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Molecular Characterization of the R Factors Implicated in the Carbenicillin Resistance of a Sequence of *Pseudomonas aeruginosa* Strains Isolated from Burns

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An outbreak of R-factor-mediated carbenicillin resistance in *Pseudomonas aeruginosa* in burned patients in March 1969 was followed by a second outbreak 6 months later. No R-factor-carrying *P. aeruginosa* strains were detected in the intervening period but R-factor-determined lactamase was commonly encountered, particularly in *Klebsiella aerogenes* strains. A comparison of the molecular properties of the R factors in pseudomonads from the first and second phases with those in the *Klebsiella* strains from the intervening period showed them to be very closely related. A single R-factor type therefore may have been maintained in the Burns Unit between the two *Pseudomonas* outbreaks as a plasmid conferring resistance to ampicillin in *K. aerogenes*.

R-factor-mediated resistance to carbenicillin among strains of *Pseudomonas aeruginosa* was first encountered in the Medical Research Council's Industrial Injuries and Burns Unit at the Birmingham Accident Hospital, Birmingham, England (9, 11) but subsequently has appeared elsewhere (2). In the Birmingham outbreak, the strains occurred in two phases: the first (from about the beginning of March to the middle of June 1969) ended only when carbenicillin was withdrawn from use in the Unit, and the second (from 1 December 1969 to the end of January 1970) coincided with a period when the use of this antibiotic was resumed for a short while before being discontinued once again. Strains of *P. aeruginosa* showing characteristics of the carbenicillin R factor were very rarely isolated in the Unit between the outbreaks, and none had been present for several weeks before the second outbreak, although carefully looked for. Nevertheless, ampicillin-resistant enteric bacteria carrying R factors continued to be found throughout the whole period (4, 11).

The *P. aeruginosa* strains examined in detail in the work described here were all taken from the Birmingham outbreaks. The sequence of events and the date of isolation of the various strains are shown in Table 1. Three of the isolates form a sequence from a single patient in the second phase: *P. aeruginosa* Ps 8830 (carbenicillin-susceptible and R^-) was isolated on 17 November and Ps 9169 (of the same typing pattern as Ps 8830 but carbenicillin-resistant and R^+) appeared in the patient on 1 December 1969 (see Table 1). *Klebsiella aerogenes* strain K8834 (carbenicillin/ampicillin resistant and R^+) was also isolated on 17 November. Thus, on that date the patient in question was carrying a carbenicillin-susceptible R^- strain of *P. aeruginosa* and a carbenicillin-resistant, R^+ strain of *K. aerogenes* in his burns. This situation persisted until 1 December 1969 when an R^+, carbenicillin-resistant strain of *P. aeruginosa*, of the same typing pattern as the pseudomonad originally present, joined the *K. aerogenes* strain in the burns (9, 11). The first stage of this investigation, therefore, was to examine the molecular nature of the R factor found in the *Klebsiella* strain K8834 and to compare its properties with the R factor subsequently detected in *P. aeruginosa* Ps 9169 to see whether the *Pseudomonas* strain could have obtained its R factor, and hence its carbenicillin resistance, by R factor transfer from the *Klebsiella* strain within the patient.

The second objective was to compare the R factors that caused the first phase of carbenicillin resistance in the Burns Unit with those that caused the second and also to examine the relationship of these R factors with other plasmids carried in enteric bacteria in the intervening period. *P. aeruginosa* Ps 1822 was taken as a typical example of a strain implicated in the first phase of the outbreak of carbenicillin resistance (Table 1). This strain was chosen because the R

TABLE 1. *Sequences of isolation, source, and some characteristics of the strains of Pseudomonas aeruginosa and Klebsiella aerogenes used*

Date of isolation	Strain	Phase	Phage pattern	R factor	R factor designation	β-Lactamase formation[a]	Patient
4 March 1969	*P. aeruginosa* Ps1822	I	7/21/68/119X	+	RP1	Type Id + type IIIa*	A
21 September 1969	*K. aerogenes* K7418	Intermediate	—	+	RK3	Type IIIa*	B
17 November 1969	*P. aeruginosa* Ps 8830	II	7/44/F8/F10/109/352/1214/M6/Col 11	−	—	Type Id	H
17 November 1969	*K. aerogenes* K8834	II	—	+	RK1	Type IIIa	H
17 November 1969	*K. aerogenes* K8841	II	—	+	RK2	Type IIIa*	C
1 December 1969	*P. aeruginosa* Ps 9169	II	7/44/F8/109/352/1214/M6/Col 11	+	RP9	Type Id + type IIIa*	H

[a] Type IIIa β-lactamase is characteristic of the R factors involved in these experiments (16, 17), and type Id enzyme is found in most isolates of *P. aeruginosa* regardless of the presence of an R factor (17). The ampicillin/carbenicillin resistance of the strains is primarily due to the type IIIa component.

factor carried (RP1) has already been examined in some detail (6, 7). Two other strains of *K. aerogenes* were also studied: the first strain (K8841, R^+ and ampicillin/carbenicillin resistant) was isolated on 17 November 1969, but from a different patient than K8834 (see Table 1 and above); the second strain (K7418, R^+ and ampicillin/carbenicillin resistant) was isolated on 21 September 1969, that is, at a point almost 2 months before the onset of the second phase of resistance in the Unit.

The R factors found in the different *Klebsiella* strains and in *P. aeruginosa* 9169 were found to be very similar and to be closely related to the R factor RP1 from *P. aeruginosa* Ps 1822. These results suggest that the necessary genetic information to confer resistance to carbenicillin may well have been conserved in the hospital between the two outbreaks as an R factor carried in strains of enteric bacteria such as *K. aerogenes*.

MATERIALS AND METHODS

Bacterial strains, media, and buffers. The *K. aeruginosa* strains examined in this paper were all isolated from patients in the Birmingham Accident Hospital by E. J. L. Lowbury and his colleagues. Some of their properties together with their date of isolation are shown in Table 1. *P. aeruginosa* strain S8 was obtained from R. W. A. Girdwood at the Glasgow Royal Infirmary (see 2). All of the other bacterial strains used here, together with the composition of CY medium and of the "low-phosphate" medium, have been described (6, 7).

SET buffer contains NaCl (0.15 M), disodium ethylenediaminetetraacetate (0.1 M), and tris(hydroxymethyl)aminomethane (0.05 M) adjusted to *p*H 8.0 with HCl. SSC solution contains NaCl (0.15 M) and trisodium citrate (0.015 M).

Determination of antibiotic susceptibility. The minimal inhibitory concentration (MIC) of the various antibiotics used was determined by plating single colony-forming units of the test organism on agar containing a suitable range of antibiotic concentrations. The MIC values were recorded after overnight growth at 37 C, as the lowest antibiotic concentration at which no colonies were obtained.

Genetic transfer. Transfer of resistance determinants was carried out routinely as described previously (6). Rifampin (100 μg/ml) and either carbenicillin (500 μg/ml) or chloramphenicol (50 μg/ml) were used for selection as appropriate. Transcipient clones that appeared on selective agar were tested for their resistance to neomycin (250 μg/ml), kanamycin (500 μg/ml), and tetracycline (100 μg/ml) by replica plating.

In the out-crossing experiments to discover the extent of genetic linkage between carbenicillin and chloramphenicol resistance, mating was carried out as described previously (6) except that the donor concentration was reduced to one-tenth of that of the recipient and the mating was only continued for 40 min before selection was applied.

Radiochemical labeling of bacterial deoxyribonucleic acid (DNA). Cultures (10 ml) of *Escherichia coli* or *K. aerogenes* growing exponentially in CY medium were incubated at 37 C for three to four generations in the presence of adenosine (250 μg/ml) and ^{3}H-thymidine (10

μCi/ml; 5 Ci/mmole). *P. aeruginosa* cultures were grown in low-phosphate medium (7) containing ^{32}P-labeled inorganic phosphate (100 μCi/ml.). In all cases, tracer was added at a culture density of about 5×10^7 bacteria/ml.

Isolation of bacterial DNA. Bacteria were harvested by centrifugation after growth in radioactive precursors, washed with SET buffer, and then resuspended in 1.0 ml of SET buffer (containing lysozyme [1 mg] and ribonuclease A [0.5 mg]). The bacteria were then incubated at 37 C for 15 min, sodium sarcosinate solution (0.25 ml; 0.04 g/ml) was added, and the lysate was sheared by passing through a 21-gauge needle. Preparations were then made up to 4.8 ml with water, 2.0 ml of ethidium bromide solution (700 μg/ml) and 6.55 g of CsCl were added, and the mixture was centrifuged at 20 C for 40 hr at 105,000 × *g*.

CsCl gradients containing ^{3}H-labeled DNA were fractionated (0.2-ml fractions), and 20-μliter samples were counted directly after addition to scintillant in a counting vial. Similar-size fractions were also taken from gradients containing ^{32}P-labeled DNA, but each fraction was first incubated with 1.0 ml of 2 N NaOH for at least 5 hr and neutralized with HCl; DNA was precipitated by adding ribonucleic acid (RNA) carrier (20 μg of yeast RNA) and trichloroacetic acid (1.0 ml of 25%, w/v). The DNA precipitates were collected by filtration through glass-fiber filters (Whatman GF/C: Reeve Angel & Co., London, England). Fractions containing covalently closed circular DNA molecules (6) were dialyzed against double-strength SSC buffer, treated with 10 μg of ribonuclease A and 1,000 units of ribonuclease T1 (Sigma Chemical Co.), and dialyzed again. The dialyzed material was then used for further studies as required.

Determination of S value. DNA solutions (0.02 or 0.05 ml) were layered on 5 to 20% (w/v) linear sucrose gradients (4.8 ml) which were then centrifuged at 20 C in the swinging-bucket rotor (3 × 5 ml) of a Superspeed 50 ultracentrifuge at 40,000 rev/min. The gradients were fractionated as above.

A sample of RP1 DNA was included as a molecular weight standard, and *S* values were calculated by assuming values of 62 and 43*S* for the two forms of this plasmid (6).

DNA/DNA hybridization. The method used (a membrane-filter technique), together with the method of preparation of the reference RP1 DNA, was described in detail previously (7).

Materials. We are indebted to F. Knüsel (CIBA/Geigy, Basel, Switzerland) for a generous gift of rifampin and to R. Sutherland (Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, England) for a similar gift of carbenicillin. All other antibiotics were purchased as proprietary brands.

RESULTS

Marker pattern and transfer properties of the strains. *P. aeruginosa* strains Ps 1822 and Ps 9169 were resistant to carbenicillin (5,000 μg/ml), neomycin (625 μg/ml), kanamycin (1,250 μg/ml), and tetracycline (250 μg/ml) when tested for single-cell susceptibility on agar (see Materials and Methods). *P. aeruginosa* Ps 8830, on the other hand, was susceptible to all of these antibiotics (Table 2). All of the *Klebsiella* strains were resistant to the same antibiotics as Ps 1822 and 9169 but were resistant to chloramphenicol (500 μg/ml) and ampicillin (5,000 μg/ml) as well (Table 2).

Conjugation experiments carried out between strains of *P. aeruginosa* and *K. aerogenes* on the one hand and a rifampin-resistant mutant of *Escherichia coli* K-12 R$^-$ on the other showed that R factors were present in all strains except Ps 8830. Transcipients were obtained at a frequency between 10^{-3} and 10^{-5}/donor on agar containing 500 μg of carbenicillin and 100 μg of rifampin/ml; that is, transfer of carbenicillin resistance to *E. coli* R$^-$ occurred at a typical repressed rate (Table 3). Replica plating of the transcipients showed that all that were carbenicillin resistant had also received resistance to ampicillin, to neomycin and kanamycin, and to tetracycline, and that about 10% of transcipients also expressed chloramphenicol resistance when this character was present in the donor bacteria (Table 3).

TABLE 2. *Resistance pattern of the strains examined*

Strain	Minimal inhibitory concn (μg/ml)					
	Carbenicillin	Ampicillin	Neomycin	Kanamycin	Tetracycline	Chloramphenicol
P. aeruginosa Ps 1822	5,000	5,000	625	1,250	250	80
P. aeruginosa Ps 8830	80	400	40	80	2.5	80
P. aeruginosa Ps 9169	5,000	7,500	1,250	1,250	250	160
K. aerogenes K7418	5,000	5,000	625	625	160	625
K. aerogenes K8834	5,000	7,500	625	1,250	160	625
K. aerogenes K8841	5,000	5,000	1,250	1,250	160	625

TABLE 3. *Transfer patterns obtained when a range of donor cultures was crossed with E. coli W3110 rif-r (R⁻) and the transcipient colonies were selected on plates containing 500 μg of carbenicillin and 100 μg of rifampin/ml*

Donor	Marker pattern transferred[a]	Plasmid designation	No. of transcipients	Frequency of transfer
Ps 1822	P N/K T	RP1	1.4×10^3	7×10^{-4}
Ps 8830	P N/K T	—	0	$<10^{-8}$
	P N/K T C	—	0	$<10^{-8}$
Ps 1969	P N/K T	RP9	2.5×10^2	4×10^{-5}
	P N/KT C	—	0	$<10^{-8}$
K7148	P N/K T	RK3	1×10^3	1×10^{-5}
	P N/K T C	—	100	1×10^{-6}
K8834	P N/K T	RK1	5×10^2	2×10^{-5}
	P N/K T C	—	35	3×10^{-6}
K8841	P N/K T	RK2	1.1×10^3	8×10^{-4}
	P N/K T C	—	50	2×10^{-6}

[a] P, β-lactamase production; N/K, neomycin/kanamycin phosphorylating enzyme; T, tetracycline resistance; C, chloramphenicol resistance.

A second series of experiments in which the recipient was *P. aeruginosa* R⁻ *rif-r* gave a similar picture (Table 4). Transfer occurred at a frequency of 10^{-3} to 10^{-5}/donor in all experiments (except those in which Ps 8830 was the donor), but resistance to carbenicillin, to neomycin and kanamycin, and to tetracycline was the only pattern found among the transcipients, even when chloramphenicol resistance was part of the donor phenotype. These results suggest strongly that an R factor with a pattern *carb-r.neo/kana-r.tet-r* and capable of promoting transfer to both enteric bacteria and to pseudomonads is present in *P. aeruginosa* strains Ps 1822 and Ps 9169 and also in *K. aerogenes* strains K8834, K8841, and K7418. These R factors are designated RP1, RP9, RK1, RK2, and RK3, respectively (Table 1).

The transfer of chloramphenicol resistance from the *Klebsiella* strains to *E. coli* probably reflects the presence of a second plasmid specifying resistance to that antibiotic alone which can only promote transfer between members of the *Enterobacteriaceae*. This conclusion was supported by the transfer pattern obtained when transcipients were selected for transfer of chloramphenicol resistance. With the *Klebsiella* strains as donors and *Escherichia coli* R⁻*.rif-r* as recipient, colonies were obtained on agar containing 200 μg of chloramphenicol and 100 μg of rifampin/ml at a frequency between 10^{-6} and 10^{-8}/donor; that is, transfer of chloramphenicol resistance occurs at about 10^{-3} the frequency found for carbenicillin resistance with the same recipient (Table 5; cf. Table 3). Replica plating of the chloramphenicol-resistant transcipients onto other antibiotics showed that all also expressed resistance to carbenicillin, ampicillin, neomycin, kanamycin, and tetracycline. This pattern is, however, quite compatible with successive rather than linked transfer of carbenicillin and chloramphenicol resistance because transfer of the former occurs about 1,000 times more frequently than the latter (cf. Tables 3 and 5). Attempts to transfer chloramphenicol resistance from any of the strains of *K. aerogenes* to *P. aeruginosa* R⁻ have uniformly failed.

R-factor-mediated gene products. All strains of *P. aeruginosa* that have been examined previously in this laboratory (about 65 in all) produced an inducible β-lactamase predominantly active against cephalosporins (β-lactamase type 1d; see 10, 17), and strains Ps 1822 and Ps 9169 were found to be no exception. However, this enzyme was not detected in any of the *Klebsiella* strains examined. All of the strains carrying R factors (Ps 1822, Ps 9169, K7418, K9934, and K8841) also synthesized a second type of β-lactamase which was identified as type IIIa (10) by means of its substrate profile (10, 16) and reaction with specific antiserum (8). The type 1d enzyme, when present, did not interfere with these tests since it is present in insignificant amounts in uninduced cultures (17). The type IIIa enzyme is the β-lactamase primarily responsible for carbenicillin resistance among stains of *P. aeruginosa* and for both carbenicillin and ampicillin resistance in *Klebsiella* strains, hence the designation of the gene concerned as *amp/carb-r* (16).

Neomycin and kanamycin resistance in the R⁺ strains was due to the presence of an aminoglycoside phosphorylating enzyme (J. Arrand, *unpublished data*). A similar enzyme has al-

TABLE 4. *Transfer patterns obtained when a range of donor cultures was crossed with Pseudomonas aeruginosa rif-r (R⁻) and the transcipient colonies were selected on plates containing 500 μg of carbenicillin and 100 μg of rifampin/ml*

Donor	Marker pattern transferred[a]	Plasmid designation	No. of transcipients	Frequency of transfer
Ps 1822	P N/K T	RP1	2×10^3	5×10^{-4}
Ps 8830	P N/K T	—	0	$<10^{-8}$
Ps 9169	P N/K T	RP9	1.1×10^3	8×10^{-4}
K 7148	P N/K T	RK3	1×10^3	1×10^{-5}
K 8834	P N/K T	RK1	1.4×10^3	7×10^{-4}
K8841	P N/K T	RK2	1.1×10^3	9×10^{-4}

[a] P, β-lactamase production; N/K, neomycin/kanamycin phosphorylating enzyme; T, tetracycline resistance; C, chloramphenicol resistance.

TABLE 5. *Transfer patterns obtained when a range of donor cultures was crossed with E. coli W3110 rif-r (R⁻) and the transcipient colonies were selected on plates containing 250 μg of chloramphenicol and 100 μg of rifampin/ml*

Donor	Marker pattern transferred[a]	Plasmid designation	No. of transcipients	Frequency of transfer
Ps 1822	P N/K T C	—	0	$>10^{-8}$
Ps 8830	P N/K T C	—	0	$>10^{-8}$
Ps 9169	P N/K T C	—	0	$>10^{-8}$
K7418	P N/K T C	—	20	5×10^{-6}
K8834	P N/K T C	—	14	7×10^{-6}
K8841	P N/K T C	—	25	4×10^{-6}

[a] P, β-lactamase production; N/K, neomycin/kanamycin phosphorylation; T, tetracycline resistance; C, chloramphenicol acetyl transference.

ready been described in R^+ enteric bacteria by Umezawa (18) and Davies (5).

Isolation of extrachromosomal DNA. Plasmid DNA was isolated as covalently closed, circular (CCC) DNA as described in Materials and Methods. The CCC DNA in strains Ps 1822 and Ps 9169 was examined directly after labeling of the strains with ^{32}P, and the plasmids conferring resistance to penicillins, to neomycin and kanamycin, and to tetracycline (plasmids RK1, RK2, and RK3) in the *Klebsiella* strains K8834, K8841, and K7418 were studied after transfer of the plasmids to *E. coli* K-12 (see Materials and Methods). This step was introduced to avoid the difficulty of distinguishing CCC DNA derived from the plasmids conferring resistance to penicillin, to neomycin and kanamycin, and to tetracycline from the element responsible for chloramphenicol resistance in the *Klebsiella* strains. Lysates prepared from cultures of *P. aeruginosa* Ps 9169 and from *E. coli* strains K-12(RK1), K-12(RK2), and K-12(RK3) all contained between 3 and 5% of their total DNA in the CCC form (Fig. 1). This value agrees closely with the composition of similar extracts made from *P. aeruginosa* Ps 1822, the strain that carries RP1 (6). These satellite DNA preparations were further purified and characterized as detailed below.

Sucrose gradient sedimentation of the R factors. The S values of the satellite DNA prepared from Ps 9169 and from *E. coli* strains K-12(RK1), K-12(RK2), and K-12(RK3) were determined by sedimentation through sucrose gradients. All of the satellite DNA preparations (RP9, RK1, RK2, and RK3) separated into two components during this treatment (Fig. 2), as did standard preparation of the R factor RP1 prepared from Ps 1822 (6). The faster sedimenting peak in the preparation from strain Ps 9169 was about 65*S* compared with 62*S* for a similar fraction in preparations of RP1 and RK1 DNA. The 65 and 62*S* components could be converted to slower (approximately 44 and 43*S*) material by treatment with small quantities of deoxyribonuclease (data not shown). This observation supports the identification of the 65 and 62*S* material in these preparations as CCC DNA.

Buoyant density of the R factors. The

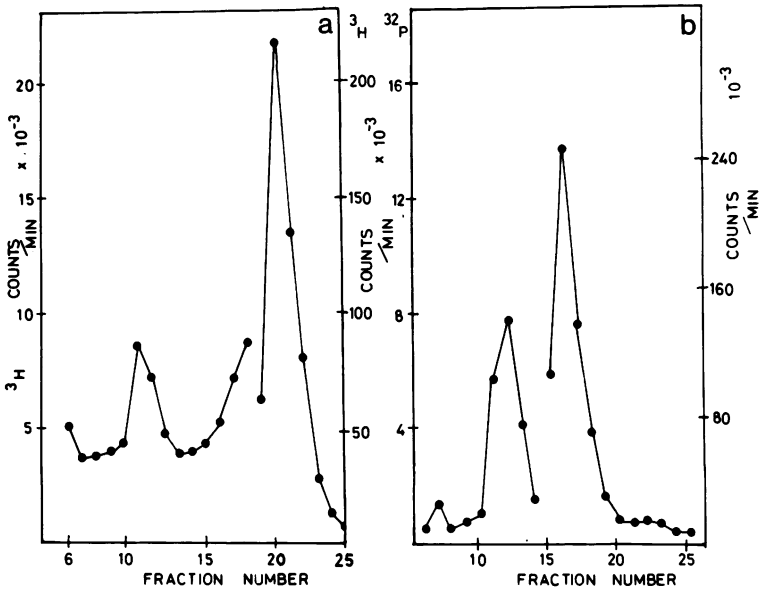

FIG. 1. *Isolation of covalently closed circular DNA. Radioactive lysates from cultures of (a) Escherichia coli K-12 (RK1) labeled with ^3H-thymidine and (b) Pseudomonas aeruginosa Ps 9169 labeled with ^{32}P were centrifuged to equilibrium in ethidium bromide CsCl gradients, fractionated, and assayed as described in the text. Note the 10-fold change in scale at fractions 20 and 25.*

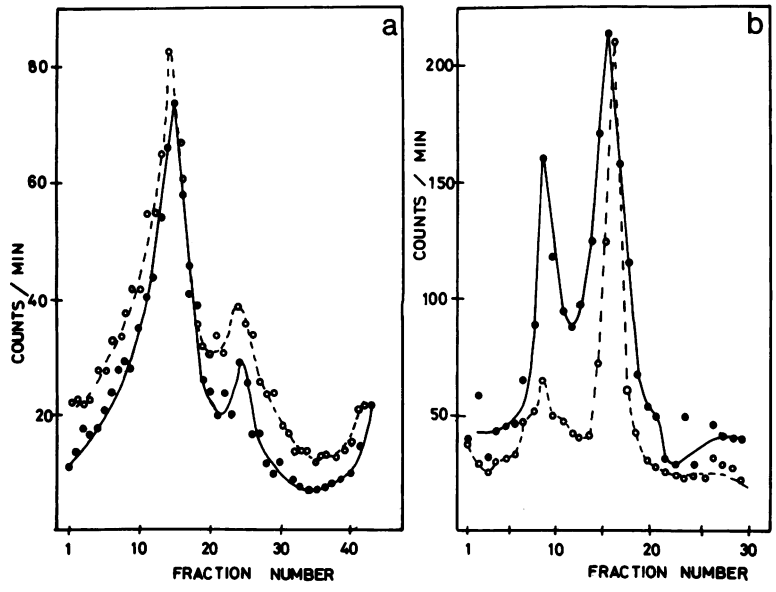

FIG. 2. *Sedimentation velocity of plasmid DNA. Purified, covalently closed, circular (CCC) DNA (containing some nicked circular DNA) was sedimented through sucrose gradients as described in the text. (a) ^3H RK1 CCC DNA with ^{14}C-RP1 DNA as marker; (b) ^{32}P RP9 CCC DNA with ^3H RP1 DNA. Symbols: ○, ^3H; ●, ^{14}C or ^{32}P.*

CCC DNA preparations from Ps 9169 and from *E. coli* strains K-12(RK1), K-12(RK2), and K-12(RK3) were analyzed on CsCl gradients in the presence of standard DNA preparations. Figure 3a shows that RK1 DNA had a density of 1.719 g/cc in CsCl, which is identical with RP1 DNA (6) and with RK2 and RK3 DNA (data not shown). Similarly, CCC DNA from

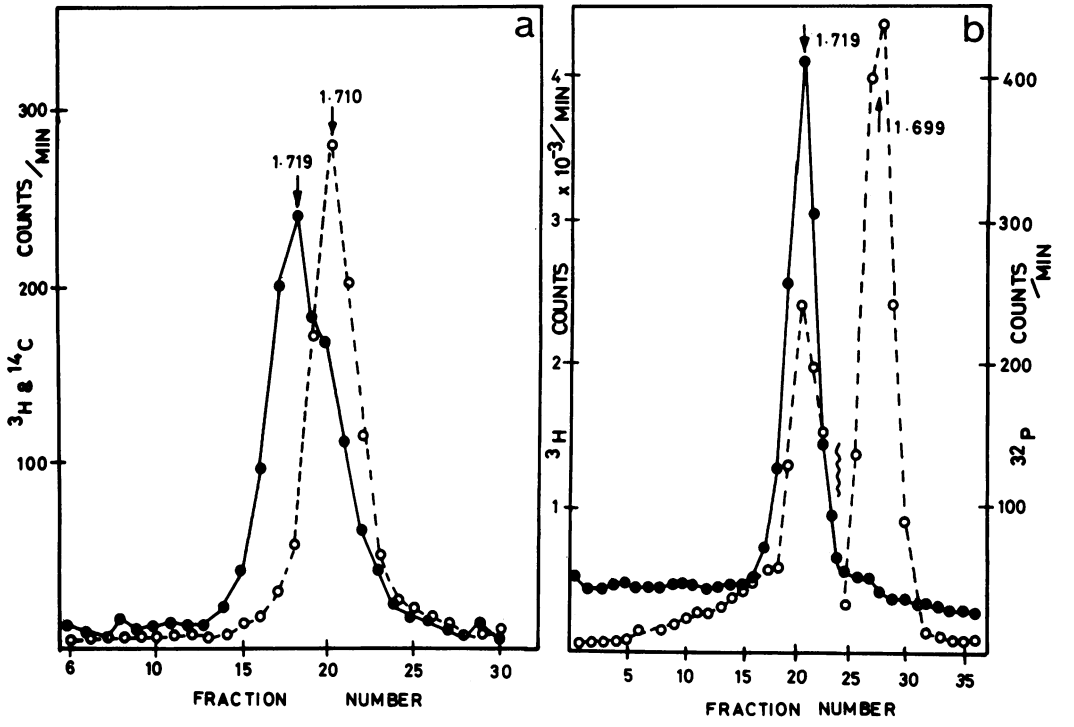

FIG. 3. *Buoyant density of plasmid DNA. Purified, covalently closed circular (CCC) DNA was mixed with CsCl and centrifuged to equilibrium as described in the text. (a) ^3H RK1 CCC DNA with ^{14}C Escherichia coli chromosomal DNA as marker; (b) ^{32}P RP9 CCC DNA with ^3H Proteus mirabilis Pm-1 (RP1) DNA as marker. Note that the latter gives peaks of RP1 DNA and of P. mirabilis chromosomal DNA; the ^{32}P counts in fractions 25–36 have been reduced by a factor of 10. Symbols: ●, CCC DNA; ○, marker DNA.*

Ps 9169 (RP9 DNA) gave a single peak with the same density, and Fig. 3b also shows that the CsCl gradient resolved this peak clearly from the chromosomal DNA of *Proteus mirabilis* (density, 1.699 g/cc).

DNA/DNA hybridization. CCC DNA molecules from bacteria containing the R factors RP9, RK1, RK2, and RK3 were tested for homology with RP1 DNA. Table 6 shows the extent to which each preparation would bind to excess RP1 DNA. The behavior of the plasmid RP4 (12)—a plasmid originally isolated in this laboratory (15) from *P. aeruginosa* strain S8 (2) and also specifying resistance to penicillin, neomycin, kanamycin, and tetracycline—is included for comparison. The plasmids RP9, RK1, RK2, and RK3 contained DNA which could bind to 80 or 90% of the DNA of RP1. This level of binding was, however, significantly less than the 100% binding obtained when ^{32}P-labeled RP1 DNA was tested against an excess of ^3H-DNA prepared from the same strain. RP4 DNA, on the other hand, only hybridized with 63% of RP1 DNA under the same conditions. The fact that RP9, RK1, RK2, and RK3 are approximately the same molecular size as RP1 (see Table 7) but contain

TABLE 6. *Similarity of plasmids RP9, RK1, RK3, and RP4 as measured by DNA/DNA hybridization*[a]

Plasmid	Hybridized with	Percent similarity[b]	Standard deviation	Experiments (no.)
RP1	RP1	100	7	5
RK1	RP1	83	5	4
RK2	RP1	90	6	3
RK3	RP1	81	6	3
RP4	RP1	63	8	7

[a] Hybridization was carried out as described in reference 7 by measuring the binding of isolated, covalently closed, circular DNA to filters carrying 0.6 μg of RP1 DNA. The efficiency of RP1 binding to RP1 filters was assayed in the same vial with the use of ^{14}C-labeled RP1.

[b] Percent similarity = (radioactivity bound to test filter − radioactivity bound to blank filter)/radio-activity added to test vial × 100/efficiency of homologous system.

TABLE 7. *Sedimentation coefficients (covalently closed circular form) and approximate molecular weights of R factors*[a]

Plasmid	$S_{20,w}$[b]	Approx molecular wt[c] (daltons)	Reference
RP1.....	62	40×10^6	6, 7
RP4.....	75	62×10^6	12
RP9.....	66	48×10^6	—
RK1.....	65	46×10^6	—
RK2.....	66	48×10^6	—
RK3.....	65	46×10^6	—

[a] The density of the purified plasmid DNA, determined by equilibrium centrifugation in CsCl solution as described in the text, was 1.719 for all plasmids. The percent guanine plus cytosine, calculated as described in reference 13, was 60 ± 1 (approximate standard deviation).

[b] The $S_{20,w}$ of the covalently closed circular form was measured by centrifugation through a sucrose gradient as described in the text.

[c] Calculated from the relationship: log (molecular weight) = [log ($S_{20,w}$ of the linear duplex) − log (0.0882)]/0.346 (14) with the assumptions that covalently closed circular DNA sediments 1.44 times as fast as nicked covalently closed circular DNA, which in turn sediments 1.14 times as fast as linear duplex DNA.

only 80% of their DNA sequences in common suggests that these plasmids cannot simply be a large fragment of RP1 but must also contain small amounts of DNA derived from elsewhere.

DISCUSSION

The second phase of R-factor-mediated carbenicillin resistance among strains of *P. aeruginosa* in the Medical Research Council's Industrial Injuries and Burns Unit was recognized initially by the isolation of strains Ps 9168 and Ps 9169 on 1 December 1969. These two isolates were obtained at the same time from the infected burns of a single patient (patient H) but came from different sites. These two strains were identical in terms of typing pattern and antibiotic resistance, and there is little doubt that they represent duplicate isolations of the "same" strain. These strains are now known to owe their carbenicillin resistance to type IIIa β-lactamase specified by the plasmid RP9. Patient H had carried a strain of *P. aeruginosa* in large quantities in his burns from 17 November, but this strain (Ps 8830 was the initial isolate) remained carbenicillin-susceptible and R^- up to 1 December. A strain of *K. aerogenes* was also present in the burns during this period, however, and this strain (K8834 was the initial isolate made on 17 November) expressed type IIIa β-lactamase and carried an R factor (RK1). A comparison of the properties of the plasmids RP9 and RK1 shows them to be extremely similar. Both specify resistance to tetracycline, to neomycin and kanamycin, and to carbenicillin; in both cases, resistance to the aminoglycoside antibiotics was due to a phosphorylating enzyme and carbenicillin resistance was caused by type IIIa β-lactamase; in both cases, the plasmid molecular weight was close to 50×10^6 and the guanine plus cytosine content was 60%. Hybridization between the two plasmids shows such a high interaction that their sequences must have been almost identical (Table 8). Plasmid RK1 can be transferred to *P. aeruginosa* in laboratory experiments (Table 4), and transfer between *Klebsiella aerogenes* and *Pseudomonas aeruginosa* has been shown to take place in burn fluid under artificial conditions (1). Under these circumstances, it seems extremely probable that the plasmid in *K. aerogenes* K8834 was the source—or at least extremely closely related phylogenetically to the source—of the plasmid that gave rise to the carbenicillin-resistant *P. aeruginosa* strains that were detected in the same patient 13 days later.

RP1 is typical of the R factors responsible for the first outbreak of carbenicillin-resistant pseudomonads in the Burns Unit, and consequently it was interesting to compare this plasmid with those isolated later, RP9 and RK1. All three share the markers *amp/carb-r.neo/kana-r.tet-r* and have an $S_{20,w}$ close to 65. However, hybridization studies show that only about

Table 8. *Homology between plasmids RP9 and RK1 as measured by DNA/DNA hybridization*[a]

Plasmid	Hybridized with	Percent similarity[b]
RK1	RP9	82
RP9	RK1	85

[a] The method described in Table 6 was modified so that the test filter carried 20 μg of DNA from strain K-12 (RP9) or K-12 (RK1) and the blank filter carried 20 μg of DNA from strain K-12 (R^-). The binding of RK1 DNA to K-12 (RP9) filters and of RP9 DNA to K-12 filters was corrected for the binding to the K-12 (R^-) filters (less than 10%) and compared with the efficiency of the homologous system—RP9 binding to K-12 (RP9) or RK1 binding to K-12 (RK1)—measured in the same vial by a double label technique.

[b] Results are based on three experiments. The standard deviation was 3.

80 to 90% of RP9 and RK1 DNA is represented in RP1 (Table 6), despite the very similar molecular weight of all of the plasmids. This argues that a limited amount of genetic recombination may have occurred between the two phases of resistance. Alternatively, the two phases may have been due to infection with two distinct plasmids which were nevertheless very similar.

In view of the similarity between the RP plasmids found in the two phases of resistance and the fact that a very similar plasmid, RK1, was found in a strain of *K. aerogenes* just before the onset of phase II, it was important to see whether any plasmids similar to RP1, RP9, and RK1 were present in the Burns Unit between the two phases of carbenicillin resistance. A survey of the marker patterns found among enteric bacteria during this period showed that the pattern *amp/carb-r.neo/kana-r.tet-r* was rare but that a relatively large number of strains of *K. aerogenes* had the pattern *amp/carb-r.neo/kana-r.tet-r.cam-r*—the pattern also found in strain K8834 on isolation from patient H (4, 11). The two *K. aerogenes* strains chosen for further study were strain K8841, a strain isolated on the same day as K8834 but from a different patient, and strain K7418, a strain isolated 2 months before the onset of the second phase. Both of these strains carried a plasmid specifying production of type IIIa β-lactamase and the aminoglycoside phosphorylating enzyme as well as resistance to tetracycline. These plasmids (RK2 and RK3) could be transferred to *P. aeruginosa* by conjugation. Furthermore, the molecular characteristics of these two elements were indistinguishable from RP9 and RK1.

It seems, therefore, highly probable that a plasmid very similar to RP1 and RP9 was present in the Burns Unit during the period between the two outbreaks of resistance. No carbenicillin was used in the Unit during this period, but ampicillin was employed extensively, and this antibiotic may well have served to maintain a plasmid that was capable of giving rise to carbenicillin-resistant pseudomonads by transfer at any time. Indeed, transfer may well have been occurring throughout the period, but the resistant pseudomonads were never selected since ampicillin is an ineffective antibiotic against *P. aeruginosa*.

One possible argument against this pattern of events is to claim that the only plasmids capable of giving rise to carbenicillin resistance in *P. aeruginosa* are similar to RP1 and RP9. Indeed this may not be so improbable, since plasmids that can bridge the gap between pseudomonads and the enteric bacteria form a minority among R factors (3). Examination of the plasmid (RP4) which was responsible for the outbreak of carbenicillin-resistant strains of *P. aeruginosa* in Glasgow (2) goes some way to refute this possibility. RP4 has a molecular weight of about 62×10^6 and a guanine plus cytosine content of about 61%, but shows only 63% homology when hybridized with excess RP1 DNA (12).

These molecular studies cannot prove that transfer from *K. aerogenes* was the source of the R factors that caused carbenicillin destruction in the resistant *Pseudomonas* strains. The similarity of the plasmids found in the various strains does, however, suggest strongly that transfer by this route is likely to have occurred in patient H. If this is indeed so, the sequence of events illustrates some of the pitfalls surrounding antibiotic therapy when R factors are involved. In this case, the existence of a plasmid that can confer resistance to both ampicillin and carbenicillin, and which can also readily transfer from enteric bacteria to strains of *P. aeruginosa*, means that the use of both antibiotics must be discontinued to remove selection pressure on that plasmid in a clinical unit. Withdrawal of carbenicillin alone does little to lower the pressure for survival of the R factor in enteric bacteria, and consequently these R-factor-carrying organisms survive to be a potential source of carbenicillin resistance in *P. aeruginosa*, by gene transfer, should carbenicillin be used again therapeutically.

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