SpxB Regulates O-Acetylation-dependent Resistance of Lactococcus lactis Peptidoglycan to Hydrolysis

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Endogenous peptidoglycan (PG)-hydrolizing enzymes, the autolysins, are needed to relax the rigid PG sacculus to allow bacterial cell growth and separation. PGs of pathogens and commensal bacteria may also be degraded by hydrolyses of animal origin (lysozymes), which act as antimicrobials. The genetic mechanisms regulating PG resistance to hydrolytic degradation were dissected in the Gram-positive bacterium Lactococcus lactis. We found that the ability of L. lactis to counteract PG hydrolysis depends on the degree of acetylation. Overexpression of PG O-acetylase (encoded by oatA) led to bacterial growth arrest, indicating the potential lethality of oatA and a need for its tight regulation. A novel regulatory factor, SpxB (previously denoted as YneH), exerted a positive effect on oatA expression. Our results indicate that SpxB binding to RNA polymerase constitutes a previously missing link in the multistep response to cell envelope stress, provoked by PG hydrolysis with lysozyme. We suggest that the two-component system CesSR responds to this stress by inducing SpxB, thus favoring its interactions with RNA polymerase. Induction of PG O-acetylation by this cascade renders it resistant to hydrolysis.

Peptidoglycan (PG)7 is the major and essential component of the bacterial cell envelope, the main function of which is to preserve cell integrity by withstanding internal osmotic pressure (1, 2). It is also responsible for cell shape, participates in cell division, and serves as support for attachment of other cell wall molecules such as teichoic acids, proteins, and exopolysaccharides (3). The bacterial PG is a giant multilayer polymer that envelops the cell as a rigid sacculus. It is composed of N-acetylglucosamine-N-acetylmuramic acid disaccharide pentapeptide blocks that are synthesized intracellularly and transported through the cytoplasmic membrane as lipid-disaccharide pentapeptides. These blocks are covalently linked to the pre-existing PG polymers by high molecular weight penicillin-binding proteins (4).

To allow cell division and surface expansion, the rigid PG sacculus has to be relaxed. This is achieved by PG ruptures, which could be introduced in several ways. First, not all possible covalent bonds are formed during PG synthesis; for example, only 36% of possible PG cross-links between stem peptides are formed in Lactococcus lactis (5). Bacteria also possess a number of endogenous bacterial enzymes (collectively called autolysins) that disrupt PG and can result in cell lysis. According to their hydrolytic bond specificity and products, autolysins are classified as muramidases, lytic transglycosylases, glucosaminidases, amidases, and peptidases (6). Bacteria often possess several autolysins, e.g. L. lactis encodes a main autolysin (N-acetylglucosaminidase AcmA) (7, 8) and four minor PG hydrolases (9, 10) as well as prophase-encoded bacteriolytic enzymes (11).

In the human or animal host, antimicrobial PG lytic enzymes such as lysozyme constitute a first line of defense against infection. Bactericidal properties of lysozyme are attributed to its N-acetylmuramidase activity. Human body fluids such as tears, saliva, and milk contain 2.6, 0.13, and 0.2 mg/ml lysozyme, respectively (12).

As the name suggests, autolysins have potentially suicidal activities and must be exquisitely well regulated. Regulation of PG hydrolysis is also important with regard to the proposed role of PG hydrolases in resuscitation from the dormant state (13, 14). Numerous studies indicate that PG resistance to hydrolysis is effectuated via acetylation. PG acetylation occurs at N-2 of N-acetylglucosamine and N-acetylmuramyl residues.
(N-acetylation) or at the C-6 hydroxyl group of N-acetylmuramyl residues (O-acetylation) (15). N-Acetylation proceeds during precursor synthesis prior to transport across the cytoplasmic membrane and incorporation into PG. However, >80% of the glucosamine and 10% of the muramic acid residues are not acetylated in Streptococcus pneumoniae PG, suggesting the presence of deacetylation activities. This was confirmed, as a pgdA gene was identified as encoding the PG N-acetylmuraminyl deacetylase; the pgdA mutant produced fully N-acetylated PG and became hypersensitive to lysozyme (16). PG N-acetylmuraminyl deacetylase was also described in Bacillus cereus (17). Bacillus subtilis was shown to possess the PG N-acetylmuraminyl acetylase PdaA (18).

In contrast to N-acetylation, O-acetylation occurs after nascent PG strands are attached to the cell wall. The gene oatA (encoding PG O-acetyltransferase) was identified in Staphylococcus aureus; an oatA mutant showed increased sensitivity to lysozyme (19). The analogous mutant was later isolated in S. pneumoniae and named adr for its attenuation of the drug (penicillin) resistance phenotype (20). The concerted action of PG O-acetyltransferases and O-acetylpentaglycosamine esterases was proposed to participate in the control of PG degradation (21), although the genetic mechanisms of such regulation remain unknown.

In this study, we developed an in vivo screen for functions affecting PG modifications, which allowed us to identify a homolog of the staphylococcal oatA gene, encoding an O-acetylase in the non-pathogenic Gram-positive food bacterium L. lactis. Control of O-acetylation was found to occur via a newly identified regulator, SpxB (previously designated as YneH). Our results indicate that SpxB is induced by the two-component regulatory system CesSR, which reacts to cell envelope stress.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions and Bacterial Strains**—The bacterial strains and plasmids used in this study are listed in Table 1. L. lactis was grown at 30 °C in M17 medium (BD Biosciences, Le Pont-de-Clai, France) supplemented with 0.5% glucose (GM17 medium). Erythromycin (2.5 μg/ml), tetracycline (2.5 μg/ml), and chloramphenicol (5 μg/ml; all from Sigma, Saint-Quentin, France) were added as needed. Nisin was prepared in Me2SO (Sigma) and added at a final concentration of 2 ng/ml. For growth curve measurements, overnight cultures grown in LB medium without antibiotic, distributed in Serowell microtiter plates (200 μl/well; Barloworld Scientific Ltd., Stone, UK), and incubated at 30 °C. Growth was followed by spectrophotometric absorbance measurements at 600 nm (Model EL808 spectrophotometer, BioTek Instruments, Inc., Saint-Quentin-en-Yvelines, France). Escherichia coli was grown in LB medium (BD Biosciences) at 37 °C in the presence of 50 μg/ml ampicillin or 10 μg/ml chloramphenicol as needed.

**Viability and Lysozyme Resistance Tests**—Bacterial viability was determined using a ViaGram Red™ Gram stain and viability kit (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations. For the lysozyme resistance test, a 10-fold concentrated hen egg white lysozyme solution (Fluka, Buchs, Switzerland) was freshly prepared in GM17 medium and then diluted 10-fold in melted GM17 agar (1.5%) at 45 °C. Overnight bacterial cultures were successively diluted 10-fold, and 5 μl of each dilution was spotted on GM17 agar plates supplemented with different concentrations of lysozyme.

**Preparation of L. lactis Genomic DNA Libraries**—Three genomic libraries were constructed after chromosomal DNA digestions with HindIII, NspI, or TaqI restriction endonuclease (New England Biolabs, Beverly, MA). Digested genomic DNA fragments were inserted into the recipient vector pVE3916, a pNZ8020 derivative that replicates in both E. coli and Gram-positive bacteria and that has a copy number of ~30 in L. lactis. For this purpose, genomic DNA was totally digested with NspI or HindIII or partially digested with TaqI for 5 min at 37 °C and ligated with linearized HindIII, NspI, or Clal and dephosphorylated pVE3916. T4 DNA ligase and calf intestine alkaline phosphatase (Fermentas, Vilnius, Lithuania) were used according to the manufacturers’ recommendations. Ligation mixtures were used to transform E. coli TG1, and transformants were selected on LB agar supplemented with 10 μg/ml chloramphenicol. Colony PCR was performed on transformants to estimate genomic DNA insertion frequency. For the HindIII and TaqI libraries, we used primers BankS (5’-TGAGATAATGGCGACTGTA-3’) and BankAS (5’-CATGCTGAAGAGCATACT-3’). For the NspI library, we used primers BankS and BankAS2 (5’-ACGCTCAAGGGCTTTTACG-3’). Insertion frequencies were estimated to be 50, 88, and 67% for the HindIII, NspI, and TaqI libraries, respectively, whereas average insert sizes were estimated to be 1.3, 1.6, and 1.7 kilobase pairs, respectively. Approximately 13,000, 16,200, and 12,000 clones for the HindIII, NspI, and TaqI libraries, respectively, were harvested from plates in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 25% sucrose. Plasmid extraction from each library was performed using a Midi-Prep kit (Qiagen GmbH, Hilden, Germany).

**Selection of Plasmids Carrying L. lactis Lysozyme Resistance Determinants**—L. lactis MG1363 was transformed with each genomic library and plated on GM17 agar supplemented with 3 mg/ml lysozyme (Fluka) and 5 μg/ml chloramphenicol. We selected 32, 32, and 14 lysozyme-resistant clones resulting from transformation with the NspI, HindIII, and TaqI libraries, respectively. For each clone, the genomic DNA insert was verified by colony PCR using primers BankS and BankAS2. 22 and 26 of the 32 tested clones from the HindIII and NspI libraries were shown to have the same insert size (~1870 and 3138 bp, respectively). Plasmid DNA corresponding to one clone of each library was sequenced using primers BankS and BankAS2. Reactions for DNA sequence determination were performed according to the protocol of the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) using a MegaBACE 1000 automated capillary sequencer (Amersham Biosciences, Orsay, France). The repeated 1870-bp insert from the NspI library contained the xynD gene, and the repeated 3138-bp insert from the HindIII library contained the pabB and spxB yneG genes. The plasmids carrying these inserts were named

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8 T. Rochat and P. Langella, unpublished data.
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pVES3787 and pVES3801, respectively, and retained for further work. Other isolated lysozyme-resistant clones were confirmed to carry synD and spxB yneG inserts by colony PCR using BankS and BankA52 as vector-specific primers and a specific primer for the synD or spxB gene.

To obtain in-frame deletions of the spxB, yneG, and pabB genes in plasmid pVES3801, we used an inverse PCR strategy. For deletion of pabB, the fragment of pVES3801 was PCR-amplified using primers YGH1-Xmal (5'-AAAAACCCGGGCCTTGGATGGTTCGG-3'; restriction sites present on primers are underlined) and YGH2-Xmal (5'-AAATCCGGGTATACTTGTGTTTGGG-3') and the Phusion high fidelity DNA polymerase (Finnzymes, Espoo, Finland). The PCR fragment was digested with Xmal, self-ligated using T4 DNA ligase, and transformed into L. lactis MG1363. Plasmid pVES3902 deleted for pabB was obtained from a chloramphenicol-resistant clone. The same strategy was used to delete the spxB or yneG gene from pVES3902. We used primers iYH1-BamHI (5'-AAAAAGATTCCTTGGATGGGTTTCCG-3') and iYH2 (5'-AAAAAAGATCCGCCGGTTTCCGTGTTTGGG-3') to inactivate spxB and primers iYG1-BamHI (5'-AAAAAGATCCGCCGGTTTCCGTGTTTGGG-3') and iYG2-BamHI (5'-AAAAAAGATCCGCCGGTTTCCGTGTTTGGG-3') to inactivate yneG. The PCR fragments obtained were digested with BamHI, self-ligated, and transformed into MG1363, giving rise to plasmids pVES3908 (spxB- yneG+) and pVES3910 (spxB+ yneG+), respectively.

Chromosomal Gene Inactivation— For inactivation of the mtlA, pepN, oatA, and synD genes in the L. lactis MG1363 chromosome, we used a strategy based on gene disruption by single cross-over plasmid insertion. For this, internal fragments of each gene were PCR-amplified from L. lactis MG1363 chromosomal DNA using the following primer pairs: mtlA10-EcoRI (5'-ATGATGGAATTCGACCCGGCCCTTGGATGCCTC-3') and mtlA11-Xmal (5'-ATGATGGAATTCGACCCGGCCTTGGATGCCTC-3') to inactivate mtlA and primers pabB1-EcoRI (5'-ATGATGGAATTCGACCCGGCCCTTGGATGCCTC-3') and pabB2-Xmal (5'-ATGATGGAATTCGACCCGGCCCTTGGATGCCTC-3') to inactivate pabB, respectively. The PCR fragments obtained were digested with EcoRI and Xmal, self-ligated, and transformed into MG1363, giving rise to plasmids pVES4260 (mtlA- yneG+ spxB- pabB+) and pVES4533 (mtlA- yneG+ spxB- pabB+). The resulting plasmid pVES4196 was transformed into an MG1363 derivative carrying pVE6007, a thermosensitive plasmid encoding a chloramphenicol resistance determinant, selecting for erythromycin (2.5 μg/ml) and chloramphenicol (2.5 μg/ml). Integration of plasmid pVES4196 in the resulting strain was obtained after overnight growth in GM17 liquid medium supplemented with erythromycin at 37 °C, a non-permissive temperature for pVE6007 replication. The culture was then plated on GM17 agar with erythromycin, and four independent chloramphenicol-resistant clones were isolated and grown on GM17 medium without antibiotics for at least 100 generations. Strain VES4284 (∆spxB) was then selected as a white colony on GM17 agar supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Euromedex, Souffelweyersheim, France) and verified by PCR. For construction of the MG1363ΔtrmA strain, primer pair F1-forward (5'-GGAGATCCGCTTGGATGCCTC-3') and F1-reverse (5'-GGAGATCCGCTTGGATGCCTC-3') was used to amplify the 720-bp fragment just upstream of the trmA start codon (called FN). Primer pair F2-forward (5'-GGAGATCCGCTTGGATGCCTC-3') and F2-reverse (5'-GGAGATCCGCTTGGATGCCTC-3') was used to amplify a 535-bp fragment including the end of trmA and the downstream region (called FC). The PCR fragments were digested with EcoRI, BamHI, and Xmal, respectively, and ligated to EcoRI-Xmal-digested plasmid pBluescript-KS+ (Stratagene, Amsterdam, The Netherlands). The resulting plasmid containing the ligated FN-FC fragment was denoted pΔtrmA. Subsequently, a 1.1-kb EcoRI fragment containing the erythromycin resistance gene was cloned into pΔtrmA, and the resulting plasmid was transformed into MG1363. One transformant resulting from plasmid integration via FC was selected and verified by PCR. Inactivation of trmA increases the heat tolerance of MG1363 (25). Therefore, to enrich for bacteria carrying a chromosomal deletion of trmA as a result of plasmid excision (via FN), bacteria were grown for 3 h at 40 °C, serially diluted, and spread on GM17 plates that were incubated overnight at 38 °C. This procedure resulted in a high proportion of erythromycin-sensitive colonies that all carried the chromosomal...
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The former gene names of cesR, spxB, oatA, and pgdA were tldD, yneH, ynhB, and yneD, respectively. SCO, single cross-over.

### TABLE 1

| Strains and plasmids used in this study and their relevant genetic properties |
|-----------------------------------------------|
| The former gene names of cesR, spxB, oatA, and pgdA were tldD, yneH, ynhB, and yneD, respectively. SCO, single cross-over. |

| Relevant genotype | Ref./source |
|-------------------|-------------|
| L. lactis         | Plasmid-free strain |
| MG1363            | MG1363 poniA |
| NZ9000            | MG1363 pepN-nisB |
| DF4clpP           | MG1363 clpP |
| HI3605            | MG1363 clpP trmA |
| MG1363°traMA      | MG1363 trmA deletion mutant This work |
| VES1842           | MG1363 ponA |
| VES4284           | MG1363 spxB in-frame deletion mutant This work |
| VES3757           | MG1363 carrying pVES787 (pgdA) This work |
| VES3801           | MG1363 carrying pVES801 (yneG+ spxB+ pabB+) This work |
| VES3902           | MG1363 carrying pVES3902 (spxB+ yneG+) This work |
| VES3910           | MG1363 carrying pVES3910 (spxB+) This work |
| VES4433           | VES1842 carrying pVES3910 (spxB+) This work |
| VES3908           | MG1363 carrying pVES908 (yneG+) This work |
| VES4299           | mtlA mutant obtained by pVES4258 SCO insertion in MG1363 This work |
| VES4534           | pglA mutant obtained by pVES4533 SCO insertion in MG1363 This work |
| VES4289           | oatA mutant obtained by pVES4260 SCO insertion in MG1363 This work |
| VES4372           | pglP mutant obtained by pVES4341 SCO insertion in MG1363 This work |
| VES3915           | cesR mutant obtained by pRV30002D SCO insertion in MG1363 This work |
| VES4075           | MG1363 derivative carrying pVE916 |
| VES4379           | VES4372 (pepN) carrying pVES910 (spxB+) |
| VES4382           | VES46534 (pgdA) carrying pVES910 (spxB+) |
| VES4320           | VES4289 (oatA) carrying pVES910 (spxB+) |
| VES4317           | VES4299 (mtlA) carrying pVES910 (spxB+) |
| VES4476           | VES5508 (cesR) carrying pVES910 (spxB+) |
| VES5556           | MG1363°traMA derivative carrying pVE916 |
| VES5566           | MG1363°traMA carrying pVES3910 (spxB+) |
| VES5558           | MG1363°traMA mutant obtained by pVES4258 SCO insertion in MG1363 |
| VES5573           | VES4375 (pepN) carrying pVES5540 |
| VES5577           | TE050 carrying pVES910 (spxB+) |

| E. coli          | |
|------------------|-------------|
| JIM4646          | PG1 with chromosomal copy of repA gene |
| HB101            | F'-mcrB mrr lsd20 recA13 leuB6 ara-14 proA2 lacI1 galK2 xyl-5 mtl-l rpsL20 glnV44 |
| TG1              | F'-tra386 lacP3 lacZ M15 proAB5 (spxB ΔntrA-mcrB5 thi Δlac-proAB) |

### Plasmids

| Name              | Description                                                                 | Source          |
|-------------------|-------------------------------------------------------------------------------|-----------------|
| pRV300            | Erythromycin-resistant pBluescript derivative                                 | 22              |
| pVE6007           | Replication-thermosensitive derivative of broad host range replicon pWV01    | 66              |
| pNZ3004           | Plasmid carrying nisin-inducible promoter                                     | 24              |
| pOR210            | repA-negative lacZ' derivative of pWV01                                     | 29              |
| pGBDU-C3          | Expression vector for use in two-hybrid analysis                              | 29              |
| pGAD-C3           | Expression vector for use in two-hybrid analysis                              | 29              |
| pRV3000lR         | prV300 carrying 500 bp cesR fragment                                          | 61              |
| pVE916            | Derivative of broad host range replicon pWV01                                 | T. Rochat and P. Langella |
| pVES3916          | pRV300 derivative carrying 527 bp mtlA fragment                              | This work       |
| pVES4260          | pRV300 derivative carrying 316 bp oatA fragment                              | This work       |
| pVES4341          | pRV300 derivative carrying 313 bp pepN fragment                               | This work       |
| pVES4533          | pRV300 derivative carrying 459 bp pgdA fragment                               | This work       |
| pVES3787          | pVE3916 derivative carrying pgdA gene                                         | This work       |
| pVES3801          | pVES3916 derivative carrying pabB, spxB, and yneG genes                       | This work       |
| pVES3902          | pVES3801 derivative carrying deletion of pabB gene                           | This work       |
| pVES3908          | pVES3902 derivative carrying deletion of spxB gene                           | This work       |
| pVES3910          | pVES3801 derivative carrying deletion of yneG gene                           | This work       |
| pVES4375          | pVE3916 derivative carrying oatA gene with its own promoter                  | This work       |
| pVES5540          | pVE3916 derivative carrying oatA, the expression of which is controlled by Pnis promoter | This work       |
| pVES4548          | pGBDU-C3 derivative carrying trmA gene                                        | This work       |
| pVES4640          | pGBDU-C3 derivative carrying spxB gene                                       | This work       |
| pVES4642          | pGBDU-C3 derivative encoding RpoA-(123–312)                                 | This work       |
| pVES4644          | pGBDU-C3 derivative encoding RpoA-(211–289)                                  | This work       |
| pVES4450          | pGAD-C3 derivative carrying trmA gene                                        | This work       |
| pVES4452          | pGAD-C3 derivative carrying spxB gene                                       | This work       |
| pVES4454          | pGAD-C3 derivative encoding RpoA-(123–312)                                 | This work       |
| pVES4456          | pGAD-C3 derivative encoding RpoA-(211–289)                                 | This work       |

deletion of trmA as verified by PCR using primers clpP-tag (5'-CCACTGCTATCGTCGTCGTCCTTGTAGTCTTTTAGATCAATTATTCCATTTCCATATTGTCGCTTG-3') and hypC (5'-GGCTAGGCAATCGAGCTCGC-3').

Cloning of oatA—A DNA fragment containing the oatA gene and its promoter region was PCR-amplified using the high fidelity enzyme Pfu (Invitrogen, Paisley, UK) and primers yvhB5-kpnl (5'-GGGCGGATCCTAGCTGACTGAGAAATTACTCCAG-3') and yvhB6-BamHI (5'-GGGCGGATCCTCGCAGGATCC-3'). The PCR fragment was inserted into the pCRII-TOPO vector (Invitrogen) following the manufacturer's recommendations, resulting in plasmid pVES4000, with XhoI and NspI restriction sites around the inserted fragment. The XhoI-NspI fragment of pVES4000 was ligated to an XhoI-NspI digest of pVE3916, with subsequent transformation into JIM4646. The resulting plasmid (carrying the oatA gene) was obtained as a chloramphenicol-resistant clone named pVES4375.
**Regulation of Peptidoglycan Hydrolysis by SpxB**

To substitute the oatA native promoter with the Pnis promoter (26), pVES4448 minus the oatA native promoter sequence was amplified by inverse PCR with primers pVE1-BglII (5’-ATGATGAAAGATCTGGAGCTGTAATATAA-3’) and pVE2-Xhol (5’-ATGATGCTCTAGTGAAACGTTACGTCCAGG-3’) using Phusion high fidelity enzyme. PnisA was PCR-amplified from a derivative of pNZ8048 using primers Pnis1-BglII (5’-TACAGCTCAAGATCTAGTC-3’) and Pnis2-Xhol (5’-ATGATGCTGAGTGCTCTTATAATTAT-3’). Both PCR fragments were digested with BglII and Xhol, mixed, and ligated with T4 DNA ligase. The ligation mixture was transformed into E. coli TG1 repA, and plasmid pVES5540 (carrying oatA under the control of the Pnis promoter) was obtained as a chloramphenicol-resistant clone.

**DNA Microarray Analysis**—DNA microarray experiments were performed as described (27, 28). Briefly, RNA was isolated from three independent cultures of L. lactis MG1363/pVE3916 (control strain VES4075) and L. lactis MG1363/psspB+ (strain VES3910). Slide data were processed and normalized using MicroPrep software (27, 28) as described (28). Expression of a gene was considered to be significantly altered when the Cyber-T Bayesian p value was ≥0.001. All DNA microarray data obtained in this study are available at www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc (series GSE7386).

**Yeast Two-hybrid Assay**—The yeast two-hybrid assay and medium were used as described (65). The genes trmA and spxB were cloned as translational fusions with the Gal4 DNA-bind (Gal4BD) and Gal4 activation (Gal4AD) domains in recipient vectors pGBDU-C3 (bait) and pGAD-C3 (prey), respectively (Gal4BD) and Gal4 activation (Gal4AD) domains in recipient vectors pGBDU-C3 (bait) and pGAD-C3 (prey), respectively (29). The lactococcal rpoA fragment encoding the distal part of the gene (RpoA amino acids 123–312) was PCR-amplified and cloned into the same recipient vectors as described above. Plasmids pVES4458 (trmA-Gal4BD), pVES4450 (trmA-Gal4AD), pVES4460 (sspB-Gal4BD), pVES4452 (sspB-Gal4AD), pVES4462 (rpoA-Gal4BD), and pVES4454 (rpoA-Gal4AD) were verified by DNA sequencing, and yeast strains P669-4a and P669-4a (29) were transformed by the bait and prey vectors, respectively. Bait-containing cells were selected on synthetic complete medium lacking uracil, and prey-containing cells were selected on synthetic complete medium lacking leucine. Prey- and bait-containing strains were grown as linear streaks on fresh selective plates at 30 °C for 48 h. Matings were carried out by replica plating prey- and bait-containing cells on yeast extract/peptone/dextrose plates. Cells were transferred onto synthetic complete medium lacking leucine and uracil for diploid selection, and plates were incubated for 2–3 days at 30 °C. Diploid cells were then transferred onto medium selecting for the expression of the HIS3 and ADE2 interaction reporters (synthetic complete medium lacking leucine, uracil, and histidine or adenine, respectively). Interaction phenotypes were scored after 5–12 days of growth at 30 °C. Control matings with Gal4BD and Gal4AD were used to detect self-activation and as negative controls for interaction. The Gal4BD-RpoA (211–289) fusion protein exhibited a strong self-activation phenotype and was not used in this assay. Every Gal4BD/Gal4AD combination was tested in triplicate.

**PG Structure Analysis**—L. lactis peptidoglycan structure was analyzed by reverse phase high pressure liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as described (5). The m/z values were measured as described (10).

**Microscopy**—Transmission electron microscopy was performed as described (30). Microscopy images were obtained with a phase-contrast microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Induscam image analysis system (Andersa, Palaiseau, France).

**Quantitative Real-time (QRT) PCR**—RNA extraction and the QRT-PCR approach were carried out using first-strand cDNA as template, which was synthesized from 20-µg RNA samples as described (31). The tuf gene (coding for elongation factor TU) was used as internal control and for normalization of results. Specific primers for spxB and tuf were designed using the EPrimer3 software (http://bioweb.pasteur.fr/seqanal/interfaces/epimer3.html). For cell wall stress induction, lysozyme (5 mg/ml) was added to exponentially growing cells (A = 0.5). Cells were incubated for 20 min, washed with GM17 medium, and used for RNA extractions. PCRs were carried out as described previously (32) using RNA from three independent cultures. Measurements were performed in triplicate for each sample.

**RESULTS**

**Overexpression of pgdA and spxB (yneH) Confers Lactococcal Resistance to Lysozyme**—Genomic libraries of L. lactis strain MG1363 were used to transform the MG1363 strain with selection for lysozyme-resistant clones, assuming that the lysozyme-resistant phenotype would be due to increased expression of genes cloned on the multicopy plasmid. Using this screening procedure, we isolated several plasmids carrying a 1870-bp DNA fragment (Fig. 1) that encodes the gene annotated as yynD in L. lactis strain IL1403 (33). This gene was renamed pgdA9 in accordance with its pneumococcal ortholog encoding N-acetylglucosamine deacetylase (16). Because this gene was shown to be involved in the lysozyme-resistant phenotype in pneumococci, we assumed that lysozyme resistance in L. lactis is due to increased PG deacetylation by the pgdA gene product. The 3138-bp DNA fragment, which was also repeatedly cloned, contained the yneH yneG operon and pabB, encoding component 1 of a p-aminobenzoate synthase (33). To determine which of the cloned genes is responsible for the lysozyme-resistant phenotype, we inactivated pabB, yneH, and yneG on pVES3801 (Fig. 1). Only inactivation of yneH (pVES3908) completely abolished the lysozyme-resistant phenotype, indicating that yneH is mainly responsible for lysozyme resistance. We therefore concentrated our efforts on investigation of its function. On the basis of the results presented below, we renamed yneH as spxB.

SpxB is one of seven paralogs of L. lactis that bear homology to the regulator protein SpxB of B. subtilis (34). Another paralog, TrmA of L. lactis MG1363, is involved in the heat-sensitive phenotypes of recA and clpP mutants (25, 35). The homology of SpxB to both TrmA and B. subtilis SpxB further suggested its possible regulatory function. By means of MEME motif software (36), we detected a sequence in the spxB promoter region

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9 M.-P. Chapot-Chartier, manuscript in preparation.
that is similar to a sequence reportedly regulating the expression of the two-component system (TCS) LiaRS in \textit{B. subtilis} (37, 38) or CesSR in \textit{L. lactis}, both of which respond to cell envelope stress (Fig. 1).

Whole Genome Transcriptional Analysis of the Strain Over-expressing \textit{spxB}—To investigate the possible role of \textit{spxB} in the regulation of gene expression and to understand how this might confer the lysozyme-resistant phenotype, we compared the transcriptional profiles of \textit{VES3910} (\textit{pspxB}+) and the control strain \textit{MG1363} (Table 2). As expected, \textit{spxB} itself was among the genes showing markedly increased expression in \textit{VES3910}, thus confirming that multicopy cloning leads to its overexpression (20-fold). Among the up-regulated genes, only \textit{yvhB} had a predicted function that could be directly related to the cell wall and consequently to the lysozyme-resistant phenotype; the encoded protein showed 30\% identity to OatA, the PG \textit{O-acetylation} of \textit{S. aureus} (19), and 23\% identity to its pneumococcal ortholog Adr (20). On the basis of this observation and enzyme activity assays detailed below, we assigned \textit{yvhB} the name \textit{oatA}. Because the cloned \textit{spxB} gene showed 20-fold higher expression levels in \textit{VES3910} than in \textit{MG1363}, we hypothesized that high \textit{spxB} expression leads to \textit{oatA} induction, resulting in increased \textit{PG O-acetylation} and resistance to lysozyme. This reasoning suggests that \textit{spxB} may act as a positive regulator of \textit{oatA}.

Among the other overexpressed genes with known functions were \textit{mtlARF}, belonging to the \textit{mtl} operon, responsible for mannitol transport and metabolism (39), and the gene encoding the PepN peptidase (40). Among the down-regulated genes that may be related to the lysozyme-resistant phenotype were the main lactococcal autolysin \textit{acmA} and the genes belonging to the \textit{opp} operon, \textit{B. subtilis} homologs of which were reported to import PG degradation products (41).

\textit{L. lactis TrmA} and \textit{spxB} Both Physically Interact with \textit{RpoA}—\textit{B. subtilis} \textit{spxB} interacts with a subdomain of \textit{RpoA} (amino acids 213–291) in a yeast two-hybrid assay (34). As \textit{L. lactis} \textit{TrmA} and \textit{spxB} are orthologs of \textit{B. subtilis} \textit{spxB}, we examined the potential binary interactions between \textit{L. lactis} \textit{RpoA}, \textit{TrmA}, and \textit{spxB} using the yeast two-hybrid assay. A domain of \textit{L. lactis} \textit{RpoA} (amino acids 211–289) corresponding to the \textit{spxB}-interacting domain in \textit{B. subtilis} \textit{RpoA} and a larger C-terminal part of \textit{RpoA} (amino acids 123–312) encompassing the above domain were fused to Gal4AD and Gal4BD and expressed in yeast. Full-length \textit{TrmA} and \textit{spxB} proteins were fused to Gal4AD and Gal4BD and also expressed in yeast. Interactions were tested by a yeast two-hybrid mating assay as described under “Experimental Procedures.” The result of a typical mating experiment is shown in Fig. 2. Interestingly, both Gal4BD-\textit{TrmA} and Gal4BD-\textit{spxB} interacted specifically with the C-terminal part of \textit{RpoA}. In the reciprocal cross, Gal4BD-\textit{RpoA}-(123–312) interacted with Gal4AD-\textit{TrmA}, but not with Gal4AD-\textit{spxB}. The interaction between Gal4AD-\textit{spxB} and Gal4BD-\textit{RpoA}-(123–312) might be below the detection level of the two-hybrid assay under the stringent selection conditions used. The interactions of \textit{TrmA} and \textit{spxB} with \textit{RpoA} suggest that \textit{spxB} can affect the expression of genes such as \textit{oatA} through modulation of \textit{RpoA} transcriptional efficiency.

Lysozyme Resistance of \textit{pgdA}, \textit{mtlA}, \textit{pepN}, \textit{oatA}, \textit{spxB}, and \textit{trmA} Mutants—To examine the possible links between up-regulated genes in the MG1363/\textit{pspxB}+ strain and lysozyme resistance, we inactivated the genes \textit{mtlA}, \textit{pepN}, \textit{oatA}, \textit{spxB}, and \textit{trmA} and tested mutants for lysozyme sensitivity, cell morphology, and PG structure. In a wild-type (WT) background, mutations in \textit{pepN} and \textit{mtlA} did not influence bacterial lysozyme sensitivity. Surprisingly, \textit{spxB} inactivation had only a slight effect on lysozyme sensitivity compared with the WT parent. The sensitivity of \textit{oatA} and \textit{pgdA} mutants was more pronounced (Fig. 3B). In bacteria overexpressing \textit{spxB} (carrying pVES3910), inactivation of only \textit{oatA} markedly decreased resistance to lysozyme. This could indicate that \textit{oatA} activity alone is responsible for increased lysozyme resistance in the \textit{pspxB}+ context. However, strain VES4320 (\textit{oatA}/\textit{pspxB}+) showed higher resistance to lysozyme than did the WT strain (Fig. 3D), indicating that factors other than \textit{oatA} may influence resistance to lysozyme.

Interestingly, in this test, we observed that mutational inactivation of \textit{TrmA}, which, like \textit{spxB}, interacted with \textit{RpoA} in the yeast two-hybrid system, resulted in a marked increase in lysozyme resistance in both the WT and \textit{pspxB}+ -carrying strains (Fig. 3, C, E, and F). This suggests that \textit{TrmA} and \textit{spxB} may compete for \textit{RpoA} binding \textit{in vivo}. In keeping with this hypothesis, inactivation of \textit{clpP} (encoding a protease putatively responsible for degradation of \textit{TrmA}) decreased resistance to lysozyme. The \textit{clpP \textit{trmA}} double mutant expressed intermediate lysozyme resistance between \textit{trmA} and the WT strain. The
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### Table 2

Up- and down-regulated genes of VES3910 (pspxB*) compared with MG1363 (WT)

Values represent the expression ratio of open reading frames (ORFs) whose expression was >-2-fold higher in VES3910 than in the WT strain. PTS, phosphotransferase; ABC, ATP-binding cassette.

| ORF identifier<sup>a</sup> | Gene<sup>b</sup> | Expression ratio (n-fold) | p value | Putative function<sup>c</sup> |
|--------------------------|----------------|--------------------------|---------|-------------------------------|
| **Up-regulated genes**   |               |                          |         |                               |
| Central metabolism and energy |               |                          |         |                               |
| ilmg0022<sup>a</sup> | mtlA | 21.5 | 10^-7 | PTS system, mannitol-specific IIB component |
| ilmg0023 | mtlB | 28.0 | 10^-6 | Transcription regulator of mtl operon |
| ilmg0024 | mtlF | 5.0 | 10^-5 | PTS system, mannitol-specific IIA component |
| ilmg0271 | ycgD | 2.2 | 10^-7 | Oxidoreductase (COG0673, MvIM, predicted dehydrogenases) |
| ilmg0273 | luxX | 2.5 | 10^-7 | S'-Riboisohomocysteinatease |
| ilmg0319 | pepN | 8.3 | 10^-9 | Aminopeptidase N |
| ilmg0415 | yeiB | 2.2 | 10^-4 | Thymidylate kinase |
| ilmg0945 | ybaI | 2.3 | 10^-4 | Putative glycerol dehydrogenase |
| ilmg2046 | prsB | 2.0 | 10^-4 | Ribose-phosphate pyrophosphokinase |
| ilmg2226 | yueE | 2.7 | 10^-5 | Putative protease (COG0612, predicted Zn-dependent peptidases) |
| ilmg2432 | adhE | 2.0 | 10^-5 | Alcohol-acetaldehyde dehydrogenase |
| DNA replication and repair |               |                          |         |                               |
| ilmg0478 | yedD | 2.5 | 10^-6 | Hypothetical protein (COG3613, nucleoside 2-deoxyribosyltransferase) |
| ilmg0479 | yedE | 3.8 | 10^-6 | Conserved hypothetical protein (COG1611, predicted Rossmann fold nucleotide-binding protein) |
| ilmg0480 | dskA | 2.5 | 10^-6 | Deoxynucleoside kinase |
| ilmg0762 | udk | 2.3 | 10^-5 | Uridine kinase |
| Regulatory functions |               |                          |         |                               |
| ilmg1155 | yneH (spxB) | 20.1 | 10^-11 | Peptidoglycan O-acetylas activator (this work; homology to Spx of B. subtilis) (34) |
| ilmg1930 | ysaA | 2.1 | 10^-6 | Hypothetical protein (homology to VanZ of Enterococcus faecium) (62) |
| ilmg1929 | lrgG | 2.1 | 10^-7 | TCS regulator |
| Transport and binding proteins |               |                          |         |                               |
| ilmg0322 | ydaE | 2.3 | 10^-7 | Cation transporter |
| ilmg0666 | oxT | 2.5 | 10^-4 | Oxalate/formate Antipporter |
| ilmg1993 | yshA | 2.3 | 10^-7 | Putative amino acid permease |
| Cell wall-related protein |               |                          |         |                               |
| ilmg2391 | yvhB (oatA) | 13.3 | 10^-7 | Peptidoglycan O-acetylas activator (this work; homology to OatA of S. aureus) (19) |
| Miscellaneous and unknown proteins |               |                          |         |                               |
| ilmg0731 | yrgA | 2.0 | 10^-6 | Hypothetical protein (pfam01746, LysM domain) |
| ilmg0808 | p346 | 2.4 | 10^-5 | DNA replication protein |
| ilmg0848<sup>a</sup> | ilmg1498 | 2.4 | 10^-5 | Conserved hypothetical protein |
| ilmg2227 | yueF | 3.1 | 10^-5 | Putative protease (COG0612, predicted Zn-dependent peptidases) |
| ilmg2423 | ywal | 2.6 | 10^-4 | Conserved hypothetical protein (COG0840, methyl-accepting chemotaxis protein) |
| ilmg2520 | yxcD | 4.4 | 10^-5 | Hypothetical protein |
| ilmg2563 | ysfC | 2.3 | 10^-7 | Hypothetical protein (COG1242, predicted Fe/S oxidoreductase) |
| **Down-regulated genes**   |               |                          |         |                               |
| Central metabolism and energy |               |                          |         |                               |
| ilmg1551 | yijF | -2.0 | 10^-5 | Transporter (COG2116, formate/nitrite family of transporters) |
| ilmg1642 | batB | -2.6 | 10^-6 | 2,3-Butanediol dehydrogenase |
| ilmg2309 | arcC2 | -2.9 | 10^-5 | Carbohydrate kinase |
| ilmg2310 | arcC1 | -2.7 | 10^-4 | Carbohydrate kinase |
| ilmg2311 | arcD1 | -2.6 | 10^-4 | Arginine/ornithine antiporter |
| ilmg2312 | arcB | -3.3 | 10^-5 | Ornithine carbamoyltransferase |
| ilmg2313 | arcA | -3.9 | 10^-7 | Arginine deiminase |
| Cell wall-related protein |               |                          |         |                               |
| ilmg0280 | acmA | -2.6 | 10^-8 | N'-Acetylglucosaminidase |
| Regulatory functions |               |                          |         |                               |
| ilmg0439 | yecA | -2.2 | 10^-4 | Transcription regulator, LacI family |
| Transport and binding proteins |               |                          |         |                               |
| ilmg0320 | napC | -3.6 | 10^-7 | Multidrug efflux transporter |
| ilmg0454 | yedF | -4.5 | 10^-7 | Putative β-glucoside-specific IIBC component |
| ilmg0650<sup>a</sup> | ilmg0697 | -2.1 | 10^-7 | Branched-chain amino acid transport system II carrier protein |
| ilmg0698 | oppD | -2.4 | 10^-6 | Oligopeptide transport ATP-binding protein |
| ilmg0699 | oppF | -2.4 | 10^-7 | Oligopeptide transport ATP-binding protein |
| ilmg0700 | oppC | 2.2 | 10^-7 | Oligopeptide transport system permease protein |
| ilmg0701 | oppA | -2.9 | 10^-9 | Oligopeptide-binding protein |
| ilmg1012<sup>a</sup> | ilmg2024 | -2.4 | 10^-5 | Putative ABC transporter substrate-binding protein |
| Miscellaneous and unknown proteins |               |                          |         |                               |
| ilmg0165 | ybF | -2.7 | 10^-7 | Conserved hypothetical protein |
| ilmg0173<sup>a</sup> | ilmg0852<sup>a</sup> | -4.4 | 10^-6 | Hypothetical protein |
| ilmg1092 | yngG | -2.5 | 10^-6 | Hypothetical protein |
| ilmg2107 | yslO | -2.1 | 10^-4 | Hypothetical protein |
| ilmg2406 | comGC | -2.0 | 10^-5 | Putative competence protein ComGC |

<sup>a</sup> Open reading frames were identified on the L. lactis MG1363 genome sequence (67).
<sup>b</sup> Gene annotation is based on the L. lactis IL1403 genome (33), except for those genes not present in L. lactis.
<sup>c</sup> Putative function is based on the annotation of the L. lactis IL1403 genome (33) and homology to known proteins or conserved domains from the Clusters of Orthologous Groups (COG) of Proteins (63) and Pfam (64) Databases.
<sup>d</sup> These genes are not present in the IL1403 genome. The proposed functions and names of these genes are based on the MG1363 genome sequence (67).
introduction of pspxB into the WT strain, trmA, and the clpP trmA double mutant increased lysozyme resistance in all three strains (Fig. 3, C–F). An alternative explanation for its function as a SpxB competitor is that TrmA controls the expression of spxB. We used QRT-PCR to exclude this possibility: the introduction of a trmA deletion did not affect spxB expression in the WT and pspxB backgrounds (Fig. 4A).

The ces Operon and spxB Genes Are Induced by Cell Wall Hydrolysis with Lysozyme—We also used QRT-PCR to verify induction of spxB and the ces operon (consisting of three genes: yjbB of unknown function; a histidine kinase, cesS; and a response regulator, cesR) in response to cell envelope damage. As would be predicted, all these genes were induced in the WT strain after lysozyme treatment. In contrast, this response was completely abolished in the cesR mutant (Fig. 4B). In this experiment, we also verified the possible involvement of the TCS cesSR in spxB induction. We compared the expression of spxB in the WT strain and a cesR mutant (VES4476), both carrying pspxB/H11001, and observed a clear decrease in spxB expression in the latter strain, strongly indicating a requirement of CesR for spxB expression (Fig. 4A). These results further suggest that spxB may be part of the cesSR regulatory network and, as such, is induced in response to cell envelope damage.

Growth Arrest and Loss of Viability upon oatA Overexpression—Unexpectedly, the transformation efficiency of MG1363 with pOatA/H11001 was poor (~3000-fold lower than that of the control vector pVE3916). We assumed that overexpression of oatA may lead to excessive PG O-acetylation and eventually to an overly rigid cell wall and that this could have an effect on the colony-forming ability of transformants. To confirm the lethality of oatA overexpression, we placed oatA under the control of the nisin-inducible promoter (26) on plasmid pVE5540. This plasmid transformed L. lactis ~100-fold more efficiently when nisin was not added in the selective medium.

FIGURE 2. TrmA and SpxB both interact with RpoA in a yeast two-hybrid assay. Diploid yeast cells expressing the indicated combinations of proteins fused to Gal4BD (BD) and Gal4AD (AD) were subjected to selection for expression of the HIS3 interaction reporter. Binary interactions appeared as growing colonies on synthetic complete medium lacking leucine, uracil, and histidine. The Gal4AD and Gal4BD fusions are indicated on top and to the left side of the matrix, respectively. Two different RpoA fragments with the indicated amino acid coordinates were used. Control matings with Gal4BD and Gal4AD were used to detect self-activation and as negative controls for interaction.

FIGURE 3. Comparison of lysozyme resistance of the L. lactis WT MG1363 strain and its mutants involved in the lysozyme-resistant phenotype at different lysozyme concentrations. All strains showed identical growth rates without lysozyme, and for this reason, only growth of the WT strain is shown. A, 0 mg/ml; B, 0.25 mg/ml; C, 1 mg/ml; D, 1.5 mg/ml; E, 2 mg/ml; F, 3 mg/ml.

FIGURE 4. Expression of the ces operon and spxB genes as evaluated by QRT-PCR. A, induction levels of spxB (white bars) in the VES3910 (WT/pspxB+), VES4476 (cesR/pspxB+), MG1363 ΔtrmA, and VESS5556 (trmA/pspxB+) strains in comparison with the WT MG1363 strain. B, induction levels of the yjbB (black bars), cesS (dark gray bars), cesR (light gray bars), and spxB (white bars) genes in the MG1363 and VES3915 (cesF) strains after lysozyme treatment in comparison with the untreated MG1363 strain.
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FIGURE 5. Effect of nisin-induced oatA overexpression on bacterial growth. Growth was measured by following the absorbance at 600 nm (OD 600nm) and by plating cfu/ml, where “cfu” is colony-forming units; filled symbols). At A = 0.05, the culture was divided in two subcultures; a sample to which nisin was added at a final concentration of 2 ng/ml (○ and ■) and an untreated sample (■ and □).

Using strain NZ9000/pVES5540, we observed that the cell count dropped ~1000-fold after addition of nisin to the growth medium, and the absorbance stopped increasing and remained constant, indicating the absence of cell lysis (Fig. 5). Surprisingly, despite a 1000-fold drop in cell counts, determination of viability by SYTOX coloration showed that only ~50% of the cells were dead 2.5 h after nisin addition. Low plating efficiency may signify that a proportion of these live cells are in a dormant non-culturable state.

Overexpression of oatA and spxB Alters Cell Morphology—Strains carrying cloned spxB or pgdA (VES3910 and VES3787, respectively) formed long chains (Fig. 6), which is a phenotypic indication of decreased activity of the main lactococcal autolysin (glucosaminidase) AcmA (7). This observation suggests that spxB or pgdA overexpression, in addition to conferring resistance to the muramidase lysozyme, is also responsible for resistance to autolysis, which are mainly glucosaminidases in L. lactis.

We observed severe cell morphology anomalies in electron transmission microphotographs of strains carrying the cloned spxB or pgdA gene (VES3910 and VES3787, respectively): cells had “shrunken” surfaces and were rounder (Fig. 7, E, F, I, and J) than in the control strain VES4075, carrying the empty vector (Fig. 7A). Because strains overexpressing spxB or pgdA are lysozyme-resistant, it is possible that such anomalies are related to PG acetylation-mediated cell wall resistance to hydrolysis. This interpretation is supported by data showing that oatA inactivation (strain VES4320, oatA/pspxB−) abolished the abnormal morphology phenotype (Fig. 7, G and H). Probably high expression of oatA leads to resistance to autolysis and an overly rigid cell wall, which would interfere with normal cell shape determination. In keeping with this reasoning, a ponA mutation, which abolishes PB1A activity and results in the appearance of PG breaks in L. lactis (42), completely suppressed the irregular morphology associated with spxB overexpression (strain VES4433) (Fig. 7, K and L). Direct involvement of oatA in cell shape formation was demonstrated in microphotographs of strain VES5558 expressing nisin-inducible oatA. In the absence of inducer, most of the cells of this strain had a normal morphology, whereas nisin addition resulted in marked cell shape changes. We also observed that the strain expressing nisin-inducible oatA had a thicker PG layer (Fig. 7D) compared with the WT or ponA/pspxB− and oatA/pspxB− strains (Fig. 7, H and L). A thicker cell wall could be the reason for increased resistance to PG autolytic hydrolases.

SpxB, OatA, and TrmA Affect O-Acetylation of PG—To confirm the function of OatA as a PG O-acetylase and SpxB as its regulatory protein, we determined the PG structures of L. lactis VES3910 (pspxB+) and the oatA mutant strain VES4289 by RP-HPLC and MALDI-TOF mass spectrometry and compared their muropeptide profiles with that of the L. lactis MG1363
The amounts of OatA expression when spxB is overproduced. Thus, the lysozyme resistance of VES3910 is correlated with greater PG O-acetylation, which is consistent with transcriptomic analysis showing increased oatA expression when spxB is highly expressed. Furthermore, PG O-acetylation was increased in strains carrying the trmA mutation, in keeping with their elevated lysozyme resistance. These results prove that SpxB is a positive regulator of OatA expression and that its regulation is modulated by TrmA.

**DISCUSSION**

The introduction of bacterial PG breaks by endogenous potentially lethal enzymes is part of a natural process that is reportedly involved in the growth and turnover of the rigid PG sacculus, cell separation, spore germination, autolysis, and biofilm formation (42, 43). The function of PG hydrolases was recently associated with resuscitation from dormancy (13, 14). PGs of pathogens and commensal bacteria may also be degraded by lysozymes, the hydrolases of animal origin.

Despite extensive studies on the molecular mechanisms of bacterial resistance to PG hydrolysis, little is known about the regulation of these processes. We designed a screen to identify possible regulators of PG hydrolysis among genes whose increased expression led to lysozyme resistance. This screen led to the identification of two genes, one of which is pgdA, encoding a PG deacetylase. PgdA inactivation was previously shown to confer lysozyme sensitivity in *S. pneumoniae* (16). The second is a previously uncharacterized gene, *yneH* (renamed here as spxB). Increased spxB expression led to induction of *YvhB*, which we demonstrated by RP-HPLC analysis to be a PG O-acetylase and which we renamed OatA. Our results show that oatA and spxB are components of a regulatory cascade that starts with induction of the TCS CesSR in response to cell wall stress and ends with O-acetylation of PG by OatA as a means of rendering it more resistant to hydrolytic damage. SpxB appears to be a missing link between response to cell envelope stress and PG modification.

Our results indicate that *L. lactis* lysozyme resistance depends on the degree of PG acetylation: higher expression of the PG N-deacetylase PgdA or O-acetylase OatA conferred resistance to lysozyme and also to lactococcal autolysins. On the other hand, their depletion led to lysozyme sensitivity. The ability of orthologs of both enzymes to influence PG resistance

![FIGURE 8. RP-HPLC separation of muropeptides from L. lactis PG. A, MG1363 (WT); B, VES4289 (oatA); C, VES3910 (psxB−). Peaks corresponding to O-acetylated muropeptides were previously identified (5) and are marked a−e.](image)

**TABLE 3**

| Peak | Proposed structure | m/z value | Area | Area | Area | Area |
|------|-------------------|-----------|------|------|------|------|
|      |                   | Actual    | WT   | WT/psxB | trmA/pVE3916 | trmA/psxB | VES5558 − nisin | VES5558 + nisin |
|      |                   | value     |      |         |                |            |                |                |
| a    | Tri-N (Ac)        | 1004.47   | 0.6  | 0.6     | 0.5            | 0.7         | 0.5             | 1.0            |
| b    | Tetra-D (Ac)      | 1076.49   | 0.3  | 0.4     | 0.4            | 0.9         | 0.2             | 0.4            |
| c    | Tetra-N (Ac)      | 1075.88   | 0.3  | 0.4     | 0.5            | 0.5         | 0.2             | 0.3            |
| d    | Tri-N-Tetra-N (Ac)| 1996.89   | 1.3  | 1.5     | 1.2            | 1.4         | 1.1             | 1.6            |
| e    | Tri-N-Tetra-N-Tetra-N (Ac)| 2989.35 | 0.7  | 0.9     | 0.6            | 0.9         | 0.6             | 0.9            |
| Sum  |                   | 3.1       | 3.8  | 3.2     | 3.4            | 4.4         | 2.6             | 4.2            |

**Note:** Peak numbers refer to Fig. 8.

The structures are those proposed in Ref. 5: Tri, disaccharide tripeptide (L-Ala–iGln–L-Lys, where iGln is isoglutaminyl); Tetra, disaccharide tetrapeptide (L-Ala–iGln–L-Lys–D-Ala); disaccharide, GlcNAc–MurNAc; D, D-Asp; N, D-Asn; Ac, acetylation.

*m/z* values correspond to monoisotopic masses. Sodiated molecular ions were the most abundant in MALDI-TOF mass spectra for all muropeptides.

Percentage of each peak was calculated as the ratio of the peak area over the sum of areas of all peaks in the chromatogram.
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to hydrolysis was previously reported, and in some cases, mutants have been reported as lysozyme-sensitive (15–17, 19, 20, 44). The present and previous studies suggest that the activities of only two enzymes, PgdA and/or OatA, are directly needed for lysozyme resistance in a variety of bacteria.

Increased spxB expression led to severe cell morphology anomalies (Fig. 7). The introduction of the oatA mutation into a spxB-overexpressing strain completely abolished defective cell morphology, whereas the induction of OatA expression resulted in the appearance of the morphology defect and a thicker cell wall. These results indicate that the cell morphology changes are due to an excess of O-acetylation, which most likely prevents PG hydrolyses from introducing the required breaks in PG to relax the sacculus. The introduction of PG breaks by other means, notably by inactivation of the ponA gene, also suppressed abnormal cell morphology in the spxB-overexpressing strain, indicating that PG breaks are indeed missing in cells with abnormal morphology. We therefore consider that a reason for cell anomalies in spxB is a greater resistance to hydrolysis of the PG sacculus, which becomes too rigid and too thick to allow normal cell growth and division.

In view of the observed role of OatA in lysozyme resistance, why was it not among the lysozyme-resistant clones in our initial screening? This can be explained by growth arrest due to OatA overexpression. First, the transformation efficiency of WT lactococci with an oatA-carrying plasmid decreased ~3000-fold. Second, OatA overexpression from an inducible promoter resulted in a 1000-fold drop in cell counts while the cell density remained stable, reflecting the absence of cell lysis (Fig. 5). Recent studies in Enterococcus faecalis showed a correlation between increased PG O-acetylation and the viable but non-culturable state (45). Interestingly, the results from a viability test suggested that ~50% of non-culturable OatA-overproducing lactococci were not dead, possibly suggesting that, similarly, a subpopulation had undergone a transition to the viable but non-culturable state. Further evaluation of cell viability by other methods will be needed to confirm this observation.

The potential of oatA to cause growth arrest implies a strong need for its tight regulation. Nevertheless, despite investigations of the role of O-acetylation in lysozyme resistance for different bacteria, no regulators were identified. Our results indicate that SpxB is a novel positive regulator of OatA expression. Interestingly, the spxB promoter region contains a sequence similar to that reportedly recognized by the TCS LiaRS in B. subtilis (38) or CesSR in L. lactis. Both TCSs are members of the envelope stress sensor family and mediate the cellular response to cell wall synthesis-directed antimicrobial treatments in low G + C Gram-positive bacteria (37, 46–48). The involvement of the lactococcal TCS cesSR in cell wall stress is supported by our finding that the ces operon and spxB are induced by PG hydrolysis with lysozyme and that cesR is needed for induction (Fig. 4). In Group A streptococcus, a TCS was shown to be induced by human saliva, which could also cause cell wall damage because it is rich in lysozyme (49). A link between spxB and the cell wall stress response is supported by recent transcriptome results on L. lactis strain IL1403 that had acquired resistance to the cell wall-targeted antimicrobial peptide nisin: in this strain, spxB (yneH), oatA (yvhB), and cesR (kind llrD) were among the up-regulated genes (50).

Among seven lactococcal paralogs of the spx/trmA family, only spxB has the signature motif in its promoter region that is recognized by CesSR and thus may be specifically induced in response to cell wall damage (38). As would be predicted, we found that spxB present on a multicopy plasmid is not overexpressed in the CesR mutant (Fig. 4A). Induction of the ces operon by lysozyme treatment and the need for cesR in spxB induction suggest that spxB may be part of the cesSR regulatory network that leads to increased expression of oatA in response to cell wall damage. Apparently, bacterial resistance to PG hydrolysis is achieved not by diminishing expression of its own autolysins, but by modification of the PG target. This may be a logical response to cell wall damage and may prove efficient against extracellular PG hydrolyses whose activities cannot be regulated by known intracellular control pathways. However, such a strategy has its limits: although SpxB-mediated induction leads to greater PG O-acetylation and resistance to hydrolysis, a further increase in O-acetylation could lead to an excessively rigid cell wall, changes in cell morphology, and eventually to cell death or switch to the dormant state.

How does spxB affect the expression of oatA? In B. subtilis, the regulatory protein Spx is involved in positive and negative gene regulation by interacting with RpoA and, in this way, influences its transcriptional efficiency (51). We have shown that Spx homologs in lactococci (SpxB and TrmA) also interact with RpoA in an ex vivo (yeast two-hybrid) assay. Interactions between SpxB and RpoA could thus be a molecular link explaining induction of oatA by overexpression of spxB.

The suspected interactions between SpxB, TrmA, and RpoA were confirmed as relevant in vivo: the marked increase in lysozyme resistance of the trmA mutant in the WT and spxBB− backgrounds suggests that SpxB and TrmA both interact with RpoA in lactococcal cells and that these interactions are competitive. TrmA inactivation eliminates competition for RpoA and, in this way, may allow better access by SpxB. In B. subtilis, SpxB is degraded by ClpP (52). In L. lactis, the thermosensitivity of a ClpP mutation is alleviated by trmA inactivation, suggesting that, like B. subtilis SpxB, TrmA is also degraded by ClpP (25). In agreement with this interpretation, the lysozyme-sensitive phenotype of the clpP mutant can be explained by accumulation of TrmA. Interestingly, a clpP trmA double mutant carrying spxB+ exhibited lower levels of lysozyme resistance compared with a trmA mutant, possibly indicating that ClpP may also affect the levels of other factors (possibly other spxB orthologs) that compete with SpxB for RpoA binding.

Other genes markedly up-regulated in the spxb-overexpressing strain were pepN and the mtl operon. Strains carrying the mtlA and pepN mutations were not sensitive to lysozyme, and we did not observe any changes in their PG structure and integrity compared with the WT strain (data not shown). Their induction may be a secondary effect not directly related to PG modification or to cell wall reconstruction upon damage. Induction of the mtl operon might be related to osmotic pressure changes due to PG hydrolysis because mannitol is an osmoproprotectant (53, 54). It is likely that not all SpxB-regulated genes are related to the cell wall damage response, as spxB-de-
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TrmA-TrmA, such that each combination might direct the complex to different promoters.

spx-like genes are present in other Gram-positive bacteria, including pathogens such as S. pneumoniae and Streptococcus agalactiae (four paralogs) and Bacillus anthracis (three paralogs). Spx of S. aureus was recently shown to fulfill an important role in growth, general stress protection, and biofilm formation (56). The existence of several Spx-like paralogs in a bacterium may indicate that competitive (or synergistic) interactions between these proteins and RpoA in response to cell envelope stress may be a general phenomenon.

A multistep organization of the response to cell envelope damage may have evolved to allow overlap with other regulatory networks (mediated, for example, by TrmA or CodY) to counteract multiple environmental stresses. Such complex regulation could also be a means of achieving bistability, in which a genetically unique bacterial population can differentiate into phenotypically distinct subpopulations (57, 58).

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