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Identification and Analysis of a Gene Encoding L-2,4-Diaminobutyrate:2-Ketoglutarate 4-Aminotransferase Involved in the 1,3-Diaminopropane Production Pathway in *Acinetobacter baumannii*

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The ca. 2.2-kbp region upstream of the *ddc* gene encoding L-2,4-diaminobutyrate decarboxylase in *Acinetobacter baumannii* was sequenced and found to contain another open reading frame of 1,338 nucleotides encoding a protein with a deduced molecular mass of 47,423 Da. Analysis of the homologies observed from the deduced amino acid sequence indicated that the gene product is an enzyme belonging to subgroup II of the aminotransferases. This was first verified when examination of the crude extracts from *Escherichia coli* transformants led to detection of a novel aminotransferase activity catalyzing the following reversible reactions: L-2,4-diaminobutyric acid + 2-ketoglutaric acid↔L-glutamic acid + L-aspartic β-semialdehyde. Further confirmation was obtained when the gene was overexpressed in *E. coli* and the corresponding protein was purified to homogeneity. It catalyzed the same reactions and its N-terminal amino acid sequence was consistent with that deduced from the nucleotide sequence. Therefore, the gene and its product were named *dat* and L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase (DABA AT), respectively. Feeding experiments of *A. baumannii* with L-[U-^{14}C]aspartic acid resulted in the incorporation of the label into 1,3-diaminopropane. Apparent homologs of *dat* and DABA AT were detected in other *Acinetobacter* species by PCR amplification and Western blotting. These results indicate that the *dat* gene (as well as the *ddc* gene) participates in the synthesis of 1,3-diaminopropane, the only diamine found in this genus. However, the biological role, if one exists, of 1,3-diaminopropane synthesis is unknown.

Members of the genus *Acinetobacter* are nonfermentative, aerobic gram-negative coccobacilli that are widely distributed in nature and can be part of the normal flora of the human skin (2). *Acinetobacter* species have also been isolated from various types of opportunistic infections, including septicemia, pneumonia, endocarditis, meningitis, skin and wound sepsis, and urinary tract infections (for a recent review, see reference 4). There are indications that most clinically important isolates of *Acinetobacter* species belong to the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex. In addition, recent reports showed that many clinical isolates of this complex have developed widespread and increasing resistance to many commonly used antibiotics (38, 39, 41).

On the other hand, *Acinetobacter* species are unique in that when grown in a chemically defined medium, they produce large amounts of 1,3-diaminopropane (DAP) but not the prototypic polyamines, such as putrescine, spermidine, and cadaverine (14, 47). We previously purified and characterized a novel enzyme, L-2,4-diaminobutyrate decarboxylase (DABA DC), yielding DAP from *A. calcoaceticus* (48), although the biosynthetic pathway for DABA as a precursor of DAP remained to be clarified. This finding is rather unexpected, since the oxidative cleavage of spermidine through the action of spermidine dehydrogenase is generally accepted to be the sole pathway for the formation of DAP in bacteria (40). Recently, the gene (*ddc*) encoding DABA DC has been identified in a 14-kbp *Bam*HI genomic fragment of *A. baumannii*, which also conferred upon *Escherichia coli* hosts an ability to produce DAP (19). This suggests that the insert also contains the gene(s) relevant to the biosynthesis of DABA, in addition to the *ddc* gene, which has previously been sequenced and analyzed (20).

In the present report, we demonstrate that this locus contains a second open reading frame (ORF) (named *dat*), which encodes a novel aminotransferase, DABA:2-ketoglutarate 4-aminotransferase (DABA AT), involved in DAP biosynthesis. The absence of a recognizable transcriptional terminator for the *dat* gene suggests that the gene is cotranscribed with the *ddc* gene. Furthermore, we show properties of the recombinant enzyme purified from *E. coli* cells and the distribution and expression of the genes encoding DABA AT-like proteins in other *Acinetobacter* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. All type strains of *Acinetobacter* species were obtained from the American Type Culture Collection (Rockville, Md.), except for that of *Acinetobacter radioresistens*, which was obtained from the Institute of Applied Microbiology, University of Tokyo (Tokyo, Japan), and were grown as previously described (48). *E. coli* HB101 (8) was used as a host for all manipulations including the generation of double-stranded DNA templates prior to DNA sequencing. DNA inserts of the recombinant plasmids used throughout this study are shown in Fig. 1 and were contained either in pUC18 or in pUC19 (42). *E. coli* strains carrying a plasmid were grown aerobically at 37°C in Luria-Bertani (LB) broth or LB agar (37) containing 100 μg of ampicillin per ml.

DNA manipulations. Standard molecular biology techniques were performed according to the methods described by Sambrook et al. (37). Plasmid isolation from *E. coli* cells was performed as previously described by Birnboim and Doly (5). For PCR amplification, genomic DNA was prepared from eight type strains of *Acinetobacter* species by the procedure of Murray and Thompson (30), with the inclusion of 1% (wt/vol) hexadecyltrimethylammonium bromide in the cell lysis buffer. Restriction and modifying enzymes (Takara Shuzo, Osaka, Japan, and Nippon Gene, Tokyo, Japan) were used under the conditions recommended by the manufacturers. Restriction fragments were isolated, as required, from

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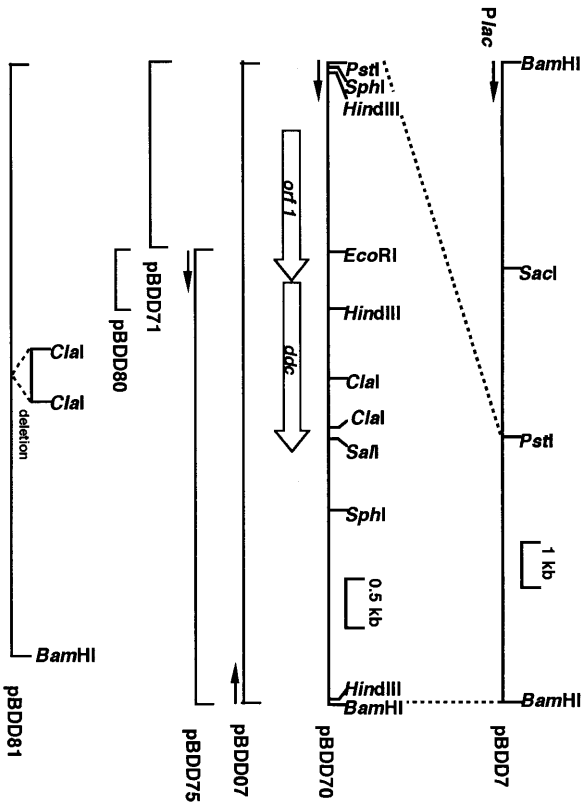

FIG. 1. Restriction map of the plasmids, constructed from the 14-kbp insert of pBDD7 containing *A. baumannii* ORF1 (*dat*) and *ddc* (19) used in this study. The relevant restriction sites are shown, and relative positions and directions of transcription of ORF1 (*dat*) and *ddc* are indicated by open arrows. The relevant plasmid inserts are shown diagrammatically, and the direction of the external *lacZ* transcription, where relevant, is indicated by filled arrows.

agarose gels with the Prep-A-Gene DNA purification kit (Bio-Rad) according to the manufacturer's instructions.

Sequencing and protein analysis. The 1.75-kbp *Pst*I-*Eco*RI and 0.5-kbp *Eco*RI-*Hin*dIII (the second) fragments were each subcloned in pBluescript KS^+ (Stratagene) from pBDD70 (19) to generate pBDD71 and pBDD80, respectively. Unidirectional deletion plasmids were constructed in Bluescript KS^+ from pBDD70 by exonuclease III and mung bean nuclease digestion (Kilo-Sequence deletion kit; Takara Shuzo) with the appropriate sets of restriction enzymes according to the manufacturer's recommendations. After purification of the nested plasmids with a Prep-A-Gene DNA Miniprep kit (Bio-Rad), the DNA fragments were sequenced in both orientations with a *Bca*BEST Dideoxy sequencing kit (Takara Shuzo). Synthetic oligonucleotides designed on the basis of the determined sequence were used as primers to complete the sequence of the nonoverlapping regions. Sequence analysis and alignments were performed with GENETYX-Mac software (version 32.0; Software Development, Tokyo, Japan). The deduced amino acid sequence was compared with other sequences in the GenBank (3) and SwissProt databases.

Enzyme assay methods. Dialyzed crude extracts of various strains in which the DABA AT and DABA DC activities were measured were prepared as described below. DABA AT activity was measured by quantifying glutamic acid formed in the assay mixture (1 ml), containing 100 mM Tris (pH 8.5), 10 mM DABA, 10 mM ketoglutarate (2-KG), 0.2 mM pyridoxal phosphate, and enzyme protein (5 μg to 1 mg), incubated at 37°C for 30 min. The reaction was terminated with 0.4 ml of 20% (wt/vol) perchloric acid, and the appropriate amount of proline (25 to 100 nmol) was added as an internal standard. After centrifugation at 1,500 × *g* for 10 min, the supernatant was transferred to a reaction vial for derivatization of glutamic acid to its *N*-isobutyloxycarbonyl methyl ester derivative, which was analyzed by flame ionization detection-gas chromatography (GC) (44). The reproducibility of the assay method was assessed by calculating the recoveries of known amounts of glutamic acid added to the assay mixtures denaturated by the prior addition of perchloric acid. The kinetic parameters were obtained from Lineweaver-Burk plots for 0.5 to 10.0 mM DABA and 0.1 to 5 mM 2-KG in the presence of 10 mM 2-KG and 10 mM DABA, respectively. 4-Aminobutyric acid, L-2,3-diaminopropionic acid (Fluka), β-alanine, L-ornithine, and L-lysine (each 10 mM) were examined for their activities as amino group donors in the presence of 10 mM 2-KG. Likewise, pyruvic acid and oxalacetic acid (each 10 mM) were examined as amino group acceptors in the presence of 10 mM DABA. Alanine and aspartic acid formed from pyruvic acid and oxalacetic acid, respectively, were analyzed by the same method as described for glutamic acid. L-Aspartic β-semialdehyde used in the reverse reaction to form DABA was prepared by ozonolysis of L-C-allylglycine (Sigma) and purified (6). The DABA DC activity was assayed as described elsewhere (48). Protein concentrations were determined by the method of Lowry et al. (27) or with a commercial protein assay (Bio-Rad) with bovine serum albumin as the standard.

Purification of recombinant DABA AT. Unless otherwise noted, all operations were done at 0 to 5°C, and all dialyses were done for 12 to 16 h against 100 volumes of the specified buffers with two changes. When the enzyme was eluted with buffers without pyridoxal phosphate, an appropriate aliquot of 4 mM pyridoxal phosphate was added to each reservoir tube in advance to make a final concentration of 40 μM. Twenty-four grams (wet weight) of *E. coli* HB101 cells carrying pBDD81 was suspended in 5 volumes of 20 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA and 40 μM pyridoxal phosphate (buffer A) and disrupted with an ultrasonic homogenizer. The suspension was centrifuged for 30 min at 40,000 × *g*, and a small portion of the supernatant was dialyzed against the same buffer to determine the activity (dialyzed crude extract). The supernatant was fractionated with ammonium sulfate, and the precipitate (35 to 50% saturation) was dissolved in a minimum volume of buffer A and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Sepharose CL 6B (Pharmacia) column (2.6 by 22 cm) equilibrated with buffer A without pyridoxal phosphate. After the column was washed with two column volumes of the same buffer, the enzyme was eluted with a 500-ml linear gradient from 0 to 1 M NaCl at a flow rate of 20 ml per h. The active fractions eluting at 0.52 to 0.60 M NaCl were pooled, dialyzed for 4 h against buffer A, and rechromatographed on the same column under identical conditions to remove large interfering peaks proximate to the active peak as effectively as possible. The combined active fractions were concentrated to ca. 5 ml with an 8050 ultrafiltration cell equipped with a PM-10 membrane filter (Amicon) and loaded onto a Sephacryl S-200 HR (Pharmacia) column (2.1 by 87 cm) equilibrated with buffer A containing 400 mM NaCl and 0.02% sodium azide. The enzyme was eluted with the same buffer at a flow rate of 10 ml per h, and the active fractions eluting between 75 to 85 ml were combined and dialyzed against 20 mM potassium phosphate buffer, pH 7.5 (buffer B). The enzyme solution was applied to a Bio-Scale CHT-2 Hydroxyapatite column (7 by 52 mm; Bio-Rad) equilibrated with buffer B. The enzyme activity was found in the washing fractions, which were concentrated to ca. 5 ml with a Centricon-10 tube (Amicon). The enzyme solution was applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer B. After the column was washed with 10 ml of the same buffer, the enzyme was eluted with an 80-ml linear gradient from 0 to 1 M NaCl at a flow rate of 0.5 ml per min. The active fractions eluting at around 0.23 M NaCl were combined and stored at 1°C after dialysis against buffer B containing 40 μM pyridoxal phosphate. As required, this solution was concentrated with a Centricon-10 tube.

GC-MS. GC-mass spectrometry (MS) analysis was performed for the identification of the enzyme reaction products as their *N*-isobutyloxycarbonyl methyl ester derivatives with a VG Analytical 70SE Mass Spectrometer equipped with a Quadrex bonded-fused silica capillary column coated with Silicon OV-1 (25 m by 0.25 mm). Mass spectra obtained were compared with those of authentic compounds.

N-terminal sequence analysis. Recombinant DABA AT purified as described above was analyzed with an Applied Biosystems 477A protein sequencer equipped with an on-line high-pressure liquid chromatography apparatus, model 120A.

Gel filtration and SDS-PAGE. The native M_r for the recombinant enzyme was estimated with a Sephacryl S-200 HR column (2.1 by 88 cm; Pharmacia). Isocratic elution was done with buffer A containing 400 mM NaCl and 0.02% sodium azide. The column was standardized with gel filtration molecular-weight markers from 12,000 to 200,000 (Sigma). All sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with buffers as described by Laemmli (26) and with a 10% (wt/vol) polyacrylamide resolving gel with a 4.5% (wt/vol) polyacrylamide stacking gel. Samples were denatured at 100°C for 5 min in buffer containing 4% (wt/vol) SDS, 10% (wt/vol) β-mercaptoethanol, and 14% (wt/vol) glycerol. Gels were stained with Coomassie brilliant blue or (gels selected for Western blotting) were processed to transfer proteins to a Hybond-C membrane (Amersham) with a Trans-Blot SD electrophoretic blotting cell (Bio-Rad) according to the manufacturer's protocol.

Radiotracer experiments with [^{14}C]aspartic acid. *A. baumannii* ATCC 19606 cells grown for 5 h in a chemically defined medium (46) were suspended at an optical density at 660 nm of 0.5 in 50 ml of the fresh medium containing 5 mM L-aspartic acid (185 kBq L-[U-^{14}C]aspartic acid), and incubated for 1 h at 30°C. To the culture was added 60% (wt/vol) perchloric acid at a final concentration of 2%, and this mixture was left overnight at room temperature. After centrifugation, the supernatant was applied to a column of Dowex 50W× 8 (Dow Chemical Co.), and the amine fraction was collected, dansylated, and thin-layer chromatographed on a precoated silica gel 60 plate (Merck), as described elsewhere (43). The dansylated amines were visualized as fluorescent spots under UV light. Radioactive bands were detected on the same plate with a thin-layer chromatogram scanner (Aloka, Tokyo, Japan).

PCR amplification. Two oligonucleotides, 5′GCGATAGCGAAAGCACAA GG3′ (bases 763 to 782) and 5′CCACGGAAAGTACCAGTGTG3′ (bases 1610 to 1591), were prepared on the basis of the determined nucleotide sequence of *dat* and used as PCR primers. PCR was performed with 1 μg of *Acinetobacter* chromosomal DNA in a final volume of 100 μl containing deoxynucleoside triphosphates (200 μM each), primers (0.5 μM), 2.5 U of recombinant *Taq* DNA polymerase (Takara Shuzo), and the buffer supplied with the enzyme. Amplification was performed with a PCR Thermal Cycler TP2000 (Takara Shuzo) as follows: cycle 1, 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min; and cycles 2 to 40, 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

Preparation of polyclonal antibodies and Western blotting. Polyclonal antibodies against purified recombinant DABA AT were raised in a rabbit as previously described (48). The antiserum was absorbed with sonicated HB101 cells prior to use. Crude extracts of various *Acinetobacter* strains were analyzed by Western blotting (immunoblotting), in which the rabbit antiserum was employed as the primary antibody and goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Boehringer Mannheim) was employed as the secondary antibody. The blot was developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride (37).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB001599.

RESULTS

Sequencing of the region upstream of the *ddc* gene. A 14-kbp *Bam*HI fragment from *A. baumannii* DNA has previously been cloned in pUC19 as plasmid pBDD7, which conferred upon *E. coli* host cells an ability to produce DAP (Fig. 1) (19). *E. coli* cells harboring pBDD70, which contains a 5.8-kbp *Pst*I-*Bam*HI fragment, specified the region to produce DAP. *E. coli* cells harboring pBDD07, in which the 5.8-kbp *Pst*I-*Bam*HI was inserted into pUC18 in an opposite orientation, showed productivity of DAP similar to pBDD70, suggesting that the gene(s) contains a promoter region for its self-expression. However, *E. coli* cells transformed with pBDD75 failed to produce DAP, in spite of its expression of DABA DC, suggesting that the gene(s) responsible for DABA biosynthesis is contained within the 1.75-kbp *Pst*I-*Eco*RI region of pBDD70 or pBDD07 (the sequence of the fragment encompassing the complete *ddc* gene has been reported elsewhere [20]). Then, in order to verify the actual presence of the gene(s) involved in DABA synthesis, the *Pst*I-*Eco*RI region as well as the ca. 0.5-kbp *Eco*RI-*Hin*dII (the second) region, was sequenced with several deletion plasmids constructed from pBDD71 and pBDD80. Sequence analysis revealed the presence of an ORF, ORF1, of 1,338 nucleotides in length, which encodes a potential protein of 445 amino acids with a deduced M_r of 47,423 (Fig. 2). A putative promoter region was found by its similarity to the *E. coli* consensus −35 and −10 sequences (17). These two regions are separated by 16 nucleotides, which is comparable to the spacing in *E. coli* (17 ± 1 bp [15]). The translation initiation codon of ORF1 is probably an ATG triplet, which is preceded by a potential ribosome-binding site (GGT) complementary to the 16S rRNA of *A. calcoaceticus* (5′GATCACCTCCTT3′) (24), with a spacing of 8 bp fitted to the optimal distance of 7 ± 2 bp in *E. coli* (15, 25). An apparent translation stop codon, TAA, delineates the 3′ end of ORF1, which is followed by the ATG translation initiation codon of the *ddc* gene, which is only 2 bp away. However, no potential transcriptional terminator sequence for ORF1 was found within the 5′ region of the *ddc* gene, suggesting that both ORF1 and the *ddc* genes are cotranscribed from a common promoter upstream of ORF1 to the ρ-independent transcriptional terminator located 15 bp downstream from the translation stop codon of the *ddc* gene (20). The calculated G+C content of ORF1 was 43.3%, which falls within the G+C content of 38 to 47% determined for the chromosomal DNA of *Acinetobacter* species (18).

The ORF1 product is similar to subgroup II of the aminotransferases. A search in the GenBank data libraries for proteins with similar sequences revealed that the amino acid sequence deduced from ORF1 has similarity with and is similar in size to several bacterial proteins, which are mainly the aminotransferases belonging to subgroup II proposed by Mehta et al. (28). Interestingly, the ORF1 protein showed the greatest similarity (identity score, 70%; similarity score, 92%) with the *Haemophilus influenzae* Rd gene HI0949 (GenBank accession no. U32776), which has been tentatively assigned to the 4-aminobutyrate aminotransferase gene (*gabT*) because of its homology with the *E. coli gabT* gene (identity score, 34%; similarity score, 56%) (10). In this regard, our recent data have suggested that some *Haemophilus* species possess both the DABA DC and DABA AT activities to produce DAP (21). Other proteins, whose functions have been clearly characterized, with significant similarity to the ORF1 product are *E. coli* 4-aminobutyrate aminotransferase (1), *Anabaena* sp. *N*-acetylornithine aminotransferase (11), and *Bacillus subtilis* ornithine aminotransferase (12), which show identity (and similarity) scores of 33% (74%), 31% (72%), and 30% (70%), respectively. In Fig. 3, the amino acid sequence of the ORF1 product is aligned with these three proteins, with a high degree of homology in some regions. In this analysis, the four invariant amino acid residues, i.e., Gly-234, Asp-260, Lys-289, and Arg-412 in the ORF1 product, playing a specific structural or functional role in aminotransferases as discussed by Mehta et al. (28), can be aligned with the equivalent positions in the three proteins. Lys-289 in the ORF1 product corresponds to Lys-258 in the *E. coli* 4-aminobutyrate aminotransferase (1), which has been proposed to be the pyridoxal phosphate-binding residue, in comparison with the cytosolic chicken aspartate aminotransferase, whose three-dimensional structure and catalytic site are known (23). On the basis of these findings, we expect that ORF1 encodes an enzyme similar to subgroup II of the pyridoxal phosphate-dependent aminotransferases, whose substrates possess the unique feature of a distal amino group which undergoes the transamination reaction (28).

Detection of DABA AT activity both in *E. coli* transformants and in *Acinetobacter* species. To elucidate which enzyme ORF1 encodes, the *E. coli* transformant cells carrying the 5.8-kbp *Pst*I-*Bam*HI fragment (pBDD70) (Fig. 1) were examined for aminotransferase activity, with DABA as a fixed amino group donor and with 2-KG, pyruvate, and oxalacetate as amino group acceptors. As a result, the highest levels of glutamate formation activity were observed in the dialyzed crude extracts, when 2-KG was used as the amino group acceptor, the activity being ca. 29-fold higher than that for *A. baumannii* (Table 1). Omission of pyridoxal phosphate from the assay mixture resulted in almost the complete disappearance of enzyme activity. Furthermore, the construct pBDD07 exhibited half the activity of pBDD70, indicating that expression of ORF1 in pBDD07 is entirely dependent on its own promoter. Such an enzyme activity was absent from *E. coli* cells transformed with pBDD75 or pUC19 as a control. Moreover, a similar enzyme activity was consistently detected in dialyzed crude extracts of all the *Acinetobacter* species tested (Table 1), and the highest activity was observed for each crude enzyme when DABA and 2-ketoglutarate were used as the substrates, as for the purified recombinant DABA AT described below.

DABA can lose either the α-amino group or the γ-amino group during transamination, forming either aspartic β-semialdehyde or 4-amino-2-ketobutyric acid. It has been reported that these two compounds can be distinguished by oxidation with hydrogen peroxide, i.e., the former gives aspartic acid and the latter gives β-alanine (36). When such experiments were carried out with appropriate controls, aspartic acid was invari-

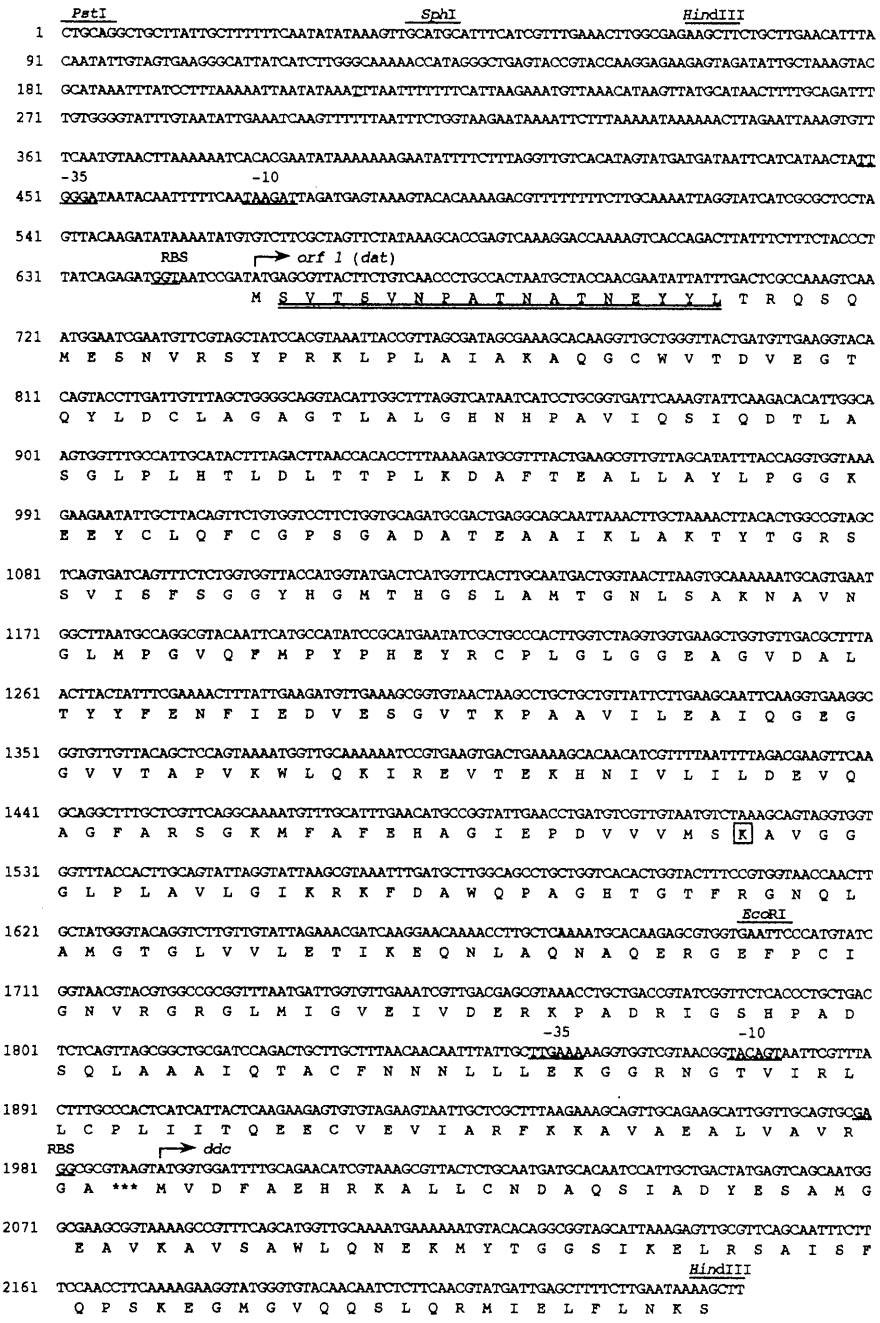

FIG. 2. Nucleotide and deduced amino acid sequences of the 2.2-kbp *Pst*I-*Hin*dIII region. The complete nucleotide sequence of ORF1 (*dat* [this work]) and the nucleotide sequence of the 5′ region of *ddc* (20) are shown. The start and direction of the two genes are indicated by horizontal arrows, and the −10 and −35 regions of putative promoter sequences and a putative ribosome-binding site (RBS) are underlined. The translation stop codon is marked with asterisks. Relevant restriction sites are indicated above their corresponding recognition sequences. The N-terminal amino acid sequence which was consistent with that of the recombinant enzyme is double underlined. The K residue, a putative pyridoxal phosphate-binding site, is boxed.

ably formed by treatment of the reaction mixture with 3% (wt/vol) H_2O_2 (final concentration) for 30 min at room temperature, and its identity was confirmed by GC-MS (data not shown). Furthermore, the reverse reactions with L-glutamic acid and L-aspartic β-semialdehyde provided DABA, which was identified by GC-MS (data not shown), although the reaction rate was not linear as a function of the incubation time or the amount of enzyme protein used, probably due to the lability of aspartic β-semialdehyde at alkaline pH (36). Therefore, the transamination reaction between L-DABA and 2-KG was employed for the DABA AT activity assay. Replacement of L-glutamic acid with the D-isomer failed to give rise to DABA, suggesting at least stereospecificity in the α-amino group transfer of glutamic acid. Since DABA DC is specific to L-DABA (48), only the L-configuration of aspartic β-semialdehyde must serve as an amino group donor. An experiment with D-aspartic β-semialdehyde, however, was not performed because it was unavailable commercially.

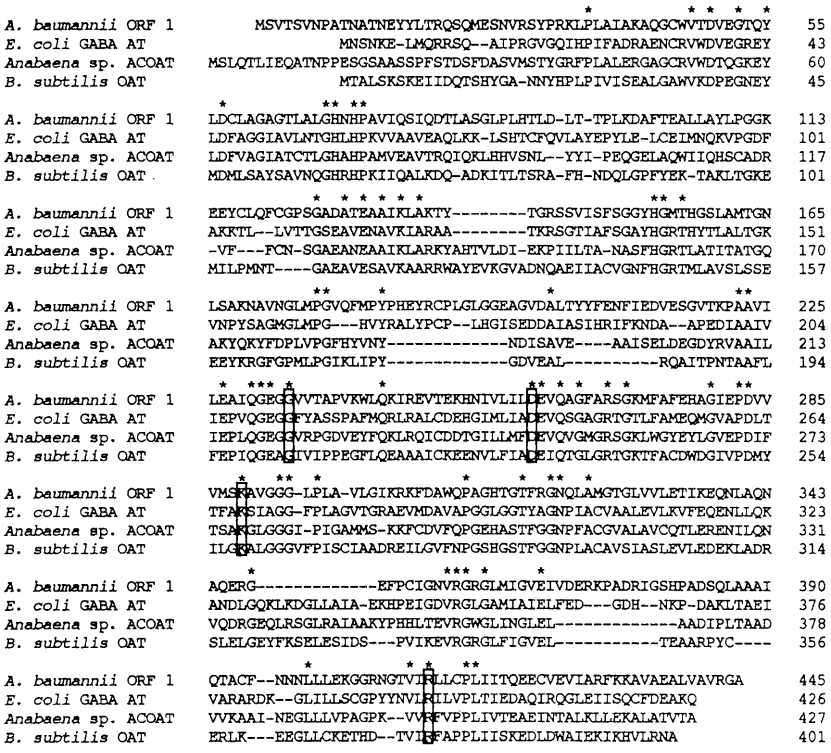

FIG. 3. Multiple alignment of the deduced amino acid sequences of the *A. baumannii* ORF1 (*dat*) to those of aminotransferases of bacterial sources. The residues that are identical in all the displayed sequences are marked by asterisks, and the four invariant residues (G, D, K, and R) proposed for aminotransferases by Mehta et al. (28) are boxed. Gaps, indicated by dashes, were introduced to allow for an optimal alignment. Abbreviations are as follows, with GenBank accession numbers in parentheses: GABA AT, 4-aminobutyrate aminotransferase (M88334); ACOAT, *N*-acetylornithine aminotransferase (X78854); and OAT, ornithine aminotransferase (X81802).

Purification and characterization of DABA AT expressed in *E. coli*. In order to confirm the identity of ORF1 as the gene encoding DABA AT, we attempted to purify the protein that it encodes to homogeneity and to determine its N-terminal sequence. Unfortunately, *E. coli* cells harboring either pBDD7, pBDD70, or pBDD07 exhibited significant cell lysis, leading to pronounced reduction in growth. DAP produced may affect the intracellular environment of the host strain because it was mostly found in the cells, in contrast to *A. baumannii*. Therefore, the 456-bp *Cla*I region of the *ddc* gene in pBDD70 was deleted to generate an in-frame construct, pBDD81, with the native translation stop codon and transcriptional terminator (Fig. 1). *E. coli* cells transformed with pBDD81 showed normal growth with DABA AT activity ca. 25-fold higher than that of *A. baumannii* (Table 1) but failed to express the active DABA DC protein. A 35-fold purification of recombinant DABA AT was achieved with a 2.6% overall recovery in total activity; a yield of ca. 2 mg of the purified enzyme with a specific activity of 765 μmol of glutamic acid formed per h per mg of protein was obtained from 24 g (wet weight) of the *E. coli* transformant cells. The purification was followed by SDS-PAGE, and the heat-treated sample appeared as a single band of 45 kDa in Coomassie (data not shown), which is in reasonable agreement with the M_r predicted from the nucleotide sequence.

TABLE 1. DABA AT activity of *E. coli* HB101 carrying a recombinant plasmid and of type strains of *Acinetobacter* spp.

Plasmid or strain	Sp act[a]
pBDD70	25.6
pBDD07	12.8
pBDD75	0
pBDD81	22.1
pUC19	0
A. baumannii ATCC 19606	0.89
A. calcoaceticus ATCC 23055	0.38
Acinetobacter genospecies 3 ATCC 19004	1.0
A. lwoffii ATCC 15309	0.78
A. haemolyticus ATCC 17906	1.1
A. junii ATCC 17908	2.1
A. johnsonii ATCC 17909	1.3
A. radioresistens IAM13186	0.59

[a] Transformants carrying either a recombinant plasmid or the plasmid vector as a control were grown in LB broth in the presence of 100 μg of ampicillin per ml at 37°C for 8 h, and the type strains of *Acinetobacter* sp. were grown in nutrient broth at 30°C for 8 h. Enzyme activity was determined in dialyzed crude extracts. Specific activity is defined as micromoles of glutamic acid formed per hour per milligram of protein.

N-terminal sequence analysis of the first 17 amino acid residues of purified enzyme confirmed the translational start of the *dat* gene and the predicted protein sequence (Fig. 2), although processing of the initial methionine was observed. The native M_r was determined by gel filtration on a Sephacryl S-200 HR column to be 188,000 (data not shown), suggesting that the enzyme is a tetramer. The K_m values for L-DABA and 2-KG were determined to be 4.30 and 1.46 mM, respectively. The enzyme was active in the high-pH region, with a maximum activity at pH 8.25 to 8.75. Although the enzyme was active towards some structurally related compounds, the highest activity was observed for the combination of DABA and 2-KG (Table 2). Interestingly, lysine repeatedly showed a higher activity than did ornithine. On the basis of the substrate specificity together with the data described above, the enzyme and

TABLE 2. Substrate specificity of purified recombinant DABA AT

Substrate	Relative activity (%)[a] for:	
	2-KG	L-DABA
Amino group donor		
L-DABA	100	
4-Aminobutyric acid	44	
L-2,3-Diaminopropionic acid	4	
β-Alanine	0	
L-Ornithine	19	
L-Lysine	27	
Amino group acceptor		
2-KG		100
Pyruvic acid		4
Oxalacetic acid		5

[a] 2-KG and L-DABA were used as an amino group acceptor and an amino group donor, respectively, at a final concentration of 10 mM.

ORF1 were named DABA:2-KG 4-aminotransferase (DABA AT) and *dat*, respectively.

Conversion of radiolabeled aspartic acid into DAP. To confirm that DAP is actually derived from aspartic acid in *Acinetobacter*, we analyzed an amine fraction from the culture of *A. baumannii* ATCC 19606 incubated with radiolabeled aspartic acid. Only a single radioactive band was detected throughout the lane of the radiochromatogram, whose location corresponded to that of the authentic DAP (Fig. 4), suggesting that DAP is produced from aspartic acid by its conversion into aspartic β-semialdehyde, followed by the actions of the two enzymes DABA AT and DABA DC.

Detection of *dat* homologs in other *Acinetobacter* species. Samples of genomic DNA from the type strain of each *Acinetobacter* species were PCR amplified with a primer set prepared according to the known sequence of *dat* and analyzed by agarose gel electrophoresis. The expected amplicon band of 848 bp was found for each strain (Fig. 5). In agreement with this result, a Western blot of the crude cell extract from each of these strains exhibited a band with an M_r similar to that of *A. baumannii* DABA AT, when proteins on membranes were probed with antibodies against the purified recombinant enzyme (Fig. 5). These data indicate that the homologs of *dat* are widely distributed and expressed in the genus *Acinetobacter*.

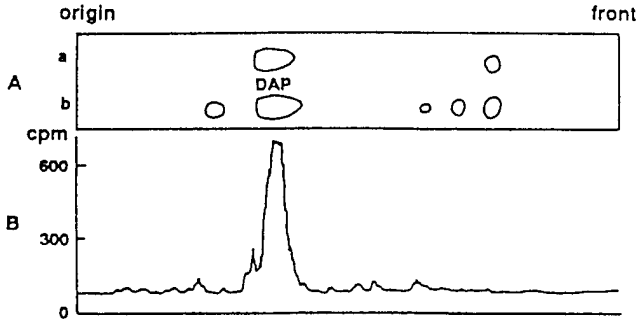

FIG. 4. Thin-layer and radiochromatograms showing the conversion of L-aspartic acid into DAP. *A. baumannii* ATCC 19606 cells were incubated for 1 h in a chemically defined medium containing 5 mM L-aspartic acid (185 kBq of L-[U-^{14}C]aspartic acid), and the prepared amine fraction was dansylated and separated with a precoated silica gel 60 plate (see Materials and Methods for details). (A) Thin-layer chromatograms of the authentic dansylated DAP (a) and the dansylated amine fraction from *A. baumannii* (b). The fluorescent spots under UV light are shown. (B) Radiochromatogram of row b in panel A.

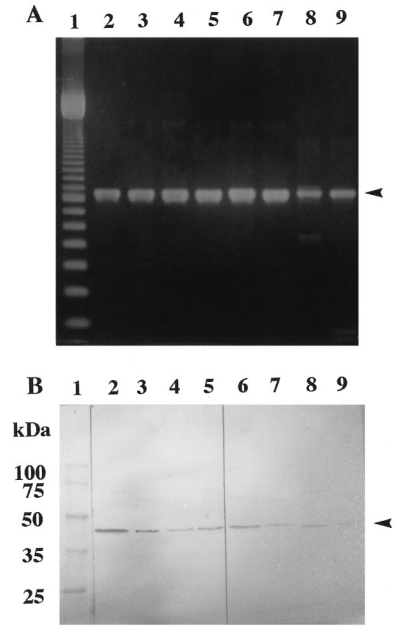

FIG. 5. Detection of the homologs of *dat* and DABA AT in various *Acinetobacter* species by PCR (A) and Western blotting (B). (A) A primer set prepared according to the known nucleotide sequence of the *A. baumannii dat* gene was used for PCR amplification of the chromosomal DNAs from *Acinetobacter* species. (B) The DABA AT protein was probed with the antibodies raised against the recombinant DABA AT purified from *E. coli* cells carrying pBDD81. See Materials and Methods for details. Lane 1, 100-bp ladder (Pharmacia) (A) and M_r marker proteins (Novagen) (B); lane 2, *A. baumannii* ATCC 19606; lane 3, *A. calcoaceticus* ATCC 23055; lane 4, *Acinetobacter* genospecies 3 ATCC 19004; lane 5, *A. lwoffii* ATCC 15309; lane 6, *Acinetobacter junii* ATCC 17908; lane 7, *Acinetobacter haemolyticus* ATCC 17906; lane 8, *Acinetobacter johnsonii* ATCC 17909; lane 9, *Acinetobacter radioresistens* IAM13186. Arrowheads in panels A and B indicate amplicon bands (848 bp for *A. baumannii*) and DABA AT proteins (45 kDa for *A. baumannii*), respectively.

DISCUSSION

DABA, one of the unusual amino acids, occurs naturally, for example, in the peptide antibiotics polymyxins (33) and pseudomucins (16), in cell walls of *Corynebacteria* (34) and *Agrococcus jenensis* (13), and in plants (reference 32 and references cited therein). On the other hand, enzymes catalyzing the formation of DABA from L-glutamic acid and L-aspartic β-semialdehyde have been reported in *Bacillus colistinus* (22) and in *Ectothiorhodospira halochloris* and *Halomonas elongata* (35), where DABA serves as a component of a peptide antibiotic and a precursor of ectoine, an osmoprotectant, respectively. An enzyme capable of transferring the amino group to aspartic β-semialdehyde to form DABA has also been described in *Xanthomonas* species, but only L-alanine is accepted as an amino group donor (36). However, as far as we know, these enzymes and the genes responsible have not been addressed at the genetic or molecular level.

Our previous work (19) suggested that the cloned *Bam*HI DNA fragment from *A. baumannii* contains the gene(s) relevant to the formation of DABA in addition to the *ddc* gene encoding the DABA DC protein. In the present report, we have demonstrated that a second 1,388-nucleotide ORF (*dat*) identified just upstream of *ddc* codes for a novel enzyme, DABA AT, catalyzing the following reversible reactions: L-DABA + 2-KG↔L-glutamic acid + L-aspartic β-semialdehyde. The absence of a recognizable transcriptional terminator signal for *dat* indicates that the promoter upstream of the *dat* gene drives the cotranscription of both *dat* and *ddc*, ending at

the transcriptional terminator situated just downstream of the *ddc* gene (20).

Our sequence comparison studies revealed significant homology of *A. baumannii* DABA AT with 4-aminobutyrate aminotransferase, *N*-acetylornithine aminotransferase, and ornithine aminotransferase of bacterial origin (1, 11, 12). In this context, it is interesting that 4-aminobutyric acid and, to a lesser extent, ornithine and lysine are accepted as substrates by DABA AT. Thus, the structural relatedness of these aminotransferases may reflect functional similarity of the proteins. The activities of these enzymes are characteristic of catalyzing the transfer of ω-amino groups, and hence DABA AT can be classified into group II of the aminotransferases (28). Moreover, the sequence alignment of these enzymes shows that DABA AT shares the conserved sequence segments containing four important invariant residues with the family of aminotransferases (28). In particular, it is worth noting that K-289 in *A. baumannii* DABA AT is aligned with the lysine residue proposed to be a pyridoxal phosphate-binding site in many other aminotransferases (28). However, in contrast to *E. coli* 4-aminobutyrate aminotransferase, which is a homodimer (1), DABA AT may be a homotetramer.

It is known that aspartic acid is converted by two enzymatic steps into aspartic β-semialdehyde, an intermediate common to lysine, threonine, and methionine syntheses (9). This explains why *E. coli* transformants carrying both *dat* and *ddc* genes produce DAP. A similar pathway probably functions in *Acinetobacter*. The rapid incorporation of the radioisotope derived from aspartic acid into DAP supports this hypothesis. Most likely, *Acinetobacter* DABA AT participates exclusively in the synthesis of DABA, which is subsequently decarboxylated by DABA DC yielding DAP. PCR and Western blot analyses revealed the existence of the *dat* (this study) and *ddc* homologs (20) in several other *Acinetobacter* species. Together with these findings, the synthesis of DABA in a coordinated form with its conversion to DAP by DABA DC suggests the possible biological importance of DAP to *Acinetobacter*. It has been reported that the growth of the *E. coli* mutants defective in putrescine synthesis can be fully sustained by the addition of DAP to the medium (29). This is expected due to the growth promotion activity of DAP itself, because the *E. coli* putrescine aminopropyltransferase cannot accept DAP at all as a substrate to synthesize a triamine analog (7).

We have previously purified the DABA DCs from *Vibrio alginolyticus* (31) and some enterobacterial species, such as *Enterobacter aerogenes* and *Serratia marcescens* (45). It is conceivable that these species have an enzyme similar to DABA AT of *Acinetobacter* species to synthesize DABA as the substrate for these decarboxylases. The present findings might be useful for the elucidation of such enzymes and the genes responsible in these species as well as in other species in which DABA has been detected as described above. It would be interesting from evolutionary and structural standpoints to compare the genes responsible for DABA synthesis among these bacteria.

In conclusion, we have demonstrated the existence of a novel enzyme, DABA AT, in *A. baumannii* by analyzing the nucleotide sequence of the gene responsible and have shown that *A. baumannii dat* is potentially translationally coupled with the downstream gene *ddc*. At the present time, the physiological roles of the DABA-DAP synthetic pathway are not clear, and further investigation to clarify them is needed. Considering that in bacteria, genes with related functions often are grouped together in operons, it is possible that these genes play physiologically important roles in *Acinetobacter.*

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