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Ordering of the Flagellar Genes in *Pseudomonas aeruginosa* by Insertions of Mercury Transposon Tn*501*

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The flagellar genes of *Pseudomonas aeruginosa* PAO cluster on the chromosome at two distinct regions, region I and region II. The order of the flagellar cistrons in this organism was established by using transducing phage G101 and plasmids FP5 and R68.45. A method to insert transposon Tn*501* near the *fla* genes was devised. We obtained two strains in which Tn*501* was inserted at sites close to the flagellar cistrons in region II. We isolated Fla$^-$ mutants in which the chromosomal segment between the two Tn*501* insertion sites was deleted. Using Tn*501*-encoded mercury resistance as an outside marker, we determined the order of 9 of the 11 flagellar cistrons in region II as follows: *puuF*-region I-*flaG-flaC-flaI-flaH-flaD-flaB-flaA-flaF-flaE-pur-67*. By using phage G101-mediated transduction, the mutation converting monoflagellated bacteria into the multiflagellated (*mfl*) form was closely linked to the five *fla* cistrons in region I. Using *mfl* as an outside marker, we determined the order of the five cistrons as follows: *puuF-flaV-flaZ-flaW-flaX-flaY*-region II. The *mfl* mutation was shown to be either located within the *flaV* cistron or linked very closely to this cistron. No linkage was observed in transductions between any of the *fla* cistrons in region I and any of the *fla* cistrons in region II.

Each *Pseudomonas aeruginosa* PAO cell possesses a single flagellum at one pole (2). We have been interested in the flagellation of this organism. Why does each cell not possess a the single flagellum protruding from the lateral side of the cell surface, and why does it not possess multiple flagella at one pole?

Suzuki and Iino (28) isolated multiflagellate (*mfl*) mutants from a monotrichously flagellated parent and mapped the *mfl* mutation in a *fla* gene cluster. However, these authors could not identify the cistron responsible for the Mfl$^-$ phenotype since information concerning the *fla* genes was not available at that time. A more detailed analysis of flagellar formation and functions has been desired for elucidation of the control mechanism(s) involved in the flagellation of *P. aeruginosa*.

Previously (29, 30), we showed that the flagellar genes of this organism are clustered in two distinct regions (region I and region II), which are located in the very late region of the linkage map of the chromosome (12, 23), with an order of *puuF*-region I-region II-*oruI*. Neither region was cotransducible with *puuF* or *oruI* (30)(Fig. 1). One *mot* and five *fla* cistrons were identified in region I, and ten *fla* and two *che* cistrons (one of which was presumed to be identical to an *fla* cistron) were identified in region II. Some of the functions of these cistrons were inferred from the mutant phenotypes which they exhibited (29).

In this paper, we report the order of the flagellar cistrons in these regions. An *mfl* mutation was found to be linked to the region I *fla* cistrons. Therefore, the *mfl* mutation was used as a marker for determining the order of the *fla* cistrons in region I. Since selective markers not concerned with flagellar formation and functions were not available for this region of the chromosome, we inserted Tn*501*, which confers resistance to mercuric ions (1, 27), near the *fla* genes. Using chromosomally inserted Tn*501*, we determined the order of most of the flagellar cistrons in region II.

MATERIALS AND METHODS

Strains. All of the bacterial strains used in this study were derivatives of *P. aeruginosa* PAO (10). Together with the plasmids used, these strains are listed in Table 1. To determine the order of the flagellar cistrons, we used primarily the flagellar mutants derived from strain MT350 (29). E79 *tv-1* (20), F116L (16), G101 (13), and G101 *h2* (4) were used as the transducing phages.

Media. L broth, L-broth agar, nutrient gelatin agar, nutrient agar, nutrient broth top agar, and minimal media were used as described previously (29, 30). The soft agar used for examining the multiflagellate mutation has also been described previously (28). Antibiotics were added to the selection media at the following

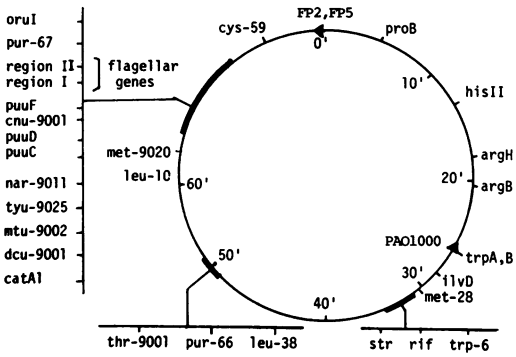

FIG. 1. Chromosome map of *P. aeruginosa* PAO showing the positions of the relevant markers. The gene symbols are described in Table 1, footnote *a*. The map distance between *leu-10* and the FP5 origin is arbitrary.

concentrations: kanamycin, 250 μg/ml; tetracycline, 200 μg/ml; carbenicillin, 200 μg/ml; spectinomycin, 1,000 μg/ml; rifampin, 200 μg/ml; streptomycin, 500 μg/ml; and sulfonamide, 1,000 μg/ml. $HgCl_2$ was added at a concentration of 15 μg/ml.

Isolation of mutants. Spontaneous mutants resistant to streptomycin, rifampin, or spectinomycin were isolated by spreading 5×10^9 cells onto an L-broth agar plate containing the corresponding antibiotic. The flagellar mutants were isolated by the procedure described previously (30).

Conjugation and R-prime plasmid construction. FP5- and R68.45-mediated conjugations were carried out as described previously (29). An R*pur* plasmid, pMT101, was constructed by patch mating (11, 30) of MT1100(R68.45) and MT2505; 10-μl portions of the donor and recipient cultures were spotted at the same place on an L-broth agar plate, which was incubated overnight at 37°C. The cells grown on the plate were suspended in saline (0.8% [wt/vol] NaCl; 10^9 cells per ml) and were plated onto minimal glucose medium containing cysteine, proline, tetracycline, and kanamycin.

Transduction. F116L-, G101-, or G101 *h2*-mediated transduction was carried out at a multiplicity of infection of 5, and E79 *tv-1*-mediated transduction was carried out at a multiplicity of infection of 1. Detailed procedures have been described previously (29, 30). When E79 *tv-1* was used as the transducing phage, recipient cells carrying plasmid R38 were used to prevent multiplication of this phage (20).

Insertion of Tn*501* near the *fla* genes. Two plasmids, pMT6 and pVS1 carrying Tn*501*, were introduced into Rec^- Fla^- strain MT506 Spc^r. The resulting strain was cultivated overnight at 37°C in L broth containing kanamycin and then mated with MT506 Rif^r, and the transconjugants were selected on L-broth agar plates containing rifampin, tetracycline, and $HgCl_2$. More than 3,000 of the resulting colonies were pooled without purification and grown in L broth containing kanamycin. This culture was then mated with MT506 Spc^r and plated onto L-broth agar plates containing spectinomycin, tetracycline, and $HgCl_2$. More than 3,000 of the resulting transconjugants were again pooled without purification and grown. Using this pooled culture, we prepared a G101 *h2* lysate. This lysate was mixed with cells of various flagellar mutants in region II as the recipients for transduction, and the mixtures were inoculated as lines on chilled nutrient gelatin agar plates containing $HgCl_2$. The motile Hg^r transductants appeared at a frequency of 10^{-9}/PFU and were examined for resistance to sulfonamide. Then the linkage of *fla* with the Hg^r determinant in these Su^S clones was examined by transduction or R68.45-mediated conjugation. A similar procedure was carried out with pMT19, pVS1, MT519, and the flagellar mutants in region I.

The procedure used to isolate *leu-8001*::Tn*501* (in strain MT2090) and *flaE2052*::Tn*501* (in strain MT2052) mutations will be described elsewhere.

RESULTS

Linkage of *mfl-110* with the flagellar cistrons. We examined the linkage of *mfl-110* with each of the flagellar cistrons identified in a previous study (29). Transduction with G101 was carried out from an *mfl-110* strain (strain MT903) to each of the flagellar mutants. Motile transductants were selected on nutrient gelatin agar plates, and cotransduction of *mfl-110* was examined (Table 2). The five *fla* cistrons in region I exhibited high linkage values (>70%) with the *mfl-110* mutation, and no linkage was observed between *mfl-110* and any of the known flagellar cistrons in region II.

Determining the order of the flagellar cistrons in region I. To determine the order of the five *fla* cistrons in region I, we first carried out three-factor conjugation experiments, in which we combined an *fla* (FP5) donor derived from strain MT903 and an *fla cnu-9001* recipient derived from strain MT350 (Tables 3 and 4 and Fig. 2). In the cross between *flaX* (FP5) and *flaZ cnu-9001*, approximately 70% of the Fla^+ transconjugants inherited the donor cnu^+ marker. In the reciprocal cross between *flaZ* (FP5) and *flaX cnu-9001*, none of the Fla^+ transconjugants received the donor cnu^+ marker. Hence, the order must be *cnu-9001-flaZ-flaX* (Table 3 and Fig. 2). Furthermore, our results suggested that *flaV* was located closer to *cnu-9001* than *flaZ* was, since many of the Fla^+ transconjugants resulting from the cross between *flaZ* (FP5) and *flaV cnu-9001* inherited the donor cnu^+ marker (Table 4). Similarly (Table 4), the following order was suggested by our conjugation experiments: *cnu-9001-flaV-flaZ-(flaX-flaY)* (the order of the cistrons in parentheses could not be determined in these conjugation experiments).

Further experiments for establishing the order of the five *fla* cistrons and the *mfl-110* mutation site were performed by three-factor transductional crosses in which *mfl-110 fla* donors and *fla* recipients were used (Table 5). The *mfl-110* mutation site was determined as follows. In a

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant genotype and/or phenotype[a]	Construction, source, or reference(s)
Strains		
PAO18	*pur-66 proB64*	16
PAO1000	*trpA,B*::pME319 *his-301 str-1*[b]	9
PAO1042	*pur-67 thr-9001 cys-59 proB65*	23
PAO3385	*his-308 ilvD309 trp-6 rec-301*[b]	M. Kageyama
PJ110	*leu-38 ade-301 str-302 mfl-110* FP2$^+$	28
MT315	*flaZ315* derivative of MT318	30
MT318	*oruI325 puuC10 tyu-9018 ben-9010 rif-8002*[b]	30
MT350	*met-9020 catA1 nar-9011 cnu-9001 puuE8 tyu-9025 rif-8003*	30
MT506	*argH32 rec-2 str-39 flaI506*	30
MT519	*argH32 rec-2 str-39 mot-519*	30
MT903	*met-28 ilv-202 met-9020 catA1 nar-9011 rif-8005 mfl-110*	PJ110 × MT1201
MT1100	*argB18 chl-2 rif-8001*	30
MT1200	*met-28 ilv-202 met-9020 catA1 nar-9011 cnu-9001 rif-8005*	30
MT1201	*flaI1201* derivative of MT1200	29
MT1501	*chr-1055*::Tn*501* derivative of MT1100[c]	This study
MT1502	*chr-1061*::Tn*501* derivative of MT1100[c]	This study
MT1551	*flaH1054 chr-1061*::Tn*501* derivative of MT1100	This study
MT1561	*flaC303 chr-1061*::Tn*501* derivative of MT1100	This study
MT1591	*flaB353 chr-1055*::Tn*501 str-8005* derivative of MT903	This study
MT1592	*flaG1045 chr-1055*::Tn*501 str-8005* derivative of MT903	This study
MT2052	*str-8006 flaE2052*::Tn*501*	This study
MT2090	*str-8006 leu-8001*::Tn*501*[b]	This study
MT2501	*proB64 rec-301*	PAO3385(FP5) × PAO18
MT2502	*trpA,B*::pME319 *his-301 str-1 leu-8001*::Tn*501*[b]	F116L(MT2090) × PAO1000
MT2503	*trpA,B*::pME319 *his-301 str-1 rec-301*[b]	MT2501(FP5) × MT2502
MT2504	*flaE2052*::Tn*501* derivative of MT318	G101(MT2052) × MT318
MT2505	*pur-67 cys-59 proB65 rec-301*	MT2503 × PAO1042
Plasmids		
FP2	Hg Tra Cma[d]	10
FP5	Hg Tra Cma	19
R38	Hg Tc Sm Su Phi Tra	26
RP4	Cb Tc Km Tra	6
R68.45	Cb Tc Km Tra Cma	8
pMT6	Cb Tc Km Tra Cma *fla*$^+$	30
pMT19	Cb Tc Km Tra Cma *fla*$^+$	30
pMT101	Cb Tc Km Tra Cma *pur-67*$^+$	This study
pVS1	Hg Su Mob	25, 27

[a] The following gene symbols are used (12, 23). Anabolic markers: *ade*, adenine; *arg*, arginine; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine and valine; *leu*, leucine; *met*, methionine; *pro*, proline; *pur*, purine; *thr*, threonine; *trp*, tryptophan. Catabolic markers: *ben*, benzoate; *cat*, catechol; *cnu*, carnosine; *oru*, ornithine; *puu*, purine; *tyu*, tyrosine. Resistance markers: *chl*, chloramphenicol; *rif*, rifampin; *str*, streptomycin. Other markers: *fla*, flagellar formation; *mfl*, multiflagellation; *mot*, motility; *nar*, nitrate reductase; *rec*, recombination.

[b] *his-301* is at the *hisII* locus (9), and *his-308* is at the *hisI* locus (M. Kageyama, personal communication). *ben-9010* is at the *catB* locus and is closely linked to *catA*, as determined by conjugational analysis (H. Matsumoto, personal communication). *ilvD309* is an *ilvD* mutation (M. Kageyama, personal communication). *leu-8001* is allelic with *leu-10* (Tsuda, unpublished data).

[c] *chr-1055*::Tn*501* and *chr-1061*::Tn*501* are Tn*501* insertions on the chromosome. For designations of transposon insertions, see reference 3.

[d] The following phenotype symbols are used (21): Cb, carbenicillin resistance; Km, kanamycin resistance; Tc, tetracycline resistance; Sm, streptomycin resistance; Su, sulfonamide resistance; Hg, mercuric ion resistance; Tra, transfer; Mob, mobilization; Phi, phage inhibition; Cma, chromosome mobilization ability.

transduction between G101 (*mfl flaX*) and *flaZ*, the majority of the Fla$^+$ transductants received the donor *mfl* marker, and in the reciprocal transduction between G101 (*mfl flaZ*) and *flaX*, no transductant received the donor *mfl* marker. Similar results were obtained when *flaY* mutations were used instead of *flaX* mutations. Hence, the *mfl-110* mutation site was located between *cnu-9001* and *flaX-flaY*, but not in the region distal to *flaX* and *flaY* from *flaZ*. A more precise location for the *mfl-110* mutation site was determined by transductions in which the

TABLE 2. Cotransduction of the flagellar mutations with *mfl-110*, *chr-1055*::Tn*501*, and *chr-1061*::Tn*501*[a]

fla mutation	% Cotransduction with:		
	mfl-110	*chr-1061*::Tn*501*	*chr-1055*::Tn*501*
Region I			
flaV314	97	<1	<1
flaZ354	90	<1	<1
flaW312	84	<1	<1
flaX307	72	<1	<1
flaY351	76	<1	<1
Region II			
flaG1045	<1	23	25
flaC303	<1	21	48
flaI1062	<1	25	67
flaH1054	<1	22	67
flaD306	<1	5	79
flaB353	<1	1	86
flaA352	<1	<1	91
flaF1009	<1	<1	94
cheA1211	<1	<1	93
flaE407	<1	<1	95
flaJ1068	<1	<1	<1
cheB1008	<1	<1	<1

[a] G101 lysates were prepared on strains MT903 (*mfl-110*), MT1502 (*chr-1061*::Tn*501*), and MT1501 (*chr-1055*::Tn*501*) and used to transduce strains to Fla$^+$; 100 to more than 400 transductants were scored in each case.

flaV mutant was used as the recipient. With the *mfl flaX* donor, 96% of the Fla$^+$ transductants exhibited the Mfl$^-$ phenotype, whereas 34% exhibited the same phenotype when the *mfl flaZ* donor was used. Taking these two values into consideration together with the cotransduction frequency of the *mfl-110* mutation with each of the *fla* mutations, we suggest that the *mfl-110* mutation is located between the mutation sites of *flaV* and *flaZ*. Since the *mfl-110* mutation site was deduced, the order of *flaZ*, *flaX*, and *flaY* was determined to be *flaZ-flaX-flaY*, although the linkage of *mfl-110* with *flaY* was a little higher than the linkage of *mtl-110* with *flaX* (Table 2). Furthermore, *flaW* was located between *flaZ* and *flaX*.

In summary, we concluded that the five *fla* cistrons in region I are in the order *cnu-9001-puuF-flaV-flaZ-flaW-flaX-flaY*-region II and that the *mfl-110* mutation is either located within *flaV* cistron or linked very closely to it. The *mot* cistron, which is also located in region I (29), was not ordered in this present study, since this mutant was isolated from a Rec$^-$ parent (30) and we could not transfer this *mot* mutation into other appropriate Rec$^+$ strains.

Insertion of Tn*501* near the *fla* genes. In G101-mediated transductions, none of the flagellar cistrons in region I or region II were cotransducible with either *puuF* or *orul*, which were the two nearby markers identified so far (30). Hence, we attempted to insert Tn*501* near the *fla* genes on the host chromosome by using the procedure described above. We obtained two strains in which Tn*501* was inserted near the *fla* cistrons in region II. These were designated *chr-1055*::Tn*501* and *chr-1061*::Tn*501*. These two Tn*501* insertions retained the capacity to transpose to a plasmid, RP4 (data not shown). We could isolate no strain in which Tn*501* was inserted near the *fla* cistrons in region I.

Transduction with G101 was carried out from an *fla*$^+$ *chr-1055*::Tn*501* (MT1501) or *fla*$^+$ *chr-1061*::Tn*501* (MT1502) strain to each of the flagellar mutants, and cotransduction of *fla* with *chr-1055*::Tn*501* or *chr-1061*::Tn*501* was examined (Table 2). Neither *chr-1055*::Tn*501* nor *chr-1061*::Tn*501* was cotransduced with any of the flagellar cistrons in region I. *chr-1055*::Tn*501* was not cotransduced with either *flaJ* or *cheB* in region II but was cotransduced with the other cistrons in region II at frequencies of 25 to 95%, whereas *chr-1061*::Tn*501* was cotransduced with *flaB*, *flaC*, *flaD*, *flaG*, *flaH*, and *flaI* at frequencies of 1 to 25%.

Based on these two-factor crosses, the order of the flagellar cistrons in region II was determined tentatively (Table 2). For example, we inferred that *flaC* and *flaH* are arranged in the following order: *chr-1061*::Tn*501-flaC-flaH-chr-1055*::Tn*501*. The precise order of the 10 cis-

TABLE 3. FP5-mediated three-factor crosses used to determine the order of *flaX*, *flaZ*, and *cnu-9001*[a]

Cross	Donor	Recipient	Selected marker	Coinheritance of *cnu*	No. of recombinants	Minimum no. of crossovers required for:	
						Order I[b]	Order II
1	MT1252(FP5) (*flaX1252*)	MT354 Str^r (*flaZ354 cnu-9001*)	*fla*$^+$	+	54	2(A-C)	4(A′-B′-C′-D′)
				−	20	2(B-C)	2(C′-D′)
2	MT1251(FP5) (*flaZ1251*)	MT307 Str^r (*flaX307 cnu-9001*)	*fla*$^+$	+	0	4(A″-B″-C″-D″)	2(A‴-C‴)
				−	45	2(C″-D″)	2(B‴-C‴)

[a] The donors were the FP5$^+$ derivatives of the *fla* mutants isolated from strain MT903. The recipients were the Str^r derivatives of the *fla* mutants isolated from strain MT350. The Fla$^+$ transconjugants were selected on nutrient gelatin agar plates containing streptomycin, and the coinheritance of *cnu* was examined.

[b] See Fig. 2.

TABLE 4. FP5-mediated three-factor crosses used to determine the order of region I flagellar cistrons[a]

Recipient	Donor (FP5$^+$ *fla*)	
	flaZ1251	*flaX1252*
flaV314	73	80
flaZ354	NT[c]	73
flaX307	<2	NT
flaY351	<4	NT

[a] The characteristics of the donors and the recipients were the same as those described in Table 3, footnote *a*. The Fla$^+$ transconjugants were selected on nutrient gelatin agar plates containing streptomycin.

[b] Percent coinheritance of the donor *cnu*$^+$ marker in the Fla$^+$ recombinants.

[c] NT, Not tested.

TABLE 5. G101-mediated three-factor crosses used to determine the order of region I flagellar cistrons[a]

Recipient	Donor (*mfl-110 fla*)		
	flaZ1251	*flaX1252*	*flaY1253*
flaV314	34[b]	96	100
flaZ354	NT[c]	88	80
flaW312	9	70	90
flaX307	<1	NT	48
flaY351	<1	<4	NT

[a] The donors and the recipients were the same as those used in the experiments shown in Table 4, and the donors carried an *mfl-110* mutation. G101 lysates were prepared on the donor strains and were used to transduce the recipient strains to Fla$^+$.

[b] Percent coinheritance of the donor *mfl* character in the Fla$^+$ transductants.

[c] NT, Not tested.

trons in region I could not be determined completely from these crosses since the insertion sites of the two transposons had not been determined yet.

Isolation of deletion mutants. An *flaH1054 chr-1055*::Tn*501* strain (MT1551) was transduced to Fla$^+$ on nutrient gelatin agar plates containing $HgCl_2$ with the G101 lysate prepared on an *fla*$^+$ *chr-1055*::Tn*501* (MT1501) or *flaC303 chr-1061*::Tn*501* (MT1561) strain. From the resulting Fla$^+$ Hg^r transductants, spontaneous Fla$^-$ mutants were isolated on nutrient gelatin agar plates. Some but not all of the Fla$^+$ transductants produced Fla$^-$ mutants at higher frequencies than Fla$^+$ strains carrying only one copy of Tn*501* on the chromosome (for example, strain MT1501).

Six of the Fla$^-$ mutants isolated from independent Fla$^+$ transductants were selected [strains MT1552, MT1553, and MT1554 derived from Fla$^+$ transductants by the cross between G101(MT1501) and MT1551 and strains MT1562, MT1563, and MT1564 derived from Fla$^+$ transductants by the cross between G101(MT1561) and MT1551] and were examined for resistance to Hg and for defects in the flagellar cistrons (Table 6). When four Hg^r mutants (strains MT1552, MT1553, MT1562, and MT1563) were used as donors, they gave neither swarms nor trails in transductions to *flaA*, *flaB*, *flaC*, *flaD*, *flaE*, *flaF*, *flaG*, *flaH*, *flaI*, and *cheA* mutants in region II but did give swarms and

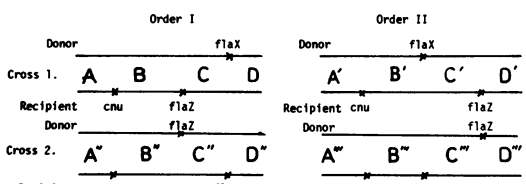

FIG. 2. Possible orders of *flaX*, *flaZ*, and *cnu-9001*. See Table 3.

trails in transductions to *flaJ* and *cheB* mutants in region II and with the six flagellar mutants in region I. These results indicated that these four Hg^r mutants carried deletions in region II. The remaining two Hg^S mutants, strains MT1554 and MT1564, gave MT1054 (*flaH1054*) neither swarms nor trails. However, these two Hg^S mutants produced swarms, but not trails, with another *flaH* mutant, strain MT1056 (*flaH1056*). Hence, the mutation sites in strains MT1554 and MT1564 were located in the *flaH* cistron. It seems likely that both *flaH* mutation sites are the same as in strain MT1054.

The generation of these two types of Fla$^-$ mutants may be best interpreted by the processes shown in Fig. 3, and we suggest that *chr-1055*::Tn*501* and *chr-1061*::Tn*501* were inserted in the chromosome with the same orientation and that cistrons *flaA* to *flaI* and *cheA* were located between the two transposons (see below).

Determining the order of the flagellar cistrons in region II. Since the two Tn*501* insertion sites were determined, the order of the flagellar cistrons in region II was examined by three-factor crosses with G101. The recipients were *fla* strains, and the donors were *fla chr-1055*::Tn*501* or *fla chr-1061*::Tn*501* strains. Selection was for Fla$^+$, and coinheritance of the donor Tn*501* was examined (Table 7). In the transduction between G101 (*chr-1055*::Tn*501 flaH*) and *flaC*, only 1% of the Fla$^+$ transductants were Hg^r, and in the reciprocal transduction between G101 (*chr-1055*::Tn*501 flaC*) and *flaH*, 80% of the transductants received the Hg^r determinant from the donor. Since both the *flaC* cistron and the *flaH* cistron were located between *chr-1055*::Tn*501* and *chr-1061*::Tn*501*, we concluded that the order is *chr-1061*::Tn*501-flaC-flaH-chr-1055*::Tn*501*. Similarly, we concluded that the *cheA* cistron and nine *fla* cistrons are arranged in the following way: *chr-1061*::Tn*501-flaG-*

TABLE 6. Characterization of the defects of the flagellar cistrons in the spontaneously isolated Fla⁻ mutants[a]

Recipient	Donor[b]					
	MT1552	MT1553	MT1554	MT1562	MT1563	MT1564
Region I						
flaV314	+	+	+	+	+	+
flaZ354	+	+	+	+	+	+
flaW312	+	+	+	+	+	+
flaX307	+	+	+	+	+	+
flaY351	+	+	+	+	+	+
mot-519	+	+	+	+	+	+
Region II						
flaG1045	−	−	+	−	−	+
flaC303	−	−	+	−	−	+
flaI1062	−	−	+	−	−	+
flaH1054	−	−	−	−	−	−
flaD306	−	−	+	−	−	+
flaB353	−	−	+	−	−	+
flaA352	−	−	+	−	−	+
flaE1009	−	−	+	−	−	+
cheA1211	−	−	+	−	−	+
flaE407	−	−	+	−	−	+
flaJ1068	+	+	+	+	+	+
cheB1008	+	+	+	+	+	+

[a] Transduction was carried out with E79 *tv-1* on nutrient gelatin agar plates. +, Presence of complementation and recombination; −, absence of complementation and recombination.

[b] MT1552, MT1553, and MT1554 were derived from the Fla⁺ transductants from the cross between G101(MT1501) and MT1551. MT1562, MT1563, and MT1564 were derived from the Fla⁺ transductants from the cross between G101(MT1561) and MT1551.

flaC- flaI - flaH - flaD- flaB- flaA-(flaF-cheA)-flaE-chr-1055::Tn*501* (The order of the cistrons in parentheses could not be determined clearly from this analysis). In these crosses, Hg^r transductants were also selected at the same time on L-broth agar plates containing $HgCl_2$, and the motilities of these transductants were examined. The order of the cistrons deduced from this procedure agreed with the order described above (data not shown). The *flaF* and *cheA* mutations have been shown previously to belong to the same complementation group (29). The results in Table 7 suggest that the *flaF* and *cheA* mutants carry the defects in the same locus.

The orientation of the 10 flagellar cistrons in region II relative to region I was determined by FP5-mediated three-factor conjugations in which *mfl flaB*, and *flaG* mutations were used as the markers (Table 8). The *flaB353* and *flaG1045* mutations were each introduced into an *mfl* mutant, strain MT903 Str^r, by cotransduction with *chr-1055*::Tn*501*, and the resulting derivatives (strains MT1591 and MT1592, respectively) were used as recipients. The FP5⁺

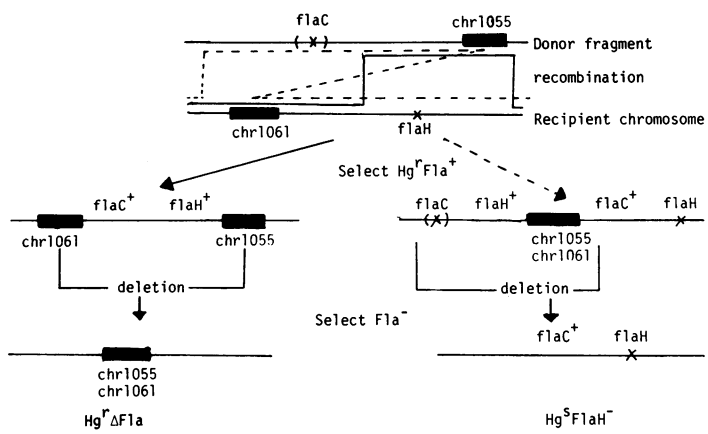

FIG. 3. Isolation of Hg^r Δ*fla* and Hg^s *flaH* mutants by using Tn*501* insertions. See text for details.

TABLE 7. Determining the order of the flagellar cistrons in region II[a]

Recipient	*chr-1055*::Tn*501 fla* donors								*chr-1061*::Tn*501 fla* donors			
	flaG1045	*flaC303*	*flaI1062*	*flaH1054*	*flaD306*	*flaB353*	*flaA352*	*flaF1009*	*cheA1211*	*flaE407*	*flaG1045*	*flaC303*	*flaI1062*	*flaH1054*
flaG1045	NT[b]	7[c]	3	2	1	NT	1	1	NT	NT	NT	44[b]	55	76
flaC303	44	NT	3	1	2	NT	1	NT	NT	NT	3	NT	45	51
flaI1062	41	57	NT	1	3	1	1	1	1	1	3	2	NT	NT
flaH1054	72	80	73	NT	2	1	2	1	1	1	2	2	NT	NT
flaD306	83	68	84	55	NT	1	3	1	1	1				
flaB353	NT	NT	85	75	54	NT	2	1	1	4				
flaA352	95	95	97	93	86	82	NT	2	5	6				
flaF1009	NT	NT	93	94	77	77	13	NT	4	3				
cheA1211	NT	NT	79	74	NT	NT	NT	NT	NT	NT				
flaF407	NT	NT	93	94	80	92	80	40	96	NT				

[a] G101 lysates were prepared on the donor strains and were used to transduce the recipient strains to Fla$^+$, and the coinheritance of Tn*501* in the transductants was examined.
[b] NT, Not tested.
[c] Percent coinheritance of the donor Hg^r determinant in Tn*501* in the Fla$^+$ transductants.

derivatives of the *flaB353* and *flaG1045* mutants (strains MT353 and MT1045, respectively) were used as donors. In the cross between *flaB* (FP5) and *mfl*, *flaG*, all of the Fla$^+$ transconjugants inherited the donor *mfl*$^+$ character. In the reciprocal cross between *flaG* (FP5) and *mfl flaB*, the majority of the Fla$^+$ transconjugants retained the recipient *mfl* character. Hence, we concluded that the order was *mfl-flaG-flaB*.

In summary, the order of the flagellar cistrons in region II was as follows: region I-*chr*-1061::Tn*501*-*flaG*-*flaC*-*flaI*-*flaH*-*flaD*-*flaB*-*flaA*-*flaF* (=*cheA*)-*flaE*-*chr-1055* ::Tn*501*-*oruI*. The remaining two region II cistrons, *flaJ* and *cheB* (29), were not linked to this *fla* cluster, as determined by a transductional analysis, and the locations of these cistrons were not determined.

Linkage of the *fla* regions to *pur-67*. We reported previously the order *puuF*-region I-region II-*oruI* (30). Recently, D. Haas (personal communication) established the marker order of this very late region as *puuF-pur-67-*(*hisI-cys-59*)-*proB* by using sex factors (the order of the markers in parentheses has not been determined, but these markers are closely linked to each other, as determined by transduction). Therefore, we examined the linkage of the *fla* regions to *pur-67* by using an R-prime plasmid and R68.45-mediated conjugation.

R-prime plasmid pMT101, which complemented the *pur-67* lesion on the chromosome, was isolated from R68.45. This plasmid did not complement either the region I *fla* mutation or the region II *fla* mutation and did not complement the *oruI* lesion.

The linkage between *pur-67* and the *fla* regions was examined by the following two kinds of matings: MT315 (R68.45) × PAO1042 Str^r and MT2504 (R68.45) × PAO1042 Str^r (Table 9). The former donor carried a region I *flaZ* mutation, and the latter carried a region II *flaE* mutation. A recombinant analysis in which these crosses were used indicated that neither *flaE* nor *flaZ* is located between *pur-67* and *cys-59* and that both *fla* genes are located earlier than *pur-67* on the linkage map of the chromosome. We concluded that the two flagellar regions are in the following order: *puuF*-region I-region II-*pur-67-oruI*.

The order of the flagellar cistrons defined in this study is summarized in Fig. 4.

DISCUSSION

Of 15 *fla*, 1 *mot*, and 2 *che* cistrons so far identified in *P. aeruginosa* (29), we determined the order of *cheA* and 14 *fla* cistrons on the chromosome by using *mfl* and mercury resistance in Tn*501* (Fig. 4). The order of these cistrons was confirmed by using the deletion mutations Δ(*flaVZWXY*), Δ(*flaWXY*), Δ(*flaBD*),

TABLE 8. Determining the order of *mfl*, *flaB*, and *flaG* mutations by PF5-mediated conjugation[a]

Donor	Recipient	Selected marker	Coinheritance of *mfl*	No. of recombinants
MT353(PF5) (*flaB353*)	MT1592 (*mfl flaG1045*)	*fla*$^+$	+	116
			−	0
MT1045(FP5) (*flaG1045*)	MT1591 (*mfl flaB353*)	*fla*$^+$	+	14
			−	74

[a] Selection and counterselection were for Fla$^+$ and Sm^r, respectively, and the coinheritance of the donor *mfl*$^+$ marker in the Fla$^+$ transconjugants was examined.

and Δ(*flaAB*) (29). Five *fla* cistrons in region I were tightly linked to each other, as were nine *fla* cistrons in region II (Table 2). Using transduction, we found that *flaJ* and *cheB* cistrons in region II were not closely linked to the other *fla* cistrons in region II. The location of the *mot* cistron in region I was not determined. In two-factor transductional crosses, the *mfl* mutation located in region I did not exhibit any linkage with region II, and none of the Tn*501* insertions in region II exhibited any linkage with region I (Table 2). Furthermore, some of the genes responsible for transport of the branched-chain amino acid (*bra*) were mapped between *flaG* and *chr-1061*::Tn*501* (Fig. 4) (T. Hoshino, M. Tsuda, T. Iino, K. Nishio, and M. Kageyama, in press). Hence, region I and region II are not contiguous on the linkage map, as we had previously imagined (30).

In the accompanying paper (29) we show that the *cheA* and *flaF* mutations constitute one complementation group. The data from the two- and three-factor crosses in this study (Tables 2 and 7) support the idea that *flaF* and *cheA* are the same cistron.

We found that the *mfl* mutation is either located within the *flaV* cistron or linked very closely to it (Tables 2 and 5). A further analysis of the genetic properties of the *mfl* mutation is being undertaken.

In both *Escherichia coli* and *Salmonella*, more than 30 cistrons are involved in flagellar formation and functions, and these cistrons are clustered in three distinct regions on the chromosome (14, 15, 18, 22, 24). In this study we show that the flagellar genes of *P. aeruginosa* are clustered on the chromosome. The remarkable clustering of the flagellar genes in this organism is only one exceptional example of the gene organization in the genome (12, 23). In *P. aeruginosa*, genes coding for related functions do not show the close linkage characteristic of enterobacteria. The clustering of the *fla* cistrons implies that the expression of each *fla* cistron might be coordinately regulated (for example, constituting operons, as has been shown for the *fla* cistrons of *E. coli* and *Salmonella*) (14, 15, 18, 22, 24).

We obtained the two strains in which Tn*501* was inserted near the *fla* genes on the host chromosome by the procedure described above. The step where Tn*501* was transposed from pVS1 to *chr-1055* or *chr-1061* on the host chromosome during this procedure was one of the following: (i) transposition from pVS1 to the chromosomal portion on the R*fla* plasmid; (ii) transposition directly from pVS1 to the host chromosome just before the phage lysate was prepared; or (iii) transposition from pVS1 to the phage genome and then to the host chromosome

TABLE 9. Coinheritance of *fla*, *pur-67*, *oruI*, *cys-59*, and *pro-65*[a]

Cross	Selected marker	No. of recombinants examined	% Coinheritance of unselected markers				
			fla$^-$	*pur*$^+$	*oru*$^-$	*cys*$^+$	*pro*$^+$
MT315(R68.45) × PAO1042 Str^r							
	pur-67$^+$	111	4	100	44	4	2
	cys-59$^+$	121	1	10	42	100	56
	pro-65$^+$	70	0	19	ND[b]	76	100
MT2504(R68.45) × PAO1042 Str^r							
	pur-67$^+$	109	6	100	ND	11	4
	cys-59$^+$	20	10	100	ND	100	10
	pro-65$^+$	77	4	31	ND	77	100

[a] MT315 and MT2504 were *flaZ* (region I) and *flaE* (region II) derivatives of MT318, respectively. Counterselection was for Str^r.

[b] ND, Not determined.

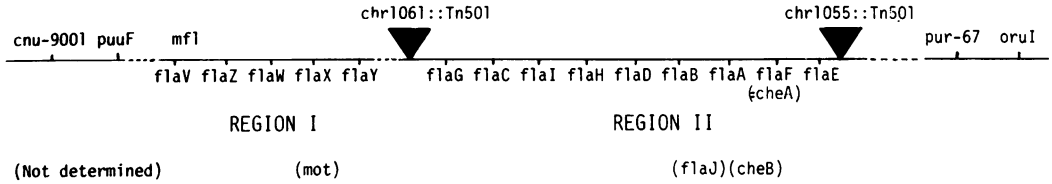

FIG. 4. Order of the flagellar cistrons in *P. aeruginosa* PAO. The distances between the cistrons are arbitrary. Three cistrons, *mot*, *flaJ*, and *cheB*, were not mapped. *flaF* = *cheA*. The *mfl* mutation either is located within *flaV* cistron or is very closely linked to it. See text for details.

in the recipient in transduction. Expecting the occurrence of the first step described above, we used the procedure which would enrich the strains carrying R*fla*::Tn*501* plasmids. However, plasmid pVS1 is mobilized by other conjugative plasmids, such as those belonging to the Inc-P1 group (27). Therefore, in a mating between the donor carrying both R*fla* and pVS1 and a Rec$^-$ recipient, almost all of the resulting Tc^r Hg^r transconjugants received both R*fla* and pVS1, but not R*fla*::Tn*501* (data not shown). There have been only a few genetic analyses of *Pseudomonas* plasmids in which transposon insertions in *P. aeruginosa* were used. Krishnapillai (17) inserted Tn*7* and Tn*501* into an R plasmid, R91-5, and Fennewald et al. (7) inserted Tn*7* into a degradative plasmid, OCT. In both cases, the transposons were inserted by the mating out procedure, where the donor retained a Mob$^-$ plasmid carrying a transposon and the target plasmid. If Mob$^-$ derivatives of pVS1 were used in our transposition technique, we would be able to insert Tn*501* near the *fla* genes with higher efficiency.

In *Salmonella typhimurium*, Chumley and Roth (5) inserted Tn*10* into the chromosome region near the histidine (*his*) operon. Combining two such strains, each carrying Tn*10* inserted at a different site with the same orientation, these authors constructed two classes of strains. One class contained the structure Tn*10*-*his*-Tn*10* (i.e., a structure in which two copies of Tn*10* were retained on the same chromosome with the same orientation and the *his* region was flanked by the two insertions). The other class contained the structure *his*-Tn*10*-*his* (i.e., a structure in which the *his* region was duplicated and the two copies of the *his* region were joined by a single copy of Tn*10*). Chumley and Roth also observed that both the structures, Tn*10*-*his*-Tn*10* and *his*-Tn*10*-*his*, were unstable. The first class segregated Tc^r derivatives in which the chromosomal segment between the two Tn*10* insertions was deleted by homologous recombination between the two insertions. The second class segregated Tc^S derivatives which were generated by homologous recombination between the duplicated *his* regions.

By introducing the chromosomal region around *chr-1055*::Tn*501* into cells carrying *chr-1061*::Tn*501* by transduction, we could also induce rearrangement of the genome (e.g., formation of deletions)(Table 6). Some of the Fla$^+$ Hg^r transductants of *flaH chr-1061*::Tn*501*(MT1551) constructed by using phage lysate prepared on *flaC chr-1055*::Tn*501*(MT1561) produced Fla$^-$ mutants spontaneously at higher frequencies. The resulting Fla$^-$ mutants were categorized into two classes, Hg^r Δ*fla* and Hg^S *flaH* (Table 6). Taking the study by Chumley and Roth described above into consideration, we explain the generation of the genetic structures in the two classes of Fla$^-$ mutants and the unstable Fla$^+$ phenotype in the parental strains as follows (Fig. 3). (i) The stable Fla$^+$ transductants do not receive *chr-1055*::Tn*501* from the donor. (ii) The unstable transductants that segregate *fla* deletion mutants carry two copies of Tn*501* on the same chromosome with the same orientation. By recombination between the two Tn*501* insertions on the same chromosome, the region between these transposons may be deleted at a high frequency. However, we could not completely rule out the possibility that the deletion endpoints were not the two Tn*501* insertion sites. Successful isolation of these deletion mutants implies that no gene essential for the viability of the cells is located within the deletion region. (iii) The last group of Fla$^+$ transductants that segregate Hg^S FlaH$^-$ mutants carry a single copy of Tn*501*, and the region around this Tn*501* insertion is duplicated. This duplication occurs when Hg^r Fla$^+$ transductants are formed by homologous recombination at two sites, one site between the two Tn*501* insertions in the donor and the recipient and the other site between the intristic DNA sequences of the donor and the recipient. These duplicated strains should also segregate Hg^S Fla$^-$ derivatives at high frequencies. The disappearance of the Hg^r phenotype in these Fla$^-$ strains could be explained by this duplication-deletion mechanism, since once inserted into the chromosome, a single copy of Tn*501* is hardly ever excised (Tsuda, unpublished data). The Fla$^+$ strains, in which both *flaC* and *flaH* duplicated, segregated only *flaH*

mutants; these strains did not segregate *flaC* mutants as far as we examined (Table 6). This phenomenon could be explained by the fact that the distance between *flaH* and *chr-1055*::Tn*501* is shorter than the distance between *flaC* and *chr-1061*::Tn*501*(Table 2). As discussed above, some of the *bra* genes have been mapped between *flaG* and *chr-1061*::Tn*501* (Hoshino et al., in press). Our Hg^r Δ*fla* mutants were *bra*, and Hg^S *flaH* mutants were bra^+ (data not shown). These data conform with our interpretation discussed above (Fig. 3).

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