

Selectivity for D-Lactate Incorporation into the Peptidoglycan Precursors of *Lactobacillus plantarum*: Role of Aad, a VanX-Like D-Alanyl-D-Alanine Dipeptidase^{∇†}

Marie Deghorain,¹ Philippe Goffin,^{1,‡} Laetitia Fontaine,¹ Jean-Luc Mainardi,² Richard Daniel,³ Jeff Errington,³ Bernard Hallet,¹ and Pascal Hols^{1*}

Unité de Génétique, Institut des Sciences de la Vie, Université catholique de Louvain, 1348 Louvain-la-Neuve, Belgium¹; INSERM, U655-LRMA, Université Paris 6, Université Paris-Descartes, Centre de Recherche Biomedicales des Cordeliers, Paris, F-75006, France²; and Institute for Cell and Molecular Biosciences, The Medical School, University of New Castle upon Tyne, New Castle upon Tyne NE2 4HH, United Kingdom³

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***Lactobacillus plantarum* produces peptidoglycan precursors ending in D-lactate instead of D-alanine, making the bacterium intrinsically resistant to vancomycin. The ligase Ddl of *L. plantarum* plays a central role in this specificity by synthesizing D-alanyl-D-lactate depsipeptides that are added to the precursor peptide chain by the enzyme MurF. Here we show that *L. plantarum* also encodes a D-Ala-D-Ala dipeptidase, Aad, which eliminates D-alanyl-D-alanine dipeptides that are produced by the Ddl ligase, thereby preventing their incorporation into the precursors. Although D-alanine-ended precursors can be incorporated into the cell wall, inactivation of Aad failed to suppress growth defects of *L. plantarum* mutants deficient in D-lactate-ended precursor synthesis.**

The antibiotic vancomycin binds to the usual D-alanyl-D-alanine (D-Ala-D-Ala) termini of the peptidoglycan precursor side chain, thereby inhibiting peptidoglycan synthesis by interfering with the penicillin-binding proteins (PBPs) (27). In the majority of vancomycin-resistant enterococci, the synthesis of peptidoglycan precursors is reprogrammed from the formation of D-Ala-D-Ala termini to D-alanyl-D-lactate (D-Ala-D-Lac) termini, to which vancomycin binds with an ~1,000-fold-lower affinity (for reviews, see references 10 and 15). This resistance mechanism results from the acquisition of at least three genes (*vanH*, *vanA* [or *vanB*], and *vanX*) generally clustered in an operon (2, 3). The *vanH* gene encodes a D-lactate dehydrogenase responsible for the conversion of pyruvate into D-Lac (5); *vanA* (or *vanB*) encodes a specific D-Ala-D-Lac ligase required for the formation of D-Ala-D-Lac depsipeptides, which are incorporated at the end of the modified precursors (2, 7, 12); and the product of *vanX* is a highly specific dipeptidase needed to selectively eliminate D-Ala-D-Ala dipeptides produced by the host endogenous D-Ala-D-Ala ligase Ddl (1, 25) (Fig. 1). Additional accessory genes have been reported to contribute to vancomycin resistance in different enterococcal strains (10, 15). For example, *vanY* encodes a D,D-carboxypeptidase capable of hydrolyzing the terminal D-Ala of the UDP-*N*-acetylmuramyl (MurNAc) pentapeptide precursors, further increasing the resistance level (1, 2) (Fig. 1).

Highly homologous and similarly organized *van* operons

have been discovered in bacteria producing vancomycin and other structurally related glycopeptides (e.g., *Amycolatopsis orientalis* and *Streptomyces toyocaensis*), suggesting a common origin for the resistance mechanisms used by the vancomycin producers and those subsequently acquired by vancomycin-resistant enterococci (20, 21).

Genes homologous to the resistance genes *vanH*, *vanA* (or *vanB*), and *vanX* are also found together or separately in the genomes of other organisms, where they appear to have distinct physiological roles. In particular, homologues of the VanX dipeptidase were identified in a wide variety of bacterial species, including gram-negative bacteria, which are normally not challenged by vancomycin because of the failure of the antibiotic to penetrate the outer membrane barrier. However, only a few of these VanX variants have been studied at the functional level (20).

In *Escherichia coli*, *ddpX*, encoding a VanX-related dipeptidase, is cotranscribed with a dipeptide transport system (*ddpABCDF*) during the stationary phase of growth. It was proposed that D-Ala-D-Ala dipeptides released from cell wall peptidoglycan can be reimported from the periplasm through the Ddp transporter and subsequently hydrolyzed by DdpX to provide D-Ala as a nitrogen and carbon source (18, 29). Likewise, the D-Ala-D-Ala dipeptidase PcgL, found in the pathogen species *Salmonella enterica*, confers the ability to use D-Ala-D-Ala as a sole carbon source and is involved in survival under starvation conditions (16, 23).

Lactobacillus plantarum NCIMB8826 is an intrinsically vancomycin-resistant lactic acid bacterium that produces D-Lac-ended peptidoglycan precursors (13). This bacterium possesses the requisite D-Ala-D-Lac ligase (Ddl_{Lp}) for depsipeptide synthesis (13) and a D-Lac dehydrogenase for D-Lac production (13) (Fig. 1). We recently reported that D-Lac is also produced by L-lactate racemization in *L. plantarum* (14) (Fig. 1). Racemization seems to represent an important rescue pathway to

* Corresponding author. Mailing address: Unité de Génétique, Institut des Sciences de la Vie, Université catholique de Louvain, 1348 Louvain-la-Neuve, Belgium. Phone: 32-10-47 88 96. Fax: 32-10-47 31 09. E-mail: hols@gene.ucl.ac.be.

‡ Present address: Wageningen Centre for Food Sciences, NIZO Food Research, 6710 BA Ede, The Netherlands.

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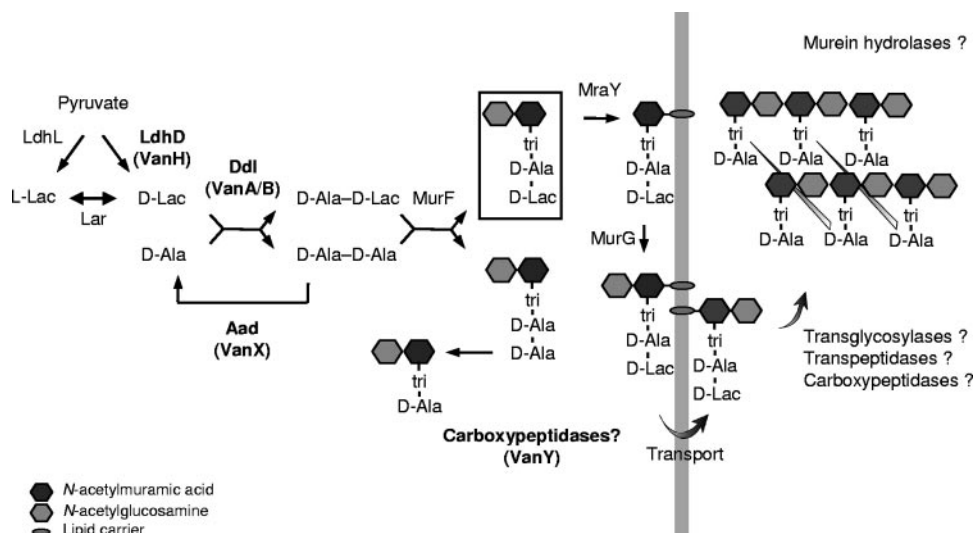


FIG. 1. Selectivity for D-lactate incorporation in peptidoglycan biosynthesis of *L. plantarum* WCFS1. *L. plantarum* enzymes for which orthologues are found in vancomycin-resistant enterococci (in parentheses) are in bold. LdhD, NAD-dependent D-lactate dehydrogenase; LdhL, NAD-dependent L-lactate dehydrogenase; Lar, lactate racemase; Ddl, D-Ala-D-Lac ligase; Aad, D-Ala-D-Ala dipeptidase; MurF, UDP-MurNAc-tripeptide:D-Ala-D-Lac ligase; MraY, phospho-N-acetylmuramoyl-pentapeptide transferase; MurG, N-acetylglucosamyl transferase; tri, L-Ala-D-Glu-m-DAP.

supply the cell with D-Lac for peptidoglycan synthesis. An *L. plantarum* mutant totally impaired in D-Lac production strictly requires exogenous D-Lac in the medium for growth, reflecting a strong selectivity for D-Lac incorporation into the peptidoglycan of *L. plantarum* (14).

In the present study, we show that *L. plantarum* also contains a functional D-Ala-D-Ala dipeptidase belonging to the VanX family. The biological function of this endogenous dipeptidase and its implications for intrinsic vancomycin resistance and D-Lac selectivity for peptidoglycan synthesis were investigated.

Identification of a vanX-like gene in *L. plantarum*. Analysis of the genome of *L. plantarum* WCFS1 (17) revealed the presence of a gene homologous to the resistance gene *vanX* (lp_0769; designated *aad* for D-alanyl-D-alanine dipeptidase). This gene is not located in the same locus as the gene coding for the D-Ala-D-Lac ligase (Ddl_{lp}) of *L. plantarum*, nor is it in a locus involved in D-Lac production. Inspection of the surrounding sequences indicates that *aad* is likely to be transcribed as a monocistronic unit, with the adjacent open reading frames being on the complementary strand (data not shown).

Phylogenetic analysis of VanX-like sequences from gram-positive and gram-negative bacteria reveals that they form a rather heterogeneous family of enzymes (see Fig. S1A in the supplemental material). The Aad protein clearly belongs to a distinct subgroup, displaying only 30% identity with VanX_A from *Enterococcus faecium* BM4147 (3, 4) and 32% and 28% identity with the dipeptidases DdpX and PcgL from *E. coli* (18) and *S. enterica* (16), respectively. This Aad subgroup contains VanX-like proteins identified in the genomes of *Lactobacillus brevis* ATCC 367 (accession number YP_796214) and *Lactobacillus salivarius* UCC118 (accession number YP_536261) (75% and 72% identical to Aad, respectively; see Fig. S1A in the supplemental material). These two VanX-like-protein-encoding genes are present in a different genetic context than

aad, but, as found in *L. plantarum*, they are not located in loci involved in peptidoglycan biosynthesis or D-Lac production.

Enzymes of the VanX family are metalloproteases that use Zn²⁺ as a cofactor. Despite a relatively low level of overall similarity, members of the family are characterized by several highly conserved residues involved in catalysis (E181 and R71 in *E. faecium* VanX_A) and Zn²⁺ cofactor binding (H116, D123, and H184) (8, 18, 19, 22). In the *L. plantarum* protein Aad, all these amino acids are conserved except H184, which is replaced by an aspartate (see Fig. S1B in the supplemental material).

The *L. plantarum* *aad* gene encodes a functional D-Ala-D-Ala dipeptidase. To determine whether the *L. plantarum* *aad* gene encodes a functional D-Ala-D-Ala dipeptidase, the levels of D-Ala-D-Ala hydrolysis were examined in the wild-type strain, an *aad* knockout strain, and an Aad-overproducing strain.

Overexpression was achieved by cloning *aad* under the control of the nisin-inducible promoter P_{nisA} from *Lactococcus lactis* (24). The resulting plasmid (pGIM023) was introduced into *L. plantarum* strain NZ7100, a WCFS1 derivative carrying the *nisR* and *nisK* regulatory genes required for nisin induction (M. Kleerebezem, unpublished data). The *aad* knockout mutant (MD119) was constructed in NZ7100 using a two-step homologous recombination procedure as previously described (14). Aad inactivation resulted in the stable replacement of the *aad* open reading frame (encoding 180 amino acid residues out of 185) by an erythromycin resistance cassette. For details on the construction of these recombinant strains, see the supplemental text and Table S1 in the supplemental material.

The D-Ala-D-Ala dipeptidase activities of the different strains were measured in crude cell extracts prepared from exponentially growing cultures (MRS broth [Becton Dickinson, Cockeysville, MD]; optical density at 600 nm [OD₆₀₀] = 2). Nisin (50 ng/ml) was added when required to allow expression from

TABLE 1. D-Ala-D-Ala dipeptidase activity of Aad in *L. plantarum*

Strain	Description	D-Ala-D-Ala dipeptidase activity ^a	
		–Zn ²⁺ ^b	+Zn ²⁺
NZ7100	Wild type	ND ^c	1.9 ± 0.6
MD119	<i>aad::erm</i>	ND	ND
NZ7100(pGIM023) ^d	<i>P_{nisA}::aad</i>	1.5 ± 0.4	387.2 ± 20.7
NZ7100(pGIM020) ^d	Empty expression vector	ND	1.8 ± 0.3

^a The assay was carried out on fresh cell extracts resuspended in Tris-maleate buffer (50 mM, pH 7.0) in the presence of 100 mM D-Ala-D-Ala (Sigma, Bornem, Belgium). Protein concentrations were determined by the method of Bradford (6) using the Bio-Rad Laboratories (Munich, Germany) protein assay. Specific activity is expressed as nmol of D-Ala released per minute per mg of total proteins in the cell extract (values are means ± standard deviations from a minimum of three independent repetitions).

^b Extracts were preincubated on ice in the presence of 15 mM ZnCl₂ for 1 h (final concentration in the assay, 1.5 mM).

^c ND, not detected (below the detection level of the assay).

^d Nisin (50 ng/ml) was added to the culture medium 5 h before the cells were collected.

the *P_{nisA}* promoter. The enzymatic assay consisted of measurement of the amount of D-Ala released from the D-Ala-D-Ala substrate (100 mM) using a D-amino acid oxidase coupled to a peroxidase (2).

In the presence of Zn²⁺, low but significant D-Ala-D-Ala dipeptidase activity was measured in extracts of the wild-type strain, whereas D-Ala-D-Ala hydrolysis detected in cell extracts of the *aad* mutant was in the range of the background level measured in the absence of substrate or cosubstrate (Table 1). Overexpression of *aad* increased the observed activity about 200-fold compared to the activity in the wild-type strain or the control strain containing the empty expression vector pGIM020 (Table 1).

These results clearly demonstrate that the *vanX*-like gene *aad* of *L. plantarum* encodes a functional dipeptidase capable of converting D-Ala-D-Ala substrates into D-Ala and that, under the conditions of this study, no other enzyme with detectable D-Ala-D-Ala hydrolysis activity is present in *L. plantarum*.

In contrast to what has been observed for the enterococcal dipeptidase VanX_A and other enzymes of the same family,

which remain sufficiently charged in cofactors when extracted from the cells (18; M. Deghorain, unpublished data), the optimal activity of *L. plantarum* Aad required the addition of Zn²⁺ to the in vitro reaction mixture (Table 1). This may reflect a lower affinity for the cofactor as a consequence of the substitution of the conserved histidine (H184) in the Zn²⁺-binding motif of the protein.

The D-Ala-D-Ala dipeptidase specific activity of the wild-type strain remained constant throughout growth (data not shown), suggesting that the expression of *aad* is not regulated by a growth-dependent mechanism as was reported for the dipeptidase DdpX of *E. coli* (18).

Aad contributes to the selectivity for the incorporation of D-Ala-D-Lac at the terminal position of the peptidoglycan precursor side chain. As mentioned above, *L. plantarum* is highly selective for the production and incorporation of D-Ala-D-Lac-ended peptidoglycan precursors in the cell wall (13, 14). A key enzyme for this selectivity is Ddl_{LP}, which is responsible for the production of D-Ala-D-Lac depsipeptides that are incorporated at the end of the peptidoglycan precursor peptide chain by MurF (Fig. 1).

To determine whether Aad contributes to this selectivity, the intracellular pools of UDP-*N*-acetylmuramyl (UDP-MurNAc) pentadepsipeptides and UDP-MurNAc pentapeptides were compared in exponentially growing cells of the wild type and an *aad* knockout strain. Peptidoglycan precursors were extracted as reported before (14).

As previously observed (13), precursors ending in D-Ala-D-Ala were undetectable in the wild-type strain, whereas they accounted for 7% of the mature precursors found in the *aad* mutant (Table 2). This result shows that the Ddl_{LP} ligase produces both D-Ala-D-Lac depsipeptides and D-Ala-D-Ala dipeptides for their incorporation into the peptidoglycan precursors by MurF and that Aad prevents the formation of D-Ala-D-Ala-ended precursors in the wild-type strain by eliminating the D-Ala-D-Ala dipeptides. Production of UDP-MurNAc-pentapeptide precursors ending in D-Ala-D-Ala had already been observed in an *L. plantarum* mutant deficient for the *l*- and *D*-lactate dehydrogenases (TF103; *ldhL ldhD* double mutant)

TABLE 2. Effect of *aad* inactivation on peptidoglycan precursor composition, vancomycin resistance, and Van-FL staining of *L. plantarum* in the absence and presence of the D-Ala-D-Ala ligase (Ddl_{LC}) from *L. lactis*

Strain	Description	% D-Ala-D-Ala-ended PG precursors ^a	Vancomycin MIC (μg/ml) ^b	% Van-FL labeled cells
NZ7100	Wild type	0 ^c	>256 (>1,536)	0
MD119	<i>aad::erm</i>	7	>256 (>1,536)	0
NZ7100(pGIM021)	<i>P_{nisA}::ddl_{LC}</i> transcriptional fusion ^d	5	>256	~20 ^e
MD119(pGIM021)	<i>P_{nisA}::ddl_{LC}</i> transcriptional fusion ^d	23	32–128	~30 ^e
NZ7100(pGIM121)	<i>P_{nisA}::ddl_{LC}</i> translational fusion ^d	58	1–3	>75
MD119(pGIM121)	<i>P_{nisA}::ddl_{LC}</i> translational fusion ^d	97	1–3	>75

^a Percentage of penta-substituted UDP-MurNAc-penta(depsi)peptide precursors ending in D-Ala-D-Ala. Peptidoglycan (PG) precursors were prepared from *L. plantarum* cells grown in MRS broth to an OD₆₀₀ of 0.7 and treated with ramoplanin (10 μg/ml) for 90 min (2). Precursors were then extracted with 20% trichloroacetic acid and analyzed by reverse-phase high-pressure liquid chromatography (14).

^b Determined by the E-test method (AB-Biodisk, Solna, Sweden) in MRS broth. Values in parentheses are MICs determined by measuring the OD₆₀₀ reached after 16 h of growth in liquid MRS broth containing increasing concentrations of vancomycin (from 0 to 1536 μg/ml).

^c No UDP-MurNAc-tri-D-Ala-D-Ala could be detected.

^d Basal, noninduced expression of the *ddl_{LC}* ligase from a fusion with the *P_{nisA}* promoter on a multicopy plasmid.

^e Weak fluorescence signal observed in isolated cells, most likely due to the stochastic expression of the *P_{nisA}::ddl_{LC}* fusions into these cells in the absence of the nisin inducer.

(13). This mutant is impaired in D-Lac production, which was not the case with the *aad* mutant.

Determination of vancomycin MICs revealed that the *aad* mutant remained resistant to high concentrations of the antibiotic, similar to those determined for the wild-type strain. This indicates that the amount of D-Ala-D-Ala-ended precursors that accumulated in the absence of Aad was insufficient to result in sensitivity to vancomycin, suggesting that these precursors are not transferred or poorly transferred to the outside of the cell or are not incorporated efficiently into the cell wall.

D-Ala-D-Ala-ended peptidoglycan precursors can be transported to the external face of the plasma membrane. In order to further investigate the ability of Aad to control the intracellular composition in peptidoglycan precursors and its impact on cell wall biosynthesis, the monospecific D-Ala-D-Ala ligase from *L. lactis* (encoded by *ddl_{Lc}*), a vancomycin-sensitive gram-positive bacterium, was expressed in the *L. plantarum* wild-type strain (NZ7100) and in the *aad* mutant. It was shown previously that Ddl_{Lc} is capable of promoting the synthesis of peptidoglycan precursors ending in D-Ala-D-Ala in *L. plantarum* (14).

Different levels of *ddl_{Lc}* expression were obtained by cloning the *ddl_{Lc}* gene as a transcriptional and a translational fusion with the P_{*nisA*} promoter, on a multicopy plasmid, yielding pGIM021 and pGIM121, respectively (see the supplemental text and Table S1 in the supplemental material). Dipeptidase activity in cell extracts from strains expressing the ligase gene *ddl_{Lc}* was unchanged compared to that of the wild-type strain, indicating that Aad activity is not regulated by the presence of D-Ala-D-Ala dipeptides (data not shown).

For both constructs, induction of the P_{*nisA*} promoter by adding nisin in the culture medium had toxic effects, making the observations difficult to reproduce. In the absence of nisin, basal expression of the *ddl_{Lc}* gene from the transcriptional and translational fusion in the wild-type strain led to the accumulation of 5% and 58% of D-Ala-D-Ala-ended peptidoglycan precursors, respectively [NZ7100 (pGIM021) and NZ7100 (pGIM121)] (Table 2). This proportion was increased to about 97% in the *aad* deletion mutant expressing the P_{*nisA*}::*ddl_{Lc}* translational fusion [MD119 (pGIM121)] (Table 2).

These results further demonstrate the contribution of the Aad dipeptidase in preventing the accumulation of peptidoglycan precursors ending in D-Ala-D-Ala for cell wall synthesis.

Strains producing increasing amounts of D-Ala-D-Ala-ended precursors were found to be gradually less resistant to vancomycin, with the strains expressing the P_{*nisA*}::*ddl_{Lc}* translational fusion being completely sensitive to the antibiotic (Table 2). This indicates that noncanonical D-Ala-D-Ala-ended UDP-MurNAc-pentapeptides are correctly transported to the external face of the plasma membrane to serve as a substrate for the PBPs at the outside of the cell, where they become accessible to the antibiotic in the form of the lipid II complex.

To confirm this, and to visualize the sites of precursor incorporation into the growing peptidoglycan network, exponentially growing cells from the different strains were incubated with a fluorescent derivative of vancomycin (Van-FL) and examined by epifluorescence microscopy as previously reported (11). Consistent with the high level of resistance to vancomycin and the absence of detectable peptidoglycan precursors ending in D-Ala-D-Ala, cells of the wild-type strain NZ7100 were not

stained by the Van-FL probe (Table 2; Fig. 2A). In contrast, strains expressing the *ddl_{Lc}* gene were labeled to different extents, depending on the ratio of D-Ala-D-Ala- and D-Ala-D-Lac-ended precursors that they produced (Table 2). Fluorescence was specifically localized at the division sites and along the longitudinal axis of the cells, forming peripheral spots and transversal bands suggestive of the formation of a short spiral around the cell (Fig. 2B), as previously reported for *Bacillus subtilis* (11).

D-Ala-D-Ala-ended peptidoglycan precursors are not suitable substrates for optimal growth of *L. plantarum*. The results presented above show that under conditions where D-Ala-D-Ala-ended precursors are produced, the cell wall biosynthesis machinery of *L. plantarum* is able to further process these molecules into lipid II complexes that are exposed on the outside of the cell.

Since the strains described above still produce peptidoglycan precursors ending in D-Ala-D-Lac (Table 2), it cannot be ruled out that their growth exclusively relies on the incorporation of D-Ala-D-Lac-ended precursors into the growing peptidoglycan. This is not the case for the *ldhD lar* double mutant of *L. plantarum* (strain PG1104, a derivative of PG6212 *ldhD lar* containing a chromosomal integration of the empty expression plasmid pMec10), which is totally impaired in D-Lac production. This mutant is unable to grow in a chemically defined medium (MPL) (9) deprived of D-Lac (Fig. 3) (14). However, expression of *ddl_{Lc}* from *L. lactis* can partially rescue this growth defect through the production of D-Ala-D-Ala-ended peptidoglycan precursors (Fig. 3) (strain PG1174, a derivative of PG6212 containing the chromosomally integrated P_{*nisA*}::*ddl_{Lc}* fusion) (14).

Based on the results presented above, we hypothesized that the D-Ala-D-Ala dipeptidase activity of *L. plantarum* could be responsible for depleting the pool of D-Ala-D-Ala produced by the endogenous ligase Ddl_{Lp} (strain PG1104) or by the heterologous Ddl_{Lc} ligase (strain PG1174). To test this hypothesis, the *aad* gene was inactivated in these two genetic backgrounds, yielding strains MD1104 and MD1174, respectively.

In the presence of D-Lac in the culture medium, growth of both strains was identical to the growth of the similarly D-Lac-supplemented parental *ldhD lar* mutant (Fig. 3 and data not shown). In a D-Lac-deprived medium, inactivation of the *aad* gene neither suppressed the growth defect of the *ldhD lar* mutant nor improved the growth of the mutant expressing *ddl_{Lc}* (Fig. 3). Thus, it appears that the pool of D-Ala-D-Ala is not the limiting growth factor in this background but rather that D-Ala-ended peptidoglycan precursors are not optimal substrates for cell wall synthesis in *L. plantarum*.

Concluding remarks. This study reports the characterization of a VanX-like dipeptidase, Aad, in *L. plantarum* and its role in resistance to vancomycin. This enzyme was found to direct incorporation of D-lac into peptidoglycan precursors, rather than D-Ala, through the specific hydrolysis of D-Ala-D-Ala peptides. However, a second key enzyme in promoting the synthesis of this specific form of peptidoglycan precursor is the D-Ala-D-Lac ligase Ddl_{Lp}. We show here that Ddl_{Lp} produces D-Ala-D-Ala dipeptide in *L. plantarum*, even under conditions where D-Lac is not limiting. Therefore, Aad contributes to the exclusive formation of D-Ala-D-Lac-ended precursors by hydrolyzing D-Ala-D-Ala arising from this dual

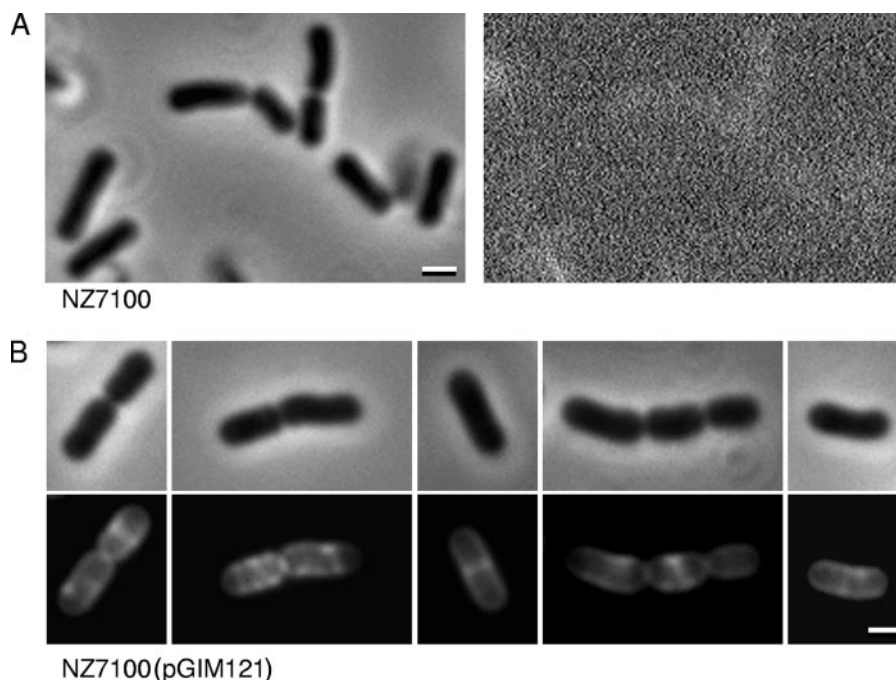


FIG. 2. Van-FL staining of *L. plantarum* cells. Cells were harvested in early exponential phase (MRS broth, $OD_{600} = 0.2$) at 30°C. A mixture of equal amounts of vancomycin BODIPY-FL conjugate (Molecular Probes) and unlabeled vancomycin (Sigma) was added to the cultures to a final vancomycin–Van-FL concentration of 3 $\mu\text{g/ml}$ (30 min of incubation). Fixed cells were visualized by fluorescence microscopy (484-nm set filter), and images were taken and analyzed as described previously by Daniel and Errington (11). Images of wild-type and NZ7100(pGIM121) cells were treated similarly. (A) *L. plantarum* wild-type cells. (Left) Phase contrast; (right) Van-FL staining. (B) Cells from NZ7100(pGIM121) expressing the *L. lactis* ligase gene *ddl_{Lc}* from the translational fusion with the P_{nisA} promoter, cultured without nisin. (Upper panels) Phase contrast; (lower panels) Van-FL staining. Bars, 1 μm .

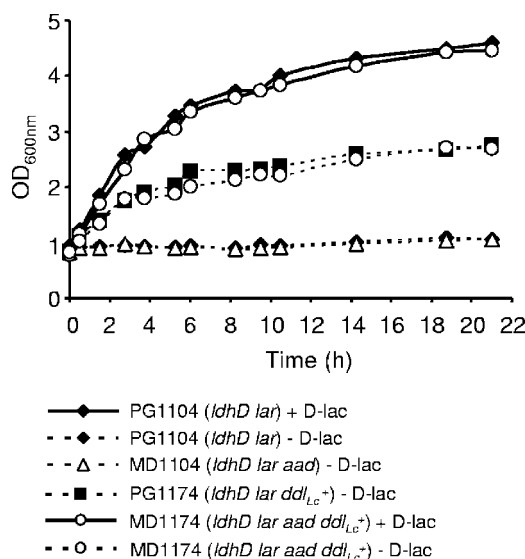


FIG. 3. Effect of *aad* deletion on the growth of the *L. plantarum* *ldhD lar* mutant (PG1104) and the derivative strain expressing the *L. lactis* *ddl_{Lc}* ligase under the control of the P_{nisA} promoter (PG1174). Filled symbols, PG1104 (diamonds) and PG1174 (squares) (14); open symbols, corresponding *aad* deletion mutants MD1104 (triangles) and MD1174 (circles). Strains were grown in MPL broth containing nisin (50 ng/ml) and D-lactate (20 mM) to an OD_{600} of 0.5, washed twice in MPL broth, and resuspended in MPL containing 50 ng/ml of nisin and 20 mM of D-lactate (solid lines) or containing 50 ng/ml of nisin only (dashed lines).

specificity (Fig. 1). This conclusion does not rule out the possibility that Aad may have additional functions, such as providing D-Ala as a carbon or nitrogen source, as was found in *E. coli* and *S. enterica* (16, 18, 23, 29).

The biological reason for the selective incorporation of D-Ala-D-Lac-ended precursors in the cell wall of *L. plantarum* remains unclear. The possibility that it may provide *L. plantarum* with a selective advantage in specific ecological niches by conferring resistance to vancomycin cannot be ruled out. However, it is unlikely that the genes involved in D-Lac production and precursor incorporation (i.e., *ldhD*, *ddl*, and *aad*) were transferred from glycopeptide producers to *L. plantarum* as was proposed for other vancomycin-resistant species, since they are not clustered in the genome and do not display obvious signs of recent lateral exchange. Instead, it is tempting to speculate that the resistance genes that were acquired by vancomycin producers, and subsequently by enterococci, initially originated from other organisms, where they contribute to a separate physiological function, as proposed here for *L. plantarum*.

In the absence of Aad, D-Ala-D-Ala dipeptides can be processed and incorporated into peptidoglycan precursors. Furthermore, the use of a Van-FL probe showed that D-Ala-D-Ala-ended precursors can be exported across the cell membrane, supporting the view that the enzymes catalyzing the corresponding reactions (i.e., MurF, MraY, and MurG) do not have an absolute specificity for the fifth position of the peptidoglycan peptide (26) (Fig. 1).

The observation that inactivation of *aad* failed to rescue a

mutant strain unable to synthesize D-Lac (*ldhD lar* double mutant) shows that the amount of D-Ala-D-Ala-ended precursors erroneously synthesized by Ddl of *L. plantarum* is insufficient to support cell wall synthesis. Surprisingly, only a partial restoration of growth is obtained by the overexpression of the *ddl_{Lc}* ligase gene of *L. lactis* in the triple mutant (*ldhD lar aad*), which should exclusively produce D-Ala-D-Ala dipeptides. This suggests that there is selectivity for the D-Ala-D-Lac-ended precursors in the later stages of peptidoglycan biosynthesis (Fig. 1). In particular, carboxypeptidases may play a role in this specificity. Five genes encoding potential carboxypeptidases are present in the *L. plantarum* genome, including one homologue of the enterococcal gene *vanY* (lp_1010; designated *dacB*; 27% identity with the VanY_A gene from *E. faecium* BM4147) (28). High-molecular-weight PBPs catalyzing the transglycosylation and transpeptidation reactions, murein hydrolases, and carboxypeptidases involved in peptidoglycan maturation may also have a high specificity for D-Lac in the last position of the peptide chain (Fig. 1).

Although D-Lac is required for growth of the *ldhD lar* mutant, suppressor mutations can be selected, restoring growth in the absence of D-Lac based on the production of peptidoglycan precursors ending in D-Ala-D-Ala (14). Genetic analysis of these suppressor mutants in the presence and absence of *aad* will help to determine the key enzymes controlling the nature of the last residue of the peptide chain and perhaps elucidate the biological significance of this specificity.

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