Transferable Drug Resistance in *Pseudomonas aeruginosa*¹

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Received for publication 5 October 1971

Three strains of *Pseudomonas aeruginosa* were demonstrated to transfer double-drug resistance by conjugation to a *P. aeruginosa* recipient at frequencies of 10^{-4} to 10^{-2} per recipient cell. Two of the three strains also transferred to *Escherichia coli* at frequencies which were 10^3 - to 10^5 -fold lower, but the third strain could not be demonstrated to do so. The latter strain, however, conferred maleness on the *Pseudomonas* recipient. The transfer of streptomycin resistance was associated with the acquisition of streptomycin phosphorylase by both *P. aeruginosa* and *E. coli* recipients. Maximal broth mating frequencies were obtained with nonagitated cultures less than 1 mm in depth. A pyocine selection system based on donor sensitivity and recipient resistance is described and appears to have future value as a generalized selective device for use after matings.

Transfer of multiple-drug resistance in the genus *Pseudomonas* has been examined by utilizing *Escherichia coli* F⁻ recipients (7, 20, 21) or by an indirect method involving the use of burned mice and again requiring *E. coli* F⁻ strains (18). Direct transfer between two strains of *Pseudomonas* has not been reported. The most important reasons for this are the difficulty in obtaining a drug-susceptible recipient or a satisfactory selection method applicable on a wide basis, and the occurrence of recipient- or donor-induced death of the mating partner (10).

The transfer of drug resistance from *Pseudomonas* to *E. coli* occurs at very low frequencies (19) except in a few instances (7, 21). The reliability of surveys of the incidence of transferable drug resistance in *P. aeruginosa* based on intergeneric matings (with *E. coli* recipients) is doubtful, owing to the low frequencies generally obtained under these conditions. There would seem to be sufficient dissimilarity between the two organisms to account for this. For example, the guanine plus cytosine (GC) content of *E. coli* deoxyribonucleic acid (DNA) is about 50 to 52% (11) and that of *P. aeruginosa* is 68%. The latter is an oxidative organism whereas the former is facultative, and *P. aeruginosa* produces slime whereas *E. coli* does not. Thus, though *E. coli* is a convenient organism to use as a recipient, it does not appear to be a good physiological

choice. To explore the possibility that intrageneric matings would permit higher frequency of drug-resistance transfer, and patterns not detected by intergeneric matings, a study of mating and transfer between a *P. aeruginosa* donor and recipient was undertaken. The objectives of this work included the development of a selection system for use in survey work, the demonstration that direct intrageneric transfer occurs, the examination of optimal mating conditions, and the comparison of the frequencies of transfer obtained in the *E. coli* and *Pseudomonas* systems.

MATERIALS AND METHODS

Organisms. *E. coli* K-12 F: ATCC 14948 was used as recipient in intergeneric matings. *P. aeruginosa* strains obtained as isolates from clinical specimens were as follows: recipient, 280; donors, 931, 679, and 1162; and pyocine-producing strains, 371, 1363, and 280. Mutant strains 280 met⁻, 280 trp⁻, and 679 trp⁻ were isolated by the method of Ornston et al. (16). Strain 280 cb^r su^r was isolated as a carbenicillin-resistant (50 µg/ml) colony. *E. coli* CSH-2 containing R factor NR 3 (known to produce streptomycin phosphorylase) was obtained from J. Davies, Department of Biochemistry, University of Wisconsin.

Media. The minimal medium used was that of Norris and Campbell (15) modified to contain 500 mg of MgSO₄·7H₂O/liter. Complex media were Brain Heart Infusion (BHI; Difco), Trypticase Soy (TS; BBL), and MacConkey agar (Difco).

Strain characterization. All methods used to characterize *P. aeruginosa* were those of Cowan and Steel (5), except acetamide hydrolysis (3) and growth

¹ Presented in part at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

in triphenyl tetrazolium chloride (23). The DNA base ratios were determined by the method of Owen et al. (17). Electron microscopy was performed with a Siemens model Elmiskope 1 electron microscope.

Mating systems. Mating mixture was prepared with equal volumes of logarithmic-phase donor and recipient strains grown in BHI and adjusted to a cell density of $0.5 A_{600}$ unit (Beckman DBG spectrophotometer) prior to mixing. Amounts of 2 or 4 ml of that mixture were used in filter matings (14) with incubation of the filter on TS agar. Broth matings were performed at 37 C by adding 5 ml of mating mixture to a 150-ml medicine bottle which was then positioned flat side down. Samples were removed from filters with a sterile loop to TS or minimal broth and from broths to BHI broth; mating was interrupted by vigorous agitation, and the cell density was adjusted to 0.5 absorbance units at 600 nm (10^8 colony-forming units) with the appropriate broth.

Selection methods. Selection was carried out by conventional auxotrophic methods for $679 \text{ trp}^- \times 280 \text{ met}^-$, $679 \text{ trp}^- \times E. coli \text{ K-12 F}^-$, and $280 \text{ met}^- \text{ R931}$ (the recipient of $280 \text{ met}^- \times 931$) $\times 280 \text{ cb}^+ \text{ su}^+$ matings by plating resuspended samples of filter matings on minimal medium or minimal medium plus DL-methionine (100 $\mu\text{g}/\text{ml}$). The number of organisms plated was assessed from the initial absorbance (0.5 at 600 nm) and by duplicate plate counts.

Pyocine selection was used for the matings $931 \times 280 \text{ met}^-$, $931 \times E. coli \text{ K-12 F}^-$, $1162 \times 280 \text{ met}^-$, and $1162 \times E. coli \text{ K-12 F}^-$.

Pyocines were produced by the method of Farmer and Herman (6) and stored at 4 C. Donor organisms were screened for initial sensitivity to pyocines by applying 25- μliter drops of undiluted pyocine to a lawn of the test organism on TS agar prepared exactly as for Kirby-Bauer disc antibiotic testing (1). The plates were incubated at 37 C for 18 and 42 hr, and if fewer than 20 colonies grew in the pyocine drop zone that pyocine was considered satisfactory for selection, provided it failed to inhibit the recipient strain.

For use in pyocine selection after mating, the pyocine was titrated with donor and recipient strains. Volumes of 0.1 ml containing 10^8 organisms were added to 1.9-ml volumes of serial twofold dilutions of the pyocine (in TS broth) and incubated in 50-ml Erlenmeyer flasks at 37 C for 20 min with vigorous shaking; 0.1-ml volumes (diluted in the case of the insensitive recipient strain 280 or *E. coli*) were applied to TS agar and incubated as above. The pyocine dilution producing less than 10% killing of strain 280 (*E. coli* K-12 F⁻ was totally insensitive) and allowing growth of fewer than 20 colonies of the donor after 42 hr of incubation was used for selection after mating.

Pyocine selection of the resuspended filter or broth matings in BHI ($A_{600} = 0.5$) was carried out by adding 0.1 ml of that material to 1.9 ml of the appropriate pyocine dilution selected as above and incubated as for the pyocine titration. Appropriately diluted 0.1-ml volumes were added to TS agar or MacConkey agar (the latter for *E. coli* matings) containing strep-

tomycin (16.5 $\mu\text{g}/\text{ml}$) and to TS or MacConkey agar for plate counts. *E. coli* platings were, in addition, incubated anaerobically. In some cases, before final plating the *E. coli* mixture was concentrated 20-fold by centrifugation at $7,000 \times g$. Strain 280 was readily differentiated from the rare breakthrough of donor cells by an intense brown pigment and from *E. coli* by lactose fermentation on the MacConkey medium.

After selection by either method, about 10 colonies from each plate were picked and tested for antibiotic susceptibility by the disc method (1) and by determination of minimal inhibitory concentrations (MIC). The MIC was also determined for donors. All *Pseudomonas* colonies showing transfer were typed by the methods of Govan and Gillies (8) and Jackson et al. (A. W. Jackson, M. M. Hodgkin, and J. E. Peart, *Bacteriol. Proc.*, p. 79, 1971) to confirm their identity as strain 280.

Transduction. Examination for transduction was carried out on filtrates of strain 931 and the pyocine of 1363 with strain 280 as described by Chakrabarty and Gunsalus (4) except that 0.22 μm filters were used.

Transformation. To rule out transformation, broth matings of 280×931 were incubated in the presence of 1, 10, and 50 μg of deoxyribonuclease I per ml (Worthington Biochemical Corp.; 2,000 units/mg) for 1, 4, and 20 hr.

Mating kinetics in broth. Broth matings (duplicates) were performed as given except that timed samples were taken from separate mating mixtures in 150-ml medicine bottles.

Streptomycin phosphorylase. Cell extracts to be examined for streptomycin phosphorylase (streptomycin and dihydrostreptomycin are used interchangeably in this context) were prepared by a method similar to that of Umezawa et al. (22) except that streptomycin (0.5 $\mu\text{g}/\text{ml}$) was contained in the growth medium (TS broth) and the resuspended cells (5-ml volumes) were ruptured by ultrasonic treatment [Bronson Biosonik III, 0.375-inch (0.95-cm) probe] for 2 min and subsequently centrifuged at $25,000 \times g$ for 20 min.

The method was further modified so that reaction mixtures consisted of the following: 1 ml of cell extract, 1 mg of adenosine triphosphate (disodium), 100 μg of dihydrostreptomycin, and 2×10^6 disintegrations per min of (^3H)-dihydrostreptomycin (Amersham Searle; 2.1 mCi/mg) in a total volume of 1.05 ml. A 50- μliter amount of the supernatant fluid obtained after incubation and centrifugation ($5,000 \times g$ for 30 min) was chromatographed on a Dowex 50W column (0.9 by 4.2 cm) with the use of a stepwise gradient of 0.25 to 4 M ammonium acetate, and the radioactivity was determined on 0.4-ml samples of each 4-ml fraction in 5 ml of Bray's (2) scintillation fluid (Beckman model LS-250 liquid scintillation counter). The dihydrostreptomycin standard was chromatographed by use of a reaction mixture in which water replaced the cell extract.

Dephosphorylating conditions were as follows: 100 μliters of the reaction mixture after 10 hr at 37 C, but prior to chromatography, was again incubated

at 37 C for 8 hr with either 20 μ g of snake venom phosphodiesterase (*Crotalus admanteus*; Sigma Chemical Co.; 0.29 units/mg) or *E. coli* alkaline phosphatase (Worthington Biochemical Corp.; 26 units/mg) in 0.1 M borate buffer (pH 8.7) in a final volume of 0.4 ml. Dowex 50W chromatography was then carried out as described above.

RESULTS

Strain characterization. All of the *Pseudomonas* strains were confirmed as *P. aeruginosa* by the procedures given in Table 1. Strains 931 and 280 were further confirmed by DNA base ratios (68% GC) and by the presence of one to three polar flagella on electron microscopy.

Pyocine selection. Three pyocine-producing strains (371, 1363, and 280) were examined for production of selective pyocines which would extensively inhibit the donor organisms. The pyocine of strain 1363 proved the most efficacious, inhibiting strains 931 and 1162 but not 679. No pyocine examined inhibited strain 679 to a satisfactory degree. Further examination of the pyocine of strain 1363 by the screening procedure on other strains of *P. aeruginosa* demonstrated inhibition of 19 of 24 strains, suggesting the possibility that this pyocine would be useful for selective purposes on a broad scale.

The effectiveness of the pyocine of strain 1363 in selectively inhibiting strain 931 after the mating $931 \times 280 \text{ met}^-$ was determined by replicate plating (12). TS agar-streptomycin plates containing colonies considered to be recipients of drug resistance were replicated to minimal agar which supported the growth of strain 931 but not 280 met^- . No 931 colonies could be detected on any plate examined (10^{-1} , 10^{-2} , and 10^{-3} dilutions). Thus, the pyocine selection method

was judged satisfactory for use provided the procedure as outlined in Materials and Methods was strictly adhered to. The use of 280 met^- as a recipient and replication to minimal agar allowed an accurate assessment of donor breakthrough after pyocine selection. Strain 1162 ($1162 \times 280 \text{ met}^-$) frequently exhibited breakthrough, but by use of the above replication technique transfer of drug resistance could be detected.

Transfer of drug resistance. Strain 931 was mated with 280 met^- by both broth and filter methods, and strains 1162 and 679 trp^- were mated with 280 met^- by the filter method. In each of these cases, the recipient was confirmed as described in Materials and Methods and by auxotrophy for methionine.

Strain 931 transferred resistance to streptomycin and to tetracycline; strains 1162 and 679 trp^- transferred resistance to streptomycin and sulfonamide. The MIC values for donor and hybrid strains are shown in Table 2. The high level of streptomycin resistance is noteworthy. The hybrid strains obtained generally demonstrated a slightly lower resistance to streptomycin than the donor [though of a level considerably higher than similar resistance in *E. coli* (9)] and a similar resistance level for tetracycline in the case of strains 931 and 280 met^- R931 (recipient of $931 \times 280 \text{ met}^-$). The sulfonamide resistance was examined only by the disc susceptibility method (Table 2). The zone diameter for that drug was <10 mm for the donors and for the recipients after transfer. The resistance transferred to *E. coli* R1162 and *E. coli* R679 was to sulfonamides and streptomycin. Susceptibility to penicillin, ampicillin, cephaloridine, gentamicin, kanamycin, tetracycline, chloramphenicol,

TABLE 1. Characterization of *P. aeruginosa* strains

Method	Strains			
	679	1162	931	280
Oxidase	+	+	+	+
Oxidation-fermentation (O-F) test	0+, F-	0+, F-	0+, F-	0+, F-
Acetamide hydrolysis	+	+	+	+
Pigment				
King A medium	+	+	+	+
King B medium	+	+	+	+
Motility	+	+	+	+
Gluconate oxidation	+	+	+	+/-
Serial growth at 42 C ^a	+	+	+	+
Arginine dihydrolase	+	+	+	+
Growth in triphenyltetrazolium chloride	+	+	+	+
DNA base ratio	-	-	68%	68%
Flagella	-	-	Polar, 1-3	Polar, 1-3

^a Three consecutive transfers.

TABLE 2. MIC, disc zone to sulfonamide, and presence of streptomycin phosphorylase for strains before and after drug-resistance transfer

Strain	MIC ^a (μg/ml)		Disc zone to sulfonamide (mm)	Streptomycin phosphorylase
	Tetracycline	Streptomycin		
Recipients				
<i>P. aeruginosa</i> 280	3	2-4	35	—
<i>E. coli</i> K-12 F ⁻	—	2.5	30-35	—
Donors				
<i>P. aeruginosa</i> 931	100	1,600	35	+
<i>P. aeruginosa</i> 679	—	>3,200	<10	+
<i>P. aeruginosa</i> 1162	—	>3,200	<10	+
Hybrids ^b				
<i>P. aeruginosa</i> 280 R931	100	400	35	+
<i>P. aeruginosa</i> 280 R679	—	1,600	<10	+
<i>P. aeruginosa</i> 280 R1162	—	2,000	<10	+
<i>P. aeruginosa</i> 280 cb ^r su ^r R931	100	400	<10	+
<i>E. coli</i> K-12 F ⁻ R679	—	40	<10	+
<i>E. coli</i> K-12 F ⁻ R1162	—	400	<10	+

^a Inoculum was 10⁷ organisms; grown for 18 hr.^b Hybrids are designated by the number of the recipient strain followed by R (resistance transfer) and the number of the donor strain.TABLE 3. Transfer frequency of double-drug resistance to *E. coli* and *Pseudomonas* strains^a

Donor	Recipients	
	<i>E. coli</i> K 12 F ⁻	<i>P. aeruginosa</i> 280 met ⁻ or 280 cb ^r su ^r ^b
<i>P. aeruginosa</i> 931	<5 × 10 ⁻⁸	10 ⁻² -10 ⁻³
<i>P. aeruginosa</i> 679	2 × 10 ⁻⁸ -6 × 10 ⁻⁸	10 ⁻³
<i>P. aeruginosa</i> 1162	5 × 10 ⁻⁷	10 ⁻⁴
<i>P. aeruginosa</i> 280 met ⁻ R931 ^b	—	10 ⁻⁴

^a Based on 20-hr filter matings. Results are given as the frequency of double-drug resistance per recipient cell.^b Strain 280 cb^r su^r was used as the recipient only in the mating *P. aeruginosa* 280 met⁻ R931 × 280 cb^r su^r.

carbenicillin, and polymyxin (disc) was not changed.

Frequency of drug-resistance transfer. The frequencies obtained after 20-hr matings on filters are shown in Table 3 for both transfer to *E. coli* and to *P. aeruginosa* 280. Strain 931 in six repetitions did not transfer drug resistance to *E. coli*, whereas 679 trp⁻ or 679 and 1162 transferred at very low frequencies. The order of difference between the *Pseudomonas* and *E. coli* recipient is at least 10³ to 10⁵. The survival of *E. coli* was unaffected by pyocine treatment or auxotrophic selection methods. An extremely marked difference in the ability of *E. coli* K-12 F⁻ or strain 280 to act as a recipient was thus demonstrated. The MIC values produced for streptomycin resistance in *E. coli* hybrids were also very much reduced relative to those seen in the

donors, particularly in the case of strain 679 and *E. coli* R679 (Table 2).

Hybrid transfer of drug resistance. Matings of the hybrid strain 280 met⁻ R931 with 280 trp⁻ and 280 cb^r su^r (a strain resistant to 50 μg of carbenicillin/ml and with a disc zone of <10 mm for sulfonamides) clearly showed that the hybrid had acquired the ability to act as a donor (male-ness). Resistance to tetracycline and sulfonamides was transferred at a frequency of 2.5 × 10⁻³ in 20-hr matings with 280 trp⁻; Table 3 illustrates the transfer frequency for those drugs with 280 cb^r su^r as a recipient; that frequency is at least 10³ times the observed mutational frequency of 280 R931 to combined carbenicillin-sulfonamide resistance (10⁻⁷ or less). As shown in Table 2, 280 cb^r su^r also acquired streptomycin phosphorylase in the above mating.

FIG. 1. Effect of cell extracts on phosphorylation of dihydrostreptomycin. Cell extracts of a strain producing streptomycin phosphorylase (*E. coli* CSH-2 with R factor NR3; ●), *P. aeruginosa* strain 280 (▲), or *P. aeruginosa* 280 R931 (■) were incubated under phosphorylating conditions, and then applied and eluted from Dowex 50W as described in Materials and Methods. Identical treatment of a reaction mixture containing no cell-free extract determined the elution position of a [3 H]-dihydrostreptomycin standard.

Transfer of streptomycin phosphorylase. Figure 1 illustrates the elution profile of [3 H]-dihydrostreptomycin after incubation under phosphorylating conditions with various cell extracts. After incubation with the cell extract of strain 280 *met*⁻, it was eluted at 3 to 4 M ammonium acetate, essentially identical to that of a [3 H]-dihydrostreptomycin standard; after exposure to the cell extract of *E. coli* CSH 2 with R factor NR 3 (known to produce streptomycin phosphorylase) or of *P. aeruginosa* 280 *met*⁻ R931, the [3 H]-dihydrostreptomycin was eluted with 0.75 to 1.0 M ammonium acetate. The hybrid, thus, has acquired streptomycin phosphorylase.

To confirm that the product was dihydrostreptomycin phosphate and not dihydrostreptomycin adenylate, the cell extract of strain 931, after incubation under phosphorylating conditions, was chromatographed without further treatment or after treatment with either snake venom phosphodiesterase or *E. coli* alkaline phosphatase under dephosphorylating conditions (see Materials and Methods). The results in Fig. 2 demonstrate that strain 931 produces streptomycin phosphate.

FIG. 2. Product of phosphorylating conditions obtained with the cell extract of *P. aeruginosa* 931 (●) and the effect on that product of dephosphorylating conditions with snake venom phosphodiesterase (■) or *E. coli* alkaline phosphatase (▲).

Strains 1162, 679 *trp*⁻, 280 R1162, 280 R679 *trp*⁻, *E. coli* K-12 F⁻, *E. coli* hybrids of 679 *trp*⁻ and 1162, and 280 *cb*⁺ *su*⁺ R931 (obtained by mating 280 *met*⁻ R931 × 280 *cb*⁺ *su*⁺) were also examined for the production of streptomycin phosphorylase. The results given in Table 2 show that the donors 1162 and 679 produced streptomycin phosphorylase and that this property was acquired by *P. aeruginosa* 280 *met*⁻ and *E. coli* K-12 F⁻ after filter matings with those strains. The sequential hybrid 280 *cb*⁺ *su*⁺ R931 also contained streptomycin phosphorylase. In all cases, the phosphorylating conditions (see Materials and Methods) were sufficient to convert all of the dihydrostreptomycin to the phosphorylated derivative with the exception of *E. coli* K-12 F⁻ recipients which converted only about 70% (Fig. 3). The MIC of streptomycin for those strains was considerably lower (Table 2) than for the parent donor. Though the assay is not quantitative, this observation suggests a lower streptomycin phosphorylase complement in *E. coli* recipients, perhaps resulting from poorer expression of the transferred gene or from an impaired replication of the transferred genetic material in the *E. coli* recipient.

Optimal conditions for transfer of resistance between *Pseudomonas* strains. Table 4 clearly shows a marked difference in broth matings de-

FIG. 3. Presence or absence of dihydrostreptomycin phosphorylating activity in *E. coli* K-12 F⁻ ATCC 14948 (Δ), *E. coli* K-12 F⁻ R1162 ($\bullet$), and *E. coli* K-12 F⁻ R679 ($\blacksquare$) strains. Cell extracts of those strains were incubated under phosphorylating conditions, and then applied and eluted from Dowex 50W as described in Materials and Methods.

TABLE 4. Optimal broth conditions for drug-resistance transfer between *Pseudomonas* strains^a

Conditions (20 hr matings)	Doubly resistant colonies/10 ⁸ recipient cells
Stationary	
Medium depth 1.3 cm	
+KNO ₃ ^b	455
-KNO ₃	460
Medium depth < 1 mm	
+KNO ₃	3,150
-KNO ₃	2,960
Shaking	
Medium depth < 1 mm	
+KNO ₃	1,000-2,100
-KNO ₃	1,020-2,110

^a Strains 931 and 280.

^b KNO₃, 0.4% (w/v).

pending on the aeration conditions. *Pseudomonas* 931 $\times$ 280 matings carried out in non-agitated broths of a depth of 1.3 cm, which results in relatively poor aeration, produced a 10-fold lower number of doubly resistant recipients than did matings with an average depth of less than 1 mm. As noted in Materials and Methods, all matings were adjusted to a constant cell density prior to cell selection, so that the relatively greater growth rate in the latter case (doubling time was about one-half the former) cannot account for the differences in numbers. Shaking mating mixtures less than 1 mm in depth produced variable results. However, the

TABLE 5. Kinetics of mating of *P. aeruginosa* 931 $\times$ 280 mat^a

Mating system	Time (min)	Resistant colonies/10 ⁸ recipient cells	Frequency of double resistance ^a (%)
Broth	30	64	90-95
	60	175	95-100
	90	450	100
	120	745	100
	180	935	100
	240	1,150	100
	1,200	1,800	100
Filter	30	4	95 ^b
	60	63	95-100
	90	94	100
	120	129	100
	180	480	100
	240	705	100
	1,200	1,440	100

^a Identical samples as given in Materials and Methods were selected both on tetracycline (25 μ g/ml) and streptomycin (16.5 μ g/ml) agar; colonies on tetracycline plates were subsequently replicated to streptomycin plates after 48 hr of incubation (37 C) and vice versa.

^b In one of four experiments, this value at 30 min was 50% and at 180 min was 80%.

frequencies obtained were always two to three times those obtained with matings 1.3 cm in depth and from one-fourth to four-fifths those of equivalent stationary mixtures. The growth rates of cells in shallow matings (<1 mm) were essentially identical. It is, thus, possible that the shaking disturbs the formation or maintenance of mating pairs. Loutit et al. noted that KNO₃ enhanced recombinant frequencies in a *P. aeruginosa* mating system (13), but that reagent had no significant effect on frequency of drug-resistance transfer (Table 4) in our experiments.

Shown in Table 5 are frequencies of filter matings which were similar to those of the broth matings.

Kinetics of drug-resistance transfer. Kinetics of drug-resistance transfer for strain 931 to 280 are given in Table 5 for broth and filter matings. The increase in frequency of doubly resistant colonies with time was not due to multiplication of resistant colonies produced by a rare conjugal event, as the sampled cells were resuspended to a constant density ($A_{600} = 0.5$) before selection. No selective process occurred during the matings as they were not subjected to antibiotics, and the growth rate of the recipients was the same before or after the acquisition of drug resistance. The transfer of resistance to both tetracycline and streptomycin occurs in the ma-

TABLE 6. Broth matings of *P. aeruginosa* 931 $\times$ 280 containing deoxyribonuclease I and of *P. aeruginosa* 931 filtrate or 1363 pyocine filtrate $\times$ *P. aeruginosa* 280

Conditions ^a	Doubly resistant colonies/10 ⁸ recipient cells
Control <i>P. aeruginosa</i> 931 $\times$ 280 . .	630
Control + deoxyribonuclease I	
1 μ g/ml.	540
10 μ g/ml.	640
50 μ g/ml.	910
Filtrate of 931 + strain 280.	0
Filtrate of pyocine 1363 + strain 280	0

^a Mating conditions are specified in Materials and Methods. The duration of mating was 20 hr.

jority of cases simultaneously. These experiments utilized selection on both tetracycline (25 μ g/ml) in TS agar)- and streptomycin-containing plates; tetracycline plates were replicated to streptomycin and vice versa. In a few instances, but only early in the mating period, it was possible to select colonies which carried resistance to streptomycin only. The reverse situation was not encountered. Such colonies never represented greater than 5% of all colonies of any timed sample from broth matings, and with one exception that was also true of filter matings. In the one exception, the frequency of such colonies was about 50% at 30 min and 20% at 180 min. This particular experiment was repeated several times, but such a difference in frequency was not detected again nor was the reason for that single observation apparent.

Transformation and transduction. The slight possibility that transduction or transformation accounted for the transfer of drug resistance was excluded by the results tabulated in Table 6.

DISCUSSION

The transfer of drug resistance directly between two strains of *P. aeruginosa* has not been documented previously. The demonstration of such transfer was feasible only because of the unique characteristics of the recipient strain 280. It was susceptible to seven antibiotics (carbencillin, gentamicin, kanamycin, streptomycin, sulfonamide, tetracycline, and polymyxin), was resistant to most pyocines, and produced a brown pigment uncommon among *P. aeruginosa* strains. Unfortunately, it was resistant to cephaloridine and ampicillin and moderately resistant to chloramphenicol, but in spite of these problems it is a useful strain to utilize as a recipient for transfer of the resistance to other drugs. The remarkable difference demonstrated by the *Pseudomonas* strain (280) in recipient potential relative to the

E. coli strain is of considerable significance. The utilization of *E. coli* as a recipient, particularly in survey techniques for transferable drug resistance, is, in our opinion, unwise in view of the possibility and even likelihood that many examples of transferable drug resistance would not be detected. A positive result under these conditions would be acceptable, but it would be hard to justify a negative result.

The reason for the difference in transfer frequency (of drug resistance) has not been established. Examination of our donor, recipient, and mating mixtures of *Pseudomonas* by electron microscopy has failed to demonstrate any obvious mating pili or other unusual morphological features. It is, however, not surprising that severe mating incompatibility exists between *Pseudomonas* and *E. coli* because of the significant differences between these members of distinct genera.

The nature of the transferable drug resistance has not been completely established by the present work, but a situation analogous to R factors in *E. coli*, for example, is likely. The simultaneous transfer of two drug markers, the ability to confer maleness on the recipient, the acquisition of enzymatic phosphorylation, and the ability to transfer drug resistance to *E. coli*, albeit at a low frequency, are all compatible with an R factor. Transduction and transformation appear extremely remote in view of the data presented here. Chromosomal recombination cannot be excluded but seems unlikely in view of the two transfers to *E. coli*. Work is presently in progress to examine for satellite banding of DNA in donors and recipients.

Pyocine selection was judged to be a useful technique. In the case of strain 931, the results were excellent, but for strain 1162 breakthrough was troublesome. However, in combination with an auxotrophic recipient it also proved a useful method. Pyocine selection appears to be as good or better than auxotrophic methods, at least in the case of strain 931, but will probably find most use as a selection device for surveying large numbers of strains for transferable drug resistance, which we are presently doing.

The streptomycin resistance mediated by transferable drug resistance in *Pseudomonas* is very much greater than most similar examples in *E. coli*. However, when introduced into *E. coli*, the *Pseudomonas* streptomycin phosphorylase is associated with much lower streptomycin resistance (400 μ g/ml versus 3,000 μ g/ml for 1162; 40 μ g/ml versus 3,000 μ g/ml for 679) than in the donor.

The enhancing effect of aeration in broth matings is in keeping with the oxidative metabolism

of *P. aeruginosa* and suggests that the rate of transfer of drug resistance is dependent on the rate of metabolism. This suggestion is further supported by the observation that mating in minimal broth reduced the frequency of drug-resistance transfer considerably below that in complex medium.

In the strains examined in this work, donor- or recipient-induced death of the mating partner was not a problem, though it has been documented by others (10), and in other mating experiments not reported here we have on occasion encountered this problem. In spite of these problems and the lack of a total antibiotic-susceptibility spectrum in a *P. aeruginosa* recipient, we believe that considerable progress in obtaining a good recipient, defining a selection system, and assessing mating conditions and dynamics has been made and that a workable system has been achieved. In view of the apparent marked ineffectiveness of *E. coli* as a recipient with our three strains, a problem also reported by others (19), it seems mandatory that such methods be further investigated and defined to allow a proper evaluation of the state of transferable drug resistance in *P. aeruginosa*.

ACKNOWLEDGMENTS

This work was supported by Medical Research Council of Canada grant MA-4350.

ADDENDUM IN PROOF

A recent paper by V. A. Stanicsh and B. W. Holloway (Genet. Res. 17:169-172, 1971) has come to our attention. This article reports transfer of R factors carrying resistance to carbenicillin (18) between *P. aeruginosa* strains.

LITERATURE CITED

- Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Amer. J. Clin. Pathol.* 45:493-496.
- Bray, G. A. 1960. A simple and efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1:279-285.
- Bühlman, X., W. A. Vischer, and H. Bruhin. 1961. Identification of apocyanogenic strains of *Pseudomonas aeruginosa*. *J. Bacteriol.* 82:787-788.
- Chakrabarty, A. M., and I. C. Gunsalus. 1969. Defective phage and chromosome mobilization in *Pseudomonas putida*. *Proc. Nat. Acad. Sci. U.S.A.* 64:1217-1223.
- Cowan, S. T., and K. J. Steel. 1965. Manual for the identification of medical bacteria. Cambridge University Press, London.
- Farmer, J. J., III, and L. G. Herman. 1969. Epidemiological fingerprinting of *Pseudomonas aeruginosa* by the production of and sensitivity to pyocin and bacteriophage. *Appl. Microbiol.* 18:760-765.
- Fullbrook, P. D., S. W. Elson, and B. Slocumbe. 1970. R factor mediated β -lactamase in *Pseudomonas aeruginosa*. *Nature (London)* 226:1054-1056.
- Govan, J. R. W., and R. R. Gillies. 1969. Further studies in the pyocine typing of *Pseudomonas pyocyanea*. *J. Med. Microbiol.* 2:17-25.
- Harwood, J. H., and D. H. Smith. 1969. Resistance factor-mediated streptomycin resistance. *J. Bacteriol.* 97:1262-1271.
- Herman, A. I., and P. S. Griffin. 1968. Viability in fertile stocks of *Pseudomonas aeruginosa*. *J. Bacteriol.* 95:1758-1763.
- Hill, L. R. 1966. An index to deoxyribonucleic acid base compositions of bacterial species. *J. Gen. Microbiol.* 44:419-437.
- Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. *J. Bacteriol.* 63:399-406.
- Loutit, J. S., L. E. Pearce, and M. G. Marinus. 1968. Investigation of the mating system of *Pseudomonas aeruginosa*. *Genet. Res.* 12:29-36.
- Matney, T. S., and N. E. Achenbach. 1962. New uses for membrane filters. III. Bacterial mating procedure. *J. Bacteriol.* 84:874-875.
- Norris, F. C., and J. J. R. Campbell. 1949. The intermediate metabolism of *Pseudomonas aeruginosa*. III. The application of paper chromatography to the identification of gluconic and 2-keto-gluconic acids, intermediates in glucose metabolism. *Can. J. Res.* 27:253-268.
- Ornston, L. N., M. K. Ornston, and G. Chou. 1969. Isolation of spontaneous mutant strains of *Pseudomonas putida*. *Biochem. Biophys. Res. Commun.* 36:179-184.
- Owen, R. J., L. R. Hill, and S. P. Lapage. 1969. Determination of DNA compositions from melting profiles in dilute buffers. *Bipolymers* 7:503-510.
- Roe, E., R. J. Jones, and E. J. L. Lowbury. 1971. Transfer of antibiotic resistance between *Pseudomonas aeruginosa*, *Escherichia coli*, and other gram negative bacilli in burns. *Lancet* 1:149-152.
- Smith, D. H. 1967. Drug resistance of enteric bacteria mediated by R factors. *Antimicrob. Ag. Chemother.* 1966, p. 274-280.
- Smith, D. H., and S. E. Armour. 1966. Transferable R factors in enteric bacteria causing infection of the genito-urinary tract. *Lancet* 2:15-18.
- Sykes, R. B., and M. H. Richmond. 1970. Intergeneric transfer of a β -lactamase gene between *Ps. aeruginosa* and *E. coli*. *Nature (London)* 226:952-954.
- Umezawa, H., M. Okanishi, S. Kondo, K. Hamana, R. Utahara, and K. Maeda. 1967. Phosphorylative inactivation of aminoglycosidic antibiotics by *Escherichia coli*-carrying R factor. *Science* 157:1559-1561.
- Wahba, A. H., and J. H. Darrell. 1965. The identification of atypical strains of *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* 38:329-342.