Synthesis of Mosaic Peptidoglycan Cross-bridges by Hybrid Peptidoglycan Assembly Pathways in Gram-positive Bacteria*

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Ana Arbeloa‡‡, Jean-Emmanuel Hugonnet‡, Anne-Charlotte Sentilhes‡, Nathalie Josseaume‡, Lionnel Dubost‡, Christelle Monsenpes‡, Didier Blanot‡, Jean-Paul Brouard‡, and Michel Arthur‡‡‡

From ‡INSERM E0004, Laboratoire de Recherche Moléculaire sur les Antibiotiques, 15 rue de l’Ecole de Médecine, 75270 Paris cedex 06, France. §Développement et Diversité Moléculaire, Muséum National d’Histoire Naturelle, USM0562-CNRS UMR8041, 75005 Paris, France, and ¶Enveloppes Bactériennes et Antibiotiques, UMR 8619 CNRS, Bâtiment 430, Université de Paris-Sud, 91405 Orsay, France

The peptidoglycan cross-bridges of Staphylococcus aureus, Enterococcus faecalis, and Enterococcus faecium consist of the sequences GlyX3, L-AlaX2, and D-AxX, respectively. Expression of the fnmB, femA, and femB genes of S. aureus in E. faecalis led to the production of peptidoglycan precursors substituted by mosaic side chains that were efficiently used by the penicillin-binding proteins for cross-bridge formation. The Fem transfers were specific for incorporation of glycoly residues at defined positions of the side chains in the absence of any additional S. aureus factors such as tRNAs used for amino acid activation. The PBPs of E. faecalis displayed a broad substrate specificity because mosaic side chains containing from 1 to 5 residues and Gly instead of D-Ala were used for peptidoglycan cross-linking. Low affinity PBP2a of S. aureus conferred β-lactam resistance in E. faecalis and E. faecium, thereby indicating that there was no barrier to heterospecific expression of resistance caused by variations in the structure of peptidoglycan precursors. Thus, conservation of the structure of the peptidoglycan cross-bridges in members of the same species reflects the high specificity of the enzymes for side chain synthesis, although this is not essential for the activity of the PBPs.

The peptidoglycan cross-bridges of S. aureus, E. faecalis, and E. faecium are inefficiently acylated by D,L,D-transpeptidases that cross-link glycan strands (4). The latter reaction is catalyzed by essential high molecular weight penicillin-binding proteins (PBPs) that cleave the C-terminal residue (D-Ala) of a donor stem peptide and link the carboxyl group of the penultimate residue (D-Ala) to the side chain amino group of an acceptor stem peptide (5) (see Fig. 1). This two-step reaction involves formation of a covalent adduct between the β-hydroxyl of the active site serine of the PBPs and the carboxylate of D-Ala of the donor stem peptide (acyl-enzyme) (5). β-Lactam antibiotics are structural analogues of the D-Ala-D-Ala, extremity of peptidoglycan precursors that irreversibly inactivate the PBPs in a similar acylation reaction. Most bacterial species produce multiple PBPs that have partially overlapping functions (6). Multimodular PBPs associate a C-terminal D,L,D-transpeptidase module to N-terminal glycosyltransferase (class A) or non catalytic (class B) modules. Clinically relevant β-lactam resistance phenotypes in staphylococci and enterococci involve production of class B D,L,D-transpeptidases that are inefficiently acylated by β-lactams (commonly referred to as low affinity PBPs). Methicillin-resistant Staphylococcus aureus has acquired an additional php gene (mecA encoding low affinity PBP2a) presumably from a related staphylococcal species (7). Resistance is an intrinsic property of Enterococcus faecalis and Enterococcus faecium because virtually all isolates are resistant to moderate (e.g. ampicillin) or high (e.g. ceftriaxone) levels of β-lactams and produce species-specific low affinity PBPs designated PBP5α and PBP5β, respectively (8–10).

Diversification of the side chain structure during speciation (see Fig. 1) is potentially associated with diversification of the substrate specificity of the D,L,D-transpeptidases. In S. aureus, the Fmhb transferase for incorporation of the first residue of the pentaglycine side chain (see Fig. 1) is an essential enzyme. This indicates that unsubstituted pentapeptide stems cannot be cross-linked (11). In addition, femA and femB mutants of methicillin-resistant S. aureus are susceptible to methicillin. This suggests that a complete pentaglycine side chain is essential for the D,L,D-transpeptidase activity of PBP2a (12). Similarly, the side chain is essential for penicillin resistance in Streptococcus pneumoniae (13). Inactivation of bppA1 encoding the transferase for incorporation of the first residue of the L-Ala-L-Ala side chain in E. faecalis has not been obtained (14). Deletion of bppA2 led to production of precursors substituted by a single L-Ala and to impaired expression of intrinsic β-lactam
resistance (14). For these reasons, the Fem family are considered to be potential targets for the development of novel antibiotics active against β-lactam-resistant Gram-positive cocci (15).

In this study, we have further investigated the synthesis of the side chains of peptidoglycan precursors by transpeptidases of the Fem family and their use by the PBPs in the cross-linking reaction. The study was designed to test the hypothesis that a narrow specificity of the PBPs could account for the essential role of transferases of the Fem family in peptidoglycan synthesis and β-lactam resistance. Heterospecific expression of genes encoding transpeptidases and PBPs was used to manipulate the structure of the side chain of the peptidoglycan precursors and the PBPs responsible for their polymerization. We first determined whether heterospecific expression of the E. aerus \textit{fmhB}, \textit{femA}, and \textit{femB} genes in \textit{E. faecalis} leads to synthesis of muropeptide dimers. Mosaic mass spectrometry and tandem mass spectrometry (MS/MS) analyses of uncross-linked muropeptide monomers revealed that the Fem transferences of \textit{E. aerus} were functional in \textit{E. faecalis} and retained their substrate specificity as displayed in the original host. The participation of \textit{E. faecalis} femA and femB genes in \textit{E. faecium} –mecA and –mecB–femA femB was used to manipulate the activity of the PBPs.

Experimental Procedures

Bacterial Strains and Growth Conditions—Bacterial strains were grown in brain heart infusion (BHI) broth or agar (Becton Dickinson, le Pont de Clairs, France) at 37°C. Population analysis profiles were performed as previously described (16). Bacteria were grown on BHI agar containing increasing concentrations of ceftriaxone (Roche Applied Science). Colony forming units were determined after 48 h of incubation at 37°C on BHI agar containing increasing concentrations of ceftriaxone (Roche Applied Science). Colony forming units were determined after 48 h of incubation at 37°C on BHI agar containing increasing concentrations of ceftriaxone (Roche Applied Science).

Preparation of Lactoyl Peptides—The ether link internal to MurNac was cleaved under alkaline conditions (23) to produce lactoyl peptides. Peptidoglycan fragments were used to generate lactoyl peptides by hydrolysis with 20% orthophosphoric acid.

Purification of Peptidoglycan Fragments—Reduced disaccharide peptides and lactoyl peptides monomers were obtained by alkaline hydrolysis (23) to produce lactoyl peptides.

Preparation of Hybrid Pathways of Peptidoglycan Synthesis

The open reading frame and ribosome-bind-
Determination of the Structure of Multimers—Tandem mass spectrometry performed on reduced disaccharide peptide dimers provided little information on the peptide moiety of the molecules because fragmentation occurred mainly at the \(\alpha\)-1,4-GlcNAc-MurNAc bonds. In contrast, the entire peptide sequence could be determined by analyzing lactoyl peptide peptidoglycan fragments. In a first set of experiments, fragmentation was performed on the doubly charged ions of the molecules \([M+2H]^2+\), which gave a higher current intensity than singly charged ions \([M+H]^+\). The declustering potential was set to 60 V, and the collision energy was typically of 26–35 eV. Fragments resulting from loss of D-Lac-L-Ala\(_1\) moieties of the molecules (loss of 143 atomic mass units) and additional \(\text{NH}_3\) (17 atomic mass units) or \(\text{NH}_3-\text{CONH}_3\) (17 + 45 atomic mass units) in various combinations gave ions of high current intensities. Fragments generated by cleavage within the cross-bridges gave ions of lower intensity. The fragmentation pattern was also complicated by the presence of singly and doubly charged forms of certain fragments.
Optimization of the fragmentation conditions for lactoyl peptide dimers was achieved by raising the declustering potential to 100 V, which increased the current intensity of the singly charged form of the molecules ([M/H]+100). Fragmentation performed with a declustering potential of 100 V on singly charged ions with a collision energy of 57–65 eV exclusively produced singly charged fragments. The highest intensities were observed for cleavage within the cross-bridges. The data reported under “Results” were performed under these conditions by using 7–35 μl of the purified fractions.

RESULTS

Structure of the Main Monomer Resulting from Heterospecific Expression of femA in E. faecalis JH2–2ΔbppA2/pNJ21femA. The femA gene, encoding the transferase required for incorporation of glycyl residues at the second and third positions of the pentaglycine side chain in S. aureus (Fig. 1C), was cloned into the expression vector pNJ2 to generate plasmid pNJ21femA and introduced into E. faecalis JH2–2ΔbppA2. The latter host produces precursors substituted by a single L-alanyl residue following deletion of the bppA2 gene (Fig. 1A). The most abundant monomers of JH2–2ΔbppA2/pNJ21femA (Fig. 2, Peak 6) had a monoisotopic mass of 744.6, which matched the calculated value for a D-lactoyl-pentapeptide stem substituted by a side chain consisting of one L-alanyl and two glycyl residues. The structure of this branched peptide was solved by MS/MS, based on the detection of specific ions generated by loss of residues from the N terminus of the side chain (L-Ala-Gly-Gly-Nter) and...
FIG. 3. Analysis of the main monomer from JH2–2ΔbppA2/pNJ2ΔfemA by tandem mass spectrometry. A, fragmentation was performed on the ion at m/z 745.6 corresponding to the [M+H]^+ from the major monomer. B, structure of the major monomer and inferred fragmentation pattern. The boxed m/z values in A originate from cleavage at single peptide bonds as represented in B. Peaks at m/z 631.5 and 560.4 matched the predicted values for loss of two N-terminal glycyl residues and of an additional l-alanyl residue, respectively. Loss of one and two l-Ala from the C terminus of the pentapeptide stem gave ions at m/z 656.5 and 585.4. Further loss of NH₃ gave peaks at m/z 639.4 and 568.4. Fragmentation of the L-Lac-L-Ala₃ amide bond was not observed. The peak at m/z 602.4 matched the predicted value for loss of L-Lac-L-Ala₃. Further loss of NH₃ and additional CONH₃ led to peaks at m/z 585.4 and 540.4. Cleavage of the same peptide bond also produced peaks at m/z 144.0 and 116.0 corresponding to the L-Lac-L-Ala₃ moiety of the molecule and loss of CO, respectively. Fragmentation at the D-iGln₂-L-Lys₃ peptide bond produced ions at 272.1 and 474.3. Additional loss of NH₃ from ion at 474.3 gave an ion at 457.3. Additional ions could be accounted for by
from the carboxyl (D-Ala,G-Ala,Cter) or hydroxyl (OH,D-Lac,G-Ala,iGln) extremities of the lactoyl-pentapeptide stem (Fig. 3). The interpretation of the fragmentation pattern (Fig. 3B) was confirmed by MS/MS performed on fragments of the molecule, as exemplified by fragmentation of the ion at m/z 474.3 (Fig. 4). The structure of the main monomer determined by these approaches (Figs. 3 and 4) indicates that FemA of S. aureus is functional in E. faecalis because it catalyzes the addition of two glycyl residues after the L-alanyl residue incorporated by BppA1.

Structure of Secondary Monomers from JH2–2bppA2/pNJ2ΩfemA—Muropeptide diversity was thoroughly investigated based on MS/MS analysis of the monomers (Fig. 2). In the order of decreased abundance, the first polymorphism was generated by the presence of side chains consisting of a single L-Ala (peak 4, 15.7%) instead of the sequence L-Ala-Gly-Gly (peak 6, 18.5%; see above). Side chains generated by BppA1 alone (L-Ala) or BppA1 and FemA (L-Ala-Gly-Gly) were therefore both produced by JH2–2bppA2/pNJ2ΩfemA. Lactoyl peptides with side chains consisting of the sequence L-Ala-Gly-Gly were present in small amounts. Residues other than L-Ala were not detected at the first position of the side chains. Residues other than Gly were not detected at the second and third positions. Side chains containing glycyl residues were not detected in JH2–2 and JH2–2bppA2 (14). Thus, BppA1 and FemA were highly specific both for the type (L-Ala versus Gly) and position (first second versus third) of the residues incorporated into the side chains.

A second polymorphism was generated by the presence of stem peptides lacking one (tetrapeptide) or both (tripeptide) C-terminal D-alanyl residues (Fig. 2). As previously described (14), these stem peptides could be generated by D,D-carboxypeptidases and L,D-carboxypeptidases that cleave the D-Ala,D-Ala,G and L-Lys,G-D-Ala peptide bonds, respectively.

A third polymorphism was generated by the presence of Gly instead of D-Ala at the fifth position of lactoyl-pentapeptide stems. Pentapeptide stems with a C-terminal glycyl residue were also detected in E. faecalis JH2–2 and JH2–2bppA2 and are therefore unrelated to the activity of FemA. The PBPs are likely to be responsible for exchange of the C-terminal D-Ala by Gly because cytoplasmic UDP-MurNAc-pentapeptide ending in a glycyl residue were not detected in the cytoplasm (data not shown).

A fourth polymorphism was generated by the lack of amidation of the D-carboxyl group of the D-isoglutaminyl residue at the second position of the stem peptide. This polymorphism is unrelated to the activity of FemA because it was also detected in the control strains JH2–2 and JH2–2bppA2.

Structures of Multimers of E. faecalis JH2–2bppA2/pNJ2ΩfemA—Expression of femA in JH2–2bppA2 led to production of novel dimers differing from those present in preparations from the JH2–2bppA2 host by an increment of 228 atomic mass units, as expected for the presence of a total of four additional glycyl residues (Fig. 2). Tandem mass spectrometry
provided the sequence of the cross-bridge and of the free N-terminal side chain, which were both found to contain the sequence L-Ala-Gly-Gly (see Fig. 5 for the analysis of the main dimer in peak 11). As described for the monomers, MS/MS was also performed on fragments of the molecules to confirm the interpretation of the fragmentation patterns. The structure of FIG.5:

A. Analysis of the main dimer from JH2–2AhpA2/pNJR25fomA by tandem mass spectrometry. A, fragmentation was performed on the ion at m/z 1400.7 corresponding to the [M+H]⁺ form of the major dimer. B, the structure of the major dimer was inferred from the fragmentation pattern. The boxed m/z values in A originate from cleavage at single peptide bonds as represented in B. Other peaks could originate from multiple cleavage. For example, the loss of additional D-Lac-L-Ala1 from ions at m/z 1240.6, 841.4, 656.3, and 585.3 gave ions at m/z 1097.5, 698.3, 513.2, and 442.2, respectively. The loss of additional NH3 and CONH3 from ion at m/z 513.2 gave an ion at m/z 451.2. The loss of additional NH3 from ion at m/z 1097.5 gave an ion at m/z 1080.5. Loss of additional D-Lac-L-Ala1-D-iGln2 from ions at m/z 1240.6 gave an ion at m/z 989.5.
the main dimer (Fig. 5B) indicates that the D,D-transpeptidases of E. faecalis cross-linked glycol-containing precursors. Peptide stems substituted by l-Ala-Gly-Gly had participated in the transpeptidation reaction both as an acceptor and a donor. Polymorphism in the multimers of JH2–2ΔbppA2/pNJ2Ω femA was generated by the presence of dimers, trimers, and tetramers containing pentapeptide (major form) and tripeptide (of lesser abundance) acceptor stems (Fig. 2). Other polymorphisms could be accounted for by variations already described in detail for the monomers.

Expression of femA in E. faecalis JH2–2—Analysis of the monomers of E. faecalis JH2–2/pNJ2ΩfemA indicated the participation of BppA1, BppA2, and FemA in side chain synthesis in various combinations (Table I). The wild-type sequence (l-Ala-l-Ala) was generated by BppA1 and BppA2. The sequence l-Ala-Gly-Gly, also observed in JH2–2ΔbppA2/pNJ2ΩfemA (see above), was generated by BppA1 and FemA. The sequence l-Ala-l-Ala-Gly involved the participation of BppA1, BppA2, and FemA. Thus, FemA and BppA2 competitively added Gly and l-Ala, respectively, at the second position of the side chain. The sequence l-Ala-l-Ala-Gly also indicated that FemA can add a single residue. The three types of sequences (l-Ala-Gly-Gly, l-Ala-l-Ala, and l-Ala-l-Ala-Gly) were present both in the cross-bridges and in the free side chains of multimers.

FmhB-mediated Incorporation of Gly at the First Position of the Side Chain—Co-expression of the fmhB and bpp genes led to the production of monomers substituted by Gly or l-Ala in JH2–2ΔbppA2 and by the sequence Gly-l-Ala or l-Ala-l-Ala in JH2–2 (Table I). Thus, FmhB and BppA1 competitively added glycol and l-allyl residues at the first position of the side chain, respectively. Detection of the sequence Gly-l-Ala indicates the sequential participation of FmhB and BppA2 to side chain synthesis. BppA2 was therefore able to elongate a side chain consisting of Gly, although its natural substrate in E. faecalis contains l-Ala. The structure of the dimers indicated that glycol-containing precursors were used as donors and acceptors in the transpeptidation reaction.

Expression of femB—The FemB transferase is required for incorporation of glycol residues at the fourth and fifth positions of the pentaglycine side chain in S. aureus (Fig. 1). Expression of femB did not lead to incorporation of glycol residues in the peptidoglycan of JH2–2 or JH2–2ΔbppA2 (Table I). Thus, FemB was unable to elongate side chains comprising one or two l-allyl residues.

Synthesis of Pentaglycine Side Chains by FmhB, FemA, and FemB—Co-expression of the fmhB, femA, and femB genes in JH2–2ΔbppA2 led to production of muropeptides monomers substituted by five glycol residues (Table I). Thus, synthesis of the complete pentaglycine side chain of S. aureus was observed in E. faecalis. Side chains consisting of one l-alanyl and four glycol residues were also present because of competitive incorporation of l-Ala and Gly by BppA1 and FmhB at the first position. Amino acid residues other than Gly were not observed at positions 2–5. Incomplete side chains were also present. Analysis of multimers indicated that precursors containing pentaglycine side chains were used as donors and acceptors in the cross-linking reaction. Expression of fmhB, femA, and femB in JH2–2 gave essentially the same results except that Gly and l-Ala were competitively incorporated also at the second position of the side chain (Table I).

Heterospecific Expression of Genes Encoding Low Affinity PBPs—Resistance to β-lactams mediated by heterospecific expression of mecA, pbp5_R, and pbp5_M was used as a screen to evaluate the capacity of the corresponding PBPs to catalyze cross-linking of pentapeptide stems substituted by l-Ala-l-Ala in E. faecalis and by D-Asx in S. aureus. pbp5_M, and pbp5_R were cloned into the expression vector pNJ2 and introduced into E. faecalis JH2–2ΔbppB and E. faecium D344S. The latter hosts are previously characterized mutants susceptible to β-lactams because of deletion of their respective species specific pbp5 genes (17, 19). Ceftriaxone, a third generation cephalosporin, was used to monitor expression of β-lactam resistance, because deletion of the pbp5 genes produced large decreases (>1000-fold) in the minimal inhibitory concentration of this antibiotic both in E. faecium and E. faecalis.

Resistance Phenotype Mediated by mecA in E. faecalis and E. faecium—Population analysis profiles indicated that the mecA gene of S. aureus can confer resistance to ceftriaxone both in E. faecalis JH2–2ΔbppB (Fig. 6A) and E. faecium D344S (Fig. 6B). The resistance phenotypes were characterized in both hosts by the presence of subpopulations of bacteria highly resistant to the drug. Representatives of the resistant subpopulations obtained on agar containing ceftriaxone were homogeneously resistant to high levels of ceftriaxone. The resistance trait remained stable after five serial subcultures in the absence of the drug. Sequencing of the mecA open reading frame and upstream sequences comprising the relevant promoter and ribosome-binding site did not reveal any mutation. Moreover, introduction of pNJ2ΩmecaA isolated from representatives of the resistant subpopulations into E. faecalis JH2–2ΔbppB or E. faecium D344S resulted in expression of high level ceftriaxone resistance only in subpopulations of the bacteria, as initially observed for the wild-type plasmid. Thus, plasmid pNJ2ΩmecaA of homogeneously resistant variants did not harbor any mutation affecting mecA or cis-acting sequences required for its expression. Together, these observations indicate that expression of wild-type mecA of S. aureus can confer high level resistance to ceftriaxone in E. faecalis and E. faecium. Modification of an unknown host factor was required for full expression of resistance, as also observed for the introduction of mecA in methicillin susceptible S. aureus (24).

Analysis of Peptidoglycan Structure of E. faecalis and E. faecium Strains Expressing mecA—To determine the contribution of PBP2a to peptidoglycan cross-linking, the resistant variants of E. faecalis JH2–2ΔbppB harboring pNJ2ΩmecaA grown in the presence of ceftriaxone (100 μg/ml) were used as the host PBPs, and the peptidoglycan was analyzed by mass spectrometry and tandem mass spectrometry. Complementation of the chromosomal pbp5_R deletion of E. faecalis JH2–2ΔbppB by mecA or by pbp5_M led to synthesis of similarly cross-linked peptidoglycan in the presence of ceftriaxone (Fig. 7). Likewise, no significant difference in the peptidoglycan structure was observed for complementation of the chromosomal pbp5_M deletion of E. faecium D344S by mecA or by

| Table I |
| --- |
| Sequence of the side chains and cross-bridges in E. faecalis strains expressing fem genes of S. aureus |

| E. faecalis strains | None | JH2-2 (BppA1 and BppA2) | JH2-2ΔbppA2 (BppA1) |
| --- | --- | --- | --- |
| fmnB | t-Ala-t-Ala | t-Ala | t-Ala |
| femA | Gly-l-Ala | Gly-l-Ala | Gly-l-Ala |
| femB | t-Ala-t-Ala | t-Ala | t-Ala |
| femB femA femB | Gly-Gly-Gly-Gly-Gly | Gly-Gly-Gly-Gly-Gly | Gly-Gly-Gly-Gly-Gly |

The same sequences were detected in the free side chains of the monomers and in the cross-bridges of the multimers, indicating that all the mosaic side chains where used in the transpeptidation reaction.
Heterospecific Expression of pbp5fs and pbp5fm—Derivatives of E. faecalis JH2–2Δpbp5 harboring the expression vector pNJ2 or plasmid pNJ2Ωpbp5m were uniformly susceptible to ceftriaxone (Fig. 6A). Thus, the pbp5fs gene of E. faecium did not confer β-lactam resistance in E. faecalis. In contrast, the pbp5fm gene of E. faecalis conferred resistance to ceftriaxone in E. faecalis (Fig. 6C). As found for mecA, expression of high level resistance was only detected in a fraction of the bacteria. Derivatives of the resistant subpopulation stably expressed homogenous resistance in the absence of mutational alteration of pNJ2Ωpbp5fs.

DISCUSSION

The Fem proteins form a family of highly diverse tRNA-dependent aminoacyl-transferases with respect to the type, number, and position of the amino acids added to the side chain of peptidoglycan precursors (Fig. 1) (25). The catalytic activity of the transferases has been assessed only of purified FemXp from Weissella viridescens (26, 27) and the Bpp transferases from E. faecalis (14, 28). In S. aureus, transferases essential for incorporation of residues at specific positions of the pentaglycine side chain (Fig. 1) have been identified based on gene inactivation (femA and femB) and conditional gene expression (fmbB) (11, 12). Individual inactivation of two additional fem-related genes had little impact on viability and amino acid composition of the peptidoglycan (29). We show here that FmhB competes with BppA1 for the incorporation of Gly at the first position of the side chain in E. faecalis JH2–2 and JH2–2ΔbppA2. FemA was sufficient for addition of two residues because expression of femA in JH2–2ΔbppA2 led to the incorporation of two Gly in the absence of the other four fem genes present in the chromosome of S. aureus (Figs. 2 and 3). The two reactions can occur independently because FemA mediated incorporation of one or two Gly to generate the sequence L-Ala–L-Ala–Gly and L-Ala–Gly–Gly in E. faecalis JH2–2 because of competition between FemA and BppA2 at the second position (Table 1). FemB added two Gly and required a side chain composed of three residues, indicating that this transferase was highly specific for the fourth and fifth positions. Co-expression of fmbB, femA, and femB led to the production of stem peptides substituted by a pentaglycyl moiety, showing for the first time that synthesis of the complete side chain of S. aureus can be reproduced in a heterologous host.

The Fem transferases of S. aureus incorporated glycyld residues in E. faecalis, indicating that the enzymes retained the specificity displayed in the original host (Fig. 2 and Table I). This implies that E. faecalis produces glycyld-tRNAs, which were efficiently used by the Fem transferases. Fractionation of the tRNAs isoacceptor of glycine and sequence analysis have raised the possibility that staphylococci produce unusual tRNAs dedicated to peptidoglycan synthesis (30, 31). Our analysis indicates that these putative tRNAs are unlikely to play a key role in the specificity of the Fem transferases for the amino acids incorporated into the side chains of peptidoglycan precursors.

A narrow substrate specificity of the D,D-transpeptidases for the acceptor side chain was proposed to account for the essential role of the transferases in viability (e.g. FmhB) and β-lactam resistance (e.g. FemA and FemB) (11, 12). However, this has not been directly established by kinetic analyses of purified PBPs, because the enzymes were not active in vitro, except in very special cases involving highly reactive substrates (e.g. thiostrepton) or atypical enzymes (e.g. the soluble R61 D,D-peptidase from Streptomyces spp.) (see Ref. 32 for a recent discussion). For this reason, heterospecific expression of pbp and fem genes was used in the current study to gain insight into the specificity of the PBP in vitro. The D,D-transpeptidases of E. faecalis tolerated variations in the number of residues in the side chains (from 1 to 5) and substitution of L-Ala by Gly at the N-terminal position both in the donor and the acceptor (Fig. 2 and Table I). The substrate specificity of the D,D-transpeptidases was also explored based on heterospecific expression of pbp genes encoding low affinity PBPs. The mecA gene of S. aureus conferred resistance to ceftriaxone in E. faecalis JH2–2Δpbp5 and E. faecium D344S (Fig. 6). In neither host was the expression of resistance associated with a modification of peptidoglycan structure (Fig. 7 and data not shown). Because the D,D-transpeptidases are the essential target of β-lactams (5, 6), PBP2a acted as a surrogate of the host D,D-transpeptidases and therefore catalyzed peptidoglycan cross-linking. This implies a low substrate specificity of PBP2a, because the amino group of the acceptor participating in the transpeptidation reaction was located on side chains consisting of five Gly,
L-Ala-L-Ala, and D-Asx in *S. aureus*, *E. faecalis*, and *E. faecium*, respectively. These observations establish for the first time that *mecA* of *S. aureus* can confer β-lactam resistance in distantly related hosts belonging to the genus *Enterococcus*, despite substantial diversity in the structure of peptidoglycan precursors. Similarly, PBP5fs from *E. faecalis* conferred resistance to ceftriaxone in *E. faecium* D344S (Fig. 6C), indicating that substitution of L-Ala-L-Ala by D-Asx was also tolerated by this D,D-transpeptidase. Horizontal gene transfer in natural conditions has been documented for *mecA* between related species belonging to the genus *Staphylococcus*. Our data indicate that there is no barrier to heterospecific expression of β-lactam resistance.

**Fig. 7.** Structural analysis of peptidoglycan from derivatives of *E. faecalis* JH2-2Δpβp5 grown in the presence of ceftriaxone at 100 μg/ml. Purified peptidoglycan was digested with muramidases and treated with ammonium hydroxide producing β-lactam peptide fragments that were separated by reversed-phase HPLC. A, *E. faecalis* JH2-2Δpβp5/pN21pβp5a. B, *E. faecalis* JH2-2Δpβp5/pN21mecA (representative of the subpopulation highly resistant to ceftriaxone). C, mass spectrometry was performed on individually collected peaks. The calculated mass (not shown) of the proposed structure for the β-lactam peptides differed from the observed monoisotopic mass by less than 0.2 atomic mass unit. The structure of the monomers and dimers was determined by tandem mass spectrometry. The relative abundance (%) of material in peaks 1–10 was calculated by integration of the absorbance at 210 nm. NA, not applicable.
resistance mediated by the low affinity PBP2a and PBP5fs in more distantly related bacterial species, and thus, intergeneric transfers of low affinity PBPs should be anticipated.

Among the three low affinity PBPs that were tested in the current study (PBP2a, PBP5fs, and PBP5fm), only PBP5fm from E. faecium did not confer ceftriaxone resistance in a heterologous host (Fig. 6). To explore the basis for the lack of expression of pbp5fm-mediated resistance in E. faecalis, the bppA1 gene of E. faecalis was expressed in wild-type E. faecium BM4107 harboring ppb5fm. The BppA1 transferase mediated incorporation of L-Ala instead of D-Asp in the E. faecium host, and the modified precursors were used as donors and acceptors in the cross-linking reaction (data not shown). Ceftriaxone did not inhibit the formation of the L-alanyl-containing cross-bridges. Thus, the lack of heterospecific expression of resistance to ceftriaxone, which inhibits all PBPs except PBP5fm, did not appear to due to the incapacity of E. faecium PBP5fm to cross-link L-alanyl-containing peptidoglycan precursors.

In conclusion, we have established that diversification of the structure of the side chains of peptidoglycan precursors associated with speciation in Gram-positive bacteria (Fig. 1) did not correlate with diversification of the substrate specificity of the PBPs. The cross-bridges of wild-type S. aureus, E. faecalis, and E. faecium were found to exclusively contain Gly, l-Ala, and d-Asx, respectively. This reflects the high in vitro efficacy and specificity of the enzymes for side chain synthesis rather than a narrow specificity of the d,D-transpeptidases.

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Synthesis of Mosaic Peptidoglycan Cross-bridges by Hybrid Peptidoglycan Assembly Pathways in Gram-positive Bacteria

Ana Arbeloa, Jean-Emmanuel Hugonnet, Anne-Charlotte Sentilhes, Nathalie Josseaume, Lionnel Dubost, Christelle Monsempes, Didier Blanot, Jean-Paul Brouard and Michel Arthur

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