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Further Evidence that a Cell Wall Precursor [C_{55}-MurNAc-(Peptide)-GlcNAc] Serves as an Acceptor in a Sorting Reaction

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Previous studies suggested that a Gly-containing branch of cell wall precursor [C_{55}-MurNAc-(peptide)-GlcNAc], which is often referred to as lipid II, might serve as a nucleophilic acceptor in sortase-catalyzed anchoring of surface proteins in *Staphylococcus aureus*. To test this hypothesis, we first simplified the procedure for in vitro biosynthesis of Gly-containing lipid II by using branched UDP-MurNAc-hexapeptide isolated from the cytoplasm of *Streptomyces* spp. Second, we designed a thin-layer chromatography-based assay in which the mobility of branched but not linear lipid II is shifted in the presence of both sortase and LPSTG-containing peptide. These results and those of additional experiments presented in this study further suggest that lipid II indeed serves as a natural substrate in a sorting reaction.

Surface proteins of *Staphylococcus aureus* and other gram-positive bacteria are considered to be essential for the initial establishment of infection, as they are involved in both attachment of bacteria to host tissue matrix proteins and evasion of the host immune response (17). Most of the known staphylococcal surface proteins are covalently attached to the cell wall by a mechanism that requires a well-conserved C-terminal LPXTG motif (17, 23). Both genetic and biochemical data suggest that a specific enzyme, sortase, catalyzes both the proteolytic cleavage of the LPXTG motif between Thr and Gly residues and the transpeptidation reaction that links the carboxyl group of Thr to the amino group of the pentaglycine cross-bridge in *S. aureus* (14, 27). Analysis of the cell wall anchor structures from various *fem* mutants showed that surface proteins were linked to cross-bridges containing one, three, or five glycyl residues but not directly to the ε-amino group of lysine (26). As shown recently, sortase utilizes NH_2-$(Gly)_n$ (n = 1 to 3) as an in vitro nucleophile acceptor, confirming the ability of sortase to catalyze a transpeptidation reaction by using the NH_2 group of glycine as a specific acceptor site (28).

Previous experiments left unresolved the identity of the natural acceptor molecule for the sortase-catalyzed transpeptidation reaction. It has been shown that the sorting reaction is inhibited by vancomycin and to a lesser extent by moenomycin but not by penicillin (29). In staphylococcal protoplasts, cleavage of surface protein precursors and formation of putative transpeptidation products were observed (29). These results suggested that the sorting reaction might not require assembled cell wall to proceed and that the Gly-containing branch of membrane-anchored peptidoglycan (PG) precursor lipid II [C_{55}-MurNAc-(peptide)-GlcNAc] might serve as an acceptor site in a sortase-catalyzed transpeptidation reaction.

Lipid II is a minor component of bacterial cell membrane which can be detected by thin-layer chromatography (TLC) separation of presolubilized membranes supplied with the cytoplasmic precursors, UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and [^{14}C]UDP-*N*-acetylglucosamine ([^{14}C]UDP-GlcNAc) (25). In *S. aureus*, the formation of branched lipid II molecules by the sequential addition of glycine residues to the third position of the peptide moiety is catalyzed at the membrane level by the FemABX family of enzymes (12). It has been postulated that in *S. aureus* the addition of the first glycine to the conserved lysine residue of UDP-MurNAc-pentapeptide precursor occurs by FemX activity preferentially after conjugation of the precursor to the lipid carrier (13), although traces of UDP-MurNAc-hexapeptide could be detected in the cytoplasm (2). FmhB is considered to be the primary candidate for FemX, even though the enzymatic activity of FmhB remains to be demonstrated (21).

The in vitro biosynthesis of branched lipid II of *S. aureus* requires whole-cell membranes, cytoplasmic PG precursors, glycine (^{14}C labeled for detection of reaction products), purified tRNA, and an intracellular fraction that contains tRNA-activating enzymes (13). Therefore, the quantitative isolation of staphylococcal glycine-containing lipid II from the complex whole-cell mixtures is a tedious procedure. One way to facilitate this procedure is to use cytoplasmic Gly-containing branched UDP-MurNac-hexapeptide precursor. The Ala-containing branched UDP-MurNac-hexapeptide is relatively abundant in the cytoplasm of *Weissella viridescens* (formerly *Lactobacillus viridescens*) (10). Recombinant FemX protein from this organism was purified, and its in vitro activity was demonstrated (10). The distinctive feature of this enzyme is that it catalyzes the addition of a branching amino acid (Ala for *W. viridescens*) before the formation of membrane-bound cell wall precursor. This prompted the search for bacterial species

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that would contain similar Gly-containing branched precursors in their cytoplasm.

The present study reports the isolation of Gly-containing branched UDP-MurNac-hexapeptide from the cytoplasm of *Streptomyces* spp. The use of this precursor facilitated the in vitro biosynthesis of branched lipid II and allowed the testing of both branched and linear forms of lipid II in the sortase-catalyzed transpeptidation reaction. This study presents the evidence that branched but not linear lipid II serves as a natural acceptor in the sorting reaction.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study were *Staphylococcus aureus* ATCC 29213, *Streptomyces lydicus* ATCC 25470, *Streptomyces lividans* 1326 (John Innes Center, Norwich, United Kingdom), and *Streptomyces hygroscopicus* NS17. *Streptomyces* strains were grown in Trypticase soy broth (TSB) at 28°C with aeration. *S. aureus* ATCC 29213 was grown in brain heart infusion (BHI) broth at 37°C with aeration.

Isolation of PG nucleotide precursors from *Streptomyces*. The pool of cytoplasmic UDP-linked PG precursors was extracted by a modification of a previously described method (19). Cells were grown to mid-logarithmic phase, chilled, harvested by centrifugation, washed in 0.9% NaCl, and extracted with cold trichloroacetic acid (TCA) (final concentration of 5%) for 30 min at 4°C. The extract was desalted on Sephadex G-25 and concentrated by rotary evaporation. High-pressure liquid chromatography (HPLC) separation of precursors was performed essentially by the method of Flouret et al. (8) with some modifications. The precursors were separated by reverse-phase HPLC on a μBondapak C_{18} column (3.9 by 300 mm; Waters) in 50 mM ammonium formate (pH 3.9) at a flow rate of 0.5 ml/min. The elution of precursors was monitored at a wavelength of 254 nm. The approximate yields were 35 nM UDP-MurNAc-Ala-Glu-A_2pm (L,L-diaminopimelic acid)-Ala-Ala and 4 nM UDP-MurNAc-Ala-Glu-A_2pm (Gly)-Ala-Ala per gram of bacterial pellet (dry weight).

MS. Electrospray ionization tandem mass spectrometry (ESI/MS/MS) was performed using a Micromass Q-ToF mass spectrometer equipped with a nanoelectrospray source. Samples, adjusted to a pH of 3, were sprayed from a metal-coated capillary purchased from New Objectives. Instrumental settings were as follows: capillary voltage, 900 V; cone voltage, 30 V; and collision energy, 40 eV. Doubly charged ions, $(M + 2H)^{2+}$, were selected as a precursor ions for MS/MS studies.

In vitro PG biosynthesis assay. The in vitro PG biosynthesis assay was performed as described previously (1, 25) with some modifications including the addition of 0.1% Triton X-100 to the reaction mixture and the preincubation of membranes both prior to the addition of UDP-MurNAc-peptides (30 min on ice) and prior to the addition of UDP-[^{14}C]GlcNAc (10 min, room temperature). The reaction mixture contained 0.05 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1% Triton X-100 in a final volume of 14 μl, cell membrane fractions isolated from mid-exponential cultures (20 μg of protein), 33 μM appropriate UDP-MurNAc-peptide precursor (when added), and 5.7 μM UDP-[^{14}C]GlcNAc (250 mCi/mmol; American Radiolabeled Chemicals, Inc.). The A_2pm-containing precursors were isolated from *S. lividans* as described above, and the Lys-containing precursor was isolated by a similar procedure from TCA extracts of *S. aureus* ATCC 29213. When required, the samples were preincubated with 50 μg of ramoplanin (kindly provided by Franco Parenti, Biosearch Italia) per ml for 10 min at room temperature prior to the addition of UDP-[^{14}C]GlcNAc. Upon the addition of all reagents, the reaction mixtures were incubated for 30 min at room temperature and placed in a boiling water bath for 2 min to inactivate enzymes and thus prevent degradation of lipid II. Aliquots (2 μl) of the samples were separated by TLC on silica gel plates (K6; Whatman) for 2 h in isobutyric acid–1 M NH_4OH (5:3, vol/vol). After the aliquots were separated, the plates were dried and exposed to X-ray film (BioMax MS; Kodak). The final concentration of lipid II in the samples was estimated to be ≤0.5 μM, based on the estimate that ≤10% of added UDP-[^{14}C]GlcNAc was converted to lipid II.

The nature of the radioactive material retained on TLC start line was investigated by treating the material with *N*-acetylmuramidase by the previously published procedure (11, 13), except that mutanolysin was used instead of egg white lysozyme. The radioactive material was scraped from the TLC plate start line and resuspended in 25 mM phosphate buffer (pH 5.5). The samples were incubated overnight at 37°C either with or without mutanolysin (final concentration, 200 μg/ml; Sigma) and separated by TLC as described above.

In vitro sortase-catalyzed transpeptidation reaction. The LPXTG-containing peptide α-Dabsyl-KRQLPSTGET[E-Edans]NPFF (kindly provided by C. Hal Jones, Siga Pharmaceuticals) (which we will refer to as LPSTG peptide hereafter), where Dabsyl and E-dans stand for (4-4′-dimethylaminophenazo)benzoyl and 5-[(2′-aminoethyl)-amino]-naphthalene-1-sulfonic acid, respectively, was incubated in the presence and absence of purified sortase (Siga Pharmaceuticals) with membrane preparations, containing either linear or branched lipid II. A typical sample contained a 4-μl aliquot of an appropriate heat-inactivated PG biosynthesis reaction mixture (see above), 20 mM HEPES (pH 8.0), 10 mM $CaCl_2$, 44.45 μM LPSTG peptide, and 15 μg of sortase (when added) in a final volume of 20 μl. When required, the reaction mixture also contained either 10 mM NH_2-$(Gly)_3$ or 0 to 100 μg of vancomycin per ml. Samples were incubated for 1 h at room temperature, and 12-μl aliquots of the samples were separated by TLC as described above. After the samples were separated, the plates were dried and exposed to X-ray film.

RESULTS

Isolation of glycine-containing branched muropeptide. To identify bacterial species that might contain branched PG precursors in their cytoplasm, a search for homologs of FemX of *W. viridescens* was performed. The deduced amino acid sequence similarity search in the GenBank database identified FemX of *W. viridescens* as most similar to three putative proteins SCH24.26c, SCF55.26, and SC66T3.04 of *Streptomyces coelicolor* (24 to 25% identity and 36 to 40% similarity over a 357- to 365-amino-acid stretch), followed by MurM of *Streptococcus pneumoniae* (24% identity and 37% similarity over a 223-amino-acid stretch). This suggested that *S. coelicolor* and possibly other *Streptomyces* spp. might be able to synthesize branched UDP-MurNAc-peptide precursors in their cytoplasm. Because the cell walls of *Streptomyces* spp. contain A_2pm and, as suggested, are cross-linked via single Gly residues (22), the pools of cytoplasmic UDP-linked precursors of several *Streptomyces* species, such as *S. lividans*, *S. lydicus*, and *S. hygroscopicus*, were tested for the presence of UDP-MurNAc-Ala-Glu-A_2pm(Gly)-Ala-Ala. The HPLC profile of precursors isolated from *S. lividans* is shown in Fig. 1. Similar profiles were obtained for *S. lydicus* and *S. hygroscopicus* (data not shown). The two peaks with retention times of 24.2 min (peak 1) and 37.5 min (peak 2) have molecular weights of 1,193 and 1,250, respectively, as determined by ESI/MS. Based upon their ESI/MS/MS, the structures for peaks 1 and 2 were assigned to UDP-MurNAc-Ala-Glu-A_2pm-Ala-Ala (referred to as linear precursor hereafter) and UDP-MurNAc-Ala-Glu-A_2pm(Gly)-Ala-Ala (referred to as branched precursor hereafter), respectively. Figure 2 shows the positive-ion ESI/MS/MS and structural assignments for the doubly charged parent ion for peak 2. Two product ions of particular importance were the fragment ions with *m/z* of 390.1 and 687.4, demonstrating that the Gly residue is attached to the A_2pm residue. These results suggest the presence of a FemX-like activity in the cytoplasm of *Streptomyces* species.

In vitro biosynthesis of Gly-containing branched lipid II. In order to test whether both linear and branched precursors could be incorporated into lipid II, both precursors as well as staphylococcal UDP-MurNAc-Ala-Glu-Lys-Ala-Ala were used in an in vitro PG biosynthesis assay with membranes isolated from either *S. lividans* or *S. aureus*. The membranes of *S. lividans* were completely inactive in the PG biosynthesis assay (data not shown). This could possibly be due to nonoptimal reaction conditions and/or due to the lack of some important

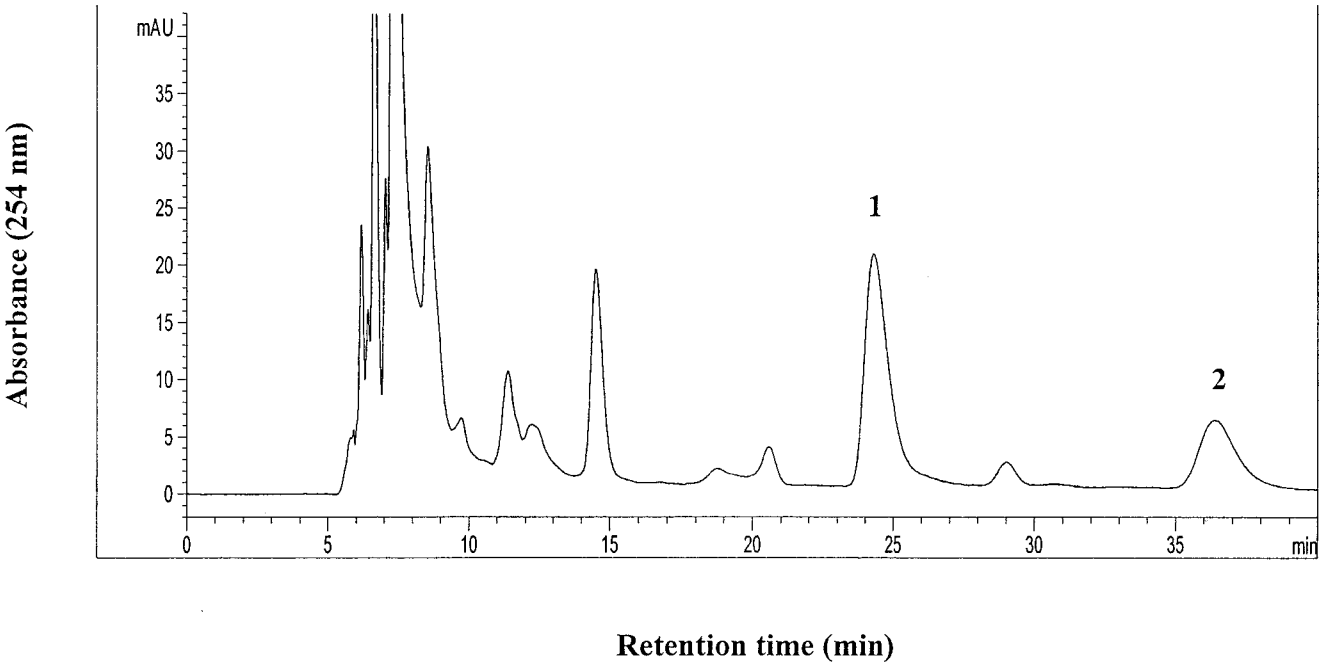

FIG. 1. HPLC profile of cytoplasmic PG precursors isolated from *S. lividans.* Shown above is the separation of the pool of cytoplasmic UDP-linked PG precursors by reverse-phase HPLC. Peaks 1 and 2 with retention times of 24.2 and 37.5 min correspond to UDP-MurNAc-Ala-Glu-A_2pm-Ala-Ala and UDP-MurNAc-Ala-Glu-A_2pm(Gly)-Ala-Ala, respectively.

cofactor(s). In contrast, staphylococcal membranes synthesized both lipid II and PG regardless of the type of precursor supplied (Fig. 3). The assignments of lipid II and PG spots were confirmed by treatment with ramoplanin, a known lipid II synthesis inhibitor (25), as none of those spots were present on TLC autoradiogram of ramoplanin-treated samples (Fig. 3). This result is consistent with the previously reported data on the lack of stringent specificity of both transferases and transglycosylases for the amino acid composition of peptide chain (in this case the only difference is A_2pm in *Streptomyces* species versus Lys in *S. aureus*) (3, 16, 18, 20, 30, 31). With the one exception of having A_2pm instead of Lys, which results in a slight decrease in TLC mobility (Fig. 3), the lipid II molecules that are synthesized by using precursors from *Streptomyces* are identical to the native staphylococcal lipid II.

The previous studies established that PG material remains on the start line during TLC separation (11, 13). Because in the present study staphylococcal cell membranes were supplied with either their native (Lys-containing) or nonnative (A_2pm-containing) UDP-linked peptide precursors, it was necessary to confirm the PG nature of the radioactive material retained on the TLC start line. This was performed by treatment with mutanolysin, *N*-acetylmuramidase from *Streptomyces globisporus* (6, 7, 24), which resulted in the release of TLC-mobile low-molecular-weight radioactive species (data not shown) similar to those observed in previous studies (11, 13). This result implies that the radioactivity retained on the TLC start line indeed represents PG. It also suggests, consistent with the results of previous experiments (11, 13), that this PG material lacks peptide cross-links, since cross-linked PG would remain polymerized (and would be retained on the TLC start line) even when glycosidic bonds were cleaved.

In vitro sortase-catalyzed transpeptidation reaction. Both linear and branched lipid II molecules were tested in a mobility shift sortase activity assay to determined whether those molecules might serve as the acceptors in the sortase-catalyzed transpeptidation reaction. It should be noted that prior to this assay all enzymatic activities that are present in the lipid II-containing membrane preparations and that are relevant to PG biosynthesis were inactivated by heating at 100°C. This ensured the stability of lipid II molecules and the lack of a possible competition between the sorting reaction and PG biosynthesis. In addition, the ability of staphylococcal sortase to cleave the T/G bond of LPSTG peptide and to catalyze a transpeptidation reaction with NH_2-Gly_3 was confirmed by ESI/MS analysis as described previously (28) (K. Tabei and M. M. Siegel, unpublished data). As shown in Fig. 4, an upward shift in lipid II mobility was detected when both sortase and LPSTG peptide were present in membrane preparations containing branched but not linear lipid II molecules (see lanes 2, 5, and 8). No mobility shift was detected either in the absence of sortase (Fig. 4, lanes 1, 4, and 7) or in the absence of LPSTG peptide substrate (not shown). Based on the previous studies (28), these results suggested that an observed shift in lipid II mobility is a result of a sortase-catalyzed proteolytic cleavage/transpeptidation reaction during which α-Dabsyl-KRQLPST (a cleavage product) is covalently linked to a Gly residue of branched lipid II. The increased mobility of the putative transpeptidation product versus that of lipid II

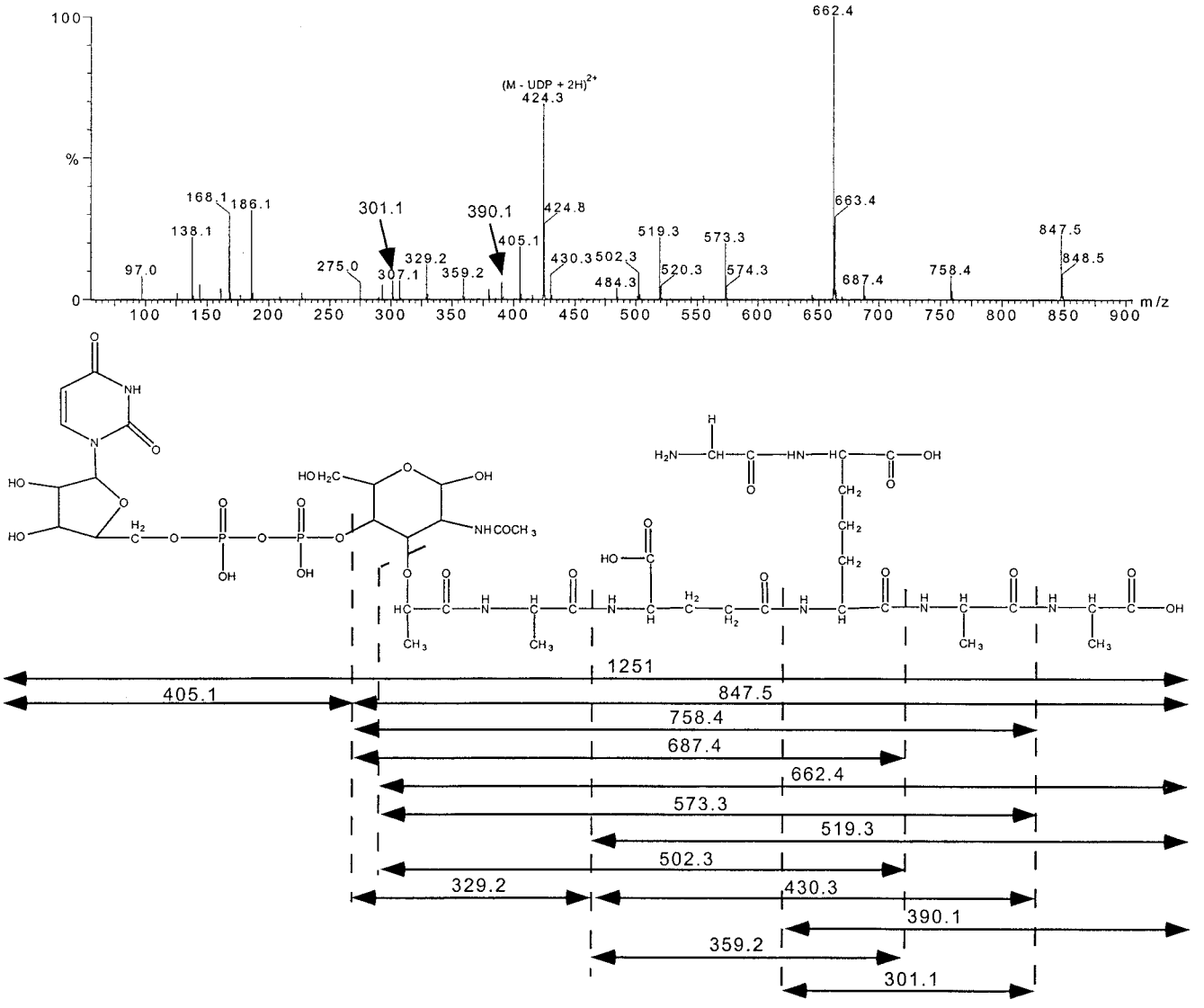

FIG. 2. Positive-ion ESI/MS/MS analysis of HPLC peaks. Peaks 1 and 2 were collected during HPLC of PG precursors (Fig. 1) and analyzed by ESI/MS/MS. The ESI/MS/MS of peak 2 and the structural assignments for the detected fragment ions for UDP-MurNAc-Ala-Glu-A_2pm(Gly)-Ala-Ala are shown. Note that the masses of the observed fragment ions (numbers above arrows) are 1 Da greater than the masses of the neutral structures due to the addition of H^+. Similar analysis was performed for peak 1 (data not shown).

most likely reflects the increase in hydrophobicity due to the covalent addition of a Dabsyl-containing peptide moiety. The fact that putative α-Dabsyl-KRQLPST-lipid II is detected only with branched lipid II but not with linear lipid II implies that the NH_2 group of branching Gly but not the NH_2 group of A_2pm (or Lys in *S. aureus*) serves as a specific nucleophile acceptor in the sorting reaction. Further supporting this hypothesis was the observation that excess NH_2-$(Gly)_3$ inhibited the formation of the putative transpeptidation product (Fig. 4, lane 3) presumably due to the competition between NH_2-$(Gly)_3$ and NH_2-Gly of the branched lipid II for the formation of amide linkage with α-Dabsyl-KRQLPST. In an additional test for the acceptor site specificity of the sorting reaction, staphylococcal sortase was substituted for that of *Streptococcus pyogenes* (kindly provided by Stephen Olmsted). Both staphylococcal and streptococcal sortases were confirmed by ESI/MS to cleave the LPSTG peptide at the T/G bond (Tabei and Siegel, unpublished). However, no mobility shift was detected with streptococcal sortase (data not shown), most likely due to the lack of specificity of this enzyme for NH_2-Gly, since the PG of *S. pyogenes* is cross-linked via Ala residues (22).

As shown previously, vancomycin inhibits the anchoring of surface proteins, presumably by altering "the physiological concentration of PG precursor molecules that may serve as a substrate for the sorting reaction" (29). Vancomycin binds to the D-Ala–D-Ala terminus of the lipid II peptide moiety (5, 9). The positioning of a bulky vancomycin molecule near the Gly-containing branch might cause a steric interference with the sortase-catalyzed transpeptidation reaction. Indeed, in this study vancomycin inhibits the formation of the putative transpeptidation product in a concentration-dependent manner

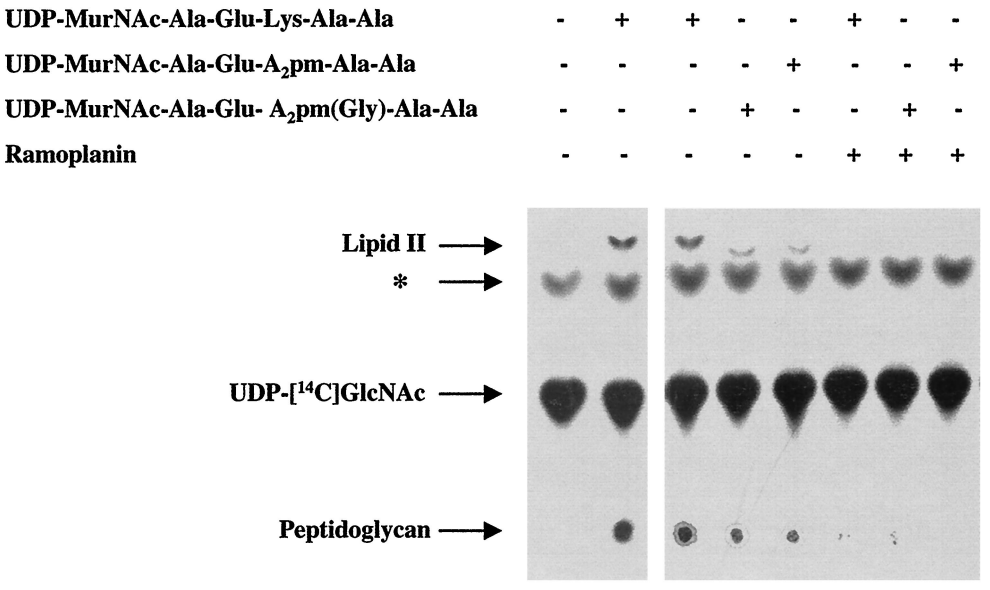

FIG. 3. TLC separation of in vitro PG biosynthesis reaction mixtures. Cell membranes were prepared from *S. aureus* ATCC 29213, solubilized, and supplied with UDP-[^{14}C]GlcNAc and an appropriate UDP-MurNAc precursor. Where indicated, the samples were treated with ramoplanin prior to the addition of UDP-[^{14}C]GlcNAc. Following incubation and inactivation by boiling, 2-μl aliquots of samples were separated by TLC on silica gel plates and the plates were dried and autoradiographed. The band of unknown nature (indicated by an asterisk) that is detected regardless of the presence (+) or absence (−) of UDP-MurNAc-peptide precursors might result from previously described translocation of [^{14}C]*N*-acetylglucosamine-1-phosphate from UDP-[^{14}C]GlcNAc to C_{55} lipid carrier (4, 15).

(Fig. 4, lanes 9, 10, and 11), supporting previous data and further suggesting that lipid II serves as a substrate for the sortase-catalyzed transpeptidation reaction.

It should be noted that according to densitometry analysis, only about 50% of branched lipid II molecules undergo a mobility shift in this assay (Fig. 4, lanes 2 and 8). This might be a consequence of either suboptimal reaction conditions or the reversible nature of the transpeptidation reaction.

DISCUSSION

The present study addressed a previously suggested hypothesis on the use of bacterial cell wall precursor lipid II as a natural substrate in the sortase-catalyzed anchoring reaction (28, 29). First, an in vitro biosynthesis of Gly-containing branched lipid II, which is required for sortase activity assay, was simplified by using a branched precursor, UDP-MurNAc-Ala-Glu-A_2pm(Gly)-Ala-Ala, isolated from TCA extracts of several *Streptomyces* species. The presence of such a precursor in the cytoplasm of *Streptomyces* spp. was predicted based on the presence of putative proteins that are similar to FemX from *W. viridescens*, a known cytoplasmic branching enzyme. Further work is required for isolation and characterization of the FemX-like activity from *Streptomyces*. Also, additional experiments are necessary to determine the roles of both branched and linear UDP-MurNAc-peptide precursors in the biosynthesis of *Streptomyces* cell wall. This might be achieved by both an in vitro PG biosynthesis assay and by analysis of the cell wall structure. Interestingly, *Streptomyces* membranes did not synthesize PG with either branched or linear cognate precursors, whereas staphylococcal membranes successfully utilized both of these precursors for lipid II and PG biosynthesis. This may indicate that standard conditions for the in vitro PG biosynthesis reaction, which were previously optimized for membranes of other organisms, such as *Bacillus megaterium*, *Gaffkya homari*, and *S. aureus* (13, 25), are not suitable for *Streptomyces* membranes. It is possible that some important cofactor(s) might be lost during membrane preparation.

Second, the need for lipid II purification was alleviated by using the lipid II-containing membrane preparation in a TLC-based mobility shift assay. In this assay radioactively labeled lipid II was easily detected in the context of the whole-cell membranes and the shift in its mobility was correlated with the onset of the sortase-catalyzed cleavage/transpeptidation reaction. The data from this study indicated that the NH_2 group of branching Gly of the lipid II molecule serves as a specific nucleophilic acceptor site for this reaction and thus supported the previously published hypothesis that lipid II serves as a natural substrate in the anchoring of surface proteins (28, 29). In this study, sortase was able to recognize lipid II from a complex whole-cell membrane mixture. This could be regarded as an additional demonstration of sortase specificity. However, the existence of additional natural acceptor sites, such as N-terminal branching Gly residues of the growing non-cross-linked PG chains, could not be ruled out. It appeared that the previous studies with cell wall inhibitors and staphylococcal protoplasts did not completely rule out that possibility either (29). In fact, the anchoring of a bulky protein molecule on lipid II might create a steric interference with subsequent transglycosylation reaction, and thus, the sorting reaction might preferably proceed at the branches of nonmature PG. Alternatively, lipid II and PG chains may both serve as natural acceptors in the anchoring of surface proteins. Further experiments are currently under way to test these possibilities.

	1	2	3	4	5	6	7	8	9	10	11
LPSTG-Peptide	+	+	+	+	+	+	+	+	+	+	+
$CaCl_2$	+	+	+	+	+	+	+	+	+	+	+
Sortase	-	+	+	-	+	+	-	+	+	+	+
$(Gly)_3$	-	-	+	-	-	+	-	-	-	-	-
LipidII (Ala-Glu-A_2pm(Gly)-Ala-Ala)	+	+	+	-	-	-	+	+	+	+	+
LipidII (Ala-Glu-A_2pm-Ala-Ala)	-	-	-	+	+	+	-	-	-	-	-
Vancomycin (μg/ml)	-	-	-	-	-	-	-	-	100	10	1

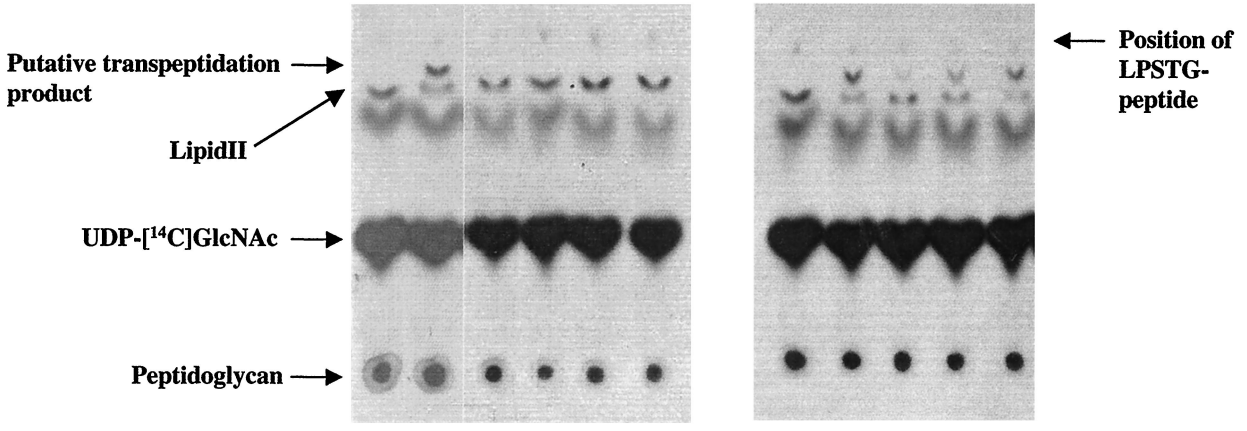

FIG. 4. TLC-based mobility shift assay. The aliquots of heat-inactivated PG biosynthesis reaction mixtures (Fig. 3) were incubated in the presence (+) or absence (−) of LPSTG peptide, sortase, NH_2-$(Gly)_3$, and vancomycin as indicated above the lanes. Following incubation, the samples were separated by TLC, and the plates were dried and autoradiographed. The position of nonradioactive LPSTG peptide was visualized by its intrinsic orange color.

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REFERENCES

1. **Anderson, J. S., P. M. Meadow, M. A. Haskin, and J. L. Strominger.** 1966. Biosynthesis of the peptidoglycan of bacterial cell walls. I. Utilization of uridine diphosphate acetylmuramyl pentapeptide and uridine diphosphate acetylglucosamine for peptidoglycan synthesis by particulate enzymes from *Staphylococcus aureus* and *Micrococcus lysodeikticus*. Arch. Biochem. Biophys. **116:**487–515.
2. **Billot-Klein, D., D. Shlaes, D. Bryant, D. Bell, R. Legrand, L. Gutmann, and J. van Heijenoort.** 1997. Presence of UDP-*N*-acetylmuramyl-hexapeptides and -heptapeptides in enterococci and staphylococci after treatment with ramoplanin, tunicamycin, or vancomycin. J. Bacteriol. **179:**4684–4688.
3. **Bouhss, A., D. Mengin-Lecreulx, D. Le Beller, and J. Van Heijenoort.** 1999. Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. Mol. Microbiol. **34:**576–585.
4. **Bracha, R., and L. Glaser.** 1976. An intermediate in teichoic acid biosynthesis. Biochem. Biophys. Res. Commun. **72:**1091–1098.
5. **Bugg, T. D., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408–10415.
6. **Croux, C., B. Canard, G. Goma, and P. Soucaille.** 1992. Purification and characterization of an extracellular muramidase of *Clostridium acetobutylicum* ATCC 824 that acts on non-*N*-acetylated peptidoglycan. Appl. Environ. Microbiol. **58:**1075–1081.
7. **Croux, C., and J. L. Garcia.** 1991. Sequence of the *lyc* gene encoding the autolytic lysozyme of *Clostridium acetobutylicum* ATCC824: comparison with other lytic enzymes. Gene **104:**25–31.
8. **Flouret, B., D. Mengin-Lecreulx, and J. van Heijenoort.** 1981. Reverse-phase high-pressure liquid chromatography of uridine diphosphate N-acetylmuramyl peptide precursors of bacterial cell wall peptidoglycan. Anal. Biochem. **114:**59–63.
9. **Handwerger, S., M. J. Pucci, K. J. Volk, J. Liu, and M. S. Lee.** 1992. The cytoplasmic peptidoglycan precursor of vancomycin-resistant *Enterococcus faecalis* terminates in lactate. J. Bacteriol. **174:**5982–5984.
10. **Hegde, S. S., and T. E. Shrader.** 2001. FemABX family members are novel nonribosomal peptidyltransferases and important pathogen-specific drug targets. J. Biol. Chem. **276:**6998–7003.
11. **Katz, W., M. Matsuhashi, C. P. Dietrich, and J. L. Strominger.** 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. IV. Incorporation of glycine in *Micrococcus lysodeikticus*. J. Biol. Chem. **242:**3207–3217.
12. **Kopp, U., M. Roos, J. Wecke, and H. Labischinski.** 1996. Staphylococcal peptidoglycan interpeptide bridge biosynthesis: a novel antistaphylococcal target? Microb. Drug Resist. **2:**29–41.
13. **Matsuhashi, M., C. P. Dietrich, and J. L. Strominger.** 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. III. The role of soluble ribonucleic acid and of lipid intermediates in glycine incorporation in *Staphylococcus aureus*. J. Biol. Chem. **242:**3191–3206.
14. **Mazmanian, S. K., G. Liu, H. Ton-That, and O. Schneewind.** 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science **285:**760–763.
15. **McArthur, H. A., F. M. Roberts, I. C. Hancock, and J. Baddiley.** 1978. Lipid intermediates in the biosynthesis of the linkage unit between teichoic acids and peptidoglycan. FEBS Lett. **86:**193–200.
16. **Mengin-Lecreulx, D., T. Falla, D. Blanot, J. van Heijenoort, D. J. Adams, and I. Chopra.** 1999. Expression of the *Staphylococcus aureus* UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate:L-lysine ligase in *Escherichia coli* and effects on peptidoglycan biosynthesis and cell growth. J. Bacteriol. **181:**5909–5914.
17. **Navarre, W. W., and O. Schneewind.** 1999. Surface proteins of gram-positive

bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. **63:**174–229.
18. **Ornelas-Soares, A., H. de Lencastre, B. L. de Jonge, and A. Tomasz.** 1994. Reduced methicillin resistance in a new *Staphylococcus aureus* transposon mutant that incorporates muramyl dipeptides into the cell wall peptidoglycan. J. Biol. Chem. **269:**27246–27250.
19. **Park, J. T.** 1966. Membrane associated reaction involved in bacterial cell wall mucopeptide synthesis. Methods Enzymol. **8:**466–472.
20. **Pisabarro, A. G., R. Prats, D. Vaquez, and A. Rodriguez-Tebar.** 1986. Activity of penicillin-binding protein 3 from *Escherichia coli*. J. Bacteriol. **168:** 199–206.
21. **Rohrer, S., K. Ehlert, M. Tschierske, H. Labischinski, and B. Berger-Bachi.** 1999. The essential *Staphylococcus aureus* gene *fmhB* is involved in the first step of peptidoglycan pentaglycine interpeptide formation. Proc. Natl. Acad. Sci. USA **96:**9351–9356.
22. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
23. **Schneewind, O., P. Model, and V. A. Fischetti.** 1992. Sorting of protein A to the staphylococcal cell wall. Cell **70:**267–281.
24. **Siegel, J. L., S. F. Hurst, E. S. Liberman, S. E. Coleman, and A. S. Bleiweis.** 1981. Mutanolysin-induced spheroplasts of *Streptococcus mutans* are true protoplasts. Infect. Immun. **31:**808–815.
25. **Somner, E. A., and P. E. Reynolds.** 1990. Inhibition of peptidoglycan biosynthesis by ramoplanin. Antimicrob. Agents Chemother. **34:**413–419.
26. **Ton-That, H., H. Labischinski, B. Berger-Bachi, and O. Schneewind.** 1998. Anchor structure of staphylococcal surface proteins. III. Role of the FemA, FemB, and FemX factors in anchoring surface proteins to the bacterial cell wall. J. Biol. Chem. **273:**29143–29149.
27. **Ton-That, H., G. Liu, S. K. Mazmanian, K. F. Faull, and O. Schneewind.** 1999. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. Proc. Natl. Acad. Sci. USA **96:**12424–12429.
28. **Ton-That, H., S. K. Mazmanian, K. F. Faull, and O. Schneewind.** 2000. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Sortase catalyzed *in vitro* transpeptidation reaction using LPXTG peptide and NH_2-Gly_3 substrates. J. Biol. Chem. **275:**9876–9881.
29. **Ton-That, H., and O. Schneewind.** 1999. Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction. J. Biol. Chem. **274:**24316–24320.
30. **van Heijenoort, Y., M. Gomez, M. Derrien, J. Ayala, and J. van Heijenoort.** 1992. Membrane intermediates in the peptidoglycan metabolism of *Escherichia coli*: possible roles of PBP 1b and PBP 3. J. Bacteriol. **174:** 3549–3557.
31. **Weppner, W. A., and F. C. Neuhaus.** 1977. Fluorescent substrate for nascent peptidoglycan synthesis. Uridine diphosphate-N-acetylmuramyl-(Nepsilon-5-dimethylaminonaphthalene-1-sulfonyl)pentapeptide. J. Biol. Chem. **252:** 2296–2303.