JOURNAL OF BACTERIOLOGY, Sept. 1980, p. 1466-1470
0021-9193/80/09-1466/05$02.00/0

Vol. 143, No. 3

Specification of Surface Mating Systems Among Conjugative Drug Resistance Plasmids in *Escherichia coli* K-12

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Representative plasmids for most incompatibility groups in *Escherichia coli* K-12 were transferred to a "bald" strain to compare transfer frequencies for liquid and solid media. Standard broth matings were used for a liquid environment, but for solid surface mating, conjugation was allowed to take place on nutrient plates before washing off the cells for transconjugant selection on plates containing appropriate drugs. Plasmids that determine rigid pili transferred at least 2,000× better on plates than in broth. Some plasmids that determine thick flexible pili transferred 45 to 470× better, whereas others transferred equally well in both environments, as did plasmids of the I complex, which determine thin flexible pili. These results clearly distinguished a number of surface mating systems where most plasmids were derepressed for transfer and determined conjugative pili constitutively. The temperature-independent IncH2 plasmid R831b transferred best on plates, but other IncH plasmids transferred equally well in broth. This inconsistency led to the reclassification of R831b as IncM.

Transferable drug resistance plasmids are classified according to their inability to coexist in the same cell (incompatibility). Plasmids within an incompatibility group (Inc) determine morphologically similar serologically related pili. There are three basic morphological forms: thin (6-nm diameter) flexible, thick (9-nm diameter) flexible, and rigid (4; D. E. Bradley, Plasmid, in press). Representative plasmids of incompatibility groups N and P, which determine rigid pili, were reported to transfer better on solid surfaces than in liquids (9). Using a delayed transconjugant selection procedure, Dennison and Baumberg (9) obtained transfer frequencies of 8.3×10^{-4} transconjugants per donor for R46 (IncN) and 2.4×10^{-3} transconjugants per donor for RP4 (IncP) in 30-min matings, representing 55- and 32-fold improvements, respectively, over normal immediate transconjugant selection. However, Bradley and Chaudhuri (5) obtained frequencies near 1 transconjugant per donor for N3 (IncN) and RP1 (IncP) in a preliminary investigation with a simple 1-h surface mating procedure. Our study applies this procedure to representative plasmids for the majority of incompatibility groups so far identified.

It was thought that all plasmids determining flexible pili would transfer equally well on a solid or in a liquid, whereas all plasmids with rigid pili would transfer best on a solid surface. However, like plasmids with rigid pili, some with flexible pili also transferred best on solid media. The characterization of preferred mating environment, together with other properties such as pilus morphology, should provide a quick means of identifying a plasmid by limiting the number of possible incompatibility groups for which tests would be required. We include an example of this application.

MATERIALS AND METHODS

Plasmids. The plasmids used are listed in Table 1. Because of the large number, references are not cited individually, since they have been listed by Shapiro (16) with the following exceptions: pTM559 (13), MIP233 (15; supplied by L. Le Minor), TP228 (isolated and supplied by J. Frost; see reference 4), RA3 (1), and R831b (14). N. Datta and R. W. Hedges supplied all plasmids other than those indicated above together with N3 (from R. V. Iyer), RP1 (from R. H. Olsen), and TP114 (from H. R. Smith). The possible effects on transfer frequencies of type I pili and flagella were eliminated by transferring all plasmids to the nalidixic acid-sensitive *Escherichia coli* K-12 "bald" strain JE2571 Nal^s (*leu thr str fla pil*).

Matings. Plasmid-carrying JE2571 Nal^s strains were used as donors; the recipient for all matings was a nalidixic acid-resistant derivative of JE2571 Nal^s (JE2571 Nal^r). Transfer frequencies were compared for liquid and solid environments for each plasmid as follows. All matings (and growth) were in or upon brain heart infusion broth or agar (BBL Microbiology Systems). The selecting drugs for the plasmids are listed in Table 1, and all counterselections were carried out with nalidixic acid (20 μg/ml), which is an immediate inhibitor of bacterial DNA synthesis, and con-

TABLE 1. *Comparison of transfer frequencies for plate and broth matings*

Pilus morphology[a]	Inc	Plasmid	Selecting drug[b]	Pilus synthesis[c]	Transfer frequencies[d]		Ratio of frequencies plate/broth	Average frequency ratio[e]
					Plate	Broth		
Thin flexible	I_α	R64	Tc	Repressed	1.0×10^{-3}	1.4×10^{-3}	0.7	0.9
					1.0×10^{-3}	9.2×10^{-4}	1.1	
	I_2	TP114	Km	Repressed	1.6×10^{-2}	1.5×10^{-2}	1.1	1.1
					1.9×10^{-2}	1.7×10^{-2}	1.1	
	K	pTM559	Km	Repressed	3.7×10^{-4}	7.7×10^{-4}	0.48	0.51
					5.8×10^{-4}	1.1×10^{-3}	0.53	
Thick flexible	C	RA1	Tc	Repressed	8.7×10^{-3}	9.8×10^{-5}	89	45[e]
					1.7×10^{-4}	5.9×10^{-6}	29	
	D	R711b	Km	Constitutive	6.4×10^{-4}	4.7×10^{-6}	140	180
					3.2×10^{-3}	1.5×10^{-5}	210	
	FII	R100	Tc	Repressed	1.3×10^{-4}	1.3×10^{-4}	1.0	0.73
					2.9×10^{-4}	6.5×10^{-4}	0.45	
	H1	R27[f]	Tc	Repressed	2.0×10^{-7}	6.0×10^{-8}	3.0	5.5
					1.6×10^{-7}	2.0×10^{-8}	8.0	
	H2	R478[f]	Tc	Repressed	6.6×10^{-5}	2.0×10^{-4}	0.33	0.29
					1.0×10^{-4}	4.0×10^{-4}	0.25	
	H3	MIP233[f]	Te	Repressed	4.7×10^{-6}	1.0×10^{-6}	4.7	2.5
					2.2×10^{-6}	6.2×10^{-6}	0.35	
	J	R391	Km	Repressed	1.9×10^{-6}	1.7×10^{-6}	1.1	0.9
					1.4×10^{-6}	2.1×10^{-6}	0.7	
	T	Rts1[f]	Km	Constitutive	2.8×10^{-2}	7.4×10^{-5}	380	265
					7.6×10^{-3}	5.1×10^{-5}	150	
	T	R394	Km	Constitutive	5.4×10^{-1}	6.5×10^{-3}	83	46[e]
					1.8×10^{-1}	7.7×10^{-3}	23	
	V	R753	Cm	Repressed	1.3×10^{-6}	2.8×10^{-6}	0.46	0.35
					3.3×10^{-6}	1.4×10^{-5}	0.24	
	X	R6K	Ap	Constitutive	2.9×10^{-1}	2.2×10^{-3}	130	250
					6.7×10^{-1}	1.8×10^{-3}	370	
	X	TP228	Km	Repressed	4.4×10^{-2}	1.3×10^{-4}	340	470
					5.7×10^{-2}	9.5×10^{-5}	600	
	9[g]	R71	Cm	Repressed	3.2×10^{-4}	2.0×10^{-4}	1.6	1.55
					1.1×10^{-4}	7.5×10^{-5}	1.5	
Rigid	M	R446b	Tc	Repressed	4.8×10^{-2}	1.6×10^{-5}	3,000	16,150
					2.4×10^{-1}	8.2×10^{-6}	29,300	
	M[h]	R831b	Hg	Constitutive	2.0×10^{0}	2.0×10^{-4}	10,000	6,150
					5.0×10^{-1}	2.2×10^{-4}	2,300	
	N	N3	Tc	Constitutive	9.3×10^{-1}	6.4×10^{-5}	14,500	10,200[e]
					7.8×10^{-1}	2.3×10^{-4}	3,400	
	P	RP1	Ap	Constitutive	1.2×10^{0}	4.0×10^{-4}	3,300	2,100[e]
					1.2×10^{0}	2.2×10^{-3}	540	
	W	Sa	Cm	Constitutive	7.4×10^{-1}	2.7×10^{-5}	27,400	36,450
					1.0×10^{0}	2.2×10^{-5}	45,500	
	—	RA3	Cm	Constitutive	1.1×10^{-1}	1.1×10^{-5}	10,000	7,900[e]
					2.6×10^{-1}	4.6×10^{-5}	5,650	

[a] There are three basic morphological forms of pili: thin (6-nm diameter) flexible, thick (9-nm diameter) flexible, and rigid, based on original descriptions (4).

[b] Abbreviations and concentrations: Ap, ampicillin (400 μg/ml); Cm, chloramphenicol (25 μg/ml); Hg, mercuric chloride (12 μg/ml); Km, kanamycin (100 μg/ml); Tc, tetracycline (10 μg/ml); Te, potassium tellurite (20 μM).

[c] As determined by electron microscopy (data from Bradley, Plasmid, in press; permission granted from Academic Press, Inc.).

[d] Where more than two experiments were done, the frequencies giving the highest and lowest plate/broth ratios are quoted.

[e] Where more than two experiments were done, the average frequency ratio for all the determinations (not for the two quoted) is given.

[f] Matings for IncH plasmids were done at 30°C, and those for Rts1 were at 27°C. MIP233 pili not yet found.

[g] Inc9 = com9 (7).

[h] Originally IncH2, reclassified as IncM in this paper.

[i] Unclassified, but constitutes the "type plasmid" of a new Inc group since it has been found compatible with plasmids representing all existing groups (1; R. W. Hedges, personal communication).

jugal transfer of the chromosome (reviewed in reference 8). Its rapid action is such that it can be used to interrupt mating on plates (10), so it was employed here to eliminate erroneous results due to plate mating on the transconjugant-selecting plates. Restriction was absent because all matings were between JE2571 strains with and without the plasmids. Overnight broth cultures from single colonies of donor and recipient strains were diluted 1 in 20 with nutrient broth and grown with shaking for about 3 h to an absorbance of 1.0 at a wavelength of 620 nm (ca. 2×10^9 cells per ml, late exponential phase), or adjusted to this level. Dilutions of the donor culture were spread on nutrient plates for viable counts and on nutrient plates with

plasmid-selecting drugs to check plasmid stability. A 0.3-ml amount of a mixture of equal volumes of donor and recipient cultures was added to 1.0 ml of broth in a tube for the liquid mating. The dilution brought the cell concentration close to that normally employed in liquid matings. After incubation at 37°C for 1 h, the volume was adjusted to 3 ml with broth, and 0.1-ml amounts of serial dilutions were spread on transconjugant-selecting plates for overnight incubation. For the plate mating, 0.3 ml of the mixture of donor and recipient cultures was spread on a nutrient plate (poured 1 or 2 days previously and predried at 37°C for 20 min inverted with the lid off) without selective drugs by using a wire spreader. It was allowed to dry at 37°C not inverted with the lid off (5 to 10 min). The objective was to ensure that every cell was in contact with another. Light microscopy confirmed that the minimum volume of mating mixture required to achieve this at an absorbance of 1 was 0.3 ml. The plate was incubated for 1 h, the time being taken from when it was about half dry (it was covered as soon as it was completely dry). It was then flooded with 1 ml of nutrient broth, and the cells were resuspended by scraping with a wire spreader. Two further treatments ensured quantitative removal of the bacteria. The volume of the suspension was adjusted to 3 ml by weighing, and the mixture was blended briefly in a Vortex mixer. Serial dilutions were spread in 0.1-ml amounts on transconjugant-selecting plates for overnight incubation. Broth and plate matings were carried out concurrently. This method was considered to provide a more favorable mating environment than the commonly used membrane filter mating procedure. Transfer frequencies were calculated as the number of transconjugants per donor at the start of mating. Incubations were at 27 or 30°C for temperature-sensitive plasmids and at 37°C for the remainder.

Electron microscopy. Grid labeling (12) for immune electron microscopy was used to test the interactions of pili with various anti-pilus sera. Strains under test were grown on selective plates by using a growth method giving temporary derepression with plasmids repressed for pilus synthesis (4). Carbon-coated electron microscope grids were touched on the surface of concentrated bacterial suspensions in 0.1 M ammonium acetate solution. After washing off the cells by dabbing on the surface of successive ammonium acetate baths, grids were floated on 1:1 dilutions of appropriate antisera with 0.1 M ammonium acetate (see reference 6 for antiserum preparation). Normally, treatments were for 5 min at room temperature, but 1.5 h at 37°C was used for the weak anti-H2 serum. Preparations were negatively stained with 1% sodium phosphotungstate solution for electron microscopy.

Data on the state of pilus synthesis are reproduced from a previous publication (D. E. Bradley, Plasmid, in press, with permission of Academic Press, Inc.). By definition, constitutive pilus synthesis implies the presence of one or more organelles on each cell. However, since the preparation method removed pili from cells, the state of pilus synthesis was ascertained from the number of free pili found on each electron microscope specimen support grid square (>20 pili per grid square indicated derepression, and <1 pilus per grid square indicated repression).

RESULTS AND DISCUSSION

Comparison of transfer frequencies for broth and plate matings. The results in Table 1 are arranged according to pilus morphological type, since it was thought that the form of the pilus (rigid or flexible) might determine the optimum mating environment of a plasmid (liquid or solid). The criterion used to indicate this was the ratio plate mating frequency/broth mating frequency. Usually, two sets of matings were carried out for each plasmid, but where more determinations were done, the highest and lowest ratios have been included, the average ratios being calculated from all the experiments.

Frequency ratios near 1 were obtained from representative plasmids determining thin flexible pili ($IncI_\alpha$, $IncI_2$, IncK), indicating that transfer was as efficient in broth as on plates. The same applied to most of the plasmids which determined thick flexible pili (IncFII, IncH1, IncH2, IncJ, IncV, com9; pili have not yet been found for IncH3), where the highest frequency ratio obtained was 5.5 for R27 (IncH1); this was not considered significant. Unexpectedly, plasmids representing IncC, IncD, IncT, and IncX transferred significantly better on plates; their frequency ratios were in the range 45 to 470. All plasmids determining rigid pili (IncM, IncN, IncP, IncW, and RA3) had very high ratios (2,100 to 36,450), transferring very poorly in broth. We did not expect rigid pili to function well in liquids since they are apparently fragile and are probably broken by brownian motion or convection forces which would be absent on plates. However, it was unexpected that a significant number of plasmids determining flexible pili transferred best on plates, although their plate/broth transfer frequency ratios were not as high as for plasmids with rigid pili. This could be explained by a weak linkage between the pili and either the donor cells (at the bases), or the recipients (at the tips), or both.

We have demonstrated the existence of at least two different types of mating system where transfer efficiency depends upon environment. Thus, the F transfer system must no longer be considered to typify all such systems. Until now it was suspected that some important plasmids such as those of incompatibility groups N and P might transfer better on solid media than in liquids (9). Our results not only confirm this, but also show that many plasmids previously thought to be repressed for transfer are, in fact, naturally derepressed. Some practical applications emerge: certain plasmids originally characterized as nontransferable in broth might transfer at detectable frequencies on plates. Also, some surface mating plasmids at present

restricted to a single bacterial species, genus, or family might be transferable to a wider range of hosts by plate mating. The existence of different mating systems should also be useful in identifying or classifying plasmids (see R831b below).

Correlation of state of pilus synthesis (constitutive or repressed) with maximum transfer frequency. It has not always been possible to clearly define a plasmid as being repressed or derepressed for transfer. Transfer frequencies, which have almost always been measured in liquid matings, are sometimes much lower than would be expected in cases in which the organelles of transfer (pili) are determined constitutively (5, 9). We have shown that in many cases the frequencies obtained in broth do not reflect the true transferability of a plasmid. Therefore, we have endeavored to correlate the state of pilus synthesis as revealed by electron microscopy (data from D. E. Bradley, Plasmid, in press) with the maximum transfer frequency obtained (Table 1). In the majority of cases where conjugative pili were produced constitutively, transfer frequencies were $>10^{-1}$ transconjugants per donor under optimum conditions. Where pilus synthesis was repressed, transfer frequencies were significantly less than this value, although there were exceptions to the general rule (e.g., R711b, R446b). Notable examples within the same incompatibility group conforming to the rule were the IncX plasmids R6K and TP228, the former being derepressed for transfer (average frequency, 4.8×10^{-1} transconjugants per donor) and pilus synthesis, and the latter repressed (average frequency, 5.1×10^{-2} transconjugants per donor). R6K was not originally thought to be naturally derepressed since it was transferred in broth by other investigators, whereas, like TP228, it transferred much better on plates. It may be that the derepressed characteristic of R6K is because it is a multicopy plasmid (11). The supposed derepressed version of R6K, TEM*drd* (4), has not been included here since it did not transfer any better than the original R6K on plates, so is the same plasmid.

Classification of R831b. R831b was tested for optimum mating environment since it was important as a temperature-independent, supposedly IncH2 plasmid (3, 14, 17), which apparently determined conjugative pili constitutively. However, unlike other IncH plasmids, we found that it transferred 6,150× better on plates than in broth (Table 1). This suggested that it determined rigid pili rather than the flexible pili of IncH plasmids and might not be IncH2 after all. R831b pili were therefore studied by immune electron microscopy to find out whether or not they were serologically related to H2 pili. There was no serological reaction between R831b pili and anti-H2 (R478) or H2 pili (R478) and anti-R831b (reciprocal test), showing that R831b pili were not the same as H2 pili. However, when R831b pili were tested with antisera to various types of rigid pili, a strong reaction occurred with anti-M (R471a; Fig. 1). In the reciprocal reaction, M pili were clearly labeled with anti-R831b (Fig. 2). Our subsequent incompatibility tests have shown that R831b is compatible with the IncH2 plasmid pSD114, both in a Rec^+ host and in the *recA E. coli* strain JC1659 (2). Therefore, its previous designation (3, 14, 17) is incorrect. In addition, H. Richards and M. M. McConnell (personal communications) find that the plasmid is incompatible with IncM plasmids.

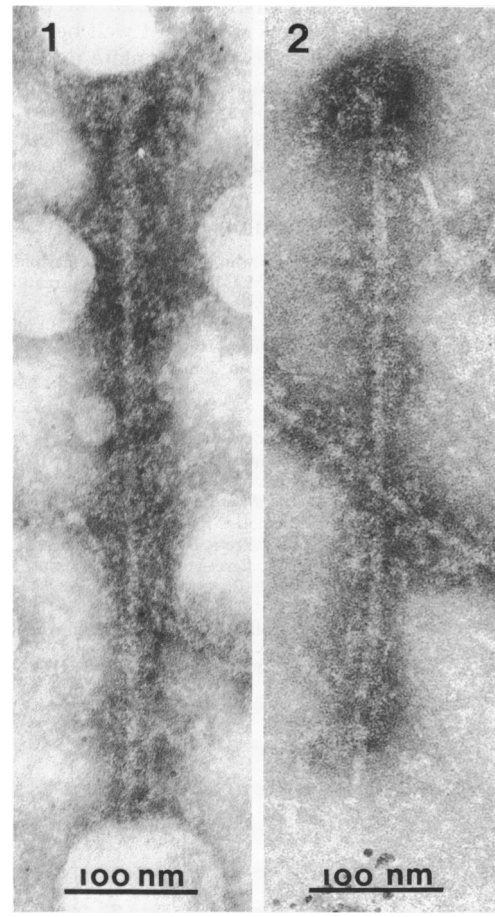

FIG. 1. *R831b pilus labeled with antiserum to M pili determined by R471a, showing a strong reaction.*

FIG. 2. *M pilus (determined by R446b) labeled with antiserum to R831b pili. Fewer antibodies were adsorbed from the weaker antiserum.*

R831b is thus the first definite example of a derepressed IncM plasmid.

These results demonstrate that the characterization of a plasmid's mating system from an environmental standpoint is useful in identifying or classifying it by the following suggested procedure. A plate mating test would indicate whether a plasmid transferred equally well in liquids or on plates, or whether surface mating was more efficient. The transfer frequency ratio (plate/broth) would indicate whether it determined rigid or flexible pili. The relatively few appropriate incompatibility tests would then be done. The full range might only be considered desirable if the plasmid seemed to belong to a new incompatibility group or was found to determine a new transfer system. Future incompatibility testing should obviously be done with the optimum mating environment for the plasmid under test. However, it might well be prudent to perform all tests by using plate mating, since with every plasmid studied here, a solid environment was as good as or better than a liquid one. In this way, surface mating reference plasmids would be automatically accommodated.

ACKNOWLEDGMENTS

We are grateful to all those who kindly supplied plasmids, and to H. Richards and M. M. McConnell for information concerning new incompatibility tests for R831b.

This work was supported by grants MA5608 (D.E.B.) and MA6200 (D.E.T.) from the Medical Research Council of Canada. D.E.T. is a Medical Research Council Scholar.

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