.) Ltd.
Tetracycline:	Tetracycline base and Tetrex discs; a gift from Bristol Laboratories.
Vancomycin :	"Vancocin" - vancomycin hydrochloride (500 mg ampoule); a gift from Lilly Laboratories (S.A.) (Pty.) Ltd.
Multodiscs :	1788E Oxoid 1789E Oxoid 30-2H Oxoid

Apparatus

Bench centrifuge	: MSE centrifuge
Colorimeter:	: Eel colorimeter
Ultraviolet Lamp	: Hanovia UV lamp
Anaerobic Jars	: Baird and Tatlock
Shoulder length rubber gloves	: Baird and Tatlock
UV lamp in cabinet	: Phillips 15W
Deoxo catalyst cartridge:	Engelhard Industries
Gas cylinders	: Afrox Ltd.

List of basic items required in cabinet

Alcohol

Cotton wool

Waste tin

Sterile pipettes - open lid of cannister during flushing of cabinet to ensure O_2 is not trapped inside.

Sterile glass spreaders arranged in layers on tray so that one can be picked up without contaminating others.

Marker pens and ruler

Pumpette

Container for dirty pipettes

Electric inoculating loop

Spirit lamp and matches

Tweezers

Sandpaper for cleaning inoculating loop

Palladium catalyst

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