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Effect of R-Factor-Mediated Genes on Some Surface Properties of *Escherichia coli*

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The plasmid RP1 was shown to contain a genetic region (the *irp* region) responsible for influencing the intrinsic resistance of *Escherichia coli* to penicillins but not to cephalosporins. Mutants in which the *irp* genes are inactive were isolated. RP1 carrying functional *irp* genes protected *E. coli* AS19 against lysozyme lysis and also enhanced resistance to actinomycin D, to nalidixic acid, and to rifampin. This plasmid also phenotypically repaired the hypersensitivity to penicillins of strain AS19, and also that of *E. coli envA* mutants. Similar regions were not detected on the plasmids R1 and R55.

R-factor-mediated resistance to tetracycline has been shown to involve an altered bacterial permeability to the antibiotic (2, 3) and a similar mechanism has been proposed for sulfonamide resistance in gram-negative organisms (8). Up to this time, however, all R-factor-mediated resistance to penicillins and cephalosporins has been thought to involve β-lactamase production (12) with a consequent destruction of the antibiotics.

The discovery of a group of R-factors which conferred high levels of carbenicillin resistance on strains of *Pseudomonas aeruginosa* (6, 14) gave a hint that certain R-factors might specify enhanced intrinsic resistance to β-lactam antibiotics since it was difficult to reconcile the very high resistance (6) with the relatively poor rate of carbenicillin hydrolysis afforded by these plasmids (12). The presence of genes on these plasmids which affected the intrinsic resistance of bacteria was confirmed by the isolation of mutant R-factors in which the β-lactamase gene had been deleted (1). This paper extends preliminary studies and describes some of the changes in surface properties that R-factors induce in bacteria carrying them.

METHODS AND MATERIALS

Organisms and media. The strains used in these studies together with their source and derivation are shown in Table 1. They include two mutants of *Escherichia coli* with unusual properties. The first is the lysozyme-susceptible mutant AS19, a derivative of *E. coli* B, which is also inordinately susceptible to rifampin, nalidixic acid, and actinomycin D (13), as well as to many penicillins and cephalosporins. The second is the *envA* mutant originally isolated by Normark and his colleagues from *E. coli* K-12 as being relatively susceptible to ampicillin (9).

All experiments were carried out in nutrient broth with antibiotic additions as necessary.

R-factors and their mutants. The plasmids used in this study, together with their source and derivation, are shown in Table 1. The plasmid RP1 was originally detected in *P. aeruginosa* 1822 (6) and its properties have been studied in detail (5). It specifies the marker pattern ApNe/KmTc. The mutant RP1*amp1* was isolated after treating *E. coli* NC21 carrying RP1 with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine as described previously (1). No revertants that specify β-lactamase production have yet been obtained from this mutant plasmid.

A further mutant of the plasmid RP1*amp1* was made by treatment with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine under conditions identical to those used to isolate RP1*amp1* itself. Treated bacteria were plated on nutrient agar, and the resulting colonies were replicated on to further nutrient agar plates and on to plates containing carbenicillin, although in this case the antibiotic concentration in the agar was 500 μg/ml (1). Those colonies which grew on unsupplemented agar, but would not grow when ampicillin was present, were tested to see whether or not they contained a plasmid that no longer specified resistance to penicillins. One such mutant was obtained by this method and is designated RP1 (*amp1irp1*).

The plasmid R1*drd*19 was obtained from Naomi Datta (Bacteriology Department, Royal Postgraduate Medical School, Ducane Road, Hammersmith, London, England). It specifies the marker pattern ApCmKmSmSu (7). A mutant plasmid which no longer specified β-lactamase synthesis was isolated from R1*drd*19 after treatment of *E. coli* NC21 carrying this plasmid with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. In this case, however, the mutant was not detected by replication on to plates containing carbenicillin (as was the case with RP1*amp1* (1) but by spraying mutated colonies with the chromogenic cephalosporin 87/312 (11). Colonies that synthesised no β-lactamase were then picked. One of these was found to specify no β-lactamase (limits of detection:

less than 0.03 U of β-lactamase per mg [dry weight] when measured with either ampicillin or cephaloridine as substrate). It has been designated R1*drd*19*amp*2. It was found to revert spontaneously to a β-lactamase-producing phenotype at a frequency of about 10^{-9}. R55 was obtained from D. Bouanchaud (Service de Bacteriologie Medicale, Institut Pasteur, Paris). Its marker pattern is Ap.Cm.Su.GenK (16). The non-β-lactamase mutant of R55 (R55*amp*4) was produced in the same way as R1*drd*19*amp*2.

Non-plasmid bacteria were obtained by growth in sodium dodecyl sulphate as described by Tomoeda et al. (15).

Lysozyme susceptibility. Lysozyme susceptibility was measured by following the change in optical density of a bacterial suspension after the addition of lysozyme. For this purpose the bacteria were collected from their culture medium by centrifugation and resuspended in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, to give an optical density value of 1.3 at 450 nm. This bacterial suspension was then placed in a Unicam SP800 spectrophotometer (Pye Unicam Instruments, Cambridge, England) and the temperature of the cuvette was allowed to reach 25 C. The reaction was started by adding lysozyme (final concentration: 10 μg/ml), and the rate of lysis was followed by measuring the optical density at intervals at 600 nm. A sample incubated without added lysozyme acted as control.

Antibiotic susceptibilities. Bacterial antibiotic susceptibilities were measured by plating single-colony-forming units on nutrient agar containing antibiotics incorporated at appropriate concentrations.

Chromogenic cephalosporin. The chromogenic cephalosporin 87/312 was the gift of Glaxo Research Ltd. (11).

TABLE 1. *Bacterial strains and plasmids used in this work and their source*[a]

Plasmid and strain	Reference	Source
E. coli strain no.		
NC21		Laboratory stock strain (U.B.)
D21	(8)	H. Boman (U.U)
D21 *envA*	(8)	S. Normark (U.U.)
B		Laboratory stock strain (U.B.)
AS19	(13)	P. M. Bennett (U.B.)
Plasmid no.		
RP1	(14)	This laboratory
R1 *drd* 19	(16)	D. Datta (RPGMS)
R55	(17)	D. Bouanchaud (IP)

[a] U.U., Department of Microbiology, University of Umeå, Sweden; U.B., Department of Bacteriology, University of Bristol; I.P., Institut Pasteur, Paris; RPGMS., Royal Postgraduate Medical School, Hammersmith, London.

RESULTS

Resistance caused by RP1, RP1amp1, and RP1amp1irp1. The resistance of *E. coli* NC21 carrying RP1, RP1*amp*1, or RP1*amp*1*irp*1 to a range of penicillins and cephalosporins was compared with an R^- variant of the same strain and with the strain from which RP1 had been cured by growth in the presence of sodium dodecyl sulphate (15). The results are shown in Table 2. Resistance to penicillins in strains carrying RP1 is very high and this is particularly noticeable with carbenicillin. In contrast, resistance to cephalosporins is much less, a result consistent with the known activity of these β-lactam antibiotics against many strains producing this type of enzyme (10).

Examination of the strains which carry no R factors give single cell susceptibility values as little as one-thousandth of those found when RP1 is present regardless of whether the strain has been "cured" of its plasmid or never carried one, but the difference between the $RP1^+$ and $RP1^-$ cultures is less for cephalosporins than with penicillins. Strains carrying RP1*amp*1 do not have the susceptibility of R^- strains but intermediate levels of penicillin resistance. The loss of the β-lactamase gene from RP1 does not therefore completely destroy the ability of this plasmid to afford resistance to penicillins, but this intermediate degree of resistance does not extend to cephaloridine.

This conclusion is reinforced by the properties of bacteria carrying the plasmid

TABLE 2. *The effect of RP1 and its derivatives RP1amp1 and RP1amp1irp1 on the resistance of Escherichia coli NC21 to penicillins and cephalosporins and to tetracycline*

Strain	Single cell susceptibilities[a] (μg/ml)				
	PenG	Amp	Carb	CER	Tet
Escherichia coli NC21 (R^-)	16	4	2	2	1
E. coli NC21 (RP1)	10,000	10,000	25,000	8	125
E. coli NC21 (RP1*amp*1)	625	625	2,500	8	125
E. coli NC21 (RP1*amp*1*irp*1)	16	2	1	1	125
E. coli NC21 (R^-) ("cured")	16	4	1	1	1

[a] PenG, benzyl penicillin; Amp, ampicillin; Carb, carbenicillin; CER, cephaloridine; Tet, tetracycline.

RP1*amp1irp1*. Such bacteria have resistance to tetracyclines and to neomycin and kanamycin characteristic of RP1- and RP1*amp1*-carrying strains but do not show resistance to penicillins over and above that characteristic or non-R-factor organisms of this strain (Table 2).

Non-β-lactamase mutants of R1drd19 and R55. The existence on RP1 of genes seeming to specify intrinsic resistance raises the question as to whether similar genes are found on all R-factors that confer resistance to ampicillin. To investigate this point non-β-lactamase mutants were obtained from R1*drd*19 and R55 (see Materials and Methods). Comparison of single cell susceptibilities obtained against a range of penicillins and cephalosporins with *E. coli* carrying R1*drd*19 or its non-β-lactamase derivative (R1*drd*19 *amp1*) with R^- variants of the same host strain gave rather different results from those obtained with RP1 and its derivatives (Tables 2 and 3). First, the level of resistance to carbenicillin specified by R1*drd*19 was only about 1 mg/ml as opposed to values of about 25 mg/ml when RP1 was present, and similar differences were seen for other penicillins. Secondly, the presence of R1*drd*19*amp2* conferred no additional resistance to penicillins or to cephalosporins over that found in the R^- state. Similar results were obtained when the effects of R55 and R55*amp4* on *E. coli* were compared (Table 3).

One possible explanation of the differences between RP1*amp1* and R1*drd*19*amp2* is that mutation may have deleted all genes concerned with penicillin resistance in the latter plasmid but only affected the β-lactamase gene in the former. This interpretation seems unlikely since R1*drd*19*amp2* was found to revert to an Amp^+ phenotype at a frequency of about 10^{-9}, suggesting that the mutation in R1*drd*19*amp2* was probably a point mutation. Such a change is unlikely to inactivate both the β-lactamase gene and an intrinsic resistance marker in one step. It seems therefore that RP1 has one or more genes specifying intrinsic resistance to penicillins which are lacking in R1, and probably also in R55. This interpretation is certainly consistent with the lower level of resistance to penicillins, and particularly to carbenicillin, specified by R1 when compared with RP1.

The effect of R-factors and their mutant derivatives on the properties of E. coli AS19. *E. coli* is not normally susceptible to lysozyme unless the surface layers of the bacteria have been damaged by treatment with ethylenediaminetetraacetic acid or detergents. Cultures of the mutant strain AS19, however, are rapidly lysed to about 50% of their initial opacity by concentrations of 10 μg of lysozyme per ml without the help of any other agent. This mutant also shows single cell susceptibilities of 0.2, 0.6, and 1.6 μg/ml against rifampin, nalidixic acid, and actinomycin D, whereas the equivalent values for wild-type *E. coli* B are about 25, 12.5, and 16 μg/ml.

Since the behavior of mutant AS19 suggests strongly that the surface layers of the bacteria have been modified, it was interesting to know whether RP1, or any of its mutant derivatives, affected any properties of this strain. Susceptibility to lysozyme was the first character tested. Cultures of *E. coli* AS19(RP1), *E. coli* AS19(RP1*amp1*), *E. coli* AS19(RP1*amp1irp1*) as well as *E. coli* AS19(R^-) were grown in nutrient broth to a density of about 5×10^8 bacteria/ml. The bacteria were then collected by centrifugation and tested for their susceptibility to lysozyme as described in the Materials and Methods section. Bacterial suspensions incubated under identical conditions without lysozyme acted as controls. The change in optical density of these preparations is shown in Fig. 1. As expected *E. coli* AS19(R^-) lysed in the presence of lysozyme while the untreated control culture maintained its density. The *E. coli* cultures carrying RP1 and RP1*amp1*, on the

TABLE 3. *The effect of R1drd19, and R55 and their mutant derivatives R1drd19amp2 and R55amp4 on the resistance of E. coli NC21 to penicillins, cephalosporins, and chloramphenicol*

Strain	Single cell susceptibilities (μg/ml)				
	PenG	Amp	Carb	CER	Cm
Escherichia coli NC21 (R^-)	16	4	2	2	6
E. coli NC21 (R1*drd*19)	500	500	1,000	4	250
E. coli (R1*drd*19*amp2*)	20	2	1	2	250
E. coli NC21 (R55)	120	120	160	4	250
E. coli NC21 (R55*amp4*)	20	2	1	2	250

[a] Cm, chloramphenicol; PenG, benzyl penicillin; Amp, ampicillin; Carb, carbenicillin; CER, cephaloridine.

other hand, were almost completely protected against lysozyme attack. In contrast, cultures of *E. coli* AS19(*amp1irp1*) lysed almost as rapidly as non-R-factor bacteria when treated with lysozyme. There is therefore a complete correlation between the ability of these plasmids to specify intrinsic resistance to penicillins in *E. coli* K-12 and their ability to protect strain AS19 from lysozyme action (Fig. 1 and Table 2).

E. coli NC21 was resistant to lysozyme whether it carried an R-factor or not.

If the intrinsic resistance markers of RP1 and its derivatives are responsible for conferring lysozyme resistance on *E. coli* AS19, then neither R1*drd*19 nor R55 should protect. *E. coli* AS19(R1*drd*19) and *E. coli* AS19(R55) were therefore tested for their lysozyme susceptibility. *E. coli* AS19(R$^-$) acted as a control. Neither of these plasmids was able to protect strain AS19 from lysis by 10 μg of lysozyme per ml (data not shown).

Strain AS19 is abnormally susceptibile to β-lactam antibiotics when compared to its parent, *E. coli* B. The single cell susceptibilities of AS19(RP1) to penicillins and cephalosporins was therefore compared with *E. coli* AS19 (RP1*amp*1), *E. coli* AS10(RP1*amp*1*irp*1), and *E. coli* AS19(R$^-$) (Table 4). As with *E. coli* K-12 (Table 2), RP1 and its derivatives protected strain AS19 against killing by penicillins when the *irp* gene was present. Furthermore, the effects of these plasmids was much greater in strain AS19 than it had been on *E. coli* K-12 (Tables 2 and 4).

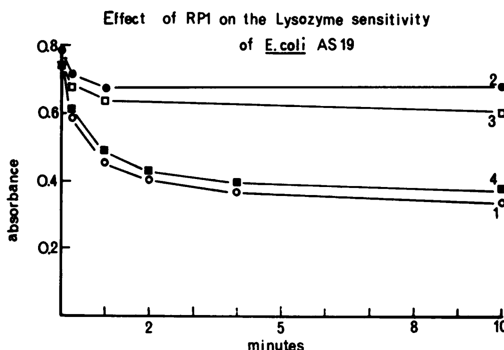

FIG. 1. *The effect of lysozyme (10 µg/ml) on E. coli B, on E. coli AS19, and on E. coli AS19 carrying RP1, and various mutant derivatives of that plasmid. Curve 1, E. coli AS19(R⁻); 2, E. coli B(R⁻); 3, E. coli AS19(RP1); 4, E. coli AS19(RP1amp1irp1). The behavior of E. coli AS19(RP1amp1) is similar to curve 3 (data not shown). E. coli B is the parent from which E. coli AS19 was derived by mutagen treatment.*

As RP1*amp*1 affects both the lysozyme susceptibility and intrinsic penicillin resistance of *E. coli* AS19, this plasmid might be expected to alter the single cell susceptibility of this strain to rifampin, nalidixic acid, and actinomycin D when compared with *E. coli* AS19(R$^-$) and *E. coli* AS19(RP1*amp*1*irp*1), since reduced resistance to these antibiotics is part of the pleiotropic phenotype produced by the mutation in strain AS19 (13). Accordingly, single cell susceptibilities for these antibiotics were determined for *E. coli* AS19(R$^-$) and for the same strain carrying RP1 and its various mutant derivatives. The results of this experiment are shown in Table 5. RP1 or RP1*amp*1 increased the resistance of strain AS19 to all three antibiotics to varying degrees, while RP1*amp*1*irp*1 had no effect. There is therefore complete correlation between the presence of the *irp* gene (or genes) on the R-factor, an increased resistance to rifampin, nalidixic acid, and actinomycin D, and resistance to lysozyme.

Neither R1*drd*19 nor R55 was able to protect strain AS19 against lysozyme digestion, nor did they confer intrinsic resistance to penicillins in *E. coli* AS19(R1*drd*19*amp*2) and *E. coli* AS19 (R55*amp*4). Examination of the resistance of these two strains to rifampin, nalidixic acid, and actinomycin D, in separate experiments, showed that neither of these plasmids conferred any resistance to these three antibiotics in this particular strain of *E. coli*.

The effect of R-factors and their mutant derivatives on E. coli D21 envA. Mutations in

TABLE 4. *The effect of RP1 and its mutant derivatives RP1amp1 and RP1amp1irp1 on the resistance of E. coli AS19 to penicillin, cephalosporins, and tetracycline*

Strain	Single cell susceptibilities (µg/ml)				
	PenG	Amp	Carb	CER	Tet
Escherichia coli B.(R$^-$)	16	2	2	2	2
E. coli AS19 (R$^-$)	2	<	<1	1	<1
E. coli AS19 (RP1)	500	500	1,000	4	63
E. coli AS19 (RP1*amp*1)	32	8	16	1	63
E. coli AS19 (RP1*amp*1*irp*1)	2	<1	<1	1	63

[a] PenG, benzyl penicillin; Amp, ampicillin; Carb, carbenicillin; CER, cephaloridine; Tet, tetracycline.

the *env*A gene produce strains of *E. coli* with an abnormal susceptibility to a number of antibiotics including penicillins (9). The plasmids which would protect *E. coli* AS19 against lysozyme action were therefore tested to see whether they would increase the intrinsic resistance of *E. coli* D21 *envA* to levels characteristic of the wild-type *E. coli* strain. The resistance of *E. coli* D21 *envA* carrying the plasmids RP1, RP1*amp*1, and RP1*amp*1*int*1 was therefore compared with the same strain lacking a plasmid against benzyl penicillin, ampicillin, carbenicillin, cephaloridine, neomycin, kanamycin, and tetracycline (Table 6). All the strains carrying RP1, or mutant derivatives of it, showed the expected resistance to neomycin, kanamycin, and tetracycline. None of the plasmids increased the resistance to cephaloridine (Table 2) and only RP1 or RP1*amp*1 raised the resistance to benzyl penicillin, to ampicillin, and to carbenicillin. RP1*amp*1*irp*1 had no significant effect on penicillin resistance in *envA* strains (Table 6).

The effect of R1*drd*19 and R1*drd*19*amp*2, and of R55 and R55*amp*4, on the antibiotic resistance of *E. coli* D21 *envA* were also examined. In no case did any of these plasmids confer increased resistance to this strain (data not shown).

TABLE 5. *The effect of RP1 and its mutant derivatives RP1amp1 and RP1amp1irp1 on the resistance of E. coli AS19 to rifampin, nalidixic acid, and actinomycin D*

Strain	Single cell susceptibilities (μg/ml)		
	Rif	Nal	Act
Escherichia coli B (R^-)	25	12.5	16
E. coli AS19 (R^-)	0.2	0.4	1.6
E. coli AS19 (RP1)	3.2	1.6	3.2
E. coli AS19 (RP1*amp*1)	3.6	1.6	3.2
E. coli AS19 (RP1*amp*1*irp*1)	0.4	0.4	0.8

[a] Rif, rifampin; Nal, nalidixic acid; Act, actinomycin D.

DISCUSSION

The mutations in both *E. coli* AS19 and D21*envA* produce pleiotropic effects on the surface layers of *E. coli* although, as yet, the precise chemical changes caused are far from clear. In particular, the ability of a range of antibacterial agents to reach their targets in the cell is enhanced (13) and with some of the agents concerned, at least, these targets are known to lie within the inner membrane of the bacteria (4).

The results detailed in this work show that certain R-factors (notably RP1 and some of its derivatives) can repair some, at least, of the phenotypic lesions in strains AS19 and D21*envA*, whereas others (e.g., R1 and R55) cannot. Moreover the effectiveness of RP1 and its derivatives depends on the integrity of the plasmid gene (or genes) that confer intrinsic resistance to penicillins on many strains of *E. coli* and *P. aeruginosa*.

The simplest hypothesis to account for the observations described here is that the intrinsic resistance gene (or genes) of the plasmid RP1 specify one or more products that modify the envelope of *E. coli* so that resistance to penicillins, but not cephalosporins, is enhanced. The distinction between these two classes of β-lactam antibiotic is not too surprising since the latter does seem to pass freely to the periplasmic space of many naturally occurring strains of *E. coli*, whereas penicillins do not (11a, 12). In wild-type strains these plasmid-specified changes in the envelope make little significant differences to the resistance of the bacteria to

TABLE 6. *The effect of RP1 and its mutant derivatives RP1amp1 and RP1amp1irp1 on the resistance of E. coli D21 and its mutant E. coli D21envA to penicillins, cephalosporins, and tetracycline*

Strain	Single cell susceptibilities (μg/ml)				
	PenG	Amp	Carb	CER	Tet
Escherichia coli D21 (R^-)	320	63	2	4	1
E. coli D21 (RP1)	10,000	10,000	25,000	8	125
E. coli D21 (RP1*amp*1)	625	625	1,250	4	63
E. coli D21 (RP1*amp*1*irp*1)	320	63	8	4	250
E. coli D21 *env*A(R^-)	32	4	1	2	1
E. coli D21 *env*A(RP1)	320	625	1,250	4	125
E. coli D21 *env*A(RP1*amp*1)	63	63	125	4	125
E. coli D21 *env*A(RP1*amp*1*irp*1)	32	8	1	2	125

[a] PenG, benzyl penicillin; Amp, ampicillin; Carb, carbenicillin; CER, cephaloridine; Tet, tetracycline.

rifampin, nalidixic acid, actinomycin D, and to lysozyme since the bacteria are relatively resistant to these agents anyway, but the effects can be seen as enhanced penicillin resistance. In the mutants AS19 and D21*envA*, however, where there is strong circumstantial evidence for reduced protection by the cell envelope, the effect of RP1 on resistance to all these agents is readily detectable.

If RP1 does indeed specify the synthesis of material in the surface layers which impedes the access of penicillins to their target, the high levels of resistance to carbenicillin caused by this plasmid may be explained. In gram-negative bacteria the greatest protective effect by a given amount of β-lactamase is obtained when the enzyme is situated behind a barrier which provides a slow feed of substrate (penicillin in this case) to the interior of the cell (11a). Plasmids such as RP1, which provide both the enzyme and some degree of enhancement of the barrier, achieve much higher levels of penicillin resistance than do plasmids that rely on β-lactamase alone.

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