IncP-1 R Plasmids Decrease the Serum Resistance and the Virulence of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa strain PAO1 was compared to PAO1 strains containing an IncP-1 R plasmid (RP1, R68, or R68.45) in an experimental mouse burn infection model. All R plasmids tested caused a 10- to 400-fold increase in mean lethal dose (LD₅₀). The decrease in virulence produced by plasmids R68 and R68.45 was significantly greater than the decrease caused by the closely related plasmid RP1. All plasmids also led to an increased sensitivity of strain PAO1 to human serum bactericidal activity. Virulence and serum resistance of strain PAO1 were restored by curing of the entire plasmid R68.45 but not by deletions in the plasmid's transfer gene regions.

INTRODUCTION

Bacterial plasmids often carry genes for activities that allow their host to survive in adverse environments or to compete more successfully with other micro-organisms. This selective advantage appears obvious in the case of plasmids that confer resistance to antibiotics. Several plasmids that specify virulence factors have been characterized (Elwell & Shipley, 1980). Examples of such plasmid-mediated properties are production of toxins and colonization factors (Smith *et al.*, 1983), iron uptake systems (Williams, 1979), and resistance to serum bactericidal activity (Binns *et al.*, 1982). On the other hand, plasmids may be disadvantageous to their hosts. R plasmids have been associated with decreased virulence (Lacey, 1975; Daum *et al.*, 1981), and lower growth rates under nutrient limitation (Klemperer *et al.*, 1979). Gentamicin-resistant *Pseudomonas aeruginosa* strains are less virulent in experimental rat infections than sensitive strains (Khakoo & Kluge, 1978), and a recent report has indicated that gentamicin-resistant *P. aeruginosa* strains generally produce only superficial infections in patients in contrast to the more invasive gentamicin-sensitive isolates (Clark *et al.*, 1984).

P. aeruginosa strains highly resistant to carbenicillin were first isolated from burns of patients at a Birmingham hospital shortly after the introduction of carbenicillin as a therapeutic agent (Lowbury *et al.*, 1969). This antibiotic resistance was caused by the broad-host-range IncP-1 R plasmids RP1 or R68 (Grinsted *et al.*, 1972). Lowbury *et al.* (1969) concluded that the carbenicillin-resistant isolates were fully virulent for mice. Here we show that in *P. aeruginosa* strain PAO the plasmids R68, its derivative R68.45, and RP1 lead to a decrease in virulence for mice and an increased susceptibility to human serum bactericidal activity.

METHODS

Bacterial strains, plasmids and bacteriophages. *Pseudomonas aeruginosa* strain PAO1 was used as host for the R plasmids RP1, R68 and R68.45 (Grinsted *et al.*, 1972; Stanisich & Holloway, 1971; Haas & Holloway, 1976). These plasmids confer resistance to carbenicillin, kanamycin/neomycin and tetracycline. Plasmids pME106 and pME107 were derived from R68.45 (Δ [Km Tra-1]) and pME140 from R68 (Δ [Km Tra-2]) (Haas & Riess,

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1983). PAO1c was a PAO1(R68.45) strain cured of the plasmid. Curing was done by the introduction of the incompatible plasmid pME390 [Cb Tc Km^r Tp Sp Rep(ts) Tra IncP-1, a temperature-sensitive RP1 derivative carrying Tn7] and subsequent elimination of pME390 by incubation at 43 °C without antibiotic selection. The cured strain did not carry a plasmid but was resistant to trimethoprim and spectinomycin, indicating that transposon Tn7 had inserted into the genome. This can happen readily because of a hotspot for Tn7 insertion in the chromosome of strain PAO (Caruso & Shapiro, 1982). Plasmid pME390 was kindly supplied by John M. Watson, The Australian National University, Canberra City, Australia. All strains were sensitive to phages F116c, E79 and B3c. Their growth rates were tested in nutrient yeast broth (NYB) (Haas & Holloway, 1976) and their motility was assessed in NYB with 0.6% agar at 37 °C. Phages were obtained from Bruce W. Holloway, Monash University, Melbourne, Australia.

Sensitivity to serum bactericidal activity. The sensitivity of the plasmid-containing strains to human serum bactericidal activity was tested according to Thomassen & Demko (1981). The bacteria were grown in Brain Heart Infusion Broth (Difco) and harvested in the exponential phase of growth, washed once in 0.15 M-NaCl with 0.05 M-sodium phosphate pH 7.2 (PBS), and diluted to 3.5×10^8 c.f.u. ml⁻¹. The cell suspension was diluted in PBS to a cell density of 3×10^3 c.f.u. ml⁻¹ and mixed with an equal volume of fresh human serum (pooled from three donors) in a test tube. The tubes were incubated at 37 °C for 1 h. Control tubes containing bacteria and inactivated (56 °C at 30 min) serum were run in parallel. The serum-sensitive strain PA103 (Liu, 1973) was included as a reference. The number of viable PA103 cells was reduced by 97% under these conditions.

Mouse burn infection model. The mouse burn model of Stieritz & Holder (1975) has been described in some detail in previous reports (Pavlovskis & Wrettlind, 1979; Pavlovskis *et al.*, 1983). In short, the backs of female CBA mice (Alab, Stockholm, Sweden) weighing 18–20 g were shaved, and the mice were anaesthetized with halothane (Hoechst). A template with a 2.5 × 2.5 cm opening was placed on the shaved area, which was covered by 0.5 ml 95% (v/v) ethanol, and flamed for 10 s. The burn *per se* was not lethal. A bacterial suspension containing 3.5×10^8 c.f.u. ml⁻¹ was prepared as described above and serial tenfold dilutions of this suspension were prepared in PBS and stored in an ice-bath until use; 0.1 ml of the desired suspension was injected subcutaneously in the burned area immediately after the trauma. To compensate for fluid loss, 0.5 ml 0.15 M-NaCl was injected intraperitoneally. Five mice were injected for each dilution, and the mice were observed for 7 d. Strain PAO1 was included in all experiments as a reference. Cultivations of bacteria from mice that had succumbed to infection with the plasmid-bearing strains were done in order to determine loss of plasmid *in vivo*. Samples were taken from the peritoneal and pleural cavities and streaked on blood agar plates (Columbia agar, Difco, supplemented with 5%, v/v, horse blood). Antibiotic sensitivity discs (Biodisk, Solna, Sweden) containing the appropriate antibiotic (kanamycin or carbenicillin) were then placed on the agar plate.

Statistical methods. The mean lethal dose (LD₅₀) was calculated by the Spearman-Kärber method as described by Finney (1964). Student's *t* test was used to compare the LD₅₀ values of different strains.

RESULTS AND DISCUSSION

Strain PAO1 was virulent in the mouse burn infection model. On average, 15 c.f.u. were sufficient to cause a 50% lethality (Table 1). The plasmids RP1, R68 and R68.45 caused a significant 15–400-fold decrease in virulence (Table 1). The effects of R68 and R68.45 (an R86 derivative with an IS21 duplication; Willetts *et al.*, 1981) were similar whereas RP1 had a less pronounced effect (Table 1). R68 and RP1 are indistinguishable with respect to size, antibiotic resistance determinants and restriction cleavage sites (Burkardt *et al.*, 1979; Currier & Morgan, 1981) but differ in at least one phenotypic trait: strain PAO1 carrying RP1 propagates the plasmid-specific phage PRR1 whereas strain PAO1 carrying R68 or R68.45 does not (Willetts *et al.*, 1981; D. Haas, unpublished data). Thus, the surface of PAO1 may differ according to the R plasmid carried and this might conceivably influence the bacterium's survival in an animal. Strain PAO1c, which was cured of the plasmid R68.45, was as virulent as the original strain PAO1 (Table 1). This indicates that the observed decrease in virulence was due to the IncP-1 plasmid itself rather than to a plasmid-induced host mutation.

Strains PAO1 and PAO1c were resistant to serum bactericidal activity. By contrast, all plasmid-containing strains were somewhat serum-sensitive (Table 1) and this fact might account, at least in part, for their decreased virulence. Since RP1 and R68/R68.45 had a similar effect on the survival of strain PAO1 in serum there may be additional factors involved in the decreased virulence caused by R68/R68.45.

In a rich medium (NYB) all strains tested had the same doubling time (about 30 min) and showed no visible differences in motility or sensitivity to some virulent phages. In the absence of

Table 1. Virulence and serum sensitivity of *P. aeruginosa* PAO1 with and without IncP-1 plasmids

Strain	Experimental mouse burn infection				Serum sensitivity (% change in c.f.u.)
	log LD ₅₀ ± SD	d* ± SD	P†	No. of expts	
PAO1	1.2 ± 0.7	—	—	17	+3
PAO1(R68.45)	4.1 ± 0.5	2.2 ± 1.0	<0.01	6	-39
PAO1(R68)	3.9	2.6	—	1	-30
PAO1(RP1)	2.2 ± 0.5	1.2 ± 0.3	<0.001	5	-45
PAO1(pME106)	2.4 ± 1.2	1.0 ± 0.7	<0.001	11	-41
PAO1(pME107)	2.8 ± 1.2	1.4 ± 0.4	<0.001	7	-41
PAO1(pME140)	4.3 ± 1.1	1.4 ± 0.3	<0.01	4	-43
PAO1c‡	3.5 ± 1.7	0.2 ± 0.5	>0.1	2	+5

* *d* is the difference in log₁₀ LD₅₀ between the plasmid-containing strain and PAO1 in a parallel experiment. In all experiments PAO1 was included as a control to account for the natural variation in the animals' susceptibility to infection.

† *P* value for difference in LD₅₀ between PAO1(R68.45) and PAO1(RP1) <0.05; and for difference between PAO1(R68.45) and PAO1(pME106) <0.001.

‡ The LD₅₀ values for the control (PAO1) and plasmid strains were higher in the two experiments involving PAO1c than in the other 15 experiments.

such gross effects, the R plasmids might be responsible for changes of the bacterial surface and this might lead to decreased virulence and serum resistance of strain PAO. Therefore, three R68 derivatives having deletions in their transfer gene regions Tra-1 or Tra-2 (Haas & Riess, 1983) were examined; the Tra-2 region includes the surface exclusion determinant(s) (Barth, 1979). However, the plasmids pME106 and pME107 (Δ [Km Tra-1]; surface exclusion positive) and pME140 (Δ [Km Tra-2]; surface exclusion negative) still conferred serum sensitivity on strain PAO1 and virulence was not restored (Table 1).

All plasmids except pME140 were stable in strain PAO propagated in the mouse. Mice infected with PAO1(pME140) showed a mixture of carbenicillin-resistant and sensitive colonies in cultures from the peritoneal cavity. Thus, the decrease in virulence caused by this plasmid is probably underestimated (Table 1). Slight instability of pME130 was also observed in broth cultures; after approximately 60 generations in NYB without antibiotic selection 6–12% of the PAO1 cells had lost the plasmid. The 14.5 kb deletion of pME140, which spans the Tra-2 region and the kanamycin resistance gene (Haas & Riess, 1983), also removes a site that seems to be required for the resolution of plasmid multimers to monomers; deletion of this site leads to plasmid instability in *Escherichia coli* (Grinter, 1985). The Tra-1 deletions of pME106 and pME107, by contrast, did not cause any plasmid instability in *P. aeruginosa* during 60 generations.

The effect of IncP-1 plasmids on virulence is probably not restricted to strain PAO, since similar findings have been reported for plasmid RP1 in other *Pseudomonas* strains (Wright *et al.*, 1977; McManus *et al.*, 1981). Plasmid FP39 (Pemberton & Holloway, 1973; unclassified incompatibility group) caused a 100-fold decrease in virulence for *P. aeruginosa* PAO in the mouse burn model (B. Wretling, unpublished data).

No evidence could be obtained for a direct involvement of the determinant specifying resistance to kanamycin because deletion of these genes did not lead to full serum resistance or virulence (Table 1). Also, RP1 derivatives having deletions in their tetracycline or carbenicillin resistance genes still caused serum sensitivity (data not shown). In conclusion, the decrease in serum resistance and virulence of *P. aeruginosa* caused by the IncP-1 R plasmids could not be attributed to gross effects on growth rate, motility, or to a single factor such as *tra* gene-dependent alterations of the bacterial surface.

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