In Vitro Synthesis of Cross-linked Murein and Its Attachment to Sacculi by PBP1A from *Escherichia coli*[*]

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The penicillin-binding protein (PBP) 1A is a major murein (peptidoglycan) synthase in *Escherichia coli*. The murein synthesis activity of PBP1A was studied *in vitro* with radioactive lipid II substrate. PBP1A produced murein glycan strands by transglycosylation and formed peptide cross-links by transpeptidation. Time course experiments revealed that PBP1A, unlike PBP1B, required the presence of polymerized glycan strands carrying monomeric peptides for cross-linking activity. PBP1A was capable of attaching nascent murein synthesized from radioactive lipid II to nonlabeled murein sacculi. The attachment of the new material occurred by transpeptidation reactions in which monomeric tri- and tetrapeptides in the sacculi were the acceptors.

Most bacteria have a murein (peptidoglycan) sacculus that forms an uninterrupted net-like structure around the cytoplasmic membrane. The sacculus is essential for the osmotic stability of the cell (1) and consists of glycan strands that are cross-linked by short peptides (2). During growth and division, the sacculus is enlarged by the incorporation of lipid II precursor by a yet unknown mechanism, which involves the coordinated action of murein synthases, murein hydrolases, and probably regulatory proteins (3).

Escherichia coli contains six known murein synthases (4–8). However, it is not known what particular reaction these enzymes catalyze during the enlargement of the sacculus and, hence, which precise function they have in cell wall growth. Furthermore, most of the murein synthases from *E. coli* and other species are poorly or not characterized so far, perhaps because of difficulties in obtaining pure enzyme and lipid II substrate in sufficient quantities.

The penicillin-binding proteins (PBPs)[2] PBP1A and PBP1B are the major bifunctional murein synthases in *E. coli* catalyzing both the oligomerization of the glycan strands and the forma-tion of the peptide cross-links (9–11). Both PBPs form independent dimers *in vivo* (12). PBP1A and PBP1B are not essential for cell growth, but cells lacking both enzymes are not viable, indicating that both have a similar, essential function that cannot be taken over by other murein synthases (5, 13). Yet, mutants lacking either PBP1A or PBP1B show particular phenotypes, indicating that these synthases may play distinct roles during cell growth and division. For example, mutants without PBP1B are more sensitive to β-lactam antibiotics than mutants without PBP1A (14). Furthermore, PBP1B-deficient mutants, but not PBP1A-deficient mutants, lose cell integrity upon inactivation of PBP2, PBP3, or the cell division protein FtsQ (15).

The enzymatic activities of PBP1B with lipid II or artificial substrates have been studied previously (16–19). However, apart from two early studies from Matsuhashi and colleagues (20, 21), the activities of PBP1A remain uncharacterized. In this article, we report that PBP1A is capable of producing *in vitro* a cross-linked murein with glycan strands of (on average) almost 20 disaccharide units and with 18–26% of the peptides being part of cross-links. We have also studied the specificity of the PBP1A-catalyzed transpeptidation reaction. For the first time, we could demonstrate *in vitro* an attachment of nascent murein to isolated murein sacculi by a murein synthase, namely by PBP1A.

MATERIALS AND METHODS

E. coli Strains and Growth Conditions—XL1-Blue (Stratagene) and BL21(DE3) (Novagen) were used for cloning and PBP1A overproduction, respectively. All experiments were performed in Luria-Bertani (LB) medium. Kanamycin (50 μg/ml) was added to strains carrying overexpression plasmids.

Materials—All chemicals were purchased from Sigma unless indicated otherwise. [14C]GlcNAc-labeled lipid II was prepared as published (22), with modifications described previously (19), and had a specific activity of 9180 dpm/nmol. UDP-MurNAc-tripeptide, DP-MurNAc-tetrapeptide, and UDP-MurNAc-pentapeptide were prepared as published (23). Murein sacculi were prepared from *E. coli* MC1061 (24) as described (25). Cellosyl was kindly provided by Hoechst AG, Frankfurt, Germany.

Cloning of a mrcA Expression Plasmid—The *mrcA* gene encoding for PBP1A (GenBank™ accession no. AAA58193) was amplified by PCR from chromosomal DNA from *E. coli* wild type MC1061 using the primers 1A_up (5′-GGACGAATTCCC-AAATGAAACTAAATGGG-3′) and 1A_down (5′-ATTGGAT-CCATCAGAACAATTCCTGTGCC-3′). The PCR fragment was digested with EcoRI and BamHI and cloned into the plasmid

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[2] The abbreviations used are: PBP, penicillin-binding protein; HPLC, high pressure liquid chromatography; TG, transglycosylase; TP, transpeptidase.
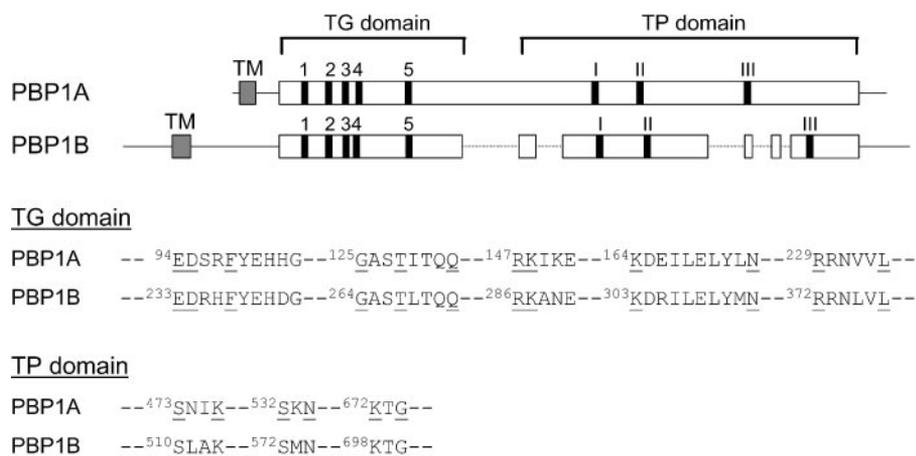
FIGURE 1. **Domain organization and signature motifs in PBP1A and PBP1B from *E. coli*.** Homologous regions are aligned and indicated as *bars*. Regions without similarity are drawn as *black lines*. Gaps in the PBP1B sequence (as compared with the PBP1A sequence) are shown as *dashed lines*. *Gray bars*, predicted transmembrane (*TM*) regions; *black bars*, signature motifs *1–5* of the TG domain and *I–III* of the TP domain. The sequences of the signature motifs are shown. The *underlined* amino acid residues are strictly conserved in class A PBPs.
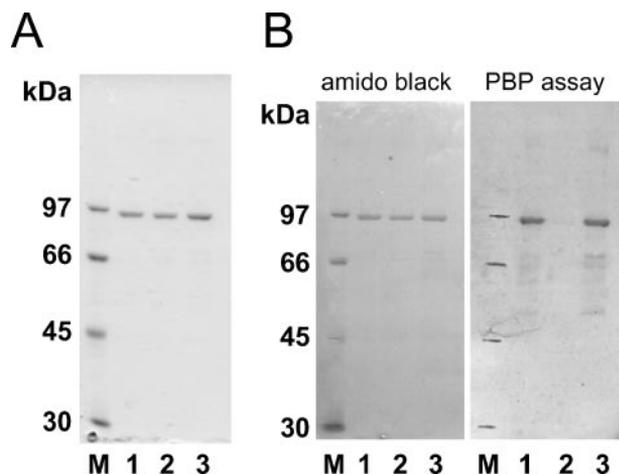

FIGURE 2. **Analysis of purified PBP1A, PBP1A(TP*), and PBP1A(TG*) by SDS-PAGE and by a β-lactam binding assay.** *A*, Coomassie-stained SDS-PAGE showing PBP1A (*lane 1*), PBP1A(TP*) (*lane 2*), and PBP1A(TG*) (*lane 3*). *B*, the proteins were incubated with biotinylated ampicillin followed by SDS-PAGE, Western blotting, and staining with Amido Black (*left side*). Then, the membrane was destained, and biotin was detected with streptavidin-horseradish peroxidase and enhanced chemoluminescence (*right side*). The lanes are numbered in the same way as in *A*. *M*, molecular weight marker. Only PBP1A and PBP1A(TG*), but not PBP1A(TP*), bound biotinylated ampicillin.

pJFK118EH (26), resulting in pTK1A. This plasmid was used as template in a PCR for the amplification of the *mrcA* gene with the primers PBP1Ahis_up (5′-GCGGCTAGCATGAAACTA-AATGGGAAAT-3′) and PBP1Ahis_down (5′-CCTGAATTC-ATCAGAACAATTCCTGTGC-3′). After digestion with NheI and EcoRI, the fragment was cloned into the overexpression plasmid pET28a(+) (Novagen), resulting in pTK1Ahis. The correctness of the *mrcA* sequence in pTK1Ahis was confirmed by sequencing.

Site-directed Mutagenesis—For the production of PBP1A-(TG*), with a Glu^{94} → Gln exchange in the first transglycosylase motif, pTK1Ahis was changed by site-directed mutagenesis using the primers 1A-TG*_up (5′-AGCCTTTA-TCGCGACACAAGACAGCCGCTTCTACG-3′) and 1A-TG*_

down (5′-TCGTAGAAGCGGCT-GTCTTGTGTCGCGATAAAGG-C-3′). For the purification of PBP1A(TP*), with a Ser^{473} → Ala exchange in the first transpeptidase motif, pTK1Ahis was changed by site-directed mutagenesis using the primers 1A-TP*_up (5′-TGCGTC-AGGTGGGTGCCAACATCAAA-CCGTTCC-3′) and 1A-TP*_down (5′-AACGGTTTGATGTTGGCAC-CCACCTGACGCAGTGC-3′). Mutagenesis was performed according to the protocol of the manufacturer employing the QuikChange site-directed mutagenesis kit (Stratagene). The correct sequences of the inserts in the resulting plasmids, pTK1Ahis(TG*) and pTK1Ahis(TP*), were confirmed.

Preparation of Cell Extracts—For the overproduction of the His-tagged form of the PBP1A variants, *E. coli* BL21(DE3) carrying the appropriate overexpression plasmid was grown in 4 liters of LB medium at 30 °C until an optical density (578 nm) of 0.3 was reached. The cells were induced with 0.05 mM isopropyl 1-thio-β-D-galactopyranoside (Gerbu, Gailberg, Germany). After 90 min of further growth, the cells were chilled on ice for 10 min and harvested by centrifugation (4200 × *g*, 20 min, 4 °C). The pellet was resuspended in 30 ml of 25 mM Tris/HCl, 10 mM MgCl$_2$, 100 mM NaCl, 0.02% NaN$_3$, pH 7.5. After addition of DNase and 1 mM phenylmethylsulfonyl fluoride, the cells were broken in a French pressure cell at 700 psi. After centrifugation (62,000 × *g*, 1 h, 4 °C), the pellet was resuspended in 25 mM Tris/HCl, 10 mM MgCl$_2$, 0.02% NaN$_3$, 1 M NaCl, 20% glycerol, pH 7.5, and stirred for 2 h at 6 °C. After another centrifugation (see above), the pellet was resuspended with 20 ml of 25 mM Tris/HCl, 10 mM MgCl$_2$, 0.02% NaN$_3$, 1 M NaCl, 2% Triton X-100, 20% glycerol, pH 7.5 (buffer A), and stirred for 18 h at 6 °C. The supernatant obtained after a third centrifugation (see above) contained the solubilized membrane proteins.

Purification of PBP1A Variants—The purification was performed in two chromatography steps. First, 20 ml of membrane extract (see above) was incubated with 2 ml of washed and equilibrated Ni^{2+}-nitrilotriacetic acid Superflow beads (Qiagen) for 2 h at 6 °C. The beads were spun down (4200 × *g*, 30 min, 4 °C) and washed twice with buffer A containing 5 mM imidazole. His-tagged PBP1A variants were eluted with buffer A containing 500 mM imidazole for 30 min at 6 °C. The eluate was dialyzed against 25 mM Tris/HCl, 10 mM MgCl$_2$, 0.02% NaN$_3$, 1 M NaCl, 10% glycerol, pH 7.5, for 4 h. For the removal of the His tag, 16 units of thrombin (restriction grade, Novagen) were added, and dialysis continued for 16–20 h. Then the sample was dialyzed for 1.5 h against 2 liters of buffer I (10 mM sodium acetate, 10 mM MgCl$_2$, 0.02% NaN$_3$, 500 mM NaCl, 10% glycerol, pH 4.8) and for 16 h against buffer I containing 200 mM NaCl. The sample was diluted 1-fold with 10 mM sodium acetate, 10 mM MgCl$_2$, 0.02% NaN$_3$, pH 4.8, and the protein was purified on a 1-ml HiTrap SP HP column
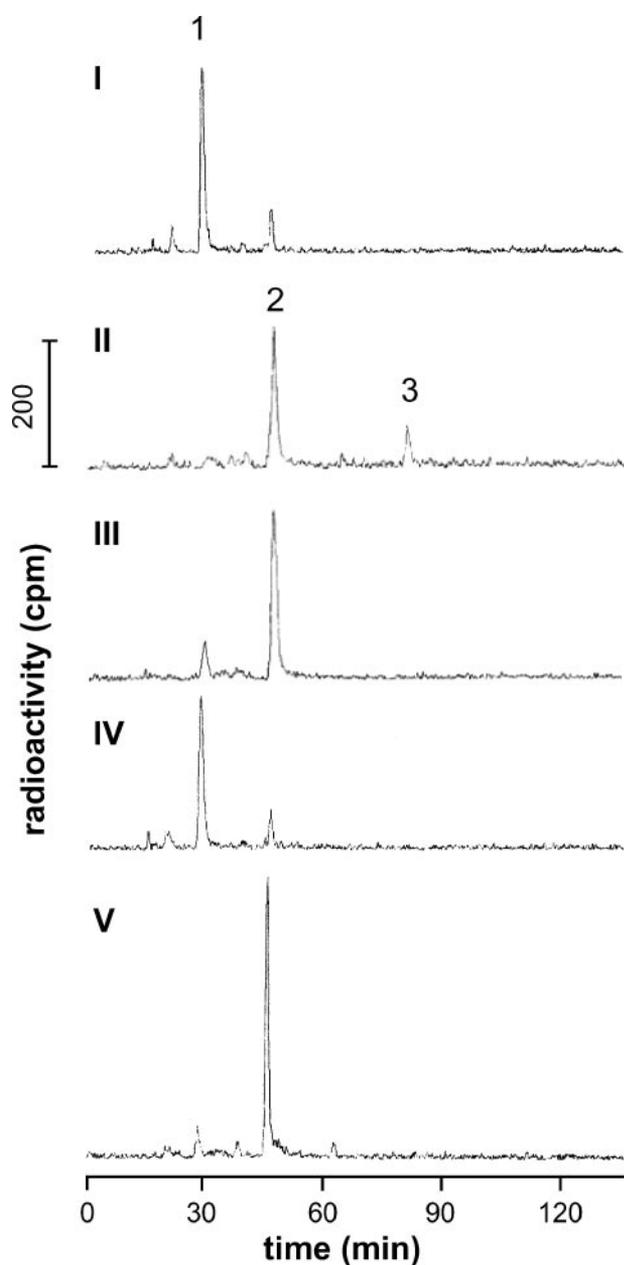
FIGURE 3. **Reaction of PBP1A variants with lipid II.** The reaction mixture contained radioactively labeled lipid II (24 μM) and either no enzyme (*I*) or PBP1A (0.8 μM) (*II*), PBP1A (0.8 μM) and 50 μg/ml penicillin G (*III*), PBP1A(TG*) (0.5 μM) with inactive transglycosylase domain (*IV*), or PBP1A(TP*) (0.5 μM) with inactive transpeptidase domain (*V*). The reaction products were boiled, digested with cellosyl, reduced with NaBH$_4$, and separated by HPLC with detection of radioactivity. Native PBP1A oligomerized lipid II to glycan strands and formed peptide cross-links (product *peaks 2* and *3*; see Fig. 4 for structures). The cross-linked product was not formed upon inactivation of PBP1A with penicillin G or with PBP1A(TP*). PBP1A(TG*) was inactive.

β-*Lactam Binding (PBP Assay)*—β-Lactam binding was assayed with biotinylated ampicillin as described previously (27).

Transglycosylation and Transpeptidation Reaction of PBP1A with Lipid II—Lipid II (1.2 nmol, 11,000 dpm) was vacuum dried and dissolved in 5 μl of methanol. The reaction was performed in a total volume of 50 μl in 25 mM Tris/HCl, 10 mM MgCl$_2$, 100 mM NaCl, 0.05% Triton X-100, pH 7.5 (buffer B), with 0.5–0.8 μM PBP1A variant. In different experiments, the precursor UDP-MurNAc-tripeptide, UDP-MurNAc-tetrapeptide, or UDP-MurNAc-pentapeptide was added 1 mM. In one experiment, the transpeptidation domain of PBP1A was blocked with 50 μg/ml penicillin G. The reaction was performed for 1 h at 30 °C. The pH of the samples was adjusted to 4.8 prior to boiling for 5 min followed by a digestion with 50 μg/ml cellosyl for 1 h at 37 °C and boiling for 5 min.

Time Course Experiments—In a total volume of 325 μl in buffer B, lipid II (24 μM) and PBP1A (0.8 μM) were incubated at 30 °C. At different time points (0, 5, 10, 15, 30, and 60 min), 50 μl-aliquots were taken and boiled for 5 min. The samples were chilled on ice, and cellosyl digestion was done as described above.

In a second experiment, glycan strands with monomeric peptide side chains were preformed with PBP1A(TP*) in a total volume of 425 μl for 1 h. The sample was boiled for 10 min, and PBP1A (0.8 μM) was added followed by an incubation for 10 min on ice. After the addition of lipid II (24 μM), the time course experiment was performed as described above.

Attachment of Newly Synthesized Material to Sacculi—PBP1A (0.8 μM) was preincubated for 10 min on ice with 0.5 mg/ml murein sacculi (isolated from MC1061) in buffer B. Lipid II (4.8 μM) was added, and the reaction proceeded in a final volume of 100 μl for 1 h at 30 °C. The sacculi were sedimented by centrifugation (14,000 × g, 10 min), and the pellet was washed twice with 200 μl of buffer A containing 0.2% Triton X-100. The pellet samples were digested for 18 h at 37 °C with 35 μg/ml cellosyl. The radioactivity of each sample (digested pellet, two wash fractions, and supernatant) was determined by scintillation counting.

For HPLC analysis, the reaction was performed in a final volume of 200 μl. PBP1A (0.8 μM) was incubated for 10 min on ice with or without 1 mg/ml sacculi (from MC1061). Lipid II (12 μM) was added, and the reaction was performed for 1 h at 30 °C. After sedimentation (14,000 × g, 10 min), the pellets were washed twice with 200 μl of buffer A containing 0.2% Triton X-100 and resuspended in 200 μl of 20 mM sodium phosphate, pH 4.8. The pH of the supernatant and of the washes was adjusted to 4.8, and the samples were digested with 17.5 μg/ml cellosyl for 18 h at 37 °C. Samples were boiled for 5 min.

Analysis of the Reaction Products—Prior to HPLC analysis, the samples were reduced with NaBH$_4$ as described (28). The reaction products were separated by HPLC according to a published method (25, 28), using a radioactivity flow-through detector (Canberra). The separated muropeptides were identified by their retention times with the help of muropeptide standards (19, 28). Compound 1 (Fig. 4*C*) could be dephosphorylated to compound 2 either by extended boiling or by treatment with an acidic phosphatase followed by reduction with NaBH$_4$ (19).

(GE Healthcare) in a 20-ml gradient from 100 mM NaCl and 0.2% Triton X-100 to 1 M NaCl and 0.2% Triton X-100. The PBP1A-containing fractions were dialyzed against 25 mM HEPES, 10 mM MgCl$_2$, 0.02% NaN$_3$, 200 mM NaCl, 10% glycerol, pH 7.5, and stored at −74 °C. The recombinant PBP1A obtained after removal of the His tag has an N-terminal extension of six amino acids and has the following sequence, GSHMAS-PBP1A(M1-F858).
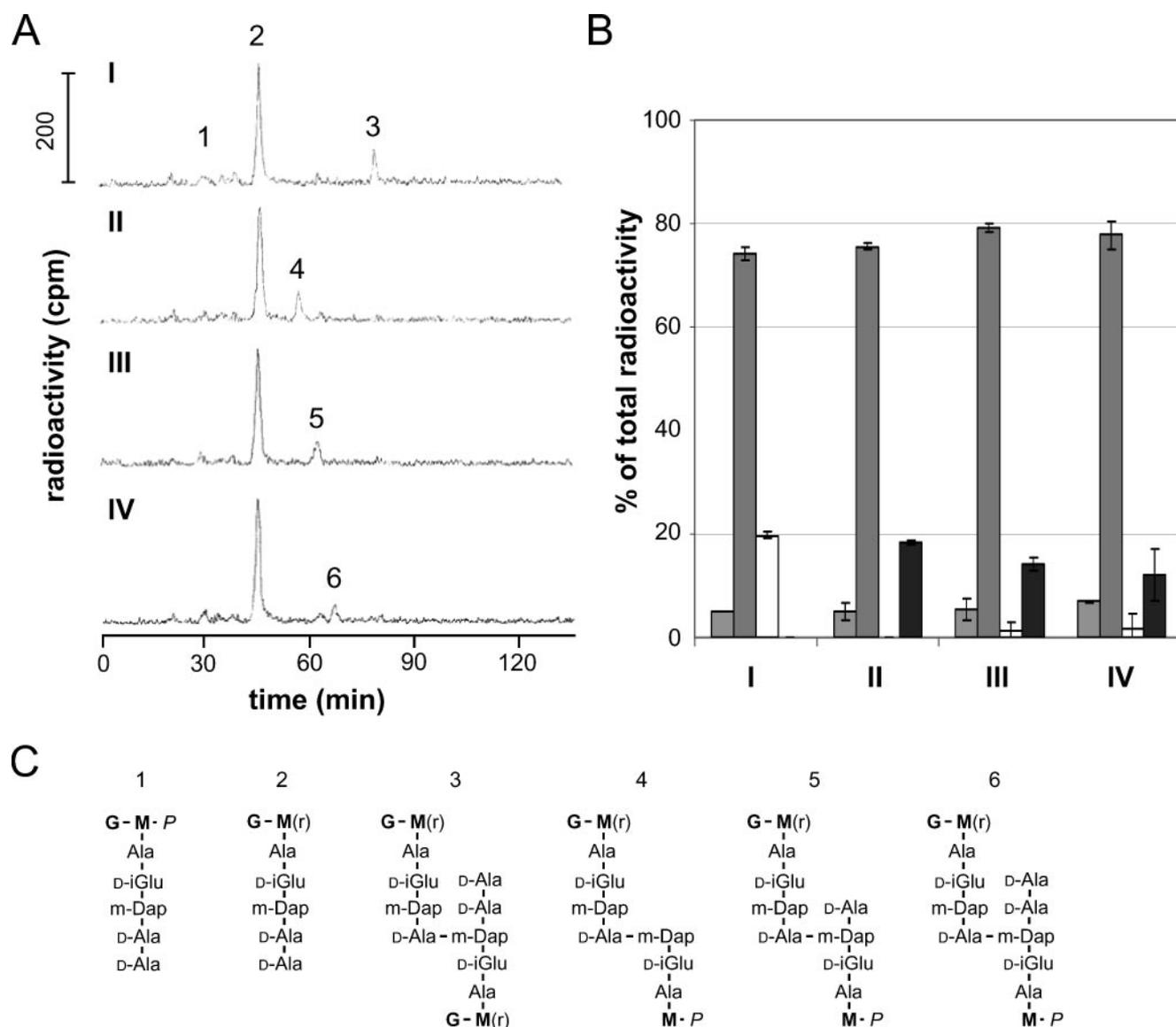
FIGURE 4. **Specificity of the transpeptidation reaction catalyzed by PBP1A.** PBP1A (0.8 μM) was incubated with lipid II (24 μM) in the absence or in the presence of different soluble murein precursors (1 mM) followed by cellosyl digestion, boiling for 10 min, and reduction with $NaBH_4$. The reaction products were analyzed by HPLC. *A*, chromatograms obtained from the sample with PBP1A and lipid II alone (*I*); PBP1A, lipid II, and UDP-MurNAc-tripeptide (*II*); PBP1A, lipid II, and UDP-MurNAc-tetrapeptide (*III*); and PBP1A, lipid II, and UDP-MurNAc-pentapeptide (*IV*). The different peaks are numbered. *B*, quantification of the reaction products (average of three independent experiments). *Numbers I–IV* correspond to the chromatograms *I-IV* in *A*. *Light gray bars*, peak 1; *dark gray bars*, peak 2; and *white bars*, peak 3. The *black bars* represent the percentage of compounds 5, 6, and 7. *C*, proposed structures of the muropeptides 1– 6. **G**, GlcNAc; **M**(r), reduced MurNAc; and *P*, phosphate. Note that compounds 1 and 2 are monomeric, and compounds 3– 6 are dimeric muropeptides. PBP1A was able to transfer the disaccharide tetrapeptide to tri-, tetra-, or pentapeptide structures yielding tetratri-, tetratetra-, or tetrapenta products.

RESULTS

PBP1A belongs to the class A PBPs and is anchored to the cytoplasmic membrane via a (predicted) transmembrane helix (amino acids 14–34). Most of the enzyme (amino acids 35– 858) is located in the periplasm, where it contains a transglycosylase and a transpeptidase domain. The modular domain organization of PBP1A is similar to that of PBP1B (Fig. 1). The five typical signature motifs in the transglycosylase domain and the three signature motifs in the transpeptidase domain were present in both enzymes. In PBP1B, Glu[233] and Ser[510] are essential for transglycosylation and transpeptidation activity, respectively (16). The corresponding residues in PBP1A are Glu[94] and Ser[473] (Fig. 1).

To study the enzymatic reactions of PBP1A, we cloned pET28a(+)-based expression plasmids for the purification of PBP1A and of two variants with single amino acid exchanges, PBP1A(TG*) with a Glu[94] $\rightarrow$ Gln exchange and PBP1A(TP*) with a Ser[473] $\rightarrow$ Ala exchange. Purification involved the preparation of membrane fraction, Ni^{2+}-nitrilotriacetic acid affinity chromatography, cleavage of the N-terminal oligo-histidine tag by thrombin, and cation exchange chromatography. Fig. 2 shows the purified proteins and a β-lactam binding assay. As expected, PBP1A and PBP1A(TG*), but not PBP1A(TP*), bound biotinylated ampicillin.

The activities of the PBP1A variants were studied with radioactive lipid II substrate employing a recently developed *in vitro*
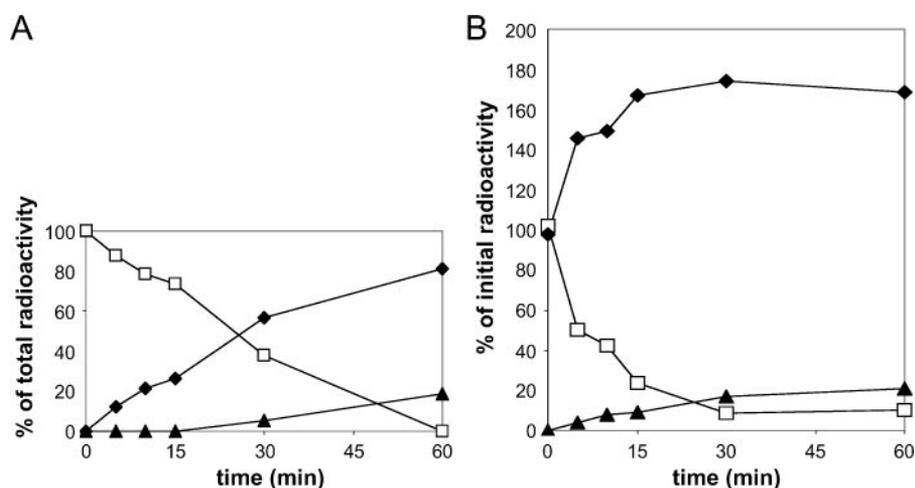
FIGURE 5. **Time course of the PBP1A-catalyzed transglycosylation and transpeptidation reactions.** *A*, radioactively labeled lipid II (24 μM) was incubated with PBP1A (0.8 μM) in a total volume of 325 μl at 30 °C. At different time points, 50 μl of aliquots were withdrawn, boiled for 5 min, digested with cellosyl, reduced by NaBH$_4$, and analyzed by HPLC. *Open squares*, compound 1 (Fig. 4C), substrate plus chain ends; *closed diamonds*, compound 2 (Fig. 4C), transglycosylation product; *closed triangles*, compound 3 (Fig. 4C), dimeric transglycosylation-transpeptidation product. Initially, only transglycosylation product was detected. The transpeptidation product appeared after 15 min of reaction time. *B*, the mixture contained transglycosylation product (preformed by PBP1A(TP*) and lipid II), radioactively labeled lipid II, and PBP1A. Sampling and sample processing occurred as described in *A*. Symbols, as defined in *A*. In the presence of murein glycan strands, the transpeptidation reaction started immediately.
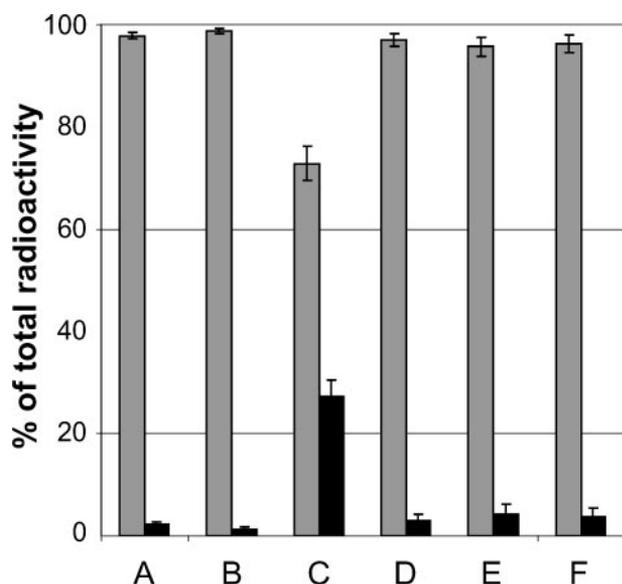

FIGURE 6. **Attachment of newly synthesized murein to sacculi by PBP1A.** PBP1A (0.8 μM) was incubated with radioactive lipid II (4.8 μM) in the presence of nonlabeled murein sacculi (0.5 mg/ml). The sacculi were sedimented by centrifugation and washed twice. The radioactivity in the supernatant and washes (*gray bars*) and in the murein sacculi (*black bars*) was determined by scintillation counting. The values were obtained in three independent experiments. Control samples: *A*, only lipid II and sacculi; *B*, only PBP1A and lipid II. *C*, sample with PBP1A, lipid II, and sacculi; *D*, PBP1A(TP*), lipid II, and sacculi; *E*, PBP1A(TG*), lipid II, and sacculi; *F*, PBP1A blocked with penicillin G, lipid II, and sacculi. PBP1A attached a fraction of the newly synthesized murein to sacculi.

murein synthesis assay (Fig. 3; see Ref. 19 for a scheme). Briefly, boiling of lipid II yielded the phosphorylated disaccharide pentapeptide (Fig. 4C, compound 1). Because of partial dephosphorylation during the boiling and reduction steps, a small fraction (0–15%) of nonphosphorylated disaccharide pentapeptide was formed. Upon reaction with a transglycosylase, glycan strands were produced. Digestion with cellosyl and boiling yielded

nonphosphorylated disaccharide pentapeptide (Fig. 4C, compound 2) and phosphorylated disaccharide pentapeptide, the latter originating from glycan chain ends and nonreacted lipid II. If a transpeptidase is present, the procedure also yields a dimeric (cross-linked) muropeptide, the bisdisaccharide tetrapentapeptide (Fig. 4C, compound 3). PBP1A oligomerized glycan strands with an average length of 19.6 disaccharide units and formed peptide cross-links (Fig. 3). In different experiments, between 18 and 26% of the peptides became part of cross-links. PBP1A(TP*) produced glycan strands with an average of 10.4 disaccharide units and completely lacked peptide cross-linking activity, as did PBP1A that was blocked with penicillin G. PBP1A(TG*) did not produce glycan strands and did not form peptide cross-links.

The specificity of the transpeptidation reaction was studied by adding to the reaction of PBP1A with lipid II a 42-fold molar excess (over lipid II) of artificial substrates, the murein precursors UDP-MurNAc-tripeptide, UDP-MurNAc-tetrapeptide, or UDP-MurNAc-pentapeptide (Fig. 4). Addition of the UDP compounds almost completely prevented cross-linking reactions between the two lipid II molecules, as demonstrated by the small fraction of the normal bisdisaccharide tetrapentapeptide (Fig. 4, compound 3). PBP1A could use tri-, tetra-, and pentapeptide substrates as acceptors in transpeptidation reactions, leading to the formation of "mixed" transpeptidation products (Fig. 4C, compounds 4, 5, and 6). As expected, the formation of the mixed compound 4 (Fig. 4C) could be completely prevented by the preincubation of PBP1A with penicillin G, and compound 4 was not formed from lipid II and UDP-MurNAc-tripeptide by PBP1A(TP*) (data not shown).

Subsequently, we studied the time course of the reaction of PBP1A with lipid II. Strikingly, only transglycosylation (but not transpeptidation) occurred within the first 15 min of the reaction under consumption of ~25% of the available lipid II (Fig. 5A) and with a rate of lipid II consumption of ~0.75 mol/(mol PBP1A · min). Between 15 and 60 min, transglycosylation and transpeptidation reactions proceeded until almost all lipid II was consumed. Peptide cross-links were formed at a rate of ~0.06 mol/(mol PBP1A · min). The absence of initial transpeptidation activity indicates that PBP1A requires the presence of oligomerized glycan strands carrying monomeric peptides for cross-linking activity. Therefore, the time course experiment was repeated but this time in the presence of oligomerized glycan strands that had been preformed from lipid II by PBP1A(TP*). Indeed, if glycan strands with monomeric peptides were present, the transpeptidation reaction started imme-

diately, with cross-links being formed with a rate of ~0.06 mol/(mol PBP1A · min) (Fig. 5*B*).

The time course experiments indicated that polymeric murein and not lipid II precursor could be the preferred acceptor for the PBP1A-catalyzed cross-linking reactions. Therefore, we tested in a pull-down experiment whether PBP1A was able to attach newly synthesized murein to isolated sacculi (Fig. 6). In the absence of murein sacculi, the reaction product of PBP1A and lipid II was not sedimented by centrifugation (Fig. 6, sample *B*). In the presence of sacculi, ~25% of the total radioactivity sedimented together with the sacculi (Fig. 6, sample *C*), indicating that a fraction of the newly synthesized murein had been covalently attached to the sacculi. No attachment to sacculi occurred with PBP1A(TG*) or with PBP1A(TP*) (Fig. 6, samples *D* and *E*). The PBP1A-catalyzed attachment of new material to sacculi could be blocked with penicillin G (Fig. 6, sample *F*), suggesting that the attachment occurred via transpeptidation reactions.

To determine the structure(s) of the attachment site(s), the reaction products were sedimented by centrifugation, and the supernatant, as well as the sedimented murein sacculi, was digested with cellosyl followed by reduction and HPLC analysis (Fig. 7). The reaction of PBP1A and lipid II was performed in the presence and absence of murein sacculi. In the absence of murein sacculi (Fig. 7*A*, *left two bars*), 98.1% of the radioactivity was present in the supernatant, and the produced murein had an average glycan strand length of 18.2 disaccharide units, with 26.4% of the peptides being part of cross-links. In the presence of murein sacculi (Fig. 7*A*, *right two bars*), ~44% of the total radioactivity was sedimented together with the sacculi, and ~56% of the total radioactivity was present in the supernatant. HPLC analysis revealed a different muropeptide composition in the supernatant and in the pellet (Fig. 7*B*); the quantification of the respective compounds is depicted in *C*. The supernatant (Fig. 7*B*, chromatogram *III*) contained a similar muropeptide profile as the sample without murein sacculi (chromatogram *I*), except that the average glycan strand length was decreased to 6.1 disaccharide units. In the pellet, the average glycan strand length was >40 disaccharide units, and it contained additional radioactive cross-linked compounds (Fig. 7*B*, chromatogram *IV*), the bisdisaccharide tetratripeptide and the bisdisaccharide tetratetrapeptide. These compounds could only be produced by cross-linking reactions between new pentapeptides (from radioactive lipid II) as donors and monomeric tri- and tetrapeptides in the sacculi as acceptors. Thus, the attachment of new material to sacculi by PBP1A occurred by transpeptidation reactions with monomeric (and not with cross-linked dimeric or trimeric) acceptors.

DISCUSSION

PBP1A formed *in vitro* a cross-linked, oligomeric murein from lipid II precursor. As predicted from sequence comparison (Fig. 1), the Glu[94] and Ser[473] residues are essential for the catalytic activities. Although the PBP1A(TP*) variant with inactive transpeptidase domain could still catalyze transglycosylation reactions, a PBP1A(TG*) variant with inactive transglycosylase domain lacked both transglycosylase and transpeptidase activities. Similar observations have been made with inactive variants of PBP1B from *E. coli* (16). Transpeptidation by PBP1A(TG*) occurred neither in the presence of an oligomeric reaction product produced by PBP1A(TP*) nor in a reaction mixture containing PBP1A(TG*), PBP1A(TP*), and lipid II (data not shown). Therefore, it is possible that the transpeptidase domain of PBP1A is activated by ongoing transglycosylation reactions and that the cross-linking occurs preferentially with pentapeptide donors in the glycan strands produced by the transglycosylase domain.

The time course experiments of the reaction of PBP1A with lipid II revealed that the cross-linking reaction was delayed and started only after glycan strands with monomeric pentapeptides had been produced by transglycosylation. If such material was added initially to PBP1A and lipid II, formation of cross-links started immediately and proceeded until 21% of the newly added peptides became part of cross-links. On the other hand, only 2% of the peptides became part of cross-links if oligomerized glycan strands were incubated with PBP1A alone (without lipid II) for 60 min (not shown). Apparently, transpeptidation requires ongoing transglycosylation (see above) and, in addition, the presence of oligomerized glycan strands carrying monomeric peptides. Either binding of the enzyme to this oligomeric material activates the transpeptidase function or the oligomeric material is a substrate for the transpeptidation reaction (or both possibilities). We have obtained evidence that monomeric peptides in high molecular weight murein function as acceptors in PBP1A-catalyzed cross-linking reactions. First, a fraction of newly synthesized murein was covalently attached to murein sacculi, and this attachment was inhibited by penicillin G, an inhibitor of the transpeptidation reaction (Fig. 6). Second, we identified dimeric muropeptides with tetratripeptide and tetratetrapeptide cross-links as products, proving that cross-linking reactions had occurred between pentapeptides (from lipid II and/or newly synthesized glycan strands) as donors and tri- and tetrapeptides in the sacculi as acceptors (Fig. 7). The observed cross-linking reactions of PBP1A, with acceptor peptides in nascent murein or in murein sacculi, are depicted schematically in Fig. 8. Interestingly, the formation of

FIGURE 7. **Attachment of newly synthesized murein to sacculi by PBP1A-catalyzed transpeptidation reactions.** PBP1A (0.8 μM) reacted with radioactive lipid II (12 μM) in the absence and presence of nonlabeled murein sacculi (1 mg/ml). The samples were centrifuged, and the supernatant and pellet were treated with cellosyl. *A*, an aliquot of the samples was taken to quantify the radioactivity in the supernatants (*gray bars*) and pellets (*black bars*). *B*, the samples were reduced by NaBH$_4$ and analyzed by HPLC using a radioactivity detector. Chromatograms *I–IV* correspond to the samples *I–IV* in *A*. Chromatogram *V* shows the UV profile of a digestion of sacculi with cellosyl. Note that sacculi contained only minor amounts of pentapeptide compounds. *C*, quantification of the radioactive peaks in chromatograms *I–IV*. *Dashed bar*, peak 1; *light gray bar*, peak 2; *white bar*, peak 3; *black bar*, peak C; *dark gray bar*, peak B. *D*, proposed structures of *peaks 1, 2, 3, A, B*, and *C*. *G*, GlcNAc; *M(r)*, reduced MurNAc; *P*, phosphate. In the absence of sacculi, the radioactive transglycosylase and transpeptidase products were present only in the supernatant. In the presence of sacculi, 44% of the newly synthesized murein was present in the pellet. Attachment of newly synthesized murein to sacculi occurred via cross-linking (transpeptidation) reactions to tri- and tetrapeptides in the sacculi, yielding radioactive compounds *B* and *C* (chromatogram *IV*).
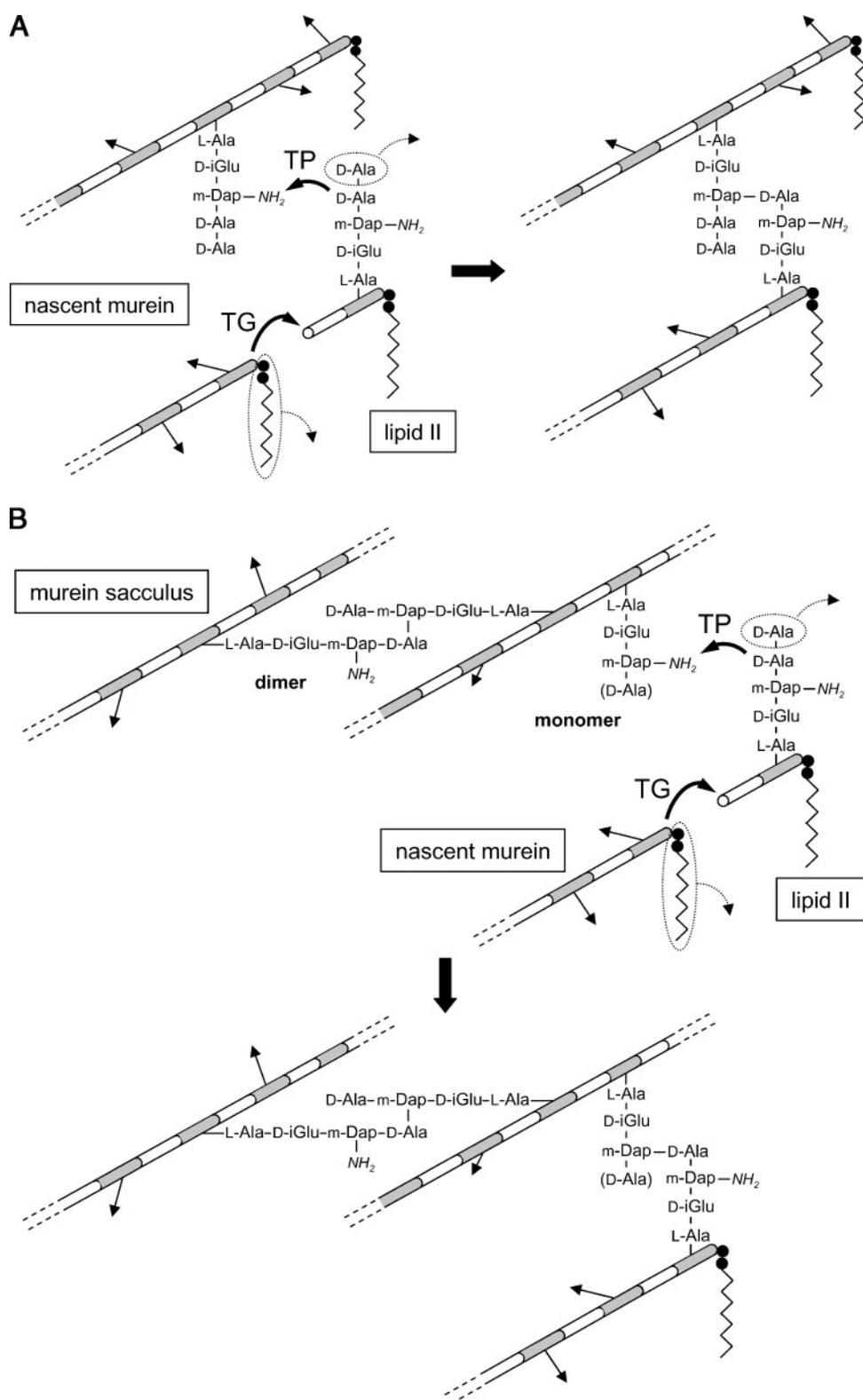
In Vitro Murein Synthesis by PBP1A

FIGURE 8. **Schemes for TG and TP reactions catalyzed by PBP1A.** PBP1A polymerizes lipid II by transglycosylation and simultaneously attaches the growing glycan strand by transpeptidation to monomeric peptides, present either in another newly synthesized glycan strand (*A*) or in a murein sacculus (*B*). The leaving groups (undecaprenyl pyrophosphate and D-alanine) are encircled by a *dashed line*. The cross-linking reaction with two pentapeptides (*A*) yields a tetrapentapeptide product, whereas the cross-linking reaction between a pentapeptide (donor) and a tri- or tetrapeptide acceptor yields a tetratri- or tetratetrapeptide cross-link. Free amino groups at meso-diaminopimelic acid residues that represent possible acceptor sites for transpeptidation reactions are indicated (NH_2). Glycan strands are shown as *rods* with *gray* (MurNAc) and *white* (GlcNAc) parts. *Straight arrow*, peptide; *black circle*, phosphate group; *zigzag line*, undecaprenyl residue.

cross-links between nascent murein and sacculi did not take place at the expense of the formation of cross-links between new (pentapeptide) donors and acceptors; in the presence of sacculi, ~23% of the peptides became part of tetrapentapeptide cross-links (in the attached material), which was only slightly less than in the murein produced in the absence of sacculi (~26%) (Fig. 7). In this experiment, a total of ~30% of the pentapeptides (originating from lipid II) had participated as donors in transpeptidation reactions, of which 11.5% reacted with pentapeptides (in lipid II) and 19% with tri- and tetrapeptides (in sacculi) as acceptors.

PBP1A and PBP1B are similar in their *in vitro* activities in that both can use artificial tri-, tetra-, and pentapeptides (in UDP-linked precursors) as acceptors for transpeptidation reactions (19). Such reactions have been observed before in ether-permeabilized cells (29, 30). On the other hand, PBP1A and PBP1B are different with respect to several properties. (i) The transglycosylation and transpeptidation rates were significantly higher for PBP1B. (ii) Under optimum condition, PBP1B produced a murein with almost 50% of the peptides being present in cross-links. With PBP1A, we observed a maximum of 26% of the peptides becoming part of cross-links. (iii) PBP1B produced a trimeric compound by cross-linkage of a pentapeptide donor and a tetrapentapeptide acceptor. A previous study reported the production of a trimeric cross-linked product by PBP1A (21). We have observed in some experiments the formation of a putative trimeric product only in small quantities (Fig. 7*B*, chromatogram *I*, see peak at 100 min). It is possible that the differences in the enzyme preparation methods and, hence, in the purities of the enzyme account for this discrepancy. (iv) Although both PBP1A and PBP1B required the presence of Triton X-100 for solubilization, the activities of PBP1A, but not of PBP1B, significantly decreased if the concen-

trations of the detergent exceeded 0.2% (data not shown). (v) PBP1B was most active as a dimer, whereas dimerization of purified PBP1A was not observed. (vi) Unlike in the case of PBP1A, time course experiments did not reveal a preference of PBP1B for polymerized murein glycan strands with monomeric peptides as acceptors for the transpeptidation reaction. In addition, we have observed that PBP1B attached a significantly smaller fraction of nascent murein to sacculi *in vitro* as compared with PBP1A (data not shown). It is possible that differences in the activities of both enzymes account for the different phenotypes of mutants lacking either PBP1A or PBP1B (see the Introduction).

For the first time, we could demonstrate *in vitro* an attachment of nascent murein to sacculi, a reaction that occurs in living cells during the growth of the sacculus. Similar to our *in vitro* experiments, it has been shown that incorporation of new material *in vivo* involves the formation of new cross-links; during cell division, cross-links are formed mainly between two new peptides, whereas during cell elongation, cross-links are also formed between new peptides and peptides in the existing sacculus (31–34). *In vitro*, PBP1A most likely attached new material without increasing the surface of the sacculi. The enlargement of the sacculus *in vivo* is a more complex process and presumably requires a coordinated interplay between different bifunctional and monofunctional murein synthases, murein hydrolases, and regulatory proteins. The precise role of PBP1A in the enlargement of the sacculus during cell elongation and division remains to be determined. Our results indicate that PBP1A might synthesize patches of nascent murein and attach them to the sacculus.

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