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Isolation of the *braZ* Gene Encoding the Carrier for a Novel Branched-Chain Amino Acid Transport System in *Pseudomonas aeruginosa* PAO

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The *braZ* gene for a novel branched-chain amino acid transport system in *Pseudomonas aeruginosa* PAO was isolated and characterized. Determination of the nucleotide sequence showed that the *braZ* gene comprises 1,311 nucleotides specifying a protein of 437 amino acids. Hydropathy analysis suggested that the product is an integral membrane protein with 12 membrane-spanning segments. The amino acid sequence showed extensive homology to those of the *braB* and *brnQ* gene products, branched-chain amino acid carriers of *P. aeruginosa* and *Salmonella typhimurium*, respectively. By using the T7 RNA polymerase-promoter system, the *braZ* gene product was identified as a protein of an apparent M_r of 34,000 on a sodium dodecyl sulfate-polyacrylamide gel. Properties of the transport system encoded by *braZ* were studied by using *P. aeruginosa* PAO3537, defective in both the high- and low-affinity branched-chain amino acid transport systems (LIV-I and LIV-II, respectively). The transport system encoded by *braZ* was found to be another effective branched-chain amino acid transport system in *P. aeruginosa* PAO and was thus designated as LIV-III. This system is specific for isoleucine and valine, giving the same K_m value of 12 μM for these amino acids. The system was found, however, to have a very low affinity for leucine, with a K_m value of 150 μM, which contrasts with the substrate specificities of LIV-I and LIV-II.

The transport of branched-chain amino acids by *Pseudomonas aeruginosa* is mediated by two distinct systems, LIV-I and LIV-II (6, 11). The high-affinity LIV-I system is dependent on the periplasmic binding protein for branched-chain amino acids and is specific for alanine and threonine in addition to branched-chain amino acids (6). The low-affinity LIV-II system is a carrier-mediated transport system coupled to Na^+ or Li^+ ions (7, 30). The genes required for these transport systems have recently been cloned, and their nucleotide sequences were determined (8–10). A *P. aeruginosa* PAO mutant defective in both LIV-I and LIV-II showed almost complete loss of leucine uptake (10), suggesting that LIV-I and LIV-II were the only systems for the transport of branched-chain amino acids in this organism. In the course of cloning the *braB* gene for the LIV-II carrier (with a phenotype requiring a high concentration of branched-chain amino acids [Hrb^-] [10]), however, we incidentally isolated a gene showing a branched-chain amino acid transport activity with properties different from the known LIV transport systems. The gene was designated *braZ* and further characterized.

In this report, we describe the cloning and nucleotide sequence of *braZ* and the deduced amino acid sequence and identification of the gene product. We also describe the properties of the transport system encoded by *braZ*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* HB101 and JM109 were used as hosts for plasmids derived from RSF1010 and those from pBR322, respectively. All strains were grown aerobically at 37°C as described previously (8) unless otherwise indicated.

Screening for Hrb^+. The *P. aeruginosa* PAO3012 genomic library constructed in cosmid pMMB34 and deposited in *E. coli* HB101(RP4-4) (8) was screened for Hrb^+ as described previously (10) with the following modifications. Each *P. aeruginosa* PAO3536 exconjugate was tested for phenotypes of Hrb^+ (growth at 5 μg of leucine per ml) and Leu^+ on plates based on G medium (7) in place of those on D medium (6).

Manipulation of DNA. Procedures for the preparation of plasmid DNA, cloning, and restriction analysis were basically the same as described previously (8) or by Maniatis et al. (16). 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. (24). Plasmids having unidirectional deletions of the DNA fragments cloned to pUC18 were generated with the Deletion Kit (Takara Shuzo) as described by Yanisch-Perron et al. (33).

Identification of the *braZ* gene product. The T7 RNA polymerase-promoter system of Tabor and Richardson (26) was used for exclusive labeling of the *braZ* gene product. *E. coli* cells containing both pGP1-2 and a pT7 recombinant plasmid were pulsed with [^{35}S]methionine as described previously (9). The proteins labeled with [^{35}S]methionine were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (14) and analyzed with a bioimage analyzer (BAS2000; Fuji Film Co., Tokyo, Japan).

Other methods. Initial rates of branched-chain amino acid uptake by *P. aeruginosa* cells suspended in 10 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM $MgCl_2$ and 1 mM KCl were determined at 37°C with 10 mM glucose and a uniformly ^{14}C-labeled branched-chain amino acid as described elsewhere (6). The protein content of the preparations was determined by the method of Lowry et al. (15), with bovine serum albumin used as a standard.

Enzymes and chemicals. Enzymes used for DNA manipulation were purchased from Toyobo (Osaka, Japan) or

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
Escherichia coli		
HB101	F^- *recA13 hsdR hsdM* λ^-	2
JM109	*recA1 endA1 gyrA1 hsdR17* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *Z*ΔM15) λ^-	33
K38	HfrC	23
Pseudomonas aeruginosa		
PAO3012	*trp-6*	12
PAO3536	*trp-6 braB307 braC317 leu-8001*::Tn*501*	10
PAO3537[a]	*trp-6 braB307 braC317 rec-301*	This study
MT2503	*trpAB*::pME*319 his-301 str-1 rec-301*	28
Plasmids		
pUC18	Cb^r (Ap^r)	33
pMMB34	Km^r *cos*λ	5
pKT240	Cb^r (Ap^r) Km^r	1
pGP1-2	Km^r *cI857 1*[b]	26
pT7-5	Cb^r (Ap^r)	S. Tabor
pT7-6	Cb^r (Ap^r)	S. Tabor

[a] *rec-301 leu*$^+$ derivative of PAO3536; constructed by conjugation with MT2503.

[b] Structural gene for bacteriophage T7 RNA polymerase, which is under the control of the bacteriophage promoter p_L in pGP1-2.

Takara Shuzo. Rifampin was from Sigma Chemical Co. (St. Louis, Mo.). Uniformly ^{14}C-labeled branched-chain amino acids, [^{35}S]methionine, and [α-^{35}S]dCTP were obtained from Amersham (Little Chalfont, Buckinghamshire, England). All other chemicals used were commercial products of analytical grade.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession no. D90222.

RESULTS

Isolation of the *braZ* gene. The *braB* gene for the Na^+-coupled LIV-II carrier has been isolated with *P. aeruginosa* PAO3536 defective in both LIV-I and LIV-II by the selection for Hrb$^+$ on D medium-based agar plates (10). In order to isolate the *braB* gene, we first screened the *P. aeruginosa* PAO3012 genomic library for Hrb$^+$ on G medium-based agar plates, another synthetic medium for *P. aeruginosa*, since this medium contains a high concentration of Na^+ ions, which might be better for LIV-II to operate in the cells. We failed, however, to isolate the *braB* gene with this medium. Instead, among 576 clones examined, we identified several clones showing Hrb$^+$ on G medium-based agar plates and having leucine transport activities independent of Na^+ ions. Restriction analysis showed that plasmids pTH6, pTH7, and pTH8, which are such Hrb$^+$ clones, contain 30- to 40-kb DNA segments, which overlapped considerably. The analysis further showed that these plasmids contain no chromosomal region homologous to that for LIV-I or LIV-II (8, 10). Thus, we designated a gene for the Hrb$^+$ phenotype retained in this overlapped region *braZ*. To locate the *braZ* gene, various *Eco*RI fragments were subcloned into pKT240 and tested for their Hrb phenotypes. The analysis showed that the *braZ* gene is located on the 9.2-kb *Eco*RI fragment, where pTH8 has a terminus of the inserted DNA segment. Plasmid pTH8S was generated from pTH8 by deleting most of the insert with *Sac*I and was found large enough to restore the Hrb$^+$ phenotype to PAO3536. A restriction map of pTH8S is shown in Fig. 1. The insert of pTH8S was subcloned to pUC18, generating pUHZ1 and pUHZ2, and then to pKT240, giving pKHZ11 (Fig. 1). Plasmid pKHZ11 restored Hrb$^+$ to PAO3536, confirming that the *braZ* gene is retained in the cloned insert. The 1.8-kb *Sma*I-*Aat*I segment of the insert was subcloned into the *Sma*I site of pKT240 in both orientations, giving pKHZ2 and pKHZ3 (Fig. 1). Plasmid pKHZ2 restored Hrb$^+$ to PAO3536, whereas pKHZ3 failed to restore the phenotype. The *Sma*I site is in the kanamycin resistance (Km^r) gene of pKT240 (1), suggesting that the expression of *braZ* in pKHZ2 is due to transcriptional or translational readthrough from the Km^r gene. Plasmids pKHZ12 and pKHZ13 having unidirectional deletions from the *Aat*I site towards the *Sma*I site were further constructed and tested for their Hrb phenotypes (Fig. 1). Plasmid pKHZ12, unlike pKHZ13, gave the Hrb$^+$ phenotype to PAO3536, suggesting that the *braZ* gene terminates in the DNA segment retained by pKHZ12 but not by pKHZ13.

Properties of the transport system encoded by *braZ*. The branched-chain amino acid transport activities of PAO3537, another PAO strain defective in both LIV-I and LIV-II, and its derivatives transformed with various pKHZ plasmids were examined (Table 2). Strain PAO3537 was almost completely defective in leucine uptake as expected but showed considerable isoleucine and valine uptake, suggesting that PAO3537 retains an as yet unidentified transport system specific for isoleucine and valine. As shown clearly in Table 2, branched-chain amino acid transport activities of the strains transformed with pKHZ plasmids paralleled their Hrb phenotypes. Hrb$^+$ plasmids, pKHZ11 and pKHZ12, restored or markedly enhanced branched-chain amino acid transport to PAO3537, while restoration or stimulation by pKHZ2 was less effective. The Hrb$^-$ plasmids, on the other hand, failed to enhance branched-chain amino acid transport in PAO3537. Stimulation by the Hrb$^+$ plasmids was much more effective for the transport of isoleucine and valine than that of leucine. This fact suggests that the transport system enhanced by the Hrb$^+$ plasmids is the same as that retained by PAO3537. To make this point clear, the effects of various amino acids on isoleucine uptake by PAO3537 or by PAO3537(pKHZ11) were examined (Table 3). Addition of a fivefold amount of cold isoleucine or valine caused 80% inhibition of [^{14}C]isoleucine uptake by the strains, suggesting strongly that the transport system is a common system to isoleucine and valine, with similar affinities for these amino acids. The addition of leucine also showed considerable inhibition of isoleucine uptake, although about 10 times as much was required to give the same extent of inhibition as with isoleucine or valine. The other amino acids caused no or little inhibition of isoleucine uptake even at 1 mM. The extent of inhibition by each amino acid was the same between PAO3537 and PAO3537(pKHZ11) (Table 3), confirming the idea that the transport system enhanced by *braZ* is the same as that retained by PAO3537.

Kinetic analysis of branched-chain amino acid transport by PAO3537 and PAO3537(pKHZ11) was further carried out. The initial rates of uptake were determined with the concentration range of 0.95 to 61 μM leucine, isoleucine, or

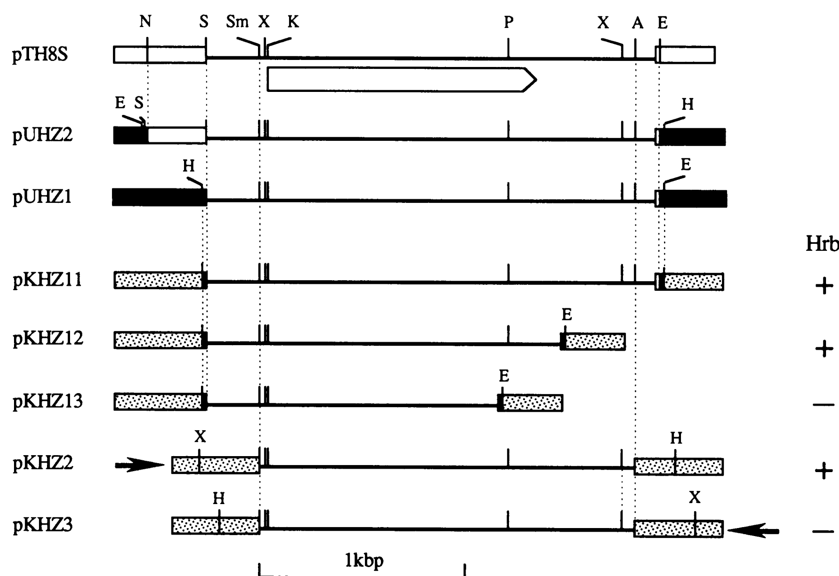

FIG. 1. Subcloning and localization of the *braZ* gene. The restriction map of pTH8S is shown at the top. The open arrow under the map indicates the location and direction of transcription of the *braZ* gene. The open, solid, and stippled boxes represent portions of plasmids pMMB34, pUC18, and pKT240, respectively. The cloned DNA segment of pTH8S was transferred into pUC18, generating pUHZ1 and pUHZ2. The inserts of pUHZ1 and its derivatives with unidirectional deletions from the *Eco*RI site were subcloned to pKT240, giving pKHZ11, pKHZ12, and pKHZ13. The 1.8-kb *Sma*I-*Aat*I fragment was also subcloned into the *Sma*I site of pKT240 in both orientations, generating pKHZ2 and pKHZ3. Restoration of growth at a low leucine concentration (Hrb) to PAO3536 was examined as described in the text. The solid arrows denote the directions of transcription with the promoter of the kanamycin resistance gene carried on pKT240. Restriction site abbreviations: A, *Aat*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; X, *Xho*I.

valine, and the results are presented in a double-reciprocal fashion (Fig. 2). The uptake of isoleucine and valine by PAO3537 cells gave straight lines, with the same K_m and V_{max} values of 12 μM and 9 nmol/mg of protein per min, respectively. Kinetic parameters for leucine uptake by PAO3537 were not obtained, however, due to very low leucine uptake compared with nonspecific binding of leucine to membrane filters. Strain PAO3537 carrying pKHZ11, on the other hand, exhibited considerable leucine uptake, giving a very high K_m value of 150 μM. Double-reciprocal plots of isoleucine and valine uptake by PAO3537(pKHZ11) also gave straight lines, with the same K_m value of 12 μM, which was also the same as those for isoleucine and valine uptake by PAO3537. The V_{max} values for leucine, isoleucine, and valine uptake by PAO3537(pKHZ11) were the same, showing 100 nmol/mg of protein per min. This value was 10 times bigger than that for isoleucine or valine uptake by PAO3537. These findings further confirm the possibility that the transport system identified in PAO3537 is the same as that affected by the *braZ* gene and that the transport system is common with branched-chain amino acids.

Nucleotide sequence of *braZ* and its flanking regions. To determine the nucleotide sequence of the *braZ* gene, various derivatives of pUHZ1 and pUHZ2 (Fig. 1) having unidirectional deletions in the inserts from the *Eco*RI site to the *Hin*dIII site were constructed and used for sequencing. Sequencing with each clone was carried out at least twice, and the entire sequences of both strands were determined.

TABLE 2. Branched-chain amino acid transport by various strains

Strain	Uptake (nmol/mg of protein per min) of[a]:		
	Leu	Ile	Val
PAO3012	13.8	14.5	14.4
PAO3537	0.1	5.2	5.8
PAO3537(pKHZ11)	12.1	62.2	68.8
PAO3537(pKHZ12)	8.9	56.5	64.5
PAO3537(pKHZ13)	0.3	5.0	4.9
PAO3537(pKHZ2)	1.3	17.7	17.5
PAO3537(pKHZ3)	0.4	7.3	6.9

[a] Initial rates were determined with 20 μM of the indicated amino acid.

TABLE 3. Effect of various amino acids on isoleucine uptake by PAO3537 and PAO3537(pKHZ11) cells

Amino acid (concn)[a]	% Isoleucine uptake[b]	
	PAO3537	PAO3537(pKHZ11)
None	100	100
Leucine (0.1)	82	79
Leucine (1)	35	26
Isoleucine (0.1)	21	22
Isoleucine (1)	2.3	3.0
Valine (0.1)	18	24
Valine (1)	2.7	4.2
Glycine (1)	90	93
Alanine (1)	78	69
Serine (1)	92	92
Threonine (1)	87	78
Methionine (1)	65	80
Proline (1)	92	98

[a] Each amino acid was added concomitantly with 20 μM [^{14}C]isoleucine at the final concentration given in parentheses (millimolar).

[b] Expressed as a percentage of the control. Control values for [^{14}C]isoleucine uptake by PAO3537 and PAO3537(pKHZ11) were 5.4 and 56.7 nmol/mg of protein per min, respectively.

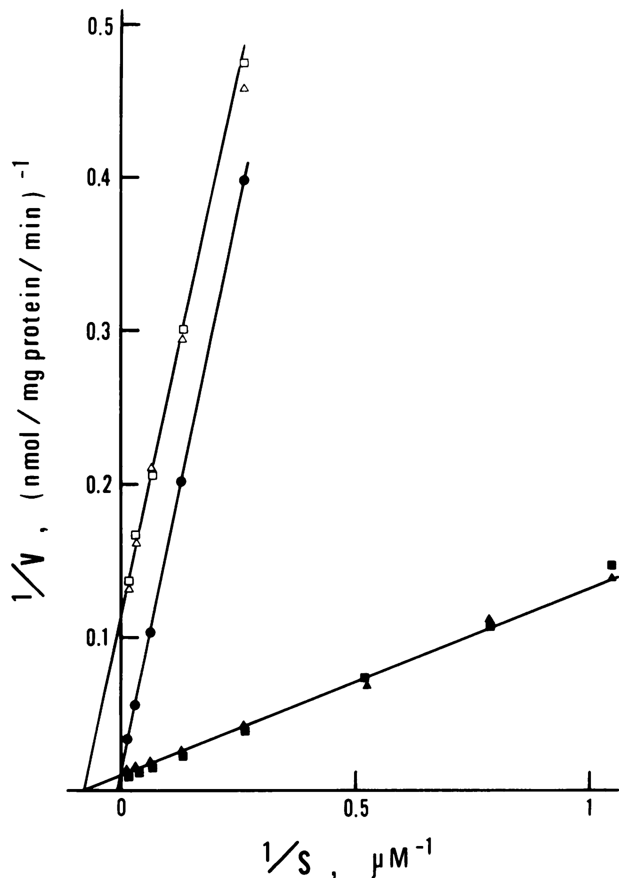

FIG. 2. Double-reciprocal plots of initial rates of leucine (circle), isoleucine (triangle), and valine (rectangle) uptake. Initial rates of uptake by strain PAO3537 (open symbols) or by PAO3537(pKHZ11) (solid symbols) were determined with a concentration range of 0.95 to 61 μM as described in the text.

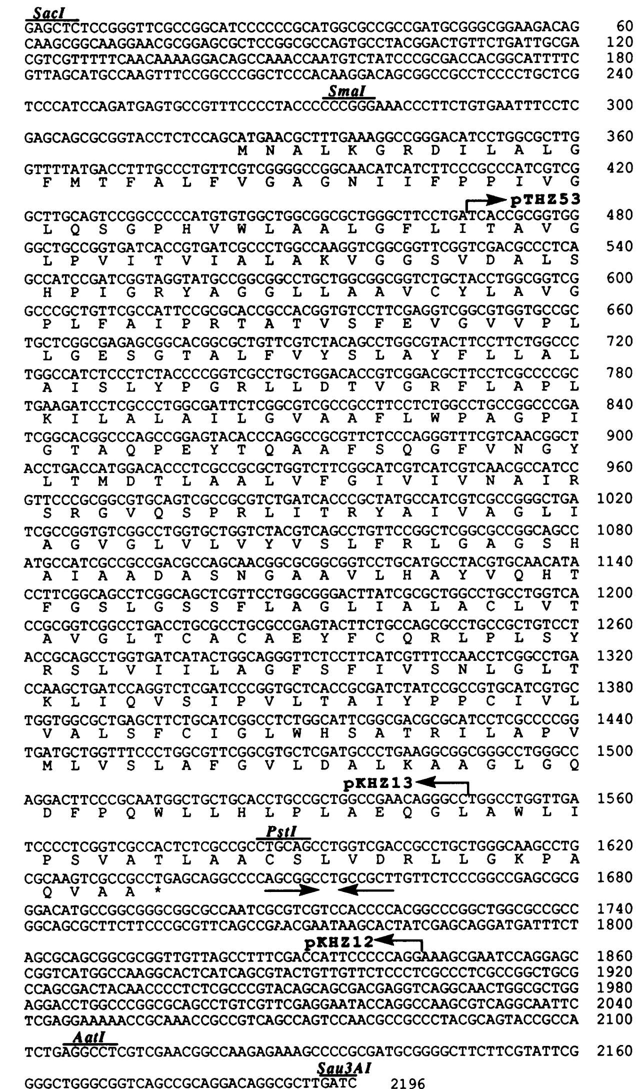

FIG. 3. Nucleotide sequence of the *braZ* gene and deduced amino acid sequence of the gene product. Nucleotide numbering (at right) begins with the first base of the *Sac*I site. The putative transcriptional termination signal in the 3'-flanking region is shown by the converging arrows. The 5' or 3' ends of the inserts of pTHZ53, pKHZ12, and pKHZ13 are indicated by bent arrows. Locations of the *Aat*I, *Pst*I, and *Sma*I restriction sites are presented for comparison with Fig. 1.

Figure 3 shows the nucleotide sequence of the *braZ* gene and its flanking regions.

As described above, it is suggested that the *braZ* gene is transcribed from the *Sac*I site towards the *Aat*I site (Fig. 1). Thus, the nucleotide sequence shown in Fig. 2 was inspected for the open reading frame for *braZ*. A long open reading frame (positions 324 to 1,634) was found to be the only reading frame that is consistent with deletion mapping for *braZ* (Fig. 1). This open reading frame is contained by the Hrb$^+$ plasmids and terminates in the DNA segment retained by pKHZ12 but not by pKHZ13. Thus, we conclude that the open reading frame is that for the *braZ* gene, although the open reading frame is not preceded by a Shine-Dalgarno sequence (25) typical of ribosome-binding sites. A G+C-rich dyad symmetry with a T cluster (positions 1,647–1,666) typical of the ρ-independent transcriptional termination signals (22) is present in the 3'-flanking region of the *braZ* gene. This palindromic sequence is able to form a stable stem-loop structure in the corresponding transcript with a calculated free energy value (27) of −12.4 kcal (52.0 kJ).

Amino acid sequence and identification of the *braZ* gene product. The *braZ* gene specifies a protein of 437 amino acid residues with a calculated M_r of 45,271. The deduced amino acid sequence of the BraZ protein is shown in Fig. 3. The amino acid composition of BraZ shows that this protein is extremely hydrophobic (76% nonpolar). The BraZ protein contains only 33 charged amino acid residues, 13 acidic and 20 basic, giving an excess of seven positive charges at neutral pH. The hydropathy profile of the BraZ protein was obtained with a nine-residue span by the method of Kyte and Doolittle (13) (Fig. 4). The profile reveals that the BraZ protein contains 12 membrane-spanning segments flanked by short hydrophilic stretches.

To identify the BraZ protein by controlled expression with the T7 RNA polymerase-promoter system of Tabor and Richardson (26), portions of the *braZ* region on pUHZ2 were subcloned into pT7-5 and pT7-6, giving the pTHZ plasmids (Fig. 5A). *E. coli* K38(pGP1-2) was transformed by these plasmids and tested for the expression of the *braZ* gene (Fig. 5B). When the strain harboring pTHZ51 or pTHZ52 was heat

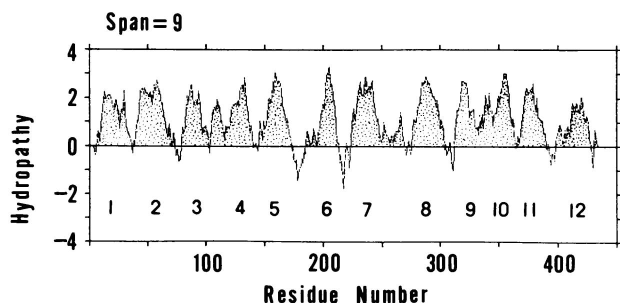

FIG. 4. Hydropathy profile of the *braZ* gene product. Hydropathy was calculated with a window of nine residues along the amino acid sequence. Twelve hydrophobic segments capable of spanning the membrane are indicated.

induced, a protein band with an apparent M_r of 34,000 was detected on an SDS-polyacrylamide gel. The protein was not synthesized, however, by these strains incubated at 30°C (noninduced) or by the strain with pTHZ61 incubated at 42°C (induced). The strain with pTHZ53 lacking the N-terminal region of the open reading frame for *braZ* also failed to produce this protein. These findings clearly show that the protein of the apparent M_r of 34,000 is the *braZ* gene product.

DISCUSSION

The present study shows that *P. aeruginosa* PAO has another branched-chain amino acid transport system in addition to the LIV-I and LIV-II transport systems. We designate the new system as LIV-III. The LIV-III transport system was found by kinetic analysis to be comparable to LIV-I and LIV-II in isoleucine and valine uptake by PAO cells (Table 2; Fig. 2). The LIV-III system, however, was shown to be inefficient in leucine uptake because of its very low affinity for leucine, which is in contrast to LIV-I and LIV-II in substrate specificity (6). This inefficiency of LIV-III in leucine uptake by PAO3536, a *leu* auxotroph defective in both LIV-I and LIV-II, enabled us to isolate the gene *braZ* encoding the LIV-III transport system by the selection for Hrb^+ (growth at a low leucine concentration). The *braZ* gene has recently been mapped to the *cys-54* region of the *P. aeruginosa* PAO chromosome by Southern hybridization (12a), confirming that *braZ* is an authentic gene of *P. aeruginosa* PAO.

Determination of the nucleotide sequence of the *braZ* gene and its flanking regions revealed an open reading frame of 1,311 nucleotides that was capable of coding for a protein of 437 amino acid residues. The G+C content of the third position in the codons used for *braZ* is 91%, showing a high bias for G or C in the wobble base as determined for other *Pseudomonas* genes (4, 9). This open reading frame is the only one which is consistent with the results of deletion mapping (Fig. 1) and controlled gene expression by the T7 RNA polymerase-promoter system (Fig. 5). The amino acid composition of the *braZ* gene product indicates that this protein is highly hydrophobic (76% nonpolar). The hydropathy profile of the BraZ protein further shows that the protein contains 12 membrane-spanning segments (Fig. 4). These properties are typical of carrier proteins (3, 9, 19). Plasmid pKHZ3 lacking the *SacI-SmaI* fragment (positions 1 to 275) failed to enhance branched-chain amino acid uptake by PAO3537 cells (Table 2), while the plasmid contains the

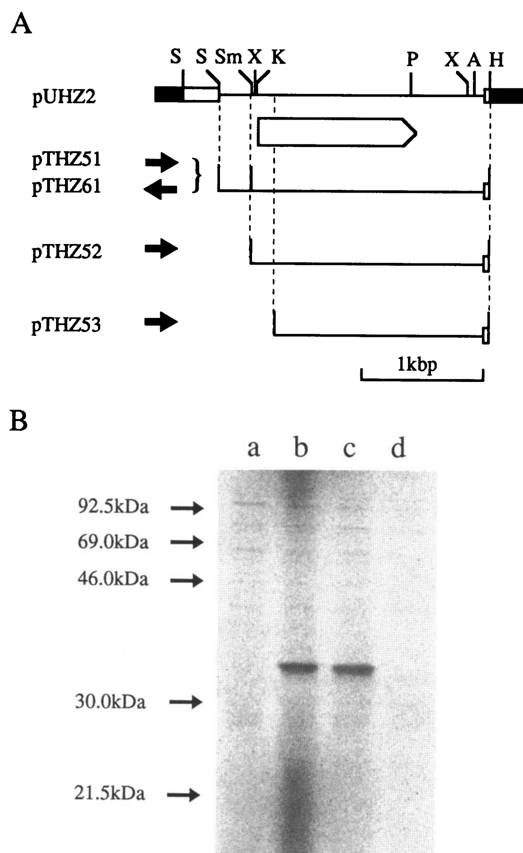

FIG. 5. Identification of the *braZ* gene product. (A) Construction of pT7 derivatives for controlled expression of *braZ*. The 2.2-kb *SacI-HindIII* fragment of pUHZ2 was subcloned into pT7-5 and pT7-6, generating pTHZ51 and pTHZ61, respectively. Plasmids pTHZ52 and pTHZ53 are the derivatives of pTHZ51, having unidirectional deletions from the *SacI* site. The open arrow indicates the location and direction of transcription of the *braZ* gene. Solid arrows denote the directions of transcription by the T7 promoter derived from pT7 plasmids. Restriction site abbreviations are as described in the legend to Fig. 1. (B) SDS-PAGE patterns of proteins synthesized by *E. coli* K38(pGP1-2) carrying the pTHZ plasmids. Samples for SDS-PAGE were prepared as described in the text. After electrophoresis, proteins labeled with [^{35}S]methionine were analyzed with a bioimage analyzer (BAS2000). Lanes: a, pTHZ61; b, pTHZ51; c, pTHZ52; d, pTHZ53. The arrows on the left indicate the positions of rainbow colored protein molecular weight markers (Amersham).

entire open reading frame for *braZ*, suggesting that the promoter for *braZ* is retained in the *SacI-SmaI* region.

The apparent molecular weight of the BraZ protein estimated from SDS-PAGE is 34,000, which is considerably smaller than the M_r of 45,271 calculated from the deduced amino acid sequence. Such discrepancies seem to be common for intrinsic membrane proteins, presumably because of an abnormally higher capacity for binding of SDS (3). It is not likely that the difference in molecular weights is the result of cleavage of the signal peptide, as the BraZ protein does not have a sequence typical of signal peptides (21). If translation of *braZ* starts at an ATG or GTG codon located inside the sequence, the molecular weight would be closer to that estimated from SDS-PAGE. Considering the homology analysis described below, however, we conclude that the ATG codon at positions 324 to 326 is the initiation codon for

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1   MTH-LKQFDLLALGFMTFALFLQAGNIIFPPPSAGMAAGEHVWSAAFGFLL    49
2   MNA-LKGRDILALGFMTFALFVGAGNIIFPPPIVGLQSGPHVWLAALGFLI    49
3   MTHQLKSRDIIALAPMTFALFVGRGNIIFPPPMVGLQAGEHVWTARIGFLI    50

1   TGVGLPLLTVVALARVGGGIGRLTQPIGRRAGVAFAIAVVLAIGPLFATP    99
2   TAVGLPVITVIALAKVGGSVDALSHPIGRYAGGLLAAVCYLAVGPLFAIP    99
3   TAVGLPVLTVVALAKVGGGVDSLSTPIGKVAGLLLATVCYLAVGPLFATP   100

1   RTAVVSFEMGVAPFTGDGGVPLLIYTVAYFSVVLFLVLNPGRLVDRVGKV   149
2   RTATVSFEVGVVPLLGESGTALFVVSLAYFLLALAISLYPGRLLDTVGRF   149
3   RTATVSFEVGIAPLTGDSAMPLLIYSVVYFAIVILVSLYPGKLLDTVGNF   150

1   ITPVLLSALLVLGGAAIFAPAGEIGSSSGEYQSAPLVQGFLQGYLTMDTL   199
2   LAPLKILALAILGVAAFLWPAGPIGTAQPKYTQAAFSQGFVNGYLTMDTL   199
3   LAPLKIIALVILSVAAIVWPAGPISNALDAYQNAAFSNGFVNGYLTMDDW   200

1   GALVFGIVIATAIRDRGISDSRLVTRYSMIAGVIAATGLSLVYLALFYLG   249
2   AALVFGIVIVNAIRSRGVQSPRLITRYAIVAGLIAGVGLVLVYVSLFRLG   249
3   VAMVFGIVIVNAARSRGVTEARLLTRYTVWAGLMAGVGLTLLYLALFRLG   250

                                              (A)
1   ATSQGIAGDAQNGVQILTAYVQQTFGVSGSLLLAVVITLACLTTAVGLIT   299
2   AGSHAIAADASNGAAVLHAYVQHTFGSLGSSFLAGLIALACLVTAVGLTC   299
3   SDSATLVDQSANGAAILHAYVQHTFGGAGSFLLAALIFIACLVTAVGLTC   300

1   ACGEPFSDLLPVSYKTVVIVFSLFSLLVANQGLTQLISLSVPVLVGLYPL   349
2   ACAEYFCQRLPLSYRSLVIILAGPSFIVSNLGLTKLIQVSIPVLTAIYPP   349
3   ACAEPFAQYIPLSYRTLVFILGQPSMVVSNLGLSHLIQISIPVLTAIYPP   350

1   AIVLIALSLFDRLWVSAPRVFVPVMIVALLPGIVDGLGAAKLNGWVPDVF   399
2   CIVLVALSFCIGLWHSATRILAPVMLVSLAFGVLDALKAAGLGQDFPQWL   399
3   CIALVVLSFTRSWWHNSTRIIAPAMFISLLFGILDGIKASAFGDMLPAWS   400

1   AKLPLADQSLGWLLPVSIALVLAVVCDRLLGKPREAVA-              437
2   LHLPLAEQGLAWLIPSVATLAACSLVDRLLGKPAQVAA-              437
3   QRLPLAEQGLAWLMPTVVMVILAIIWDRAAGRQVTSSAH              439
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FIG. 6. Alignment of the deduced amino acid sequences of the LIV-II (rows 1) and LIV-III (rows 2) carriers of *P. aeruginosa* PAO and the LIV-II carrier of *S. typhimurium* (rows 3). Identical amino acids are shadowed. The amino acid at position 292 substituted in the *P. aeruginosa* PML LIV-II carrier is shown in parentheses.

braZ in spite of its lack of a Shine-Dalgarno sequence (25) typical of ribosome-binding sites.

Comparison of the amino acid sequence of the BraZ protein with those of the BraB (10) and BrnQ (20) proteins, the LIV-II carriers in *P. aeruginosa* and *S. typhimurium*, respectively, revealed extensive homology among the proteins (Fig. 6). The BraB, BraZ, and BrnQ proteins are of similar sizes, containing 437, 437, and 439 amino acid residues, respectively. These proteins are 46% identical over the entire regions. In particular, the homology in an N-terminal fourth of the sequences is remarkable, giving 61% identity. If the conservative substitutions (18) are considered, the three proteins show 72% homology. The homology between BraZ and BrnQ is the highest among the carrier proteins. The BraZ and BrnQ proteins give 291 identical residues (67%), while BraB and BraZ or BraB and BrnQ show 56 or 53% identity, respectively. As shown in this report, the LIV-III (BraZ) system is quite different in substrate specificity from the LIV-II transport systems in *P. aeruginosa* and *S. typhimurium*, which are both specific for leucine and isoleucine and less specific for valine (6, 17). Thus, the higher homology between BraZ and BrnQ in the primary structure does not seem to reflect their substrate specificities.

The *P. aeruginosa* LIV-II transport system is shown to be coupled to an electrochemical potential of Na^+ or Li^+ ions (7, 30), while the transport system coded for by the *E. coli brnQ/hrbA* gene, which is homologous to *brnQ* of *S. typhimurium*, is suggested to be an H^+-coupled transport system (31, 32). Isoleucine uptake by the *P. aeruginosa* LIV-III system was not affected by the addition of Na^+ or Li^+ ions (data not shown), suggesting that the system is an H^+-coupled transport system. Thus, the higher homology between BraZ and BrnQ may reflect the nature of energy coupling of these transport systems. Our previous report (29) showed that the difference in Na^+ requirement of the LIV-II carrier between *P. aeruginosa* PAO and PML strains is due to the substitution of an amino acid at position 292, suggesting that the amino acid residue at this position plays an important role in determining specificity for coupling cations. In this sense, it seems worth noting that both the BraZ and BrnQ proteins contain valine at position 292. This amino

acid is different from those of the PAO and PML BraB proteins (Thr and Ala, respectively), while the homology of the amino acid sequence around position 292 is retained among the BraB, BraZ, and BrnQ proteins (Fig. 6).

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