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Characterization of the Genes Encoding D-Amino Acid Transaminase and Glutamate Racemase, Two D-Glutamate Biosynthetic Enzymes of *Bacillus sphaericus* ATCC 10208

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In *Bacillus sphaericus* and other *Bacillus* spp., D-amino acid transaminase has been considered solely responsible for biosynthesis of D-glutamate, an essential component of cell wall peptidoglycan, in contrast to the glutamate racemase employed by many other bacteria. We report here the cloning of the *dat* gene encoding D-amino acid transaminase and the *glr* gene encoding a glutamate racemase from *B. sphaericus* ATCC 10208. The *glr* gene encodes a 28.8-kDa protein with 40 to 50% sequence identity to the glutamate racemases of *Lactobacillus*, *Pediococcus*, and *Staphylococcus* species. The *dat* gene encodes a 31.4-kDa peptide with 67% primary sequence homology to the D-amino acid transaminase of the thermophilic *Bacillus* sp. strain YM1.

Bacteria normally synthesize D-glutamate and D-alanine as essential components of cell wall peptidoglycan (24). In addition, certain species such as the bacilli synthesize a number of other D-amino acids, including D-phenylalanine, D-asparagine, and D-ornithine, as intermediates in the production of secondary metabolites such as peptide-based antibiotics (2, 13, 36). D-Alanine biosynthesis from L-alanine by alanine racemase appears to be ubiquitous in bacteria (1, 34), but two distinct enzymatic routes have been identified for bacterial D-glutamate biosynthesis. In organisms such as *Escherichia coli*, *Lactobacillus* spp., and *Pediococcus* spp., specific racemases are responsible for the direct biosynthesis of D-glutamate and D-alanine from their L-amino acids (6, 9, 20). Conversely, *Bacillus* spp., such as *Bacillus sphaericus* and *Bacillus licheniformis*, possess a D-amino acid transaminase capable of synthesizing D-glutamate among a broad range of D-amino acids from keto acid precursors, using D-alanine as the amino donor (13, 35). The D-amino acid transaminases of *B. sphaericus* ATCC 10208 and *Bacillus* sp. strain YM1 have been particularly well studied (18, 27, 31, 35). The bacilli have generally been considered to synthesize only D-alanine through the action of a racemase, with D-glutamate biosynthesis attributed to the D-amino acid transaminase using α-ketoglutarate and D-alanine as substrates (5, 20), although a strain of *Bacillus pumilus* which lacks D-amino acid transaminase does possess glutamate racemase activity (14). Only one organism, *Staphylococcus haemolyticus*, has been shown to possess both D-amino acid transaminase and glutamate racemase activities (22).

We have cloned and characterized genes encoding two distinct D-amino acid biosynthetic enzymes from *B. sphaericus* ATCC 10208 by using a complementation screen with *E. coli* WM335 (15). This mutant, dependent on exogenously supplied D-glutamate has been used previously to isolate genes encoding D-amino acid transaminase and glutamate racemase (14, 22, 32). Enzymatic assays indicate that the genes isolated in this work encode the D-amino acid transaminase purified from this strain earlier and a previously undetected glutamate racemase. The presence of the two enzymes in *B. sphaericus* suggests that the bacilli, like *Staphylococcus* spp., possess two biosynthetic routes to D-glutamate.

Cloning of D-glutamate biosynthetic genes from *B. sphaericus*. Liquid cultures were grown with aeration at 37°C in Lennox broth (LB) (Gibco/BRL, Gaithersburg, Md.). Plate cultures were grown at 37°C on LB agar or M9 (23) minimal salts–1.5% agar supplemented with 0.2% glucose. *E. coli* WM335 cultures were supplemented with D-glutamate and thymine, both at 50 μg/ml. Where appropriate, chloramphenicol (10 μg/ml) and ampicillin (200 μg/ml) were added. pBR322 DNA was obtained from New England Biolabs (Beverly, Mass.). Partially *Mbo*I-digested chromosomal DNA (16) of *B. sphaericus* ATCC 10208 was size fractionated on a 0.8% agarose gel, and 2- to 10-kb fragments were ligated to *Bam*HI- and *Bgl*II-cleaved pIF306. Plasmid pIF306 is a pBR322 (3) derivative containing a unique *Bgl*II site inserted between the vector *Bam*HI and *Sph*I sites. Genes inserted between the *Bam*HI and *Bgl*II sites are transcribed from a strong constitutive promoter (21) derived from the *pheA* (10) promoter of *E. coli* K-12, located immediately upstream between the unique *Hin*dIII and *Bam*HI sites of the vector. The plasmid library was used to transform the restriction-deficient strain XL1 Blue (Stratagene), and approximately 20,000 colonies were obtained. Plasmid DNA prepared from pooled isolates was then introduced into *E. coli* WM335 and plated on LB-thymine medium in the absence of supplemental D-glutamic acid. Approximately 50 clones which were able to complement the D-glutamic acid deficiency of the host were identified. Restriction analysis of these clones identified two classes of clones represented by the individual plasmid isolates pIF1001 and pIF1002. Each of these plasmids was used to retransform fresh cells of WM335 which were cultured and assayed for D-amino acid transaminase and glutamate racemase activity.

Characterization of the *B. sphaericus glr* gene. Plasmid DNA isolation, restriction analysis, and ligation were performed by standard methodology as previously described (16). Plasmid pIF1001 carried a 2.34-kb *Mbo*I fragment of *B. sphaericus* chromosome. Sequence analysis of this fragment (25) revealed a single large open reading frame of 684 bp encoding a 28,876-Da protein (GenBank accession no. U26733). Strong primary sequence identity was observed between this protein sequence and the glutamate racemases of *B. pumilus* (50%), *S. haemolyticus* (50%), *Pediococcus pentosaceus* (43%), *Lacto-*

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B. sphaericus     ---------- ------MNA- P------IGV IDSGVGGLTV AKEIIKRLPN ETIYYIGDTA RCPYGPRTRQ EVRNF    52
B. pumilus        ---------- ------MLDQ P------IGV IDSGVGGLTV AKEIMRQLPK EKIIYVGDTK RCPYGPRKEE EVLHY    53
S. haemolyticus   M--------- ------KMNK P------IGV IDSGVGGLTV AKEIMRQLPN ETIYYLGDIA RCPYGPRPGD EVKQF    54
P. pentosaceus    MDVCIILFSG GIK---MDNR P------IGF MDSGVGGLTV VKTAQKLLPN EEIIFIGDEA RMPYGPRPTA EVVEF    66
L. brevis         ---------- ------MLDQ P------IGV IDSGVGGLTV AKEIMRQLPK EKIIYVGDTK RCPYGPRKEE EVLHY    53
L. fermentus      ---------- ------MDNR P------IGV MDSGLGGLSV VRVIQQKLPN EEVIFVGDQG HFPYGTKDQA EVRQL    53
E. coli           MRQSMATKLQ DGNTPCLAAT PSEPRPTVLV FDSGVGGLSV YDEIRHLLPD LHYIYAFDNV AFPYGEKSEA FIVER    75
B. subtilis RacE  ---------- ------MLEQ P------IGV IDSGVGGLTV AKEIMRQLPK ENIIYVGDTK RCPYGPRPEE EVLQY    53
B. subtilis YrpC  ---------- ------M--- K------IGF FDSGIGGMTV LYEAIKVLPY EDYIFYADTL NVPYGEKSKG KVKEY    50
Consensus         ---------- ------M... P------IGV IDSGVGGLTV AKEI...LP. E.IIY.GDT. RCPYGPR... EV...    75

B. sphaericus     TWQMAKALEK MN-IKMLVIA CNTATAVALE SLQRNMPFPV LGVINRGARA AVK-KTKRHE VVVLATEGTI KSGAY   125
B. pumilus        TWEMAHYLLK HHHIKMLVIA CNTATAIALD EIKATLDIPV IGVIQPGART AIK-VTNNQH IGVIGTINTI KSEAY   127
S. haemolyticus   TTQLANKLMQ FD-IKMLVIA CNTATAVALE HLQQMLPIPV IGVIEPGSRT AIM-TTKNQN VLILGTEGTI KSEAY   127
P. pentosaceus    SRQMASFLMT KN-IKALVIA CNTATNAALA VLQAELPIPV IGVILPGAIA ANR-QTKNQK IGVIATLGTI KSEAY   139
L. brevis         TWEMAHYLLK HHHIKMLVIA CNTATAIALD EIKATLDIPV IGVIQPGART AIK-VTNNQH IGVIGTINTI KSEAY   127
L. fermentus      ALSIGAFLLK HD-VKMMVVA CNTATAAALP ALQAALPIPV IGVIEPGARA ALA-QDKKGP IGVIATTATT TAGAY   126
E. coli           VVAIVTAVQE RYPLALAVVA CNTASTVSLP ALREKFDFPV VGVV-PAIKP AAR-LTANGI VGLLATRGTV KRSYT   148
B. subtilis RacE  TWELTNYLLE NHHIKMLVIA CNTATAIALD DIQRSVGIPV VGVIQPGARA AIK-VTDNQH IGVIGTENTI KSNAY   127
B. subtilis YrpC  IFNAAEFL-A SQNIKALVIA CNTATSIAIE DLRRNFDFPI IGIEPAVKPA INKCTEERKR VLVVATNLTL KEEKF   124
Consensus         T...A..L.. ...IKMLVIA CNTATA.AL. .LQ..L.IPV IGVI.PGARA A.K-.T.NQ. IGVIAT..TI KSEAY   150

B. sphaericus     EEALLSLNTS THIIPLACPT FVPLVESGEY KGQFANN-LI AEGLKPLK-N EQFDTVILGC THYPILQKQI EAVVG   198
B. pumilus        KEALLSLKAG LTVQSLACPL LVPFVESGTF LDQTAEAVVK D-SLEPMK-E TGIDTLILGC THYPILKEPI QRFMG   200
S. haemolyticus   RHHIKHINPN VHVLWCGLPG FVPLVEQMRY DDPTITSIVI HQTLKQWR-N TDADTIILGC THYPLLYKPI NDYFG   201
P. pentosaceus    PKALAEINTK LRAYPVACQE FVEIAEKNEL HTTAAQK-VM NEKLAEFR-Q DQIDTLILGC THFPLLEEGI QAAVG   212
L. brevis         KEALLSLKAG LTVQSLACPL LVPFVESGTF LDQTAEAVVK D-SLEPMK-E TGIDTLILGC THYPILKEPI QRFMG   200
L. fermentus      PATIERLAPG TPVIAKATQP MVEIVEHGQT GTAKAQE-VV SEQLMTFK-E HPVKTLIMGC THFPFLAPEI SKAVG   199
E. coli           HELIARFANE CQIEMLGSAE MVELAEAKLH GEDVSLDALK RILRPWLRMK EPPDTVVLGC THFPLLQEEL LQVLP   223
B. subtilis RacE  EEALLALNPD LKVENLACPL LVPFVESGKF LDQTADEIVK T-SLYPLK-D TSIDSLILGC THYPILKEAI QRYMG   200
B. subtilis YrpC  HNLVKEIDHH DLVDCLALPG LVEFAENFDF SEDKIIKYLK N-ELSSFD-L KQYGTIVLGC THFPFFKNSF EKLFG   197
Consensus         .EAL..L... ..V..LACP. .VP.VE.G.. ....A...VK ...L...K-. ...DTLILGC THYP.L.E.I ....G   225

B. sphaericus     EDVFVLSSAE ETAKDVEEML AYNGTLADTN AKPAHKFYAT GSVPIFRSIA ENWLEQGTLD IHRITLK--- --      265
B. pumilus        SDVSIISSGD ETAREASTIL SYKGLLNTSK ETPVHTFYTT GQQQNFQNIA RDWFGYLPGK VETVSLEHIY QQ      272
S. haemolyticus   GEKKVISSGL ETAREVSALL TFSNEHASYT QHPEHRFFAT GDTVHIKNII LQWL-KLDVE VERISVDE-- --      268
P. pentosaceus    PDVTLVDPGV ETVHQLIEIL TKQALQHAEG PKAQDQVYST GNIKNF-EEI ARTFLNQDLR VEEVKID--- --      278
L. brevis         SDVSIISSGD ETAREASTIL SYKGLLNTSK ETPVHTFYTT GQQQNFQNIA RDWFGYLPGK VETVSLEHIY QQ      272
L. fermentus      PTVALVDPAK ETVATAKSWL EQHQ-AMGNH AHPNYHLYST GNLPDLRAGV NKWLLSGHFD LGTAQIEE-- GD      268
E. coli           EGTRLVDSGA AIARRTAWLL EHEA--PDAK SADANIAFCM AMTPGAEQLL PVLQRYGFET LEKLAVLG-- --      289
B. subtilis RacE  EHVNIISSGD ETAREVSTIL SYKGLLNQSP IAPDHQFLTT GARDQFAKIA DDWFGHEVGH VECISLQEPI KR      272
B. subtilis YrpC  IKVDMISGSV GTAKQLKKVL ADRNQL--GK GSGSITFFNS GHKIVDQEVI SK-YKRLFEI LDETQRSHV- GH      265
Consensus         ..V..ISSG. ETAR.....L .....L.... ..P.H.FY.T G....F..I. ..W..... VE......-- ..      297
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FIG. 1. Primary sequence homology between glutamate racemase of *B. sphaericus*, glutamate racemases from other bacteria, and the putative gene products of the *B. subtilis racE* and *yrpC* genes. The consensus sequence shows the amino acids that are conserved in four or more sequences. Sequence identities (percentages) between *B. sphaericus* glutamate racemase and the individual enzymes are as follows: *B. pumilis*, 50%; *S. haemolyticus*, 50%; *P. pentosaceus*, 43%; *L. brevis*, 40%; *L. fermentus*, 37%; *E. coli*, 26%; *B. subtilis* RacE, 50%; and *B. subtilis* YrpC, 30%.

bacillus fermentus (37%), *Lactobacillus brevis* (40%), and to a lesser extent *E. coli* (26%). Strong sequence identity (50 and 30%, respectively) was also observed between *B. sphaericus* glutamate racemase and the putative products of the *racE* (GenBank accession no. Z75208) and *yrpC* (GenBank accession no. U93875) genes identified in the *B. subtilis* genomic sequence. The sequence alignment is shown in Fig. 1. The peptide also displayed a low level of primary sequence identity (21%) to the aspartate racemases of *Streptococcus thermophilus* and *Desulfurococcus* sp., but no significant identity was observed with alanine racemase of *B. subtilis* or *Bacillus stearothermophilus* or with *B. subtilis* amino acid racemase (GenBank accession no. Z94043). Identification of the ATG start codon of the protein was strongly supported by the sequence homology between the protein's N terminus and the other known glutamate racemase sequences, the presence of a consensus *Bacillus* ribosome binding site GAGG 7 bp upstream of the ATG codon, and the absence of upstream ATG, TTG, and GTG start codons capable of initiating the same open reading frame. Downstream of the coding sequence, there is a region of strong dyad symmetry typical of a bacterial transcription terminator.

Glutamate racemase activity of pIF1001. Cultures of strains to be assayed were inoculated from single colonies into 10 ml of LB and grown with shaking at 37°C. These were used to inoculate 50-ml LB cultures which were grown for 8 h in a 1-liter shake flask from an initial optical density at 600 nm of 0.05. Cells for glutamate racemase assay were washed, resuspended in 50 mM Tris HCl buffer (pH 8.0), and lysed in a French pressure cell at 1,000 lb/in^2. Supernatant fluid was recovered for the assay following centrifugation at 14,000 $\times$ *g* for 30 min at 4°C. Glutamate racemase activity was assayed by an L-glutamate dehydrogenase-coupled assay as described previously (22). Assay results were corrected for nonspecific reduction of NADP, which was determined from control assays carried out in the absence of D-glutamate. One unit is defined as 1 μmol of NADP reduced per min at 37°C. The results are

TABLE 1. D-Amino acid transaminase and glutamate racemase activity of *dat* and *glr* clones[a]

Strain	D-Amino acid transaminase sp act (U[b]/mg of protein)	Glutamate racemase sp act (U[c]/mg of protein)	Complementation of WM335[d]
WM335/pIF306	0.0080	0.007	−
WM335/pIF1001 (*glr*)	0.0065	0.025	+
WM335/pIF1002 (*dat*)	7.09	0.005	+
WM335/pIF372 (*dat*)	0.3	ND[e]	+

[a] Results represent averages of three assays corrected for endogenous rates.
[b] One unit of activity defined as 1 mmol of pyruvate (NAD) produced per min at 37°C.
[c] One unit of activity defined as 1 mmol of NADPH produced per min at 37°C.
[d] Complementation being normal overnight growth on LB agar. Symbols: −, no growth; +, growth.
[e] ND, not determined.

summarized in Table 1. Measurements of glutamate racemase activity in pIF1001-bearing cells were consistently three to four times higher than those of the control cells (WM335 carrying pIF306), while the activity of WM335 carrying pIF1002 was not higher than that of the control. The protein encoded on pIF1001 was concluded to be a glutamate racemase of *B. sphaericus* ATCC 10208, and the gene was designated *glr*. The *glr* gene does not appear to be part of an operon, as there are no significant open reading frames upstream or downstream of the gene. Moreover, *glr* is oriented on pIF1001 such that expression is convergent with the upstream *pheA*-derived promoter region. This suggests that the native *glr* promoter is present on the fragment cloned in pIF1001, although we have not yet identified the transcription start site in *B. sphaericus*. Analysis of the DNA sequence upstream of the coding region did not reveal sequences with significant homology to the consensus *Bacillus* σ^a −35 (TTGACA) and −10 (TATAAT) regions reported for several *B. sphaericus* genes expressed during vegetative growth (4, 33). The codon usage of *glr* is generally consistent with that of moderately expressed *E. coli* genes (7).

Characterization of the *B. sphaericus dat* gene. Plasmid pIF1002 contains a 2.5-kb insert, and sequence analysis revealed an open reading frame of 848 bp encoding a protein of 31,392 Da (GenBank accession no. U26732). The N-terminal coding sequence was subsequently determined by using additional primers and was found to agree entirely with that of the purified enzyme (data not shown). This confirmed that the gene cloned in pIF1002 encodes D-amino acid transaminase, and we have named it *dat*. The C-terminal amino acid sequence of the *B. sphaericus* ATCC 10208 D-amino acid transaminase has been previously reported to consist of the peptide sequence VI (FY)LAL-COOH (5). The VI(FY)LAL peptide does not occur anywhere in the peptide sequence, and the origin of this stretch of sequence is unknown. The coding sequence is preceded by a strong *Bacillus* ribosome binding site (AAAGGA) located 9 bp upstream of the ATG start codon. The gene is followed by a region of extensive dyad symmetry typical of bacterial transcription terminators.

D-Amino acid transaminase activity of pIF1002. Cells for D-amino acid transaminase assay were prepared as described above for the glutamate racemase assay but were washed and resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 8.5). Extracts of *E. coli* WM335 carrying pIF1002 were assayed for D-amino acid transaminase activity by the lactate dehydrogenase-coupled assay as described previously (11). Assay results were corrected for nonspecific oxidation of NADH, which was determined by assaying in the absence of D-alanine. One unit of activity is defined as that which produces 1 μmol of pyruvate (NAD) per min at 37°C. The results shown in Table 1 indicated levels of D-alanine-dependent NADH oxidation approximately 1,000-fold higher than those of control cultures carrying only the vector pIF306. Similar assays conducted with WM335 transformed with pIF1001 had shown no increase in D-alanine-dependent NADH oxidation over the levels in the controls. The specific activity of the purified *B. sphaericus* D-amino acid transaminase has been reported to be 116 to 160 U/mg, indicating that the WM335/pIF1002 cells express Dat to approximately 5% of releasable cell protein. The high level of *dat* expression may be due in part to the colinear orientation of the *dat* coding sequence with the constitutive *pheA* promoter located upstream on the vector. To determine if transcription occurred in the absence of a heterologous promoter, the *dat* gene and 400 bp of upstream untranslated sequence were isolated by PCR and subcloned onto the low-copy-number vector pLG338 (28) between the unique *Sal*I and *Eco*RI sites. Oligonucleotides used to isolate the *B. sphaericus dat* gene by PCR had the sequences 5′-GATGTCGACGTTAATCCAAACGT TAGC-3′ and 5′-GACGAATTCTTTTAGGTAGCTCTTTTT AATC-3′. The resulting plasmid, pIF372, was able to confer wild-type growth upon WM335 in the absence of exogenous D-glutamate and showed D-amino acid transaminase activity of 0.3 U/mg. These results indicated that *dat* expression could originate from the 400-bp upstream sequence. However, neither this transcription start site nor that used in *B. sphaericus* has been identified.

Primary sequence conservation among known D-amino acid transaminase enzymes. Genes encoding D-amino acid transaminases have previously been isolated from the thermophilic *Bacillus* sp. strain YM1 (30) and from *S. haemolyticus* (22). In addition, we have previously identified and characterized the *dat* gene of *B. licheniformis* (32) (GenBank accession no. U26947). The sequence identity between the enzymes encoded by these genes and the D-amino acid transaminase of *B. sphaericus* was examined as shown in Fig. 2. The *B. sphaericus* D-amino acid transaminase shows 67% identity to that of the thermophilic *Bacillus* sp. strain YM1, 48% identity to the D-amino acid transaminase of *S. haemolyticus*, and 42% identity to the *B. licheniformis* D-amino acid transaminase. The sequence also shows 42% identity to the putative D-amino acid transaminase encoded by the *B. subtilis yheM* gene (GenBank accession no. Y14082) identified by the sequencing of the genome. The crystal structure of the YM1 enzyme has been determined to 1.9-Å resolution, identifying a novel enzyme fold for a transaminase and enabling the assignment of residues believed to be important to the catalytic mechanism (29). Alignment of the primary sequence of *B. sphaericus* D-amino acid transaminase with those of the YM1 enzyme and the other D-amino acid transaminases not surprisingly shows complete conservation of K145 (important for substrate-cofactor proton transfer) and residues Y31, R50, E177, I204, and T205, which are all involved in cofactor ion pairing. There is also partial conservation of the serine residues S179 to S181, which contribute to cofactor positioning. Interestingly, the region from Ser240 to Ser243, which has been suggested to be important in substrate specificity through side chain discrimination, shows sequence variations in the known D-amino acid transaminase enzymes.

Conclusions. In this report we describe the isolation from *B. sphaericus* ATCC 10208 of the *dat* gene encoding the D-amino acid transaminase and a second gene we have designated *glr*, which encodes a hitherto unknown glutamate racemase. This is the first clear report of a *Bacillus* strain possessing both D-glutamate biosynthetic enzymes, although the genomic sequence of *B. subtilis* indicates that it may also possess both

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B. sphaericus      MAYSLWNDQI VEEGSITISP EDRGYQFGDG IYEVIKVYNG HMFTAQEHID RFYASAEKIR LVIPYTKDVL HKLLH     75
Bacillus sp. YM1   MGYTLWNDQI VKDEEVKIDK EDRGYQFGDG VYEVVKVYNG EMFTVNEHID RLYASAEKIR ITIPYTKDKF HQLLH     75
S. haemolyticus    MTKVFINGEF IDQNEAKVSY EDRGYVFGDG IYEYIRAYDG KLFTVTEHFE RFIRSASEIQ LDLGYTVEEL IDVVR     75
B. licheniformis   MK-VLFNGRL MERSECAVDI EDRGYQFGDG VYEVIRIYNG ILFTLDEHIA RLYKSAAEIG IDLSFSEAEL KSQLK     74
B. subtilis YheM   MK-VLVNGRL IGRSEASIDL EDRGYQFGDG IYEVIRVYKG VLFGLREHAE RFFRSAAEIG ISLPFSIEDL EWDLQ     74

Consensus          M..VL.NG.. ....E..ID. EDRGYQFGDG IYEVIRVYNG .LFT..EHI. RFY.SA.EI. I.LPYT...L ...L.     75

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B. sphaericus      DLIEKNNLNT GHVYFQITRG TTSRNHIFPD ASVPAVLTGN VKTGERSIEN FEKGVKATLV EDVRWLRCDI KSLNL    150
Bacillus sp. YM1   ELVEKNELNT GHIYFQVTRG TSPRAHQFPE NTVKPVIIGY TKENPRPLEN LEKGVKATFV EDIRWLRCDI KSLNL    150
S. haemolyticus    ELLKVNNIQN GGIYIQATRG VAPRNHSFPT PEVKPVIMAF AKSYDRPYDD LENGINAATV EDIRWLRCDI KSLNL    150
B. licheniformis   ELVDINQRRD GGLYLQVTRG KAPRKHQY-G AGLTPQVTAY TFPIQKPEKE QQNGVSAITA DDMRWLRCDI KSLNL    148
B. subtilis YheM   KLVQENAVSE GAVYIQTTRG VAPRKHQY-E AGLEPQTTAY TFTVKKPEQE QAYGVAAITD EDLRWLRCDI KSLNL    148

Consensus          ELV..N.... G..Y.Q.TRG .APR.HQFP. A.V.PV.TAY TK...RP... .E.GV.A.TV ED.RWLRCDI KSLNL    150

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B. sphaericus      LGAVLAKQEA SEKGCYEAIL HRGDIITECS SANVYGIKDG KLYTHPANNY ILNGITRQVI LKCAAEINLP VIEEP    225
Bacillus sp. YM1   LGAVLAKQEA HEKGCYEAIL HRNNTVTEGS SSNVFGIKDG ILYTHPANNM ILKGITRDVV IACANEINMP VKEIP    225
S. haemolyticus    LGNVLAKEYA VKYNAGEAIQ HRGETVTEGA SSNVYAIKDG AIYTHPVNNY ILNGITRKVI KWISEDEDIP FKEET    225
B. licheniformis   LYNVMIKQKA QEASAFEAIL IRDGLVTEGT SSNVYVAKQN VIYTHPVTTL ILNGITRMKV LQLCEENGLN YEEKA    223
B. subtilis YheM   LYNVMTKQRA YEAGAFEAIL LRDGVVTEGT SSNVYAVING TVRTHPANRL ILNGITRMNI LGLIEKNGIK LDETP    223

Consensus          LGNVLAKQ.A .E.GA.EAIL HR...VTEG. SSNVY.IKDG ..YTHPANN. ILNGITR.VI L...EE...P ..E.P    225

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B. sphaericus      MTKGDLLTMD EIIVSSVSSE VTPVIDVDGQ QIGAGVPGEW TRKLQKAFEA KL--PISINA                      283
Bacillus sp. YM1   FTTHEALKMD ELFVTSTTSE ITPVIEIDGK LIRDGKVGEW TRKLQKQFET KI--PKPLHI                      283
S. haemolyticus    FTVEFLKNAD EVIVSSTSAE VTPVVKIDGE QVGDGKVGPV TRQLQEGFNK YI--E-SRSS                      282
B. licheniformis   VTKDELLNAD EVFITSTTAE VIPVTSIDGQ TIGSGAPGPL TKNVQTALQN SILSETAKTV                      283
B. subtilis YheM   VSEEELKQAE EIFISSTTAE IIPVVTLDGQ SIGSGKPGPV TKQLQAAFQE SI-QQAASIS                      282

Consensus          .T..ELL.AD E.FVSSTTAE VTPV..IDGQ .IG.GKPGP. TR.LQ.AF.. .I--......                      285
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FIG. 2. Primary sequence homology between the D-amino acid transaminase, other known microbial D-amino acid transaminases, and the gene product of the *B. subtilis yheM* gene. The consensus sequence shows the amino acids that are conserved in three or more sequences. Individual percentages for sequence conservation with *B. sphaericus* D-amino acid transaminase are as follows: *Bacillus* sp. strain YM1, 67%; *S. haemolyticus*, 48%; *B. licheniformis*, 42%; and *B. subtilis* YheM, 42%. Asterisks indicate residues assigned active functions in the YM1 enzyme (29): substrate-cofactor proton transfer (K145), cofactor ion pairing (Y31, R50, E177, S179 to S181, I204, and T205), and substrate side chain recognition (S240 to S243).

enzymes. The *B. sphaericus* enzymes display high primary sequence identity to other known D-amino acid transaminases and glutamate racemases.

The physiological significance of the utilization of two independent types of D-amino acid biosynthetic enzymes and the relative contribution and regulation of the two *B. sphaericus* enzymes in D-glutamate biosynthesis remain undetermined. Pucci et al. (22) have demonstrated that the D-amino acid transaminase and glutamate racemase of *S. haemolyticus* can each complement the D-glutamate auxotrophy of *E. coli* WM335 when these genes are present on low-copy-number plasmid vectors. Similarly, we have observed complementation of WM335 by both *B. sphaericus* genes on the low-copy-number plasmid vector pLG338, suggesting that either enzyme may be able to synthesize sufficient D-glutamate to sustain cell growth. Definitive experiments to disable each gene independently in the chromosome of *B. sphaericus* are required to determine the respective roles of the enzymes in D-glutamate biosynthesis. It is interesting to speculate that the glutamate racemase, by analogy to the alanine racemase, may be sufficient to provide the necessary D-glutamate for peptidoglycan synthesis, whereas the D-amino acid transaminase is required to provide a broader range of D-amino acids necessary for secondary metabolite biosynthesis. This would be consistent with the narrow substrate specificity typically displayed by glutamate and alanine racemases in contrast to the much broader substrate profile of the D-amino acid transaminases. Bacilli are known to produce a number of antibiotics, such as bacillomycins and bacitracins, which incorporate a variety of D-amino acids, such as D-phenylalanine, D-tyrosine, and D-asparagine (2, 13, 36).

The function of D-amino acid transaminase in the biosynthesis of D-glutamic acid (18) has been well documented, as has the ability of the enzyme to synthesize a wide range of D-amino acids from keto acid substrates (35). D-Amino acid transaminases from *B. subtilis* and *B. licheniformis* as well as the enzymes from *Bacillus* sp. strain YM1 and *B. sphaericus* have been shown to display diverse but distinct substrate specificities. The recent determination of the YM1 enzyme backbone fold and the identity of residues participating in the catalytic mechanism has provided a clearer understanding of the effects of the site-directed enzyme mutants created earlier (17, 19, 29). The isolation of the *B. sphaericus dat* gene now facilitates structure-function analyses between two well characterized and highly homologous *Bacillus* D-amino acid transaminases and potentiates three-dimensional molecular modeling studies. Molecular modeling of the *B. sphaericus* D-amino acid transaminase sequence on the YM1 enzyme backbone may permit mutagenesis studies to probe differences in the properties of the enzymes. The 67% exact sequence identity between the YM1 and *B. sphaericus* D-amino acid transaminases is considerably greater than the 43% identity used successfully to conduct earlier studies using *E. coli* L-amino acid transaminases in which molecular models of the aromatic transaminase encoded by the *tyrB* gene (8) were derived from the known three-dimensional structure of the aspartate transaminase (26) encoded by *aspC*. Site-directed mutagenesis studies of *aspC* and *tyrB* were then used to test predictions regarding the residues influencing the substrate specificity of those enzymes towards aromatic and dicarboxylic substrates (12).

Distinct differences in substrate preferences have been observed between the D-amino acid transaminases of *Bacillus* sp.

strain YM1 and *B. sphaericus*. Amino acids, such as D-methionine, D-phenylalanine, and D-norleucine, which are good substrates for the *B. sphaericus* enzyme, are poor substrates for the YM1 enzyme (31). Many of the D-amino acid transaminase residues implicated in the active-site architecture of the YM1 D-amino acid transaminase are conserved in the *B. sphaericus* D-amino acid transaminase, but there are differences in the Ser240-to-Ser243 region proposed as part of a side chain binding pocket for D-amino acid substrate side chains. It will be interesting to explore this observation and to probe the differences in D-amino acid transaminase substrate specificities through mutagenesis selection procedures, site-directed mutagenesis, and gene shuffling experiments.

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