Expression and Characterization of the Isolated Glycosyltransferase Module of *Escherichia coli* PBP1b[†]

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ABSTRACT: The enzymes involved in the biosynthesis of peptidoglycan are targets for the development of new antibiotics. The bifunctional high molecular weight (HMW) penicillin-binding proteins (PBPs), which contain both glycosyltransferase (GTase) and transpeptidase (TPase) activities, are particularly attractive targets because of their extracellular location. However, there is limited mechanistic or structural information about the GTase modules of these enzymes. In this paper, we describe the overexpression and characterization of the GTase module of *Escherichia coli* PBP1b, a paradigm of the HMW PBPs. We define the C-terminal boundary of the GTase module and show that the isolated module can be overexpressed at significantly higher levels than the full-length protein. The catalytic efficiency and other characteristics of the isolated module are comparable in most respects to the full-length enzyme. This work lays the groundwork for mechanistic and structural analysis of GTase modules.

The increasing frequency of resistance to existing antibiotics is a serious problem. Structural and mechanistic information on essential bacterial enzymes could lead to the development of antibiotics that are active against resistant microorganisms. Both Gram-positive and Gram-negative bacterial cells are surrounded by layers of peptidoglycan, a cross-linked carbohydrate polymer that prevents the cells from rupturing under high internal osmotic pressures (*1*). Many antibiotics function by inhibiting peptidoglycan synthesis (*2*), and intense effort has been focused recently on obtaining structures for the enzymes that synthesize peptidoglycan because each is a potential target for the design of new antibiotics (*3*).

Peptidoglycan is synthesized in a three-stage process (Figure 1) (*4*). The first stage takes place in the cytoplasm and involves the synthesis of UDP-*N*-acetylmuramic acid (UDP-MurNAc) peptide from UDP-*N*-acetylglucosamine (UDP-GlcNAc). The second stage takes place on the cytoplasmic surface of the bacterial membrane and starts with the transfer of phospho-MurNAc peptide to undecaprenyl phosphate in the bacterial membrane to form a lipid-anchored monosaccharide known as Lipid I. A glycosyltransferase (GTase),[1] MurG, then transfers GlcNAc from UDP-GlcNAc to the C4 sugar hydroxyl of Lipid I to form a lipid-anchored β-(1,4)-linked GlcNAc-MurNAc peptide known as Lipid II. This disaccharide peptide unit is the basic building block of peptidoglycan. Once formed, the third stage of peptidoglycan biosynthesis begins with the translocation of the disaccharide portion of Lipid II across the membrane, where it is polymerized to form alternating β-linked GlcNAc-MurNAc polysaccharide chains, which are then cross-linked through peptide chains on alternating glycan strands. It is believed that membrane-associated, bifunctional enzymes containing both GTase and transpeptidase (TPase) activities are responsible for the majority of peptidoglycan synthesis (*5*, *6*). Because these bifunctional enzymes were first identified based on the ability of the TPase domains to react with penicillin, they are called penicillin-binding proteins (PBPs). Bacterial cells typically contain several high molecular weight (HMW) PBPs, some of which contain active GTase domains and some of which appear to contain vestigial GTase domains in addition to the TPase domains (*7*, *8*). There are also several low molecular weight PBPs containing only endopeptidase or carboxypeptidase activities (*9*). In addition, some bacterial strains have been found to contain monofunctional glycosyltransferases (MGTs) that do not have TPase domains (*10*−*12*). The cellular roles of these monofunctional enzymes are not understood.

In the last 10−15 years, an enormous amount of information has been learned about the individual enzymes involved in peptidoglycan biosynthesis. Crystal structures exist for all of the enzymes involved in the first stage of peptidoglycan biosynthesis (MurA, MurB, MurC, MurD, MurE, MurF, and D-Ala-D-Ala ligase) (*13*−*18*), for MurG (*19*) and for several different D-Ala-D-Ala carboxypeptidases or TPases (*20*−*22*). There are also crystal structures of two nonclassical low molecular weight PBPs, which share homology to β-lactamases (*23*, *24*). Finally, there are structures for two HMW PBPs that contain what appear to be vestigial GTase domains (PBP2x from *Streptococcus pneumoniae* R6 and PBP2a from methicillin-resistant *Staphylococcus aureus*) (*25*−*28*). The functions of these vestigial GTase domains are not known, and the sequence homologies between these domains and active GTase domains are so low that structural relationships are difficult to determine.

There remain two groups of enzymes in the pathway to peptidoglycan for which no structural information exists. These enzymes include MraY, the translocase that catalyzes

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[1] Abbreviations: HMW, high molecular weight; MGT, monofunctional transglycosylase; PBP, penicillin-binding protein; GTase, glycosyltransferase; TPase, transpeptidase.

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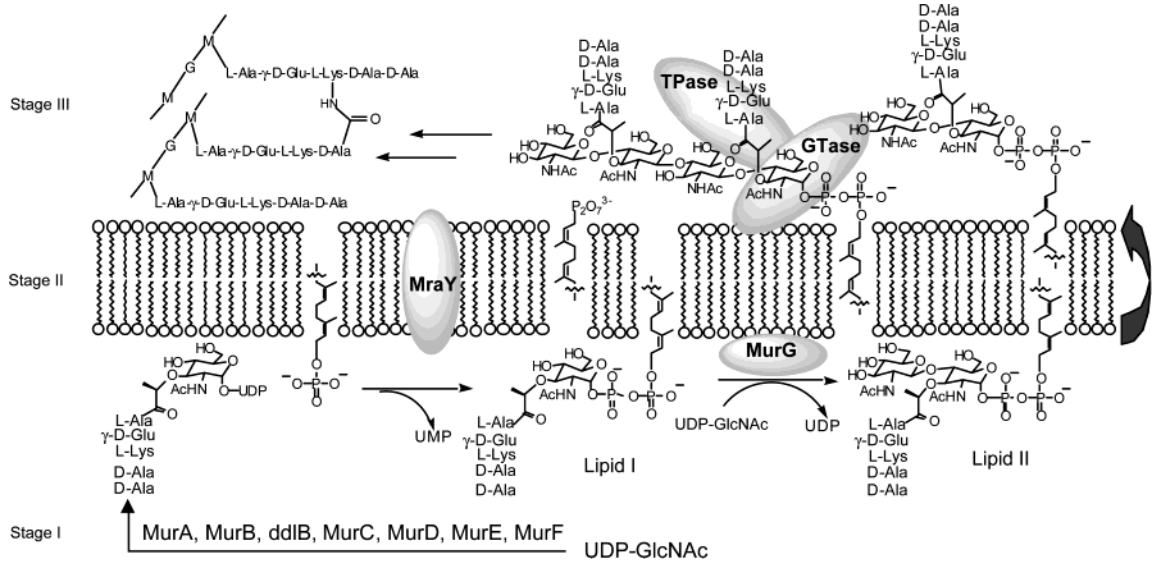

FIGURE 1: Diagram depicting the three stages of peptidoglycan biosynthesis. GTase and TPase represent the GTase module and TPase module, respectively, of HMW PBPs.

the attachment of phospho-MurNAc pentapeptide to its lipid anchor in the bacterial membrane, and the bacterial GTases that polymerize Lipid II to form the glycan chains of the peptidoglycan polymer. MraY poses significant challenges for structural and mechanistic analysis because it contains several membrane-spanning domains (*29*). The glycan-synthesizing GTases are predicted to contain only a single transmembrane helix, but they have also proven difficult to study, albeit for other reasons. The single biggest reason is that the substrate for these enzymes, Lipid II, is difficult to isolate from natural sources because of its low abundance. It is also difficult to handle because it contains a 55-carbon undecaprenyl chain, the lipid anchor, which renders it insoluble in water. In recent years, we (*30*) and others (*31*, *32*) have developed chemical approaches to the synthesis of Lipid II and analogues to overcome the substrate limitations impeding the study of bacterial GTases. Breukink and co-workers have developed a complementary biological method to produce Lipid II using bacterial membranes supplemented with appropriate enzymes and starting materials (*33*). We have compared the relative rates of reaction of natural Lipid II and analogues containing modified lipid chains and have identified a heptaprenyl Lipid II analogue as the optimal substrate for monitoring peptidoglycan synthesis activity in vitro (*30*). Access to Lipid II analogues now makes it possible to monitor the enzymatic activity of various GTase modules, and it is hoped that a better understanding of the biochemical behavior of these enzymes will lead to a structure.

Here, we report studies characterizing the activity of the isolated GTase domain of *Escherichia coli* PBP1b. Although the existence of MGTs suggests that GTase domain activity does not depend on TPase activity, no MGTs have been kinetically characterized (*10*−*12*). Furthermore, there are questions about the functional similarity of these enzymes to the GTase domains found in HMW PBPs. For example, it has been reported that the MGT from *E. coli* is not inhibited by moenomycin, a well-known inhibitor of the GTase domains in HMW PBPs (*10*). In addition, for reasons that are not yet clear, it has proven difficult to overexpress active MGTs at high levels (*10*, *11*). There have been other reported attempts to make truncated GTases from HMW PBPs to address the behavior of the isolated domains, but the resulting constructs were unstable and the proteins were not characterized (*34*). Below, we define the C-terminal boundary of the GTase domain of *E. coli* PBP1b and show that the truncated GTase module is both active and stable, although its activity differs in key ways from that of the full-length protein. Notably, the activity of the isolated GTase domain is not enhanced by the addition of micelle-forming detergent. We note that it is possible to predict the C-terminal boundaries of the GTase domains of other HMW PBPs based on the C-terminal boundary of *E. coli* PBP1b. Access to isolated GTase modules that can be overexpressed in large amounts should facilitate structural analysis of this important class of peptidoglycan-synthesizing enzymes.

MATERIALS AND METHODS

Materials. *E. coli* PBP1b, expressed from pET21b:ponB, a pET21b plasmid encoding the *ponB* gene from *E. coli*, was partially purified as previously described (*35*) and stored as a 50% glycerol stock at −78 °C. The C35−Lipid I analogue was prepared as previously described (*30*), converted to the corresponding Lipid II analogue using *E. coli* MurG, and stored in dimethylsulfoxide (DMSO) at −20 °C.

Plasmid Construction. Truncated variants were constructed by PCR amplification of pET21b:ponB. All constructs are under T7 promoter control and include an ampicillin-resistant gene. The first construct (L433a), encoding the C-terminal truncated variant at leucine 433 with no tags, was made by PCR amplification of pET21b:ponB using primers NponBpet21b and CponBL433a (Table 1). The PCR product was ligated into pET21b (Novagen) between the *Nde*I and *Xho*I restriction sites. Similarly, L433b, S409, and I385 were made by PCR amplification of pET21b:ponB using the appropriate primers (Table 1). The products were then digested with *Nco*I and *Bam*HI and ligated into pET32b vectors, each containing an N-terminal thioredoxin tag (Trx tag), S tag, and His_6 tag. Constructs S409 and I385 also have a C-terminal His_6 tag. The gene sequences were verified by MWG Biotech Inc.

Protein Expression and Purification. The plasmids were transformed into Rosetta-gami(DE3) (Novagen) for protein

Table 1: Primers Used To Make Plasmids for the Truncated Enzymes

construct (amino acids)	primer	primer sequence
L433a	NponBpet21b	5′-gagcatgtcccatggccgggaatgaccgc-3′
(1−433)	CponBL433a	5′-cactgcagtctcgagtcagagatcttttaccttatcgcc-3′
L433b	NponBpet32b	5′-gagcatgtcccatggccgggaatgaccgc-3′
(1−433)	CponBL433b	5′-cactgcagtggatcctcagagatcttttaccttatcgcc-3′
S409	NponBpet32b	5′-gagcatgtcccatggccgggaatgaccgc-3′
(1−409)	CponBS409	5′-cactgcagtggatccggagagatcaccccaccg-3′
I385	NponBpet32b	5′-gagcatgtcccatggccgggaatgaccgc-3′
(1−385)	CponBI385	5′-cactgcagtggatccaatctgttgctgttgcagcag-3′

expression except for S409 and I385, which were transformed into Origami(DE3) (Novagen). Agar plates and liquid cultures for cloning and protein expression consisted of Luria Bertani (LB) media containing 50 μg/mL carbenicillin. Bacterial cultures were grown at 37 °C to an OD_{600} of 0.6, cooled on ice, and induced by adding 1 mM isopropyl-β-D-thiogalatoside. After 14 h at 18 °C, the cells were pelleted and stored at −78 °C. For a typical purification, thawed pellets were resuspended in buffer A (20 mM Tris-HCl, 150 mM NaCl, and 1.5% CHAPS at pH 7.4), lysed by sonication, and centrifuged at 39000*g* for 20 min. The supernatant was then filtered through a 0.45-μm membrane and loaded onto a His-tag column, and the protein was purified by Ni^{2+}-charged IDA agarose (Novagen) chromatography. The column was washed with 10 column volumes of buffer B (20 mM Tris-HCl, 500 mM NaCl, and 1.5% CHAPS at pH 7.9) containing 5 mM imidazole, 6 column volumes of buffer B containing 60 mM imidazole, and 6 column volumes of buffer B containing 100 mM imidazole. The protein was eluted with buffer B containing 200−500 mM imidazole and then dialyzed overnight against detergent-free buffer A (20 mM Tris-HCl and 150 mM NaCl at pH 7.4).

N-Terminal tags were removed by cleavage with enterokinase. Cleaved proteins were purified using the S-tag Purification Kit (Novagen). Lysis of the cells was similar to the above-mentioned procedure, except that the filtered supernatant was incubated with S-protein agarose at room temperature for 30 min on an orbital shaker. The mixture was then centrifuged at 500*g* for 10 min, and the supernatant was decanted. Resuspending the resin in buffer A and repeating the centrifugation washed unbound protein away. The protein was then released from the S-agarose by overnight incubation at room temperature with recombinant enterokinase, rEK (Novagen). The protease was removed by incubation with Ekapture agarose (Novagen). The isolated GTase modules were concentrated on Ultra-15 membranes (30-kDa molecular mass cutoff; Amicon), flash-frozen, and stored in 50% glycerol at −78 °C. Protein concentrations were measured using the modified Lowry Assay (BioRad).

Measurement of Decyl PEG Dependence. The assay for the full-length PBP1b enzyme was performed as previously described in reference *35*. Reactions (10 μL each) were carried out in Eppendorf tubes containing 4 μM [^{14}C]-GlcNAc-labeled Lipid II analogue, 1000 units/mL penicillin G, buffer C [50 mM Hepes (pH 7.5), 10 mM $CaCl_2$, and 11% DMSO] and 0, 0.2, 0.4, and 1 mM octaethylene glycol monodecyl ether (decyl PEG). The reactions were initiated by adding 1 μL of enzyme and quenched with 10 μL of ice-cold buffer D [10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.2% Triton X-100]. Reactions were left on ice until they were spotted on 3MM Whatman chromatography paper. Products were separated using chromatography (isobutyric acid/1 M NH_4OH, 5:3) and quantitated by scintillation counting. The assays for the truncated PBP1b variants were performed as previously described for *E. coli* PBP1b, except penicillin G was not added. The reactions were initiated by adding 1−2 μL of enzyme, corresponding to a final enzyme concentration of 70 nM, quenched, and analyzed as previously described.

Measurement of Metal Ion Dependence. Reactions (10 μL each) were carried out in Eppendorf tubes containing 4 μM [^{14}C]GlcNAc-labeled Lipid II analogue, buffer C, 0 or 10 mM concentrations of $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$, and 17.3 mM KCl. The reactions were initiated by adding 1−2 μL of enzyme and quenched after 20 min. The values for k_{rel} were obtained by dividing the values for the rate of formation of peptidoglycan by the rate with no metal ion present in the reaction buffer for L433b and S409.

Measurement of Time Course for Truncated Variant Catalyzed Reactions. Reactions (70 μL each) were carried out in Eppendorf tubes containing 4 μM [^{14}C]GlcNAc-labeled Lipid II analogue in buffer C and initiated by adding 1−2 μL of enzyme. Aliquots (10 μL) of the reaction mixtures were quenched with an equal volume of ice-cold buffer D at 5, 10, 15, 20, 30, 40, and 60 min. The conversion of the Lipid II analogue was plotted against the reaction time, and the linear range was determined for subsequent kinetic analysis.

Kinetic Analysis of Peptidoglycan Formation by the Truncated Variants. Reactions (10 μL each) were carried out in Eppendorf tubes containing 1−2 μL of enzyme, buffer C, and varying amounts of [^{14}C]GlcNAc-labeled Lipid II analogue ranging from 0.2 to 15 μM (specific concentrations are indicated in the figure captions). The reactions were initiated by adding enzyme and quenched after 20 min, in the linear region of the reaction time course.

Moenomycin Titrations. Reactions (10 μL each) were carried out in Eppendorf tubes containing 4 μM [^{14}C]-GlcNAc-labeled Lipid II analogue, buffer C, and either 0, 10, 20, 30, 40, 60, 80, 100, or 200 nM concentrations of moenomycin A. The reactions were initiated by adding 1−2 μL of enzyme and quenched after 20 min. The conversion of the Lipid II analogue was plotted against the moenomycin A concentration. The enzyme concentration was determined as the moenomycin A concentration at which complete inhibition occurred.

RESULTS

Design of Truncated PBP1b Variants. Our goals were to establish the C-terminal boundary of the GTase domain and to determine to what extent the presence of the TPase domain

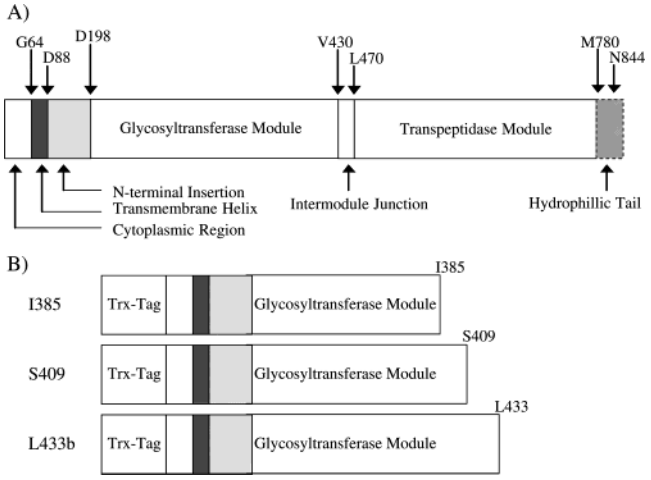

FIGURE 2: (A) Schematic diagram depicting the proposed topology of PBP1b and its modules. The residues are numbered according to the sequence for *E. coli* PBP1b K12 and shown above the figure. (B) Truncated forms of the enzyme (described in the text) and plasmid names indicated at the left of each diagram. The C-terminal amino acids are shown.

influences enzymatic activity. *E. coli* PBP1b, which is encoded by the *ponB* (or *mrcB*) gene, contains 844 amino acids (94 kDa). The proposed topology of *E. coli* PBP1b (Figure 2A) consists of a cytoplasmic tail, a hydrophobic transmembrane helix, and a periplasmic module with an N-terminal GTase domain and a C-terminal TPase domain (*36*). Sequence alignments reveal five conserved motifs that are characteristic of all functional GTases (*7*). On the basis of sequence alignments by Goffin et al. and alanine-stretch-scanning mutagenesis by Masson et al., the junction between the GTase and TPase domains was proposed to be located between V430 and L470. However, Zijderveld et al. made a hybrid protein containing the GTase domain of *E. coli* PBP1b and the TPase module of *E. coli* PBP3 (*37*). This hybrid protein, which contained only the first 423 amino acids of *E. coli* PBP1b, was shown to have GTase activity, suggesting that the C-terminal boundary is before the proposed junction. Unfortunately, efforts to isolate and characterize a GTase domain terminating at amino acid 423 were not successful because of proteolytic instability of the protein (*34*).

To establish the C-terminal boundary of *E. coli* PBP1b, we prepared truncation mutants terminating at amino acids 433, 409, and 385 (Figure 2B). These truncation sites were selected based on predicted secondary structure alignments of a set of GTases, including an MGT from *E. coli* (Figure 3). Truncations were made in predicted coil regions in an effort to minimize effects on protein folding. The L433 truncation lies near the start of the proposed junction between the GTase and TPase modules; the S409 truncation lies in a predicted coil region just beyond the aligned C terminus of the GTase module of *E. coli* MGT; and I385 lies inside the aligned C-terminal boundary of MGT.

The truncated GTase modules were constructed by PCR amplification of the parent *ponB* gene and expressed from pET21b (L433a). Although the expression of L433a was better than that of the full-length enzyme, all of the protein pelleted with the insoluble fraction even in the presence of detergent (data not shown). We tested a pET32b construct in which the N terminus of the truncated protein was fused to a thioredoxin tag. The pET32b:L433b variant showed improved solubility as well as significantly improved expression (Figure 4) over full-length PBP1b, with a yield in the soluble fraction estimated to be greater than 30 mg/L. Therefore, the shorter truncated proteins, S409 and I385, were also expressed as fusions. The recombinant proteins were purified to >85% purity using a Ni^{2+}-IDA column. For some experiments, the N-terminal tags were removed by treatment with enterokinase, while the fusion proteins were bound to S-agarose beads, resulting in the release of soluble truncated enzymes into the supernatant. The truncated enzymes were then assayed in the GTase assay to determine if they retained their activity.

Influence of Detergent on Enzymatic Activity. Schwartz and co-workers reported in 2002 that decyl PEG is required for the enzymatic activity of full-length *E. coli* PBP1b and that there is an optimal concentration beyond which inhibition is observed (*38*). We have also found that the addition of decyl PEG increases the enzymatic activity of *E. coli* PBP1b (*35*). The activity increases by a factor of at least 5-fold at the optimal decyl PEG concentration, which under our assay conditions is 0.2 mM or about 2 times the critical micelle concentration (Figure 5). We investigated the influence of decyl PEG on the enzymatic activities of L433b and S409 as a prelude to kinetic analysis of these truncated enzymes. In preliminary experiments, we compared the activity of full-length PBP1b, L433b, and S409 under identical reaction conditions but using different concentrations of decyl PEG. As shown in Figure 5, decyl PEG does not enhance the activity of the truncated proteins as it does for the full-length protein. In fact, it has an inhibitory effect, and therefore we omitted it from subsequent reactions involving the truncated proteins.

Kinetic Parameters. Preliminary experiments were carried out to establish conditions for evaluating the kinetic parameters of the truncated enzymes. In these experiments, I385 was found to be completely inactive, which indicates that the C-terminal boundary of the GTase module is located beyond amino acid 385. For the other proteins, reaction rates were found to be linear with time, up to relatively high conversions (greater than 50%). Although Schwartz and co-workers reported a significant lag phase with full-length PBP1b (*38*), we have found that full-length PBP1b stored in 50% glycerol does not display lag phase kinetics. Furthermore, we did not observe a lag phase with L433b or S409 that had been stored in glycerol.

Reaction rates were measured as a function of the C35−Lipid II concentration and analyzed to determine kinetic parameters. The concentration of the active enzyme was determined by titration with moenomycin, an antibiotic that binds extremely tight to the GTase module and is assumed to inhibit the enzyme in a stoichiometric manner. Both L433b and S409 could be completely inhibited by moenomycin, and we used the moeonomycin concentrations at which full inhibition was achieved as a measure of the active enzyme. The total protein concentration, estimated by colorimetric methods, was approximately 10-fold higher than the active (i.e., inhibited by moenomycin A) enzyme concentration. These results are comparable to those obtained with full-length PBP1b.

The results of a Michaelis−Menten analysis of S409 and L433b (Table 2) showed that the GTase activity is modestly affected by the removal of the TPase module. The catalytic

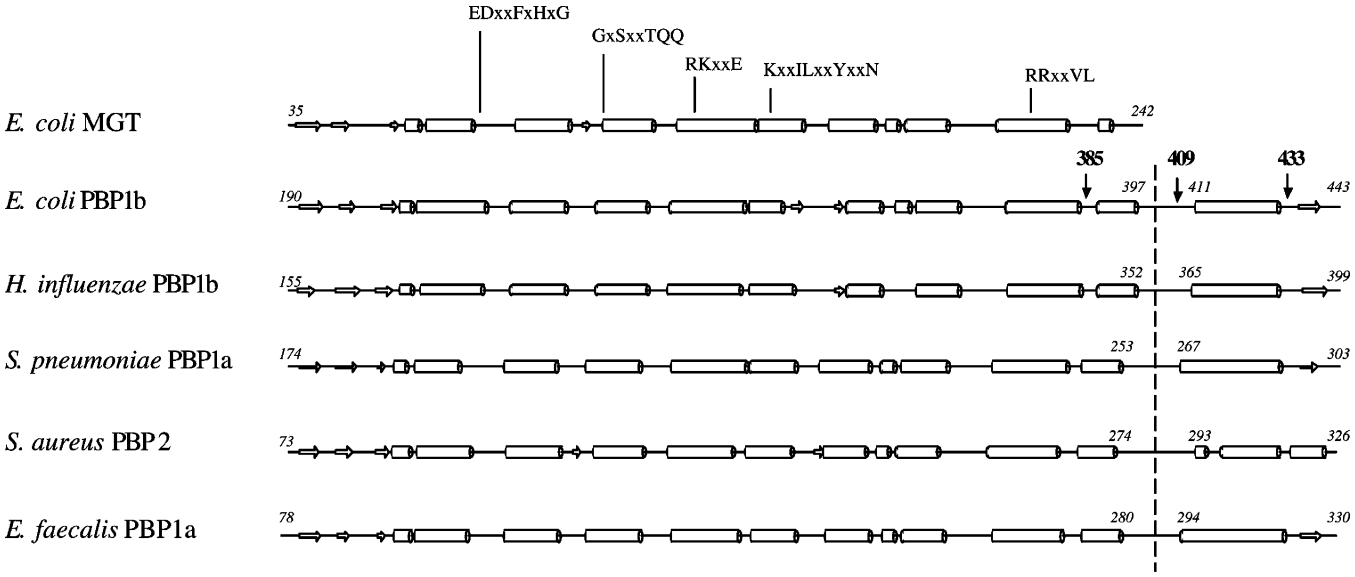

FIGURE 3: Predicted secondary structures from a range of HMW PBPs were aligned using the MGT from *E. coli* as a paradigm. Secondary structure predictions were generated using PSIPRED and aligned manually by the conserved sequence motifs. The dashed line indicates the region of the proposed C-terminal boundary of the GTase module. The arrows point to the sites of truncation in *E. coli* PBP1b (see the text for details). Amino acid positions in each protein are indicated in italics.

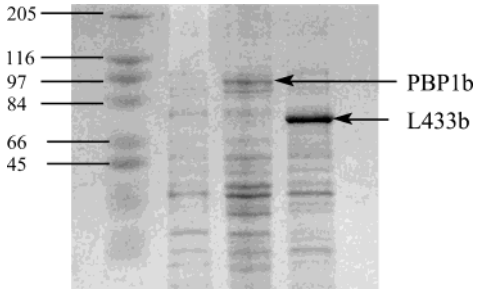

FIGURE 4: Expression levels of full-length PBP1b (lane 3) with L433b (lane 4) relative to the uninduced sample (lane 2) are shown on an SDS−PAGE 10% Tris-HCl gel. Cells were grown to an OD_{600} of 0.6 before being induced with 1 mM IPTG for 14 h at 14 °C. The cells for each sample were lysed in 3 mL of buffer A (see the Materials and Methods) and centrifuged at 39000*g*, and the pellet was resuspended in 3 mL of buffer A with 0.5% Sarkosyl. The mixture was then recentrifuged, and the supernatant was loaded onto the gel. The samples were normalized by their OD_{600} relative to the uninduced sample.

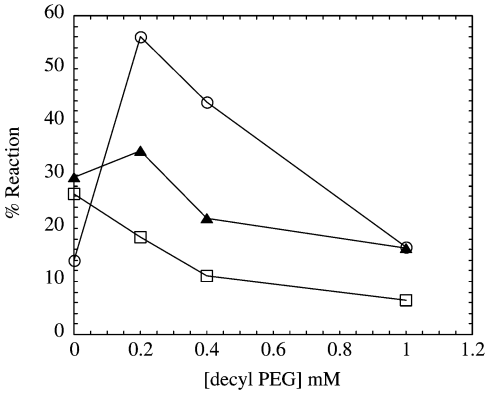

FIGURE 5: Decyl PEG dependence of full-length PBP1b (○), L433b (▲), and S409 (□).

efficiencies of L433b and S409 were comparable to each other and approximately 4−5-fold lower than that of the full-length protein. This difference in enzymatic activity was not due to the presence of the N-terminal Trx tag because enterokinase cleavage of the tag yielded tag-free modules

Table 2: Kinetic Parameters for the Truncated GTase Variants

constructs	Trx tag	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
L433b	absent	2.6	1.2	0.43
	present	1.1	0.46	0.42
S409	absent	0.87	0.50	0.57
	present	2.3	1.0	0.4
full-length PBP1b[a]	absent	2.0	0.43	2.1

[a] See ref *35*.

with catalytic efficiencies similar to the parent fusion proteins. The difference in catalytic efficiency between the full-length protein and the truncated proteins is mainly due to a reduction in k_{cat}, which suggests that the presence of the TPase module, while not required for activity, nevertheless influences the turnover rate. Polymerization of Lipid II to form the glycan chains of peptidoglycan occurs before crosslinking, and the TPase module apparently can only cross link oligosaccharide chains and not disaccharide starting material (*39*). Perhaps intermodular transfer of the growing glycan chain to the TPase module helps free the GTase active site for incoming substrate.

Metal Ion Dependence. Schwartz and co-workers have reported that *E. coli* PBP1b activity depends on divalent, oxophilic cations, with a 6-fold preference for Ca^{2+} over Mg^{2+} (*38*). We evaluated the activity of the truncated proteins in the presence of several different metal ions. The results show that L433b and S409 behave similarly to the full-length enzyme in the presence of divalent cations (Table 3). Thus, cations such as Ca^{2+}, Mn^{2+}, and Mg^{2+} enhance enzymatic activity up to 9-fold relative to the activity in the presence of no added metal ions. This improvement in activity is not due to ionic strength because the addition of K^+ at a similar ionic strength did not significantly affect the reaction rate. Furthermore, the activating effects are limited to a subset of divalent cations, suggesting specific requirements for coordination.

It has been proposed that Ca^{2+} plays a key role in the catalytic mechanism by chelating the diphosphate and

Table 3: Metal Ion Dependence of the Truncated Enzymes

metal ion[a]	L433b		S409	
	rate (μM min^{-1})	k_{rel}[b]	rate (min^{-1})	k_{rel}[b]
Ca^{2+}	0.11	11.0	0.11	11.0
Mg^{2+}	0.03	3.0	0.03	3.0
Mn^{2+}	0.05	5.0	0.07	7.0
Zn^{+}	0.02	2.0	0.01	1.0
K^{+}	0.02	2.0	0.01	1.0
none	0.01	1.0	0.01	1.0

[a] Metal ions were present as chloride salts at 10 mM concentration, except for K^{+}, which was present at 17.3 mM. [b] The values for k_{rel} were obtained by dividing the values for the rate of formation of peptidoglycan by the rate with no metal ion present in the reaction buffer.

assisting it to leave. There is precedence for the involvement of divalent cations in the mechanism of other types of GTases. For example, in many families of nucleotide-GTases, active-site-bound divalent cations such as Mg^{2+} or Mn^{2+} play an important role in substrate binding by coordinating the diphosphate group of the nucleotide-sugar substrate (*40*). Ca^{2+} does not normally play this type of role, and Ca^{2+} levels in cells are tightly regulated. It has been reported, however, that calcium accumulates in the periplasmic space, raising the possibility that calcium plays a special role in the mechanism of peptidoglycan-synthesizing GTases (*41*). However, divalent cations may also function to stabilize the folded protein, as has been postulated for a lytic transglycosylase (GTase), Slt35, which was crystallized with a bound Ca^{2+} (*42*).

DISCUSSION AND CONCLUSION

Efforts to characterize bacterial GTases have been ongoing since 1966 when Strominger and co-workers first reported that bacterial membranes contain enzymes that catalyze the polymerization of Lipid II to form the glycan chains of peptidoglycan (*43*). However, progress in studying the GTases was slow, and for many years, *E. coli* PBP1b was the only GTase demonstrated to have activity in vitro. Eventually, it was shown that *E. coli* PBP1a and PBP1c as well as a few MGTs were competent to polymerize Lipid II in in vitro assays, but no detailed kinetic analysis of any purified peptidoglycan-synthesizing GTase was reported until 2001 when Schwartz and co-workers reported a careful study of *E. coli* PBP1b. For the studies reported here, which involve examining the properties of an isolated GTase module, we chose *E. coli* PBP1b as a model system because the kinetic behavior of the full-length protein had previously been described. Our goals were to determine where the C-terminal boundary of the GTase module lies and whether the activity of the isolated module depends on the presence of the TPase module. Answering these questions is important for engineering soluble GTase modules for structural analysis.

The results reported above show that the activity of the GTase module does not depend on the presence of the TPase module. The converse is not true; several studies have indicated that the TPase module of PBP1b depends on the presence of the transglycosylase module. In *E. coli*, the glycan chains are formed before cross linking occurs, and it is believed that the substrates for the TPase module are at least oligomers of the Lipid II disaccharide (*34*, *39*). We have found that the catalytic activity of the isolated GTase module is lower than that of the full-length protein, which suggests that an interaction between the GTase module and the TPase module influences catalytic activity. Nevertheless, in many respects, including the metal ion dependence, the transglycosylase module is comparable to the full-length protein, and it may thus serve as a simplified model system for mechanistic investigations.

The major difference between the isolated module and the full-length protein is that the former does not benefit from the addition of the micelle-forming detergent decyl PEG. When *E. coli* PBP1b is compared to other transglycosylases, *E. coli* PBP1b contains a large stretch of amino acids of unknown function between the end of the transmembrane helix and the start of the transglycosylase module. Nicholas and co-workers have suggested that this stretch of amino acids comprises a membrane association site that is independent of the transmembrane helical anchor (*44*). We thus anticipated that the isolated transglycosylase module would have poorer solubility properties than the full-length protein. However, even when the Trx tag is removed, the truncated proteins do not require decyl PEG for catalytic activity. The beneficial effects of detergent in the case of the full-length protein are thus related to the presence of the TPase module. The ability to omit detergents from the reaction mixture may be advantageous for some studies.

We have established that the C-terminal boundary of the GTase module of *E. coli* PBP1b lies between amino acids 385 and 409. The GTase module is shorter than predicted from other experiments or from sequence alignments of bifunctional PBPs. We anticipate that the C-terminal boundaries of other HMW PBPs can be predicted based on the results reported here, making it possible to express a range of different transglycosylase modules to identify enzymes with suitable properties for structural analysis. We note that the isolated GTase module of PBP1b can be expressed at higher levels than the full-length protein and obtained as a soluble and stable enzyme. If the same trend holds for other HMW PBPs, it should be possible to obtain suitable GTase modules for structural analysis.

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