Balance between Two Transpeptidation Mechanisms Determines the Expression of \( \beta \)-Lactam Resistance in Enterococcus faecium*

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The \( \text{D,D}- \) transpeptidase activity of high molecular weight penicillin-binding proteins (PBPs) is essential to maintain cell wall integrity as it catalyzes the final cross-linking step of bacterial peptidoglycan synthesis. We investigated a novel \( \beta \)-lactam resistance mechanism involving by-pass of the essential PBPs by \( \text{L,D}- \) transpeptidation in Enterococcus faecium. Determination of the peptidoglycan structure by reverse phase high performance liquid chromatography coupled to mass spectrometry revealed that stepwise selection for ampicillin resistance led to the gradual replacement of the usual cross-links generated by the PBPs (\( \text{D-Ala} \rightarrow \text{D-Asx-Lys}_3 \)) by cross-links resulting from \( \text{L,D}- \) transpeptidation (\( \text{L-Lys}_3 \rightarrow \text{D-Asx-Lys}_3 \)). This was associated with no modification of the level of production of the PBPs or of their affinity for \( \beta \)-lactams, indicating that altered PBP activity was not required for ampicillin resistance. A \( \beta \)-lactam-insensitive \( \text{L,D}- \) transpeptidase was detected in membrane preparations of the parental susceptible strain. Acquisition of resistance was not because of variation of this activity. Instead, selection led to production of a \( \beta \)-lactam-insensitive \( \text{D,D}- \) carboxypeptidase that cleaved the C-terminal D-Ala residue of pentapeptide stems in vitro and caused massive accumulation of cytoplasmic precursors containing a tetrapeptide stem in vivo. The parallel dramatic increase in the proportion of \( \text{L-Lys}_3 \rightarrow \text{D-Asx-Lys}_3 \) cross-links showed that the enzyme was activating the resistance pathway by generating the substrate for the \( \text{L,D}- \) transpeptidase.

The synthesis of bacterial cell wall peptidoglycan is a two-stage process. First, the disaccharide peptide monomer unit is assembled in a series of cytoplasmic and membrane reactions (1). In Enterococcus faecium, the resulting unit is composed of N-acetylmuramic acid (GlcNAc)\(^1\) and N-acetylmuramiduronic acid (MurNAc) substituted by the l-alanyl-\( \gamma \)-d-glutamyl-\( \eta \)-d-aspartyl\( \gamma \)-D-alanyl-D-alanine or l-alanyl-\( \gamma \)-d-glutamyl-\( \eta \)-d-asparaginyl\( \gamma \)-D-alanyl-D-alanine stem hexapeptide (D-Asx-pentapeptidase) (2–4). The final steps of peptidoglycan synthesis involve its transfer through the cytoplasmic membrane, its polymerization to glycan strands by glycosyltransferases, and the cross-linking of stem peptides by \( \text{D,D}- \) transpeptidases. These latter enzymes catalyze the formation of a peptide bond between the carboxyl of D-Ala at position 4 of a donor stem peptide and the amino group of the D-asparagine or D-aspartate linked to the \( \varepsilon \)-amino group of L-Lys at position 3 of an acceptor peptide stem (3–5). The \( \text{D,D}- \) transpeptidases of \( E. \) faecium thus catalyze the formation of \( \text{D-Ala}_4 \rightarrow \text{D-Asx-L-Lys}_3 \) cross-links after release of the C-terminal D-Ala of the donor peptide stem (Fig. 1A).

\( \beta \)-Lactam antibiotics, which are structural analogues of the C-terminal D-Ala, D-Ala end of the peptide stem, act as suicide substrates of the \( \text{D,D}- \) transpeptidases in an acylation reaction (6). Because transpeptidation is essential to the integrity of the cell wall, these enzymes are the killing target of \( \beta \)-lactams (7). \( \text{D,D}- \) transpeptidases are multimeric enzymes that combine a C-terminal penicillin-binding domain to an N-terminal glycosyltransferase (class A) or morphogenic (class B) domain (8). Penicillin-binding-proteins (PBPs) also include monomodular enzymes with \( \text{D,D}- \) carboxypeptidase or \( \text{D,D}- \) endopeptidase activity (8). Among the \( \text{D,D}- \) transpeptidases of \( E. \) faecium, low-affinity PBP5 (class B) is responsible for intrinsic low-level \( \beta \)-lactam resistance. In clinical isolates, acquired high-level resistance to these antibiotics is generally associated with increased production of PBP5 or with amino acid substitutions near the conserved motifs of this protein (9–13). Recently, we searched for other resistance mechanisms and obtained after five selection steps a highly ampicillin-resistant mutant, designated D344M512, or briefly M512, from the hypersusceptible \( E. \) faecium D344S that does not harbor the \( \text{ppbp} \) gene. Analysis of the peptidoglycan structure by reverse-phase HPLC (RP-HPLC) coupled to mass spectrometry revealed substitution of D-Ala\(_4 \) \( \rightarrow \) D-Asx-L-Lys\(_3 \) cross-links (Fig. 1A) by L-Lys\(_3 \) \( \rightarrow \) D-Asx-L-Lys\(_3 \) cross-links (Fig. 1B) establishing for the first time that \( \text{L,D}- \) transpeptidation could by-pass the essential \( \beta \)-lactam-sensitive \( \text{D,D}- \) transpeptidases (14).

The presence of the unusual L-Lys\(_3 \) \( \rightarrow \) D-Asx-L-Lys\(_3 \) cross-links in M512 implies that an \( \text{L,D}- \) transpeptidase cleaves the L-Lys\(_3 \)-D-Ala\(_4 \) peptide bond of a donor peptide stem and links the \( \alpha \)-carboxyl of its L-Lys\(_3 \) to the amino group of the D-Asx...
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| A. D,D-transpeptidase (PBP) | B. L,D-transpeptidase |
|-----------------------------|------------------------|
| D-Ala 1                      | D-Ala 1                |
| D-Ala 2                      | D-Ala 3                |
| D-Ala 3                      | D-Ala 4                |
| D-Ala 4                      |                        |
| D-Axl-Lys 1                  | D-Axl-Lys 1            |
| D-Axl-Lys 1                  | D-Axl-Lys 2            |
| L-Lys 1                      | L-Lys 2                |
| L-Lys 2                      |                         |
| R                           | R                      |

**Donor** | **Acceptor**
---|---

**Fig. 1.** Cross-links generated by D,D-transpeptidases (A) and L,D-transpeptidases (B). D,D-Transpeptidases cleave the d-Ala-d-Ala- peptide bond of the donor and forms a d-Ala → d-Axl-Lys, cross-link (boxed). L,D-Transpeptidases cleave the L-Lys, d-Ala bond and forms a L-Lys → d-Axl-Lys, cross-link (boxed). R, glycan strands made of alternating GlcNAc and MurNAc residues.

were about 10⁻⁸, 10⁻⁸, and 10⁻⁶, at the third, fourth, and fifth selection steps, respectively.

**Susceptibility Tests**—Minimal inhibitory concentration (MICs) were determined on brain heart infusion agar containing 2-fold dilutions of ampicillin (Bristol-Myers Squibb) (10). Plates were incubated at 37°C for 24 h, with MICs being recorded (21). Growth in brain heart infusion (Difco) agar containing increasing concentrations of ampicillin (0.06, 0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) was obtained on the highest ampicillin concentration (0.5 μg/ml). The frequencies of ampicillin-resistant mutants derived from M1 were observed at the same frequency as for the parental strain.

**Analysis of Peptidoglycan Structure**—Peptidoglycan was extracted at 100°C with SDS (4%) from exponentially growing bacteria (Aₕₒₒ = 0.7), purified after treatment with Pronase and trypsin, and digested with lysozyme and mutanolysin (14). The resulting muramopentapeptides were reduced with sodium borohydride and separated by RP-HPLC coupled to mass spectrometry (MS and MS/MS) as previously described (14, 23). The linear RP-HPLC gradient (0–100% B) was applied between 5 and 45 min and elution in buffer B was continued for an additional 5 min (buffer A, 0.05% trifluoroacetic acid in water; buffer B, 0.05% trifluoroacetic acid and 20% acetonitrile in water) at a flow rate of 0.5 ml/min.

**Preparation of Cytoplasmic and Membrane Extracts**—Bacteria were grown on an Aₕₒₒ of 0.7, harvested by centrifugation (4,000 x g for 10 min at 4°C), and washed twice in 10 mM sodium phosphate (pH 7.0). Bacteria were disrupted with glass beads in a cell disintegrator (The Mickle Laboratory Engineering Co., Gromeshall, United Kingdom) for 2 h at 4°C. The extract was centrifuged (5,000 x g for 10 min at 4°C) to remove cell debris and the supernatant was ultracentrifuged at 40,000 x g for 30 min at 4°C. The supernatant was saved (cytoplasmic fraction) and the pellet was washed twice in 10 mM sodium phosphate buffer (pH 7.0) (membrane fraction). The protein contents were determined with the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as standard.

**Synthesis of the Dipeptide N,N'-Acetyl-L-lysyl-D-alanine (Ac₂-L-Lys-D-Ala)”—Boc₂-L-Lys nm-nitrophenylester (24) was coupled overnight with 1 equivalent of d-Ala-OBn p-toluensulphonate (Novabiochem, Laufelfingen, Switzerland) in tetrahydrofuran and in the presence of 1.1 equivalent of triethylamine; after the usual work-up, the protected dipeptide derivative Boc₂-L-Lys-d-Ala-OBn was obtained as a yellowish solid (yield, 99%). The Boc groups were removed by acidolysis (trifluoroacetic acid/anisole, 10:1 (v/v) for 30 min; the resulting compound, 1-Lys-D-Ala, was used for in vivo acceptor residue. Although the peptidoglycan was not reduced (21), the L,D-transpeptidase was thought to form L-Lys → d-Axl-Lys, cross-links in vivo.

We have now characterized specific cellular and biochemical aspects of the peptidoglycan metabolism of the highly resistant mutant E. faecium M512 and of the four intermediary mutants M1, M2, M3, and M4. This included the identification of ampicillin-resistant D,D-carboxypeptidase and L,D-transpeptidase activities, the HPLC and mass spectrometry analyses of the peptidoglycan and of the cytoplasmic precursor pools, the examination of cells by electron microscopy, and the study of their proneness to autolysis. The contribution of Lys₈₂ → d-Axl-Lys₈₂ cross-links to peptidoglycan synthesis was found to increase with the level of ampicillin resistance, although no variation of the L,D-transpeptidase activity was detected. Selection for high-level resistance led to production of a β-lactam-insensitive D,D-carboxypeptidase, indicating that the availability of tetrapeptide donor stems was one of the limiting factors for L,D-transpeptidation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Parental strain E. faecium D344S is highly susceptible to ampicillin and derives from E. faecium D344 (10) by a spontaneous deletion of pbr26 encoding low-affinity PBPs (22). E. faecium M1, M2, M3, M4, and M512 are spontaneous mutants of D344S obtained by five successive selection steps on brain heart infusion (Difco) agar containing increasing concentrations of ampicillin as follows. An inoculum of 4 × 10⁶ colony forming units of D344S was plated on agar containing 2-fold increasing concentrations of ampicillin (0.06–4 μg/ml). Mutants appeared after 72 h of incubation on plates containing 0.06, 0.12, 0.25, and 0.5 μg/ml ampicillin (2–10 colonies per plate, frequency of about 10⁻⁶). The selection procedure was repeated for one of these mutants, designated M1, that was obtained on the highest ampicillin concentration (0.5 μg/ml). Second step mutants derived from M1 were observed at the same frequency (about 10⁻⁵) up to 1 μg/ml ampicillin. One mutant, M2, growing at the latter concentration, was chosen for further selection steps. Using this approach, mutants M3, M4, and M512 were sequentially obtained from plates containing 2, 4, and 512 μg/ml, respectively. The frequencies of the peptidoglycan metabolism of the highly resistant D,D-carboxypeptidase and L,D-transpeptidase M1, M2, M3, and M4. This included the identification of ampicillin-resistant D,D-carboxypeptidase, indicating that the availability of tetrapeptide donor stems was one of the limiting factors for L,D-transpeptidation.

**Inhibition of L,D-Transpeptidase Activity by Ampicillin**—E. faecium membranes (300 μg of proteins) were preincubated for 20 min with ampicillin at 0, 50, 100, 200, 400, 800, 1600, 3200, and 6400 μg/ml in sodium cacodylate (10 mM, pH 7.0) and Triton X-100 (0.1% v/v) (preincubation volume, 25 μl). Kinetics of the reaction catalyzed by the L,D-transpeptidase was performed by adding 25 μl of a solution containing Ac₂-L-Lys-d-Ala (final concentration, 5 μM) and p-[¹⁴C]Ala (0.05 μM) in 10 mM sodium cacodylate (final volume, 30 μl) final ampicillin concentration, 0–3200 μg/ml). Aliquots were taken at 0, 25, 45, and 120 min, boiled at 100°C for 3 min to stop the reaction, and Ac₂-L-Lys-d-[¹⁴C]Ala was determined by RP-HPLC as described above. The IC₅₀ was defined as the ampicillin concentration that inhibited the reaction by 50% and was derived from v/v, v₀ = f(μ) plots, where vᵣ and v₀ are the velocity in the presence and absence of ampicillin, respectively, and [F

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TABLE I

| Strains (MIC of ampicillin in µg/ml) | Concentration of ampicillin in the culture medium | Muropeptides |
|-------------------------------------|-----------------------------------------------|--------------|
|                                     | µg/ml                                         | 13<sup>a</sup> | 6<sup>b</sup> | G<sup>c</sup> |
| D344S (0.06)                        | 0                                            | 96.9         | 1.2          | 1.9          |
| M1 (0.5)                            | 0.03                                          | 75.0         | 11.5         | 13.5         |
| M2 (1)                              | 0.12                                          | 88.6         | 8.4          | 3.0          |
| M3 (2)                              | 0.25                                          | 36.0         | 34.0         | 30.0         |
| M4 (125)                            | 0.25                                          | 88.1         | 9.2          | 2.7          |
| M512 (>2000)                        | 0.25                                          | 33.0         | 34.0         | 33.0         |
|                                     | 32.0<sup>d</sup>                              | 28.5         | 42.9         | 28.6         |
|                                     | 1000                                          | 6.6          | 50.5         | 42.9         |
|                                     |                                                | 0.0          | 69.4         | 30.6         |
|                                     |                                                | 0.0          | 76.0         | 24.0         |

<sup>a</sup> Disaccharide-asparagine-tetrapeptide-asparagine-tripeptide-disaccharide.
<sup>b</sup> Disaccharide-asparagine-tripeptide-asparagine-tripeptide-disaccharide.
<sup>c</sup> D,L-asparagine-tripeptide-asparagine-tripeptide-disaccharide.
<sup>d</sup> These conditions reproduce those reported in our previous study (14) that were mistakenly described as growth in the absence of antibiotic since the carry over of the antibiotic from the preculture was not taken into account.

RESULTS

Muropeptide Composition of the Peptidoglycans from E. faecium D344S, M1, M2, M3, M4, and M512—We previously reported identification and quantitative comparison of 34 muropeptides from D344S and M512 by RP-HPLC, MS, and MS-MS (14). In the present paper, this analysis was extended to the four intermediary mutants (M1, M2, M3, and M4) and to the comparison of the muropeptide composition of peptidoglycan from bacteria grown in the presence or absence of ampicillin. The profiles of monomers were almost identical for the mutants and the parental strain. Variations in the relative proportions of dimer muropeptides generated by D,L-transpeptidation (D-Ala<sub>4</sub>→D-Asx-L-Lys) versus L,D-transpeptidation (L-Lys<sub>4</sub>→D-Asx-L-Lys) was the main difference between the muropeptide profiles. For the sake of simplicity, Table I indicates the proportions of muropeptides 13, E, and G that were the most abundant dimers. Muropeptide 13 was the major dimer generated by D,L-transpeptidation and contained a donor tetrapeptide stem and an acceptor tripeptide stem with a D-asparagine branched on the ε-amino group of both lysine residues (Asn-tetra-Asn-tri). Peaks E (Asn-tri-Asn-tri) and G (Asn-tri-Asn-tetra) were the major dimers generated by L,D-transpeptidation.

In the absence of ampicillin (Table I), muropeptides E and G generated by L,D-transpeptidation were detected in small amounts (3.1%) in D344S, indicating that this mode of transpeptidation was pre-existing in the parental strain. Stepwise increases in the proportion of muropeptides E and G and in the MICs of ampicillin were only detected for the 1st (M1), 4th (M4), and 5th (M512) selection steps. Specifically, the proportion of muropeptides E and G decreased from 3.1 (D344S) to 11.4% (M1) at the 1st step, from 11.7% (M3) to 28.7% (M4) at the 4th step, and from 32.0% (M512) to 24.0% (M512).
4th step, and from 28.7 (M4) to 71.5% (M512) at the 5th step. Selection led to parallel large increases of the ampicillin MICs at each of these steps (8-, 64-, and 32-fold, respectively). In contrast, marginal increases of the MICs (2-fold) were observed for the 2nd and 3rd selection steps and the muropeptide composition of mutants M1, M2, and M3 were similar. These observations indicate that three of the five selection steps led to increased L,D-transpeptidation to the detriment of D,D-transpeptidation. Activation of the L,D-transpeptidation pathway at these steps was associated with large increases in the ampicillin-resistance level.

The peptidoglycan structure analysis was repeated for the strains grown in the presence of subinhibitory concentrations of ampicillin to test the effect of PBP inhibition (Table I). For D344S, M1, M2, and M3, the concentration of ampicillin added to culture medium corresponded to the maximum concentration allowing growth. Partial inhibition of the D,D-transpeptidases by ampicillin increased the proportion of dimers generated by L,D-transpeptidation in D344S (from 3.1 to 25%, 8-fold) and in mutants M1, M2, and M3 (6-fold). However, the PBPs remained essential targets in these mutants because higher concentrations of ampicillin inhibited growth. For M4 and M512, the D,D-transpeptidase pathway was almost completely inhibited by ampicillin at 0.5 μg/ml, which corresponds to the subinhibitory concentration tested for M3. Under these conditions, and in contrast to M3, the proportion of muropeptides generated by L,D-transpeptidation reached 82.0 and 93.4% for M4 and M512, respectively. No muropeptides generated by D,D-transpeptidation were detected in the peptidoglycan of M512 grown in the presence of 32 or 1000 μg/ml ampicillin.

**L,D-Transpeptidase Activity**—Dipeptide Ac$_2$L-Lys-D-Ala was synthesized as described under “Experimental Procedures” to detect L,D-transpeptidase activity based on the exchange reaction (Ac$_2$L-Lys-D-Ala $\rightarrow$ [14C]Ala $\rightarrow$ [14C]Ala $\rightarrow$ D-Ala) (21). L,D-Transpeptidase activity was detected in membrane preparations of D344S (Fig. 2), which contained minor amounts of dimers with a L-Lys$_3$→D-Asx-L-Lys$_3$ cross-link in its peptidoglycan. This activity was similar for D344S and M512 (23 ± 4 and 32 ± 6 pmol/min/mg of protein, respectively). The concentrations of ampicillin required to inhibit the L,D-transpeptidase activity by 50% ($IC_{50}$) were also similar for D344S and M512 (about 105 and 110 μg/ml, respectively). Residual activity (about 15–25%) was detected at 1600 and 3200 μg/ml for both strains. Thus, increased synthesis of L-Lys$_3$→D-Asx-L-Lys$_3$ cross-links in mutant M512 was not associated with increased L,D-transpeptidase activity. Neither the L,D-transpeptidase produced by susceptible strain D344S nor that of M512 were inhibited by low concentrations of ampicillin and the $IC_{50}$ of the antibiotic were similar for the two enzyme preparations.

**D,D-Carboxypeptidase Activity**—In the presence of ampicillin (20 μg/ml), D,D-carboxypeptidase activity was detected only in membrane preparations from M4 and M512 (Table II). This enzyme was not inhibited by ampicillin at 2000 μg/ml (data not shown). Membrane preparations from D344S, M1, M2, and M3 contained a 10-fold lower D,D-carboxypeptidase activity that was totally (>95%) inhibited by ampicillin at 20 μg/ml. These results indicate that the fourth selection step, which generated mutant M4, resulted in high-level production of a β-lactam-insensitive D,D-carboxypeptidase in addition to the D,D-carboxypeptidase activity of the putative monofunctional PBPs.

Low amounts (<10%) of UDP-MurNAc-tetrapeptide were detected in the cytoplasm of D344S and M3 (Table III). In contrast, UDP-MurNAc-tetrapeptide accounted for about half of the cytoplasmic peptidoglycan precursors of M4 (49.5%) and M512 (59.0%). Thus, the β-lactam-insensitive D,D-carboxypeptidase, specifically detected in these mutants, generated tetrapeptide stems in vivo.

**Expression of a Heterologous D,D-Carboxypeptidase Gene in E. faecium D344S and M3**—The vanY gene encodes a metallo-D,D-carboxypeptidase-insensitive to β-lactam inhibition and
was originally detected in transposon Tn1546 that confers glycopeptide resistance in enterococci (26). Plasmid pJC1 was constructed by inserting the vanY gene under the control of the P2 promoter of the shuttle vector pNJ3 to test the influence of elevated D,D-carboxypeptidase activity on β-lactam resistance. Introduction of pJC1(P2\textasciitildevanY) into mutant M3 led to an increase in the MIC of ampicillin (from 2 to 256 μg/ml). A similar increase in the MIC (from 2 to 128 μg/ml) was obtained at the fourth selection step that generated M4 from M3 (Table I). The vector alone had no effect on the level of resistance. Thus, elevated D,D-carboxypeptidase activity was responsible for increased resistance observed at the fourth selection step.

Plasmid pJC1(P2\textasciitildevanY) did not increase the MIC of ampicillin in D344S. Selection of ampicillin-resistant mutants from D344S/pJC1(P2\textasciitildevanY) resulted in a highly resistant mutant M3, indicating that their level of production was not modified (data not shown). Based on the competition assay, ampicillin at 4 μg/ml saturated all PBPs of D344S and M512. Partial saturation at lower drug concentrations showed that the affinity of the PBPs were not modified in the highly resistant mutant M512.

**Table II**

| Strains | Activity (pmol/min/mg protein) |
|---------|-------------------------------|
|         | Without ampicillin | With ampicillin (20 μg/ml) |
| D344S   | 42 ± 23                  | <1                       |
| M1      | 19 ± 8                  | <1                       |
| M2      | 20 ± 5                  | <1                       |
| M3      | 25 ± 15                 | <1                       |
| M4      | 250 ± 20                | 210 ± 21                |
| M512    | 340 ± 67                | 290 ± 72                |

*Calculated as percentage of the sum of the peak areas of UDP-MurNAc-pentapeptide and UDP-MurNAc-tetrapeptide.

**Table III**

| Strains | UDP-MurNAc-pentapeptide (/%) |
|---------|-----------------------------|
|         | Tetrapeptide | Pentapeptide |
| D344S   | 8.6          | 91.4         |
| M3      | 4.7          | 95.3         |
| M4      | 49.5         | 50.5         |
| M512    | 59.0         | 41.0         |

**DISCUSSION**

In this report, we show that the level of ampicillin resistance in the mutants derived from *E. faecium* D344S is determined by a balance between the D,D- and L,D-transpeptidation pathways for peptidoglycan cross-linking (Table I). The 1st, 4th, and 5th selection steps that led to mutants M1, M4, and M512, respectively, resulted in large increases in the proportion of muropeptides containing the L-Lys3 → D-Asx-Lys3 cross-links generated by L,D-transpeptidation. Large increases of the ampicillin MICs were observed at these steps. Partial inhibition of the D,D-transpeptidases by ampicillin resistant mutants in enterococci (26). Plasmid pJC1 was constructed by inserting the vanY gene under the control of the P2 promoter of the shuttle vector pNJ3 to test the influence of elevated D,D-carboxypeptidase activity on β-lactam resistance. Introduction of pJC1(P2\textasciitildevanY) into mutant M3 led to an increase in the MIC of ampicillin (from 2 to 256 μg/ml). A similar increase in the MIC (from 2 to 128 μg/ml) was obtained at the fourth selection step that generated M4 from M3 (Table I). The vector alone had no effect on the level of resistance. Thus, elevated D,D-carboxypeptidase activity was responsible for increased resistance observed at the fourth selection step.

Plasmid pJC1(P2\textasciitildevanY) did not increase the MIC of ampicillin in D344S. Selection of ampicillin-resistant mutants from D344S/pJC1(P2\textasciitildevanY) resulted in a highly resistant mutant M3, indicating that their level of production was not modified (data not shown). Based on the competition assay, ampicillin at 4 μg/ml saturated all PBPs of D344S and M512. Partial saturation at lower drug concentrations showed that the affinity of the PBPs were not modified in the highly resistant mutant M512.

**Binding of β-Lactams to PBPs—**PBP labeling with benzylpenicillin revealed similar SDS-PAGE patterns for D344S and M512, indicating that their level of production was not modified (data not shown). Based on the competition assay, ampicillin at 4 μg/ml saturated all PBPs of D344S and M512. Partial saturation at lower drug concentrations showed that the affinity of the PBPs were not modified in the highly resistant mutant M512.

**Cell Autolysis and Detection of Autolysins—**Autolysis of D344S cells collected from exponential and stationary phases led to a 50% decrease of the *A*~490~ after 9 and 24 h of incubation at 37 °C in phosphate buffer, respectively. In contrast, no significant autolysis of M512 was observed after 36 h. Crude extracts from D344S and M512 contained two major autolysins active on heat-killed *E. faecium* D344S (data not shown). These enzymes were not active on heat-killed M512 cells. These observations suggested that the mutant M512 was not prone to autolysis because its peptidoglycan containing cross-links generated by L,D-transpeptidation was no longer well recognized by the major *E. faecium* autolysins.

**Growth Rate and Abnormal Morphology—**The stepwise increase in ampicillin resistance was accompanied by a decrease in growth rate. In the absence of ampicillin in the culture medium, the generation time was 27, 48, 60, 72, 84, and 90 min for D344S, M1, M2, M3, M4, and M512, respectively. For M512, the generation time increased from 90 to 180 min when the ampicillin concentration increased from 750 to 4000 μg/ml. In comparison with parental strain D344S (Fig 3a), electron microscopy of thin sections of M1 and M2 grown in antibiotic-free medium showed no difference in morphology (data not shown). For M3, thickening of the cell wall and abnormalities of the septa were present in about 30% of the cells (Fig 3b). For M4 and M512, severe abnormalities were observed in more than 80% of the cells, including cell wall thickening and formation of cell aggregates resulting from disordered septation (Fig 3, c and d).

**FIG. 3.** Electron microscopy of thin sections of parental strain D344S and the ampicillin-resistant mutants. D344S cells displayed regular coccoidal shape, symmetric septation, and a thin cell wall (a). Mutants M3 (b), M4 (c), and M512 (d) showed increasing numbers of cells with ultrastructural abnormalities, including formation of cell aggregates, anarchic septation, and cell wall thickening especially at the septum level (arrow). Bar = 0.5 μm.
(less or equal to 0.5 μg/ml) increased the proportion of muropeptides with an L-Lys₃ → D-Asx-Lys₃ cross-link in all strains. Higher drug concentrations inhibited growth of D344S, M1, M2, and M3, indicating that at least one PBP remained essential in these strains. In contrast, the PBPs were not contributing to peptidoglycan cross-linking in M512 as growth in the presence of a saturating concentration of ampicillin (32 or 1000 μg/ml) led to exclusive synthesis of L-Lys₃ → D-Asx-Lys₃ cross-links. In the absence of ampicillin, the two types of cross-links were detected, indicating that the L,D- and D,D-transpeptidation pathways were functional in all strains although their relative contribution varied. Several factors that could affect this balance were examined, revealing that activation of the resistance pathway was not associated with any variation of the L,D-transpeptidase activity or of the PBPs, but to production of a D,D-carboxypeptidase that generated the tetrapeptide donor stems for L,D-transpeptidation.

The L,D-transpeptidase activity measured by the exchange reaction was similar in D344S and M512 (Fig. 2), indicating that increased production of the enzyme was not required for by-pass of the D,D-transpeptidases. The L,D-transpeptidase activity was only partially inhibited by high concentrations of ampicillin. The drug concentrations required for 50% inhibition were also similar for the enzyme present in membrane preparations from D344S and M512 (IC₅₀ of about 105 and 110 μg/ml, respectively). Thus, emergence of high-level resistance to ampicillin did not appear to involve any alteration of the L,D-transpeptidase. The following observations may account for the apparent discrepancy between the IC₅₀ (110 μg/ml) and the MIC (>2,000 μg/ml) observed for M512. The generation time of this strain increased from 90 to 180 min when the ampicillin concentration increased from 750 to 4,000 μg/ml, indicating that the drug significantly slowed growth despite the high MIC. A residual L,D-transpeptidase activity was detected in vitro at ampicillin concentrations of 1600 (25%) and 3,200 μg/ml (15%) in membrane preparations of M512. This residual activity could be sufficient to sustain bacterial growth in M512 although at a lower rate. As previously discussed (14, 21, 31), the L,D-transpeptidase and D,D-transpeptidase are not expected to be inhibited by β-lactams by the same mechanism. The drugs are suicide substrates of the D,D-transpeptidases because the β-lactam ring is structurally related to the D-Ala-D-Ala moiety of peptidoglycan precursors (32). The L,D-transpeptidases, which cleave the L-Lys-D-Ala rather than D-Ala-D-Ala peptide bond, are therefore not expected to be acylated by the same mechanism.

Penicillin-binding studies did not reveal any modification of the PBP patterns in M512 (data not shown). This observation implies that in this strain the resistance pathway is dominant as the D,D-transpeptidase appeared to remain functional based on β-lactam acylation. Competition experiments indicated that the acylation of PBPs by ampicillin was not affected by the five-step selection for resistance to this antibiotic because all PBPs were saturated by low concentrations of ampicillin (<4 μg/ml). This observation implies that the β-lactam-sensitive D,D-transpeptidase and D,D-carboxypeptidase activities of the PBPs were not playing any essential role in the high level of resistance expressed by M512 (MIC > 2000 μg/ml). In Staphylococcus aureus, methicillin resistance mediated by class B PBPA2 requires the glycosyltransferase activity of class A PBPA2 (33). Site-directed mutagenesis of the active-site serine residue of PBPA2 showed that the glycosyltransferase module of this protein can function in the absence of its catalytically active D,D-transpeptidase module (33). In our system, peptidoglycan synthesis may similarly involve cooperation between the L,D-transpeptidase and the glycosyltransferase module of a class A PBP.

The fourth selection step that generated mutant M4 led to production of a β-lactam-insensitive D,D-carboxypeptidase (Table II) that was active in vivo as shown by the accumulation of UDP-MurNAc-tetrapeptide in the cytoplasm of M4 and M512 (Table III). Selection may have activated a cryptic gene encoding a metallo-D,D-carboxypeptidase because this type of enzyme is not inhibited by β-lactams (26, 34, 35) and no modification of the patterns of the PBP was observed. In agreement, production of the heterologous D,D-carboxypeptidase VanY increased the level of ampicillin resistance in mutant M3. This effect was not observed in D344S, suggesting that elevated D,D-carboxypeptidase activity can only increase the level of resistance after modification of other unknown function(s). High-level accumulation of UDP-MurNAc-tetrapeptide (Table III) suggests that the enzyme hydrolyzes the C-terminal D-Ala residue of UDP-MurNAc-pentapeptide in vivo. However, because formation of lipid intermediate I is reversible (1) and translocation of this intermediate may also be reversible (36), hydrolysis of pentapeptide stems at the outer surface of the cytoplasmic membrane cannot be excluded, as previously discussed for a D,D-carboxypeptidase involved in glycopeptide resistance (36).

Taking in account all these observations, production of the
β-lactam-sensitive D,D-carboxypeptidase is expected to increase synthesis of L-Lys₃ → D-Asx-Lys₂ to the detriment of D-Ala₄ → D-Asx-Lys₂ cross-links by two mechanisms (Fig. 4). First, hydrolysis of the C-terminal D-Ala₄ reduces D,D-transpeptidation because the PBP requires a pentapeptide donor stem. Second, the D,D-carboxypeptidase by generating the tetratetptide stem donor substrate acts as a new source to increase L,D-transpeptidation. The large increase in the MIC of ampicillin observed at the fourth selection step indicates that the acquisition of resistance led to impaired growth and cell wall abnormalities (Fig. 3). Impaired activity of the autolysins could be involved in these defects because peptidoglycan polymerization is thought to involve multienzyme complexes that include glycosyltransferases, D,D-transpeptidases, and hydrolases (20). Therefore, it is perhaps not surprising that acquisition of resistance led to impaired growth and required multiple mutations that did not directly affect the catalytic activity of the transpeptidases.

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