A Carbenicillin R Factor from *Pseudomonas aeruginosa*

A. J. VAN RENSBURG, M. J. DE KOCK

SUMMARY

Of 64 carbenicillin-resistant *Pseudomonas aeruginosa* strains 40 transferred this resistance to *Escherichia coli*. R factor RP-638 isolated from *Ps. aeruginosa* strain 638 conferred resistance to ampicillin, carbenicillin, kanamycin, neomycin and tetracycline. This R factor was transferred at frequencies of 10^{-7} to 10^{-4} between various strains of *Pseudomonas*, *Proteus* and *Alcaligenes*, and coexisted stably with both fi^+ and fi^- R factors. Electron microscopy showed the presence of circular DNA molecules with an average contour length of 19,3 ±0,6 μm.

S. Afr. Med. J., **48**, 1187 (1974).

R factors have been isolated from carbenicillin-resistant strains of *Pseudomonas aeruginosa*[1-6] and the genetic and physical properties of two of these R factors have been reported.[3-5]

This article describes some of the transfer and physical properties of an R factor isolated from a resistant *Ps. aeruginosa* strain.

METHODS

Bacterial Strains

Resistant strains were obtained from routine isolates of *Ps. aeruginosa* from patients in the intensive care units at the National and Pelonomi Hospitals. *Proteus mirabilis* strain 13,[6] *P. vulgaris* strain 69,[7] *P. morganii* NCTC 2815, Providence NCTC 9211, *Alcaligenes faecalis* ATCC 9220 and *Escherichia coli* K12 strain J53 were used for studies on the transfer properties of the R factor. Reference R factors were obtained from Dr Naomi Datta.

Media

MacConkey agar was Oxoid CM76 and nutrient broth was Oxoid No. 2 (CM67). Welco sensitivity test agar (CM48) was used to determine minimum inhibitory concentrations of antibiotics.

Antibiotics

Antibiotics were used as freshly prepared solutions added to media at the following final concentrations: carbenicillin 1 000 μg/ml, ampicillin 50 μg/ml, streptomycin 15 μg/ml, chloramphenicol 30 μg/ml, kanamycin 25 μg/ml, tetracycline 15 μg/ml and sulphonamide 100 μg/ml. Mast sensitivity discs were used to determine resistance patterns and were confirmed by plating on media containing drugs at these concentrations.

Department of Microbiology, University of the Orange Free State, Bloemfontein, OFS
A. J. VAN RENSBURG
M. J. DE KOCK

Date received: 7 January 1974.

Determination of Minimum Inhibitory Concentrations

Overnight cultures in nutrient broth were diluted to about 10^3 bacteria/ml with nutrient broth. Small drops (0,1 ml) were spotted on Welco sensitivity test agar plates which contained doubling dilution of the appropriate antibiotic. The minimum inhibitory concentration was taken as the lowest concentration of the antibiotic that prevented the formation of colonies.

Genetic Transfer

Transfer of R factors was done as described by Datta *et al.*[4] Donors were late log-phase cultures in nutrient broth No. 2, incubated without shaking. Recipients were late log-phase, shaken-broth cultures in the same nutrient broth. One part of donor was mixed with 4,5 parts of recipient and 4,5 parts of fresh broth. Mixtures were incubated for 1 hour at 37°C, and 0,1 ml volumes of appropriate dilutions were then plated on selective media. Frequency of transfer was calculated relative to the number of donors.

In transfer of R factors from *Pseudomonas aeruginosa* to *Escherichia coli* and *Proteus spp.* the mixture were incubated anaerobically after plating on selective media. When *Alcaligenes faecalis* was used as recipient it was shaken for 6 hours and mating was prolonged to 90 min.

Analytical CsCl Gradient Centrifugation

DNA was prepared and examined in caesium chloride density gradients by a previously described method.[8,9] The chromosomal DNA of the strain being examined was used as density marker.

Labelling of Cultures

Tris-buffered minimal medium[10] was used and labelling performed as described by Bazaral and Helinski.[11] Deoxyadenosine (250 μg/ml) was added to media when ^{3}H-

thymidine was used for labelling. Bacteria were grown at 37°C with vigorous aeration and 0,1 ml of ^{3}H-thymidine (1 mCi/ml, 6,7 Ci/m-mole) or 0,2 ml. ^{14}C-thymidine (0,1 mCi/ml, 30 mCi/m-mole) was added to 15 ml of the culture before the last two generations before harvesting. ^{32}P-labelling was accomplished by adding carrier-free inorganic ^{32}P (20 μCi/ml) to the culture at least three generations before harvesting.

Ethidium Bromide - CsCl Gradient Centrifugation

Labelled cultures were harvested at 4°C and washed twice with TES buffer (0,05M-NaCl + 0,05M-tris + 0,005M-EDTA, pH 8,0) at 0°C. The final pellet was resuspended in TES buffer containing lysozyme (1,0 mg/ml) and ribonuclease A (500 μg/ml). After incubation at 37°C for 15 min sodium dodecyl sarcosinate was added to a final concentration of 0,8% (w/v) for *E. coli* and 1,6% (w/v) for *P. mirabilis*. Shearing of the resulting lysate was performed by passing it 4 or 5 times through a 21-gauge needle at about 0,2 ml/ sec.

The sheared lysate (2,0 ml) was diluted with 7,6 ml water and then added to a vial containing 13,1 g CsCl and 4,0 ml ethidium bromide solution (700 μg/ml in 0,1M phosphate buffer, pH 7,0). The mixtures were pipetted into centrifuge tubes and overlaid with light mineral oil. Samples were centrifuged for 42 hours at 44 000 rev/min and 20°C in a Ti-60 fixed-angle centrifuge rotor, using a Beckman Spinco model L4. The bottoms of the tubes were pierced with a hollow needle and the gradients were fractionated by collecting 9-drop fractions.

Counting of radio-isotopes was done in a Packard Tri-Carb scintillation spectrometer.[11] Purified plasmid DNA was used for electron microscopic studies.

Electron Microscopy of R Factor DNA

Electron microscopy was done as previously described.[9] Grids were shadowed with 40% palladium-gold and examined in a Phillips EM-200 electron microscope. Electron micrographs were enlarged and contour lengths of the DNA molecules measured. Molecular weights were calculated from the contour lengths.

RESULTS

Forty (62,5%) of the 64 resistant strains examined were able to transfer this resistance to *Escherichia coli* or *Proteus mirabilis* recipients. These R factors conferred resistance to carbenicillin at levels of 2 500 μg/ml or more.

Transfer Characteristics of RP-638

R factor RP-638, which determines resistance to ampicillin, kanamycin, neomycin and the tetracyclines, showed stable existence in all species and was elected for further studies. It could be transferred between various strains of *Ps. aeruginosa, E. coli, Proteus spp.* and *Alcaligenes faecalis* at frequencies of 10^{-7} to 10^{-4} (Table I).

TABLE I. TRANSFER CHARACTERISTICS OF RP-638

Donor	Recipient	Frequency of transfer*
Pseudomonas aeruginosa **638**	*E. coli* **J53**	$1,7 \times 10^{-4}$
	P. mirabilis **13**	$1,3 \times 10^{-4}$
E. coli **strain J53 (RP-638)**	*P. mirabilis* **13**	$3,4 \times 10^{-4}$
	P. vulgaris **69**	$2,7 \times 10^{-4}$
	P. vulgaris **45**	$3,8 \times 10^{-6}$
	P. morganii **NCTC 2815**	$1,9 \times 10^{-4}$
	Providence NCTC 9211	$2,2 \times 10^{-4}$
	A. faecalis **ATCC 9220**	$2,9 \times 10^{-4}$
Proteus mirabilis **13 (RP-638)**	*E. coli* **J53**	$1,9 \times 10^{-4}$
	P. vulgaris **69**	$1,8 \times 10^{-4}$
	P. morganii **NCTC 2815**	$1,8 \times 10^{-4}$
	Providence NCTC 9211	$1,6 \times 10^{-4}$
	A. faecalis **ATCC 9220**	$1,3 \times 10^{-7}$

*Frequency of transfer was calculated relative to the number of donors.

Resistance Characteristics Conferred by RP-638

In all recipients the acquisition of RP-638 resulted in higher levels of resistance to the antibiotics tested (Table II). In *E. coli* J53 this increase was spectacular, but less impressive in *P. mirabilis* and *A. faecalis*. R factor-containing strains showed differences in relative resistance to carbenicillin and ampicillin. *Ps. aeruginosa* 638 was equally resistant to both antibiotics, but in *E. coli* J53 resistance to carbenicillin was about ten times higher than to ampicillin.

Superinfection Immunity

RP-638 could be transferred to R factor-containing strains of both *E. coli* J53 and *P. mirabilis* 13 (Table III).

When the fi^{+} R factor R1 or fi^{-} R factor R64 was present in the *E. coli* recipient, an increase in transfer frequencies was observed. R64 could not be transferred to *P. mirabilis* 13, but when this strain contained the fi^{+} R factor R1 the frequency of transfer remained unchanged.

Analytical CsCl Gradient Centrifugation

Equilibrium centrifugation profiles of DNA isolated from *E. coli* J53 and *P. mirabilis* 13 with and without R factor RP-638 are shown in Figs 1 and 2. These microdensitometer tracings show the presence of a satellite peak of DNA in the R factor-containing strains. In Fig. 1 *E. coli* chromosomal DNA banded at 1,710 g/cm^{3} while the plasmid DNA formed a satellite peak at a density of 1,720

TABLE II. ANTIBIOTIC RESISTANCE IN VARIOUS STRAINS WITH AND WITHOUT R FACTOR RP-638: MINIMUM INHIBITORY CONCENTRATIONS (μg/ml)

Strain	Carbenicillin	Ampicillin	Tetracycline	Neomycin	Kanamycin
E. coli **J53**	2	2	2	2	2
E. coli **J53 (RP-638)**	20 000	2 500	128	1 024	1 024
P. mirabilis **13**	32	16	32	32	32
P. mirabilis **13 (RP-638)**	2 048	1 024	256	512	512
P. vulgaris **45**	32	16	64	32	32
P. vulgaris **45 (RP-638)**	2 048	1 024	512	512	512
A. faecalis **ATCC 9220**	32	16	8	32	32
A. faecalis **ATCC 9220 (RP-638)**	2 048	1 024	256	512	512
Ps. aeruginosa **(RP-638)**	64	64	4	32	64
Ps. aeruginosa **638 (RP-638)**	2 500	2 500	256	512	1 024
E. coli **J53 (R64)**	16	16	256	4	4
E. coli **J53 (R64, RP-638)**	20 000	2 048	512	1 024	1 024
E. coli **J53 (R1)**	2 048	2 048	8	512	512
E. coli **J53 (R1, RP-638)**	20 000	5 000	512	2 048	2 048
E. coli **J53 (R192)**	32	16	256	4	4
E. coli **J53 (R192, RP-638)**	2 500	1 024	512	2 048	2 048
E. coli **J53 (R348)**	32	16	256	512	512
E. coli **J53 (R348, RP-638)**	15 000	2 048	1 048	2 048	2 048

TABLE III. COMPATIBILITY OF RP-638

Donor	Recipient	Frequency of transfer
E. coli **J53 (RP-638)**	*E. coli* **J53**	$1{,}7 \times 10^{-4}$
	E. coli **J53 (R1)**	$1{,}3 \times 10^{-2}$
	E. coli **J53 (R64)**	$1{,}7 \times 10^{-1}$
	E. coli **J53 (R192)**	$1{,}1 \times 10^{-5}$
	E. coli **J53 (R348)**	$6{,}0 \times 10^{-3}$
	P. mirabilis **13**	$3{,}4 \times 10^{-4}$
	P. mirabilis **13 (R1)**	$1{,}8 \times 10^{-4}$

g/cm³. This satellite peak was absent in the normal wild strain.

Fig. 2 represents microdensitometer tracings of DNA from strains of *P. mirabilis*. DNA from the sensitive strain banded in a single peak at a density of 1,700 g/cm³. In the R factor-containing strain a peak of satellite DNA with a buoyant density of 1,720 g/cm³ appeared. The satellite DNA accounted for about 4% of the total amount of DNA in the *E. coli* (R^+) strain. In the $R^\pm$ *P. mirabilis* strain this value was about 10%.

Fig. 3 shows a microdensitometer tracing after CsCl gradient centrifugation of DNA from *Ps. aeruginosa* strain 638. In both the sensitive and the R factor-containing strain only a single peak of DNA with a density of 1,721 g/cm³ was observed.

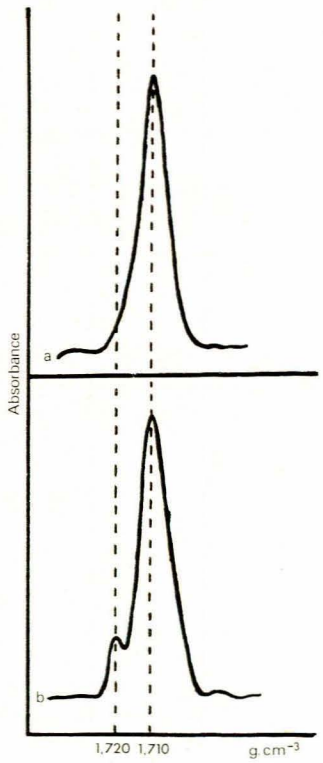

Fig. 1. Density profiles of DNA isolated from *Escherichia coli* strain J53. Trace a — DNA from *E. coli* J53 (R^-); trace b — DNA from *E. coli* J53 (R-638).

Preparative Ethidium Bromide-CsCl Gradient Centrifugation

Covalently closed circular DNA can be separated from other conformations of DNA on ethidium bromide-CsCl gradients.[12] Cultures of *E. coli* strains J53 and J53 (RP-638), *P. mirabilis* strains 13 and 13 (RP-638) and *Ps. aeruginosa* strains $638R^-$ and $638R^+$ were examined by this method. Lysates from strains J53, 13 and 638 showed only one peak of radioactive material. RP-638-containing derivatives, however, showed an additional denser satellite band. The ratio of radioactivity in the satellite band was about 2% in *E. coli* J53, 4% in *P. mirabilis* 13 and 1% in *Ps. aeruginosa* 638.

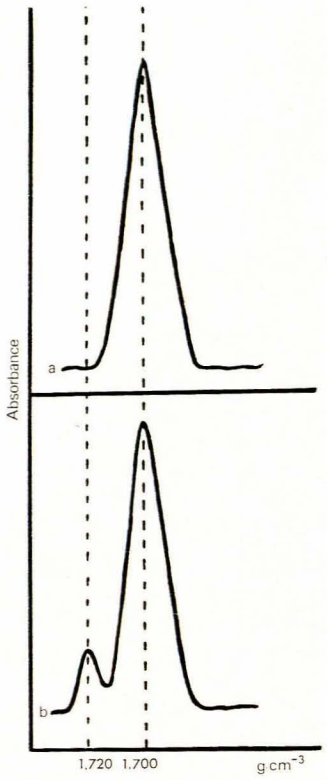

Fig. 2. Density profiles of DNA isolated from *Proteus mirabilis* strain 13. Trace a — DNA from *P. mirabilis* 13 13 (R^-); trace b — DNA from *P. mirabilis* 13 (R-638).

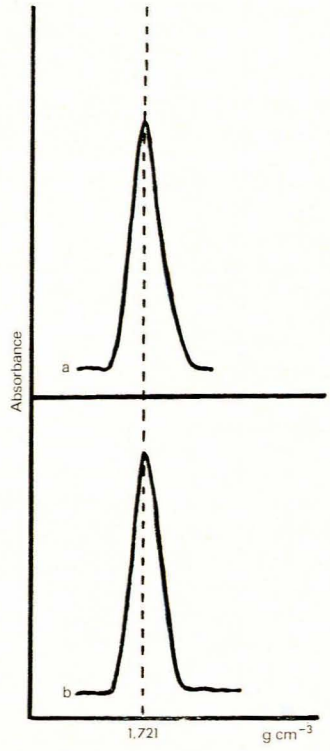

Fig. 3. Density profiles of DNA isolated from *Pseudomonas aeruginosa* strain 638. Trace a — DNA from *Ps. aeruginosa* 638 (R^-); trace b — DNA from *Ps. aeruginosa* 638 (R-638).

Electron Microscopy

After fractionation the samples containing the satellite DNA were pooled and examined by electron microscopy. In all 3 strains examined this revealed the presence of covalently closed as well as relaxed circular molecules. Average contour lengths were obtained from measuring 42 of the relaxed molecules. This yielded an average value of 19,3 ±0,6 μm. Assuming a linear density of 207 daltons/Å this corresponds to a molecular weight of about 40 × 10^6 daltons.[18]

DISCUSSION

In *Escherichia coli* and *Proteus mirabilis* acquisition of R factor RP-638 was accompanied by the appearance of a band of satellite DNA with a buoyant density of 1,720 g/cm³. In *Pseudomonas aeruginosa* the absence of this satellite DNA may be ascribed to limitations in the technique used.

Electron microscopy showed that this satellite DNA consisted of covalently closed and relaxed circular molecules. From the contour lengths of these molecules a molecular weight of about 40 × 10^6 daltons was calculated. This agrees closely with similar data for another *Pseudomonas* R factor.[5]

R factor RP-638 was transferred to all strains tested and showed stable coexistence with known fi^+ and fi^- R factors. This suggests that RP-638 may belong to the P compatibility group of R factors[4] but further proof is required. It is of interest that 10 other *Pseudomonas* R factors examined in our laboratory produced similar results. These R factors not only determined resistance to the same group of antibiotics but were also alike in physical and chemical properties. However, since completion of this report a number of R factors determining resistance to gentamicin were isolated and are at present being investigated.

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

REFERENCES

1. Lowbury, E. J. L., Kidson, A., Lilly, H. A., Ayliffe, G. A. J. and Jones, R. J. (1969): Lancet, **2**, 448.
2. Fullbrook, P. D., Elson, S. W. and Slocombe, B. (1970): Nature (Lond.), **226**, 1054.
3. Sykes, R. B. and Richmond, M. H. (1970): *Ibid.*, **226**, 952.
4. Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B. and Richmond, M. H. (1971): J. Bacteriol., **108**, 1244.
5. Grinsted, J., Saunders, J. R., Ingram, L. C., Sykes, R. B. and Richmond, M. H. (1972): *Ibid.*, **110**, 529.
6. Coetzee, J. N. and Sacks, T. G. (1960): J. Gen. Microbiol., **23**, 445.
7. Coetzee, J. N. (1963): *Ibid.*, **31**, 219.
8. Van Rensburg, A. J. (1972): S. Afr. Med. J., **46**, 200.
9. Van Rensburg, A. J. and Hugo, N. (1969): J. Gen. Microbiol., **58**, 421.
10. Clewell, D. B. and Helinski, D. R. (1969): Proc. Nat. Acad. Sci., (Wash.), **62**, 1159.
11. Bazaral, M. and Helinski, D. R. (1968): J. Mol. Biol., **36**, 185.
12. Radloff, R., Bauer, W. and Vinograd, J. (1967): Proc. Nat. Acad. Sci. (Wash.), **57**, 1515.
13. Kontomichalou, P., Mitani, M. and Clowes, R. C. (1970): J. Bacteriol., **104**, 34.