Characteristics and Function of Thick and Thin Conjugative Pili Determined by Transfer-derepressed Plasmids of Incompatibility Groups I₁, I₂, I₅, B, K and Z

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Eleven transfer-derepressed plasmids from incompatibility groups I₁, I₅, B, K and Z were constructed using the *dnaG3* mutant *Escherichia coli* strain BW86. All were found to determine thin flexible and thick rigid pili constitutively. Immune electron microscopy was used to relate thick and thin pilus serotypes with incompatibility grouping. Mutant plasmids that determined only thick pili constitutively transferred efficiently on an agar surface but not in a liquid, whereas plasmids with both kinds of pili transferred equally well in both environments. A mutant of the IncI₂ plasmid R721 determined thin pili constitutively, and thick pili at a repressed level, as indicated by electron microscopy. Experiments with this indicated that thin pili were apparently not involved directly in conjugation but were only used to stabilize mating aggregates.

INTRODUCTION

Plasmids belonging to the I complex of incompatibility (Inc) groups have been identified by their specification of sensitivity to the similar filamentous bacteriophages If1 (Meynell & Lawn, 1968; Datta, 1979) and PR64FS (Coetzee *et al.*, 1980). Phage receptors are the tips of thin flexible I pili. When it was discovered that IncB and IncK plasmids determined morphologically and serologically similar pili (Bradley, 1980*a*), they were added to the I complex despite their apparent inability to determine If1 and PR64FS sensitivity. The seven original groups have now been reduced to IncI₁, IncI₂, IncB and IncK, with some plasmids determining both I₁ and B phenotypes (R. W. Hedges and N. Datta, personal communications). Two new groups, IncI₅ (Tschäpe & Tietze, 1981) and IncZ (Tschäpe & Tietze, 1983) have recently been added. The thin, 6.0 nm diameter flexible pili of I complex plasmids, excluding the untested ones of incompatibility groups I₅ and Z, form two distinct serotypes: I₁ (I₁, B and K pili) and I₂ (I₂ pili only) (Bradley, 1980*a*). In addition to the 6.0 nm flexible pili, R64*drd11* (IncI₁) and R144*drd3* (IncI₁ + B) determine thick pili (Bradley, 1983*a*), apparently of the rigid kind (Bradley, 1980*a*). Likewise, thick pili were found for three IncI₂ plasmids, pIN2b, R721*pil^c*, and TP114*drp1* (Bradley & Coetzee, 1982). They have been designated thick I₁ and thick I₂ pili, and are morphologically distinct. The tips of thick I₂ pili are receptors for lipid phage PR4 (Bradley & Coetzee, 1982) and the filamentous phage IKE (Bradley *et al.*, 1983), and the sides (shafts) of thin I₁ pili for the RNA phage Iα (Coetzee *et al.*, 1982).

Wild-type transfer-repressed plasmids determine very few conjugative pili (Bradley, 1980*a, b*). Plasmids that synthesize pili constitutively (*pil^c*, R. W. Hedges, personal communication; Coetzee *et al.*, 1982), which may or may not be transfer-derepressed, are required for the effective investigation of the structure and occurrence of thick and thin I-type pili. Those prepared by conventional mutagenesis are designated 'authentic transfer-derepressed mutants' (Sasakawa & Yoshikawa, 1978). A better construction method is the use of an *Escherichia coli*

plasmid host strain carrying the temperature-sensitive *dnaG3* mutation (Wilkins, 1975; Sasakawa & Yoshikawa, 1978). Such mutants lack the *dnaG* gene product, a DNA primase. A suitable primase is determined by certain plasmids, but under repression by the transfer regulator. When this repression is lifted, adequate primase synthesis permits the organism to grow at up to 43 °C instead of at a maximum of about 30 °C (see Discussion and Wilkins, 1975; Sasakawa & Yoshikawa, 1978; Dalrymple & Williams, 1982). 'Spontaneous transfer-derepressed mutants' as they are designated here, are thus selected by high temperature growth. Sasakawa & Yoshikawa (1978) and Dalrymple & Williams (1982) point out that an authentic transfer-derepressed mutant plasmid in a host with the *dnaG3* mutation suppresses temperature-sensitivity more efficiently than its wild-type repressed parent. However, wild-type repressed plasmids were deliberately chosen here to ensure that the derepressed plasmids produced were the genuine results of temperature-selection and not merely authentic derepressed plasmids in naturally reverted clones of the *dnaG3* host. Until now, the only plasmids found capable of suppressing temperature-sensitivity on becoming transfer-derepressed have belonged to incompatibility groups I₁, B or a combination of the two. Claims that an IncI₂ (= I δ) plasmid, R724, had this capability (Dalrymple *et al.*, 1982) are incorrect because of revised classification. R724 is now, as earlier, IncB (R. W. Hedges and N. Datta, personal communications; Jacob *et al.*, 1977). The main objectives of this work have been to screen other I complex plasmids for thick pili, and to define the roles of both thick and thin I-type pili in conjugation. To achieve the former, it has been necessary to test the ability of IncK, IncI₅ and IncZ plasmids to suppress temperature-sensitivity. The spontaneous derepressed mutant plasmids constructed have been characterized for their degree of transfer-derepression (transfer frequency), their type of conjugation system (universal or surface-obligatory; Bradley *et al.*, 1980), specified sensitivity to bacteriophages, and stability in a host without the *dnaG3* mutation.

METHODS

Bacterial strains, plasmids and bacteriophages. The following bacteria, or mutants with nalidixic acid-resistance (suffix -1) or rifampicin-resistance (suffix -2), were used: *E. coli* K12 strains J53 (*pro met*) (Bachmann, 1972), JE2571 (*leu thr str fla pil*) (Bradley, 1980b), BW86 [*dnaG3 leu thyA deoB rpsL (chlA-uvrB) ColI^r*] (Boulnois & Wilkins, 1979), *Salmonella typhimurium* LT2 strains SQ1139 (*purC proA ilv str fla pil*) (Bradley, 1980b), M827 (*pro his*) (Spratt *et al.*, 1973). Most of the plasmids used are listed in Table 1. Those designated 'drd86' or 'drd86.1' were constructed for this work (see below). The majority of parent plasmids and those not so designated are listed by Jacob *et al.* (1977), although some incompatibility groupings may have been changed. Jacob *et al.* (1977) do not list: pTM559 (Monti-Bragadin *et al.*, 1979), pIE360 (Tschäpe & Tietze, 1981), pIE545 (Tschäpe & Tietze, 1983), R721 *pil^c* (constructed by R. W. Hedges; Coetzee *et al.*, 1982), R721 *pil^c.4* (constructed by R. W. Hedges; Bradley *et al.*, 1983), and the IncI₁ plasmids R1535 and R1539 (R. W. Hedges, unpublished). Derivatives of the prototype IncI₂ plasmid TP114 were not used as they expressed conjugative pili poorly in strain JE2571 (see Bradley & Coetzee, 1982), although antiserum to TP114 pili (Bradley, 1980a) was used for immune electron microscopy (see below). Likewise R64drd11, an authentic transfer-derepressed mutant of the prototype IncI₁ plasmid, reverted to wild-type when transferred into strain JE2571 so that R64 derivatives were not used in some experiments. Bacteriophages were If1 (Meynell & Lawn, 1968), PR64FS (Coetzee *et al.*, 1980), I α (Coetzee *et al.*, 1982), and PR4 (Stanisich, 1974; Bradley & Rutherford, 1975).

Media, bacteriophage techniques and matings. The growth medium was Brain Heart Infusion Broth (BBL) with or without 2% (w/v) agar, incubation temperatures being 30 °C, 37 °C or 42 °C, according to the experiment. Drug concentrations were: ampicillin, 400 µg ml⁻¹; chloramphenicol, 25 µg ml⁻¹; kanamycin, 100 µg ml⁻¹; streptomycin, 200 µg ml⁻¹; sulphonamides, 1 mg ml⁻¹; tetracycline, 25 µg ml⁻¹; trimethoprim, 1 mg ml⁻¹; rifampicin, 100 µg ml⁻¹; nalidixic acid, 20 µg ml⁻¹. Standard methods were used for bacteriophage growth, and spot or cross-streak tests for sensitivity to them (see Bradley, 1967; Bradley & Coetzee, 1982). Comparative plate and broth mating methods were as described by Bradley *et al.* (1980), the cross-streak mating method was that of Barth (1979), and crosses described as plate matings were done by the temporary derepression growth method (Bradley, 1980b).

Construction of spontaneous transfer-derepressed plasmids using *E. coli* BW86-1. These plasmids were designated 'drd86'. The technique was based on that of Sasakawa & Yoshikawa (1978). Several minor variations were introduced, and some of the transfer-derepressed (pilus-constitutive) mutant plasmids were isolated using these slightly modified procedures. The method which is considered optimal is described below, though some small changes would be required for plasmids determining β -lactamase synthesis (not applicable here). The day-by-day procedure should not be interrupted by storage over week-ends, etc.

(i) Day 1: plate mating. *Escherichia coli* strains BW86-1 and J53(R⁺) (R⁺ denotes carrying a transfer-repressed wild-type plasmid) were heavily streaked from fresh overnight plates (containing selective drugs for R⁺ strains) to non-selective plates and incubated for 4 h at 30 °C and 37 °C, respectively. About 1 µl of each culture was transferred to another non-selective plate, mixed in a drop of broth, then spread and dried. This mating plate was incubated for 2 h at 30 °C. The mixture was then streaked on a transconjugant-selecting plate (nalidixic acid 20 µg ml⁻¹ with plasmid-selecting drugs in Table 1) for confluent and single-colony growth at 30 °C overnight.

(ii) Day 2: selection at 42 °C. The thinnest transconjugant growth or > 10 single colonies (should be rough) was heavily streaked on a non-selective plate and incubated for 6 h at 30 °C. About 0.5 µl bacteria was then spread in 0.1 ml broth on a non-selective plate roughly grading the density from heavy to thin across the plate. After drying, incubation was for 18–24 h at 42 °C.

(iii) Day 3: isolation of BW86-1 strains possibly carrying transfer-derepressed plasmids. (a) If small colonies were visible, > 20 smooth ones [BW86-1(R⁻) is rough] were streaked on a plate containing the plasmid-selecting drug (Table 1) with 200 µg streptomycin ml⁻¹ to remove possible nalidixic acid-resistant J53 donors from day 1 (omitted if streptomycin resistance was determined by the plasmid). Overnight incubation was at 42 °C. (b) If few or no colonies were visible, the plate from day 2 was replica-plated onto a plate with streptomycin and incubated overnight at 37 °C.

(iv) Day 4: transfer of possible transfer-derepressed plasmids to strain JE2571-2. Growth on both plates from day 3 usually contained a proportion of colonies of BW86-1 carrying transfer-derepressed plasmids and some with wild-type (repressed) plasmids. Single colonies were tested for transfer-derepression by cross-streak mating (Barth, 1979) with strain JE2571-2 on plates containing 200 µg rifampicin ml⁻¹ with a plasmid-selecting drug; incubation was overnight at 37 °C.

(v) Day 5: selection of JE2571 strains with potential derepressed plasmids. Derepressed transfer on cross-streak mating plates was identified by heavy transconjugant growth. Smooth single colonies (if not available, bacteria from growth areas with smooth margins) were subcultured onto the same transconjugant-selecting medium for overnight incubation at 37 °C.

(vi) Day 6: identification of JE2571 strains with transfer-derepressed plasmids. Cultures were examined with a lens under oblique illumination. Usually, rough and smooth, or opaque and transparent colonies were found. The latter kinds carried transfer-derepressed plasmids, but a uniform colony morphology did not preclude their presence. Single colonies were subcultured onto plates with the plasmid-selecting drug only, and incubated overnight at 37 °C.

(vii) Day 7 onwards: screening for transfer-derepressed plasmids. Colonies of BW86-1 strains from day 4 that gave derepressed transfer on day 5 were restreaked on plasmid-selecting plates and tested by electron microscopy for constitutive conjugative pilus production (type I pili were distinguished by labelling with homologous antibodies). Alternatively, phage spot tests were done, lysis indicating the presence of transfer-derepressed plasmids. Sometimes the derepressed character did not appear to be expressed in strain BW86-1 although it was transferred to strain JE2571-2. Excessive type I pilus production was believed to interfere with conjugative pilus production in these cases. BW86-1 strains with smooth small colonies generally carried transfer-derepressed plasmids. Because the derepressed character was under temperature-mediated selective pressure in strain BW86-1, it was probably more stable than in strain JE2571-2 so that storage was more successful.

Construction of transfer-derepressed plasmids with one morphological form of pilus only. Those spontaneous and authentic transfer-derepressed plasmids specifying sensitivity to RNA phage Iα determined thick and thin I₁-type pili constitutively. Mutants of spontaneous derepressed plasmids without thin pilus determinants (designated 'drd86.1') were selected by confluent lysing soft agar layer plates with phage Iα, then picking phage-resistant colonies from the surface. Incubation at 37 °C maintained temperature-selection of the derepressed character in strain BW86-1, constitutive thick pilus synthesis being stably transferable to strain JE2571-2 in all cases except R64drd86.1. Unfortunately it was impossible to make similar authentic derepressed mutants since selection for resistance to phage Iα resulted in reversion to wild-type (loss of constitutive pilus synthesis and reduction of transfer frequencies to repressed levels, i.e. < 1 × 10⁻¹ transconjugants per donor h⁻¹). No phage for thick I₁ pili was available, so that plasmids determining thin I₁ pili only could not be made. Conversely, the only phages specific for thin I₂ pili (If1, PR64FS) are filamentous and unsuitable for the selection of mutants without thin I₂ pili. R721pil^c.4, which determined only thin pili constitutively, was constructed by R. W. Hedges (personal communication) by transferring R721pil^c in the presence of phage PR4 (adsorbs to thick I₂ pili).

Stability of derepressed character. For assay, at least 25 colonies from viable counts on non-selective plates (overnight static culture in drug-free broth grown in drug-free shake culture to 2 × 10⁹ organisms ml⁻¹) were tested for sensitivity to appropriate bacteriophages by the cross-streak method. Lysis indicated constitutive pilus synthesis and transfer-derepression. Where no phage was available, electron microscopy of at least five clones verified that smooth colony morphology was associated with constitutive pilus synthesis. The numbers of smooth and rough colonies in a sample of > 100 were then determined.

Electron microscopy. Pili were mounted for electron microscopy (Bradley, 1980a, b) by thoroughly suspending a loopful of bacteria from a plate culture in a drop of 0.1 M-ammonium acetate solution. A carbon-coated electron

microscope specimen support grid was touched on the surface and excess bacteria washed off before negatively staining with 1% (w/v) sodium phosphotungstate solution. Antisera for immune electron microscopy were prepared from pilus suspensions made as follows (Bradley, 1977). The plasmid-carrying host organism was harvested from six heavily inoculated plates into 2 ml phosphate-buffered saline. The suspension was homogenized in a Thomas Teflon pestle tissue grinder for 2 min at about 500 r.p.m. Large debris was removed by centrifugation at 7000 *g* for 15 min and the supernatant was inoculated into rabbits intravenously. Immune electron microscopy showed that the antisera raised contained antibodies to small cell and membrane fragments as well as to pili. Immune electron microscopy was by the grid-labelling method of Lawn (1967). Initially treatment with antiserum was for 10 min, but if no adsorbed antibodies were found in the electron microscope, this negative result was confirmed by treatment for 30 min. The 30 min period also permitted the detection of definite but weak labelling of heterologous pili.

RESULTS

Characteristics of transfer-derepressed plasmids

The method described for isolating derepressed plasmids was not successful with those of incompatibility group I₂, but was surprisingly efficient with most representatives of other I complex groups (Table 1). For example, R387 (IncK) gave 22/30 clones of BW86-1(R387) that

Table 1. *Characteristics of plasmids*

Inc group*	Plasmid†	Drug resistance markers‡	Host lysis by bacteriophages§			Stability of derepressed character (%)
			PR64FS	Iα	PR4	
I ₁	R64drd86	Sm Tc	+	+	-	100
I ₁ + B	R144	Km Tc	-	-	-	NT
I ₁ + B	R144drd3	Km Tc	+	+	-	100
I ₁ + B	R144drd86	Km Tc	+	+	-	100
I ₁ + B	R144drd86.1	Km Tc	-	-	-	100
I ₁ + B	R864adr86	Km	+	+	-	98
I ₁ + B	R864adr86.1	Km	-	-	-	100
I ₁ + B	R805adr86	Km	(+)¶	-	-	92
B	R621a	Tc	-	-	-	NT
B	R621pil ^c	Tc	(+)	+	-	99.1
B	R621adr86	Tc	+	+	-	100
B	R621adr86.1	Tc	-	-	-	100
B	R16drd86	Ap Sm Su Tc	-	-	-	100
B	RIP72drd86	Km	-	-	-	51
K	R387drd86	Cm Sm	(+)	-	-	16
K	pTM559drd86	Km	+	-	-	100
I ₅	pIE360drd86	Km Sm Su Tp	+	-	-	100
Z	pIE545drd86	Km Su	+	-	-	100
I ₂	R721	Sm Tp	-	-	-	NT
I ₂	R721pil ^c	Sm Tp	(+)	-	+	100
I ₂	R721pil ^c .4	Sm Tp	(+)	-	-	99.7
P	RP4	Ap Km Tc	-	-	+	100
W	Sa	Cm Km Sm Su	-	-	+	100

* The incompatibility groups listed do not all correspond with those given by Jacob *et al.* (1977) due to the reclassification of the I complex (see Introduction). Incompatibility groups are based on those of parent plasmids, derivatives presumably retaining the same phenotype.

† Designations: *drd86*, spontaneous transfer-derepressed plasmid prepared by temperature-selection in *E. coli* host strain BW86-1 (see Methods); suffix .1, host strain selected for resistance to RNA phage Iα so that no thin I₁ pili were determined; suffix .4, host strain selected for resistance to lipid phage PR4 so that no thick I₂ pili were determined; *pil^c*, 'pili-constitutive' mutant plasmids (authentic transfer-derepressed for R721*pil^c* only, see Results) isolated by R. W. Hedges; host strains, *E. coli* JE2571-1 or JE2571-2 (see Methods for phenotypes) for all tests in this table.

‡ Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Su, sulphonamides; Tc, tetracycline; Tp, trimethoprim.

§ By standard spot test (see Methods): +, complete or almost complete clearing; (+), partial distinctly hazy clearing; -, no clearing. For details of phages see Methods.

|| Percentage of clones with constitutive pilus synthesis after overnight culture in non-selective medium. For assay procedures see Methods. NT, not tested.

¶ Partial inhibition of growth by phage PR64FS was obtained using the cross-streak method for testing sensitivity (see Methods). This weak sensitivity was not detectable by the spot test.

Table 2. Transfer frequencies and conjugation systems of I-type plasmids

Inc group	Plasmid*	Selection†	Transfer frequencies‡		Frequency ratio plate/broth§
			Plate	Broth	
I ₁ + B	R144	Km	5.1×10^{-3}	4.6×10^{-3}	1.1
	R144 <i>drd</i> 3	Km	1.9×10^0	5.4×10^{-1}	3.5
	R144 <i>drd</i> 86	Km	1.2×10^0	9.3×10^{-1}	1.1
	R144 <i>drd</i> 86.1	Km	1.5×10^0	3.2×10^{-5}	46900
	R864 <i>adr</i> 86	Km	9.2×10^{-1}	6.1×10^{-1}	1.5
	R864 <i>adr</i> 86.1	Km	1.5×10^0	2.4×10^{-5}	62500
B	R621a	Tc	1.4×10^{-2}	1.2×10^{-2}	1.2
	R621 <i>apil</i> ^c	Tc	1.1×10^0	8.2×10^{-1}	1.3
	R621 <i>adr</i> 86	Tc	5.9×10^{-1}	3.7×10^{-4}	1594
	R621 <i>adr</i> 86.1	Tc	7.6×10^{-1}	3.4×10^{-5}	22400
	R16 <i>drd</i> 86¶	Tc	2.7×10^0	9.2×10^{-1}	2.9
	RIP72 <i>drd</i> 86	Km	2.8×10^{-1}	2.4×10^{-1}	1.2
K	pTM559 <i>drd</i> 86	Km	7.6×10^{-1}	6.6×10^{-1}	1.2
I ₅	pIE360 <i>drd</i> 86	Km	4.2×10^{-1}	7.2×10^{-1}	0.6
Z	pIE545 <i>drd</i> 86	Km	5.6×10^{-1}	6.6×10^{-1}	0.9
I ₂	R721	Tp	2.1×10^{-2}	1.5×10^{-3}	14
	R721 <i>pil</i> ^c	Tp	4.4×10^{-1}	6.6×10^{-2}	6.7
	R721 <i>pil</i> ^c .4	Tp	3.3×10^{-3}	3.0×10^{-4}	11

* Only plasmids designated *drd* or *pil*^c determined pili constitutively. Suffixes: .1, resistance to RNA phage Iα determined (no thin pili detectable); .4, resistance to lipid phage RP4 determined (no thick pili detectable).

† Abbreviations: Km, kanamycin ($100 \mu\text{g ml}^{-1}$); Tc, tetracycline ($25 \mu\text{g ml}^{-1}$, but $10 \mu\text{g ml}^{-1}$ for R16*drd*86); Tp, trimethoprim (1 mg ml^{-1}). Counterselection against donors was with nalidixic acid ($20 \mu\text{g ml}^{-1}$).

‡ Average frequencies from at least two experiments, transconjugants per donor h⁻¹. The recipient organism was JE2571-1 in all cases.

§ A high ratio indicated poor transfer in broth (surface-obligatory conjugation system); a ratio near 1 showed equal transfer efficiency in both environments (universal conjugation system).

¶ Approximately 90% of JE2571-2(R16*drd*86) clones were found to have lost all drug resistance markers when viable counts were carried out on non-selective plates, although constitutive pilus synthesis was retained (see text). Transfer frequencies were therefore calculated from viable counts on selective (tetracycline, $10 \mu\text{g ml}^{-1}$) plates.

transferred at a derepressed level by cross-streak mating with JE2571-2 (see Methods). With IncI₁ or IncI₁ + B plasmids, at least 15/20 clones of BW86-1(R⁺) growing at 37 °C were lysed by phages PR64FS or Iα indicating derepression. The method used more bacteria than other workers use, enabling a very low frequency of reversion to temperature-sensitivity to be detected.

Characteristics affecting this investigation were sensitivity to plasmid-specific bacteriophages (Table 1), stability of the derepressed character in host strain JE2571 (Table 1), and transfer frequency (Table 2). Plasmids not specifying sensitivity to phage PR64FS (similar to If1; Coetzee *et al.*, 1980) were: those which were constructed such that they did not determine thin flexible pili (suffix.1), RP4 (IncP), Sa (IncW), and the IncB plasmids R16*drd*86 and RIP72*drd*86. The last result agreed with previous findings (Coetzee *et al.*, 1982), but the unexpected specification of PR64FS sensitivity by IncK plasmids did not. Most plasmids found to suppress temperature-sensitivity in *E. coli dnaG3* mutants (Wilkins, 1975; Sasakawa & Yoshikawa, 1978; Dalrymple & Williams, 1982; Dalrymple *et al.*, 1982) provided stable spontaneous transfer-derepressed mutant plasmids. A curious exception was R16*drd*86. When tested for the stability of its derepressed character (see Methods), it was found that 90% of the JE2571-2(R16*drd*86) clones had lost all their drug resistance markers and yet retained the determinants of constitutive pilus synthesis. These variants of R16*drd*86 were presumably discrete transfer operons and might be equated with transferable plasmids isolated from cultures that had been stored since before the advent of antibiotics and had similar properties (Hughes & Datta, 1983). Predictably, plasmids that were less than 100% stable did not specify complete lysis (using the spot test) by phage PR64FS (Table 1), indicating a lack of sufficient phage receptors (thin flexible pili). The IncI₂ plasmids R721*pil*^c and R721*pil*^c.4 (authentic transfer-derepressed mutants) were exceptions. '*drd*86' plasmids transferred at derepressed frequencies

(Table 2) including some which were significantly unstable such as RIP72*drd86*, although its transfer frequencies were much less than those of stable plasmids.

The derepressed characters of spontaneous transfer-derepressed plasmids without thin I₁ pili (designated *drd86.1*) were stable in host strain JE2571, though as in most other cases, purification of strains was required after storage by freezing. In order to ascertain whether the determinants for thin I₁ pilus synthesis had become repressed or whether they were completely inoperative (e.g. deleted), a temporary derepression spot test was done (Bradley & Fleming, 1983). A drop of phage I α suspension was placed on a transconjugant-selecting plate (kanamycin + rifampicin) covered with a soft agar layer containing a mixture of strains J53(R144*drd86.1*) and J53-2. There was no lysis, showing that the possible repression of the phage I α receptors, thin I₁ pili, was not lifted temporarily as was the case with J53(R144) (repressed wild-type), where phage I α lysed the plate. Plasmids determining thin I₁ pili only could not be isolated (see Methods).

Occurrence of thick I₁-type pili

Straight knobbed thick I₁-type pili like those described by Bradley (1983*a*) were determined by all plasmids listed in Table 1 in host strain *E. coli* JE2571-2, and occasionally in *E. coli* BW86-1 (examples illustrated below). Exceptions were R721 (IncI₂) and derivatives, RP4 (IncP) and Sa (IncW). R721 *pil^c* determined thick I₂ pili which were morphologically different from thick I₁ pili (Bradley & Coetzee, 1982). pIE360 and pIE545 represented the new groups IncI₅ and IncZ, respectively (Tschäpe & Tietze, 1981, 1983); preliminary tests indicated that thin I-type pili were determined by both plasmids (Fig. 1*c*, pIE360). Spontaneous transfer-derepressed mutants specified sensitivity to phage PR64FS, confirming this. Thick I₁-type pili were also determined by both plasmids (Fig. 1*c*, *d*, pIE360). In addition, thick I₁-type pili were synthesized by *S. typhimurium* M827-2(R483*drd3*), *E. coli* J53-1(R483*drd3*), *S. typhimurium* M827-2(R163*drd7*) (Fig. 1*b*), and *S. typhimurium* SQ1139-2(R621*apil^c*), to quote but a few examples of authentic transfer-derepressed mutants.

The undescribed wild-type plasmid R1535 determined thick and thin I₁-type pili in fairly large numbers in host strain *S. typhimurium* SQ1139 (not illustrated), but not in *E. coli* strains. SQ1139-1(R1535) alone was visibly lysed in the spot test by RNA phage I α , which adsorbs specifically to thin I₁-type pili (Coetzee *et al.*, 1982), indicating that the plasmid determined them constitutively and so was presumably transfer-derepressed in this host. Its IncI₁ designation (R. W. Hedges, personal communication) was also confirmed. Using the temporary derepression growth method (Bradley, 1980*b*), R1539 (IncI₁, R. W. Hedges, personal communication) also determined thin and thick I₁-type pili. A transfer system with two different pilus types is thus probably typical for all I complex plasmids including those of incompatibility groups B, K and Z. The I₁ morphological form of thick pilus is common to all except IncI₂ plasmids, regardless of the bacterial host species.

Morphology of thick I₁-type pili

The pili of all the I complex plasmids studied here were basically similar in form except for those determined by R721 *pil^c* (IncI₂). Designated as thick I₁-type pili, their principal features were a large terminal structure of variable shape, a straight thick shaft, and a pointed distal tip. They were undoubtedly of the 'rigid' category (Bradley, 1980*a*). While the following illustrations were derived from representative plasmids of a few incompatibility groups (wild-type parent plasmids are indicated after the figure numbers), most features could be found amongst the pili of all. A small proportion of thick I₁-type pili were short nail-like objects (Fig. 1*a*, RIP72) like some M pili (Bradley, 1980*b*) and *Pseudomonas* conjugative pili (Bradley, 1983*b*). Small knobs were also infrequent (Fig. 1*b*, R163); large round knobs were most common (Fig. 1*c*, pIE360). Two examples of a single knob with two pili were found (not illustrated), determined by R144*drd86.1*, which was constructed such that it did not determine thin I pili. Rarely, the round knobs had membraneous structures attached. Membrane-like structures without knobs were common, some having a solid appearance (Fig. 1*d*, pIE360), and some resembling vesicles formed from a unit membrane, being closed off or open at one or both ends (Fig. 1*e, f*, R144;

Fig. 1. Thick I_1 -type pili determined by various plasmids. (a) An unusual thick B pilus resembling a nail from confluent transconjugant growth of the plate mating *E. coli* BW86-1(RIP72*drd*86) $\times$ JE2571-2 (see Methods). (b) Pilus with a small knob determined by *S. typhimurium* M827-2(R163*drd*7). (c) A typical thick I_1 -type pilus and thin I pili synthesized by *E. coli* JE2571-2(pIE360*drd*86). The thick pilus is about twice the diameter of the thin ones. (d) An asymmetrical knob on a thick pilus from the same preparation. (e) Thick I_1 pilus from the transconjugant growth of the cross-streak mating (see Methods) of *E. coli* BW86-1(R144*drd*86.1) $\times$ JE2571-2. The knob is some form of membrane open at one end. (f) As (e) with the membranous knob open at both ends. Bar represents 100 nm for all micrographs.

Fig. 2, RIP72). The distinctive membrane from which pili arose allowed the detection of very short ones (Fig. 2 illustrates a series). The pilus in Fig. 2(c) is better described as a stylus (at the left hand end of the vesicle). The existence of very short pili or stubs was suspected by Dennison & Baumberg (1975) for IncN plasmids, but only long ones were subsequently identified

Fig. 2. Thick I_1 -type pili from confluent transconjugant growth of the plate mating *E. coli* BW86-I (RIP72*drd*86) $\times$ JE2571-2. The series shows pili of decreasing lengths attached to membraneous structures. The shortest, in (c), a 'stylus', can be clearly identified at the left hand end of the membrane. Bar represents 100 nm for all micrographs.

(Bradley, 1979). This is therefore the first illustration of pilus stubs or styli, which were common. A stylus is best defined as the tapered point of a pilus with no visible length of parallel-sided shaft.

A preponderance of pili with large vesicles was found in pilus preparations from plates using the temporary derepression growth procedure (Bradley, 1980*b*); there were relatively few with normal plate cultures. The selecting drugs used with the former method often caused some cell lysis which probably mediated the release of pili with vesicles attached.

Serological relationships of thin and thick I pili

Immune electron microscopy tests were done: (i) to confirm previous tests with thin I-type pili (Bradley, 1980*a*) using the larger numbers and cleaner preparations available with spontaneous transfer-derepressed mutant plasmids; (ii) to ascertain what relationship existed between the thin pili of pIE360 (IncI₅) and pIE545 (IncZ) with those of the I complex, IncB and IncK plasmids already investigated (Bradley, 1980*a*); and (iii) to ascertain whether or not serological relationships amongst thick I-type pili corresponded with those for thin I-type pili. Tests were limited to a manageable number, and also because some plasmids determined too few thick pili. It is extremely unlikely that two morphologically different types of pilus could be serologically related, nevertheless serological differentiation was confirmed for thick and thin I_1 -type pili with the following examples. Antiserum containing antibodies to only thick R144 pili did not label thin R144 pili. pIE360*drd*86 determined relatively few thick pili, and its antiserum labelled only thin, not thick, pIE360 pili.

The results shown in Table 3 confirmed the existence of the I_1 and I_2 serotypes (Bradley, 1980*a*). However, the relationships originally observed between R64 (IncI₁) and R16 (IncB) thin pili were not confirmed satisfactorily, although the low adsorption level of R64 pilus antibodies by R16 pili was increased by extending treatment with antiserum from 10 to 30 min. The thin pili of pIE360 (IncI₅) and pIE545 (IncZ) were related to one another and to R16 (IncB) and pTM559 (IncK) pili, but not to thin I_2 pili or the thin pili of several plasmids with the IncI₁ phenotype. Results with thick pili gave three clear serotypes: the I_1 serotype contained thick pili

Table 3. Serological relationships of pili

Serological relationships between the pili of well-studied plasmids were assessed by immune electron microscopy (see Methods): +, level of antibody labelling as with homologous pili; (+), less labelling than with homologous pili; -, no labelling.

Inc group	Plasmid	Antiserum to thin pili of*:				Antiserum to thick pili of*:		
		R64 (I ₁)	R16 (B)	pIE545 (Z)	TP114 (I ₂)	R144 (I ₁ + B)	R16 (B)	pIE545 (Z)
I ₁	R64 <i>drd86</i>	+	-	-	-	+	-	-
I ₁ + B	R144 <i>drd86</i>	+	+	-	-	+	-	-
I ₁ + B	R864 <i>adrd86</i>	+	-	-	-	(+)	-	-
I ₁ + B	R805 <i>adrd86</i>	(+)†	+	-	(+)†	-	+	(+)
B	R621 <i>adrd86</i>	+	+	-	-	(+)	-	-
B	R16 <i>drd86</i>	(+)	+	(+)†	-	-	+	+
K	pTM559 <i>drd86</i> ‡	(+)	+	+	-	-	+	+
I ₅	pIE360 <i>drd86</i>	-	+	+	-	-	+	+
Z	pIE545 <i>drd86</i>	-	(+)	+	-	-	+	+
I ₂	R721 <i>pil</i> ^c	-	-	-	+	-	-	-

* Incompatibility groups indicated in parentheses. With the exception of antiserum to R144 thick pili, antisera contained antibodies to both thick and thin pili, and the results are separated for clarity. Previously published reactions between the thin pili of R64, R805a, R621a, R16, pTM559 and R721 with antisera to the thin pili of R64, R16 and TP114 (Bradley, 1980*a*) were repeated using the thin pili determined by spontaneous (*drd86*) transfer-derepressed mutant plasmids.

† Reactions detectable only after labelling for 30 min.

‡ The prototype IncK plasmid mutant R387*drd86* did not determine enough thick pili for the test because of instability (see Table 1).

of R64 (IncI₁), R144 (IncI₁ + B), R864a (IncI₁ + B) and R621a (IncB); the B serotype contained pili of R805a (IncI₁ + B), R16 (IncB), pTM559 (IncK), pIE360 (IncI₅) and pIE545 (IncZ); thick I₂ pili, which are morphologically different from thick I₁-type pili (Bradley & Coetzee, 1982), were unrelated to all the others (for relevance to classification, see Discussion).

Additional tests were done to establish whether or not thick I-type pili were related to any other type of rigid pilus. The thick pili of R64, R864a, R805a, R621a, R16 and R721 were each tested against previously described antisera (Bradley, 1980*a*) to rigid pili of R831b (IncM), N3 (IncN), pAr-32 (IncU), Sa (IncW) and R775 (IncR775). In no case was there any antibody-labelling, indicating that the three serotypes of thick I-type pili were unrelated to any other known rigid type.

Key tests are illustrated in Fig. 3. They show the degree of antibody adsorption obtained, and that, regardless of whether or not the pilus shafts were labelled with antibodies, the pilus knobs or terminal membrane structures always were (sera contained antibodies to bacterial membranes; see Methods). Therefore the knobs were not specifically plasmid-determined (see Discussion).

Plasmid transfer frequencies for liquid and solid environments

Bradley *et al.* (1980) identified three types of conjugation system: surface-obligatory, surface-preferred and universal (equal transfer in liquid or on solid). Table 2 identifies such systems for the present plasmids by the ratios of their transfer frequencies on plates and in broth; the higher the ratio, the more surface-dependent the system. Two 'sets' of mutant plasmids were available for R144 and R621a: the wild-type (transfer-repressed), an authentic transfer-derepressed mutant, a spontaneous transfer-derepressed mutant (*drd86*), and a similar mutant with thick pili only (suffix 1, see Methods). For R144, the three plasmids determining both thick and thin pili had universal conjugation systems (transfer frequency ratios near 1), but when thick pili only were present (R144*drd86*.1), a surface-obligatory system was found (Table 2). Spontaneous transfer-derepressed mutants of R864a with and without thin pili gave similar results. However, with the R621a set of plasmids, a surface-obligatory conjugation system was determined by R621a*drd86* in spite of the fact that both thick and thin pili were synthesized constitutively. All

Fig. 3. Key examples showing serological relationships of thick I_1 -type pili. (a) Pilus synthesized by *E. coli* JE2571-2(R621adrd86.1) weakly labelled with antibodies to R144 thick pili. (b) Thick B pilus synthesized by JE2571-2(R16drd86) treated with antiserum to R144 thick pili. Antibodies are only adsorbed to the pilus knob. (c) Pilus synthesized by JE2571-2(R805adrd86) treated with antiserum to R144 thick pili. Again only the pilus knob is labelled. (d) Thick K pilus synthesized by JE2571-2(pTM559drd86) treated with antiserum to pIE545 thick (and thin) pili for 10 min showing antibody adsorption to the pilus at the same level as to homologous pili. Only a small part of the large knob is included. (e) Pilus synthesized by JE2571-2(pIE360drd86) treated with antiserum to pIE545 thick pili for 30 min showing more antibody adsorption than in (d). Bar represents 100 nm for all micrographs.

other spontaneous transfer-derepressed plasmids had both pilus types and specified universal conjugation systems.

The Inc I_2 plasmid R721 did not form a spontaneous derepressed mutant in strain BW86-1, but the authentic derepressed mutant R721c and the wild-type R721 had universal conjugation systems, albeit with rather high plate/broth transfer frequency ratios. R721c.4, selected by resistance to thick I_2 pilus phage PR4 (see Methods), retained constitutive synthesis of thin I_2 pili, but there were no thick I_2 pili visible in the electron microscope on strain JE2571-2(R721c.4). However, when this strain was crossed with JE2571-1 using the temporary derepression method of growth (Bradley, 1980b), one or two thick pili were found on each electron microscope specimen support grid square. Their synthesis was thus repressed. R721c.4 transferred as a universal conjugation system, but at low frequencies (Table 2), even though its numerous thin pili were functional as indicated by specified sensitivity to phage

Table 4. Effect of constitutively synthesized thin I_2 pili on the surface mating characteristics of RP4 and Sa

Donor	Recipient	Selection*	Transfer frequencies†		Frequency ratio plate/broth‡
			Plate	Broth	
JE2571(RP4)	JE2571-1	Km	1.4×10^0	5.6×10^{-4}	2500
JE2571(RP4)	JE2571-1(R721pil ^c .4)§	Km	9.8×10^{-1}	4.9×10^{-2}	20
JE2571-2(R721pil ^c .4, RP4)	JE2571-1¶	Km	2.0×10^{-1}	4.2×10^{-3}	48
JE2571-2(R721pil ^c .4, RP4)	JE2571-1(R721pil ^c .4)	Km	1.1×10^{-1}	6.4×10^{-3}	17
JE2571-2(Sa)†	JE2571-1	Cm	1.2×10^0	5.0×10^{-5}	24000
JE2571-2(Sa)	JE2571-1(R721pil ^c .4)	Cm	2.2×10^0	2.0×10^{-1}	11
JE2571-2(R721pil ^c .4, Sa)	JE2571-1¶	Cm	1.0×10^0	2.3×10^{-2}	43
JE2571-2(R721pil ^c .4, Sa)	JE2571-1(R721pil ^c .4)	Cm	1.6×10^0	1.1×10^{-1}	15
JE2571-2(R721pil ^c .4)§	JE2571-1	Tp	3.3×10^{-3}	3.0×10^{-4}	11

* Abbreviations: Cm, chloramphenicol ($25 \mu\text{g ml}^{-1}$); Km, kanamycin ($100 \mu\text{g ml}^{-1}$); Tp, trimethoprim (1 mg ml^{-1}). Counterselection against donors was with nalidixic acid ($20 \mu\text{g ml}^{-1}$).

† Average frequencies from at least two experiments, transconjugants per donor h^{-1} . The four determinations for JE2571-2(Sa) $\times$ JE2571-1 included two published previously (Bradley *et al.*, 1980).

‡ A high ratio indicated a greater efficiency for surface mating, and a lower one demonstrated a proportional improvement for liquid matings when the thin I_2 pili of R721pil^c.4 were present.

§ R721pil^c.4 determined thin I_2 pili constitutively (by electron microscopy) and yet transferred at low frequencies (results from Table 2). No thick I_2 pili could be identified.

¶ These were the only instances where R721pil^c.4 might have contributed to the transfer of RP4 or Sa. R721pil^c.4-mediated transfer was detected by testing transconjugants for resistance to trimethoprim; the very small corrections to the transfer frequencies have been included (see text). They were consistent with the low transfer frequencies of the plasmid on its own (see footnote §).

PR64FS (Table 1). These observations indicate that thick I_2 pili rather than thin I_2 pili functioned as mating organelles. If thin I_2 pili had mediated plasmid transfer, derepressed frequencies should have been obtained. Also, thick I_1 -type pili on their own mediated typical surface-obligatory conjugation systems, and thin flexible I_1 -type pili converted these to universal systems.

Conjugation systems of RP4 (IncP) and Sa (IncW) in the presence and absence of constitutively-synthesized thin I_2 pili

Plasmids RP4 (IncP) and Sa (IncW) determine surface-obligatory conjugation systems, the former with thin rigid pili, transferring about $2000 \times$ better on plates than in broth, and the latter with thicker rigid pili, transferring about $36000 \times$ better (Bradley *et al.*, 1980). To test the hypothesis that the thin I_2 pili of R721pil^c.4 could reduce these ratios significantly, comparative surface and liquid matings were done for RP4 and Sa with thin pili on the recipient strains only, the donors only, and on both recipients and donors. The results in Table 4 show that thin I_2 pili completely altered the characteristics of the original conjugation systems. Transfer efficiency in broth became almost as good as on plates [transfer frequency ratios were about 10-fold greater than for normal universal conjugation systems (Bradley *et al.*, 1980), but close to that of R721pil^c.4 on its own]. Thin I_2 pili thus acted as stabilizers for the RP4 and Sa mating systems.

The concern with these experiments was that R721pil^c.4 was self-transmissible, although at low (repressed) frequencies. It could have contributed to the transfer frequencies of the other plasmids, but only when it was not present in the recipients (Table 4). To check these two results [JE2571-2(R721pil^c.4, RP4) $\times$ JE2571-1 and JE2571-2(R721pil^c.4, Sa) $\times$ JE2571-1], 100–700 transconjugants were scored for resistance to trimethoprim (R721pil^c.4 drug resistance phenotype) using replica plating. With the plate matings, none was detected, but with the broth matings about 2% of RP4 transconjugants and 4% of Sa transconjugants were resistant to trimethoprim (corrections included in Table 4). It would have been better to use a non-transferable plasmid determining only thin pili constitutively, but this could not be constructed.

DISCUSSION

The present objective was to study pilus function, but spontaneous transfer-derepressed versions of an unexpectedly large number of plasmids were isolated. The genetic basis for this is as follows (see Dalrymple *et al.*, 1982). During conjugal DNA transfer, a single strand of plasmid DNA moves from donor to recipient through a membrane-located transfer complex (Willets, 1981). Synthesis of the cDNA strand to regenerate the double-stranded plasmid molecule requires a DNA primase, which is normally that synthesized by the bacterial *dnaG* gene product for chromosomal DNA replication. However, certain plasmids determine a similar primase, normally under repression by the transfer regulator, to replace that lacking in *dnaG3* mutants which are temperature-sensitive (only residual DNA synthesis can occur at low temperatures). Complete suppression of temperature-sensitivity requires constitutive synthesis of the primase, and a degree of derepression of the transfer genes greater than that normally found in authentic transfer-derepressed plasmid mutants (Sasakawa & Yoshikawa, 1978). Thus selection for growth at 42 °C of a plasmid-carrying *dnaG3* mutant with 100% growth efficiency also selects a spontaneous transfer-derepressed plasmid; such derepression should be stable so long as temperature-selective pressure is maintained in that host. Remarkably, a high degree of spontaneous derepression stability was found in strain JE2571, a host without any *dnaG* mutation, for the majority of plasmids. This resulted in the constitutive synthesis of both thin and thick pili and a manifestation of plasmid-determined sensitivity to bacteriophages.

Unexpectedly, IncK plasmids determined sensitivity to phage PR64FS, which defines the I complex plasmids. Plasmids of the new groups IncI₅ and IncZ also determined PR64FS sensitivity, so their inclusion in the I complex is valid.

Pilus serotypes are related to incompatibility phenotypes (Bradley, 1980*a*) and so are a useful criterion in classification. Previous extensive tests on the thin pili of I complex plasmids (Bradley, 1980*a*) were not all confirmed. The equal serological relationship between B (R16) and I₁ (R64) pili was only found with anti-R64 pilus serum at a low level of antibody adsorption. Likewise, the reactions of R805a (IncI₁ + B) pili with antisera to both thin I₁ and thin I₂ pili were not as clearcut as before. I₂ pili alone were serologically distinctive, being different from all others. Relationships within the original I₁ serotype were vague and inconsistent. This may be because thin I-type pili appear to be poor antigens and immune electron microscopy is difficult (Bradley, 1980*a*), nevertheless serological relationships of pili do reflect the often equally vague incompatibility relationships amongst I complex plasmids outside the I₂ group. However, better-defined results with thick I-type pilus serotypes indicated a similarity between the IncZ representative and IncB, IncK and IncI₅ plasmids. IncI₁ and IncI₁ + B plasmids, with the exception of R805a, did not determine thick pili of the B serotype, but of the I₁ serotype; this is consistent with their dual incompatibility.

The appearance of thick I₁-type pili (includes thick B, I₅, K and Z pili) was unlike that of any other conjugative pili because of the pleomorphic vesicular structures from which they arose. Their membrane-like characteristics suggested that they consisted of outer, or more likely inner, bacterial membrane, perhaps fragments of mesosomes. The only reasonably certain aspect of their origin was that they were not plasmid-determined since the vesicles all adsorbed antibodies regardless of antiserum. F pili often have terminal knobs consisting of cell envelope material (Lawn & Meynell, 1970).

Plate mating experiments demonstrated clearly that thick I-type pili mediated surface mating and that thin I-type pili stabilized mating aggregates to the extent that conjugation could occur equally efficiently in the less favourable liquid environment. Indeed it seems highly likely that thin I-type pili are not directly involved in the formation of a conjugation bridge; fertile cell contact is almost certainly mediated by the thick pili only. The one instance where thin pili did not provide a universal conjugation system with equal transfer on solid and in liquid was with R621*adrd*86. This could be explained by the presence of too few thin pili for the numbers of thick pili, but pilus counts could not be done because thin pili aggregated strongly. While adding further evidence for the basic hypothesis that conjugative pili are in fact involved in conjugation, which has only been indicated directly for a few plasmids (Ippen & Valentine, 1967; Bradley, 1978), these results confirm the existence of a kind of conjugation system

requiring two types of pilus, and occurring in two stages. The plasmids investigated here were artificially derepressed for transfer whereas in nature they are repressed. However, the relevance of the present work lies in the temporary lifting of repressor function for a significant number of generations after transfer (see Bradley & Fleming, 1983) during which time individual cells would contain plasmids expected to behave like authentic or spontaneous transfer-derepressed mutants. Thus the first stage of transfer would consist of conjugation at low frequency mediated by cells that produced only a few pili. One cannot speculate as to whether sufficient thin pili would be synthesized to stabilize mating pairs. Derepressed expression of the plasmid transfer operon would then occur in the newly infected cells to give constitutive pilus synthesis and high frequency transfer.

In effect, three different mechanisms have now been identified for achieving cell contact in conjugation: by rigid pili in surface mating, by a combination of rigid pili and thin flexible pili (universal mating), and by thick flexible pili (usually universal mating). Thus, thick flexible pili combine the functions of rigid and thin flexible pili into one type of organelle. An important question to be answered is whether the same receptors are used by all the pili or whether two pilus systems require different receptors.

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REFERENCES

- BACHMANN, B. M. (1972). Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriological Reviews* **36**, 525-557.
- BARTH, P. T. (1979). Plasmid RP4, with *Escherichia coli* DNA inserted *in vitro*, mediates chromosomal transfer. *Plasmid* **2**, 130-136.
- BOULNOIS, G. J. & WILKINS, B. M. (1979). A novel priming system for conjugal synthesis of an IncI plasmid in recipients. *Molecular and General Genetics* **175**, 275-279.
- BRADLEY, D. E. (1967). Ultrastructure of bacteriophages and bacteriocins. *Bacteriological Reviews* **31**, 230-314.
- BRADLEY, D. E. (1977). Characterization of pili determined by drug resistance plasmids R711b and R778b. *Journal of General Microbiology* **102**, 349-363.
- BRADLEY, D. E. (1978). W pili: characteristics and interaction with lipid phages specific for N, P-1, and W group plasmids. In *Pili*, pp. 339-353. Edited by D. E. Bradley, E. Raizen, P. Fives-Taylor & J. Ou. Washington, DC: International Conferences on Pili.
- BRADLEY, D. E. (1979). Morphology of pili determined by the N incompatibility group plasmid N3, and interaction with bacteriophages PR4 and IKe. *Plasmid* **2**, 632-636.
- BRADLEY, D. E. (1980a). Morphological and serological relationships of conjugative pili. *Plasmid* **4**, 155-169.
- BRADLEY, D. E. (1980b). Determination of pili by conjugative bacterial drug resistance plasmids of incompatibility groups B, C, H, J, K, M, V, and X. *Journal of Bacteriology* **141**, 828-837.
- BRADLEY, D. E. (1983a). Derepressed plasmids of incompatibility group I₁ determine two different morphological forms of pilus. *Plasmid* **9**, 331-334.
- BRADLEY, D. E. (1983b). Specification of the conjugative pili and surface mating systems of *Pseudomonas* plasmids. *Journal of General Microbiology* **129**, 2545-2556.
- BRADLEY, D. E. & COETZEE, J. N. (1982). The determination of two morphologically distinct types of pilus by plasmids of incompatibility group I₂. *Journal of General Microbiology* **128**, 1923-1926.
- BRADLEY, D. E. & FLEMING, J. (1983). Incompatibility group identification for repressed plasmids using host cell lysis by specific bacteriophages. *Journal of Microbiological Methods* **1**, 171-176.
- BRADLEY, D. E. & RUTHERFORD, E. L. (1975). Basic characterization of a bacteriophage specific for plasmids of the P, N and W compatibility groups. *Canadian Journal of Microbiology* **21**, 152-163.
- BRADLEY, D. E., TAYLOR, D. E. & COHEN, D. R. (1980). Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. *Journal of Bacteriology* **143**, 1466-1470.
- BRADLEY, D. E., COETZEE, J. N. & HEDGES, R. W. (1983). IncI₂ plasmids specify sensitivity to filamentous phage IKe. *Journal of Bacteriology* **154**, 505-507.
- COETZEE, J. N., ŠIRGEL, F. A. & LECATSAS, G. (1980). Properties of a filamentous phage which adsorbs to pili coded by plasmids of the IncI complex. *Journal of General Microbiology* **117**, 547-551.
- COETZEE, J. N., BRADLEY, D. E. & HEDGES, R. W. (1982). Phages I_α and I₂-2: plasmid-dependent bacteriophages. *Journal of General Microbiology* **128**, 2797-2804.
- DALRYMPLE, B. P. & WILLIAMS, P. H. (1982). Detection of primase specified by IncB plasmid R864a. *Journal of Bacteriology* **152**, 901-903.

- DALRYMPLE, B. P., BOULNOIS, G. J., WILKINS, B. M., ORR, E. & WILLIAMS, P. H. (1982). Evidence for two genetically distinct DNA primase activities specified by plasmids of the B and I incompatibility groups. *Journal of Bacteriology* **151**, 1-7.
- DATTA, N. (1979). Plasmid classification: incompatibility grouping. In *Plasmids of Medical, Environmental and Commercial Importance*, pp. 3-12. Edited by K. N. Timmis & A. Puhler. Amsterdam: Elsevier/North-Holland.
- DENNISON, S. & BAUMBERG, S. (1975). Conjugational behaviour of N plasmids in *Escherichia coli* K12. *Molecular and General Genetics* **138**, 323-331.
- HUGHES, V. M. & DATTA, N. (1983). Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature, London* **302**, 725-726.
- IPPEN, K. A. & VALENTINE, R. C. (1967). The sex hair of *E. coli* as a sensory fiber, conjugation tube or mating arm? *Biochemical and Biophysical Research Communications* **27**, 674-680.
- JACOB, A. E., SHAPIRO, J. A., YAMAMOTO, L., SMITH, I. D., COHEN, S. N. & BERG, D. (1977). Appendix B: bacterial plasmids. In *DNA Insertion Elements, Plasmids and Episomes*, pp. 606-704. Edited by A. I. Bukhari, J. A. Shapiro & S. L. Adhya. Cold Spring Harbor: Cold Spring Harbor Laboratory.
- LAWN, A. M. (1967). Simple immunological labelling method for electron microscopy and its application to the study of filamentous appendages of bacteria. *Nature, London* **214**, 1151-1152.
- LAWN, A. M. & MEYNELL, E. (1970). Serotypes of sex pili. *Journal of Hygiene* **68**, 683-694.
- MEYNELL, G. G. & LAWN, A. M. (1968). Filamentous phages specific for the I sex factor. *Nature, London* **217**, 1184-1186.
- MONTI-BRAGADIN, C., CINCO, C. M.-B. C., BABUDRI, N. & MANZO, A. (1979). Incompatibility of R plasmids from clinical material. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* (Abteilung I, Originale A) **245**, 490-494.
- SASAKAWA, C. & YOSHIKAWA, M. (1978). Requirements for suppression of a *dnaG* mutation by an I-type plasmid. *Journal of Bacteriology* **133**, 485-491.
- SPRATT, B. G., ROWBURY, R. H. & MEYNELL, G. G. (1973). The plasmid of *Salmonella typhimurium* LT2. *Molecular and General Genetics* **121**, 347-353.
- STANISICH, V. (1974). The properties and host range of male-specific bacteriophages of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **84**, 332-342.
- TSCHÄPE, H. & TIETZE, E. (1981). Genetische und molekulare Grundlagen der Plasmid-Spezies-Hypothese. *Biologisches Zentralblatt* **100**, 353-384.
- TSCHÄPE, H. & TIETZE, E. (1983). Characterization of conjugative plasmids belonging to a new incompatibility group (IncZ). *Zeitschrift für Allgemeine Mikrobiologie* **23**, 393-401.
- WILKINS, B. M. (1975). Partial suppression of the phenotype of *Escherichia coli* K-12 *dnaG* mutants by some I-like conjugative plasmids. *Journal of Bacteriology* **122**, 899-904.
- WILLETTS, N. (1981). Sites and systems for conjugal DNA transfer in bacteria. In *Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids*, pp. 207-215. Edited by S. B. Levy, R. C. Clowes & E. L. Koenig. New York: Plenum Press.