Asl$_{fm}$, the d-Aspartate Ligase Responsible for the Addition of d-Aspartic Acid onto the Peptidoglycan Precursor of Enterococcus faecium*

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D-Aspartate ligase has remained the last unidentified peptide bond-forming enzyme in the peptidoglycan assembly pathway of Gram-positive bacteria. Here we show that a two-gene cluster of Enterococcus faecium encodes aspartate racemase (Rac$_{fm}$) and ligase (Asl$_{fm}$) for incorporation of D-Asp into the side chain of the peptidoglycan precursor. Asl$_{fm}$ was identified as a new member of the ATP-grasp protein superfamily, which includes a diverse set of enzymes catalyzing ATP-dependent carboxylate-amino ligation reactions. Asl$_{fm}$ specifically ligated the β-carboxylate of D-Asp to the ε-amino group of L-Lys in the nucleotide precursor UDP-N-acetylmuramyl-pentapeptide. D-iso-Asparaginase was not a substrate of Asl$_{fm}$, indicating that the presence of this amino acid in the peptidoglycan of E. faecium results from amidaion of the α-carboxyl of D-Asp after its addition to the precursor. Heterospecific expression of the genes encoding Rac$_{fm}$ and Asl$_{fm}$ in Enterococcus faecalis led to production of stem peptides substituted by D-Asp instead of L-Ala$_2$, providing evidence for the in vivo specificity and function of these enzymes. Strikingly, sequencing of the cross-bridges revealed that substitution of L-Ala$_2$ by D-Asp is tolerated by the D,D-transpeptidase activity of the penicillin-binding proteins both in the acceptor and in the donor substrates. The Asl$_{fm}$ ligase appears as an attractive target for the development of narrow spectrum antibiotics active against multiresistant E. faecium.

Peptidoglycan is a macromolecule found on the outer face of the cytoplasmic membrane of all eubacteria except certain halophilic bacteria, such as Halobacterium halobium, and intracellular parasites, such as Mycoplasma pneumoniae (1). This structure is essential to protect bacteria against the internal osmotic pressure and plays a key role in cell division. The basic unit of peptidoglycan is a disaccharide peptide G-pentapeptide. D,-amino group of L-Lys3 in the cytoplasmic precursor UDP-D-glutamyl-L-lysyl-D-alanyl-D-alanine ligase (ADP-forming) (EC.6.3.1.1); Rac, aspartate racemase; IPTG, isopropyl-β-D-thiogalactopyranoside; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high-pressure liquid chromatography; RP-HPLC, reverse-phase HPLC; PBp, penicillin-binding protein; iGln, iso-Gln.

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2 The abbreviations used are: MurNAc, N-acetylmuramic acid; Asl, β,β-aspartic:UDP-N-acetylmuramoyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanylatedano amino ligase (ADP-forming) (EC.6.3.1.1); Rac, aspartate racemase; IPTG, isopropyl-β-D-thiogalactopyranoside; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high-pressure liquid chromatography; RP-HPLC, reverse-phase HPLC; PBp, penicillin-binding protein; iGln, iso-Gln.
The D-Aspartate Ligase of Enterococcus faecium

FIGURE 1. Incorporation of D-Asp into the peptidoglycan precursor and cross-bridge formation in E. faecium. A, D-Asp is added to the ε-amino group of ε-Lys by the Aslfm, D-aspartate ligase. The reaction proceeds through formation of a β-aspartyl-phosphate intermediate (11). D-Asp is produced by the Racfm, aspartate racemase. B, peptidoglycan is polymerized from a di-saccharide-peptide subunit containing β-1,4-linked GlcNAc and MurNAc, a pentapeptide stem (L-Ala-L-Gln-L-Lys-D-Ala-D-Ala), and a side chain D-Asp residue linked to the ε-amino group of ε-Lys. The ε-carboxyl group of ε-Asx is partially amidated in mature peptidoglycan (ε-Asx). The ε-carboxyl group of ε-iso-Glu is fully amidated (ε-iGln).

D,-transpeptidases

Computers of E. faecium. Heterospecific expression of the cluster in E. faecalis was used to demonstrate the function of the proteins in vivo and to study the consequences of substitution of L-Ala2 by D-Asp on peptidoglycan cross-linking by the D:D-transpeptidases.

EXPERIMENTAL PROCEDURES

D-Aspartate Ligase Assays—The standard assay was performed in a total volume of 25 μl containing Tris-HCl (100 mM, pH 8.5), MgCl₂ (50 mM), ATP (20 mM), D-[¹⁴C]aspartic acid (0.11 mM, 2 GBq/mmol, Isobio, Fleurs, Belgium), and UDP-MurNAc-pentapeptide (0.15 mM) prepared from S. aureus (16). The reaction was incubated for 15–120 min at 37 °C and stopped by boiling for 3 min. UDP-MurNAc-pentapeptide-D-[¹⁴C]Asp was separated from D-[¹⁴C]Asp by descending paper chromatography (Whatman 4MM, Elancourt, France) with a mobile phase composed of isobutyric acid and 1 M ammonia (5:3, v/v). Alternatively, the products of the reaction were separated by reverse phase high-pressure liquid chromatography (rp-HPLC) on a Hypersil C18 column (3 μm, 4.6 × 250 nm, Interchrom, Montluçon, France) using isocratic elution (10 mM ammonium acetate, pH 5.0) at a flow rate of 0.5 ml/min. The products were detected by the absorbance at 262 nm and liquid scintillation with a Radiolinker Detector (LB508; PerkinElmer Life Sciences, Courtabœuf, France) coupled to the HPLC apparatus (L-62000A; Merck, Nogent-Sur-Marne, France).

The specificity of the D-aspartate ligase was studied by replacing D-[¹⁴C]Asp by D-Asp, L-Asp, D-iso-asparagine, D-Glu, D-Ala, and D-malic acid (5 mM; Sigma, Saint-Quentin Fallavier, France). The products of the addition reactions were detected by tandem mass spectrometry, and their structures were determined by tandem mass spectrometry (MS/MS) (7).

Identification of the D-aspartate Ligase by Reverse Genetics—The D-aspartate ligase (Aslfm) of E. faecium D359 (17) was partially purified in three chromatographic steps and identified as a member of the ATP-grasp protein family by MS/MS amino acid sequencing. Briefly, E. faecium D359 was grown to an OD₆₅₀ of 0.7 in 20 liters of brain heart infusion broth (Difco, Elancourt, France), harvested by centrifugation (6,000 × g for 20 min at 4 °C), and washed twice in 50 mM sodium phosphate buffer (pH 7.0). Bacteria were disrupted with glass beads in a refrigerated cell disintegrator (Sartorius, Palaissau, France) for 3 × 30 s. The extract was centrifuged at 7,000 × g for 10 min at 4 °C to remove cell debris, and the supernatant was ultracentrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was dialyzed against 50 mM phosphate buffer (pH 6.0) containing 200 mM NaCl (buffer A). Proteins (1.3 g) were loaded onto a cation exchange column equilibrated with buffer A (HiLoad 26/10 SP Sepharose HP; Amersham Biosciences, Saclay, France). Active fractions, eluting between 0.8 and 0.9 M NaCl, were pooled (12 mg of proteins), concentrated in a dialysis bag with solid polyethylene glycol, and loaded onto a gel filtration column (Superdex 75 HR26/60, Amersham Biosciences) equilibrated with buffer A. Active fractions (1.8 mg of proteins) were loaded onto a 1-m1 cation exchange column (HiTrap SP Sepharose fast flow, Amersham Biosciences) equilibrated with buffer A. Proteins (200 μg) eluting between 0.8 and 0.95 M NaCl were dialyzed against buffer A, concentrated by lyophilization, and analyzed by SDS-PAGE. The native enzyme was stored at −20 °C in 50 mM phosphate buffer (pH 6.0) containing 200 mM NaCl.

For amino acid sequencing, protein bands were excised from the SDS-PAGE gel, reduced with dithiothreitol (Sigma, Poole, UK), alkylated with iodoacetamide, and digested overnight at 37 °C with trypsin (modified trypsin, sequencing grade, Roche Applied Science). Tryptic digests were dried under vacuum, resuspended in 4 μl of 0.1% formic acid, and analyzed by HPLC (LC Packing system, LC Packing, San Francisco, CA) coupled to mass spectrometry. Chromatography was performed at a flow rate of 200 nl/min in 0.1% formic acid with three consecutive linear acetonitrile gradients (0–2% for 1 min, 2–50% for 40 min, and 50–90% for 10 min). The LC system was connected to an ion trap mass spectrometer (LCQ Deca, Finnigan Corp., San Jose, CA). The spray voltage was set at 2.1 kV, the temperature of the ion transfer tube
The D-Aspartate Ligase of Enterococcus faecium

was set at 180 °C, and the normalized collision energies were set at 35% for MS/MS. The sequences of the uninterpreted spectra were identified by correlation with the peptide sequences from the National Center for Biotechnology Information (NCBI) non-redundant protein data base using the SpectrumMill program (Millenium Pharmaceuticals, Cambridge, MA). The α-aspartate ligase was identified as a member of the ATP-grasp protein family present in the major 49-kDa protein band.

Production of the D-Aspartate Ligase in Escherichia coli and Purification of the Protein—DNA of E. faecium D359 was amplified with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) using primers Asl1 (5′-GAGAGACCATGGTGAAACGTATTGCCAAAATGAGG-3′) and Asl2 (5′-CTCCATGGCTAGGTCCTTTCCATGAAATATCCTTTTGTTG-3′). The PCR product was digested with Ncol and BamHI (underlined) and cloned into pET2818 (18), and the resulting plasmid (pSJL1) was introduced into E. coli BL21 (DE3) harboring the pREP4 plasmid (19). For protein production, bacteria were grown at 37 °C to an optical density at 600 nm of 0.7 in 2 liters of brain heart infusion broth containing kanamycin (50 μg/ml) and ampicillin (100 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added (0.5 mM), and incubation was continued for 3.5 h at 37 °C. Aslm was purified from a clarified lysate by affinity chromatography on Ni²⁺-nitrilotriacetateagarose resin (Qiagen GmbH, Hilden, Germany) and cation exchange chromatography (HiTrap SP-Sepharose fast flow, Amersham Biosciences). The protein was concentrated by ultrafiltration and stored at −20 °C in 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 50% glycerol.

Western Blot Analysis—Anti-Aslαm antiserum was obtained by three subcutaneous injections at 3-week intervals of 200 μg of purified Aslm in a New Zealand rabbit. Proteins were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane (Hybond, Amersham Biosciences, Little Chalfont, UK), and incubated with the anti-Aslαm antiserum at a 1/1000 dilution. Goat anti-rabbit IgG coupled to peroxidase (SouthernBiotech, Birmingham, AL) was used as a secondary antibody, and Aslαm was detected by chemiluminescence (ECL kit, Pierce and Amersham Biosciences). Tagged proteins were alternatively detected using polyclonal anti-Hisα rabbit antibodies (Ebiosciences, San Diego, CA).

Heterospecific Expression of the D-Aspartate Ligase Gene (aslαm) in E. faecalis—The shuttle expression vector pJEH11 (our laboratory collection) confers gentamicin resistance and replicates in E. coli and in Gram-positive hosts. The NcoI site used for cloning in pJEH11 is preceded by a constitutive promoter (20) and a ribosome binding site active in enterococci. The BamHI site is followed by in-frame codons specifying a Hisα tag. The Ncol-BamHI fragment of pSJL1 (above) containing the aslαm open reading frame was cloned into pJEH11. The resulting plasmid pSJL2(aslαm) was introduced into E. faecalis H2-2 by electroporation (20), and clones were selected and subcultured in brain heart infusion containing 128 μg/ml gentamicin.

Co-expression of the D-Aspartate Ligase (aslαm) and Aspartate Racemase (racαm) Genes in E. faecalis—The racαm open reading frame was amplified with primers rac1 (5′-AAAGAAGATCTAGCCATGGAGAATTATTTTCATATTATGGC-3′), containing BamHI (underlined) and a stop codon (bolded), and rac2 (5′-AAAGGATTCTTCTTTGCTGTCATCTCCAC-3′), containing BamHI. The PCR fragment was cloned into the BamHI restriction site of pSJL2(aslαm) (above). Insertion of the PCR product introduced a stop codon at the end of aslαm (TAG present in primer rac1) and generated an in-frame fusion between the 3′ end of racαm and the sequence of the vector pJEH11 specifying the Hisα tag. The resulting plasmid, pSJL3(aslαm racαm), carried a bi-cistronic operon encoding Aslαm and Racαm.

Peptidoglycan Structure Analysis—The structure of the peptidoglycan was determined by mass spectrometry as described previously (21).
Briefly, derivatives of *E. faecalis* JH2-2 harboring plasmids pJEH11, pSJL2(asfm) and pSJL4(asfm,racf) were grown at 37 °C to an optical density of 0.7 in 250 ml of brain heart infusion broth in the presence or absence of D-aspartate (50 mM). Peptidoglycan was extracted with boiling SDS and digested with mutanolysin and lysozyme (Sigma). The resulting muropeptides were cleaved under alkaline conditions to generate lactoyl-peptides, separated by rp-HPLC, and analyzed by MS and MS/MS using an electrospray time-of-flight mass spectrometer operating in positive mode (Qstar Pulsar I, Applied Biosystems, Courtaboeuf, France).

**RESULTS**

**Aslfm Is a Member of the ATP-Grasp Protein Family—**D-Aspartate ligase activity was detected in a cytoplasmic fraction of *E. faecium* D359 using a radioactive assay (Fig. 2A) and partially purified in three chromatographic steps (Fig. 2B). Amino acid sequencing was performed by MS/MS on individual protein bands digested with trypsin. The 49-kDa protein (Fig. 2B) was encoded by the EfaeDRAFT_0086 nucleotide sequence in the *E. faecium* genome database (U.S. Department of Energy (DOE) Joint Genome Institute). Sequence data obtained by MS/MS included the N terminus of the 49-kDa protein, revealing that the methionine specified by the proposed translation initiation codon had been cleaved. The protein was produced in *E. coli* with a C-terminal His6 tag and purified by affinity and cation exchange chromatographies (Fig. 2C). The recombinant protein was active in the radioactive assay (Fig. 3A), indicating that the gene encoding the D-aspartate ligase (*asfm*) had been successfully identified. Aslfm belonged to the ATP-grasp protein superfamily composed of highly diverse enzymes that catalyze ATP-dependent carboxylate-amine ligation reactions (22) and form acyl-phosphate intermediates (23, 24).

**Characterization of Aslfm Activity—**To determine the structure of the reaction product, the assay was scaled up using non-radioactive D-Asp for mass spectrometry (Fig. 3B) and tandem mass spectrometry (Fig. 3, C and D) analyses. The monoisotopic mass of the compound eluting
The D-Aspartate Ligase of Enterococcus faecium

**TABLE 1**

Muropeptides from *E. faecalis* JH2–2/pSJL2(asfm) and *E. faecalis* JH2–2/pJEH11 grown in the presence of D-aspartate (50 mM)

The relative abundance (% of the material in the peaks was calculated by integration of the absorbance at 210 nm. The sequence of the side chain and of the cross-bridge was determined by tandem mass spectrometry for peaks A to G and 1 to 4. Assignment of amino acids to the side chain and to the cross-bridge(s) is arbitrary for other muropeptides. The abbreviations are as follows: Tri, tripeptide; L-Ala–D-iGln–L-Lys; Tetra, tetrapeptide; L-Ala–D-iGln–L-Lys–D-Ala; Penta, pentapeptide; L-Ala–D-iGln–L-Lys–D-Ala–D-Ala; —, not detected; NA, not applicable.

| Peak | Monoisotopic mass | Stem | Side chain | Cross-bridge |
|------|-------------------|------|------------|--------------|
| JH2–2/pSJL2(asfm) | JH2–2/pJEH11 |
| **Monomers** | | | |
| A | 38.8 | 22.0 |
| B | 6.6 | — |
| C | 4.2 | — |
| D | 23.4 | — |
| E | 1.8 | 6.0 |
| F | 2.8 | 16.0 |
| **Dimers** | | | |
| D | 39.9 | 36.5 |
| E | 2.2 | — |
| F | 18.6 | 1,117.5 |
| G | 2.6 | 1,215.6 |
| H | 0.7 | — |
| I | 3.1 | 1,286.7 |
| J | 2.0 | 10.4 |
| K | 3.0 | 26.1 |
| **Trimers** | | | |
| D | 17.4 | 25.2 |
| E | 4.3 | — |
| F | 0.8 | — |
| G | 6.4 | — |
| H | 0.7 | 1,729.8 |
| I | 1.0 | — |
| J | 0.2 | 1,756.9 |
| K | 1.4 | 1,899.0 |
| L | 2.0 | 17.1 |
| M | 3.9 | 16.3 |
| N | 0.6 | — |
| O | 3.3 | — |
| P | — | 3.2 |
| Q | — | 3.2 |
| R | — | 3.2 |
| S | 1.3 | 3,150.7 |
| **Tetramers and pentamers** | | | |
| O | 3.9 | 16.3 |
| Q | 2,288.1 |
| R | 2,430.2 |
| S | 2,396.2 |
| T | 2,538.3 |
| U | 3,008.6 |
| V | 3,150.7 |

**Notes:**

- To assess the in vivo activity of the d-aspartate ligase, plasmid pSJL2(asfm) was introduced into *E. faecalis* JH2–2, which produces two transferases of the Fem family (BppA1 and BppA2) for the addition of an L-Ala side chain to the peptidoglycan precursors. This approach was used as versatile tools for gene inactivation have not been developed for *E. faecium*. Expression of the *asfm* d-aspartate ligase gene under the control of a constitutive promoter led to production of the protein as determined by Western blot analysis with anti-Aslm (Fig. 4A) and anti-His6 (Fig. 4B) antisera.

- D-Aspartate ligase activity was detected in extracts from *E. faecalis* JH2–2/pSJL2(asfm) but not in the control strain harboring the vector pJEH11 used to construct pSJL2 (data not shown).

**To investigate the impact of Aslm ligase activity on growth rate and peptidoglycan composition, *E. faecalis* JH2–2/pSJL2(asfm) and JH2–2/pJEH11 were grown in the presence or absence of D-Asp. The addition of D-Asp (50 mM) to the culture medium had no significant impact on the growth rate of the control strain *E. faecalis* JH2–2/pJEH11.**
profile was drastically altered (Fig. 5B). The L-Ala,-containing muropeptides were only detected as minor peaks (peaks 1–6), and a novel series of peaks (peaks A–R) was detected. The main monomer (peak C) had a monoisotopic mass of 674.3, which matched the calculated value for a d-lactoyl-pentapeptide stem substituted by a side chain consisting of one d-aspartyl residue. The structure was confirmed by MS/MS (Fig. 6), indicating that Aslfm catalyzed the addition of d-Asp to the peptidoglycan precursor of E. faecalis JH2-2 in competition with the addition of L-Ala by the BppA1 and BppA2 transferases of the host.

Incorporation of d-Asp by Aslfm was efficient only 12% of the monomers contained the usual L-Ala, side chain (Table 1). Hybrid side chains containing both L-Ala and d-Asp were not detected. This indicates that Aslfm did not elongate L-alanyl-containing side chains to form d-Asp-L-Ala or d-Asp-L-Ala2 side chains. This also indicates that the host transferases did not add L-Ala to d-Asp-containing precursors to form d-Asp-L-Ala or d-Asp-L-Ala2 side chains.

**DISCUSSION**

Incorporation of d-amino acids and glycine into the side chains of peptidoglycan precursors by the Fem transferases involves activation of the substrate by the aminoacyl-tRNA synthetases of the translation machinery, a pathway that cannot be directly tailored for incorporation of d-amino acids. Instead, d-aspartate has been shown in 1972 to be activated as β-aspartyl-phosphate (Fig. 1) in an ATP-dependent reaction, although the identity of the enzyme remained unknown for the following 33 years. Using reverse genetics (Fig. 2), we have identified the gene encoding the d-aspartate ligase of E. faecium (Aslfm). The UDP-MurNac-pentapeptide-d-Asp ligase activity of Aslfm (Fig. 1) has been demonstrated in vitro based on purification of the protein produced in E. coli and determination of the structure of the hexapeptide product by tandem mass spectrometry (Fig. 3). The catalytic activity of Aslfm has also been demonstrated in vivo based on heterospecific expression of the aslfm gene in E. faecalis and detection of d-Asp in the peptidoglycan cross-bridges of the recombinant strain (Figs. 5 and 6).

Genes encoding close homologues of Aslfm (Fig. 7) are present in the 10 known genomes of bacteria producing precursors substituted by d-Asp. These genes were present at a single copy per genome except for the chromosome of Lactobacillus delbrueckii, which encodes two aslfm-related proteins (81% identity). Close homologues of Aslfm were not detected in bacteria producing directly cross-linked peptidoglycan or cross-bridges containing l-amino acids and glycine. Thus, incorporation of d-amino acids into peptidoglycan precursors appears to be mediated by a single family of closely related proteins that are specialized in this function.

Low level similarity was also detected with the ubiquitous carboxamidylphosphate synthases and β-alanine β-alanine ligases, indicating that Aslfm is a novel member of the ATP-grasp superfamily. Members of the family, which also includes glutathione synthetases, biotin carboxylases, and succinyl-CoA synthases, form acylphosphate intermediates and catalyze ATP-dependent ligation of a carboxyl group carbon to an amino or imino nitrogen, a hydroxyl oxygen, or a thiol sulfur (22, 23, 29).
The δ-Aspartate Ligase of Enterococcus faecium

FIGURE 7. Alignment of Aslfm with putative δ-aspartate ligases from lactobacilli, lactococci, and pediococci. Conserved residues in the ATP-grasp protein family that interact with ATP are indicated by an asterisk. Ent. faec, Enterococcus faecalis; E. faecium, Enterococcus faecium; Ltc lacti, Lactococcus lactis subsp. Lactis IL1403; Ltc crem, Lactococcus lactis subsp. cremoris SK11; Ltb gasse, Lactobacillus gasseri ATCC 33332; Ltb johns, Lactobacillus johnsonii NCC 533; Ltb delbr, L. delbrueckii subsp. bulgaricus ATCC BAA-365; Ltb brevi, Lactobacillus brevis ATCC 354; Ltb acido, Lactobacillus acidophilus NCFM; Ltb sakei, Lactobacillus sakei subsp. sakei 23K; Pe. pento, Pediococcus pentosaceus ATCC 25452.
The D-Ala-D-Ala ligases (24) and closely related enzymes for synthesis of D-Ala-D-lactate (30) and D-Ala-D-Ser (31) in glycopeptide-resistant Gram-positive bacteria are the only members of the family known to be involved in peptidoglycan synthesis. Assembly of the linear pentapeptide stem is performed by a distinct family of enzymes, the Mur synthetases, and the tRNA-dependent transferases of the Fem family (or amide) bond-forming enzymes, Aslfm and the D-Ala:D-Ala ligases, the Mur synthetases, and the tRNA-dependent transferases of the Fem family.

The racfm gene, located downstream from aslfm, appeared as a likely candidate for D-Asp production in E. faecium since it encoded a protein related to pyridoxal-independent amino acid racemase (25–28). The function of Racfm in D-Asp production was confirmed based on co-expression of the racfm and aslfm genes in E. faecalis H12-2. Close homologues of Racfm were detected in the 10 species listed in Fig. 7, and the linkage with aslfm-related genes was conserved in six genomes. This protein subfamily is related to the glutamate racemases that generate D-Glu present at the second position of the pentapeptide stem of peptidoglycan precursors (34). Since synthesis of modified precursor by Aslfm in E. faecalis required either expression of the racfm gene or the addition of D-Asp in the culture medium, racemization of aspartate and glutamate is performed by distinct enzymes.

Analysis of the specificity of Aslfm for its amino acid substrate indicated that minor modifications of D-Asp were not tolerated by the enzyme, including an increase in the length of the side chain (D-Glu), substitution of the α-amino group by a hydroxyl group (D-malic acid), and substitution of the α-carboxyl group by CONH (D-isosapragine). The latter observation indicates that the presence of D-isosapragine in the cross-bridges of E. faecium exclusively originates from amidation of the α-carboxyl group of D-Asp after its incorporation into the precursors by Aslfm. The enzyme is also highly specific in vivo since the peptidoglycan cross-bridges exclusively contain D-Asp and its amidated form in E. faecium (35), and heterospecific expression of aslfm in E. faecalis H12-2 led only to the incorporation of D-Asp (Fig. 5).

Heterospecific expression of aslfm revealed that the PBPs of E. faecalis catalyzed peptidoglycan cross-linking with acceptor and donor stem peptides substituted by D-Asp. Thus, the presence of a free carboxyl group on the α-carbon of D-Asp did not prevent recognition of the α-amino group of the acceptor, although the reacting group is normally located at the extremity of an l-Ala2 side chain in this host. A similar observation has been previously reported for the low affinity PBPs responsible for β-lactam resistance in E. faecalis (PPB5) and in methicillin-resistant S. aureus (PPB2a) (21). Both low affinity PBPs conferred β-lactam resistance in E. faecium, indicating that these D,D-transpeptidases catalyzed the cross-linking of D-Asp-substituted stem peptides, although they function with l-Ala2- and Gly5-substituted precursors in their original hosts, respectively. Full expression of β-lactam resistance mediated by PBP5 and PBP2a in E. faecium required a mutation in an unknown locus of the host. In the current study, we designed two systems for conditional production of D-Asp-substituted precursors based on the addition of this amino acid in the culture medium of a recombinant E. faecalis strain expressing only the aslfm gene or inducible expression of a bi-cistronic operon comprising both aslfm and racfm. These systems provided direct evidence that the peptidoglycan synthesis machinery tolerates the replacement of l-Ala2 by D-Asp in the absence of any mutation.

Vancomycin-resistant enterococci have emerged as important nosocomial pathogens in the U. S. A. and more recently in Europe, where hospital outbreaks are being reported with increasing frequency (36). Co-resistance to glycopeptides and β-lactams, mostly in E. faecium, has complicated the management of enterococcal infections (37), and the optimal antimicrobial therapy is not yet defined since the use of the newest agents is limited by emerging resistance and toxicity. Thus, the Aslfm ligase appears as an attractive target for the design of new narrow spectrum antibacterial agents active against multiresistant E. faecium.

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The D-Aspartate Ligase of Enterococcus faecium

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