Serological Classification of Conjugative Plasmids by Indirect Haemagglutination

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The indirect haemagglutination reaction was evaluated in the classification of conjugative plasmids. A simple and sensitive method was worked out using pili and in some cases whole bacteria as antigens. Antibodies were prepared against pili coded for by plasmids from incompatibility groups IncFI, IncFII, IncI α and IncN. The antisera were tested against pili from 35 strains harbouring plasmids. The test differentiated clearly between plasmids from unrelated incompatibility groups, whereas cross-reaction occurred with closely related groups such as FI and FII. Minor antigenic variation could be seen within the IncFII group.

INTRODUCTION

Incompatibility testing is a widely used method for the classification of conjugative (self-transmissible) plasmids (Datta, 1979). This test is based on an examination of the stable coexistence of two plasmids (a plasmid to be tested and a reference plasmid) in a bacterial strain (clone) without selective pressure. Incompatibility is recognized by the loss of one of the plasmids. Therefore the plasmid to be tested must possess an easily identifiable character different from the reference plasmid.

Conjugation pili are specialized hair-like structures on the surface of bacterial cells. They serve an important role in the transfer of plasmid DNA from one bacterial cell to another. The conjugation pili seem to be unique for each incompatibility group, although the conjugation pili of a few incompatibility groups are related both genetically and in their molecular structure (Achtman & Skurray, 1977).

Serological methods have seldom been used in the classification of conjugative pili, or the plasmids determining them. Ørskov & Ørskov (1960) showed that cells of *Escherichia coli* K12 with an F plasmid were agglutinated by homologous antisera absorbed with F⁻ bacteria. The antigen responsible for the reaction was later found to be identical with the F pilus (Ishibashi, 1967).

The early serological investigations on conjugation pili were based on slide or tube agglutination studies (Hirota *et al.*, 1974; Kétyi & Ørskov, 1969). Electronmicroscopic visualization of the reaction between antibodies and pili (Lawn, 1967; Lawn *et al.*, 1967; Lawn & Meynell, 1970; Bradley, 1980; Bradley *et al.*, 1980), and the blocking of gene transfer caused by homologous anti-pili antibodies (Harden & Meynell, 1972), have also been used in serological studies.

This paper is a report on the utility of the passive indirect haemagglutination reaction in a serological classification of conjugative plasmids. The serological investigations were performed with antibodies against pili coded for by plasmids belonging to the incompatibility groups IncFI, IncFII, IncI α and IncN.

METHODS

Bacterial host strains and plasmids. An autotrophic pilus-free and flagellum-free derivative of *Escherichia coli* K12, JE2217 (Helmuth & Achtman, 1978) was used as the only host strain for the plasmids in the immunization

procedure. Incompatibility reactions and haemagglutination assays were performed using *E. coli* J62-1 (Pro⁻ His⁻ Trp⁻ Str^R NaI^R Lac⁻) obtained from Dr N. Datta, Hammersmith Hospital, London, as the plasmid host strain. In a few experiments the original wild-type strain as well as JE2217 were also used. *Salmonella panama* 1 was obtained from J. van Embden, Rijks Instituut voor de Volkgezondheid, Bilthoven, The Netherlands. The other wild-type strains were isolated in our laboratory (Møller, 1980). These strains were used only when they contained plasmids.

The plasmids are reference type plasmids or plasmids isolated and characterized in our laboratory. They were transferred from the original host to the new host as described by Møller (1980). The F plasmid was provided by Dr I. Ørskov, State Serum Institute, Copenhagen, Denmark; R386, NR1, *Idrd16*, R144, R64*drd11* and N3T were obtained from Dr N. Datta; P307 from Dr H. Williams Smith, Houghton Poultry Research Station, Houghton, U.K.; SPI from Dr J. van Embden; and R1*drd19* from Dr K. Nordstrøm, University of Odense, Denmark. The other plasmids were isolated in our laboratory (Møller *et al.*, 1976).

Production of pilus-specific antisera. Antigens for the immunization of rabbits were prepared from tryptone broth cultures of JE2217 possessing one of the four reference plasmids [R386 (IncFI), R1*drd19* (IncFII), *Idrd16* (IncI α), N3T (IncN)]. The bacteria were grown under aeration to late exponential phase, collected by centrifugation (5 min at 3000 g, 4 °C), gently washed twice in phosphate-buffered saline (PBS) pH 7.4, containing 0.02% (w/v) sodium azide, and then suspended at a concentration of 5×10^8 bacteria ml⁻¹ in PBS pH 7.4, containing 0.5% (v/v) formalin.

Albino rabbits were immunized according to the method of Ernø *et al.* (1973). The serum was absorbed with JE2217 repeatedly until no reaction was seen in an indirect haemagglutination reaction with undiluted serum or serum diluted 1/10; it was inactivated (30 min, 56 °C) and absorbed with sheep erythrocytes. All absorption experiments were done by conventional methods.

Preparation of antigens for haemagglutination assay. For antigen preparations bacteria were grown under aeration in tryptone broth with 0.2% (w/v) glucose and necessary amino acids for 8 h or on the same medium plus agar for 18 h. Broth cultures were centrifuged and the sediment suspended in PBS, washed twice and finally suspended in PBS with 0.02% sodium azide. The bacteria grown on agar were scraped off and treated in the same way. The pili were liberated from the bacterial cells by mixing with sucrose (30%, w/v of the bacterial suspension) in a rotatory shaker (200 r.p.m., 30 min), removing the cells by centrifugation (15 min at 2000 g, 4 °C), and the sucrose by dialysis against PBS. The protein concentration of the pilus suspension was determined by the Lowry method, and adjusted to at least 50 µg ml⁻¹.

Indirect haemagglutination assay. Freshly drawn sheep erythrocytes (in Alsevers solution) were washed three times in PBG (0.114 M-Na₂HPO₄, 0.036 M-KH₂PO₄ and 2.6% glucose, pH 7.2) and suspended in PBG to give a 20% solution. Equal volumes of sheep erythrocytes and 0.2% (v/v) glutaric aldehyde were mixed for 15 min at 37 °C, washed in 0.9% (w/v) sodium chloride and PBS, and suspended at a concentration of 25% in PBS supplemented with 0.1% sodium azide. Before use the erythrocytes were washed twice in PBS and resuspended in the same volume of PBS with 0.1% sodium azide and coated with antigen overnight while rotating slowly at room temperature.

The amount of pilus antigen needed for optimal coating of the erythrocytes was determined in a check-board titration using homologous antisera undiluted, diluted to 1/10 and to 1/100, and twofold dilutions of homologous antigen (pili). When whole bacterial cells were used the amount was determined in a similar way.

After coating, excess antigen was removed by three gentle washes with PBS. If not used immediately (2% mixture in PBS), the erythrocytes were kept in PBS with 50% (v/v) glycerol for no more than a week.

The assay was performed in a dispensable microvial (Nunc). The dilution liquid was PBS with 1% rabbit serum (1% SA) inactivated and absorbed against sheep erythrocytes; 25 µl 1% SA was dripped into each vial. Antiserum (25 µl) was added to the first vial and serial dilutions were made. Then, 25 µl of coated erythrocytes were added to each of the vials. Positive and negative controls were included.

The results were read visually after 2 h at room temperature. The end point was taken as being when the erythrocytes had formed a carpet covering more than three-quarters of the bottom of the vial.

RESULTS

The serological results are recorded in Tables 1, 2, 3 and 4. Table 1 gives the results obtained when different antigen preparations from strains with plasmids belonging to two incompatibility groups (N and I) were tested against N pilus-specific antibodies. The pilus-free strain (JE2217) and the K12 (J62-1) strain that possesses chromosomally-coded pili (Salit & Gotschlich, 1977) were also included as antigens. Plasmid pJM14 does not belong to IncN or IncI, but is otherwise unclassified. The highest titres were obtained with homologous pilus antigens (N3T and pJM18, IncN). The titres varied with the antigen preparation; the highest

Table 1. *Indirect haemagglutination assay using N pilus-specific antibodies and different antigen preparations from Escherichia coli strains with homologous and heterologous plasmids*

Antigen source		Incompatibility group	Antibody titres against antigen preparations of:			
Strain	Plasmid		Broth-grown bacteria	Pili from broth-grown bacteria	Agar-grown bacteria	Pili from agar-grown bacteria
JE2217	N3T	IncN	384	768	96	768
J62-1	N3T	IncN	384	768	192	768
J62-1	pJM18	IncN	192	768	96	768
J62-1	pJM21	IncI α	96	96	6	0
JE2217	pJM14	Not IncN or Inc α	24	48	12	6
J62-1	pJM14	Not IncN or Inc α	0	ND	0	ND
JE2217	Plasmid-free	—	0	—	0	—
J62-1	Plasmid-free	—	48	48	0	0

ND, Not done.

Table 2. *Indirect haemagglutination reaction between N pili antigens from different host strains and N pili antibodies*

Host strain	Plasmid	Incompatibility group	N pili antibody titre
<i>Escherichia coli</i> K12 (J62-1)	N3T	IncN	768
<i>Escherichia coli</i> K12 (JE2217)	N3T	IncN	768
<i>Escherichia coli</i> K12 (CR34)	SP1	IncN	384
<i>Salmonella panama</i> type I (MMCA 496)*	SP1	IncN	192
<i>Enterobacter cloacae</i> (MMCA 268)*	pJM18	IncN	384
<i>Escherichia coli</i> K12 (JE2217)	pJM18	IncN	384
<i>Escherichia coli</i> K12 (J62-1)	pJM18	IncN	384
<i>Escherichia coli</i> K12 (J62-1)	pJM56	IncN	384
<i>Escherichia coli</i> K12 (J62-1)	pJM57	IncFII	0
<i>Escherichia coli</i> wild-type (MMCA 255)*	pJM56, pJM57	IncN, IncFII	192

* Abbreviation: MMCA, Medical Microbiological Collection, Aarhus, Denmark.

titres were found with pili as antigens. Broth-grown bacteria showed a higher titre than bacteria grown on agar. With the heterologous plasmid (pJM21, IncI α) and the unclassified plasmid pJM14, reactions were not significantly different from those obtained with the plasmid-free strain (J62-1). With the plasmid-free strain JE2217 no reaction was seen. With broth-grown J62-1 as antigen a low titre was recorded, whether with whole bacterial cells, or preparations of the chromosomally-coded pili. This may be caused by non-specific adherence of the erythrocytes and the chromosomally-coded pili (Salit & Gotschlich, 1977).

Table 2 contains the results of experiments using antibodies against IncN pili, and antigens which consisted of pilus preparations made from eight different strains, all containing (with one exception) IncN plasmids. Only minor variations could be seen in titres regardless of the host strain and IncN plasmid. The presence of an unrelated plasmid (pJM57, IncFII) with which there was no reaction, together with an IncN plasmid (pJM56), did not have a significant influence on the titre.

The value of the indirect haemagglutination assay was examined further using IncFI, IncFII, IncI α and IncN antisera against pilus antigens isolated from a number of strains harbouring conjugative plasmids. These were reference plasmids with known incompatibility grouping, and plasmids isolated from wild-type enterobacterial strains in our laboratory.

The results of experiments using pili coded by Fi⁺ (F) plasmids as antigens are listed in Table 3, and those using the Fi⁻ (F) plasmid-determined pili are listed in Table 4. From Table 3 it can be seen that no reaction could be detected between IncI α and IncN antisera and the antigens

Table 3. *Indirect haemagglutination reaction of F⁺ (F) plasmids*

Pilus antigens	Incompatibility group	Antibody titres against conjugation pili from:			
		IncFI	IncFII	IncI α	IncN
F	IncFI	768	192	0	0
R386	IncFI	768	192	0	0
P307	IncFI	768	192	0	0
pJM11	IncFI	768	192	0	0
pJM26	IncFI	384	192	0	0
R1 <i>drd</i> 19	IncFII	384	768	0	0
NRI	IncFII	384	192	0	0
pJM3	IncFII	192	768	0	0
pJM6	IncFII	192	192	0	0
pJM12	IncFII	96	192	0	0
pJM19	IncFII	192	384	0	0
pJM20	IncFII	96	192	0	0
pJM22	IncFII	192	768	0	0
pJM24	IncFII	192	384	0	0
pJM15	IncFII and IncT	96	384	0	0
pJM17	IncFII and IncT	384	768	0	0
pJM7	Not IncFI or IncFII	96	96	0	0
pJM8	Not IncFI or IncFII	192	384	0	0
pJM25	Not IncFI or IncFII	192	768	0	0
pJM55	Not IncFI or IncFII	192	96	0	0
pJM60	Not IncFI or IncFII	192	380	0	0

Table 4. *Indirect haemagglutination reactions of F⁻ (F) plasmids*

Pilus antigens	Incompatibility group	Antibody titres against conjugation pili from:			
		IncFI	IncFII	IncI α	IncN
I <i>drd</i> 16	IncI α	0	0	768	0
R144	IncI α	0	0	768	0
R64 <i>drd</i> 11	IncI α	0	0	192	0
pJM21	IncI α	0	0	768	0
pJM23	IncI? [*]	0	0	96	0
N3T	IncN	0	0	0	768
SP1	IncN	0	0	0	768
pJM18	IncN	0	0	0	384
pJM56	IncN	0	0	0	384
pJM61	IncN	0	0	0	768
pJM4	Inc? [†]	0	0	0	0
pJM9	Inc? [†]	0	0	0	0
pJM14	Inc? [†]	0	0	0	0
pJM16	Inc? [†]	0	0	0	0

* Strains possessing this plasmid multiply the IncI-specific phage If1, but are compatible with plasmids from IncI α or IncI γ .

† Compatible with IncI α , IncI γ , IncN, IncP, IncT, and IncW plasmids.

used. A high titre was always observed between IncFI antisera and pilus antigens of IncFI plasmids (a homologous reaction). Significantly different titres were sometimes observed in the homologous reaction between IncFII pili and the corresponding antisera. The heterologous reactions (IncFI antigens versus IncFII antisera or IncFII antigens versus IncFI antisera) generally gave lower titres than in the homologous reactions. Five plasmids (pJM7, pJM8,

pJM25, pJM55 and pJM60) were compatible with IncFI and IncFII plasmids, but otherwise untypable (Møller, 1980). The titres obtained indicate that these plasmids belong to the F complex but to other incompatibility groups within the complex.

The results in Table 4 show that no reaction could be detected between IncFI and IncFII antisera and the antigens used. With one exception (R64*drd11* pili versus IncI α antisera) the same high titres were observed between IncI α antisera and pili of the IncI α plasmids as were obtained with IncN antisera of pili of IncN plasmids. A significantly lower titre was seen when pili from a strain harbouring plasmid pJM23 was probed against IncI α antiserum. Plasmid pJM23 does not belong to the I α or the I γ incompatibility groups; however, strains with pJM23 are sensitive towards the I pilus specific phage If1, and this plasmid has a high percentage of DNA homology with the IncI α reference plasmid *Idrd16* (Møller *et al.*, 1978).

No reaction could be found in the assay with plasmids pJM4, pJM9, pJM14 and pJM16, which are of unknown incompatibility group, but do not belong to IncFI, IncFII, IncI α , IncI γ , IncN, IncP, IncT or IncW.

DISCUSSION

Serological methods have seldom been applied in the classification of conjugative plasmids, and have been limited exclusively to derepressed plasmids. Thus, slide agglutination of bacteria as well as electron microscopic visualization of the reactions have been used, but with little success.

Indirect haemagglutination has apparently never been applied before in the serological classification of conjugative plasmids. It is, however, easily performed. By using pilus preparations as antigens the method is independent of whether the plasmids are repressed or derepressed. The non-specific adherence between chromosomally-coded pili and the erythrocytes is considered to be negligible. The method may be performed with pili liberated from wild-type strains and is unaffected by either the presence of a coexisting unrelated plasmid or the simultaneous presence of two incompatibility determinants on one plasmid (e.g. the IncFII and IncT determinants on pJM15).

The method unequivocally distinguishes between groups of plasmids from different incompatibility complexes (IncI, IncF and IncN). These groups were originally chosen because of their frequent occurrence and were later shown to represent three morphological groups (Bradley, 1980). There are, however, limitations in distinguishing between plasmids from different incompatibility groups within the F complex. This is not surprising because these pilus proteins are very much alike (Kennedy *et al.*, 1977; Achtman *et al.*, 1978). The variable results within the F complex and in a single case in the I complex (R64*drd11*) may be explained by minor antigenic variations (Lawn & Meynell, 1970; Lawn *et al.*, 1967; Bradley, 1980). Further investigations will show whether it is possible to use different combinations of absorbed antisera to define plasmids within such groups. However, we are dealing with two different biological phenomena. The pilus is active in transfer and as a receptor for specific phage, whereas incompatibility is closely associated with replication.

The Fi⁺ (F) plasmids not belonging to groups IncFI or IncFII, which showed positive reactions with FI and FII antisera, must belong to other groups in the F complex. The surprisingly high titre noted in one instance (pJM25) might support the assumption that two different incompatibility groups share a common pilus.

The F⁻ (F) plasmids not belonging to IncFI, IncFII, IncI α or IncN, which showed no reaction with the antisera, cannot possess pili related to groups F, I or N. Neither do they possess a significant DNA homology with plasmids from these groups (Møller *et al.*, 1978; N. H. Feilberg Jørgensen, unpublished results).

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REFERENCES

- ACHTMAN, M. & SKURRAY, R. (1977). A redefinition of the mating phenomenon in bacteria. In *Microbial Interactions (Receptors and Recognition)*, Series B, vol. 3, pp. 235–279. Edited by J. L. Reissig. London: Chapman & Hall.
- ACHTMAN, M., KUSECEK, B. & TIMMIS, K. (1978). Tra cistrons and proteins encoded by *Escherichia coli* antibiotic resistance plasmid R6-5. *Molecular and General Genetics* **63**, 169–179.
- BRADLEY, D. E. (1980). Morphological and serological relationship of conjugative pili. *Plasmid* **4**, 155–169.
- 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.
- DATTA, N. (1979). Plasmid classification: incompatibility grouping. In *Plasmids of Medical, Environmental and Commercial Importance*, pp. 3–12. Edited by K. N. Timmis & A. Pühler. Amsterdam: Elsevier-North Holland.
- ERNØ, H., JURMANOVÁ, K. & LEACH, R. H. (1973). Bovine mycoplasmas: a serological study by the metabolic inhibition test. *Acta veterinaria scandinavica* **14**, 511–523.
- HARDEN, V. & MEYNELL, E. W. (1972). Inhibition of gene transfer by antiserum and identification of serotype of sex pili. *Journal of Bacteriology* **109**, 1067–1074.
- HELMUTH, R. & ACHTMAN, M. (1978). Cell-cell interactions in conjugating *Escherichia coli*: purification of F pili with biological activity. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 1237–1241.
- HIROTA, Y., NISHIMURA, Y., ØRSKOV, F. & ØRSKOV, I. (1964). Effect of drug-resistance factor R on the F properties of *Escherichia coli*. *Journal of Bacteriology* **87**, 341–351.
- ISHIBASHI, M. (1967). F pilus as f⁺ antigen. *Journal of Bacteriology* **93**, 379–389.
- KENNEDY, N., BENTIN, L., ACHTMAN, M. & SKURRAY, R. (1977). Conjugation proteins encoded by the F sex factor. *Nature, London* **270**, 580–585.
- KÉTYI, I. & ØRSKOV, I. (1969). Studies on the antigenic structure of sex fimbriae carried by a strain of *Shigella flexneri* 4b. *Acta pathologica et microbiologica scandinavica* **77**, 299–308.
- 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.
- LAWN, A. M., MEYNELL, E., MEYNELL, G. & DATTA, N. (1967). Sex pili and the classification of sex factors in *Enterobacteriaceae*. *Nature, London* **216**, 343–346.
- MØLLER, J. K. (1980). *Infectious drug resistance*. Ph.D. thesis (in Danish), University of Aarhus, Denmark.
- MØLLER, J. K., BAK, A. L., CHRISTIANSEN, C., CHRISTIANSEN, G. & STENDERUP, A. (1976). Extrachromosomal deoxyribonucleic acid in R factor-harboring *Enterobacteriaceae*. *Journal of Bacteriology* **125**, 398–403.
- MØLLER, J. K., JØRGENSEN, N. H. F., CHRISTIANSEN, C., CHRISTIANSEN, G., BAK, A. L. & STENDERUP, A. (1978). Characterization of plasmids from wild-type *Enterobacteriaceae*. In *Microbiology – 1978*, pp. 257–261. Edited by D. Schlessinger. Washington, D.C.: American Society for Microbiology.
- ØRSKOV, I. & ØRSKOV, F. (1960). An antigen termed f⁺ occurring in F⁺ *E. coli* strains. *Acta pathologica et microbiologica scandinavica* **48**, 37–46.
- SALIT, I. E. & GOTSCHLICH, E. C. (1977). Hemagglutination by purified type 1 *Escherichia coli* pili. *Journal of Experimental Medicine* **146**, 1169–1181.