The Properties and Host Range of Male-specific Bacteriophages of *Pseudomonas aeruginosa*

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SUMMARY

Three *Pseudomonas aeruginosa* phages specific for bacteria harbouring the P-group plasmid RP₁ have been isolated, and their properties compared with those of a previously described sex-specific phage, PRR₁ (Olsen & Shipley, 1973). These phages are distinguishable from each other by various criteria, although in terms of host range to FP⁺ and RP⁺ lines of *P. aeruginosa* PAO they comprise broadly two groups. Thus all the phages infect bacteria harbouring any of a group of plasmids with similar properties to those of RP₁, but whereas the filamentous phage Pf₃ is specific for this group, the host ranges of PRR₁, PR₃ and PR₄ are considerably wider. Nevertheless, with one exception, this does not extend to plasmids isolated outside the United Kingdom, which suggests that all these plasmids share a common ancestry even though by other criteria they constitute three fairly discrete sub-groups. Of the plasmids that fail to allow phage propagation, three, when present in the same cell as RP₁, reduce its susceptibility to phage infection. This inhibition may reflect a relationship between these elements, similar to that found among plasmids of Enterobacteria. A correlation is observed between the susceptibility of bacteria to phage infection and their ability to mediate plasmid transfer, such that these phages can conveniently be used to isolate both derepressed or transfer-defective mutants of various R factors.

INTRODUCTION

The characteristics of R factors carried by clinical strains of *Pseudomonas aeruginosa* isolated from different geographical areas have been described by a number of investigators (Datta *et al.* 1971; Stanisich & Holloway, 1971; Chabbert *et al.* 1972; Grinsted *et al.* 1972; Ingram *et al.* 1972; Kawakami *et al.* 1972; Saunders & Grinsted, 1972; Bryan *et al.* 1973; Ingram, 1973; Morgan, Saunders, Stanisich & Richmond, 1973; Olsen & Shipley, 1973; Chandler & Krishnapillai, 1974*a, b*; Stanisich, 1974). One of these strains, *P. aeruginosa* 1822 (Lowbury *et al.* 1969), has been used widely in both inter- and intra-generic conjugations, and yields R⁺ transciipients resistant to the antibiotics carbenicillin (CB), neomycin/kanamycin (NM/KM), and tetracycline (TC). The R factors carried by these sub-lines have been variously designated RP₁ (Grinsted *et al.* 1972), RP₄ (Datta *et al.* 1971; Holloway & Richmond, 1973), R1822 (Olsen & Shipley, 1973) and R18 (Chandler & Krishnapillai, 1974*a*), and although isolated in independent experiments may well represent similar, if not identical, R factors. Olsen & Shipley (1973) used the R1822⁺ sub-line to isolate an RNA-containing sex-specific phage, and this also infects bacteria harbouring RP₄. The phage, designated PRR₁, has physical characteristics similar to those described for the icosahedral RNA phages of *Escherichia coli* and *P. aeruginosa* (Olsen & Thomas, 1973),

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Table 1. Derivation and phenotypes of plasmid-carrying sub-lines of *P. aeruginosa* PAO

Plasmid	<i>P. aeruginosa</i> carrier strain and geographical origin	Reference(s)	Phenotype conferred in <i>P. aeruginosa</i> PAO	Derivation of <i>P. aeruginosa</i> PAO sub-line
RP1-I	1822 (U.K.)	Lowbury <i>et al.</i> (1969), Ingram <i>et al.</i> (1972)	CB	From <i>P. aeruginosa</i> 18s(RP1-I) ⁺ (provided by M. H. Richmond) via <i>P. aeruginosa</i> PAT
R18-I	1822 (U.K.)	Lowbury <i>et al.</i> (1969), Chandler & Krishnapillai (1974 <i>b</i>)	CB	From <i>P. aeruginosa</i> 1822; Chandler & Krishnapillai (1974 <i>a</i>)
R9I	9169* (U.K.)	Stanisich & Holloway (1971)	CB†	From <i>P. aeruginosa</i> 9169 via <i>P. aeruginosa</i> PAT; Stanisich & Holloway (1971)
R19	1954* (U.K.)	Chandler & Krishnapillai (1974 <i>b</i>)	CB†	From <i>P. aeruginosa</i> 1954; Chandler & Krishnapillai (1974 <i>b</i>)
RP8	s8 (U.K.)	Black & Girdwood (1969), Saunders & Grinstead (1972)	CB	From <i>P. aeruginosa</i> s8 (provided by M. H. Richmond)
RP1	1822 (U.K.)	Lowbury <i>et al.</i> (1969), Grinstead <i>et al.</i> (1972)	CB NM/KM TC	From <i>P. aeruginosa</i> 18s(RP1) ⁺ (provided by M. H. Richmond) via <i>P. aeruginosa</i> PAT
RP4	1822 (U.K.)	Datta <i>et al.</i> (1971), Holloway & Richmond (1973)	CB NM/KM TC	From <i>E. coli</i> 154 (carrier strain provided by B. Davey)
R1822	1822 (U.K.)	Olsen & Shipley (1973)	CB NM/KM TC	From <i>P. aeruginosa</i> PAO67(R1822) ⁺ (provided by R. Olsen)
R18	1822 (U.K.)	Chandler & Krishnapillai (1974 <i>a</i>)	CB NM/KM TC	From <i>P. aeruginosa</i> 1822; Chandler & Krishnapillai (1974 <i>a</i>)
R30	3098* (U.K.)	Stanisich (1974)	CB NM/KM TC	From <i>P. aeruginosa</i> 3098; Stanisich (1974)
R68	6886* (U.K.)	Chandler & Krishnapillai (1974 <i>a</i>)	CB NM/KM TC	From <i>P. aeruginosa</i> 6886, P14 and 6888 respectively; Chandler & Krishnapillai (1974 <i>a</i>)
R74	P14* (U.K.)	Chandler & Krishnapillai (1974 <i>a</i>)	CB NM/KM TC	
R88	6888* (U.K.)	Chandler & Krishnapillai (1974 <i>a</i>)	CB NM/KM TC	
R2	R2-72 (Japan)	Kawakami <i>et al.</i> (1972)	CB KM SM	From <i>P. aeruginosa</i> R2-72, R38-72 and R39-72 respectively (carrier strains provided by H. Matsumoto)
R38	R38-72 (Japan)	Kawakami <i>et al.</i> (1972)	SM TC Hgt	
R39	R39-72 (Japan)	Kawakami <i>et al.</i> (1972)	SM TC Hgt	
R716	716 (Canada)	Bryan <i>et al.</i> (1973)	SM	
R679	679 (Canada)	Bryan <i>et al.</i> (1973)	SM Su	From <i>P. aeruginosa</i> 716, 679, 130 and 931 respectively (carrier strains provided by L. E. Bryan)
R130	130 (Canada)	Bryan <i>et al.</i> (1973)	SM Su GM Hgt	
R931	931 (Canada)	Bryan <i>et al.</i> (1973)	SM TC Hgt	
FP2	PAT (South Africa)	Holloway (1969)	Hg	From <i>P. aeruginosa</i> PAO8 (FP2) ⁺
FP5	ps5-70 (Japan)	Matsumoto & Tazaki (1973)	Hg	From <i>P. aeruginosa</i> ps5-70 (sub-line constructed by B. W. Holloway)
FP8	8 (Australia)	Pemberton (1971)	Hg	From <i>P. aeruginosa</i> 8; Pemberton (1971)
FP39	39 (Australia)	Pemberton & Holloway (1973)	Leu ⁺	From <i>P. aeruginosa</i> PAO1264 (sub-line constructed by B. W. Holloway)

Antibiotic resistances: CB, carbenicillin; NM, neomycin; KM, kanamycin; TC, tetracycline; SM, streptomycin; Su, sulphonamide; GM, gentamycin. Metal resistance: Hg, mercuric ions.

* Strain isolation numbers assigned by E. J. L. Lowbury (personal communication).

† Chandler & Krishnapillai (1974*b*) have shown that NM/KM TC resistance is conferred by these R factors in *E. coli* K12.

‡ Resistance to mercuric ions is an additional determinant carried by these R factors (Stanisich, unpublished data).

and like them shares a requirement for bacterial pili for adsorption (unpublished data of C. H. To and C. Brinton cited in Olsen & Thomas, 1973; Bradley, 1974).

In the present investigation three phages specific for bacteria harbouring RPI have been isolated and their properties compared with those of PRR1. The ability of these phages to infect various plasmid-carrying derivatives of *P. aeruginosa* PAO was studied as a means of determining the genetic similarity, and hence possible relationships, between these plasmids. These include elements conferring resistance to antibiotics (R or RP factors), as well as those initially detected because of their ability to mediate host chromosome transfer (FP factors). The use of these phages to isolate derepressed or transfer-defective mutants of the R factors was also investigated.

METHODS

Bacterial strains. All *P. aeruginosa* lines are derivatives of strain PAO (Holloway, 1969). These were: PAO8 *met-28 ilv-202 str-1* (Isaac & Holloway, 1968) and its sub-lines harbouring RPI-1, RI8-1, RI9, R91, RP8, RPI, RP4, RI822, RI8, R30, R68, R74 or R88; PAO1670 *leu-8 pur-136 chl-3 rif-1* (Chandler & Krishnapillai, 1974c) and its sub-lines harbouring R2, R38, R39, R716, R679, RI30, R931, FP2, FP5, or FP39, or the 'double male' lines shown in Table 4 below; PAO3 *ser-3* (Holloway collection) and its sub-line harbouring FP8. The derivation and phenotypes of the sub-lines are given in Table 1. The double male strains were constructed from the appropriate PAO1670 lines and inherited RPI from PAO2604, i.e. PAO8 (RPI)⁺.

Escherichia coli C str (strain C411 of Wiman, Bertani, Kelly & Sasaki, 1970) was obtained from P. M. Chandler and an RPI⁺ sub-line constructed by mating with PAO1670 (RPI)⁺.

Escherichia coli K12 *tonB trpΔAB* was obtained from C. Yanofsky and an RPI⁺ sub-line constructed by mating with PAO2604.

Abbreviations conform to the nomenclature of Demerec, Adelberg, Clark & Hartman (1966).

Media. Nutrient broth (NB) and nutrient agar (NA) were as previously described (Stanisich & Holloway, 1972). Supplements to NA were carbenicillin (Beecham Research Labs) and streptomycin (BDH), 250 µg/ml, rifampicin (Ciba-Geigy), 200 µg/ml, and mercuric chloride, 15 to 30 µg/ml.

Phage assay or plate mating by spot method. Sensitive bacteria or recipients for mating were seeded on to the surface of nutrient or selective medium by flooding the agar surface with several millilitres of a log phase culture, then removing the excess fluid. When the surface had dried, 0.01 ml of serially diluted phage preparations or donor bacteria was spotted on to the lawn. Phage plaques or conjugal transcipts developed after 24 and 48 h respectively at 37 °C.

Multiplication of phage in liquid medium. Log phase cultures (10⁷ to 10⁸ bacteria/ml) were infected with phage at a final titre of 10⁴ to 10⁵ p.f.u./ml. The mixture was incubated at 37 °C with aeration for about 6 h, then centrifuged and the phage titre of the supernatant determined.

Preparation of derepressed cultures. Log phase cultures of the recipient bacterium and its FP⁺ or RP⁺ sub-line were inoculated into 10 ml NB at a ratio of 50 or 100 to 1 respectively, and at an initial density of about 5 × 10⁷ bacteria/ml. The mixture was incubated at 37 °C with aeration for about 3 h or until the cell density had increased to about 10⁹/ml.

Isolation of derepressed or transfer-defective R factor mutants. Details of the mutagenesis procedure are described by Curtis, Richmond & Stanisich (1973). Immediately after exposure to nitrosoguanidine, R⁺ cultures were mated with a recipient strain and the mixture plated to appropriate selective medium to isolate R⁺ transcipts. Derepressed mutants of

R91 were isolated by cross-streaking single clones of such transciipients against PRR1 (titre about 10^9 p.f.u./ml). Colonies that were phage-sensitive were selected as possible derepressed mutants. Non-siblings were those obtained from independent experiments. Transfer-defective mutants of RP1 were isolated by testing transciipients for their ability to transfer CB-resistance to a recipient by replica plate mating (Stanisich & Holloway, 1972). Colonies that failed to yield CB-r progeny were selected as possible transfer-defective mutants.

Preparation of phage antiserum. Six injections of phage PRR1 (0.5 to 1.5 ml of 2×10^{10} p.f.u./ml) were made intravenously (except for the first which was intramuscular) at 3 to 4 day intervals. After resting the animal for one week, blood was collected and the serum separated by centrifugation after clot formation had occurred.

RESULTS

Isolation of bacteriophages specific for bacteria harbouring RP1

The isolation of RP1-specific bacteriophages from Melbourne, Australia, sewage followed a procedure similar to that described by Olsen & Shipley (1973). Two bacterial strains were used, *P. aeruginosa* PAO (sensitive to streptomycin) and *E. coli* C (resistant to streptomycin), both harbouring the R factor RP1 (Grinsted *et al.* 1972). Sewage effluent from two Melbourne localities was Millipore-filtered and samples plated to the *P. aeruginosa* indicator using the agar layer method (Adams, 1959). The plaques that developed after 24 h incubation at 37 °C (about 40/ml) were tested for lytic activity on the *E. coli* host by inoculating samples of the plaques to the indicator lawn on streptomycin-containing NA. Approximately 60% of the plaques tested produced lysis of both indicators, and further testing showed that the phages were specific for R⁺ bacteria in that they failed to produce plaques on the R⁻ parent strains. Three phages, which appeared to differ in plaque morphology, were chosen for further study and designated PR3 (from locality 1) and PR4 and Pf3 (from locality 2). Their lytic activity on various hosts is shown in Table 2.

Properties of R-specific bacteriophages

Bradley (1974) has carried out a preliminary investigation of the four phages used in this study to determine their general morphology and sites of adsorption to R⁺ hosts. The phages are of three types. PRR1 is an isometric RNA-containing phage of approx. 26 nm diam, which adsorbs to R-coded pili in *E. coli* and is assumed to adsorb to similar structures in *P. aeruginosa* although these have yet to be demonstrated (Olsen & Shipley, 1973; Olsen & Thomas, 1973; unpublished data of C. H. To and C. Brinton cited in Olsen & Thomas, 1973; Bradley, 1974). PR3 and PR4 are larger (approx. 65 nm diam) hexagonal-shaped virions, possibly with an easily detached tail, and these, in contrast to PRR1, adsorb to the cell wall of R⁺ *E. coli* and *P. aeruginosa*. However, the possibility that very short or retractable pili provide the adsorption sites has not been disproved. Pf3 is a short filamentous virus (approx. 760 nm length) which also appears to infect the host directly through the cell wall rather than via R-coded pili (Bradley, 1974).

A comparison of the plaque morphologies of these phages, produced after 18 h growth in layers using an indicator such as *P. aeruginosa* PAO2604, allowed the filamentous Pf3 to be readily distinguished from the other phages. Thus Pf3 produces small (1 to 1.5 mm) turbid plaques with ill-defined edges, in contrast to the clear, sharply defined plaques of the other phages. The turbidity of the former probably reflects the ability of Pf3-infected cultures to show a simultaneous increase, with time, of both phage titre and bacterial viable count, a characteristic common among filamentous phages (Marvin & Hohn, 1969; Stanisich,

Table 2. *Lytic properties of R-specific bacteriophage on various bacterial hosts*

Strain	Phage			
	PRR1	PR3	PR4	Pf3
<i>P. aeruginosa</i> PAO8(RP1) ⁺	+	+	+	+
<i>E. coli</i> C(RP1) ⁺	+	+	+	(-)
<i>E. coli</i> K12(RP1) ⁺	+	(+)	+	-
<i>P. aeruginosa</i> PAO	-	-	-	-
<i>E. coli</i> C				
<i>E. coli</i> K12				

+, Complete lysis at high phage titre ($> 10^4$ p.f.u./ml) and characteristic plaques at lower titre (see text); efficiency of plating (e.o.p.) on the various hosts is identical. (+), Complete lysis at high phage titre ($> 10^6$ p.f.u./ml) but plaque size is markedly reduced in comparison to other hosts; e.o.p. may be reduced but this was difficult to determine. (-), Partial lysis at high phage titre, although individual plaques are not observed at lower titre. -, No lysis evident at phage titres of 10^8 p.f.u./ml.

unpublished data). The plaques of PRR1 and PR4 are indistinguishable from each other, and both are considerably larger than those produced by PR3 (3 to 4 mm and 1 to 1.5 mm respectively). A similar plaquing difference is seen when an RP1⁺ line of *E. coli* K12 is used as indicator (Table 2). PRR1 and PR4 can be distinguished by their action on *P. aeruginosa* derivatives harbouring the R factor R2 (see below, Table 3). However, among other plasmid-carrying lines of *P. aeruginosa* the host range of these phages is identical.

Rabbit antiserum prepared against PRR1 with an inactivation constant of 11 min⁻¹ failed to inactivate any of the other phages even when undiluted antiserum was used. Although it is possible that some inactivation might occur with antiserum of greater potency, these results suggest a serological distinction between PRR1 and the newly isolated R-specific phage. On these various criteria it is concluded that the four phages are not identical.

Host range of the R-specific bacteriophages

Plaque-forming ability. The transferable plasmids used in this study are harboured by three auxotrophic sub-lines of *P. aeruginosa* PAO: PAO8, PAO1670 and PAO3. Strain PAO is a particularly suitable host since a number of its plasmid-carrying derivatives are stable during about 15 generations' growth in non-selective medium (Stanisich & Holloway, 1971; Chandler & Krishnapillai, 1974*a, b*; Pemberton & Holloway, 1973; Stanisich, 1974), and this is also true for the R factors RP1, RP1-1 and RP8, those described by Bryan *et al.* (1973), Kawakami *et al.* (1972) and the FP factors FP5 and FP8 (Stanisich, unpublished data; P. Dry, personal communication). The genealogy of the various derivatives and the characteristics conferred by the individual plasmids are shown in Table 1.

The host ranges of the R1822-specific phage PRR1 and of the three RP1-specific phages PR3, PR4 and Pf3 were determined by spot phage assays on lawns of RP⁺ and FP⁺ bacteria on NA (Table 3). Only one series of plasmid-carrying bacteria acted as efficient hosts for the multiplication of these phages leading to plaque formation. This comprised bacteria harbouring the plasmids RP1, RP4, R18 and R1822, all derived from *P. aeruginosa* 1822, and R30, R68, R74 and R88, derived from *P. aeruginosa* strains isolated from the same clinical environment as *P. aeruginosa* 1822 (Lowbury *et al.* 1969; E. J. L. Lowbury, personal communication). The efficiency of plating (e.o.p.) of these phages was constant on the various R⁺ hosts except for R68 which yielded turbid rather than clear plaques with an e.o.p. reduced 10- to 100-fold. This difference is probably associated with the slightly lower conjugal transferability observed with this R factor (Chandler & Krishnapillai, 1974*a*;

Table 3. Host range of sex-specific phages on FP⁺ and RP⁺ lines of *P. aeruginosa* PAO

Plasmid carried by host bacterium*	Plaque formation†				Increase in phage titre in liquid medium‡			
	PRR1	PR3	PR4	Pf3	PRR1	PR3	PR4	Pf3
RP1-1, R18-1	—	—	—	—	+	+	+	—
RP8	(+)	(+)	(+)	—	+	+	+	—
R91, R19	—	—	—	—	+	+	+	—
RP1, RP4, R18, R1822	+	+	+	+	+	+	+	+
R30, R68, R74, R88	+	+	+	+	+	+	+	+
R2	—	—	(+)	—	(+)	(+)	+	—
R38, R39	—	—	—	—	—	—	—	—
R130, R931	—	—	—	—	—	—	—	—
R679, R716	—	—	—	—	—	—	—	—
FP2, FP5, FP8, FP39	—	—	—	—	—	—	—	—

* For simplicity, the FP and RP factors are arranged in groups. These generally reflect the geographical origin of these elements (see Table 1) or the groupings determined on the basis of plating of 'female-specific' phages (Krishnapillai, 1974). The latter are in good agreement with the results obtained in other investigations.

† Determined by spot phage assay (see Methods). +, Single plaques at low phage titres; (+), partial lysis at high phage titre but generally no plaques at lower titre; —, no lysis at high phage titre.

‡ Procedure described in Methods. +, Increase in phage titre of 10³ to 10⁵; (+), increase in phage titre of 10- to 20-fold; —, no increase in phage titre.

also see below). The host range of PRR1 is known to include RP4⁺ hosts (Olsen & Shipley, 1973) so the cross-sensitivity observed within the first group of bacteria is perhaps not surprising. However, the identical behaviour of the second group suggests a similarity between the two. This has already been proposed by Chandler & Krishnapillai (1974a) whose study of the latter group, and of R18, indicated that these plasmids comprise a discrete collection, conferring a range of similar properties on their host cells. Most striking of these is the ability of these plasmids to infect bacteria of different genera, as has been particularly studied for RP4 (Datta *et al.* 1971; Datta & Hedges, 1972) and R1822 (Olsen & Shipley, 1973). Furthermore, the observation of both exclusion and incompatibility between RP1 and the remaining plasmids (Stanisich, unpublished data) and their common ability to prevent multiplication of the temperate phage G101 (Krishnapillai, 1974) leaves little doubt that plasmids in this entire collection are closely similar.

Of the other R⁺ bacteria studied only two, those harbouring R2 and RP8, showed evidence of lysis by these phages, though the response was variable and dependent on both cultural conditions and the particular sub-line employed. Thus at high phage concentrations, PRR1, PR3 and PR4 inhibited bacterial growth, but never completely, and if individual plaques were observed at lower concentrations these were generally extremely indistinct (Table 3).

Phage multiplication in liquid medium. In the preceding section the host range of the R-specific phages was determined by their gross lysis and plaque formation on plasmid-carrying bacteria. This procedure detects populations in which the majority of bacteria are sensitive to phage infection, but gives little information on those that appear 'resistant'. The latter could comprise bacteria that fail to act as host under any circumstances, or those that appear resistant because only a fraction of the population, at any particular time, is susceptible to infection. To distinguish between these two possibilities, combinations of the various phage and bacterial strains were cultured in liquid medium and the supernatants tested after a period of time to determine whether any increase in phage titre had occurred (Table 3).

With the exception of the R2⁺ host, all increases in phage titre were of the order 10³ to 10⁵. The host ranges of PRR1, PR3 and PR4 extended to the entire series of hosts derived from the Lowbury collection, and their activity on RP8⁺ hosts was confirmed. Multiplication of all the phages was evident on RP1⁺ hosts (and on the similar series of R⁺ hosts), and it was of interest that Pf3 was quite specific for this group. In contrast, neither the FP-carrying strains nor those harbouring R38, R39 or the R factors described by Bryan *et al.* (1973) acted as phage hosts. The results obtained with the R2⁺ line allow a distinction to be made between PR4 and the pair PRR1 and PR3. It appears that some aspect of phage adsorption and/or multiplication occurs far more efficiently with PR4 leading to an increase in phage titre of 10² to 10³ compared with an increase of only 10- to 20-fold with PRR1 and PR3. One may conclude that RP8 and all the Lowbury R factors share properties that are necessary for the propagation of phages PRR1, PR3 and PR4. At least some of these are also carried by R2, but not by any of the other RP or FP factors studied. The former two groups are, however, heterogeneous, and can be further subdivided on the basis of Pf3 plating and the conditions under which phage multiplication can be detected.

Concerning the latter point, it is known that in *P. aeruginosa* PAO certain of the R factors, e.g. R91 and R38, are repressed for transfer function since populations newly inheriting these plasmids behave as more efficient donors (Chandler & Krishnapillai, 1974*b*; Stanisich, unpublished data). Such 'derepressed' populations of the various FP⁺ and RP⁺ strains, when tested for plaque formation by the R-specific phages, showed precisely the same lytic response as was found for their multiplication in liquid medium (Table 3). This suggests that the plasmids RP1-1, R18-1, RP8, R19, R91 and R2 differ from the RP1 group in being repressed for those functions necessary for phage propagation, but that derepression can readily be effected by transfer of the plasmids to a new host.

Relationship between phage susceptibility and plasmid transferability

Three experimental approaches were employed to demonstrate a relationship between the susceptibility of R⁺ bacteria to phage infection and their efficiency in promoting plasmid transfer. At least superficially, this relationship seems analogous to that described for certain plasmids of Enterobacteria (Meynell, Meynell & Datta, 1968).

Isolation of derepressed mutants by selection for bacteria sensitive to PRR1. R91⁺ derivatives of *P. aeruginosa* PAO are repressed for transfer function and show no evidence of lysis by the R-specific phages when tested on solid medium, in contrast to the results obtained with populations newly infected with this plasmid. Bacteria harbouring mutants of R91, which were lysed by PRR1 on solid medium, were isolated following the mutagenesis procedure of Curtis *et al.* (1973). Three non-sibling mutants were studied for their frequency of R factor transfer and for sensitivity to the other sex-specific phages.

In 25 min broth matings, using equal numbers of donor and recipient bacteria, transfer of the wild-type R91 occurred at frequencies of 10⁻²/donor, whereas transfer of the mutant R factors was at an increased frequency of 0.5 to 1/donor. Moreover, although plaquing of PRR1, PR3 and PR4 was not evident on the R91⁺ host, the e.o.p. of these phages on the mutants and on an RP1⁺ host was identical. In similar experiments, Chandler & Krishnapillai (1974*b*) isolated derepressed mutants of R19 and R91 by the method of Edwards & Meynell (1968) and observed that in all instances increased R factor transfer was accompanied by increased sensitivity to phage. It is therefore concluded that the same function(s) is required both for efficient plasmid transfer and phage susceptibility, or that these properties are under the same regulatory control.

Properties of phage-resistant and transfer-defective mutants of R⁺ bacteria. Bacteria

Table 4. Phage susceptibility and R factor transfer from double male strains of *P. aeruginosa*

Plasmid(s) harboured by male strain	Lysis by male- specific phages*	Frequency of RP1 transfer†
RP1	+	1.2×10^{-2}
RP1.FP2	+	1.1×10^{-3}
RP1.FP5	—	$< 10^{-7}$
RP1.FP39	+	1.6×10^{-2}
RP1.R38	—	$< 10^{-7}$
RP1.R39	—	$< 10^{-7}$
RP1.R130	+	1.0×10^{-2}
RP1.R716	(+)	9×10^{-5}

* Determined by spotting 10^7 to 10^8 p.f.u. of the four male-specific phages to lawns of the male bacteria.
+, Complete lysis; —, no lysis; (+), partial lysis.

† Represents the frequency of CB-r transipients recovered by spot plate matings.

harbouring RP1 or a derepressed mutant of R91 were made resistant to one of the R-specific phages. Ten mutants of each resistance class showed cross-resistance to the remaining phages (not including Pf3 in the case of R91), and the transfer frequency of their R factors, as determined by spot plate matings, was invariably reduced in comparison with the parent strain. The reduction was however variable, ranging from 10^{-2} to 10^{-4} of the control level. Furthermore, 63 to 65 transfer-defective mutants of RP1, selected because of their low conjugal transfer of CB resistance (at least 10^{-4} of the wild-type level), showed resistance to all four sex-specific phages. The two sensitive strains retained sensitivity to all phages. These results are in accord with the conclusion of the preceding section, since they demonstrate that mutations affecting either phage plating or plasmid transfer generally produce concomitant changes in the alternate character.

Properties of double male strains. From Table 3 it was evident that the R factors R2, RP8 and those derived from the Lowbury collection were similar in their sensitivity to some or all of the R-specific phages. Although this similarity did not extend to R factors isolated outside the United Kingdom, nor to the four FP factors studied, an interaction was observed between RP1 and several of these plasmids which simultaneously affected both phage sensitivity and plasmid transferability. Table 4 compares the properties of various double male strains harbouring RP1 together with other FP and RP factors. Coexistence in the same cell with either FP5, R38 or R39 reduced the susceptibility of these RP1⁺ bacteria to infection by sex-specific phage, and this reduced sensitivity was accompanied by a dramatic reduction in transferability of the RP1 component. These same phenomena were observed with the other RP1-like elements, i.e. RP4, R1822, R30, etc. (data not shown), supporting the previous conclusion that these plasmids are very similar. No such effect was apparent in the presence of FP2, FP39 or R130, although these plasmids coexist stably with RP1 (Stanisich, unpublished data), but a slight inhibitory effect occurred with R716. The mechanisms by which these interactions are manifest are not yet clear; however, the important point is that once again an effect on phage plating was accompanied by a corresponding effect on plasmid transfer.

DISCUSSION

In this paper the isolation and properties of three sex-specific bacteriophages of *P. aeruginosa* have been described. By various criteria, including their electron microscopic characteristics (Bradley, 1974), these phages are distinguishable from each other and from the previously described sex-specific phage PRR1 (Olsen & Shipley, 1973; Olsen & Thomas,

1973). However, in terms of host range to FP⁺ and RP⁺ lines of *P. aeruginosa* PAO, they comprise broadly two groups: PRR1, PR3 and PR4 infect a variety of R⁺ hosts, whereas the filamentous Pf3 is more restricted. With a single exception, that of the plasmid R2, only bacteria harbouring R factors isolated in the United Kingdom are susceptible to these phages. This finding is of interest as it suggests that this entire series of plasmids shares a common ancestry. Such a possibility is not unlikely since most of these elements were obtained from the same clinical environment, where the maintenance and interaction of plasmids would be favoured under the selective pressure provided by antibiotic usage. However, the three fairly discrete subgroups apparent from the phenotypic characteristics of these plasmids (Chandler & Krishnapillai, 1974*a, b*) is further supported by the plating responses to 'female-specific' phages (Krishnapillai, 1974; T. Morgan, personal communication), and to a lesser extent to the male-specific phages. Thus RP1 and its relatives are distinguishable from the two groups comprising RP8, R19, R91 and RP1-I, R18-I. In spite of these differences, studies of the DNA sequence homology of RP1-I, RP8 and RP1, which are representative of these subgroups, has revealed similarities (Ingram *et al.* 1972; Saunders & Grinsted, 1972). Admittedly, in the case of RP1-I and RP1 this may simply reflect similarity of the β -lactamase genes and those required for propagation of R-specific phage, but between RP8 and RP1 the 100% homology suggests that all the sequences of RP1 are represented in the larger molecule of RP8 (Saunders & Grinsted, 1972).

This approach is undoubtedly the most satisfactory in establishing evolutionary relationships, since it overcomes the difficulty that similar phenotypic properties may merely reflect the identity of one or a small number of genes between otherwise quite dissimilar elements. For this reason it is difficult to assess the possible relationship between R2 and the U.K. isolates suggested by their susceptibility to R-specific phages and determination of CB resistance, and that between FP5, R38, R39 and the RP1-like elements indicated by the inhibitory effect of the first three plasmids on the latter in double male strains. As far as has been studied, pairs of these elements show neither exclusion nor incompatibility with each other (Morgan *et al.* 1973; Stanisich, unpublished data), and the most obvious distinction (at least between R38, R39 and RP1) likely to result in the observed inhibition, is the repressed nature of plasmid transfer of R38 and R39 (Chandler & Krishnapillai, 1974*a*; Stanisich, unpublished data). Their interaction could therefore reflect a similarity of the repressor and operator genes controlling transferability of these plasmids, such as occurs between fi⁺ R factors and F (Meynell *et al.* 1968). Although this commonly involves R factors that confer susceptibility to F-specific phages, exceptions are known (Grindley & Anderson, 1971). However, other explanations are equally likely. For example, the presence of R38 and R39 reduces the susceptibility of *P. aeruginosa* PAO to a variety of serologically unrelated phages (Krishnapillai, 1974), suggesting that these elements may cause changes in cell surface. Such an effect could indirectly inhibit RP1 functions without indicating any genetic similarity between the plasmids.

Pseudomonas male-specific phages can be used to isolate transfer-defective and derepressed R factor mutants in a manner similar to that used with the male-specific phages of the Enterobacteria. It therefore seemed likely that the relationship between plasmid transferability and phage susceptibility could be an indirect one, stemming from a requirement for R-coded sex-pili for both these processes. This seems not to be the case from the electron microscope studies of Bradley (1974). Only PRR1 is pilus-specific; the remaining phages, including the filamentous Pf3, apparently infect the cell directly through the cell wall. A dilemma arises when one considers that a single mutation can affect both plasmid transfer and susceptibility to all four R-specific phages, even though the latter may reflect changes at

one of at least two possible adsorption sites – cell wall and pilus. It may be that a common component such as a pilin subunit is present at both sites and that this is an essential precursor for plasmid transfer. Adsorptional specificity could then be provided by the polymerized or unpolymerized state of the subunits at these locations.

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