Specification of the Conjugative Pili and Surface Mating Systems of *Pseudomonas* Plasmids

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Conjugative pili were identified for representative *Pseudomonas* plasmids of incompatibility groups P-2, P-3, P-5, P-7, P-8, P-10, P-11, and P-13, pili for groups P-1 and P-9 having already been described in detail. FP5 pili (unclassified) were also found. In most cases pili could be characterized by electron microscopy as rigid or flexible. The majority of *Pseudomonas* plasmids transferred significantly better on a surface than in a liquid. Examples of all incompatibility groups were tested.

INTRODUCTION

Conjugative pili have been identified for all plasmid incompatibility (Inc) groups in *Escherichia coli* K12 (Bradley, 1980*a, b*). Electron microscopy revealed that they fell into three morphological categories: thin (6 nm) flexible, thick (9 nm) flexible, and rigid. The type of pilus influenced the optimum mating environment (liquid or surface) of the plasmid, three conjugation systems being specified (Bradley *et al.*, 1980): surface obligatory (surface transfer > 2000 times better than liquid transfer), surface preferred (surface transfer 45–450 times better than liquid transfer), and universal (equal transfer in both environments). For *Pseudomonas* species, some conjugative pili have been identified (Bradley, 1974, 1981), but optimum plasmid mating environments have not been specified.

Like *E. coli* K12 plasmids, those of *Pseudomonas* are classified by incompatibility, groups IncP-1 to IncP-13 being recognized (Jacoby, 1977, 1980; Jacoby & Matthew, 1979; G. A. Jacoby, personal communication). IncP-4 and IncP-6 plasmids are not self-transmissible. IncP-1 pili, which are synthesized constitutively (in large numbers), are well-characterized as thin rigid rods (Bradley & Chaudhuri, 1980). Thick flexible pili are determined constitutively by the IncP-9 degradative plasmid TOL in its wild-type *Pseudomonas putida* host (Bradley & Williams, 1982). Pili for Rms148 (IncP-7) and R91.5 (IncP-10) have been provisionally identified (Bradley, 1981). The present objective has been to identify conjugative pili for as many additional *Pseudomonas* plasmids as possible, and also to confirm the rigid or flexible form of the known pili. In addition, an attempt has been made to correlate pilus type with possible surface mating systems.

METHODS

Bacterial strains and plasmids. 'Bald' plasmid host strains were *E. coli* K12 strain JE2571 (*leu thr str fla pil*) (Bradley, 1980*a*), *Pseudomonas aeruginosa* PAO1150.1 (*fla pil*) (Bradley, 1980*c*), *Pseudomonas putida* PaW340.3 (*trp str fla*) (Bradley & Williams, 1982); *P. putida* does not synthesize somatic pili. In addition, *P. aeruginosa* strain 280 (Bryan *et al.*, 1973), which was available with various nutritional markers, was used with R931. Rifampicin-resistant derivatives of these strains were used for the temporary derepression method (see below). *Pseudomonas aeruginosa* strain PU1, which carries plasmid FP2 (OT15 in Loutit *et al.*, 1968), was used to illustrate filamentous objects which resembled pili. Most plasmids were originally supplied in *P. aeruginosa* strain PU21 (*ilv leu str rif*) (Jacoby & Matthew, 1979), being transferred to a more suitable 'bald' host later. The plasmids used are listed and

Table 1. *Plasmids used and the morphology of their conjugative pili*

Inc group	Plasmid	Resistance and other phenotypic markers*	Pilus morphology†	Aggregation‡	References§
P-1	RP1	Cb Km Tc	Rigid	+	1, 2
P-2	R931	Sm Tc Hg Te	Rigid	—	3
P-2	CAM	Cam ⁺ Te	Rigid	?	4
P-3	RIP64	Cb Cm Gm Su Tm Hg	Flexible?	?	5
P-5	Rms163	Cm Su Tc	Flexible?	?	6
P-7	Rms148	Sm	Rigid	—	6
P-8	FP2	Hg	Flexible	—	7
P-9	R2	Cb Km Sm Su	Flexible	?	8
P-10	R91.5	Cb	Rigid	+	9
P-11	RP1-1	Cb	Rigid	—	6, 10
P-12	R716	Sm Hg	?	?	3
P-13	pMG26	Cb Cm Gm Km Sm Su Tm	Flexible¶	+	11
?	FP5	Hg	Flexible	?	12

* Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Tm, tobramycin; Hg, mercury; Te, tellurium; Cam⁺, camphor metabolizing ability.

† ?, too few pili to be certain of morphology, probable category given.

‡ +, aggregation; —, no aggregation; ?, insufficient pili (see Methods).

§ References: 1, Grinsted *et al.* (1972); 2, Bradley & Chaudhuri (1980); 3, Bryan *et al.* (1973); 4, Rheinwald *et al.* (1973); 5, Witchitz & Gerbaud (1972); 6, Jacoby (1977); 7, Holloway *et al.* (1971); 8, Kawakami *et al.* (1972); 9, Chandler & Krishnapillai (1977); 10, Lowbury *et al.* (1969); 11, Jacoby (1980); 12, Matsumoto & Tazaki (1973).

¶ Thin and thick flexible pili were found. Both aggregated.

referred to in Table 1, further references being given by Jacoby & Shapiro (1977). In addition, R91 (Chandler & Krishnapillai, 1977), and TOL::Tn401 (Benson & Shapiro, 1978) derived from TOL (Williams & Murray, 1974; Wong & Dunn, 1974), were used.

Media, culture methods, matings. Oxoid tryptone soya broth or BBL Brain Heart Infusion Broth were used, 2% (w/v) agar being added for plates. Incubation was at 37 °C, except for *P. putida* PaW340.3 and *P. aeruginosa* strains carrying the CAM or TOL plasmids when 30 °C was used. Plasmid transfer from *P. aeruginosa* PU21 to PAO1150.1 was by conventional plate mating using M9 minimal salts medium supplemented with 0.25% (w/v) glucose for selection of the prototrophic recipient. Drug concentrations used for plasmid selection are listed in Table 2. Sometimes, transconjugant-selecting plates were made with BBL Brain Heart Infusion agar containing 100 µg rifampicin ml⁻¹ together with an appropriate plasmid-selecting drug. Comparative plate and broth matings were done isogenically using strain PAO1150.1 containing the plasmid, with its rifampicin-resistant derivative as recipient. This strain had no surface appendages which might affect the transfer frequencies obtained. The method was that of Bradley *et al.* (1980), at least two experiments being done, and average values being given in the Results.

Electron microscopy. Bacteria were usually grown by the temporary derepression method (Bradley, 1980a), which provided confluent transconjugant growth on a nutrient plate containing a plasmid-selecting drug and 100 µg rifampicin ml⁻¹ for donor counterselection. Bacteria from these plates, and also those from non-selective nutrient plates incubated overnight (used for derepressed plasmids), were mounted for electron microscopy as described by Bradley (1980a, b). Immune electron microscopy was done by the grid-labelling method of Lawn (1967) using antiserum to pure somatic *P. aeruginosa* PAO1 pili kindly supplied by Dr W. Paranchych. Negative staining was with 1% (w/v) sodium phosphotungstate solution. Pilus thickness was not usually measured since it varied with local negative staining conditions and was not meaningful save for the comparison of two different types of pilus adjacent to one another.

The aggregation of pili was assessed by observing whether or not they formed bundles as in Fig. 4, or when fewer were present, whether or not they formed pairs (Fig. 16). A lack of aggregation could not be demonstrated with low densities of pili on the electron microscope specimen support grid. Examples of a few areas where several detached pili were close together with no pairs or bundles were required (Fig. 9).

RESULTS

Pilus-like structures produced by cultures of Pseudomonas species

The search for conjugative pili was hampered by the production of spurious structures, some of which are illustrated here so that others may recognize them. Filamentous metabolic

Fig. 1. Polymerized bacteriocin core protein from *P. aeruginosa* strain PU1. The bar marker represents 100 nm.

Fig. 2. Flexible bacteriocin-like structure from *P. aeruginosa* strain PU1. The bar marker represents 100 nm.

Fig. 3. Pilus-like filament with faint helical structure from *P. aeruginosa* strain PAO1150.1. Structures in Figs 1–3 were found in most strains of *P. aeruginosa* whether or not a plasmid was carried. The bar marker represents 100 nm.

products, which were easily identified by their variable thickness and high contrast (not illustrated), were produced by *P. aeruginosa* and *P. putida* (Bradley & Williams, 1982). The following objects found in association with some lines of *P. aeruginosa* had to be reliably distinguished from conjugative pili. Polymerized aeruginocin core protein (Fig. 1), associated with a contractile bacteriocin in the form of a headless phage tail (Bradley, 1967), closely resembled rigid pili but was thinner and always had a hollow appearance. Flexible aeruginocin-like structures (Fig. 2) were distinguishable by cross-striations visible at high magnification. *Pseudomonas aeruginosa* strain PU1, which was used to illustrate these structures, carried plasmid FP2 and synthesized P-8 pili as well (see below). A thinner pilus-like filament (Fig. 3) had a less obvious periodic structure. *Pseudomonas aeruginosa* somatic pili were thinner than most conjugative pili (not illustrated; see Bradley, 1972). Although the strains used here did not normally synthesize them, when production did occur, identification was made by immune electron microscopy (see Methods).

Morphology of conjugative pili determined by Pseudomonas plasmids

Table 1 summarizes the results of an electron microscopic survey of *P. aeruginosa* strain PAO1150.1 containing plasmids representing each *Pseudomonas* incompatibility group. Most conjugative pili could be recognized as being rigid or flexible with reasonable certainty (Bradley, 1980*b*). Whether or not pili aggregated could not always be determined because sometimes too few pili were produced (Table 1). A difference in the aggregation characteristics (see Methods) of two morphologically similar types of pili can distinguish them since a chemical difference in the pilin (pilus protein) is indicated (Bradley, 1980*a*).

IncP-1 pili are typified by those of RP1 which were synthesized in very large numbers (Fig. 4) by *E. coli* K12 strain JE2571 (RP1). *Pseudomonas aeruginosa* PAO1150.1 (RP1) produced comparatively few pili, aggregates of two or three being the rule. P-1 pili were about 8 nm thick with one pointed end, a feature not previously noted (Fig. 5).

P-2 is the largest and commonest incompatibility group. Pili of the repressed plasmid R931 were sought using *P. aeruginosa* strain 280 as host (Bryan *et al.*, 1973). In one experiment only, the temporary derepression method of growth gave many rigid rods (Fig. 6), the shorter ones probably being fragments of longer ones. Plasmid transfer was poor, and only one out of several repeat experiments produced a few pili. The plasmid CAM was therefore studied in host strain *P. putida* PaW340.3 using tellurium plus streptomycin selection for the temporary derepression method. One or two rigid rods similar to R931 pili were found on each electron microscope specimen support grid square (not illustrated).

IncP-3 plasmids can be transferred to *E. coli* (Witchitz & Chabbert, 1971) where they are classified as IncC and determine thick flexible pili (Bradley, 1980*a*). *Pseudomonas aeruginosa* PAO1150.1 (RIP64) gave no pili by the temporary derepression method using either mercury or gentamicin for selection. However, a very few were found with cultures on non-selective plates incubated either overnight or for 4 h. Only five pili were counted on three support grids, each being examined in the electron microscope for about 15 min. P-3 pili were easily recognizable and were probably of the thick flexible kind (Fig. 7); they could not be confused with any other structure.

Rms163 is representative of incompatibility group P-5. Despite reasonable transfer when grown by the temporary derepression method, strain PAO1150.1 (Rms163) provided only about five pili in several preparations. These were probably of the thick flexible type (Fig. 8).

Rms148 (IncP-7) is naturally derepressed for transfer and an overnight culture on a non-selective plate gave many non-aggregating short nail-like rods (Fig. 9). A few long pili allowed their identification as rigid (not illustrated).

Incompatibility group P-8 consists of the single important plasmid FP2, which mobilizes the *P. aeruginosa* chromosome. Figure 10 shows that its pili, synthesized by PAO1150.1 (FP2), are thick and flexible with a terminal structure, 1-4 being found on each specimen support grid square using the temporary derepression method (mercury selection). They did not aggregate. Strain PU1, which also carries FP2, synthesized similar pili (not illustrated).

While P-9 pili, exemplified by those of the TOL plasmid, have been described in detail elsewhere (Bradley & Williams, 1982), those of another plasmid in the group were sought for comparison. R2 was the only one of several different repressed plasmids (TOL is naturally derepressed) that provided pili; only ten were found in several different preparations (temporary derepression method, carbenicillin selection). They were thick and flexible (Fig. 11) like TOL pili.

P-10 is the only incompatibility group containing a plasmid that has been made derepressed in the laboratory. R91.5 (parent R91) determined very numerous thick rigid pili which aggregated. They had sharp-pointed ends (Fig. 12), and what was presumed to be a basal structure sometimes in the form of a ring (Fig. 13).

Incompatibility group P-11 contains the plasmid RP1-1 that transferred very well (see Table 2). However, it did not determine conjugative pili constitutively, only one or two being found on each specimen support grid square. Longer pili (not illustrated) were identified as rigid, but the majority were short non-aggregating nail-like structures (Fig. 14).

Fig. 4. *Escherichia coli* K12 strain JE2571 (RP1) synthesized these large aggregates of P-1 pili in considerable quantity. The bar marker represents 100 nm.

Fig. 5. *Pseudomonas aeruginosa* strain PAO1150.1 (RP1) synthesized fewer conjugative pili than *E. coli*, small aggregates being found. P-1 pili have been described before (Bradley, 1974; Bradley & Chaudhuri, 1980) but are included here for completeness. The bar marker represents 100 nm.

Fig. 6. P-2 pili determined by R931 in host strain *P. aeruginosa* 280. The bar marker represents 100 nm.

Fig. 7. Only about five P-3 pili were found in an extensive search using *P. aeruginosa* strain PAO1150.1 (RIP64), but they were unmistakable. The bar marker represents 100 nm.

Fig. 8. Again only five P-5 pili were found using strain PAO1150.1 (Rms163), and these were less obvious than P-3 pili. The bar marker represents 100 nm.

Fig. 9. PAO1150.1 (Rms148) synthesized many short rods. The bar marker represents 100 nm.

Fig. 10. P-8 pili of FP2 (host PAO1150.1) were thick, flexible, and quite numerous. The bar marker represents 100 nm.

Fig. 11. *Pseudomonas aeruginosa* strain PAO1150.1 (R2) only produced very few pili (P-9) which were thick flexible. The bar marker represents 100 nm.

Fig. 12. IncP-10 plasmid R91.5 is derepressed for transfer and determined many rigid pointed rods in host strain PAO1150.1. The bar marker represents 100 nm.

Fig. 13. P-10 pili often had ring-like structures at one end, probably representing the pilus base. The bar marker represents 100 nm.

Fig. 14. PAO1150.1 (RP1-1) determined rigid pili some of which were longer than the short rods illustrated (IncP-11). The bar marker represents 100 nm.

Table 2. Comparison of transfer frequencies for surface and liquid matings*

Pilus morphology†	Inc group	Plasmid	Selection‡	Pilus synthesis§	Transfer frequencies¶		Frequency ratio plate/broth
					Plate	Broth	
Flexible	P-3	RIP64	Gm ₁₀	Repressed	3.7×10^{-4}	2.1×10^{-5}	18
	P-5	Rms163	Tc ₅₀	Repressed	6.8×10^{-5}	2.4×10^{-5}	2.8
	P-8	FP2	Hg ₁₂	Repressed	3.4×10^{-4}	1.3×10^{-4}	2.6
	P-9	R2	Cb ₂₀₀₀	Repressed	4.9×10^{-2}	1.1×10^{-4}	445
	P-9	TOL‡	Cb ₂₀₀₀	Repressed	2.6×10^{-2}	1.4×10^{-4}	186
	P-13	pMG26	Sm ₂₀₀	Repressed	5.2×10^{-5}	2.1×10^{-6}	25
	?	FP5	Hg ₁₂	Repressed	2.9×10^{-4}	1.7×10^{-5}	17
Rigid	P-1	RP1	Cb ₂₀₀₀	Constitutive	6.8×10^{-1}	8.8×10^{-4}	773
	P-2	R931	Sm ₂₀₀	Repressed	4.3×10^{-5}	9.0×10^{-7}	48
	P-2	CAM	Te ₂₅	Repressed	1.0×10^{-2}	9.2×10^{-5}	109
	P-7	Rms148	Sm ₂₀₀	Constitutive	5.6×10^{-2}	8.5×10^{-4}	66
	P-10	R91	Cb ₅₀₀	Repressed	5.2×10^{-2}	2.6×10^{-4}	200
	P-10	R91.5	Cb ₅₀₀	Constitutive	3.0×10^0	6.5×10^{-2}	46
	P-11	RP1-1	Cb ₂₀₀₀	Repressed	2.6×10^{-1}	1.3×10^{-3}	200
Unknown	P-12	R716	Sm ₂₀₀	Repressed	3.2×10^{-5}	1.2×10^{-6}	27

* The host organism for all plasmids was *P. aeruginosa* strain PAO1150.1 (*fla pil*).

† The plasmids studied determined only thick flexible pili, not thick or thin flexible pili as was the case with *E. coli* K12 plasmids (Bradley, 1980*a, b*). There was one exception, pMG26, which had both thick and thin flexible pili. No pili were found for R716. See also Table 1.

‡ Cb, carbenicillin; Gm, gentamicin; Hg, mercury (as chloride); Sm, streptomycin; Tc, tetracycline; Te, tellurium (as potassium tellurite). TOL was selected with carbenicillin because this example of the plasmid contained transposon Tn401 (Benson & Shapiro, 1978), which specifies resistance to the drug. Numbers represent concentrations in $\mu\text{g ml}^{-1}$.

§ As judged by electron microscopy (Bradley, 1980*b*).

¶ Average frequencies obtained from a minimum of two experiments (see Methods), transconjugants per donor h^{-1} .

Plasmid R716 (IncP-12) was the only example where no pili could be found in the course of an extensive search. Attempts to make a derepressed version using conventional mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine failed. This result provided a 'negative control' for the experimental data described.

Group P-13 was represented by pMG26 which determined flexible pili of two different thicknesses using the temporary derepression method. The thick ones were typical flexible pili (Fig. 15). The thin ones (Fig. 16) were fairly short and about 6 nm in diameter. Because their thickness was similar to that of the much longer somatic pili, they were tested with antiserum to somatic pili by immune electron microscopy. No antibody adsorption was detected and there was thus no doubt that pMG26 determined two kinds of filament. Both aggregated separately and together.

FP5, though unclassified, was included because of its importance in chromosomal mapping. The temporary derepression method yielded 1–5 thick flexible non-aggregating pili on each support grid square (Fig. 17). They were similar to FP2 pili.

Surface mating systems of *Pseudomonas* plasmids

Table 2 compares transfer frequencies in liquid and surface environments for *Pseudomonas* plasmids. Results are arranged according to pilus morphological type. In order to ascertain a plasmid's degree of preference for a surface environment, the plate/broth transfer frequency ratio was calculated. A high ratio indicated a greater efficiency for a plate mating, and a lower one demonstrated that the plasmid transferred equally well in a liquid environment. The latter (universal conjugation system) was exemplified only by Rms163 (IncP-5) and FP2 (IncP-8). Thereafter, ratios increased from 17 (FP5) to a maximum of 773 (RP1) there being no obvious classes of surface mating systems as was the case with *E. coli* K12 plasmids (see Introduction).

Fig. 15. *Pseudomonas aeruginosa* strain PAO1150.1 (pMG26) determined two kinds of pili both flexible. The figure shows the thickest (10 nm). The bar marker represents 100 nm.

Fig. 16. The thin (6 nm) pili shown here were close to those in Fig. 15. pMG26 belongs to group IncP-13. The bar marker represents 100 nm.

Fig. 17. The only unclassified plasmid studied was FP5 in host strain PAO1150.1. It had thick flexible pili. The bar marker represents 100 nm.

Plasmids with flexible pili exhibited almost as large a range of frequency ratios as did those with rigid pili.

These results also correlate the state of pilus synthesis (repressed or derepressed) with transfer frequency in the optimum environment. It was unexpected that RP1-1, which was repressed for pilus synthesis, transferred at a derepressed frequency (Bradley *et al.*, 1980).

DISCUSSION

One of the present objectives has been to ascertain whether or not a large number of *Pseudomonas* plasmids determined conjugative pili like *E. coli* K12 plasmids. Only one of the fifteen examined failed to show pili. Since the exception, R716, had a very low transfer frequency, the existence of pili cannot be ruled out. Though not unexpected, the overall results show that *Pseudomonas* and *E. coli* conjugative pili are structurally generally similar with the same rigid and flexible forms. There is no definite evidence of a *Pseudomonas* or *E. coli* transfer system without any pili.

The comparison of surface and liquid matings has shown that only FP2 and Rms163 transferred more or less equally well in both environments (universal conjugation systems; Bradley *et al.*, 1980). All the others were significantly better on an agar surface. However, the surface mating systems did not appear to fall into two distinct classes, surface obligatory and surface-preferred, which in *E. coli* gave plate/broth transfer frequency ratios of > 2000 and 45–450, respectively. *Pseudomonas* plasmids must therefore be described as having surface preferred or universal conjugation systems. The highest ratio was 773 for RP1, about 1/3 of the value for *E. coli* (Bradley *et al.*, 1980), which was 2100. It had rigid pili. Unlike *E. coli* K12 plasmids with rigid pili, all of which had very high plate/broth transfer frequency ratios (surface obligatory systems), some *Pseudomonas* plasmids with rigid pili (R91.5, R931, Rms148) had quite low ratios. These results need not reflect any fundamental differences in the mating types of *Pseudomonas* and *E. coli* K12 plasmids, but the less well-defined results with *Pseudomonas* may be due to interference with pilus function by the large amount of filamentous metabolic material produced by the organism.

It should be possible to correlate the state of pilus synthesis (repressed or derepressed) as revealed by electron microscopy with transfer frequency in the optimum environment. For *E. coli* K12 plasmids, all those derepressed for transfer (frequency $> 10^{-1}$ transconjugants per donor h^{-1}) determined conjugative pili constitutively (Bradley *et al.*, 1980). Here, there was an exception, RP1-1 (Table 2); the plasmid transferred at 2.6×10^{-1} transconjugants per donor h^{-1} but few pili could be found in the electron microscope. Since only those pili detached from cells could be seen, a likely explanation is that those of RP1-1 remained attached to the cells, perhaps due to the metabolic products produced by the organism. Indeed, far fewer pili were found overall for *Pseudomonas* plasmids compared with *E. coli* plasmids. With the latter organism, not all plasmids that determined pili constitutively were derepressed for transfer (Bradley *et al.*, 1980); the same applied to Rms148 here. Inefficient pilus function would have this effect.

Those plasmids with low transfer frequencies provided very few pili even by the temporary derepression method (R716 had none) with the exception of pMG26. Here the temporary derepression method gave many pili indicating the presence of an efficient repressor. pMG26 also had two kinds of pilus, thick and thin flexible. A similar situation was found for *E. coli* K12 plasmids of incompatibility group I₁ (Bradley, 1983) and I₂ (Bradley & Coetzee, 1982) though in both these cases, the thick pili were believed to be rigid and the thin ones flexible. Sufficient pili were determined by *E. coli* K12 plasmids to permit antiserum preparation and a survey of serological relationships among pili of different incompatibility groups (Bradley, 1980*b*). This is unfortunately impossible with *Pseudomonas* plasmids, too few pili being synthesized in most cases. It has been tacitly assumed throughout that *Pseudomonas* plasmid-determined pili are involved in conjugation, hence the term 'conjugative'. While this is virtually certain, it has yet to be demonstrated.

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