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Knockout of the Two *ldh* Genes Has a Major Impact on Peptidoglycan Precursor Synthesis in *Lactobacillus plantarum*

T. FERAIN,[1] J. N. HOBBS, JR.,[2] J. RICHARDSON,[2] N. BERNARD,[1] D. GARMYN,[1] P. HOLS,[1] N. E. ALLEN,[2] AND J. DELCOUR[1]*

Laboratoire de Génétique Moléculaire, Unité de Génétique, Université Catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium,[1] and Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana[2]

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Most bacteria synthesize muramyl-pentapeptide peptidoglycan precursors ending with a D-alanyl residue (e.g., UDP–*N*-acetylmuramyl–L-Ala–γ-D-Glu–L-Lys–D-Ala–D-Ala). However, it was recently demonstrated that other types of precursors, notably D-lactate-ending molecules, could be synthesized by several lactic acid bacteria. This particular feature leads to vancomycin resistance. Vancomycin is a glycopeptide antibiotic that blocks cell wall synthesis by the formation of a complex with the extremity of peptidoglycan precursors. Substitution of the terminal D-alanine by D-lactate reduces the affinity of the antibiotic for its target. *Lactobacillus plantarum* is a lactic acid bacterium naturally resistant to vancomycin. It converts most of the glycolytic pyruvate to L- and D-lactate by using stereospecific enzymes designated L- and D-lactate dehydrogenases, respectively. In the present study, we show that *L. plantarum* actually synthesizes D-lactate-ending peptidoglycan precursors. We also report the construction of a strain which is deficient for both D- and L-lactate dehydrogenase activities and which produces only trace amounts of D- and L-lactate. As a consequence, the peptidoglycan synthesis pathway is drastically affected. The wild-type precursor is still present, but a new type of D-alanine-ending precursor is also synthesized in large quantities, which results in a highly enhanced sensitivity to vancomycin.

Cell wall synthesis in gram-positive bacteria requires the production of a UDP–*N*-acetylmuramyl (MurNAc)–pentapeptide precursor (e.g., UDP–MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala) (48). This precursor is assembled in the cytoplasm and involves the addition of a D-Ala–D-Ala dipeptide at the C-terminal position of the UDP-MurNAc-tripeptide. The production of this dipeptide results from the activity of a D-Ala–D-Ala ligase. The UDP-MurNAc-pentapeptide is transferred to the outer face of the cell membrane by a lipid carrier and incorporated along with UDP–*N*-acetylglucosamine into the cell wall structure. The synthesis of other types of peptidoglycan precursors was demonstrated a few years ago in the context of several studies concerning vancomycin resistance. Vancomycin and other glycopeptide antibiotics can bind to the D-Ala–D-Ala terminus of pentapeptide-containing precursors by hydrogen bonding, thereby effectively blocking polymerization and preventing further cross-linking reactions (7, 41). Investigations of the molecular basis of vancomycin resistance started with strains of *Enterococcus faecium* and *Enterococcus faecalis* which showed inducible resistance to high levels of vancomycin and teicoplanin, another glycopeptide antibiotic. Examination of enzymes involved in cell wall synthesis in the resistant bacteria indicated that resistance to vancomycin was due to the synthesis of a novel type of peptidoglycan in which the terminal D-alanine residue was replaced by D-lactate, resulting in a drastic reduction of affinity for vancomycin (2, 4, 12, 25, 36). Two enzymes designated VanH and VanA are required for the synthesis of this alternative precursor (5). VanH is an α-keto-acid dehydrogenase that reduces pyruvate to D-lactate and that shows a high degree of homology with NAD-dependent D-lactate dehydrogenases (LDH) (6). VanA is a variant D-Ala–D-Ala ligase (17) with mixed substrate specificity that allows the synthesis of a D-Ala–D-lactate (D-Lac) depsipeptide instead of the D-Ala–D-Ala dipeptide (11, 12).

The synthesis of another type of peptidoglycan precursor has been described for *Enterococcus gallinarum*, which expresses inducible resistance to low levels of vancomycin but is susceptible to teicoplanin. In this case, the modified precursor terminates in D-serine instead of D-lactate (9). This feature results from the presence of another variant D-Ala–D-Ala ligase accepting D-serine (18).

The genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* comprise strains and species constitutively resistant to vancomycin (15, 20, 33, 39, 46, 49). Recently, peptidoglycan precursors from several of these lactic acid bacteria were analyzed. In *Pediococcus pentosaceus* and *Lactobacillus casei* (9, 26), the exclusive presence of a terminal D-lactate has been demonstrated. This presence could result from the action of a ligase which preferentially or exclusively catalyzes the synthesis of a D-Ala–D-Lac depsipeptide, as was suggested by Elisha and Courvalin (19). Analysis of *Leuconostoc mesenteroides* extracts identified a precursor that also terminates in D-lactate, but with an additional branched L-alanine (MurNAc–L-Ala–D-Glu–L-Lys–[L-Ala]–D-Ala–D-Lac) (26).

In this paper, we report that the wild-type strain *Lactobacillus plantarum* NCIMB8826 is naturally resistant to high levels of vancomycin and teicoplanin and exclusively produces D-lactate-ending peptidoglycan precursors. We describe the construction of a strain defective for both D- and L-LDH, resulting in drastically reduced production of both isomers of lactate. We show that this alteration leads to the synthesis of a new type of precursor ending with D-alanine in addition to the usual muramyl depsipentapeptide observed in the wild-type strain,

* Corresponding author. Mailing address: Laboratoire de Génétique Moléculaire, Unité de Génétique, Université Catholique de Louvain, Place Croix du Sud, 5 (bte 6), B-1348 Louvain-la-Neuve, Belgium. Phone: (32) (10) 473484. Fax: (32) (10) 473109. Electronic mail address: delcour@gene.ucl.ac.be.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
L. plantarum		
NCIMB8826	Wild-type strain	LMG[b]
NCIMB8826-D2	NCIMB8826 *ldhD*::pGIT042	This work
TF101	NCIMB8826 Δ*ldhL*	21
TF101-D2	NCIMB8826 Δ*ldhL ldhD*::pGIT042	This work
TF102	NCIMB8826 *ldhD*::*cat*	This work
TF103	NCIMB8826 Δ*ldhL ldhD*::*cat*	This work
E. coli		
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	23
Plasmids		
pGK13	Cm^r Em^r; *E. coli-L. plantarum* shuttle vector	32
pJDC9	Em^r; *lacZ*′; *E. coli* vector	14
pGIT042	Cm^r Em^r; pJDC9 derivative with a 5′ truncated copy of the *ldhD* gene from *L. plantarum* disrupted by the chloramphenicol acetyltransferase (*cat*) gene from the pGK13 plasmid	This work

[a] Cm^r and Em^r indicate resistance to chloramphenicol and erythromycin, respectively.
[b] LMG, Laboratorium voor Microbiologie Rijksuniversiteit Gent (Ghent, Belgium).

resulting in a highly enhanced sensitivity to vancomycin and teicoplanin.

MATERIALS AND METHODS

General molecular biology techniques were essentially performed according to the instructions given by Sambrook et al. (43).

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. All the plasmid constructions were performed in strain TG1 of *Escherichia coli. L. plantarum* was grown in DeMan, Rogosa, and Sharpe (MRS) broth (Difco 0881) at 37°C without shaking. As indicated, D- and/or L-lactic acid (lithium salt; Sigma) were added to the medium as a filter-sterilized aqueous solution. Antibiotics were used at the following concentrations: erythromycin, 250 μg/ml for *E. coli* and 5 μg/ml for *L. plantarum*; chloramphenicol, 50 μg/ml for *E. coli* and 10 μg/ml for *L. plantarum*. MICs of vancomycin and teicoplanin were determined with the Etest system (AB Biodisk, Solna, Sweden). Cells from the exponential growth stage were diluted in order to plate approximately 10^5 cells per dish, and MICs were read after 24 to 48 h of incubation.

Construction of pGIT042. The ′D1 and D2 regions of the *ldhD* gene were individually amplified from *L. plantarum* by PCR. The ′D1 region corresponds to the 5′ half of the gene but lacks the expression signals and the first 5 bp of the coding region. D2 is the 3′ part of the gene that ends 3 bp after the stop codon. Four oligonucleotide primers were chosen from the *ldhD* gene sequence of the closely related species *Lactobacillus pentosus* (50). Indeed, analysis of their respective *ldhL* genes revealed 91% identity for the nucleic acid sequences (21, 47). The two primers located in the central region are anticomplementary and span an *Eco*RV restriction site which was used in further construction steps. ′D1 and D2 regions were brought together in the same transcriptional orientations on the *E. coli* pJDC9 vector (14). The *cat* marker was isolated from the pGK13 plasmid (32) as a filled-in *Cla*I-*Bam*HI restriction fragment and inserted at the *Eco*RV site located between the ′D1 and D2 regions.

Transformation. Electrotransformation of *E. coli* and *L. plantarum* was performed as described by Dower et al. (16) and Josson et al. (30), respectively.

PCR amplification of DNA. *L. plantarum* chromosomal DNA was prepared by an adaptation of the method described by Posno et al. (40) for plasmid DNA preparation, with the two steps that eliminate chromosomal DNA being suppressed. PCRs were performed with 1 to 5 μg of DNA in a final volume of 100 μl containing deoxyribonucleoside triphosphates (200 μM each), oligonucleotides (25 μM each), 50 μM tetramethylammonium chloride, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim), and the buffer supplied with the enzyme. Amplification was performed with a GeneAmp PCR System 9600 (Perkin-Elmer) as follows: denaturation at 100°C for 5 min and then cycles 1 to 5, 92°C for 1 min, 30°C for 1 min, and 72°C for 2 min; cycles 6 to 30, 92°C for 1 min, 42°C for 1 min, and 72°C for 2 min; and cycle 31, 92°C for 1 min, 42°C for 1 min, and 72°C for 10 min. The two pairs of oligonucleotide primers used for amplification of the two halves of *ldhD* are 5′-AAATTATTGCATATGCTGTACG-3′ and 5′-GCAGCCATTGATATCTTCAAAGG-3′ (the 5′ half of the gene) and 5′-CCTTTGAAGATATCAATGGCTGC-3′ and 5′-AAATTAGTCAAACTTAACT-3′ (the 3′ half of the gene). In addition, universal and reverse primers (Biolabs) were used for the study of integration events.

LDH and lactate assays. Preparations of crude cell extracts and LDH assays were performed as previously described (21). Residual L-LDH activity corresponds to the activity measured after crude cell extracts were heated for 3 min at 50°C to inactivate D-LDH (37). One unit of activity corresponds to the oxidation of 1 μmol of NADH per min. The amount of total protein in the cell extracts was measured according to the method of Bradford (10), with the Bio-Rad protein assay (no. 500-0006) being used. The concentrations of L-(+)- and D-(−)-lactate in the culture supernatants were measured enzymatically with the Boehringer Mannheim kit no. 1112821.

Isolation and purification of peptidoglycan precursors. The muramyl-peptide precursors were isolated and purified from cell extracts by the method of Gorecki et al. (24), which involved Sephadex chromatography and high-performance liquid chromatography (HPLC). Lyophilized crude extracts were redissolved in distilled water, pH adjusted to 7.0, and clarified by centrifugation. The clarified extracts were purified first by Sephadex G-25 chromatography. The extracts were loaded onto a column and eluted at room temperature with 2 mM acetic acid. The eluant was monitored at A_{254}. UV absorbance peak fractions (1.0 ml) containing precursors (2) were pooled, pH adjusted to 7.0, and lyophilized. The lyophilized samples were redissolved in distilled water and further purified by HPLC with a μBondapak C_{18} column (7.8 by 300 mm). The muramyl-peptide precursors were isolated by isocratic elution at room temperature with 0.05 M ammonium acetate, pH 5.0. UV absorbance peak fractions were collected, pH adjusted to 7.0, and lyophilized.

Analysis of peptidoglycan precursors by HPLC and mass spectrometry. Samples were analyzed by HPLC with a μBondapak C_{18} column (3.9 by 300 mm). Samples (0.5 to 2 nmol) were subjected to isocratic elution at room temperature with 0.05 M ammonium acetate, pH 5.0. Muramyl-peptide precursors were detected at A_{262}. Analysis by electrospray ionization mass spectrometry was conducted with a PE Sciex API III mass spectrometer equipped with a pneumatically assisted electrospray (Ionspray) interface. Spectra were obtained by continuously infusing the sample into the interface at a rate of 10 μl/min with a Harvard infusion pump. Negative ion spectra were obtained with 50:49:1 (vol/vol/vol) acetonitrile-water-ammonium hydroxide. An inlet orifice potential of −20 V relative to the R0 potential was employed. Mass spectra were obtained with a step size of 0.1 atomic mass unit (u), a scan range of 350 to 1,400 u, and a scan time of 11 s per scan. Five scans per sample were made to provide an average final mass spectrum.

Amino acid analysis and carboxy-terminal residue identification of purified precursors. Amino acid composition of precursors was determined with a 6300 Beckman amino acid analyzer. The carboxy-terminal residue was hydrolyzed by incubation with purified D,D-carboxypeptidase from *Streptomyces* sp. strain R39 (a gift from P. Charlier) by following the method of Messer and Reynolds (36). D-Alanine was assayed by the D-amino acid oxidase–peroxidase procedure (29). D-Lactic acid was assayed with D-LDH (38).

RESULTS

Peptidoglycan precursors analysis in the wild-type NCIMB 8826 strain of *L. plantarum*. We tested the sensitivity of wild-type strain NCIMB8826 to vancomycin and teicoplanin and found the strain to be highly resistant (Table 2). Peptidoglycan precursors were identified in cell extracts prepared from the wild-type strain grown in the presence of bacitracin to facilitate the accumulation of peptidoglycan precursors in the cytoplasm. HPLC analysis revealed a single precursor molecule having a retention time of approximately 34 min (Fig. 1A, peak 1). This precursor was purified by HPLC, and structural

TABLE 2. MICs of vancomycin and teicoplanin in wild-type and mutant strains of *L. plantarum*

L. plantarum strain	MIC (μg/ml)					
	MRS		MRS + 50 mM L-lactate		MRS + 50 mM D-lactate	
	Vancomycin	Teicoplanin	Vancomycin	Teicoplanin	Vancomycin	Teicoplanin
NCIMB8826 (wild type)	>256	>256	ND[a]	ND	ND	ND
TF103 (Δ*ldhL ldhD*::*cat*)	2	0.5	2	0.5	>256	>256

[a] ND, not determined.

identification of this molecule was based on electrospray ionization mass spectrometry. This analysis indicated a compound with a molecular weight of 1,194.5 (calculated from the M-H^- ion at 1,193.5 u and the M-$2H^{2-}$ ion at 596.3 u as shown in Fig. 2A), which closely corresponds to the expected molecular weight (1,194.3) for the lactate-containing depsipentapeptide UDP–MurNAc–L-Ala–D-Glu–*meso*-diaminopimelic acid (m-Dpm)–D-Ala–D-Lac. Additionally, the sodium adduct of UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Lac (the M-H^- ion at 1,215.4 u and the M-$2H^{2-}$ ion at 607.2 u), the trifluoracetyl adduct of UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Lac (the M-$2H^{2-}$ ion at 653.2 u), and the combination sodium-trifluoracetyl adduct of UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Lac (the M-$2H^{2-}$ ion at 664.2 u) were detected (Fig. 2A). Amino acid analysis and identification of the carboxy-terminal residue of the purified precursor confirmed this predicted composition (data not shown). The presence of a D-lactate-ending precursor was previously observed in other vancomycin-resistant lactic acid bacteria (2, 4, 9, 25, 26, 36). The occurrence of m-Dpm instead of the usual lysine at the third position of the depsipentapeptide precursor was also previously described for *L. plantarum* (44).

Disruption of the *ldhD* gene in *L. plantarum*. Lactic acid bacteria produce D-lactate during lactic acid fermentation via NAD-dependent D-LDH. We decided to study the consequences of suppressing D-LDH activity for the cell wall synthesis pathway in *L. plantarum*. As was previously described for the *ldhL* gene in the same strain (21), we used a procedure which leads to stable chromosomal disruption of the *ldhD* gene through a two-step homologous recombination process. To this end, we employed the pGIT042 suicide vector bearing a 5′-

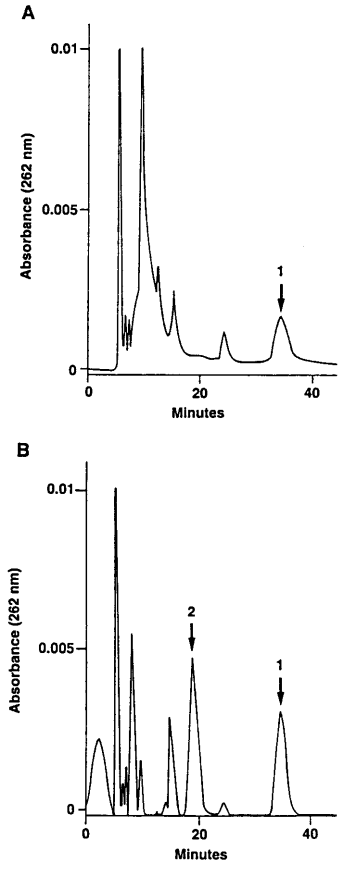

FIG. 1. HPLC analysis of UDP-MurNAc-peptide precursors in extracts of *L. plantarum*. (A) Wild-type NCIMB8826. (B) LDH-deficient strain TF103. Arrow 1 was identified as UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Lac; arrow 2 was identified as UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Ala. The ratio of peak 2 to 1 in panel B is 55/45.

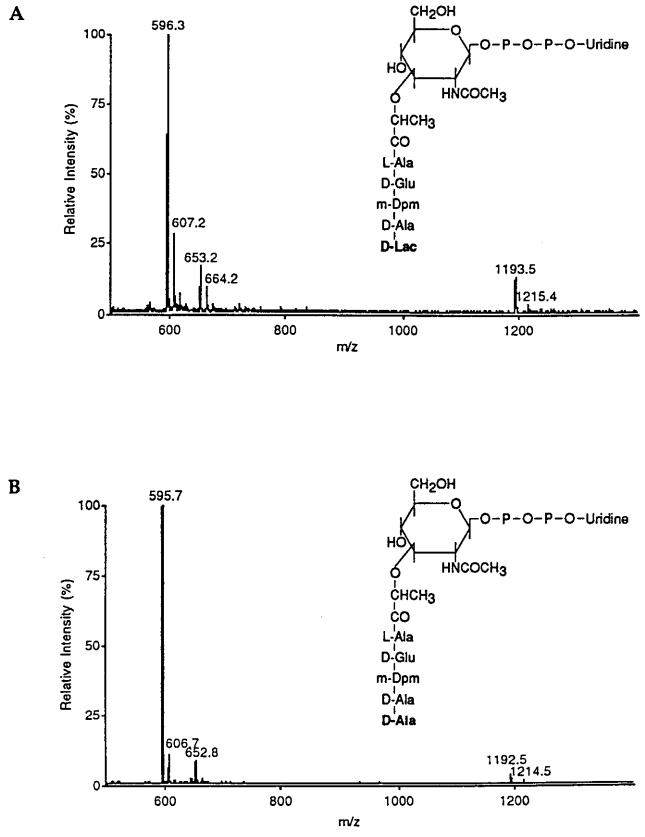

FIG. 2. Negative ion electrospray ionization mass spectra of peptidoglycan precursors observed in the wild-type strain (A) and the TF103 strain (A and B) of *L. plantarum* and proposed structures of precursors UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Lac (A) and UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Ala (B).

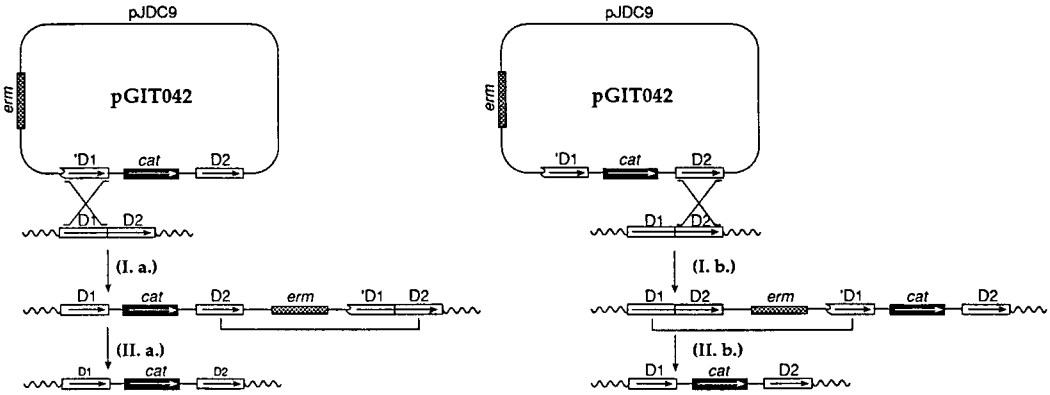

FIG. 3. Construction of a disruption in the *L. plantarum ldhD* gene. Integration plasmid pGIT042 is a pJDC9 derivative that contains a 5′-truncated copy of the *L. plantarum ldhD* gene (′D1 and D2 open boxes) disrupted by a gene conferring resistance to chloramphenicol. Campbell-like integration of this plasmid into the chromosome can take place via the D1 region (I. a.). This integration results in both a truncated and a disrupted copy of the *ldhD* gene. On the other hand, disrupted and intact copies of the gene are obtained when integration occurs via the D2 region (I. b.). A single disrupted copy of the *ldhD* gene in the chromosome can finally be obtained in both cases by appropriate secondary excision (II. a. and II. b.). The strain is stably deficient for D-LDH activity and can be identified by its chloramphenicol resistance and erythromycin sensitivity phenotype.

truncated copy of the *ldhD* gene (′D1/D2) disrupted by a chloramphenicol resistance gene (*cat*) and an erythromycin resistance marker (*erm*) located outside the disruption cassette (Fig. 3). The construction of pGIT042 is described in Materials and Methods, and the disruption strategy is illustrated in Fig. 3.

Plasmid pGIT042 was introduced into *L. plantarum* NCIMB 8826 by electroporation. Integration events among erythromycin- and chloramphenicol-resistant colonies were identified by PCR. We obtained only D2 integration events which yielded clones still possessing a functional copy of the *ldhD* gene. In order to isolate the expected excision event, one such strain (NCIMB8826-D2) was grown in MRS broth supplemented with chloramphenicol and racemic DL-lactate (20 mM). Replica plating was used to identify chloramphenicol-resistant, erythromycin-sensitive colonies. Four clones were analyzed by PCR and Southern blot hybridization (data not shown). All of them yielded the profiles expected for disruptive excision, and the resulting *ldhD*::*cat* strain was designated TF102.

Specific LDH activity measurements in crude cell extracts after 24 h of unaerated culture are listed in Table 3. These data show a 45% reduction in total LDH activity in TF102 compared with that in the wild-type strain. The thermoresistance of this activity shows that it is due to L-LDH. Additional measurements performed at several time intervals during growth gave similar results. However, despite the fact that we did not detect D-LDH activity in this strain, we measured nearly equivalent amounts of D and L isomers of lactate in supernatant from 24-h cultures (Table 3). These observations are in agreement with the involvement of a lactate racemase. Racemization has already been reported in a few species of lactobacilli: *Lactobacillus sake*, *Lactobacillus curvatus*, and *L. casei* subsp. *pseudoplantarum* (27, 45). The production of both isomers of lactate by the TF102 mutant made this strain unsuitable for our investigation of the pathway of peptidoglycan precursor synthesis.

TABLE 3. Lactate concentrations in culture supernatants and LDH activities of wild-type and mutant strains of *L. plantarum*

L. plantarum strain[a]	L-Lactate (g/liter)	D-Lactate (g/liter)	Total LDH sp act (U/mg of protein)	Residual L-LDH sp act (U/mg of protein)[b]
NCIMB8826 (wild type)	7.80	8.90	34	18
TF102 (*ldhD*::*cat*)	8.90	8.40	19	18
TF103 (Δ*ldhL ldhD*::*cat*)	0.91[c]	0.05[c]	ND[d]	ND[d]

[a] *L. plantarum* strains were grown aerobically for 24 h in MRS broth.
[b] Residual L-LDH activity was determined in crude cell extracts after a 3-min heat treatment of the sample (50°C) to inactivate D-LDH (see reference 37).
[c] Repeated measurements detected low levels of both D- and L-lactate produced by the TF103 strain by comparison with the levels in fresh MRS medium containing L- and D-lactate at 0.87 g/liter and 0.03 g/liter, respectively.
[d] ND, not detected.

Disruption of the *ldhD* gene in the *L. plantarum* TF101 (Δ*ldhL*) strain. In order to prevent D-lactate production, a strain deficient for both D-LDH and L-LDH was constructed. The two-step homologous recombination strategy described above was applied to L-LDH-deficient strain TF101 (21). Plasmid pGIT042 was transferred in TF101 by electroporation, and cells were grown on MRS agar plates. Recombinant clones were screened by antibiotic selection and then by Southern blot analysis (data not shown). Only nondisruptive integrations within the D2 region were obtained, as was observed during the construction of the TF102 strain. One such clone (TF101-D2) was grown in selective chloramphenicol MRS broth supplemented with 20 mM racemic DL-lactate. One chloramphenicol-resistant, erythromycin-sensitive clone was obtained, and analysis of this clone by PCR and Southern blot hybridization confirmed the chromosomal disruption of the *ldhD* gene (data not shown). This strain was designated TF103.

TF103 culture supernatants contained D- and L-lactate at concentrations only slightly above the background values for fresh MRS broth. However, no LDH activity was detected in crude cell extracts from the TF103 strain (Table 3). These low residual levels of lactate could result from such minor dehydrogenase activities as hydroxyisocaproate dehydrogenase (HicDH). These enzymes are able to weakly reduce pyruvate to lactate, and although they have been described in several lactic acid bacteria (8, 28, 34, 35), we could not detect hydroxyisocaproate dehydrogenase activity in *L. plantarum*. Production of L-lactate could also result from the metabolism of citrate present in MRS medium, via L-malate and malolactic fermentation (13, 31).

Peptidoglycan precursors analysis in the LDH-defective TF103 strain of *L. plantarum*. Since D-lactate production was drastically reduced in the TF103 strain, peptidoglycan precur-

sors in this mutant were analyzed by a method described previously for the wild-type strain. Two precursors were identified by HPLC: one eluted as the precursor found in the wild-type strain (retention time, 34 min [Fig. 1B, peak 1]), while the other was released after approximately 19 min (Fig. 1B, peak 2). This second peak amounted to about 55% of the total precursor pool.

The new peptidoglycan precursor was first shown to have the same HPLC retention time as the precursor isolated from *Bacillus megaterium* (1) which has the following structure: UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Ala (expected molecular weight, 1,193.3). Its identity was confirmed by mass spectrometry, which indicated a compound with a size of 1,193.5 Da (calculated from the $M\text{-}H^-$ ion at 1,192.5 u and the $M\text{-}2H^{2-}$ ion at 595.7 u as shown in Fig. 2B), which closely corresponds to the expected molecular weight. Figure 2B shows that the sodium adduct of UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Ala (the $M\text{-}H^-$ ion at 1,214.5 u and the $M\text{-}2H^{2-}$ ion at 606.7 u) was also detected, as was the trifluoracetyl adduct of UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Ala (the $M\text{-}2H^{2-}$ ion at 652.8 u). Amino acid analysis of the precursor confirmed this composition, and D-alanine was identified as the ending residue (data not shown). The presence of the wild-type precursor ending in D-lactate shows that the double mutant strain is still able to incorporate D-lactate in about 45% of the precursors, despite the drastically reduced production of lactate and the lack of detectable LDH activity.

In view of the substitution of the D-lactate terminus by D-Ala in about half of the precursors, the susceptibilities to vancomycin and teicoplanin of TF103 and wild-type strains were compared. Whereas the MICs of the antibiotics were above 256 μg/ml for the wild-type strain as indicated earlier, MICs of vancomycin and teicoplanin were reduced to 2 and 0.5 μg/ml, respectively, for TF103 (Table 2). The addition of D-lactate (50 mM) to the MRS agar plates restored resistance to both antibiotics at MICs higher than 256 μg/ml.

DISCUSSION

Several bacterial species incorporate D-lactate at the C terminus of peptidoglycan precursors instead of the usual D-alanine (2, 4, 9, 25, 26, 36). This alternative pathway is due to the particular substrate specificity of the species' D-Ala–D-Ala ligase. This enzyme was first known to synthesize D-Ala–D-Ala dipeptides, but it was shown a few years ago that several lactic acid bacteria possess a D-Ala–D-Ala ligase-related enzyme which preferentially or exclusively synthesizes D-Ala–D-Lac depsipeptides. This synthesis results in the assembly of depsipentapeptide peptidoglycan precursors ending with D-lactate which are then incorporated into the cell wall. This particular feature is linked with vancomycin and teicoplanin resistance because of the lower affinity of D-lactate-ending precursors for the antibiotics which are known to specifically bind their extremity.

L. plantarum NCIMB8826 was found to be resistant to high levels of both vancomycin and teicoplanin, as are some strains of lactic acid bacteria, including several lactobacilli (3, 15, 20, 33, 39, 46, 49). This observation prompted us to investigate the molecular basis of this phenotype. Peptidoglycan precursors from *L. plantarum* NCIMB8826 were thus analyzed by HPLC and mass spectrometry. These studies showed that the C-terminal D-alanine was indeed substituted by D-lactate. The precursor identified also contains m-Dpm in the third position, as was previously mentioned for another strain of *L. plantarum* (44), resulting in the following structure: UDP–MurNAc–L-Ala–D-Glu–m-Dpm–D-Ala–D-Lac. No other type of precursor was observed in the wild-type strain. This first investigation demonstrated the implication of D-lactate in peptidoglycan precursor synthesis in *L. plantarum.*

We then constructed a mutant strain deficient for D-lactate production, with the hope of evaluating peptidoglycan precursor synthesis in the absence of this stereoisomer. Disruption of the *ldhD* gene was achieved as previously described for the *ldhL* gene (21) and resulted in the loss of D-LDH activity. However, the mutant strain designated TF102 still produced a racemic mixture of lactate. Since Δ*ldhL* constructed previously produces only D-lactate, the racemization process observed in the *ldhD*::*cat* TF102 strain is likely to be inducible by L-lactate, as was described for lactate racemases in other lactobacilli (27, 45). This observation showed that the D-LDH-deficient strain could not be used for an investigation of peptidoglycan precursor synthesis since it still produces D-lactate.

The *ldhD* disruption strategy was repeated on the Δ*ldhL* TF101 strain, and we obtained a double mutant lacking L-LDH and D-LDH activities. This strain, designated TF103, produces only trace amounts of D- and L-lactate. It is highly affected in growth, especially under anaerobiosis, and displays a completely modified metabolism (22). Peptidoglycan precursors in the TF103 strain were analyzed by HPLC and mass spectrometry and shown to be of two types. The first precursor differs from the wild-type precursor by the substitution of the terminal D-lactate by D-alanine. The proportion of this precursor reaches 55% of the total pool. The other type is identical to the D-lactate-containing molecule observed in the wild-type strain. A high proportion of this precursor (45%) is observed, despite the highly reduced production of D-lactate in this strain. This finding evokes the exclusive incorporation of D-lactate in peptidoglycan precursors of *L. casei* (9, 26), a lactic acid bacterium producing only very small amounts of D-lactate.

We compared the vancomycin and teicoplanin sensitivities of the TF103 strain with those of the wild type, since D-lactate substitution by D-alanine restores the target bound by the antibiotics in sensitive species. TF103 was found to be highly sensitive to vancomycin and teicoplanin, a surprising finding in view of the mixed production of precursors in roughly similar proportions. It is not known whether both types of precursors are actually incorporated into peptidoglycan in the mutant strain. Nevertheless, the process of cell wall synthesis is expected to be severely impaired by vancomycin. We know that this glycopeptide antibiotic shuts down polymerization by tightly binding the undecaprenyl lipid-bound disaccharide pentapeptide intermediate. In this way, vancomycin-bound intermediates accumulate, and this accumulation exhausts the pool of free lipid carriers, which are prevented from recycling. So, even a limited amount of D-alanine-ending precursor could cause a major inhibition of cell wall synthesis in the presence of vancomycin. The consequences of a mixed production of pentapeptide and depsipentapeptide precursors have already been studied in *E. faecalis*. In this case, coproduction of similar amounts of both precursors also results in vancomycin sensitivity, and a drastic reduction of precursors ending in D-alanine (<5%) is necessary to reach high levels of resistance (2a, 42).

A question arises about the origin of the D-alanine-ending precursor in the mutant, since it is not observed at all in the wild-type strain. Its synthesis could reflect the existence of a distinct ligase that could be responsible for D-Ala–D-Ala dipeptide formation in the mutant strain. In the wild-type strain, the involvement of this enzyme would be negligible because of a low level of activity compared with that of the major D-Ala–D-Lac ligase. Alternatively, incorporation of D-alanine instead of D-lactate could reflect the mixed substrate specificity of a single *L. plantarum* ligase which would prefer D-lactate. This prefer-

ence, together with competition between abundant D-lactate and probably scarce D-alanine, could lead to exclusive use of D-lactate in the wild-type strain. On the other hand, the limited availability of D-lactate in the TF103 strain would lead to a mixed synthesis of D-lactate- and D-alanine-ending precursors. Mixed substrate specificity has been described for the inducible ligase (VanA) involved in glycopeptide resistance in *E. faecium* BM4147 (11, 12). A comparison of the ligase genes from constitutively vancomycin-resistant lactobacilli species (including *L. plantarum*) and *L. mesenteroides* has shown that their deduced amino acid sequences are more closely related to each other than to that of a sensitive strain of *Lactobacillus leichmannii* (19). Their common feature could be a preference for D-lactate. These genes could have evolved from an ancestral D-Ala–D-Ala ligase gene in species which were in contact with organisms producing glycopeptide antibiotics, and such a genesis would explain the residual activity on D-alanine.

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