Crystal Structure of a Peptidoglycan Synthesis Regulatory Factor (PBP3) from *Streptococcus pneumoniae*

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Penicillin-binding proteins (PBPs) are membrane-associated enzymes which perform critical functions in the bacterial cell division process. The single d-Ala,d-Ala (D,D)-carboxypeptidase in *Streptococcus pneumoniae*, PBP3, has been shown to play a key role in control of availability of the peptidoglycan substrate during cell growth. Here, we have biochemically characterized and solved the crystal structure of a soluble form of PBP3 to 2.8 Å resolution. PBP3 folds into an NH-terminal, d,d-carboxypeptidase-like domain, and a COOH-terminal, elongated β-rich region. The carboxypeptidase domain harbors the classic signature of the penicilloyl serine transferase superfamily, in that it contains a central, five-stranded antiparallel β-sheet surrounded by α-helices. As in other carboxypeptidases, which are present in species whose peptidoglycan stem peptide has a lysine residue at the third position, PBP3 has a 14-residue insertion at the level of its omega loop, a feature that distinguishes it from carboxypeptidases from bacteria whose peptidoglycan harbors a diaminopimelate moiety at this position. PBP3 performs substrate acylation in a highly efficient manner ($k_{cat}/K_m = 50,500 \text{ m}^{-1}\text{s}^{-1}$), an event that may be linked to its central role in control of pneumococcal peptidoglycan reticulation. A model that places PBP3 poised vertically on the bacterial membrane suggests that its COOH-terminal region could act as a pedestal, placing the active site in proximity to the peptidoglycan and allowing the protein to “skid” on the surface of the membrane, trimming pentapeptides during the cell growth and division processes.

Bacterial division is a complex phenomenon that requires the coordination of diverse processes including chromosomal segregation, FtsZ ring-dependent membrane constriction, and cell wall synthesis at the site of septation. The latter process involves the polymerization of glycan chains and transpeptidation of pentapeptidic moieties within the structure of the peptidoglycan, a highly cross-linked mesh that is crucial for maintaining bacterial shape and providing protection from osmotic shock and lysis (1). Both reactions are catalyzed by penicillin-binding proteins (PBPs), membrane-associated molecules, which can be classified as high molecular mass (hmm; often bifunctional) and low molecular mass (lmm; monofunctional) and play key roles in the bacterial life cycle. The pathogenic bacterium *Streptococcus pneumoniae* offers a unique opportunity for the study of the relationship between cell division and cell wall synthesis, since it carries a relatively simple set of six PBPs, compared with other well studied organisms which present much higher complexity (2). In this organism, PBP1a, -1b, and -2a catalyze both glycosyltransfer and transpeptidation; PBP2b and -2x only catalyze the latter reaction, and PBP3, the single lmm PBP in *S. pneumoniae*, has been shown to act as a d-Ala,d-Ala (D,D) carboxypeptidase (3).

The central role of hmm PBPs in the cell growth and division processes has been recently confirmed through the study of their localization within the cell cycle through the employment of immunofluorescence techniques (4). In *S. pneumoniae*, the constriction of the FtsZ-ring is spatially coupled to PBP2x- and PBP1a-mediated septal peptidoglycan synthesis, with the former process preceding the latter by approximately 5 min (4). At the beginning of the cell cycle, PBP3 localizes throughout the whole bacterial surface but seems to be absent from the future division site (5). Since the d,d-carboxypeptidase activity of PBP3 removes the COOH-terminal d-alanine of the peptidoglycan pentapeptide side chains, its hemispheric localization implies that the cellular region neighboring the future division site will be the only one where full-length pentapeptides will be available as substrates for other PBPs. Interestingly, a mutant pneumococcal strain which lacks PBP3 displays abnormal morphology and exhibits multiple septa initiated at aberrant locations (6). Thus, it is likely that the availability of intact pentapeptidic substrates dictates the localization of the hmm PBPs. Therefore, by guaranteeing that pentapeptides are available uniquely at the future division site, PBP3 may ensure the spatial coordination of the FtsZ-ring with the septum synthesis machinery.

PBP3 is associated to the bacterial membrane through a COOH-terminal amphiphilic helix. In the d,d-carboxypeptidase reaction catalyzed by PBP3, an active serine residue reacts with the d-Ala-d-Ala COOH terminus of a peptide chain of the peptidoglycan to form a transient acyl-enzyme complex that is subsequently hydrolyzed. The reaction results in the formation of a tetrapeptide that can only serve as an acceptor for a subsequent transpeptidation reaction by other PBPs (3). As in

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The atomic coordinates and structure factors (code 1XP4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡‡ The abbreviations used are: PBP, penicillin-binding protein; hmm, high molecular mass; lmm, low molecular mass; d,d, d-Ala,d-Ala; Ac,L-KAA, N,N-diacetyl-L-Lys-d-Ala-d-Ala; SeMet, selenomethionine; r.m.s., root mean square.
zymatic role of lmm PBPs in the cell division process. The functional homogeneity of the protein sample was determined by SDS-12% PAGE electrophoresis, and estimation of [3H]benzylpenicillin bound to proteins was monitored by two different procedures. The gel was stained with Coomassie Blue, destained, incubated with Amplify (Amersham Biosciences), dried, and either exposed to film for 16 h or cut around the protein bands. In the latter case, the gel slices were mixed with 5 ml of LSC mixture (Picofluor 15, Packard), and their contact the peptidoglycan layer throughout the two cellular
brane-interacting region may place it in optimal position to contact the peptidoglycan layer throughout the two cellular hemispheres.

MATERIALS AND METHODS

Measurement of Kinetic and Antibiotic Recognition Parameters—The construction of plasmid pGEX-sPPB3* encoding the soluble form of wild type PBPs, which lacks both the COOH-terminal helix and the signal peptide (sPPB3*), was described previously (5). d,d-Carboxypeptidase activity was assayed with N,N-diacyl-l-Lys or D-Ala-D-Ala (Ac2-KAA). sPPB3* (10 mM) was incubated at 37 °C in 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 0.5 mM bovine serum albumin, and Ac2-KAA at concentrations ranging from 37.5 to 24,000 μM. After various time intervals, the reaction was stopped by addition of penicillin G to 0.1 mM, and released D-Ala was measured by the method described by Johnson et al. (10).

The functional homogeneity of the protein sample was determined by titrating the active sites present in the preparation using [3H]benzyloacylpenicillin (20 Ci/mol, 1 μCi/ml; Amersham Biosciences) as a reporter. sPPB3* solutions at 2 and 5 μM were incubated for 15 min at 37 °C in 50 mM Tris-HCl (pH 8.0), 200 mM NaCl containing 0.01–20 μM [3H]benzyloacylpenicillin. The samples were subsequently submitted to SDS-12% PAGE electrophoresis, and estimation of [3H]benzyloacylpenicillin bound to proteins was monitored by two different procedures. The gel was stained with Coomassie Blue, destained, incubated with Amplify (Amersham Biosciences), dried, and either exposed to film for 16 h or cut around the protein bands. In the latter case, the gel slices were mixed with 5 ml of LSC mixture (Picofluor 15, Packard), and their radioactivity was measured using a liquid scintillation analyzer (Packard model 2100TR).

To analyze the kinetics of the deacylation reaction, 2 μM purified sPPB3* was labeled with 1 μM [3H]benzyloacylpenicillin at 37 °C during 15 min in 50 mM Tris-HCl (pH 8.0), 200 mM NaCl. Excess of cold benzylpenicillin (15 mM) was then added, and the reaction was continued at 37 °C. Aliquots were regularly removed, submitted to SDS-PAGE electrophoresis, and the amount of radioactivity was measured in the protein bands as mentioned above.

The ability of sPPB3* to hydrolyze the pseudo substrate N-benzoyl-d-alanylmercaptoacetic acid (S2d), which is a thioester analog of the stem wall peptide, was explored to generate a comparison profile of PBPs. Hydrolysis of S2d was followed by monitoring the amount of thiol hydrolysis rates for other, previously characterized pneumococcal PBPs: D,D-carboxypeptidase domain is highly reminiscent of that of a class A enzyme at 2.8 Å resolution. Although the general folds of pneumococcal PBP3 and solved the structure of a soluble form of the enzyme at 2.8 Å resolution. Although the general folds of wild type PBP5 were reported in wild type and mutant forms to high resolution (1.85 and 1.9 Å, respectively; Refs. 8 and 9). Wild type PBPs deacylates its acyl-enzyme complex at a very high rate, which is reminiscent of that of a class A β-lactamase (the latter with a poor substrate). Although these reports shed light on the enzymology of D,D-carboxypeptidation and the two-domain fold of the enzyme, several points still remain unclear, including the nature of the D,D-carboxypeptidase substrates and the enzymatic role of lmm PBPs in the cell division process.

In light of our previous reports on the localization of PBPs in the cell cycle (5) and in an effort to answer some of the questions above, we performed the enzymatic characterization of pneumococcal PBPs and solved the structure of a soluble form of the enzyme at 2.8 Å resolution. Although the general folds of the pneumococcal and E. coli enzymes are similar, PBPs harbor a significantly longer omega-like loop, a feature subsequently identified as a telltale motif in enzymes present in bacteria whose peptidoglycan structures contain an L-lysine group substituted sPBP3* was expressed in E. coli (11). The ability of sPBP3* to hydrolyze the pseudo substrate N-benzoyl-d-alanylmercaptoacetic acid (S2d), which is a thioester analog of the stem wall peptide, was explored to generate a comparison profile of PBPs. Hydrolysis of S2d was followed by monitoring the amount of thiol released using the method described by Zhao et al. (11). The ability of sPPB3* to hydrolyze the pseudo substrate N-benzoyl-d-alanylmercaptoacetic acid (S2d), which is a thioester analog of the stem wall peptide, was explored to generate a comparison profile of PBPs. Hydrolysis of S2d was followed by monitoring the amount of thiol released using the method described by Zhao et al. (11).

Crystallization and Structure Solution—Selenomethionine (SeMet)-substituted sPPB3* was expressed in E. coli B834. Cells were grown in LeMaster medium (12) containing 40 mg/l methionine that was progressively replaced by SeMet. Expression was induced at A600 ~ 0.3 and the purification of the protein was carried out as described previously (5), except that all of the buffers were supplemented with 10 mM dithiothreitol. Complete replacement of methionine residues by selenomethionine was confirmed by electrospray mass spectrometry. SeMet-labeled sPPB3* crystals were grown by hanging drop vapor diffusion using 1.5 μl of protein solution (4 mg/ml), 1.5 μl of well

### Table I

| Protein | kcat/|Km| Ref. |
|---------|-----|---|-----|
| sPPB3* | 5.7 | 3.38 | This work |
| PBP2x* | 3.5 | 5.80 | 26 |
| PBP1a* | 1.0 | 19.20 | 23 |
| PBP2a* | 3.2 | 6.00 | 24 |
| PBP1b* | 5.6 | 3.44 | 42 |
| SPBP5 (E. coli) | 78.0 | 0.25 | 9 |
| SPBP3* (E. coli) | 3.0 | 6.40 | |
| K15 (Streptomyces) | 10 | 1.92 | 43 |

### Table II

| Protein | kcat/|Km| Ref. |
|---------|-----|---|-----|
| sPPB3* | 50,500 ± 2500 | This work |
| PBP2x* | 2500 | 44 |
| PBP2b | 80 | 25 |
| PBP1a* | 256 | 23 |
| PBP2a* | 220 ± 20 | 24 |

### Table III

| Data collection, phasing, and refinement statistics |
|---------------------------------------------------|
| Cell dimensions (Å) | a = 87.57, b = 120.69, c = 176.92 |
| Space group | P212121 |
| Wavelength (Å) | 0.9792 |
| Resolution range (Å) | 2.8–2.8 |
| No. of unique/total reflections | 47,073/38,767 |
| completeness (%) | 99.9 (100) |
| Multiplicity | 5.4 (5.0) |
| Average Rmerge (%) | 10.2 (27.9) |
| Rfree (last shell) | 12.7 (4.7) |
| Rano (%) | 7.9 (16.0) |
| Resolution (Å) | 2.80 |
| Rwork/Rfree (%) | 21.2 / 26.2 |
| Number of residues | 369/350/368/477 |
| Number of water molecules | 477 |
| Number of iodines | 16 |
| Number of sulfates | 4 |
| Average B factor (Å²) | 29.51 |
| Protein | 33.30 |
| Solvent | 0.01 |
| r.m.s. bond deviation (Å) | 0.01 |
| r.m.s. angle deviation (°) | 2.00 |
The crystals belong to space group P2₁2₁2₁ with cell dimensions a = 87.57 Å, b = 120.69 Å, and c = 176.92 Å and have four molecules in the asymmetric unit. Prior to data collection, the crystals were cryoprotected by transfer into 20% (v/v) glycerol, 2% ethylene glycol, 0.2M K,Na-tartrate, 0.1M trisodium citrate (pH 5.6), and 1.9M (NH₄)₂SO₄.

Multiwavelength anomalous diffraction data were collected at 100 K and processed using MOSFLM (13) and CCP4 (14). Using the peak anomalous data, the sulfate atom (yellow and red) located in the active site of sPBP3* and the iodines (blue) that interact with the protein are represented. In this view, the cytoplasmic membrane is located at the bottom of the molecules, and the entrance of the active site is at the top.

The crystals belong to space group P2₁2₁2₁ with cell dimensions a = 87.57 Å, b = 120.69 Å, and c = 176.92 Å and have four molecules in the asymmetric unit. Prior to data collection, the crystals were cryoprotected by transfer into 20% (v/v) glycerol, 2% ethylene glycol, 0.2M K,Na-tartrate, 0.1M trisodium citrate (pH 5.6), and 1.9M (NH₄)₂SO₄.

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FIG. 1. Ribbon diagrams of sPBP3* and PBP5. sPBP3* from S. pneumoniae (A) and PBP5 from E. coli (B) have their NH₂-terminal domains (I) in cyan and COOH-terminal domains (II) in violet. The sulfate atom (yellow and red) located in the active site of sPBP3* and the iodines (blue) that interact with the protein are represented. In this view, the cytoplasmic membrane is located at the bottom of the molecules, and the entrance of the active site is at the top.

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which comprises residues 293–393 and is formed by a sandwich between two anti-parallel β-sheets. Comparison of the four molecules, which exist in the asymmetric unit, reveals that the greatest differences at the Cα level map to loop regions within the COOH-terminal domain, where the electron density is of poor quality in certain regions. Notably, the large area of interaction between the surfaces of domains I and II (∼800 Å², including six potential hydrogen bonds) guarantees stability for the full-length molecule, which is reflected by the slight variation (∼1°) of the angle between domains I and II in all four molecules of the asymmetric unit.

The sPBP3* Active Site—The sPBP3* active site is at the distal end from the COOH terminus of the molecule. As observed with the other carboxypeptidases and penicillin-metabolizing enzymes, the active site is mainly defined by three conserved structural motifs: SXXK (Ser56-Ile57-Thr58-Lys59), which includes the nucleophilic Ser56 residue, positioned at the NH₂-terminal end of helix α2; SXN (Ser119-Ala120-Asn121), which forms the turn between helices α4 and α5 on the left side of the cavity, and K(T/S)G (Lys239-Thr240-Gly241), which lines strand β3 (Fig. 2A). In addition, the backbone NH groups of the essential Ser26 and Thr242 residues occupy positions that are compatible with the oxyanion hole required for catalysis. The NH₂ terminus of helix α11 and the loop between α6 and β2d also contribute residues to the active site. These include Arg278, located at the right top angle of the cavity, Thr260, and the structural Gly161, present on the extended loop at the bottom of the cavity.

The hydrogen bonding network within the active site is extensive (Fig. 2A) and is identical in all four molecules in the asymmetric unit. The ε-NH₂ group of Lys59 plays a central role in this network, forming hydrogen bonds with the hydroxyl group of Ser56 and Ser119, the side chain carbonyl group of Asn121 and the backbone carbonyl group from Thr260. Two water molecules are observed within the hydrogen bonding network, one of which (O-26) is conserved in the K15 transpeptidase (29), as well as in E. coli PBP5 (8, 9). Although the architecture of the active site of the three enzymes is similar (compare Fig. 2, A–C), some significant differences can be observed in the orientation of three important catalytic residues: the side chains of Ser110, Lys213, and Thr214 of PBP5 are oriented differently from the equivalent residues in sPBP3*, Ser119, Lys239, and Thr240, respectively. In particular, in PBP5, Ser110 and Lys213 point away from the active site, and consequently the classical hydrogen bonding network within the active site is not formed in this molecule. It is of interest that all three molecules, which recognize peptidic substrates, harbor a conserved glycine residue at the bottom of the cleft (Gly161, Gly152, and Gly144 in sPBP3*, PBP5, and K15, respectively). The importance of the absence of a side chain at this position becomes evident if one considers that it is part of a binding pocket which could accommodate the penultimate
The overall structure of sPBP3* is highly reminiscent of that of PBP5 (9) (Fig. 3A). Domains I of the two structures superimpose with an r.m.s. deviation of 1.09 Å; the main differences include helix α2a, which is replaced by an irregular loop in PBP5, and the omega-like loop (residues 156–181 in sPBP3* and residues 147–158 in PBP5), which is longer in sPBP3* due to an insertion of 14 residues (yellow in Fig. 3A). A comparison of domain II for both molecules reveals greater differences including an r.m.s. deviation between Cα atoms of 2.83 Å. In sPBP3*, this region is clearly less compact than in PBP5, with shorter secondary structure elements.

A particularly outstanding feature of the sPBP3* structure is the close similarity of domain I with the general fold of the Streptomyces K15 transpeptidase (Ref. 29; Fig. 3B); this observation is surprising, since PBP3 catalyzes only carboxypeptidation, while K15 is a transpeptidase (and must first catalyze a carboxypeptidation of the terminal d-Ala residues before transpeptidating with an amino acceptor group). Domain I of sPBP3* can be superimposed onto the structure of the transpeptidase K15 (Ref. 29; Protein Data Bank code 1SKF) with an r.m.s. deviation of 1.54 Å. The same calculation performed with the structure of TEM-1 β-lactamase (Protein Data Bank code 1BTL) reveals a Cα rms deviation of 1.70 Å. As is the case for PBP5, both K15 and TEM-1 harbor shorter omega-like loops than sPBP3* (compare Fig. 3, A–C).

The secondary structural elements that harbor the conserved catalytic residues of the penicillin-binding domain of the transpeptidase PBP2x of S. pneumoniae (Ref. 28; Protein Data Bank code 1PM) PBP2a of Staphylococcus aureus (31; Protein Data Bank code 1MWR) and domain I of sPBP3* are similarly positioned (Fig. 3), with β3, α2, α4, and α5 playing important roles. However, outside of the active site, all of the other regions display large differences, as can be observed from the poor superposition results for these molecules (Fig. 3, D and E).

**The Omega-like Loop, a Key Structural Feature within d,d-Carboxypeptidases—**The omega-like loop of class A β-lactamases harbors residues that are required both for maintenance of active site topology and for enzymatic activity. The large structural deviations observed between sPBP3* and PBP5 at the level of the omega loop prompted us to explore this region within the sequences of other putative “PBP5-like” d,d-carboxypeptidase homologues in different bacterial species. Our genomic search focused on proteins with potential or demonstrated d,d-carboxypeptidase activity and for which a topology similar to sPBP3* or PBP5 was predicted, including the presence of a signal peptide, a penicillin-binding domain (analogous to domain I), a COOH-terminal extension of −100 residues (analogous to domain II), and an amphiphilic helix at the COOH terminus (Fig. 4). In addition, only carboxypeptidases harbored by bacteria whose cell wall composition is known were chosen for the study.

This primary sequence analysis revealed the existence of two groups of PBP5-like d,d-carboxypeptidases that could be differentiated based uniquely on the number of residues of their omega-like loops. Interestingly, this variation in length could be correlated to the chemical composition of the bacterial peptidoglycan. Indeed, PBP5-like d,d-carboxypeptidases from bacterial species whose peptidoglycan harbors stem peptides with a diaminopimelate moiety in the third position, such as PBP5 from E. coli or the PBP from B. subtilis, are characterized by a short omega-like loop. On the other hand, the primary sequences of PBP5-like d,d-carboxypeptidases from species whose peptidoglycan possesses stem peptides with a lysine residue in the third position, such as PBP3 from S. pneumoniae, display a much longer omega-like loop (in this case, corresponding to an insertion of 14 residues). Interestingly, the
same carboxypeptidases that carry the longer omega-like loops also harbor a 6-amino acid insertion downstream from the third catalytic motif (KTG; see Fig. 4), which, in the tertiary fold, is in proximity to the omega loop. It is thus conceivable that such structural characteristics provide differential substrate recognition within the peptidoglycan biosynthetic machinery.

**DISCUSSION**

Lmm PBPs are membrane-associated enzymes that play important roles in the maintenance of cell shape and in cellular growth and division processes (5, 6, 32–34). Here, we have biochemically and structurally characterized a soluble form of PBP3 from the human pathogen *S. pneumoniae*. This work has confirmed that the single Lmm PBP from *S. pneumoniae* catalyzes a D,D-carboxypeptidation reaction, although its catalytic domain is highly reminiscent of the structure of the K15 transpeptidase from *Streptomyces*. In addition, sPBP3* is also able to recognize β-lactam antibiotics, as expected from the presence of the three conserved penicilloyl serine transferase superfamily motifs present in domain I (SXN, SXK, K/T/S/G). Notably, both D,D-carboxypeptidase activity and β-lactam processing proceed through the formation of an acyl-enzyme intermediate followed by deacylation of the enzyme. Based on these data as well as structural and mechanistic information that is available for other PBPs and β-lactam-recognizing enzymes (8, 9, 22–31, 36), we propose that the acylation mechanism of sPBP3* may occur in four steps. Initially, there is formation of a non-covalent complex that may be stabilized by interactions between the carboxylate group of the ligand and conserved side chains of Thr240 and Arg278. Backbone nitrogen atoms of Thr242 and Ser56 could play the role of the oxanion hole required for stabilization of the intermediate reactive species. Ser 56 is activated to form the ester bond with the substrate through abstraction of a proton from neighboring Ser119 by the COOH of the ligand. This is followed by protonation of the nitrogen of the β-lactam ring by the carboxylate and subsequently ring breakage (35). An alternative concerted mechanism is possible, whereby the hydrogen from Ser119 is transferred to the nitrogen of the β-lactam ring at the same time as the hydrogen of Ser56 is transferred to Ser119, and the former forms the acyl bond with the antibiotic (37).

sPBP3* is exceptionally efficient in hydrolyzing the pseudo substrate S2d, over 20 times more active than the most efficient pneumococcal enzyme measured to date, PRP2x (Table II). In addition, the $k_{\text{cat}}/K_m$ value measured with the synthetic peptide substrate $N,N$-diacetyl-l-lys-d-Ala-d-Ala (5689 s$^{-1}$mM$^{-1}$) is 180-fold.
greater than the value measured for PBP5 with the same substrate (32 m⁻³s⁻¹; (9)), indicating a greater catalytic efficiency for the pneumococcal enzyme. This observation suggests that PBP3 plays an important role in control of peptidoglycan reticulation in *S. pneumoniae*. Indeed, Gram-positive organisms have several layers of peptidoglycan, while Gram-negative bacteria, such as *E. coli*, have much smaller amounts. In addition, *E. coli* possesses at least three D,D-carboxypeptidases (PBP5, PBP6, PBP6b), and although the precise function of each one in the cell cycle is unknown, it is conceivable that they could share peptidoglycan processing duties within the Gram-negative cell wall. Hence, it is not surprising that PBP3, the only D,D-carboxypeptidase in *S. pneumoniae*, must be an enzyme with a very high catalytic activity, since it must limit the amount of pentapeptidic stem peptides in the peptidoglycan throughout the entire bacterial cell surface, with the exception of the division site, as shown by immunofluorescence localization studies (5).

In the structures of class A β-lactamases, the omega-like loop is a 19-residue stretch comprised of amino acids which participate both in catalysis and maintenance of local topology. Insertional mutagenesis studies showed that enzymes with larger omega-like loops were still able to perform catalysis, and in addition, displayed expanded substrate specificities (38). In this study, we have identified that organisms which display a lysine residue as the third component of the peptidoglycan pentapeptide harbor D,D-carboxypeptidases with large omega-like loops as well as an insertion of six amino acids ~30 residues downstream from the catalytic KTG motif. Conversely, bacteria whose peptidoglycan displays a diaminopimelate residue at the same position carry D,D-carboxypeptidases with short omega loops (and lack the 6-residue insertion). The insertion of the amino acid at the third position in the stem peptide is catalyzed by MurE ligases, which show high specificity for their respective substrates (39); notably, if the incorrect residue is inserted at this position, one of the subsequent reactions, transpeptidation, does not take place (40). Thus, it is conceivable that, much like the transpeptidase enzymes that will not bind the incorrect amino acid at the third position of the stem peptide, D,D-carboxypeptidases also require specificity at this position for catalysis. Considering that the carboxypeptidation reaction may require the recognition of a large portion of the stem peptide, including the third moiety and maybe beyond, it is conceivable that the binding region generated by the omega-like loop, and the 6-residue insertion may be able to participate in substrate discrimination. Hence, carboxypeptidases involved in peptidoglycan metabolism may have evolved to perform optimal recognition of a large portion of the stem peptide.

**Interaction with and Accessibility to Peptidic Substrates**—Although lipid II, the natural substrate for PBP enzymes, carries only pentapeptides in its stem moiety, it is well documented that the pre-existent (available) murein can also harbor multimeric peptides (41). Although the reaction of D,D-carboxypeptidation of stem pentapeptides has been suggested as being necessary to regulate the degree of peptidoglycan reticulation, it is still unclear whether carboxypeptidases are able to interact with more complex substrates, i.e., with reticulated peptidic molecules. The close similarity between the general folds as well as the active sites of K15 and sPBP3* suggests that it may be the case. The fact that the K15 active site cleft must harbor two peptidic chains to catalyze the transpeptidation reaction suggests that the highly similar sPBP3* may be able to recognize stem peptides that are more complex than the regular pentapeptide. This idea is in agreement with the observation that PBP3 is present throughout the entire bacterial cell surface, except at the site where hmm PBPs are posi-

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