THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 42, pp. 28464–28470, October 17, 2008

Importance of the Conserved Residues in the Peptidoglycan Glycosyltransferase Module of the Class A Penicillin-binding Protein 1b of *Escherichia coli*

Received for publication, April 28, 2008, and in revised form, June 23, 2008 Published, JBC Papers in Press, August 13, 2008, DOI 10.1074/jbc.M803223200

Mohammed Terrak[‡1], Eric Sauvage[‡], Adeline Derouaux[‡], Dominique Dehareng[‡], Ahmed Bouhss[§], Eefjan Breukink[¶], Sylvie Jeanjean[‡], and Martine Nguyen-Distèche[‡]

From the [‡]Centre d'Ingénierie des Protéines, Institut de Chimie, Université de Liège, B6a, B-4000 Sart-Tilman, Belgium, the [§]Laboratoire des Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619 CNRS, Université Paris-Sud 11, 91405 Orsay, France, and the [¶]Department of Biochemistry of Membranes, Bijvoet Center for Biomolecular Research, Institute of Biomembranes, Utrecht University, Padualaan, 8, 3584 CH, Utrecht, The Netherlands

The peptidoglycan glycosyltransferase (GT) module of class A penicillin-binding proteins (PBPs) and monofunctional GTs catalyze glycan chain elongation of the bacterial cell wall. These enzymes belong to the GT51 family, are characterized by five conserved motifs, and have some fold similarity with the phage λ lysozyme. In this work, we have systematically modified all the conserved amino acid residues of the GT module of *Escherichia coli* class A PBP1b by site-directed mutagenesis and determined their importance for the *in vivo* and *in vitro* activity and the thermostability of the protein. To get an insight into the GT active site of this paradigm enzyme, a model of PBP1b GT domain was constructed based on the available crystal structures (PDB codes 2OLV and 2OLU). The data show that in addition to the essential glutamate residues Glu^{233} of motif 1 and Glu^{290} of motif 3, the residues Phe^{237} and His^{240} of motif 1 and Gly^{264}, Thr^{267}, Gln^{271}, and Lys^{274} of motif 2, all located in the catalytic cavity of the GT domain, are essential for the *in vitro* enzymatic activity of the PBP1b and for its *in vivo* functioning. Thus, the first three conserved motifs contain most of the residues that are required for the GT activity of the PBP1b. The residues Asp^{234}, Phe^{237}, His^{240}, Thr^{267}, and Gln^{271} are proposed to maintain the structure of the active site and the positioning of the catalytic Glu^{233}.

Most bacteria are surrounded by cell wall peptidoglycan, a net-like polymer consisting of glycan strands made of alternating β-1,4-linked GlcNAc and *N*-acetylmuramic acid (MurNAc)[2] residues cross-linked by peptides (1, 2). Peptidoglycan metabolism is linked to cell cycle and function to confer shape and osmotic stability to the cell (3–5). Its assembly is complex and requires the contribution of several proteins (polymerases, hydrolases, and skeletal proteins), which are believed to form protein assemblages specialized in cell elongation and division (3, 6, 7).

Multimodular penicillin-binding proteins (PBPs) are membrane-bound enzymes responsible for peptidoglycan polymerization (8, 9). They are essentially two-module proteins that belong either to class A or to class B, depending on the structure and the catalytic activity of their N-terminal module. The C-terminal penicillin-binding domain of both classes has a transpeptidase activity, catalyzing peptide cross-linking between two adjacent glycan chains. In class A, the N-terminal module is responsible for their glycosyltransferase (GT) activity. This module catalyzes the polymerization of uncross-linked glycan chains from a membrane bound intermediate called lipid II, the GlcNAc-MurNAc-(pentapeptide) linked to the undecaprenyl via a pyrophosphate bridge (Fig. 1). In class B, the N-terminal module appears to play a role in cell morphogenesis (10). Monofunctional enzymes similar to the GT module of class A PBPs also exist in some bacteria, but their exact role is still unknown (11).

Crystal structures of the *Staphylococcus aureus* class A PBP2 in apo form (PDB code 2OLU) and in complex with moenomycin (PDB code 2OLV) and the GT module of *Aquifex aeolicus* PBP1a (PDB code 2O9O) reveal that the GT module has some similarity to phage λ lysozyme and shed light on its active site (12, 13). The available structural and biochemical data provide better understanding of the mechanism of glycan chain elongation by the glycosyltransferases (12–15). The essential glutamate of motif 1 functions as active site general base and deprotonates the GlcNAc 4-OH group of lipid II acting as an acceptor for nucleophilic attack on the reducing end of the growing glycan chain acting as a donor (16, 17) (Fig. 1). The reaction results in the formation of a β-1,4 glycosidic linkage with inversion of the configuration at the anomeric C1 carbon of MurNAc. The departure of the leaving diphospho-undecaprenyl group is probably assisted by anionic interaction with the second con-

* This work was supported by grants from the European Commission within the "EUR-INTAFAR" (Inhibition of New Targets for Fighting Antibiotic Resistance) (LSHM-CT-2004-512138) network, the Belgian programme of Interuniversity Poles of Attraction (IAP) initiated by the Federal Office for Scientific Technical and Cultural Affairs (IAP Number 6/19), the Actions de Recherche Concertées (Grant 03/08-297), and the Fonds National de la Recherche Scientifique (Fonds de la Recherche Fondamentale Collective d'initiative des chercheurs (FRFC) Numbers 2.4506.08 and 2.4511.06). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

[1] A Research Associate of the National Fond for Scientific Research (FNRS, Belgium). Tel.: 32-4-3663397; Fax: 32-4-3663364; E-mail: mterrak@ulg.ac.be.

[2] The abbreviations used are: MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein; GT, glycosyltransferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PDB, Protein Data Bank.

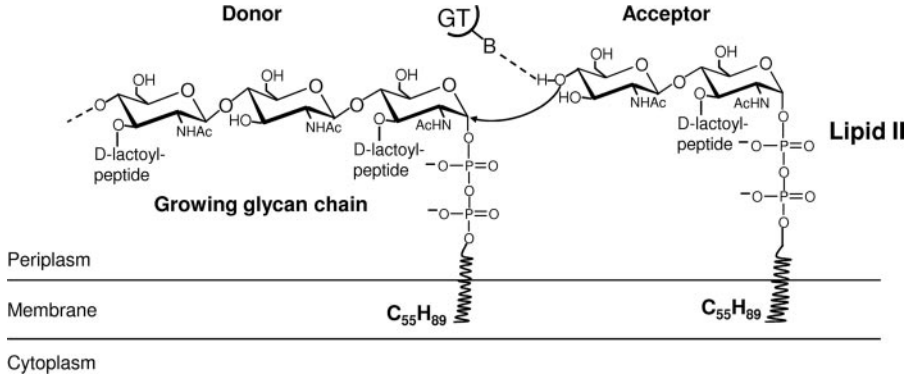

FIGURE 1. **Mechanism of glycan chain elongation by peptidoglycan glycosyltransferases.** The catalytic base (glutamate of motif 1) catalyzes the deprotonation of the GlcNAc 4-OH of lipid II, and the activated nucleophile then directly attacks the C1 of the lipid-linked MurNAc of the growing polysaccharide chain.

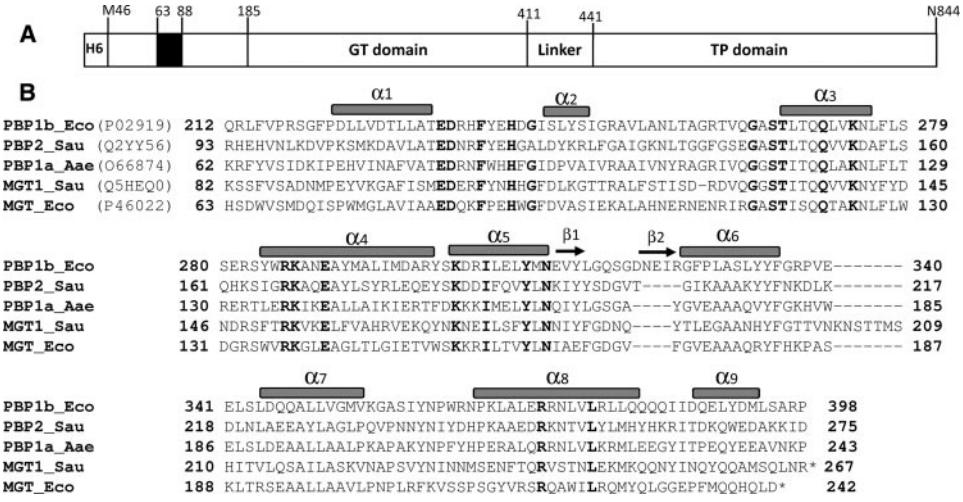

FIGURE 2. **Organization of the multimodular PBP1b and structural model of its GT module.** *A*, schematic representation of [H6]PBP1bγ. PBP1b has a cytoplasmic N-terminal tail (1–63 in PBP1bα; 46–63 in PBP1bγ) followed by a transmembrane anchor (64–87), an insertion of about 100 amino acids (88–185), and the GT (185–398) and transpeptidase (*TP*) (441–844) modules separated by a linker. *B*, sequence alignment (ClustalW) of the GT module of class A PBPs and monofunctional GTs. *Eco*, *E. coli*; *Sau*, *S. aureus*; *Aae*, *A. aeolicus*. The secondary structures of *S. aureus* PBP2 are shown on *top* of the sequences, and motifs 1–5 are *underlined* with the conserved residues in *bold type*. *Numbers* indicate the position of the first and last residues. The C-terminal end of monofunctional GT (*MGT*) is indicated by *.

served glutamate in motif 3 via a divalent metal cation. The translocation of the new product from the acceptor to the donor site may be driven by the high affinity of the pyrophosphate for the positively charged donor site (12). Apart from *S. aureus* PBP2 (18), the activity of other GTs (16, 19, 20) was dependent on metal ions, but the exact role of bivalent ions in the reaction still need to be determined.

The multimodular class A PBP1b (Fig. 2) is the major bifunctional peptidoglycan synthase in *Escherichia coli* endowed with GT and transpeptidase activities (17, 21–23). It is an essential constituent of the peptidoglycan synthesis machineries involved in cell elongation and cell division (24–26), where its function is probably modulated by other proteins, such as FtsN, which stimulates its activity (27).

E. coli PBP1b is the most studied class A PBP and exhibits the highest peptidoglycan polymerase activity *in vitro* (16, 17, 24, 25, 28). However, no tridimensional structure is available for this protein, and its active site is not well characterized. The crystal structure of the PBP1b ortholog, *S. aureus* PBP2 (PDB code 2OLU), is known (12), and the two proteins share high sequence similarity with each other. Based on the PBP2 structure, we have constructed a model of PBP1b GT domain to get an insight into its active site. The GT domains of the two PBPs, like almost all the proteins of the GT51 family (29), are characterized by five characteristic motifs containing several invariant amino acids, most of them located in the catalytic cavity of these enzymes (Figs. 2 and 3). We have modified all the conserved residues within these motifs and determined their importance for the function of the PBP1b both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Construction of the Mutants—Single amino acid mutations were introduced into the *ponB* gene using the QuikChange site-directed mutagenesis kit (Stratagene) as described previously (17). Briefly, the primers (supplemental Table S1) were used to PCR-amplify the pDML920C plasmid containing the 1300-bp NcoI-PstI sequence encoding the His tag (Met^{46}–Gln^{423}) PBP1b GT module. After DNA sequencing to check for the desired mutation and absence of any errors, the 840-bp NheI-SacII or 300-bp SacII-PstI fragments were exchanged with the corresponding unmodified segments of the expression plasmid pDML924 carrying *ponB* gene encoding the His tag PBP1bγ (Met^{46}–Asn^{844}) (17) and sequenced. Supplemental Table S1 shows the pDML924 derivatives (E to N) allowing the production of PBP1b mutants.

Expression and Purification of the His Tag PBP1bγ and the Mutants—E. coli BL21 (DE3)/pDML924-E to R transformants were grown at 37 °C in Luria Bertani (LB) medium containing 50 μg of kanamycin/ml. When the absorbance at 600 nm reached a value of 0.6, the cultures were supplemented with 1 mM IPTG and grown for two additional hours. The cells were disrupted and fractionated. The membrane-bound PBPs were solubilized in 25 mM Tris-HCl, pH 7.5, 1 M NaCl, and l % CHAPS and purified on Ni^{2+}-Sepharose column (HisTrap HP from GE healthcare) as described previously (17).

Complementation Assay—The *in vivo* activity of the PBP1b mutants was assayed by their ability to complement for the deletion of PBP1b and the thermosensitive PBP1a in *E. coli* strain EJ801 at 42 °C (30). EJ801 was transformed with the plasmids pDML924-E to R at 30 °C. The transformants were first grown at 30 °C in LB medium containing 30 μg of kanamycin/

ml. The preculture was then diluted in 20 ml of fresh LB medium in the absence and the presence of IPTG to an $A_{600\ nm}$ value of 0.1–0.3, and the absorbance of the culture was monitored for 4 h at 42 °C.

Glycosyltransferase Assay—The [^{14}C]*meso*-diaminopimelic acid (A_2pm)-labeled C_{55} lipid II intermediate (*N*-acetylglucosaminyl-*N*-acetylmuramoyl(L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala)-pyrophosphate-undecaprenol) was prepared essentially as described previously (31).

In vitro peptidoglycan polymerization was performed by incubation of the [^{14}C]lipid II (0.5–5 μM; 0.126 μCi/nmol) and the His tag PBP1bγ or the mutants (20 nM to 1 μM) in 50 mM Tris-HCl, pH 7.5; 0.046% CHAPS; 33 mM NaCl; 10 mM $MgCl_2$; 0.5% decyl polyethylene glycol; 12.5% 1-octanol; 25% dimethyl sulfoxide (DMSO) for 15–60 min at 30 °C. The reaction products were separated by an overnight chromatography on Whatman No. 1 filter paper in isobutyric acid:1 M ammonium hydroxide (5:3; V/V) and analyzed as described previously (17).

Interaction of the PBP1b Mutants with Fluorescent Ampicillin or [^{14}C]Benzylpenicillin—[^{14}C]Benzylpenicillin (54 nCi nmol^{-1}) was from Amersham Biosciences. Fluoresceyl-labeled ampicillin was prepared as described previously (32). The interaction between PBP1b and β-lactams was analyzed using the same procedure as described previously (17, 19).

Thermostability of the PBP1b Mutants—Purified PBP1b and PBP1b mutants (5 μM final concentration) were incubated at 60 °C for various times and then labeled with fluoresceyl-ampicillin (10^{-4} M final concentration) in 25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.7% CHAPS (buffer A) for 5 min at 30 °C. The samples were analyzed by SDS-PAGE, and the acyl-enzyme complex formed was detected with a Molecular Imager scanner and analyzed with the Quantity One software (Bio-Rad).

PBP1b GT Domain Modeling—A model of *E. coli* PBP1b GT domain was constructed based on *S. aureus* PBP2 crystallographic structure (Protein Data Bank code 2OLV) (12). From residue 185 to residue 400 (PBP1b numbering), *S. aureus* PBP2 side chains have been replaced by the equivalent ones of *E. coli* PBP1b and have been reoriented so as to avoid steric clashes. Side chains positions have not been modified when the side chains are common to both enzymes. Residues 255–263 have not been modeled as the structure of equivalent residues in *S. aureus* PBP2 is unknown, like residues 320–323, which are not present in *S. aureus* PBP2. The model was energy-minimized with the program Insight II® (BIOSYM Technologies, San Diego, CA; Accelrys Software Inc.) in a two-step process, on a *all-atom* framework, thus taking all the hydrogen atoms into account. In the first step, all the C_α atoms were fixed, and the rest of the protein was subject to a geometry optimization. In the second step, the geometry optimization was performed without restraint.

RESULTS

Model of the GT Domain of E. coli PBP1b—Alignment of the *E. coli* PBP1b GT module sequence, starting from residues 212–398, with that of *S. aureus* PBP2 showed 39% of identity and 65% of similarity. This high degree of conservation suggests that their three-dimensional structures must also be well conserved. A model of *E. coli* PBP1b GT domain based on the crystal structure of *S. aureus* PBP2 (PDB code 2OLV) (12) was constructed (see "Experimental Procedures"). The fold adopted by the 212–398 polypeptide model with the five conserved motifs is shown in Fig. 3*A*.

At least 18 amino acid residues within these motifs are highly or strictly conserved (Fig. 2*B*). In the *E. coli* PBP1b GT domain, four of them (Glu^{233}, Asp^{234}, Glu^{290}, and Tyr^{310}) were analyzed previously (17). The 14 additional conserved residues in the PBP1b were modified by site-directed mutagenesis, and the mutants were characterized (Table 1).

Expression, Purification, and Properties of the PBP1b Mutants—All the PBP1b mutants were expressed and purified in the same conditions as for the wild type, except that PBP1bT267A could only be expressed at 18 °C instead of 37 °C, and the PBP1b R286A was not produced even at 18 °C (Fig. 4). The PBP1b mutants were characterized in terms of penicillin binding activity, thermostability, *in vivo* activity by complementation, and GT activity using lipid II as substrate. The expression levels (2–3 mg of protein/liter of culture) and the stability of the mutants, unless otherwise mentioned, were similar to those of the wild-type protein. The penicillin binding capacity of the native PBP1b was reduced by 50% after 8–10 min of preincubation at 60 °C. The acylation rate constants of the PBP1b mutants by β-lactams were comparable with those of the native PBP1b (500–700 M^{-1} s^{-1} for [^{14}C]benzylpenicillin or 20–30 M^{-1} s^{-1} for fluoresceyl-ampicillin). The results of the complementation experiments with and without induction were similar unless otherwise indicated.

Mutations in Motif 1 (^{233}EDRHFYEHDG^{242}*), F237A, H240Q, or H240A and G242A*—Motif 1 is located in a loop between the helices α1 and α2 and contains five conserved residues, Glu^{233}, Asp^{234}, Phe^{237}, His^{240}, and Gly^{242} (Figs. 2 and 3*A*). The residues, Phe^{237}, His^{240}, and Gly^{242} were modified to Ala. In addition, His^{240} was modified to Gln. The thermostability and the penicillin binding activity of the four PBP1b mutants were similar to those of the wild type. The PBP1b F237A and H240A were inactive *in vivo* as assayed by complementation using the *ponA* (*ts*) *ponB* EJ801 *E. coli* strain producing a thermosensitive PBP1a and depleted of PBP1b (see "Experimental Procedures"), whereas PBP1b H240Q was partially functional (Fig. 5), and G242A was only active in the presence of IPTG (data not shown). The GT activity of PBP1b F237A and H240A was completely abolished. The glycan polymerase activities of PBP1b H240Q and G242A were 8- and 22-fold lower than that of the wild type with efficiencies of 4700 M^{-1} s^{-1} and 1800 M^{-1} s^{-1}, respectively, when compared with 39,000 M^{-1} s^{-1} for the wild type (Table 1).

Mutations in Motif 2 (^{264}GASTLTQQLVK^{274}*), G264L, S266A, T267A, Q271A, and K274A*—We propose that motif 2 extends to Lys^{274} since the nature (Lys/Arg) of the residue at this position is highly conserved among the hundreds of class A PBPs/monofunctional GTs (CAZy (carbohydrate-active enzymes) data base), and the three-dimensional structures of the GT domain show that it may play an important role in the binding of the substrate (12, 13). Motif 2 contains five conserved residues starting before the α3 helix (Gly^{264}, Ser^{266}, and Thr^{267}) and extends through the whole helix (Gln^{271} and Lys^{274}) (Figs. 2*B* and 3*A*). To test their importance in the function of PBP1b, the mutations G264L, S266A, T267A, Q271A,

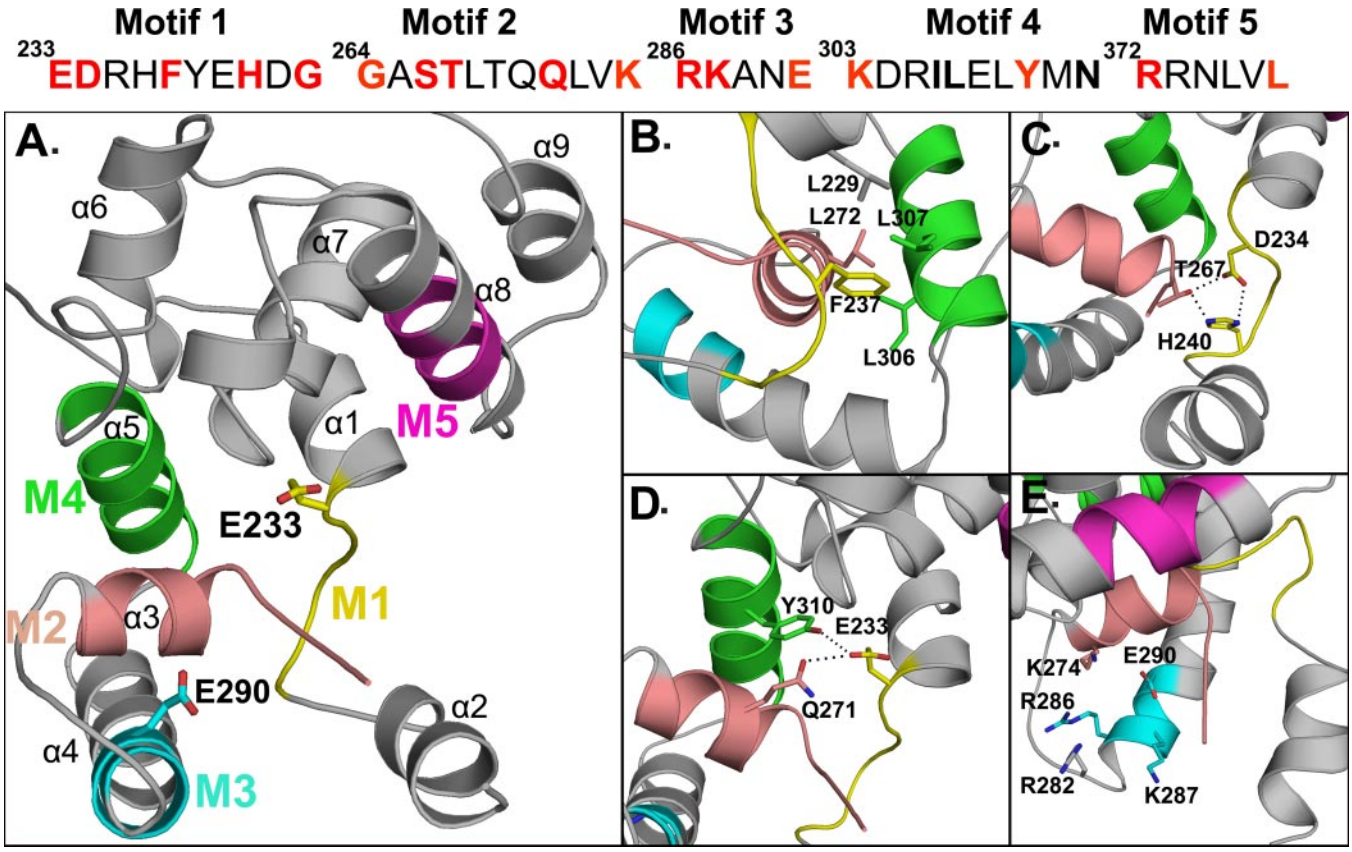

FIGURE 3. **Structural model of PBP1b GT module (212–398) and close view into the catalytic site depicting the positions of the studied conserved residues in *sticks*.** *A*, motif 1 (*M1*) is shown in *yellow*, motif 2 (*M2*) is shown in *salmon*, motif 3 (*M3*) is shown in *cyan*, motif 4 (*M4*) is shown in *green*, and motif 5 (*M5*) is shown in *magenta*. The catalytic Glu^{233} and the Glu^{290} residues are shown in *sticks*. *B*, Phe^{237} is part of a hydrophobic cluster. *C*, interaction between Asp^{234} and His^{240} of motif 1 and Thr^{267} of motif 2. *D*, Gln^{271} and Tyr^{310} are within interacting distance of the catalytic Glu^{233}. *E*, positively charged residues within the donor site close to the Glu^{290} are shown. The figures were generated with the help of the PyMOL software.

TABLE 1

Properties of *E. coli* PBP1b mutants

The kinetic parameters were determined, with lipid II as substrate. *In vivo* activities of the mutants were determined by complementation assay at 42 °C with and without IPTG induction in *ponA (ts) ponB E. coli* EJ801 double mutant. The kinetic parameters values are the mean of three to five separate experiments. + indicates that the mutant is active or stable; −, the mutant is not active or instable; ±, the mutant has intermediate activity or not less stable. ND, not determined because of low activity or instability of the protein.

	In vitro activity	Penicillin binding/thermostability	k_{cat}	K_m	k_{cat}/K_m	GT activity
			s^{-1}	μM	$M^{-1} s^{-1}$	%
PBP1b wild-type[a]	+	+/+	70 ± 13	1.8 ± 0.8	39,000	100%
I. E233Q[a]	−	+/+	ND	ND	ND	0.2
D234N[a,b]	−	+/±	9.4 ± 1.8	1.68 ± 0.65	5600	14
F237A	−	+/+	−	−	−	0
H240A	−	+/+	−	−	−	0
H240Q	±	+/+	5.2 ± 1.24	1.8 ± 0.21	4700	7
G242A	+[c]	+/+	2.9 ± 1	1.6 ± 0.9	1800	4
II. G264L	−	+/+	−	−	−	0
S266A	+	+/+	7.6 ± 2	1.2 ± 0.67	6300	11
T267A[b]	−	+/±	−	−	−	0
Q271A	−	+/+	−	−	−	0
K274A	−	+/+	−	−	−	0
III. R286A[d]	−	×/×	×	×	×	×
K287A	+	+/+	44 ± 10	1.2 ± 0.6	36,500	63
E290Q[a]	±	+/+	1.44 ± 0.4	0.4 ± 0.2	3600	2
IV. K303A[b]	−	+/−	ND	ND	ND	−
Y310F[a,d]	−	×/×	×	×	×	−
N312A	+	+/+	11 ± 3.6	1.2 ± 0.94	9200	15
V. R372A	+	+/+	13.4 ± 4	1.1 ± 0.63	12,200	19
L377G[b]	+	+/±	ND	ND	ND	+

[a] From Ref. 17.
[b] These PBP1b mutants were expressed only at lower temperature or at lower level at 37 °C when compared with the wild-type.
[c] Active only after induction with IPTG.
[d] These mutants were not expressed at all (×).

and K274A were performed. The PBP1b G264L, S266A, Q271A, and K274A mutants were expressed in stable form at 37 °C and behave as the wild-type protein. They all bound penicillin, but their GT activity was completely abolished except that of PBP1b S266A, which had a 6-fold reduced GT activity with an efficiency of 6300 $M^{-1} s^{-1}$ (Table 1) and was active in

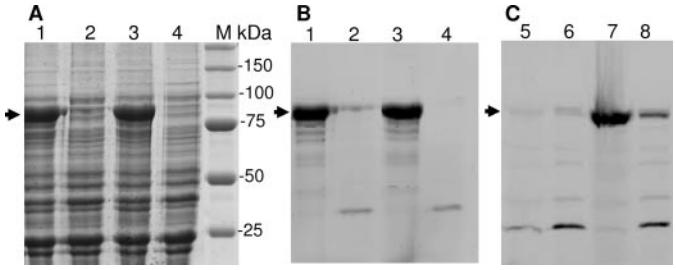

FIGURE 4. **Expression and fluoresceyl-ampicillin labeling of some PBP1b mutants.** *A* and *B*, Coomassie Blue staining (*A*) and fluoresceyl-ampicillin (50 μM) labeling (*B*) of PBPs after SDS-PAGE loaded with equivalent amount of membrane extract from cells expressing PBP1b mutants at 37 °C. *Lane 1*, F237A; *lane 2*, T267A; *lane 3*, Q271A; *lane 4*, R286A; *M* = molecular mass standard. *C*, fluorescence detection of fluoresceyl-ampicillin labeled PBPs at 30 °C. In *lanes 5* and *6*, the expression of the PBP1b R286A was realized at 18 and 37 °C, respectively. In *lanes 7* and *8*, the expression of PBP1b T267A was realized at 18 and 37 °C, respectively (no overexpression of R286A was visible after Coomassie Blue staining, not shown).

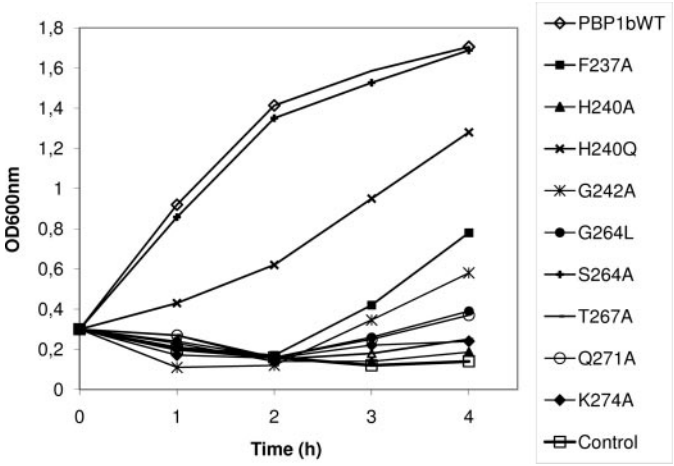

FIGURE 5. **In vivo activity of PBP1b mutants modified in motifs 1 and 2 by complementation assays at 42 °C using the *ponA(TS) ponB E. coli* strain EJ801.** *WT*, wild type.

the complementation assay (Fig. 5). PBP1b G264L, Q271A, and K274A do not support the growth of *E. coli* EJ801 *ponA* (*ts*) *ponB* at the nonpermissive temperature (Fig. 5). The PBP1b T267A was not expressed at 37 °C, but when the expression was tested at 18 °C for 16 h, the protein was produced (Fig. 4). The purified PBP1b T267A was stable and bound penicillin (Fig. 4) but was devoid of GT activity and was inactive in the complementation assay at 42 °C (Fig. 5). The ability of the PBP1b T267A to bind penicillin after incubation at 60 °C was similar to the wild type (50% labeling after 8–10 min at 60 °C), suggesting that the mutation had not affected the overall stability and the structure of the protein (other than the GT active site) but perhaps affected the folding process of the protein, which was restored at lower temperature.

Mutations in Motif 3 (^{286}RKANE^{290}), R286A and K287A—Motif 3 is located at the beginning of the α4 helix and contains three conserved residues, Arg^{286}, Lys^{287}, and Glu^{290} (Figs. 2*B* and 3*A*). Arg^{286} and Lys^{287} were modified to Ala. The PBP1b R286A mutant was not detected in induced cells using the same condition as for the wild type. Its expression was also tested at 18 °C for 3–16 h, but no protein was produced (Fig. 4). This mutation may have affected the expression or the folding and stability of the protein. Moreover, when the plasmid encoding the PBP1b R286A was used in the complementation assay, the *ponA* (*ts*) *ponB* EJ801 strain was not able to grow at 42 °C. By contrast, the behavior of the PBP1b K287A was similar to the nonmutated protein with respect to the level of production, thermostability, susceptibility to penicillin, glycan chain elongation from lipid II (k_{cat}/K_m 36,500 M^{-1} s^{-1}) (Table 1), and *in vivo* activity.

Mutations in Motif 4 (^{303}KDRILELYMN^{312}), K303A and N312A—Motif 4 holds within the entire α5 helix and contains four conserved residues, Lys^{303}, Ile^{306}, Tyr^{310}, and Asn^{312} (Figs. 2*B* and 3*A*). Lys^{303} and Asn^{312} were modified to Ala. The PBP1b K303A mutant was produced at a 3-fold lower level than the wild type. The K303A mutant from membrane preparations bound penicillin, but the CHAPS-solubilized protein underwent spontaneous precipitation during purification, making its *in vitro* characterization difficult. This mutation may have affected the stability of the protein. The plasmid, which encoded the PBP1b K303A mutant, was not able to rescue the strain EJ801 from lysis at the nonpermissive temperature, showing that the protein was not functional *in vivo*. The PBP1b N312A behaves as the wild type in terms of expression level, stability, and penicillin binding. This mutant has 4-fold lower GT activity than the wild type with an efficiency of 9200 M^{-1} s^{-1} (Table 1). *E. coli* EJ801 transformants expressing the PBP1b N312A grew at 42 °C, indicating that the mutant was active *in vivo*.

Mutations in Motif 5 (^{372}RRNLVL^{377}), R372A and L377G—Motif 5 is located in the middle of the α8 helix and contains two conserved residues, Arg^{372} and Leu^{377}, which were modified to Ala and Gly, respectively (Figs. 2 and 3*A*). The thermostability of PBP1b R372A and its affinity for penicillin were similar to those of the wild type. The mutant R372A was active *in vivo* and catalyzed glycan chain polymerization *in vitro* with an efficiency of 12,200 M^{-1} s^{-1} (Table 1). The expression level (0.3 mg/l) and thermostability (50% labeling after 4 min at 60 °C) of the PBP1b L377G were lower than those of the wild-type PBP1b. The PBP1b L377G mutant was active *in vivo* in the complementation assay and had GT activity *in vitro* with lipid II as substrate (kinetic parameters not determined). The PBP1b mutants, which retained some activity (Table 1), all have their k_{cat} values affected to different extents, but there was almost no change in the K_m values.

DISCUSSION

PBP1b is the major peptidoglycan polymerase in *E. coli*. It is a bifunctional enzyme catalyzing both glycan chain elongation and peptide cross-linking. Here we have studied the structure-function relationship and the effect of point mutations within the PBP1b GT domain on the peptidoglycan polymerase activity of the PBP1b.

The glycosyltransferase domain of the PBP1b catalyzes the transfer of the lipid linked growing glycan chain, which acts as the glycosyl donor, to the lipid II precursor, which functions as the glycosyl acceptor (12, 14) (Fig. 1). The catalytic base Glu^{233} catalyzes the deprotonation of the GlcNAc 4-OH of lipid II, and the activated nucleophile then directly attacks the C1 of the lipid-linked MurNAc of the growing polysaccharide chain (S_N2-like mechanism). In addition to the essential dicar-

boxylic acid residue, the GT module contains several other conserved residues, most of them clustered in the five conserved motifs. The importance of these residues for the function of the PBP1b was analyzed both *in vitro* and *in vivo*, and their possible role is discussed below. The results presented here show a nice correlation between the GT activity *in vitro* and the *in vivo* activity of the mutants.

A three-dimensional structural model of *E. coli* PBP1b GT domain was constructed using the x-ray structure of *S. aureus* PBP2 (12). By similarity, the motif 1 containing the essential Glu^{233} is located in a loop at the end of the α1 helix and just before the flexible region, which encompasses the α2 helix (Fig. 3*A*). Therefore, the structure of the loop, which also contains Asp^{234}, Phe^{237}, and His^{240}, must be very important for the correct positioning of the glutamate and the activity of the enzyme. The side chain of Phe^{237} is part of a hydrophobic cluster that contains Tyr^{238} of motif 1, Leu^{272} of motif 2, Ile^{306} and Leu^{307} of motif 4, and Leu^{229} on the α1 helix (Fig. 3*B*). This hydrophobic cluster is likely to play an important role in the maintenance of the structure of the motif 1 loop. In addition, the side chains of Asp^{234} and His^{240} of motif 1 and Thr^{267} of motif 2 interact with each other (Fig. 3*C*), and therefore, the mutation of one of these residues may destabilize the loop that holds Glu^{233}, resulting in the loss of activity. Consistently, the PBP1b mutants D234N, F237A, H240A, and T267A were all not functional *in vivo*, and only PBP1b D234N had 13% of the wild-type GT activity *in vitro* (17). Note that although the PBP1b H240A was completely inactive, the mutant H240Q, the activity of which was reduced about 8-fold, was partially functional in the complementation assay. These results show that the glutamine can replace the histidine and suggest that the hydrogen bonds network of the triad His-Asp-Thr (Fig. 3*C*) is partially maintained in the PBP1b H240Q mutant and is thus important for the activity.

According to the model, the Glu^{233} side chain (motif 1) interacts directly with that of Gln^{271} (motif 2) and the hydroxyl group of Tyr^{310} (motif 4) (Fig. 3*D*). The PBP1b Q271A mutant was completely inactive *in vitro* and *in vivo*, suggesting that this substitution also had an effect on the position of the glutamate. In the case of Y310F, the modified protein could not be isolated, probably because Tyr^{310} may also play a role in the folding process and stability of the protein (17).

Gly^{242} and Gly^{264} are located in the flexible region upstream and downstream of the α2 helix. PBP1b G242A mutant has low activity, and PBP1b G264L mutant was inactive, suggesting that the flexibility of the region may be important for the activity of the enzyme and that the mutations may have caused local conformational change or steric effect.

Residues Lys^{274} (motif 2), Arg^{286}, and Lys^{287} (motif 3), together with Arg^{282} (residues equivalent to Lys^{155}, Arg^{167}, Lys^{168}, and Lys^{163} in *S. aureus*), create a positively charged pocket in the donor site (Fig. 3*E*) and may play a role in the positioning of the negatively charged pyrophosphate of the donor substrate (12). Below this region lays a hydrophobic surface, which likely interacts with the membrane and where the lipid moiety would bind. Among the positively charged residues, Lys^{274} seems to play a major role in the interaction with the substrate as the modification of this residue into Ala completely abolished the *in vivo* and *in vitro* functions of the protein. The PBP1b R286A mutant was not expressed, and its exact role cannot be determined. However, the equivalent residue in *A. aeolicus* (Arg^{136}) was shown to be important for enzymatic activity (15), and therefore, Arg^{286} may also play similar function in PBP1b. Lys^{287} seems not to be important since the K287A mutant retained high GT activity and was functional *in vivo*.

The PBP1b R372A retained 20% of GT activity *in vitro* and was active *in vivo*. The equivalent residue to Arg^{372} in *S. aureus* PBP2 (Arg^{249}) was proposed to stabilize the deprotonated form of the catalytic glutamate (12). A mutant of the equivalent residue (R218A) in *A. aeolicus* PBP1a seems to be more affected as it retained only 10% of GT activity. Its *in vivo* activity is not known. However, under certain conditions (more enzyme), good turnover by *A. aeolicus* PBP1a was observed (15). One explanation is that the role of Arg^{372} in the PBP1b mutant or Arg^{218} in the PBP1a mutant may be taken over by the amino group of a lysine downstream of helix α7, which is not strictly conserved but is well positioned and could fold into the active site upon substrate binding: Lys^{355} in *E. coli* PBP1b R372A mutant and Lys^{200} in *A. aeolicus* PBP1a R218A mutant.

In contrast to *E. coli* PBP1b mutants F237A, H240A, and T267A, which were completely inactive *in vivo* and *in vitro* even when high protein concentrations (20–50 times more than the wild type) were used *in vitro* to monitor their activity, the mutants of the equivalent residues (F87A, H90A, and T117A) in *A. aeolicus* PBP1a GT domain retain residual GT activity *in vitro* (5–10%) (13, 15). These results suggest that some local variation may exist between different GT domains. However, the general tendency of the effects of mutations in *E. coli* PBP1b and *A. aeolicus* PBP1a is similar and shows that most of the conserved residues of motifs 1, 2, and 3 play an important role in the activity of their GT domains.

Consistent with the location of the first three conserved motifs in the catalytic cavity of the GT domain, most of the mutations in motifs 1 and 2 and to a lesser extent motif 3 severely affected the function of the PBP1b; only 2 of 12 mutants (S266A in motif 2 and K287A in motif 3) were fully active *in vivo*, and these residues are probably dispensable. Except from D234N (motif 1), T267A (motif 2), and R286A (motif 3), whose thermostabilities were affected to different extents when compared with the wild type, all the mutants were thermostable. On the contrary, in terms of *in vitro* GT activity, six mutants (F237A, H240A, G264L, T267A, Q271A, and K274A) were completely inactive, and D234N, G242A, and Glu^{290} have only residual GT activities estimated at 13, 4.6, and 2% of the wild-type activity, respectively.

In conclusion, motifs 1, 2, and 3 are important for the GT activity of the PBP1b. The residues Asp^{234}, Phe^{237}, His^{240}, Thr^{267}, and Gln^{271} are proposed to maintain the structure of the active site and the positioning of the catalytic Glu^{233} and may interact with the sugar moiety of the substrates as suggested (12). Residue Lys^{274}, located in a positively charged pocket of the donor site, is involved in the positioning of the pyrophosphate of the growing glycan chain. Motifs 4 and 5 seem less critical for the activity and may be involved mainly in maintaining the overall structure of the GT domain.

Acknowledgment—We thank Astrid Zervozen for the preparation of fluoresceyl-labeled ampicillin.

REFERENCES

1. van Heijenoort, J. (2001) *Glycobiology* **11,** 25R–36R
2. Vollmer, W., Blanot, D., and de Pedro, M. A. (2008) *FEMS Microbiol. Rev.* **32,** 149–167
3. Vollmer, W., and Höltje, J. V. (2001) *Curr. Opin. Microbiol.* **4,** 625–633
4. Nanninga, N. (1991) *Mol. Microbiol.* **5,** 791–795
5. Goehring, N. W., and Beckwith, J. (2005) *Curr. Biol.* **15,** R514–526
6. Höltje, J. V. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 181–203
7. den Blaauwen, T., de Pedro, M. A., Nguyen-Distèche, M., and Ayala, J. A. (2008) *FEMS Microbiol. Rev.* **32,** 321–344
8. Goffin, C., and Ghuysen, J. M. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 1079–1093
9. Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., and Charlier, P. (2008) *FEMS Microbiol. Rev.* **32,** 234–258
10. Marrec-Fairley, M., Piette, A., Gallet, X., Brasseur, R., Hara, H., Fraipont, C., Ghuysen, J. M., and Nguyen-Distèche, M. (2000) *Mol. Microbiol.* **37,** 1019–1031
11. Spratt, B. G., Zhou, J., Taylor, M., and Merrick, M. J. (1996) *Mol. Microbiol.* **19,** 639–640
12. Lovering, A. L., de Castro, L. H., Lim, D., and Strynadka, N. C. (2007) *Science* **315,** 1402–1405
13. Yuan, Y., Barrett, D., Zhang, Y., Kahne, D., Sliz, P., and Walker, S. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 5348–5353
14. Perlstein, D. L., Zhang, Y., Wang, T. S., Kahne, D. E., and Walker, S. (2007) *J. Am. Chem. Soc.* **129,** 12674–12675
15. Barrett, D., Wang, T. S., Yuan, Y., Zhang, Y., Kahne, D., and Walker, S. (2007) *J. Biol. Chem.* **282,** 31964–31971
16. Schwartz, B., Markwalder, J. A., Seitz, S. P., Wang, Y., and Stein, R. L. (2002) *Biochemistry* **41,** 12552–12561
17. Terrak, M., Ghosh, T. K., van Heijenoort, J., Van Beeumen, J., Lampilas, M., Aszodi, J., Ayala, J. A., Ghuysen, J. M., and Nguyen-Distèche, M. (1999) *Mol. Microbiol.* **34,** 350–364
18. Barrett, D., Leimkuhler, C., Chen, L., Walker, D., Kahne, D., and Walker, S. (2005) *J. Bacteriol.* **187,** 2215–2217
19. Zawadzka-Skomial, J., Markiewicz, Z., Nguyen-Distèche, M., Devreese, B., Frère, J. M., and Terrak, M. (2006) *J. Bacteriol.* **188,** 1875–1881
20. Terrak, M., and Nguyen-Distèche, M. (2006) *J. Bacteriol.* **188,** 2528–2532
21. Tamaki, S., Nakajima, S., and Matsuhashi, M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5472–5476
22. Suzuki, H., Nishimura, Y., and Hirota, Y. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 664–668
23. Suzuki, H., van Heijenoort, Y., Tamura, T., Mizoguchi, J., Hirota, Y., and van Heijenoort, J. (1980) *FEBS Lett.* **110,** 245–249
24. Bertsche, U., Breukink, E., Kast, T., and Vollmer, W. (2005) *J. Biol. Chem.* **280,** 38096–38101
25. Bertsche, U., Kast, T., Wolf, B., Fraipont, C., Aarsman, M. E., Kannenberg, K., von Rechenberg, M., Nguyen-Distèche, M., den Blaauwen, T., Höltje, J. V., and Vollmer, W. (2006) *Mol. Microbiol.* **61,** 675–690
26. Vollmer, W., von Rechenberg, M., and Höltje, J. V. (1999) *J. Biol. Chem.* **274,** 6726–6734
27. Müller, P., Ewers, C., Bertsche, U., Anstett, M., Kallis, T., Breukink, E., Fraipont, C., Terrak, M., Nguyen-Distèche, M., and Vollmer, W. (2007) *J. Biol. Chem.* **282,** 36394–36402
28. Barrett, D. S., Chen, L., Litterman, N. K., and Walker, S. (2004) *Biochemistry* **43,** 12375–12381
29. Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003) *J. Mol. Biol.* **328,** 307–317
30. Hara, H., and Suzuki, H. (1984) *FEBS Lett.* **168,** 155–160
31. van Heijenoort, Y., Gomez, M., Derrien, M., Ayala, J., and van Heijenoort, J. (1992) *J. Bacteriol.* **174,** 3549–3557
32. Lakaye, B., Damblon, C., Jamin, M., Galleni, M., Lepage, S., Joris, B., Marchand-Brynaert, J., Frydrych, C., and Frère, J. M. (1994) *Biochem. J.* **300,** 141–145