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The use of bacteriophages for differentiating plasmids of *Pseudomonas aeruginosa*

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SUMMARY

Six bacteriophages have been used in the classification of 19 plasmids (antibiotic resistance-mediating R factors and FP sex factors which promote host chromosome transfer) of *P. aeruginosa* isolated in different geographical regions. On the basis of phage-plating responses on isogenic strains of bacteria differing only in the plasmids carried, five groups of plasmids were distinguishable. In general the groups could be correlated with their geographical origin although differences between plasmids from the same region were found. The unique phage-plating responses were also useful in establishing the possible identity of plasmids isolated from the same original strain and given different designations by independent investigators. The classification of the plasmids derived here on the basis of phage-plating responses could be correlated with classifications based upon other phenotypic characteristics described elsewhere. The nature of inhibition of plating of phages B39 and G101 by R18-1 and R18-3 respectively was shown to be due to interference with some aspect of intracellular phage replication rather than to plasmid-mediated restriction.

1. INTRODUCTION

With the isolation of an increasing number of FP (i.e. sex factors promoting host chromosome transfer) and R factor (i.e. mediating antibiotic resistance) plasmids of *Pseudomonas aeruginosa* from different geographical regions (Lowbury *et al.* 1969; Black & Girdwood, 1969; Kawakami *et al.* 1972; Bryan *et al.* 1973; Chabbert *et al.* 1972; Pemberton & Holloway, 1973; Matsumoto & Tazaki, 1973), it has become essential to classify these. Although ultimately the most meaningful way of studying the genetic relationship between plasmids is DNA hybridization it is nevertheless useful to have tests which are relatively easy to perform and allow rapid identification of plasmids. This could conceivably be achieved by taking advantage of possible plasmid effects on phage replication such as plasmid restriction of phage (Watanabe *et al.* 1966; Bannister & Glover, 1968) or plasmid interference with phage transcription or translation (Nishioka & Ozeki, 1968; Morrison & Malamy, 1971; Moyer, Fu & Szabo, 1972). There are other tests, including plasmid exclusion and incompatibility, which are also being used to study plasmids of *P. aeruginosa* (Datta *et al.* 1971; Grinsted *et al.* 1972; Ingram *et al.* 1972; Olsen

& Shipley, 1973; Chabbert *et al.* 1972; Bryan *et al.* 1973; Chandler & Krishnapillai, 1974*a*, *b*).

This paper reports the use of phages to establish the relationship between independently isolated plasmids. In addition, the nature of inhibition of phage plating by certain of these plasmids is discussed.

2. MATERIALS AND METHODS

(i) *Media.* These have been described (Krishnapillai, 1971).

(ii) *Bacteriophages.* E79 is virulent (Holloway, 1969). B3, G101, F116L and D3 are all temperate but, whereas the first two are u.v.-noninducible and promote general transduction, the latter two are inducible; and although F116L transduces D3 does not (Holloway, 1969; Krishnapillai, 1971). B39 is a temperate phage newly isolated by the method described by Krishnapillai (1971) from a *P. aeruginosa* strain isolated in a Melbourne hospital. It is serologically related to B3 (neutralization constant $K = 548$ min^{-1} for B39/anti-B3 serum in comparison to $K = 2091$ min^{-1} for B3/anti-B3 serum) but hetero-immune to B3 in immunity tests. It is non-transducing and u.v.-inducible.

(iii) *Bacteria.* The following *P. aeruginosa* sublines of the PAO strain (Holloway, 1969) were used. PAO1 is prototrophic, *ch12* (Isaac & Holloway, 1968); PAO8 = *met28 ilv202 str1* (Isaac & Holloway, 1968); PAO1670 = *leu8 pur136 chl3 rif1* (Chandler & Krishnapillai, 1974*c*). The origin and derivation of various plasmid carrying derivatives of PAO are shown in Table 1.

(iv) *General phage methods.* These are described in Adams (1959).

(v) *Transduction and co-transduction tests.* These have been described (Krishnapillai, 1971).

(vi) *Measurement of adsorption of phage to bacteria.* Overnight broth cultures of bacteria were mixed with phage at a multiplicity of 0·05 at 37 °C. At intervals, aliquots were sterilized by Millipore filtration, and the filtrate assayed for non-adsorbed phage.

(vii) *Efficiency of plating (EOP) of phage tests.* Nutrient agar plates pre-flooded with overnight bacterial broth cultures were spotted (in 0·01 ml amounts) with decimal dilutions of phage. Phage assays were read after overnight incubation.

3. RESULTS

(i) *Efficiency of plating of phages on plasmid-carrying bacteria.* The EOP of the six phages G101, B3, B39, F116L, D3 and E79 tested on PAO1670 and on isogenic sublines carrying each of the different plasmids is shown in Table 2. The results divided the plasmids in five categories. (1) The plasmids R74, R18, R88, R68, R30, RP1, R1822 and RP4 which inhibited plating of phage G101, (2) R18-1, RP1-1 and R716 which inhibited B39; in the case of R716 the inhibition was much more

Abbreviations: *chl* = chloramphenicol; *str* = streptomycin; *rif* = rifampicin; *met* = methionine; *ilv* = isoleucine and valine; *leu* = leucine; *pur* = adenine).

Table 1. *Origin and characteristics of* P. aeruginosa *plasmids*

Plasmid	Initial *P. aeruginosa* strain	Plasmid phenotype*	Derivation of *P. aeruginosa* strain PAO carrying plasmid†	Reference
R18-1	1822	CB^r	1822 → PAT900 → PAO1670	Lowbury *et al.* (1969); Stanisich & Holloway (1971); Chandler & Krishnapillai (1974*a*)
R18	1822	CB^rNM/KM^rTC^r	1822 → PAO8 → PAO1670	Chandler & Krishnapillai (1974*a*)
R18-3	1822	CB^rNM/KM^rTC^r	P14 → 1822 → *E. coli* K12 → /PAT900 → PAO8	Stanisich (pers. comm.)
R30	3098	CB^rNM/KM^rTC^r	3098 → PAO8 → PAO1670	Stanisich (1974*a*)
R68	6886‡	CB^rNM/KM^rTC^r	6886 → PAO8 → PAO1670	Chandler & Krishnapillai (1974*a*)
R74	P14‡	CB^rNM/KM^rTC^r	P14 → PAO8 → PAO1670	
R88	6888‡	CB^rNM/KM^rTC^r	6888 → PAO8 → PAO1670	
R91	9169‡	$CB^r(NM/KM^rTC^r)$§	9169 → PAO8 → PAO1670	Stanisich & Holloway (1971)
R19	1954‡	$CB^r(NM/KM^rTC^r)$§	1954 → PAO8 → PAO1670	Chandler & Krishnapillai (1974*b*)
RP1-1	1822	CB^r	1822 → 18S → PAT900 → PAO1670	Lowbury *et al.* (1969); Ingram *et al.* (1972)
RP1	1822	CB^rNM/KM^rTC^r	1822 → 18S → PAT900 → PAO1670	Lowbury *et al.* (1969); Grinsted *et al.* (1972)
RP8	S8	CB^r	S8 → PAO8 → PAO1670	Black & Girdwood (1969); Saunders & Grinsted (1972)
RP4	1822	CB^rNM/KM^rTC^r	1822 → *E. coli* K12 → PAO8 → PAO1670	Datta *et al.* (1971), Holloway & Richmond (1973)
R 1822	1822	CB^rNM/KM^rTC^r	1822 → PAO67 → PAO8 → PAO1670	Olsen & Shipley (1973)
R2	R2-72	$CB^rKM^rSM^r$	R2-72 → PAO1670	Kawakami *et al.* (1972), Stanisich (pers. comm.)
R38	R38-72	SM^rTC^r	R38-72 → PAO1670	
R39	R39-72	SM^rTC^r	R39-72 → PAO1670	
R716	716	SM^r	716 → PAO1670	Bryan *et al.* (1973)
R679	679	SM^rSu^r	679 → PAO1670	
R130	130	$SM^rSu^rGM^r$	130 → PAO1670	
R931	931	SM^rTC^r	931 → PAO1670	
FP2	PAT	Hg^r	PAT → PAO8 → PAO1670	Holloway (1969)
FP5	Ps5-70	Hg^r	Ps5-70 → PFO3 → PAO1670	Matsumoto & Tazaki (1973)
FP39	PAR39	Leu^+	PAR39 → PAO1670	Pemberton & Holloway (1973)

* Resistance (r) to antibiotics or metal; CB = carbenicillin; NM = neomycin; KM = kanamycin; TC = tetracycline; SM = streptomycin; Su = sulphonamide; GM = gentamycin; Hg = mercuric chloride; Leu^+ = suppression of leucine requirement.

† All these derivatives were constructed by V. Stanisich except PAO1670 carrying FP5 or FP39 which were constructed by B. W. Holloway.

‡ Isolation number allocated by E. J. L. Lowbury (pers. comm.).

§ Refers to the observation that resistance determinants within parentheses are not expressed in *Pseudomonas* (Chandler & Krishnapillai, 1974*b*).

extreme (i.e. no plaques were detectable even when tested with 2×10^{10} pfu/ml), (3) R38 and R39 which inhibited all phages except F116L, (4) R130 and R931, which inhibited all six phages, (5) R19, R91, RP8, R2, R679 and the sex factors FP2, FP39 and FP5 which usually plated all the phages normally. Occasionally, some of the phages showed smaller plaques, sometimes associated with a tenfold reduction in EOP.

Table 2. *Efficiency of plating (EOP) of phages on isogenic strains differing in the carriage of plasmids*

Plasmid	Geographical origin	Phage* G101	B3	B39	F116L	D3	E79
R74	Birmingham, U.K.	10^{-4}†	1	1	1	1	1
R18	Birmingham, U.K.	10^{-4}	1	1	1	1	1
R88	Birmingham, U.K.	10^{-4}	1	1	1	1	1
R68	Birmingham, U.K.	10^{-4}	1	1	1	1	1
R30	Birmingham, U.K.	10^{-4}	1	1	1	1	1
RP1	Birmingham, U.K.	10^{-4}	1	1	1	1	1
R1822	Birmingham, U.K.	10^{-4}	1	1	1	1	1
RP4	Birmingham, U.K.	10^{-4}	1	1	1	1	1
R18-1	Birmingham, U.K.	1	1	10^{-6}	1	1	1
RP1-1	Birmingham, U.K.	1	1	10^{-6}	1	1	1
R19	Birmingham, U.K.	1	1	1	1	1	1
R91	Birmingham, U.K.	1	1	1	1	1	1
RP8	Glasgow, U.K.	1	1	1	1	1	1
R2	Japan	1	1	1	1	1	1
R38	Japan	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	1	$< 10^{-6}$	$< 10^{-6}$
R39	Japan	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	1	$< 10^{-6}$	$< 10^{-6}$
R679	Canada	1	1	1	1	1	1
R130	Canada	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$‡	$< 10^{-6}$	$< 10^{-6}$
R931	Canada	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$‡	$< 10^{-6}$	$< 10^{-6}$
R716	Canada	1	1	$< 10^{-7}$§	1	1	1
FP2	South Africa	1	1	1	1	1	1
FP39	Australia	0·1‖	0·1	1	1	0·1‖	1
FP5	Japan	1‖	1	1	1‖	0·1‖	1

* Propagated on PAO1 and tested at 10^9 pfu/ml.

† EOP: titre on PAO1670 carrying plasmid relative to that on PAO1670.

‡ When spotted with high concentration of phage F116L ($> 10^5$ pfu) there was lysis but no distinct plaques were visible.

§ See text. ‖ Reduced plaque size.

(ii) *Basis for the reduced EOP of phages B39 and G101.* A number of reasons may be advanced for the failure of phages to plate on R^+ bacteria. For example, the presence of the R factor may alter the cell surface and prevent phage adsorption; or, alternatively, the R factor may inhibit intracellular phage development by R factor-mediated restriction (Watanabe *et al.* 1966; Bannister & Glover, 1968) or by interference with transcription or translation of the phage genome (Morrison & Malamy, 1971; Nishioka & Ozeki, 1968; Moyer *et al.* 1972). This question was investigated for the B39/R18-1 and G101/R18-3 systems. The R factor R18-3 used in these tests is probably identical to R18 since it also lowers the EOP of G101 by

8×10^{-5} (not shown in Table 2) but, because of its different geneology (see Table 1), it is designated differently. Phage G101 adsorbed equally well to PAO8 and to PAO8 (R18-3) (71% and 69%, respectively, in 15 min), as did the poorly adsorbing phage B39 to PAO8 and PAO8 (R18-1) (45% and 54%, respectively, in 2 h).

Since lack of adsorption was not the explanation for the low EOP of the phages on R18-1$^+$ and R18-3$^+$ bacteria, respectively, the possibility of restriction or interference was tested. To distinguish between these alternatives, the phage particles arising from infection of R$^+$ bacteria were 're-cycled' on R$^-$ bacteria and the plating properties of the latter then tested on R$^+$ and R$^-$ bacteria. If restriction and modification were involved, then the EOP on R$^+$ bacteria should again be reduced relative to that on R$^-$ bacteria because of the loss of R factor-imposed modification by the 'recycling' procedure. However, it was found that, with both B39 and G101 and the respective R factors R18-1 and R18-3, the phages harvested from plaques appearing on R$^+$ bacteria when 'recycled' now plated with equal efficiency on R$^+$ and R$^-$ bacteria. Therefore it was concluded that plaques appearing initially on R$^+$ bacteria were due to mutants which had overcome the interference effects of the plasmid (Mizobuchi, Anderson & McCorquodale, 1971).

Another line of evidence which supported the view that R factor restriction was not involved for G101 plating on PAO8 (R18-3) was the observation that transduction of bacterial genes into the R$^+$ recipient was normal, since it is known that R factor restriction recognizes phage as well as bacterial DNA (Watanabe *et al.* 1966). Thus, the transduction frequency by phage G101 for *ilv202*, was $3{\cdot}4 \times 10^{-7}$ transductants/pfu when the donor was PAO1 and the recipient was PAO8 (R18-3); and similar frequencies were obtained when PAO8, PAO8 (R18-1) or PAO8 (R68) ($3{\cdot}4 \times 10^{-7}$) were used as recipients. In addition, the cotransduction frequencies for the genes *ilv202* and *met28* (Krishnapillai, 1971) in all these transductions was 40–53%. This type of analysis could not be carried out with phage B39 because it does not transduce.

(iii) *R factor suppression of prophage induction in R$^+$ lysogens.* Since R18-1 and R18-3 appear to interfere with the intracellular development of phages B39 and G101, respectively, the plasmids might also interfere with phage synthesis occurring spontaneously or following ultraviolet (u.v.) induction of lysogens harbouring the plasmid. This observation has been made with some R factors from enterobacteria (Hedges, 1972), in contrast to those R factors which restrict λ or P22 but which do not inhibit spontaneous or u.v.-induced multiplication of these phages (Watanabe *et al.* 1966). When this possibility was tested with the u.v.-inducible phage B39, R factor inhibition of phage multiplication in lysogens was indeed found. For example, the supernatant of an overnight broth culture of PAO8 (B39) had a titre of $1{\cdot}7 \times 10^8$ pfu/ml., whereas PAO8 (B39) (R18-1) had a titre of $1{\cdot}6 \times 10^6$ pfu/ml. In addition, u.v. irradiation (420 ergs/mm^2) of PAO8 (B39) increased phage titre from $6{\cdot}3 \times 10^7$ pfu/ml. to $1{\cdot}5 \times 10^{10}$ pfu/ml., whereas identical treatment only increased the titre of phage B39 in cultures of PAO8 (B39) (R18^{-1}) from $1{\cdot}7 \times 10^5$ pfu/ml to $3{\cdot}5 \times 10^5$ pfu/ml. This inhibition was specific since phage titres in overnight broth cultures of PAO8 (B39) lysogens harbouring other R factors

such as R18-3, R68, R91 or the sex factor FP2 varied only between $1{\cdot}1–2 \times 10^8$ pfu/ml. Furthermore, the presence of the R factor R18-1 in PAO8 sub-lines lysogenic for either phage F116L or D3 did not influence the level of free phage in overnight broth cultures. However, R18-3 failed to inhibit spontaneous induction of the u.v.-non-inducible phage G101 in R^+ lysogens, perhaps because G101 lysogens were relatively stable. For example, the titres of phage G101 in overnight broth cultures of strains PAO8 (G101) and PAO8 (G101) (R18-3) were $1{\cdot}2 \times 10^5$ pfu/ml and $6{\cdot}3 \times 10^4$ pfu/ml, respectively.

Inhibition of B39 phage replication by R18-1 and of G101 by R18-3 therefore appears to resemble interference exerted by such plasmids as F, ColIb-P9 and some R factors (Morrison & Malamy, 1971; Moyer *et al.* 1972; Hedges, 1972).

4. DISCUSSION

Both male-specific and female-specific bacteriophages have been found to be useful in the classification of plasmids of enteric bacteria such as *Escherichia*, *Salmonella* and *Shigella* (Meynell, Meynell & Datta, 1968; Pitton & Anderson, 1970; Watanabe *et al.* 1966; Bannister & Glover, 1968; Meynell, 1972). A similar approach using male-specific phages has been fruitful in *P. aeruginosa* (Stanisich, 1974*b*). In the present study 19 plasmids isolated from different geographical regions were grouped into five categories by the use of six phages. It was also possible to distinguish between plasmids isolated from the same geographical region. Thus, the Birmingham R factors, R74, R18, R88, R68, R30, RP1, R1822 and RP4, were distinguishable from other R factors from the same source by inhibiting only phage G101. The other Birmingham R factors R18-1, RP1-1, R19 and R91 could be divided further on the basis of inhibition of phage B39 by R18-1 and RPI-1, but not R19 and R91. Thus classification of the Birmingham R factors was consistent with classifications using other phenotypic characteristics. For example, R18, R74, R88, R68 and R30 were distinguishable from R18-1 and from R19 (and R91) on the basis of host range, sensitivity to the male-specific phage PRR1 (Olsen & Shipley, 1973), requirement for the host *recA* function for inheritance, drug-resistance pattern and its expression, stability, compatibility, derepressibility of transfer functions (Chandler & Krishnapillai, 1974*a*, *b*) and plating properties of male-specific filamentous phages (Stanisich, 1974*b*). Likewise, the Glasgow R factor RP8 (Black & Girdwood, 1969), although it shows considerable genetic similarity by DNA hybridization tests with the Birmingham R factor RP1 (Saunders & Grinsted, 1972), was nevertheless distinguishable by phage G101. This is consistent with differences between these two plasmids revealed by filamentous phages (Stanisich, 1974*b*).

The phage-plating responses also differentiated between the Japanese R factors R2, R38 and R39. On this basis the latter two R factors were distinguishable from R2, as was perhaps expected from their different drug-resistance patterns (Kawakami *et al.* 1972). On a similar basis the Canadian R factors R130 and R931 appeared related. This was also to be expected because of the relationship

established from their exclusion characteristics (Bryan *et al.* 1973). The Canadian R factor R679 was distinguishable from R130 and R931 and this could be correlated with their non-exclusion characteristics (Bryan *et al.* 1973). R716 was distinguishable from R679 and R130 (and R931), and this was consistent with differences in antibiotic resistance pattern.

The sex factors FP2, FP5 and FP39 were not distinguishable from each other, although they were from the R factors, by phage-plating responses. However, they have been shown to be distinct by other criteria such as mercury-resistance, suppression of a Leu phenotype, chromosome transfer kinetics, curing by acriflavine, and interfertility (Pemberton & Holloway, 1973; Matsumoto & Tazaki, 1973).

The phage-plating responses have also been useful in confirming the possible identity of R factors isolated from the same original strain and designated differently by different investigators. For example, RP1 (Grinsted *et al.* 1972), R1822 (Olsen & Shipley, 1973), RP4 (Datta *et al.* 1971; Holloway & Richmond, 1973) and R18 (Chandler & Krishnapillai, 1974*a*) are all probably the same R factor isolated from *P. aeruginosa* strain 1822 (Lowbury *et al.* 1969), since they have the unique ability to inhibit the plating of phage G101. Similarly RP1-1 (Ingram *et al.* 1972) and R18-1 (Chandler & Krishnapillai, 1974*a*) also isolated from 1822 are probably identical because of their unique ability to inhibit B39 plating. The Canadian R factor R716 also inhibits B39 plating but it was possible to distinguish this from RP1-1 and R18-1 because R716$^+$ bacteria failed to plate B39 at all whereas the other two R factors reduced the EOP to 10^{-6}.

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