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Peptidoglycan Structure of *Lactobacillus casei*, a Species Highly Resistant to Glycopeptide Antibiotics

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The structure of the peptidoglycan of *Lactobacillus casei* ATCC 393, a species highly resistant to glycopeptide antibiotics, was examined. After digestion, 23 muropeptides were identified; monomers represented 44.7% of all muropeptides, with monomer tetrapeptides being the major ones. Fifty-nine percent of the peptidoglycan was *O*-acetylated. The cross-bridge between D-alanine and L-lysine consisted of one asparagine, although aspartate could be found in minor quantities. Since UDP–MurNAc–tetrapeptide–D-lactate is the normal cytoplasmic precursor found in this species, monomer tetrapeptide-lactate was expected to be found. However, such a monomer was found only after exposure to penicillin, suggesting that penicillin-sensitive D,D-carboxypeptidases were very active in normal growing cells.

Resistance to glycopeptide antibiotics among gram-positive bacteria is either acquired or naturally expressed (3, 7). When acquired, as in *Enterococcus faecalis* or *Enterococcus faecium*, it is due to the presence of a new set of genes (2, 15), leading to the phenotype VanA or VanB, both of which are characterized by the synthesis of a cytoplasmic precursor containing a C-terminal D-lactate instead of the normal C-terminal D-alanine (1, 7, 14, 19, 25). The level of glycopeptide resistance of these organisms is associated with two main parameters: the lower affinity of the C-terminal D-alanyl–D-lactate of the new cytoplasmic precursors for the glycopeptides (3, 23) and the residual cell pool of the normal UDP-MurNAc-pentapeptide precursor, the D-alanyl–D-alanine terminus of which has a high affinity for glycopeptides (4, 6, 26). The natural high-level resistance of *Leuconostoc mesenteroides*, *Pediococcus* sp., and *Lactobacillus casei* to glycopeptides can be explained by the presence of a cytoplasmic precursor with a C-terminal D-lactate and the total absence of one with a C-terminal D-alanine (7, 20). The primary structure of the peptidoglycan of *L. casei*, like that of *E. faecium*, belongs to subgroup A4 (27) and has a common monomer structure, GlcNAc–MurNAc–L-Ala–γ-D-Glu–L-Lys–D-Ala, with an asparagine attached to the ε-amino group of lysine (21, 27). Thus, we were interested in determining if any difference in the overall composition of the peptidoglycan would exist between *L. casei* ATCC 393 and the VanB-type *E. faecium* D366 that we had previously described (8), both of which synthesize a lactate precursor.

Muropeptide composition of *L. casei* ATCC 393 in the absence of penicillin G. *L. casei* ATCC 393 was obtained from the Institut Pasteur collection and grown in MRS broth (Diagnostic Pasteur). The MIC of vancomycin was >512 μg/ml, and that of teicoplanin was 512 μg/ml (7). Peptidoglycan was extracted as previously described (8). Briefly, a 500-ml exponential-phase culture was grown to an A_{650} of 0.4 in the absence of vancomycin and quickly chilled in an ice-ethanol bath. After concentration by centrifugation, cells were boiled in 4% (wt/vol) sodium dodecyl sulfate (SDS) and cell walls were purified by using pronase followed by trypsin, each at 37°C for 16 h. Peptidoglycan was hydrolyzed with lysozyme and mutanolysin. We estimated that more than 95% of the lactococcal cell wall was solubilized by this treatment. For separation and analysis of muropeptides, samples were mixed with equal volumes of borate buffer (0.5 M, pH 9) and reduced with sodium borohydride for 15 min at room temperature (16). Excess borohydride was destroyed by addition of H_3PO_4 (20%, wt/vol) to a pH of

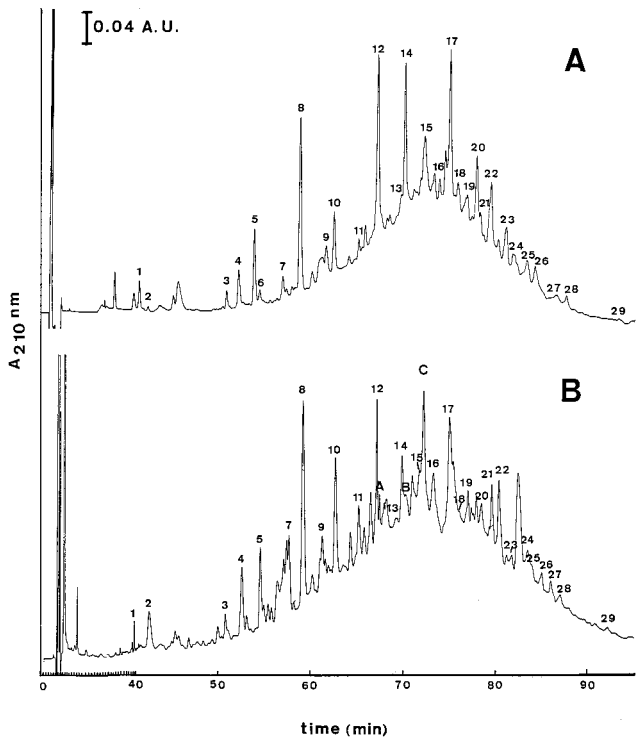

FIG. 1. Separation of *L. casei* ATCC 393 cell wall muropeptides by reverse-phase HPLC after growth in the absence (A) or presence (B) of penicillin G. Peaks with lactate-containing muropeptides are designated A, B, and C. Numbers correspond to peaks identified in Table 1. A.U., absorbance unit.

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TABLE 1. Molecular mass and composition of muropeptides from *Lactobacillus casei* ATCC 393

Peak(s)	Structure	m/z[e]		Amino acid composition[f]				% of all peaks[g]	
		Observed	Calculated	Gln	Lys	Ala	Asn	Without penicillin	With penicillin
Monomers								44.7	63.9
1	ds-di[a]	698.3	698.2					1.8	1.8
2	ds-tri	826.4	826.3					0.1	3.2
3	ds(AC)-di[b]	740.4	740.2					1	1.6
4	ds-tetra	897.7	897.4	1	0.9	2.2		2.9	4.1
5	ds-N-tri	940.6	940.5	1	0.9	1.2	1	4.6	4.3
6	ds(AC)-tri	868.7	868.4					0.7	0.6
7	ds-D-tetra	1,012.6	1,012.5					1.1	3
8	ds-N-tetra	1,011.6	1,011.5	1	1.1	2.3	0.9	11.9	13.9
9	ds(AC)-tetra[b]	939.6	939.5					1.3	2.1
10	ds(AC)-N-tri[b]	982.5	982.5					3.9	7.2
11	ds(AC)-D*-tetra	1,054.5	1,054.6					0.7	3.4
12	ds(AC)-N-tetra[b]	1,053.7	1,053.6	1	0.8	2.4	0.9	14.7	9.2
A	ds-tetra-lactate[c]	969.6	969.5						2
Dimers-trimers-oligomers								55.3	36.1
13	Bis-ds(AC)-tetra-N-tri	1,861.2	1861					0.2	0.1
14	Bis-ds-N-tetra-N-tetra	2,004.6	2,004.1	1	0.7	2	0.8	7.9	4
B	ds-D*-tetra-lactate[c]	1,084.5	1,084.6						2.5
15	Bis-ds(AC)-N-tetra-D*-tri[b,d]	1,975.8	1,976.1					4.6	2.4
C	ds-N-tetra-lactate[c]	1,083.5	1,083.6						5
16	Bis-ds(AC)-N-tetra-N-tri[b]	1,975.5	1,975.1					1.8	3.9
	Bis-ds(AC)-N-tetra-D*-tetra[b,d]	2,047.1	2,047.1						
17	Bis-ds(AC)-N-tetra-N-tetra	2,046	2,046.1					12.8	9.7
18	Ter-ds(AC)-N-tetra-N-tetra-D*-tri[b,d]	2,968.7	2,968.6					1.4	
19	Bis-ds(AC×2)-N-tetra-D*-tri[b,d]	2,018.1	2,018.1					1.1	1.1
20	Ter-ds(AC)-N-tetra-N-tetra-D*-tetra[d]	3,039.6	3,039.7					4.6	1.7
21	Bis-ds(AC×2)-N-tetra-D*-tetra[b,d]	2,089.2	2,089.1					0.4	
22	Bis-ds(AC×2)-N-tetra-N-tetra	2,088.6	2,088.1					6.1	3.4
23	Ter-ds(AC×2)-N-tetra-N-tetra-D*-tetra[d]	3,081.4	3,081.7					3.5	0.5
24–29	Oligomers	>ND[h]						10.9	9.3

[a] ds, disaccharide (GlcNAc-MurNAc); Bis, dimeric form; Ter, trimeric form; N, asparagine; D, aspartic acid; D*, putative aspartic acid; di, dipeptide (L-Ala–D-iGln); tri, tripeptide (L-Ala–D-iGln–L-Lys); tetra, tetrapeptide (L-Ala–D-iGln–L-Lys–D-Ala).

[b] AC, *O*-acetylation located on MurNAc by using MS-MS; AC×2, *O*-acetylation on both MurNAc parts of the dimer.

[c] A, B, and C, monomers with a lactate at the C terminus present only in penicillin-treated cells.

[d] Assignment of the D* and N residues to either peptide strain is arbitrary.

[e] $[M + H]^+$ ion of the reduced muropeptide.

[f] Molar ratio normalized to Gln.

[g] The values shown are percentages of the sum of all of the integrated peak areas present in the table. The percentage of the total monomer for penicillin-treated cells includes the lactate monomers A, B, and C.

[h] ND, not determined with precision.

up to 4 (a treatment which was found not to cleave the terminal lactate of the UDP–MurNAc–tetrapeptide–D-lactate precursor). Samples were kept at −20°C. Separation of the digested cell wall components was performed by the procedure of Glauner (16), with some modifications as previously described (8). Peaks were identified by liquid chromatography-mass spectrometry (MS) using a Waters 600 MS–high-pressure liquid chromatography (HPLC) pump system and a Waters PD1991 liquid chromatograph with a diode array detector system coupled to a Finningam (San Jose, Calif.) TSQ7000 triple quadrupole mass spectrometer. MS-MS was performed on singly and doubly charged protonated molecules using argon as the collision gas. The muropeptide structures deduced by MS were confirmed either by fragmentation using an MS-MS system or chemically, after purification of the peaks by HPLC. The amino acid composition of the muropeptides was analyzed as described previously (8) by using the Waters AccQTag method (Waters Corporation, Milford, Conn.).

The separation by HPLC of muropeptides derived by peptidoglycan digestion of naturally vancomycin-resistant *L. casei* ATCC 393 grown in the absence of antibiotics is presented in Fig. 1. The structures of the first 23 peaks were identified, and their respective abundances are expressed as percentages of the area under all of the muropeptide peaks by using baselines drawn by connecting the lowest A_{210} values for the peaks (Table 1).

Monomers represented about 45% of the total muropeptides. Peaks 24 to 29 were oligomers to which no precise structure was assigned, since each peak was a mixture of many components of very high molecular mass (tetramers and pentamers). Structures were generally deduced from the molecular mass obtained by MS and confirmed for many of them by MS-MS or by amino acid analysis (Table 1). Among the monomers, some were common to those found in *E. faecium* (8) (peaks 1, 2, 4, 5, 7, and 8). Others differed by an apparent molecular mass of 42 Da, suggesting an *O*-acetylation of *N*-acetylmuramyl residues which was confirmed by MS-MS on different components presented in Table 1. As an example, we give details for the MS-MS spectra of two compounds differing by a mass $[M + H]^+$ of 1 Da, i.e., 940 and 939 Da. The component with a mass $[M + H]^+$ of 940 Da that is present in peak 5 was a disaccharide-tripeptide with an additional aspar-

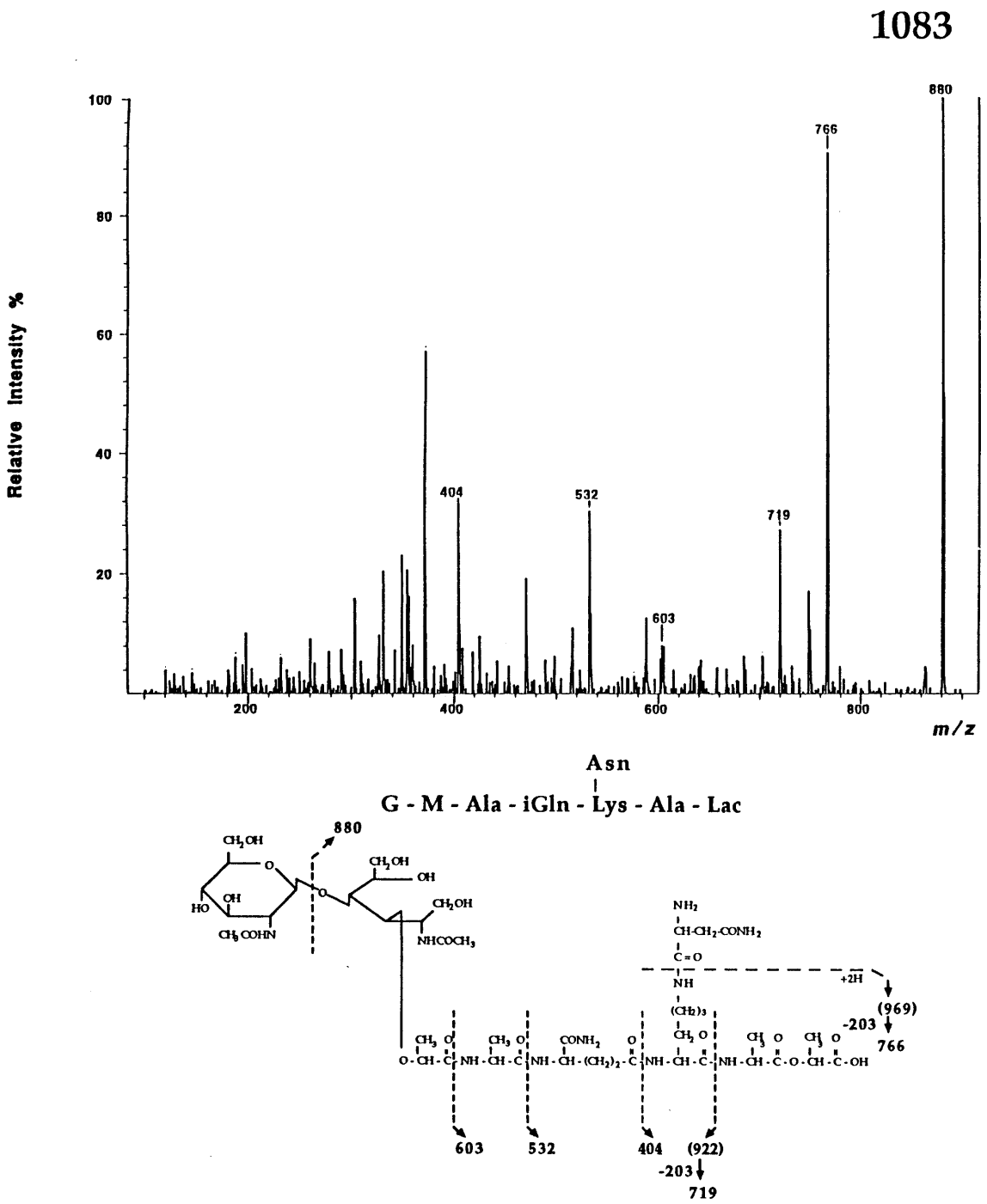

FIG. 2. Schematic representation corresponding to the MS-MS daughter spectrum of a compound present in peak C with an $[M + H]^+$ ion at *m/z* 1,083.

agine branched to the ε-amino group of lysine. The observed fragmentation pattern indicated that fragment ions at *m/z* 460, 389, and 261 were associated to the amino acid sequence Ala-iGln-(ε-amino Asn)-Lys. The presence of Asn was substantiated by the 114-Da mass difference between *m/z* 940 and *m/z* 826 (disaccharide tripeptide). This was also confirmed by chemical analysis (Table 1). The component with a molecular mass $[M + H]^+$ of 939 Da present in peak 9 was an *O*-acetylated disaccharide-tetrapeptide. The observed fragmentation pattern of *m/z* 417, 346, and 218 of the *m/z* 939 ions indicated the presence of the amino acid sequence Ala-iGln-Lys-Ala. The mass difference of 319 Da between the internal fragment ions at *m/z* 736 (*m/z* 939 component which had lost the GlcNAc moiety [*m/z* 204]) and *m/z* 417 revealed the presence of an *O*-acetylated MurNAc moiety. Such an *O*-acetylated peptidoglycan has been previously described in different gram-positive organisms, including *Lactobacillus fermentum* and *Lactobacillus acidophilus* (9). In this study, 59% of the *L. casei*

peptidoglycan was *O*-acelylated, which appears to be similar to the 60% found for *L. acidophilus* (10). Although the physiological role of *O*-acetylation remains unclear, it has been suggested that it could serve to protect the peptidoglycan strands from the hydrolytic activity of muramidase-type autolysins (9).

According to molecular mass and MS-MS data, the amino acid in position 2 was an α-amidated γ-glutamyl residue, a residue which, if present in the acceptor, would favor cross-linking (5, 29). Asparagine branched to the ε-amino group of the lysine was found in the cross-bridge with D-Ala-4 of the donor, but no direct cross-bridge between D-Ala-4 and lysine was detected. However, some of the muropeptides (peaks 7, 11, 15, 16, 18, 19, 20, 21, and 23) differed by a molecular mass of +1 Da from that expected if only glutamine and asparagine were present. This suggested that either a glutamate or an aspartate residue was present. To solve this problem, we compared by MS-MS the muropeptides with molecular masses $[M + H]^+$ of 1,011 Da in peak 8 and 1,012 Da in peak 7. These two muropeptides were disaccharide-tetrapeptides: the 1,011-Da form contained asparagine, whereas the 1,012-Da form contained aspartate (data not shown). Therefore, all muropeptides differing by a molecular mass of +1 Da from a typical muropeptide which would contain only glutamine and asparagine residues were presumed to contain an aspartate residue, a situation already suggested for some muropeptides in *E. faecium* (11).

Since UDP–MurNAc–tetrapeptide–D-lactate is the only final cytoplasmic precursor detected in *L. casei* ATCC 393 (7), only monomer tetralactate and no monomer pentapeptide was expected to be found. However, no murotetrapeptide-lactate was found. In contrast, murotetrapeptide represented 80% of the monomers, suggesting that a highly efficient D,D-carboxypeptidase activity (activities) was present. The low ratio of murotripeptides over murotetrapeptides found in the monomers and the oligomers of *L. casei* ATCC 393 contrasted with the high ratio found in *E. faecium* (8). A simple explanation, at least for the oligomers, would be that the monomer tetrapeptides, which are predominant in *L. casei*, are the preferential acceptors, a situation already demonstrated for *Gaffkya homari* in a cell-free system and suggested for vancomycin-resistant *E. faecalis* (12, 18, 29), while in *E. faecium*, the monomer tripeptides, whose quantity is twice that of the monomer tetrapeptides, would be the preferential acceptors.

Muropeptide composition of *L. casei* ATCC 393 in the presence of penicillin G. Since MurNAc–tetrapeptide–D-lactate was found neither in monomers nor in oligomers, new experimental conditions were designed to elicit its possible presence in peptidoglycan. For this purpose *L. casei* was grown overnight with penicillin G at 1/3 of its MIC (0.03 μg/ml), backdiluted, grown under the same conditions to an A_{650} of 0.2, and exposed to 100 times the MIC (10 μg/ml) of penicillin G for 2 h. We expected that carboxypeptidase and transpeptidase activities would be blocked. The profile and the relative amounts (percentages) of the muropeptides obtained under these conditions are presented in Fig. 1 and Table 1. The overall profile of the muropeptides was similar to that found in the absence of penicillin G (Fig. 1). An increase in the amounts of monomer, from 45 to 64% of all muropeptides, showed that transpeptidase activities had been inhibited to some extent. Interestingly, three new structures, A, B, and C (Fig. 1 and Table 1), were identified, the molecular masses of which suggested that they all have the same disaccharide-tetrapeptide-lactate structure. Since very small amounts of material were present in peaks A and B, MS-MS could only be performed on the muropeptide with a mass $[M + H]^+$ of 1,083 Da in peak C and showed (Fig. 2) that it was composed of a disaccharide-tetrapeptide-lactate with an asparagine substitution. As far as we are aware, this is the first lactate-containing muropeptide described.

Interestingly, when the same type of experiment was performed with MT9, a constitutive VanB-type *E. faecium* strain, a reduction of oligomers was observed with no detectable lactate muropeptides (5a). This absence of a lactate-containing muropeptide after penicillin treatment in MT9, in contrast to what was observed in *L. casei*, could be explained by the expression in MT9 of VanY, a penicillin-insensitive D,D-carboxypeptidase (17) which is acquired with the VanB-encoding operon (15) and which was not detected in *L. casei* ATCC 393 (5a).

In the presence of penicillin, only 39% *O*-acetylation was found in *L. casei*, corresponding to 33% reduction compared to that found in the absence of penicillin treatment. This is in agreement with the results of previous studies in which exposure to concentrations of β-lactams below the MICs decreased *O*-acetylation of peptidoglycan in *Neisseria gonorrhoeae*, *Proteus mirabilis*, and *Staphylococcus aureus* (13, 24, 28). In conclusion, the results indicate that in *L. casei* ATCC 393, which is naturally resistant to glycopeptides, the tetralactate precursor is readily exported by the lipid transporter and, similarly to what was found in *E. faecium* expressing vancomycin resistance, well processed to be integrated in the peptidoglycan (8). The absence of lactate-muropeptide in the cell wall synthesized by cells not treated with penicillin and the large amounts of murotetrapeptides reflect the natural efficiency of the transpeptidases and the presence of D,D-carboxypeptidase(s). Finally, the presence of tetralactate muropeptides after exposure to penicillin is very similar to the accumulation of normal pentapeptide muropeptides of various bacteria under the same conditions (22, 30, 31).

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