Site-Directed Mutagenesis of the Bacterial Metalloamidase UDP-(3-*O*-acyl)-*N*-acetylglucosamine Deacetylase (LpxC). Identification of the Zinc Binding Site†

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ABSTRACT: UDP-3-*O*-(acyl)-*N*-acetylglucosamine deacetylase (LpxC) catalyzes the second step in the biosynthesis of lipid A in Gram-negative bacteria. Compounds targeting this enzyme are proposed to chelate the single, essential zinc ion bound to LpxC and have been demonstrated to stop the growth of *Escherichia coli*. A comparison of LpxC sequences from diverse bacteria identified 10 conserved His, Asp, and Glu residues that might play catalytic roles. Each amino acid was altered in both *E. coli* and *Aquifex aeolicus* LpxC and the catalytic activities of the variants were determined. Three His and one Asp residues (H79, H238, D246, and H265) are essential for catalysis based on the low activities (<0.1% of wild-type LpxC) of mutants with alanine substitutions at these positions. H79 and H238 likely coordinate zinc; the Zn^{2+} content of the purified variant proteins is low and the specific activity is enhanced by the addition of Zn^{2+}. The third side chain to coordinate zinc is likely either H265 or D246 and a fourth ligand is likely a water molecule, as indicated by the hydroxamate inhibition, suggesting a His_3H_2O or His_2AspH_2O Zn^{2+}-polyhedron in LpxC. The decreased zinc inhibition of LpxC mutants at E78 suggests that this side chain may coordinate a second, inhibitory Zn^{2+} ion. Given the absence of any known Zn^{2+} binding motifs, the active site of LpxC may have evolved differently than other well-studied zinc metalloamidases, a feature that should aid in the design of safe antibiotics.

Lipopolysaccharide (LPS)[1] is the major lipid component of the outer leaflet of the outer membranes of Gram-negative bacteria (*1*). The hydrophobic anchor of LPS is lipid A, a unique amphiphilic lipid that is required for bacterial growth (*1*, *2*). The enzyme, UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase (LpxC), catalyzes the second reaction in the biosynthetic pathway for lipid A. Because of the unfavorable equilibrium constant for the acylation of UDP-GlcNAc, deacetylation of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc catalyzed by LpxC is the committed step of lipid A biosynthesis (Figure 1) (*3*−*5*).

LpxC proteins from *Escherichia coli*, *Pseudomonas aeruginosa* and *Aquifex aeolicus* have been overexpressed and purified (*3*, *6*, *7*). *E. coli* LpxC contains a single zinc ion that is required for catalytic activity (*8*). Hydroxamate-containing inhibitors of LpxC with antibiotic activity were first described for the *E. coli* enzyme (*9*), consistent with the presence of a bound Zn^{2+}. However, these compounds are relatively inactive against some divergent LpxC enzymes, particularly that of *A. aeolicus* (*7*). Recently, novel substrate-based hydroxamate and phosphinate compounds that inhibit both *E. coli* and *A. aeolicus* LpxC have been reported (*7*). In other families of zinc metalloamidases, hydroxamates inhibit by direct coordination with the active site zinc ion, displacing the zinc-bound water ligand (*10*, *11*). Phosphinates, phosphonates, and phosphoramidates similarly inhibit zinc metalloamidases by binding to the active site zinc ion and also by mimicking the proposed transition-state for catalysis (*12*−*15*). The discovery that substrate-based hydroxamates and phosphinates not only inhibit *E. coli* LpxC but also *P. aeruginosa* and *A. aeolicus* LpxC suggests that an essential active site Zn^{2+} ion is present in all LpxCs. Interestingly, *E. coli* and *A. aeolicus* LpxC can be inhibited by excess Zn^{2+}. Many metalloamidases are also inhibited by zinc ions; for example, in carboxypeptidase A, a second inhibitory Zn^{2+} ion binds very close to the essential active site Zn^{2+} (*16*−*18*).

Despite the similarities with regard to inhibition and proposed mechanism between bacterial LpxCs and many other Zn^{2+} metalloamidases, they share no obvious sequence homology. In the neutral endopeptidases, the carboxypeptidases, and the pitrilysins, the protein residues that coordinate the active site Zn^{2+} have been delineated, with all three

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[1] Abbreviations: bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; BSA, bovine serum albumin; DTT, dithiothreitol; FPLC, fast-protein liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); ICP, inductively coupled plasma emission spectroscopy; IPTG, isopropyl β-D-thiogalactopyranoside; LpxC, UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase.

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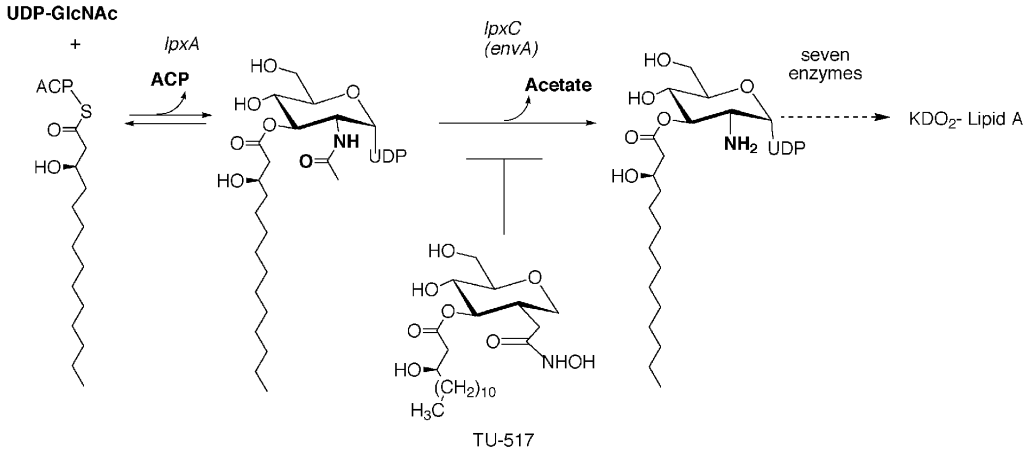

FIGURE 1: Reaction catalyzed by LpxC. The committed step in lipid A biosynthesis is the second step, catalyzed by the LpxC protein.

classes of enzymes containing a His_2Glu metal polyhedron (*19*−*22*). These metal sites can be readily identified by the consensus sequences HEXXH in neutral endopeptidases, HXXE in carboxypeptidases, and HXXEH in pitrilysins (*23*−*25*). The first and last H or E residues of the consensus sequences coordinate the active site zinc ion, and in all cases the third ligand is provided by a downstream His or Glu residue. Not all of the Zn^{2+} metalloproteases are coordinated by a His_2Glu binding site; the Zn^{2+} binding site found in the astacin and the interstitial collagenase families of Zn^{2+}-metalloamidases consists of three His residues (*26*, *27*). However, these enzymes also contain the HEXXH consensus motif that provides the first two closely spaced His ligands that coordinate Zn^{2+}. These consensus zinc-binding sequences have been used successfully in several cases to identify residues that coordinate Zn^{2+} in proteins that had not been characterized structurally by X-ray crystallography or NMR (*28*−*30*). None of these patterns exist in the primary amino acid sequence of any of the known LpxC enzymes.

Given the lack of an obvious Zn^{2+} binding site, we have used sequence comparisons and site-directed mutagenesis to identify amino acid side chains in LpxC that might coordinate the active site Zn^{2+}. Only 33 of the 280−306 amino acid residues that are present in the LpxC sequences of 11 diverse Gram-negative bacteria are completely conserved. Recent analyses of the zinc coordination spheres in single, catalytic zinc binding sites in protein crystal structures indicates that histidine is the most frequently observed ligand, followed by Glu, Asp, and Cys (*31*). However, other amino acids, including tyrosine in astacin (*27*) and glutamine in phosphomannose isomerase (*32*), have been observed to coordinate zinc in mononuclear sites. These data suggest that only 10 of the completely conserved amino acids, including four His, three Glu, and three Asp residues (Figure 2), could potentially coordinate Zn^{2+} with high affinity. In the present study, single amino acid substitutions were generated at each of these positions to identify groups that coordinate zinc. The resulting variant proteins were assayed for LpxC activity, Zn^{2+} stimulation and/or inhibition of catalysis, and the amount of bound Zn^{2+} in the purified mutant proteins. These data indicate that two His residues likely coordinate the active site Zn^{2+} in LpxC. A third zinc ligand is likely either a conserved His residue that is absolutely essential for catalytic activity or a conserved Asp residue. Both of these side chains clearly play critical catalytic or structural roles. Furthermore, the data suggest that one conserved Glu residue binds a second inhibitory Zn^{2+} ion. Our findings suggest that the zinc coordination polyhedron in LpxC, although similar in composition, is different in sequence context from those of other previously characterized metalloamidases.

MATERIALS AND METHODS

Buffers and Reagents. [α-^{32}P]UTP was purchased from NEN Dupont. PEI−Cellulose TLC plates were from E. Merck, Darmstadt, Germany. Bis-tris buffer (Ultrapure reagent) and bovine serum albumin (BSA), essentially fatty acid free, were purchased from Sigma. Restriction enzymes (*Bam*HI, *Nde*I, and *Eco*RI) were purchased from New England Biolabs.

Mutagenesis. E. coli and *A. aeolicus lpxC* genes encoding single amino acid substitutions were prepared by oligonucleotide-directed mutagenesis (*33*) of the cloned *lpxC* genes in the plasmids pEcLpxC and pAaLpxC, respectively (*7*). Oligonucleotides (25−32 nucleotides) were made such that the codon for each conserved His, Glu or Asp residue was replaced by either Ala (GCA), Gln (CAA), Asn (AAC), Ser (TCT), or Tyr (TAC). T4 DNA polymerase (Amersham) and T4 DNA ligase (generously provided by Sharon M. Crary, Duke University) were used to polymerize and covalently close the plasmids containing each variant *lpxC* gene, and the resulting DNA was transformed into XL-1 Blue cells (*34*). After isolation of each plasmid, the entire *lpxC* gene was sequenced by the method of Sanger (*35*) to ensure that only the desired mutation was introduced. Plasmids were named according to the LpxC species (Ec for *E. coli* or Aa for *A. aeolicus*) followed by the actual substitution that was made. The numbering always refers to the position of the conserved amino acid in the *E. coli* LpxC sequence. For example, the plasmid in which the His residue at position 19 of *E. coli* LpxC has been changed to Ala is named pEcH19A.

Preparation of Extracts Overexpressing LpxC Variants. Each of the plasmids containing a specific *lpxC* point mutation was transformed into *E. coli* strain BL21(DE3)-pLysS (*36*) for overexpression of the desired *E. coli* LpxC variant. Overnight cultures (5 mL) were grown at 37 °C in LB broth containing 50 μg/mL ampicillin and 30 μg/mL

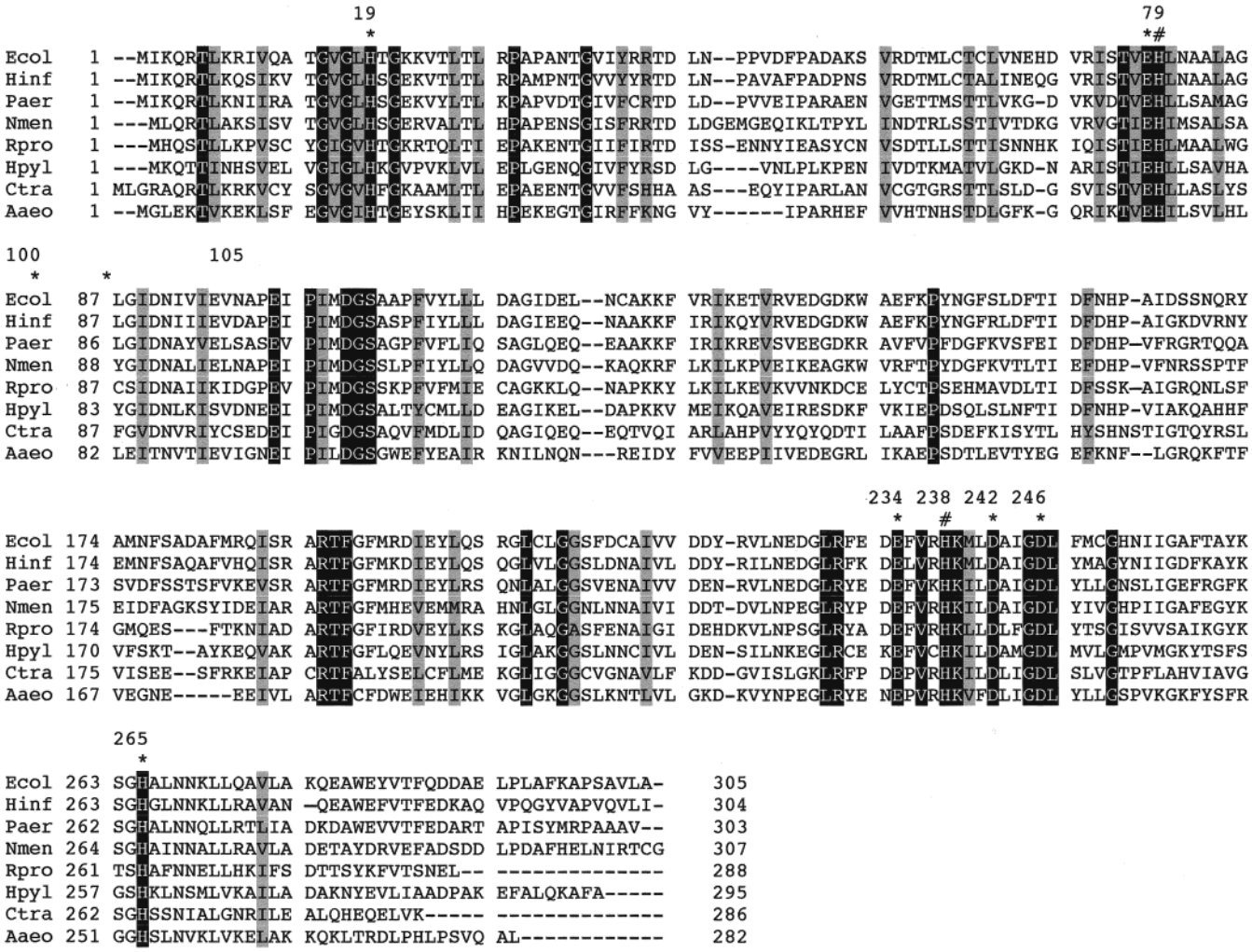

FIGURE 2: Multiple sequence alignment of LpxC proteins from Gram-negative bacteria. Alignment of LpxC sequences [*E. coli* (accession no. M19211), *Haemophilus influenzae* (U32794), *Pseudomonas aeruginosa* (U67855), *Neisseria meningitidis* (AL162752), *Helicobacter pylori* (AE000613), *Rickettsia prowazekii* (AJ235271), *Chlamydia trachomatis* (AE001324), *A. aeolicus* (AE000755), *Synechocystis sp.* PCC6803 (D90902), *Chlamydophila pneumoniae* (AE001648), and *Campylobacter jejuni* (AL139074) (last three sequences are not shown)] was generated using the CLUSTAL W (v. 1.8) multiple sequence alignment program (*40*). Residues highlighted in black are conserved and residues in gray are similar in all eleven LpxC sequences. Marked residues (# or *) are conserved amino acids in LpxC that are capable of coordinating Zn^{2+}. Mutagenesis studies implicate two His residues (indicated by #) in the formation of the zinc polyhedron. Data also suggest that either Asp246 or His265 also coordinates zinc in LpxC.

chloramphenicol, and then used to inoculate 50 mL cultures of rich induction medium (*37*). These cultures were grown with shaking at 30 °C until they reached $A_{620} = 0.4-0.5$, at which point 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 200 μM $ZnSO_4$ were added to induce expression from the T7 expression plasmid. A duplicate culture for each variant was also grown in the same way except that IPTG was not added. The cultures were allowed to grow for an additional 3−4 h at 30 °C and were then harvested by centrifugation at 6000*g* for 10 min at 4 °C. The cell pellets were washed with 25 mL of 25 mM HEPES, pH 7, containing 20% glycerol and 0.2 mM dithiothreitol (DTT). The washed cells were again collected by centrifugation, and then resuspended in 5 mL of the same buffer containing 2 mM DTT. The cells were lysed by passage through a French pressure cell at 18 000 psi and then clarified by centrifugation at 2000*g* for 20 min at 4 °C. These cell-free extracts were stored at −80 °C for use in activity assays.

Plasmids containing the *A. aeolicus lpxC* variant genes were transformed into *E. coli* strain BL21(DE3)pLysS (*36*) for overexpression and preparation of crude extracts. Cell cultures were grown as described for *E. coli* LpxC, except that the growth temperature was increased to 34 °C (from 30 °C) and the concentration of Zn^{2+} added at induction was reduced to 100 μM. The cell-free extracts were also prepared and stored as described for *E. coli* LpxC, except that all of the buffers contained 2 mM DTT.

The protein concentration of each cell-free extract was quantified using the BCA protein assay (Pierce) with BSA as the protein standard. The amount of each overexpressed variant LpxC protein was determined by comparison of the intensity of the band corresponding to LpxC in each extract on 12% polyacrylamide gels stained with Coomassie blue dye (*38*).

LpxC Variant Activity Assays. LpxC activity was assayed (*8*) using UDP-3-*O*-(*R*-3-hydroxymyristoyl)GlcNAc (UDP-3-*O*-acyl-GlcNAc) as the substrate, prepared as previously described (*8*, *39*). Specific activity measurements are expressed in units of picomoles of product per minute per milligrams of total protein present in each assay. Extracts

expressing variant *E. coli* LpxC proteins were assayed in 40 mM bis-tris, pH 5.9 at 30 °C with 3 μM UDP-3-*O*-acyl-GlcNAc, 1 mg/mL BSA, and 0.5 mM AMP. Extracts of cells overexpressing variant *A. aeolicus* LpxC proteins were assayed under the same conditions except at 60 °C where the pH of the buffer is 5.5. The concentration of each extract in the assay was chosen so that the total conversion to product during the time course of the reaction was less than 10%, typically, 0.4−1.0 mg/mL extract for the uninduced cell extracts and 0.1 μg/mL to 1.0 mg/mL for the induced extracts. Time courses were also chosen so that the enzyme activity remained stable under the conditions of the assay. The activity of *E. coli* LpxC is stable for up to 6 h at 30 °C, and the activity of *A. aeolicus* LpxC is stable for up to 20 min at 60 °C.

Purification of A. aeolicus LpxC Variants. Large cultures (3 L) of rich induction medium were inoculated with 100 mL of overnight cultures of BL21(DE3)/pLysS cells that had been transformed individually with each of the indicated *A. aeolicus* variant plasmids. The cells were grown at 34 °C to $A_{620} = 0.4-0.5$. At this point, expression of each *A. aeolicus* LpxC was induced by the addition of 1 mM IPTG only, with no added Zn^{2+}. After 6 h, the cells were harvested, and the resulting wet cell paste (15−20 g) was resuspended in 60−80 mL of 10 mM sodium phosphate buffer, pH 6.2, containing 2 mM DTT, and frozen in two batches at −80 °C. Each batch was processed separately at 4 °C. The resuspended cell pellet from one batch was lysed by passage through a French pressure cell at 18 000 psi. The cellular debris was removed by ultra-centrifugation in a Beckman Ti-70 rotor at 43 000 rpm for 60 min. The supernatant (typically 30−40 mL) was diluted to approximately 50 mL with 25 mM Hepes, pH 7.0, containing 2 mM DTT, and loaded at 0.5 mL/min onto a Q-Sepharose Fast-Flow (Pharmacia) FPLC column (HR16/10, 20 mL), equilibrated in 25 mM Hepes, pH 7.0, with 2 mM DTT. After loading the protein, the column was washed with an additional 100 mL of the equilibration buffer, and then a linear gradient (500 mL) of 0 to 0.3 M NaCl in 25 mM Hepes, pH 7.0, containing 2 mM DTT was used to elute the column. Each of the LpxC proteins emerged in a peak at 0.08 to 0.12 M NaCl. The LpxC-containing fractions were pooled and loaded directly onto a 25 mL Reactive Red120 Fast-flow column (Sigma) that had been preequilibrated in 25 mM Hepes, pH 7.0, containing 2 mM DTT and 0.3 M NaCl. The Red120 column was washed with an additional 100−125 mL of the same buffer and then with 125−150 mL of 25 mM Hepes, pH 7.0, containing 2 mM DTT and 1.0 M NaCl. The majority of the LpxC protein was then eluted with 100−150 mL of 25 mM Hepes, pH 7.0, containing 2 mM DTT and 2.5 M NaCl. The 2.5 M NaCl eluate from this column was concentrated and desalted in an Amicon ultrafiltration device fitted with a YM-10 membrane. The concentration of each protein purified in this manner was determined using the BCA protein assay (Pierce) with BSA as the protein standard. Each LpxC protein was judged to be approximately 80−90% pure by SDS−PAGE analysis visualized by Coomassie Blue staining. The variant LpxC proteins were all stored at −80 °C at concentrations greater than 2 mg/ml in 25 mM Hepes, pH 7.0, containing 2 mM DTT and 50−150 mM NaCl.

Metal Analysis. Inductively coupled plasma emission spectroscopy (ICP) was performed at the Garratt-Callahan Company, Millbrae, CA. Each of the purified *A. aeolicus* LpxC enzymes, including a buffer reference sample, was analyzed for the presence of the following 22 elements: Al, Ba, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, K, Si, Ag, Na, Sr, V, and Zn. Zinc standard solutions were analyzed simultaneously to ensure the accuracy of this method. A concentrated solution of each protein (4−20 mg/mL as measured by the BCA protein assay) was sent for analysis. The measured amount of zinc in each *A. aeolicus* LpxC preparation was normalized to the amount of protein, expressed as milligrams of Zn per gram of protein.

RESULTS

Identification of Possible Zinc Binding Residues. A sequence alignment of 11 LpxC protein sequences was generated by the CLUSTAL W (v.1.8) multiple sequence alignment program (*40*). This alignment identified 33 out of the 280−306 amino acids from diverse LpxC enzymes that are completely conserved in the primary amino acid sequence (Figure 2). The same conserved residues were identified independently by using another multiple sequence alignment program (Multalin) (*41*). Cys, His, Asp, and Glu residues have previously been observed as the most frequent amino acids to coordinate a single, catalytic Zn^{2+} bound to a protein although tyrosine, asparagine and glutamine have also been observed (*25*, *31*, *42*−*46*). Of the 33 invariant residues in LpxC, there are no conserved cysteine, tyrosine, asparagine, or glutamine residues, leaving only 10 candidates for Zn^{2+} ligands in LpxC: H19, E78, H79, E100, D105, E234, H238, D242, D246, and H265 (according to the numbering in the *E. coli* sequence). Site-directed mutagenesis was used to change the potential Zn^{2+} binding residue at each of these positions in *E. coli* LpxC to alanine which cannot coordinate Zn^{2+} or to glutamine which retains hydrogen bonding characteristics similar to histidine but may coordinate zinc with decreased affinity (*47*). Also, H19 was changed to Y to investigate the properties of this variant LpxC protein that is found in the mutant LpxC strain *envA1* (*3*, *48*). Disruption of Zn^{2+} binding sites by targeted mutagenesis of amino acids that coordinate Zn^{2+} decreases enzyme activity 100−10^5-fold in other enzyme systems (*29*, *30*, *49*−*51*).

Analysis of Extracts Overexpressing E. coli LpxC Variants. Cell-free extracts of *E. coli* cells overexpressing each of the *E. coli* LpxC variants were made and analyzed by SDS−PAGE to determine the level of overexpression of each variant enzyme. In the cell extracts of LpxC variants at positions E100, D105, and E234, the overexpressed proteins remained in the pellet after French pressure lysis of the cells, most likely due to the formation of inclusion bodies (data not shown). The formation of inclusion bodies is often due to aggregation of improperly folded intermediates (*52*, *53*), suggesting that mutations at these positions in *E. coli LpxC* hinder the correct folding of the enzyme. However, the soluble crude extracts made from cells overexpressing wild-type and variants at positions H19, E78, H79, H238, D242, D246, and H265 contained bands of similar intensity migrating with an *E. coli* LpxC standard on an SDS−polyacrylamide gel (data not shown). This high level of expression of the mutants suggests that these structural

Table 1: Activity of *E. coli* LpxC Variants Overexpressed in *E. coli* BL21(DE3)pLysS

plasmid	specific activity[a] (pmol/min/mg)	% wild-type activity
pJEJ1 (wild-type)	840 000	
pEcH19A	600	0.07
pEcH19Q	9300	1.1
pEcH19Y	4000	0.5
pEcE78A	1200	0.1
pEcE78Q	280	0.03
pEcH79A	370	0.04
pEcH79Q	670	0.08
pEcH238A	760	0.09
pEcD242A	80 000	9.5
pEcD242Q	370	0.04
pEcD246A	480	0.06
pEcH265A	220	0.03
pEcH265Q	150	0.02
pET21a	170	0.02

[a] Activity was measured using 3 μM UDP-3-*O*-acyl-GlcNAc, 1 mg/mL BSA, 0.5 mM AMP in 40 mM bis-tris, pH 5.9, 30 °C, as described in the Materials and Methods. The values listed are ±20% error based on multiple measurements with the same extracts.

Table 2: Activity of *A. aeolicus* LpxC Variants Overexpressed in *E. coli* BL21(DE3)pLysS

plasmid	specific activity[a] (pmol/min/mg)	% wild-type activity
pJSG (wild-type)	180 000	
pAaH19A	9100	5.0
pAaH19Q	81 000	45
pAaH19Y	8000	4.4
pAaE78A	18 000	9.8
pAaE78Q	<1[b]	<0.0005
pAaH79A	10	0.006
pAaH79Q	15	0.008
pAaE100A	130 000	72
pAaE100N	110 000	61
pAaE100S	150 000	83
pAaD105A	100 000	56
pAaD105N	120 000	67
pAaD105S	140 000	78
pAaE234A	8600	4.8
pAaE234N	<1[b]	<0.0005
pAaE234S	12 000	6.7
pAaH238A	250	0.1
pAaD246A	2.4	0.001
pAaD246N	6300	3.5
pAaD246S	<1[b]	<0.0005
pAaH265A	<1[b]	<0.0005
pAaH265Q	<1[b]	<0.0005
pET21a	<1[b]	<0.0005

[a] Specific activity of extracts of *E. coli* overexpressing the indicated *A. aeolicus* LpxC variants was determined using 3 μM UDP-3-*O*-acyl-GlcNAc in assays that also contained 1 mg/mL BSA and 0.5 mM AMP in 40 mM bis-tris, pH 5.5, at 60 °C. The values listed are ±20% error based on multiple measurements with the same extracts. [b] No activity detected, even assaying 1.0 mg/mL extract.

alterations have little significant effect on the stability of these proteins.

Since the variant *E. coli* LpxC proteins were all expressed to levels comparable to wild-type LpxC, the specific LpxC activity in each of the extracts was measured and compared with the specific activity of the wild-type *E. coli* LpxC extract (Table 1). The extract overexpressing D242A LpxC exhibited ~10% of the specific activity of the wild-type extract. Activity in all of the remaining extracts was very low (0.02−1.1% of wild-type) (Table 1). However, in *E. coli* extracts, the observed activity includes the background LpxC activity from the wild-type chromosomal copy of *E. coli lpxC*. The activity of the pET21a vector control extract suggests that the background activity is small, normally about 0.02% of the activity of overexpressed wild-type *E. coli* LpxC (Table 1). Since the D242A extract is partially active, the background constitutes a small fraction of this observed activity as well. For the low activity variants, the actual specific activity of the variant LpxC cannot be reliably estimated due to this chromosomal background. Therefore, the activity of overexpressed *E. coli* LpxC variants only rules out one residue, D242, as a Zn^{2+} ligand, due to the high activity of the D242A LpxC extract when this side chain is removed.

The activity of the H19Y mutant, a variation observed in the mutant LpxC strain *envA1* (*3*, *48*), is decreased 200-fold (Table 1). While deletion of LpxC is lethal in Gram-negative bacteria, the *envA1* strain (*48*, *54*) grows at all temperatures, albeit with increased sensitivity to antibiotics. This phenotype indicates that H19Y LpxC must function well enough in vivo to allow for bacterial survival. Recently, this same amino acid substitution (H19Y) has been observed in the sequence of the *lpxC* gene from *Cyanidium caldarium* (accession no. NC_001840), indicating both that H19 is not completely conserved in all LpxC sequences and that tyrosine can be an adequate substitute for histidine at this position.

Analysis of Extracts Overexpressing A. aeolicus LpxC Variants. To eliminate interference from the activity of the chromosomally encoded *E. coli* LpxC, we overexpressed *A. aeolicus* LpxC in *E. coli* and made alterations in potential Zn^{2+} binding residues in this protein using site-directed mutagenesis. *A. aeolicus* LpxC is active at high temperatures whereas *E. coli* LpxC is not (*7*). No LpxC activity is detected at 60 °C in extracts of *E. coli* containing the pET21a vector control at the highest concentration of extract that can be used in these assays (1 mg/mL) (Table 2). Therefore, when extracts of *E. coli* overexpressing *A. aeolicus* LpxC are assayed at 60 °C, the background activity from the chromosomally encoded *E. coli* LpxC is negligible. The sensitivity of the assay is enhanced, allowing for the detection of activity as low as 0.0005% of wild-type *A. aeolicus* LpxC activity with these variants.

Mutations were made at all of the positions in *A. aeolicus* LpxC that were altered in the *E. coli* enzyme, except for D242, which had already been eliminated as a potential Zn^{2+} ligand. Also, at positions where variations caused the formation of insoluble *E. coli* protein (E100, D105, and E234), Asn, Ser, and Ala were substituted for the original amino acid. These three amino acids were also substituted for Asp at position 246. In contrast to the results with the *E. coli* variants, all of the overexpressed *A. aeolicus* proteins were found in the soluble portion of the cell-free crude extracts (data not shown). Furthermore, as with the soluble *E. coli* variants, all of the *A. aeolicus* LpxC variants expressed at levels comparable to wild-type *A. aeolicus* LpxC.

The specific activity of each *A. aeolicus* variant LpxC in cell-free extracts was measured at 60 °C and compared to the specific activity of extracts overexpressing wild-type *A. aeolicus* LpxC. The variants are numbered according to their positions in *E. coli* LpxC for clarity. At position E100 or D105, deletion of the side chain by substitution with Ala or substitution with Asn or Ser decreases the specific activity

less than 2-fold, suggesting that these side chains are not important for activity and, therefore, do not coordinate Zn^{2+} in LpxC (Table 2). At position H19, E78, or E234, substitution with Ala decreases the specific activity 5−20-fold, indicating that these side chains enhance catalytic activity but are not absolutely essential (Table 2). These decreases are modest compared to the 10^2-10^5-fold decreases in catalytic activity observed for the removal of zinc ligands in other catalytic zinc sites (*29*, *30*, *49*−*51*), suggesting that these side chains also do not coordinate the metal. Consistent with this conclusion, the recently determined LpxC sequence from *Cyanidium caldarium* contains a tyrosine substituted for histidine at position 19 (accession no. NC_001840). Interestingly, for both E78 and E234, larger decreases in activity ($>10^5$-fold) are observed for the substitution of the carboxylate with a carboxamide side chain (N or Q) rather than an alanine side chain.

Alanine substitutions at four positions enormously decrease the observed specific activity of *A. aeolicus* LpxC. The specific activity of extracts overexpressing variants with an alanine substituted at position H79, H238, or D246 is reduced 10^3-10^5-fold (Table 2). Additionally, no significant activity is detected in an extract containing H265A LpxC at the highest concentration tested (1 mg/mL), indicating that the specific activity decreases more than 10^5-fold (Table 2). Alterations at positions H79, H238, and D246 cause larger decreases in the specific activities of *A. aeolicus* LpxC compared to *E. coli* LpxC (Tables 1 and 2), perhaps partly due to the decreased background activity from the chromosomal copy of *lpxC* in the *A. aeolicus* extracts. The undetectable activity of H265A LpxC in the *A. aeolicus* extracts confirms that this protein has little or no catalytic activity. At positions 79 and 265, mutants with a glutamine substitution also retain little catalytic activity; however, the specific activity of the D246N variant is increased significantly compared to the alanine substitution (Tables 1 and 2). Nonetheless, the large decreases in specific activity for alanine substitutions at positions H79, H238, D246, and H265 indicate that these side chains likely play important catalytic roles in this enzyme, and could perhaps directly coordinate the catalytic zinc ion.

Response to Addition of Divalent Metal Ions. To test further which amino acids might directly coordinate Zn^{2+}, the specific activities of extracts containing Ala substitutions at various positions in *A. aeolicus* LpxC were determined in the presence of added $ZnSO_4$ (Table 3). The activity in the majority of the extracts (H19A, E100A, D105A, E234A, and D246A) was inhibited 3−4-fold by the addition of 500 μM Zn^{2+}, similar to wild-type *A. aeolicus* LpxC (Table 3). These data suggest that the decreased activity in these mutants is not due to decreased zinc affinity caused by loss of a zinc ligand. However, the specific activity of the E78A LpxC variant was neither inhibited nor activated by the addition of up to 1 mM Zn^{2+} (Table 3, data not shown), suggesting that this mutation may decrease the apparent affinity of the inhibitory zinc site (*8*). In contrast, the extracts overexpressing H79A or H238A LpxC were each stimulated approximately 20-fold by the addition of $ZnSO_4$ (Table 3). Maximal stimulation of the activity of the H79A and H238A *A. aquifex* variants in extracts was observed at 500−1000 μM Zn^{2+}. At higher concentrations the activity decreases, presumably due to Zn^{2+} inhibition (Figure 3A). These data indicate that removal of the H79 or H238 side chain decreases the zinc affinity of LpxC, suggesting that these side chains coordinate Zn^{2+} in the wild-type enzyme. The activity in extracts overexpressing H265A LpxC remains

Table 3: Zn^{2+} Addition to *A. aeolicus* LpxC Variants

plasmid	SA_{Zn}/SA_{ctrl}[a]
pJSG (wild-type)	0.25
pAaH19A	0.24
pAaE78A	1.0
pAaH79A	24
pAaE100A	0.24
pAaD105A	0.34
pAaE234A	0.36
pAaH238A	19
pAaD246A	0.3
pAaH265A	ND[b]

[a] Extracts of *E. coli* BL21(DE3)pLysS that contain the indicated overexpressed *A. aeolicus* LpxC proteins were assayed in the presence and absence of 500 μM $ZnSO_4$. Assays were performed at 60 °C and included 3 μM UDP-3-*O*-acylGlcNAc, 1 mg/mL BSA, and 0.5 mM AMP in 40 mM bis-tris, pH 5.5. The number reported here is the ratio of the specific activity of the extract in the presence of 500 μM Zn^{2+} to the specific activity of the extract when assayed in the absence of added Zn^{2+}. [b] No activity detected, even assaying 1.0 mg/mL extract.

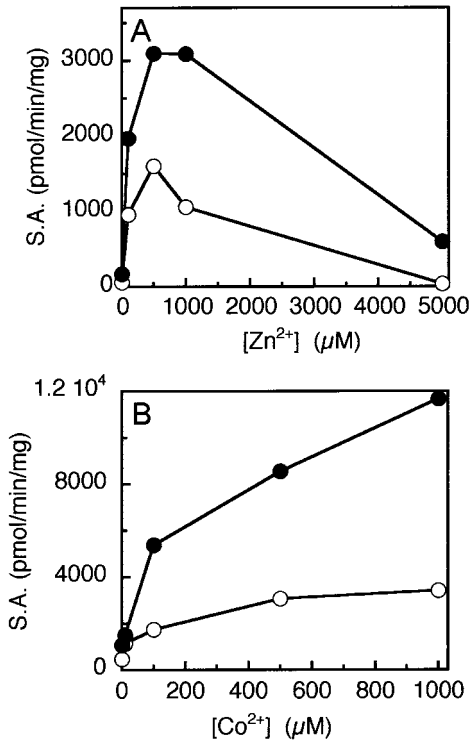

FIGURE 3: Stimulation of LpxC activity in the presence of divalent metal ions. (A) Cell-free crude extracts of *E. coli* BL21(DE3)pLysS overexpressing either H79A (○) or H238A (●) *A. aeolicus* LpxC were incubated with varied concentrations of $ZnSO_4$ (0−5000 μM) for 20−40 min prior to initiation of each assay. Initial rates of LpxC activity were determined in assays containing either 1 mg/mL H79A extract or 0.4 mg/mL H238A extract and 3 μM UDP-3-*O*-acyl-GlcNAc, 1 mg/mL BSA, and 0.5 mM AMP in 40 mM bis-tris, pH 5.5 at 60 °C. (B) Cell-free crude extracts of *E. coli* BL21(DE3)-pLysS overexpressing either H79A (○) or H238A (●) *E. coli* LpxC were incubated with varied concentrations of $CoSO_4$ (0−1000 μM) for 20−40 min prior to initiation of each assay. Assays contained either 0.05 mg/mL H79A extract or 0.02 mg/mL H238A extract and 3 μM UDP-3-*O*-acyl-GlcNAc, 1 mg/mL BSA and 0.5 mM AMP in 40 mM bis-tris, pH 5.9, at 30 °C.

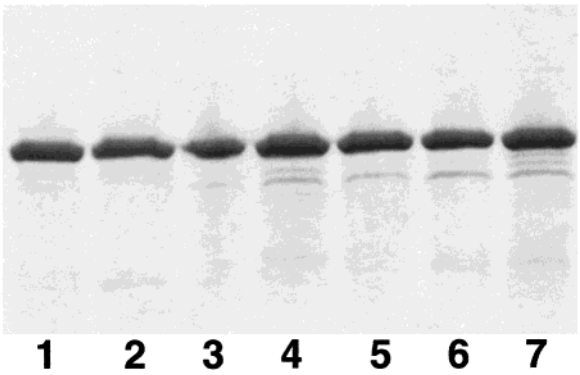

FIGURE 4: SDS−PAGE analysis of purified *A. aeolicus* LpxC variants. Samples of each purified *A. aeolicus* LpxC variant were boiled for 5 min in SDS−PAGE sample buffer and loaded onto a 12% SDS−PAGE gel for analysis. Each lane contains 3 μg total protein. Lane 1, wild-type *A. aeolicus* LpxC. Lane 2, H19A LpxC. Lane 3, E78A LpxC. Lane 4, H79A LpxC. Lane 5, H238A LpxC. Lane 6, D246A LpxC. Lane 7, H265A LpxC.

undetectable (<1 pmol/min/mg), even after the addition of 1−500 μM Zn^{2+}.

Stimulation of the activity of *E. coli* LpxC variants by addition of Zn^{2+} is not likely to be observed at 500 μM zinc since Zn^{2+} inhibits *E. coli* LpxC at significantly lower concentrations than it inhibits *A. aeolicus* LpxC (data not shown) (*8*). However, Co^{2+} does not inhibit *E. coli* LpxC at these concentrations and is able to substitute for the active site Zn^{2+} in wild-type *E. coli* LpxC (*8*). Therefore, the specific activities of extracts of *E. coli* overexpressing *E. coli* H79A and H238A LpxC were determined in the presence of increasing concentrations of $CoSO_4$ (10 μM to 1 mM) (Figure 3B). As with the variants in *A. aeolicus* LpxC, addition of divalent metal ions increases the specific activities of the *E. coli* H79A and H238A variant proteins about 10-fold.

Zinc Content of Purified A. aeolicus LpxC Variants. To measure the Zn^{2+} content of the *A. aeolicus* LpxC variants directly, each variant was purified to near homogeneity, and the metal content was determined using inductively coupled plasma emission (ICP) spectroscopic analysis. The purification scheme used for the wild-type and variant *A. aeolicus* LpxCs is similar to the previously described method (*7*), except that the S-200 gel-filtration chromatographic step is replaced with a reactive dye affinity column. *A. aeolicus* LpxC binds tightly to Reactive Red 120 dye resin and can be eluted by the addition of 2.5 M NaCl. The wild-type *A. aeolicus* LpxC, as well as the H19A, E78A, H79A, H238A, D234A, and H265A *A. aeolicus* LpxC variants, were purified by this procedure. The resulting proteins are approximately 80−90% pure, as judged by SDS−PAGE (Figure 4). The specific activities of the purified proteins, compared to the activity of purified wild-type *A. aeolicus* LpxC, agree well with the percentage of wild-type activity that was measured in crude extracts for each variant (Table 4). Moreover, the wild-type and the mutant proteins all elute under similar conditions from both the Q-Sepharose anion exchange and the Reactive Red 120 columns, suggesting that amino acid substitutions at these positions do not substantially affect the structure of each variant protein. Concentrated samples of each of these purified variants (4−20 mg/mL, as measured by the BCA protein assay using BSA as the protein standard) were analyzed for zinc content by ICP, yielding the milligrams of Zn^{2+} per grams of total protein in each sample (Table 4). Since this protein assay does not measure the molar concentration of LpxC active sites directly (*8*), the Zn^{2+}:LpxC stoichiometry cannot be determined accurately. The zinc content of the purified variants is lower than for wild-type LpxC in each case (Table 3), probably reflecting both the decreased zinc affinity of the mutants and the fact that no additional zinc was added during the overexpression and purification of these enzymes. However, the amount of zinc bound to the H79A and H238A LpxC variants is the lowest of all the proteins, ≤10% of that in the wild-type enzyme. This decreased zinc stoichiometry supports the hypothesis that the zinc affinity of these proteins is decreased due to the removal of a protein side chain that directly coordinates the active site zinc ion in wild-type LpxC.

Table 4: Zn^{2+} Content of Purified *A. aeolicus* LpxC Variants

variant	% wild-type activity (in extracts)[a]	% wild-type activity (as purified)[b]	mg of Zn^{2+}/g of protein[c]	% wild-type Zn^{2+} content
wild-type	100	100	0.98	
H19A	5.0	4.0	0.50	51
E78A	9.8	16	0.27	28
H79A	0.006	0.2	0.098	10
H238A	0.1	0.3	0.059	6
D246A	0.001	0.002	0.63	64
H265A	<0.0005	<0.002	0.23	23

[a] Specific activity measurements as described in Table 1. [b] Specific activity of purified *A. aeolicus* LpxC variants was determined using 3 μM UDP-3-*O*-acyl-GlcNAc in assays that also contained 1 mg/mL BSA and 0.5 mM AMP in 40 mM bis-tris, pH 5.5, at 60 °C. [c] Zinc concentration of each *A. aeolicus* LpxC variant was determined by ICP analysis (Garratt-Callahan Co., Millbrae, CA) as described in the Materials and Methods. The measurements of zinc content are ±20% error based on the zinc content determined for a $ZnSO_4$ standard solution. The amount of total protein in each sample submitted for ICP analysis was quantified by the BCA protein assay (Pierce).

DISCUSSION

As a metalloamidase that requires a single Zn^{2+} for enzyme activity, LpxC shares many characteristics of other Zn^{2+} metalloamidases, including inhibition by hydroxamate and phosphinate compounds and the ability of only Co^{2+} and Ni^{2+} to replace the active site metal ion (*7*, *8*). However, a consensus Zn^{2+} binding sequence in LpxC, such as the ones observed with other Zn^{2+} metalloamidases (*31*), is not present. Therefore, to identify amino acids that coordinate the zinc ion required for optimal activity, we made alterations in all of the conserved His, Glu, and Asp residues in LpxC. We have analyzed the activity of all of these variants and have identified the functional importance of some of these residues.

Coordination of Zn^{2+} in LpxC. In other Zn^{2+} metalloenzymes, the residues that coordinate a catalytic Zn^{2+} are completely conserved among enzymes from different species (*43*). Furthermore, alteration of one of the amino acids that coordinates Zn^{2+} decreases enzyme activity 100−10^5-fold due to either or both decreased Zn^{2+} affinity and decreased activity of the Zn^{2+}-bound enzyme (*29*, *30*, *49*, *50*). Of the 10 conserved Asp, Glu, and His residues, deletion of the side chain (alanine substitution) at six residues (H19, E78, E100, D105, E234, and D242) decreases activity <20-fold,

and this activity is not stimulated by addition of Zn^{2+}. Therefore, these side chains likely do not directly coordinate Zn^{2+} (Tables 1, 2, and 3). However, substitution of the side chains by alanine indicates four residues that are essential for catalysis (H79, H238, D246, and H265) and could potentially coordinate the bound zinc ion.

Identification of two of the three His residues, H79 and H238, as Zn^{2+} ligands is supported by the stimulation of activity seen in these variant extracts upon the addition of Zn^{2+} or Co^{2+} (Figure 3) and the decreased Zn^{2+} content (<10% of wild-type) in purified H79A and H238A variants (Table 4). The loss of Zn^{2+} binding residues in other zinc metalloenzymes does not usually completely abolish the ability of the enzyme to bind zinc but often leads to a decreased affinity for Zn^{2+} by 10^3-10^5-fold (*49*). The addition of higher concentrations of Zn^{2+} to variants lacking one or more metal-coordinating groups can restore partial activity by saturating the Zn^{2+} binding site (*50*, *55*, *56*). The decreased Zn^{2+} affinity in the H79A and H238A variants is not likely caused by large perturbations in protein structure, since the proteins are overexpressed as soluble proteins and since both of these proteins were purified similarly to the wild-type *A. aeolicus* enzyme (Table 4). Therefore, it is likely that these two His residues coordinate the Zn^{2+} that is required for LpxC catalytic activity.

Since all of the known high affinity catalytic zinc ions in enzymes are coordinated by at least three protein ligands (*31*, *43*, *57*), it is likely that at least one additional protein residue also coordinates the Zn^{2+} in LpxC. Our data indicate that either D246 or H265 likely coordinates the zinc ion, although the evidence may be slightly stronger for H265. The His at position 265 is a completely conserved residue that is absolutely essential for enzyme activity, as would be expected of an amino acid that coordinates Zn^{2+}. The purified H265A variant has the next lowest zinc content (23% of wild-type LpxC), after H79A and H238A, of all of the mutants analyzed (Table 4). However, the LpxC activity of the H265A variant cannot be restored by addition of Zn^{2+}, perhaps due to distortion of the Zn^{2+}-binding site in the H265A and H265Q variants. Similarly, an alanine substitution at D246 decreases activity substantially, although not completely. However, the bound zinc content of the D246A variant is only decreased 2-fold (Table 4) and added zinc ions inhibit, rather than activate, the observed activity (Table 3). Furthermore, significant activity (3%) is observed for the *A. aeolicus* D246N LpxC mutant, indicating that Asp at this position is not absolutely essential. The side-chain carbonyl of Asn could potentially coordinate Zn^{2+} in this variant, as previously observed in other systems; however, the decreased affinity of Asn compared with Asp for Zn^{2+} would predict that addition of Zn^{2+} to this variant protein would stimulate activity, as seen with the H79A and H238A variants (*32*, *47*, *51*). Such stimulation was not observed with the D246N variant extract (data not shown). The high activity of the D246N variant, the lack of stimulation by Zn^{2+}, and the relatively high Zn^{2+} content of the purified D246A LpxC all argue, but do not definitively prove, that the D246 side chain does not coordinate Zn^{2+} (Tables 3 and 4, data not shown). Therefore, the three His residues at positions 79, 238, and 265 are the best candidates for amino acid side chains that coordinate Zn^{2+} in LpxC. The structure of a zinc-dependent histone deacetylase, which catalyzes a similar reaction but has no observable sequence identity with LpxC, indicates that this enzyme has a $HisAsp_2$ metal polyhedron with the following spacing $DXHX_{88}D$ (*58*).

Our data indicate that LpxC contains a His_2X metal polyhedron where X is most likely His-265 or Asp-246. Zn^{2+} binding sites composed of three His residues are found in several families of Zn^{2+} metalloamidases, including the interstitial collagenases and the astacin family (*26*, *27*). However, LpxC does not share the HEXXH consensus Zn^{2+} binding sequences of these families or the HXXE or HXXEH consensus sequences of other Zn^{2+} metalloamidases (*31*, *43*). Aside from the Zn^{2+} metalloamidases, several other zinc metalloenzymes contain His_3 Zn^{2+} binding sites, including carbonic anhydrase II (CAII) and adenosine deaminase (*59*−*61*). In all of these Zn^{2+} sites, at least two of the Zn^{2+} ligand residues are located close together, often separated by only one to three amino acids in the primary sequence of the enzyme (*24*). In fact, the largest separations between the two closest Zn^{2+} ligand residues in a mononuclear site are the 21 amino acid separation between Cys and His in alcohol dehydrogenase (*62*) and the 24 amino acid separation between Glu and Asp in enolase (*63*). Therefore, if the three His residues identified in this study (H79, H238, and H265) are the Zn^{2+} ligands in LpxC, they represent an atypical Zn^{2+} site, with the closest two ligands separated by 26 amino acids. Similarly, if the third ligand is D246 the amino acid spacing between the ligands in LpxC ($HX_{159}HX_7D$) is farther apart than typical His_2Asp metal coordination polyhedra identified in neutral proteases (HX_3HX_5D) (*43*) or sonic hedgehog ($HX_4DX_{14}H$) (*64*).

Despite some differences in the identity of zinc ligand residues between many of the Zn^{2+} metalloamidases, almost all that have been described to date share a common mechanism for catalyzing the hydrolysis of amide bonds. The single Zn^{2+} in these enzymes can be characterized as a catalytic Zn^{2+}, participating directly in catalysis through direct coordination of a Zn^{2+}-bound water molecule that acts as a nucleophile. The presence of a water molecule as a ligand to the Zn^{2+} ion is one hallmark feature of catalytic Zn^{2+} binding sites (*46*, *65*, *66*). This differentiates them from structural Zn^{2+} sites that are composed entirely of protein ligands to the metal ion, such as the Cys_4 and Cys_2His_2 sites in alcohol dehydrogenase and Xenopus transcription factor IIIA, respectively (*25*, *44*). The Lewis acidity of the catalytic Zn^{2+} in metalloamidases lowers the pK_a of the Zn^{2+}-bound water to facilitate nucleophilic attack at the carbonyl carbon of the scissile peptide bond, creating a tetrahedral transition state (*67*). An active site glutamate residue that forms a hydrogen bond with the Zn^{2+}-bound water molecule has been proposed to enhance catalysis by functioning as a general base and accepting the proton that is released during formation of the tetrahedral transition state (*11*, *67*). This mechanism has been well documented for the metalloproteases but has not yet been demonstrated for metal-dependent deacetylases. However, the Zn^{2+} ion in LpxC exhibits features that suggest that it is a catalytic Zn^{2+}, including complete inhibition by hydroxamate and phosphinate-containing compounds (*7*). These compounds inhibit catalytic Zn^{2+} sites in metalloamidases by binding to the Zn^{2+} and displacing the Zn^{2+}-bound water molecule (*12*, *13*, *68*). Therefore, by analogy to the other single-Zn^{2+} metalloamidases, it is likely that a water molecule serves as a fourth

ligand to the catalytic Zn^{2+} in LpxC, completing a four-coordinate His_3-H_2O or $His_2-Asp-H_2O$ coordination sphere.

Potential Functions of Other Amino Acids. If the catalytic mechanism of the LpxC deacetylase is similar to the metalloproteinases, an amino acid is predicted to hydrogen bond with the zinc-bound water. In most metalloproteinases this group is a conserved glutamate (*11*, *67*), while a histidine residue is proposed to carry out this function in the histone deacetylases (*58*). As previously discussed, the largest decreases in activity are observed for alanine substitutions at D246 or H265, consistent with the $100-10^5$-fold decreased activity expected for the removal of either a catalytic general base (*69*, *70*) or a zinc ligand (*29*, *30*, *49*−*51*). However, the high activity of the D246N variant suggests that Asp246 may not function as a general base.

Intriguingly, in the *A. aeolicus* E78A LpxC variant, the inhibition by zinc is decreased significantly (Table 3) with no inhibition detected up to 1 mM Zn^{2+}. This decrease in zinc inhibition suggests that Glu78 in *A. aeolicus* LpxC may directly coordinate the inhibitory Zn^{2+} bound to LpxC. Other metalloproteinases, including CPA and thermolysin (*18*, *71*), are inhibited by Zn^{2+}. In CPA, kinetic and X-ray crystallographic data (*16*−*18*) indicate that the second zinc ion coordinates both the active site Zn^{2+}-bound hydroxide, creating a bridged hydroxide between the catalytic and inhibitory zinc ions, and the amino acid that forms a hydrogen bond with the zinc-water, Glu270. Since E78 may play an analogous role to E270 of CPA in binding the inhibitory Zn^{2+} ion, it is possible that this residue also functions similarly to E270 as a general base in catalysis. However, the *A. aeolicus* E78A variant retains 10% of wild-type LpxC activity, higher than the decreased activity expected for the removal of a catalytic general base (*69*, *70*).

A third possible role for conserved Asp or Glu residues (such as E78, E234, and/or D246) is to form a hydrogen bond with one of the direct histidine ligands, as observed in all zinc enzymes (*31*, *72*). In carbonic anhydrase, removal of one of these hydrogen bonding interactions decreases catalytic activity modestly and zinc affinity about 10-fold (*73*), consistent with the properties of the E78A LpxC variant. However, determination of the exact roles of these amino acids will require additional mechanistic studies.

Conclusions. We have demonstrated that several of the highly conserved residues in *E. coli* and *A. aeolicus* LpxC are important for LpxC catalysis. The low activity of LpxC variants at positions H79 and H238, coupled with the ability of Zn^{2+} to stimulate the activity of these enzymes and the low Zn^{2+} content of the purified variant enzymes suggests that these residues directly coordinate a catalytic Zn^{2+} in LpxC. The variants with alanine substituted at H265 or D246 also exhibit large ($>10^4$-fold) decreases in LpxC activity, suggesting that one of these residues may constitute a third protein ligand to the active-site Zn^{2+}, although additional evidence for this hypothesis remains to be garnered. Finally, *A. aeolicus* variants with substitutions at E78 are not inhibited by Zn^{2+}, consistent with a function for this residue as a ligand to the inhibitory Zn^{2+} in LpxC. Structural confirmation of these results by X-ray crystallography or NMR spectroscopy will be an important step in the elucidation of mechanistic information regarding the action of LpxC.

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