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Genetic Mapping of *bra* Genes Affecting Branched-Chain Amino Acid Transport in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa PAO mutants defective in the transport systems for branched-chain amino acids were isolated and characterized. Two mutations in strains selected for trifluoroleucine resistance, *braA300* and *braB307*, were mapped in the *met-9020–dcu-9108* and the *nar-9011–puuC10* region, respectively. The mutation loci in strains selected for azaleucine resistance, *braC310* and *bra-311* through *bra-314*, were all located near the *fla* genes, with an order of region I *fla-bra*–region II *fla*. Strains with *braA300* showed a marked reduction in the high-affinity branched-chain amino acid transport system (LIV-I) and a considerable decrease in the lower-affinity system (LIV-II). Strains with *braB307* were found to be defective in the LIV-II system. Strains selected for azaleucine resistance were all defective only in the LIV-I system and fell into three phenotypically distinct classes. Strains with *braC310* produced a binding protein for leucine, isoleucine, valine, alanine, and threonine (LIVAT-BP) altered in binding ability, indicating that the *braC* gene is the structural one for the LIVAT-BP. Strains with *bra-311* or *bra-312* showed a complete loss of production of the LIVAT-BP. Strains with *bra-313* or *bra-314* produced normal levels of functional LIVAT-BP, suggesting that these mutations are located in a gene(s) other than *braC*.

The transport of the branched-chain amino acids L-leucine, L-isoleucine, and L-valine in *Pseudomonas aeruginosa* is mediated by two kinetically distinct systems, LIV-I and LIV-II (12). The LIV-I system, with a high affinity, is specific for alanine and threonine in addition to the branched-chain amino acids. The LIV-II system, with a low affinity, is specific for branched-chain amino acids alone and is operative only when Na^+ is present. Osmotic shock treatment of cells causes a preferential decrease in the LIV-I system alone and releases a binding protein for leucine, isoleucine, valine, alanine, and threonine (LIVAT-BP) (12, 14). The kinetic properties of the LIVAT-BP are very similar to those of the LIV-I system (14). Characterization of mutants and their revertants has further shown the parallelism between the level of LIVAT-BP in the shock fluid and that of LIV-I transport activity (14). In contrast, the LIV-II system resists osmotic shock (12) and is mutationally separable from the LIV-I system (15). This system alone is responsible for the branched-chain amino acid transport by membrane vesicles (12, 13, 15).

The selection for resistance to 5′,5′,5′-trifluoro-DL-leucine (TFL), a leucine analog, has proved to be very useful in isolating *P. aeruginosa* PML14 mutants defective in the transport systems for branched-chain amino acids (14, 15). Mutants defective in LIV-I, LIV-II, or both systems were selected for resistance to TFL without Na^+, with Na^+ and an excess of alanine, or with Na^+ alone, respectively. No gene, however, has been located that participates in the LIV transport systems, as there is little information on the genetic properties of the PML14 strains so obtained. To identify the genes responsible for the LIV transport systems and to clarify the role of each of these genes, we attempted to isolate *P. aeruginosa* PAO mutants defective in the LIV transport systems.

In this report, we describe the isolation of *P. aeruginosa* PAO mutants by selection for resistance to TFL or 4-aza-DL-leucine, another leucine analog. Genetic analysis of these mutants by conjugation and transduction was performed. The phenotypic characteristics of the mutants are also described.

MATERIALS AND METHODS

Bacteria and phages. The bacterial strains used in this study were all derivatives of *P. aeruginosa* PAO (10) and are listed in Table 1. Strains carrying plasmid FP5 (24) or R68.45 (7), except those naturally contain-

TABLE 1. *P. aeruginosa* PAO strains used in this study

Strain	Relevant genotype[a]	Derivation	Reference or source
PAO1042	*pur-67 thr-9001 cys-59 pro-65*		28
PAO2354	*orul325 puuC10 tyu-9018 ben-9010*		H. Matsumoto
PAO2369	*met-9020 catA1 nar-9011 cnu-9001 puuE8 tyu-9025*		H. Matsumoto
PAO3012	*trp-6 chl-301 fla-301*		18
PAO3501	*his-308 fla-301*		16
PAO3504	*catA1 nar-9011 cnu-9001 puuE8 tyu-9025*		16
PAO3510	*trp-6 fla-301 braA300*	NTG[b] mutagenesis of PAO3012	This paper
PAO3517	*trp-6 fla-301 braB307*	NTG mutagenesis of PAO3012	This paper
PAO3520	*his-308 fla-301 braA300*	*trp*$^+$ *his* recombinant of PAO3510	This paper
PAO3527	*his-308 fla-301 braB307*	*trp*$^+$ *his* recombinant of PAO3517	This paper
PAO3530	*his-308 fla-301 braC310*		16
PAO3531	*his-308 fla-301 bra-311*	NTG mutagenesis of PAO3501	This paper
PAO3532	*his-308 fla-301 bra-312*	NTG mutagenesis of PAO3501	This paper
PAO3533	*his-308 fla-301 bra-313*	NTG mutagenesis of PAO3501	This paper
PAO3534	*his-308 fla-301 bra-314*	NTG mutagenesis of PAO3501	This paper
PAO3535	*his-308 braC310*	*fla*$^+$ revertant of PAO3530	This paper
PAO3536	*his-308 braC310 fon-301*	*fon* derivative of PAO3535	This paper
PAO4043	*met-9020 catA1 nar-9011 mtu-9002 tyu-9030 dcu-9108*		H. Matsumoto
MT1517	*met-28 ilvD202 str-1 flaG1045 chr-1055*::Tn*501*		32
MT1523	*flaG1045 chr-1061*::Tn*501*		32
MT1552	*argB18 chl-2 rif-8001* Δ(*chr-1061*::Tn*501*–*chr-1055*::Tn*501*)[c]		32
MT1562	*argB18 chl-2 rif-8001* Δ(*chr-1061*::Tn*501*–*chr-1055*::Tn*501*)[c]		32

[a] Symbols for genetic markers are those used in previous papers (16, 22, 28, 33). Markers *chr-1055*::Tn*501* and *chr-1061*::Tn*501* represent the chromosomal sites at which transposon Tn*501* is inserted. *ben-9010* is probably *catB* and is closely linked to *catA* (H. Matsumoto, personal communication). *tyu-9030* and *tyu-9025* are probably allelic with *tyu-9018*, and *dcu-9108* is allelic with *dcu-9013* (H. Matsumoto, personal communication). *fon* is a symbol for resistance to phage F116.

[b] NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

[c] Deletion of the chromosomal region between *chr-1061*::Tn*501* and *chr-1055*::Tn*501*. Both strains MT1552 and MT1562 retain resistance to 0.2 mM $HgCl_2$.

ing such a plasmid, are designated PAO3531(FP5) or PAO3531(R68.45). Phages G101 (11) and E79*tv-1* (25) were used for transduction experiments. Phage F116c, a clear-plaque mutant of F116, was used for the isolation of F116-resistant derivatives of PAO strains.

Media. Nutrient broth and agar (17), nutrient gelatin agar (33), and two minimal media, G-medium (18) and D-medium (12), were used. Nutrient gelatin agar plates were used for the motility assay. G-medium-based agar plates were used to test for auxotrophy and sensitivity to antibiotics or $HgCl_2$. D-medium-based agar plates were used to test for sensitivity to leucine analogs and for the utilization of substrates such as benzoate, mannitol, tyrosine, or purine as a sole carbon source (final concentration, 0.2%). Amino acids, when needed, were added to a final concentration of 50 μg/ml. Drugs were added at the following concentrations: carbenicillin (Fujisawa Pharmaceuticals Co.), 500 μg/ml; kanamycin (Meiji Seika Co.), 250 μg/ml; tetracycline (Lederle Japan Ltd.), 100 μg/ml; $HgCl_2$, 0.2 mM.

Mutagenesis. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was done by the procedure of Fargie and Holloway (6) with modifications (14).

Isolation of mutants. Spontaneous mutants resistant to phage F116 (*fon*) were isolated by plating about 10^8 cells with 7×10^7 PFU of phage F116c on a nutrient agar plate.

Selection for resistance to TFL was carried out as follows. After strain PAO3012 cells were mutagenized with nitrosoguanidine, the surviving cells were plated on a D-medium-based agar plate containing 50 μg of TFL per ml or a mixture of 10 μg of TFL per ml, 20 mM NaCl, and 10 mM alanine. Colonies resistant to TFL appeared at a frequency of 10^{-4} under both conditions after cultivation for 48 h at 37°C. About half of the colonies showed stable resistance to TFL. Six colonies resistant to 50 μg of TFL per ml were streaked out and checked for LIV transport activities. Only one strain, designated PAO3510 (*braA300*), showed a marked decrease in LIV-I activity. Eleven colonies resistant to 10 μg of TFL per ml in the presence of NaCl and alanine were also streaked out and checked for LIV-I and LIV-II activity. Seven colonies showed marked reduction in LIV-II activity (less than 10% of that of their parent). One of these, strain PAO3517 (*braB307*), was chosen for further characterization.

Selection for resistance to azaleucine was used for the preferential isolation of *P. aeruginosa* PAO mutants defective in the LIV-I transport system, as described previously (16). Five mutants, PAO3530 through PAO3534, were chosen for detailed characterization. The properties of PAO3530 (*braC310*) were described previously (16).

Assays of transport and binding activities. L-Leucine transport activity via the LIV-I or LIV-II system was assayed without Na^+ or with 20 mM NaCl and 10 mM L-alanine, respectively (12). The binding activities of shock fluids were measured at 4°C by the equilibrium dialysis method (3, 12), with the cap part of an Eppendorf type 3810 micro test tube used as a dialysis capsule (16).

Preparation of shock fluids. Cells grown in D-medium supplemented with 0.5% D-glucose as a carbon source were harvested at the mid-exponential phase and subjected to cold shock (14). The suspension was centrifuged twice at 20,000 × *g* for 15 min, and the resulting fluid was dialyzed overnight against 10 mM Tris-hydrochloride buffer (pH 7.4) containing 0.02% NaN_3. The fluid was stored at 0°C until used.

Solubilization of whole-cell proteins. To separate cellular proteins on a slab gel, we solubilized whole cells as follows. Cells grown in 5 ml of D-medium were centrifuged at room temperature and suspended in 75 μl of 10 mM Tris-hydrochloride buffer (pH 7.4). After being boiled for 20 min, the suspension was supplemented with 20 μl of 100-μg/ml DNase and 10 μl of 10 mM $MgCl_2$ and incubated at 37°C for 60 min to digest the cellular DNA. The total cellular proteins were solubilized from the sample by adding 100 μl of 125 mM Tris-hydrochloride (pH 6.8)–4% β-mercaptoethanol–4% sodium dodecyl sulfate (SDS)–20% sucrose.

SDS-polyacrylamide gel electrophoresis. SDS-slab gel electrophoresis was carried out by the method of Laemmli (19). The gels used were 0.75 mm thick.

Immunological methods. The detection of immunologically cross-reactive protein(s) in whole-cell extracts and shock fluids with the LIVAT-BP (16) was performed as follows. After separation on an SDS-polyacrylamide slab gel, the proteins were transferred onto nitrocellulose sheets (type BA85; Schleicher & Schuell Co.) at 30°C by the method of Bowen et al. (5), giving two replicas of the original gel pattern. The replicas were treated with antiserum against the purified LIVAT-BP of *P. aeruginosa* PAO3012 (wild type) (16). Protein bands that cross-reacted with LIVAT-BP were stained by peroxidase reaction with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G serum (31).

Patch mating. The transfer of a plasmid such as FP5 or R68.45 from one strain to another was carried out by a method similar to that described by Holloway (8). Ten microliters each of donor and recipient cultures were mixed at the center of a nutrient agar plate and incubated overnight at 37°C. Cells grown on the plate were suspended in dilution buffer (29) (about 10^9 cells per ml) and spread on a G-medium-based agar plate supplemented with the appropriate drugs.

Conjugation. R68.45-mediated conjugation was carried out by mating strains on plates by the method of Stanisich and Holloway (30), with modifications (16). Conjugation mediated by FP5 was performed by the method of Holloway and Fargie (9), with modifications as described previously (16).

Transduction. Transduction mediated by phage G101 was carried out as follows. Recipient cells from the late exponential phase (10^9 cells per ml) were mixed with an equal volume of a phage lysate containing about 3×10^{10} to 5×10^{10} PFU/ml and kept at 37°C for 30 min. The mixture (0.1 to 0.15 ml) was then spread on selection plates. After incubation at 37°C for 2 days, colonies appearing on the plates were streaked out and tested for their phenotypes. When a recipient strain was resistant to G101, phage E79*tv-1* was used for transduction, with a plasmid R38-carrying derivative as the recipient (25).

Other methods. Elimination of Na^+ from agar powder (Iwai Kagaku Co., Tokyo, Japan) was carried out by washing the powder with 1 M KCl, as described previously (14). This washed powder was used for the preparation of D-medium-based agar plates containing TFL.

Protein concentrations were determined by the method of Lowry et al. (21), with bovine serum albumin used as a standard.

Chemicals. *U*-^{14}C-labeled L-leucine was purchased from the Radiochemical Centre, Amersham, United Kingdom. Acrylamide was from Eastman Kodak Co., Rochester, N.Y. SDS and Coomassie brilliant blue were products of E. Merck AG, Darmstadt, West Germany. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Aldrich Chemical Co., Milwaukee, Wis. TFL was from Fairfield Chemical Co., Blythewood, S.C. Azaleucine, bovine serum albumin (fraction V), and DNase (DN-25) were obtained from Sigma Chemical Co., Saint Louis, Mo. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G serum was a product of Miles Yeda Ltd., Rehovot, Israel. All other compounds used were of reagent grade.

RESULTS

Mapping of *braA300* and *braB307*. Resistance to TFL (10 μg/ml) with 20 mM NaCl was used as a phenotype for the *braA300* and *braB307* mutations. As the addition of one of the branched-chain amino acids suppressed the TFL sensitivity of wild-type strains, the auxotrophic markers *leu* and *ilv* were not available for use as unselected markers. Preliminary conjugational crosses were carried out with PAO3520(FP2) (*braA300*) or PAO3527(FP2) (*braB307*) as donors and strains having auxotrophic markers earlier than 60 min as recipients (Fig. 1). No linkage was observed between *braA300* or *braB307* and *arg-32*, *trp-6*, or *leu-38*. However, the *bra* mutations were coinherited with *lys-9015* and *met-9020*. Linkage values of 13 and 59% or 4 and 9% were obtained for *braA300* and *braB307*, respectively, suggesting that these mutation loci are located near or later than 60 min.

Mapping of *braA300*. To determine the location of *braA300* on the chromosome of *P. aeruginosa* PAO, we carried out conjugation experiments with PAO3520(R68.45) (*braA300*) as the donor and PAO4043 as the recipient (Table 2). Strain PAO4043 carries a variety of catabolic

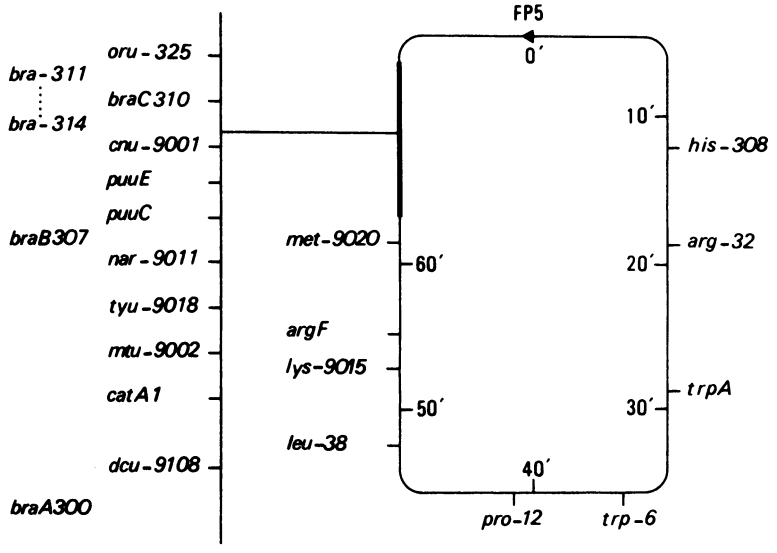

FIG. 1. Chromosome map of *P. aeruginosa* PAO, showing the positions of relevant markers and the point of FP5 origin. After *met-9020*, only the order of markers is shown.

markers later than *met-9020*. The results showed that *braA300* is located between *met-9020* and *dcu-9108*. Conjugational crosses with the *braA*$^+$ strain PAO3501(R68.45) as the donor and PAO3505, a *braA300* derivative of PAO4043, as the recipient further showed that *braA300* is located in the *met-9020–catA1* region (data not shown). The frequencies of the coinheritance of *braA300* with *met-9020* and *catA1* approximated those obtained by crosses with the *braA300* strain as the donor. We concluded from these results that the *braA300* mutation is located near the *met-9020* locus, with an order *met-9020–braA300–dcu-9108–catA1*. Transductional analyses with phage G101 or E79*tv-1* showed no linkage (<1%) between *braA300* and either *met-9020*, *dcu-9108*, or *pyrD*, which seems to be located in the *met-9020–catA1* region (28), however.

Mapping of *braB307*. To determine the location of *braB307* on the *P. aeruginosa* PAO chromosome, we carried out conjugation experiments with PAO3527(R68.45) (*braB307*) as the donor and PAO4043 as the recipient. No linkage was observed between *braB307* and either *met-9020*, *dcu-9108*, *catA1*, or *mtu-9002*. However, 4% coinheritance of *braB307* with *tyu-9030* was found, indicating that the *bra* mutation is located later than *mtu-9002*. Conjugational crosses were further carried out with PAO2369 as the recipient (Table 3). The *braB307* mutation was coinherited with *nar-9011* and *cnu-9001* at relatively high frequencies. The marker order was determined by these crosses to be *tyu-9025–nar-9011–braB307–cnu-9001*. Markers near this region have been mapped in the order *nar-9011–puuC–puuE–cnu-9001–puuF* (23, 28). The cotransduction frequencies of *puuC* and *puuE*, *puuE* and *cnu-9001*, and *cnu-9001* and *puuF* by phage G101 have been determined as 80, 15, and 36%, respectively (20, 28). We obtained values of 20 (20 of 100) and 39% (28 of 72) for cotransduction frequencies by phage G101 between *puuE* and *cnu-9001* and *cnu-9001* and *puuF*, respectively,

TABLE 2. Coinheritance of *braA300* with other late markers[a,b]

Selected marker	% Coinheritance of unselected markers							No. of recombinants examined
	met$^+$	*bra*	*dcu*$^+$	*cat*$^+$	*mtu*$^+$	*tyu*$^+$	*nar*$^+$	
met-9020$^+$	(100)	17	5	1	0	0	0	100
dcu-9108$^+$	6	16	(100)	56	28	3	3	100
catA1$^+$	1	2	18	(100)	41	2	1	100
mtu-9002$^+$	0	2	30	54	(100)	8	5	62
tyu-9030$^+$	1	2	4	7	6	(100)	43	97

[a] Strains PAO3520(R68.45) and PAO4043 were used as donor and recipient, respectively. Counterselections were made with *his-308*$^+$.

[b] *braA300* was scored as resistance to 10 μg of TFL per ml in the presence of 20 mM NaCl.

TABLE 3. Coinheritance of *braB307* with the late markers[a,b]

Selected marker	% Coinheritance of unselected markers						No. of recombinants examined
	met$^+$	*cat*$^+$	*tyu*$^+$	*nar*$^+$	*bra*	*cnu*$^+$	
PAO3527(R68.45) × PAO2369							
tyu-9025$^+$	1	3	(100)	39	18	8	99
nar-9011$^+$	0	0	30	(100)	49	9	79
PAO3517(R68.45) × PAO2369							
cnu-9001$^+$	0	0	13	23	64	(100)	100

[a] Counterselections were made with *his-308*$^+$ for PAO3527(R68.45) and with *trp-6*$^+$ for PAO3517(R68.45).
[b] *braB307* was scored as resistance to 10 μg of TFL per ml in the presence of 20 mM NaCl.

confirming the above reports. On the other hand, no linkage (<1%) was observed by G101-mediated transduction between *braB307* and either *nar-9011*, *puuE*, *cnu-9001*, or *puuF*. Taking these results into consideration, we concluded that the marker order is *nar-9011–braB307–puuC–puuE–cnu-9001*.

Mapping of *braC310* and *bra-311* through *bra-314*. As shown in the previous paper (16), azaleucine preferentially selects mutants of *P. aeruginosa* PAO defective only in the LIV-I system. The *braC310* mutation in strain PAO3530, one of the mutants thus selected, has been mapped between *cnu-9001* and *oruI325* (16). To determine the approximate locations of *bra-311* through *bra-314*, we carried out conjugation experiments mediated by FP5 or R68.45, with azaleucine resistance as an unselected marker. One of the *bra* mutants carrying either FP5 or R68.45 was mated with strain PAO2354 or PAO3504. These strains carry several markers later than *met-9020* (Table 1, Fig. 1). From the results obtained with PAO2354 as the recipient (data not shown), it was presumed that *bra-311* through *bra-314* are located between *puuC10* and *oruI325*. The results obtained with PAO3504 as the recipient (data not shown) showed that *bra-311* through *bra-314* are situated further from *puuE* than *cnu-9001*, suggesting the marker order *puuE–cnu-9001–bra–oruI325*.

Tsuda et al. (33) recently showed that the flagellar (*fla*) genes are divided into two clusters, region I and region II, and that these two regions are located on the *P. aeruginosa* PAO chromosome in the order *puuF*-region I-region II-*oruI325*. These findings suggested that the *bra* mutations selected for azaleucine resistance are closely linked with *fla* mutations. Two sites, *chr-1061* and *chr-1055*, at which Tn*501*, carrying mercury resistance (4), is inserted, are also located near the region II *fla*, in the order region I–*chr-1061*–region II–*chr-1055* (32). To locate the *bra* mutations more precisely, we carried out two-point analyses by G101-mediated transduction, with mercury resistance as a selected marker. The *bra* mutations showed between 45 and 55% linkage to *chr-1061*::Tn*501* and between 2.7 and 4.5% linkage to *chr-1055*::Tn*501* (Fig. 2). No linkage was observed by G101 transduction between *chr-1061*::Tn*501* and *flaE*, the nearest gene of the region II *fla* genes to *chr-1055*::Tn*501* (32), indicating that the *bra* mutations are situated in the *chr-1061–chr-1055* region. The region II *fla* genes, such as *flaG* and *flaI*, showed lower or higher linkage to *chr-1061*::Tn*501* or *chr-1055*::Tn*501* than the *bra* mutations, respectively (Fig. 2). This fact suggested that the *bra* mutations are located between *chr-1061* and the region II *fla* genes. To confirm this, we performed three-point analyses by G101 transduction (Table 4). The three markers chosen for this purpose were Hg^r (*chr-1061* or *chr-1055*), *braC310* (which seems to be the most distant of the *bra* mutations from *chr-1061* [Fig. 2]), and *flaG*, which is the nearest of the region II *fla* genes to *chr-1061* (32). In the crosses with *chr-1061*::Tn*501*, all of the transductants having donor-type mercury resistance and Fla phenotypes together showed a donor-type Bra phenotype. In the crosses with *chr-1055*::Tn*501*, however, *braC310* and *chr-1055*::Tn*501* segregated when selection was made for Fla$^+$. From these results, we concluded that the order of *braC310* and the nearby markers is *chr-1061–braC310–flaG–chr-1055*.

Phenotypes of *bra* mutants. The characteristics of the *bra* mutants in analog sensitivity, LIV transport activities, and LIVAT-BP production were examined (Table 5, Fig. 3). The transport activity of the LIV-I and LIV-II systems was assayed separately. Strain PAO3517 (*braB307*), selected for resistance to TFL, showed a complete loss of leucine uptake by LIV-II and a normal level of leucine uptake by LIV-I, indicating that the *braB* gene is responsible for the LIV-II system alone. This strain showed resistance to TFL with Na^+ but not to azaleucine. Strain PAO3510 (*braA300*), another TFL-resistant mutant, lost most of the LIV-I activity. A partial loss of leucine uptake by LIV-II, however, was observed in the *braA* mutant. This strain, unlike PAO3517 (*braB307*), showed resistance to aza-

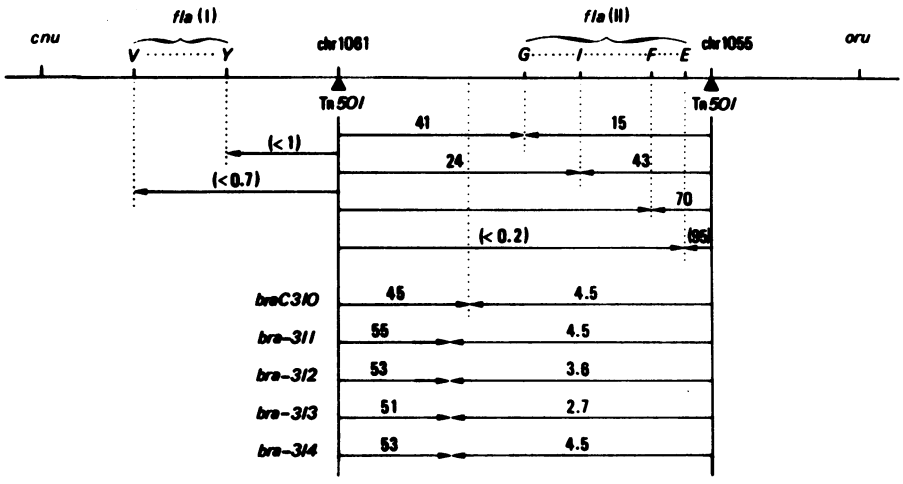

FIG. 2. Linkage of *bra* and *fla* mutations to *chr-1055*::Tn*501* and *chr-1061*::Tn*501* sites. Phage G101, propagated on strain MT1517 or MT1523, was used to select for *chr-1055*::Tn*501* or *chr-1061*::Tn*501* in *fon* derivatives of *bra* strains, respectively. The *chr* transductants were scored for *bra* mutations by testing for growth with 10 μg of azaleucine per ml. Phage G101, propagated on each of the *chr fla* strains, was employed to select for *chr* in strain PAO3506, a *fon* derivative of strain PAO1042. The *chr* transductants were scored for the respective *fla* mutations by testing for motility on nutrient gelatin agar plates. Results are expressed as percentage cotransduction. The values in parentheses are taken from Tsuda and Iino (32).

leucine in addition to TFL with Na^+. Strains PAO3530 through PAO3534, all selected for azaleucine resistance, showed very low levels of LIV-I transport and retained normal levels of LIV-II activity. This finding indicates that the gene(s) specified by mutations *braC310* and *bra-311* through *bra-314* is responsible only for the LIV-I system. Strains MT1552 and MT1562, mutants with a chromosomal deletion between *chr-1061* and *chr-1055* (32), were found to be defective in the LIV-I system alone, confirming that some genes responsible for LIV-I but not for LIV-II are located in the *chr-1061–chr-1055* region. In contrast to the *braA* mutant, these LIV-I-defective strains, including the deletion mutants, were still sensitive to TFL with Na^+.

Our previous reports (14, 16) suggested that the LIVAT-BP found in the shock fluid of *P. aeruginosa* cells participates in the LIV-I transport system. LIVAT-BP production by the *bra* strains was examined. Analysis by SDS-polyacrylamide gel electrophoresis (Fig. 3A) and by binding assay (Table 5) of mutant shock fluids showed that strains PAO3533 (*bra-313*) and PAO3534 (*bra-314*) retained normal levels of LIVAT-BP, as shown by the wild-type strain PAO3501. The *braC* strain PAO3530 was found to produce a LIVAT-BP altered in leucine binding, confirming the previous report (16). On the other hand, strains PAO3510 (*braA300*), PAO3531 (*bra-311*), and PAO3532 (*bra-312*) and deletion mutants MT1552 and MT1562 were defective in the band corresponding to LIVAT-BP, although very low levels of protein(s) were still observed at the same position (Fig. 3A). Since strains MT1552 and MT1562 carry a deletion in a part of the chromosome where the structural gene for the LIVAT-BP is situated, the faint band should not be LIVAT-BP itself but some other protein(s). To make this point clear we performed immunological staining (Fig. 3B). Complete loss of the cross-reacting material at the LIVAT-BP band was revealed in the shock fluids from strains PAO3531, PAO3532, MT1552, and MT1562. However, a low level of cross-reactive material at the LIVAT-BP band was detected in the shock fluid from the *braA* strain PAO3510. To ascertain whether the loss or decrease of LIVAT-BP in shock fluids was due to a reduction in synthesis of the LIVAT-BP or to the alteration of posttranslational processes, the cross-reactive material in total cellular proteins was determined. The production of LIVAT-BP by the cells was completely eliminated by the mutations *bra-311* or *bra-312* or by the deletion of the *chr-1061–chr-1055* region (Fig. 3C). The results further suggests that the reduced level of LIVAT-BP in the shock fluid from the *braA* strain PAO3510 was caused by the low expression of *braC* at the transcriptional or translational level.

DISCUSSION

P. aeruginosa PAO mutants defective in the LIV transport systems showed somewhat differ-

TABLE 4. Three-point crosses by phage G101 transduction for determination of the order of *braC* and nearby markers[a,b]

Donor	Recipient	Selected marker	No. of transductants examined	Transductant classes	% of total
MT1523 (*chr-1061*::Tn*501* *flaG1045*)	PAO3536 (*braC310*)	Hg^r	160	Bra^- Fla^+	56
				Bra^+ Fla^+	0
				Bra^- Fla^-	3
				Bra^+ Fla^-	42
PAO3535 (*braC310*)	MT1523 (*chr-1061*::Tn*501* *flaG1045*)	Fla^+	80	Hg^r Bra^+	8
				Hg^s Bra^+	0
				Hg^r Bra^-	63
				Hg^s Bra^-	30
MT1517 (*chr-1055*::Tn*501* *flaG1045*)	PAO3536 (*braC310*)	Hg^r	160	Bra^- Fla^+	85
				Bra^+ Fla^+	0
				Bra^- Fla^-	8
				Bra^+ Fla^-	7
PAO3535 (*braC310*)	MT1517 (*chr-1055*::Tn*501* *flaG1045*)	Fla^+	79	Hg^r Bra^+	3
				Hg^s Bra^+	6
				Hg^r Bra^-	57
				Hg^s Bra^-	34

[a] *chr-1055*::Tn*501* and *chr-1061*::Tn*501* were examined by mercury resistance. Hg^s and Hg^r signify sensitivity and resistance to 0.2 mM $HgCl_2$, respectively.

[b] *braC310* genotype was examined by azaleucine resistance (20 μg/ml), except for the cross of PAO3535 and MT1517, in which the LIV-I activity of the transductants was measured for determination of the *bra* genotype.

ent TFL resistance patterns than do mutants obtained from strain PML14. In strain PML14, mutants defective in LIV-I or LIV-II alone were sensitive to TFL in the presence of Na^+ (15). Only those defective in both of the LIV transport systems grew under those conditions (15). In strain PAO, a defect in LIV-II alone seems to be enough to confer resistance to TFL with Na^+. Actually, the LIV-II-defective strain PAO3517, with no defect in LIV-I, showed resistance to TFL with Na^+. Strain PAO3510, which lacked LIV-I activity, also showed resist-

TABLE 5. Summary of phenotypes of the LIV transport mutants of *P. aeruginosa* PAO

Strain	Genotype[a]	Growth with:		L-Leucine transport (%)[b] by:		L-Leucine binding activity of shock fluid (%)[c]
		TFL (10 μg/ml) + 20 mM NaCl	Azaleucine (10 μg/ml)	LIV-I	LIV-II	
PAO3501	*bra*$^+$	−	−	100	100	100
PAO3510	*braA300*	+	+	12	72	11
PAO3517	*braB307*	+	−	102	0	115
PAO3530	*braC310*	−	+	14	115	1
PAO3531	*bra-311*	−	+	16	108	0
PAO3532	*bra-312*	−	+	11	94	0
PAO3533	*bra-313*	−	+	9	93	69
PAO3534	*bra-314*	−	+	12	108	66
MT1552	Δ	−	+	14	126	0
MT1562	Δ	−	+	14	132	0

[a] Δ, Deletion of the chromosomal region between *chr-1061*::Tn*501* and *chr-1055*::Tn*501*.

[b] Expressed as percentage of the control (strain PAO3501). Control values (100%) for LIV-I and LIV-II are 14.1 and 16.1 nmol/mg of protein per min, respectively.

[c] Expressed as percentage of the control (strain PAO3501). The control value (100%) is 1.18 nmol/mg of protein.

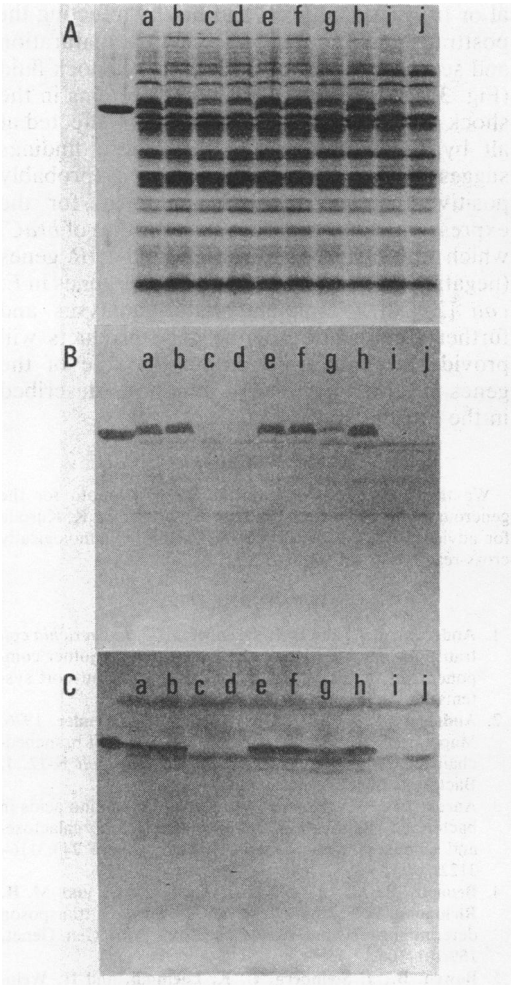

FIG. 3. (A) SDS-polyacrylamide gel electrophoresis of shock fluids. (B) Detection of protein bands in shock fluids immunologically cross-reactive with the LIVAT-BP. (C) Detection of protein bands in whole-cell extracts immunologically cross-reactive with the LIVAT-BP. Strains: (a) PAO3501; (b) PAO3530; (c) PAO3531; (d) PAO3532; (e) PAO3533; (f) PAO3534; (g) PAO3510; (h) PAO3517; (i) MT1552; (j) MT1562. Samples containing about 8 or 150 μg of protein were loaded on a 12% polyacrylamide gel for shock fluids and whole-cell extracts, respectively. Purified LIVAT-BP (0.5 μg) from strain PAO3012 was loaded on the left-end lane.

ance to TFL with Na^+. The resistance, however, should not be due to the loss of LIV-I activity because strains defective in LIV-I alone, including deletion mutants, are still sensitive to TFL with Na^+ (Table 5). Therefore, a partial loss of LIV-II activity may be responsible for the resistance of strain PAO3510 to TFL with Na^+. In contrast, it is clear that sensitivity to azaleucine parallels LIV-I activity, which is consistent with the previous finding that azaleucine is taken up via the LIV-I and not the LIV-II system (16).

Using PAO mutants, we identified at least three genes, *braA*, *braB*, and *braC*, responsible for the LIV transport systems, with resistance to TFL with Na^+ or to azaleucine as their phenotypes. All of the recombinants tested that received resistance to TFL with Na^+ or to azaleucine showed the same properties in the LIV transport systems and in LIVAT-BP production as their donors (data not shown). Genetic mapping showed that the *bra* genes are not clustered on the *P. aeruginosa* PAO chromosome: *braA* is located in the *met-9020–dcu-9108* region; *braB* in the *nar-9011–puuC10* region; *braC* in a region near the *fla* genes. Previously we isolated *P. aeruginosa* PML14 mutants defective in LIV-I or LIV-II alone, suggesting that the LIV-I and LIV-II systems are genetically separate (15). No gene, however, was identified in strain PML14 mutants that might be responsible for the LIV transport systems, since it is very difficult to analyze PML14 strains genetically. Therefore, we could not obtain conclusive evidence for PML14 mutants to show the genetic separation of the LIV transport systems. In contrast, this study, with PAO mutants, clearly shows the genetic separation of the LIV-I and LIV-II systems. Namely, the *braB* gene is responsible for LIV-II alone and the *braC* gene is responsible for LIV-I alone.

Our previous report (16) showed that strain PAO3530 (*braC310*), a mutant selected for resistance to azaleucine, produces an LIVAT-BP altered in substrate binding, suggesting that *braC* is the structural gene for the LIVAT-BP. In this study, we isolated LIV-I-defective mutants with properties different from strain PAO3530 by selecting for resistance to azaleucine. Strains PAO3533 (*bra-313*) and PAO3534 (*bra-314*) produced functional LIVAT-BP, as far as could be determined by the binding assay and polyacrylamide gel electrophoresis (Table 5, Fig. 3). Therefore, mutations *bra-313* and *bra-314* seem to be located in a gene(s) for an additional component(s) for the LIV-I system. Strains PAO3531 (*bra-311*) and PAO3532 (*bra-312*) showed a complete loss of the LIVAT-BP in their shock fluids. Furthermore, these strains synthesized no material that was immunologically cross-reactive with the LIVAT-BP (Fig. 3C). These findings suggest that mutations *bra-311* and *bra-312* are located not in the structural region of the *braC* gene but in the regulatory gene(s) or region of *braC*.

Anderson and Oxender (1) identified four genes, *livJ*, *livK*, *livH*, and *livG*, responsible for the high-affinity transport system for branched-chain amino acids (LIV-I) in *Escherichia coli*. *livJ* and livK encode the LIV- and leucine-

specific binding proteins, respectively. *livH* and *livG* seem to code for additional components of the transport system. Yamato and Anraku (34) recently reported that *hrbC* is responsible for the LIV-1 system (probably identical to LIV-I by Anderson and Oxender) and have suggested that *hrbC* is allelic with either *livH* or *livG*. These genes are all located near *malT* on the *E. coli* genetic map (1, 34) and have been cloned into λ phage and a plasmid vector (26, 27). The present study suggests that the genes for LIV-I are also clustered in the *P. aeruginosa* PAO chromosome. Transduction analyses have shown that all of *braC310* and *bra-311* through *bra-314* are closely linked and are located between *chr-1061* and *flaG* (Fig. 2, Table 4). The fact that strains with a chromosomal deletion between *chr-1061* and *chr-1055* are defective in the LIV-I system and in LIVAT-BP production supports the idea that the genes responsible for the LIV-I system are clustered at the *chr-1061–flaG* region. However, we do not yet have conclusive evidence that mutations *bra-311* through *bra-314* specify gene(s) for other component(s) than the LIVAT-BP.

In *E. coli*, the *livP* gene, which is responsible for the low-affinity nonrepressible transport system for branched-chain amino acids (LIV-II), is closely linked to *livJKHG* (1), although *hrbA*, another gene responsible for the system, is located in the *proC* region apart from the *liv* genes (35). In *P. aeruginosa*, however, it is not likely that a gene for LIV-II is located near the genes for LIV-I, because strains with a chromosomal deletion between *chr-1061* and *chr-1055* still retain normal LIV-II activities compared with the wild-type strain PAO3501 (Table 5). Only one gene, *braB*, has been identified for the *P. aeruginosa* LIV-II system.

Strain PAO3510 (*braA300*) has a marked defect in LIV-I and a partial defect in LIV-II and shows resistance to TFL with Na^+ (Table 5). As far as was investigated, with TFL resistance used as a phenotypic marker of the mutation, *braA* was mapped at the *met-9020–dcu-9108* region, apart from the other *bra* genes. When Met^+ recombinants were selected after the cross between met^+ *braA* and *met-9020* bra^+ strains, all of the recombinants with the TFL^r phenotype showed decreases in both LIV transport system activities (data not shown). In reverse, the recombinants sensitive to TFL with Na^+ retained both activities. Therefore, we assume that the *braA* gene is required for both LIV transport systems, although the defect in LIV-I itself is not responsible for the TFL^r phenotype of the *braA* mutation. We found that the *braA310* mutation causes a decrease in the level of LIVAT-BP in shock fluid by reducing the expression of the *braC* gene at the transcriptional or translational level and not by affecting the posttranslational processes, such as maturation and secretion of LIVAT-BP into the shock fluid (Fig. 3). Production of the other proteins in the shock fluid, on the other hand, is not affected at all by the mutation (Fig. 3). These findings suggest that *braA* is a gene for a (probably positively acting) regulatory element for the expression of *bra* genes, at least for that of *braC*, which contrasts with the *livR* and *lstR* genes (negatively acting elements for the *liv* genes in *E. coli* [2, 20]). Complementation analyses and further identification of the gene products will provide more information on the role of the genes specified by the *bra* mutations described in the present study.

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