JOURNAL OF BACTERIOLOGY, Mar. 1978, p. 1113-1125
0021-9193/78/0133-1113$02.00/0

Vol. 133, No. 3
Printed in U.S.A.

Chromosome Mapping in *Pseudomonas aeruginosa* PAT

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Received for publication 16 August 1977

A linkage map of *Pseudomonas aeruginosa* PAT has been derived from the results of conjugation experiments using the plasmids FP2-2, R68, R91-5, and R68.45. FP2-2 and R68 each mobilize the chromosome from single, distinct transfer origins. R91-5 appears to mobilize the chromosome from two such origins, and R68.45 utilizes a number of transfer origins. R68 and R91-5 have both been shown to mobilize the chromosome with a polarity opposite to that by FP2-2. The locations of the transfer origins of these plasmids are such that it has not been possible to demonstrate chromosomal circularity by means of interrupted mating experiments. However, the available time-of-entry data combined with linkage data from plate mating experiments support the conclusion that the chromosome of *P. aeruginosa* is circular.

The plasmid F in *Escherichia coli* has many properties of interest which have stimulated its detailed study (1, 27). The ability of this plasmid to mobilize the chromosome from a range of sites around the *E. coli* genome (15) has greatly facilitated chromosome mapping in this organism. In *Pseudomonas aeruginosa* PAO, a number of plasmids have been shown to mobilize the chromosome (7, 16, 18, 19). However, with the exception of R68.45 (7), these plasmids mobilize the chromosome from one predominant origin (10). Although the R68.45 plasmid is able to mobilize the chromosome from a number of origins (7), it has not been possible to isolate donor lines analogous to the Hfr donors of *E. coli*.

Genetic analysis of strain PAO by means of conjugation and transduction has resulted in the definition of a linear map of the chromosome showing the location of about fifty genes (2, 7, 8, 14, 18, 19, 22). The relative positions of the transfer origins of the available conjugative plasmids are such that it has not been possible to demonstrate chromosomal circularity or to accurately locate markers that are transferred later than about 50 min from the transfer origin of the FP2 plasmid (8).

The donor strain PAT, which harbors the conjugative plasmid FP2, was isolated in South Africa, and the recipient strain PAO came from a hospital in Sydney (10). Conjugational analysis in strain PAT became possible with the isolation of a recipient line which could form recombinants with PAT donors and which could accept and maintain the FP2 plasmid, thereby regaining donor functions (24). Recent studies (21) have shown that the recipient line of strain PAT harbors a nonconjugative plasmid (pVS1) which determines resistance to mercuric ions and sulfonamides but which appears to be unrelated to FP2. The recipient line of strain PAT was used by Stanisich and Holloway (23) to show that the plasmids R68 and R91 are able to mobilize the chromosome of this strain.

This paper describes the mapping of the chromosome of strain PAT using the plasmids FP2-2 (24), R68, R91-5 (5), and R68.45. New origins of chromosome transfer have been identified, and a preliminary map of strain PAT is presented.

(A preliminary account of these results was presented at the Third International Symposium on Antibiotic Resistance, Smolenice, Czechoslovakia, June 1976).

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The bacterial strains used in this study are shown in Table 1. All the PAT recipient strains (FP^-) were derived from the prototroph PAT964 (24). Donor strains were constructed by introducing the plasmids FP2-2 (24), R68 (3, 23), R91-5 (5), or R68.45 (7) into the appropriate recipient strain. Strains carrying R68, R68.45, or R91-5 were maintained on heart infusion agar containing 250 μg of carbenicillin per ml. Phage F116L (13) was used for all transductions.

Media. Nutrient broth, heart infusion agar, and minimal medium have been described previously (26). Nitrate heart infusion broth was Difco heart infusion broth containing 0.4% (wt/vol) potassium nitrate. The antibiotics carbenicillin (Pyopen; Beecham) and nalidixic acid (Sterling) were added to heart infusion agar at the concentrations indicated. Amino acids were added to minimal medium as required to a final con-

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TABLE 1. *Bacterial strains used in this study*[a]

Strain	Genotype	Reference
PAT2001	*leu-2104*	This paper
PAT2002	*met-3109*	This paper
PAT2003	*lys-1105*	This paper
PAT2005	*thr-1100*	This paper
PAT2040	*his-1111*	This paper
PAT2109	*arg-2119*	This paper
PAT2111	*arg-3121*	This paper
PAT2115	*arg-5125*	This paper
PAT2148	*leu-2104, lys-1115, trp-3114, pur-1118, pro-2108,* (Ts)*1102*	This paper
PAT2178	*leu-2104, lys-1115, trp-3114, pur-1118, pro-2108, ilv-1118, his-1116, nalB-115*	This paper
PAT2179	*leu-2104, lys-1115, trp-3114, pur-1118, pro-2108, ilv-1118, his-1116, nalA-116*	This paper
PAT2186	*arg-3121, thr-2111*	This paper
PAT2242	*leu-2104, lys-1115, trp-3114,* (Ts)*1102, thr-1100, pur-4119*	This paper
PAT2245	*leu-2104, lys-1115, trp-3114, met-1129, pro-1111, nalB-119*	This paper
PAO8	*ilv-202, met-28, str-1*	11
PAO18	*pro-64, pur-66*	13
PAO227	*met-28, trp-6, lys-12, his-4, pro-82, ilv-226, leu-13*	This paper
PAO1376	*pur-154, leu-38*	19
GMA253	*his-5075, cys-5605*	17

[a] All strains are recipients (FP^-). Abbreviations: *arg*, arginine requirement; *his*, histidine requirement; *ilv*, isoleucine and valine requirement; *leu*, leucine requirement; *lys*, lysine requirement; *met*, methionine requirement; *nal*, nalidixic acid resistance; *pro*, proline requirement; *pur*, adenine requirement; *thr*, threonine (*thr1*) or homoserine (*thr2*) requirement; *trp*, tryptophan requirement; Ts, temperature sensitive at 43°C. Strain PAT markers are designated according to the nomenclature of Watson and Holloway (26). The first number following each gene symbol indicates the arbitrary locus designation, whereas the following three numbers refer to the allele number; e.g., *leu-2104* indicates allele 104 of the *leu-2* locus.

centration of 1 mM, except isoleucine, which was used at 0.5 mM. Stocks of amino acid solutions (50 mM) were kept over chloroform.

Isolation of mutants. Spontaneous nalidixic acid-resistant mutants were selected on heart infusion agar containing nalidixic acid at a concentration of either 1 mg/ml (for *nalA* mutants) or 400 μg/ml (for *nalB* mutants).

Auxotrophic mutants were isolated after ethyl methane sulfonate mutagenesis (26) and carbenicillin enrichment as follows. After allowing for the expression of mutations, the culture was centrifuged, washed twice, and suspended to the same volume in saline. The washed cells were then diluted 1 in 100 into nitrogen-free minimal medium and starved by incubating for 6 to 8 h at 37°C without shaking. The starved cells were then diluted 1 in 10 into parental minimal medium containing 2 mg of carbenicillin per ml. After overnight incubation at 37°C with shaking, residual carbenicillin was inactivated by adding penicillinase (300 Levy units per ml) and incubating for 1 h at 37°C with shaking. Surviving clones were screened for auxotrophic mutants by replica plating.

Plate mating. The procedure for plate mating has been described previously (22).

Interrupted mating. (i) In liquid. Overnight cultures of the recipient and donor strains in nitrate heart infusion broth were diluted 1 in 30 into the same medium and incubated for 6 h (recipient) or 2 to 3 h (donor) at 37°C with shaking. The mating was initiated by adding 1 volume each of recipient and donor cultures to 2 volumes of saline. After mixing gently, 1-ml aliquots were dispensed into test tubes. At the time of interruption, 1 ml of saline containing 800 μg of nalidixic acid per ml was added and the mixture was blended in a Vortex mixer vigorously for 5 to 10 s. Aliquots of 0.2 ml of the mixture were then spread on selective minimal medium containing 400 μg of nalidixic acid per ml. The plates were then incubated at 37°C for 48 to 72 h and scored. All operations were carried out in a 37°C constant-temperature room, and all materials were prewarmed to 37°C.

(ii) On solid media. The procedure followed for interrupted mating on solid media was that of Haas and Holloway (7), except that matings were interrupted by spreading 0.5 ml of nalidixic acid solution (8 mg/ml, pH 10) over the entire surface of the plate.

Transduction. The procedure for transduction has been described previously (13).

RESULTS

On the assumption that the relative locations of genes are similar in strains PAO and PAT, we sought preliminary evidence for the locations of PAT markers by means of transduction between strains PAO and PAT. In these transductions (Table 2), a range of PAO recipients carrying markers of known map location were transduced with F116L propagated on strain PAT2166. Close linkage between phenotypically similar mutations in both strains was indicated by a significant reduction in the yield of prototrophic transductants for the marker under examination. Such close linkage is indicative of similar map locations of the apparently homologous markers in strains PAO and PAT.

Confirmation of the locations of these PAT markers was achieved by means of interrupted

matings using appropriate multiply marked recipients and donors carrying FP2-2, R68, or R91-5.

Interrupted matings using FP2-2. In the first of these matings, PAT2178 (a *nalA* derivative of PAT2166) was crossed with PAT2002 (FP2-2) using the procedure for interrupted mating in liquid (see Materials and Methods). The results of this cross are shown in Fig. 1.

It can be seen that each marker has a well defined time of entry which is similar to that of the homologous marker in strain PAO (see Table 2). The results of a number of interrupted matings using other PAT recipients (data not shown) are consistent with the view that FP2 mobilizes the chromosome from the same site and with the same polarity in strains PAO and PAT.

As can be seen from Fig. 1, the gradient of entry of late markers is considerably reduced relative to that of early markers. In this particular experiment, *pur-1118*$^+$ recombinants were not recovered even after 90 min of mating. The donor contraselective marker (*met-3109*) is

TABLE 2. *Comparison of markers in strains PAO and PAT by reduction in prototrophic transductant formation*[a]

Recipient	Selected marker	Map position (min)	Transductants per 0.2 ml with donor:		Homologous PAT2116 marker
			PAT2116	PAT2	
GMA253	*his-5075*$^+$	12	20	68	*his-1116*
	cys-5605$^+$	12	58	30	None
PAO8	*ilv-202*$^+$	30	11	152	*ilv-1118*
	met-28$^+$	30	75	33	None
PAO18	*pro-64*$^+$	4	0	32	*pro-2108*
	pur-66$^+$	~50	2	34	*pur-1118*
PAO227	*lys-12*$^+$	20	1	24	*lys-1115*
	trp-6$^+$	35	2	34	*trp-3114*
	leu-13$^+$	~60	1	14	*leu-2104*
	pro-82$^+$		25	44	None
PAO1376	*pur-154*$^+$		4	42	*pur-1118*
	leu-38$^+$		85	98	None

[a] Various PAO recipients were transduced with phage F116L propagated on strain PAT2166 and the wild-type strain PAT2. Failure to produce prototrophic transductants at normal frequencies using PAT2166 as donor is evidence of close linkage of the phenotypically similar mutations in both strains. Map positions of the PAO markers are as determined by Haas and Holloway (7) and Haas et al. (8).

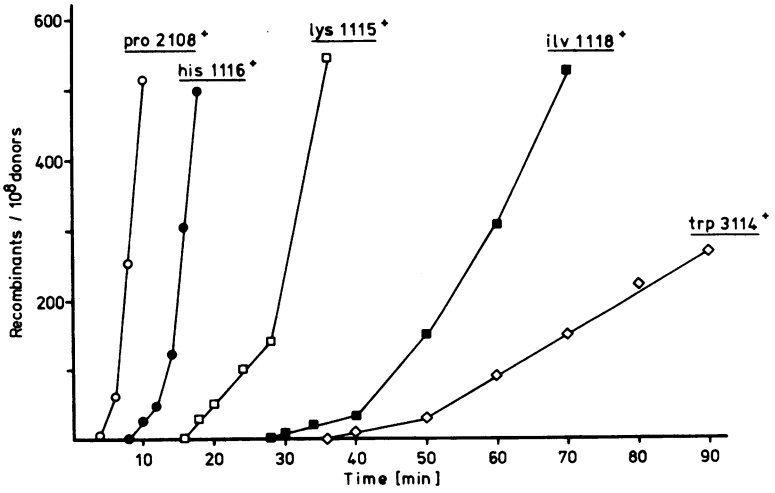

FIG. 1. *Interrupted mating between PAT2178 and PAT2002 (FP2-2). No recovery was observed when selection was made for either pur*$^+$ *or leu*$^+$ *recombinants up to 90 min after mating commenced.*

known to be cotransducible with *leu-2104* (6), and hence recombinants for the latter marker were not selected. The observed decrease in the number of recombinants for later markers indicates that donors carrying FP2-2 are unsuitable for the mapping of markers that enter later than about 40 min. The results of the above experiment confirm the locations of some of the PAT markers as suggested by the transduction data shown in Table 2.

Chromosome mobilization by R68 and R91-5. Because of the limited potential of FP2-2 for mapping markers in the "late" region of the chromosome, we examined the chromosome mobilization properties of R68 and R91-5 in strain PAT. In preliminary experiments, a number of plate matings were carried out using singly auxotrophic recipients and otherwise isogenic R68 and R91-5 donors. For comparison, the same recipients were mated with an FP2-2 donor. The results of these crosses are shown in Table 3.

It can be seen that the gradient of recovery of the various selected markers in the R68 and R91-5 matings is opposite to that in the FP2-2 matings. For example, *leu-2104*$^+$ is recovered at low frequency with the FP2-2 donor but at relatively high frequency with the R68 and R91-5 donors. In strain PAO, the homologous marker (*leu-10*) is also recovered at low frequency in FP2 crosses and has been mapped at 55 to 60 min on the chromosome (8). In general, markers that are recovered at relatively high frequency in crosses with the FP2-2 donor are recovered at low frequency in crosses with the R68 and R91-5 donors and vice versa. This is prima facie evidence that R68 and R91-5 mobilize the chromosome from an origin (or origins) different from that of FP2-2, assuming that the decline in recombination frequencies for the markers reflects their increasing distance from the origin of transfer. Further evidence to support this conclusion was obtained from interrupted mating experiments using R68 and R91-5 donors.

Interrupted matings using R68 and R91-5. There are a number of factors that will affect the recovery of recombinants in interrupted matings. In addition to the location of the transfer origin (or origins) of the conjugative plasmid relative to the marker under examination, the position of the donor contraselective markers will also influence the recovery of recombinants. As with R68.45 crosses in strain PAO (7), we have found it necessary to use a procedure for interrupted mating on solid media for crosses using R68 and R91-5 donors in strain PAT. Since nalidixic acid is used as the interrupting agent in these experiments, the recovery of certain classes of recombinants may be influenced by the relative positions of the selected marker and the locus determining nalidixic acid resistance in the recipient. It has been shown in *E. coli* (9) that nalidixic acid sensitivity (*nal*$^+$) is dominant to resistance (*nal*) and, while we have not examined the dominance relationship of these alleles in detail, our observations indicate that the same situation occurs in *P. aeruginosa*. In strain PAT, we have found that the *nalA* locus is situated between *leu-2104* and *pur-1118*, while the *nalB* locus is cotransducible by phage F116L with *pro-3106* (42%) and *met-2105* (43%) and is very closely linked to *ilv-1118* (J. M. Watson and B. W. Holloway, manuscript in preparation). In designing some of the experiments discussed below, we have used donor contraselective markers in desired positions to examine the mobilization of chromosome by R91-5. The locations of these markers (*thr-1100* and *arg-5125*) have been determined by transduction. *thr-1100* was previously shown to be 20% cotransducible with *pur-1118* (26), and *arg-5125* is 1% cotransducible with *lys-1115* using phage F116L.

The results of a mating between PAT2179 (a *nalB* derivative of PAT2166) and PAT2115 (R68) are shown in Fig. 2. The observed entry times for the markers recovered in this cross are 11 min for *leu-2104*, 15 min for *pur-1118*, and 29

TABLE 3. *Plate matings using singly auxotrophic recipients*[a]

Recipient	Selected marker	No. of prototrophic recombinants per 10^8 donors		
		× PAT2005 (FP2-2)	× PAT2005 (R68)	× PAT2005 (R91-5)
PAT2040	*his-1111*$^+$	ca. 200,000	3,500	45
PAT2003	*lys-1105*$^+$	56,800	19,000	145
PAT2115	*arg-5125*$^+$	50,000	21,000	225
PAT2111	*arg-3121*$^+$	11,500	23,000	440
PAT2109	*arg-2119*$^+$	1,480	160,000	24,200
PAT2001	*leu-2104*$^+$	6,360	421,000	30,600
PAT2002	*met-3109*$^+$	2,020	433,000	32,600

[a] A series of recipient strains, each with a single auxotrophic marker, were mated on the plate with three donor strains each with the same contraselective marker (*thr-1100*) but carrying different conjugative plasmids.

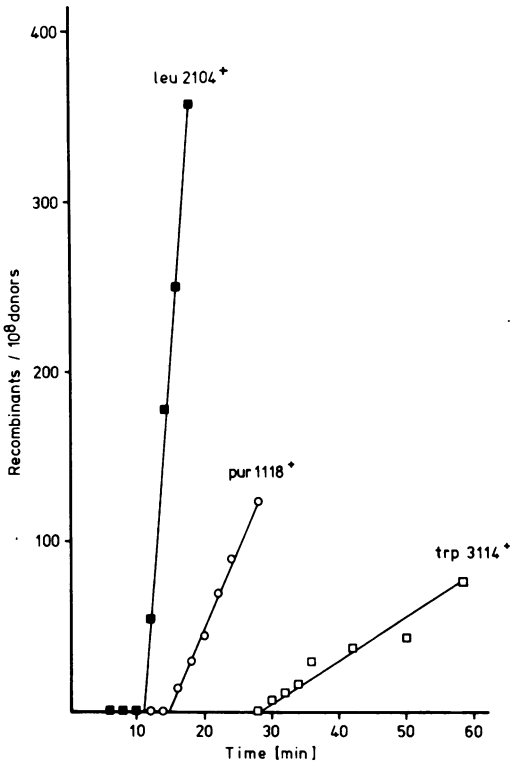

FIG. 2. *Interrupted mating between PAT2179 and PAT2115 (R68). No recovery was observed when selection was made for either lys+, pro+, ilv+, or his+ recombinants up to 90 min after mating commenced.*

min for *trp-3114*. No *ilv-1118*+ or *lys-1115*+ recombinants were recovered even after 60 min of mating, which is most likely due to the high linkage of the *nalB*+ and *ilv-1118*+ markers (see above). The recovery of *leu-2104*+ and *pur-1118*+ recombinants in this cross, but not in the equivalent cross using the FP2-2 donor (see Fig. 1), and the recovery of *trp-3114*+ recombinants in both crosses indicate that R68 mobilizes the chromosome from a different transfer origin to that of FP2-2, and furthermore the polarities of chromosome mobilization by these two plasmids are different. The results of other interrupted matings (data not shown) support these conclusions and show that, like FP2, R68 mobilizes the chromosome of strain PAT from a single origin which is situated 65 min clockwise from the FP2 origin (see below).

The results of interrupted matings between PAT2245 and otherwise isogenic R68 and R91-5 donors are shown in Fig. 3. Whereas *leu-2104*+ enters at about 12 min and *pro-1111*+ enters at 23 min in both crosses, the *trp-3114*+ marker enters at different times in these two crosses. The interval of 16 min between *leu-2104*+ and *trp-3114*+ observed in the R68 cross (Fig. 3A) is close to the interval of 18 min observed in the previous cross (see Fig. 2). The fact that *leu-2104*+ and *trp-3114*+ both enter at about 13 min in the R91-5 cross (Fig. 3B) suggests that the origin from which the chromosome is mobilized by R91-5 is different from that of R68.

A number of hypotheses can be suggested to explain the similar entry times of *leu-2104*+ and *trp-3114*+ in the R91-5 cross. First, R91-5 could mobilize the chromosome in both directions from a single origin located between *leu-2104* and *trp-3114* such that both markers show the same entry time. Alternatively, the chromosome could be mobilized in opposite directions from two distinct origins located between *leu-2104* and *trp-3114*. Neither of these hypotheses is supported by the observation that the *pro-1111*+ marker, which is located between *leu-2104*+ and *trp-3114*+ (see Fig. 3A), enters later than the latter two markers in the R91-5 cross (see Fig. 3B). Another hypothesis is that R91-5 mobilizes the chromosome from two origins (Fig. 4). To account for the similar entry times of *leu-2104*+ in R68 and R91-5 matings (Fig. 3A and B), one of these postulated origins (OR1) would have to be close to, or the same as, that of R68. The polarity of chromosome mobilization from this origin would also have to be the same as that of R68, since *pro-1111*+ enters 11 min after *leu-2104*+ in both the R91-5 and R68 crosses (Fig. 3A and B). Mobilization of the chromosome from the second of these postulated origins (OR2) would explain the entry time of *trp-3114*+. The location of OR2 could be 12 min to the left of *trp-3114* with the direction of chromosome mobilization being the same as that of R68 (as shown in Fig. 4). Alternatively, OR2 could be located 12 min to the right of *trp-3114* with the polarity of mobilization being opposite to that of R68. The results of experiments to be described below are inconsistent with the second of these alternatives and support the model as shown in Fig. 4.

Evidence for the two transfer origins of R91-5. Further crosses were carried out to test the hypothesis that R91-5 mobilizes the chromosome from two origins and to locate these origins. In these experiments it has been assumed that R91-5 can mobilize the chromosome with equal frequency from either origin and that, in any single mating pair, chromosome mobilization occurs from only one of these origins. The following crosses were arranged so that the entry times of markers mobilized from either origin could be measured under selective conditions that minimized the recovery of markers mobilized from the alternative origin. This can be

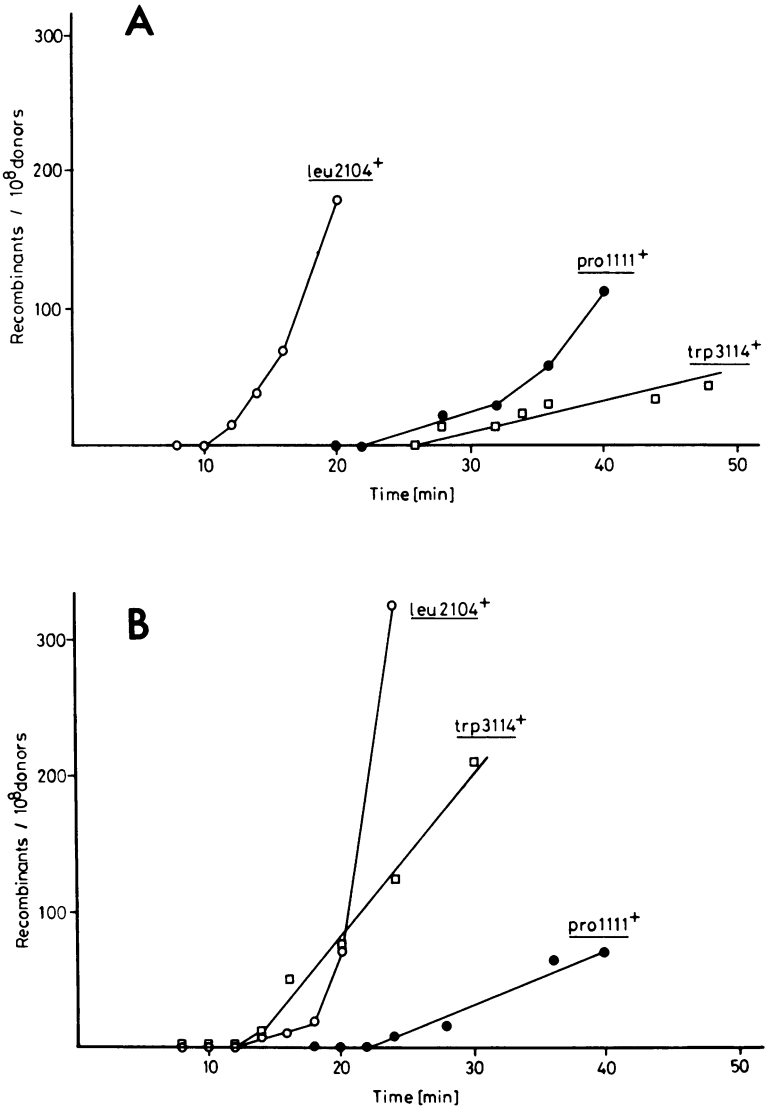

FIG. 3. *Interrupted matings between PAT2245 and (A) PAT2115 (R68) or (B) PAT 2115 (R91-5). No recovery was observed when selection was made for either lys+ or met+ recombinants up to 90 min after mating commenced.*

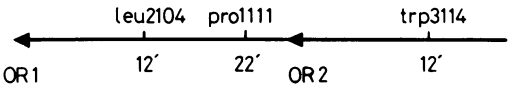

FIG. 4. *The postulated locations of the two transfer origins (OR1 and OR2) of R91-5.*

done in two ways. First, either *nalA* or *nalB* recipients can be used so that entry of the corresponding *nal*+ (sensitivity) allele of the donor will reduce the recovery of recombinants where the selected marker is closely linked to, or transferred later than, the *nal*+ allele. Similarly, the recovery of particular recombinants will be reduced if the auxotrophic contraselective marker of the donor is closely linked to the marker being selected. Figure 5 shows the results of four matings using *nalA* or *nalB* recipients and R91-5 donors carrying either *arg-5125* or *thr-1100* as the contraselective marker.

In Fig. 5A, where the recipient carries a *nalA* mutation, the recovery of *leu-2104*+ was unaffected, whereas *pur-1118*+ recombinants were not recovered (compare Fig. 5C). The observed entry times of *trp-3114*+ and *ilv-1118*+ were 8 and 11 min, respectively. The recovery of these markers would be unaffected by the entry of

nalA$^+$ if OR2 is to the right of *nalA*. The earlier entry time of *trp-3114*$^+$ relative to *ilv-1118*$^+$ is not consistent with OR2 being located to the right of *trp-3114*.

In Fig. 5B, *leu-2104*$^+$ recombinants were recovered at somewhat lower frequency; however, the recovery of *pur-1118*$^+$ recombinants was prevented by coinheritance of the donor contraselective marker (*thr-1100*). However, if OR2 is located to the right of *thr-1100*, then the recovery of *trp-3114*$^+$ recombinants, as a result of chromosome mobilization from OR2, should be unaffected since *thr-1100* would be mobilized distally, with respect to *trp-3114*$^+$, from this origin. The location of *nalB* close to *ilv-1118* severely reduces the recovery of recombinants for the latter marker (compare Fig. 5D) and also the recovery of *lys-1115*$^+$ and *his-1116*$^+$ recombinants, which were not detected even after 60 min of mating.

In Fig. 5C, both *leu-2104*$^+$ and *pur-1118*$^+$ recombinants were recovered, with *leu-2104*$^+$ entering at 11 min and *pur-1118*$^+$ at 19 min as a result of mobilization from OR1. The order of entry of these two markers indicates that R91-5 mobilizes the chromosome from OR1 with the same polarity as that of R68 (see Fig. 2). Although *trp-3114*$^+$ recombinants were recovered in this cross, the recovery of *ilv-1118*$^+$ recombinants was affected by the close linkage of *nalB* (compare Fig. 5D).

In Fig. 5D, *leu-2104*$^+$ recombinants were recovered as a result of chromosome mobilization from OR1; however, *pur-1118*$^+$ recombinants were not recovered because of the close linkage of this marker to *nalA* and *thr-1100*. The location of these two markers, however, had no effect on the recovery of *trp-3114*$^+$, *ilv-1118*$^+$, *lys-1115*$^+$, and *his1116*$^+$ recombinants (compare Fig. 5A and B), which is further evidence for the location of OR2 to the left of these markers, but to the right of *nalA* and *thr-1100*. The order of entry of the markers *trp-3114*$^+$, *ilv-1118*$^+$, *lys-1115*$^+$, and *his-1116*$^+$ (Fig. 5D) indicates that chromosome mobilization from OR2 occurs in the opposite direction to that by FP2-2 (see Fig. 1).

The results of the above crosses are consistent with the following conclusions. The chromosome of strain PAT can be mobilized by R91-5 from an origin (OR1) which is located about 11 to 12 min to the left of *leu-2104*, since the recovery of *leu-2104*$^+$ recombinants was not greatly affected by the donor markers *nalA*$^+$, *nalB*$^+$, *thr-1100*, or *arg-5125*. The fact that *pur-1118*$^+$ is mobilized later than *leu-2104*$^+$ (Fig. 5C) indicates that chromosome mobilization from OR1 by R91-5 occurs in the same direction as that by R68 (see Fig. 2). The order of entry of these two markers is also inconsistent with the hypothesis that R91-5 mobilizes the chromosome in both directions from an origin (or origins) located between *leu-2104* and *trp-3114*. The observation that *trp-3114*$^+$ recombinants can be recovered in the cross where *pur-1118*$^+$ recombinants are not detected, as a result of coinheritance of the donor markers *nalA*$^+$ and *thr-1100* (Fig. 5D), is consistent with the hypothesis that R91-5 can mobilize the chromosome from a second origin (OR2). From the above results, OR2 must be located 11 to 12 min to the left of *trp-3114* (as shown in Fig. 4), since *trp-3114*$^+$ was mobilized before *ilv-1118*$^+$.

One feature of the above crosses for which we have no satisfactory explanation is the biphasic entry kinetics observed with certain markers. This is not an uncommon feature of interrupted matings using various conjugative plasmids in *P. aeruginosa* (7, 8, 22). While it may affect the accuracy of some of the chromosome distances involved in the above arguments, it does not invalidate the conclusion that R91-5 mobilizes the chromosome from two origins.

Chromosome mobilization by R68.45. The plasmid variant R68.45 has been shown to mobilize the strain PAO chromosome from a number of origins (7; D. Haas and B. W. Holloway, submitted for publication). A similar pattern of chromosome mobilization by R68.45 is apparent in strain PAT (Table 4). It can be seen that there is no obvious correlation between the recombination frequency of a given marker and its map location. This suggests that, as in strain PAO (7), R68.45 mobilizes the PAT chromosome from a number of origins, although no data have been obtained from time-of-entry experiments to support this.

Genetic circularity of the PAT chromosome. Genetic circularity of the *E. coli* chromosome could be demonstrated because of the availability of a range of Hfr strains which mobilized the chromosome from different origins and in different directions (12). The demonstration of chromosomal circularity in *P. aeruginosa* by means of time-of-entry experiments is not possible because of the limited number of sites from which the chromosome can be mobilized. We have sought indirect evidence for chromosomal circularity in strain PAT by means of linkage data.

To show that FP2-2 and R68 mobilized the chromosome from different origins, it was necessary to isolate markers which were located between these postulated origins. Such markers would be expected to show low recombination frequencies in crosses with both FP2-2 and R68

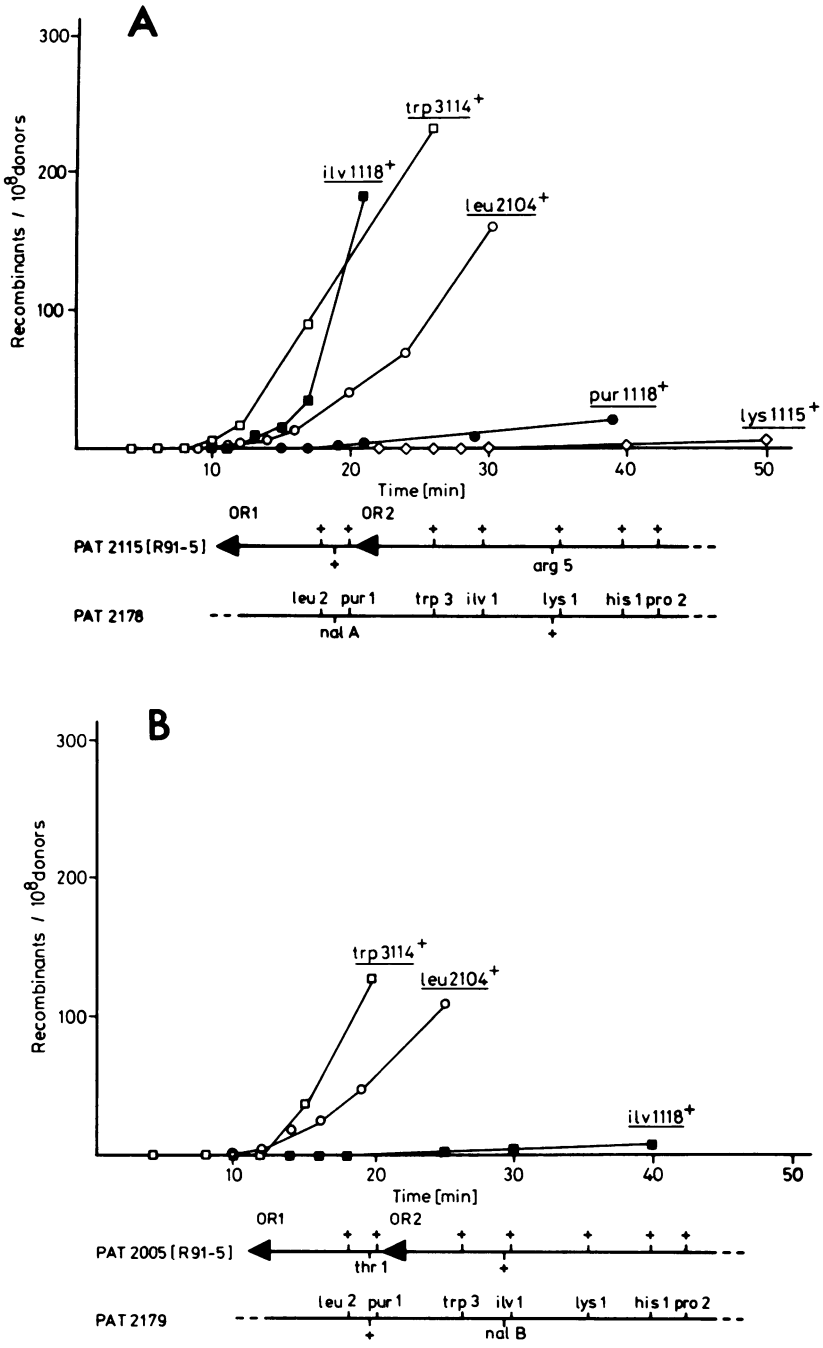

FIG. 5. *Interrupted matings between the recipient strains PAT2178 or PAT2179 and the donor strains PAT2005 (R91-5) or PAT2115 (R91-5). The markers involved in each cross (indicated by locus designation only) are shown diagrammatically below each figure. Arrowheads indicate the postulated locations of the transfer origins of R91-5. The wild-type allele of each marker is indicated by +. In each of these crosses, selection was made for leu-2104$^+$, pur-1118$^+$, trp-3114$^+$, ilv-1118$^+$, lys-1115$^+$, and his-1116$^+$ recombinants at various time intervals up to 90 min. In some of the crosses, certain classes of recombinants were not recovered after 90 min of mating and hence are not shown on the corresponding figure.*

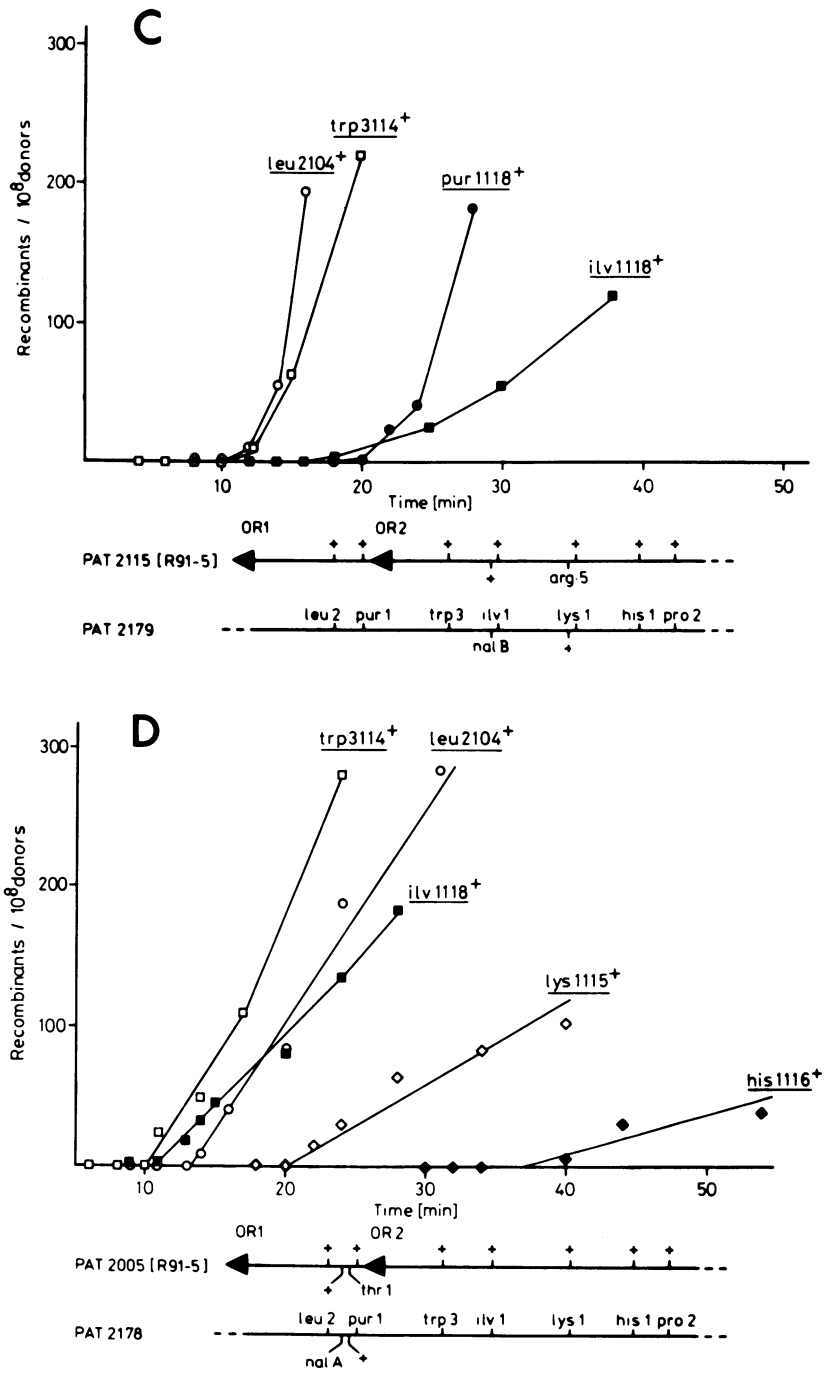

FIG. 5*C* AND *D*

donors. Therefore, a range of temperature-sensitive markers was screened for low recombination frequencies in crosses using both these plasmids, and one such marker, (Ts)*1102*, was found. A similar screening of other markers revealed that *pur-4119* also yielded low numbers of recombinants in crosses with FP2-2 and R68 donors. In plate mating experiments (data not

shown), both (Ts)*1102*$^+$ and *pur-4119*$^+$ recombinants were recovered at lower frequencies than *leu-2104*$^+$ recombinants in FP2-2 crosses, and at lower frequencies than *pro-2108*$^+$ recombinants in R68 crosses. These observations suggested that (Ts)*1102* and *pur-4119* are located between *leu-2104* and *pro-2108*. The results of a plate mating using an R91-5 donor (Table 5) showed that *pur-4119* and (Ts)*1102* are closely linked and indicate the marker order *lys-1115–pro-2108–pur-4119–*(Ts)*1102*. The *leu-2104*$^+$ recombinants selected in this cross failed to show linkage of any of the remaining unselected markers.

To show that (Ts)*1102* and *pur-4119* were linked to *leu-2104*, and hence to confirm their location between *leu-2104* and *pro-2108*, a cross was carried out using an R68.45 donor. In strain PAO, R68.45 donors have been shown to mobilize chromosomal segments equivalent in length to about 10 min although, less frequently, transfer of segments of about 20-min length is also observed (7; Haas and Holloway, submitted for publication). Since this plasmid is able to mobilize such chromosomal segments from a number of origins (7), it can be used to examine the linkage relationships between markers which show poor linkage to each other in crosses with donors carrying other conjugative plasmids.

The results of a cross between PAT2242 and an R68.45 donor are shown in Table 6. As in the previous cross (see Table 5), (Ts)*1102* and *pur-4119* both show linkage to *pro-2108* but negligible linkage to *leu-2104*. These observations suggest that *pro-2108* is considerably closer to the (Ts)*1102–pur-4119* pair of markers than is *leu-2104*. When recombinants which have inherited both *pro-2108*$^+$ and *leu-2104*$^+$ are selected, the coinheritances of the unselected markers (Ts)*1102*$^+$ and *pur-4119*$^+$ are considerably greater than when either *pro-2108*$^+$ or *leu-2104*$^+$ recombinants are selected individually. This result confirms that (Ts)*1102* and *pur-4119* are located between *pro-2108* and *leu-2104* and, taken together with the results of the R91-5 cross (Table 5), suggests the marker order *pro-2108–*(Ts)*1102–pur-4119–leu-2104*.

The marker orders O(FP2)-*pro-2108–his-1116–lys-1115–ilv-1118–trp-3114* and O(R68)-*leu-2104–pur-1118–trp-3114* were established from the results of interrupted matings (see Fig. 1 and 2, respectively). Similarly, the order OR2 (R91-5)-*trp-3114–ilv-1118–lys-1116–his-1116* was observed in the cross shown in Fig. 5D. Analysis of recombinants derived from plate mating experiments using FP2-2 and R68 donors (Fig. 6) indicates that each of the above markers is linked.

The above data, together with those shown in Table 6, support the conclusion that all the

TABLE 4. *Plate mating between PAT2166 and PAT2115 (R68.45)*

Selected marker	Map location (min)	Recombination frequency per 10^8 donor cells	% Coinheritance of unselected markers[a]						
			pro-2108$^+$	*his-1116*$^+$	*lys-1115*$^+$	*ilv-1118*$^+$	*trp-3114*$^+$	*pur-1118*$^+$	*leu-2104*$^+$
pro-2108$^+$	4	8,250		76	4	3	2	0	0
his-1116$^+$	9	23,000	73		3	4	3	1	0
lys-1115$^+$	19	3,250	5	4		2	0	0	0
ilv-1118$^+$	29	14,000	3	3	2		22	1	1
trp-3114$^+$	36	25,500	0	0	0	60		14	4
pur-1118$^+$	47	64,000	0	0	0	1	12		18
leu-2104$^+$	52	4,250	0	0	0	4	2	6	

[a] 100 recombinants of each class scored.

TABLE 5. *Recombinant analysis of the plate mating PAT2242 × PAT2186 (R91-5)*

Selected marker	Recombinants per 10^8 donor cells	% Coinheritance of unselected markers[a]				
		leu-2104$^+$	*lys-1115*$^+$	*pro-2108*$^+$	*pur-4119*$^+$	(Ts)*1102*$^+$
leu-2104$^+$	9,550		0	0	0	0
lys-1115$^+$	790	2		9	4	5
pro-2108$^+$	320	2	23		11	12
pur-4119$^+$	22	11	34	45		71
(Ts)*1102*$^+$	17	2	29	41	89	

[a] 100 recombinants of each class scored.

markers of *P. aeruginosa* PAT used in this study are located on the one circular linkage group. The data presented above have been arranged in the form of a chromosome map (Fig. 7). In deriving this map, we have assumed that the rates of chromosome mobilization by FP2-2, R68, and R91-5 are similar. In strain PAO, data have been obtained (Haas and Holloway, submitted for publication) which show that the rates of chromosome mobilization by FP2 and R68.45 are very similar.

DISCUSSION

Chromosome mapping in *P. aeruginosa* PAT has been achieved by using several different plasmids. This approach has been necessary, since, with one exception, plasmids that do mobilize the chromosome in *P. aeruginosa* do not do so from a range of origins as is the case with F in *E. coli*. In *P. aeruginosa* PAO, FP2, FP39, and FP5 (16, 18, 19) each have only one origin, while R68.45 has been shown to mobilize the chromosome from a number of origins (7). In strain PAT, we have shown that FP2-2 and R68 each mobilize the chromosome from only one distinct origin.

The fact that the markers *leu-2104* and *pur-1118* enter at similar times in R68 and R91-5 matings indicates that one of the transfer origins (OR1) of R91-5 is located close to, or may be the same as, that of R68. The available data indicate that the second transfer origin (OR2) of R91-5 is located between *pro-1111* and *trp-3114*.

There is a strain specificity in the ability of R68 to mobilize chromosome in *P. aeruginosa*. In strain PAT, recombination frequencies of up to 10^{-3} per donor cell are observed in R68 crosses. In strain PAO, however, the frequencies of recombination are less than 10^{-8} for all the markers tested, whereas the frequency of transfer of R68 itself is similar in both strains (V. A. Stanisich, Ph.D. thesis, Monash University, Clayton, Victoria, Australia, 1972). This suggests that there is some difference between strains PAO and PAT with respect to a DNA sequence that interacts with the plasmid itself, or with a plasmid gene product, to bring about chromosome mobilization.

TABLE 6. *Recombinant analysis of the plate mating PAT2242 × PAT2186 (R68.45)*

Selected marker(s)	Recombinants per 10^8 donor cells	% Coinheritance[a]			
		pro-2108$^+$	*pur-4119*$^+$	(Ts)*1102*$^+$	*leu-2104*$^+$
pro-2108$^+$	22,600		24	26	2
pur-4119$^+$	7,450	18		66	1
(Ts)*1102*$^+$	33,450	32	71		1
leu-2104$^+$	10,150	1	2	2	
pro-2108$^+$ *pur-4119*$^+$	2,020			98	1
pro-2108$^+$ (Ts)*1102*$^+$	6,430		66		0
pro-2108$^+$ *leu-2104*$^+$	365		72	75	
leu-2104$^+$ *pur-4119*$^+$	145	48		86	
leu-2104$^+$ (Ts)*1102*$^+$	450	66	96		

[a] 100 recombinants of each class scored.

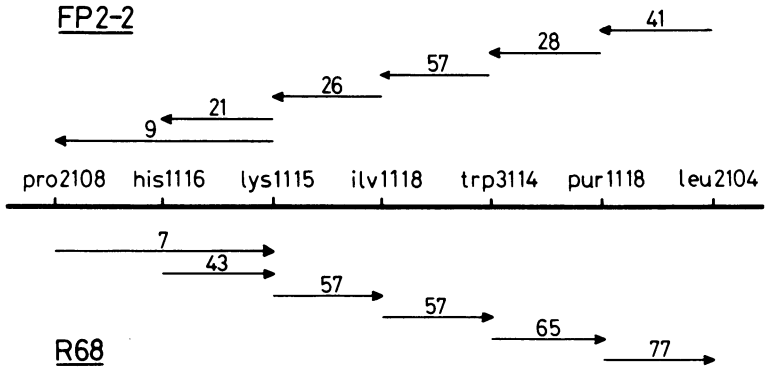

FIG. 6. *Linkages between markers in FP2-2 and R68 crosses. Arrowheads indicate unselected markers. Linkage values are expressed as the percentage of selected recombinants which coinherited the unselected marker. Values shown above the markers were obtained from FP2-2 crosses, and those shown below the markers were obtained from R68 crosses.*

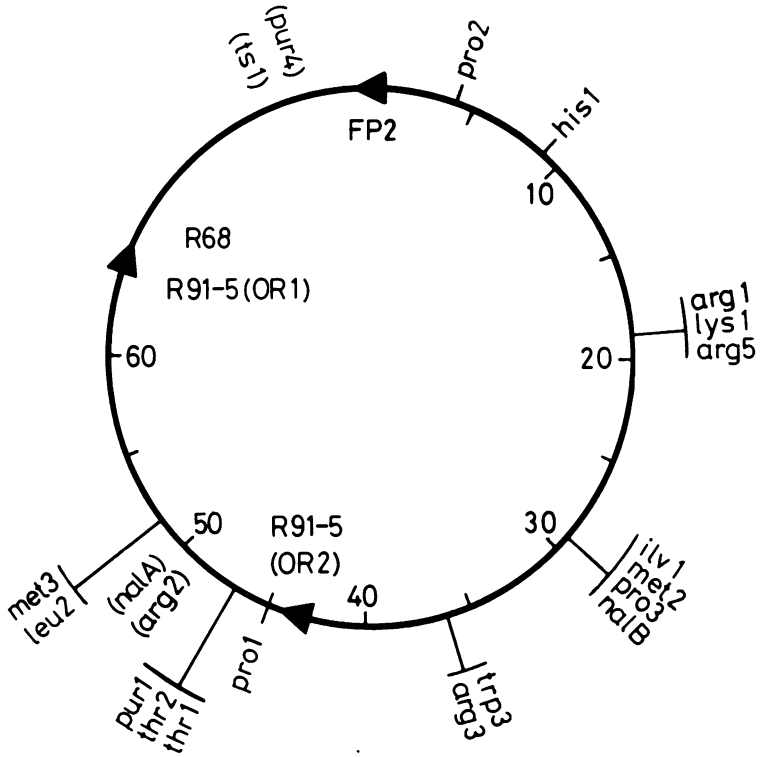

FIG. 7. *Chromosome map of P. aeruginosa PAT. The genes are displayed on a circular linkage group which is calibrated in minutes of transfer time with the transfer origin of FP2 arbitrarily designated as zero. Arrowheads indicate the sites of the transfer origins of the conjugative plasmids. Markers displayed on outer arcs of the circle are cotransducible by phage F116L. Markers in parentheses are only approximately mapped. The length of the chromosomal region clockwise between the R68 and FP2 origins is unknown.*

The conclusion that the strain PAT chromosome is circular is based solely on linkage data. Confirmation of chromosomal circularity by time-of-entry data has not been possible because of the relative locations of the transfer origins of FP2-2, R68, and R91-5. This has precluded an estimation of the total length of the chromosome in terms of transfer time. The length of the chromosomal region clockwise between the R68 and FP2 origins remains to be determined. This will require the isolation of a conjugative plasmid which is able to mobilize this region proximally.

ACKNOWLEDGMENTS

This work was supported by a grant from the Australian Research Grants Committee. J.M.W. was the recipient of a Commonwealth Postgraduate Award.

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