THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 27, pp. 19342–19354, July 6, 2007

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

Received for publication, December 11, 2006, and in revised form, March 30, 2007 Published, JBC Papers in Press, May 7, 2007, DOI 10.1074/jbc.M611308200

Patrick Veiga[‡1,2], Carmen Bulbarela-Sampieri[‡1,3], Sylviane Furlan[‡], Aurélie Maisons[‡], Marie-Pierre Chapot-Chartier[§], Michael Erkelenz[§4], Peggy Mervelet[¶], Philippe Noirot[¶], Dorte Frees[‖], Oscar P. Kuipers[**], Jan Kok[**], Alexandra Gruss[‡], Girbe Buist[5], and Saulius Kulakauskas[‡6]**

*From the [‡]Unité Bactéries Lactiques et Pathogènes Opportunistes, [§]Unité de Biochimie Bactérienne, and [¶]Génétique Microbienne, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France, the [‖]Department of Veterinary Pathobiology, Royal Veterinary and Agricultural University, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark, and the **Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

Endogenous peptidoglycan (PG)-hydrolyzing 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 hydrolases 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 prophage-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 either *N*-acetylglucosaminyl or N-acetylmuramyl residues

* This work was supported in part by an Aard- en Levenswetenschappen middelgrote apparatuur grant for a transcriptomics facility from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (to O. P. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Both authors contributed equally to this work.
[2] Supported by a thesis grant from the Region Ile de France and Institut National de la Recherche Agronomique and by European Molecular Biology Organization Fellowship ASTF 116-05.
[3] Supported by Thesis Grant 68184 from the Consejo Nacional de Ciencia y Tecnologia.
[4] Recipient of a fellowship from LabHealth under Marie Curie Contract MEST-CT-2004-514428.
[5] Supported by Innovatiegerichte Onderzoeksprogramma Genomics Grant IGE01018. Present address: Dept. of Medical Microbiology, University Medical Center Groningen, University of Groningen, 9700 RB Groningen, The Netherlands.
[6] To whom correspondence should be addressed. Tel.: 33-1-3465-2073; Fax: 33-1-3465-2065; E-mail: saulius.kulakauskas@jouy.inra.fr.
[7] The abbreviations used are: PG, peptidoglycan; Gal4BD, Gal4 DNA-binding domain; Gal4AD, Gal4 activation domain; RP-HPLC, reverse phase high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; QRT, quantitative real-time; TCS, two-component system; WT, wild-type.

(*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*-acetylglucosamine deacetylase; the *pgdA* mutant produced fully *N*-acetylated PG and became hypersensitive to lysozyme (16). PG *N*-acetylglucosamine deacetylase was also described in *Bacillus cereus* (17). *Bacillus subtilis* was shown to possess the PG *N*-acetylmuramic acid deacetylase 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*-acetylpeptidoglycan 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-Claix, 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 Me_2SO (Sigma) and added at a final concentration of 2 ng/ml. For growth curve measurements, overnight cultures grown in the presence of antibiotics were diluted 100-fold in fresh GM17 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*.[8] 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 ClaI 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′-TGAGATAATGCCGACTGTA-3′) and BankAS (5′-CATGCTGAAGAGCATCTC-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

[8] T. Rochat and P. Langella, unpublished data.

pVES3787 and pVES3801, respectively, and retained for further work. Other isolated lysozyme-resistant clones were confirmed to carry *xynD* and *spxB yneG* inserts by colony PCR using BankS and BankAS2 as vector-specific primers and a specific primer for the *xynD* 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-XmaI (5′-AAAAACCCGGGCGTAAAAGCCCTTGAGCG-3′; restriction sites present on primers are underlined throughout) and YGH2-XmaI (5′-AAAAACCCGGGTACTTATTTGGATTGGTTCGG-3′) and the Phusion high fidelity DNA polymerase (Finnzymes, Espoo, Finland). The PCR fragment was digested with XmaI, 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′-AAAAAGGATCCCTCAGCTGACTGATTTGAAC-3′) and iYH2 (5′-AAAAAGGATCCCGTACTTTTATTCCTTGGCG-3′) to inactivate *spxB* and primers iYG1-BamHI (5′-AAAAAGGATCCGAATTCGGGAATTTCTCTCTC-3′) and iYG2-BamHI (5′-AAAAAGGATCCGGTTGTCAGGATTGTTCTTG-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 *xynD* 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′-ATGATGGAATTCGTGTTGACGATGTTCTTCAT-3′) and mtlA11-XmaI (5′-ATGATGCCCGGGCATTTCAAGGCCCGCTTTAA-3′), pepN1-XmaI (5′-ATGATGCCCGGGACTTCAACAAAGTTCGCGCT-3′) and pepN2-EcoRI (5′-ATGATGGAATTCACAATGATGTCGCCTTCTTC-3′), yvhB1-EcoRI (5′-ATGATGGAATTCCTTTATCACCTCTGGCCAAAT-3′) and yvhB2-XmaI (5′-ATGATGCCCGGGCAGCGAAGTAAGAACTTCCT-3′), and xynD1-SmaI (5′-AAACCCGGGAGTTCGTAAGCAGA-3′) and xynD2-EcoRI (5′-CCCGAATTCTGAGGAGTGGTAGT-3′). PCR products were digested with EcoRI and XmaI, ligated to pRV300 (22) previously digested with EcoRI and XmaI enzymes, and transformed into *E. coli* HB101. Plasmids pVES4258, pVES4341, pVES4260, and pVES4533, carrying fragments of *mtlA* (327 bp), *pepN* (313 bp), *oatA* (316 bp), and *pdgA* (459 bp), respectively, were isolated as ampicillin-resistant transformants (Table 1). Plasmids pVES4258, pVES4341, and pVES4260, containing an internal fragment of *mtlA*, *pepN*, and *oatA*, respectively, were transformed into *L. lactis* strain MG1363. Single cross-over insertion mutants were selected on GM17 agar plates containing erythromycin (5 μg/ml) and verified by PCR.

An in-frame deletion of the internal 425-bp fragment of the *spxB* gene was constructed using the pORI280 (*lacZ*$^+$)/pVE6007 two-plasmid system (23, 24). First, a fragment of the *spxB* gene situated upstream of the deletion site was PCR-amplified from *L. lactis* MG1363 genomic DNA using primer pair yneGH6-XbaI (5′-ATGATATCTAGACAAACTTTCAGGTCCTGATT-3′) and yneGH5-XmaI (5′-ATGATACCCGGGCTCAGCTGACTGATTTGAAC-3′). The PCR-amplified region was ligated to an XbaI-XmaI digest of pORI280, and the ligation mixture was transformed into *E. coli* JIM4646, which carries a chromosomal copy of *repA*, required for pORI280 replication. A derivative of pORI280 carrying an *spxB* upstream fragment was obtained as an erythromycin (100 μg/ml)-resistant transformant. Second, a fragment of the *spxB* gene situated downstream of the deletion site was PCR-amplified from MG1363 genomic DNA using primer pair yneGH4-XmaI (5′-ATGATACCCGGG TTTATTCCTTGGCGCCTTC-3′) and yneGH1-BglII (5′-ATGATATAGATCTCGAAAGAACAAAAAATGCGC-3′). The amplified region was digested with the corresponding restriction endonucleases and then ligated to a BglII-XmaI digest of the pORI280 derivative containing the *spxB* upstream region, and the ligation mixture was transformed into *E. coli* JIM4646. 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-sensitive clones were isolated and grown on GM17 medium without antibiotics for at least 100 generations. Strain VES4284 (Δ*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 MG1363Δ*trmA* strain, primer pair F1-forward (5′-CGGAATTCGGCTTGATTGTTCAATAACGG-3′) and F1-reverse (5′-CGGGATCCTTTGCCTTCTGTAACTCTCG-3′) was used to amplify the 720-bp fragment just upstream of the *trmA* start codon (called FN). Primer pair F2-forward (5′-CGGGATCCGACGAAAAACGTCTTCATGTAGG-3′) and F2-reverse (5′-GCTCTAGAGTTGTTCAAGAAGAAAGTGAATGG-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 BamHI-XbaI, respectively, and ligated to EcoRI-XbaI-digested pBluescript-KS$^+$ (Stratagene, Amsterdam, The Netherlands). The resulting plasmid containing the ligated FN-FC fragment was denoted pΔ*trmA*. Subsequently, a 1.1-kb EcoRI fragment containing the erythromycin resistance gene was cloned into pΔ*trmA*, 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

TABLE 1

Strains and plasmids used in this study and their relevant genetic properties

The former gene names of *cesR*, *spxB*, *oatA*, and *pdgA* were *llrD*, *yneH*, *yvhB*, and *xynD*, respectively. SCO, single cross-over.

	Relevant genotype	Ref./source
Strain		
L. lactis		
MG1363	Plasmid-free strain	59
NZ9000	MG1363 *pepN*::*nisRK*	26
DFΔclpP	MG1363 Δ*clpP*	25
HI2050	MG1363 *clpP trmA*	25
MG1363Δ*trmA*	MG1363 *trmA* deletion mutant	This work
VES1842	MG1363 *ponA*	42
VES4284	MG1363 *spxB* in-frame deletion mutant	This work
VES3787	MG1363 carrying pVES3787 (*pgdA*$^+$)	This work
VES3801	MG1363 carrying pVES3801 (*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 pVES3908 (*yneG*$^+$)	This work
VES4299	*mtlA* mutant obtained by pVES4258 SCO insertion in MG1363	This work
VES4534	*pgdA* mutant obtained by pVES4533 SCO insertion in MG1363	This work
VES4289	*oatA* mutant obtained by pVES4260 SCO insertion in MG1363	This work
VES4372	*pepN* mutant obtained by pVES4341 SCO insertion in MG1363	This work
VES3915	*cesR* mutant obtained by pRV300llrD SCO insertion in MG1363	This work
VES4075	MG1363 derivative carrying pVE3916	This work
VES4379	VES4372 (*pepN*) carrying pVES3910 (*spxB*$^+$)	This work
VES4382	VES4534 (*pgdA*) carrying pVES3910 (*spxB*$^+$)	This work
VES4320	VES4289 (*oatA*) carrying pVES3910 (*spxB*$^+$)	This work
VES4317	VES4299 (*mtlA*) carrying pVES3910 (*spxB*$^+$)	This work
VES4476	VES5508 (*cesR*) carrying pVES3910 (*spxB*)	This work
VES5565	MG1363Δ*trmA* derivative carrying pVE3916	This work
VES5566	MG1363Δ*trmA* carrying pVES3910 (*spxB*$^+$)	This work
VES5558	NZ9000 carrying pVES5540	This work
VES5573	DFΔ*clpP* carrying pVES3910 (*spxB*$^+$)	This work
VES5575	HI2050 carrying pVES3910 (*spxB*$^+$)	This work
E. coli		
JIM4646	TG1 with chromosomal copy of *repA* gene	P. Renault
HB101	F$^-$ *mcrB mrr hsdS20 recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 glnV44*	60
TG1	F′ *traD*36 *lacI*^{q} Δ*lacZ* M15 *proAB*$^+$/*supE* Δ(*hsdM-mcrB*)5 *thi* Δ(*lac-proAB*)	Laboratory collection
Plasmids		
pRV300	Erythromycin-resistant pBluescript derivative	22
pVE6007	Replication-thermosensitive derivative of broad host range replicon pWV01	66
pNZ8048	Plasmid carrying nisin-inducible promoter	26
pORI280	*repA*-negative *lacZ*$^+$ derivative of pWV01	24
pGBDU-C3	Expression vector for use in two-hybrid analysis	29
pGAD-C3	Expression vector for use in two-hybrid analysis	29
pRV300llrD	pRV300 carrying 500-bp *cesR* fragment	61
pVE3916	Derivative of broad host range replicon pWV01	T. Rochat and P. Langella
pVES4258	pRV300 derivative carrying 327-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 P*nis* promoter	This work
pVES4458	pGBDU-C3 derivative carrying *trmA* gene	This work
pVES4460	pGBDU-C3 derivative carrying *spxB* gene	This work
pVES4462	pGBDU-C3 derivative encoding RpoA-(123–312)	This work
pVES4464	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′-CCTCACTTGTCATCGTCGTCCTTGTAGTCTTTTA-ATCAATTATTTTCCATAATTTCATC-3′) and hypC (5′-GCTGAACGCAATCGAGCTCCAC-3′).

Cloning of oatA—A DNA fragment containing the *oatA* gene and its promoter region was PCR-amplified using the high fidelity enzyme *Pfx* (Invitrogen, Paisley, UK) and primers yvhB5-kpnI (5′-CGGCGGGGTACCTACGACTGGAAATA-CTCCAG-3′) and yvhB6-BamHI (5′-CGGCGGGGATCCCT-TTCGCTACTGATGAAAGA-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 and named pVES4375.

To substitute the *oatA* native promoter with the P*nis* promoter (26), pVES4448 minus the *oatA* native promoter sequence was amplified by inverse PCR with primers pVE1-BglII (5′-ATGATGAAGATCTGGAGCTGTAATATAA-3′) and pVE2-XhoI (5′-ATGATGCTCGAGTGAAGCGTTACGTCACAGG-3′) using Phusion high fidelity enzyme. P*nisA* was PCR-amplified from a derivative of pNZ8048 using primers Pnis1-BglII (5′-TACAGCTCCAAGATCTAGTC-3′) and Pnis2-XhoI (5′-ATGATGCTCGAGTGCCTCCTTATAATTTAT-3′). Both PCR fragments were digested with BglII and XhoI, 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 P*nis* 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/p*spxB*$^+$ (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-binding (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 (*spxB*-Gal4BD), pVES4452 (*spxB*-Gal4AD), pVES4462 (*rpoA*-Gal4BD), and pVES4454 (*rpoA*-Gal4AD) were verified by DNA sequencing, and yeast strains PJ69-4a and PJ69-4α (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/eprimer3.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 *xynD* in *L. lactis* strain IL1403 (33). This gene was renamed *pgdA*[9] 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 I 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 Spx 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* Spx further suggested its possible regulatory function. By means of MEME motif software (36), we detected a sequence in the *spxB* promoter region

[9] M.-P. Chapot-Chartier, manuscript in preparation.

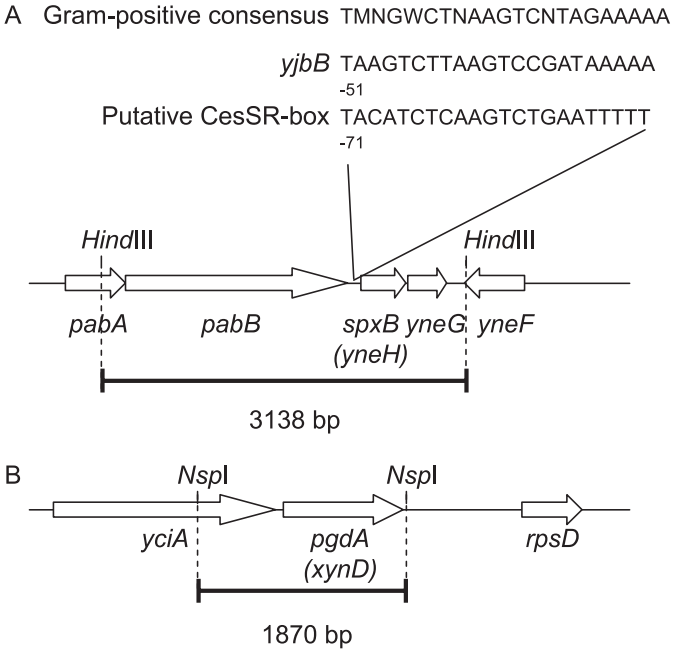

FIGURE 1. **Schematic representation of cloned *L. lactis* chromosomal regions that confer resistance to lysozyme.** *A*, the 3138-bp HindIII fragment, cloned into plasmid pVE3801, comprises the operon including *spxB* (*yneH*), which confers lysozyme resistance. A sequence just upstream of *spxB* shows sequence similarity to the box recognized by CesR, as identified upstream of the *yjbB cesS cesR* operon in *L. lactis* IL1403. The Gram-positive consensus (38) is also indicated. *B*, the 1870-bp NspI fragment, cloned into plasmid pVE3787, comprises *pgdA*, which also confers lysozyme resistance.

that is similar to a sequence reportedly regulating the expression of the two-component system (TCS) LiaRS in *B. subtilis* (37, 38) or CesSR in *L. lactis*,[10] both of which respond to cell envelope stress (Fig. 1).

Whole Genome Transcriptional Analysis of the Strain Overexpressing spxB—To investigate the possible role of SpxB in the regulation of gene expression and to understand how this might confer the lysozyme-resistant phenotype, we compared the transcriptional profiles of VES3910 (p*spxB*$^+$) and the control strain MG1363 (Table 2). As expected, *spxB* itself was among the genes showing markedly increased expression in VES3910, thus confirming that multicopy cloning leads to its overexpression (20-fold). Among the up-regulated genes, only *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 *O*-acetylase of *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 *yvhB* the name *oatA*. Because the cloned *spxB* gene showed 20-fold higher expression levels in VES3910 than in MG1363, we hypothesized that high *spxB* expression leads to *oatA* induction, resulting in increased PG *O*-acetylation and resistance to lysozyme. This reasoning suggests that SpxB may act as a positive regulator of *oatA*.

Among the other overexpressed genes with known functions were *mtlARF*, belonging to the *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 *acmA* and the genes belonging to the *opp* operon, *B. subtilis* homologs of which were reported to import PG degradation products (41).

L. lactis TrmA and SpxB Both Physically Interact with RpoA—*B. subtilis* Spx interacts with a subdomain of RpoA (amino acids 213–291) in a yeast two-hybrid assay (34). As *L. lactis* TrmA and SpxB are orthologs of *B. subtilis* Spx, we examined the potential binary interactions between *L. lactis* RpoA, TrmA, and SpxB using the yeast two-hybrid assay. A domain of *L. lactis* RpoA (amino acids 211–289) corresponding to the Spx-interacting domain in *B. subtilis* RpoA and a larger C-terminal part of RpoA (amino acids 123–312) encompassing the above domain were fused to Gal4AD and Gal4BD and expressed in yeast. Full-length TrmA and 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-TrmA and Gal4BD-SpxB interacted specifically with the C-terminal part of RpoA. In the reciprocal cross, Gal4BD-RpoA-(123–312) interacted with Gal4AD-TrmA, but not with Gal4AD-SpxB. The interaction between Gal4AD-SpxB and Gal4BD-RpoA-(123–312) might be below the detection level of the two-hybrid assay under the stringent selection conditions used. The interactions of TrmA and SpxB with RpoA suggest that SpxB can affect the expression of genes such as *oatA* through modulation of RpoA transcriptional efficiency.

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

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

[10] Martinez, B., Zomer, A. L., Rodriguez, A., Kok, J., and Kuipers, O. P. (2007) *Mol. Microbiol.* **64,** 473–486

TABLE 2

Up- and down-regulated genes of VES3910 (p*spxB*$^+$) 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[a]	Gene[b]	Expression ratio (*n*-fold)	*p* value	Putative function[c]
Up-regulated genes				
Central metabolism and energy				
llmg0022[d]	*mtlA*	21.5	10^{-7}	PTS system, mannitol-specific IIBC component
llmg0023	*mtlR*	28.0	10^{-6}	Transcription regulator of *mtl* operon
llmg0024	*mtlF*	5.0	10^{-5}	PTS system, mannitol-specific IIA component
llmg0271	*ycgD*	2.2	10^{-7}	Oxidoreductase (COG0673, MviM, predicted dehydrogenases)
llmg0273	*luxS*	2.5	10^{-7}	*S*-Ribosylhomocysteinase
llmg0319	*pepN*	8.3	10^{-9}	Aminopeptidase N
llmg0415	*yeaB*	2.2	10^{-4}	Thymidylate kinase
llmg0945	*ybaI*	2.3	10^{-4}	Putative glycerol dehydrogenase
llmg2046	*prsB*	2.0	10^{-4}	Ribose-phosphate pyrophosphokinase
llmg2226	*yueE*	2.7	10^{-5}	Putative protease (COG0612, predicted Zn-dependent peptidases)
llmg2432	*adhE*	2.0	10^{-5}	Alcohol-acetaldehyde dehydrogenase
DNA replication and repair				
llmg0478	*yejD*	2.5	10^{-6}	Hypothetical protein (COG3613, nucleoside 2-deoxyribosyltransferase)
llmg0479	*yejE*	3.8	10^{-6}	Conserved hypothetical protein (COG1611, predicted Rossmann fold nucleotide-binding protein)
llmg0480	*dukA*	2.5	10^{-6}	Deoxynucleoside kinase
llmg0762	*udk*	2.3	10^{-5}	Uridine kinase
Regulatory functions				
llmg1155	*yneH* (*spxB*)	20.1	10^{-11}	Peptidoglycan *O*-acetylase activator (this work; homology to Spx of *B. subtilis*) (34)
llmg1930	*ysaA*	2.1	10^{-6}	Hypothetical protein (homology to VanZ of *Enterococcus faecium*) (62)
llmg1929	*llrG*	2.1	10^{-7}	TCS regulator
Transport and binding proteins				
llmg0322	*ydaE*	2.3	10^{-7}	Cation transporter
llmg0666[d]	*oxlT*	2.5	10^{-4}	Oxalate/formate Antiporter
llmg1993	*yshA*	2.3	10^{-7}	Putative amino acid permease
Cell wall-related protein				
llmg2391	*yvhB* (*oatA*)	13.3	10^{-7}	Peptidoglycan *O*-acetylase (this work; homology to OatA of *S. aureus*) (19)
Miscellaneous and unknown proteins				
llmg0731	*yrgA*	2.0	10^{-6}	Hypothetical protein (pfam01476, LysM domain)
llmg0808	*pi346*	2.4	10^{-5}	DNA replication protein
llmg0848[d]		2.4	10^{-6}	Conserved hypothetical protein
llmg1498	*ykiE*	3.8	10^{-4}	Conserved hypothetical protein
llmg2227	*yueF*	3.1	10^{-5}	Putative protease (COG0612, predicted Zn-dependent peptidases)
llmg2423	*ywaI*	2.6	10^{-4}	Conserved hypothetical protein (COG0840, methyl-accepting chemotaxis protein)
llmg2520	*yxcD*	4.4	10^{-5}	Hypothetical protein
llmg2563	*yxfC*	2.3	10^{-7}	Hypothetical protein (COG1242, predicted Fe/S oxidoreductase)
Down-regulated genes				
Central metabolism and energy				
llmg1551	*yjjF*	−2.0	10^{-5}	Transporter (COG2116, formate/nitrite family of transporters)
llmg1642	*butB*	−2.6	10^{-6}	2,3-Butanediol dehydrogenase
llmg2309	*arcC2*	−2.9	10^{-5}	Carbamate kinase
llmg2310	*arcC1*	−2.7	10^{-4}	Carbamate kinase
llmg2311	*arcD1*	−2.6	10^{-4}	Arginine/ornithine antiporter
llmg2312	*arcB*	−3.3	10^{-5}	Ornithine carbamoyltransferase
llmg2313	*arcA*	−3.9	10^{-7}	Arginine deiminase
Cell wall-related protein				
llmg0280	*acmA*	−2.6	10^{-8}	*N*-Acetylglucosaminidase
Regulatory functions				
llmg0439	*yecA*	−2.2	10^{-4}	Transcription regulator, LacI family
Transport and binding proteins				
llmg0320	*napC*	−3.6	10^{-7}	Multidrug efflux transporter
llmg0454	*yedF*	−4.5	10^{-7}	Putative β-glucoside-specific IIBC component
llmg0650[d]	*brnQ*	−2.1	10^{-7}	Branched-chain amino acid transport system II carrier protein
llmg0697	*oppD*	−2.4	10^{-6}	Oligopeptide transport ATP-binding protein
llmg0698	*oppF*	−2.4	10^{-7}	Oligopeptide transport ATP-binding protein
llmg0700	*oppC*	2.2	10^{-7}	Oligopeptide transport system permease protein
llmg0701	*oppA*	−2.9	10^{-9}	Oligopeptide-binding protein
llmg1012[d]		−2.4	10^{-5}	Putative ABC transporter substrate-binding protein
llmg2024	*oppA2*	−2.2	10^{-6}	Oligopeptide-binding protein
Miscellaneous and unknown proteins				
llmg0165	*ybfA*	−2.7	10^{-7}	Conserved hypothetical protein
llmg0173[d]		−4.4	10^{-6}	Hypothetical protein
llmg0852[d]	*ps357*	−3.6	10^{-7}	Conserved hypothetical protein
llmg1092	*ynjG*	−2.5	10^{-6}	Hypothetical protein
llmg2007	*ysiD*	−2.1	10^{-4}	Hypothetical protein
llmg2406	*comGC*	−2.0	10^{-5}	Putative competence protein ComGC

[a] Open reading frames were identified on the *L. lactis* MG1363 genome sequence (67).
[b] Gene annotation is based on the *L. lactis* IL1403 genome (33), except for those genes not present in *L. lactis*.
[c] Putation 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.
[d] 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).

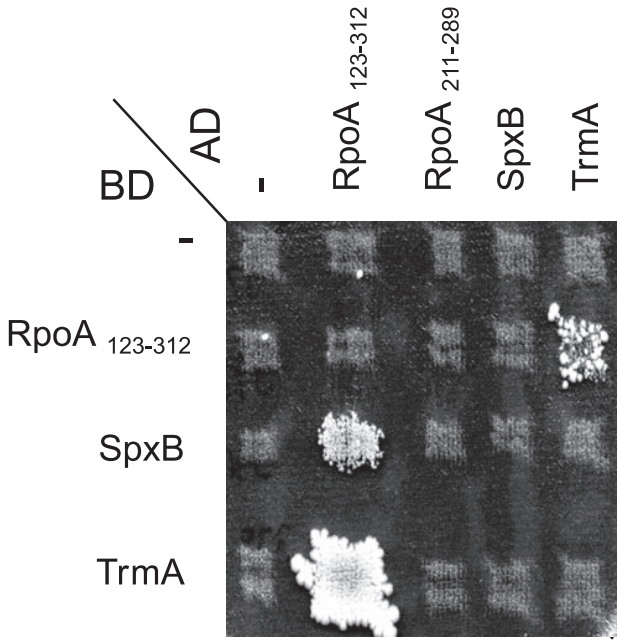

FIGURE 2. **TrmA and SpxB both interact with RpoA in a yeast two-hybrid assay.** Diploid yeast cells expressing the indicated combinations of proteins fused to Gal4BD (*BD*) and Gal4AD (*AD*) were subjected to selection for expression of the *HIS3* interaction reporter. Binary interactions appeared as 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.

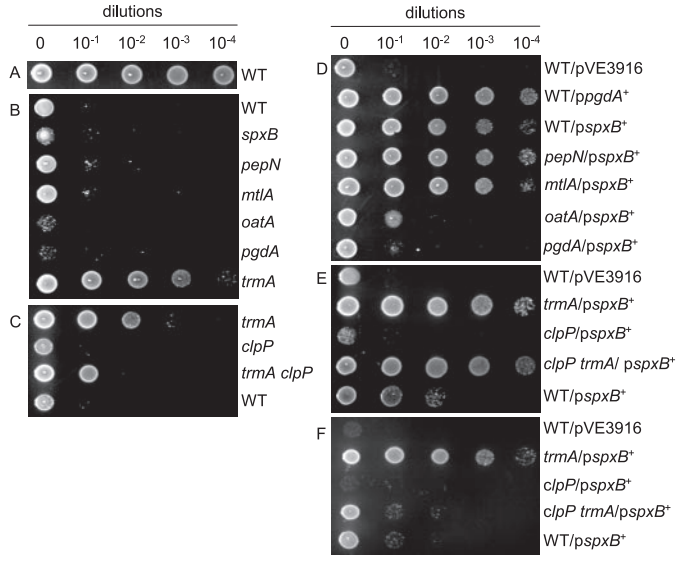

FIGURE 3. **Comparison of lysozyme resistance of the *L. lactis* WT MG1363 strain and its mutants involved in the lysozyme-resistant phenotype at different lysozyme concentrations.** All strains showed identical growth rates without lysozyme, and for this reason, only growth of the WT strain is shown. *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.

introduction of p*spxB*$^+$ 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 p*spxB*$^+$ backgrounds (Fig. 4*A*).

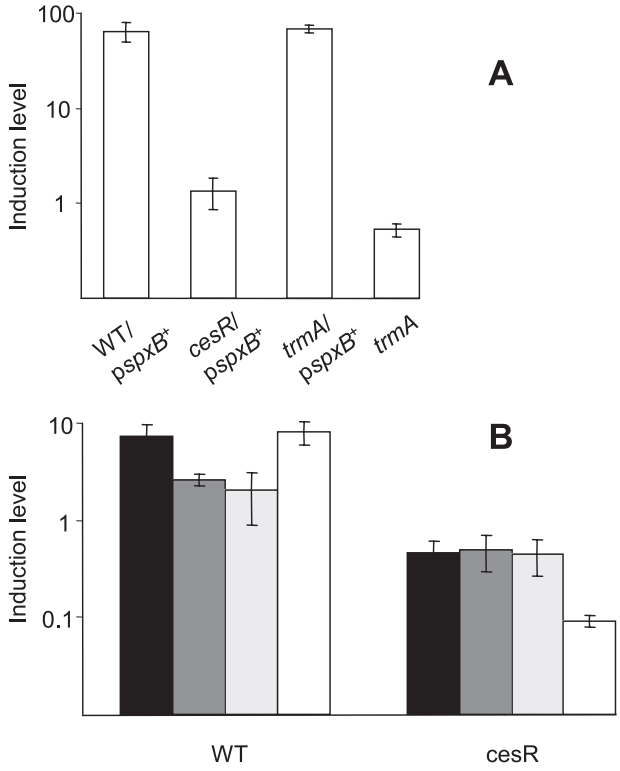

FIGURE 4. **Expression of the *ces* operon and *spxB* genes as evaluated by QRT-PCR.** *A*, induction levels of *spxB* (*white bars*) in the VES3910 (WT/p*spxB*$^+$), VES4476 (*cesR*/p*spxB*$^+$), MG1363Δ*trmA*, and VES5556 (*trmA*/p*spxB*$^+$) strains in comparison with the WT MG1363 strain. *B*, induction levels of the *yjbB* (*black bars*), *cesS* (*dark gray bars*), *cesR* (*light gray bars*), and *spxB* (*white bars*) genes in the MG1363 and VES3915 (*cesR*) strains after lysozyme treatment in comparison with the untreated MG1363 strain.

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 wall hydrolysis by lysozyme. 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. 4*B*). 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 p*spxB*$^+$, and observed a clear decrease in *spxB* expression in the latter strain, strongly indicating a requirement of CesR for *spxB* expression (Fig. 4*A*). 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 p*oatA*$^+$ 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.

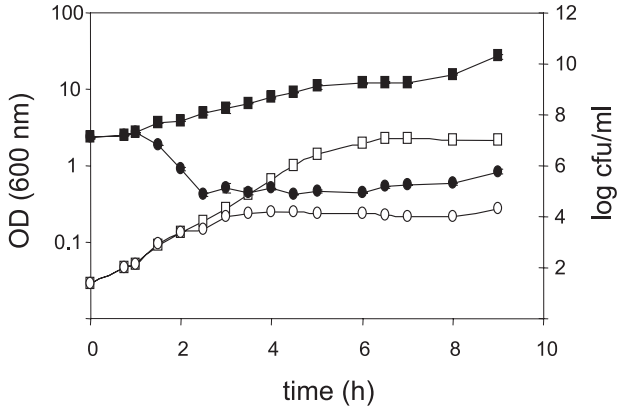

FIGURE 5. **Effect of nisin-induced *oatA* overexpression on bacterial growth.** Growth was measured by following the absorbance at 600 nm (*OD (600nm)*; *empty symbols*) and by plating (*log cfu/ml*, where "cfu" is colony-forming units; *filled symbols*). At $A = 0.05$, the culture was divided in two subcultures: a sample to which nisin was added at a final concentration of 2 ng/ml (● and ○) and an untreated sample (■ and □).

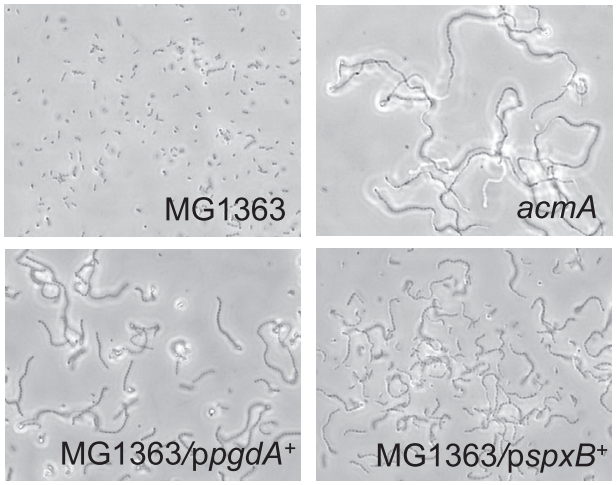

FIGURE 6. **Phase-contrast microphotographs of *L. lactis* MG1363, its *acmA* deletion derivative, and the PgdA (MG1363/p*pgdA*$^+$)- and SpxB (MG1363/ p*spxB*$^+$)-overexpressing strains.**

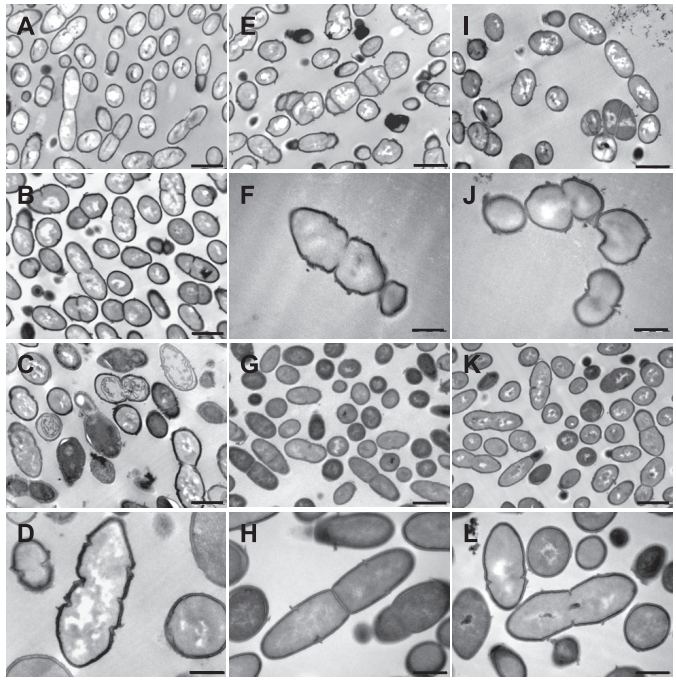

FIGURE 7. **Electron transmission microphotographs of *L. lactis* control strain VES4075 (WT/pVE3916; *A*), VES5558 carrying nisin-inducible *oatA* without (*B*) and with (*C* and *D*) nisin, VES3910 (p*spxB*$^+$; *E* and *F*), VES4320 (*oatA*/p*spxB*$^+$; *G* and *H*), VES3787 (p*pgdA*$^+$; *I* and *J*), and VES4315 (*ponA*/p*spxB*$^+$; *K* and *L*).** *Scale bars* = 1 μm (*A–C*, *E*, *G*, *I*, and *K*) and 0. 5 μm (*D*, *F*, *H*, *J*, and *L*).

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 autolysins, 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. 7*A*). 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*/p*spxB*$^+$) 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 PBP1A 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. 7*D*) compared with the WT or *ponA*/p*spxB*$^+$ and *oatA*/p*spxB*$^+$ 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 (p*spxB*$^+$) 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

parental strain (Fig. 8). The muropeptide profile of the *oatA* mutant lacked the peaks corresponding to *O*-acetylated muropeptides (peaks a– e in Table 3), which were present in the MG1363 PG (5). Furthermore, a strain expressing nisin-inducible *oatA* displayed a marked increase in *O*-acetylated muropeptide peaks in the presence of inducer (Table 3). These results confirm our prediction, based on DNA homology, that OatA is the lactococcal PG *O*-acetylase. We also compared the levels of *O*-acetylation in the different genetic backgrounds. The amounts of *O*-acetylated muropeptides were found to be greater in strain VES3910 (p*spxB*$^+$) than in the WT strain (3.8% *versus* 3.1%) (Table 3), indicating that PG *O*-acetylation is increased by SpxB overproduction. 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.

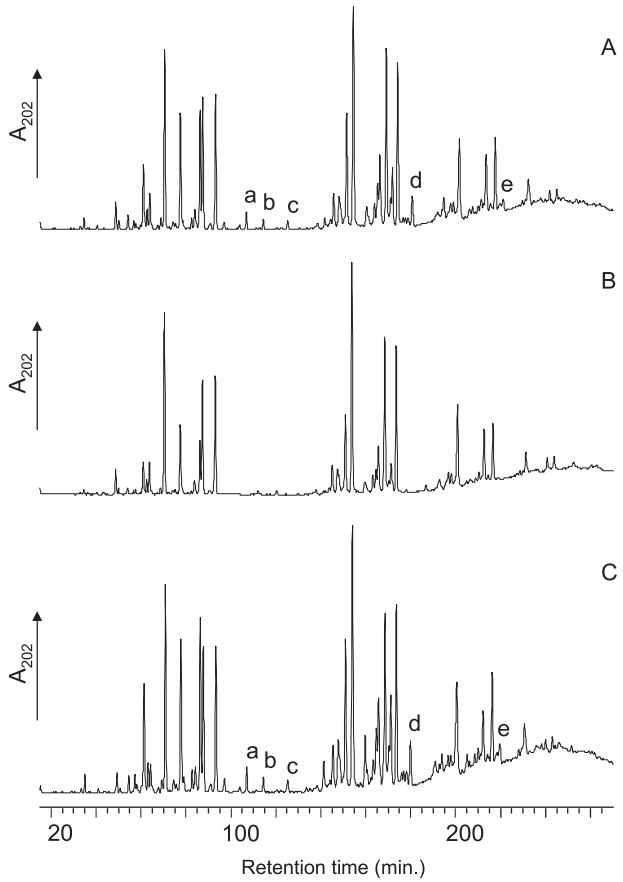

FIGURE 8. **RP-HPLC separation of muropeptides from *L. lactis* PG.** *A*, MG1363 (WT); *B*, VES4289 (*oatA*); *C*, VES3910 (p*spxB*$^+$). Peaks corresponding to *O*-acetylated muropeptides were previously identified (5) and are marked *a*–*e*.

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

TABLE 3

Structures and quantities of *O*-acetylated muropeptides from *L. lactis* MG1363 (WT), VES3910 (WT/p*spxB*$^+$), VES5565 (*trmA*/pVE3916), VES5566 (*trmA*/p*spxB*), and VES5558 with and without nisin

Peak[a]	Proposed structure[b]	*m*/*z* value[c]	Area[d]					
			WT	WT/p*spxB*	*trmA*/pVE3916	*trmA*/p*spxB*	VES5558 − nisin	VES5558 + nisin
						%		
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)	1075.48	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	4.4	2.6	4.2

[a] Peak numbers refer to Fig. 8.
[b] The structures are those proposed in Ref. 5: Tri, disaccharide tripeptide (L-Ala-D-iGln-L-Lys, where iGln is isoglutaminyl); Tetra, disaccharide tetrapeptide (L-Ala-D-iGln-L-Lys-D-Ala); disaccharide, GlcNAc-MurNAc; D, D-Asp; N, D-Asn; Ac, acetylation.
[c] *m*/*z* values correspond to monoisotopic masses. Sodiated molecular ions were the most abundant in MALDI-TOF mass spectra for all muropeptides.
[d] Percentage of each peak was calculated as the ratio of the peak area over the sum of areas of all peaks in the chromatogram.

to hydrolysis was previously reported, and in some cases, mutants have been reported as lysozyme-sensitive (15–17, 19, 20, 44).[9] 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 hydrolases 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*.[10] 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 *cesSR* (*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).[10] As would be predicted, we found that *spxB* present on a multicopy plasmid is not overexpressed in the *CesR* mutant (Fig. 4*A*). 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 hydrolases 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 p*spxB*$^+$ 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*, Spx is degraded by ClpP (52). In *L. lactis*, the thermosensitivity of a ClpP mutation is alleviated by *trmA* inactivation, suggesting that, like *B. subtilis* Spx, 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 p*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 osmoprotectant (53, 54). It is likely that not all SpxB-regulated genes are related to the cell wall damage response, as *spxB*-de-

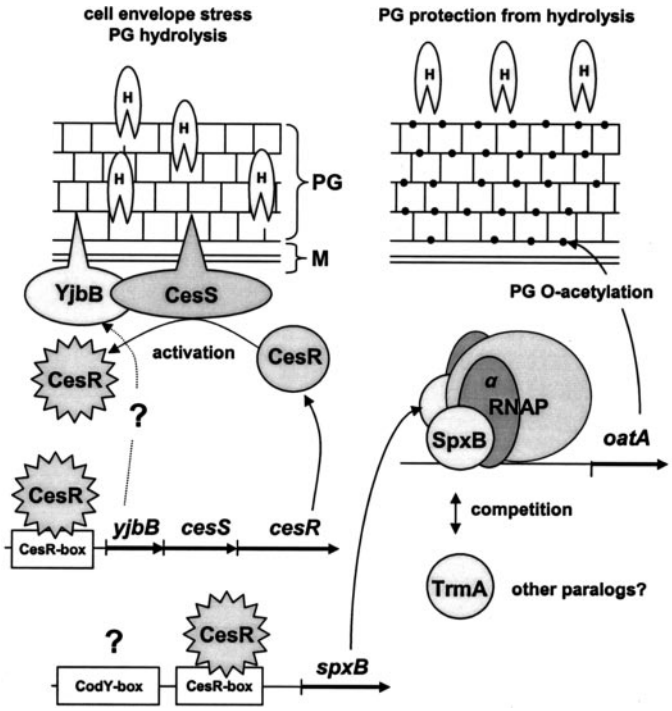

FIGURE 9. **Model for the lactococcal cell wall stress regulon.** For details, see "Discussion." The *black dots* in the cell wall indicate sites of PG *O*-acetylation. *M*, membrane; *H*, PG hydrolase enzyme (lysozyme); *RNAP*, RNA polymerase; α, α-subunit of RNA polymerase.

pendent regulation may be complicated by cross-talk with other paralogs of the same family. Also, *spxB* (*yneH*) was reported to be part of the CodY regulon because it has a CodY DNA-binding box in its promoter region (30 bp upstream from the CesR box) (Fig. 9) and was shown to be induced in a *codY* mutant (31), further suggesting that *mtlA* and *pepN* may be important in the context of other stress situations.

In conclusion, the *L. lactis* response to cell-surface stress provoked by PG hydrolase lysozyme treatment appears to involve a cascade of events (schematized in Fig. 9). First, the CesSR regulon genes are induced: the membrane-located CesS sensor kinase activates the transcription activator CesR, most probably by autophosphorylation and subsequent phosphate transfer (37). The first gene of the *ces* operon (*yjbB*) possibly encodes an inhibitor of CesR activation, similar to LiaF of *B. subtilis* (38). Activated CesR recognizes the CesR box sequence, which is present in the promoter region of its own operon and in that of the *spxB* gene. CesR induces increased expression of SpxB, which, similarly to Spx of *B. subtilis* (51), associates with RpoA, the α-subunit of RNA polymerase (Fig. 9). We postulate that SpxB binding to RNA polymerase activates *oatA* expression, the activity of which might increase PG resistance to hydrolysis.

This regulatory scheme may be fine-tuned by competition of SpxB with TrmA and possibly other Spx-like paralogs of *L. lactis.* Alternatively, the ability of both SpxB and TrmA to bind RpoA may present an even more sophisticated example of regulation; as the RNA polymerase complex contains two RpoA subunits (55), it may bind two Spx-like molecules. This raises the possibility that two regulatory proteins (*e.g.* SpxB and TrmA) simultaneously bind RpoA, suggesting the possibility of three different binding combinations, SpxB-SpxB, SpxB-TrmA, and 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).

Acknowledgments—We thank M.-A. Petit for critical reading of the manuscript; P. Serror, M. El Karoui, M. F. Noirot, S. A. van Hijum, A. de Jong, and A. L. Zomer for discussions; and E. Guédon for help with QRT-PCR. We are indebted to B. Martinez for valuable discussions and communications of results before publication. We are grateful to T. Rochat and P. Langella for providing the pVE3916 plasmid. We thank C. Longin and S. Chat (Microscopie et Imagerie des Micro-organismes, Animaux et Aliments, Plateau de Microscopie Electronique, Unité Génomique et Physiologie de la Lactation, Jouy-en-Josas, France) for electronic photography.

REFERENCES

1. Shockman, G. D., Daneo-Moore, L., Kariyama, R., and Massidda, O. (1996) *Microb. Drug Resist.* **2,** 95–98
2. van Heijenoort, J. (2001) *Glycobiology* **11,** 25R–36R
3. Delcour, J., Ferain, T., Deghorain, M., Palumbo, E., and Hols, P. (1999) *Antonie Leeuwenhoek* **76,** 159–184
4. Matsuhashi, M. (1994) in *Bacterial Cell Wall* (Ghuysen, J. M., and Hakenbeck, R., eds) pp. 55–71, Elsevier Science Publishers B. V., Amsterdam
5. Courtin, P., Miranda, G., Guillot, A., Wessner, F., Mezange, C., Domakova, E., Kulakauskas, S., and Chapot-Chartier, M.-P. (2006) *J. Bacteriol.* **188,** 5293–5298
6. Foster, S. J., and Popham, D. L. (2002) in *Bacillus subtilis and Its Closest Relatives: From Genes to Cells* (Sonenstein, A. L., ed.) pp. 21–41, ASM Press, Washington, D. C.
7. Buist, G., Kok, J., Leenhouts, K. J., Dabrowska, M., Venema, G., and Haandrikman, A. J. (1995) *J. Bacteriol.* **177,** 1554–1563
8. Steen, A., Buist, G., Horsburgh, G. J., Venema, G., Kuipers, O. P., Foster, S. J., and Kok, J. (2005) *FEBS J.* **272,** 2854–2868
9. Huard, C., Miranda, G., Wessner, F., Bolotin, A., Hansen, J., Foster, S. J., and Chapot-Chartier, M.-P. (2003) *Microbiology* (*Read.*) **149,** 695–705
10. Huard, C., Miranda, G., Redko, Y., Wessner, F., Foster, S. J., and Chapot-Chartier, M.-P. (2004) *Appl. Environ. Microbiol.* **70,** 3493–3499
11. Lepeuple, A. S., Van Gemert, E., and Chapot-Chartier, M.-P. (1998) *Appl. Environ. Microbiol.* **64,** 4142–4148
12. Masschalck, B., and Michiels, C. W. (2003) *Crit. Rev. Microbiol.* **29,** 191–214
13. Telkov, M. V., Demina, G. R., Voloshin, S. A., Salina, E. G., Dudik, T. V., Stekhanova, T. N., Mukamolova, G. V., Kazaryan, K. A., Goncharenko, A. V., Young, M., and Kaprelyants, A. S. (2006) *Biochemistry* (*Mosc.*) **71,** 414–422
14. Mukamolova, G. V., Murzin, A. G., Salina, E. G., Demina, G. R., Kell, D. B., Kaprelyants, A. S., and Young, M. (2006) *Mol. Microbiol.* **59,** 84–98

15. Clarke, A. J., and Dupont, C. (1992) *Can. J. Microbiol.* **38,** 85–91
16. Vollmer, W., and Tomasz, A. (2000) *J. Biol. Chem.* **275,** 20496–20501
17. Psylinakis, E., Boneca, I. G., Mavromatis, K., Deli, A., Hayhurst, E., Foster, S. J., Varum, K. M., and Bouriotis, V. (2005) *J. Biol. Chem.* **280,** 30856–30863
18. Fukushima, T., Kitajima, T., and Sekiguchi, J. (2005) *J. Bacteriol.* **187,** 1287–1292
19. Bera, A., Herbert, S., Jakob, A., Vollmer, W., and Gotz, F. (2005) *Mol. Microbiol.* **55,** 778–787
20. Crisostomo, M. I., Vollmer, W., Kharat, A. S., Inhulsen, S., Gehre, F., Buckenmaier, S., and Tomasz, A. (2006) *Mol. Microbiol.* **61,** 1497–1509
21. Weadge, J. T., Pfeffer, J. M., and Clarke, A. J. (2005) *BMC Microbiol.* **5,** 49
22. Leloup, L., Ehrlich, S. D., Zagorec, M., and Morel-Deville, F. (1997) *Appl. Environ. Microbiol.* **63,** 2117–2123
23. Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G., and Leenhouts, K. (1995) *J. Bacteriol.* **177,** 7011–7018
24. Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G., and Kok, J. (1996) *Mol. Gen. Genet.* **253,** 217–224
25. Frees, D., Varmanen, P., and Ingmer, H. (2001) *Mol. Microbiol.* **41,** 93–103
26. Kuipers, O. P., de Ruyter, P., Kleerebezem, M., and de Vos, W. M. (1998) *J. Biotechnol.* **64,** 15–21
27. van Hijum, S. A., de Jong, A., Baerends, R. J., Karsens, H. A., Kramer, N. E., Larsen, R., den Hengst, C. D., Albers, C. J., Kok, J., and Kuipers, O. P. (2005) *BMC Genomics* **6,** 77
28. Zomer, A. L., Buist, G., Larsen, R., Kok, J., and Kuipers, O. P. (2006) *J. Bacteriol.* **189,** 3256–3270
29. James, P., Halladay, J., and Craig, E. A. (1996) *Genetics* **144,** 1425–1436
30. Veiga, P., Piquet, S., Maisons, A., Furlan, S., Courtin, P., Chapot-Chartier, M.-P., and Kulakauskas, S. (2006) *Mol. Microbiol.* **62,** 1713–1724
31. Guédon, E., Sperandio, B., Pons, N., Ehrlich, S. D., and Renault, P. (2005) *Microbiology* (*Read.*) **151,** 3895–3909
32. Sperandio, B., Polard, P., Ehrlich, D. S., Renault, P., and Guédon, E. (2005) *J. Bacteriol.* **187,** 3762–3778
33. Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S. D., and Sorokin, A. (2001) *Genome Res.* **11,** 731–753
34. Nakano, S., Nakano, M. M., Zhang, Y., Leelakriangsak, M., and Zuber, P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4233–4238
35. Duwat, P., Ehrlich, S. D., and Gruss, A. (1999) *Mol. Microbiol.* **31,** 845–858
36. Bailey, T. L., and Elkan, C. (1994) *Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology*, pp. 28–36, AAAI Press, Menlo Park, CA
37. Mascher, T. (2006) *FEMS Microbiol. Lett.* **264,** 133–144
38. Jordan, S., Junker, A., Helmann, J. D., and Mascher, T. (2006) *J. Bacteriol.* **188,** 5153–5166
39. Gaspar, P., Neves, A. R., Ramos, A., Gasson, M. J., Shearman, C. A., and Santos, H. (2004) *Appl. Environ. Microbiol.* **70,** 1466–1474
40. Tan, P. S., van Alen-Boerrigter, I. J., Poolman, B., Siezen, R. J., de Vos, W. M., and Konings, W. N. (1992) *FEBS Lett.* **306,** 9–16
41. Goodell, E. W., and Higgins, C. F. (1987) *J. Bacteriol.* **169,** 3861–3865
42. Mercier, C., Durrieu, C., Briandet, R., Domakova, E., Tremblay, J., Buist, G., and Kulakauskas, S. (2002) *Mol. Microbiol.* **46,** 235–243
43. Shockman, G. D., and Höltje, J.-V. (1994) *New Compr. Biochem.* **27,** 131–166
44. Brumfitt, W., Wardlaw, A. C., and Park, J. T. (1958) *Nature* **181,** 1783–1784
45. Pfeffer, J. M., Strating, H., Weadge, J. T., and Clarke, A. J. (2006) *J. Bacteriol.* **188,** 902–908
46. Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. (2003) *Mol. Microbiol.* **49,** 807–821
47. McAleese, F., Wu, S. W., Sieradzki, K., Dunman, P., Murphy, E., Projan, S., and Tomasz, A. (2006) *J. Bacteriol.* **188,** 1120–1133
48. Utaida, S., Dunman, P. M., Macapagal, D., Murphy, E., Projan, S. J., Singh, V. K., Jayaswal, R. K., and Wilkinson, B. J. (2003) *Microbiology* (*Read.*) **149,** 2719–2732
49. Shelburne, S. A., III, Sumby, P., Sitkiewicz, I., Granville, C., DeLeo, F. R., and Musser, J. M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 16037–16042
50. Kramer, N. E., van Hijum, S. A., Knol, J., Kok, J., and Kuipers, O. P. (2006) *Antimicrob. Agents Chemother.* **50,** 1753–1761
51. Zuber, P. (2004) *J. Bacteriol.* **186,** 1911–1918
52. Nakano, S., Zheng, G., Nakano, M. M., and Zuber, P. (2002) *J. Bacteriol.* **184,** 3664–3670
53. Iwamoto, K., and Shiraiwa, Y. (2005) *Mar. Biotechnol.* **7,** 407–415
54. Kets, E. P., Galinski, E. A., de Wit, M., de Bont, J. A., and Heipieper, H. J. (1996) *J. Bacteriol.* **178,** 6665–6670
55. Darst, S. A. (2001) *Curr. Opin. Struct. Biol.* **11,** 155–162
56. Pamp, S. J., Frees, D., Engelmann, S., Hecker, M., and Ingmer, H. (2006) *J. Bacteriol.* **188,** 4861–4870
57. Smits, W. K., Kuipers, O. P., and Veening, J. W. (2006) *Nat. Rev. Microbiol.* **4,** 259–271
58. Dubnau, D., and Losick, R. (2006) *Mol. Microbiol.* **61,** 564–572
59. Gasson, M. J. (1983) *J. Bacteriol.* **154,** 1–9
60. Boyer, H. W., and Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–472
61. O'Connell-Motherway, M., van Sinderen, D., Morel-Deville, F., Fitzgerald, G. F., Ehrlich, S. D., and Morel, P. (2000) *Microbiology* (*Read.*) **146,** 935–947
62. Arthur, M., Depardieu, F., Molinas, C., Reynolds, P., and Courvalin, P. (1995) *Gene* (*Amst.*) **54,** 87–92
63. Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997) *Science* **278,** 631–637
64. Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L., Studholme, D. J., Yeats, C., and Eddy, S. R. (2004) *Nucleic Acids Res.* **32,** 138–141
65. Noirot-Gros, M. F., Soultanas, P., Wigley, D. B., Ehrlich, S. D., Noirot, P., and Petit, M. A. (2002) *Mol. Genet. Genomics* **267,** 391–400
66. Maguin, E., Duwat, P., Hege, T., Ehrlich, D., and Gruss, A. (1992) *J. Bacteriol.* **174,** 5633–5638
67. Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M. J., Kuipers, O. P., van Sinderen, D., and Kok, J. (2007) *J. Bacteriol.* **189,** 3256–3270