R Factor Transfer to Obligate Anaerobes from *Escherichia coli*

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(Received 25 June 1975; revised 31 October 1975)

INTRODUCTION

The environment in the mammalian gastrointestinal tract is mainly anaerobic, as demonstrated by the abundance of obligate anaerobic bacteria such as *Bacteroides* species (Finegold, 1970; Moore, Cato & Holdeman, 1969) and direct studies of intestinal gas composition (Askevold, 1956). Stallions & Curtiss (1972), Moodie & Woods (1973*a*) and Anderson (1975) have shown that both chromosomal and R factor transfer can occur between facultative *Escherichia coli* strains under stringent anaerobic conditions but this phenomenon has not been demonstrated in faecal obligate anaerobes. We have investigated R factor transfer in mating experiments between 30 faecal obligate anaerobes and between the anaerobes and known donor and recipient *E. coli* strains.

METHODS

Bacteria. The anaerobic glove box and techniques described by Moodie and Woods (1973*b*) were used to isolate 30 obligate anaerobic bacteria from human faecal samples. The anaerobes were isolated on blood agar media containing either kanamycin plus vancomycin or rifamycin plus vancomycin, which are selective for *Bacteroides* and *Fusobacterium* species, respectively (Finegold, Sugihara & Sutter, 1971). All were Gram-negative, non-sporeforming rods, and their ability to grow in media containing desoxycholate (0.1 %, w/v) and bile (20 %, w/v) or desoxycholate alone was determined (Shimada, Sutter & Finegold, 1970). The following abbreviations are used for genetic markers: *amp* (ampicillin), *kan* (kanamycin), *nal* (nalidixic acid), *rif* (rifamycin), *tet* (tetracycline) and *van* (vancomycin). Anaerobes that were *kan*^r *rif*^s *van*^r and were able to grow in medium containing desoxycholate and bile were regarded as *Bacteroides fragilis* (strains 1 to 10) and those that were inhibited by desoxycholate, with or without bile, as *Bacteroides* spp. (strains 1 to 4). Anaerobes that were *kan*^s *rif*^r *van*^r and grew in medium containing desoxycholate in the presence or absence of bile were regarded as *Fusobacterium* spp. (strains 1 to 7) (Moore & Holdeman, 1972). A fourth group included obligate anaerobes selected on antibiotic media but which could not be grouped in the above scheme (anaerobic strains 1 to 9). The obligate anaerobes were maintained under anaerobic conditions and tested routinely for aerobic growth. Two facultative *E. coli* strains were isolated from human faecal specimens: an R factor-containing strain A₁ (*amp*^r) and a recipient strain B (*nal*^r, *rif*^r) (Moodie & Woods, 1973*a*).

Media. (Note: all percentage compositions are w/v unless otherwise stated.) All media contained 0.4 % Na₂CO₃ and 0.05 % cysteine-HCl. The liquid medium (BHI-S) and agar medium (BHIA-S) contained brain-heart infusion broth (Difco), supplemented with 0.005 % haemin, 0.002 % menadione and 0.5 % yeast extract. Antibiotics were incorporated anaerobically in the agar medium at the following concentrations (μg ml⁻¹): ampicillin, 25; kanamycin, 1000; nalidixic acid, 30; rifamycin, 50; tetracycline, 50; vancomycin, 7.5. Incubation was at 37 °C.

Table 1. *Minimal inhibitory concentrations of ampicillin for the obligate anaerobic bacteria used as recipients in the transfer experiments with the E coli A₁ donor strain*

M.i.c. of ampicillin (μ g/ml)	<i>B. fragilis</i> strain no.	<i>Bacteroides</i> spp. strain no.	<i>Fusobacterium</i> spp. strain no.	Anaerobic species strain no.
0.8	8	—	—	—
1.6	—	—	2	—
3.1	6, 9	3	3, 5, 6	4, 9
6.2	7	4	—	6
12.5	2, 3, 5, 10	1, 2	1, 4, 7	1, 2, 5, 7, 8
25.0	1, 4,	—	—	3

Antibiotic sensitivity tests. Antibiotic susceptibility patterns of the obligate anaerobes were determined by the sensitivity disc method (Sapico *et al.*, 1972). Minimal inhibitory concentration (m.i.c.) values for ampicillin were determined by streaking broth cultures on BHIA-S medium containing doubling dilutions of ampicillin. The m.i.c. was defined as the lowest concentration which completely inhibited growth after incubation for 24 h.

Resistance transfer experiments. Overnight cultures of the presumptive donor and recipient cultures were mixed in 4 ml double-strength BHI-S medium. After incubation for a further 2 h exconjugants were selected by plating on BHIA-S agar media containing the appropriate antibiotics. In some experiments the obligate anaerobes were held at 50 °C for 20 min immediately before mating (Schell & Glover, 1966; Mojica-a & Middleton, 1971). In all mating experiments each strain was plated separately on the selective media as a control for measuring the rate of spontaneous mutation. A further control was introduced in which the recipient culture was plated, in close proximity to the mating mixture, on a section of the same plate.

β -Lactamase assay. Overnight broth cultures of the strains under test were adjusted to a standard turbidity and harvested by centrifugation. Cells were resuspended in 0.1 M-phosphate buffer pH 5.9 and treated for 5 min at 4 °C in an ultrasonic disintegrator (MSE) at maximum amplitude (Anderson & Sykes, 1973). The disintegrated material was clarified by centrifugation at 35000 g for 30 min at 4 °C. The supernatant was assayed for β -lactamase activity by measuring the rate of hydrolysis of ampicillin at 30 °C with the microiodometric method of Sykes & Nordström (1972). The protein concentration of the supernatants was determined by the method of Lowry *et al.* (1951) as described by Chase & Williams (1968) and the β -lactamase activity expressed as specific activity (rate of decolorization of starch-iodine/mg protein at 30 °C).

RESULTS AND DISCUSSION

Intragenic matings

Transfer experiments between obligate anaerobes involved strains that were *tet^r rif^s* and strains that were *tet^s rif^r* with selection on medium containing tetracycline and rifamycin. No evidence of transfer of either marker was found.

Intergeneric matings

All anaerobes were *amp^s*, with m.i.c. values ranging from 0.8 to 25 μ g ml⁻¹ (Table 1), and were *nal^r*. The *E. coli* donor was *amp^r* (m.i.c. > 10³ μ g ml⁻¹) and *nal^s*. Exconjugants were therefore sought on medium containing ampicillin and nalidixic acid. None were found when the anaerobes were not heated. However, 29 of the 30 anaerobes yielded *amp^r* exconjugants

Table 2. *Transfer frequencies from E. coli donor strain (amp^r nal^s) to obligate anaerobic recipient bacteria (amp^s nal^r) after heating the recipients at 50 °C for 20 min*

Frequencies were calculated as the number of amp^r nal^r exconjugants/donor cell added.

Anaerobes	Strain no.	10 ⁻⁶ × transfer frequency	Stability of exconjugants on subculture
<i>B. fragilis</i>	1	100	+
	2	100	+
	3	12	+
	4	500	+
	5	11	+
	6	39	—
	7	210	—
	8	39	—
	9	430	—
	10	0	—
<i>Bacteroides</i> spp.	1	250	+
	2	410	+
	3	280	—
	4	100	—
<i>Fusobacterium</i> spp.	1	100	+
	2	100	+
	3	100	+
	4	380	+
	5	100	+
	6	100	+
	7	25	—
<i>Anaerobic</i> spp.	1	43	+
	2	100	+
	3	100	+
	4	100	+
	5	100	+
	6	100	+
	7	50	—
	8	1	—
	9	100	—

at frequencies $> 1 \times 10^6$ /donor cell after heat treatment (Table 2). No spontaneous amp^r mutants were found on control plates. All amp^r exconjugants were obligate anaerobes and either kan^r, van^r or rif^r, in agreement with their susceptibility patterns before mating. The changes in antibiotic resistance patterns were therefore not due to mutation of the donor facultative *E. coli* strain or to contamination.

Since ampicillin resistance in the *E. coli* R factor donor strain is due to the production of β -lactamase, the production of β -lactamase by five of the obligate anaerobic amp^r exconjugant strains and their parent amp^s strains was determined and compared with the β -lactamase activity of the amp^s *E. coli* B recipient and an amp^r exconjugant of this strain (Table 3). The levels of β -lactamase activity of the anaerobic amp^s recipient strains and the amp^s *E. coli* B recipient strain were low (between 0.007 and 0.035). All the exconjugant amp^r strains showed a statistically significant increase in β -lactamase activity. This is further evidence that the amp^r exconjugants received the amp^r R factor by conjugation from the *E. coli* donor strain and that their resistance was due to enzymic destruction of ampicillin rather than to an increase in intrinsic resistance.

Exconjugants from ten of the obligate anaerobes isolated on the nalidixic acid plus

Table 3. β -Lactamase activity of the amp^s recipient strains and the amp^r exconjugant strains obtained after mating with the *E. coli* amp^r R factor-containing strain

Specific activity of β -lactamase is expressed as the rate of decolorization of starch-iodine/mg protein.

Bacterial strain	Specific activity of β -lactamase	
	Recipient amp^s	Exconjugant amp^r
<i>B. fragilis</i> 1	0.033	6.612
<i>B. fragilis</i> 2	0.007	0.255
<i>B. fragilis</i> 3	0.022	0.636
<i>Bacteroides</i> spp. 1	0.009	0.162
<i>Fusobacterium</i> spp. 1	0.035	0.851
<i>E. coli</i> B	0.011	1.213

ampicillin selective medium were unstable on subculture on to medium containing these two antibiotics (Table 2), whereas those from 19 other strains were stable. The average rate of segregation of amp^s in three of the stable amp^r strains varied between 0.50 and 0.94/cell/generation. Three stable anaerobic exconjugants ($amp^r nal^r rif^s$) were tested for their ability to retransfer the amp^r marker in mating experiments with 11 obligate anaerobes ($amp^s nal^r rif^r$) and with the *E. coli* B recipient strain ($amp^s nal^r rif^r$). No $amp^r nal^r rif^r$ exconjugants were isolated.

It was possible that the ten exconjugants that were unstable on subculture, were obtained as a result of the donor strain producing β -lactamase, which reduced the concentration of ampicillin in the selective medium to a level that failed to inhibit the recipient. This is unlikely, however, since the recipient control cultures failed to produce colonies when they were plated on separate sections of the same plate and in close proximity to mating mixtures which produced confluent growth.

Our results show that R factor transfer from *E. coli* to *B. fragilis*, *Bacteroides* spp., *Fusobacterium* spp. and other faecal obligate anaerobic bacteria is possible after heat treatment of the recipients, which presumably inhibits restriction systems of the anaerobes, as in *E. coli* (Schell & Glover, 1966) and in *Salmonella typhimurium* (Mojica-a & Middleton, 1971). The anaerobic exconjugants segregated amp^s cells at a frequency characteristic of cytoplasmic genes and were unable to retransfer the amp^r marker. No transferable resistance factors were detected in 14 *Bacteroides* strains, seven *Fusobacterium* strains and nine other obligate anaerobic strains, all of which were resistant to a number of antibiotics. This is in agreement with the study of ten *Bacteroides* strains reported by Anderson & Sykes (1973). Failure to detect R factor transfer to obligate anaerobes under normal conditions suggests that R factor-mediated antibiotic resistance in anaerobes is not clinically significant. Nevertheless, our results show that R factor transfer to anaerobes is possible and there may be other more natural conditions which might enhance R factor transfer to anaerobes under clinical conditions.

This work was supported by a research grant from the South African Medical Research Council.

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