Structure of the LdcB LD-Carboxypeptidase Reveals the Molecular Basis of Peptidoglycan Recognition

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SUMMARY

Peptidoglycan surrounds the bacterial cytoplasmic membrane to protect the cell against osmolysis. The biosynthesis of peptidoglycan, made of glycan strands crosslinked by short peptides, is the target of antibiotics like β-lactams and glycopeptides. Nascent peptidoglycan contains pentapeptides that are trimmed by carboxypeptidases to tetra- and tripeptides. The well-characterized DD-carboxypeptidases hydrolyze the terminal D-alanine from the stem pentapeptide to produce a tetrapeptide. However, few LD-carboxypeptidases that produce tripeptides have been identified, and nothing is known about substrate specificity in these enzymes. We report biochemical properties and crystal structures of the LD-carboxypeptidase LdcB from Streptococcus pneumoniae, Bacillus anthracis, and Bacillus subtilis. The enzymes are active against bacterial cell wall tetrapiptides and adopt a zinc-carboxypeptidase fold characteristic of the LAS superfamily. We have also solved the structure of S. pneumoniae LdcB with a product mimic, elucidating the residues essential for peptidoglycan recognition and the conformational changes that occur on ligand binding.

INTRODUCTION

Bacteria surround their cytoplasmic membrane with a large, semipermeable barrier, the peptidoglycan (PG) sacculus, which not only protects the cell from lysis from turgor but also helps to maintain cell shape. PG comprises repeating units of the N-acetylglucosamine-N-acetylmuramic acid (GlcNAc−MurNAc) disaccharide that polymerizes into long glycan chains. Pentapeptides, usually comprising L-Ala−D-γ-Glu[Gln]−L-Lys−D-Ala−D-Ala, extend from the MurNAc moiety. In Gram-negative bacteria and some Gram-positive Firmicutes, the L-Lys at position 3 is replaced by meso-diaminopimelamic acid (A2pm), which is amidated in Bacillus subtilis (Vollmer et al., 2008a). Peptides protruding from adjacent glycan chains are crosslinked by transpeptidases to form the net-like PG sacculus, which the cell has to enlarge in order to grow and to divide (Sauvage et al., 2008). Newly inserted PG matures and is turned over by PG hydrolases, and the balance between synthesis and degradation of PG is key to normal cell division (Vollmer et al., 2008b). The importance of PG to the healthy physiology of a bacterium is highlighted by the fact that its biosynthesis is the target of some of our most successful antibiotics, β-lactams such as penicillin and glycopeptides such as vancomycin, resistance to which in pathogens is an urgent and currently unmet human health challenge.

In most species, the terminal D-alanine of the nascent pentapeptide stem is hydrolysed by DD-carboxypeptidases from the class C penicillin binding protein (PBP) family (Lovering et al., 2012). LD-carboxypeptidases subsequently hydrolyze the L-Lys[A2pm]−D-Ala bond to release the tripeptide. Though tripeptides are found in many bacteria, very few of the corresponding LD-carboxypeptidases have been identified. Membrane preparations of B. subtilis have shown strong LD-carboxypeptidase activity against cell-wall-derived tetrapeptides (Arminjon et al., 1977), though the specific enzyme involved has not been isolated. The best characterized LD-carboxypeptidase is LD-carboxypeptidase A (LdcA), a cytoplasmic, penicillin-insensitive member of the S66 family of serine peptides that is involved in PG recycling and is essential for cells entering the stationary phase (Metz et al., 1986; Templin et al., 1999). The cytoplasmic localization of LdcA precludes its involvement in the production of cell wall tripeptides. Other LD-carboxypeptidases either must be active within the periplasm of Gram-negative bacteria or are anchored to the exterior of the cell membrane in Gram-positives.

Several LD-carboxypeptidase genes have been identified recently, including dacB from Lactococcus lactis and Streptococcus pneumoniae (Barendt et al., 2011; Courtin et al., 2006), csd6 from Helicobacter pylori (Sycuro et al., 2012) and pgp2
from Campylobacter jejuni (Fridrich et al., 2014), though it should be noted that csd6 and pgp2 share no sequence similarity with dacB. The corresponding null mutants exhibited increased amounts of tetrapeptides in the cell wall and a concomitant decrease in tripeptide content (Sycuro et al., 2012; Fridrich et al., 2014). The S. pneumoniae dacB mutant also displayed severe morphological defects, developing into small, spherical cells or elongating into long, thick cells, as a result of division asymmetry produced by random septum localization (Barendt et al., 2011). Both the H. pylori csd6 and the C. jejuni pgp2 mutants have a morphological defect, displaying a straight shape instead of the normal helical morphology, indicating that csd6 and pgp2 also contribute to cell shape maintenance (Fridrich et al., 2014; Sycuro et al., 2012). In C. jejuni (Fridrich et al., 2012) and H. pylori (Sycuro et al., 2010), cell shape is also linked to pathogenicity, and PG fragments released by growing cells are important signaling molecules for immune recognition and modulation in animals ranging from insects to man (Royet et al., 2011). Therefore, the PG plays an important role in bacterial shape maintenance, viability, virulence and immune signaling.

In this study, we have investigated the biochemical activity of S. pneumoniae and B. subtilis DacB against cell wall saccull and synthetic tetrapeptides. Both proteins are shown to be active against cell-wall-derived tetrapeptides and synthetic tetrapeptides lacking the sugar moiety but are inactive against tetrapeptides terminating in L-alanine, confirming their classification as LD-carboxypeptidases. We therefore propose to rename these enzymes LD-carboxypeptidase B (LdcB). We present the crystal structures of both LdcB enzymes and the ortholog from Bacillus anthracis, all of which belong to the LAS (lysostaphin, D-Ala-D-Ala metallopeptides, sonic hedgehog) family of zinc-dependent peptidases. The structure of S. pneumoniae LdcB with MurNAc—L-Ala—D-γ-Gln—L-Lys—(D-Asn) bound reveals the molecular basis of PG recognition and the key roles played in catalysis by active site elements. It is surprising that the active site is reminiscent, yet reversed, of that found in pancreatic carboxypeptidase A, suggesting that the enzymes have converged on a common catalytic mechanism.

### RESULTS

#### Role for LdcB In Vivo

The mature PG of S. pneumoniae and B. subtilis contains mostly tripeptides, indicating the presence of PG-active peptidases in these organisms (Atrih et al., 1999; Bui et al., 2012). Both species contain DacA orthologs, DD-carboxypeptidases that cleave pentapeptides to tetrapeptides; however, the LD-carboxypeptidases that produce tripeptides from the tetrapeptides have remained elusive until recently. The PG of a S. pneumoniae dacB mutant contained mainly tetrapeptides, suggesting strongly that SpDacB is an LD-carboxypeptidase (hereinafter renamed as SpLdcB) (Barendt et al., 2011). Single mutants of the dacA and dacB orthologs in B. subtilis, dacA, and yodJ (hereinafter renamed as ldcB), had no obvious phenotype during vegetative growth, indicating that the trimming of peptides or the released D-Ala is not required for growth under laboratory conditions (Atrih et al., 1999). To test whether the released D-Ala becomes important under D-Ala limiting conditions, we constructed dacA and ldcB null mutants in a B. subtilis strain auxotrophic for D-Ala (an alanine racemase [airA] null mutant) and assayed growth in the presence of D-Ala. The D-Ala-dependent strain, RD180, required at least 450 μM D-Ala for normal growth, and it grew more slowly at lower D-Ala concentrations (Figure 1). However, strains also bearing either the dacA (KS03) or the ldcB (KS02) alleles exhibited even more severe growth rate dependencies on D-Ala, with the dacA mutant the most severely affected. These data suggest strongly that LdcB is an LD-carboxypeptidase that releases D-Ala from the cell wall, which can be used to support growth under D-Ala-limiting conditions.

To test directly whether LdcB functions as an LD-carboxypeptidase, we generated S. pneumoniae and B. subtilis ldcb mutants and isolated their cell wall PG. Muropeptides were released with cellosyl and analyzed by HPLC (Figures 2A and 2B). The ldcB mutant strains lacked tripeptides and contained mainly tetrapeptides in their PGs, consistent with previous work indicating that LdcB is the main enzyme trimming cell wall tetrapeptides (Barendt et al., 2011). To obtain more direct evidence for an enzymatic activity, purified, recombinant LdcB enzymes from S. pneumoniae and B. subtilis (termed SpLdcB and BsLdcB) were incubated with PG isolated from the respective ldcb mutant strains before digesting with cellosyl and analyzing the resulting muropeptides by HPLC (Figures 2A and 2B). Both SpLdcB and BsLdcB almost quantitatively digested PG tetrapeptides with a
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carboxy-terminal D-Ala producing the tripeptides, proving their LD-carboxypeptidase activity. SpLdcB was active in the presence of Zn\(^{2+}\) ions and inactive in the presence of EDTA, suggesting a metal-ion-dependent peptidase mechanism.

**SpLdcB and BsLdcB Are Catalytically Active against Synthetic Cell Wall Tetrapeptides**

In addition to removing the terminal D-Ala from *S. pneumoniae* cell wall PG tetrypeptides (Figure 2A), SpLdcB was also tested against synthetic tetrapeptides (L-Ala—D-Glu—L-Lys—D-Ala [tetra-D]) and its L-isomer, L-Ala—D-Gln—L-Lys—L-Ala [tetra-L]). Though these substrates lack the glycan component and differ further from the natural substrate by the replacement of D-glutamine with D-glutamine, they have the potential to engage a metal-ion-dependent peptidase mechanism. SpLdcB was active solely against the tetra-D ligand (Figure 2C), an activity that was dependent on the presence of zinc and abrogated totally in the presence of EDTA (Figure 2C).

**Overall Structure of LdcB**

The crystal structures of SpLdcB and *Bacillus anthracis* LdcB (BaLdcB) were solved by single wavelength anomalous dispersion (SAD); BsLdcB was solved by molecular replacement. The LdcB enzymes are single domain proteins comprising two subdomains separated by a V-shaped cleft (Figures 3A–3C). The first subdomain comprises a four-stranded, antiparallel \(\beta\) sheet (\(\beta_{4}–\beta_{7}\) that opposes the cleft, and the outside face of the \(\beta\) sheet is flanked by three \(\alpha\) helices (\(\alpha_{2}, \alpha_{4}, \text{and} \ \alpha_{6}\)). The second subdomain comprises an N-terminal, three-stranded, antiparallel \(\beta\) sheet (\(\beta_{1}–\beta_{3}\), somewhat discrete from the four helical bundle (\(\alpha_{2}, \alpha_{3}, \alpha_{5}, \text{and} \ \alpha_{7}\)) that makes up the rest of the subdomain. Though the BaLdcB structure contains the same subdomain, it is less well structured in this region than BsLdcB and SpLdcB, and, as a consequence, the \(\beta_{1}–\beta_{3}\) sheet is not formed. Neither subdomain is formed by a contiguous, linear part of the primary sequence; six loops (\(\beta_{3}–\alpha_{1}, \beta_{4}–\alpha_{2}, \alpha_{3}–\beta_{5}, \beta_{6}–\alpha_{5}, \alpha_{5}–\beta_{7}, \text{and} \ \alpha_{6}–\alpha_{7}\)) connect the two subdomains.

The cleft between subdomains is 15–18 Å wide at the top and 15 Å deep and narrows down to the base where a Zn\(^{2+}\) ion, added to the enzyme during purification, is located (Figures 3A–3C), which is coordinated in a tetrahedral manner by three amino acids. In SpLdcB, the liganding residues are His153, Asp160, and His207 (Figure 3D); the equivalents in BaLdcB are His185/His160, Asp192/Asp167, and His244/His219 (Figures 3E and 3F). These zinc ligands are universally conserved in LdcB sequences, consistent with an unattributed electron density that opposes the cleft, and the outside face of the \(\beta\) sheet is flanked by three \(\alpha\) helices (\(\alpha_{1}, \alpha_{4}, \text{and} \ \alpha_{6}\)). The second subdomain is of a size, shape, and location corresponding to that of alanine. The ligand is stabilized in this position by van der Waals’ contacts between the alanyl methyl group

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**Figure 2. Activity of SpLdcB and BsLdcB against Peptidoglycan and Peptides**

(A) HPLC chromatograms of muropeptides from *S. pneumoniae* strains, obtained after incubating PG with or without SpLdcB or SpLdcB(E204A) in the presence of Zn\(^{2+}\) or EDTA, followed by digestion with cellosyl and reduction with sodium borohydride. The muropeptides are: Tri, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala; TetraTri, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala; Tetra, GlcNAC—MurNAc(r)—L-Ala—D-Glu—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala—L-Lys—D-Ala; deAc, deacetylation at GlcNAc; SA, L-Ser—L-Ala branch (at L-Lys), GlcNAc, N-acetylgulosamine; MurNAc(r), N-acetylmuramitol.

(B) HPLC chromatograms of muropeptides from *B. subtilis* strains, obtained after incubating PG with or without BsLdcB, followed by digestion with cellosyl and reduction with sodium borohydride. Muropeptides: Tri, GlcNAc—MurNAc(r)—L-Ala—D-Glu—mesoDap(NH\(_3\)) ; Tetra, GlcNAc—MurNAc(r)—L-Ala—D-Glu—mesoDap(NH\(_3\))—D-Ala; TetraTri, GlcNAc—MurNAc(r)—L-Ala—D-Glu—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala; Tetra, GlcNAc—MurNAc(r)—L-Ala—D-Glu—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala—mesoDap(NH\(_3\))—D-Ala; deAc, deacetylation at GlcNAc; SA, L-Ser—L-Ala branch (at L-Lys), GlcNAc, N-acetylgulosamine; MurNAc(r), N-acetylmuramitol.

(C) TLC of SpLdcB incubated with various substrates.
and the phenolic side chains of Tyr191 and Tyr201 and by hydrogen bonds to the side chains of Glu204 and the main chain amide nitrogen of Tyr144. Despite the high resolution of the SpLdcB diffraction data, it was not possible to discriminate between either alanyl stereoisomer, both of which are present in the crystallization conditions. We have modeled D-Ala because it is the enzymatic product of these enzymes. However, residual electron density adjacent to the zinc ion could not be modeled satisfactorily with any ligand arising from the enzymatic activity, the crystallization media, or purification media; consequently, the remaining electron density has been left unmodeled. Mass differences between native and denatured protein samples also failed to identify candidate molecules corresponding to the residual electron density. Residual electron density in the vicinity of the fourth zinc ligand in BsLdcB was fulfilled by a phosphate anion, a component of the crystallization conditions (Figure 3E), which refined satisfactorily. The phosphate is located so as to mimic the tetrahedral transition state. A second feature in the S′ subsite is unmodeled. The residual difference density features are shown contoured at a level of 3σ and hydrogen bonds are drawn as dashed black lines.

**Comparison of LdcB Structures**

SpLdcB, BaLdcB, and BsLdcB share pairwise sequence identities of ~30%, and they superimpose on ~155 common Cα atoms with a root-mean-square deviation (rmsd) of 1.5 Å–1.8 Å (Figures 3A–3C). The major differences between the LdcB structures are limited to the rather flexible and unconstrained N- and C-termini and the occasional longer loop in one structure in comparison to the other, as a direct consequence of sequence insertions. The active sites of SpLdcB, BaLdcB, and BsLdcB are, to all extents and purposes, indistinguishable (Figures 3D–3F). A further BaLdcB structure has been solved that contains no bound zinc ion, but there are negligible differences between it and its counterpart with zinc bound (Figure S1 available online). The active site of the LdcB enzymes is, therefore, sculpted in a robust fashion, competent for peptidolysis once the catalytic machinery is completed by the incorporation of zinc.

**Structural Comparison with Other Peptidases**

Using SpLdcB as a search model in DALI (Holm and Rosenström, 2010), over 100 significantly similar structures were identified in the Protein Data Bank (PDB), including the structure of VanXYG, a dipeptidase/DD-carboxypeptidase (PDB ID 4f78 [Meziane-Cherif et al., 2014]; DALI Z score = 17.0); an endolysin from the Listeria bacteriophage A500 (PDB ID 2vo9 [Korndörfer et al., 2008]; Z = 9.5); VanX, a DD-dipeptidase (PDB ID 1r44 [Bussiere et al., 1998]; Z = 6.6); and the N-terminal domain of sonic hedgehog, a protease involved in embryogenesis (PDB ID 2wq4 [Bishop et al., 2009]; Z = 5.3). Equivalent results were obtained using the structures of BaLdcB and BsLdcB as queries in DALI. The matched structures are all members of the wider LAS superfamily of proteases (Bochtler et al., 2004), which, although they share very little sequence identity, possess markedly similar active sites (Figure 4). By comparison with the VanXYG structure (Meziane-Cherif et al., 2014), the active site of the LdcB proteins is open and more solvent accessible. The open nature of the active site is a direct consequence of the absence of the β4–α5/bisubstrate selectivity loop in LdcB, which enables the recognition of PG, a molecule far greater in size than the dipeptides utilized by VanXYG (Meziane-Cherif et al., 2014).
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Figure 4. A Comparison of the Active Sites of LdcB and Other LAS Family Members
LdcB shares an active site closely superimposable on those of other LAS family enzymes. The zinc is coordinated by a H-X(3–6)-D-X(not conserved)-H motif. A glutamate that plays a key role in catalysis is also shown; note that, in D-Ala-D-Ala carboxypeptidase from Streptomyces albus, the equivalent amino acid is histidine, the first amino acid in the H-X-H motif common to most LAS carboxypeptidases. The structures shown, distinguished by the coloring of carbon atoms, are SpLdcB (white), BsLdcB (green), BeLdcB (orange), Enterococcus faecalis D,D-dipeptidase/D,D-carboxypeptidase VanX(3) (yellow; PDB ID 4I78), Enterococcus faecium aminopeptidase VanX (blue; PDB ID 1r44) and S. albus D-Ala-D-Ala carboxypeptidase (pink; PDB ID 1bu).

As with other LAS superfamily members, LdcB contains the characteristic zinc binding motif (Figure 4), with a consensus sequence of H-X(3–6)-D-X(not conserved)-H. The fourth zinc ligand in the structures of other LAS family members tends to be a solute from the crystallization milieu or a water that is likely to act as the nucleophile in the reaction (Bussiere et al., 1998). A second conserved motif in the LAS superfamily, H-X-H, of which the first histidine is the catalytic residue (Bochtler et al., 2004), is missing from LdcB enzymes; the catalytic histidine is replaced spatially by glutamate (i.e., Glu204 in SpLdcB).

SpLdcB Co-crystallized with a Reaction Product
Despite attempting to crystallise LdcB enzymes with a range of synthetic ligands, one crystal structure, that of SpLdcB in complex with MurNAc-L-Ala-D-γ-Gln-L-Lys—(D-Asn) (Figure 5A), this ligand is a close chemical analog of the true reaction product, as it contains half of the repeating unit of the glycan component of PG and comprises a tripeptide stem peptide that is branched at the L-lysine, the location of the crosslink position in natural PG (Figures 5F and 5G). There are two chemical differences between this synthetic mimic and the true reaction product. First, the ligand lacks GlcNAc, the second sugar moiety of the glycan repeating unit in PG. Second, the terminal D-Asn group is covalently attached by a peptide bond between the main chain carbonyl of the terminal D-Asn and the side chain Nɛ of the penultimate lysine. In crosslinks between peptide stems, the side chain Nɛ of the penultimate lysine is linked by a peptide bond to the main chain carbonyl of a D-Ala. Hence, the L-Lys—(D-Asn) linkage is a faithful mimic of the natural L-Lys—(D-Ala), albeit the chemistry of the side chains of the terminal D-amino acids clearly differ.

There are six molecules of SpLdcB in this crystallographic asymmetric unit, though none of the interfaces represent a stable multimeric form. Five of six protein chains could be built and refined satisfactorily; the electron density for the sixth copy could not be improved by any procedure to permit its reliable modeling, and its absence from the model contributes directly to the slightly inflated final Rwork and Rfree values (Table 1). The five built molecules are highly superimposable, with each other and with the apo SpLdcB structure (rmsds between 0.5 Å and 1.1 Å; Table 2). In chains B, C, and E, the zinc is coordinated by a likely nucleophilic water to form tetrahedral coordination geometry. In chain D, the fourth zinc ligand is not evident, perhaps due to local disorder. Residual electron density was retained throughout refinement in only one of the five molecules, chain A, into which MurNAc-L-Ala-D-γ-Gln-L-Lys—(D-Asn) could be built and refined unambiguously (Figure 5A).

The structure of the ligand-bound form of SpLdcB is entirely consistent with its LD-carboxypeptidase function. First, the MurNAc moiety is situated at the very top of the intersubdomain cavity and makes little contact with the protein. This part of the ligand has the highest B factors (a mean of 93 Å², versus a mean of 83 Å² for the intact ligand and a mean of 45 Å² for all atoms modeled) and, thus, the weakest electron density. The MurNAc O1 and O4 atoms, which define the location of the flanking β1- to β4-linked GlcNAc sugars in PG, project away from the protein surface so that the glycan chain would be accommodated by the protein without steric clashes. The N-acetyl group at C1 is stabilized against the side chain of Met202 by van der Waals’ forces (Figure 5B).

The Cα and Cβ atoms of L-alanine at position 1 of the peptide pack against the face of Tyr144, a conserved amino acid in many LdcB sequences, to discriminate against the D-stereoisomer in this subsite, S3. L-alanine is favored by contacts with Tyr144 and Met202, which discriminate against all L-amino acids bulkier than alanine (Figure 5B). The NH2 group of the D-γ-Gln of position 2 is located in subsite S2, 2.3 Å away from and in hydrogen bonding distance of Glu204 (Figure 5C), an invariant amino acid in LdcB sequences. Stereoselectivity at this position is maintained because L-γ-glutamine would clash with Tyr144. The γ-glutamine is selected for by specific interactions with the main chain carbonyl of Ala203 and the side chain of Glu204 while ensuring that the scissile peptide bond is in the catalytic position. These key aspects of molecular recognition could not occur with commonly occurring amino acids. Canonical glutamine can be accommodated at this position in the synthetic peptide, tetra-D, because the absence of contacts with one face of the peptide provides scope for the conformational changes needed to accommodate glutamine in the absence of the glycan strands of the natural substrate.

Position 3 of the peptide is occupied by L-lysine. D-lysine could not be accommodated at this position because of the presence nearby of Glu107. As expected of a reaction product complex, the lysine carboxylate is located adjacent to the active site zinc in subsite S1 (Figure 5D). One of the carboxylic oxygens completes the zinc tetrahedral geometry with His153/Asp160/His207, and the second oxygen forms a salt bridge with
Arg120, which is either arginine or lysine in LdcB orthologs. The L-lysine carboxylate thus represents a posthydrolysis state, placed appropriately for the peptide bond of the substrate to be hydrolyzed by the water molecule that fulfils the fourth zinc ligand in the absence of substrate.

The side chain of the L-lysine twists back against the rest of the peptide and projects into the protein core to place the terminal D-Asn branch in subsite S\(_{10}\), against the main chain atoms of Tyr144 and the side chains of Tyr191, Tyr201, and His207 (Figure 5E). The carbonyl of the L-Lys—(D-Asn) peptide bond is contacted by the side chain of Ser151. However, it should be noted that subsite S\(_{10}\) would be occupied by the scissile D-alanine in the presence of the reaction substrate, and support for this hypothesis comes from the structure of VanXYG solved in complex with a phosphinate transition state mimic (Figure 6A) (PDB ID 4muq) (Meziane-Cherif et al., 2014). The amide nitrogen of Ala146 also participates in stereoselectivity, but as this is a main chain atom, it is perhaps no surprise that sequence conservation is not maintained at this position. However, crosslinks between peptide stems cannot be accommodated in the observed conformation because of the burial of the branched amino acid in subsite S\(_{10}\) (Figure 6B). Mere rotation of the side chain torsion angles of the L-lysine would result in a more thermodynamically favored extended conformation for this side chain to result in it passing beyond Arg120, Leu128, and Tyr132 to project beyond the surface of the protein (Figure 6B; Figure S2). In this conformation, crosslinked peptide stems attached to the terminal N\(_{z}\) would not be sterically occluded by the body of the protein, consistent with the observed cleavage of crosslinked peptides (Figure 2A). In
some bacteria, the lysine is replaced with diaminopimelic acid, a carboxy derivative of lysine that is amidated in bacilli. While the amidocarboxy derivative could be stabilized in the observed conformation by a similar set of specific interactions, it would also sterically occlude crosslinked peptide stems. Diaminopimelic acid is thus likely to follow the same path predicted for lysine by forming a simple, extended conformation to project into solvent.

Several key structural changes occur on ligand binding in SpLdcB (Figure 7; Movie S1). First and foremost, substrates are occluded in the empty form of the enzyme by a loop between residues Gly163 and Glu171, which blocks access to the active site. Most of these residues (Asp167 to Glu171) move by 10–15 Å in the presence of ligand to open up the cleft by refolding into an additional turn at the N terminus of α-helix 4. The rearrangement of these residues is accompanied by further conformational changes in Trp206, the indole ring of which moves by up to 9 Å, which not only aids to open up the active site of the enzyme but also forces the side chain of Glu204 to rearrange to take up a position capable of interacting with the substrate. The importance of Glu204 and the zinc ion for catalysis is highlighted by the observation that tetra-D is a much poorer substrate for the E204A variant of SpLdcB (Figure 2A) and by the absence of activity in the presence of EDTA (Figures 2A and 2C). The conformational changes seen on ligand binding to SpLdcB contrasts with the absence of significant conformational changes described for VanXY G in complex with phosphinate (PDB ID 4muq) (Meziane-Cherif et al., 2014). Like LdcB, VanXY G maintains the H-X (3–6)-D-X(not conserved)-H motif, and as also observed in LdcB, the catalytic H-X-H motif is replaced by a glutamate. The resculpting of the N terminus of α-helix 4 in SpLdcB to accommodate the tetrapeptide is not necessary in VanXY G, because this enzyme hydrolyzes a much smaller substrate, D-Ala-D-Ala dipeptide, which is accommodated by the enzyme without significant rearrangement.

Table 1. Summary of X-Ray Data Collection and Refinement Statistics

| Parameters          | SpLdcB | BsLdcB | SpLdcB with Ligand | BaLdcB | BaLdcB with Zinc |
|---------------------|--------|--------|-------------------|--------|-----------------|
| PDB ID              | 4OX5   | 4OX3   | 4OXD              | 4JID   | 4MPH            |
| Space group         | I222   | P212121| C2                | I222   | I222            |
| Unit Cell Dimensions|        |        |                   |        |                 |
| a, b, c (Å)         | 48.8, 60.4, 138.2 | 42.7, 53.7, 102.4 | 346.0, 42.5, 79.3 | 76.6, 113.0, 124.6 | 76.8, 112.3, 125.7 |
| α, β, γ (°)         | 90, 90, 90 | 90, 90, 90 | 90, 93.1, 90 | 90, 90, 90 | 90, 90, 90 |
| Wavelength (Å)      | 0.98   | 0.97   | 0.92              | 0.97856 | 1.27696         |
| Resolution (Å)      | 20.0–1.80 (1.84–1.80) | 47.6–1.70 (1.73–1.70) | 47.8–2.80 (2.95–2.80) | 29.7–2.30 (2.36–2.30) | 28.3–2.03 (2.06–2.03) |
| Multiplicity        | 24.3 (14.2) | 3.2 (1.7) | 3.4 (3.4) | 4.2 (3.6) | 11.8 (9.9) |
| Rmerge              | 0.077 (0.108) | 0.072 (0.235) | 0.134 (0.635) | 0.093 (0.542) | 0.101 (0.511) |
| I/σf                | 34.4 (2.4) | 11.9 (2.4) | 7.8 (1.9) | 13.5 (2.4) | 32.1 (4.2) |
| Completeness (%)    | 98.6 (86.9) | 95.7 (89.3) | 98.3 (98.3) | 98.3 (94.3) | 99.8 (96.9) |
| Refinement          |        |        |                   |        |                 |
| Resolution (Å)      | 20.0–1.80 | 47.6–2.0 | 47.8–2.80 | 29.7–2.3 | 28.3–2.03 |
| No. of reflections  | 25,656 (1,157) | 35,526 (1,057) | 47,327 (1,979) | 22,730 (1,226) | 67,494 (3,374) |
| Rwork/Rfree         | 0.191/0.253 | 0.176/0.247 | 0.273/0.335 | 0.194/0.259 | 0.151/0.189 |
| No. of Atoms        |        |        |                   |        |                 |
| Protein             | 1,476  | 1,753  | 7,014             | 3,040  | 2,970           |
| No. of proteins/AU  | 1      | 1      | 6                 | 2      | 2               |
| Iono/ligand         | 3/22   | 1/5    | 21/51             | -/4    | 2/12            |
| Water               | 197    | 230    | 111               | 424    | 466             |
| B Factors (Å²)      |        |        |                   |        |                 |
| Protein             | 41.8   | 16.0   | 44.3              | 45.3   | 35.8            |
| Iono/ligand         | 36.9/50.7 | 10.7/23.8 | 38.7/69.7 | —/49.8 | 17.9/60.0     |
| Water               | 48.2   | 31.6   | 28.7              | 43.6   | 46.2            |
| Rmsd                |        |        |                   |        |                 |
| Bond lengths (Å)    | 0.018  | 0.015  | 0.005             | 0.013  | 0.0012         |
| Bond angles (°)     | 1.96   | 1.76   | 0.84              | 1.36   | 0.991          |
| Ramachandran        |        |        |                   |        |                 |
| Favored (%)         | 96.1   | 96.7   | 95.8              | 97.6   | 98.0            |
| Allowed (%)         | 100    | 100    | 99.6              | 99.7   | 100             |

Highest resolution shell shown in parentheses. AU, asymmetric units.

\[ R_{merge} = \frac{\sum_{i \neq j} |I_i(h) - \langle I_i(h) \rangle|}{\sum_{i \neq j} I_i(h)}, \]

\[ R_{work}/R_{free} = \frac{\sum_{i \neq j} \left| F_{p \text{obs}} - F_{p \text{calc}} \right|}{\sum_{i \neq j} F_{p \text{obs}}}, \]
DISCUSSION

The presence of tripeptides in the bacterial cell wall implies that LD-carboxypeptidase activity must be present in the extracellular milieu. In this study, we have shown that LdcB, from both S. pneumoniae and B. subtilis, are active against tetrapeptides found within the bacterial cell wall and are specific for LD linkages, confirming the LD-carboxypeptidase classification with a role in cell wall maturation. The precise in vivo role for LdcB remains to be determined; one possibility is that the enzyme allows recycling of D-Ala from the cell wall to support further cell wall synthesis and, thus, growth. Alternatively, the cleavage of D-Ala from the cell wall might permit the differentiation between old and new cell walls, a potentially powerful way to ensure the correct localization of new cell wall synthesis. The correct localization of new PG synthesis is likely to be more important to bacteria that have polarized cell growth, such as Bacilli, in comparison to those that have dispersed growth along the length of a cylinder (e.g., Bacilli) or that utilize a division-only mechanism, such as Staphylococci. The cell morphological defect observed in the S. pneumoniae ldcB mutant, and the absence of defect in the B. subtilis ldcB mutant, could be consistent with this premise. Further studies should distinguish these possibilities.

We also present crystal structures of BaLdcB, BsLdcB, and SpLdcB, the latter also in complex with a PG-fragment analog that has enabled the identification of amino acids key to molecular recognition of the substrate and a potential tunnel through which crosslinked PG peptides may pass during proteolysis. LdcB belongs to the LAS group of zinc metalloproteases; several members of this family are active against PG, albeit with different substrate specificities to LdcB, including the Gly-Gly endopeptidase lysostaphin (Korndörfer et al., 2008) and the DD-/LD-endopeptidase MepA (Marcyjaniak et al., 2004). LdcB is dissimilar to other groups of LD-carboxypeptidases, e.g., Pseudomonas aeruginosa LD-carboxypeptidase LdcA (PDB ID 1zrs) (Korza and Bochtler, 2005) that degrades cell wall tetrapeptides in the cytoplasm. LdcA utilizes a Ser-His-Glu catalytic triad in catalysis and belongs to the S66 serine protease family of enzymes, a group with which LdcB shares no similarity (Korza and Bochtler, 2005). Likewise, LdcB shares no sequence similarity with Csd6 and Pgp2, two recently discovered LD-carboxypeptidases that aid to maintain the helical shape of H. pylori (Sycuro et al., 2012) and C. jejuni (Fridrich et al., 2014). Csd6 and Pgp2 belong to an LD-transpeptidase family that utilizes a conserved Cys/His motif for catalysis (Barrotte-Sorin et al., 2006), and are, Structure of the LdcB LD-Carboxypeptidase

Table 2. Rmsd Values between Ligand-Bound SpLdcB Chains (4oxd) and apo SpLdcB (4ox5)

| 4ox5 Chain | 4oxd Chain A | 4oxd Chain B | 4oxd Chain C | 4oxd Chain D | 4oxd Chain E |
|------------|--------------|--------------|--------------|--------------|--------------|
| 4oxd Chain A | – | 0.73 | 0.57 | 0.66 | 0.69 | 0.80 |
| 4oxd Chain B | 0.73 | – | 0.63 | 0.64 | 0.76 | 1.03 |
| 4oxd Chain C | 0.57 | 0.63 | – | 0.56 | 0.65 | 0.86 |
| 4oxd Chain D | 0.66 | 0.64 | 0.56 | – | 0.56 | 0.64 |
| 4oxd Chain E | 0.69 | 0.76 | 0.65 | 0.56 | – | 0.61 |

Figure 6. A Comparison of the Ligand-Bound Active Sites Carboxypeptidases

(A) The active site of SpLdcB (white) with bound MurNaC—L-Ala—D-γ-Gln—L-Lys—(D-Asn) (turquoise carbons) is compared to that of VanXYg (blue) containing a phosphinate transition state analog (pink carbons) of D-Ala—D-Ala (PDB ID 4muq). The phosphinate in VanXYg coordinates the active site zinc in a similar manner to that of the lysyl carboxylate in the MurNaC—L-Ala—D-γ-Gln—L-Lys—(D-Asn) bound to SpLdcB. The D-alanyl moiety of the phosphinate ligand in VanXYg, which occupies subsite S1', is matched by the D-alanine (green carbons) in SpLdcB (white) and the D-Asn of the MurNaC—L-Ala—D-γ-Gln—L-Lys—(D-Asn) ligand. The zinc ions are shown as gray spheres.

(B) The proposed positions of the two posthydrolysis ligands (orange carbons) in the active site of SpLdcB (white). The D-alanine from the SpLdcB structure occupies the S1' site of the protein, while the lysine side chain has been rotated and modeled in an extended conformation. The altered conformation of the lysine presents the Nζ to the surface of the protein, in an ideal position to accommodate peptide crosslinks. The zinc ions are shown as gray spheres.

(C) The active sites of SpLdcB and pancreatic carboxypeptidase A (PDB ID 1thu [Cho et al., 2002]) are compared. In each structure, the Zn2+ ion is coordinated by two histidines (His69 and His196 in carboxypeptidase A; His153 and His207 in SpLdcB) and an acidic amino acid (Glu72 in carboxypeptidase A; Asp160 in SpLdcB). The reversed locations of Arg127 and Glu270 in SpLdcB are matched by a reversal of the path of the respective substrates through the active sites and the likely retention of a common catalytic mechanism.
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Figure 7. Conformational Changes in SpLdcB on Ligand Binding

(A and B) Cartoon representations of (A) apo SpLdcB and (B) SpLdcB with bound MurNAc- Ala-D-γ-Gln-Lys-(D-Asn) using the same color scheme as in Figure 3. Key residues that move on ligand binding are shown in stick representation.

Experimental Procedures

Cloning

*B. subtilis* ldcB and *S. pneumoniae* ldcB were amplified by PCR from *B. subtilis* 168 and *S. pneumoniae* R6 genomic DNA, respectively, to generate fragments with Ndel and Xhol restriction sites. Both fragments were digested with Ndel and Xhol and ligated with Ndel/Xhol-digested pET28a. *B. anthracis* ldcB was PCR amplified from *B. anthracis* str. Ames ancestor genomic DNA and cloned into p15TV-LJC (Eschenfeldt et al., 2009) that codes for a N-terminal His6 tag, followed by a tobacco etch virus (TEV) protease cleavage site. In all cases, the recombinant constructs were designed to remove membrane-anchoring amino acids to result in final clones encoding SpLdcB amino acids 56–238; BsLdcB, 61–273; and BslLdcB, 55–243.

Strain Construction

*J. pneumoniae* R6LdcB was constructed by transforming strain R6 with a PCR product covering the ldcB-deletion region from strain IU8880 (ΔSpLdcB) amplified with primers P136 and P137 (Barendt et al., 2011). Following selection on tryptic soy broth blood agar plates containing 250 μg/ml kanamycin. All strains were grown in C + Y medium (Horne and Tomasz, 1993), containing kanamycin if required. Competent bacteria were obtained as described elsewhere, with the addition of competence-stimulating peptide (Vollmer and Tomasz, 2001).

*B. subtilis* strains were constructed by transformation of chromosomal DNA (Anagnostopoulos and Spizizen, 1961; Young and Spizizen, 1961) from strains bearing null mutations for dacA (MC01, tcpC2 ΔdacA cat) or yodC (MC05, tcpC2 ΔyodC neo) into strain RD180 (tcpC2 ΔtalA A zeo), selecting for the appropriate resistance markers (chloramphenicol or kanamycin at 5 μg/ml) in the presence of 40 μg/ml D-alanine, yielding strains KS03 and KS02, respectively.

Growth Curves

*B. subtilis* strains RD180, KS02, and KS03 were grown overnight at 30°C in Luria broth (LB) supplemented with 40 μg/ml D-Ala. The overnight cultures were diluted 1:10 with LB supplemented with 40 μg/ml D-Ala and grown at 37°C for 2 hr. The cells were collected by centrifugation and suspended in D-Ala-free media at 37°C. This suspension was used to inoculate media with various concentrations of D-Ala to an optical density 600 (OD600) of 0.05, and the subsequent growth of each culture at 37°C was monitored by determining the optical density at 600 nm every 20 min for 3 hr. The growth rate was determined during the exponential growth phase only.

Mutagenesis

A modified QuickChange protocol, incorporating “touchdown” (Don et al., 1991), was used for site-directed mutagenesis. The annealing temperature of the first 10 cycles was reduced by one degree per cycle, from 68°C to 58°C, before a further ten cycles were performed at a constant annealing temperature of 58°C. The extension temperature was maintained at 72°C for 11 min per cycle. Mutagenic primers (0.3 μM) were mixed with 50 ng of template DNA, 0.5 mM dNTPs, and 2.5 U of Pfu DNA polymerase in a total reaction volume of 20 μL. The template plasmid was digested using DpnI.

Peptidoglycan Activity Assays

Cell wall and PG from *S. pneumoniae* and *B. subtilis* strains were isolated by published procedures (Bu et al., 2012; Garcia-Bustos et al., 1988). To test for LD-carboxypeptidase activity, PG from *S. pneumoniae* R6LdcB
and SpLdcB were obtained by sitting drop vapor diffusion at 20 °C. The best SpLdcB crystals were obtained from a 0.1 M mixture of amino acids (glycine, L-glutamate, and racemates of alanine, serine, and lysine). 0.1 M Morpheus buffer system 2, pH 7.5, and 37.5% 2-Methyl-2,4-pentanediol (MPD)/polyethylene glycol (PEG) 1000/PEG 3350 (Gorevic, 2009). A single SpLdcB crystal grew from 0.1 M phosphate/citrate buffer, pH 4.2, 40% PEG 300 over a period of ~2 months. Crystals of SpLdcB with bound MurNAc-L-Ala-D-β-Glu-L-Lys-(D-Ala) (InivvoGen) were obtained by incubating the protein with the ligand at 2 mM final concentration for 30 min at room temperature prior to crystallization against a mother liquor of 0.1 M ammonium chloride, 0.1 M HEPES/NaOH, pH 8.0, and 20% PEG 6000. Crystals of BaLdcB (zinc free) were grown at 23 °C using hanging-drop vapor diffusion by mixing 20 mg/ml protein with reservoir solution containing 0.3 M sodium chloride, 0.1 M HEPES, pH 7.5. As soaking of zinc chloride into BaLdcB apo-protein crystals did not provide sufficient occupancy of zinc into the active site, crystals of BaLdcB (zinc bound) were grown at 23 °C by hanging-drop vapor diffusion by mixing 20 mg/ml protein and 5 mM zinc chloride prior to crystallization with 0.2 M ammonium acetate, 0.1 M sodium acetate, and 30% PEG 4000.

**Data Collection and Processing**

All crystals were cryoprotected with paratone-N before flash cooling in liquid nitrogen prior to data collection at 100 K. Diffraction data for BaLdcB and SpLdcB were on beamline IO2 of the Diamond Light Source. For selenomethionine-labeled SpLdcB, diffraction data were collected at a wavelength of 0.98 Å to permit phasing by selenomethionine-SAD (Se-SAD). Diffraction data were integrated with XDS (Kabsch, 2010) and reduced with SCALA (Evans, 2006). For BaLdcB, diffraction data were collected on beamlines 21-ID-G at Life Sciences Collaborative Access Team, Advanced Photon Source, at 0.97856 Å (selenomethionine peak; zinc free) and 1.27696 Å (Zn²⁺ peak; zinc bound). The BaLdcB diffraction data were integrated and reduced with HKL-3000 (Minor et al., 2006).

**Structure Solution**

The structure of SpLdcB was solved using SHELX (Sheldrick, 2008) as implemented in the HKL2MAP interface (Pape and Schneider, 2004). An initial model was built by PHENIX.AUTO SOLVE (Adams et al., 2011). The completed SpLdcB structure was used as the molecular replacement search model in Phaser (McCoy et al., 2007) to solve BaLdcB and the SpLdcB-ligand complex. The BaLdcB apo-Zn²⁺-bound structure was solved by SAD phasing using PHENIX.solve (Adams et al., 2011), which identified the positions of the two bound zinc ions. The occupancies of the zinc atoms were refined during model refinement. The BaLdcB Zn²⁺-free structure was solved by molecular replacement using the D,D-peptidase domain of the apo structure of VanXYα (PDB ID 4787) (Meziane-Cherif et al., 2014).

For all structures, manual building cycles in COOT (Emsley et al., 2010) were interspersed with restrained refinement in PHENIX.REFINE (Afonine et al., 2012). B factors were refined isotropically for all structures apart from BaLdcB, which was refined anisotropically. Noncrystallographic symmetry refinement was not used. Average B factor and geometric parameters were calculated using PHENIX and verified with PHENIX.refine and the Research Collaboratory for Structural Bioinformatics PDB Validation server. Data collection and final model refinement statistics for all structures can be found in Table 1. The graphics programs UCSF Chimera and PyMOL were used to generate all molecular figures presented.

**ACCESSION NUMBERS**

The PDB accession numbers for SpLdcB, BaLdcB, SpLdcB with bound ligand, apo BaLdcB, and BaLdcB with bound zinc reported in this paper are 4O5X, 4O3X, 4OXD, 4JD, and 4MPH, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.str.2014.04.015.
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Supplemental Information

Structure of the LdcB LD-carboxypeptidase Reveals the Molecular Basis of Peptidoglycan Recognition

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Fig. S1, related to Fig. 3. Comparison between apo and zinc-bound *B*αLdcB.

The active sites of zinc-bound (A) and apo (B) *B*αLdcB are shown in “stick” format, with a semi-transparent cartoon representation in the background. There are no major conformational changes in the absence of bound metal ion.
Fig. S2, related to Fig. 6. Proposed path of cross-linked cell wall peptides.
The terminal D-Asn of the ligand when bound to SpLdcB occupies the S_1' subsite rather than projecting to the surface of the protein. Simple rotation of the lysyl's torsion angles presents the terminal D-asparagine to the surface of the protein to mimic the L-Lys-D-Ala (or L-Lys-Gly_n) cross-link observed between stem peptides. The path of the lysine is flanked by two highly conserved amino acids in LdcB sequences, Arg120 and Ser117, in addition to the functionally conserved Leu128. Tyr132 also flanks the lysine, but this amino acid is not conserved between sequences.
**Supplementary Video**

**Video S1, related to Fig. 8. Conformational changes in SpLdcB on ligand binding.**

Morph interpolation (UCSF Chimera) of the structural changes that occur upon ligand binding in SpLdcB. α-helices are shown in red, β-strands in yellow and loops between secondary structure elements are coloured silver. The bound zinc ion is shown as a grey sphere. Key residues that move upon ligand binding are shown in stick representation and the movie was rendered in PyMOL.