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# Cloning, Nucleotide Sequences, and Identification of Products of the *Pseudomonas aeruginosa* PAO *bra* Genes, Which Encode the High-Affinity Branched-Chain Amino Acid Transport System

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**A DNA fragment of *Pseudomonas aeruginosa* PAO containing genes specifying the high-affinity branched-chain amino acid transport system (LIV-I) was isolated. The fragment contained the *braC* gene, encoding the binding protein for branched-chain amino acids, and the 4-kilobase DNA segment adjacent to 3′ of *braC*. The nucleotide sequence of the 4-kilobase DNA fragment was determined and found to contain four open reading frames, designated *braD*, *braE*, *braF*, and *braG*. The *braD* and *braE* genes specify very hydrophobic proteins of 307 and 417 amino acid residues, respectively. The *braD* gene product showed extensive homology (67% identical) to the *livH* gene product, a component required for the *Escherichia coli* high-affinity branched-chain amino acid transport systems. The *braF* and *braG* genes encode proteins of 255 and 233 amino acids, respectively, both containing amino acid sequences typical of proteins with ATP-binding sites. By using a T7 RNA polymerase/promoter system together with plasmids having various deletions in the *braDEFG* region, the *braD*, *braE*, *braF*, and *braG* gene products were identified as proteins with apparent $M_r$s of 25,500, 34,000, 30,000, and 27,000, respectively. These proteins were found among cell membrane proteins on a sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie blue.**

Active transport of the branched-chain amino acids L-leucine, L-isoleucine, and L-valine across the *Pseudomonas aeruginosa* cytoplasmic membrane is mediated by two distinct systems, LIV-I and LIV-II (13). The low-affinity LIV-II transport system is specific for branched-chain amino acids alone and is mediated by a $Na^+$-coupled carrier, the product of the *braB* gene, which has been cloned (14, 18, 20). The high-affinity LIV-I transport system is operative without $Na^+$ and is specific for alanine and threonine in addition to branched-chain amino acids (13). Several lines of evidence suggest that a periplasmic binding protein (BP) is associated with the LIV-I transport system. Osmotic shock treatment of cells causes a preferential decrease in LIV-I transport activity (13). The system is lost in membrane vesicles (14). The properties of the BP for branched-chain amino acids (LIVAT-BP) purified from the shock fluid of *P. aeruginosa* cells are similar to those of the LIV-I system in substrate specificity and affinity (15). The LIVAT-BP and LIV-I transport system are concomitantly altered in a *braC310* mutant of *P. aeruginosa* PAO (19). Both defects in the *braC* mutant have recently been shown to be complemented by the cloned *braC* gene (16), confirming the involvement of LIVAT-BP in the LIV-I transport system.

*P. aeruginosa* PAO mutants defective in LIV-I with phenotypes different from that of the *braC310* mutant have been isolated (20). Transductional analysis shows that all of the mutations, including *braC310*, are closely linked and are located between the *chr-1061*::Tn*501* and *chr-1055*::Tn*501* loci on the *P. aeruginosa* PAO chromosome (20). The LIV-I defect in MT1562, a PAO strain with a chromosomal deletion between *chr-1061* and *chr-1055*, is not complemented by the cloned *braC* gene (16). These facts strongly suggest that genes for components other than LIVAT-BP for the LIV-I transport system are also located between the *chr-1061* and *chr-1055* loci.

In this report, we describe the cloning and nucleotide sequence of the DNA segment required for complementation of the LIV-I defect in MT1562. We also present the deduced amino acid sequences and identification of the products of the genes, *braD*, *braE*, *braF*, and *braG*, contained in the cloned DNA fragment. A rationale for the requirement of these genes for the LIV-I transport system is described in the accompanying paper (17).

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* HB101 and JM109 were used as hosts for plasmids derived from RSF1010 and pBR322, respectively.

**Media and growth conditions.** LB broth and agar (27) were used to grow *E. coli* cells. Nutrient broth and agar (21) and two minimal media, G medium (14) and D medium (13), were used to grow *P. aeruginosa* strains. Amino acids or antibiotics, when needed, were added to final concentrations as described previously (16). All strains were grown aerobically at 37°C unless otherwise indicated.

**Manipulation of DNA.** RSF1010-derived plasmid DNA was isolated by the boiling method of Holmes and Quigley (12), and pBR322-derived plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (5). Procedures for cloning and restriction analysis were basically as described by Maniatis et al. (27). Transformation of *P. aeruginosa* strains was carried out by the method of Sano and Kageyama (36).

**DNA sequencing.** The DNA sequence was determined with the 7-Deaza Sequencing Kit (Takara Shuzo, Kyoto, Japan) by the dideoxy-chain termination method of Sanger et al. (35). Plasmids having unidirectional deletions of the DNA fragments cloned to pUC18 or pUC19 were generated with the Deletion Kit (Takara Shuzo) as described by Yanisch-

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TABLE 1. Strains and plasmids used

| Strain or plasmid | Relevant genotype | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| HB101 | F⁻ *recA13 hsdR hsdM leu pro* λ⁻ | 6 |
| JM109 | *recA1 endA1 gyrA1 hsdR17* Δ(*lac-proAB*)(F′ *traD36 proAB lacI*$^q$ ZΔM15) λ⁻ | 45 |
| B7634 | *ileA hrbA hrbB hrbC hrbD* | 44 |
| K38 | HfrC | 34 |
| *P. aeruginosa* | | |
| PAO3012 | *trp-6* | 22 |
| MT1562 | *argB18 chl-2 rif-8001* Δ(*chr-1061*::Tn*501-chr-1055*::Tn*501*) | 41 |
| Plasmids | | |
| pUC18 | Cb$^r$ (Ap$^r$) | 45 |
| pUC19 | Cb$^r$ (Ap$^r$) | 45 |
| pKT240 | Cb$^r$ (Ap$^r$) Km$^r$ | 2 |
| pTH1 | Km$^r$ λ *cos braC* | 16 |
| pGP1-2 | Km$^r$ cI857 *1*$^a$ | 39 |
| PT7-5 | Cb$^r$ (Ap$^r$) | S. Tabor |
| PT7-6 | Cb$^r$ (Ap$^r$) | S. Tabor |

[a] The structural gene for the bacteriophage T7 RNA polymerase, which is under the control of the bacteriophage promoter $p_L$ in pGP1-2.

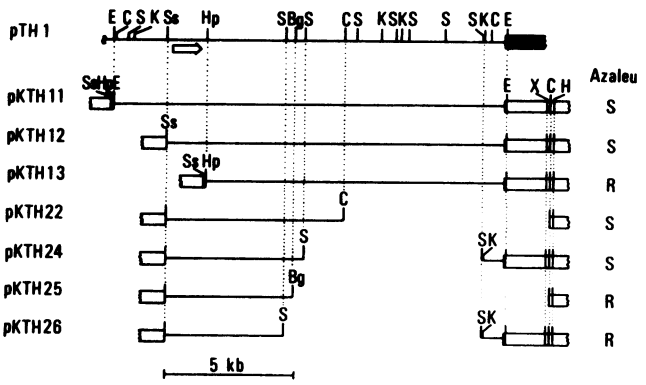


FIG. 1. Cloning and localization of genes necessary for restoration of the LIV-I transport activity to *P. aeruginosa* MT1562. The restriction map of part of the DNA fragment carried on pTH1 (16) is shown at the top. The open arrow under the map indicates the location and direction of transcription of *braC*, the structural gene for LIVAT-BP (16). The 15-kb *Eco*RI fragment of pTH1 was cloned into pKT240, generating pKTH11. The other pKTH plasmids shown are the deletion derivatives of pKTH11. Restoration of azaleucine sensitivity to MT1562 transformed by the plasmids was examined as described in the text. S, Sensitive; R, resistant. Restriction sites are abbreviated as follows: Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; S, *Sal*I; Ss, *Sac*I; X, *Xho*I.

Perron et al. (45). DNA templates for sequencing were prepared from the double-stranded plasmids by the method of Hattori and Sakaki (9).

**Exclusive labeling of plasmid proteins by using a T7 RNA polymerase/promoter system.** *E. coli* cells containing both pGP1-2 and a pT7 recombinant plasmid grown at 30°C to the mid-exponential phase were harvested, washed once, and suspended in the same volume of Vogel-Bonner medium (42) supplemented with 20 μg of thiamine and 50 μg of each amino acid except cysteine and methionine per ml. A 1-ml sample of cells was incubated at 30°C for 30 min and then at 42°C for 15 min. The cell suspension was supplemented with rifampin (20 μg/ml) and left at 42°C for additional 10 min. The cells were incubated at 30°C for 20 min and then pulsed with 15 μCi of [$^{35}$S]methionine for 5 min. Cells were collected by centrifugation for 1 min and suspended in 100 μl of sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (24). The samples were left overnight at room temperature and incubated at 37°C for 30 min before electrophoresis.

**Preparation of membrane fractions.** Membranes from a small-scale culture (5 ml) were prepared at 4°C or ice-chilled temperature unless otherwise stated. Harvested cells were washed once with 10 ml of 10 mM Tris hydrochloride (pH 7.8) containing 0.1 M NaCl and 0.1 mM dithiothreitol (TND buffer) and resuspended in 1 ml of TND buffer. Cells were disrupted by sonication with a Branson Sonifier. The sonicated suspension was centrifuged twice at 6,000 × *g* for 10 min to remove unbroken cells. The supernatant was centrifuged at 40,000 × *g* for 1 h to sediment membranes. Fractionation of inner and outer membranes was carried out with a 100-ml-scale culture by the method of Osborn et al. (32), using a 25 to 55% sucrose density gradient.

**Other methods.** Transport activities of *P. aeruginosa* and *E. coli* strains were assayed with whole cells grown in G medium and LB broth, respectively. The initial rates of leucine transport via the LIV-I system were determined at 37°C with 10 mM glucose and 2 μM [U-$^{14}$C]leucine as described previously (13). SDS-PAGE was carried out by the method of Laemmli (24) on a 12% gel. The protein content of the preparations was determined by the method of Lowry et al. (26), with bovine serum albumin used as a standard.

**Enzymes and chemicals.** Restriction endonucleases were purchased from Toyobo (Osaka, Japan) or Takara Shuzo. A large fragment of *E. coli* DNA polymerase (Klenow fragment) and T4 DNA ligase were also from Takara Shuzo. Azaleucine and rifampin were from Sigma Chemical Co. (St. Louis, Mo.). [U-$^{14}$C]leucine (342 mCi/mmol), [$^{35}$S]methionine (1,000 Ci/mmol), and [α-$^{32}$P]dCTP (400 Ci/mmol) were obtained from Amersham (Buckinghamshire, England). All other chemicals used were commercial products of analytical grade.

## RESULTS

**Cloning of the DNA fragment containing a gene cluster for LIV-I.** Plasmid pTH1, derived from pMMB34, contains a 40-kilobase-pair (kb) chromosomal DNA fragment around the *braC* gene (16). We initially attempted to determine whether pTH1 or its derivatives could confer the LIV-I transport activity on *P. aeruginosa* MT1562, which has an extensive chromosomal deletion around the *braC* gene. This strain was found, however, to be rather resistant to kanamycin, making it difficult to select transformants or exconjugates carrying the pMMB34-derived plasmids. Thus, various *Eco*RI fragments of pTH1 were subcloned into pKT240 having a Cb$^r$ marker, another shuttle vector derived from RSF1010. *P. aeruginosa* MT1562 transformants of these plasmids were tested for sensitivity to azaleucine, a toxic leucine analog specific for the LIV-I transport system (19). Plasmid pKTH11, carrying the 15-kb *Eco*RI fragment, was found to confer sensitivity on MT1562. A restriction map of the *Eco*RI fragment carried on pKTH11 is shown in Fig. 1. To locate precisely the genes capable of conferring azaleucine sensitivity, various deletion derivatives of pKTH11 were constructed (Fig. 1). Elimination of the DNA region upstream of the *braC* gene did not affect the ability to confer azaleucine sensitivity on MT1562, suggesting that no gene

TABLE 2. Leucine uptake by various *P. aeruginosa* and *E. coli* strains

| Strain | Leucine uptake (nmol/mg of protein per min) |
|---|---|
| PAO3012(pKT240) | 8.6 |
| PAO3012(pKTH24) | 41.3 |
| MT1562(pKT240) | 0.7 |
| MT1562(pKTH11) | 24.1 |
| MT1562(pKTH12) | 21.5 |
| MT1562(pKTH13) | 1.0 |
| MT1562(pKTH22) | 21.8 |
| MT1562(pKTH24) | 25.1 |
| MT1562(pKTH25) | 0.9 |
| MT1562(pKTH26) | 0.8 |
| B7634(pUC18) | 0.4 |
| B7634(pUBR8) | 30.1 |
| B7634(pUBR9) | 0.3 |

required for the LIV-I transport system is involved in this region. The 5.3-kb *Sac*I-*Sal*I fragment (i.e., the *braC* gene and the 4-kb fragment adjacent to 3′ of *braC*) was found to be large enough to restore azaleucine sensitivity to strain MT1562, whereas the 5.0-kb *Sac*I-*Bgl*II fragment failed to do so.

Leucine transport activities of the strains transformed with pKTH11 and its derivatives were determined under the LIV-I assay conditions (Table 2). All of the plasmids conferring azaleucine sensitivity regained the leucine uptake by LIV-I in strain MT1562 to a level three- to fivefold higher than that in wild-type strain PAO3012. Plasmid pKTH24 also enhanced severalfold the LIV-I transport activity in PAO3012, showing a gene dosage effect. The 5.3-kb *Sac*I-*Kpn*I fragment carried on pKTH24 was further subcloned into pUC18 and pUC19, generating pUBR8 and pUBR9, respectively. Plasmid pUBR8 restored leucine uptake to strain B7634, an *E. coli* mutant defective in the branched-chain amino acid transport systems (Table 2), suggesting strongly that all of the genes necessary for the LIV-I system are retained in the 5.3-kb *Sac*I-*Sal*I fragment carried on pKTH24. The fact that plasmid pUBR9 failed to confer leucine transport activity on B7634 suggests that expression of the genes for LIV-I carried on pUBR8 is due to the *lac* promoter of pUC18.

**Nucleotide sequence of the *bra* gene cluster for LIV-I.** The nucleotide sequence of the 5.3-kb *Sac*I-*Sal*I fragment was determined by the dideoxy-chain termination method (35). The sequencing strategy and the relevant restriction sites are shown in Fig. 2. Sequencing with each clone having a unidirectional deletion in the cloned fragment was carried out at least twice, and the entire sequences of both strands were determined. Figure 3 shows the nucleotide sequence of the *bra* gene cluster for the LIV-I transport system. Four long open reading frames, positions 1538 to 2458, 2458 to 3708, 3708 to 4472, and 4478 to 5176, were found in the region downstream from the *braC* gene (Fig. 2 and 3). These reading frames are preceded by possible ribosome-binding sites (Shine-Dalgarno sequences). We designated them *braD*, *braE*, *braF*, and *braG*, respectively, on the basis of the observations described below.

The intercistronic region between the *braC* and *braD* genes is 260 base pairs (bp) in length and is the only extensive intercistronic region within the *bra* gene cluster. The *braF* and *braG* genes are separated by only 2 bp excluding the stop codon, whereas no intercistronic space was found between the *braD*, *braE*, and *braF* genes. The

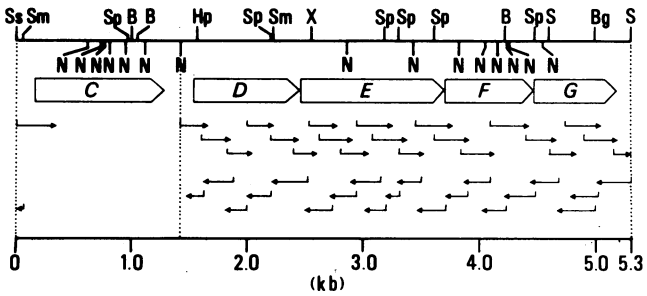


FIG. 2. Restriction map and sequencing strategy for the *bra* genes and their flanking regions. The nucleotide sequence of the *Sma*I-*Hpa*I fragment containing the *braC* gene has been described previously (16). The arrows indicate sequencing directions (5′ to 3′) and lengths of sequences determined by the dideoxy-chain termination method. The plasmid clones for sequencing were constructed by unidirectional deletion of the insert of pUBR8 or by subcloning appropriate restriction fragments into pUC18. The open arrows under the map show the location and direction of transcription of the open reading frames for the *braC*, *braD*, *braE*, *braF*, and *braG* genes. Plasmid pUBR8 contains the additional *Sal*I-*Kpn*I fragment consisting of 39 bp (Fig. 1), which is not shown. Restriction site abbreviations are as for Fig. 1, with the following additions: B, *Bam*HI; N, *Nae*I; Sm, *Sma*I; Sp, *Sph*I.

initiation codons of *braE* and *braF* overlap the termination codons of *braD* and *braE*, respectively. We showed previously (16) that there exists in the 3′-flanking region of *braC* a palindromic sequence with several T bases (positions 1295 to 1325) typical of the Rho-independent transcriptional termination signals (33). Such a sequence was also found in the 3′-flanking region of *braG*. This dyad symmetry with a T cluster (positions 5212 to 5237) could lead to the formation of a stable stem-loop structure in the corresponding transcript with a calculated free energy value of −27.0 kcal (ca. 113.0 kJ) (40).

**Amino acid sequences of the *bra* gene products.** The *braD*, *braE*, *braF*, and *braG* genes specify proteins of 307, 417, 255, and 233 amino acid residues with molecular masses of 32,511, 45,558, 28,281, and 25,590 daltons, respectively. The amino acid sequences of these gene products deduced from the nucleotide sequences are shown in Fig. 3. Amino acid compositions indicate that the BraD and BraE proteins are highly hydrophobic (74% nonpolar). The BraE protein contains 21 acidic and 33 basic amino acid residues, giving an excess of 12 positive charges at neutral pH, showing that the BraE protein is extremely basic. The BraD, BraF, or BraG protein, on the other hand, gives an excess of only one basic, two basic, or two acidic residues, respectively. The hydropathy profiles of the Bra proteins were obtained by a nine-residue span by the method of Kyte and Doolittle (23) (Fig. 4). The profiles clearly show that the BraD and BraE proteins have about 10 hydrophobic segments of an average length of 20 amino acid residues which are very likely to span the cell membrane, whereas the BraF and BraG proteins are hydrophilic throughout their sequences.

Comparison of the amino acid sequences of the Bra proteins revealed extensive identity over the entire regions of BraF and BraG: 34% of the total residues of BraG were identical with those of BraF when gaps of 5 and 15 residues were introduced in the N-terminal and middle regions, respectively, of the BraG sequence. If the conservative substitutions (29) were considered, the BraG protein showed 56% identity with the BraF protein. Both proteins contain the sequences G-X-X-G-X-G-K-T/S and h-h-h-h-D-E (h rep-

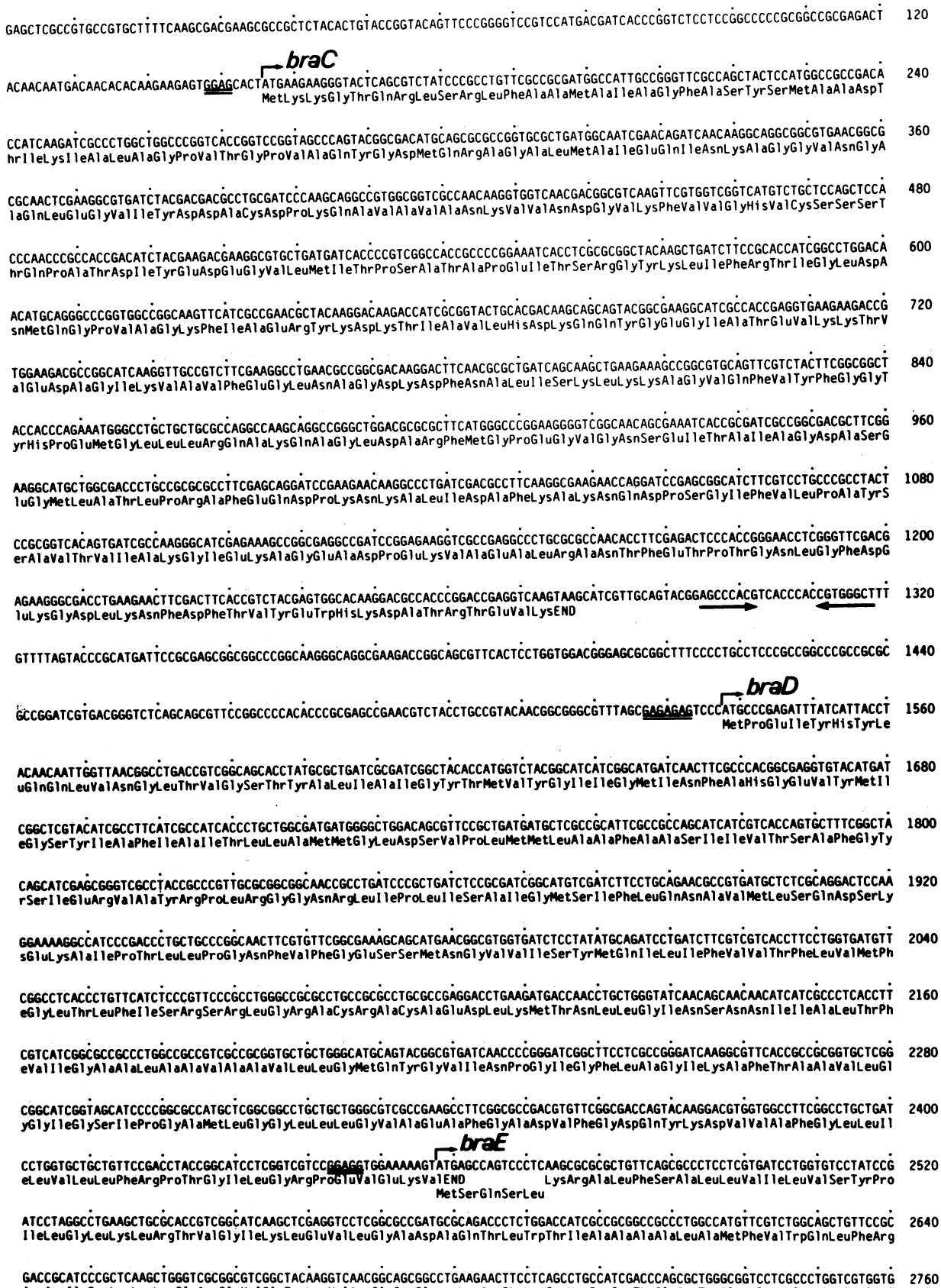


FIG. 3. Complete nucleotide sequence of the 5.3-kb *Sac*I-*Sal*I fragment and the deduced amino acid sequences of the *braC*, *braD*, *braE*, *braF*, and *braG* gene products. Nucleotide numbering begins with the first base of the *Sac*I site. The probable ribosome-binding sites (Shine-Dalgarno sequences) of the *bra* genes are shown by the double lines. The putative transcriptional termination signal in the intercistronic region between *braC* and *braD* and that in the 3′-flanking region of *braG* are indicated by the converging arrows.

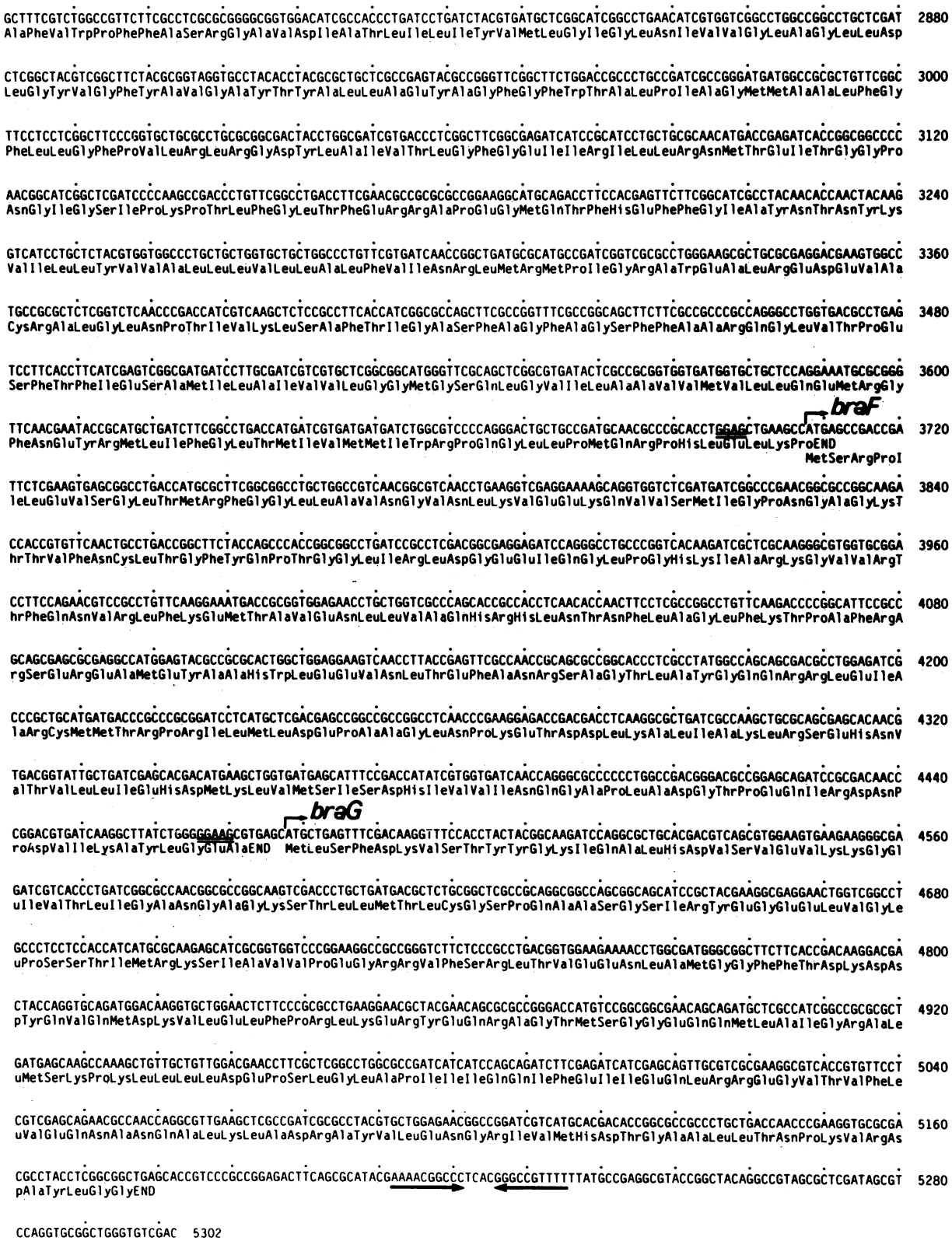


FIG. 3—*Continued.*

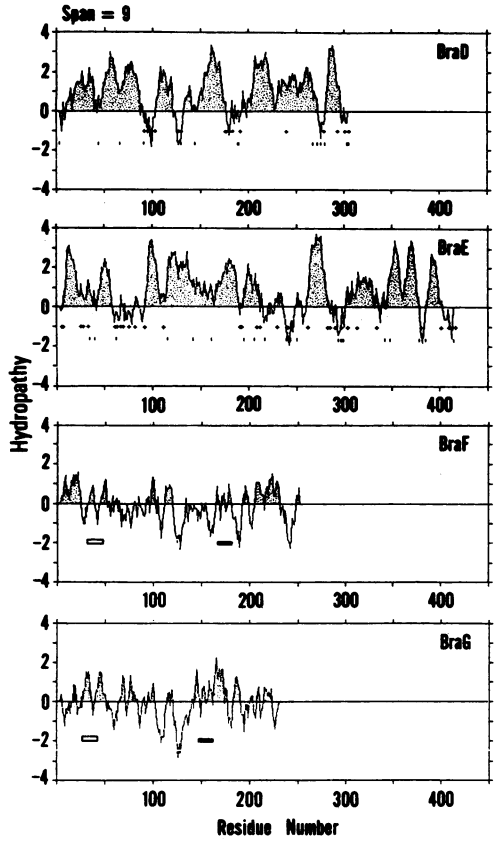


FIG. 4. Hydropathy profiles of the BraD, BraE, BraF, and BraG proteins. Positions of the charged amino acid residues of the BraD and BraE proteins are also shown: +, Lys or Arg; |, Asp or Glu. The open and solid bars in the profiles of BraF and BraG indicate the locations of the sequences containing G-X-X-G-X-G-K-T/S and h-h-h-h-D-E (h represents hydrophobic amino acid), respectively, both of which are considerably conserved among the ATP-binding components involved in periplasmic BP-dependent transport systems in *E. coli* and *S. typhimurium* (1, 11).

resents hydrophobic amino acid) (Fig. 3), which are known to be consensus sequences for ATP-binding proteins (1, 43). No significant homology was found between BraD and BraE, the intrinsic membrane proteins. However, the BraD protein showed striking homology with the *livH* gene product, a component required for the *E. coli* high-affinity branched-chain amino acid transport systems (LIV-I and Ls) (30). The BraD and LivH proteins are of a similar size, containing 307 and 308 amino acids, respectively. The amino acid sequences are extensively conserved, giving 205 identical amino acid residues (67%) and 59 conservative substitutions (19%).

**Identification and localization of the *bra* gene products.** To identify the *bra* gene products by controlled expression with the T7 RNA polymerase/promoter system of Tabor and Richardson (39), plasmid pT7-5 and pT7-6 derivatives carrying various portions of the *bra* gene cluster were constructed to generate the pTDG plasmids (Fig. 5). These plasmids were introduced into strain K38(pGP1-2) and tested for expression of the *bra* genes. When the strain carrying pTDG50 was heat induced, four proteins with apparent $M_r$s of 25,500, 27,000, 30,000, and 34,000 were detected (Fig. 6, lane a). On the other hand, none of the proteins were synthesized by the strain carrying pTDG50 incubated at 30°C

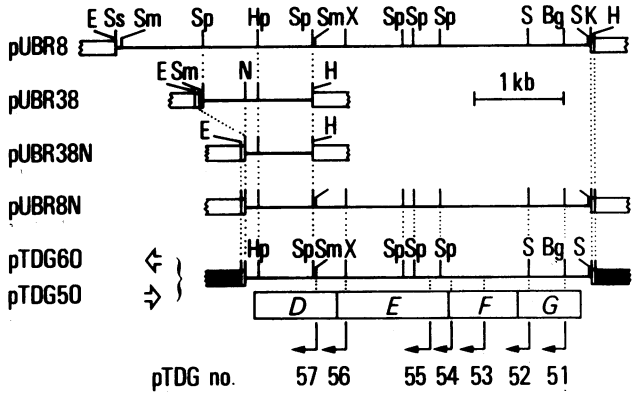


FIG. 5. Construction of pT7 derivatives for controlled expression of the *braD*, *braE*, *braF*, and *braG* genes. The 1.2-kb *Sph*I fragment of pUBR8 was subcloned into pUC18, generating pUBR38. Plasmid pUBR38N is a deletion derivative of pUBR38 lacking the 0.7-kb *Sma*I-*Nae*I fragment. Plasmid pUBR8N was constructed from pUBR38N by replacing the 0.7-kb *Hpa*I-*Hin*dIII fragment with the 3.7-kb *Hpa*I-*Hin*dIII fragment of pUBR8. The 3.9-kb *Eco*RI-*Hin*dIII fragment of pUBR8N was subcloned into pT7-5 and pT7-6, generating pTDG50 and pTDG60, respectively. Derivatives of pTDG50 having unidirectional deletions from *braG* toward *braD* were further constructed. pTDG51, -52, -56, and -57 were generated by digestion with appropriate restriction endonucleases. pTDG53, -54, and -55 were constructed by replacing the *Xho*I-*Hin*dIII fragment of pTDG50 with those of the pUBR8 derivatives having unidirectional deletions from the *Kpn*I site toward *Sac*I site. Bent arrows indicate the 3′ endpoints of the inserts retained by the pTDG plasmids shown. Open arrows mark the directions of transcription by the T7 promoter derived from pT-7 plasmids.

(noninduced) or by the strains carrying pT7-5 and pTDG60 incubated at 42°C (induced) (data not shown). These results show that the genes for these four proteins are retained in the 3.9-kb *Nae*I-*Sal*I fragment of pTDG50 and are transcribed

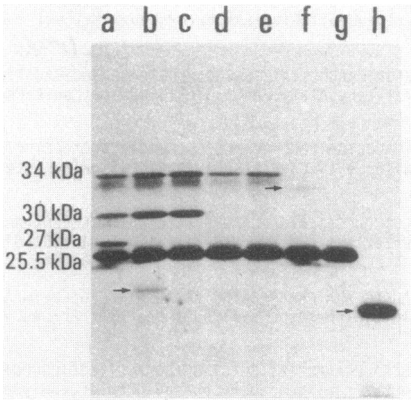


FIG. 6. Controlled expression of the *braD*, *braE*, *braF*, and *braG* gene products. Samples for SDS-PAGE were prepared from *E. coli* K38 cells harboring pGP1-2 and a pTDG plasmid as described in the text. After electrophoresis, the polyacrylamide gel was treated with Enlightning (Dupont, NEN Research Products, Boston, Mass.), dried under vacuum, and exposed to a Kodak X-Omat film at room temperature. Molecular weights of the *bra* gene products were calibrated with Rainbow colored protein molecular weight markers (Amersham). Lanes: a, pTDG50; b, pTDG51; c, pTDG52; d, pTDG53; e, pTDG54; f, pTDG55; g, pTDG56; h, pTDG57. The arrows for the protein bands in lanes b, f, and h indicate the truncated *braG*, *braE*, and *braD* gene products, respectively.

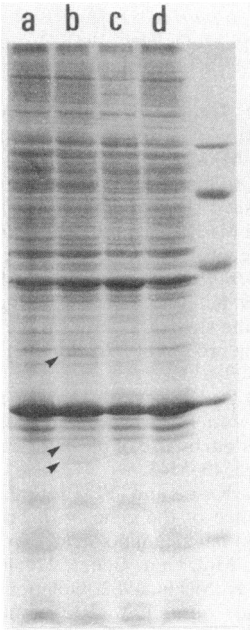


FIG. 7. SDS-PAGE of membrane proteins. Cell membranes were prepared from *E. coli* K38(pGP1-2) carrying pT7-5 (lane a), pTDG50 (lane b), pTDG60 (lane c), and pT7-6 (lane d) as described in the text. Samples containing about 50 μg of protein were electrophoresed and stained with Coomassie brilliant blue R250. Protein bands corresponding to BraD (25,500), BraG (27,000), and BraE (34,000) are indicated by arrowheads. Molecular weight standards used were trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), and phosphorylase *b* (94,000) and are shown in the rightmost lane.

from the *Nae*I site toward the *Sal*I site. The open reading frames designated *braD*, *braE*, *braF*, and *braG* are the only frames large enough to encode the detected proteins. Expression of the genes retained in the various clones having unidirectional deletions from the *Sal*I site was analyzed to assign the four proteins to the *bra* genes (Fig. 6). The protein band of apparent $M_r$ 27,000 was missing in the clones carrying pTDG51 and pTDG52 with 3′ endpoints within the *braG* gene, indicating that the protein is coded for by *braG*. In the same way, the proteins with apparent $M_r$s of 25,500, 30,000, and 34,000 were shown to be the products of the *braD*, *braF*, and *braE* genes, respectively (Fig. 5 and 6).

Cell membranes were prepared from strain K38(pGP1-2) carrying pT7-5, pT7-6, pTDG50, or pTDG60 grown at 42°C for 30 min and then at 37°C for 2 h with rifampin. Analysis of membrane proteins by SDS-PAGE showed the existence of proteins corresponding to the products of *braD*, *braE*, and *braG* only in the membrane from the strain carrying pTDG50 (Fig. 7). Detection of the *braF* product (apparent $M_r$ of 30,000) was difficult because of migration of a major membrane protein to the same position as the *braF* product. On the other hand, no difference was found in the patterns of cytoplasmic proteins among the strains (data not shown), confirming that the *bra* products are located on the cell membrane. Fractionation of the inner and outer membranes further suggested that the *bra* products are localized on the cytoplasmic membrane (data not shown).

## DISCUSSION

This study shows that the DNA fragment required for restoration of the LIV-I transport activity to *P. aeruginosa* MT1562 contains four open reading frames, designated *braD*, *braE*, *braF*, and *braG*, in addition to *braC*, the structural gene for the LIVAT-BP (16). The G+C contents of the *braD*, *braE*, *braF*, and *braG* genes were calculated as 63.0, 65.9, 65.4, and 64.4%, respectively, similar to that (65%) reported previously (38) for the average G+C content of the *P. aeruginosa* genome. Recent compilation of *P. aeruginosa* genes shows that codons with G or C at the third position are preferentially utilized in this organism (4, 8, 16). The G+C contents of the third positions in the codons used for *braD*, *braE*, *braF*, and *braG* are 91.5, 93.5, 92.9, and 92.3%, respectively, showing the codon usage typical of *P. aeruginosa* genes. Analysis of the controlled expression of genes with the T7 RNA polymerase/promoter system (Fig. 5 and 6) revealed that the open reading frames for *braD*, *braE*, *braF*, and *braG* are used to encode proteins. The fact that plasmid pKTH25 lacking the *Bgl*II-*Sal*I fragment (positions 4994 to 5297) failed to confer LIV-I transport activity to *P. aeruginosa* MT1562 (Table 2) strongly suggests the involvement of the *braG* product in the LIV-I transport system. The genetic analysis in the accompanying paper (17) further confirms that all of the *bra* genes identified in this study are required for the LIV-I transport system.

The apparent $M_r$s of the *braF* and *braG* products estimated from SDS-PAGE are 30,000 and 27,000, respectively, similar to those from the deduced amino acid sequences. On the other hand, the apparent $M_r$s of the *braD* and *braE* products from SDS-PAGE are 25,500 and 34,000, respectively, which are considerably smaller than the $M_r$s 32,511 and 45,558 from the deduced amino acid sequences. Such discrepancies seem to be common for intrinsic membrane proteins such as these *bra* products, presumably because of an abnormally higher capacity for binding of SDS (7). If translation of these genes starts at an ATG or GTG codon located inside the sequences, for example, ATG for Met-30 or Met-37 in the *braD* gene, the molecular weights would be much closer to those estimated from SDS-PAGE. However, *phoA* fusions to the *braD* and *braE* genes with the Tn*phoA* transposon (28) suggest that the first ATG codons in their respective reading frames are the likely translation initiation sites (unpublished results).

Recent genetic studies combined with recombinant DNA techniques have revealed extensively the molecular basis of the periplasmic BP-dependent transport systems in *E. coli* and *Salmonella typhimurium* (1, 10, 11). Most of the BP-dependent systems seem to require membrane components consisting of two intrinsic membrane proteins and a protein having consensus sequences for ATP-binding proteins. However, the ribose and arabinose transport systems in *E. coli* have been found to require a gene product having two ATP-binding domains (3, 37). In addition, the oligopeptide transport system in *S. typhimurium* has recently been shown to require two gene products, each of which contains an ATP-binding domain (11). The *P. aeruginosa* LIV-I transport system provides another example of the requirement of two ATP-binding proteins for the periplasmic BP-dependent transport systems: the *braF* and *braG* genes encode ATP-binding proteins (Fig. 3), both of which are necessary for LIV-I function (17). These facts strongly support the following view: the oligopeptide and LIV-I transport systems require two ATP-binding proteins as a heterodimer, whereas the other systems, represented by the *S. typhimurium* histidine transport system (10), with a single gene for an ATP-binding protein, require such proteins as a homodimer.

Nazos and colleagues (30, 31) have suggested that three more genes, designated *livH*, *livM*, and *livG*, are necessary

for the LIV-I and Ls transport systems in *E. coli*, in addition to *livJ* and *livK*, encoding the LIV- and Ls-BPs, respectively. The nucleotide sequences of *livJ*, *livK*, and *livH* have been determined (25, 30). We previously showed the striking homology among the *braC*, *livJ*, and *livK* products, BPs for branched-chain amino acids (16). The study presented here shows that the *braD* gene product is homologous to the *livH* gene product. These findings strongly suggest that the molecular organization of the *P. aeruginosa* LIV-I transport system is analogous to that of the *E. coli* transport system. Although no sequence data are yet available for the *livM* and *livG* genes, it seems likely that these *liv* products are the *E. coli* counterparts of two of the *braE*, *braF*, and *braG* products. In this sense, it will be interesting to determine whether a gene other than the known *liv* genes is necessary for the *E. coli* LIV-I and Ls transport systems.

Use of the T7 RNA polymerase/promoter system enabled us to overproduce the *braD*, *braE*, *braF*, and *braG* products in *E. coli* to the level detectable among membrane proteins by staining with Coomassie brilliant blue (Fig. 7). Thus, this system together with the information from the nucleotide sequences of the *bra* genes will provide the basis for elucidating biochemically the structure and function of the *P. aeruginosa* LIV-I transport system.

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