The β/α-barrel fold type basic amino acid decarboxylases include eukaryotic ornithine decarboxylases (ODC) and bacterial and plant enzymes with activity on l-arginine and meso-diaminopimelate. These enzymes catalyze essential steps in polyamine and lysine biosynthesis. Phylogenetic analysis suggests that diverse bacterial species also contain ODC-like enzymes from this fold type. However, in comparison with the eukaryotic ODCs, amino acid differences were identified in the sequence of the 310-helix that forms a key specificity element in the active site, suggesting they might function on novel substrates. Putative decarboxylases from a phylogenetically diverse range of bacteria were characterized to determine their substrate preference. Enzymes from species within Methanosarcina, Pseudomonas, Bartonella, Nitrosomonas, Thermotoga, and Aquifex showed a strong preference for l-ornithine, whereas the enzyme from Vibrio vulnificus (VvL/ODC) had dual specificity functioning well on both l-ornithine and l-lysine. The x-ray structure of VvL/ODC was solved in the presence of the reaction products putrescine and cadaverine to 1.7 and 2.1 Å, respectively. The overall structure is similar to eukaryotic ODC; however, reorientation of the 310-helix enlarging the substrate binding pocket allows l-lysine to be accommodated. The structure of the putrescine-bound enzyme suggests that a bridging water molecule between the shorter l-ornithine and key active site residues provides the structural basis for VvL/ODC to also function on this substrate. Our data demonstrate that there is greater structural and functional diversity in bacterial polyamine biosynthetic decarboxylases than previously suspected.

Pyridoxal 5'-phosphate (PLP)2-dependent enzymes that catalyze the decarboxylation of basic amino acids evolved within two distinct structural classes as follows: those with structural homology to aspartate aminotransferase (AAT-fold/group III decarboxylases), and a second family (β/α-barrel fold/group IV decarboxylases) that are homologs of alanine racemase (1, 2). Enzymes belonging to the AAT-fold type are largely prokaryotic, whereas the β/α-barrel fold enzymes are extensively represented in both eukaryotes and prokaryotes and include the eukaryotic ornithine decarboxylases (ODC), plant and eubacterial arginine decarboxylases (ADC), prokaryotic diaminopimelate decarboxylases (DAPDC), and based on one report, a bacterial decarboxylase from Selenomonas ruminantium with dual specificity for l-lysine and l-ornithine (L/ODC) (3). A group of bacterial sequences from the family have also been annotated as carboxynorspermidine decarboxylases (CANSDCs) (4), although direct enzymatic proof of this is lacking. Finally, an enzyme from this family was identified in Paramecium bursaria chlorella virus cVADC, and although this enzyme groups in phylogenetic analysis with the eukaryotic ODCs, it has specificity for l-arginine (5, 6).

The basic amino acid decarboxylases catalyze essential steps in two important metabolic pathways, polyamine (ODC, ADC, and CANSDC) and lysine biosynthesis (DAPDC) (Scheme 1). ODC catalyzes the decarboxylation of l-ornithine to putrescine, which is the first committed step in polyamine biosynthesis in the metazoans, fungi, and many protozoans. Polyamines are essential for cell growth and in mammals play important roles in cell cycle (7), cancer (8, 9), and embryonic development (10). In addition to their roles in growth, they function in biofilm formation (11, 12) and motility (13) in bacteria. ODC is a drug target for therapeutic intervention in the polyamine pathway, and DL-α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, is a clinically proven treatment for African sleeping sickness caused by Trypanosoma brucei (14, 15).

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2 The abbreviations used are: PLP, pyridoxal 5'-phosphate; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; DAPDC, diaminopimelate decarboxylase; CANSDC, carboxynorspermidine decarboxylase; L/ODC, dual function lysine and ornithine decarboxylase; AAT, aspartate aminotransferase; DFMO, α-difluoromethylornithine; VvL/ODC, V. vulnificus lysine/ornithine decarboxylase; cVADC, P. bursaria chlorella virus-1 arginine decarboxylase; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviation; PDB, Protein Data Bank; ORF, open reading frame.
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\[
\begin{align*}
\text{L-ornithine} & \xrightarrow{\text{ODC}} \text{putrescine} \\
\text{L-lysine} & \xrightarrow{\text{LDC}} \text{cadaverine} \\
\text{meso-diaminopimelate} & \xrightarrow{\text{ADC}} \text{agmatine} \\
\text{L-arginine} & \xrightarrow{\text{CANSDC}} \text{norspermidine}
\end{align*}
\]

SCHEME 1. Enzymatic reactions catalyzed by the β/α-barrel fold decarboxylases.

X-ray structures of several eukaryotic ODCs (16–19), bacterial DAPDCs (20, 21), and cvADC (6) have been solved. The enzymes are obligate homodimers, and the monomers are composed of a β/α-barrel N-terminal domain and a β-sheet C-terminal domain. Two identical active sites are formed at the dimer interface between the N-terminal domain from one subunit and the C-terminal domain from the other. In these structures substrate analogs make key contacts with residues from both monomers. In *T. brucei* ODC, the ω-amino group of the co-crystallized ligand (e.g. putrescine, DFMO, and β-ornithine) interacts with Asp-361 from one monomer and with Asp-332 from the other. Both of these residues are invariant among the eukaryotic ODCs; however, Asp-332 is not conserved in other members of the β/α-barrel fold decarboxylases. Comparison of the structures for enzymes with different substrate preferences (ODC, DAPDC, and cvADC) identified the 310-helix that contains Asp-332 as the key specificity element (6). Amino acid residues that project from the 310-helix interact with substrate, and both the amino acid composition of the helix and its position are variable. Substrates of different size are accommodated by changes in the distance from the 310-helix to PLP, whereas changes in amino acid composition allow specific interactions to occur with the full range of substrates.

Classical studies based on *Escherichia coli* had suggested that the L-ornithine-specific decarboxylase activity in bacteria was limited to the AAT-fold enzymes, and that the dual specificity enzyme from *S. ruminantium* represented an isolated example of a β/α-barrel fold decarboxylase in bacteria with activity on L-ornithine. However, a number of novel bacterial sequences with homology to the β/α-barrel fold decarboxylases were recently identified in sequenced bacterial genomes (22). In this study, phylogenetic and experimental analysis suggests that the β/α-barrel fold decarboxylases segregate into four distinct groups containing ADCs, DAPDCs, ODCs, and putative CANSDCs (Fig. 1). The ODCs can be further classified into three subclades as follows: the eukaryotic ODCs, bacterial sequences containing the *S. ruminantium* dual specificity L/ODC, and a third group of uncharacterized bacterial ODC-like sequences. Here we have experimentally determined the substrate preferences of these putative ODCs from a diverse group of bacteria for the first time. Despite the presence of key amino acid changes in the 310-helix specificity element relative to the eukaryotic ODCs, these enzymes utilize L-ornithine as their primary substrate. In addition, the study provides experimental confirmation for a distinct clade of dual function enzymes with specificity for both L-ornithine and L-lysine, similar to that reported for the enzyme from *S. ruminantium*.

EXPERIMENTAL PROCEDURES

Materials—L-Ornithine, L-lysine, L-arginine, meso-diaminopimelic acid, PLP, and all other reagents were purchased from Sigma unless noted otherwise. L-[1-14C]Ornithine hydrochloride (52.0 mCi/mmol), L-[U-14C]lysine monohydrochloride (304 mCi/mmol), and L-[U-14C]arginine monohydrochloride (308 mCi/mmol) were purchased from Amersham Biosciences. DL-DFMO was obtained from Marion Merrell Dow Inc. (Cincinnati, OH). Infinity™ carbon dioxide detection reagent was from Thermo Electron Corp. (Louisville, CO).

Genomic DNA from *Methanosarcina mazei* (ATCC BAA-159D), *Nitrosomonas europaea* (ATCC 19718), *Bartonella henselae* (ATCC 49882), and *Thermotoga maritima* (ATCC 43589) were purchased from ATCC (Manassas, VA). Genomic DNA from *Giardia lamblia*, *Pseudomonas aeruginosa* (ATCC 47085D), *V. vulnificus* CMCP6, and *Aquifex aeolicus* were kindly provided by Drs. Ching C. Wang (University of California San Francisco), Hong Zhang (University of Texas Southwestern Medical Center, Dallas), Joon Haeng Rhee (Chonnam University, Kwangju, Korea), and Michael Thomm (Universität Regensburg, Regensburg, Germany), respectively.

Phylogenetic Analysis—Mouse ODC, *E. coli* ADC, *S. ruminantium* L/ODC, and *V. alginolyticus* CANSDC were each used to search the nonredundant protein data base at the National
Center for Biotechnology Information, GenBank™, using BLAST. Sequences were chosen to optimally represent diverse bacterial phyla and to reduce the number of members from the same phylum for the phylogenetic analysis. Sequences were aligned using ClustalX (version 1.8) (23) and edited for display with GeneDoc (version 2.6.002) (24). Sequences from the N and C termini were removed to facilitate the alignment. Phylogenetic analysis of the aligned sequences was performed in PAUP* (25). A neighbor-joining tree with nodes assessed by 1000 bootstrap replicates was constructed and imported into TreeView (26). Taxonomic descriptions were obtained from the NCBI (ncbi.nlm.nih.gov). Secondary structure predictions were performed on the sequences using the PSIPRED program.

Cloning of the Putative Decarboxylase Genes—The genes for the selected ODC homologs were amplified by PCR from the genomic DNA using the primers described in supplemental Table I and cloned into one of the following His6 tag expression vectors: pET-15b (Novagen), pET-22b (Novagen), or a modified pRSET B vector (Invitrogen). The pRSET construct was generated from pDHODH/pRSET (27) provided by Dr. John Clardy (Harvard Medical School, Boston), and the BamHI restriction site in pDHODH/pRSET was mutated by QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the primers mtpRSET_F and mtpRSET_R to introduce an NcoI site.

Primers mtpRSET_F and mtpRSET_R were used for mutagenesis before the final cloning step could be accomplished. The DAPDC and ADC genes of B. henselae were cloned into the pRSET B vector; ORFs from M. mazei were cloned into the NcoI and HindIII sites of the modified pRSET B (Invitrogen). The pRSET construct was generated described previously (5, 30). Assays were conducted using L-[1-14C]ornithine, L-[U-14C]lysine, or L-[U-14C]arginine at 63 or 80 °C in assay buffer (50 mM NaCl, 5 mM DTT, 250 μM PLP, 20 mM HEPES, pH 8.2 and pH 7.9, at 63 and 80 °C, respectively). Assay buffers for pH profile determination utilized pH appropriate buffers (MES, pH 6.0; HEPES, pH 7.0 and pH 8.1; glycine-NaOH, pH 9.0 and 10.0; pH values were adjusted at the corresponding reaction temperature).

Enzyme Inhibition by dl-DFMO—Enzymes with activity on l-ornithine were characterized to determine whether DFMO inhibited their activity in a time-dependent manner. ODCs were incubated at 37 °C with racemic dl-DFMO in buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 20 μM PLP). At different time intervals the enzyme/DFMO mixtures were diluted 100-fold into assay mixture to determine the fraction of enzyme activity remaining. Assay mixtures contained the carbon dioxide detection reagent premixed with 10 mM l-ornithine, 2.5 mM DTT, and 50 μM PLP. The data were analyzed by the method of Kitz and Wilson (31). The observed rate constant (kobs) for the enzyme inactivation was determined at different inhibitor concentrations using Equation 1. These values were then fitted to Equation 2 to determine the inactivation rate (kinact) and the apparent Ki for the reaction.

\[ \frac{V}{V_0} = \exp(-k_{\text{obs}} \times t) \]  
\[ k_{\text{obs}} = \frac{k_{\text{inact}} [I]}{K_m[I] + [I]} \]

Statistical Methods—GraphPad Prism4 (GraphPad Software, Inc.) was used for graphing and analyzing data for kinetic parameters. Steady-state kinetic data were fitted to the Michaelis-Menten equation by nonlinear regression to obtain kcat and Km. Data for DFMO inactivation were fitted similarly to Equation 2.

Crystallization of the V. vulnificus Dual Specificity Enzyme (VvL/ODC) and Data Collection—Initial crystallization conditions were identified in Hampton Research crystallization screens using the hanging drop method (Riverside, CA). Clusters of thick plate crystals formed upon mixing equal volumes (1.5 μl) of reservoir buffer (0.1 mM Tris-HCl, pH 8.5, 30% PEG-
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4000, 0.2 M MgCl2) with VvL/ODC (10 mg/ml) in buffer (0.1 M HEPES, pH 7.5, 0.3 M NaCl, 0.5 mM EDTA, 10% w/v glycerol, 0.03% w/v Brij35 and 1 mM DTT) plus 10 mM L-ornithine at 16°C. Indexing of the resulting crystals demonstrated that they had a large unit cell dimension (6–8 molecules per asymmetric unit). To search for a crystal condition that would yield a smaller unit cell, additive screening was performed using Hampton Research Additive Screen™ kit. The inclusion of n-octyl-β-D-glucoside (0.5%) yielded single rod crystals of VvL/ODC-putrescine. Diffraction to 2.0 Å revealed the space group P212121 with only two molecules per asymmetric unit. Diffraction quality VvL/ODC-putrescine crystals were obtained with microseeding at 4°C by incubation of VvL/ODC (1.5 μl of 10 mg/ml) preincubated with 20 mM L-ornithine, 1.5 μl of reservoir buffer (0.1 M Tris-HCl, pH 8.4, 35% PEG-4000, 0.2 M MgCl2), and 0.3 μl of 5% w/v n-octyl-β-D-glucoside for 1 week. VvL/ODC-cadaverine crystals formed without microseeding by mixing 1.5 μl of 20 mg/ml protein preincubated with L-lysine (20–30 mM), 1.5 μl of the reservoir buffer (except 30% PEG-4000 was used instead of 35%), and 0.3 μl of 5% w/v n-octyl-β-D-glucoside. The crystals were flash-frozen and stored in liquid N2 until data collection. Diffraction data for the crystals of VvL/ODC-putrescine at 1.7 Å resolution and VvL/ODC-cadaverine at 1.65 and 2.15 Å resolution were collected at 190 BM beamline of Advanced Photon Source (Argonne National Laboratory, Argonne, IL). Data were processed and scaled using the HKL3000 package (32). The statistics are listed in Table 3.

Phasing and Refinement of the VvL/ODC Structures—Crystals of putrescine-bound VvL/ODC and cadaverine-bound VvL/ODC were formed in the P212121 space group with slightly different unit cell parameters (a = 82.08 Å, b = 88.68 Å, c = 111.84 Å, α = β = γ = 90°) for VvL/ODC-putrescine and a = 84.02 Å, b = 88.30 Å, c = 100.10 Å, α = β = γ = 90° for VvL/ODC-cadaverine), indicating one dimer per asymmetric unit in both structures. A monomer of the T. brucei ODC (PDB entry 1F3T) with putrescine removed was modified by Chainsaw (33) and used as the search model in the MolRep (34) program of the CCP4-6.0 package. This analysis yielded initial solutions with R-factors of 52 and 55% and correlation coefficients of 34 and 33% for VvL/ODC-putrescine and VvL/ODC-cadaverine, respectively. Phases were improved by refinement using REFMAC5 (35) resulting in density-modified maps of decent quality. The initial models for the VvL/ODC-putrescine and VvL/ODC-cadaverine were built by ARP/wARP (36). Strong electron density for the product putrescine bound to the PLP cofactor was observed in the 2Fo – Fc map of VvL/ODC-putrescine structure. Crystals were grown in the presence of the substrates L-ornithine and L-lysine; however, the substrates were efficiently decarboxylated during the time frame of the crystallization, and the ligand electron density that is observed in both structures is for the decarboxylated products putrescine and cadaverine. Putrescine-PLP was modeled into the VvL/ODC-putrescine structure, and the model was rebuilt manually in Coot (37), followed by refinement using REFMAC5. Water molecules were automatically added by ARP/warp during the early stages of refinement, and additional waters were added during manual refinement. TLS parameters were applied in the final rounds of refinement, resulting in a final model with a working R-factor of 18.2% and an Rfree of 21.3% for VvL/ODC-putrescine structure. Electron density for the product cadaverine bound to PLP appeared only in one active site in the map of VvL/ODC-cadaverine structure. The 1.65 Å-resolution data set of VvL/ODC-cadaverine was collected from a crystal that was incubated with 10 mM cadaverine in the final drop. A second data set at lower resolution (2.15 Å) was also obtained from a crystal that was incubated with 15 mM cadaverine. It had very similar unit cell parameters (a = 88.24 Å, b = 88.03 Å, c = 99.27 Å, α = β = γ = 90°) to the 1.65 Å data set. The model of 1.65 Å was applied to 2.15 Å data set and rebuilt in Coot. Electron density for the cadaverine-PLP was observed in both active sites. A good quality model was produced after several rounds of manual model building in Coot, followed by REFMAC5 refinement. In the final rounds, TLS parameters were applied for refinement, yielding the final model with a working R-factor of 17.5% and an Rfree of 24.0% for VvL/ODC-cadaverine structure. For both structures, PROCHECK (38) confirmed excellent stereochemistry. The modeling statistics are reported in Table 3.

The final refined model of VvL/ODC-putrescine contains two monomers (chain A and B) in the asymmetric unit. Good electron density is observed for PLP-putrescine in both active sites, and for all amino acid residues in the protein with the following exceptions: chain A, residues 12–15 and 155–163 and chain B, residues 155–163 and 325–326 are missing. The final refined structure contains 614 water molecules. The VvL/ODC-cadaverine structure also contains two monomers in the asymmetric unit, with good electron density observed for PLP-cadaverine in both chains, and for all amino acid residues, except that chain A, residues 11–17 and 155–164, and chain B, residues 155–163, are missing. The structure contains 352 bound water molecules.

Molecular Modeling—Structures were displayed using the graphics program PyMol. All r.m.s.d. calculations were based on structural alignment of the monomers by PyMOL and calculated within the program.

RESULTS

Phylogenetic Analysis of the β/α-Barrel Fold Decarboxylase Family—Protein sequences exhibiting similarity to the β/α-barrel fold decarboxylases were identified by PSI-BLAST analysis of the GenBank™ protein data base using selected query sequences of proteins with biochemically confirmed substrate specificity, including mouse ODC, E. coli ADC, and S. ruminantium L/ODC. V. alginolyticus CANSDC was included as a representative of this putative specificity. Phylogenetic analysis was performed for a subset of the sequences identified in the BLAST searches, representative of different bacterial phyla and each type of decarboxylase (Fig. 1). The sequence alignment used to build the Neighbor-Joining tree is shown in supplemen-
tal Fig. 1. Prokaryotic sequences segregated into four distinct groups. These include clades of sequences with strong similarity to the well characterized DAPDCs and ADCs, sequences that group with the putative CANSDC, and a group that is most closely related to the eukaryotic ODCs. The prokaryotic ODC-like sequences share high sequence identity to eukaryotic ODCs (28–33%), whereas the bacterial decarboxylase
sequences from ADCs, DAPDCs, and CANSDCs show relatively low sequence identity to eukaryotic ODCs (14–23%). Within the prokaryotic ODC-like sequences bootstrap analysis provided some support for a separate clade that contains the dual function L/ODC from S. ruminantium, as well as several predicted decarboxylases from within the /H9253-Proteobacteria, Bacteroidetes/Chlorobi, and Planctomycetes phyla. The L/ODC clade of sequences groups more closely with the eukaryotic ODCs than with the other bacterial ODC-like sequences. The ADCs do not form a distinct clade, probably because of the lack of an outgroup in the analysis, but the outliers of the ADC group are functionally characterized ADCs (e.g. E. coli (40) and Nicotiana tabacum (41)). None of the β/α-barrel fold basic amino acid decarboxylases were found in the archaean subkingdom Crenarcheota, and only the DAPDC family was widely distributed in both eubacteria and Archaia. Archaean and eubacterial sequences in the DAPDC clade are not clearly segregated. ADC sequences are found only in plants within the Eukaryota, and although they are found in diverse phyla within the eubacteria, they are particularly ubiquitous within the Cyanobacteria (results not shown).

In contrast to the well-characterized ADC, DAPDC, and eukaryotic ODCs, no biochemical analysis has been reported for the prokaryotic ODC-like enzymes. Comparison of the amino acid sequences of the ODC-like prokaryotic enzymes with the three-dimensional structure of the eukaryotic ODCs (e.g. T. brucei ODC and the ODC-like cvADC) was undertaken to provide insight into the function of these proteins (Fig. 2). The amino acid residues found in the substrate-binding site (e.g. Asp-361 and Asp-332) of the eukaryotic ODCs are conserved in the clade of enzymes that groups with S. ruminantium L/ODC, consistent with the finding that this enzyme can catalyze the decarboxylation of L-ornithine (3). In contrast the prokaryotic ODC-like enzymes that segregate outside of the L/ODC clade have evolved significant changes in the predicted substrate-binding pocket. Specifically, Asp-332 in the 310-helix specificity element is replaced either with a Glu similarly to cvADC (e.g. P. aeruginosa, Treponema denticola, M. mazei, and B. henselae) or in other cases an acidic residue at this position is missing altogether (T. maritima, Streptomyces coelicolor, N. europaea, Nitrospira multiformis, and A. aeolicus). Sequence alignment in this region is of good quality, and it is further supported by secondary structure predictions that suggest the bacterial sequences also contain a short helix in the position of the eukaryotic ODC “specificity element.” The data predict that either the active site organization of these enzymes differs from the eukaryotic ODCs or that these enzymes have evolved novel substrate specificity. The Glu at position 332 is suggestive of L-arginine as a possible substrate based on similarity to cvADC, whereas the sequences that have substituted...
neutral amino acids at this position might be predicted to have even larger changes in function.

**Functional Analysis of the Putative Prokaryotic β/α-Barrel Fold Decarboxylases**—To determine the substrate specificity and function of the putative prokaryotic ODCs, we selected enzymes from eight microorganisms for biochemical analysis. One eukaryotic organism was selected (G. lamblia) because it represents a basal eukaryotic lineage, and the others were of prokaryotic origin selected to represent diverse phyla. These consist of B. henselae, a pathogenic α-proteobacterium, P. aeruginosa, a pathogenic γ-proteobacterium, N. europaea, a β-proteobacterium, M. mazi, representing the only archaean genus containing a putative ODC-like gene, two thermophilic bacteria, A. aeolicus and T. maritima, and finally V. vulnificus, a pathogenic γ-proteobacterium, which contains a predicted dual specificity L/ODC. In addition, the ADC and DAPDC from V. vulnificus were characterized to serve as controls for enzymes that should have *bona fide* activity on L-arginine and meso-diaminopimelate. The seven putative ODCs, and the putative V. vulnificus L/ODC (VvL/ODC), ADC, and DAPDC were cloned into bacterial expression vectors, and the proteins were expressed and purified as described under “Experimental Procedures.” Steady-state kinetic analysis was then undertaken using a range of basic amino acid substrates (e.g., L-ornithine, L-lysine, L-arginine, and meso-diaminopimelate).

All seven of the prokaryotic putative ODCs and the enzyme from G. lamblia are able to catalyze the decarboxylation of L-ornithine with similar kinetic parameters (Table 1; $k_{cat}$ ranges from 4 to 12 s⁻¹; $K_m$ ranges from 0.12–2.3 mM) to those reported for the well characterized eukaryotic enzymes from mouse (42) and T. brucei (28, 29). They also function on L-lysine, L-arginine, and meso-diaminopimelate.

### Table 1

| Organism          | Reaction temperature | Reaction pH | Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-------------------|----------------------|-------------|-----------|-----------|-------|---------------|
|                   |                      |             | L-Ornithine | $\alpha$  |        | $\omega$      |
|                   |                      |             | L-Lysine   | $\alpha$  |        | $\omega$      |
| G. lamblia        | 25°C                 | 6.0         | 2.2       | 21        | 1.0 X 10² | 370           |
| M. mazi           | 37°C                 | 8.0         | 0.01      | 2.5       | 1.5 X 10³ | 16            |
| P. aeruginosa     | 37°C                 | 8.0         | 0.01      | 2.5       | 1.5 X 10³ | 16            |
| N. europaea       | 37°C                 | 8.0         | 0.01      | 2.5       | 1.5 X 10³ | 16            |
| A. aeolicus       | 37°C                 | 8.0         | 0.01      | 2.5       | 1.5 X 10³ | 16            |
| T. maritima       | 37°C                 | 8.0         | 0.01      | 2.5       | 1.5 X 10³ | 16            |
| V. vulnificus     | 37°C                 | 8.0         | 0.01      | 2.5       | 1.5 X 10³ | 16            |

* Data were taken from Ref. 29. For comparison, the $k_{cat}/K_m$ for S. ruminantium L/ODC on L-arginine and L-lysine are reported to be 10⁻² to 10⁻³ over the range of pH 6.0 to 7.0 and for cvADC the $k_{cat}/K_m$ for L-ornithine and L-arginine are reported to be 1.2 to 10⁻³ over the range of pH 6.0 to 7.0.
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than for L-ornithine. Activity on L-arginine could not be detected, thus demonstrating that these enzymes are bona fide ODCs. The ODC homologs from the two thermophiles (T. maritima and A. aeolicus) were characterized over a range of pH and temperatures (Table 1). Both enzymes displayed maximal activity at 80 °C. The $k_{cat}/K_m$ for T. maritima ODC on L-ornithine as a substrate was comparable with that observed for the enzyme from other bacterial species, and like the other enzymes it also had a strong preference for L-ornithine over L-lysine. In contrast, the $k_{cat}/K_m$ for the decarboxylation of L-ornithine by the A. aeolicus enzyme was about 100-fold lower than for the other ODCs even at the optimal temperature and pH which occurred over a broad range from pH 6–10. This difference is mostly reflected in the $K_m$ (>100 mM at 80 °C), which is significantly elevated relative to the other enzymes in the study. The A. aeolicus enzyme also had detectable activity on L-lysine, although it was significantly less active than for L-ornithine. Neither L-arginine nor meso-diaminopimelate are substrates.

VvL/ODC efficiently catalyzes the decarboxylation of both L-ornithine and L-lysine confirming that it is a dual specificity enzyme (Table 1). In addition, the putative ADC and DAPDC from V. vulnificus catalyzed the decarboxylation of their expected substrates L-arginine and diaminopimelate, further confirming the validity of the phylogenetically determined decarboxylase clades. V. vulnificus DAPDC was active only toward meso-diaminopimelate ($k_{cat}$ 5.9 ± 0.3 s$^{-1}$ and $K_m = 2.5 ± 0.4$ mM), and showed similar catalytic efficiency to the enzyme from other species (21, 43). The V. vulnificus ADC activity was measured in the presence and absence of 5 mM Mg$^{2+}$ based on several reports that bacterial ADC activity is activated by Mg$^{2+}$ (40). However, the V. vulnificus ADC efficiently catalyzed the decarboxylation of L-arginine in the absence of Mg$^{2+}$, and the kinetic constants were the same in both conditions ($k_{cat} = 20 ± 0.7$ s$^{-1}$ and $K_m = 0.20 ± 0.04$ mM).

Inhibition of the Bacterial ODCs with DFMO—Eukaryotic ODCs from human, mouse, and T. brucei are known to be inactivated by DFMO, an enzyme-activated irreversible inhibitor (42, 44, 45). To determine whether the enzymes characterized in this study are also inhibited by DFMO, the V. vulnificus L/ODC and the ODCs from M. mazei, P. aeruginosa, and N. europaea were incubated with various concentrations of DFMO over a range of time. DFMO inhibited all four enzymes with $K_{inact}/K_{app}$ ranging from 32 to 180 m$^{-1}$ s$^{-1}$ (Table 2). These values are comparable with those measured for mammalian and T. brucei ODC.

**Table 2**

| Source organism of the enzyme | $k_{inact}$ | $K_{app}$ | $k_{inact}/K_{app}$ | $K_m$ | $v_{max}$ |
|------------------------------|-------------|-----------|---------------------|-------|----------|
| Mouse*                       | 0.32        | 13        | 4.1 × 10$^2$        | 0.69  | 1.2 × 10$^{-3}$ |
| Human*                       | 0.15        | 2.2       | 1.3 × 10$^2$        | 0.86  | 1.1 × 10$^{-2}$ |
| T. brucei                    | 3.6         | 160       | 2.0 × 10$^3$        | 1.7   | 4.6 × 10$^{-2}$ |
| P. aeruginosa                | 0.81        | 420       | 3.5 × 10$^2$        | 2.1   | 3.0 × 10$^{-2}$ |
| M. mazei                     | 1.7         | 170       | 9.7 × 10$^2$        | 2.5   | 9.5 × 10$^{-2}$ |
| N. europaea                  | 0.97        | 230       | 2.3 × 10$^2$        | 1.7   | 1.4 × 10$^{-1}$ |
| V. vulnificus                | 2.4         | 220       | 1.0 × 10$^3$        | 1.7   | 1.1 × 10$^{-1}$ |

* Data are from Ref. 42.
* Data are from Ref. 44.
* Data are from Ref. 45.

**Table 3**

| VvL/ODC-putrescine | VvL/ODC-cadaverine |
|--------------------|--------------------|
| Data collection and processing | |
| Data source | BM |
| Wavelength | 0.979 Å |
| Space group | P2,2,2$_1$ |
| Unit cell dimensions | $a = 82.08$ Å, $b = 88.68$ Å, $c = 111.84$ Å |
| Resolution | 20.0 to 1.7 |
| Total reflections | 633,221 |
| Unique reflections | 90,404 |
| Completeness | 99.9% |
| Redundancy | 7.0 |
| Intensities I/σ(last shell) | 35.9 (2.5) |
| $R_{merge}$ values, last shell | 0.074 (0.63) |

| Refinement | |
| No. of non-hydrogen atoms | 6406 |
| No. of water molecules | 614 |
| $R_{work}$ | 18.2 |
| $R_{free}$ | 21.3 |
| r.m.s. bond lengths | 0.014 Å |
| r.m.s. bond angles (degree) | 1.4 |
| Average $B$-value | 20.9 Å$^2$ |

**Notes:**

- a Data are from Ref. 42.
- b Data are from Ref. 44.
- c Data are from Ref. 45.
site lid (residues 153–170), which was observed for the first time in the closed position in the cvADC structure (6), is partially disordered (residues 155–163) in the VvL/ODC structure, similar to what has been observed in the structures of T. brucei ODC.

Active site structures of the VvL/ODC bound to putrescine and cadaverine were compared to gain insight into the structural basis for the dual substrate specificity (Fig. 3). Structures were aligned by a single monomer, and the r.m.s.d. (≈0.25 Å) was calculated for the dimer demonstrating that no significant structural rearrangements were induced by the different ligands. The active site structures are nearly identical, with the following exceptions: 1) Lys-66 sits in a somewhat different position reflective of the fact that it participates in a gem-diamine in the putrescine-bound structure but not in the cadaverine-bound structure; 2) a water molecule (W5 in Fig. 3 and Scheme 2) is observed in the VvL/ODC-putrescine structure, positioned between the ε-amino group of putrescine and Asp-340 (equivalent to Asp-361 in T. brucei ODC). In contrast, in the VvL/ODC cadaverine-bound structure the ε-amino group
of cadaverine makes a direct interaction with Asp-340, and W5 is missing from the structure. The distance between W5 in the VvL/ODC-putrescine structure and the ε-amino group of cadaverine in the VvL/ODC-cadaverine structure is only 1.6 Å, suggesting that a water molecule in this position is displaced from the cadaverine-bound active site because of steric hindrance.

VvL/ODC complexed to putrescine and cadaverine were compared with the structures of T. brucei ODC bound to putrescine and to cvADC bound to agmatine (Fig. 4). Alignment of the VvL/ODC and T. brucei ODC (PDB 1F3T) or of cvADC (PDB 2NVA) monomers gave r.m.s.d. values of 1.26 and 1.3 Å calculated for the overall dimer structure. Several helices and loops toward the surface of the structure assume somewhat different positions between these structures, but the changes do not have an apparent influence on the active site structure. With respect to the active site, the most significant difference not have an apparent influence on the active site structure. These data demonstrate that bacterial enzymes with activity on l-ornithine are widely distributed in both the AAT- and β/α-barrel families, and thus are not restricted to the AAT-fold as believed previously (1, 2). In addition, we identify a second member (VvL/ODC) of the dual function enzymes that is evolutionarily distant from S. ruminantium, suggesting that other sequences that group in this subclade will also catalyze decarboxylation of both l-ornithine and l-lysine.

The bacterial ODCs display robust catalytic activity against l-ornithine as a substrate with $k_{cat}/K_m$ values equivalent to the eukaryotic enzymes from the family (Table 1). In addition, like the eukaryotic ODCs they are strongly specific for l-ornithine over l-lysine. One exception is the A. aeolicus enzyme, which has a high $K_m$ value for l-ornithine, suggesting that it may prefer another substrate that remains unidentified. However, none of the tested basic amino acids other than l-ornithine served as substrates. Bacterial ODCs from the AAT-fold, including the enzymes from E. coli are reported to be insensitive to inhibition by DFMO (46). In contrast, we demonstrate here that the bacterial enzymes from the β/α-barrel fold, including VvL/ODC, are inhibited by DFMO with similar kinetics of inactivation to values reported for the eukaryotic enzymes (Table 2). Thus in contrast to the situation in E. coli, DFMO may be an effective agent for depleting polyamines in bacterial species that contain only β/α-barrel fold ODC. The one eukaryotic ODC characterized in this study, G. lambila, was also inhibited by DFMO providing a rationale for the observation that parasite growth is sensitive to DFMO (47).

The structural basis for the dual function of VvL/ODC was analyzed by x-ray structure determination of the enzyme in complex with both products putrescine and cadaverine. Overall, the structures are similar to the eukaryotic ODC structures that have been determined previously (e.g. T. brucei (17), mouse (19), and human (16)). The active sites were remarkably similar with the exception of the position of the 310-helix (specifi city element) at the back of the binding pocket. This helix sits slightly further away from the PLP cofactor in the VvL/ODC structure compared with T. brucei ODC, thus accommodating the larger l-lysine side chain. Key contacts between ligand and the conserved Asp at position 332 (Asp-314 VvL/ODC) are preserved, however. The ability of VvL/ODC to also efficiently interact with l-ornithine despite this enlarged pocket appears to result from the positioning of a bridging water molecule that participates in the binding of the shorter putrescine ligand but which is absent in the cadaverine-bound structure. A water molecule was previously implicated in the catalysis of multiple substrates by the protease trypsin (48). Trypsin hydrolyzes both lysine-containing peptide ligands, where a water forms a bridge between the ε-amino group and Asp-189, and arginine-containing peptides where the water has been displaced and the substrate interacts directly with the substrate binding pocket.
Phylogenetic Diversity of Ornithine Decarboxylase

To understand how the bacterial ODCs function on L-ornithine despite the absence of key substrate-binding residues, the amino acid sequence alignments of the prokaryotic ODCs were compared with the available structural data (e.g. the eukaryotic ODCs, VvL/ODC, the bacterial DAPDCs, and cvADC). This analysis identifies consensus sequences within the 3_{10}-helix that are predictive of the function of these enzymes (Fig. 2). Asp-332 is an invariant residue in the eukaryotic ODCs and the L/ODCs, but it does not appear to be important in the prokaryotic ODCs where it is substituted with Glu, Gly, or Asn (Fig. 2). However, in the prokaryotic ODCs an invariant Glu at position 328 is present instead. Although the equivalent residue (Cys-328) in the eukaryotic ODCs does not contact ligand, the structure of both cvADC and DAPDC demonstrate that ligand-binding residues may originate from different positions within the 3_{10}-helix. In cvADC the residue at position 328 (Asn-292 cvADC) forms an H-bond with the guanidinium group of the bound product agmatine, and in DAPDC an invariant Arg residue in position 327 interacts with the carbonylate of the meso substrate (6). These data suggest that in the prokaryotic ODCs, Glu-328 replaces the function of Asp-332 in the eukaryotic ODCs despite originating from a different position in the 3_{10}-helix specificity element. Finally, although the L/ODCs and the eukaryotic ODCs both have an invariant Asp at position 332, the eukaryotic ODC consensus is Asn/Cys at positions 327/328, and the dual function L/ODCs contain Ser/Gly.

It is clear from this study that the \( \beta/\alpha \)-barrel fold ODC is not exclusively eukaryotic and that it has evolved from the bacterial form. Among the \( \beta/\alpha \)-barrel decarboxylases DAPDC is the most phylogenetically widespread and is the only one found throughout the Euryarchaeota. It is also the only \( \beta/\alpha \)-barrel fold decarboxylase found in the Chloroflexi, a eubacterial phylum recently postulated to be the most ancient bacterial lineage (49). Thus, a parsimonious explanation for the origin of the \( \beta/\alpha \)-barrel decarboxylases is that they arose from DAPDC by gene duplication and subsequent functional diversification to generate different substrate specificities. In support, the other activities in the family are less widely distributed. The \( \beta/\alpha \)-barrel fold ADC seems to be excluded from the single-membraned Firmicutes and Actinobacteria phyla, although it is found in the double-membraned E. coli, a member of the \( \gamma \)-Proteobacteria (50). It is also ubiquitous in the Cyanobacteria, which explains why only plants contain ADC among the eukaryotes (the chloroplast is derived from a cyanobacterial endosymbiont). ODC is the only \( \beta/\alpha \)-barrel enzyme to be found extensively throughout the Eukaryota, and in the Metazoa and Fungi, it is the only route for polyamine biosynthesis.

Bacteria possess at least five routes for polyamine biosynthesis, including ADC and ODC, which are represented in both the AAT and \( \beta/\alpha \)-barrel fold classes, and a pyruvyl-dependent ADC found in Archaea (39). Convergent evolution has played an unusually important role in bacterial polyamine biosynthesis. A consequence of this is that a variety of AAT-/\( \beta/\alpha \)-barrel fold, ODC/ADC enzyme combinations can be found in sequenced bacterial genomes leading to the potential of redundant function. It is not clear how the various decarboxylase permutations in a given genome facilitate physiological adaptation, suggesting the possibility that the overlapping enzyme activities may be functionally compartmentalized for different cellular processes. Our data suggest that a systematic bioinformatic correlation of the polyamine biosynthetic gene configuration in individual bacterial strains, correlated with other genomic attributes, will provide a better understanding of the diversity of polyamine function.

In summary, our functional data on the prokaryotic DCs provides direct support for the activities of each class of basic amino acid decarboxylases from the \( \beta/\alpha \)-barrel fold, with the exception of the putative CANSDCs. These data show that the phylogenetic analysis provides strong predictive indication of the substrate specificity of enzymes from each clade in this family, including the identification of prokaryotic ODCs and a subclade containing the dual function L/ODCs. X-ray structural analysis of VvL/ODC provides insight into the structural basis for this dual function.

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