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Chromosome Mobilization by the R Plasmid R68.45: A Tool in *Pseudomonas* Genetics

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Summary. The conjugative plasmid R68.45 mobilizes the chromosome of *Pseudomonas aeruginosa* strain PAO from multiple sites located in different chromosome regions. In interrupted matings on the plate, selection for any single marker tested resulted in entry times of 3–5 min. When selection was imposed for two markers linked in R68.45-mediated conjugation, double recombinants appeared after a delay which corresponded approximately to the map distance between the two markers as measured by the sex factor FP2. Thus, R68.45 and FP2 appear to promote chromosome transfer at similar rates, but R68.45, unlike FP2, seems to give non-polarized transfer. R68.45 may be used to estimate map distances between linked markers located in those chromosome regions where other sex factors do not produce enough recombinants to permit accurate measurement of entry times.

In R68.45 matings on the plate, most recombinants inherited short donor chromosome fragments (usually less than 10 min long) and lost the R plasmid during purification. Used like a "large" generalized transducing phage, R68.45 has proved valuable in construction of PAO strains with desired genotypes.

Introduction

The conjugative plasmid FP2 promotes polarized chromosome transfer in *Pseudomonas aeruginosa* strains PAO and PAT (Holloway, 1955, 1969; Watson, 1977). Mapping studies have shown one origin of transfer, the zero-minute-site on the genetic map (Pemberton and Holloway, 1972; Haas, Holloway, Schamböck and Leisinger, 1977; Watson, 1977). Other sex factors such as FP5 (Matsumoto and Tazaki, 1973) and FP39 (Pemberton and Holloway, 1973) appear to mobilize the chromosome from a single site close to or identical with the FP2 site. This limitation on origin sites and the low recovery of recombinants for markers located later than 40 minutes from the FP2 site prevented the demonstration of a circular linkage map.

Recently, we described an R plasmid, R68.45, which is able to mobilize the *P. aeruginosa* chromosome from multiple origins; chromosomal recombinants are formed at frequencies of about 10^{-4} per donor cell on solid media (Haas and Holloway, 1976). Several interesting characteristics of R68.45 have been found.

(i) Origins of transfer are widely spaced on the chromosome. This property has permitted Watson (1977) to obtain evidence for genetic circularity in strain PAT.

(ii) R68.45 appears to have retained the broad host range of its parent R68, which belongs to the P1 incompatibility group (Summers and Jacoby, 1977). Chromosome mobilization by R68.45 has been observed in a variety of Gram-negative micro-organisms, such as *P. putida* (Martinez and Clarke, 1975), *Escherichia coli* and several species of *Rhizobium* (Beringer and Hopwood, 1976; Johnston and Beringer, 1977; Kondorosi et al., 1977). R68.45 may have a considerable potential in the genetic analysis of some micro-organisms where lack of suitable conjugative plasmids has prevented establishment of genetic maps.

(iii) It is possible to isolate derivatives of R68.45 which carry segments of chromosomal DNA (Hedges, Jacob and Crawford, 1977; Holloway, manu-

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script in preparation). Such R prime plasmids are useful in studies of gene expression. For the first time, partial diploids can be constructed in *P. aeruginosa*. In addition, R68.45 prime plasmids permit gene transfer between different bacterial genera in vivo.

In this study we examine the kinetics of chromosome mobilization by R68.45, taking advantage of the technique of interrupted matings on the plate (Haas and Holloway, 1976). We show that R68.45 in *P. aeruginosa* and the F plasmid in *E. coli* appear to mobilize chromosomal markers in a similar way and we describe how R68.45 can be used to construct *P. aeruginosa* strains with desired genotypes.

Materials and Methods

Bacterial Strains and Plasmids. These are listed in Table 1.

Media and Growth Conditions. Minimal medium (MM), nutrient agar (NA), nutrient yeast broth (NYB), and the concentration of antibiotics and supplements were the same as described previously (Haas and Holloway, 1976). Aeruginocin 41 was prepared as described by Holloway et al. (1974).

Interrupted Matings. Chromosome transfer was interrupted with nalidixic acid. When the sex factor FP2 was used, matings were performed in liquid medium (Haas et al., 1977). Matings mediated by R68.45 were carried out on solid media (Haas and Holloway, 1976); usually the donor concentration was higher in double selection experiments than in single selection crosses to compensate for the lower recovery of double recombinants. The viable count of the recipient was $2–6 \times 10^9$ cells/ml and in excess of the donor cell concentration.

Strain Construction. A desired chromosomal marker *A* in an R68.45 donor strain can be introduced into a recipient carrying a marker *B*, provided selection for B^+ is possible and *A* and *B* are sufficiently linked (see Table 2). Contraselection of the donor is best achieved by an unlinked auxotrophic marker. Matings on the plate and testing of recombinants were done according to the following protocol. NYB is used as the growth medium, with good aeration at 37° C. Grow the recipient (A^+, *B*) to stationary phase (about 2×10^9 cells/ml) and the R68.45 donor (*A*, B^+) to exponential phase (about 5×10^8 cells/ml). Centrifuge the cultures and resuspend them in saline (0.9 % (w/v) NaCl) at room temperature. Dilute donor 10^{-1} and 10^{-2}, mix equal volumes of donor and recipient. Plate 0.2 ml of the mixture on selective MM, which permits growth of *A*, B^+ recombinants. Controls consist of 0.1 ml of the donor and the recipient alone. Incubate plates at 37° C for 2–3 days. Depending on the degree of linkage of *A* to *B*, purify 50–400 recombinants by patching clones onto selective medium. Examine coinheritance of *A* by replica plating onto appropriately supplemented MM and co-transfer of R68.45 (or its fragments) by replica plating onto NA containing antibiotics (carbenicillin, 250 µg/ml; or tetracycline, 250 µg/ml; or kanamycin, 500 µg/ml). Clones which have inherited *A* and are sensitive to all three antibiotics are purified to single colonies on NA. Sensitivity to antibiotics can manifest itself in two ways: No growth of patch (indicating the probable absence of the R plasmid) or growth of isolated colonies within the patch (indicating instability of the R plasmid, which is likely to be lost during subsequent purification on NA). After purification, single colonies are tested for stable inheritance of *A*, sensitivity to antibiotics and absence of superinfection inhibition. (We have observed occasional loss of *A* in certain classes of recombinants, but we have not studied this phenomenon further.) Superinfection inhibition is tested by using the newly constructed strain as a recipient in a mating with an R68.45 donor, with selection for carbenicillin-resistant transcon-

Table 1. Strains of *P. aeruginosa* and plasmids

Strain or plasmid	Genotype or phenotype	Reference
Strain		
PAO1	prototroph, *chl-2*, FP^-	Holloway (1969)
PAO12	*pur-136*, *leu-8*, *chl-3*, FP^-	Pemberton and Holloway (1972)
PAO18	*pro-64*, *pur-66*, FP^-	Krishnapillai (1971)
PAO25	*leu-10*, *argF10*, FP^-	Haas and Holloway (1976)
PAO236	*met-28*, *trp-6*, *lys-12*, *his-4*, *pro-82*, *ilv-226*, *nalA2*, FP^-	Haas and Holloway (1976)
PAO381	*leu-38*, *str-2*, $FP2^+$	Stanisich and Holloway (1969)
PAO477	*met-28*, *ilv-202*, *argB1*, *str-1*, *nal-12*, FP^-	Haas et al. (1977)
PAO516	*car-161*, *thi-1*, *pur-66*, FP^-	Haas et al. (1977)
PAO642	*trpB4*, *car-9*, *ilv-219*, *nal-9*, FP^-	Haas et al. (1977)
PAO643	*trpB4*, *car-9*, *pro-64*, *nal-9*, FP^-	$Ilv^+ Pro^- R^-$ recombinant of PAO642 × PAO18(R68.45)
Plasmid		
R68	Cb, Km/Nm, Tc, Aer, Tra	Holloway and Richmond (1973)
R68.45	Cb, Km/Nm, Tc, Aer, Tra, Cma	Haas and Holloway (1976)
pMO15	Cb, Km^s/Nm^s, Tc, Aer, Tra^-, Cma^-	This paper

Genotype symbols are the same as those used for *Escherichia coli* (Bachmann, Low and Taylor, 1976), except that *str* designates streptomycin resistance and *chl* chloramphenicol resistance. Plasmid phenotype symbols are used according to the proposals of Novick et al. (1976). Aer = tolerance to aeruginocin 41. Cma = chromosome mobilizing ability. In a previous communication we used the symbol Cda for "chromosome donor ability" (Haas and Holloway, 1976). To avoid confusion with the symbol Cda for production of colicin D (Novick et al., 1976), we now propose the symbol Cma for the ability of R68.45 and other plasmids to mobilize the host chromosome

jugants or chromosomal recombinants. Typical transfer frequencies in the presence or absence of superinfection inhibition are given in "Results" (Table 3).

Results

Kinetics and Polarity of Chromosome Mobilization

Kinetic experiments were designed to distinguish between two conceivable modes of chromosome transfer promoted by R68.45. The two modes are schematically represented in Figure 1. According to the first model contemplated, R68.45 mobilizes the chromosome from a relatively small number of sites and chromosome transfer proceeds in a polarized way ("clockwise" or "anticlockwise"). Thus, in Figure 1 the chromosomal marker A^+ may be transferred from only one site (site *1*), giving an entry time, for example, of 5 min in an interrupted mating. The entry time of marker B^+, which is linked to *A*, would be 5 min plus the time required to transfer the segment *A–B*. A^+B^+ double recombinants are expected to enter at the same time as B^+ recombinants. The second model assumes a considerable number (e.g. 50) of transfer origins with similar, but not necessarily identical "affinities" for R68.45. Four such sites are designated *1–4* in Figure 1. The earliest A^+ recombinants formed are due to chromosome transfer from sites *1* and *2*, while the earliest B^+ recombinants originate from sites *3* and *4*. Entry times of A^+ and B^+ may be similar. The first A^+B^+ double recombinants can be produced from origins *1* and *4*. The entry time A^+B^+ is equal to the entry time of the marker transferred first plus the time necessary for the transfer of the segment *A–B*. As a consequence of this model chromosomal markers will *appear* to be transferred in a non-polarized manner.

We chose several pairs of markers which gave 10% or more linkage in R68.45 matings on the plate. Figure 2 shows the map location of the loci used. Map distances between two linked markers were measured by interrupted matings using the sex factor FP2. With R68.45 donors, time of entry kinetics were

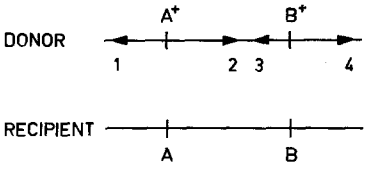

Fig. 1. Scheme of chromosome mobilization by R68.45. *A* and *B* are two chromosomal markers linked in R68.45 – mediated conjugation. The arrow-heads *1*, *2*, *3*, and *4* indicate arbitrary origins of chromosome transfer in the R68.45 donor. For further explanation see text

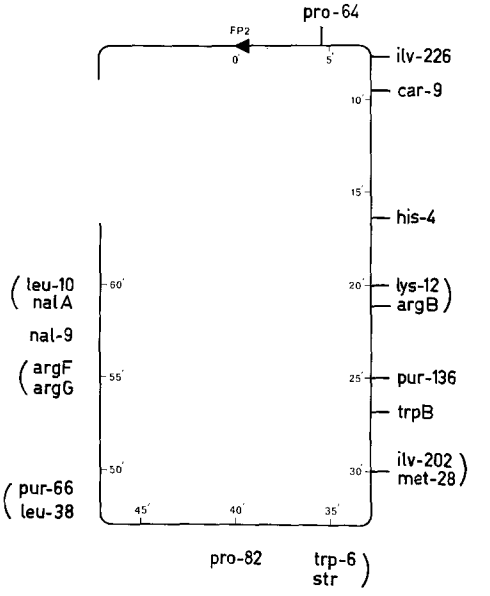

Fig. 2. Chromosome map of *Pseudomonas aeruginosa* strain PAO. The position of some markers shown was determined by interrupted matings in this study (Figs. 3–6). Map locations of other markers are taken from earlier work (Haas et al., 1977; Chakrabarty and Holloway, in the press). Very close linkage exists between the following pairs of markers: *ilv-226* and *ilv-219*, *leu-38* and *leu-8*, *car-9* and *car-161*, *nal-9* and *nal-12*

determined for each marker alone (single selection) and for both markers together (double selection). An example is given in Figure 3. In an R68.45 mating *pro-64* as well as *car-9* showed entry times of 4–5 min while pro^+car^+ double recombinants were first observed at 9–10 min (Fig. 3b). Single recombinants were about 5 times more frequent than double recombinants. These results are consistent with the second model proposed above, i.e., it appears that *pro-64* and *car-9* can each be mobilized from different origins and double recombinants occur after transfer of the *pro-car* segment. Transfer of this segment may proceed in both clockwise and anticlockwise directions and takes about 5 min. The same time is required for mobilization of the *pro-car* segment of chromosome by FP2 (entry time of *pro-64:* 4–5 min; *car-9:* 8–10 min, Fig. 3a). Hence, in this chromosome region, R68.45 and FP2 promote chromosome mobilization at a similar rate.

In a further example, entry times of *argB* and *ilv-202* were examined. The distance between the two loci was about 9 min, as measured with FP2 (Fig. 4a). The position of *argB* at about 21 min on the chromosome map is in agreement with previous results (Haas et al., 1977). The entry time of *ilv-202* (about 30 min) could not be determined with an accuracy better than ±4 min due to the low number of ilv^+ recombinants obtained. In an R68.45 mating, *argB* and *ilv-202* had

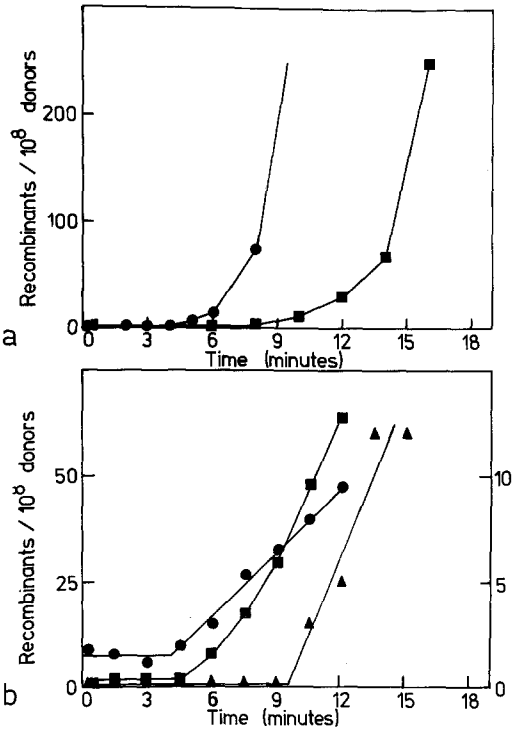

Fig. 3a and b. Time of entry kinetics of *pro-64* and *car-9*. **a** Interrupted mating in liquid PAO643 (*pro-64, car-9, trpB4, nal-9*) × PAO381 (*leu-38, str-2*; $FP2^+$). **b** Interrupted mating on the plate PAO643 × PAO25(R68.45) (*leu-10, argF10*; $R68.45^+$). The viable count of PAO25(R68.45) was 2.0×10^9 cells/ml for single and double selection, that of PAO643 was 5.6×10^9 cells/ml. ● *pro-64*$^+$; ■ *car-9*$^+$ (left ordinates); ▲ *pro64*$^+$ *car-9*$^+$ (right ordinate)

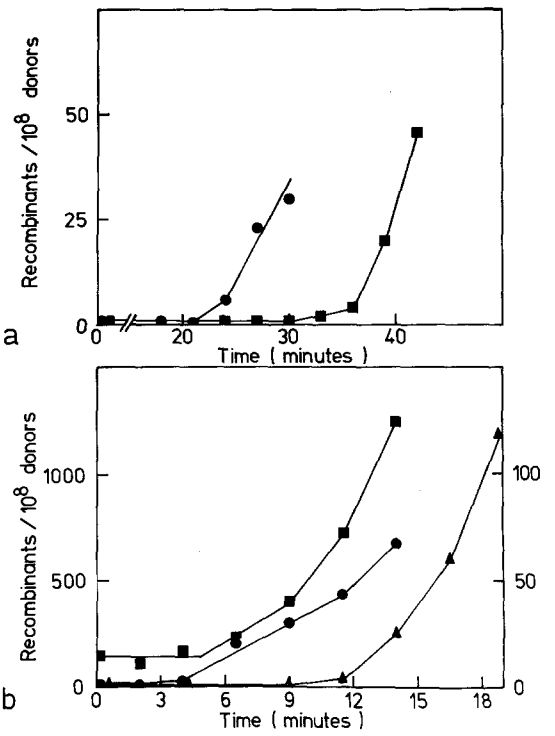

Fig. 4a and b. Time of entry kinetics of *argB* and *ilv-202*. **a** Interrupted mating in liquid PAO477 (*argB1, ilv-202, met-28, str-1, nal-12*) × PAO381($FP2^+$). **b** Interrupted mating on the plate PAO477 × PAO25(R68.45). The viable counts of PAO25(R68.45) were 1.7×10^8 cells/ml in the single selection experiment and 2.0×10^9 cells/ml in the double selection experiment. The viable count of PAO477 was 4.9×10^9 cells/ml. ● *argB1*$^+$; ■ *ilv-202*$^+$ (left ordinates); ▲ *argB1*$^+$ *ilv-202*$^+$ (right ordinate)

individual entry times of 3–4 min. Double recombinants (*argB*$^+$ *ilv*$^+$) appeared after 9–11 min, indicative of a distance of about 7 min between *argB* and *ilv-202* (Fig. 4b). Thus, similar map distances were obtained with FP2 and R68.45 for markers located in the 20–30 min chromosome region.

The distance between *his-4* and *lys-12* was about 3 min, that between *ilv-226* and *his-4* about 9 min, these distances being obtained in FP2 matings (Fig. 5a). Figure 5b illustrates the entry time kinetics of *his-4* and *lys-12* mobilized by R68.45. These two markers, although separated, appeared closely linked. The distance was probably less than 3 min, but an accurate measurement was not possible within the resolution of the double selection technique. When double selection was made for *ilv-226*$^+$ *his-4*$^+$ recombinants in an R68.45 mating, the first recombinants were produced after the mating had proceeded for 12–14 min (data not shown), corresponding to a distance of 9 min approximately and in agreement with the FP2 data.

Taken together, the results of the kinetic experiments suggest that R68.45 mobilizes the chromosome from a large number of sites and that transfer is not polar. The latter conclusion was confirmed by an analysis of linkage data. In numerous reciprocal crosses between multiply marked strains the coinheritance of unselected markers showed no sign of polarity (Haas and Holloway, 1976; our unpublished experiments).

R68.45 May Be Used to Measure Map Distances

Since it appears that FP2 and R68.45 mobilize different parts of the *P. aeruginosa* chromosome at a similar speed, interrupted matings using R68.45 and the double selection technique should have potential application in chromosome mapping. The method may be especially useful for mapping markers which are located later than about 30 min from the FP2 origin and which are difficult to map accurately by interrupted matings using FP2 because of low recovery of recombinants. As an example we have

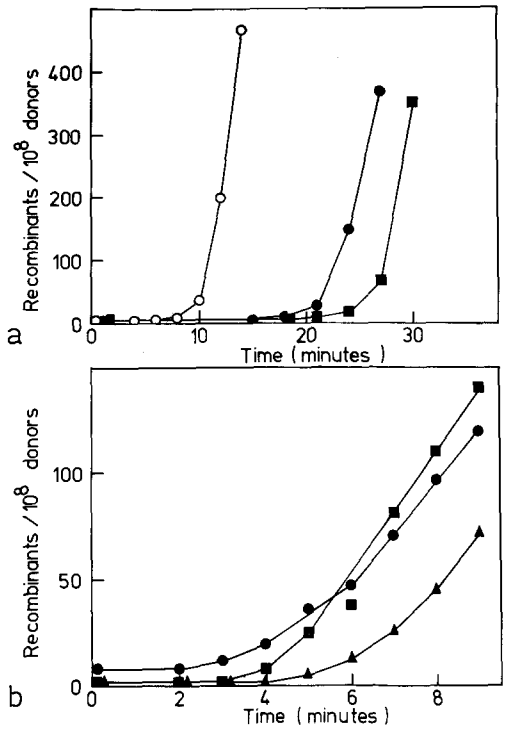

Fig. 5a and b. Time of entry kinetics of *his-4* and *lys-12*. **a** Interrupted mating in liquid PAO236 (*ilv-226, his-4, lys-12, met-28, trp-6, pro-82, nalA2*) × PAO381(FP2$^+$). **b** Interrupted mating on the plate PAO236 × PAO25(R68.45). The viable counts of PAO25(R68.45) were 1.0×10^9 cells/ml in the single selection experiment and 1.7×10^9 cells/ml in the double selection experiment. The viable count of PAO236 was 2.8×10^9 cells/ml. ● *his-4*$^+$; ■ *lys-12*$^+$; ▲ *his-4*$^+$ *lys-12*$^+$; ○ *ilv-226*$^+$ (used as a control marker in **a**)

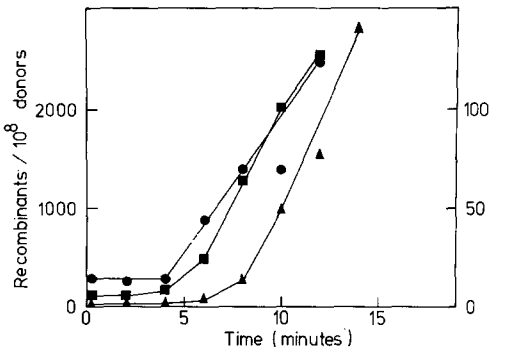

Fig. 6. Time of entry kinetics of *trp-6* and *pro-82*. Interrupted mating on the plate PAO236 (*ilv-226, his-4, lys-12, met-28, trp-6, pro-82, nalA2*) × PAO25(R68.45). The viable counts of the donor were 1.0×10^8 cells/ml in the single selection and 1.6×10^9 cells/ml in the double selection experiment. The viable count of PAO236 was 5.6×10^9 cells/ml. ● *trp-6*$^+$; ■ *pro-82*$^+$ (left ordinate); ▲ *trp-6*$^+$ *pro-82*$^+$ (right ordinate)

Table 2. Map distance *versus* linkage in R68.45 two-factor-crosses

Approximate map distance[a] (min)	Coinheritance of unselected marker[b] (%)	Number of independent crosses[c]
<2[d]	>70	7
5	25	6
10	11	7
15	6	4

[a] Map distances were measured by interrupted matings using FP2. An error of ±2 min should be assumed for the majority of markers investigated here

[b] Two-factor-crosses between R68.45 donors and R$^-$ recipients were performed on plates; mating time was unrestricted. In each cross 100–400 recombinants were scored for coinheritance of the unselected marker. The linkage values are averages of all crosses

[c] In each cross a different pair of markers was examined. In most cases, crosses were done reciprocally

[d] These markers were co-transducible with the phages F116L or G101 (Holloway, 1969)

chosen *trp-6* and *pro-82*, two markers situated in the 30–45 min region of the chromosome. An interrupted mating performed with R68.45 showed that the distance between the two markers was of the order of 3–4 min (Fig. 6). It is interesting to note that *trp*$^+$*pro*$^+$ double recombinants were about 20 times rarer than single recombinants; in other words, linkage between the two markers was about 5% (Haas and Holloway, 1976). The relation between map distance and linkage in R68.45 matings is examined in the following paragraph.

Size of Chromosome Fragments Inherited

In a previous communication (Haas and Holloway, 1976) we have shown that transconjugants of R68.45 matings tended to inherit relatively short pieces of the donor chromosome. In an attempt to quantitate this finding we carried out a large number of matings on the plate using multiply marked recipients and R68.45 donors and without interruption of conjugation. The coinheritance of unselected markers was determined in two-factor crosses, mostly reciprocal, for pairs of markers located in different chromosome regions. In Table 2 we compare linkage (coinheritance) values to map distances. The linkage values are averages of all crosses performed. A considerable variation was found; for instance, markers located about 5 min apart were linked 5–55% (average 25%), depending on the particular pair of markers. For loci separated by about 10 min linkage varied between 5–20% (average 11%). Two points are clear: The length of a chromosome segment inherited does not normally exceed 15–20 min and co-transducible markers are highly linked in R68.45 crosses. For example,

argF and *argG* were 55% linked in F116L transduction (Haas et al., 1977) and 80% linked in R68.45 conjugation.

Strain Construction with R8.45

For many reasons it is frequently necessary to construct bacterial strains with particular combinations of mutant alleles. In *P. aeruginosa*, clear-plaque mutants of the transducing phages F116L and G101 are particularly useful in strain construction because transductants are not lysogenized. The use of these phages is limited to chromosome regions where cotransducible genes have been mapped (Chakrabarty and Holloway, in the press). In many instances, loci of interest are known to be linked only by conjugation, and are not close enough to be cotransducible. In such cases R68.45 has proved a valuable tool for strain construction. Three properties of R68.45 are important in this respect. (i) Chromosome mobilization is initiated from a large number of sites; preferential transfer of any part of the chromosome has not been found. (ii) The chromosome piece inherited by the recipient is short, but several times the size of a DNA fragment carried by a transducing particle. Therefore, there is no need to interrupt an R68.45 mating to prevent the entry of long undesired chromosome pieces. (iii) In most recombinants R68.45 becomes unstable and is lost during purification (Holloway et al., 1977). As will be shown below, R^- recombinants can be used in further R68.45 crosses without difficulty. In "Materials and Methods" we describe a procedure which we have used successfully in about ten strain construction experiments. The method involves a plate mating with an R68.45 donor. Recombinants which had inherited a desired marker were purified by streaking serially on nutrient agar. After some 50 generations 40–70% of all single clones showed no resistance to carbenicillin, tetracycline and kanamycin and presumably had lost the R plasmid. The precise percentage of R^- clones appears to depend on the chromosomal marker selected, but was >40% after single colony isolation. Only few chromosomal recombinants inherited the entire R68.45 stably (Haas and Holloway, 1976) and fragmentation of the plasmid was observed. A fragment which had a Cb Tc Aer phenotype but had lost Km/Nm, Tra and Cma (see Table 1 for definition of phenotype symbols) was isolated and called pMO15. This type of fragment was stable in strain PAO and formed in about 10% of all chromosomal recombinants (average of 6 independent crosses).

Table 3 shows that R68, R68.45, and pMO15 cause superinfection inhibition in R68.45 matings. The frequency of Leu^+ recombinants was about 10^{-4} when PAO12(R^-) was the recipient. The recombination frequency was reduced approximately 1000-fold when PAO12 harboured R68, R68.45, or pMO15. The phenomenon of superinfection inhibition is probably mainly due to entry exclusion, as shown by Chandler and Krishnapillai (1977) for similar *P. aeruginosa* R plasmids. Other mechanisms such as incompatibility may contribute to the superinfection inhibition phenotype, but their role is difficult to assess since it is not clear whether chromosome mobilization involves obligatory transfer of R68.45 or a fragment thereof.

Table 3. Superinfection inhibition by R68.45 and related plasmids in *P. aeruginosa*

Donor	Recipient	Frequency of chromosomal recombinants[a]	Frequency of Cb^r transconjugants[b]
PAO25(R68.45)	PAO12	1.2×10^{-4}[c]	6.1×10^{-1}
	PAO12(R68.45)	1.1×10^{-7}	n.d.[d]
	PAO12(R68)	1.0×10^{-7}	n.d.
	PAO12(pMO15)	1.5×10^{-7}	n.d.
	PAO1	n.d.	4.0×10^{-1}
	PAO516	7.0×10^{-4}[e]	4.4×10^{-1}

[a] Expressed per donor cell. Matings were performed on the plate

[b] Expressed per donor cell. Transfer of R68.45 was measured on solid medium as described previously (Haas and Holloway, 1976)

[c] Selection was made for *leu-8*$^+$

[d] n.d. = not determined

[e] Selection was made for *pur-66*$^+$

Any strains that were constructed with R68.45 and showed no resistance to carbenicillin, tetracycline and kanamycin lacked superinfection inhibition. An example is given in Table 3: strain PAO516, a recombinant of an R68.45 cross (Haas et al., 1977), behaved as a normal R^- recipient in further R68.45 matings, whether selection was made for a chromosomal gene (*pur-66*) or a plasmid gene (resistance to carbenicillin).

Discussion

The incP-1 plasmid R68 mobilizes the chromosome of *P. aeruginosa* strain PAO at a very low frequency, ca. 10^{-8} recombinants/donor cell. Among those rare chromosomal recombinants clones were detected which contained R68 variants capable of mobilizing the chromosome at frequencies of about 10^{-4}/donor cell (Haas and Holloway, 1976). Initially, such R plasmid variants were only found when selection was made for *argB*$^+$ (map location 21 min). Recently,

selection for a few other markers located at 12, 30, 55 and 60 min on the map has also yielded R68.45 – type plasmids (C. Crowther and B.W. Holloway, unpublished experiments). We assume that R68 variants with enhanced chromosome mobilizing ability arise by recombination of plasmid DNA with particular regions of the bacterial chromosome. Jacob, Cresswell and Hedges (1977) have shown that the molecular weight of R68.45 is 37.4 ± 0.7 Mdal as compared to 36 Mdal found for R68. Thus, R68.45 has acquired an additional piece of DNA, likely by insertion of chromosomal DNA into plasmid DNA. This addition to the plasmid contains a nucleotide sequence which enables chromosome mobilization to occur, either directly by a synaptic process with chromosomal nucleotide sequences or, less likely, by coding for a protein. The molecular mechanism of chromosome mobilization remains largely unknown. The kinetic experiments described in this study allow certain comparisons between R68.45 and the better characterized F plasmid of *E. coli*.

We found that eight markers located at different sites on the *P. aeruginosa* chromosome had entry times of 3–5 min in R68.45 interrupted matings (Figs. 3–6)[1]. If we take into account the results of our previous study (Haas and Holloway, 1976) then a total of ten markers located in the 0–45 min region of the chromosome had entry times of 3–5 min. Some markers are closely linked (*ilv-226* and *car-9*, *lys-12* and *argB*, *ilv-202* and *met-28*) and it is not possible to decide whether separate transfer origins exist for those pairs of markers. We conclude that in the chromosome region examined there must be at least seven transfer origins. This number is probably an underestimate since some markers may be mobilized from several sites. There is circumstantial evidence for such an assumption: Time of entry curves had different shapes depending on the marker selected. The length of the *P. aeruginosa* chromosome (in time units) is not known, but may be similar to that of *E. coli* (100 min; Bachmann et al., 1976). Assuming that markers located outside the 0–45 min region of the *Pseudomonas* chromosome are mobilized by R68.45 from multiple origins as well, we speculate that the total number of transfer origins is perhaps of the order of 20–50.

Chromosomal transfer by R68.45 was not polarized. Thus, R68.45 strongly resembles the autonomous F plasmid in *E. coli* (Curtiss and Renshaw, 1969) with respect to the mode of chromosome mobilization. A further analogy between F and R68.45 is evident: the conjugational transfer of both plasmids is naturally derepressed in their indigenous hosts (Willetts, 1972; Haas and Holloway, 1976).

The F plasmid can integrate into the chromosome of *E. coli* spontaneously, resulting in the formation of Hfr donor strains. The integration depends on insertion sequences on the plasmid and the chromosome (Deonier and Davidson, 1976). In $F^+ \times F^-$ crosses only about 15% of the chromosomal recombinants are due to the presence of stable Hfr donors in the F^+ population (Curtiss and Stallions, 1969). The remaining 85% of the recombinants are formed by unknown mechanisms. In order to obtain a better understanding of the mobilization process, Crisona and Clark (1977) incorporated various *Eco*RI fragments of F into the plasmid pSC101, which is not self-transmissible. Upon integration of a particular F fragment into pSC101 the resulting plasmid hybrids could be mobilized by F-like plasmids at increased frequencies. Similarly, the isolation of the sequence found in R68.45, but absent in R68, might provide some information on the requirements for chromosome mobilization in *P. aeruginosa* as well as in those other bacterial species where R68.45 acts as a sex factor.

R68.45 differs from certain other R plasmids which, analogous to F′ plasmids in *E. coli* (Pittard, Loutit and Adelberg, 1963), promote polarized chromosome transfer of particular chromosome regions. The R plasmids R68 and R91-5 transfer the chromosome of *P. aeruginosa* strain PAT in one direction; there is evidence that R68 recognizes one site, whereas R91-5 utilizes two primary origins on the PAT chromosome (Watson, 1977). Polarized chromosome transfer by some R plasmids in *E. coli* C was described by Hedén and Rutberg (1976), and Towner and Vivian (1976) found variants of the incP1 plasmid RP4 which donated the *Acinetobacter* chromosome in different directions and from different origins. By analogy with F′ plasmids, these R factors may have extended sequence homologies with specific host DNA sites.

R68.45 can "pick up" chromosomal genes to form R prime structures (Hedges et al., 1977; Holloway, manuscript in preparation). In PAO(*recA*, R^-) × PAO(R68.45) crosses very few recombinants are recovered and some of these rare clones carry stable plasmids which are hybrids between R68.45 and segments of the bacterial chromosome. It is not known whether in PAO($recA^+$) × PAO(R68.45) crosses chromosome mobilization involves transient formation of R′ plasmids; if such structures are formed, the great majority of them must be highly unstable or cannot be expressed in *recA* recipients.

[1] We have shown elsewhere that nalidixic acid arrests chromosome transfer in FP2 matings within 30 s (Haas et al., 1977). We assume tacitly that nalidixic acid acts similarly in R68.45 matings, but our conclusions regarding the mode of chromosome transfer would not be affected if nalidixic acid acted only after some delay

Plasmids of the incompatibility group P-1 are playing an increasing role in the genetic analysis of Gram-negative bacteria for which previously conjugational systems did not exist or were unsatisfactory. Chromosome mobilization by incP-1 plasmids has been demonstrated in *Acinetobacter* (Towner and Vivian, 1976), *Caulobacter* (Alexander and Jollick, 1977), *Rhizobium* (Johnston and Beringer, 1977; Meade and Signer, 1977; Kondorosi et al., 1977), *Rhodopseudomonas sphaeroides* (Sistrom, 1977) and phytopathogenic *Pseudomonas* (Lacy and Leary, 1976). Often, mating conditions appear critical. In *E. coli* (Dennison and Baumberg, 1975) and *P. aeruginosa* (Haas and Holloway, 1976) as well as in *Rhizobium meliloti* (Meade and Signer, 1977) and *Acinetobacter calcoaceticus* (Towner and Vivian, 1976) solid media – agar surfaces or Millipore filters – are essential for transfer of incP-1 plasmids and effective chromosome mobilization. The technique of interrupted mating on the plate, which has provided valuable information on the *P. aeruginosa* genome, might be modified for use in those bacterial species for which there are effective chromosome donor plasmids of the incP-1 type.

A striking aspect of R68.45 crosses in *P. aeruginosa* is that most chromosome fragments inherited by the recipient are short. We do not know whether the DNA transfer from an R68.45 donor can only proceed for a limited amount of time under our experimental conditions or whether long DNA pieces can be transferred but become degraded before they are integrated into the recipient chromosome. The rates of degradation and integration might depend on the donor fragment transferred and transfer origins might have a non-random distribution on the chromosome. This could explain, in part, our inability to find a quantitative relation between map distance (in time units) and linkage. For instance, the linkage between *trp-6* and *pro-82* was very low in spite of the apparent proximity of the two markers (Fig. 6).

Nevertheless, it is clear that linkage values obtained in R68.45 crosses can be used to deduce marker orders and to construct genetic maps in *Pseudomonas* (Haas and Holloway, 1976; Watson, 1977; A. Morgan and B.W. Holloway, unpublished experiments with *P. putida*) and in *Rhizobium* (Beringer and Hopwood, 1976; Kondorosi et al., 1977). Currently we find that R68.45 is most useful in the construction of *P. aeruginosa* strains.

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Watson, J.M., Holloway, B.W.: Chromosome mapping in *Pseudomonas aeruginosa* strain PAT. J. Bact. (in the press)