Synthesis of the L-alanyl-L-alanine Cross-bridge of Enterococcus faecalis Peptidoglycan

Ahmed Bouhss*, Nathalie Josseaume*, Anatoly Severin§, Keiko Tabei§, Jean-Emmanuel Hugonnet*, David Shlaes##, Dominique Mengin-Lecreulx‡, Jean van Heijenoort‡, and Michel Arthur*¥

*: Laboratoire de Recherche Moléculaire sur les Antibiotiques, UFR Broussais-Hôtel Dieu, Université Paris VI-INSERM E0004, 15 rue de l’Ecole de Médecine, 75270 Paris, cedex 06, France.
‡: Institut de Biochimie, Biophysique Moléculaire et Cellulaire, UMR 8619 CNRS, Université Paris-Sud, 91405 Orsay, France.
§: Wyeth Research, 401 N. Middletown Rd., Pearl River, NY 10965, USA.
# Present address: Idenix Pharmaceuticals, 125 Cambridge Park Dr., Cambridge, MA, 02140.
¥ Corresponding author: Michel Arthur
LRMA, Université Paris VI
15 rue de l’Ecole de Médecine
75270 Paris cedex 06
France
Phone: 33 1 43 25 00 33
Fax: 33 1 43 25 68 12
e-mail: michel.arthur@bhdc.jussieu.fr
Running title: Peptidoglycan synthesis in Enterococcus faecalis
The enzymatic synthesis of the complete L-alanyl₁-L-alanine₂ side chain of the peptidoglycan precursors of *Enterococcus faecalis* was obtained *in vitro* using purified enzymes. The pathway involved alanyl-tRNA synthetase and two ligases, BppA1 and BppA2, that specifically transfer alanine from Ala-tRNA to the first and second positions of the side chain, respectively. The structure of the UDP-N-acetylmuramoyl-L-Ala-γ-D-Glu-L-Lys(NεL-Ala₁L-Ala₂)-D-Ala-D-Ala product of BppA1 and BppA2 was confirmed by mass spectrometry (MS) and MS/MS analyses. The peptidoglycan structure of the wild-type *E. faecalis* strain JH2-2 was determined by tandem rpHPLC-MS revealing that most muropeptides contained two L-alanyl residues in the cross-bridges and in the free N-terminal ends. Deletion of the *bppA2* gene was associated with production of muropeptides containing a single alanyl residue at these positions. The relative abundance of monomers, dimers, trimers, and tetramers in the peptidoglycan of the *bppA2* mutant indicated that precursors containing an incomplete side chain were efficiently used by the D,D-transpeptidases in the cross-linking reaction. However, the *bppA2* deletion impaired expression of intrinsic β-lactam resistance suggesting that the low-affinity penicillin-binding protein 5 did not function optimally with precursors substituted by a single alanine.
Variations in the structure of peptidoglycan from Gram-positive bacteria involve mainly the third and fifth (C-terminal) position of the pentapeptide stem linked to the disaccharide GlcNAc-MurNAc (Fig. 1). Variation at the C-terminus of the peptide stem by incorporation of D-lactate (D-Lac) instead of D-Ala is responsible for resistance to the glycopeptide antibiotics vancomycin and teicoplanin in enterococci that have acquired the vanA gene cluster (1). The D-Lac residue is not found in mature peptidoglycan since it is cleaved off by the D,D-transpeptidases and D,D-carboxypeptidases (2). Variation at the third position of the peptide stem concerns both the nature of the diamino acid present at this position (e.g. L-lysine or meso-diaminopimelic acid) and the presence or absence of a side chain linked to the ε-amino group of L-lysine (3). Such side chains consist of two L-Ala in Enterococcus faecalis, five Gly in Staphylococcus aureus, D-Asn or D-Asp in E. faecium, and the sequence L-Ser-L-Ala or L-Ala-L-Ala in Streptococcus pneumoniae (3). These amino acids form cross-bridges between L-Lys3 and D-Ala4 after cross-linking of the stem peptides by the D,D-transpeptidase activity of multimodular penicillin-binding proteins (PBP) (Fig. 1). Factors essential for methicillin resistance (fem) in S. aureus include the femA and femB genes that are required for synthesis of the pentaglycine side chain of the peptidoglycan precursors (4).

In the late sixties, the ligases for addition of glycine and L-amino acids to the ε-amino group of L-lysine were shown to use aminoacyl-tRNAs as substrates whereas D-amino acids are added in a tRNA-independent reaction (5,6). More recently, the ligase for incorporation of the first L-alanine of the side chain of the E. faecalis (7) and Weissella viridescens (8) peptidoglycan precursors has been identified based on cloning of fem-related genes in Escherichia coli and assays of the purified gene products for UDP-MurNAc-pentapeptide:L-alanine ligase activity. The assay developed in our laboratory relies on in vitro synthesis of
Ala-tRNA by the purified *E. faecalis* alanyl-tRNA synthetase (AlaS) and subsequent transfer of L-Ala from the aminoacyl-tRNA to the nucleotide precursor UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) (7). In the current study, we used this approach to identify the ligase for incorporation of the second L-alanine in the side chain of *E. faecalis* peptidoglycan precursors. We report *in vitro* synthesis of the complete L-alanyl-L-alanine side chain, deletion of the gene encoding the ligase for incorporation of the second L-alanine from the chromosome of *E. faecalis* JH2-2, and the analysis of the impact of production of incomplete side chains on peptidoglycan cross-linking and β-lactam resistance.

The abbreviations are: BHI, Brain heart infusion; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; PBP, penicillin-binding protein; rPHPLC, reverse-phase high pressure liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.
EXPERIMENTAL PROCEDURES

Protein purification—We previously reported (7) detailed procedures for purification of the UDP-MurNAc-pentapeptide:L-alanine ligase of *E. faecalis* from extracts of *E. coli* JM83/pDA29(*bppA1*) by anion exchange, hydrophobic interaction, affinity (heparin), and exclusion chromatography. The same procedures were used to purify BppA2 from extracts of *E. coli* JM83 harboring plasmid pDA28(*bppA2*) (7) except that hydrophobic interaction chromatography was omitted since the enzyme was not soluble in high concentrations of ammonium sulfate. Briefly, *E. coli* JM83/pDA28(*bppA2*) was grown to an OD<sub>600</sub> of 0.7 in 1 liter of brain heart infusion (BHI) broth containing 100 µg/ml ampicillin, and induction was performed with 1 mM IPTG for 2 hours at 37 °C. Bacteria were disrupted by sonication, centrifuged (27,000 g for 30 min at 4 °C), and the supernatant was loaded at a flow rate of 1 ml/min onto a 10 ml Bio-Scale Q anion exchange column (Bio-Rad, Ivry sur Seine, France) equilibrated in buffer A (50 mM Tris-HCl, pH 7.2; 2 mM 2-mercaptoethanol). Elution was performed with a 0-1 M NaCl gradient in buffer A. A 5 ml fraction eluting at ca. 360 mM NaCl was diluted by addition of 20 ml of buffer B (50 mM potassium phosphate, pH 7.0; 2 mM 2-mercaptoethanol) and loaded onto a HiTrap heparin affinity column (1 ml, Amersham Biotech Pharmacia, Orsay, France) equilibrated in buffer B at a flow rate of 0.25 ml/min. Elution was performed with a 0-2 M NaCl gradient in buffer B providing a 0.25 ml fraction eluting at ca. 850 mM NaCl. Gel filtration was performed with a Superdex 75 HR10/30 column (Amersham Biotech Pharmacia) equilibrated with buffer A containing 200 mM NaCl at a flow rate 0.5 ml/min. The *bppA2* gene product eluted between 8.5 to 9.0 ml (300 µg of protein) and was estimated to be >95 % pure by SDS-PAGE. Proteins were determined by the Bio-Rad assay with bovine serum albumin as a standard.
The *E. faecalis* alanyl-tRNA synthetase containing a C-terminal 6 His tag was purified in two steps based on affinity chromatography on a nickel column and exclusion chromatography as previously described (7).

In vitro addition of L-alanine onto UDP-MurNAc-pentapeptide—The assay was performed in a total volume of 90 µl containing Tris-HCl (50 mM, pH 7.2), MgCl₂ (12.5 mM), ATP (2.5 mM), L-[¹⁴C]alanine (200 µM, 1.1 GBq/m mole; ICN, Costa Mesa, Ca.), UDP-MurNAc-pentapeptide (67 µM), tRNA (24 µg), alanyl-tRNA synthetase (10 µg), and either or both the BppA1 and BppA2 ligases (10 µg). UDP-MurNAc-pentapeptide was purified from *S. aureus* as previously described (9). In certain experiments, UDP-MurNAc-pentapeptide was replaced by UDP-MurNAc-hexapeptide (67 µM) prepared as previously described (7). The reaction mixture was incubated at 30 °C, 15 µl aliquots were taken at 0, 30, 60, 90, 120, 180, and 240 min, and heated at 96 °C for 2 min to stop the reaction. The products of the reactions were separated by high pressure liquid chromatography (HPLC) on a Hypersil C18 column (3 µm, 4.6 x 250 mm, Interchrom, Montluçon, France) at a flow rate of 0.5 ml/min with a 0 to 4 % acetonitrile gradient in 10 mM ammonium acetate pH 5.0 (3). Products were detected by the absorbance at 262 nm and liquid scintillation with a Radioflow Detector (LB508, Perkin Elmer, Courtaboeuf, France) coupled to the HPLC apparatus (L-62000A, Merck, Nogent-sur-Marne, France).

Analysis of UDP-MurNAc-peptides by mass spectrometry—Samples of the UDP-MurNAc-peptide products were isolated by rpHPLC, lyophilized, and dissolved in H₂O:CH₃CN (50:50, v/v). The sample was injected at a flow rate of 10 µl/min in a Micromass (Manchester, UK) Q-TOF electrospray mass spectrometer operating in the positive mode. Nozzle-skimmer experiments, performed with a cone voltage of 55V and argon at a pressure of 15 psi as the collision gas (energy 30-35 eV), produced ions
corresponding to the MurNAc-peptide or lactyl-peptide moieties of the molecules. These fragments were subjected to an additional stage of MS/MS using argon at a pressure of 15 psi as the collision gas (energy 30-35 eV).

Construction of the E. faecalis strains—The bppA2 gene of E. faecalis JH2-2 (10) was replaced by an *erm* erythromycin resistance (EmR) gene cassette (11) by homologous recombination. Briefly, standard recombinant DNA techniques were used to construct plasmid pNJ25 which consists of the vector pH51 (rep* oriTn916 GentR, to be described elsewhere) and the *erm* gene flanked by 543-bp and 489-bp sequences originally located upstream and downstream of bppA2 in the chromosome of E. faecalis JH2-2. Plasmid pNJ25 was introduced into E. faecalis JH2-2 by electroporation with selection for erythromycin resistance at permissive temperature for plasmid replication (28 °C). Replacement of the bppA2 gene by *erm* was obtained by selecting clones resistant to erythromycin at non-permissive temperature (39 °C) and screening for the loss of the gentamicin resistance (GentR) marker carried by the vector pH51. The bppA2 locus of the parental strain E. faecalis JH2-2 and of an EmR GentS derivative, designated E. faecalis JH2-2ΔbppA2, was analyzed by PCR and Southern blot hybridization to confirm replacement of bppA2 by *erm* (data not shown). E. faecalis JH2-2(vanA+) and JH2-2ΔbppA2(vanA+) were constructed by introducing a self-transferable plasmid containing the vanA vancomycin resistance gene cluster into JH2-2 and JH2-2ΔbppA2 by conjugation, respectively (12).

Peptidoglycan structure analysis—Bacteria were grown at 37 °C to an optical density of 0.8 in BHI broth, containing 50 μg/ml of vancomycin for JH2-2(vanA+) and JH2-2ΔbppA2(vanA+). Peptidoglycan was extracted with 4% sodium dodecyl sulfate at 100° C and treated with pronase (200 μg/ml) and trypsin (200 μg/ml), as described (13). Muropeptides were obtained by digestion of the peptidoglycan with lysozyme (200 μg/ml)
and mutanolysin (200 µg/ml) for 16 h at 37 °C. Muropeptides were reduced with sodium borohydrate, and separated by rpHPLC on C18 column (3 µm, 4.6 x 250 mm, Interchrom) with a 0 to 20 % gradient applied between 10 and 90 min (buffer A: 0.05 % trifluoroacetic acid in water; buffer B: 0.035 % trifluoroacetic acid in acetonitrile). The relative abundance of muropeptides was estimated by the % of the integrate area of peaks detected by the absorbance at 206 nm. Co-injection of muropeptide preparations from different strains was used to confirm differences in the retention times. The same HPLC conditions were used for liquid chromatography coupled to mass spectrometry except that the C18 column was an ODS Hypersil (3 µm, 4.6 x 250 mm, Keystone Scientific, Inc., USA). Mass spectral data were obtained using a Micromass Q-TOF equipped with an electrospray ion source. The mass spectrometer was interfaced with the C18 column using a flow splitter to reduce the flow rate to the ion source to 50 µl/min. The mass scan range was from $m/z$ 100 to $m/z$ 2500. The time of flight mode with a scan cycle of 3 sec. was used to analyze ions. The data were acquired with a capillary voltage of 3200 V and a cone voltage of 25 V. The source temperature and desolvation temperature were kept at 80 °C and 150 °C, respectively.
RESULTS

In vitro synthesis of the L-alanyl-L-alanine side chain—Sequence comparisons have indicated that the chromosome of *E. faecalis* encodes two proteins, BppA1 and BppA2 (formerly designated ORF2 and ORF1, respectively), that are related to the Fem proteins of *S. aureus* (7). The UDP-MurNAc-pentapeptide:L-alanine ligase activity of BppA1 was detected based on addition of L-[14C]Ala to UDP-MurNAc-pentapeptide followed by detection of radioactive UDP-MurNAc-hexapeptide by rpHPLC coupled to liquid scintillation (7). The assay contained tRNA, Mg2+, ATP, and purified *E. faecalis* alanyl tRNA synthetase to generate the Ala-tRNA substrate of the ligase (7). In this report, we have purified the *bppA2* gene product (Experimental Procedures) and shown that addition of the purified protein to the assay resulted in the appearance of a novel radioactive product (peak B in Fig. 2A), in addition to the UDP-MurNAc-hexapeptide product of BppA1 (peak A in Fig. 2A). Kinetics (data not shown) revealed that peak B increased slowly between 60 and 240 min after appearance of peak A.

Structure of the product in peak B—The reaction was scaled up, L-[14C]Ala was replaced by L-Ala, and the material in peaks A and B was purified for mass-spectrometry and MS/MS analysis. The molecular mass of compound B was determined to be 1291.4 Da from the peaks at \( m/z \) 1292.4, 646.7 and 665.7, that were assigned to be [M+H]+, [M+2H]2+ and [M+H+K]2+ ions, respectively (Fig. 2B). These molecular masses match the predicted values of 1291.4 Da for UDP-MurNAc-heptapeptide. The same analysis performed on the nucleotide substrate and the material in peak A revealed the predicted values of 1149.4 Da and 1220.4 Da for UDP-MurNAc-pentapeptide and UDP-MurNAc-hexapeptide, respectively (data not shown).
In addition to the parent ion, the mass spectrum of UDP-MurNAc-heptapeptide displayed in Fig. 2B contained two peaks at \( m/z \) 888.4 and 703.3 that were subjected to an additional stage of MS/MS (Fig. 2C and D, respectively). These ions, corresponding to the MurNAc-heptapeptide and 2-hydroxy propionyl (lactyl) heptapeptide moieties of the molecules, were expected to contain an L-lysyl residue substituted by four alanyl residues that can be distinguished as follows. Two of the four alanyl residues are linked to the \( \varepsilon \)-amino group of L-lysine and form the L-Ala-L-Ala side chain of the precursor. Fragmentation in the side chain should lead to a mass difference of 71 and 142 Da for the loss of the N-terminal and both L-alanyl residues, respectively. The remaining two alanyl residues are linked to the \( \alpha \)-carboxyl of L-lysine and form the C-terminal D-Ala-D-Ala end of the pentapeptide stem. Loss of the C-terminal and both D-alanyl residues should lead to a mass difference of 89 and 160 Da, respectively.

Peaks at \( m/z \) 746.3 (Fig. 2C) and 561.3 (Fig. 2D) matched the predicted value for loss of two N-terminal alanyl residues from MurNAc- and lactyl-heptapeptide, respectively. Presence of these ions is consistent with addition of two L-alanines to the \( \varepsilon \)-amino group of L-lysine. Loss of one and two C-terminal D-alanyl residues gave ions at \( m/z \) 799.4 and 728.4 for MurNAc-heptapeptide (Fig. 2C) and ions at 614.4 and 543.3 for lactyl-heptapeptide (Fig. 2D). Loss of additional alanyl residues gave ion at \( m/z \) 657.4 for MurNAc-heptapeptide (Fig. 2C) and ions at 472.3 and 401.2 for lactyl-heptapeptide, the latter ion corresponding to the lactyl-L-Ala-\( \gamma \)-D-Glu-L-Lys moiety of the molecule. Fragmentation of MurNAc-heptapeptide generated lactyl-heptapeptide (peak at \( m/z \) 703.4 in Fig. 2C) and, as described above, derivatives at \( m/z \) 614.4, 543.3, and 472.3. Finally, loss of H\(_2\)O, CO, and HCOOH from the ions described above could account for peaks at \( m/z \) 870.4, and 525.3 (Fig. 2C) and at \( m/z \) 685.4, 586.4, 525.3, 515.3, 497.3, 454.3, 383.2, and 355.2 (Fig. 2D).
Peaks at \textit{m/z} 560.3 and 431.3 matched the expected mass of the D-Glu-L-Lys(\textit{ε}-L-Ala-L-Ala)-D-Ala-D-Ala and L-Lys(\textit{ε}-L-Ala-L-Ala)-D-Ala-D-Ala moieties of MurNAc- and lactyl-heptapeptide, respectively (Fig 2C and 2D). These ions confirmed that four alanyl residues are branched to the L-lysyl residue. Further loss of one to four alanyl residues from the ion at \textit{m/z} 560.3 resulted in the peaks at \textit{m/z} 471.3, 400.3, 329.2, and 258.2. Additional derivatives of the ion at \textit{m/z} 258.2 (Glu-Lys) could be generated by the loss of H$_2$O (240.2) or of NH$_3$ and HCOOH (195.1). In the case of the ion at \textit{m/z} 431.3, a lysine substituted by four alanyl residues, further fragmentation resulted in the loss of NH$_3$ (peak at \textit{m/z} 414.2), a C-terminal D-alanyl residue (342.3), both C-terminal residues (271.2), an N-terminal L-alanyl residue and NH$_3$ (343.3), or both N-terminal L-alanyl residues (289.2).

MS/MS experiments were also similarly performed on the MurNAc-peptide and lactyl-peptide fragments of the substrate of the reaction (UDP-MurNAc-pentapeptide) and the product of BppA1 (UDP-MurNAc-hexapeptide, peak A in Fig. 2A). The results (data not shown) confirmed several aspects of the fragmentation patterns of UDP-MurNAc-heptapeptide. Together, these results established that the BppA1 and BppA2 ligases add two L-alanyl residues to the \textit{ε}-amino group of L-lysine in the pentapeptide stem of the nucleotide UDP-MurNAc-L-Ala-\textit{γ}-D-Glu-L-Lys-D-Ala-D-Ala.

Respective roles of BppA1 and BppA2 in side chain synthesis—In the experiments depicted in Fig. 2A, the reaction catalyzed by the BppA1 and BppA2 ligases were coupled. To determine whether the two ligases can function independently from each other, the UDP-MurNAc-hexapeptide product of BppA1 was purified by rpHPLC and incubated with BppA2 and the reagents for production of Ala-tRNA. Under such conditions, BppA2 catalyzed formation of UDP-MurNAc-heptapeptide from UDP-MurNAc-hexapeptide in the absence of BppA1 (data not shown). Incubation of UDP-MurNAc-pentapeptide with BppA2 did not
result in addition of L-\[^{14}\text{C}]\text{alanine to this nucleotide. Finally, no radioactive peak appeared when BppA1 was incubated with UDP-MurNAc-hexapeptide. Thus, the BppA1 and BppA2 ligases specifically add the first and second L-alanyl residues of the side chain of peptidoglycan precursors, respectively (Fig. 2E). The ligases can function independently from each other.

**Deletion of the bppA2 gene**—The bppA2 gene was deleted from the chromosome of *E. faecalis* JH2-2 by replacing the corresponding open reading frame by an *erm* erythromycin resistance gene cassette. The generation time of JH2-2 (43.0±2.7 min) and JH2-2\(\Delta\)bppA2 (46.0±2.1 min) were similar in BHI broth at 37 °C (five independent experiments). The minimal inhibitory concentration (MIC) of ceftriaxone and ampicillin were determined three times with an inoculum of \(10^5\) colony forming unit on BHI agar after 48 h of incubation at 37 °C. Deletion of bppA2 was associated with an 8-fold reduction of the MIC of ceftriaxone (from 1000 \(\mu\)g/ml for JH2-2 to 128 \(\mu\)g/ml for JH2-2\(\Delta\)bppA2). The MIC of ampicillin was less affected (1 and 0.5 \(\mu\)g/ml for JH2-2 and JH2-2\(\Delta\)bppA2, respectively).

**Analysis of peptidoglycan structure**—The peptidoglycan of *E. faecalis* JH2-2\(\Delta\)bppA2 and of the parent strain JH2-2 was analyzed by liquid chromatography coupled to mass spectrometry to evaluate the impact of the deletion of the bppA2 gene on the structure of the cross-bridge. Since isomers containing the same number of alanyl residues cannot be distinguished based on mass determination, the peptidoglycan of derivatives of *E. faecalis* JH2-2 and JH2-2\(\Delta\)bppA2 harboring the vanA gene cluster was also analyzed to identify muropeptides containing a tetrapeptide stem (Fig. 1). Alone or in combination, introduction of the vanA gene cluster and the \(\Delta\)bppA2 deletion into *E. faecalis* JH2-2 generated four peptidoglycan types differing by the number of alanyl residues in the C-terminus of the stem peptides, in the cross-bridges, and in the free N-terminal side chains (Fig. 3).
Based on previous analyses (2,13), the majority of the muropeptides from the wild-type strain was expected to contain two D-alanyl residues at the free C-terminus of the peptide stems (a = 2 in Fig. 3A) and two L-alanyl residues both in the cross-bridges (b = 2) and in the free N-terminal side chains (c=2). Depending upon the degree of oligomerization (n), the number of alanyl residues (k), defined as k = a + n*b + c, should be equal to 4, 6, 8, and 10 for n = 0 (monomer), n=1 (dimer), n=2 (trimer), and n=3 (tetramer) (Fig. 3B). The calculated masses of these structures (Fig. 3B) matched the observed masses (Fig. 3C and data not shown) of the most abundant monomer, dimer, trimer, and tetramer of JH2-2 (Table I).

Muropeptides from JH2-2(vanA+) were expected to contain a tetrapeptide stem (a = 1) due to hydrolysis of the D-Ala-D-Lac ester bond (2). The observed mass of the most abundant muropeptides of JH2-2(vanA+) matched the mass of the monomer, dimer, trimer, and tetramer calculated with k values of 3, 5, 7, and 9, respectively. The major muropeptides of JH2-2 and JH2-2(vanA+) were not detected in JH2-2ΔbppA2 and JH2-2ΔbppA2(vanA+), since deletion of the bppA2 gene reduced the number of L-alanyl residues to one, both in the cross-bridge (b = 1) and in the free side chain (c = 1). This led to two novel series of monomers, dimers, trimers and tetramers with k values of 3, 4, 5, and 6 for JH2-2ΔbppA2 and k values of 2, 3, 4, and 5 for JH2-2ΔbppA2(vanA+).

The second most abundant muropeptides contained a tripeptide stem (a = 0) (Fig. 3 and Table I). In these series, muropeptides with the same retention time and the same mass were detected in JH2-2 and JH2-2(vanA+) or in JH2-2ΔbppA2 and JH2-2ΔbppA2(vanA+). Muropeptides containing a tripeptide stem may be generated by an L,D-carboxypeptidase cleaving the L-Lys3-D-Ala4 peptide bond (14) or originate from incorporation into the cell wall of incomplete peptidoglycan precursors lacking the C-terminal D-Ala-D-Ala extremity.
Additional muropeptides of lower abundance were accounted for by the following modifications of the main structures described above (Fig. 3 and data not shown). In agreement with previous analyses (2,13,15), sugar O-acetylation led to a mass increase of 42 Da. A mass difference of 480.2 Da was assigned to the loss of the disaccharide GlcNAc-MurNAc. These muropeptides may have been generated by amidases (14) produced by the four *E. faecalis* strains or present as a contaminating activity in the lysozyme and mutanolysin preparations used to digest the peptidoglycan *in vitro*. The remaining muropeptides included monomers with an unsubstituted L-lysyl residue. Minor peaks, that could correspond to dimers with an unsubstituted acceptor stem, were also detected. These unsubstituted muropeptides may originate from incorporation into the wall of incomplete peptidoglycan precursors or from cleavage of the L-Lys-ε-L-Ala1 peptide bound by as yet unidentified hydrolases.
DISCUSSION

Many species of Gram-positive bacteria produce branched peptidoglycan precursors resulting from the addition of various amino acids to the ε-amino group of L-lysine in the pentapeptide stem L-alanyl-γ-D-glutaminyl-L-lysyl-D-alanyl-D-alanine (3). The N-terminus of these side chains is linked to D-Ala₄ of another stem peptide by the D,D-transpeptidases in the final cross-linking step of peptidoglycan synthesis (Fig. 1). The ligases for addition of the first residue of the cross-bridge of *E. faecalis* (BppA1) and *Weissella viridescens* (FemX) were shown to transfer an alanyl residue from L-alanyl-tRNA to UDP-MurNAc-pentapeptide (7,8). In this study, we have identified the ligase for incorporation of the second residue of the \( N^\varepsilon \)-L-Ala₁-L-Ala₂ side chain of the *E. faecalis* peptidoglycan precursors based on purification of the *bppA2* gene product, demonstration of its UDP-MurNAc-hexapeptide:L-alanine ligase activity, and analysis of the structure of the heptapeptide stem by MS/MS (Fig. 2). The BppA1 and BppA2 ligases were able to function independently from each other and specifically added the first and second residue of the side chain, respectively. This is the first report of the enzymatic synthesis of a complete branched peptidoglycan precursor *in vitro*.

In wild-type *E. faecalis* JH2-2, the substantial majority of the muropeptides contained two L-alanyl residues in the cross-bridge and in the free N-terminal side chain (Fig. 3 and Table I). This observation implies that the BppA1 and BppA2 ligases efficiently synthesized the L-alanyl-L-alanine side chain prior to the translocation of the peptidoglycan precursors to the cell surface. Deletion of the *bppA2* gene was associated with production of muropeptides containing a single alanyl residue both in the cross-bridge and in the free N-terminal side chain. Thus, *E. faecalis* JH2-2 did not produce any enzyme that could substitute for BppA2. In agreement, BppA1 was unable to add *in vitro* the second alanyl residue of the side chain and no additional *bpp* homologue was detected in the genome of *E. faecalis* V583.
Analysis of the structure of mature peptidoglycan in mutants of *S. pneumoniae* have established that the *murM* and *murN* gene products are required for incorporation of the 1st (L-Ala or L-Ser) and 2nd (L-Ala) amino acid of the side chain (16,17). Amino acid sequence identity between BppA1 and MurM (39%) and between BppA2 and MurN (38%) indicates that the proteins necessary for incorporation of the first and second residues of the side chain in *E. faecalis* and *S. pneumoniae* may be considered as orthologues (24-25 % if paralogues are compared, ref. 7). The relationships between the Bpp ligases and more distantly related homologues from *S. aureus* are less obvious (7). In the latter bacterium, inactivation or impaired expression of the *fmhB*, *femA*, and *femB* genes leads to an increase in the relative abundance of muropeptides containing 0, 1, and 3 glycyl residues, respectively (4,18). These observations indicate that the corresponding gene products are required for incorporation of glycyl residues at the 1st (FmhB), 2nd and 3rd (FemA), and 4th and 5th positions (FemB) of the pentaglycine side chain. Thus, FemA and FemB may each be responsible for incorporation of two residues. If this is the case, elongation of the side chain should proceed by sequential addition of glycyl residues from glycyl-tRNA to peptidoglycan precursors, since the dipeptide glycyl-glycine is not incorporated into peptidoglycan by particulate enzyme preparations from *S. aureus* and synthesis of glycyl-glycyl-tRNA was not detected (19). Alternatively, FemA and FemB may only add the 2nd and 4th residues. This would imply that the 3rd and 5th residues are added to the side chain by as yet uncharacterized enzymes. Candidate genes for these functions do exist in *S. aureus* since the genome contains a total of five *bpp* homologues (20), a number of genes that matches the number of glycyl residues in the cross-bridge.

Structural variations at the C-terminus of the stem peptide and in the side chain are expected to affect interaction of the D,D-transpeptidases with their donor and acceptor substrates, respectively (Fig. 1). However, expression of the *vanA* gene cluster and deletion of
the \textit{bppA2} gene, alone or in combination, had little impact on peptidoglycan cross-linking (Table I). Thus, both modifications of the structure of the peptidoglycan precursors were tolerated by the D,D-transpeptidases of \textit{E. faecalis}.

In \textit{S. aureus}, inactivation of \textit{femA} or \textit{femB} abolishes methicillin resistance mediated by the low affinity PBP2a (4), whereas \textit{fmhB} is essential for viability (18). In \textit{S. pneumoniae}, \textit{murM} and \textit{murN} are both unessential genes (16,17). Inactivation of \textit{murM} prevents expression of \(\beta\)-lactam resistance mediated by mosaic PBPs whereas disruption of \textit{murN} only produces a modest decrease in the level of resistance to these antibiotics (16,21). \textit{Enterococcus faecalis} is intrinsically resistant to third generation cephalosporins, such as ceftriaxone, due to production of a low-affinity D,D-transpeptidase (PBP5) which is produced by nearly all members of the species (22). The \textit{bppA2} deletion led to a moderate decrease (8-fold) in the minimal inhibitory concentration of ceftriaxone. This observation suggests that PBP5 did not function optimally with incomplete peptidoglycan precursors as proposed for the low-affinity penicillin-binding proteins responsible for acquired resistance to \(\beta\)-lactam antibiotics in \textit{S. aureus} and \textit{S. pneumoniae} (4,16,21).

Screening of bacterial genomes (data not shown) revealed the presence of \textit{bpp} homologues in all bacteria for which production of branched peptidoglycan precursors containing L-amino acid or glycy1 residues can be inferred from existing data on the structure of the cross-bridges (3). Conversely, \textit{bpp} homologues were not detected if the cross-bridges contained D-amino acids (e.g. \textit{E. faecium} and \textit{Lactococcus lactis}) or if the peptidoglycan was directly cross-linked (e.g \textit{E. coli} and \textit{Bacillus subtilis}). Thus, the ligases for incorporation of L-amino-acids into the side chain of peptidoglycan precursors appear to form a unique family of non-ribosomal peptide bond synthesizing enzymes that use aminoacyl-tRNAs as substrates. However, the proteins do not possess a unique fold as the recently solved X-ray crystal structure of FemA revealed striking similarities with a histone acetyltransferase (23).
Together, these observations suggest that the ligases for synthesis of branched peptidoglycan precursors could be useful targets for the development of narrow-spectrum antibacterial agents active against certain β-lactam resistant Gram-positive bacteria. Such drugs would be expected to restore the activity of β-lactam antibiotics against strains producing low-affinity PBPs and to be active alone if one of the ligases is essential, as this is the case for FmhB in *S. aureus*. 
Acknowledgments—This work was supported by Wyeth Research, by the Programme de Recherche Fondamentale en Microbiologie et Maladies Infectieuses et Parasitaires (MENRT) and by the Fondation pour la Recherche Médicale. *E. faecalis* genome sequence data were kindly provided by The Institute for Genomic Research as publicly released at http://www.tigr.org.
REFERENCES

1. Arthur, M., and Courvalin, P. (1993) *Antimicrob. Agents Chemother.* **37**, 1563-1571

2. Boudewijn, L., De Jonge, M., Handwerger, S., and Gage, D. (1996) *Antimicrob. Agents Chemother.* **40**, 863-869

3. Schleifer, K. H., and Kandler, O. (1972) *Bacteriol. Rev.* **36**, 407-477

4. Stranden, A. M., Ehlert, K., Labischinski, H., and Berger-Bachi, B. (1997) *J. Bacteriol.* **179**, 9-16

5. Plapp, R., and Strominger, J. L. (1970) *J. Biol. Chem.* **245**, 3667-3674

6. Staudenbauer, W., and Strominger, J. L. (1972) *J. Biol. Chem.* **247**, 5095-5102

7. Bouhss, A., Josseaume, N., Allanic, D., Crouvoisier, M., Gutmann, L., Mainardi, J.-L., Mengin-Lecreulx, D., van Heijenoort, J., and Arthur, M. (2001) *J. Bacteriol.* **183**, 5122-5127

8. Hegde, S. S., and Shrader, T. E. (2001) *J. Biol. Chem.* **276**, 6998-7003

9. Arthur, M., Depardieu, F., Cabanie, L., Reynolds, P., and Courvalin, P. (1998) *Mol. Microbiol.* **30**, 819-830

10. Jacob, A. E., and Hobbs, S. J. (1974) *J. Bacteriol.* **117**, 360-372

11. Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C., and Courvalin, P. (1990) *Nucleic Acids Res.* **18**, 3660

12. Arthur, M., Depardieu, F., Gerbaud, G., Galimand, M., Leclercq, R., and Courvalin, P. (1997) *J. Bacteriol.* **179**, 97-106

13. Mainardi, J.-L., Billot-Klein, D., Coutrot, A., Legrand, R., Schoot, B., and Gutmann, L. (1998) *Microbiology* **144**, 2679-2685

14. Holtje, J. V. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 181-203
15. Mainardi, J.-L., Legrand, R., Arthur, M., Schoot, B., van Heijenoort, J., and Gutmann, L. (2000) *J. Biol. Chem.* **275**, 16490-16496

16. Filipe, S. R., Pinho, M. G., and Tomasz, A. (2000) *J. Biol. Chem.* **275**, 27768-27774

17. Filipe, S. R., and Tomasz, A. (2000) *Proc. Natl. Acad. Sci. USA.* **97**, 4891-4896

18. Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H., and Berger-Bachi, B. (1999) *Proc. Natl. Acad. Sci. U S A* **96**, 9351-9356

19. Matsuhashi, M., Dietrich, C. P., and Strominger, J. L. (1967) *J. Biol. Chem.* **242**, 3191-3206

20. Tschierske, M., Mori, C., Rohrer, S., Ehlert, K., Shaw, K. J., and Berger-Bachi, B. (1999) *FEMS Microbiol Lett* **171**, 97-102

21. Weber, B., Ehlert, K., Diehl, A., Reichmann, P., Labischinski, H., and Hakenbeck, R. (2000) *FEMS Microbiol. Lett.* **188**, 81-85

22. Murray, B. E. (1990) *Clin. Microbiol. Rev.* **3**, 46-65

23. Benson, T., Prince, D., Mutchler, V., Curry, K., Ho, A., Sarver, R., Hagadorn, J., Choi, G., and Garlick, R. (2002) *Structure (Camb)* **10**, 1107-1115
Legends to figures

FIG. 1. Schematic representation of peptidoglycan cross-linking in E. faecalis. A and B, glycopeptide-susceptible and -resistant strains, respectively. Leaving groups are circled. The cross-bridge is boxed. D-glutamic acid is incorporated into the precursors and secondarily α-amidated. D-Lac, D-lactate; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid. The orientation of the CO-NH peptide bonds is indicated by arrows.

FIG. 2. Structure of UDP-MurNAc-heptapeptide produced by the BppA1 and BppA2 ligases. A, Separation of [14C]-labeled L-alanine, UDP-MurNAc-hexapeptide (peak A), and UDP-MurNAc-heptapeptide (peak B) by rpHPLC. B, MS analysis of UDP-MurNAc-heptapeptide showing peaks at m/z 1292.4, 646.7 and 665.7, that were assigned to be [M+H]^+, [M+2H]^{2+} and [M+H+K]^{2+} ions, respectively. Peaks at m/z 888.4 and 703.3, labeled with a circled m and y, were assigned to be the N-acetyl-muramyl-heptapeptide and lactyl-heptapeptide moieties of the molecule, respectively. C and D, MS/MS analysis of peak y and m, respectively. E, Reactions catalyzed by alanyl-tRNA synthetase (AlaS) and the BppA1 and BppA2 ligases. The position of the cleavage generating the MurNAc-heptapeptide (m) and lactyl heptapeptide (y) moieties of UDP-MurNAc-heptapeptide is indicated by a double arrow.

FIG. 3. Structure of muropeptides. A, Model muropeptide showing all possible variations in the number of alanyl residues in the free C-terminus of peptide stems (a), in the cross-bridges (b), and in the free N-terminal side chains (c). The number of cross bridge is equal to n. The number of Ala (k) is defined as k = a + n*b + c. B, Calculated mass of muropeptides. C, Structure of the most abundant dimers detected in the four strains of E. faecalis. The relative
abundance and the observed mass (Da) are indicated for the main forms and derivatives carrying an O-acetylated sugar or lacking one GlcNAc-MurNAc disaccharide. A, l-Ala or d-Ala; G, N-acetylglucosamine; K, l-Lys; M, N-acetylmuramic acid; Q, d-isoglutamine.
Fig. 1
\[
\begin{array}{|c|c|c|c|}
\hline
\text{Strain} & \text{N° of Ala at position:} & \text{Abundance (k)} \\
& a & b & c & \text{Monomers n=0} & \text{Dimers n=1} & \text{Trimers n=2} & \text{Tetramers n=3} \\
\hline
\text{JH2-2} & 2 & 2 & 2 & 17.3 (4) & 22.6 (6) & 12.0 (8) & 5.6 (10) \\
\text{JH2-2 (vanA+)} & 1 & 2 & 2 & 23.4 (3) & 22.0 (5) & 9.7 (7) & 0.0 (9) \\
\text{JH2-2ΔbppA2} & 2 & 1 & 1 & 18.7 (3) & 31.6 (4) & 19.4 (5) & 5.0 (6) \\
\text{JH2-2ΔbppA2 (vanA+)} & 1 & 1 & 1 & 19.2 (2) & 24.9 (3) & 11.8 (4) & 3.6 (5) \\
\text{JH2-2} & 0 & 2 & 2 & 5.0 (2) & 7.2 (4) & 4.0 (6) & 1.0 (8) \\
\text{JH2-2 (vanA+)} & 0 & 2 & 2 & 2.1 (2) & 7.5 (4) & 3.7 (6) & 0.0 (8) \\
\text{JH2-2ΔbppA2} & 0 & 1 & 1 & 5.4 (1) & 8.9 (2) & 3.8 (3) & 1.3 (4) \\
\text{JH2-2ΔbppA2 (vanA+)} & 0 & 1 & 1 & 4.8 (1) & 4.5 (2) & 0.2 (3) & 0.0 (4) \\
\hline
\end{array}
\]

TABLE I

*Relative abundance of muropeptides from the four E. faecalis strains analyzed*

The relative abundance of the most abundant forms of each oligomeric state is indicated in %. The muropeptides of the strains differ by the number of alanyl residues (k) present in the cross-bridge and at the C- and N-terminal positions.
Synthesis of the L-alanyl-L-alanine cross-bridge of enterococcus faecalis peptidoglycan  
Ahmed Bouhss, Nathalie Josseaume, Anatoly Severin, Keiko Tabei, Jean-Emmanuel Hugonnet, David Shlaes, Dominique Mengin-Lecreulx, Jean van Heijenoort and Michel Arthur

*J. Biol. Chem.* published online September 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207449200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts