Identification of the Asparagine Synthase Responsible for D-Asp Amidation in the *Lactococcus lactis* Peptidoglycan Interpeptide Crossbridge[▽]

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We show that in *Lactococcus lactis*, the gene *asnH* encodes the asparagine synthase involved in amidation of D-Asp present in peptidoglycan side chains and crossbridges. The level of D-Asp amidation in peptidoglycan has a strong effect on the sensitivity of bacteria to endogenous autolysins and to the cationic antimicrobials nisin and lysozyme.

Peptidoglycan (PG), the main component of the cell wall in gram-positive bacteria, is a heteropolymer composed of glycan strands made of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc) to which is attached a stem peptide (16). In the model lactic acid bacterium *Lactococcus lactis*, the stem peptide is composed of the pentapeptide chain L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala. These peptidic chains can be cross-linked with a D-Asp residue (4, 11). D-Asp is transferred to the PG precursors by an aspartate ligase which ligates the β-carboxylate group of D-Asp to the ε-amino group of Lys (15). In the final mature *L. lactis* PG, about 75% of the side chain and crossbridge residues are amidated (4). However, neither D-iso-Asn nor D-Asn was shown to be a substrate for aspartate ligase (1, 15), indicating that amidation of the

α-carboxyl group of D-Asp occurs after its incorporation into the PG precursor by aspartate ligase. Amidation could take place on the D-iso-Asp-containing lipid or on nascent PG (13). In this study, we identified for the first time the gene encoding the enzyme responsible for D-Asp amidation of the bacterial PG crossbridge.

Analysis of the PG structure of an *L. lactis* *asnH* mutant. In the *L. lactis* MG1363 genome sequence, a gene, *asnH* (1,878 bp), encoding a putative asparagine synthase, is the first gene of the operon which also includes *aslA*, encoding the aspartate ligase involved in D-Asp incorporation into the PG interpeptide crossbridge. To investigate *asnH* function in *L. lactis*, we analyzed the PG structure of an *asnH*-negative mutant. This mutation was constructed in *L. lactis* MG1363 by single cross

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant genotype or phenotype	Source or reference
<i>L. lactis</i> strains		
MG1363	Plasmid-free strain	5
VES4254	<i>asnH</i> mutant of MG1363, obtained by SCO and allowing expression of the downstream genes due to insertion of the P ₅₉ promoter; Em ^r	15
VES5609	MG1363 derivative carrying the pAT18 (<i>asnH</i> ⁺) plasmid; Cm ^r	This study
VES4075	MG1363 derivative carrying pVES3916; Cm ^r	14
VES5622	VES4254 derivative carrying the pAT18 (<i>asnH</i> ⁺) plasmid; Em ^r Cm ^r	This study
Plasmids		
pVE3916	Derivative of broad-host-range replicon pWV01; Cm ^r	14
pAT18	Derivative of pVE3916, carrying a 2,407-bp fragment of the MG1363 chromosome with the <i>asnH</i> gene under the control of its own promoter	This study

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FIG. 1. RP-HPLC separation of mucopeptides from *L. lactis* MG1363 (A), the *asnH*-negative mutant (B), and the *asnH*-overexpressing strain (C). Peaks numbered on chromatograms were analyzed by MALDI-TOF MS. The mucopeptide structures were assigned according to reference 4 and are listed in Table 2. In panel C, peaks containing mucopeptides with only Asn and no Asp are indicated with an asterisk.

TABLE 2. Structures, molecular masses, and quantities of mucopeptides for *L. lactis* MG1363, the MG1363 *asnH* mutant, and the *asnH*-overexpressing strain

Group and peak no. ^a	Proposed structure ^b	Observed <i>m/z</i>	Calculated ^c [M+Na] ⁺	% of all peaks ^d		
				MG1363	<i>asnH</i> mutant	<i>asnH</i> -overexpressing strain
Monomers						
1	Tri	848.35	848.39	0.6	0.6	0.2
2	Tetra	919.35	919.42	1.5	2.1	1.2
3	Tri-D missing GlcNac	760.28	760.34	0.1	0.0	0.2
4	Tri*-D	964.40	964.39	0.3	1.5	0.2
5	Tri-D	963.36	963.41	2.7	6.0	1.8
6	Tri*-N	963.36	963.41	1.6	0.0	3.3
7	Tri-N	962.40	962.43	7.3	0.0	13.2
8	Tetra-D	1,034.44	1,034.45	6.5	10.0	2.7
9	Penta-D	1,105.37	1,105.49	5.3	11.6	1.2
10	Tetra-N	1,033.56	1,033.47	4.8	0.0	6.8
11	Penta-N	1,104.56	1,104.50	6.9	0.0	5.0
12	Tri-N (Ac)	1,004.47	1,004.44	0.7	0.0	0.5
13	Tetra-D (Ac)	1,076.43	1,076.46	0.8	1.1	0.1
14	Tetra-N (Ac)	1,075.48	1,075.48	0.3	0.0	0.2
33	Tetra missing GlcNac	716.32	716.35	0.0	0.2	0.0
34	Tetra (deAc)	877.39	877.41	0.0	0.4	0.0
35	Tri*-D missing GlcNac	761.33	761.32	0.0	0.1	0.0
36	Tri-D (deAc)	921.43	921.40	0.0	1.4	0.0
37	Penta	990.53	990.46	0.0	0.9	0.0
38	Tetra-D missing GlcNac	831.28	831.38	0.0	0.4	0.0
39	Tri (Ac)	890.40	890.39	0.0	0.3	0.0
40	Tetra-D (deAc)	992.38	992.44	0.0	1.1	0.0
41	Penta-D (deAc)	1,063.49	1,063.48	0.0	1.4	0.0
42	Tri-D (Ac)	1,005.40	1,005.42	0.0	0.7	0.0
44	Penta-D (Ac)	1,147.60	1,147.50	0.0	0.5	0.0
Dimers						
15	Tri-N-Tetra	1,841.00	1,840.85	0.4	0.0	0.6
16	Tri-N-Tetra-N (deAc)	1,912.90	1,912.88	0.5	0.0	2.1
17	Tri-N-Tetra-N missing GlcNac	1,751.82	1,751.82	1.6	0.0	3.5
18	Tri-N-Tetra-D	1,955.86	1,955.88	5.6	0.0	5.9
19	Tri-N-Tetra-N	1,954.87	1,954.90	7.8	0.0	17.3
20	Tetra-N-Tetra-D	2,026.90	2,026.91	6.4	0.0	3.0
21	Tetra-N-Tetra-N	2,025.80	2,025.93	8.3	0.0	8.0
22	Penta-N-Tetra-D	2,097.88	2,097.95	4.0	0.0	0.9
23	Penta-N-Tetra-N	2,096.95	2,096.97	9.3	0.0	5.5
24	Tri-N-Tetra-N (Ac)	1,996.89	1,996.90	1.3	0.0	1.1
25	Tetra-N-Tetra-N (Ac)	2,067.92	2,067.94	1.6	0.0	1.6
43	Tri-D-Tetra missing GlcNac	1,638.66	1,638.13	0.0	0.3	0.0
45	Tri-D-Tetra*-D missing GlcNac	1,754.80	1,754.76	0.0	0.6	0.0
46	Tri-D-Tetra	1,841.84	1,841.83	0.0	0.5	0.0
47	Tri-D-Tetra-D missing GlcNac	1,753.65	1,753.79	0.0	2.4	0.0
48	Tri-D-Tetra-D (deAc)	1,914.83	1,914.85	0.0	4.5	0.0
49	Tri-D-Tetra-D	1,956.83	1,956.86	0.0	4.5	0.0
50	Tetra-D-Tetra	1,912.88	1,912.87	0.0	1.3	0.0
51	Tetra-D-Tetra-D missing GlcNac	1,824.75	1,824.83	0.0	1.7	0.0
52	Tetra-D-Tetra-D	2,027.95	2,027.90	0.0	12.1	0.0
53	Tetra-D-Tetra-D (deAc)	1,985.87	1,985.89	0.0	1.7	0.0
54	Penta-D-Tetra-D	2,098.98	2,098.93	0.0	10.9	0.0
55	Penta-D-Tetra-D (deAc)	2,057.05	2,056.82	0.0	2.3	0.0
56	Penta-D-Tetra-D (deAc)	2,056.76	2,056.92	0.0	1.6	0.0
57	Tri-D-Tetra-D (Ac)	1,998.84	1,998.87	0.0	1.8	0.0
58	Tetra-D-Tetra-D (Ac)	2,069.85	2,069.91	0.0	3.5	0.0
Trimers						
26	Tri-N-Tetra-N-Tetra-N	2,947.01	2,947.36	2.7	0.0	6.5
27	Tetra-N-Tetra-N-Tetra-N	3,018.50	3,018.40	3.8	0.0	2.5
28	Penta-N-Tetra-N-Tetra-N	3,089.29	3,089.44	4.5	0.0	2.1
29	Tetra-N-Tetra-N-Tetra-N (Ac)	3,060.39	3,060.41	0.7	0.0	0.9
59	Tri-D-Tetra-D-Tetra-D	2,950.19	2,950.31	0.0	2.0	0.0
60	Tri-D-Tetra-D-Tetra-D (deAc)	2,908.36	2,908.30	0.0	1.6	0.0
61	Tetra-D-Tetra-D-Tetra-D	3,021.18	3,021.35	0.0	3.2	0.0
62	Penta-D-Tetra-D-Tetra-D	3,092.33	3,092.38	0.0	3.1	0.0
Tetramers						
30	Tri-N-Tetra-N-Tetra-N-Tetra-N	3,939.74	3,939.83	0.7	0.0	0.9
31	Tetra-N-Tetra-N-Tetra-N-Tetra-N	4,010.14	4,010.87	0.7	0.0	0.4
32	Penta-N-Tetra-N-Tetra-N-Tetra-N	4,081.37	4,081.90	0.7	0.0	0.4

^a Peak numbers refer to Fig. 1.^b Tri, disaccharide tripeptide (L-Ala-D-iGln-L-Lys); Tetra, disaccharide tetrapeptide (L-Ala-D-iGln-L-Lys-D-Ala); Penta, disaccharide pentapeptide (L-Ala-D-iGln-L-Lys-D-Ala-D-Ala); Tri*, disaccharide tripeptide without amidation (L-Ala-D-iGlu-L-Lys); Disaccharide, GlcNac-MurNac; D, Asp; N, Asn; Ac, acetylation; deAc, deacetylation. Mucopeptides containing only Asn and no Asp are indicated in bold. The positions of N and D in the peptide chains of dimers and trimers are arbitrary.^c Sodiated molecular ions were the most abundant ions detected on the positive-ion mass spectra obtained by MALDI-TOF MS for all mucopeptides.^d The percentage of each peak was calculated as the ratio of the peak area over the sum of areas of all the peaks identified in the corresponding chromatogram.

FIG. 2. MS-MS analysis of the mucopeptide purified in peak 8 from *asnH* mutant chromatogram (B) and desalted. The $[M+Na]^+$ parental ion (m/z of 1,034.46) was selected for fragmentation. The fragments were detected as sodiated ions.

over SCO insertional inactivation, using a nonreplicative plasmid vector, pRV300, carrying an internal fragment of *asnH* under the control of the P_{59} promoter (15) (Table 1). The mutant was viable, indicating that the gene was not essential in *L. lactis* and that, in addition, the P_{59} promoter allows expression of *aslA* and *racD*, the essential genes encoding aspartate ligase and aspartate racemase, which are located downstream from *asnH* (15). To evaluate the D-Asp amidation level, PG was extracted and digested with mutanolysin, and the resulting mucopeptides were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) after reduction, as described previously (4, 9). The mucopeptide profile of the *asnH* mutant was found to be strikingly different from that of the wild-type MG1363 strain (Fig. 1A and B), with several peaks apparently missing from the *asnH* mutant chromatogram. The mucopeptides separated by RP-HPLC with an am-

monium phosphate buffer system were analyzed directly, without desalting, by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) as described previously (4) (Table 2). In wild-type *L. lactis* MG1363, as already shown previously (4), PG contained both D-Asp and D-Asn in side chains and interpeptide crossbridges with 74% of D-Asn. In contrast, according to the MS results, no mucopeptides with D-Asn were present in the *asnH* mutant (Table 2).

The structure of the main monomers of the *asnH* mutant chromatogram (Fig. 1B, peaks 5, 8, and 9, containing mucopeptides with m/z values of 963.36, 1,034.44, and 1,105.37, respectively) was further analyzed by tandem MS (MS-MS) with a MALDI Q-TOF mass spectrometer (Q-Star XL; Applied Biosystems). The mass spectra confirmed the presence of an Asp residue and the absence of Asn in these three monomers (Fig. 2 and data not shown). Indeed, several ion frag-

FIG. 3. Autolysis of *L. lactis* MG1363 and its derivative strains in buffer solution. Cells were grown in M17 medium up to an optical density at 600 nm (OD_{600}) of 0.8, washed, resuspended at an OD_{600} of 1 in 50 mM sodium phosphate (pH 7.0), and incubated at 30°C. Lysis of MG1363 ($\blacktriangle$), the *asnH* mutant ($\blacksquare$), the complemented strain ($\triangle$), the *asnH*-overexpressing strain VES5609 ($\square$), and the control strain with empty plasmid pVE3916 ($\circ$) was monitored by measuring the OD_{600} decreases in the cell suspensions. The results shown are representative of three independent experiments.

ments result from the loss of one D-Asp residue (mass defect of 115.02), whereas the loss of one D-Asn residue would result in a mass defect of 114.04.

Complementation of the *asnH* mutant with a multicopy plasmid, pVE3916, containing the cloned *asnH* gene (Table 1) led to restoration of the Asn-containing muropeptides, confirming the *asnH* gene function (data not shown). Thus, we can conclude that *asnH* encodes the only asparagine synthase responsible for D-Asp amidation in *L. lactis*. In strain VES5609 (Table 1), which overexpresses *asnH* due to amplification on a multicopy plasmid, Asn is present in 87% of the muropeptide side chains and crossbridges, compared to 74% in the wild-type strain (Fig. 1C and Table 2).

It is noteworthy that a second putative glutamine-hydrolyzing asparagine synthase (EC 6.3.5.4), AsnB, which exhibits 28% sequence similarity with AsnH, is encoded by the *L. lactis* MG1363 genome sequence. Since mutation in *asnH* results in the complete absence of D-Asn in PG, we consider that *asnB* is not involved in D-Asp amidation and probably encodes the metabolic enzyme involved in L-Asn synthesis, like AsnB of *Escherichia coli* (12) and *Bacillus subtilis* (17).

Influence of the PG D-Asp amidation level on *L. lactis* autolysis. To evaluate the involvement of D-Asp amidation in the autolytic potential of lactococci, we compared the autolysis of MG1363 and its derivative strains after transfer of bacteria in

sodium phosphate buffer solution. As shown in Fig. 3, the *asnH* mutant exhibited a higher autolysis rate than the wild-type strain MG1363. In contrast, the complemented mutant as well as the overexpressing strain exhibited a lower autolysis rate. The control MG1363 strain carrying an empty plasmid (VES4075) autolyzed at the same rate as MG1363. These results indicate that the autolysis rate in buffer solution decreased concomitantly to the increase of D-Asp amidation.

Increased resistance of the *asnH* mutant to nisin and lysozyme. It was previously shown that in gram-positive bacteria and in particular in *L. lactis*, the reduction of the cell wall-negative electric charges as a result of D-alanylation of teichoic acids leads to an increase in bacterial resistance to cationic antimicrobial peptides, such as nisin (6, 8, 10).

We hypothesized that the increase of the D-Asp amidation level in PG would also decrease the number of negative charges inside the cell wall and thus modify the bacterial sensitivity to cationic antimicrobials. Therefore, we tested the resistance of the *asnH* mutant and of the overexpressing strain VES5609 to nisin and lysozyme. Culture dilutions of each strain were spotted on M17 medium plates containing 100 ng/ml of nisin or 250 μ g/ml of lysozyme. As shown in Fig. 4, the *asnH* mutant was much more sensitive to both nisin and lysozyme than either the wild type or the overexpressing strain. *asnH* overexpression had no clear effect on the resistance to either compound, despite our results showing a moderate increase of D-Asp amidation (from 74 to 87%) (Fig. 1C).

Concluding remarks. We have identified for the first time the gene *asnH*, encoding the asparagine synthase involved in amidation of D-Asp present in the interpeptide crossbridge of *L. lactis* PG. Homologous genes can be found by homology searches of several other gram-positive bacterial species which possess D-Asp in their crossbridges. In particular, we found a gene exhibiting 65% sequence identity with *L. lactis* *asnH* in *Enterococcus faecium*. Note that in contrast to what was found for *L. lactis*, this gene is not present in the same operon as the corresponding aspartate ligase gene in *E. faecium* (*asl_{fm}*) (1).

Sequence analysis by SignalP (2) and TMMOD (7) indicates that AsnH contains neither a putative signal peptide nor a transmembrane segment, suggesting that AsnH is localized inside the bacterial cytoplasm and that this protein is most probably active on PG precursors.

We showed that in *L. lactis*, D-Asp amidation increases bacterial resistance to the activities of both endogenous autolysins and the cationic antimicrobials lysozyme and nisin. Bacterial killing by lysozyme is a major factor of the host innate immune

FIG. 4. Comparison of the lysozyme (LYS) and nisin (NIS) resistance levels of *L. lactis* MG1363 (WT), the control strain with empty plasmid pVE3916 (WT/pVE3916), the *asnH* mutant, and the *asnH*-overexpressing strain VES5609.

system, used by neutrophils, monocytes, macrophages, and epithelial cells (3). Since some gram-positive bacterial pathogens (such as *E. faecium*) appear to carry AsnH homologs and possess D-Asp in their PG crossbridges, our results may thus provide insight into the role of D-Asp amidation in host defense during infection.

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