R-factor Mediated Resistance to Penicillins which does not Involve a β -Lactamase

By N. A. C. CURTIS, M. H. RICHMOND AND V. STANISICH*

Department of Bacteriology, University of Bristol, University Walk, Bristol BS8 1TH

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The R-factor RP 1 was originally detected in *Pseudomonas aeruginosa* strain 1822 (Lowbury, Kidson, Lilly, Ayliffe & Jones, 1969; Sykes & Richmond, 1970; Fullbrook, Elson & Slocombe, 1970), where it confers resistance to tetracycline, neomycin, kanamycin and a wide range of β -lactam antibiotics including carbenicillin (Grinsted, Saunders, Ingram, Sykes & Richmond, 1972). This plasmid is a member of the P-class of R-factors and specifies its own transfer between *P. aeruginosa* and a number of species of enteric bacteria (Datta, Hedges, Shaw, Sykes & Richmond, 1972). When in enteric species, it expresses the same marker pattern as found in *P. aeruginosa* (Table 1).

Much of the interest in this plasmid has centred on its protection of clinical strains of *Pseudomonas aeruginosa* against carbenicillin therapy (Lowbury *et al.* 1969; Ingram, Richmond & Sykes, 1973), a property ascribed to the production of the type IIIa β -lactamase (Richmond & Sykes, 1973) specified by the R-factor. However, it has always seemed slightly surprising that an enzyme that hydrolyses carbenicillin relatively poorly (Sykes & Richmond, 1971) should nevertheless produce at least a 1000-fold increase in resistance to the bacteria expressing it (Table 1). The isolation of a mutant of RP 1 which no longer codes for β -lactamase synthesis but which nevertheless still confers penicillin resistance to the bacteria that carry it, argues that RP 1 specifies intrinsic resistance to penicillins as well as β -lactamase production and that these two characters co-operate to produce the carbenicillin resistant phenotypes found in *P. aeruginosa*.

Pseudomonas aeruginosa 1822 (RP 1) was grown exponentially in nutrient broth and the bacteria collected by centrifuging when the culture density had reached about 10^8 organisms/ml. The organisms were resuspended in 0.1 M citrate buffer, pH 5.0, containing 100 μ g *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine/ml and maintained at 37 °C without shaking. After 15 min the culture was centrifuged and the bacteria resuspended in fresh nutrient broth to a density of about 10^8 organisms/ml. This culture was immediately mated with *P. aeruginosa* ps18sade Δ str-r using equal volumes of donor and recipient bacteria each at a density of about 10^8 bacteria/ml. The mating was continued for 15 min at 37 °C and the mixture was then treated briefly with a vortex mixer to separate mating pairs. Samples were plated at appropriate dilutions on agar containing 250 μ g streptomycin and 200 μ g tetracycline/ml. Colonies obtained after overnight growth were spotted with tooth-picks on to nutrient agar containing one of the following antibiotics: carbenicillin (250 μ g/ml), streptomycin (250 μ g/ml) or tetracycline (200 μ g/ml). One isolate resistant to tetracycline and streptomycin but relatively sensitive to carbenicillin was obtained from the 1200 colonies tested.

The isolated mutant colony was compared with the parental strain, and with its plasmidless variant, for production of β -lactamase both iodometrically (Novick, 1962) and spectrophotometrically with the chromogenic cephalosporin substrate (O'Callaghan, Morris,

* Present address: Department of Genetics, Monash University, Clayton, Australia.

Table 1. Effect of RP 1 and of the mutated RP 1 plasmid on the resistance of *Pseudomonas aeruginosa* and *Escherichia coli* to a range of antibiotics

Strain	Plasmid	MIC ($\mu\text{g/ml}$)							
		Neo	Kana	Tet	PenG	Amp	Carb	CER	6APA
<i>Pseudomonas aeruginosa</i> 1822	—	8	(NT)	16	625	160	80	1 250	1 250
	RP 1	1 000	(NT)	500	5 000	5 000	5 000	5 000	2 500
	RP 1 mutant	1 000	(NT)	500	1 250	625	160	2 500	2 500
	— (cured)	4	(NT)	8	625	160	40	1 250	1 250
<i>Escherichia coli</i> W3110	—	2	4	2	20	4	4	2	80
	RP 1	63	250	125	10 000	10 000	10 000	8	2 500
	RP 1 mutant	63	250	125	625	625	2 500	8	625
	— cured	4	2	2	20	5	5	5	80

Neo, neomycin; Kana, kanamycin; Tet, tetracycline; PenG, benzyl penicillin; Amp, ampicillin; Carb, carbenicillin; CER, cephaloridine; 6APA, 6-amino-penicillanic acid; (NT), not tested.

Kirby & Shingler, 1972). While the parent synthesized about 120 enzyme units/mg dry wt bacteria with benzyl penicillin as substrate, the mutant and the R⁻ variant produced less than 0.1 unit/mg dry wt. Furthermore, tests with specific anti-type IIIa serum (Datta & Richmond, 1966) showed no enzyme production either by the mutant or the R⁻ variant while the parent expressed relatively large amounts of type IIIa enzyme.

Pseudomonas aeruginosa strain Ps18s carrying the mutant plasmid was mated with *Escherichia coli* K12 (rif-r) and the cross selected on plates containing 100 μg tetracycline and 100 μg rifampicin/ml. Colonies of *E. coli* carrying the mutated plasmid obtained in this way were then tested for the type and amount of β -lactamase synthesis, *E. coli* RP 1 and *E. coli* R⁻ being included for comparison. As with the equivalent *P. aeruginosa* strains, *E. coli* K12 R⁻ and *E. coli* K12 carrying the mutated version of RP 1 synthesized less than 0.1 enzyme unit/mg dry wt bacteria when measured with benzyl penicillin as substrate, while *E. coli* K12 RP 1 expressed about 450 units/mg dry wt bacteria.

Attempts to revert the strains carrying the mutant plasmid (whether carried in *Escherichia coli* or *Pseudomonas aeruginosa*) by further nitrosoguanidine treatment failed, nor have any β -lactamase producing revertants ever arisen from this mutant version of RP 1. The original mutagenesis has therefore probably deleted the β -lactamase gene but left the genes specifying resistance to neomycin, kanamycin and tetracycline intact.

Examination of the resistance pattern of these strains shows that in both *Pseudomonas aeruginosa* and *Escherichia coli* the loss of the plasmid-mediated β -lactamase gene did not significantly alter the mean inhibitory concentration (MIC) values obtained with neomycin, kanamycin and tetracycline. MIC values against a range of penicillins, however, were decreased, but not to the levels characteristic of R⁻ cultures. Strains of *E. coli* carrying the mutant plasmid were still at least 30-fold more resistant than the plasmid-less controls, although this difference was not so great in *P. aeruginosa* (Table 1). The plasmid RP 1 confers little resistance to cephaloridine and the MIC values obtained against this antibiotic were unchanged when the mutant plasmid was present. It follows that the β -lactamase produced by strains carrying RP 1 cannot be the only factor influencing their penicillin resistance. Furthermore, no destruction of either benzylpenicillin or cephaloridine could be detected even after prolonged incubation with broken cell preparations (Jack & Richmond, 1970) of either *P. aeruginosa* or *E. coli* carrying the mutated R-factor. The partial resistance of these strains could not therefore be due to a penicillin or cephalosporin amidase.

To confirm that the relatively high level of residual resistance to penicillins was due to an

R-factor mediated character and not to some cell-linked gene, both the *Escherichia coli* and the *Pseudomonas aeruginosa* strains carrying the mutant plasmid were treated with sodium dodecyl sulphate by the method of Tomoeda, Inuzuka, Kubo & Nakamura (1968) and tetracycline-sensitive variants isolated. All these variants were also sensitive to neomycin and kanamycin, and moreover their resistance to penicillins had fallen to values characteristic of the R⁻ variant of the strains in question (Table 1). Reinsertion of RP 1 or mutant RP 1 into R⁻ recipients re-established the resistant phenotypes at the expected levels.

Plasmid-free strains of *Pseudomonas aeruginosa* and *Escherichia coli* K12 produce very small amounts of β -lactamase when free of R-factors (Lindström, Boman & Steele, 1970; Sykes & Richmond, 1971), the enzymes being of types Id and Ia respectively (Richmond & Sykes, 1973). The high penicillin resistance of the strains carrying the mutated R-factor might therefore be due to an alteration in the amount of these enzymes expressed by the strains. All type I enzymes are predominantly active against cephalosporins and inactive against carbenicillin, which inhibits them (Richmond & Sykes, 1973). Examination of the residual enzyme activity in both the *E. coli* and the *P. aeruginosa* strains carrying the mutant R-factor showed that the low level of β -lactamase present (always less than 0.1 unit/mg dry wt bacteria) was predominantly active against cephalosporins and inhibited by carbenicillin, and therefore could not itself be contributing to the carbenicillin resistance of the strains lacking type IIIa enzyme.

Although these preliminary studies show that RP 1 seems to carry a locus that specifies penicillin resistance by a mechanism other than enzyme destruction, the biochemical basis of the resistance is unknown. R-factors certainly can modify the permeability properties of membranes (Franklin, 1967) and perhaps this process is involved here. Nor is it yet clear how widespread this R-factor mediated intrinsic resistance may be. The answer to that point must await the isolation of appropriate mutants from a range of other R-factors.

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