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Characteristics of R931 and Other *Pseudomonas aeruginosa* R Factors

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R factors were detected in 3.3% of 233 hospital isolates of *Pseudomonas aeruginosa* using *P. aeruginosa* recipients in conjugations. Transferred markers included streptomycin, tetracycline, and sulfonamide resistance. Gentamicin resistance was transferred from two strains previously shown to acetylate gentamicin. A group of R factors exemplified by R931 were characterized by failure to transfer to *Escherichia coli* recipients. Such R factors formed a single compatibility group when examined in a *P. aeruginosa* recipient. Other *P. aeruginosa* R factors, including RP4, showed stable coexistence with the R931 group. It is proposed that RP4 and similar R factors be members of the P-1 compatibility group and that R931, R3108, R209, and R130 be members of a group termed P-2. The buoyant densities of all R factors examined were similar, about 1.716 to 1.719 g/cm^3. The content of R-factor deoxyribonucleic acid (DNA) relative to the total DNA varied among the different R factors, ranging from about 18 ± 2% in log-phase cells of 931 (R931) to undetectable for 679 (R679). However, R679, which transferred from strain 679 at extremely low and irregular frequencies to an *E. coli* host, was shown to represent about 4% R-factor DNA in that host. The relative DNA content of R931 appeared to decline in the stationary growth phase of 931 (R931) or 280 (R931). R931 covalently closed circular DNA was isolated by ethidium bromide-CsCl gradient centrifugation and examined by electron microscopy. Two major molecular distributions existed, having contour lengths of 0.5 and 12.4 μm. The molecular weights were estimated to be 10^6 and 25×10^6. Both molecules were under relaxed replication control. R factor R931 exists as a naturally occurring high-frequency transfer system in *P. aeruginosa* strains 931 and 1310. However, in strain 280 it acts as if subject to fertility repression. Other members of the P-2 compatibility group also are high-frequency transfer systems in the natural host and in strain 1310. RP4 is restricted from recipient strain 1310. Some additional recipient effects were noted in that strains 1310 or 280 sometimes differed in recipient effectiveness with a given donor. Agglutination reactions with absorbed antiserum were able to distinguish between two members of the same R-factor compatibility group, R931 and R3108.

Previous evidence (5) strongly suggested that at least some *Pseudomonas* R factors, including R931, are host-restricted. Such R factors contrast with other *Pseudomonas* R factors, described by several workers (11, 14, 17, 27, 31), which seem to be freely transferable between *P. aeruginosa* and members of the *Enterobacteriaceae*. Grinsted et al. (17) and Datta et al. (11) examined some of the properties of the latter R factors, but the former have not been described in detail. The work in this paper describes some of the characteristics of partially or completely host-restricted *Pseudomonas* R factors.

MATERIALS AND METHODS

Organisms. All *P. aeruginosa* strains were originally obtained from clinical specimens and identified as previously described (5). Recipient *P. aeruginosa* strains used in conjugations were rifampin (280 rif^r)-, kanamycin (280 kan^r)-, or carbenicillin (280 cbn^r)-resistant mutants of methionine, or tryptophan auxotrophs of strain 280 (5), and similar mutants (1310 rif^r, 1310 cbn^r) of pyocine type 5 (16) strain 1310. Other recipients were *Escherichia coli* (16) strain 1310 nal^r pro$^-$ met$^-$ (a nalidixic acid-resistant mutant kindly provided by K. Sanderson, University of Calgary), its rifampin-resistant derivative (*E. coli* nal^r rif^r pro$^-$ met$^-$), and polymyxin- or streptomycin-resistant mutants of *Proteus mirabilis* (ATCC 15290).

Media. Medium used for growth and mating experiments was Trypticase soy (TS;BBL) broth. Selection plates were composed of TS with 1.5% agar (TSA; Difco) or for *E. coli* selection, MacConkey agar (Difco). Minimal medium used was described previously (5).

Mating methods. Broth matings described previously (5) were for 2 h and used a 1:1 or 1:10 donor-to-recipient cell ratio. Frequencies are based on the total number of donor cells present at the initiation of mating. Selection was carried out by conventional counter selection with either rifampin (100 μg/ml), kanamycin (50 μg/ml), carbenicillin (100 μg/ml), nalidixic acid (50 μg/ml), or streptomycin (16 or 33 μg/ml) combined with an antimicrobial agent for which resistance was carried on the R factor. In some of the survey matings, pyocine selection was used (5). Recipients were additionally confirmed by auxotrophy for methionine or tryptophan or by pyocine typing (16).

Compatibility and stability studies of R factors. The frequency with which R-factor resistance markers were transferred to R^- and R^+ strains was determined as follows. A strain containing one or two R factors was subcultured 10 or more times in drug-free TS broth. One hundred to 500 colonies were plated and grown overnight at 37 C on TS agar or minimal agar supplemented with the appropriate amino acid. These plates were replica plated (23) to TS or supplemented minimal agar with or without antimicrobial agents. Colonies which failed to grow on plates containing antimicrobial agents were examined for their antimicrobial susceptibility pattern (2).

High-frequency transfer system. A conventional mating using 5×10^7 cells/ml of strain 931 (R931) (minimal inhibitory concentration [MIC] for streptomycin, 1,500 μg/ml) and 5×10^8 cells/ml of strain 280 met^- rif^r was prepared and incubated for 90 min at 37 C. At that time, strain 931 (R931) was eliminated by the addition of one part of pyocine from strain 1363 (prepared as previously described and used undiluted [5]) to three parts of mating mixture. This was incubated at 37 C for 60 min, centrifuged at $2,000 \times g$ for 5 min and suspended with TS broth to original volume. Strain 931 (R931) survival under these conditions was determined to be 300 colony-forming units per ml. Strain 280 was totally resistant to the pyocine under the conditions used. A 0.1-ml amount of serial 10-fold dilutions of this mixture was plated on TS agar with streptomycin (16 μg/ml) or TS agar with streptomycin (16 μg/ml) and rifampin (100 μg/ml) to select, respectively, for donor survival and for R^+ strain 280 and for R^+ strain 280 only. Colonies growing on streptomycin plates were confirmed to be R^+ strain 280 by replica plating to rifampin (100 μg/ml) and streptomycin (16.5 μg) TSA plates. Equal volumes of the pyocine-treated resuspended mating (containing 4.2×10^7 R^+ strain 280/ml) and strain 280 kan^r (10^9 cells/ml) were mixed and mated for 60 min. A 0.1-ml amount of serial 10-fold dilutions was plated on TSA containing kanamycin (50 μg) and streptomycin (33 μg/ml). A control mating containing the same cell concentration of strain 280 kan^r, and a strain 280 (R931) allowed to undergo more than 10 generations in drug-free nonmating environment (TS broth) was examined under identical mating conditions.

Antiserum to 280 (R931). Antiserum was prepared by standard methods. Absorption with strain 280 was repeated until the agglutination titer for the strain was less than 1:20.

Agglutination. Agglutination titers were determined in serological tubes with 0.5 ml of serial twofold dilutions of serum and 0.5 ml of the bacterial strain ($A_{600} = 0.3$). The tubes were incubated at 50 C for 1 h and kept overnight at 4 C. The titer represented the last tube containing visible agglutination.

Analytical CsCl gradient centrifugation. Cultures were grown in TS broth to an absorbance at 600 nm of 0.35 to 0.5 for exponential-phase cells. Stationary cultures were grown overnight. Total number of bacteria used was 5×10^{10} cells. Deoxyribonucleic acid (DNA) was isolated and subjected to CsCl (Fisher Scientific, British Drug Houses) gradient centrifugation as described by Cohen and Miller (8). Centrifugations were performed in a Beckman model E analytical ultracentrifuge equipped with a standard ultraviolet light source for 25 h or longer at 44,000 rev/min at 25 C. A charcoal-filled Epon centerpiece, 12-mm 4° single sector was used; the upper quartz window was a 1° negative wedge. The relative DNA content of satellite bands was measured by planimetry. Buoyant density and DNA base composition were determined by the method of Schildkraut, Marmur, and Doty (29).

Isolation of covalently closed circular (CCC) DNA. Cultures of *P. aeruginosa* grown in low-phosphate medium (5) with the addition of 10^{-3} M potassium phosphate were labeled with ^{32}P as described by Grinsted et al. (17). DNA was isolated by the method of Bazaral and Helinski (4) and centrifuged with a Ti-60 fixed-angle rotor in a Beckman model L-2 centrifuge at $100,000 \times g$ for 60 h at 20 C. Fractions were collected and treated as described by Grinsted et al. (17).

Electron microscopic examination of CCC DNA. Freshly isolated CCC DNA was dialyzed against 0.15 M ammonium acetate for 6 h at 4 C. Samples containing 2 μg/ml were treated with 0.1 ng of deoxyribonuclease (DNase) I (Worthington Biochemical Corp.; 2,400 μg/mg) per ml for 20 min at 37 C or allowed to age for 7 days or more at 4 C. These and untreated samples of CCC DNA were examined with a Philips 300 electron microscope. Grids for electron microscopy were prepared by the technique of Kleinschmidt (20) and stained with uranyl acetate. Contour lengths of DNA preparations were determined by projection of photographic negatives onto a screen and measurement of contours with a map-measuring device. Molecular weight of satellite DNA was estimated by using a factor of $2.07 \pm 0.04 \times 10^6$ daltons/μm (22).

RESULTS

Prevalence of R factors and transferable gentamicin resistance. A total of 233 strains of hospital isolates of *P. aeruginosa* were examined

for evidence of R factors. As shown in Tables 1 and 2, R factors were detected in seven strains, or 3.3% of those examined. The maximum number of resistance determinants on a single R factor is three. All carry streptomycin resistance and four of the seven carry sulfonamide resistance (Table 2). Table 1 does not include two strains obtained from J. Davies, University of Wisconsin, which were demonstrated to acetylate gentamicin (6) but not tobramycin. These strains are of interest, because the gentamicin resistance is transferable with streptomycin- and sulfonamide-resistance markers.

Table 3 illustrates the gentamicin-resistance transfer obtained with strains PS-130 (R130) and PS-209 (R209). The MIC for gentamicin increased up to 40-fold in the case of R^+ 280 strains and 10-fold for R^+ 1310 strains. The MIC values obtained with the R^+280 and 1310 strains were less than those of the donor strains. We also noted that the MIC for gentamicin of R^+1310 or 280 may vary depending on which antimicrobial agent is used in selection plates. Thus, if streptomycin is used for selection, the MIC of 280 (R209) for gentamicin may be as low as 2 μg/ml. The explanation for the relatively low MIC values for gentamicin in these strains has not yet been determined. However, an MIC of 10 μg of gentamicin per ml would be regarded as clinically resistant to gentamicin.

TABLE 1. *Prevalence of R factors in hospital isolates of P. aeruginosa*[a]

Agents used in selection plates	Strains examined (no.)	R factors	
		No.	Percent of strains
Streptomycin	101	7	7
Tetracycline	48[b]	2	4
Carbenicillin	48	0	0
Kanamycin	46	0	0
Gentamicin	14	0	0

[a] *P. aeruginosa* strain 280 used as a recipient in conjugations.

[b] This total includes 24 strains also examined for streptomycin resistance and which have been omitted from the total of all strains examined.

TABLE 2. *Resistance patterns of Pseudomonas R factors*

R factor	Resistance transferred[a]	Source
R931	S, T	Hospital isolate
R3108	S, T, Su	Hospital isolate
R130, R209	S, Su, G	J. Davies[b]
RP4	Cb, K, T	G. A. Jacoby[c]
R679	S, Su	Hospital isolate
R1162	S, Su	Hospital isolate
R716	S	Hospital isolate
R503	S	Hospital isolate
R5265	S, Su	Hospital isolate

[a] Abbreviations: S, streptomycin; T, tetracycline; Su, sulfonamide; Cb, carbenicillin; K, kanamycin; G, gentamicin.

[b] University of Wisconsin.

[c] Massachusetts General Hospital.

TABLE 3. *MIC of gentamicin and antimicrobial disk susceptibility for strains before and after gentamicin-resistance transfer*

Strain	MIC of gentamicin (μg/ml)	Disk zone[a] (mm)				
		S 10	T 30	K 30	Cb 100	Su 1
Recipients:						
280 met⁻ rif^r	0.25	25	18	19	40	35
1310 rif^r	1.0	12	14	12	23	15
Donors:						
PS-209 (R209)	>20	7	7	7	13	7
RS-130 (R130)	>20	7	8	7	28	7
R^+ recipients:						
280 (R209)	2 to 10	7	18	19	40	7
280 (R130)	10	7	18	19	40	7
1310 (R130)	10	7	14	13	22	7
1310 (R209)	10	7	14	14	22	7

[a] Abbreviations: S, streptomycin; T, tetracycline; K, kanamycin; Cb, carbenicillin; Su, sulfonamide. Numbers refer to micrograms per disk, except for Su, which is 1 mg/disk.

The frequency with which transfer of resistance determinants for streptomycin or gentamicin is detected varies depending on which agent is used for selection (Table 4). R^+ clones selected with gentamicin always exhibit significant streptomycin resistance. However, only about 1 to 10% of R^+ clones selected with streptomycin express gentamicin resistance which can be detected with the gentamicin concentrations given in Table 4. This results in the apparent transfer frequency for streptomycin resistance being 10- to 100-fold greater than that obtained for gentamicin resistance. Individual R^+ clones exhibit considerable heterogeneity in their level of gentamicin resistance, suggesting variation in the expression of determinants for gentamicin resistance.

It is of interest that, although these strains (PS-130, PS-209) have been shown to contain kanamycin phosphotransferase (6), there is no evidence of transfer of resistance to kanamycin to either recipient strain (Table 3). Also, a third strain acetylating gentamicin has not been found to transfer gentamicin resistance.

Intergeneric R-factor transfer. Previous

work has shown that, at least for certain R factors from *P. aeruginosa*, *E. coli* strains act as much less effective recipients than *P. aeruginosa* strains in conjugal R-factor transfer (5). Table 5 presents additional results which confirm and expand that initial observation. *E. coli* and *P. mirabilis* vary in their effectiveness as recipients, but generally mating frequencies are many-fold less than with *P. aeruginosa* strains 280 or 1310 as recipients. The exception to this is RP4, an R factor described by Datta et al. (11). The frequencies of transfer of RP4 from strain 280 to *E. coli*, *P. mirabilis*, or *P. aeruginosa* strain 280 recipients are similar. In our survey, we have not detected any similar R factor, despite examination for transferable carbenicillin and kanamycin resistance. Thus, RP4 seems to have a host range different from at least some *P. aeruginosa* R factors we have detected (R931, R130, R3108) and different transfer efficiencies to hosts in other cases.

The frequencies of transferred resistance shown for *E. coli* and *P. mirabilis* matings are the highest obtained. Frequently R factor transfer was either not detected or detected at a lower frequency than shown in Table 5.

Intrageneric R-factor transfer. Table 6 illustrates the frequency of transferable antimicrobial resistance obtained by using two different *P. aeruginosa* recipient strains in mating experiments. The frequencies obtained are, at least in some instances, influenced by the nature of the recipient. Strain 1310 is a more effective recipient for R factors R716 and R503, whereas strain 280 is a better recipient for RP4 and to a lesser extent for R5265. The observation that R factor RP4 fails to transfer to strain 1310 is surprising in view of the apparently unrestricted transfer of that R factor among *E. coli*, *P. aeruginosa*, and *P. mirabilis* (reference 11; see Table 4). Whether the recipient effect is due to defective conjugation, DNA transfer, or R-factor expression is not yet known.

Table 6 also shows that R-factor transfer is often enhanced when both donor and recipient strains are identical except for the mutations used to mark the strains. For example, R679 transfers at a higher frequency from strain 280 trp$^-$ to 280 met$^-$rif^r than to strain 1310 rif^r.

R factor R931. R931 as shown in Table 6

TABLE 4. *Transfer frequency of gentamicin and streptomycin resistance*[a]

Strains		Antimicrobial agents used in selection			
Donor	Recipient	Gentamicin (μg/ml)		Streptomycin (μg/ml)	
		1	2.5	16.6	33
PS 130 (R130)	280 met$^-$ [b]	1.6×10^{-1}		1.6	
PS 209 (R209)	280 met$^-$	1.1×10^{-1}		0.7	
PS 130 (R130)	1310		3.5×10^{-3}		1.9×10^{-1}
PS 209 (R209)	1310		6.2×10^{-3}		4.5×10^{-1}
1310 cbn^r (R130)	1310		1×10^{-3}		0.1
1310 cbn^r (R130)	280 met$^-$	1×10^{-3}		0.1	

[a] Based on 2-h matings with 1:10 donor-recipient ratio.
[b] All recipients were rifampin resistant.

TABLE 5. *Frequencies of transfer of Pseudomonas R factors to E. coli and P. mirabilis recipients*

Donor	Recipients		Transfer frequency relative to *P. aeruginosa* recipient[a]	
	E. coli	*P. mirabilis*	Strain 280	Strain 1310
931 (R931)	$<5 \times 10^{-8}$	$<5 \times 10^{-8}$	1.25×10^{-7}	5×10^{-7}
3108 (R3108)	$<5 \times 10^{-8}$	$<5 \times 10^{-8}$	2.5×10^{-8}	2.5×10^{-8}
PS130 (R130)	$<5 \times 10^{-8}$	ND[b]	5×10^{-7}	1.6×10^{-5}
679 (R679)	2×10^{-7}	$<5 \times 10^{-7}$	4.10^{-2} to 1×10^{-4}	1×10^{-1}
1162 (R1162)	5×10^{-7}	ND	1×10^{-1} to 1×10^{-3}	1×10^{-1}
5265 (R5265)	2×10^{-8}	$<5 \times 10^{-8}$	1×10^{-3}	4×10^{-3}
716 (R716)	$<5 \times 10^{-8}$	ND	7×10^{-2}	5×10^{-5}
280 (RP4)	8×10^{-4}	1×10^{-6}	1	2×10^{4}

[a] Ratio of the highest transfer frequency obtained with *E. coli* or *P. mirabilis* to an average value obtained in matings with a *P. aeruginosa* recipient. Matings contained equal numbers of donor and recipient organisms.
[b] ND, not done.

TABLE 6. *Frequency of Pseudomonas aeruginosa R-factor transfer in various conjugation mixtures*[a]

R factor	Donor strain	Recipient strains	
		280	1310
R931	931	0.5	0.15
	280	4.6×10^{-4}	4×10^{-6}
	1310	0.9	0.5
R3108	3108	0.1	2×10^{-2}
	280	9×10^{-4}	2×10^{-6}
	1310	1×10^{-4}	3.6×10^{-2}
R5265	5265	2×10^{-5}	5.5×10^{-6}
R716	716	7×10^{-7}	1×10^{-3}
	280	$<5 \times 10^{-8}$	$<5 \times 10^{-8}$
	1310	4×10^{-5}	0.6
R503	503	3×10^{-7}	4.8×10^{-4}
R1162	1162	4×10^{-6}[b]	1.6×10^{-6}
R679	679	5×10^{-6}[b]	1.6×10^{-6}
	280	5×10^{-5}	6×10^{-8}
	1310	$<1 \times 10^{-8}$	6×10^{-7}
RP4	280	4×10^{-4}	$<5 \times 10^{-8}$
	E. coli	4.5×10^{-4}	$<5 \times 10^{-8}$

[a] Based on 2-h matings with a 1:1 donor-to-recipient ratio.

[b] Transfer frequencies of R679 and R1162 have declined 50-fold during 12 months of laboratory storage.

results in high-frequency conjugal transfer of resistance markers. Our previously reported mating frequency for R931 in a 20-h mating was 10^{-2} per donor organism (5). Improvement of and experience with the *Pseudomonas* mating system have resulted in a more accurate estimation of mating frequency for R931. Frequenceis per donor cell obtained in 2 h using mating mixtures containing a ratio of one donor to one recipient cell are 0.1 to 1 (Table 6); with a donor to recipient ratio of 1:10 the frequency is greater than 1. Strain 931 containing R931 has been stored on agar slants or as freeze-dried cultures and transferred over 100 times. The mating frequency has not decreased in that time. Over 500 clones of strain 931 obtained by dilution have been tested for stability of the resistance determinants for streptomycin (S) and tetracycline (T). Less than 1% of the clones had lost either resistance marker. However, we have on occasion observed that only the S marker is transferred when conjugation is allowed to proceed for less than 30 min. Thus, the conjugating ability induced by R931 in strain 931 is analogous to that of the F factor in *E. coli*. It apparently acts as a wild type, derepressed (for conjugation and resistance transfer) sex factor.

The behavior of R931 in the two recipient strains 280 and 1310 is quite different and unusual (Table 6). When strain 1310 is used as a donor, the frequency of resistance transfer is similar to that obtained when strain 931 is used as the donor. However, the use of strain 280 as the donor results in a reduction of drug-resistance-transfer frequency in 2-h matings to about 5×10^{-4} per donor cell. Fertility mediated by R931 in strain 280 may be subject to repression. Support for that supposition is provided by the following results. In experiments in which R931 resided in strain 280 for three generations or less (see Methods), the frequency of drug-resistance transfer was 0.75. If, however, the number the generations of strain 280 (R931) exceeded 10, the transfer frequency was 9×10^{-5}. These results are very similar to those obtained in the Col I system in which conjugating ability is subject to repression (30). Thus, for R931 the apparent repression of drug-resistance transfer is not operative in strains 931 and 1310 but is operative in strain 280. The reason for this behavior is not yet known. Both R931-mediated streptomycin- and tetracycline-resistance levels decline in strain 280 (6). One possible explanation for these observations could be a reduction in the relative content of R-factor DNA in strain 280 due to its restriction to a small percentage of the population or to a reduction in copies of R factor per cell. Both possibilities are unlikely, as R931 DNA accounts for 16 ± 2% of the total DNA in strain 280 (Fig. 1c). R factor R3108 seems to act in a similar fashion to R931 (Table 6). If 280 kan^r (R3108) is allowed to mate for 2 h with 280 rif^r, the frequency of resistance transfer is 9×10^{-4} (Table 6); if allowed to mate overnight, that value increases to about 0.1 to 0.5. This suggests a repressed system (34).

Compatibility studies. The R factor RP4 has been shown to form a compatibility group distinct from that of F, I, N, or W R factors; Datta and Hedges (9) defined RP4 as a member of the P compatibility group. Determination of R factor compatibility has previously been examined using *E. coli* recipients. Such an approach is not possible with many of the R factors described in this paper due to host restriction. Table 7 gives the results of compatibility studies carried out with *Pseudomonas* recipients. In these experiments, selection was with rifampin (to which recipients were resistant) and a second drug to which the donor was resistant. Clearly, certain R factors can exclude the presence of others just as in the *Enterobacteriaceae*. The transfer frequency of R3108 and R931 to strain 280 containing a resident R factor from PS-130 or PS-209 declines to $<2 \times 10^{-8}$. Thus, it is concluded that these R factors form a single compatibility group. We propose to call this group P-2.

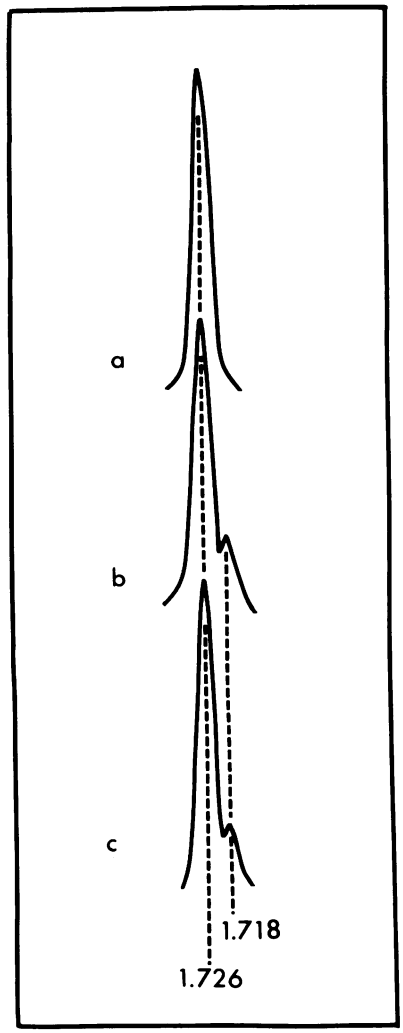

FIG. 1. *Analytical CsCl gradient centrifugation of DNA from strains of Pseudomonas aeruginosa. Samples of DNA were centrifuged to equilibrium in CsCl, and then ultraviolet photographs were taken and traced with a densitometer. Trace a, DNA from strain 280; trace b, DNA from strain 931 (R931); trace c, DNA from strain 280 (R931).*

R factor RP4, on the contrary, does not influence the drug-resistance-transfer frequency of the P-2 group nor of any other *Pseudomonas* R factor examined. It is possible that two similar R factors could initially be simultaneously expressed in a recipient, but that one would be unstable. Colonies containing P-2 R factors and RP4 were grown in drug-free medium and examined by replica plating for stability of R determinants (see Methods) to exclude that possibility. RP4 and r determinants of P-2 factors exhibit stable coexistence (Table 7).

We propose that RP4 and similar R factors be members of the P-1 compatibility group to distinguish them from other *Pseudomonas* R factors. Ingram et al. (18) recently presented evidence that RP1, an R factor very similar to RP4, is a *Pseudomonas* R factor. Further compatibility groups of *Pseudomonas* R factors could be termed P-3, P-4, etc.

Table 7 illustrates that at least one additional *Pseudomonas* compatibility group exists. Members of groups P-1 and P-2 show stable coexistence with R679, R716, and R5265. It is not possible at present to state whether this group of R factors is homologous or heterologous, due to the limited number of r determinants present. However, evidence based on CsCl density-gradient analytical centrifugation does indicate at least some dissimilarities between R5265 and R679 (Fig. 2 and 3).

Agglutination reactions can distinguish between R factors R931 and R3108. Antiserum to 280 (R931) absorbed with strain 280 (as described in Methods) has an agglutination titer for strains 280, 280 (R931), and 280 (R3108) of 20, 1,600, and 20, respectively.

Analytical CsCl gradient centrifugation. Confirmation that the R factor 931 exists as extrachromosomal DNA is seen in densitometer traces of photographs taken after gradient centrifugation (Fig. 1). Figure 1a illustrates that the recipient strain 280 contained no detectable satellite DNA whereas, in strain 931 (R931), a minor band in addition to the main chromosomal band existed (Fig. 1b). After mating of strain 931 (R931) and 280, CsCl density-gradient analysis established that a satellite band was present in strain 280 (Fig. 1c).

The buoyant density of these bands was determined by using reference values of 1.710 g/cm^3 for *E. coli* and *P. morganii* DNA (29). The main chromosomal band buoyant density was determined to be 1.725 to 1.726 g/cm^3, which corresponds to a guanine plus cytosine ratio of 67% (29). The buoyant density agrees with values generally obtained for *P. aeruginosa* strains (13, 17, 25).

Strains 280 and 931 were previously confirmed to be *P. aeruginosa* by generally accepted criteria (5).

Densitometer traces of CsCl gradient centrifugation of DNA extracted from three other *P. aeruginosa* strains which conjugally transferred resistance to antimicrobial agents are seen in Fig. 2 and 3. In the case of strains 5265 (R5265) and 3108 (R3108), satellite bands are present

TABLE 7. *Frequency of R-factor transfer to and stability of resistance determinants in R⁻ and R⁺ P. aeruginosa*

Donor	Recipient[a]	Selection for resistance to	Transfer frequency	Stability[b]
931 (R931)	1310	T[c]	0.1	210/210 (S,T)[c]
	1310 (R130)	T	2×10^{-8}	
3108 (R3108)	1310	T	0.36	190/190 (S,T)
	1310 (R130)	T	2×10^{-8}	
280 (RP4)	280	K	5×10^{-4}	335/335 (K)
	280 (R130)	K	5×10^{-4}	358/360 (K,G)
	280 (R931)	K	4×10^{-4}	220/220 (K,T)
	280 (R3108)	K	2.7×10^{-4}	194/194 (S,K)
280 (RP4)	280	K	3.5×10^{-5}	
	280 (R716)	K	2.4×10^{-5}	99/103 (K,S)
	280 (R5265)	K	5×10^{-5}	185/195 (K,S)
	280 (R679)	K	3.4×10^{-5}	76/80 (S,K)
931	280	T	0.1	
	280 (R679)	T	0.09	250/250 (T)[d]
	280 (R716)	T	0.1	
	280 (R5265)	T	0.15	1000/1000 (T)[d]

[a] All recipients were rifampin resistant

[b] Number of clones retaining resistance to the antimicrobial agent given in brackets over number of clones tested.

[c] T, tetracycline; K, kanamycin; G, gentamicin; S, streptomycin.

[d] Fifty colonies were examined by antimicrobial disk susceptibility and shown to retain sulfonamide resistance.

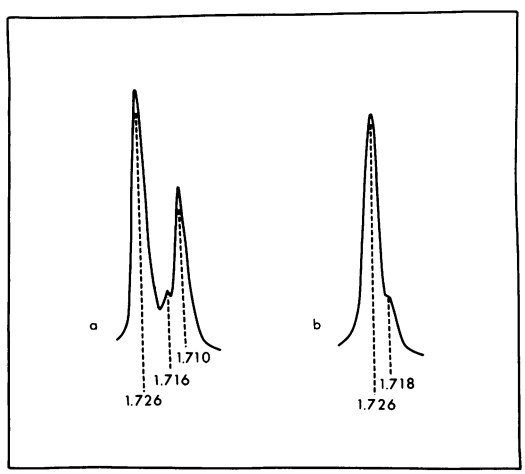

FIG. 2. *Analytical density-gradient centrifugation of DNA from Pseudomonas aeruginosa cultures. Trace a, DNA from strain 3108 (R3108) and Escherichia coli which was used as a density marker (P = 1.710 g/cm³); trace b, DNA from strain 5265 (R5265).*

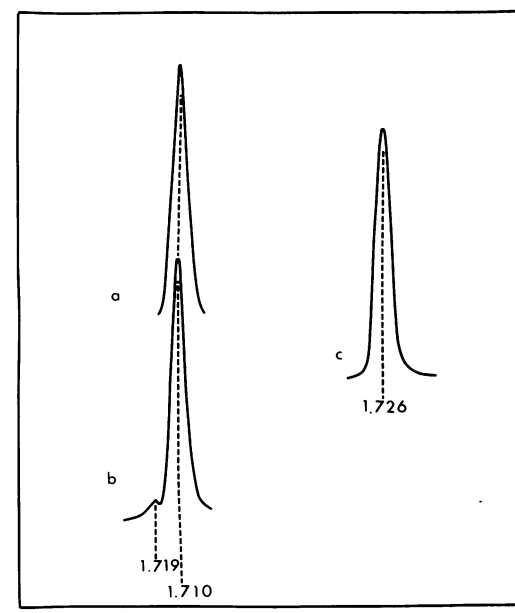

FIG. 3. *Analytical CsCl gradient centrifugation profiles of DNA isolated from Escherichia coli and Pseudomonas aeruginosa. Trace a, DNA from E. coli K-12 F⁻; trace b, DNA from E. coli R679; trace c, DNA from strain 679 (R679).*

(Fig. 2). However, strain 679 (R679) exhibited no such band (Fig. 3c). Figure 3b illustrates that a satellite band could be demonstrated in CsCl gradient centrifugation of DNA extracted from *E. coli* K-12 F⁻ (ATCC 14948) after conjugation with *P. aeruginosa* 679 (R679). The absence of a similar satellite peak in *E. coli* K-12 F⁻ prior to conjugation is seen in Fig. 3a. Conjugal transfer of R679 to *E. coli* occurs irregularly and at very low frequencies.

The buoyant densities of the R factors are seen in Fig. 1 to 4. R factors R931 and R5265 have a buoyant density of 1.718 g/cm³. This

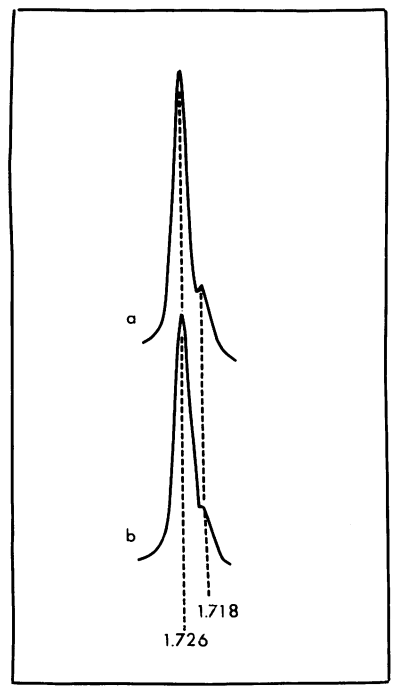

FIG. 4. *Analytical gradient centrifugation of DNA from Pseudomonas aeruginosa strain 931 (R931). Trace a, DNA from strain 931 (R931) in log-phase; trace b, DNA from strain 931 (R931) in stationary phase.*

value is similar to that described by Grinsted et al. (17) for the R factor RP1 which transfers freely between *P. aeruginosa* and *E. coli* or *P. mirabilis*. It agrees with the value obtained by Finley and Punch (13) for one of the satellite DNA bands described by those workers. A buoyant density of 1.716 g/cm^3 was obtained for R3108 (Fig. 2a).

Ethidium bromide-CsCl gradient centrifugation. The existence of R931 as a closed circular form of DNA is strongly suggested by the ethidium bromide-CsCl gradient centrifugation of lysates of *P. aeruginosa* strain 931 (R931) labeled with ^{32}P. Closed circular forms of DNA can be separated from other DNA conformations by such studies (3). Figure 5 illustrates that a minor band on the dense side of the main chromosomal band of DNA is present with strain 931 (R931). The closed circular form isolated is about 5.5% of the total DNA in our experimental system.

Electron microscopy of closed circular DNA. Electron micrographs of DNA obtained by ethidium bromide-CsCl gradient centrifugation demonstrate both closed and open circular DNA (Fig. 6 and 7). There are two major sizes of molecules which are shown in Fig. 6 and 7. Both sizes of molecules are converted to a majority of open circular forms by aging for 7 days or by DNase I treatment (Table 8). The small CCC DNA is distinguished from fragments of chromosomal DNA by the method used in its isolation, the uniformity of length, the presence of forms intermediate between CCC and open circular, DNA, and the effects of DNase I (Table 8 and Fig. 7).

The lengths of the two major sizes of DNA are given in Table 9. Based on a value of 2.07 $\times$ 10^6 daltons/μm (22), the larger molecule has a molecular weight of about 25 $\times$ 10^6 and the smaller, 10^6. The relative proportion of the sizes is about 3.6 to 4.6 small to 1 large molecule (Table 9). Estimation of relative proportions is subject to the error that larger molecules may be more susceptible to single-stranded breaks and less would be isolated by ethidium bromide-CsCl gradient centrifugation. Occasional circular molecules about 35 and 1.5 μm in size have been detected.

The relationship between the two major molecules is at present unclear. The small size

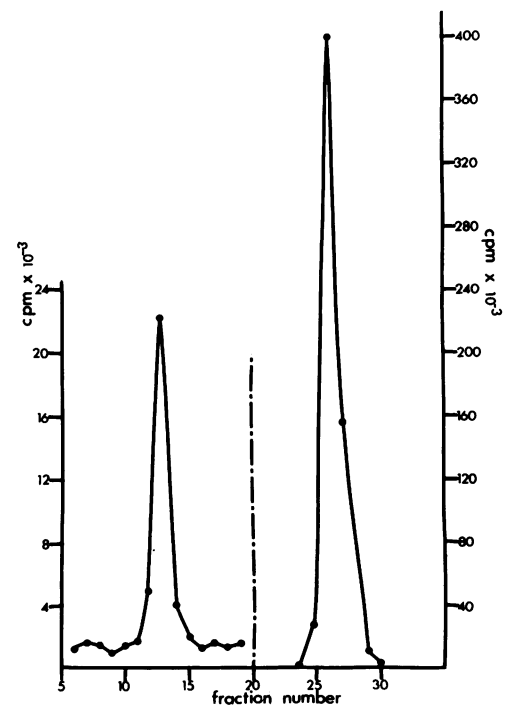

FIG. 5. *Preparative centrifugation of P. aeruginosa strain 931 (R931) labeled with ^{32}P, isolated by detergent lysis and centrifuged in CsCl in the presence of ethidium bromide. Note the change of scale at fraction 20.*

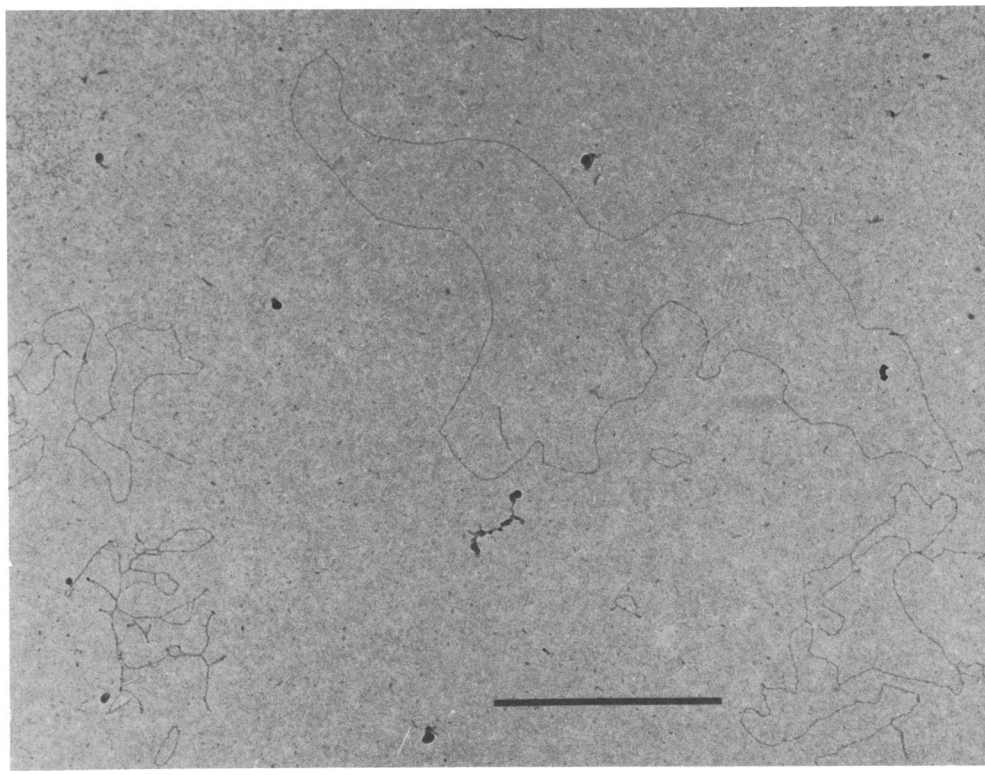

FIG. 6. *Electron microscopy of R-factor DNA taken from satellite band after dye-buoyant density centrifugation of log-phase 931 (R931) cultures. Open circular DNA molecule (12.0 μm) and supertwisted form. Bar indicates 1 μm.*

of the 0.5-μm molecule and the error of measurement of the larger molecule prevents (Table 9) any conclusions regarding the derivation of the former from the latter molecule. However, the excess of small molecules relative to the 12-μm molecule tends to rule out a 1:1 relationship between the small molecule and a larger molecule. They could, of course, also be totally unrelated plasmids.

DNA content in satellite bands under various conditions and plasmid copies per genome equivalent. Table 10 illustrates the percentage of the total DNA represented as satellite DNA as estimated by planimetry for the various R factors. Although subject to considerable error, these estimations indicate that the relative content of R931 DNA declines in the stationary phase in strains 280 (R931) and 931 (R931). The proportion of R5265 and R3108 DNA in stationary-phase hosts seems similar to that of R931. The content of R-factor DNA in strain 679 (R679) is apparently different from the above two R factors. It is not detectable in that strain and is about 4% of the total DNA in *E. coli* K-12F$^-$ stationary-phase cells. Ethidium bromide-CsCl gradient centrifugation has not yet been carried out on strain 679.

The number of plasmid copies per genome equivalent in strain 931 (R931) can be estimated based on the molecular weight of the chromosome of *P. aeruginosa*. The latter can be estimated to be similar to that of *E. coli*, 2.5×10^9 daltons. Based on a relative frequency of the small to large extrachromosomal DNA of 4.1:1 (Table 9) and a value of 18% R-factor DNA (Table 10) in log-phase cells, the number of copies per chromosome is 15 to 16 large and 65 small plasmids. The number of copies appears to decline in stationary phase. If the larger value for the *P. aeruginosa* chromosome of 6×10^9 to 7×10^9 daltons reported by Leth Bak et al. (24) is used, the number of copies per genome in each growth phase is increased by two- to three-fold. Thus both DNA molecules are under relaxed replication.

DISCUSSION

R-factor prevalence detected by our studies of hospital isolates of *P. aeruginosa* is uncommon. The statistical values given in this manuscript,

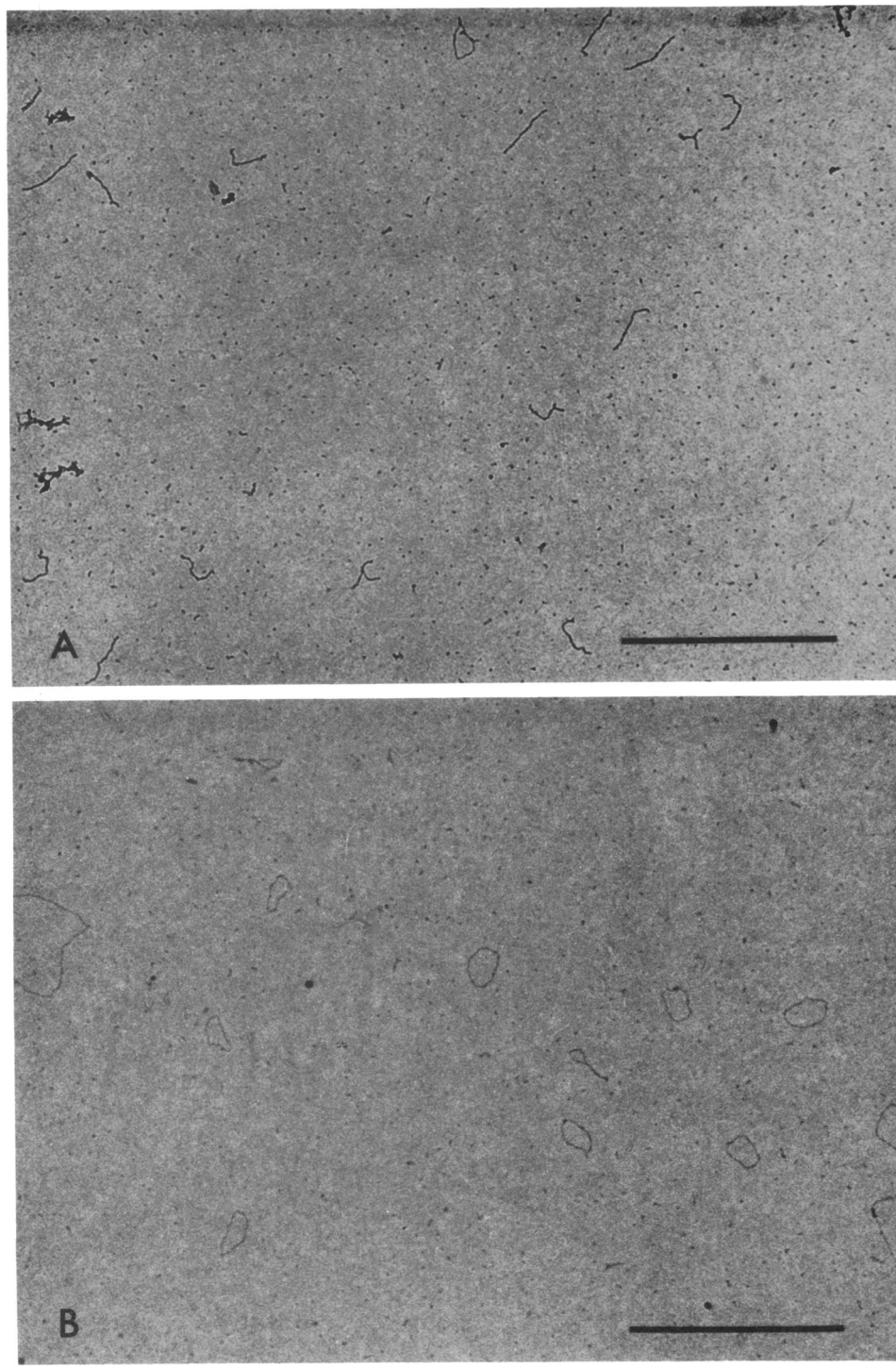

FIG. 7. *"Small" circular DNA taken from satellite band after dye-buoyant density centrifugation of log-phase 931 (R931) cultures before (A) and after (B) treatment with deoxyribonuclease. Bar indicates 1 μm.*

we feel, include R factors that would not be detected by using *E. coli* recipients. The reason for the low incidence of R factors is not known, but it is similar to values reported for *Shigella* species prior to extensive use of antimicrobial agents (26). It seems unlikely that antimicrobial agents in common use up to the time of the introduction of gentamicin and carbenicillin would exert a significant selective force on R^+ *P. aeruginosa*, because the organism is resistant for other reasons to most antimicrobial agents. Thus, for example, 90% of a series of *P. aeruginosa* strains were resistant to greater than 10 μg of streptomycin per ml because of defective permeability (33). R^+ strains would have no particular survival advantage except where streptomycin concentrations were very high. The presence of transferable gentamicin resistance reported here and elsewhere (35) may change this situation. Most *P. aeruginosa* strains are sensitive to gentamicin, and the use of the drug could select for R^+ strains, although other forms of resistance do occur (12, 32).

The R factor R931 appears to be a representative of an unique R-factor system. In our experiments, it has not been successfully transferred to *E. coli*, a very unusual property (10). R931 also possesses the unusual property for R factors of being a naturally occurring high-frequency-transfer system in at least two hosts. There is no evidence that R931 in strains 931 or 1310 is ever subject to fertility repression. However, R931 in strain 280 acts in an analogous manner to the Col I system (30) and, thus, by inference seems subject to repression of fertility. The possibility exists that the apparent repression of R931 is due to another plasmid in strain 280, although CsCl density-gradient centrifugation gives no evidence of additional plasmids in strain 280. It is also possible that the repression mechanism is not expressed in strains 931 and 1310. R931 does not seem to be a naturally occurring derepressed mutant because of its behavior in strain 280.

R factor R931 seems to represent a significant group of *Pseudomonas* R factors. Three other naturally occurring R factors belong to the same compatibility group. As noted, two of the above R factors (R209, R130) carry gentamicin resistance and thus may have much practical significance.

Although the guanine plus cytosine content of R931 is similar to that of the *Pseudomonas* R factor RP1, the size of R931 is much less than that estimated for RP1 (25×10^6 daltons versus 40×10^6 daltons). There is no evidence for a small molecular weight DNA species (1×10^6 daltons) associated with RP1 (19).

TABLE 8. *Effect of DNase I on molecules isolated by ethidium bromide-cesium chloride gradient centrifugation*[a]

Molecule size	Conformation[b]	No. of molecules	
		Pre-DNase	Post-DNase
Large	CCC	80	12
	OC	159	104
Small	CCC	764	238
	OC	90	299
Total small: total large[a]		854:239 (3.6:1)	537:116 (4.6:1)
Total small OC: total large		90:239 (0.38:1)	299:116 (2.57:1)

[a] Quantitation was based on viewing about 50 nonrepeated fields.
[b] CCC, Covalently closed circular; OC, open circular.

TABLE 9. *Contour length measurements, molecular weights, and relative frequency of plasmid DNA from P. aeruginosa 931*

No. of molecules measured[a]	Contour length ± SSD (μm)[b]	Mol wt (Mdal)[c]	Relative frequency[d]
39	12.3 ± .7	25.4	1
28	0.50 ± .03	1.0	4.1 ± 0.5

[a] Rare molecules of other sizes occurred but represented <0.01 relative frequency.
[b] SSD, Sample standard deviation.
[c] Mdal, 1×10^6 daltons.
[d] Based on 653 molecules.

TABLE 10. *Content of R-factor DNA relative to chromosomal DNA*

R factor	Host strain	Growth phase	R-factor DNA (%)[a]
R931	931	Logarithmic	18 ± 2
	931	Stationary	11 ± 2
	280	Logarithmic	16 ± 2
	280	Stationary	11 ± 2
R679	679	Stationary	ND[b]
	E. coli	Stationary	4 ± 0.5
R3108	3108	Stationary	8 ± 3
R5265	5265	Stationary	12 ± 2

[a] Percentage of total DNA based on the average of five planimetry measurements of densitometer traces on each of three or more analytical CsCl gradient-centrifugation trials.
[b] Not detected.

The size of R931 is similar to that of R6K in *E. coli* (21). The latter R factor is the smallest known factor with sex-factor activity, having a molecular weight of 26×10^6, which is similar to that of R931 (25×10^6). Both R factors seem to

be under relaxed replication. However, R931 is unusual in that the DNA content does not increase in stationary phase of growth, but rather probably slightly declines. We have detected occasional dimers but no apparent higher multimers of R931. The buoyant density reported for R6K is 1.704 g/cm^3, very much less than that of R931.

It was recently reported that minicircular DNA in *E. coli* (15) shows homology with Col E1 factors. Also, Clowes (7) has demonstrated minicircular DNA associated with streptomycin and ampicillin resistance in the Δ SAT system described by Anderson and Lewis (1) in *Salmonella*. Thus, although we can only speculate with present information, it is quite possible that the 10^6-dalton molecular weight, circular DNA in *P. aeruginosa* 931 (R931) represents r determinants. DNA of similar molecular weight has been described in *Shigella dysentariae* and *paradysentariae* (19, 28) but has no known function. Studies of homology between the small and large molecular weight DNA in strain 931 (R931) would be of interest to determine their relationship.

The failure to differentiate the large and small molecules of extrachromosomal DNA into two distinct bands in CsCl density-gradient centrifugation suggests one of two major possibilities. It is possible that both molecules have very similar or identical buoyant densities or that the smaller peak is hidden in the larger satellite or chromosomal band. The extrachromosomal DNA satellite band in strains 931 (R931) and 280 (R931) (Fig. 1[c], Fig. 4[a]) is sharp and, from its appearance, represents either a single species of DNA or two species of very similar buoyant densities. Thus, the value of 1.718 g/cm^3 seems a satisfactory estimation of the buoyant density of the majority species (25 $\times$ 10^6 daltons) of extrachromosomal DNA in strain 931 (R931). The value should not be applied to the smaller species.

The relationship of *P. aeruginosa* R factors to those in the *Enterobacteriaceae* is not fully clear. It is possible that members of the third compatibility group described in this manuscript could be R factors belonging to previously described compatibility groups. However, this seems unlikely in view of the difficulty in consistently transferring such factors as R679 to *E. coli* and because of the 1.719 buoyant density. The latter value is much closer to values obtained for the *Pseudomonas* R factors, RP1 and R931. Witchitz and Gerbaud (35) recently described transferable gentamicin resistance from *P. aeruginosa* strains. The relationship of those R factors to the ones described here is as yet unknown.

ACKNOWLEDGMENTS

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