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Isolation and Characterization of an R' Plasmid in *Pseudomonas aeruginosa*

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An R' plasmid, R'PA1, carrying a 3- to 4-min segment of the *Pseudomonas aeruginosa* chromosome has been derived from the incP-1 plasmid R68.45. The chromosomal segment includes the markers *argA, argB, argH,* and *lys-12.* The plasmid retains all the properties of R68.45, including chromosome mobilization ability and wide bacterial host range. R'PA1 reverts to R68.45 in *rec*$^+$ strains of *P. aeruginosa,* but it can be maintained in a *recA* strain.

Hybrids of plasmid and bacterial chromosome, such as the F' plasmids in *Escherichia coli,* have been of considerable value in the investigation and solution of a variety of bacterial genetic problems (13). Attempts to isolate similar hybrid plasmids in *Pseudomonas aeruginosa* by use of FP plasmids have been unsuccessful (Holloway, unpublished data). However, the incP-1 plasmids have provided evidence that they may be a more likely source of R' plasmids for *P. aeruginosa.* Olsen and Gonzalez (18) isolated a complex of the histidine operon of *E. coli* and the incP-1 plasmid RP1 which could be transferred to *P. aeruginosa* where, even though the plasmid was unstable, expression of the *E. coli* histidine operon could be demonstrated. Hedges, Jacob, and Crawford (7) isolated a derivative of the plasmid R68.44 (4) which contained a 75-megadalton segment of *P. aeruginosa* chromosome, including the *trpA* and *trpB* genes which were expressed in *E. coli.* Hedges and Jacob (6) have isolated other R' plasmids by mating selected *E. coli* strains with *P. aeruginosa* carrying R68.44. An incP-1 plasmid has been successfully used for production of an R' structure in *Klebsiella,* although it was not possible to demonstrate transfer of the chromosomal segment to *P. aeruginosa* (3).

I have sought to isolate R' plasmids using *recA* strains of *P. aeruginosa,* this being a method previously described for *E. coli* (12, 21). The plasmid source selected was R68.45, a more stable variant of R68.44 (4), in the hope that any R' plasmid formed may retain the wide host properties of R68.45 and thus provide a potential means of transferring *P. aeruginosa* chromosomal DNA to a range of other bacteria.

MATERIALS AND METHODS

Bacterial and bacteriophage strains used. Table 1 shows the bacterial strains used. Bacteriophage F116L (11) was used for transduction. PRR1 (20) and PRD1 (19) were used for plasmid characterization. The incP-1 plasmid R68.45 (4) confers resistance to carbenicillin (*bla*$^+$), kanamycin (*aph*$^+$), and tetracycline (*tet*$^+$).

Media and cultural conditions. Media were as described previously from this laboratory (24): minimal medium, nutrient yeast broth, and nutrient agar. Antibiotics used were carbenicillin (Pyopen, Beecham), kanamycin, 739 units/mg (Sigma), and rifampin as Rimactane (Ciba-Geigy). Amino acid supplements were used at a concentration of 1 mM, 50 mM stocks of amino acids being kept over chloroform. Carbenicillin and kanamycin were used at 500 μg/ml.

Plate matings were carried out as described previously (24). Patch matings were performed by mixing a standard loopful of each parent spread over 1 cm^2 on the surface of a nutrient agar plate, with overnight incubation at 37°C. The resultant growth was suspended in 1 ml of saline and blended in a Vortex mixer to produce a uniform suspension of bacteria. Other procedures were as previously described from this laboratory (4).

RESULTS

Isolation of a PAO strain carrying an R' plasmid. A patch mating was made between PAO25(R68.45) and PAO2003, which is recombination deficient (2), and selection was made for *argH*$^+$ recombinants. After 3 days of incubation at 37°C, colonies occurred at a frequency of about 10^{-8}/recipient parent cell. These could have arisen through several mechanisms: (i) merodiploids, or R' plasmids derived from the R68.45 plasmid in the donor parent and transferred by conjugation to PAO2003; (ii) chromosomal recombinants for *argH*$^+$ formed because the *rec-2* mutation in PAO2003 does allow formation of a few such recombinants; (iii) reversions to prototrophy at the *argH32* locus.

If any of the colonies did arise after R' plasmid formation, they would very likely have the following properties, which would not be found in colonies formed by the two other mechanisms.

(i) If the R' retained the transfer properties of

TABLE 1. *Strains of P. aeruginosa used in this study*[a]

Strain		Reference
PAO25 (R68.45)	*leu-10 argF10* FP⁻ carrying R68.45	4
PAO222	*met-28 trp-6 lys-12 his-4*[b] *pro-82 ilv-226* FP⁻	4
PAO260	*met-28 trp-6 lys-12 his-4*[b] *pro-82 ilv-226 rif-13* FP⁻	this study
PAO357	*argB18 cml-2 rif-15* FP⁻	this study
PAO362	*argH32 cml-2* FP⁻	8
PAO372	*argH32 lys-58 cml-2* FP⁻	5
PAO373	*argH32 cml-2 rif-12* FP⁻	this study
PAO486	*pyrE79 cml-2 rif-18* FP⁻	this study
PAO1032	*argA127 lys-61 rif-19* FP⁻	this study
PAO2003	*argH32 str-39 cml-2 rec-2* FP⁻	2
PAO2033	*argH32 str-39 cml-2 rec-2 rif-35* FP⁻	this study

[a] Abbreviations: *aph*, aminoglycoside phosphorylase; *arg*, arginine; *bla*, β-lactamase; *cml*, chloramphenicol; *his*, histidine; *leu*, leucine; *lys*, lysine; *ilv*, isoleucine plus valine; *met*, methionine; *pro*, proline; *pyr*, pyrimidine; *rif*, rifampin; *tet*, tetracycline resistance; *trp*, tryptophan; *str*, streptomycin.

[b] *his-4* is at the same locus as *hisII* (15).

R68.45, then R'-containing strains could act as donors of *argH*⁺ to an *argH* recipient.

(ii) In such a cross, if selection is made for transfer of plasmid markers, there should be high-frequency co-transfer of *argH*⁺.

(iii) Strains possessing the R' should act as donors of chromosomal markers closely linked to *argH*⁺ at similar frequencies to that found for *argH*⁺, but not for other markers located more distantly from *argH*⁺.

(iv) Strains possessing the R' should act as donors of *argH*⁺ to PAO2003 which is *argH32* and recombination deficient (*rec-2*).

A number of colonies having putative R' plasmids were obtained from the cross described above, and, after preliminary studies, one was selected for detailed study and denoted PAO2003(R'PA1); this designation is in agreement with recently proposed nomenclature rules (17).

Characterization of PAO2003(R'PA1). PAO2003(R'PA1) was shown to have the same properties as PAO strains carrying R68.45 with respect to resistance to carbenicillin, kanamycin, tetracycline, tolerance to aeruginocin AR41, sensitivity to PRD1 and PRR1, chromosome mobilization ability (Cma), and the ability to transfer antibiotic resistance markers to *E. coli*. The loci used for testing Cma were located more than 10 min away from *argH32*. It has previously been shown that PAO2003 carrying FP2 has about 60% of the donor ability of *rec*⁺ donors (2). Evidently *rec* function is not needed for chromosome mobilization by either FP2 or R68.45. PAO2003(R'PA1) has the same degree of radiation sensitivity as PAO2003 (data not shown) but, unlike PAO2003, PAO2003(R'PA1) is prototrophic. However, the latter strain still contains the *argH32* locus of PAO2003, this being shown by transduction as follows. Bacteriophage F116L was propagated on PAO2003(R'PA1) and, using PAO222 as recipient, selection for *lys-12*⁺ was made on supplemented minimal medium containing arginine to detect cotransduction of *lys-12*⁺ and *argH32* from the donor. It has been previously shown (5) that *argH* and *lys-12* are 45% cotransducible, and that F116 has a molecular weight of 39 × 10^6 (22), although the size of F116L has not been determined. In the present experiment, of 89 transductants selected for *lys-12*⁺, 50 (56%) were *argH*, indicating that this allele is still located on the chromosome of PAO2003. The prototrophic phenotype of PAO2003(R'PA1) presumably comes from the presence of an *argH*⁺ allele (derived from PAO25).

This allele can be demonstrated by propagating F116L on PAO2003(R'PA1) and transducing into PAO362 as recipient; *arg*⁺ transductants are found at about 50% of the normal frequency. Thus, F116L grown on PAO2003(R'PA1) can transduce both the *argH*⁺ and *argH32* alleles, illustrating the partial diploid nature of this strain. By selecting for CB^r (carbenicillin resistance) the same transducing phage preparation transduces the R68.45 plasmid, and clones so selected for CB^r contain other markers of R68.45, including antibiotic resistances, phage sensitivities, aeruginocin tolerance, and Cma function. No evidence has been obtained of cotransduction by F116L from R'PA1 donors of bacterial chromosome markers and plasmid markers.

Transfer of *argH*⁺ from PAO2003(R'PA1). PAO2003(R'PA1) and PAO373 were patch mated, and the resulting growth was plated on minimal medium plus rifampin to select *argH*⁺ and on nutrient agar plus carbenicillin and rifampin to select CB^r. The numbers of parental cells in the suspension from the mating were 5.0 × 10^9/ml for PAO373 and 1.3 × 10^5/ml for PAO2003(R'PA1). The recovery of *argH*⁺ recombinants was 6.1 × 10^4/ml and that of CB^r recombinants was 6.0 × 10^4/ml; i.e., each type of recombinant was recovered at a frequency of about 47% of the final number of donor cells present.

There are difficulties in accurately quantifying the frequency of transfer of markers in patch matings involving PAO2003(R'PA1). PAO2003 and PAO2003(R'PA1) both grow much slower than strains such as PAO373, and this is reflected in the viable count of each parent made of the patch mating after overnight growth. Patch matings are necessary because, as has been demonstrated previously (4), transfer of

both the R68.45 plasmid and the host chromosome in crosses mediated by R68.45 is very inefficient in liquid media. The need to contraselect the prototrophic donor parent PAO2003(R'PA1) means that matings cannot be carried out on minimal medium plus rifampin, as the rifampin inhibits recombinant formation. The equivalent frequency of transfer of *argH*$^+$ and CB^r found with the PAO2003(R'PA1) mating is significant when compared to the results of mating with R68.45, where the frequency of recovery of plasmid markers in matings is 10^4-fold higher than that found for chromosomal markers (4).

Co-inheritance of *argH*$^+$ and plasmid markers. From the cross PAO2003(R'PA1) × PAO373, colonies selected for either *argH*$^+$ or the plasmid markers CB^r and KM^r (kanamycin resistance) were examined for the co-inheritance of CB^r or KM^r and *argH*$^+$, respectively (Table 2).

Although the co-inheritance of plasmid and chromosomal markers transferred by R'PA1 is not complete, the extent of joint transfer is such that both types of markers must be transferred on a single piece of DNA, and it can be concluded that R'PA1 is a modified R68.45 plasmid containing a piece of bacterial chromosome which includes *argH*$^+$. Several suggestions can be made to explain the lack of complete co-inheritance of the plasmid and chromosomal markers. It has already been shown (4) that instability of plasmid markers of R68.45 is associated with the transfer of bacterial chromosome promoted by R68.45. In addition, R'PA1 itself is unstable, with a tendency to lose the bacterial chromosome fragment (see below), and this could be occurring during the growth on nutrient agar associated with the patch mating.

Transfer of markers other than *argH*. It is likely that the length of chromosome included in R'PA1 is limited so that markers close to *argH* are also carried by R'PA1 but that more distal markers are not. Fortunately, there are other markers which are linked to *argH* (5), and the location of these is shown in Fig. 1. The various markers shown have been used in estimating the size of the chromosomal fragment transferred by R'PA1. In each case, a patch mating was made between PAO2003(R'PA1) and a rifampin-resistant recipient carrying the particular marker. The frequency of transfer was measured in terms of the number of donor cells present in the cell suspension resulting from the growth in the patch mating. In addition to the markers shown in Table 1, other more distant markers have been examined by use of the multiply marked strain PAO260 (Table 3). The pattern of markers transferred at high frequency shows that a region extending from *argA* to *argB* is likely to be the bacterial chromosome component of R'PA1. One end can be determined rather precisely as being between *argB* and *pyrE* because these markers are 65% cotransducible with phage F116L, and *argB* is transferred by R'PA1 whereas *pyrE* is not. The limited transfer of other markers outside this region is no doubt due to the Cma activity of

TABLE 2. *Co-inheritance of chromosomal (argH*$^+$*) and plasmid markers (bla*$^+$ *or aphA*$^+$*) in the cross PAO2003 (R'PA1) × PAO373*[a]

Selected marker	Unselected marker	Co-inheritance (%)
bla$^+$	*argH*$^+$	27
aphA$^+$	*argH*$^+$	39
argH$^+$	*bla*$^+$	64
argH$^+$	*aphA*$^+$	72

[a] At least 150 recombinants were scored for each selective marker used (average of three experiments).

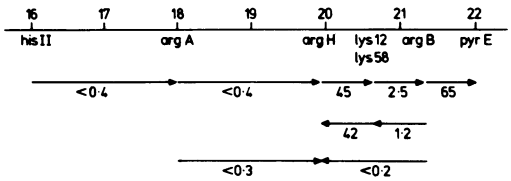

FIG. 1. *Map of the P. aeruginosa chromosome map in the vicinity of argH. The number on the top indicates the distance in minutes from the FP2 origin. Other numbers refer to the co-transduction frequencies obtained with the transducing F116L; the arrowhead indicates the marker selected. lys-12 and lys-68 are independently isolated mutants which are very closely linked, as indicated by prototroph reduction tests with F116L. The data in this figure are taken from reference 5.*

TABLE 3. *Transfer frequency by R'PA1 of various chromosomal markers*[a]

Marker	Strain	Location (min)	Transfer frequency (per donor cell)
ilv-226	PAO260	8	$<10^{-4}$
his-4	PAO260	17	$<10^{-4}$
argA	PAO1032	18	3.2×10^{-1}
argH	PAO373	20	4.7×10^{-1}
lys-12	PAO260	20	7.2×10^{-1}
argB	PAO357	21	5.1×10^{-1}
pyrE	PAO486	22	$<10^{-4}$
met-28	PAO260	30	$<10^{-4}$
trp-6	PAO260	34	$<10^{-4}$
pro-82	PAO260	40	$<10^{-4}$

[a] Various *P. aeruginosa* PAO strains were patch mated to PAO2003 (R'PA1), and the frequency of transfer of particular markers was determined. The location of the markers is given relative to the FP2 origin.

R'PA1 carried by the R68.45 component.

Transfer of R'PA1 into a *recA* recipient. PAO2003 (*recA*) shows a much reduced ability to produce chromosomal recombinants when used as a recipient in a mating with FP2 donors (2) or R68.45 donors (Holloway, unpublished data). However, when PAO2033(PAO2003 made Rif^r) is patch mated with PAO2003(R'PA1) and selection is made for *argH*$^+$ recombinants on minimal medium plus rifampin they are found to occur at the same frequency as plasmid markers are acquired by PAO2033 in the same mating, namely, 2.5×10^{-3}/donor. This figure for the acquisition of *argH*$^+$ by PAO2033 is at least 10^4-fold greater than found in the mating PAO2003 × PAO25(R68.45) and is strong evidence supporting the plasmid location of the *argH*$^+$ allele in PAO2003(R'PA1).

Maintenance of R'PA1. R'PA1 may be satisfactorily maintained in a PAO2003 background. The R' function of PAO2003(R'PA1) is progressively lost if this strain is propagated in complete media such as nutrient yeast broth or nutrient agar. This function can be satisfactorily retained by growth on minimal medium. Transfer of R'PA1 to a *rec*$^+$ strain results in loss of the bacterial chromosome portion but normal maintenance of the R68.45 plasmid. Stocks of PAO2003(R'PA1) are prepared by growth of the strain on solid minimal medium for 24 h and suspension of the resulting cells in fresh nutrient broth. This suspension is then quick frozen and sealed in thin-walled glass ampoules in an alcohol-dry ice mixture; the ampoules are maintained at −20°C until required. Then the thawed suspension should be plated out for single colonies on minimal medium.

DISCUSSION

The evidence presented above strongly supports the view that R'PA1 has arisen from the integration of a 3- to 4-min segment of the *argH* region of the *P. aeruginosa* strain PAO chromosome into the genome of R68.45. Physical data such as sedimentation values and heteroduplex studies will be necessary for confirmation of this view. The formation of *argH*$^+$ recombinants in the cross PAO2033 × PAO2003(R'PA1) is strong evidence against the view that the latter strain involves a duplication of the bacterial chromosome to give an *argH32/argH*$^+$ structure or that a variant of R68.45 with selective transfer ability for the *argH* region has been formed. The isolation of R'PA1 was possible because the hybrid plasmid can be maintained in Rec$^-$ strains. One essential feature for the isolation of R'PA1 is the patch mating of the Rec$^-$ recipient parent and the R68.45 donor parent on nutrient agar. For reasons which are not clear, it is important for the isolation of the R' plasmid that no selection for growth of strains carrying the R' plasmid be imposed immediately after the mating which produces them.

Other workers who have derived R' structures from R68.44 have used the same principle except that they have used an *E. coli* recipient, acting essentially as if it were Rec$^-$ and therefore unable to integrate *P. aeruginosa* chromosomal fragments into the *E. coli* chromosome (6, 7). An alternative method (16) involves the construction of an RP4-*trp* plasmid by restriction endonucleases using RP4 and the tryptophan operon from $\lambda trpE\text{-}A_{60\text{-}3}$. In *P. aeruginosa*, this plasmid is apparently stable. These various hybrids demonstrate that the incP-1 plasmids combine the properties of the ability to interact with host chromosome and wide bacterial host range. R68.45 appears to offer particular advantages in this respect. It promotes chromosome transfer in several unrelated genera: *P. aeruginosa* (4), *P. putida* (14; Morgan and Holloway, unpublished data), *E. coli* (1), *Rhizobium leguminosarum* (1), *R. meliloti* (10), and *Rhodopseudomonas sphaeroides* (23). The finding that R68.45 is approximately 1.6 megadaltons larger than R68 (9) and that it has a 1,800 base pair insertion of DNA near the kanamycin resistance determinant (M. van Montagu and J. Schell, personal communication) opens the way to more precise molecular explanations of how this plasmid promotes chromosome mobilization and generates R' plasmids.

One advantage of the use of a Rec$^-$ recipient of *P. aeruginosa* is that, by increasing the selective markers available in that strain, a variety of R' structures could be produced. Such hybrid plasmids will be of value for mapping *P. aeruginosa*, in studies on enzyme regulation, and in gaining an understanding of the genetic relationships of different species of *Pseudomonas* and the problems of gene expression where the DNA of one bacterial species is transferred to other unrelated species.

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