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Anaerobic Transfer of Antibiotic Resistance from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, an opportunistic pathogen that often initiates infections from a reservoir in the intestinal tract, may donate or acquire antibiotic resistance in an anaerobic environment. Only by including nitrate and nitrite in media could antibiotic-resistant and -sensitive strains of *P. aeruginosa* be cultured in a glove box isolator. These anaerobically grown cells remained sensitive to lytic phage isolated from sewage. After incubation with a phage lysate derived from *P. aeruginosa* 1822, anaerobic transfer of antibiotic resistance to recipients *P. aeruginosa* PS8EtBr and PS8EtBrR occurred at frequencies of 6.2×10^{-9} and 5.0×10^{-8} cells per plaque-forming unit, respectively. In experiments performed outside the isolator, transfer frequencies to PS8EtBr and PS8EtBrR were higher, 1.3×10^{-7} and 6.5×10^{-8} cells per plaque-forming unit, respectively. When *P. aeruginosa* 1822 was incubated aerobically with *Escherichia coli* B in medium containing nitrate and nitrite, the maximum concentration of carbenicillin-resistant *E. coli* B reached 25% of the total *E. coli* B population. This percentage declined to 0.01% of the total *E. coli* B population when anaerobically grown *P. aeruginosa* 1822 and *E. coli* B were combined and incubated in the glove box isolator. The highest concentration of the recipient population converted to antibiotic resistance occurred after 24 h of aerobic incubation, when an initially high donor/recipient ratio (>15) of cells was mixed. These data indicate that transfer of antibiotic resistance either by transduction between *Pseudomonas* spp. or by conjugation between *Pseudomonas* sp. and *E. coli* occurs under strict anaerobic conditions, although at lower frequencies than under aerobic conditions.

Infections by carbenicillin-, gentamicin-, and tobramycin-resistant strains of *Pseudomonas aeruginosa* have been reported with increasing frequency (3, 15-17). Because the anaerobic intestinal tract has been reported to be an important source of resistant *P. aeruginosa* in nosocomial infections (14), these organisms may donate or acquire antibiotic resistance in this environment. As many as 38% of a tested population were found to carry this organism in their intestines (4, 12, 24), and some of these intestinal organisms had the same pyocin types as those found in clinical isolates (9). *P. aeruginosa* can grow in an anaerobic environment by using nitrate and nitrite as terminal electron acceptors in oxidative phosphorylation (8, 10, 31). These growth factors may be provided in the human intestine in preservatives added to commercial meat products (30) and also by oxidation of reduced nitrogen compounds, such as amines, by bacteria in the small intestine (27).

Transfers of antibiotic resistance between *P. aeruginosa* by transduction (26) and from *P. aeruginosa* to various gram-negative organisms by conjugation (11) have been demonstrated in the presence of oxygen. However, no reports have been made on the transfer of genetic material between these organisms under strict anaerobic conditions. Antibiotic resistance transfer has been observed between *Escherichia coli* and *Bacteroides* strains under anaerobic conditions (6, 7, 19, 25, 29). The purpose of this study was to determine if antibiotic resistance could be transferred between strains of *P. aeruginosa* by transduction or from *P. aeruginosa* to *E. coli* by conjugation under anaerobic conditions. The capacity for genetic transfer under these conditions would be requisite for transfer of resistance in the intestine.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* 1822 (serotype 10), which contains an R factor (RP1) specifying resistance to carbenicillin, kanamycin, neomycin, and tetracycline, was provided by Ronald Olsen, University of Michigan, Ann Arbor. *P. aeruginosa* PS8EtBr (serotype 5) was a clinical isolate obtained from the Diagnostic Microbiology Laboratory, University of Mis-

souri Medical Center, Columbia, and treated with ethidium bromide (EtBr). Strain PS8EtBrR is a rough variant of PS8EtBr. *E. coli* B was obtained from the University of Missouri Medical Center Diagnostic Microbiology Laboratory. A *Pseudomonas* phage, designated ϕ9, was isolated from Columbia, Mo., sewage. Phage were propagated in soft agar overlays (0.34% agar) (1).

Materials. Carbenicillin was donated by Pfizer, Inc., New York, N.Y. The *P. aeruginosa* antisera, used for strain identification, were obtained from Difco Laboratories, Detroit, Mich.

Media. Brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) either with or without agar (Difco) and MacConkey agar (Difco) were used as the basal media in experiments. Cysteine (0.05%; Sigma Chemical Co., St. Louis, Mo.) was added to the media to lower the oxidation-reduction potential. Also added to the media were 0.1% sodium nitrate (Fisher Laboratories, Inc., Rockville, Md.) and 0.035% sodium nitrite (Mallinckrodt Inc., St. Louis, Mo.) which are the concentrations of these compounds in Sellers medium (22). Carbenicillin (300 μg/ml) was added to the scoring medium for the detection of resistant organisms. The media were designated as follows: nitrate medium, brain heart infusion broth, cysteine, nitrate, nitrite; medium I, brain heart infusion broth, cysteine, carbenicillin; medium II, nitrate medium with carbenicillin; and medium III, MacConkey agar, cysteine, carbenicillin, nitrate, nitrite.

Aerobic and anaerobic techniques. Aerobic experiments were performed by conventional methods on the open-air laboratory bench. Anaerobic experiments were performed in a glove box isolator (gas mixture, 80% N_2–10% H_2–10% CO_2) using techniques described by Aranki and Freter (2). Inocula for anaerobic experiments were obtained from plates which had been swabbed with either *P. aeruginosa* or *E. coli* and incubated in the glove box isolator.

Aerobic and anaerobic growth curves. Flasks containing nitrate broth medium were inoculated and then incubated statically at 37°C either aerobically in a water bath or anaerobically in the glove box isolator. Samples were removed at various time intervals and diluted. Inside the isolator, 0.1-ml samples from each dilution were plated and incubated either anaerobically for *E. coli* or both aerobically and anaerobically for *P. aeruginosa*.

Aerobic and anaerobic phage assays. Phage suspensions were titrated under both aerobic and anaerobic conditions by the overlay method described by Adams (1). The agar overlays used in anaerobic assays were reduced in the molten state for 48 h in a metal heating block at 42°C in the glove box isolator.

Aerobic and anaerobic adsorptions of ϕ9 to strains of *P. aeruginosa*. Inoculum cells and phage were combined in 2.0 ml of nitrate broth at a multiplicity of infection of about 10^{-2} plaque-forming units (PFU) per cell. After static incubation for 10 min, 8.0 ml of broth was added, and the cells were pelleted for 10 min at 3,000 × *g*. A phage assay was then performed on the supernatant. For anaerobic suspensions, tube caps were tightened securely, tubes were removed from the chamber and centrifuged, and an aerobic phage assay was performed on the supernatant.

Aerobic and anaerobic one-step growth curves for ϕ9. The method employed for the aerobic growth curve was similar to the one described by Adams (1). In one-step growth experiments performed in the glove box, the amount of unadsorbed phage was determined by removing a sample of cell and phage mixture from the isolator, filtering the sample through a filter (0.45 μm; Millipore Corp., Bedford, Mass.), and performing an aerobic phage assay on the filtrate.

Aerobic and anaerobic transductions. The aerobic transduction protocol was similar to the one used by Olsen and Metcalf (20). In anaerobic experiments, cell suspensions were placed in screw-capped tubes and centrifuged outside the anaerobic chamber. After centrifugation, tubes were placed in the anaerobic environment, after which the same protocol as used in the aerobic experiments was performed in the isolator. To ensure that centrifugation was anaerobic, two tubes, each containing a GasPak disposable anaerobic indicator (BBL), were run as controls. Before initiation of the anaerobic experiments, a suspension of ϕ9 was reduced for 48 h at 4°C in an anaerobic jar. The jar was moved inside the chamber and opened for an experiment.

Aerobic and anaerobic conjugations. A protocol similar to those described by Harada and Mitsuhashi (13) and Grinsted et al. (11) was used in conjugation experiments; 1.0 ml each of donor and recipient (log-phase cells) were mixed with 8.0 ml of broth and incubated statically in a water bath or in the isolator. Because there is a low recovery rate of anaerobically grown *E. coli* in the presence of oxygen (21), these cells were scored in the glove box isolator.

RESULTS

Aerobic and anaerobic growth of organisms. *P. aeruginosa* grew poorly in an anaerobic environment on standard laboratory media, even after 5 days of incubation. However, with the addition of sodium nitrate and sodium nitrite to the growth medium, colonies would form within 48 h of streak inoculation. Growth curves determined by either anaerobic or aerobic plate counts of anaerobically grown *P. aeruginosa* PS8EtBr were similar (Fig. 1). Therefore, the *P. aeruginosa* in anaerobic experiments was scored by aerobic plate counts. Growth curves indicated that *P. aeruginosa* PS8EtBr had aerobic and anaerobic generation times of 72 and 120 min, respectively, and *P. aeruginosa* 1822 had times of 84 and 150 min, respectively. Aerobic and anaerobic generation times for *E. coli* B were approximately 24 min each.

Replication of phage. Phage ϕ9 formed infective centers in lawns of *P. aeruginosa* PS8EtBr, PS8EtBrR, or 1822 growing either aerobically or anaerobically on nitrate medium. The efficiency of plating (titer determined anaerobically/titer determined aerobically) was calculated to be 1.0. Therefore, because phage titers (PFU per milliliter) determined by aerobic

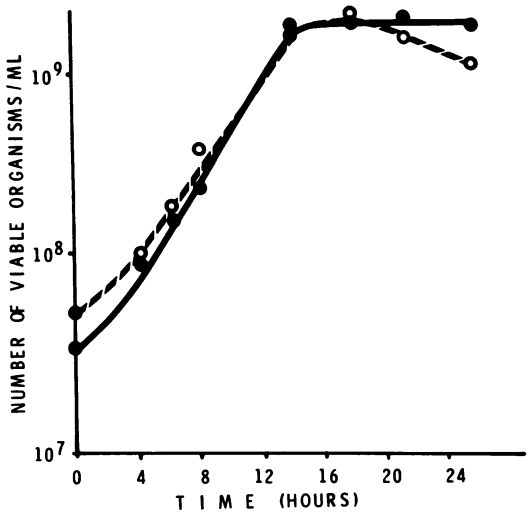

FIG. 1. *Comparison of growth curves of anaerobically grown P. aeruginosa scored by anaerobic (○) and aerobic (●) plate counts. Samples (0.1 ml) of dilutions were plated on nitrate medium and incubated either anaerobically in the glove box isolator or aerobically in a 37°C incubator outside the isolator.*

phage assay were similar to phage titers determined by anaerobic phage assay, aerobic assays were used to enumerate phage used in various experiments. Phage ϕ9 adsorbed to PS8EtBr and PS8EtBrR aerobically at rates of 94 and 95%, respectively. Under anaerobic conditions, adsorption occurred at 98 and approximately 100%, respectively. Aerobic and anaerobic adsorptions of ϕ9 to the donor strain *P. aeruginosa* 1822 were 97% each.

Aerobic and anaerobic transductions. Several attempts to transfer carbenicillin resistance from *P. aeruginosa* 1822 to *P. aeruginosa* PS8EtBr were unsuccessful due to low phage suspension concentrations. A ϕ9 titer of approximately 1.0×10^{11} PFU/ml was required before colonies grew on plates which contained carbenicillin and which had been inoculated with phage-exposed cells (Table 1). Because of the large number of phage used, the multiplicities of infection in successful transduction experiments ranged from 2.0 to 3.7 PFU/cell. However, transfer did not appear to be a function of the multiplicity of infection because a phage suspension of 10^{10} PFU/ml mixed with cells at a multiplicity of infection >1.0 did not result in transfer of antibiotic resistance.

In anaerobic transfer of carbenicillin resistance to *P. aeruginosa* PS8EtBr, there was no visible growth on medium I scoring plates after 5 days of incubation in the anaerobic chamber. Therefore, at the end of an anaerobic experiment, samples of PS8EtBr cell suspensions with and without phage were removed from the anaerobic chamber, plated on medium I, and incubated aerobically. After 48 h, colonies grew on the plates containing cells exposed to phage, resulting in a transfer frequency of 8.6×10^{-9} cell per PFU.

Carbenicillin resistance was transferred to PS8EtBr and PS8EtBrR under anaerobic conditions on medium II at frequencies of 6.2×10^{-9} and 5.0×10^{-8} cell per PFU, respectively, and under aerobic conditions at frequencies of 1.3×10^{-7} and 6.5×10^{-8} cell per PFU, respectively. Differences in frequencies observed for these two strains were not associated with the ability of phage to adsorb to cells, because ϕ9 adsorbed at similar rates to both recipient strains. Colonies produced by PS8EtBrR were discrete, with entire edges, and were easily enumerated. In contrast, the smooth colonies of PS8EtBr were spreading and sometimes produced confluent areas of growth. This resulted in lower colony counts and probably accounted for the difference in frequencies.

Aerobic or anaerobic conjugations. Carbenicillin-resistant *E. coli* B could be detected in the first samples plated from aerobic and anaerobic conjugation experiments in which *P. aeruginosa* 1822 and *E. coli* B were mixed in nitrate medium. Therefore, it appeared as if the R-factor deoxyribonucleic acid had been quickly transferred after mixing. A comparison of initial donor/recipient ratios with conjugation frequencies (number of carbenicillin-resistant *E. coli* B cells produced per *P. aeruginosa* 1822 cell) after 2 h of incubation in aerobic and anaerobic conjugation experiments is shown in Table 2. The results indicated that higher conjugation frequencies may be associated with lower donor/recipient ratios. At low donor/recipient ratios, more recipient cells were available to each donor, and the possibility of transfer of genetic material from a donor cell may have increased.

In aerobic experiments, the viable cell count for both organisms first increased, then decreased, and then increased again (Fig. 2). The

TABLE 1. *Phage and cell requirements for aerobic transduction of carbenicillin resistance from P. aeruginosa 1822 to P. aeruginosa PS8EtBr*

Phage suspension titer (PFU/ml)	Cell suspension concn (cells/ml)	Multiplicity of infection	Avg no. of colonies		Frequency (cell/PFU)
			PS8EtBr + phage	PS8EtBr	
2.0×10^{10}	4.5×10^{10}	0.5	0	0	
3.0×10^{10}	1.2×10^{10}	2.5	0	0	
3.9×10^{10}	4.0×10^{10}	1.0	0	0	
8.1×10^{10}	4.1×10^{10}	2.0	84	0	1.0×10^{-8}
1.6×10^{11}	4.3×10^{10}	3.7	163	0	1.0×10^{-8}

TABLE 2. *Comparison of conjugation frequencies with donor/recipient ratios in aerobic and anaerobic experiments*

Incubation condition	Initial donor/recipient ratio	Frequency at 2 h (conjugants per donor)
Aerobic	0.45	5.0×10^{-5}
	1.1	1.9×10^{-5}
	10.3	9.7×10^{-6}
	15.3	1.4×10^{-5}
	81.2	5.6×10^{-6}
Anaerobic	35.7	5.8×10^{-6}
	2.6×10^{3}	8.4×10^{-7}

number of carbenicillin-resistant *E. coli* B cells in the culture increased for 24 h, reaching a maximum number which was approximately 25% of the total *E. coli* B population. When anaerobically grown *P. aeruginosa* 1822 and *E. coli* B were mixed and incubated inside the glove box isolator at a similar donor/recipient ratio, the viable cell counts for both organisms increased, but thereafter did not vary considerably (Fig. 3). The number of carbenicillin-resistant *E. coli* B cells approached its maximum at the end of 12 h of incubation and only reached 0.01% of the total *E. coli* B population. In these experiments, about 4×10^2-fold more carbenicillin-resistant cells were produced in the aerobic conjugation mixture than in the anaerobic mixture.

DISCUSSION

The results of this study indicate that antibiotic resistance can be transferred under anaerobic conditions between strains of *P. aeruginosa* by transduction and from *P. aeruginosa* to *E. coli* by conjugation.

C. A. O'Bryan (unpublished data) observed that phage replication under a variety of conditions correlated directly with adenosine triphosphate synthesis in *Staphylococcus aureus* and *Clostridium* sp. A wild-type strain of *P. aeruginosa* which was grown under aerobic and anaerobic conditions exhibited phosphorous/oxygen ratios (number of molecules of adenosine triphosphate per atom of oxygen reduced) of 0.46 and 0.40, respectively (28). Because of the similarity of these ratios, a considerable difference between the numbers of *Pseudomonas* phage synthesized under aerobic and anaerobic conditions was not expected. Titers of phage ϕ9 suspensions produced under aerobic and anaerobic conditions in nitrate medium were similar. Therefore, the number of potential transducing phage produced during propagation under both conditions should be approximately equal and would not be a factor in transfer frequency.

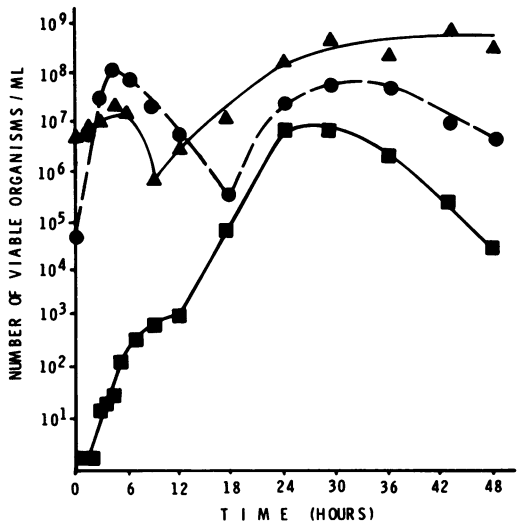

FIG. 2. *Carbenicillin-resistant conjugants of E. coli B (■) resulting from an aerobically incubated mixture of P. aeruginosa 1822 (▲) and E. coli B (●). Samples of the mating mixture were removed at various time intervals to determine the number of parental organisms and conjugants. Parental organisms were enumerated on medium III without carbenicillin, and conjugants were scored on medium III.*

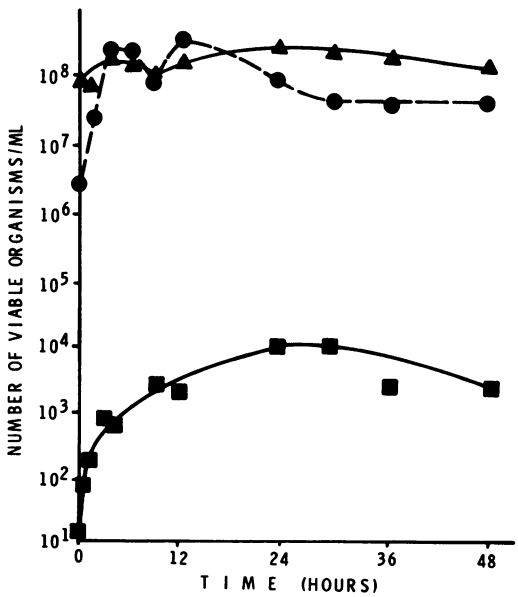

FIG. 3. *Carbenicillin-resistant conjugants of E. coli B (■) resulting from an anaerobically incubated mixture of P. aeruginosa 1822 (▲) and E. coli B (●). The mixture was incubated in the glove box isolator and sampled at various time intervals. Scoring of the different organisms was identical to that described in the legend of Fig. 2.*

The transfer frequency of carbenicillin resistance was 8.6×10^{-9} cell per PFU, which is similar to frequencies reported by other investigators for transduction. The frequency of transduction of the RP1 plasmid by phage F116L has been reported to be about 10^{-8} cell per PFU (26). Shipley and Olsen (23) observed transduction of antibiotic resistance determinants of the RP1 plasmid by phage P22 at frequencies of about 10^{-6} to 10^{-9} cell per PFU.

During aerobic incubation of mating mixtures, the carbenicillin-resistant population increased nearly 10^7-fold, whereas the increase for the total *E. coli* population was about 10^4-fold. Therefore, the carbenicillin-resistant *E. coli* B could be a consequence of conjugation during the experiment as well as reproduction of *E. coli* B which became carbenicillin resistant early in the experiment.

P. aeruginosa 1822 grew with a faster generation time under aerobic conditions than under anaerobic conditions. The growth rate for *E. coli* B was similar both aerobically and anaerobically, and the generation time was much shorter than that of *P. aeruginosa* under the same conditions. Therefore, cell-to-cell contact, formation of the conjugal bridge, or synthesis of new deoxyribonucleic acid for transfer to *E. coli* B may proceed slower anaerobically than aerobically and cause a decrease in the rate of carbenicillin resistance transfer. Few donor *P. aeruginosa* cells may have been available for conjugation.

In aerobic experiments, when a low initial donor/recipient ratio (≤ 1) was present in mixtures, a small number of the recipient *E. coli* B population became carbenicillin resistant. However, with a high donor/recipient ratio (≥ 15), a large number of the *E. coli* B population acquired carbenicillin resistance after about 24 h. Faster growing *E. coli* B may inhibit *P. aeruginosa* earlier at a low donor/recipient ratio. In the intestinal tract, where there is a large amount of resident flora and only a few *Pseudomonas* organisms, an exceptionally low number of recipients could develop carbenicillin resistance as a result of the antibiotic-resistant *Pseudomonas* organisms which are transient in the gut. However, in the germfree mouse, in the absence of a normal flora, *Pseudomonas* may achieve high populations (18). Also, with antibiotic therapy, the number of *P. aeruginosa* organisms may increase in the intestinal tract (5). Growth of *Pseudomonas* in the intestinal tract could increase the donor/recipient ratio and enhance production of antibiotic-resistant recipient populations. Harada and Mitsuhashi (13) reported that R11, a multiple drug resistance transfer factor, was acquired by a large proportion of the *E. coli* population from donor *Shigella flexneri* in vitro after 2 days of incubation at a donor/recipient ratio of less than 1. Therefore, the effect of the donor/recipient ratio on the total number of antibiotic-resistant recipients produced may depend on the specific R factor, donor, recipient, and environment.

For the antibiotic resistance transfer systems examined in this study, conjugation appeared to be a much more effective means of aerobic or anaerobic transfer. Several investigators have observed conjugation in *E. coli* in an anaerobic environment (6, 7, 19, 25), and Welch et al. (29) have described transferable resistance in *Bacteroides*. However, Harada and Mitsuhashi (13), after studying transfer of R11 from *S. flexneri* to *E. coli*, have reported that plasmid conjugation may not occur under anaerobic conditions. Burman observed that some R plasmids in *E. coli* show a moderate or strong reduction in fertility by anaerobiosis (7). *S. flexneri* and *E. coli* grew anaerobically by a fermentative metabolism. Harada and Mitsuhashi (13) state that oxidative phosphorylation is required for conjugation. Therefore, *P. aeruginosa*, which grows in an anaerobic environment by using nitrate and nitrite reductase associated with the electron transport chain (8, 10, 31), may satisfy this requirement.

P. aeruginosa cells grown in an anaerobic environment maintain similar viable counts when exposed to air. However, populations of anaerobic bacteria and facultative anaerobes decrease by a few logs when removed from anaerobiosis (21). Therefore, *P. aeruginosa* en route from the intestinal tract may have an adaptive advantage in initiating infections.

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