

## Two Homologous Agr-Like Quorum-Sensing Systems Cooperatively Control Adherence, Cell Morphology, and Cell Viability Properties in *Lactobacillus plantarum* WCFS1<sup>∇</sup>

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Received 14 September 2007/Accepted 9 September 2008

**A two-component regulatory system of *Lactobacillus plantarum*, encoded by genes designated *lamK* and *lamR* (*hpk10* and *rrp10*), was studied. The *lamK* and *lamR* genes encode proteins which are highly homologous to the quorum-sensing histidine kinase LamC and the response regulator LamA, respectively. Transcription analysis of the *lamKR* operon and the *lamBDCA* operon and liquid chromatography-mass spectrometry analysis of production of the LamD558 autoinducing peptide were performed for  $\Delta lamA$ ,  $\Delta lamR$ ,  $\Delta lamA \Delta lamR$  deletion mutants and a wild-type strain. The results suggested that *lamA* and *lamR* are cooperating genes. In addition, typical phenotypes of the  $\Delta lamA$  mutant, such as reduced adherence to glass surfaces and filamentous cell morphology, were enhanced in the  $\Delta lamA \Delta lamR$  mutant. Microarray analysis suggested that the same cell wall polysaccharide synthesis genes, stress response-related genes, and cell wall protein-encoding genes were affected in the  $\Delta lamA$  and  $\Delta lamA \Delta lamR$  mutants. However, the regulation ratio was more significant for the  $\Delta lamA \Delta lamR$  mutant, indicating the cooperative effect of LamA and LamR.**

Quorum-sensing systems are known to affect many intriguing bacterial phenotypes, such as virulence, competence, antibiotic production, and biofilm formation (10). In gram-positive bacteria quorum-sensing systems are mediated mainly by two-component systems (2CSs) consisting of a membrane-bound histidine protein kinase (HPK) and a response regulator (RR), which are regulated by autoinducing peptides (AIPs). Genes encoding these 2CSs are generally arranged in an operon consisting of the HPK-, RR-, and AIP-encoding genes (21). In a quorum-sensing 2CS, the HPK normally belongs to the subfamily designated HPK<sub>10</sub> (11), and the RR belongs to the LytTR family (25). The 2CS transmits the AIP signal through the cell membrane and eventually triggers expression of the operon encoding the 2CS and AIP itself (autoinduction), as well as different target genes involved in, for example, virulence, competence, bacteriocin production, and biofilm formation.

The accessory gene regulator system (*agr* system) is one of the best-studied quorum-sensing systems in gram-positive bacteria. In pathogenic staphylococci, the *agr* system has a critical role in biofilm formation and probably in the invasion of host cells (5, 31), and more than 100 genes are under the control of

this system (7, 26, 43–45). The *agr* gene clusters are conserved throughout the genus *Staphylococcus* and are divided into more than four groups, which are defined by the mutual inhibition by the peptides of the *agr* response in heterologous pairs. Recent genome sequencing projects have revealed that the genomes of several pathogenic bacteria, such as *Listeria monocytogenes* (3) and *Enterococcus faecalis* (12, 24), contain *agr* homologues and that these genes are important for virulence. In addition, systems homologous to the *Agr* system are present in some nonpathogenic commensal organisms, such as *Lactobacillus plantarum* (19), *Bacillus subtilis* (8), and *Roseburia inulinivorans* (30). However, the role of systems homologous to the *agr* system in nonpathogenic bacteria has not been clarified yet.

*L. plantarum* is found in fermented food products, on plant material (6), and as a natural inhabitant of the human gastrointestinal tract (2). We previously studied the function and mechanism of the *agr*-like operon (*lam*) in *Lactobacillus* using molecular analysis based on the genome sequence of *L. plantarum* WCFS1, a single-colony isolate obtained from *L. plantarum* NCIMB8826, which was originally isolated from human saliva (National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom) (13, 38). The genome sequence revealed that the *lamBDCA* operon (lp\_3582 to lp\_3580) contains an AIP-encoding gene (*lamD*), an HPK-encoding gene (*lamC*), an RR-encoding gene (*lamA*), and an AIP export/modification protein-encoding gene (*lamB*). A peptide was purified from the cultured media of a LamBD-overexpressing strain, and the most abundant peptide, which was designated LamD558, was found to be a cyclopentapeptide thiolactone (19, 37).

Global gene expression analysis using a microarray of a

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<sup>∇</sup> Published ahead of print on 19 September 2008.

TABLE 1. Bacterial strains and plasmids used in this study and relevant characteristics

Bacterial strain or plasmid	Relevant properties <sup>a</sup>	Source or reference
<i>Escherichia coli</i> JM109	Cloning host for pUC19ery and its derivatives	Promega
<i>Lactobacillus plantarum</i> WCFS1	Wild type, single-colony isolate from human saliva isolate NCIMB8826	Hayward <sup>b</sup>
<i>Lactobacillus plantarum</i> MS $\Delta$ lamA	Double-crossover mutant of <i>L. plantarum</i> WCFS1 with <i>lamA</i> replaced by <i>lamA::tag</i> gene replacement cassette	Sturme <sup>c</sup>
<i>Lactobacillus plantarum</i> TF $\Delta$ lamR	Double-crossover mutant of <i>L. plantarum</i> WCFS1 with <i>lamR</i> replaced by $\Delta$ lamR::tag gene replacement cassette	This study
<i>Lactobacillus plantarum</i> TF $\Delta$ lamA/ $\Delta$ lamR	Double-crossover mutant of <i>L. plantarum</i> WCFS1 with <i>lamA</i> and <i>lamR</i> replaced by <i>lamA::tag</i> and $\Delta$ lamR gene replacement cassettes, respectively	This study
pUC19ery	Amp <sup>r</sup> Em <sup>r</sup> , 3.8-kb derivative of pUC19 containing 1.1-kb HinPI fragment of pIL253 carrying the Ery <sup>r</sup> gene	van Kranenburg <sup>d</sup>
pDel-LamR-WCFS7	pUC19ery vector carrying knockout $\Delta$ lamR gene replacement cassette	This study

<sup>a</sup> Em<sup>r</sup>, erythromycin resistant; Cm<sup>r</sup>, chloramphenicol resistant; Amp<sup>r</sup>, ampicillin resistant.

<sup>b</sup> See reference 13.

<sup>c</sup> See reference 38.

<sup>d</sup> See reference 40.

*lamA* knockout mutant ( $\Delta$ lamA) showed that the *cps2* operon (lp<sub>1197</sub> to lp<sub>1211</sub>), which is one of the exopolysaccharide-related operons, was upregulated more than 10-fold in this mutant. Phenotypic analysis suggested that the  $\Delta$ lamA cells showed reduced adherence to glass surfaces. These results suggested that the *lamBDCA* operon was related to the cell surface functionality of *L. plantarum*.

Interestingly, the  $\Delta$ lamA mutant was able to produce 50% of the LamD558 peptide produced by the wild type and 30% of the transcripts of the *lamBDCA* operon produced by the wild type, suggesting that there was cross-regulation with other genes (4). The genome information revealed that 13 putative 2CSs were present in this strain, and 5 of them were predicted to be involved in quorum sensing, as deduced from their sequences, as previously reported (19, 22, 35, 36). In addition, the genes encoding one of these 2CSs, lp<sub>3088</sub> and lp<sub>3087</sub> (*hpk10* and *rrp10*), are highly homologous to *lamC* and *lamA*, respectively. We speculated that this operon is also related to the *lam* system and designated it *lamKR*. In this study, we constructed a *lamR* knockout mutant ( $\Delta$ lamR mutant) and a *lamA lamR* double-knockout mutant ( $\Delta$ lamA  $\Delta$ lamR mutant). The global gene expression profiles and phenotypes of these  $\Delta$ lam mutants were studied, and the results suggested that the *lamKR* operon encodes a novel quorum-sensing 2CS that controls the expression of cell surface-related genes of *L. plantarum* in concert with the *lamBDCA* operon.

#### MATERIALS AND METHODS

**Strains, plasmids, and media.** The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* JM109 was cultivated aerobically at 37°C in Luria-Bertani broth or in brain heart infusion broth when there was selection for erythromycin resistance (Difco). *L. plantarum* WCFS1 and its derivatives were cultivated in Mann-Rogosa-Shape broth (MRS) (Difco) at 30 or 37°C without agitation. Solid media contained 1.5% (wt/vol) agar. Where appropriate, erythromycin was added as follows: 150  $\mu$ g ml<sup>-1</sup> for *E. coli* or 5  $\mu$ g ml<sup>-1</sup> for *L. plantarum* WCFS1.

**DNA isolation and construction of integration plasmids.** Genomic DNA was isolated from 5 ml of an *L. plantarum* WCFS1 culture as previously described and was used as a template for PCR. PCR was performed using proofreading Platinum Pfx DNA polymerase (Invitrogen).

Primers lamR-XbaI and lamR-SmaI1 and primers lamR-SmaI2 and lamR-BamHI were used to amplify the 5' and 3' ends of *lamR* (Table 2) and the regions flanking *lamR* (approximately 1.2 kb on each side). PCR products were cloned

into the nonreplicating integration vector pUC19ery (41) after digestion of the PCR products and vector with the appropriate restriction enzymes (Gibco BRL). Plasmids were transformed into competent cells of *E. coli* JM109 by a calcium procedure as recommended by the manufacturer (Promega). This resulted in plasmid pDel-LamR-WCFS7 containing the complete gene replacement cassette with the mutated RR gene.

**Replacement of the *L. plantarum* WCFS1 *lamR* gene.** *L. plantarum* WCFS1 (13, 19) was transformed by electroporation, essentially as previously described (16), using electrocompetent cells of *L. plantarum* that were grown in MRS supplemented with 1% glycine and prepared in 30% polyethylene glycol 1450. *L. plantarum* cells were transformed with 2  $\mu$ g of integration plasmid pDel-LamR-WCFS7, and integrants were selected by plating the resulting bacteria on MRS agar supplemented with erythromycin (5  $\mu$ g ml<sup>-1</sup>) and incubating the plates in an anaerobic jar at 30°C for 2 to 4 days. Single-crossover integration was confirmed by colony PCR using primers lamR-F and lamR-BamHI (Table 2). For this purpose, single colonies were suspended in 20  $\mu$ l Tris-EDTA and cells were disrupted by microwave treatment for 3 min at 750 W and subsequent incubation for 10 min at 95°C, after which PCR analysis was performed. The single-crossover mutant was subsequently propagated for 200 generations in MRS without erythromycin to obtain the anticipated erythromycin-sensitive (Em<sup>s</sup>) *lamR* replacement mutant after a second homologous recombination event. The anticipated gene organization of the *lamR* locus in the gene replacement mutant obtained was verified by enumeration of smaller fragments by colony PCR using primers lamR-F and lamR-BamHI, as well as by Southern blot analysis using standard procedures (29).

**LC-MS analysis of culture supernatant.** *L. plantarum* strains (the wild-type strain and the  $\Delta$ lamA,  $\Delta$ lamR, and  $\Delta$ lamA  $\Delta$ lamR mutants) were grown to an optical density at 600 nm (OD<sub>600</sub>) of 4.0 in a chemically defined medium supplemented with 1% glucose (18). The culture was diluted 1:20 to obtain an OD<sub>600</sub> of 0.15 in 10 ml fresh chemically defined medium and incubated for 18 h at 30°C. Culture supernatant was partially purified with a Sep-Pak C<sub>18</sub> cartridge column and was then analyzed by liquid chromatography (LC) (Agilent HP1100 column; Agilent Zorbax Eclipse XDB-C18; 2.1 by 50 mm) and mass spectrometry (MS) (JEOL Accutof T100LC; JEOL, Tokyo, Japan) as previously described (38). After scanning for molecular ions derived from column elutes, extracted ion chromatograms were plotted with detector counts at *m/z* 559.

**Adherence assays.** A quantitative adherence assay was performed as previously described (34), with slight modifications. Briefly, cells were grown in 1 ml MRS in 24-well glass bottom plates (TPP, Switzerland) at 37°C for 48 h. The wells were washed twice with phosphate-buffered saline to remove loosely attached cells, and the remaining adherent cells were air dried for about 10 min. For staining, 0.5 ml crystal violet (0.1%) was added, and the preparations were incubated for 30 min and subsequently washed twice with water. The attached and stained cells were removed with 33% acetic acid, and the absorbance at 595 nm was determined. All the assays were performed in triplicate.

**RNA extraction.** Cultures of the wild-type strain and the  $\Delta$ lamA,  $\Delta$ lamR, and  $\Delta$ lamA  $\Delta$ lamR mutants were grown in 50 ml MRS at 37°C using a starting OD<sub>600</sub> of 0.2. Samples (25 ml) were harvested at each growth phase and immediately quenched and mixed with 4 volumes of quenching buffer (69% methanol, 66.7

TABLE 2. Primers used for construction of deletion plasmids and Q-PCR analysis

Primer	Sequence (5'-3')	Remarks
lamR-XbaI	TCTAGAATTGGGACTGGTGGTGCTGTTTAC	Deletion plasmid construction
lamR-SmaII	CCCGGGAGACGGCTTGTAGTTGGTTGCTGAC	Deletion plasmid construction
lamR-SmaII2	CCCGGGATTAGATGATGGTGAACCTGA	Deletion plasmid construction
lamR-BamHI	GAGCTCAAATAATCTTGTAAATGCCTCTTG	Deletion plasmid construction
lamR-F	CAAGTGCTTAATGGGGTAA	Deletion plasmid construction
lp_930-F	CACGGGTGGCGGATAAGGTT	Alkaline shock protein
lp_930-R	TGTCATTTTGTGGCCGCACTC	
lp_1197-2F	GACAAGCACGACCAACTCACA	Cps2A
lp_1197-2R	TAAGCCGACAACCTAGCCCAATCAA	
lp_1204-F	ACGTCATCCATTTTCGCTTTTT	Cps2H
lp_1204-R	GTCTCATTCCACGCATCTCTGT	
lp_3087-F	TATTAGCGCATGTCAAAAGTCAGC	LamR
lp_3087-R	CTCCGTATGCGTCGTCACAAAGAT	
lp_3088-F	TGAGCGCCATTTTGTTCAG	LamK
lp_3088-R	GCCCCGCTCTTGTCCCTTAG	
lp_3414-F	TGGGACGCATACGCAACCTAA	Extracellular protein
lp_3414-R	GCCAGCCAGCACCAGTCC	
lp_3575-F	CGCGTTTCAGATTGCCTTTTT	Integral membrane protein
lp_3575-R	CGTGCCAATCTTCCAACAATA	
lp_3578-F	AAGCCGGTTGTTCCAGTTCAT	Catalase
lp_3578-R	GGCATCCGGCACCTCTTCT	
lp_3579-F	GCATGCCAAGATCCCCACGAA	Negative regulator of proteolysis
lp_3579-R	TCCGGCCCATGTACAGAA	
lp_3581-F	GATTGGCCTATTTGTTCATT	LamC
lp_3581-R	GAGCTTCGATTTTCATTCA	
lp_3582-F	CACCGGCTGTTACAAGAAAGAA	LamB
lp_3582-R	AAACTAATAACAGCGATACAACAA	
lp_3583-F	GATGGGCGCTTAACGGATGGAC	ATP-dependent protease subunit ClpL
lp_3583-R	TCTGGGCGGAAAAATGGTGCTAA	
LDH-F	TGATCCTCGTTCCGTYGATG	Lactate dehydrogenase
LDH-R	CCGATGGTTGCAGTTGAGTAAG	

mM HEPES; pH 6.5) at  $-80^{\circ}\text{C}$  to stop further transcription, as described previously (27). Subsequently, samples were pelleted by centrifugation at  $-20^{\circ}\text{C}$  in a prechilled centrifuge, and cells were resuspended in 0.5 ml cold Tris-EDTA buffer. RNA was isolated using the Macaloid method, essentially as described previously (20), and purified further by on-column DNase I treatment with a High Pure RNA isolation kit (Roche). For DNA microarray analysis, 20- $\mu\text{g}$  RNA aliquots were prepared. All experiments were performed in duplicate.

**cDNA preparation, fluorescent labeling, and hybridization for microarray analysis.** Clone-based DNA microarrays were used to determine the global gene transcription levels. Total-RNA samples (20  $\mu\text{g}$ ) were extracted from log-phase cultures, and Cy3- and Cy5-labeled cDNAs were prepared using a Cyscribe postlabeling kit (GE Healthcare, United Kingdom). Unincorporated dyes were removed from labeled fragments by using Autoseq G50 columns (GE Healthcare, United Kingdom). Slides were prehybridized for 45 min at  $42^{\circ}\text{C}$  in 20 ml prehybridization solution (1% bovine serum albumin,  $5\times$  SSC, 0.1% sodium dodecyl sulfate; filtered) ( $1\times$  SSC is 0.15 NaCl plus 0.015 M sodium citrate), washed in filtered deionized water, and dried. Cohybridization with Cy5- and Cy3-labeled cDNA probes was performed overnight at  $42^{\circ}\text{C}$  for 16 h in Slidehyb#1 (Ambion, Austin, TX). The slides were then washed twice in  $1\times$  SSC-0.1% sodium dodecyl sulfate and twice in  $1\times$  SSC before they were scanned. The experiments were performed in duplicate with Cy5/Cy3 dye swaps.

**Microarray scanning and data analysis.** Slides were scanned with a ScanArray Express 4000 scanner (Perkin Elmer, Wellesley, MA), and image analysis and processing were performed using the ImaGene version 4.2 software package (BioDiscovery, El Segundo, CA). The following criteria were used for flagging spots: (i) empty spot threshold, 2.0; (ii) poor spot threshold, 0.4; and (iii) negative spots. Routinely, more than 80% of all spots met these quality criteria. Raw data were stored in BASE (28). Flagged data were discarded, and the remaining high-quality spot data were normalized using a LOWESS fit with M-A transformed data. To calculate a regulatory ratio for each gene for the genes represented by clones on the microarray, a weighted average of the M values of all clones that overlapped with the gene of interest was calculated. The weight used for each clone was equal to the square of the overlap between the gene and clone divided by the total length of the gene. Consequently, this method weighted small overlapping fragments less than proportionally compared to larger overlapping fragments. Statistical analysis was performed with the statistical software program R (14), using the Maanova analysis of variance (ANOVA) model-fitting package (17). Significant effects due to mutation (two levels) and experiment (two levels) and their interactions were observed at different levels of significance ( $P < 0.05$  and  $P < 0.01$ ). Clones displaying these effects were selected and analyzed with the Eisen CLUSTER program (<http://rana.lbl.gov/EisenSoftware.htm>).

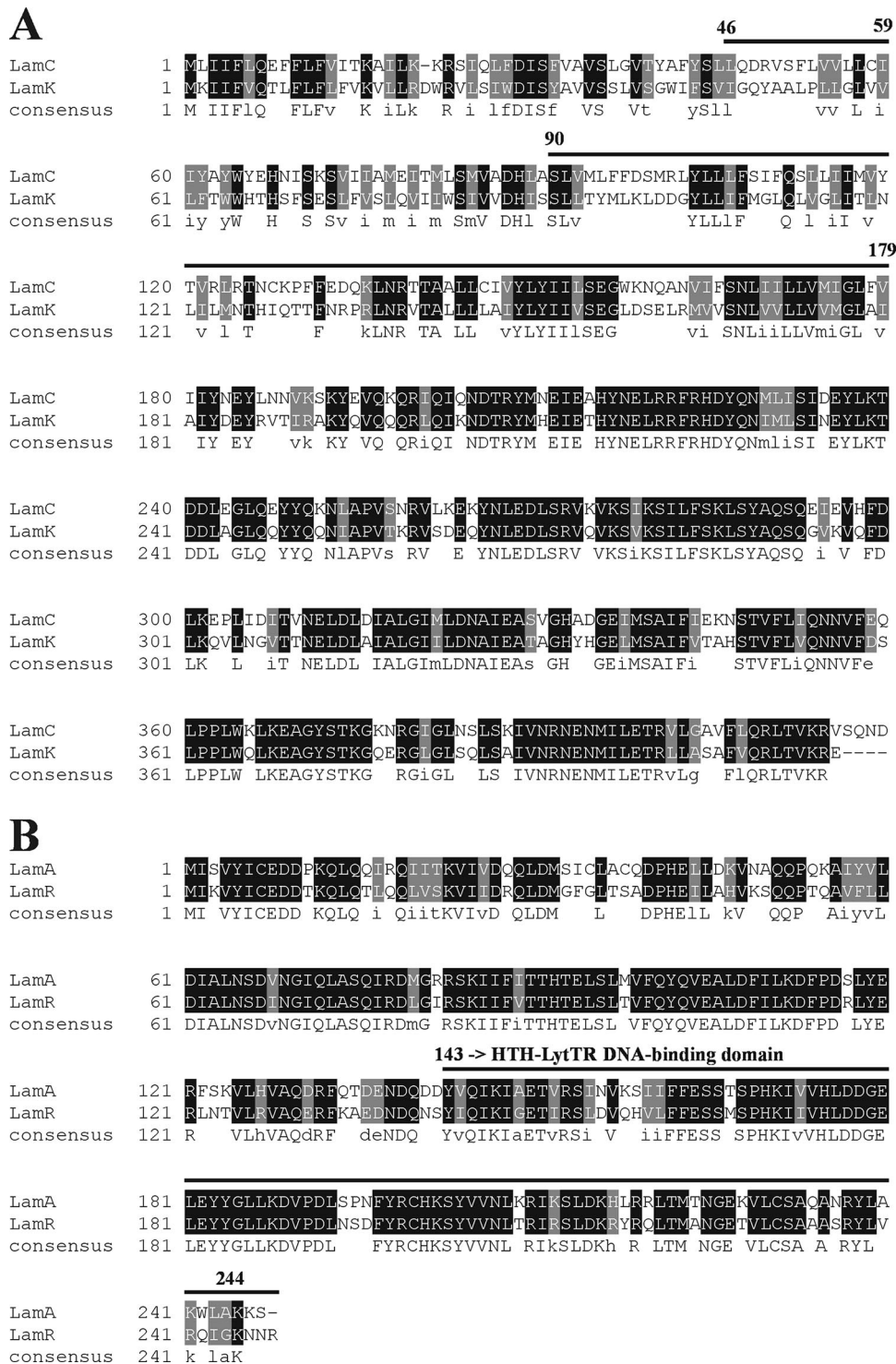


FIG. 1. Alignments of the sequences of (A) LamC and LamK and (B) LamA and LamR. Alignment was performed using ClustalW (<http://searchlauncher.bcm.tmc.edu/multi-align.html>) and BOXSHADE ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Highly conserved residues are indicated by a black (identical) or gray (similar physicochemical characteristics) background. Bold lines with residue numbers indicate regions that define AIP specificity for the staphylococcal AgrC protein (A) or the HTH-LytTR DNA-binding domain of AgrA-like proteins (B).

**Q-PCR analysis.** cDNA was synthesized from purified RNA using random hexamers as a primers and Superscript III (Invitrogen). All the procedures were performed according to the manufacturer's instructions. The quantitative PCR (Q-PCR) primers which were used are shown in Table 2. The products were quantified using an iQ5 i-Cycler (Bio-Rad), and amplification was performed

with a iQ5 Sybr green Supermix kit (Bio-Rad). All results were normalized using the level of the *L. plantarum* lactate dehydrogenase gene, and assays were performed in triplicate.

**Cell morphology analysis.** For analysis of cell morphology the strains (wild-type strain and the  $\Delta lamR$ ,  $\Delta lamA$ , and  $\Delta lamA \Delta lamR$  mutants) were grown



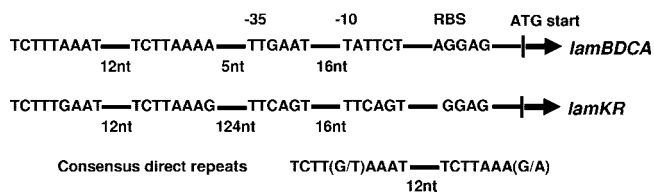


FIG. 2. Alignment of the promoter sequences of the *lamBDCA* operon and the *lamKR* operon. nt, nucleotides.

under a wide range of conditions. Liquid cultures were grown anaerobically in MRS to an  $OD_{600}$  of 0.6 (mid-log phase) or  $>3$  (late log phase). Solid-phase cultures were grown for 30 h on MRS agar or on strips of the porous ceramic Anopore (36 by 8 mm; Whatman, United Kingdom) placed on MRS agar. Cell morphology was examined immediately. Cells were visualized by staining with the fluorogenic dye Syto 9, followed by imaging with an Olympus BX-41 fluorescence microscope (15). Data were captured using an 8-bit Kappa charge-coupled device camera, and cell lengths were determined using TIFF images and ImageJ software (<http://rsb.info.nih.gov/ij/>). The lengths of at least 300 randomly selected cells were determined for each strain for each growth or storage condition, and assays were repeated at least twice. Calculations were performed using Microsoft Excel and custom scripts written for the R software package for multivariate (ANOVA) and posthoc (Tukey honestly significant difference) statistical tests (14). For the ANOVA the logarithmically transformed cell lengths were fitted to a linear model with *lamA* and *lamR* effects (two levels each; wild type and a mutant) and an incubation effect (five levels; mid-exponential growth phase, late exponential growth phase, incubation in the refrigerator for up to 21 days, incubation in the refrigerator for up to 21 days on Anopore, and incubation at room temperature for up to 7 days). The model included two- and three-way interaction effects.

**Colony phenotype analysis.** A phenotypic analysis of colonies was performed by staining the colonies with a mixture of two fluorogenic dyes, followed by imaging entire colonies from above by using low-power fluorescence microscopy. Colonies of the wild-type strain and the  $\Delta lamA$ ,  $\Delta lamR$ , and  $\Delta lamA \Delta lamR$  mutants were grown for 30 h on strips of Anopore placed on MRS agar and were then stained by transfer of the strips to microscope slides with a 1-mm-thick film of 1% (wt/vol) low-melting-point agarose containing the fluorogenic dyes Syto 9 and propidium iodide. This stained the colonies from beneath, essentially as previously described (15). Imaging and data capture were performed as described above using a  $\times 4$  Fluorotar objective lens (Olympus, The Netherlands), and images were merged using Photoshop software (Adobe). All images were taken with equivalent camera exposures.

## RESULTS

**Similarity of the *lamKR* and *lamBDCA* operons of *L. plantarum* WCFS1.** In the first part of this study, we compared the amino acid sequences of the HPKs and RRs which are encoded by the *lamBDCA* operon and the *lamKR* operon (Fig. 1). The results suggested that overall level of amino acid identity of the histidine kinase LamK and LamC was 55%, and the C-terminal ATPase domains were highly homologous. LamR

and LamA have a helix-turn-helix Lyt-TR motif, which is a structure typical of quorum-sensing RRs (25). The overall level of amino acid identity of LamR and LamA was 70%.

A comparison of flanking DNA sequences of the two operons suggested that a direct repeat with a high level of similarity was located in the 5' flanking region of both operons (Fig. 2). These results suggested that *lamKR* might be a novel quorum-sensing operon that is similar to *lamBDCA* in terms of both gene expression and function.

**Cooperation of LamA and LamR in transcription regulation and LamD558 peptide production.** To confirm that LamA and LamR are functionally related, the  $\Delta lamR$  and  $\Delta lamA \Delta lamR$  mutants were constructed. The growth of the  $\Delta lamA$ ,  $\Delta lamR$ ,  $\Delta lamA \Delta lamR$  mutants in MRS media was not significantly different from that of the wild-type strain (data not shown).

The transcription of the *lamBDCA* and *lamKR* operons and the production of the autoinducing peptide LamD558 were studied, and the results confirmed that the level of transcription of *lamKR* was much lower than that of *lamBDCA*, while the expression kinetics of *lamKR* were similar to those of *lamBDCA* in the wild-type strain (Fig. 3).

The transcription of the *lamBDCA* operon in the  $\Delta lamA$  mutant was repressed and was one-third of the transcription of the *lamBDCA* operon in wild-type strain, as previously reported (37). In the  $\Delta lamR$  mutant, the transcription of this operon was affected only slightly and was 80% of that in the wild-type strain. A dramatic reduction was observed in  $\Delta lamA \Delta lamR$  mutant, in which the transcription of the *lamBDCA* operon was less than 5% of that in the wild-type strain (Fig. 3). The transcription of the *lamKR* operon in the  $\Delta lamA$  and  $\Delta lamA \Delta lamR$  mutants was repressed and was one-third of that in the wild-type strain, and it was slightly repressed in the  $\Delta lamR$  mutant.

LC-MS analysis of culture supernatant suggested that the LamD558 production in the  $\Delta lamR$  mutant was not significantly different from that in the wild-type strain. However, the *lamR* disruption significantly affected AIP production in the  $\Delta lamA$  mutant. In the  $\Delta lamA$  mutant the level of LamD558 was about 50% of the level in the wild-type strain, but no peptide production was observed in the  $\Delta lamA \Delta lamR$  mutant (Fig. 4). Taken together, the results show that LamR is functionally similar to LamA, and these two RR proteins appear to cooperate to regulate this quorum-sensing system.

**Cooperative effect of *lamA* and *lamR* on global gene expression.** In order to study the effect of the *lamR* gene on global gene expression, microarray analyses were performed. No gene

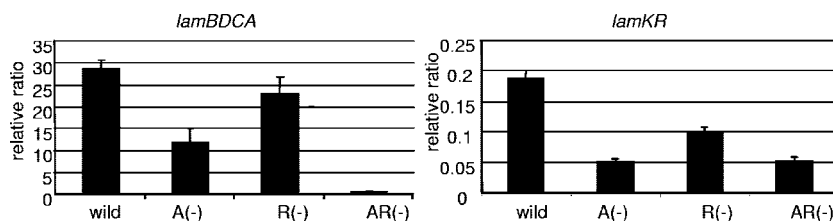


FIG. 3. Gene expression ratios for the *lamBDCA* operon and the *lamKR* operon during cell growth for the wild-type strain and the  $\Delta lamA$ ,  $\Delta lamR$ , and  $\Delta lamA \Delta lamR$  mutants. Cell cultures grown in MRS at 37°C without agitation were collected in the logarithmic growth phase. Total RNA was isolated and cDNA synthesis was performed as described in Materials and Methods. The vertical line indicates the relative level of gene expression compared to expression of the control lactate dehydrogenase gene. All Q-PCR analyses were performed in triplicate. A(-),  $\Delta lamA$  mutant; R(-),  $\Delta lamR$  mutant; AR(-),  $\Delta lamA \Delta lamR$  mutant.

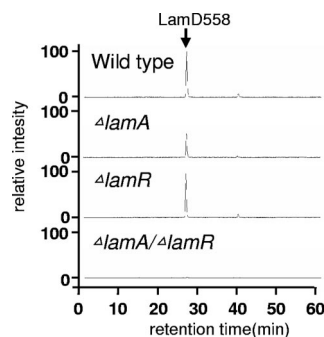


FIG. 4. LC-MS analysis of LamD558 production in the culture supernatants of wild-type and mutant strains of *L. plantarum*. The overnight culture supernatant of each strain was partially purified by using a Sep-Pak C<sub>18</sub> cartridge column and then analyzed by LC-MS as described in Materials and Methods. Extracted ion chromatograms were plotted with detector counts at *m/z* 559, corresponding to the protonated molecular ion of LamD558.

showed a significant (more than twofold) difference in the level of expression in the  $\Delta lamR$  mutant compared to the wild-type strain (data not shown). This result suggested that the lack of LamR might be functionally covered by LamA, since the level of expression of *lamR* was much lower than the level of expression of *lamA*.

The results of comparisons of the  $\Delta lamA \Delta lamR$  mutant with the wild-type strain and of the  $\Delta lamA \Delta lamR$  mutant with the  $\Delta lamA$  mutant are shown in Tables 3 and 4, respectively. Compared with the wild-type strain, 14 genes were upregulated more than threefold and 11 genes were upregulated more than fivefold in the  $\Delta lamA \Delta lamR$  mutant, while 39 genes were downregulated more than twofold and 20 genes were downregulated more than fivefold (Table 3). Most of these genes were previously reported to be regulated in the  $\Delta lamA$  mutant (38). Typical examples of the commonly regulated genes were the genes in the *cps2* operon (from lp\_1197 to lp\_1205), membrane protein-encoding genes (lp\_0926, lp\_3575, and lp\_3577),

TABLE 3. Genes whose levels of expression were significantly different between the  $\Delta lamA \Delta lamR$  mutant and the wild-type strain

Open reading frame	Gene	Product	Change (fold) <sup>a</sup>
<b>Upregulated genes</b>			
lp_0783		Lipoprotein precursor, peptide-binding protein OppA homolog	3.1
lp_1197	<i>cps2A</i>	Exopolysaccharide biosynthesis protein	27.8
lp_1198	<i>cps2B</i>	Exopolysaccharide biosynthesis protein; chain length determiner Wzz	54.9
lp_1200	<i>galE2</i>	UDP-glucose 4-epimerase	11.9
lp_1201	<i>cps2E</i>	Priming glycosyltransferase	16.0
lp_1202	<i>cps2F</i>	Glycosyltransferase	15.8
lp_1203	<i>cps2G</i>	Glycosyltransferase	11.5
lp_1204	<i>cps2H</i>	Polysaccharide polymerase	5.9
lp_1205	<i>cps2I</i>	Repeat unit transporter	6.4
lp_1207		No product defined	4.4
lp_3411		Extracellular protein	4.3
lp_3412		Extracellular protein	8.1
lp_3413		Cell surface protein precursor	13.5
lp_3414		Extracellular protein	7.4
<b>Downregulated genes</b>			
lp_0023	<i>glgA</i>	Starch (bacterial glycogen) synthase	-5.1
lp_0111		Oxidoreductase	-5.9
lp_0525	<i>kup1</i>	Potassium uptake protein	-3.3
lp_0683		Prophage P1 protein 60	-4.0
lp_0885	<i>bgIG1</i>	Transcription antiterminator	-12.4
lp_0926		Integral membrane protein	-21.4
lp_0927		Hypothetical protein	-16.9
lp_0930	<i>asp2</i>	Alkaline shock protein	-5.3
lp_1703		Hypothetical protein	-4.7
lp_2658		Glycosyltransferase (putative)	-3.2
lp_3045		Short-chain dehydrogenase/oxidoreductase	-6.1
lp_3082		Transport protein	-5.5
lp_3084		Cell surface protein, ErfK family	-7.1
lp_3085	<i>asnB2</i>	Asparagine synthase (glutamine-hydrolyzing)	-7.7
lp_3087	<i>lamR</i>	RR	-4.2
lp_3128		Stress-induced DNA binding protein	-4.0
lp_3267	<i>gshR4</i>	Glutathione reductase	-3.5
lp_3420	<i>gadB</i>	Glutamate decarboxylase	-2.6
lp_3575		Integral membrane protein	-14.4
lp_3577		Integral membrane protein	-5.0
lp_3578	<i>kat</i>	Catalase	-5.6
lp_3579	<i>nprR5</i>	Negative regulator of proteolysis	-117.8
lp_3580	<i>lamA</i>	RR; accessory gene regulator protein A	-28.7
lp_3582	<i>lamB</i>	Accessory gene regulator protein B	-14.9
lp_3583	<i>clpL</i>	ATP-dependent Clp protease, ATP-binding subunit ClpL	-10.8
lp_3586	<i>lox</i>	Lactate oxidase	-32.4

<sup>a</sup> Change in gene expression in the  $\Delta lamA \Delta lamR$  mutant compared to the wild-type strain.

TABLE 4. Genes whose levels of expression were significantly different between the  $\Delta lamA$   $\Delta lamR$  and  $\Delta lamA$  mutants

Open reading frame	Gene	Product	Change (fold) <sup>a</sup>
<b>Upregulated genes</b>			
lp_0152		Transcription regulator	2.8
lp_0177	<i>malG</i>	Maltose/maltodextrin ABC transporter, permease protein	2.2
lp_0179	<i>amy2</i>	$\alpha$ -Amylase	2.3
lp_0180	<i>msmK1</i>	Multiple-sugar ABC transporter, ATP-binding protein	3.2
lp_0302		Extracellular protein	2.4
lp_0547	<i>ftsH</i>	Cell division protein FtsH, ATP-dependent zinc metallopeptidase	2.5
lp_0578	<i>npsA</i>	Nonribosomal peptide synthetase NpsA	2.1
lp_1197	<i>cps2A</i>	Exopolysaccharide biosynthesis protein	2.1
lp_1198	<i>cps2B</i>	Exopolysaccharide biosynthesis protein; chain length determiner Wzz	2.6
lp_1200	<i>galE2</i>	UDP-glucose 4-epimerase	2.4
lp_1201	<i>cps2E</i>	Priming glycosyltransferase	2.7
lp_1203	<i>cps2G</i>	Glycosyltransferase	2.3
lp_1730	<i>map3</i>	Maltose phosphorylase	2.1
lp_2151	<i>pdhD</i>	Pyruvate dehydrogenase complex, E3 component; dihydrolipoamide dehydrogenase	2.1
lp_2352		Amino acid ABC transporter, ATP-binding protein	2.6
lp_3411		Extracellular protein	2.1
lp_3412		Extracellular protein	7.6
lp_3413		Cell surface protein precursor	9.8
lp_3414		Extracellular protein	7.2
<b>Downregulated genes</b>			
lp_0254	<i>cysE</i>	Serine <i>O</i> -acetyltransferase	-2.2
lp_0255	<i>metC1</i>	Cystathionine beta-lyase	-2.2
lp_2833		Transport protein	-2.4
lp_3084		Cell surface protein, ErfK family	-2.3
lp_3085	<i>asnB2</i>	Asparagine synthase (glutamine hydrolyzing)	-2.4
lp_3087	<i>lamK</i>	RR	-2.4
lp_3575		Integral membrane protein	-3.0
lp_3579	<i>nrpR5</i>	Negative regulator of proteolysis	-10.7
lp_3580	<i>lamA</i>	RR; accessory gene regulator protein A	-16.3
lp_3582	<i>lamB</i>	Accessory gene regulator protein B	-7.2

<sup>a</sup> Change in gene expression in the  $\Delta lamA$   $\Delta lamR$  mutant compared to the  $\Delta lamA$  mutant.

stress response genes (lp\_0930 and lp\_3578), and the *lamB-DCA* operon itself (lp\_3580 to lp\_3583).

In addition, we observed that these genes were more significantly up- or downregulated in the  $\Delta lamA$   $\Delta lamR$  mutant than in the  $\Delta lamA$  mutant. For instance, the first three genes in the *cps2* operon, lp\_1197, lp\_1198, and lp\_1199, were upregulated 8.3-, 9.1-, and 4.4-fold, respectively, in the  $\Delta lamA$  mutant compared to the wild-type strain (data not shown), while they were upregulated 19.0-, 15.7-, and 15.3-fold in the  $\Delta lamA$   $\Delta lamR$  mutant compared to the wild-type strain (Table 3).

To more clearly determine the effect of knocking out both *lam* operons, a direct comparison of the  $\Delta lamA$   $\Delta lamR$  and  $\Delta lamA$  mutants was performed. Nineteen genes were upregulated significantly (more than twofold), while 10 genes were downregulated more than twofold in the  $\Delta lamA$   $\Delta lamR$  mutant compared to the  $\Delta lamA$  mutant (Table 4). As expected, the *cps2* operon was activated nearly twofold in the  $\Delta lamA$   $\Delta lamR$  mutant compared with the  $\Delta lamA$  mutant. An additive effect was also observed for the downregulated genes, such as genes in the *lamKR* operon (lp\_3087), lp\_3084, lp\_3085, lp\_3575, and lp\_3579.

To our surprise, the operon comprising lp\_3412 to lp\_3414 was synergistically regulated in the  $\Delta lamA$   $\Delta lamR$  mutant. The levels of expression of this operon in the  $\Delta lamA$  and  $\Delta lamR$  mutants were not significantly different, but this operon was uniquely upregulated more than fivefold in the  $\Delta lamA$   $\Delta lamR$

mutant compared the wild-type strain and the  $\Delta lamR$  and  $\Delta lamA$  mutants (Tables 3 and 4).

**Q-PCR analysis.** RNA samples extracted from the mutants in the early, middle, and late log phases were analyzed by Q-PCR to confirm the observations obtained in the microarray analysis (Fig. 5). The kinetics data suggested that the *lamB-DCA* operon (lp\_3580 and lp\_3581) was completely repressed in the  $\Delta lamA$   $\Delta lamR$  mutant and partially repressed in the  $\Delta lamA$  and  $\Delta lamR$  mutants throughout the growth phases. The difference was most significant in the mid-log phase. Similar regulation was observed for the *lamKR* operon (lp\_3088) and the stress-related protein-encoding genes, such as lp\_3579 (negative regulator of proteolysis), lp\_0929 and lp\_0930 (alkaline shock protein), and lp\_3578 (catalase). Most of these genes are localized in the loci which were designated "life style adaptation regions" (22).

It was also confirmed that the operon comprising lp\_3012 to lp\_3014 was strongly activated in all growth phases and that the highest level was observed in the early growth phase in the  $\Delta lamA$   $\Delta lamR$  mutant. Neither upregulation nor growth-dependent gene expression of this operon was observed in the  $\Delta lamA$  and  $\Delta lamR$  mutants and the wild-type strain.

**Effect of *lamR* on adherence properties of *L. plantarum* WCFS1.** The glass adherence phenotype was determined for the  $\Delta lamA$ ,  $\Delta lamR$ , and  $\Delta lamA$   $\Delta lamR$  mutants and the wild-type strain. As previously reported (38), around fourfold fewer

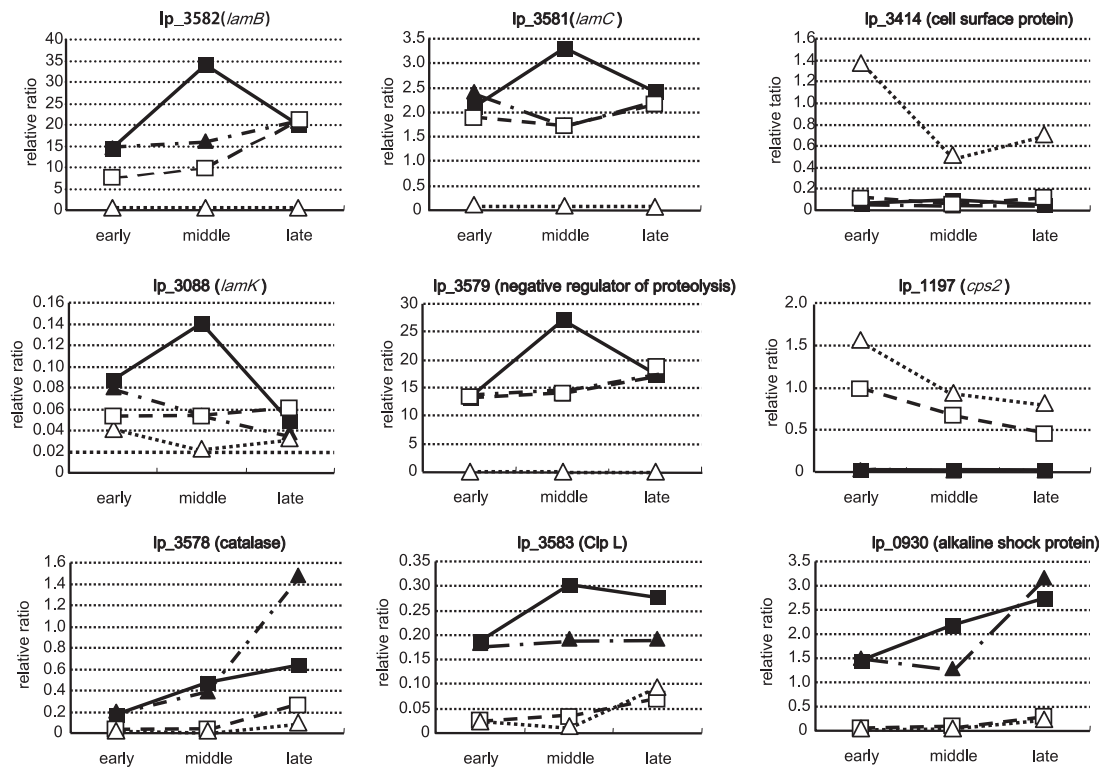


FIG. 5. Kinetics of gene expression: ratios of the wild-type strain to the  $\Delta lamA$ ,  $\Delta lamR$ , and  $\Delta lamA \Delta lamR$  mutants. Cell cultures grown in MRS at 37°C without agitation were collected in the early, middle, and late logarithmic growth phases. Then total RNA was isolated and cDNA synthesis was performed as described in Materials and Methods. The vertical line indicates the relative gene expression level compared to expression of the control lactate dehydrogenase gene. All the Q-PCR analyses were performed in triplicate. ■, wild-type strain; □,  $\Delta lamA$  mutant; ▲,  $\Delta lamR$  mutant; △,  $\Delta lamA \Delta lamR$  mutant.

cells of the  $\Delta lamA$  mutant than of the wild-type strain adhered to a glass surface. In contrast, the number of the  $\Delta lamR$  mutant cells that adhered to a glass surface was almost 75% of the number of the wild-type cells that adhered. The  $\Delta lamA \Delta lamR$  mutant cells showed the least adherence to glass. The number of cells that adhered to a glass surface was less than one-eighth of the number of wild-type cells that adhered (Fig. 6A). When the results shown in Tables 3 and 4 and Fig. 5 were compared, there was clearly a negative correlation between the number of adherent cells and the level of expression of the *cps2* operon; that is, as *cps2* expression increased, the number of adherent cells decreased. This strongly suggested that the *cps2* operon might be important primarily for the glass adherence property of *L. plantarum* cells.

In order to study the effect of the carbon source on glass adherence, the same experiments were performed using cell cultures grown in MRS in which glucose was replaced by maltose, galactose, or raffinose (Fig. 6B, 6C, and 6D). The level of glass adherence was slightly affected by the carbon source. In maltose-containing medium, the level of glass adherence was higher than that in glucose-containing medium. In all media, the *lamA* disruption was more effective in reducing the adherence than the *lamR* disruption, and the  $\Delta lamA \Delta lamR$  mutant showed the least adherence.

**Effect of the *lamA* and *lamR* genes on cell morphology and colony structure.** In the cell morphology experiments, we found that there were differences in cell length between the

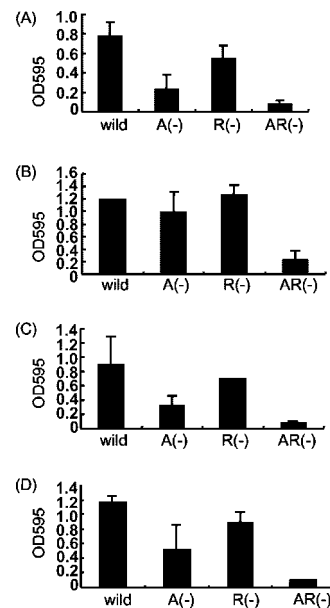


FIG. 6. Quantification of adherence of *L. plantarum* WCFS1 to a glass surface. Cell cultures were cultivated in MRS media containing (A) glucose, (B) maltose, (C) galactose, and (D) raffinose as a unique carbon source for 48 h at 37°C. The quantification methods used are described in Materials and Methods. A(-),  $\Delta lamA$  mutant; R(-),  $\Delta lamR$  mutant; AR(-),  $\Delta lamA \Delta lamR$  mutant.



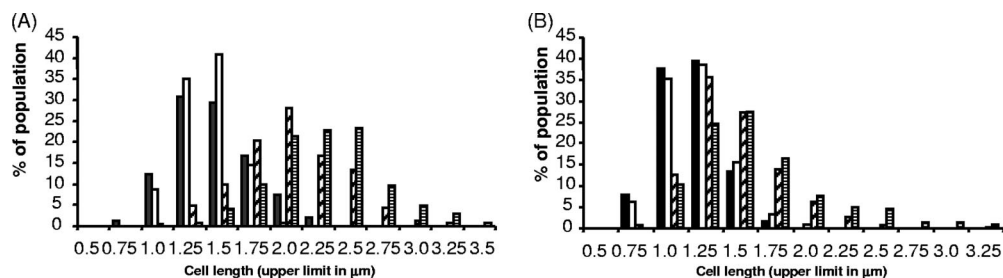


FIG. 7. Cell morphology of  $\Delta lam$  mutants: frequency plots of data used for ANOVA. Sample plots for the two most informative conditions tested, colonial growth (A) and late-log-phase liquid culture (B), are shown. The bars indicate the distributions of cell lengths for 300 cells (expressed as percentages) for the wild-type strain (filled bars), the  $\Delta lamA$  mutant (bars with diagonal lines), the  $\Delta lamR$  mutant (open bars), and the  $\Delta lamA \Delta lamR$  mutant (bars with horizontal lines).

$\Delta lamA$ ,  $\Delta lamR$ , and  $\Delta lamA \Delta lamR$  mutants and the wild-type strain. In order to confirm this, the cell length was quantified using digital images. Multiway ANOVA, using cell length, was performed to assess the cell lengths of the  $\Delta lamR$ ,  $\Delta lamA$ , and  $\Delta lamA \Delta lamR$  mutants compared with the wild-type strain under a range of different storage and growth conditions (Fig. 7). The results strongly suggested that the cell length depended on the expression of both *lam* operons. Both the  $\Delta lamA$  and  $\Delta lamR$  mutations resulted in increases in the mean cell length (ANOVA  $P_r > F$  values,  $< 2 \times 10^{-16}$  and  $3.9 \times 10^{-13}$ , respectively), and the strongest effect was observed for the  $\Delta lamA$  mutation. Again, the interaction of *lamA* and growth conditions was stronger than the interaction of *lamR* and growth conditions ( $P_r > F$  values,  $< 2 \times 10^{-16}$  and 0.01, respectively). The increase in the length of the  $\Delta lamA$  mutant cells was most pronounced under high-cell-density conditions (Fig. 7A). The effect was observed with late-log-phase cells cultured in liquid MRS (Fig. 7B). A more limited effect was also observed in mid-log-phase cells (data not shown). The trend toward more elongated cells was generally stronger for the  $\Delta lamA \Delta lamR$  mutant and weaker for the  $\Delta lamR$  mutant and wild-type strain (Fig. 7). ANOVA for the contribution that each  $\Delta lam$  mutation made to cell length supported the observation that the interaction between the two *lam* systems was significant ( $P_r > F$ ,  $3.5 \times 10^{-6}$ ), meaning that an additive effect was observed when both mutations were present. Also, a significant three-way interaction was observed, meaning that the environmental conditions influenced the degree to which the  $\Delta lam$  mutations interacted ( $P_r > F$ ,  $1.2 \times 10^{-4}$ ). In particular, the interaction was most pronounced when cells were in colony form. Although the significance of these interactions in a linear model does not provide direct evidence for a biochemical interaction between the *lam* systems, it does suggest that there is at least an indirect interaction between the two systems and that this is consistent with the gene regulation data obtained in this study.

Colonies of the  $\Delta lamA$ ,  $\Delta lamR$ , and  $\Delta lamA \Delta lamR$  mutants and the wild-type strain appeared to be similar to the eye in terms of size and morphology. However, when preparations were double stained using the fluorogenic dyes Syto 9 and propidium iodide, phenotypic differences became apparent (Fig. 8). For the wild-type strain, the colonies were stained almost completely with Syto 9. At a high magnification only rare ( $< 0.1\%$ ) filamented cells (length,  $> 20 \mu\text{m}$ ) showed strong propidium iodide staining. All three  $\Delta lam$  mutants exhibited increased propidium iodide staining in the colony center com-

pared to the wild-type strain. In some cases the difference was most extreme in the  $\Delta lamA \Delta lamR$  mutant, and in others the  $\Delta lamA$  and  $\Delta lamA \Delta lamR$  mutants appeared to be similar (Fig. 8). Propidium iodide preferentially stains cells with compromised or damaged membranes, and it is likely that the colony structure or the integrity of cells in the colony center is affected by the  $\Delta lam$  mutations.

Taken together, these data suggested qualitatively that the structure of the colonial growth of the  $\Delta lam$  mutants was affected and that functional *lam* operons play a role in the integrity of cells for colony growth and survival.

## DISCUSSION

In this study, we thoroughly investigated the function of the *lamKR* operon in terms of gene regulation, AIP production, and phenotypes. All of the results strongly suggested that the two *lam* 2CSs function cooperatively. Due to the high levels of homology of the encoded proteins, it is likely that the HPKs and RRs encoded by *lamKR* and *lamCA* cross talk with each other, as is the case in *E. coli* (48). It is also possible that LamA and LamR bind to the promoter regions of both operons and thereby regulate the expression of each other's genes at the transcriptional level, since similar direct repeats were observed in the upstream regions of *lamBDCA* and *lamKR*. PCR experiments with 22 *L. plantarum* strains using primers designed from the consensus sequence of the amino acid regions between LamA and LamR and between LamC and LamK showed that 20 strains contained *lam* genes (data not shown). This may suggest that the *lam* system is widespread among *L.*

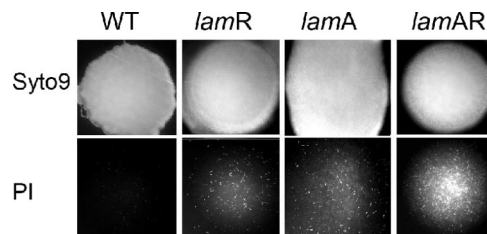


FIG. 8. Colony phenotypes of  $\Delta lam$  mutants and wild-type *L. plantarum*: merged images of typical colonies of *L. plantarum* WCFS1 and *lam* mutants (all approximately 2 mm in diameter) after double staining from beneath with Syto 9 and propidium iodide and imaging by low-power fluorescence microscopy. WT, wild type.

*plantarum* strains. However, more direct in vitro analyses, such as cross-phosphorylation assays with LamA and LamR and gel shift assays with LamA and LamR and with *lamKR* and *lamB-DCA* promoter regions, should be performed to prove these hypotheses. Such analyses are under way in our lab.

Our data also suggested that disruption of *lamR* affected the global gene expression and phenotypes, such as glass adherence and cell length. As expected, the phenotypic differences between the  $\Delta lamR$  mutant and the wild-type strain were similar to those between the  $\Delta lamA$  mutant and the wild-type strain and were most obvious in the  $\Delta lamA \Delta lamR$  mutant.

Biofilm formation is one of the typical quorum-sensing-dependent phenotypes. Several reports have suggested that bacterial biofilm formation is under the control of quorum-sensing systems. In the genus *Staphylococcus*, *agr* mutants have been reported to show increased biofilm formation and primary attachment on polystyrene (43, 45). Further, studies by Tannock et al. (39), Wen et al. (47), and Yoshida et al. (49) suggested that LuxS-based signaling affects biofilm formation. Our results provided another example of initiation of biofilm formation or adherence that was regulated by a quorum-sensing system. However, the genes that have a direct effect on these phenotypes are still unknown. Our microarray and Q-PCR analyses revealed that many gene expression differences observed for the  $\Delta lamA$  mutant were quantitatively enhanced in the  $\Delta lamA \Delta lamR$  mutant. The most probable genes that are responsible for glass adherence are the *cps2* genes, because the number of glass-adherent  $\Delta lamA \Delta lamR$  mutant cells and the level of expression of the *cps2* genes showed a strong negative correlation. It is likely that polysaccharides produced by the enzymes encoded by the *cps2* operon were responsible for the poor glass adherence phenotype.

Cell morphology was another phenotype that was affected by *lam* genes. To our knowledge, this report provides the first direct link between quorum-sensing systems and cell morphology in gram-positive bacteria, although such a link has been reported previously for gram-negative bacteria (33). Microarray analysis revealed that the level of expression of the *cps2* operon was positively correlated to the cell length, suggesting that this operon may play a role in cell length, as well as biofilm formation. The alternative possibility is that filamentous phenotypes are the result of the stress response of *L. plantarum*. Some of the stress-related genes, such as lp\_3575 (integral membrane protein), lp\_3577 (integral membrane protein), lp\_3578 (catalase), lp\_3579 (negative regulator of proteolysis), lp\_3583 (ATP-dependent Clp protease subunit ClpL), and lp\_3586 (lactate oxidase), were downregulated nearly 10-fold in the  $\Delta lamA$  or  $\Delta lamA \Delta lamR$  mutant. The results of Syto 9 and propidium iodide staining also supported the argument that a lack of *lamA* and *lamR* results in strongly stressed cells (Fig. 8). It is well known that bacterial cells have a filamentous morphology under stress conditions (1, 40, 46). A study which demonstrated that a *B. subtilis* mutant with a knockout of ClpP, a proteolytic enzyme with bacterial ATP-dependent proteases, had a filamentous cell morphology appears to be particularly relevant (23). Another study, which showed that ClpL also is involved in cold sensitivity and stress in *Streptococcus thermophilus* (42), was also intriguing since we observed that the differences in cell length of  $\Delta lam$  mutants were more pronounced after cold storage for 1 to 3 weeks (data not shown).

It is likely that expression of the *lam* system is more important at a high cell density, since no difference was observed between the  $\Delta lam$  mutants and the wild-type strain in liquid culture.

Interestingly, an operon which encodes cell surface proteins (lp\_3412, lp\_3413, and lp\_3414) was strongly upregulated throughout growth in the  $\Delta lamA \Delta lamR$  mutant (Tables 3 and 4 and Fig. 5). This operon was overexpressed in neither the  $\Delta lamA$  mutant nor the  $\Delta lamR$  mutant, suggesting that a complete lack of the *lam* system is necessary for the overexpression. Although the function of this operon has not been addressed yet, a recent bioinformatics study suggested that the lp\_3413 protein is a membrane-anchored protein and that the lp\_3412 and lp\_3414 proteins may bind to the lp\_3413 protein to form a functional complex (32). Another interesting study suggested that lp\_3412, lp\_3413, and lp\_3414 are upregulated when the *L. plantarum* strain is in the gut environment (9).

In conclusion, *lamA* and *lamR* are important for *L. plantarum* to adapt to particular environmental niches where they can grow at high densities. Future studies on the survivability and persistence of  $\Delta lam$  mutants in *L. plantarum*-specific environments, such as the gastrointestinal tract, oral cavity, or fermented foods, may provide more information about the functional role of the *lam* systems in nature.

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