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Characterization of O-Acetylation of N-Acetylglucosamine

A NOVEL STRUCTURAL VARIATION OF BACTERIAL PEPTIDOGLYCAN^{*,[5]}

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Peptidoglycan (PG) N-acetyl muramic acid (MurNAc) O-acetylation is widely spread in Gram-positive bacteria and is generally associated with resistance against lysozyme and endogenous autolysins. We report here the presence of O-acetylation on N-acetylglucosamine (GlcNAc) in *Lactobacillus plantarum* PG. This modification of glycan strands was never described in bacteria. Fine structural characterization of acetylated muropeptides released from *L. plantarum* PG demonstrated that both MurNAc and GlcNAc are O-acetylated in this species. These two PG post-modifications rely on two dedicated O-acetyltransferase encoding genes, named *oatA* and *oatB*, respectively. By analyzing the resistance to cell wall hydrolysis of mutant strains, we showed that GlcNAc O-acetylation inhibits N-acetylglucosaminidase Acm2, the major *L. plantarum* autolysin. In this bacterial species, inactivation of *oatA*, encoding MurNAc O-acetyltransferase, resulted in marked sensitivity to lysozyme. Moreover, MurNAc over-O-acetylation was shown to activate autolysis through the putative N-acetylmuramoyl-L-alanine amidase LytH enzyme. Our data indicate that in *L. plantarum*, two different O-acetyltransferases play original and antagonistic roles in the modulation of the activity of endogenous autolysins.

Peptidoglycan (PG),⁶ the major constituent of the cell envelope of Gram-positive bacteria, is a polymer of the disaccharide N-acetylmuramic acid-(β -1,4)-N-acetylglucosamine (MurNAc-GlcNAc) associated with a peptidic stem linked to MurNAc (1,

2). The composition of the peptidic stem varies from one bacterium to another and, in *Lactobacillus plantarum*, is composed of L-alanine, D-glutamic acid, meso-diaminopimelic acid, D-alanine, and a D-lactate as last moiety (see Fig. 1) (3–6). The different glycan strands are cross-linked between the fourth amino acid of the donor stem and the third amino acid of the acceptor peptide forming a three-dimensional network around the cell called the sacculus. The main functions of this sacculus are to ensure cell integrity against the internal osmotic pressure and to provide the shape of the bacteria (1, 2, 7).

Because of its rigidity, PG has to be remodeled by the PG hydrolases (PGH) to allow cell growth and cell division. PGH are divided into different classes according to their cleavage site: the carboxy- and endopeptidases that cleave the peptidic stem, the N-acetylmuramoyl-L-alanine amidases that cleave between the first amino acid and MurNAc, and finally the N-acetylglucosaminidases and N-acetylmuramidases that cut inside the glycan chain between GlcNAc and MurNAc or MurNAc and GlcNAc, respectively (7, 8).

Extracellular synthesis of PG begins with the addition of MurNAc-GlcNAc-pentadepsipeptide from lipid II to an existing glycan strand and the cross-linking of the peptidic stem, both catalyzed by the high molecular weight penicillin binding proteins (2, 9). In addition, the MurNAc-GlcNAc disaccharide can be modified by the addition of an acetyl group on the C6-OH position of MurNAc or by removing the acetyl group N-linked to MurNAc or GlcNAc (10–20). These modifications confer resistance against lysis induced by lysozyme, an N-acetylmuramidase found in human tears, saliva, or gastrointestinal tract (10, 13, 14, 18, 19). O-Acetylation inhibits lysozyme activity through steric hindrance (10) but also lytic transglycosylases that need a free –OH group at position 6 of MurNAc to form the anhydro ring concomitantly to the cleavage of GlcNAc-MurNAc linkage (20, 21).

MurNAc O-acetylation is described in Gram-negative bacteria as well as in Gram-positive bacteria but involves a different set of dedicated proteins (16, 20). Moynihan and Clarke (16) have recently demonstrated that two different proteins are involved in this process in Gram-negative bacteria: 1) an integral membrane protein called PatA is dedicated to the transport of the acetyl-donor and 2) a periplasmic membrane-anchored protein called PatB catalyzes the acetylation reaction. In contrast, in Gram-positive bacteria, MurNAc O-acetylation can be catalyzed by a unique membrane-bound protein named OatA for O-acetyltransferase. OatA is composed of two domains, 11

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[5] The on-line version of this article (available at <http://www.jbc.org/>) contains supplemental Tables S1–S4, Figs. S1–S7, and additional references.

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⁶ The abbreviations used are: PG, peptidoglycan; MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine; PGH, peptidoglycan hydrolase(s); Oat, O-acetyltransferase; RP-HPLC, reverse-phase high performance liquid chromatography; MRS, de Man, Rogosa, and Sharpe.

transmembrane helices form the first domain at the N terminus and an extracellularly exposed globular domain, which contain the catalytic site at the C terminus of the protein (10). Importantly, a recent study showed the presence of both types of O-acetyltransferases involved in PG O-acetylation in *Bacillus anthracis* (22).

Despite an extensive study of the role of MurNAc O-acetylation as a pathogenic determinant, little is known about its basic function in cell wall assembly and degradation (10, 12–14). MurNAc O-acetylation is described as a means to increase PG resistance. In *Streptococcus pneumoniae* and *Enterococcus faecalis*, it provides resistance against general autolysis (13, 23). Emirian *et al.* (23) reported a modest impact of O-acetylation on the activities of the PGH AtlA and AtlB in *E. faecalis*. Crisostomo *et al.* (13) described MurNAc O-acetylation as conferring resistance to β -lactams in *S. pneumoniae*, probably through a more robust PG.

Here, we identify the presence of O-acetylation on GlcNAc residues of PG and describe the first bacterium containing two types of O-acetylation. These two modifications play opposing roles in the control of autolysis within *L. plantarum*. Strikingly, MurNAc O-acetylation increases *L. plantarum* autolysis via LytH, a putative L-alanine-muramyl amidase, whereas GlcNAc O-acetylation provides resistance against N-acetylglucosaminidase Acm2-mediated autolysis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in the present study are listed in supplemental Table S1. Plasmids were constructed in *Escherichia coli* MC1061. *E. coli* and *Bacillus subtilis* were grown in LB medium with shaking at 37 °C. *L. plantarum* was grown in MRS broth (Difco Laboratories, Inc., Detroit, MI) at 30 °C. When required, erythromycin (250 μ g/ml for *E. coli*, 5 μ g/ml for *L. plantarum*) or chloramphenicol (10 μ g/ml for *E. coli* and *L. plantarum*) was added to the medium. Solid agar plates were prepared by adding 2% (w/v) agar to the medium. For lysozyme plate assays, chicken egg white lysozyme powder (Sigma-Aldrich) was directly added at a final concentration of 2 mg/ml. Nisin A (Sigma-Aldrich) was routinely used at a concentration of 20 ng/ml for the induction of genes under the control of the *nisA* expression signals. Cephalothin, ampicillin, and methicillin were purchased from Sigma-Aldrich.

DNA Techniques and Electrotransformation—General molecular biology techniques were performed according to the instructions given by Sambrook *et al.* (24). Electrotransformation of *E. coli* was performed as described by Dower *et al.* (25). Electrocompetent *L. plantarum* cells were prepared as described previously (26). PCRs were performed with the Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA). The primers used in this study were purchased from Eurogentec (Seraing, Belgium) and are listed in supplemental Table S2.

Construction of Deletion Mutants—Construction of the *L. plantarum* gene deletion mutants for *oatA* (*lp_0856*) and *oatB* (*lp_0925*) was performed as described previously (27). A double crossover gene replacement strategy was used to replace

the target gene(s) by a chloramphenicol resistance cassette (*lox66-P₃₂cat-lox71*; (27)). Briefly, the upstream and downstream flanking regions of the target genes were amplified by PCR using *L. plantarum* WCFS1 chromosomal DNA. Subsequently, amplicons were respectively cloned in the *SmaI* (*oatA* and *oatB*) and *SmaI* (*oatA*) or *Ecl136II* (*oatB*) restriction sites of the suicide vector pNZ5319 (27). The mutagenesis plasmids were transformed in *L. plantarum* WCFS1 and colonies displaying a chloramphenicol-resistant and erythromycin-sensitive phenotype represent candidate double crossover gene replacements. The anticipated *cat* replacement genotype was confirmed by PCR using primers flanking the sites of recombination (supplemental Table S2). Subsequently, the *lox66-P₃₂cat-lox71* cassette was excised by temporal expression of the *cre* recombinase using the unstable *cre* expression plasmid pNZ5348 as described previously (27). Erythromycin-resistant and chloramphenicol-sensitive colonies were checked by PCR for Cre-mediated recombination and correct excision of the cassette by using primers flanking the recombination locus (supplemental Table S2). This strategy was applied for the construction of *oatA::lox72* (*OatA*[−], EB002) and *oatB::lox72* (*OatB*[−], EB003) deletion mutants. To obtain the double *oatA::lox72 oatB::P₃₂cat* (*OatAB*[−], EB004) the *oatA::lox72* mutant was used for a next round of mutagenesis targeting for the deletion of *oatB* following the same procedures as described above.

Cloning and Overexpression of *oatA*^{WT} and *oatA*^{D510A/S511A}—The *oatA* gene was amplified by PCR using the primer pair 5′*oatAPstI*-3′*OatASpeI*, generating a 1990-bp DNA fragment. The *PstI*/*SpeI*-restricted fragment was ligated to a *PstI*/*SpeI*-restricted pNZ8048 (28) and transformed in *E. coli*, leading to the pGIEB003 expression plasmid. To generate a catalytic mutant enzyme of *OatA* (*OatA*(D510A/S511A)), point mutations were introduced by PCR using the primer pair *OatAS511A* and *OatAD510A*, containing the S511A and D510A mutations respectively, with pGIEB003 as a template. The PCR product was phosphorylated and then ligated, leading to the pGIEB011 expression plasmid. The integrity of *oatA* and *oatA*^{D510A/S511A} were verified by DNA sequencing.

Purification and Structural Analysis of PG—PG from *L. plantarum* strains was prepared as described previously (29) with some modifications. DNase (50 μ g/ml) and RNase (50 μ g/ml) treatment were applied before hydrofluoric acid treatment. PG was digested with mutanolysin from *Streptomyces globisporus* (Sigma-Aldrich), and the resulting muropeptides were analyzed by RP-HPLC and MALDI-TOF mass spectrometry as reported previously (29). Alkaline treatments of muropeptide solutions were performed by increasing pH to pH 13 with 5 M NaOH for 2 h at 37 °C. For lysozyme digestions, PG was incubated with chicken egg white lysozyme (Sigma-Aldrich) at a final concentration of 2 mg/ml in 50 mM Tris-HCl buffer, pH 7.0, at 37 °C with gentle agitation during 16 h. For MSⁿ structural analysis, muropeptides were desalted on a Beta-sil C18 column (4.6 \times 250 mm, Thermo Electron Corp.) with an acetonitrile/formic acid buffer system and dried with a speed vacuum. Samples were solubilized in 2% acetonitrile, 0.1% formic acid in Milli-Q water (1 μ l for 1 mAU detected at 214 nm in the previous HPLC system). Each purified muropeptide was

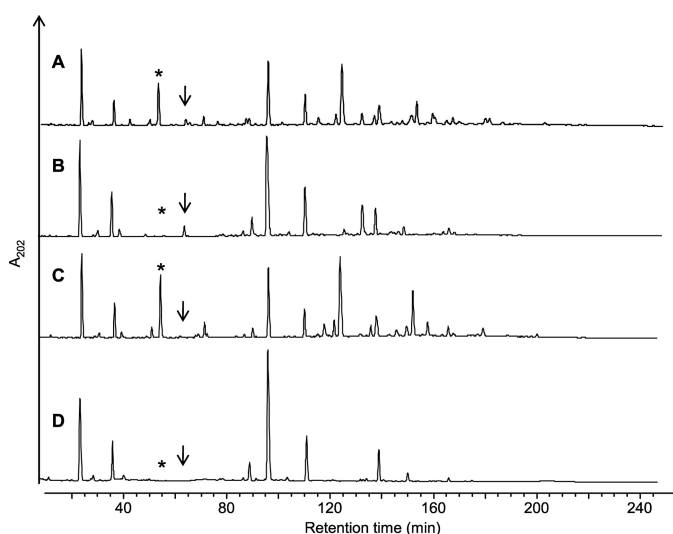


FIGURE 2. RP-HPLC separation of mucopeptides from *L. plantarum* PG. WT (A), *oatA* mutant (B), *oatB* mutant (C), and *oatA oatB* mutant (D). Peak 12 (supplemental Table S3), the disaccharide tripeptide with O-acetylated MurNAc, is indicated by an asterisk, and peak 14 (supplemental Table S3), the disaccharide tripeptide with O-acetylated GlcNAc, is indicated by an arrow.

metry (MS/MS) performed on these mucopeptides showed a different localization of the O-acetyl group in the molecules. As shown for peak 12 (Fig. 2A and supplemental Fig. S1), corresponding to the more abundant acetylated disaccharide tripeptide, the fragmentation pattern showed the presence of one GlcNAc (mass loss of 203.08 Da) and one O-acetylated MurNAc (mass loss of 319.13 Da) (Fig. 3A). In contrast, for peak 14 (Fig. 2A and supplemental Fig. S1), corresponding to a second acetylated disaccharide tripeptide, fragments resulting from the loss of one O-acetylated GlcNAc (245.10 Da) and one MurNAc (277.11 Da) were identified (Fig. 3B). All of the predicted O-acetylated mucopeptides were sensitive to alkaline pH (NaOH treatment) and lost their acetyl group due to their chemical properties (data not shown). Globally, O-acetylation of MurNAc and GlcNAc was found in ~39 and ~9% of monomers, respectively (supplemental Table S3). Disaccharide tripeptide with a double O-acetylation (peak 23, supplemental Fig. S1) as well as dimers with three O-acetylations could also be detected, showing that adjacent O-acetylations of MurNAc and GlcNAc are not mutually exclusive (supplemental Fig. S1 and Table S3). To our knowledge, *L. plantarum* is the first identified bacterium whose PG contains two types of O-acetylation on glycan strands and in particular with O-acetylation of GlcNAc that has never been reported previously.

O-Acetylation of MurNAc and GlcNAc Results from Activity of Two Dedicated O-Acetyltransferases—Identification of the O-acetyltransferase content of *L. plantarum* WCFS1 was performed using the BLASTP algorithm with the staphylococcal OatA protein (SAV2567) as the query. Two candidates encoded by *lp_0856* and *lp_0925* were found with amino acid similarity of 51 and 43%, respectively. The closer homologue was named OatA (*lp_0856*, 660 amino acids), and the most distal candidate was named OatB (*lp_0925*, 615 amino acids). These two O-acetyltransferases only displayed 21% identity to each other.

Phylogenetic analysis showed that these two O-acetyltransferases belong to two different protein clusters (supplemental Fig. S2).

OatA is closer to O-acetyltransferases present in lactobacilli and lactococci, whereas OatB seems to be related to streptococcal O-acetyltransferases. In this analysis, we also identified two other bacterial species (*Lactobacillus sakei* subsp. *sakei* 23K and *Weissella paramesenteroides* ATCC33313) that harbor two potential O-acetyltransferases belonging to the OatA and OatB subgroups as found in *L. plantarum*.

Protein sequence comparison revealed some common features between OatA and OatB. Both proteins display a similar organization with an N-terminal domain composed of 11 predicted transmembrane segments (HMMtop prediction, version 2.1), potentially involved in acyl donor transport, and a surface-exposed C-terminal region corresponding to the predicted acetyltransferase catalytic domain (Fig. 4A). Both domains exhibit sequence identity with PatA (7 and 9% for OatA and OatB, respectively) and PatB (17 and 20%), which were identified, respectively, as the acyl donor transporter and O-acetyltransferase in *Neisseria gonorrhoeae* (supplemental Fig. S3) (16). Both acetyltransferase domains of OatA and OatB were assigned to the family of SGNH/GDSL hydrolases (LOMETS prediction) as reported for PatB with a conserved Ser-Asp-His predicted catalytic triad (Fig. 4B and supplemental Fig. S4) (16).

To confirm the role of these two putative O-acetyltransferases, *oatA* and *oatB* were independently deleted and a double *oatA oatB* mutant was also constructed. PG analysis of the *oatA* mutant showed a complete disappearance of O-acetylated MurNAc containing mucopeptides and the persistence of a similar proportion of O-acetylated GlcNAc containing mucopeptides (Fig. 2B). Conversely, the deletion of *oatB* led to a complete absence of O-acetylated GlcNAc containing mucopeptides with the maintenance of O-acetylated MurNAc containing mucopeptides (Fig. 2C). The PG of the double mutant was totally depleted of both O-acetylated MurNAc and GlcNAc containing mucopeptides (Fig. 2D). A more detailed analysis of the mucopeptide content of the three mutants did not reveal any other major PG structure modification (data not shown). These results show that O-acetylation of MurNAc and GlcNAc in *L. plantarum* are separately performed by two dedicated O-acetyltransferases.

OatA Alone Confers Resistance to Lysozyme—The lysozyme resistance conferred by O-acetylation of MurNAc is well documented (10, 13, 14, 18), but nothing is known about O-acetylation of GlcNAc and its impact on lysozyme resistance. To assess their contribution to this function *in vivo*, plate assays with a fixed concentration of lysozyme (2 mg ml⁻¹) were performed with the various mutant strains (Fig. 5A, upper panel). The *oatA* mutant showed a higher sensitivity to lysozyme compared with the wild-type strain, whereas the deletion of *oatB* has no effect in both wild-type and OatA-deficient backgrounds. Complementation of the *oatA* mutant with *oatA*^{WT} on a multicopy plasmid restores lysozyme resistance (Fig. 5A, lower panel), whereas a catalytic mutant of OatA (OatA(D510A/S511A)) was not able to complement the lysozyme resistance phenotype (data not shown).

To confirm these *in vivo* observations, PG from wild-type, *oatA*, and *oatAB* mutants was digested by lysozymes, and the resulting products were analyzed by RP-HPLC (Fig. 5B). Wild-type PG was poorly digested by lysozyme, whereas PG depleted

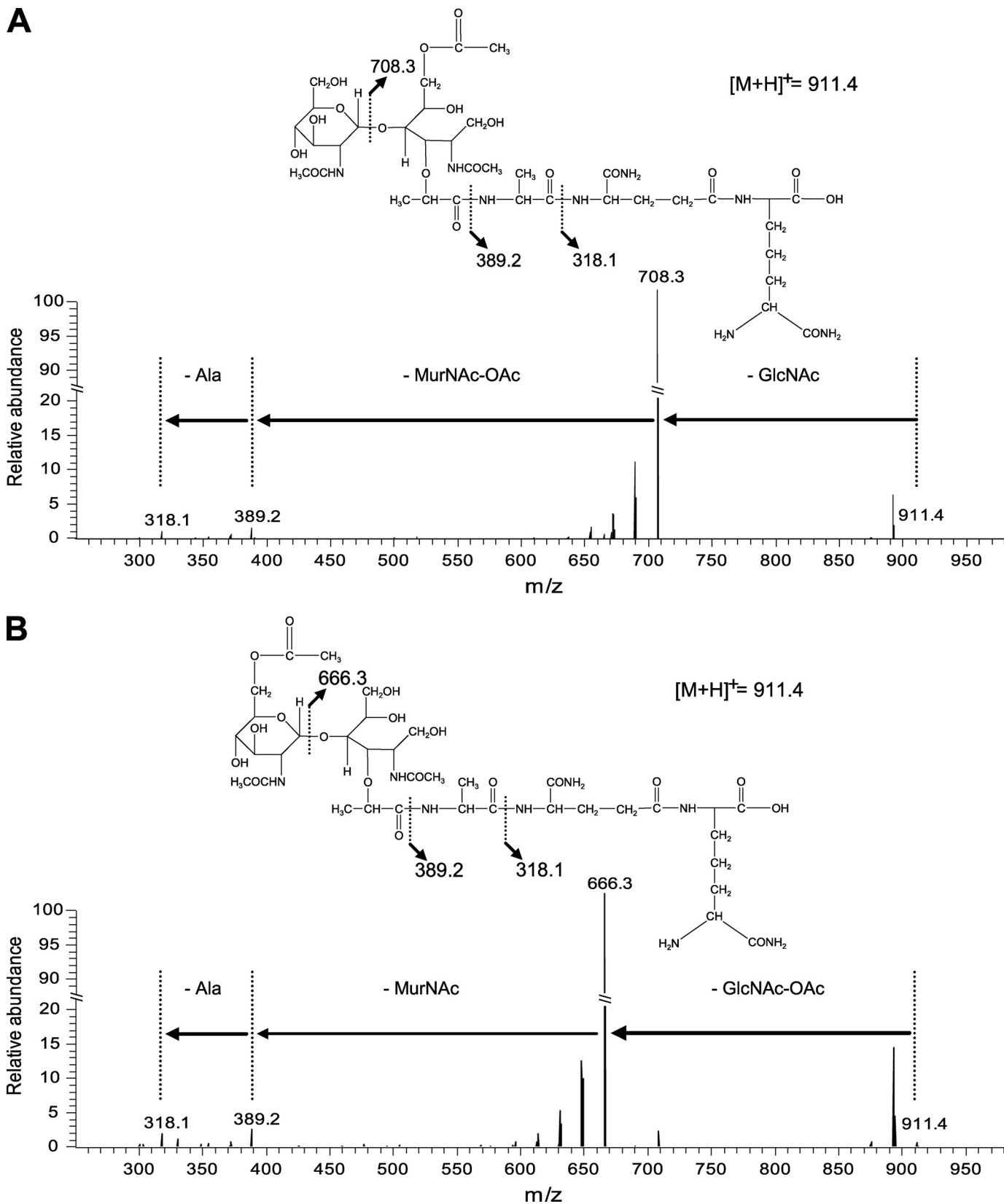


FIGURE 3. **Fragmentation of O-acetylated dissacharide tripeptides by MS-MS.** A, localization of the O-acetyl group on MurNAc. Peak 12 (supplemental Table S3) yielded fragments resulting from a mass loss of 203.08 Da (GlcNAc residue) and 319.13 Da (MurNAc-OAc residue). B, localization of the O-acetyl group on GlcNAc. Peak 14 (supplemental Table S3) yielded fragments resulting from a mass loss of 245.10 Da (GlcNAc-OAc residue) and 277.11 Da (MurNAc residue). Fragmentation was performed on the $[M + H]^+$ ion at m/z 911.4. The indicated m/z values correspond to ions obtained by cleavage of peptide bonds as represented on the chemical structures. The indicated masses correspond to $[M + H]^+$.

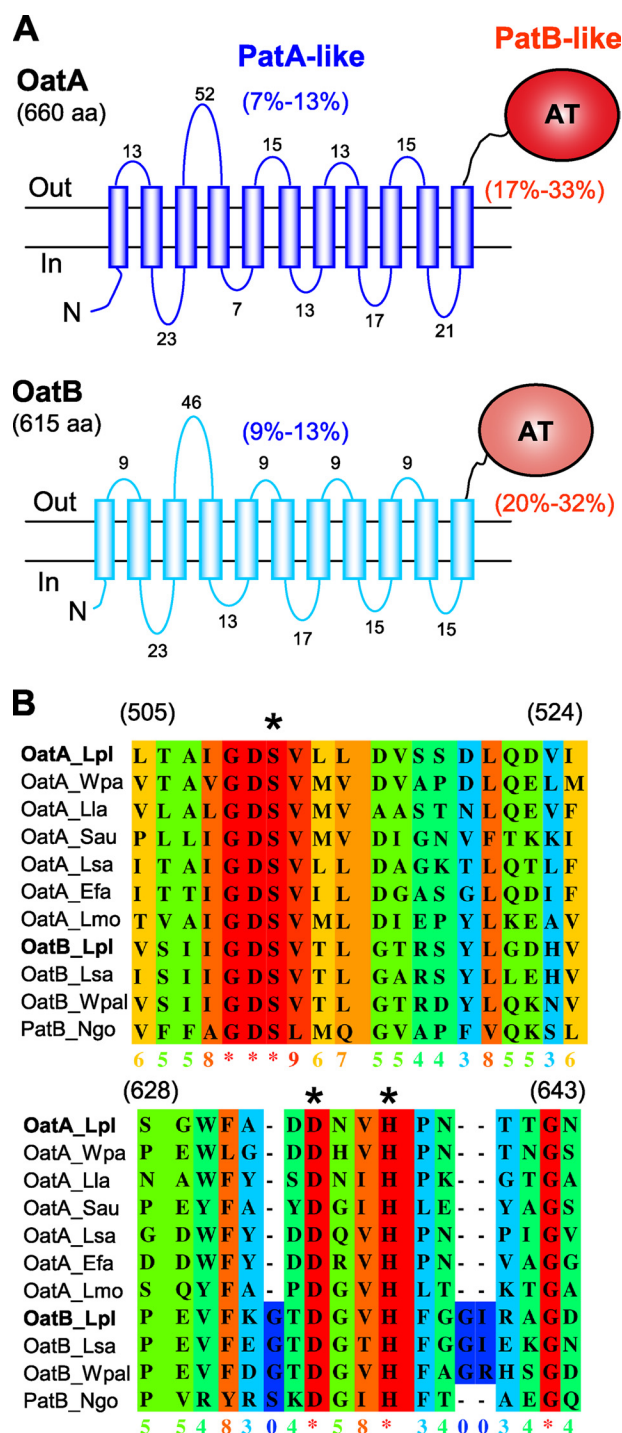


FIGURE 4. Comparison between OatA and OatB. A, schematic representation of the topology of OatA and OatB. The position and the orientation of transmembrane segments are drawn based on HMMTOP prediction. The numbers denote sizes (in amino acids) of the different loops. The putative C-terminal acetyltransferase domain (AT) is predicted as surface-exposed. Regions displaying identity and similarity (% in parentheses) with *N. gonorrhoeae* PatA and PatB are presented in blue and red, respectively. B, partial sequence alignment of the AT domain of OatA and OatB. Conservation based on the PRALINE algorithm is represented by a color code where dark blue and red represent the less and most conserved residues, respectively. Similarity scores are indicated below the alignment. Values in parentheses denote the residue number of OatA from *L. plantarum* used as reference, and black asterisks indicate the putative catalytic residues. Abbreviations (locus tag) are as follows: OatA_Lpl, *L. plantarum* (lp_0856); OatA_Wpa, *W. paramesenteroides* (HMPREF0877_0552); OatA_Lla, *L. lactis* (llmg_2391); OatA_Sau, *Staphylococcus aureus* (SAV2567); OatA_Lsa, *L. sakei* (LSA_1044); OatA_Efa, *E. faecalis*

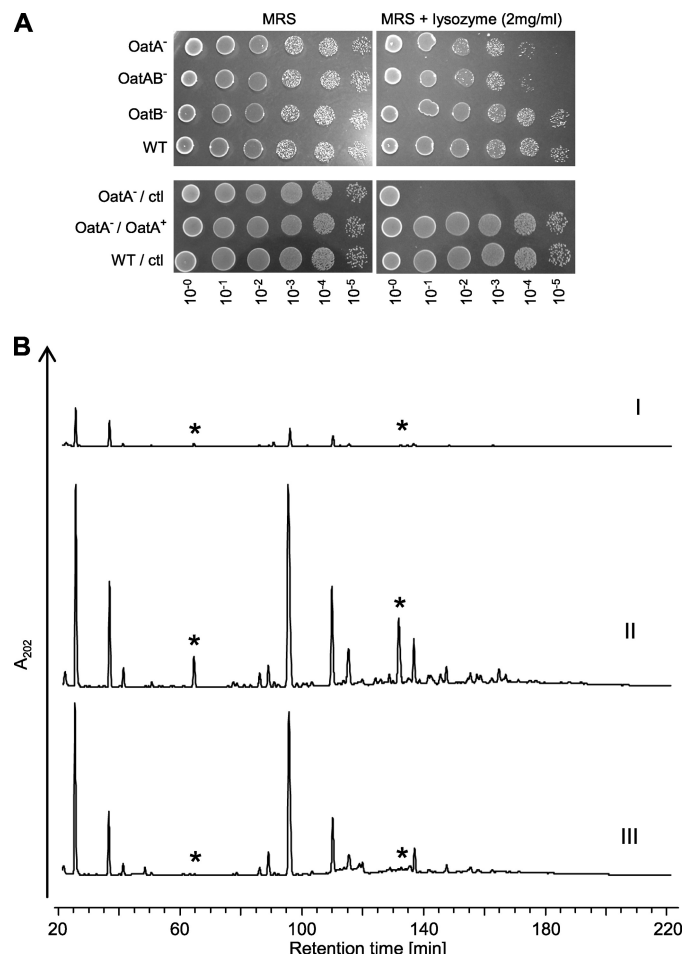


FIGURE 5. Involvement of MurNAc O-acetylation in lysozyme resistance. A, comparison of lysozyme resistance of O-acetyltransferase mutants by serial dilutions (10⁰ to 10⁻⁵) on MRS medium supplemented or not with lysozyme (2 mg/ml). For complementation experiments (bottom panels), chloramphenicol (10 μg/ml) and nisin (20 ng/ml) were added to MRS medium. OatA⁻, oatA mutant; OatB⁻, oatB mutant; OatAB⁻, oatA oatB mutant; OatA⁻/ctl, oatA mutant carrying the empty plasmid pNZ8048 (control); OatA⁻/OatA⁺, oatA mutant complemented with plasmid pGIEB003 (OatA^{WT}), WT/ctl, WT carrying the empty plasmid pNZ8048 (control). B, RP-HPLC separation of muropeptides resulting from PG digestion by lysozyme. I, WT; II, oatA mutant; III, oatB mutant. Asterisks indicate GlcNAc-OAc containing muropeptides (peaks 14 and 33, supplemental Table S3).

of O-acetylated MurNAc from the *oatA* mutant was well digested, confirming the established role of MurNAc O-acetylation in lysozyme resistance (Fig. 5B, I and II). Notably, the lysozyme was able to release O-acetylated GlcNAc containing muropeptides from the PG of the *oatA* mutant, which were absent in the digestion profile of the PG from the *oatAB* mutant (Fig. 5B, II and III). These data clearly show that the lysozyme (*N*-acetylmuramidase) is able to cleave the β-1-4-linkage next to an O-acetylated GlcNAc (Fig. 1) and that this O-acetylation is not involved in lysozyme resistance.

O-Acetylation of GlcNAc Inhibits Activity of Acm2 N-Acetylglucosaminidase—Control of autolysis by PG modifications is well established (13, 15, 17, 23, 36). O-acetylation of MurNAc in some species has been reported to confer resistance

(EF_0783); OatA_Lmo, *Listeria monocytogenes* (lmo1291); OatB_Lpl, *L. plantarum* (lp_0925); OatB_Lsa, *L. sakei* (LSA_0646); OatB_Wpa, *W. paramesenteroides* (HMPREF0877_1514); PatB_Ngo, *N. gonorrhoeae* (NGO0533).

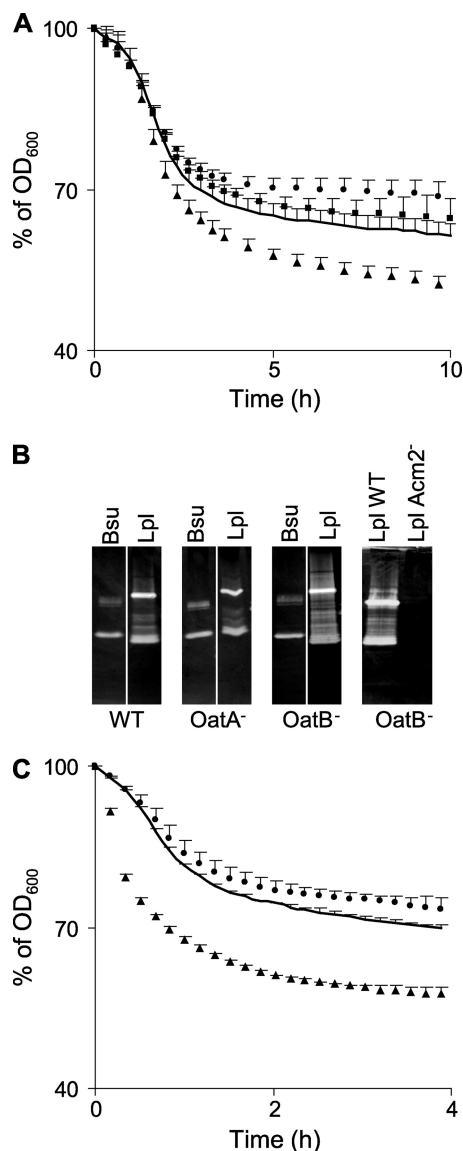


FIGURE 6. Effect of GlcNAc O-acetylation on autolysis and Acm2 activity. A, autolysis of *L. plantarum* and its mutant derivatives in presence of Triton X-100 (0.05%). Wild-type is represented by a line, *oatA* mutant by circles, *oatB* mutant by triangles, and *oatA oatB* mutant by squares. Mean values \pm S.D. of one representative experiment of three independent experiments ($n = 5$ for each). B, zymogram with cell extracts of *B. subtilis* (Bsu), *L. plantarum* wild-type (Lpl or Lpl WT), and *acm2* mutant (Lpl Acm2⁻) against dead cells of WT or derivative strains either lacking *OatA* (OatA⁻) or *OatB* (OatB⁻). C, Acm2 activity against autoclaved cells of *L. plantarum*. Mean values \pm S.D. of one representative experiment of two independent experiments ($n = 3$ for each). Symbols are as described in A.

to endogenous PGH (13, 17, 23). As *L. plantarum* PG contains two types of O-acetylation, the control of the PGH pool in this species could be unique compared with previous reports. To evaluate the impact of O-acetylation on PGH activity, Triton X-100-induced autolysis tests were performed with the different O-acetyltransferase mutants (Fig. 6A). Notably, the mutant strain depleted of O-acetylated GlcNAc (OatB⁻) was more sensitive to autolysis compared with the wild-type, whereas the mutant strain without O-acetylated MurNAc (OatA⁻) was more resistant. This opposite behavior was confirmed by the autolytic profile of the double mutant strain (OatA⁻B⁻), which is similar to the wild-type, revealing a compensatory effect.

To confirm these data, the PGH activity content of *L. plantarum* NZ7100 was evaluated by zymogram analysis using wild-type, OatA⁻, and OatB⁻ autoclaved cells as substrates (Fig. 6B). *B. subtilis* 168 cell extracts were used as control because the PG of this species is very similar to *L. plantarum* (meso-diaminopimelic acid direct linkage) and only contains O-acetylated MurNAc (22). The same amount of crude cell extracts of the two species was tested on the different substrates. The PGH activity profile of *B. subtilis* was globally similar on the different substrates. In contrast, the PGH activity was strongly increased for *L. plantarum* using OatB⁻ dead cells compared with wild-type and OatA⁻ substrates, confirming that O-acetylated GlcNAc inhibits one or more PGH in this species. We have recently shown that the N-acetylglucosaminidase Acm2 is the major autolysin of *L. plantarum* WCFS1.⁷ To evaluate its contribution to the autolysin profile observed on zymogram with the OatB⁻ substrate, the same amount of crude cell extracts from the wild-type and an *acm2*-deleted mutant were compared (Fig. 6B). All of the autolytic activity bands detected on the OatB⁻ substrate are attributed to Acm2 and its various processed forms. To definitively prove that Acm2 activity is modulated by O-acetylation of GlcNAc, purified His₆-tagged Acm2 was incubated with wild-type, OatA⁻, and OatB⁻ dead cells. As shown in Fig. 6C, Acm2 activity was similar on wild-type and OatA⁻ substrates but strongly increased with the OatB⁻ substrate deprived of O-acetylated GlcNAc, whereas the activity of mutanolysin, an N-acetylmuramidase insensitive to O-acetylation used as control, was not affected (supplemental Fig. S5). Altogether, these data show an inhibitory effect of O-acetylated GlcNAc on autolysis of *L. plantarum*, which is mediated by an inhibition of the N-acetylglucosaminidase Acm2 (Fig. 1).

O-Acetylation of MurNAc Activates LytH N-Acetylmuramoyl-L-alanine Amidase—Another strategy to evaluate the contribution of PG O-acetylation on PGH activity is to increase the O-acetylation level by O-acetyltransferase overproduction. Overproduction of OatA^{WT} and OatA(D510A/S511A) were achieved by the use of the nisin-inducible controlled expression system. Unfortunately, we were unable to clone *oatB* under the control of the nisin-inducible controlled expression system in various hosts except a truncated version deprived of the acetyltransferase domain, suggesting a toxic effect of GlcNAc O-acetylation. Concerning overproduction of OatA^{WT}, the growth rate slightly decreased in a nisin dose-dependent manner both in wild-type and OatA⁻ backgrounds, whereas overproduction of the catalytic site mutant OatA(D510A/S511A) had no impact on growth. Together, these data confirm the role of O-acetylation rather than protein overproduction in this slower growth (supplemental Fig. S6, A and B). This slight effect on growth is in sharp contrast with the major growth defect due to OatA overproduction previously reported in *Lactococcus lactis* (18). Concerning the impact on MurNAc O-acetylation, we showed that overproduction of OatA^{WT} leads to an increase of ~6% of O-acetylated MurNAc containing mucopeptides (supplemental Table S4) compared with the control strain containing the empty plasmid.

⁷ T. Rolain, E. Bernard, P. Courtin, M. P. Chapot-Chartier, and P. Hols, manuscript in preparation.

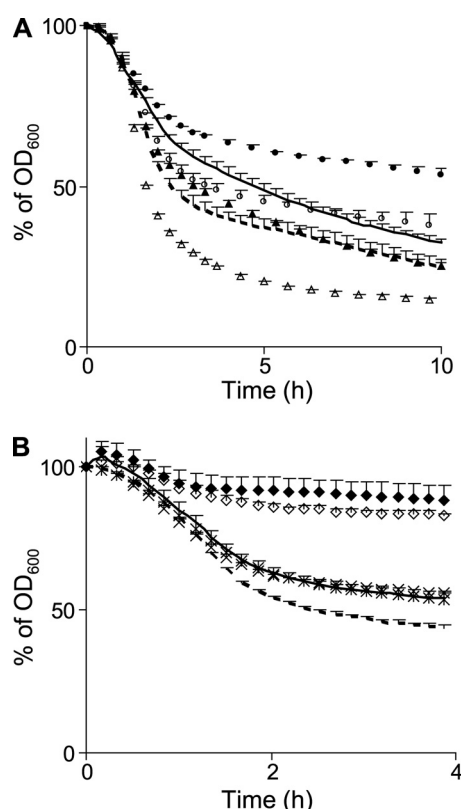


FIGURE 7. Effect of MurNAc over-O-acetylation on autolysis. A, autolysis curves (Triton X-100-induced) of *oatA*^{WT} overexpressing strains *L. plantarum* WT (dashed line), *oatA* mutant (open circles), and *oatB* mutant (open triangles) compared with control strains (empty vector) of WT (line), *oatA* mutant (filled circles), and *oatB* mutant (filled triangles). Mean values \pm S.D. ($n = 3$). B, autolysis curves of *oatA*^{WT} overexpressing strains: WT (dashed line), *acm2* mutant (open diamonds), and *lytH* mutant (asterisks) compared to control strains of WT (line), *acm2* mutant (filled diamonds), and *lytH* mutant (crosses). WT (line) and *lytH* mutant (asterisks and crosses) overlap. Mean values \pm S.D. of one representative experiment of two independent experiments ($n = 6$ for each).

To evaluate the contribution of over-O-acetylation on PGH activity, Triton X-100-induced autolysis tests were performed with wild-type and the O-acetyltransferase mutants (*OatA*[−] and *OatB*[−]) overproducing *OatA*^{WT}. An increased autolysis was observed in each overproducing strain (Fig. 7A). In contrast, overproduction of *OatA*^{D510A/S511A} did not increase autolysis confirming a direct role of over-O-acetylation rather than an indirect effect due to overproduction (data not shown). To identify which autolysin is activated by an increase of O-acetylated MurNAc, *OatA*^{WT} overproduction was achieved in a collection of eight different PGH mutants of *L. plantarum* (four predicted N-acetylmuramidases/N-acetylglucosaminidases, *Acm1*, *Acm2*, *Lys*, and *Lp_3093*; one N-acetylmuramoyl-L-alanine amidase, *LytH*; two endopeptidases, *Lp_2162* and *Lp_1242*; and the lytic transglycosylase *Lp_0302*) (37). Triton X-100-induced autolysis tests revealed that over-O-acetylation was able to increase autolysis of all strains (illustrated for the *acm2* mutant in Fig. 7B), except for the *lytH* mutant where the increased autolysis phenotype was completely abolished (Fig. 7B).

DISCUSSION

L. plantarum is the first bacterium described to harbor two different types of O-acetylation in its cell wall PG. MurNAc-

specific O-acetyltransferases are widespread among Gram-positive bacteria as well as in a range of Gram-negative bacteria (12, 20), whereas GlcNAc-specific O-acetyltransferases (*OatB*), described for the first time in this study, seem to be restricted to a smaller number of bacterial species. In Gram-positive bacteria, *OatA* proteins are derived from at least three different ancestors (supplemental Fig. S2), one of which led to two different types of O-acetyltransferases: the MurNAc-specific O-acetyltransferases mainly present in streptococci and the GlcNAc-specific O-acetyltransferases (*OatB* subgroup, supplemental Fig. S2). As the phylogeny of GlcNAc O-acetyltransferases does not reflect the phylogeny of the bacteria included in this protein cluster, we hypothesized that the *oatB* gene was acquired by horizontal gene transfer. The GC content of the *oatB* gene of *L. plantarum* (49%) is higher than that of the whole genome and of the *oatA* gene (both 44%) (37), which would be in favor of such an event. The origin of *OatB* remains unclear because the GlcNAc O-acetyltransferase could derive from a MurNAc O-acetyltransferase ancestor or the opposite.

Sequence comparison between *OatA* and *OatB* proteins exhibited a similar global organization with two predicted domains. The N-terminal transmembrane domain, composed of 11 transmembrane segments, could be involved in the transport of acetyl-CoA, as previously proposed to be the acetyl donor (10). Alignment of this domain between *OatA* and *OatB* members showed a higher similarity in five transmembrane segments (TM1, -2, -4, -6, and -9), including a range of well conserved aromatic residues (Phe, Tyr, Trp) and the conserved region Fxx(HR)RxxR (91–98 amino acids in *OatA*_{LP}) in the intracellular loop L2 (23 amino acids) (supplemental Fig. S3A). However, there is no clear clue to assign any of these conservations regarding to substrate specificity. The globular C-terminal domain, predicted to be surface exposed and assigned to the family of SGNH/GDSL hydrolases, displays a similar conservation level between *OatA*, *OatB*, and *PatB* (32–33% of similarity), the latter being reported to display a MurNAc-specific O-acetyltransferase activity (16). The two most conserved regions GDSV (509–512 amino acids in *OatA*_{LP}) and DxxH (634–637 amino acids in *OatA*_{LP}) are supposed to contain the predicted Ser-Asp-His catalytic triad (underlined amino acids) (supplemental Fig. S3B), for which we showed the importance of Asp-510 and Ser-511 as their concomitant substitution by alanine residues leads to *OatA* inactivation. The use of various modeling tools shows that the residues of the catalytic triad are indeed close to each other in *OatA*, *OatB*, and *PatB* (supplemental Fig. S4) but does not allow to define residues or structural elements involved in the recognition of MurNAc or GlcNAc as substrates.

MurNAc O-acetylation is seen as a mean for the bacteria to increase their PG resistance against lysozyme, autolysins, β -lactams, or as a step leading to the entry in a dormant state (10, 12–14, 17, 18, 23). Indeed, *L. plantarum* MurNAc O-acetylation also confers resistance to lysozyme and β -lactams (supplemental Fig. S7). However, in contrast to previous reports, *L. plantarum* MurNAc O-acetylation does not enhance the PG resistance against its own PGH, but instead induces autolysis through the *LytH* enzyme, a putative N-acetylmuramoyl-L-alanine amidase. This was observed by the over-O-acetylation of

MurNAc and corroborates the observation of a slight decrease in autolysis observed with the *oatA* mutant compared with the wild-type. Future biochemical characterization of this amidase will be needed to ascertain the effect of the O-acetyl group of MurNAc on its activity.

L. plantarum is the first species identified containing GlcNAc O-acetylation in its PG. Notably, we show that the major autolysin, N-acetylglucosaminidase Acm2, displays a higher autolytic activity on PG deprived of GlcNAc O-acetylation. The underlying mechanism could very well be a lack of access to cleavage of the β -1-4 bond between GlcNAc and MurNAc due to the presence of the acetyl group on GlcNAc as demonstrated for acetylated MurNAc regarding to lysozyme activity (Fig. 1) (10). Remarkably, GlcNAc O-acetylation in *L. plantarum* plays a similar role as MurNAc O-acetylation in other bacteria regarding inhibition of autolysins. This independent evolution toward a similar functional role of both types of O-acetylation highlights their importance for the control, probably in a localization-dependent manner, of the activity of autolysins without directly altering their intrinsic activity. Hypothetically, the regulation of autolysis by O-acetylated GlcNAc in *L. plantarum* was horizontally acquired; therefore, we propose a divergent evolutionary path for this species. Initially, the role of MurNAc O-acetylation was probably similar to other bacteria; however, the acquisition and maintenance of the second O-acetylation has led to an adaptation of synthesis/maturation PG machineries. This resulted in the co-evolution of GlcNAc O-acetylation and its dedicated PGH (Acm2). This is a novel example of the plasticity of PG synthesis/degradation machineries similar to what we have reported before regarding to D-lactate incorporation in the PG of this species (4–6).

Therefore, *L. plantarum* represents a unique model for studying the functional role of PG O-acetylation because it contains both MurNAc and GlcNAc O-acetylation, catalyzed by two dedicated proteins with a similar global organization, and both members of the Oat family. Furthermore, we show that these two modifications could independently modulate PG degradation, either by triggering the N-acetylmuramoyl-L-alanine amidase LytH for MurNAc O-acetylation or by inhibiting the N-acetylglucosaminidase Acm2 through GlcNAc O-acetylation. This affords *L. plantarum* the capacity to control PG degradation and/or remodel by modulating the abundance and localization of either O-acetylation on glycan strands. Future biochemical studies on the relationship between these modifications and their associated PGH will provide new insight into the control of cell wall assembly and degradation.

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**Characterization of *O*-Acetylation of *N*-Acetylglucosamine: A NOVEL
STRUCTURAL VARIATION OF BACTERIAL PEPTIDOGLYCAN**
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