JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 145-155
0021-9193/81/010145-11$02.00/0
Vol. 145, No. 1

Genetic Circularity of the *Pseudomonas aeruginosa* PAO Chromosome

P. L. ROYLE,[1]† H. MATSUMOTO,[2] AND B. W. HOLLOWAY[1]*

Department of Genetics, Monash University, Clayton, Victoria 3168, Australia[1]; and Department of Bacteriology, Faculty of Medicine, Shinshu University, Matsumoto 390, Japan[2]

Genetic circularity of the *Pseudomonas aeruginosa* PAO chromosome was demonstrated by a series of two- and three-factor crosses and double-selection experiments with Cma plasmids FP2, FP5, FP110, and R68.45. A range of additional markers, including catabolic markers, were located on the chromosome map. Plasmid FP2, known to have a major origin of chromosome transfer (0 min) was shown to have at least one other minor origin from which it can transfer the chromosome in the direction opposite to that found for the major origin.

Genetic studies and mapping of *Pseudomonas aeruginosa* PAO have been proceeding since 1955 by a combination of conjugation and transduction techniques. Whereas the location of over 80 genes has been determined (9), genetic circularity of the chromosome has not been established, and the locations of genes in the so-called late region (>45 min from the arbitrarily chosen 0 min, FP2 origin) have been determined with only limited accuracy. By contrast, circularity has been established in *P. aeruginosa* PAT by conjugation with plasmids R68, R68.45, and R91.5 (30, 31).

Most mapping studies of strain PAO have been done with matings using the plasmid FP2. FP2 has one predominant origin of chromosome transfer which has been designated as 0 min on the PAO chromosome map. Recovery of recombinants by using this plasmid varies from 10^{-4} per donor parent for markers within 10 min of the origin to $<10^{-7}$ per donor parent for more distal markers more than 45 min from the FP2 origin. This frequency of recovery of the distal markers is insufficient for accurate time-of-entry determinations; hence, the location of distal markers in terms of minutes from the origin has not been accurately determined.

By using plasmids FP2, FP5 (16, 17), and R68.45 (5, 6) it is possible by the analysis of recombinants obtained from plate matings to establish the relative order of some genes in the distal region. This paper summarizes the linkage data obtained from a series of crosses involving these plasmids. We report the isolation and mapping of new markers in the previously unmapped regions of the PAO chromosome from *leu-8* (ca. 48 min) in a clockwise direction to the FP2 origin. Together with the existing body of information on marker location on the PAO chromosome, the new data provide evidence to conclude that the chromosome map of *P. aeruginosa* PAO is circular.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The bacterial strains used in this study are shown in Table 1. In various strains, the same marker site may be represented by independent isolations. In almost all cases, such closely linked markers, which may be allelic, have been identified by transductional prototroph reduction tests (28). Transductions were carried out in which the mutants to be tested were transduced with phage propagated on defined mutants and on the wild-type strain. The numbers of prototrophic transductants using the mutant donors were compared with those obtained with the wild-type donor. A marked reduction in prototrophs with the mutant donors indicates close linkage and possible allelism of the marker being tested in the mutant recipient. The list of closely linked markers used is given in Table 2. Phages F116L (14), G101 (10), and E79 *tv-1* (19) were used for transductions.

Media. Nutrient broth, nutrient agar, and minimal medium have been described previously (29). The antibiotics carbenicillin (Pyopen; Beecham), rifampin (Rimactane; Ciba-Geigy), streptomycin (Sigma), and nalidixic acid (Sigma) were added at the concentrations indicated. Amino acids, purines, and pyrimidines were added to minimal medium as required to a final concentration 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 auxotrophic mutants, interrupted mating, and transduction procedures. The isolation of auxotrophic mutants, interrupted mating, and transduction procedures were as previously described (5, 30). The plate mating technique is essentially the same as that previously described (5, 30). The donor parent was grown to ca. 5 × 10^8 in nitrate nutrient broth (0.4% KNO_3), and plate matings were made with the recipient parent in the ratio 1:4 to 1:6.

† Present address: Department of Microbiology, Life Sciences Building, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, United Kingdom.

TABLE 1. *Bacterial strains used in this study*

P. aeruginosa strain	Genotype[a]	Derivation[b]	Reference or source
PAO1	Prototroph	Wild-type isolate	(8)
PAO2	*ser-3*	*ser* derivative of PAO1, MC	(8)
PAO8	*met-28 ilv-202 str-1*	*met ilv* derivative of PAO1, intermediate strain lost, MC	(11)
PAO12	*pur-136 leu-8 chl-3*	*pur leu* derivative of PAO1, intermediate strain lost, MC	(20)
PAO18	*pur-66 pro-64*	*pur pro* derivative of PAO1, intermediate strain lost, NG	(14)
PAO25	*argF leu-10*	*leu* derivative of PAO317, NG	(5)
PAO125	*pur-136 leu-8 arg-22 trp-61 ilv-230 pro-77*	Derived from PAO12, intermediate strains lost	This paper
PAO131	*thr-9001 leu-9001 met-9011 pur-67 argF23*	*arg* derivative of PAO1772	This paper
PAO133	*thr-9001 leu-9001 met-9011 pur-67 argF23 his-20*	*his* derivative of PAO131	This paper
PAO140	*pro-66 thr-9001 argF23 leu-9001 met-9011 pur-67*	*pro* derivative of PAO131	This paper
PAO161	*pyrF63 chl-2*	*pyr* derivative of PAO1, NG	(11)
PAO166	*pyrF63 leu-17*	*leu* derivative of PAO161	This paper
PAO213	*met-28 trp-6 lys-12 his-4 pro-82 ilv-226 thr-56*	*thr* derivative of PAO222	This paper
PAO220	*met-28 trp-6 lys-12 his-4 pro-82*	*pro* derivative of PAO242	Holloway collection
PAO222	*met-28 trp-6 lys-12 his-4 pro-82 ilv-226*	*ilv* derivative of PAO220	(5)
PAO227	*met-28 trp-6 lys-12 his-4 pro-82 ilv-226 leu-13*	*leu* derivative of PAO222	(30)
PAO242	*met-28 trp-6 lys-12 his-4*	Derived from PAO1, intermediate strains lost, NG	Holloway collection
PAO303	*argB18 chl-2*	*arg* derivative of PAO1, NG	(5)
PAO325	*argB18 lys-57*	*lys* derivative of PAO303	(12)
PAO349	*met-28 trp-6 his-4 lys-12 pro-82 ilv-226 pur-64*	*pur* derivative of PAO222	Holloway collection
PAO383	*pur-75*	*pur* derivative of PAO1, MC	This paper
PAO384	*aro-1 pur-75*	*aro-1* derivative of PAO383, MC	This paper
PAO388	*aro-1 pyrF69 pur-75*	*pyr* derivative of PAO384, MC	This paper
PAO389	*met-28 trp-6 lys-12 pro-82 ilv-226*	*his*$^+$ transductant of PAO222 using E79 *tv-1* propagated on PAO1	R. Crockett
PAO878	*pur-136 leu-8 pro-72*	*pro* derivative of PAO12	This paper
PAO910	*pur-67 thr-9001*	*leu*$^+$ *met*$^+$ transductant of PAO1772	Holloway collection
PAO944	*pur-67 thr-9001 cys-54*	*cys* derivative of PAO910	This paper
PAO945	*pur-67 thr-9001 cys-54 rif-96*	Spontaneous rifampicin derivative of PAO944	This paper
PAO947	*catA1 nar-9011 cnu-9001*	*met*$^+$ transductant of PAO2367	This paper
PAO948	*catA1 nar-9011 mtu-9002 tyu-9030*	*met*$^+$ transductant of PAO2376	This paper
PAO949	*pur-67 thr-9001 cys-59*	*cys* derivative of PAO910	This paper
PAO1035	*pur-67 thr-9001 cys-54 pro-63*	*pro* derivative of PAO944	This paper
PAO1039	*pur-67 cys-54 rif-96*	*thr*$^+$ transductant of PAO945 using E79 *tv-1* propagated on PAO1	This paper
PAO1042	*pur-67 thr-9001 cys-59 pro-65*	*pro* derivative of PAO949	This paper
PAO1052	*pur-67 thr-9001 cys-59 pro-65 nal-26*	Spontaneous nalidixic acid-resistant derivative of PAO1042	This paper
PAO1772	*leu-9001 thr-9001 met-9011 pur-67*	*pur* derivative of PAO1817	This paper
PAO1777	*leu-9001 met-9011 pur-67 ilv-220 thr-9001*	*ilv* derivative of PAO1772	This paper
PAO1787	*leu-9001 met-9011 pur-67 ilv-220*	*thr*$^+$ transductant of PAO1777 using E79 *tv-1*	This paper
PAO1816	*his-9004*	*his* derivative of PAO1	Matsumoto collection
PAO1817	*leu-9001 thr-9001 met-9011*	*met* derivative of PAO1809	Matsumoto collection
PAO1808	*leu-9001*	*leu* derivative of PAO1	Matsumoto collection
PAO1809	*leu-9001 thr-9001*	*thr* derivative of PAO1808	Matsumoto collection
PAO1834	*met-9020*	*met* derivative of PAO1	Matsumoto collection
PAO2152	*his-9004 nir-9006* FP5$^+$	*nir*, FP5$^+$ derivative of PAO1816	(16)
PAO2175	*met-9020 catA1*	*cat* derivative of PAO1834	(16)
PAO2178	*met-9020 catA1 nar-9011*	*nar* derivative of PAO2175	Matsumoto collection
PAO2367	*met-9020 catA1 nar-9011 cnu-9001*	*cnu* derivative of PAO2178	Matsumoto collection

TABLE 1—*Continued*

P. aeruginosa strain	Genotype[a]	Derivation[b]	Reference or source
PAO2371	*met-9020 catA1 nar-9011 cnu-9001 tyu-9026*	*tyu* derivative of PAO2367	Matsumoto collection
PAO2375	*met-9020 catA1 nar-9011 mtu-9002*	*mtu* derivative of PAO2178	Matsumoto collection
PAO2376	*met-9020 catA1 nar-9011 mtu-9002 tyu-9030*	*tyu* derivative of PAO2375	Matsumoto collection
PAO2828	*ser-3 pro-73*	*pro* derivative of PAO2	This paper
GMA075	*his-5075*	*his* derivative of PAO1	(18)
GMA253	*his-5075 cys-5605*	*cys* derivative of GMA075	(18)

[a] Anabolic markers: *arg*, arginine; *aro*, aromatic amino acids; *car*, carbamoyl; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine, valine; *leu*, leucine; *lys*, lysine; *met*, methionine; *phe*, phenylalanine; *pro*, proline; *pur*, purine; *pyr*, pyrimidine; *ser*, serine; *thr*, threonine; and *trp*, tryptophan. Catabolic markers: *ami*, amidase; *arc*, arginine; *cat*, catechol; *chu*, choline; *cnu*, carnosine; *gbu*, guanidinobutyrate; *gpu*, guanidinoproprionate; *hex*, hexose; *hut*, histidine; *mtv*, mannitol; *pca*, protocatechuate; *pob*, *p*-hydroxybenzoate; *put*, proline; *puu*, purine; *tyu*, tyrosine; and *val*, D-valine. Resistance markers: *agl*, aminoglycoside; *chl*, chloramphenicol resistance; *ese*, phage E79; *fpa*, *p*-fluorophenylalanine; *fus*, fusidic acid hypersensitivity; *nal*, nalidixic acid; *rif*, rifampin; *spc*, spectinomycin; *str*, streptomycin. Other markers: *aer*, aeruginocin production; *att*, prophage attachment site; *les*, lysogenic establishment; *nar*, nitrate reductase; *nir*, nitrite reductase; *pho*, alkaline phosphatase; *som*, somatic antigen; *sup*, suppressor activity; *tol*, aeruginocin tolerant; *xcp*, extracellular protease.

[b] Unless otherwise stated, all mutants were obtained after treatment of the parent strain indicated with ethyl methane sulfonate. NG indicates mutagenesis with *N*-methyl-*N'*-nitrosoguanidine; MC indicates mutagenesis with manganous chloride. In cases where the strain has been previously published, further information on details of isolation can be obtained from the cited reference.

TABLE 2. *Closely linked markers of P. aeruginosa PAO*

Marker[a]	Closely linked markers[b]
argA	*arg-127*
argB	*arg-22 arg-18*
argC	*arg-54*
argF	*arg-23 arg-10*
argG	*arg-9*
argH	*arg-32*
hisI	*his-20 his-9004 his-5075*
hisII	*his-4*
ilvB/C	*ilv-220 ilv-226*
ilvD	*ilv-202 ilv-230*
leu-8	*leu-38 leu-1 leu-2*
leu-10	*leu-9001 leu-13 leu-17*
lys-12	*lys-57 lys-58 lys-61 lys-67*
met-9011	*met-9020*
nalA	*nal-23 nal-2 nal-26*
proA	*pro-66 pro-82 pro-73*
proB	*pro-65 pro-64 pro-77 pro-62 pro-63*
pur-70	*pur-64 pur-67 pur-75*
pyrB	*pyr-49*
pyrE	*pyr-70*
pyrF	*pyr-78 pyr-63*
recA	*rec-2 rec-102*
rifA	*rif-1 rif-13 rif-14 rif-15 rif-19*
ser-3	*ser-31*
strA	*str-66 str-2 str-77 str-39*
thr-48	*thr-9001 thr-56*
trpA,B	*trp-61*
trpC,D,E	*trp-6*
tyu-9009	*tyu-9026 tyu-9030*

[a] See Fig. 5.

[b] Closely linked (possibly allelic), independently isolated markers of *P. aeruginosa* PAO. The linkage has been established by prototroph reduction by using the transducing phages F116, F116L, G101, and E79 *tv-1*.

Isolation of *cnu* mutants. van der Drift and Ketelaars (27) found that *P. aeruginosa* had the ability to utilize carnosine (β-alanylhistidine; Sigma Chemical Co.) as either the sole carbon source, the sole nitrogen source, or both. The metabolism of carnosine is carried out initially by carnosinase, a β-alanine-inducible peptidase which hydrolyzes the dipeptide into β-alanine and histidine. Isolation of mutants lacking the ability to utilize carnosine (*cnu*) was carried out by identifying colonies that utilized β-alanine (as the nitrogen source) and histidine (as the carbon source) but not carnosine as the sole source of nitrogen. Lack of the carnosine activity in these mutants was confirmed by assaying fluorometrically the amount of histidine released. Isolation of cnu^+ recombinants was performed on minimal plates containing carnosine as the sole source of nitrogen.

Isolation of *mtu* mutants. Utilization of mannitol by *P. aeruginosa* has been reported by Stanier et al. (24), and a biochemical and genetic study on mannitol-fructose catabolism has been described by Phibbs et al. (22). Mutants unable to utilize mannitol have been classified into four linkage groups by transduction, but chromosomal locations of the genes concerned have not been determined.

Mutants unable to use mannitol as the sole carbon source (*mtu*) were obtained by screening for colonies that could not grow on mannitol but responded to glucose and fructose; such mutants correspond to group I of Phibbs et al. (22). Identification of mtu^+ recombinants was possible by plating on minimal plates with mannitol as the sole source of carbon.

RESULTS

The experimental approach to determine marker orders in the distal region of the PAO map and the relationship of such markers to already located markers at either end of the

current linear map (9) has centered mainly on linkage analysis of recombinants derived from conjugational plate matings, with transduction being used to order closely linked genes. Multiply marked recipient strains carrying a range of different biosynthetic and catabolic markers have been crossed with donor strains carrying separately conjugative plasmids FP2, FP5, FP110, and R68.45.

By using the data from the existing map (9), a number of markers whose location was known were selected, and these were used to locate the relative map positions of previously unmapped loci.

As multiply marked strains have been built up, independent isolations of various markers have been made. The relatedness of these markers was determined by transductional prototroph reduction tests using either F116L or G101. The actual data are not shown, but Table 2 relates the markers whose location is shown on the map in Fig. 5 to other isolates of closely linked markers in different strains.

***pur-136–met-9011* region.** The results of two-factor crosses in the *pur-136–met-9011* region are shown in Fig. 1. By using markers either closely linked or allelic with those shown in Fig. 1, it has been possible to define in more detail the linkage relationships of this region by transduction. With bacteriophage F116L, *thr-48* (= *thr-9001*) and *pur-66* were 29% cotransducible, *pur-66* and *leu-8* (= *leu-38*) were 40% cotransducible, and *leu-8* and *thr-48* were not cotransducible. It can be concluded that the order of these markers is *leu-8–pur-66–thr-48*, but the orientation of these three markers with respect to the FP2 origin cannot be determined at this time. With F116L, *leu-9001* (= *leu-38*) and *met-9011* were 41% cotransducible. By means of three-factor crosses with R68.45 donors, R. Crockett (personal communication) has shown that *leu-9001* is proximal to *met-9011* with respect to the FP2 origin.

To confirm the order of the markers *proA66–thr-9001–argF23–leu-9001*, three-factor, double-selection crosses with an FP5$^+$ donor were made. The results (Table 3) show that 92% of *proA66*$^+$ *argF32*$^+$ recombinants have coinherited the *thr-9001*$^+$ allele, and 95% of the *thr-9001*$^+$ *leu-9001*$^+$ recombinants have coinherited the *argF23*$^+$ allele, confirming the order shown in Fig. 5.

An apparent anomaly (Fig. 1) arose when selection was made separately for *leu-9001*$^+$ and *met-9011*$^+$ recombinants with FP2$^+$ and FP5

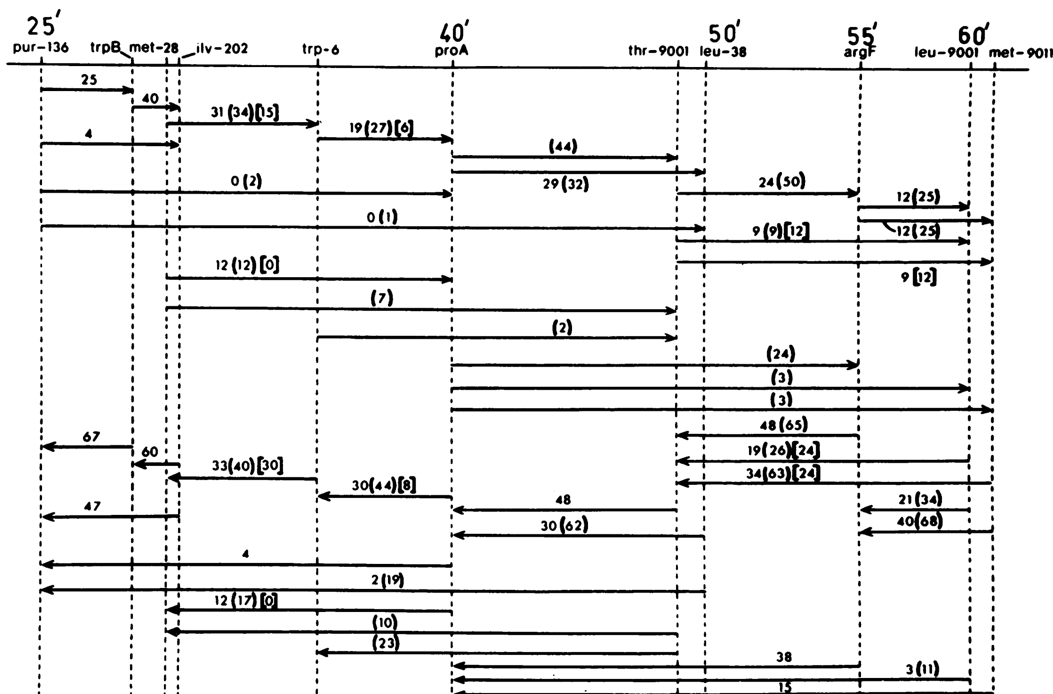

FIG. 1. *Linkage values in FP2 (unbracketed), FP5 (), and R68.45[] matings for markers in the 25- to 60-min region. Linkage values are expressed as the percentage of selected recombinants which have coinherited the unselected marker. Arrowheads indicate the unselected markers. The figure is not drawn to linear scale. The numbers above the marker symbols refer to the map locations in Fig. 5.*

donors, but not with R68.45 donors. *leu-9001* and *met-9011* were closely linked (41% cotransducible with F116L), but they showed significantly different linkage values to both *argF* and *thr-9001* when the former two markers were selected and the latter two markers were unselected. In the reciprocal situation in which *argF*$^+$ or *thr-9001*$^+$ was the selected marker, the coinheritance of the unselected markers *leu-9001*$^+$ and *met-9011*$^+$ was much the same. It is possible that the anomaly may arise due to some undetected marker in the recipient strain PAO140 or one of its ancestors which resulted in failure to isolate some recombinant types.

***thr-9001-pyrF* region.** R. Crockett (personal communication) has established the marker order *argF-leu-9001-met-9011-pyrD-pyrF* by using two- and three-factor matings with R68.45 donors. Additional linkage data for this region of the chromosome from plate matings using the donor PAO325 (FP2) and the recipients PAO166, PAO136, and PAO388, together with above mentioned data obtained with R68.45, are shown in Fig. 2.

***met-9011-cnu-9001* region.** The marker order *met-9011-catA1-tyu-9009-nar-9011-puuC-puuD-puuE-puuF* has been established (16). A new catabolic marker, *mtu-9002* (inability to use mannitol as the sole carbon source), has been mapped relative to the other markers in PAO2376. The coinheritance data (Fig. 3) are consistent with *mtu-9002* being located between *catA1* and *tyu-9030*. The data from double selection with R68.45 and FP5 donors confirm the proposed order (Table 4). The coinheritance values (Fig. 2 and 3) show that *pyrF* is more

TABLE 3. *Segregation of markers with double selection to confirm marker orders in the 40- to 60-min region*[a]

Selected markers	Respective map locations[b]	Recombination frequency[c]	% Coinheritance of unselected marker			
			proA$^+$	*thr*$^+$	*argF*$^+$	*leu*$^+$
thr-9001$^+$ *leu-9001*$^+$	48, 60	130	66		95	
proA66$^+$ *argF23*$^+$	40, 55	280		92		19

[a] Plate matings of the cross PAO2152(FP5) × PAO140 were made with selection for two markers; 100 recombinants for each selected marker pair were scored for coinheritance of unselected chromosomal markers by replica plating.

[b] Approximate minutes from FP2 origin.

[c] Per 10^8 donor cells.

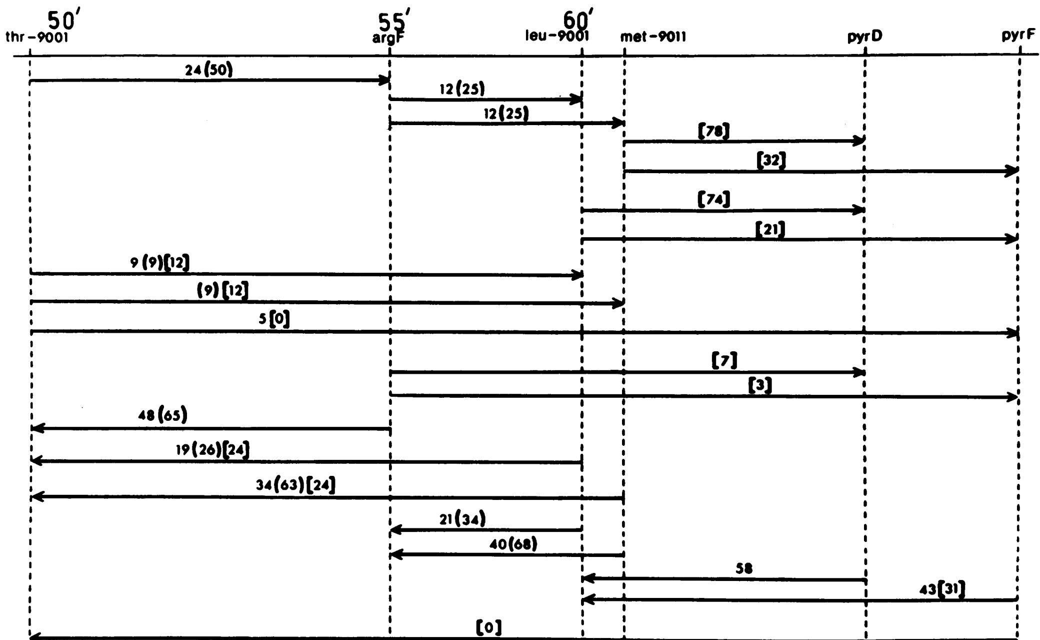

FIG. 2. *Linkage values for the thr-9001-pyrF region. Other details are as in the legend to Fig. 1.*

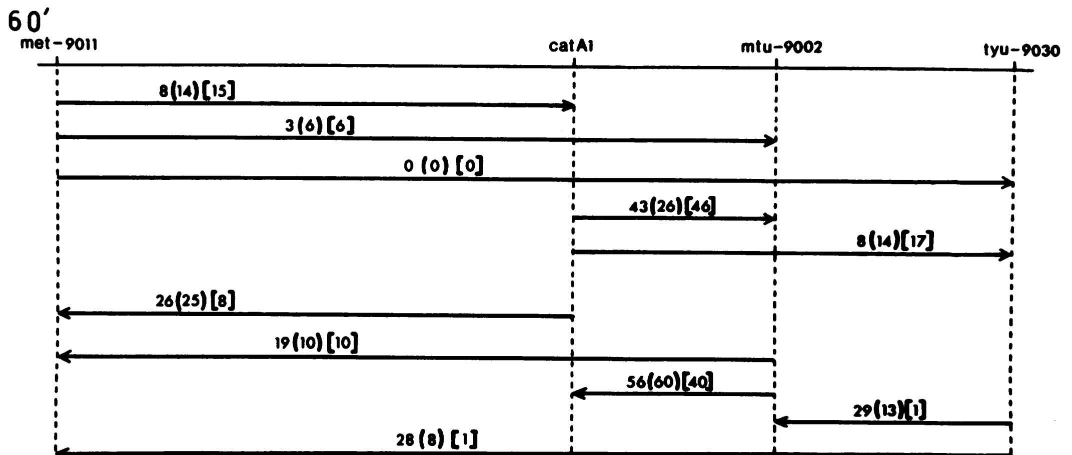

FIG. 3. *Linkage values for the met-9001-tyu-9030 region. Other details are as in the legend to Fig. 1.*

TABLE 4. *Double-selection experiments using an FP5⁺ and an R68.45⁺ donor with the recipient PAO2376 to determine marker orders in met-9011-tyu-9030 region of the PAO map*[a]

Cross	Selected markers	Recombination frequency[b]	% Coinheritance of unselected markers	
			catA⁺	*mtu-9002*⁺
PAO8[c] (R68.45) × PAO2376	*met-9011*⁺ *catA1*⁺	150		55
	met-9011⁺ *mtu-9002*⁺	40	99	
	met-9011⁺ *tyu-9030*⁺	40	73	73
PAO2152[d] (FP5) × PAO2376	*met-9011*⁺ *mtu-9002*⁺	160	84	
	met-9011⁺ *tyu-9030*⁺	100	79	80

[a] Plate matings were done by the standard technique with simultaneous selection for two markers; 100 recombinants for each selected marker were scored for coinheritance of unselected markers.
[b] Per 10^8 donor cells.
[c] Donor contraselective marker, *ilv-202* (30 min).
[d] Donor contraselective marker, *his-9004* (12 min).

closely linked to *met-9011* than is *catA1* to *met-9011*, thus making the marker order *met-9011–pyrD–pyrF–catA1*. However, this order still needs to be confirmed with three-factor crosses.

Mutants lacking carnosinase (and unable to grow on carnosine as the sole carbon and nitrogen source; these nutrients were provided by histidine and β-alanine, respectively) were isolated. *cnu-9001* was found to be located between *puuE* and *puuF* by G101 transduction. *puuE* and *cnu-9001* were 15% cotransducible, and *cnu-9001* and *puuF* were 36% cotransducible, whereas *puuE* and *puuF* were not cotransducible. It has been established that *puuE* and *puuF* are distal to *tyu-9030* (16); hence, *cnu-9001* is also distal to *tyu-9030*. The linkage between *tyu-9030* and *cnu-9001* is shown in Fig. 4.

Isolation of other markers distal to *tyu-9009*. Matings with R68.45 have failed to show any coinheritance of the markers *tyu-9030*, *cnu-9001*, or *pyrF* with the "early" marker *proB*, thus preventing any demonstration of chromosomal genetic circularity. It should be pointed out that not all combinations of markers can be studied in this way, because degradative markers (such as *tyu* or *cnu*) cannot be effectively selected if certain amino acids are present in the media to support the growth of particular auxotrophic mutants.

A search was made for new auxotrophic markers in the region distal to *tyu*. Studies with *P. aeruginosa* PAT (30) had shown the existence of an adenine-requiring mutant which mapped in this region (*pur-4*), and a variety of adenine-requiring mutants of *P. aeruginosa* PAO were examined to see whether any mapped in the region between *tyu-9009* and *proB*. An aeruginocinogenic tolerant mutant (*tolB*) was isolated which had undergone concomitant mutation at a locus which resulted in a requirement for adenine; this locus was denoted *pur-70*. Preliminary evidence was obtained which located *pur-70* in the region between *met-9011* and the FP2 origin (C. Crowther and B. W. Holloway, unpublished data). A range of adenine-requiring mutants was then isolated in other already

marked strains and shown to be closely linked to *pur-70* by prototroph reduction. These included *pur-64* (in PAO349, kindly provided by C. Crowther) and *pur-67* in PAO1772.

FP110 (23) is a plasmid with Cma which has a major origin of transfer close to *pur-136* and transfers chromosome from this origin in a direction opposite to that of FP2. The Cma properties of FP110 will be described in a separate paper (Royle and Holloway, manuscript in preparation). The results of the mating PAO2(FP110) × PAO349 (Table 5) indicated that *pur-64* is located later than *ilv-226* with respect to the FP110 origin. A recombinant analysis of the cross PAO325(R68.45) × PAO1777 (Table 6) confirms this location of *pur-67* (an independent adenine-requiring isolate closely linked to *pur-64* by prototroph reduction) as being close to the FP2 origin, but in view of the very low recovery of both *pur-67* and *pur-64* in FP2 matings, the locus in question probably does not lie between the FP2 origin and *proB*. From the table of R68.45 linkage values versus map distance (6), it can be suggested that *pur-67* and *ilv-220* map approximately 12 min apart, placing *pur-67* about 4 min distal to the FP2

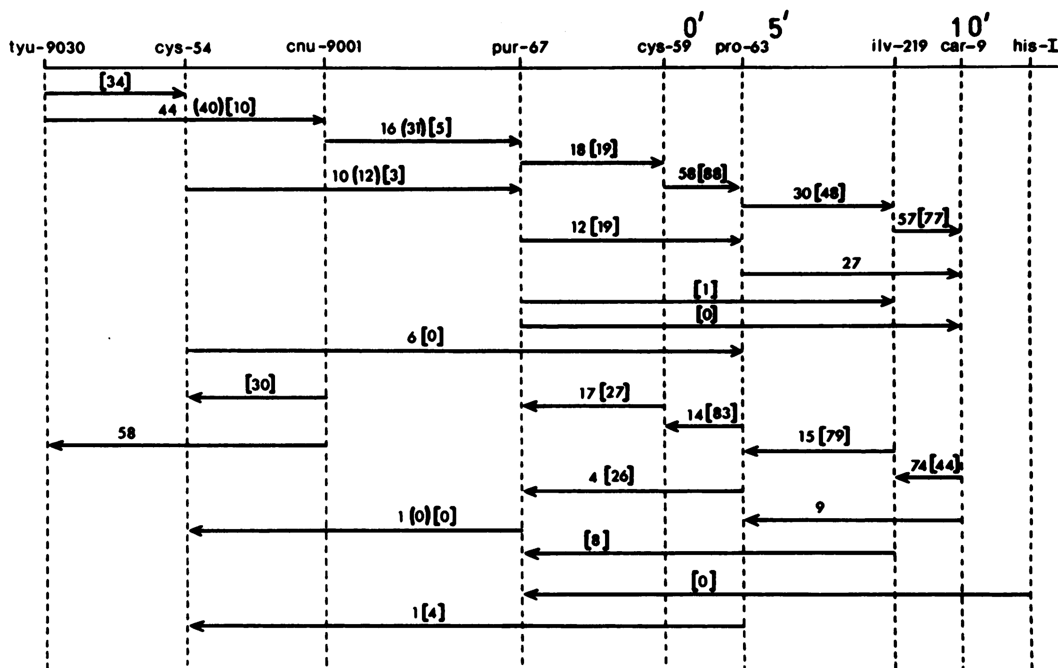

FIG. 4. *Linkage values for the tyu-9030–hisI region. Other details are as in the legend to Fig. 1.*

TABLE 5. *Recombinant analysis of a strain containing a range of chromosomal markers by using an FP110 donor to investigate the map position of the pur-64 marker*[a]

Selected marker	Map location[b]	Recombination frequency[c]	% Coinheritance of unselected markers						
			ilv$^+$	*his*$^+$	*lys*$^+$	*met*$^+$	*trp*$^+$	*pro*$^+$	*pur*$^+$
ilv-226$^+$	8	17,400		58	50	0	0	0	0
his-4$^+$	16	29,700	20		80	0	0	0	1
lys-12$^+$	20	55,900	12	58		0	0	0	1
met-28$^+$	30	40	0	0	0		0	5	0
trp-6$^+$	35	70	0	0	0	16		12	0
pro-82$^+$	40	160	0	0	0	2	27		0
pur-64$^+$		1,110	66	43	32	0	0	0	
pur-64$^+$*-his-4*$^+$		22	98		88	0	0	0	

[a] Plate mating was done by the standard technique; 100 recombinants for each selected marker were scored for coinheritance of unselected chromosomal markers by replica plating. The donor contraselective marker was *ser-3* (ca. 30 min).

[b] Minutes from the FP2 origin.

[c] Per 10^8 donor cells.

origin on the PAO map. *pur-67* and *pur-64* are very likely equivalent to *pur-4* of *P. aeruginosa* PAT.

Attempts to obtain coinheritance of *pur-67* and *leu-9001* in PAO by using R68.45 or FP2 were unsuccessful; as a consequence, attempts were made to isolate new auxotrophs between *pyrF* and *pur-67*. A range of auxotrophs were isolated from PAO901 and tested for linkage to *pur-67* by means of R68.45 matings. Two independently isolated and different *cys* mutants were obtained; both showed linkage to *pur-67*.

Mapping of *cys-54* and *cys-59*. The isolation of the cysteine-requiring mutants *cys-54* and *cys-59* was fortunate, because cysteine is one of the few amino acids which cannot be used by *P. aeruginosa* as the sole carbon source. Hence, selection could be made for cnu^+ recombinants with cysteine incorporated in the medium. The linkage of *cys-54* to *cnu-9001* was tested in recombinants from the cross PAO1039(R68.45) × PAO947 with selection for $cnu\text{-}9001^+$, and *cys-54* and *cnu-9001* were found to be 30% linked.

Linkage of *cys-54* and *tyu-9030* was demonstrated by the cross PAO948 × PAO1039-(R68.45). Selecting for $tyu\text{-}9030^+$ resulted in 34% coinheritance of *cys-54*. As the mating PAO8(R68.45) × PAO2371 indicated that *cnu-9001* and *tyu-9030* are 10% linked, the marker order must be *tyu-9030–cys-54–cnu-9001*.

Linkage between *cnu-9001* and *pur-67* was determined in R68.45, FP2, and FP5 matings (Fig. 4), indicating the order *tyu-9030–cys-54–cnu-9001–pur-67*. That *pur-67* is distal to *cys-54* was confirmed by the mating PAO8(R68.45) × PAO1035, in which double selection for $cys\text{-}54^+$ $pro\text{-}63^+$ recombinants showed a 99% unselected coinheritance of $pur\text{-}67^+$ (Table 7).

The location of *cys-59* was determined first in two-factor crosses (Fig. 4). That *cys-59* was located between *pur-67* and *proB* was confirmed when $pur\text{-}67^+$ $pro\text{-}65^+$ double recombinants from the cross PAO8(R68.45) × PAO1042 (Table 7) showed a 99% unselected coinheritance of $cys\text{-}59^+$. Figure 4 shows that all markers from *tyu-9030* to *hisI* can be linked by two-factor crosses with FP2, FP5, or R68.45; from this evidence, together with evidence from the three-factor crosses described above and the evidence for linkage in other regions of the chromosome provided here or in previous papers (5, 7, 9, 20, 25), it may be concluded that all of the markers examined are located on a single circular linkage group (Fig. 5). Haas and Holloway (5) have shown that it is possible to estimate distances between markers with R68.45 plate matings, ex-

TABLE 6. *Recombinant analysis of a strain containing a range of chromosomal markers, using an R68.45 donor to investigate the map position of the pur-67 marker*[a]

Selected marker	Map location[b]	Recombination frequency[c]	% Coinheritance of unselected markers				
			ilv^+	thr^+	leu^+	met^+	pur^+
$ilv\text{-}220^+$	8	1,170		0	0	0	8
$thr\text{-}9001^+$	ca. 48	2,571	0		12	12	0
$leu\text{-}9001^+$	ca. 60	2,142	0	24		86	0
$met\text{-}9011^+$	ca. 60	2,340	0	24	100		1
$pur\text{-}67^+$		125	1	0	0	0	

[a] Plate mating of the cross PAO325(R68.45) × PAO1777 was done by the standard technique; 100 recombinants for each selected marker were scored for coinheritance of unselected chromosomal markers by replica platings. The donor contraselective markers were *argB18* and *lys-57* (20 min).

[b] Minutes from the FP2 origin.

[c] Per 10^8 donor cells.

TABLE 7. *Double-selection experiments using R68.45 with recipients PAO1035 and PAO1042 to determine the marker order in the cys-54-pro-63 region of the PAO map*[a]

Cross	Selected markers	Recombination frequency[b]	% coinheritance of unselected markers	
			$pur\text{-}67^+$	$cys\text{-}54^+$
PAO8(R68.45) × PAO1035	$cys\text{-}54^+$ $pro\text{-}63^+$	30	99	
	$pur\text{-}67^+$ $pro\text{-}63^+$	10		0
PAO8(R68.45) × PAO1042	$pro\text{-}65^+$ $cys\text{-}59^+$	4,000	45	
	$pro\text{-}65^+$ $pur\text{-}67^+$	95		99

[a] Plate matings were done by the standard technique with simultaneous selection for two markers; 100 recombinants for each selected marker were scored for coinheritance of unselected markers. Donor contraselective markers were *met-28* (30 min) and *ilv-202* (30 min).

[b] Per 10^8 donor cells.

FIG. 5. *Chromosome map of P. aeruginosa PAO. The following symbols have been adopted. (i) Markers whose location is indicated by a bar joining the locus abbreviation to the map were located by interrupted matings with FP2 donors. (ii) Round brackets indicate that the markers are cotransducible with one or more of the bacteriophages F116, F116L, G101, and E79 tv-1. (iii) The marker abbreviation is underlined in those cases where there is evidence to locate the marker in the area in which the symbol is placed, but the relationships to flanking markers have not been determined. Marker abbreviations are listed in footnote a of Table 1.*

pressed in terms of minutes of chromosome as measured in interrupted FP2 matings. On this basis and from the data given in Fig. 1 to 4, a preliminary estimate of the length of the *P. aeruginosa* PAO chromosome is 105 min.

FP2 has additional origins of chromosome transfer. If FP2 transfers chromosome from one origin and only in one direction, then markers situated counterclockwise from 0 min should show very minimal recovery values in FP2 matings, and these markers should show little linkage with early markers. The coinheritance values of *cys-54* and *pro-63* (6%) and *pur-67* and *pro-63* (12%) in $FP2^+$ matings (Fig. 4) are not consistent with the view of only one origin and one direction of transfer. Experiments have

shown that FP2 can also transfer chromosome in the direction opposite to that previously determined.

An interrupted mating between PAO325(FP2) and PAO1052 was carried out by using nalidixic acid as the interrupting agent. Figure 6 shows the time-of-entry curves; although the nonlinearity of the curves makes it difficult to accurately determine entry times, there is no question that *cys-59* and *pur-67* entered at times other than would be expected with a single origin. The reason for the earlier than expected entry time for *pro-65* (ca. 2 min as compared with 4 to 5 min [5]) may be due to strain differences, as has been shown to occur in *Escherichia coli* (1). The discovery of an additional origin (or origins) of transfer by FP2 in no way affects the validity of the linkage data reported for FP2 in this or other papers. The major origin, in terms of frequency of transfer, accounts for the majority of recombinants produced in matings with FP2, and the other sites of transfer are minor and less frequent.

DISCUSSION

Evidence for the genetic circularity of the *P. aeruginosa* PAO chromosome has been derived from plate mating linkage data with Cma plasmids FP2, FP5, FP110, and R68.45 and from interrupted matings with FP2. Similar approaches to the establishment of genetic circularity have been successful in other bacteria, including *Rhizobium leguminosarum* (2), *Rhizobium meliloti* (13), *Acinetobacter calcoaceticus* (26), and *Proteus mirabilis* (3).

Further work is needed to precisely determine the length of the chromosome in the time units possible for the first 50 min; this information could come from work in progress in which IncP-1 plasmids have been integrated into the chromosome with the formation of donors having properties similar to *E. coli* Hfr strains (M. Sinclair, C. Crowther, N. Holmes, and B. Holloway, unpublished data).

With nearly 100 genes mapped, some general features of the *P. aeruginosa* chromosome can be discussed. In the region clockwise between 60 min and the FP2 origin, there are few auxotrophic markers, but apparently there are a number of markers affecting the dissimilation of a variety of substrates. In view of the fact that most FP plasmids have a major origin of transfer close to that of FP2 (4, 21; Royle, unpublished data), and that the ability of such plasmids to

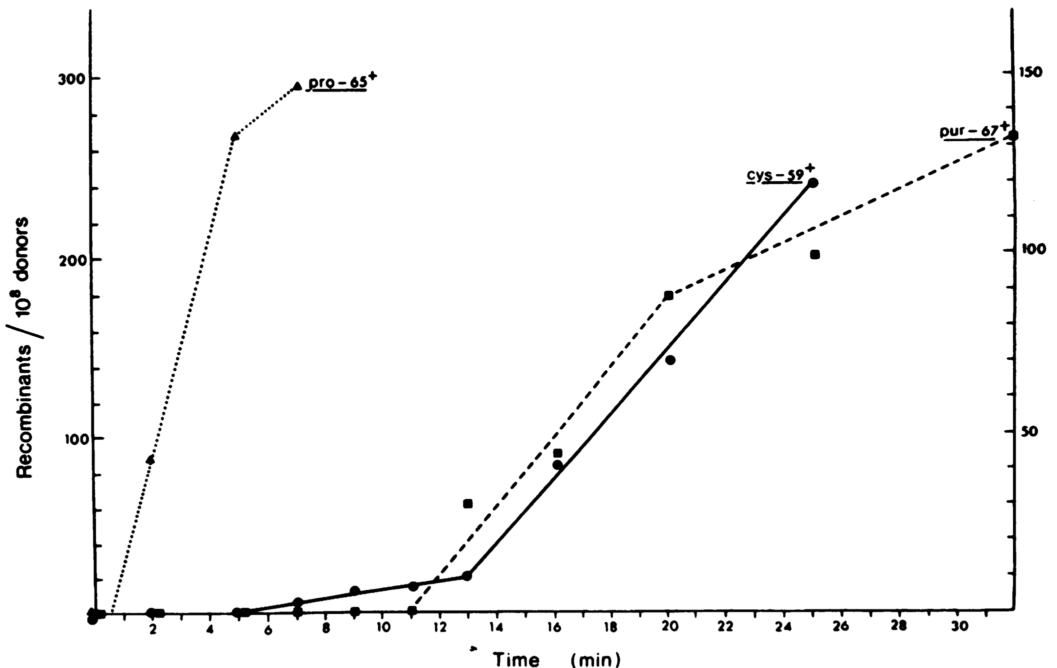

FIG. 6. *Interrupted mating was carried out with a stationary-phase recipient, PAO1052 (pur-67 pro-65 thr-9001 cys-59 nal-26), and an exponential-phase donor, PAO325(FP2) (argB18 lys-57). Donor and recipient were mated in liquid as described in the text, and at different times nalidixic acid was added to samples of the mating mixture to give a final concentration of 500 μg/ml. Samples of 0.2 ml each were plated on selective media containing 1,000 μg of nalidixic acid per ml. Symbols:* ▲, *pro-65*$^+$ *(left ordinate);* ●, *cys-59*$^+$ *and* ■, *pur-67*$^+$ *(right ordinate).*

transfer the region of the chromosome clockwise between 60 min and the FP2 origin is very low even with secondary origins as has been shown above, this region of the chromosome would tend to be conserved compared with the 0- to 30-min region which can be readily transferred in conjugation by many FP plasmids. As the gene distribution on the chromosome of *P. aeruginosa* becomes better known, it will be interesting to attempt to define the selective forces which could maintain the patterns of gene arrangement found in the chromosome.

ACKNOWLEDGMENTS

This work was supported by the Australian Research Grants Committee. P.L.R. was the recipient of a Commonwealth Postgraduate Scholarship.

LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116-167.
2. **Beringer, J. E., S. A. Hoggan, and A. W. B. Johnston.** 1978. Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J. Gen. Microbiol. **104**:201-207.
3. **Coetzee, J. N.** 1979. Genetic circularity of the *Proteus mirabilis* linkage map. J. Gen. Microbiol. **110**:171-176.
4. **Dean, H. F., P. Royle, and A. F. Morgan.** 1979. Detection of FP plasmids in hospital isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **138**:249-250.
5. **Haas, D., and B. W. Holloway.** 1976. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **144**:243-251.
6. **Haas, D., and B. W. Holloway.** 1978. Chromosome mobilization by the R plasmid R68.45: a tool in *Pseudomonas* genetics. Mol. Gen. Genet. **158**:229-237.
7. **Haas, D., B. W. Holloway, A. Schamböck, and T. Leisinger.** 1977. The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **154**:7-22.
8. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33**:419-443.
9. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43**:73-102.
10. **Holloway, B. W., and P. van de Putte.** 1968. Lysogeny and bacterial recombination, p. 175-183. *In* W. J. Peacock and R. D. Brock (ed.), Replication and recombination of genetic material. Australian Academy of Science, Canberra.
11. **Isaac, J. H., and B. W. Holloway.** 1968. Control of pyrimidine biosynthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **96**:1732-1741.
12. **Isaac, J. H., and B. W. Holloway.** 1972. Control of arginine biosynthesis in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **73**:427-438.
13. **Kondorosi, A., G. B. Kiss, T. Forrai, E. Vincze, and Z. Banfalvi.** 1977. Circular linkage map of *Rhizobium meliloti* chromosome. Nature (London) **268**:525-527.
14. **Krishnapillai, V.** 1971. A novel transducing phage:its role in recognition of a possible new host-controlled modification system in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **114**:134-143.
15. **Leisinger, T., D. Haas, and M. P. Hegarty.** 1972. Indospicine as an arginine antagonist in *Escherichia coli* and *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **262**:214-219.
16. **Matsumoto, H., S. Ohta, R. Kobayashi, and Y. Terawaki.** 1978. Chromosomal location of genes participating in the degradation of purines in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **167**:165-176.
17. **Matsumoto, H., and T. Tazaki.** 1973. FP5 factor, an undescribed sex factor of *Pseudomonas aeruginosa*. Jpn. J. Microbiol. **17**:409-417.
18. **Mee, B. J., and B. T. O. Lee.** 1969. A map order for *hisI*, one of the genetic regions controlling histidine biosynthesis in *Pseudomonas aeruginosa*, using the transducing phage F116. Genetics **62**:687-696.
19. **Morgan, A. F.** 1979. Transduction of *Pseudomonas aeruginosa* with a mutant of bacteriophage E79. J. Bacteriol. **139**:137-140.
20. **Pemberton, J. M., and B. W. Holloway.** 1972. Chromosome mapping in *Pseudomonas aeruginosa*. Genet. Res. **19**:251-260.
21. **Pemberton, J. M., and B. W. Holloway.** 1973. A new sex factor of *Pseudomonas aeruginosa*. Genet. Res. **21**: 263-272.
22. **Phibbs, P. V., S. M. McCowan, T. W. Feary, and W. T. Blevins.** 1978. Mannitol and fructose catabolite pathways of *Pseudomonas aeruginosa* carbohydrate negative mutants and pleiotropic effects of certain enzyme deficiencies. J. Bacteriol. **133**:717-728.
23. **Royle, P. L., and B. W. Holloway.** 1980. Relationship between R and FP plasmids in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **17**:293-297.
24. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159-271.
25. **Stanisich, V. A., and B. W. Holloway.** 1969. Conjugation in *Pseudomonas aeruginosa*. Genetics **61**:327-339.
26. **Towner, K. J., and A. Vivian.** 1977. Plasmids capable of transfer and chromosome mobilization in *Acinetobacter calcoaceticus*. J. Gen. Microbiol. **101**:167-171.
27. **van der Drift, C., and H. C. J. Ketelaars.** 1974. Carnosinase: its presence in *Pseudomonas aeruginosa*. Antonie van Leeuwenhoek J. Microbiol. Serol. **40**:377-384.
28. **Waltho, J. A.** 1972. Genetic analysis of phenylalanine-responding mutants of *Pseudomonas aeruginosa*. J. Bacteriol. **112**:1070-1075.
29. **Watson, J. M., and B. W. Holloway.** 1976. Suppressor mutations in *Pseudomonas aeruginosa*. J. Bacteriol. **125**:780-786.
30. **Watson, J. M., and B. W. Holloway.** 1978. Chromosome mapping in *Pseudomonas aeruginosa* PAT. J. Bacteriol. **133**:1113-1125.
31. **Watson, J. M., and B. W. Holloway.** 1978. Linkage map of *Pseudomonas aeruginosa* PAT. J. Bacteriol. **136**: 507-521.