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Superinfection inhibition by prophage B3 of some R plasmids in *Pseudomonas aeruginosa*

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SUMMARY

The prophage of the phage B3 of *P. aeruginosa* exhibits superinfection inhibition towards certain R plasmids such as R18-1 or R18-3 as shown by a severe depression (*c.* 10^{-6}) in the conjugal transfer of these plasmids into B3 lysogens. The transfer of other R plasmids into such lysogens and the transfer of R18-1 or R18-3 into bacteria lysogenic for unrelated phages was unimpaired. R18-1 or R18-3 was non-transducible into B3 lysogens although transducible into non-lysogens. The interaction appeared to be reciprocally expressed since B3 lysogenization of bacteria R^+ for R18-1 or R18-3 was also reduced. Consistent with the failure of co-existence of the prophage and the plasmids was the observation that when the transconjugants from the cross R18-1 donor × B3 lysogenic recipients were tested, transfer-deficient plasmid derivatives were encountered (about 40%). Other transconjugants were cured of prophage, or carried mutant B3 prophage with lytic, lysogenization or superinfection inhibition functions impaired.

1. INTRODUCTION

Conjugative R plasmids identified from *P. aeruginosa* strains isolated from a Birmingham hospital (Lowbury *et al.* 1969) have been extensively characterized (Datta *et al.* 1971; Ingram *et al.* 1972; Grinsted *et al.* 1972; Holloway & Richmond, 1973; Chandler & Krishnapillai, 1974*a*, *b*; Krishnapillai, 1974; Stanisich, 1974). Perhaps the best known of these plasmids are the genetically promiscuous ones such as the prototype RP1 (or RP4) which is a P incompatibility group plasmid in the *E. coli* classification scheme (Datta *et al.* 1971) or the P-1 group in the *P. aeruginosa* scheme (Jacoby, 1976). It can be shown that the Birmingham plasmids are separable into three groups on the basis of their antibiotic resistance, host range, stability, response to donor-specific phages and by their ability to interfere with the propagation of certain bacteriophages (Phi), derepressibility of transfer functions and the requirement of the host *recA* gene product for stable inheritance. In addition plasmids of the three groups are compatible with each other and do not show entry exclusion towards each other (Chandler & Krishnapillai, 1974*a*, *b*; Chandler, 1975). R18-1, R18-3 and R19 are representative of these groups and they belong to incompatibility groups P-9, P-1 and P-10 respectively, in *P. aeruginosa* (Jacoby, 1976; V. Stanisich, personal communication). R74-3, R30, R68 and R88 are other P-1 plasmids. R18-1 is probably identical to RP1-1

(Ingram *et al.* 1972; Chandler & Krishnapillai, 1974*a*; Krishnapillai, 1974) and R18-3 is probably identical to RP1 (Grinsted *et al.* 1972; Chandler & Krishnapillai, 1974*a*; Krishnapillai, 1974). These plasmids were isolated from the same initial strain but the separate designations are maintained because of their different genealogies with the possibility of genetic differences between the respective plasmids, although none has been detected so far (Krishnapillai, 1974).

While studying transmission of the Birmingham plasmids into bacterial recipients lysogenic for different prophages it was observed that bacteria lysogenic for the transducing phage B3 (Holloway, 1969) were poor recipients of certain of these plasmids. This report concerns the analysis of this phenomenon.

2. MATERIALS AND METHODS

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

(ii) *Bacterial strains, phages and plasmids.* See Table 1.

(iii) *Phage methods.* These were essentially as described in Adams (1959).

(iv) *Transduction.* The standard procedure (Krishnapillai, 1971) was used.

Table 1. *Bacterial strains, temperate phages and plasmids used*

Strain, phage or plasmid	Relevant characters*	Reference†
Strain		
P. aeruginosa		
PAO1	*cml*-2 prototroph	1
PAO8	*met*-28 *ilv*-202 *str*-1	1
Phage‡		
B3	Transducing, u.v.-non-inducible	2
D3	Non-transducing, u.v.-inducible	2
G101	Transducing, u.v.-non-inducible	2
F116L	Transducing, u.v.-inducible	3
B39	Non-transducing, u.v.-inducible	4
Plasmid alternative designation§		
R18-1 RP1-1	Cb Phi (B39) Inc P-9	4, 6, 7
R18-3 RP1	Cb Nm Km Tc Phi (G101) Rar Inc P-1	4, 6, 8
R88	Cb Nm Km Tc Phi (G101) Rar Inc P-1	4, 6
R30	Cb Nm Km Tc Phi (G101) Rar Inc P-1	4, 6
R74-3	Cb Nm Km Tc Phi (G101) Rar Inc P-1	5, 6
R68	Cb Nm Km Tc Phi (G101) Rar Inc P-1	4, 6
R19	Cb [Nm Km Tc] Inc P-10	4, 6

* Chromosomal marker abbreviations: *cml* = chloramphenicol; *str* = streptomycin; *met* = methionine; *ilv* = isoleucine plus valine. Plasmid markers are symbolized according to Novick *et al.* (1976). The markers in parenthesis with reference to R19 refers to their non-expression in *P. aeruginosa* (Chandler & Krishnapillai, 1974*b*). Rar refers to resistance to aeruginocin (Chandler & Krishnapillai, 1974*a*). The incompatibility (Inc) group designation refers to that assigned in *P. aeruginosa* (Jacoby, 1976). Inc P-9 and Inc P-10 have recently been assigned (V. Stanisich, personal communication).

† 1, Isaac & Holloway (1968); 2, Holloway (1969); 3, Krishnapillai (1971); 4, Krishnapillai (1974); 5, Chandler & Krishnapillai (1974*a*); 6, Jacoby (1976); 7, Ingram *et al.* (1972); 8, Grinsted *et al.* (1972).

‡ B3 and B39 are serologically related but are heteroimmune (Krishnapillai, 1974).

§ See Introduction.

(v) *Plasmid transfer.* Plate matings were performed by using overnight broth-grown cells (resuspended in saline) and plating donor (diluted when necessary) and recipient cells on nutrient agar supplemented with streptomycin (500 μg/ml) and carbenicillin (250 μg/ml) or on minimal agar supplemented with carbenicillin. Broth matings were performed by mixing log-phase cells of donor and recipient and incubating at 37 °C for 2 h prior to plating on nutrient agar supplemented with streptomycin and either neomycin (400 μg/ml) or tetracycline (250 μg/ml).

(vi) *Lysogen test.* Whether a strain was lysogenic or not was scored on the basis of plaque formation on indicator overlays and/or by immunity to superinfection with homologous phage.

3. RESULTS

(i) *Transfer of conjugative R plasmids into recipients lysogenic for different phages.* A number of lysogenic sublines of strain PAO8 were constructed by lysogenizing with the temperature phages B3, B39, G101, F116L or D3 and the plasmid transfer frequency into lysogenic or non-lysogenic recipients was compared (Table 2A). It is seen that the transfer frequency of R18-1, R18-3, R88, R74-3 and R30 is reduced about a million-fold or more into recipients lysogenic for B3. That this reduction in transfer frequency was not simply due to the use of the plasmid marker Cb as the selective marker was supported by similar results when two other plasmid markers were used for selection (Table 2B). In contrast, R68 and

Table 2A. *Transfer of conjugative R plasmids into lysogenic recipients by selection for Cb transfer*

	Plasmid donor*						
Recipient	R18-1	R18-3	R88	R30	R74-3	R68	R19
PAO8	2×10^{-2}†	5×10^{-2}	3×10^{-2}	3×10^{-3}	1×10^{-3}	2×10^{-3}	2×10^{-4}
PAO8 (B3)‡	3×10^{-8}	8×10^{-8}	$< 10^{-8}$	$< 10^{-8}$	$< 10^{-8}$	2×10^{-3}	2×10^{-4}
PAO8 (B39)	3×10^{-2}	5×10^{-2}					
PAO8 (G101)	3×10^{-2}	2×10^{-2}					
PAO8 (D3)	1×10^{-2}	5×10^{-2}					
PAO8 (F116L)	3×10^{-3}	2×10^{-2}					

Table 2B. *Transfer of R18-3 from PAO1 (R18-3) into B3 lysogen by selection for Nm or Tc transfer*

Recipient	Tc transfer	Nm transfer
PAO8	10^{-3}§	4×10^{-4}§
PAO8 (B3)	$< 4 \times 10^{-9}$	$< 4 \times 10^{-9}$

* PAO1 carrying the indicated plasmid.

† Cb transconjugants/donor in plate matings.

‡ When the transfer frequency into this recipient was low, the carbenicillin concentration was 1000 μg/ml.

§ Tc or Nm transconjugants/donor in 2 h broth matings.

R19 are transferable at comparable frequencies into B3 lysogens and non-lysogens. Furthermore, the transfer of R18-1 or R18-3 is unimpaired into recipients lysogenic for B39, G101, F116L or D3.

One possible explanation for the enormously reduced transfer frequency of certain of these R plasmids into B3 lysogens might simply be a reflexion of the induction of prophage in the lysogenic recipients subsequent to mating with the plasmid donors. This was discounted because the titre of phage in mating mixtures (mated for 3 h at 37 °C on an agar surface) of lysogenic recipients with R^+ or R^- donors was very similar. Furthermore, the cell count of the recipient under these conditions was also very similar consistent with non-induction of the lysogenic recipient.

(ii) *Transduction of R plasmids into B3 lysogens.* The failure of conjugal transmission of R18-1 or R18-3 (and presumably also R88, R74-3 and R30) into B3 lysogenic recipients could be due to a cellular surface alteration (analogous to entry exclusion between related plasmids) or a failure at co-existence intracellularly (analogous to incompatibility between plasmids). To distinguish between

Table 3. *Transduction of plasmid into B3 lysogenic recipients*

Transductional		Chromosomal	Plasmid
Donor	Recipient	(*ilv*-202$^+$)	(Cb)†
PAO1 (R18-1)	PAO8	$2{\cdot}1 \times 10^{-7}$*	$2{\cdot}0 \times 10^{-7}$
PAO1 (R18-3)	PAO8	$2{\cdot}2 \times 10^{-7}$	$0{\cdot}62 \times 10^{-7}$
PAO1 (R18-1)	PAO8 (B3)	$1{\cdot}4 \times 10^{-7}$	$< 2{\cdot}0 \times 10^{-10}$
PAO1 (R18-3)	PAO8 (B3)	$3{\cdot}5 \times 10^{-7}$	$< 2{\cdot}0 \times 10^{-10}$

* Transductional frequency: per pfu.

† Selected on nutrient agar supplemented with streptomycin (500 μg/ml) and carbenicillin (250 μg/ml).

these alternatives, transduction of the plasmids by the transducing phage F116L (Krishnapillai, 1971) was attempted. It was hoped that if only a surface alteration was responsible for the inability of B3 lysogens to conjugate effectively with donors carrying the plasmid then it ought to be possible to circumvent this by transfer of the plasmid via a phage particle as in transduction. As an internal control, the transduction of a chromosomal marker (*ilv*-202) was also monitored from the same set of donors into the same set of recipients. The results (Table 3) show that the frequency of transduction of *Ilv*$^+$ is very similar whether the recipient was lysogenic or otherwise and also whether the donor carried R18-1 or R18-3. By contrast the transduction of the plasmids themselves occurs at frequencies comparable to the chromosomal marker only into non-lysogenic but not into B3 lysogenic recipients.

(iii) *B3 lysogenization of bacteria harbouring R18-1 or R18-3.* Since it was shown that the failure of B3 lysogens to inherit R18-1 or R18-3 was expressed intracellularly, i.e. via the inability of B3 prophage and R18-1 or R18-3 to co-exist in the same cell, the question was asked as to whether this phenomenon is reciprocal.

That is, whether there is a reduction in the ability of B3 to lysogenize bacteria harbouring the plasmid. In qualitative lysogenization tests (i.e. by scoring purified clones surviving phage infection on agar plates) it was found that it was not possible to obtain R18-1 or R18-3 carrying derivatives which were also lysogenic for B3 (less than 5% lysogenic) whereas very efficient lysogenization (nearly 100% lysogenic) was possible with R^- isogenic bacteria. In similar tests the ability to lysogenize R18-1 or R18-3 carrying bacteria by the unrelated phages G101 or D3 was unimpaired by comparison with R^- bacteria (i.e. nearly 100% lysogenic in all cases).

(iv) *Nature of transconjugants in genetic crosses between PAO1 (R18-1) donors and PAO8 (B3) recipients.* Although the transfer of R18-1 or R18-3 (or R88, R74-3 or R30) into B3 lysogens was reduced a millionfold or more (Table 2A), it was nevertheless possible to obtain some transconjugants in these crosses. In the light of the observations that the interaction between B3 prophage and these R plasmids was inimical to their stable co-existence, the nature of the prophage and/or R plasmid derivatives in these transconjugants which occurred at very low frequency in such crosses (Table 2A) were examined. For this purpose 32 carbenicillin-resistant (Cb) transconjugants from the cross PAO1 (R18-1) donor × PAO8 (B3) recipient were studied. Two questions were asked – (1) Was the Cb determinant still transferable and if so, was it still subject to superinfection inhibition by B3 lysogens? (2) Was the B3 prophage still present and if so, was it normal (at least by the criteria of ease of lysogenization and plaque appearance in lysogen tests), or mutant (as measured by an altered superinfection inhibition response)?

Each purified transconjugant was mated with PAO1 and selected for the transfer of Cb. Where transfer occurred the PAO1 R^+ derivatives so obtained were crossed with PAO8 (B3) to determine their transfer frequency. To test the prophage, the transconjugant was streaked out on an overlay agar of PAO8 and from the plaques arising, new lysogenic derivatives of PAO8 were constructed. These derivatives were then tested for their recipient ability for R18-1 from PAO1 (R18-1) donors. Two classes of transconjugants were observed. One class of eighteen had transferable Cb as tested by transfer into PAO1 (frequency = $> 10^{-4}$ Cb/donor). However, in seven cases there was no evidence for the presence of B3 prophage in lysogen tests (i.e. these were presumably cured of prophage). In five cases the prophage was mutant with respect to superinfection inhibition, i.e. lysogenic derivatives of three of these acted as conjugal recipients at frequencies of about 10^{-3} Cb/donor to PAO1 (R18-1) donors which is only about tenfold lower than the transfer of the R plasmid into a non-lysogen (Table 2A). These recipients were nearly 100% lysogenic. Lysogenic derivatives of two of the five prophages acted as recipients at frequencies of 3×10^{-5} and 3×10^{-6} Cb/donor, respectively, for R18-1 transfer. These recipients were 30-60% lysogenic. The latter results perhaps indicate that the prophage mutations are leaky with respect to superinfection inhibition. The final group in this class were six transconjugants which had a defective prophage as judged by their inability to form discrete plaques on indicator overlays although lysis was observed and they were still immune to B3 phage (three transconjugants),

or by their inability to be lysogenized (i.e. survivors of phage infection were non-lysogenic). The R plasmids themselves appear to be normal with respect to superinfection inhibition by B3 prophage since their transfer into B3 lysogens is still similar to that seen before (Table 2A) (i.e. these eighteen derivatives of R18-1 were still nontransferable into PAO8 (B3) = $< 10^{-8}$ Cb/donor). In the other class (fourteen transconjugants) all the R plasmid derivatives were non-transferable as tested by transfer into PAO1 (transfer frequency = $< 10^{-8}$ Cb/donor) but in virtually all of the transconjugants (13/14) the prophage appears to be normal as judged by ability to form lysogenic derivatives, B3 immunity and superinfection inhibition towards R18-1 (transfer frequency of R18-1 into PAO8 (B3) = $< 10^{-8}$ Cb/donor). One transconjugant had a defective prophage (reduced incidence of lysogenization) in addition to a non-transmissible Cb determinant.

4. DISCUSSION

The R plasmids used in this study belong to three different incompatibility groups: R18-1 is a P-9 plasmid; R18-3, R74-3, R30, R68, R88 are P-1 plasmids and R19 is a P-10 plasmid (Jacoby, 1976; V. Stanisich, personal communication). However, the prophage B3 of *P. aeruginosa* fails to co-exist intracellularly with R18-1, R18-3 (and presumably also with R74-3, R30, R88) whereas it does with R68 and R19. Incompatibility has generally been used to imply that the replication machinery of homologous or genetically related plasmids share common components such that two such elements fail to co-exist intracellularly (Datta, 1975). But because there is no correlation between the R plasmid incompatibility group and the interaction with the prophage the term superinfection inhibition is used to describe it because this is a more general term (Novick *et al.* 1976). Furthermore, the efficiency of plating of phage B3 on bacteria R^+ for R18-1 or R18-3 is the same as on R^- isogenic bacteria indicating that superinfection inhibition is manifested only when B3 is a prophage (i.e. in an established lysogen) rather than when it is undergoing vegetative replication. Typical incompatibility between prophages thought to be extrachromosomal in location, has been shown between the phages ϕ AMP and PICM although lytic replication between these phages can occur in the same cell suggesting a dichotomy in the control of prophage replication and vegetative phage replication leading to cell lysis (Hedges *et al.* 1975).

Although there is evidence in *P. aeruginosa* that RP1 is extrachromosomal (Ingram *et al.* 1972) and so also possibly for R18-1 (on the basis of differential UV sensitivity of the Cb determinant relative to a known chromosomal determinant, during transduction – the so-called Arber (1960) effect (V. Krishnapillai, unpublished data), the location of B3 prophage is unknown since neither chromosome genetic mapping nor buoyant density equilibrium centrifugation indicates its location (Carey, 1974).

The superinfection inhibition described here in *P. aeruginosa* appears not to have many parallels but a superficially analogous situation exists in *Staph. aureus*

where chromosomal integration of penicillinase plasmids directed by a prophage and the occurrence of prophage–plasmid co-integrates have been reported (Schwesinger & Novick, 1975). Perhaps the non-transferable Cb transconjugants of *P. aeruginosa* are instances of chromosomal integration of the Cb determinant mediated by prophage B3. On the other hand it may be an intrinsic property of the R18-1 plasmid (and perhaps of others as well) since chromosomal integration of RP1-1 is demonstrable in *E. coli* (Richmond & Sykes, 1972). The five B3 prophage mutants which show greatly decreased superinfection inhibition to R18-1 appear to be analogous to the Inc$^-$ penicillinase plasmid mutant described in *Staph. aureus* (Wyman & Novick, 1974) or the Inc$^-$ Hfr mutant of *E. coli* (De Vries & Maas, 1973). How similar these situations are remains to be determined. These and other considerations, such as the occurrence of defective forms of the prophage or R18-1 when these elements are forced to co-exist, highlight the genetic relationship between these elements in bacteria.

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Note added in proof: Because extensive incompatibility tests with already designated incompatibility groups have not been made with R18-1 and R19 it has been decided not to use, for the present time, the P-9 and P-10 designations, respectively, for these plasmids (Jacoby, 1976; and personal communication).

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