JOURNAL OF BACTERIOLOGY, Mar. 2010, p. 1444–1454 Vol. 192, No. 5
0021-9193/10/$12.00 doi:10.1128/JB.01251-09

A Novel Pheromone Quorum-Sensing System Controls the Development of Natural Competence in *Streptococcus thermophilus* and *Streptococcus salivarius*▽†

Laetitia Fontaine,[1] Céline Boutry,[1] Marie Henry de Frahan,[1] Brigitte Delplace,[1] Christophe Fremaux,[2] Philippe Horvath,[2] Patrick Boyaval,[2] and Pascal Hols[1]*

Unité de Génétique, Institut des Sciences de la Vie, Université catholique de Louvain, Place Croix du Sud 5, B-1348 Louvain-la-Neuve, Belgium,[1] and Danisco France SAS, BP 10, 86220 Dangé-Saint-Romain, France[2]

Received 17 September 2009/Accepted 12 December 2009

In streptococcal species, the key step of competence development is the transcriptional induction of *comX*, which encodes the alternative sigma factor σ^X, which positively regulates genes necessary for DNA transformation. In *Streptococcus* species belonging to the *mitis* and *mutans* groups, induction of *comX* relies on the activation of a three-component system consisting of a secreted pheromone, a histidine kinase, and a response regulator. In *Streptococcus thermophilus*, a species belonging to the *salivarius* group, the oligopeptide transporter Ami is essential for *comX* expression under competence-inducing conditions. This suggests a different regulation pathway of competence based on the production and reimportation of a signal peptide. The objective of our work was to identify the main actors involved in the early steps of *comX* induction in *S. thermophilus* LMD-9. Using a transcriptomic approach, four highly induced early competence operons were identified. Among them, we found a Rgg-like regulator (Ster_0316) associated with a nonannotated gene encoding a 24-amino-acid hydrophobic peptide (Shp0316). Through genetic deletions, we showed that these two genes are essential for *comX* induction. Moreover, addition to the medium of synthetic peptides derived from the C-terminal part of Shp0316 restored *comX* induction and transformation of a Shp0316-deficient strain. These peptides also induced competence in *S. thermophilus* and *Streptococcus salivarius* strains that are poorly transformable or not transformable. Altogether, our results show that Ster_0316 and Shp0316, renamed ComRS, are the two members of a novel quorum-sensing system responsible for *comX* induction in species from the *salivarius* group, which differs from the classical phosphorelay three-component system identified previously in streptococci.

Among pathogenic streptococci, some species display the ability to naturally acquire exogenous DNA by entering a physiological state known as "competence for transformation." This mechanism relies on the synthesis of machinery for DNA uptake and recombination. Competence is a virulence determinant (35) and is potentially involved in a number of functions: increasing genome plasticity through the acquisition of new genes (9), DNA repair (46), and/or fulfilling nutritional requirements by supplying a carbon, nitrogen, phosphorus, and energy source (17).

In the model species *Streptococcus pneumoniae* and *Streptococcus mutans*, transformation is tightly regulated by cell density, but also by various environmental stresses. In the literature, competence is increasingly viewed as a general stress response because it is activated by the presence of reactive oxygen species, pH or temperature changes, antibiotics, or mutagens (1, 8, 41). In *S. pneumoniae* and *S. mutans*, development of the competence state involves two steps: early and late. The early step involves 5 genes, *comABCDE* (orthologous to *blpABCHR* in *S. mutans*) (38), encoding a quorum-sensing system responsible for competence activation. The precursor of the inducer peptide of competence, encoded by *comC* (*blpC*), is matured and secreted in the extracellular medium through an ABC-type transporter encoded by *comAB* (*blpAB*) (27, 38). The expression of *comC* increases with cell density and with the presence of stress signals in the medium (41, 46). When its extracellular concentration reaches a critical threshold, the mature peptide activates ComD (BlpH), a transmembrane histidine kinase, which in turn stimulates the phosphorylation of the response regulator ComE (BlpR). The phosphorylated regulator positively regulates the expression of the early genes *comABCDE* (positive feedback loop), as well as *comX*, encoding the alternative sigma factor σ^X, specifically required for activation of the late step of competence. The σ^X factor transiently associates with the RNA polymerase core, which can then bind to the promoter region of the late genes (27). The late essential genes are required for the biosynthesis of the DNA uptake machinery, protection of single-stranded DNA, and integration of the new genetic material by homologous recombination (27, 42). The σ^X factor also induces the expression of genes involved in other functions, such as adaptation to stress conditions (41, 42).

Streptococcus thermophilus, a member of the *salivarius* group, is of major economic importance, since it is widely used for the manufacture of yoghurt and cheese. *In silico* analysis of the genomes of three strains of *S. thermophilus* (LMG18311, CNRZ1066, and LMD-9) has revealed the presence of a *comX* gene and of all the late competence genes essential for natural competence (22). However, it seems to lack orthologues of the

* Corresponding author. Mailing address: Unité de Génétique, Université Catholique de Louvain, Place Croix du Sud 5, B-1348 Louvain-la-Neuve, Belgium. Phone: 32-10-478896. Fax: 32-10-473109. E-mail: hols@gene.ucl.ac.be.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 18 December 2009.

quorum-sensing system that controls *comX* expression in *S. mutans* and *S. pneumoniae*, since the *blpABCHR* genes of *S. thermophilus* were shown to control bacteriocin production in the species (18, 19). In 2006, Blomqvist et al. (4) showed the functionality of natural transformation in *S. thermophilus* LMG18311 at a remarkably high frequency (10^{-2} to 10^{-3}). The strategy was based on the overexpression of *comX* under the control of a BlpC-inducible promoter from *S. thermophilus* (4). Recently, Gardan et al. (20) showed that competence development in *S. thermophilus* can spontaneously turn on during growth in chemically defined medium (CDM). However, competence efficiency under those conditions differs between strains, since LMD-9, LMG18311, and CNRZ1066 were shown to be highly (10^6 transformants/ml), poorly (10^2 transformants/ml), and not transformable, respectively. In strain LMD-9, the oligopeptide ABC transporter Ami was shown to be absolutely required for the transcriptional induction of *comX* and activation of natural transformation under those conditions (20). Furthermore, it was shown that the two oligopeptide-binding proteins AmiA1 and AmiA3 are differentially involved in the triggering of competence, since their inactivation results in 99% and 50% decreases in the transformation rate, respectively (20). In *S. pneumoniae*, mutations in the orthologous oligopeptide binding proteins were also shown to modulate competence development, but the underlying regulatory mechanisms remained unclear (2). The Ami system of Gram-positive bacteria actively imports oligopeptides present in the extracellular medium and is known to have both nutritive and signaling functions (39). Since CDM is a peptide-free medium, Gardan et al. (20) hypothesized that *S. thermophilus* LMD-9 synthesizes and secretes a specific competence-stimulating peptide, which is then sensed and imported by the Ami system. Once internalized, this pheromone would then interact with a specific cytoplasmic regulator, leading to the transcriptional induction of *comX* (20). The pheromone and the transcriptional regulator responsible for *comX* expression and natural transformation under CDM conditions are still unknown.

The aim of the present study was to identify the genetic determinants involved in the early steps of competence development of *S. thermophilus* LMD-9 under CDM-inducing conditions. In order to reveal early competence genes, we compared the transcriptomes of Ami^- and $ComX^-$ strains, both unable to switch on late competence genes. This transcriptomic approach led to the identification of an Rgg-like transcriptional regulator and its cognate pheromone, both essential for the transcriptional control of *comX*. Moreover, we showed that this pheromone can stimulate or activate competence development in *S. thermophilus* and *Streptococcus salivarius* strains that are poorly or not transformable.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. Plasmids derived from pG^+host9 (37) were constructed in *Escherichia coli* EC1000 (32). *E. coli* was grown in LB medium with shaking at 37°C (49). *S. thermophilus* and *S. salivarius* were grown anaerobically (BBL GasPak Systems; Becton Dickinson, Franklin Lakes, NJ) at 37°C in M17 broth (Difco Laboratories Inc., Detroit, MI) or in CDM, as described by Letort and Juillard (34). Both media contain 1% (wt/vol) lactose. When required, erythromycin (250 μg/ml for *E. coli*; 2.5 μg/ml for *S. thermophilus* and *S. salivarius*) or chloramphenicol (20 μg/ml for *E. coli*; 5 μg/ml for *S. thermophilus*) was added to the media. Solid agar plates were prepared by adding 2% (wt/vol) agar to the medium.

Detection of absorbance and luminescence. Growth, luciferase activity, and transformation experiments for *S. thermophilus* were monitored in the Synergy HT multimode reader (BioTek, Winooski, VT). The reader automatically measures the optical density at 600 nm (OD_{600}) and luminescence at 595 nm (expressed in relative light units [RLU]) of culture samples at 10-min intervals.

Before the experiment was started, small volumes (300 μl) of culture samples were transferred to the wells of a prewarmed (37°C) sterile covered white microplate with a transparent bottom (Greiner, Alphen a/d Rijn, the Netherlands). The luciferase activity catalyzed by LuxAB requires the presence of nonanal as a substrate. In all experiments, we supplied nonanal (Acros Organics, Geel, Belgium) in a volatile form to the cultures by placing 50 μl of a solution containing 1% nonanal diluted in mineral oil (Sigma) in the spaces between the wells of a covered microplate as described by Bachmann et al. (3). The microplate was next transferred to the prewarmed automatic reader (37°C) (time zero [t_0]) and incubated at 37°C for 5 h.

Natural-transformation experiments. An overnight culture of *S. thermophilus* grown in M17 at 37°C was washed twice (5,000 × *g*; 9 min; room temperature) in 1 volume of CDM and resuspended in 1 volume of CDM. The washed culture was then 30-fold diluted in CDM. Small volumes (300 μl) of the culture sample were next transferred into the wells of a sterile microplate (Greiner). In one sample, 1 μg of plasmid (pGIUD0855ery or pG^+host9) or 500 ng overlap PCR fragments was added at the beginning of the experiment (t_0). The plate was next incubated at 37°C in the prewarmed Synergy HT multimode reader (see above). In the supplementation experiments, different concentrations of synthetic forms of Shp0316 (NH_2-COOH) peptides (purity > 95%) supplied by Peptide 2.0 (Chantilly, VA) were added to the 300-μl culture samples after 1 h 30 min of growth at 37°C ($t_{1.5}$). After 5 h (t_5), samples (100 μl of serial dilutions in M17 broth) containing DNA, or not (negative control), were spread on M17 plates containing erythromycin in the case of pGIUD0855ery and pG^+host9 or chloramphenicol in the case of overlap PCR fragments. The transformation rate was calculated after 30 h of incubation at 37°C or 29°C (only for pG^+host9) as the number of antibiotic-resistant CFU per ml divided by the total number of viable CFU per ml. After the transformation experiments, the integration of the antibiotic resistance cassette at the right location or acquisition of pG^+host9 was checked by PCR. For pGIUD0855ery and pG^+host9, the primer pairs Chstu0855A-Chstu0855B and pGhost1-pGhost2, respectively, were used. The primer pairs used for overlap PCR fragment are listed in Table S1 in the supplemental material.

DNA techniques and electrotransformation. General molecular biology techniques were performed according to the instructions given by Sambrook et al. (49). Electrotransformation of *E. coli* was performed as described by Dower et al. (14). Electrocompetent *S. thermophilus* cells were prepared as previously described (4). After transformation with 1 μg of pG^+host9 derivative plasmids, the cells were immediately resuspended in 1 ml M17 and incubated anaerobically for 6 h at 29°C. *S. thermophilus* chromosomal DNA was prepared as described by Ferain et al. (15). PCRs were performed with Fhusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA). The primers used in this study were purchased from Eurogentec (Seraing, Belgium) and are listed in Table S1 in the supplemental material.

Construction of plasmid pGIUD0855ery. Plasmid pGIUD0855ery was constructed to assess and compare the natural transformation rates of *S. thermophilus* strains. It allowed the replacement of the *stu0855* open reading frame (ORF) of strain LMG18311 (*ster_0891* and *str0855* in the case of strains LMD-9 and CNRZ1066, respectively) by an erythromycin resistance cassette. This plasmid was constructed as follows. In the first step, an overlapping PCR was performed to create a DNA fragment containing the erythromycin expression cassette (P_{ery}-*ery*), flanked by the 1-kb upstream (UP) and downstream (DN) regions of *stu0855*. The P_{ery}-*ery* cassette and the UP and DN fragments were separately amplified using pUC18ery and *S. thermophilus* chromosomal DNA, respectively, as templates (the primer pairs are listed in Table S1 in the supplemental material). The 3′ end of UP and the 5′ end of DN fragments, respectively, were complementary to the 5′ and 3′ ends of P_{ery}-*ery*. The three fragments were then mixed in equimolar concentrations and joined together by PCR with the external primers. In the second step, the overlap fragment obtained was digested with SphI/KpnI and cloned in the similarly digested pUC18 vector.

Construction of *luxAB* reporter strains. The reporter strains CB001 and CB002 were constructed by replacing part of the *blp* locus or *comX* ORF, respectively, in strain LMD-9 with the transcriptional fusions P_{comX}-*luxAB*. Replacements of *blp* bacteriocin operons (18) and *comX* were obtained by double homologous recombination of the pG^+host9 derivative plasmids pGICB001 and pGICB002 after

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
S. thermophilus		
LMD-9	Wild type	ATCC
LF101	LMD-9 Δ*blpR*	18
CB001	LMD-9 *blpD-blpX*::P_{comX}-*luxAB*	This study
CB002	LMD-9 *comX*::*luxAB*	This study
CB003	LMD-9 Δ*comX*	This study
LF115	LMD-9 *amiA1-amiF*::P32-*cat*	This study
LF116	CB001 *amiA1-amiF*::P32-*cat*	This study
LF117	CB001 *ster_0316*::P32-*cat*	This study
LF118	CB001 *shp0316*::P32-*cat*	This study
LF119	CB001 $IR_{shp0316}$::P32-*cat*	This study
LF120	CB001 *amiA3*::P32-*cat*	This study
LMG18311	Wild type	22
CNRZ1066	Wild type	22
S. salivarius		
ATCC25975	Wild type	ATCC[a]
JIM8777	Wild type	12
JIM8780	Wild type	12
E. coli		
EC1000	Km^r $RepA^+$; MC1000 containing a copy of the *repA* gene of pWV01 in its chromosome	32
Plasmids		
pNZ5319	Cm^r Em^r; pACYC184 derivative containing the *cat* gene under the control of the P32 constitutive promoter from *Lactococcus lactis* (P32-*cat* cassette)	30
pG^+host9	Em^r Ts	37
pUC18	Ap^r	40
pUC18ery	Ap^r Em^r; pUC18 derivative containing the erythromycin resistance marker used to amplify the erythromycin resistance cassette (called P_{ery}-*ery*)	54
pGIUD0855ery	Ap^r Em^r; pUC18ery derivative containing an erythromycin resistance cassette flanked by 1-kb fragments corresponding to the upstream and downstream regions of *stu0855*. This plasmid was constructed to assess the natural transformation rates of *S. thermophilus* strains.	This study
pJIM4900	Em^r Ts; pG^+host9 containing the *luxAB* genes of *P. luminescens* and a transcriptional terminator.	E. Guédon[b]
pGICB001	Em^r Ts; pJIM4900 derivative containing the P_{comX}-*luxAB* transcriptional fusion flanked by the upstream and downstream sequences of *blpD* and *blpX*, respectively. This plasmid was constructed to replace part of the *blp* locus of strain LMD-9 by P_{comX}-*luxAB*.	This study
pGICB002	Em^r Ts; pJIM4900 derivative containing the *luxAB* genes flanked by the upstream and downstream sequences of *comX* of *S. thermophilus* LMD-9. The upstream sequence was cloned in order to create a P_{comX}-*luxAB* transcriptional fusion. This plasmid was constructed to replace P_{comX}-*comX* of strain LMD-9 with P_{comX}-*luxAB*.	This study
pGICB003	Em^r Ts; pGICB002 plasmid in which *luxAB* genes have been removed. This plasmid was used to delete *comX* of strain LMD-9.	This study

[a] Km^r, Cm^r, Em^r, Ap^r, and Ts indicate resistance to kanamycin, chloramphenicol, erythromycin, and ampicillin and that the plasmid encodes a thermosensitive RepA protein, respectively.
[b] Plasmid pJIM4900 was kindly provided by E. Guédon (unpublished data).

two steps of temperature shift, as previously described (37). The recombinant strains were confirmed by PCR with primers located upstream and downstream of the recombination regions. The pGICB001 and pGICB002 plasmids were constructed from the pJIM4900 vector, which is a pG^+host9 derivative plasmid containing the *Photorabdus luminescens luxAB* genes (a generous gift from E. Guédon). Plasmid pGICB001 was used to create a *blpD-blpX*::P_{comX}-*luxAB* reporter strain and was obtained in two steps. First, 1-kb fragments corresponding to the UP region of *blpD* and the DN region of *blpX* were PCR amplified with specific primer pairs (see Table S1 in the supplemental material); digested by PshA1/SpeI and SalI/PvuII, respectively; and cloned upstream and downstream of the *luxAB* genes of plasmid pJIM4900, respectively, using the same restriction enzymes. Second, to create a P_{comX}-*luxAB* transcriptional fusion, the fragment containing the *comX* expression signals was amplified with the primer pair DPX1-DPX2, digested by SpeI/EcoRI, and cloned in transcriptional fusion with *luxA* in the plasmid obtained in the first step. Plasmid pGICB002 was used to create a *comX*::P_{comX}-*luxAB* reporter strain and was obtained as follows: 1-kb fragments corresponding to the UP and DN regions of *comX* were PCR amplified with specific primer pairs (see Table S1 in the supplemental material); digested by PshA1/SpeI and SalI/PvuII, respectively; and cloned upstream and downstream, respectively, of the *luxAB* genes of plasmid pJIM4900 (the same restriction enzymes were used). After two steps of homologous recombination of plasmids pGICB001 and pGICB002, the strains that had integrated the P_{comX}-*luxAB* fusion, CB001 and CB002, respectively, were confirmed by PCR.

Deletion of *comX* in strain LMD-9. Strain CB003 was constructed by deleting the *comX* ORF in strain LMD-9 by double-crossover events of plasmid pGICB003, a pG^+host9 derivative (37). Plasmid pGICB003 was constructed as follows. Plasmid pGICB002 was digested with SalI and partially digested by EcoRI to remove the *luxAB* genes. The linearized plasmid was then filled in with the Klenow fragment and ligated.

Construction of deletion mutants for *amiA1-amiF*, *ster_0316*, *shp0316*, and *amiA3*. The LMD-9 derivative strain LF115 (*amiA1-amiF*::P32-*cat*) and CB001 derivative strains LF116 (*amiA1-amiF*::P32-*cat*), LF117 (*ster_0316*::P32-*cat*), LF118 (*shp0316*::P32-*cat*), LF119 ($IR_{shp0316}$::P32-*cat*) ($IR_{shp0316}$ is the inverted repeat located upstream of *shp0316*), and LF120 (*amiA3*::P32-*cat*) were constructed by replacing the sequence between the start and stop codons of the target gene or a specific region with the chloramphenicol expression cassette P32-*cat* (double homologous recombination). DNA fragments containing P32-*cat* flanked by the UP and DN regions of the target gene or region were made *in vitro* by overlapping PCR. For this purpose, the 3′ ends of UP fragments and the 5′ ends of DN fragments are complementary to the 5′ and 3′ ends of P32-*cat*,

TABLE 2. Natural transformation rates of *S. thermophilus* and *S. salivarius* strains

Species and strain	Genotype	Plasmid	Transformation rate/μg of DNA	
			0 nM $Shp0316_{18-24}$	1 μM $Shp0316_{18-24}$
S. thermophilus				
LMD-9	Wild type	pGIUD0855ery	$4.5 \times 10^{-4} \pm 2.3 \times 10^{-4}$	NT[a]
CB001	LMD-9 *blpD-blpX*::P_{comX}-*luxAB*	pGIUD0855ery	$3.3 \times 10^{-4} \pm 1.7 \times 10^{-4}$	NT
CB002	LMD-9 *comX*::*luxAB*	pGIUD0855ery	ND[b]	NT
CB003	LMD-9 Δ*comX*	pGIUD0855ery	ND	NT
LF115	LMD-9 *amiA1-amiF*::P32-*cat*	pGIUD0855ery	ND	NT
LF116	CB001 *amiA1-amiF*::P32-*cat*	pGIUD0855ery	ND	ND
LF117	CB001 *ster_0316*::P32-*cat*	pGIUD0855ery	ND	ND
LF118	CB001 *shp0316*::P32-*cat*	pGIUD0855ery	ND	$5.1 \times 10^{-3} \pm 1.4 \times 10^{-3}$
LF119	CB001 $IR_{shp0316}$::P32-*cat*	pGIUD0855ery	$2.6 \times 10^{-4} \pm 1.3 \times 10^{-4}$	NT
LF120	CB001 *amiA3*::P32-*cat*	pGIUD0855ery	$3.6 \times 10^{-6} \pm 1{,}1 \times 10^{-6}$	$1.4 \times 10^{-5} \pm 0.3 \times 10^{-5}$
LMG18311	Wild type	pGIUD0855ery	2.1×10^{-7}	3.2×10^{-4}
CNRZ1066	Wild type	pGIUD0855ery	ND	3.7×10^{-4}
CNRZ1066	Wild type	pG^+host9	ND	6.4×10^{-4}
S. salivarius				
ATCC25975	Wild type	pG^+host9	ND	4.7×10^{-6}
JIM8777	Wild type	pG^+host9	3.0×10^{-7}	3.9×10^{-4}
JIM8770	Wild type	pG^+host9	ND	8.1×10^{-5}

[a] NT, not tested.
[b] ND indicates that the transformation rate was below the detection limit of the test ($<1 \times 10^{-8}$).

respectively. The strategy used was the following. In the first step, the UP, DN, and P32-*cat* fragments were PCR amplified separately. The P32-*cat* cassette (1.3 kb) was amplified with primers Uplox66 and DNlox71 using plasmid pNZ5319 as a template (30). The UP and DN regions (1.2 kb) were PCR amplified using the LMD-9 chromosome as a template with specific primers (UPA-UpB and DNA-DNB primers, respectively, which are listed in Table S1 in the supplemental material). In the second step, the UP, DN, and P32-*cat* fragments were mixed in equimolar concentrations and joined together by PCR using the specific UpA and DNB external primers. The resulting fragments (500 ng) were further used to transform naturally competent cells of strain LMD-9 or CB001, as described above. Transformants were selected on M17 plates containing chloramphenicol and checked by PCR using the specific primers ChA and ChB listed in Table S1 in the supplemental material.

RNA extraction and microarray experiments. The experimental procedures for RNA extraction and microarray experiments were detailed in Text S1 in the supplemental material.

RESULTS

Kinetics of *comX* induction in LMD-9 cells growing in CDM. In *S. pneumoniae* and *S. mutans*, the *comABCDE* genes responsible for *comX* induction are regulated by a ComX-independent positive feedback loop, which is initiated when a critical extracellular concentration of the competence-stimulating peptide is reached and sensed (27). In *S. thermophilus*, we postulated that a similar autoamplification mechanism governs the regulation of the early competence genes and that activation depends on the reimportation of a secreted pheromone *via* the Ami system (20). Based on this hypothesis, our leading strategy to identify the early competence genes was to compare the transcriptomes of Ami^- and $ComX^-$ strains, since early genes should be induced only in the latter. To determine the appropriate time to extract RNAs, we monitored the kinetics of *comX* induction in different *S. thermophilus* LMD-9 backgrounds, i.e., strains that contain or do not contain a functional ComX or Ami system. For this purpose, three *comX* reporter strains were constructed. Strain CB001 ($ComX^+$ Ami^+) was obtained by replacing the *blp* bacteriocin operons (18, 19) with a transcriptional fusion between the intergenic region upstream of *comX*, called P_{comX}, and the luciferase genes *luxAB* of *Photorabdus luminescens*. In strain CB002 ($ComX^-$ Ami^+), *luxAB* genes are under the control of the native P_{comX} promoter and replace the *comX* ORF (*comX*::*luxAB*). To construct strain LF116 ($ComX^+$ AmiA1-$AmiF^-$), the *amiA1-amiF* region of strain CB001 ($ComX^+$ Ami^+) was replaced by the chloramphenicol resistance cassette P32-*cat*. Prior to any experiment, we assessed the competence efficiency of the reporter strains. As expected, the transformation rate of strain CB001 ($ComX^+$) was not affected compared to that of the wild-type LMD-9 strain, while the deletion of *comX* in strain CB002 or *amiA1-amiF* in strain LF116 abolished competence (Table 2) (20). Growth and luciferase activities were monitored under CDM growth conditions at 37°C in an automatic multimode reader. The results are presented in Fig. 1A. By monitoring the number of RLU per OD_{600} unit (RLU/OD_{600}) of strain CB001 ($ComX^+$ Ami^+), we found that P_{comX} activity started to increase in the early exponential phase and reached a maximum (RLU/OD_{600} = 15,000) before mid-log phase, after 50 min of induction. The activity of P_{comX} remained stable for 10 min and then constantly decreased to reach an RLU/OD_{600} of 380 in stationary phase. These kinetics are in accordance with those of the appearance of transformed cells in CDM (20). The kinetics of P_{comX} activity in strain CB002 ($ComX^-$ Ami^+) followed a similar pattern, except that the maximum number of RLU per OD unit obtained was 3-fold higher and the activity remained stable for a longer time than with strain CB001 (Fig. 1A). As expected, the luciferase activity of strain LF116 remained low (maximum RLU/OD_{600} = 250) throughout growth, confirming the role of the Ami system in *comX* transcription. Interestingly, when we compared the growth curves of the transformable strain CB001 to those of the competence-deficient strains CB002 and LF116, we observed a ComX-dependent decrease in the growth rate and the final growth yield. Deletion of the *blp* bacteriocin operons in strain CB001 was not responsible for this phenotype, since the

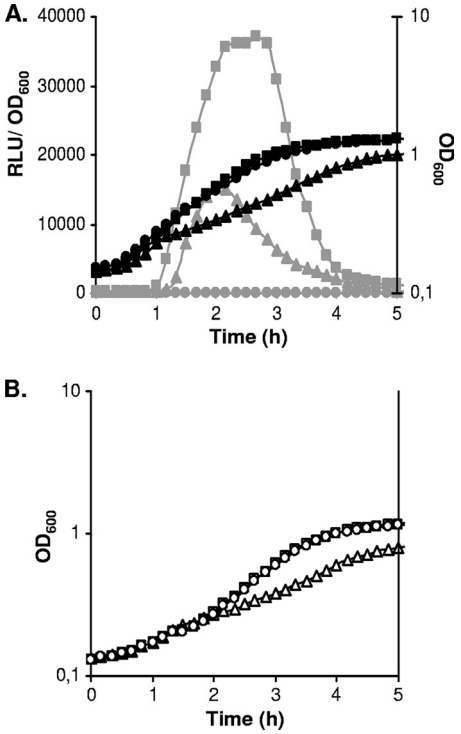

FIG. 1. Growth (OD_{600}) and luciferase activities (RLU/OD_{600}) of LMD-9 reporter strains and their isogenic mutants used in the microarray experiments. (A) Growth (black) and luciferase activities (gray) of P_{comX}-*luxAB* fusions of strains CB001 ($ComX^+$ Ami^+; triangles), CB002 ($ComX^-$ Ami^+; squares), and LF116 ($ComX^+$ Ami^-; circles). (B) Growth of strains LMD-9 (wild type [WT]; open triangles), CB003 (LMD-9 $ComX^-$ Ami^+; open squares), and LF115 (LMD-9 $ComX^+$ Ami^-; open circles).

growth of the wild-type LMD-9 strain was similar to that of CB001 (Fig. 1B). This shows that competence development of LMD-9 under CDM conditions has a deleterious effect on growth.

The early competence genes of *S. thermophilus* include four main loci that are preceded by a conserved box shared with *comX*. For transcriptomic study purposes, $ComX^-$ (CB003; LMD-9 Δ*comX*) and AmiA1-$AmiF^-$ (LF115, LMD-9 *amiA1-amiF*::P32-*cat*) strains harboring a native *blp* locus were constructed. These competence-defective strains displayed growth curves in CDM similar to those of their Blp^- isogenic strains, with an improved growth rate and yield compared to the wild-type strain (Fig. 1B). Total RNA was extracted from mid-log-phase growing cells (CDM conditions), where the RLU/OD_{600} of strain CB002 was found to be maximal. Labeled RNAs were then cohybridized to Agilent LMG18311 microarrays as described in Materials and Methods. As expected, the *ami* probes were highly induced in the $ComX^-$ strain, except for *amiA1*, for which specific probes were not present in the LMG18311 microarrays (see Table S2 in the supplemental material). In contrast, no *comX*-specific probes were repressed, confirming that *comX* is no longer induced in the AmiA1-$AmiF^-$ strain grown in CDM (data not shown).

To identify genes involved in the activation of *comX* expression, we focused our attention on genes that were induced in the $ComX^-$ strain compared to the AmiA1-$AmiF^-$ strain. We considered relevant only those genes that were represented by more than 50% induced probes with a mean absolute fold change (FC) of at least 10. Induced genes can be separated into highly ($10 <$ mean FC < 14) and very highly ($19 <$ mean FC < 100) regulated genes. Interestingly, the latter are organized into 4 loci that are related to bacteriocin production (see Table S2 in the supplemental material). The first locus (*ster_0317-ster_0319*) encodes a truncated ABC transporter specific for 2-Gly peptides. Loci 2 (*blpABC*) and 3 (*orf4-orf6*) belong to the *blp* bacteriocin cluster and, respectively, encode the induction factor and the secretion apparatus of thermophilin 9 and potential immunity proteins (18, 19). Finally, genes of locus 4 (*ster_1720-ster_1718*) include homologues of the mutacin I biosynthetic genes. Of the three genes belonging to the group of less induced genes (*blpG*, *ster_0935*, and *ster_1924*), only *blpG* is related to bacteriocin production, since it was shown to be involved in thermophilin 9 interspecies activity (19). Coprogrammed production of bacteriocins and competence development was previously reported in several transformable streptococcal species and was proposed to play an important role in the acquisition of substrate DNA for transformation (7, 21, 29, 41).

To provide further evidence that the four identified loci specifically include early competence genes, we aligned their upstream regions with the intergenic region upstream of *comX*. This allowed us to highlight a conserved organization of their promoter sequences (60 nucleotides [nt]; 43% identity). Importantly, they all contain a highly conserved motif (20 nt; 80% identity) consisting of two 9-bp inverted repeats with the following consensus sequence: 5′-TAGTGACATNTATGTCAC TA-3′, called the early competence box (ECom box). This motif is followed by a conserved T tract and a putative −10 box, which is located 21 bp downstream of the ECom box in all promoters (Fig. 2A). The ECom box could serve as a binding site for a positive transcriptional regulator specific to the early competence step. This motif is not present upstream of the three less induced genes, strongly suggesting that they are differentially regulated and probably not specific to early competence in *S. thermophilus*.

The *ster_0317* locus encodes an Rgg-like regulator and a small hydrophobic peptide essential for *comX* induction. Since we hypothesized that an autoamplification mechanism governs *comX* induction, we searched for genes coding for precursors of signaling peptides and transcriptional regulators among the four early competence operons.

None of the induced gene products displayed obvious regulator features, but two transcriptional regulators were encoded in the direct vicinity of early competence genes: BlpR and Ster_0316. They belong to the LytR and Rgg families of regulators, respectively. Strain LMD-9 Δ*blpR* (LF101) from our mutant collection (18) displayed a competence efficiency similar to that of the wild-type strain (data not shown). To assess the role of *ster_0316* in early competence induction, the *ster_0316* ORF of the P_{comX}-*luxAB* reporter strain CB001 was replaced by the P32-*cat* cassette (LF117) (Table 1 and Fig. 2B). Compared to the control strain CB001 cultivated in CDM, the low luciferase activity (maximum $RLU/OD_{600} = 160$) of strain LF117, together with its improved growth rate and yield, unequivocally showed the key role of *ster_0316* in the activation

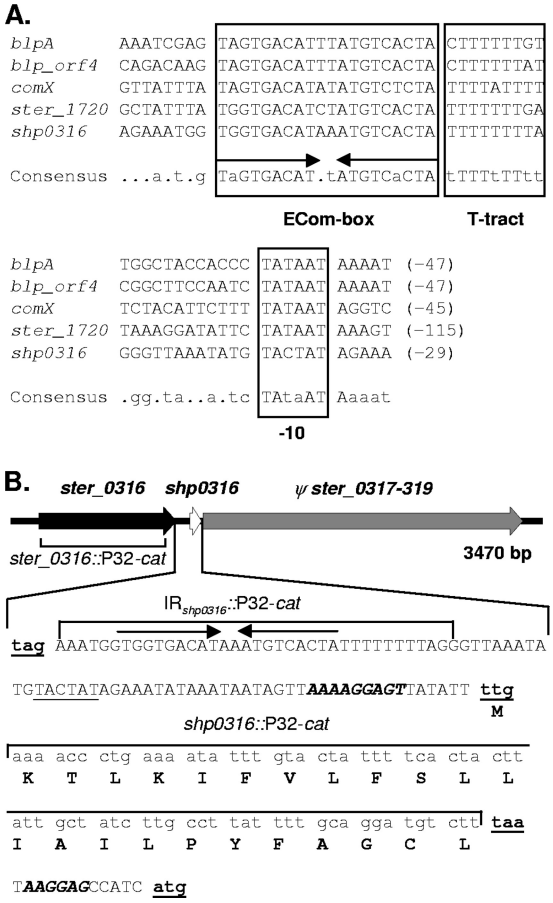

FIG. 2. Analysis of the *ster_0316-shp0316* locus in strain LMD-9. (A) Sequence alignment of the upstream regions of *comX*, *shp0316*, *blpA*, *blp_orf4* (*orf4* of the *blp* locus), and *ster_1720*. The consensus sequence is shown below the alignment: nucleotides present in each of these sequences are in uppercase, and nucleotides occurring in 3 or 4 of the sequences are in lowercase. The following conserved elements are boxed: inverted repeats (ECom box) (arrows), followed by a sequence rich in T (T tract) and a putative −10 box (−10). The numbers (in parentheses) indicate positions relative to the translational start codon. (B) Schematic representation of the locus from *ster_0316* to *ster_0319* (3,470 bp) and sequence analysis of the intergenic region (*ster_0316-ster_0317*) coding for Shp0316. Genes encoding proteins or peptides with predicted functions are represented by arrows: Rgg-like transcriptional regulator (black; *ster_0316*), small hydrophobic peptide (white; *shp0316*), and inactive peptide ABC exporter (gray; pseudogene ψ*ster_0317-319*). The following elements are annotated in the *ster_0316-ster_0317* intergenic region: the ORF for Shp0316 (24 amino acids), start and stop codons (lowercase, boldface, and underlined), Shine-Dalgarno sequences (boldface and italics), the conserved inverted repeat (arrows), and the putative −10 box (uppercase and underlined). The deletion-replacement regions of the P32-*cat* cassette of *ster_0316* (*ster_0316*::P32-*cat*), $IR_{shp0316}$ ($IR_{shp0316}$::P32-*cat*), and *shp0316* (*shp0316*::P32-*cat*) are indicated by brackets.

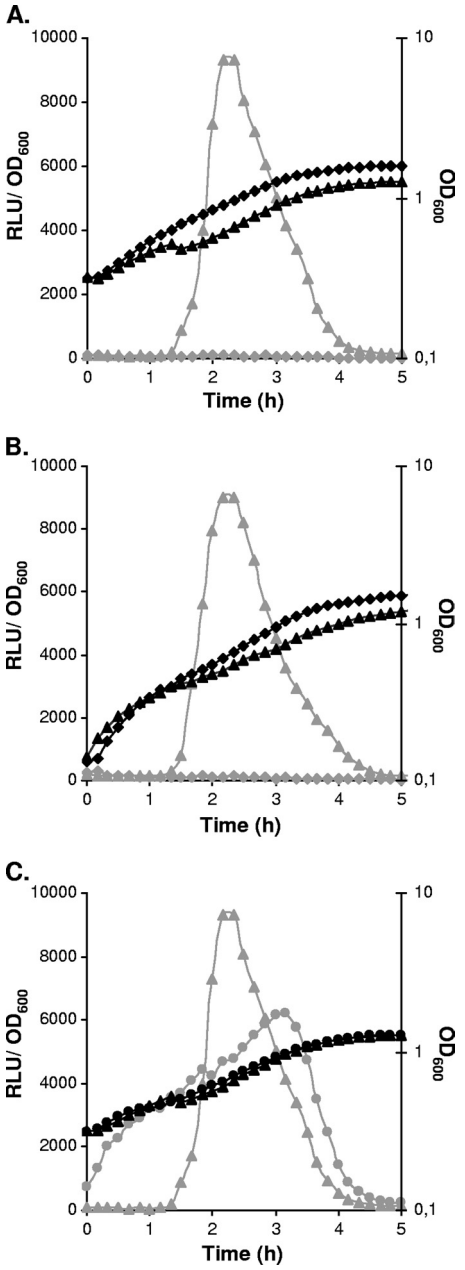

FIG. 3. Role of the *ster_0316-shp0316* locus of *S. thermophilus* LMD-9 in the activation of the *comX* promoter. (A) Role of *ster_0316* encoding a Rgg-like regulator. Shown are the growth (OD_{600}; black) and luciferase activities (RLU/OD_{600}; gray) of strains CB001 (control strain; triangles) and LF117 (Ster_0316$^-$; diamonds). (B) Role of *shp0316*. Shown are the growth and luciferase activities of strains CB001 (control strain; triangles) and LF118 (Shp0316$^-$; diamonds). (C) Role of the inverted repeat ($IR_{shp0316}$) located upstream of *shp0316*. Shown are the growth and luciferase activities of strains CB001 (control strain; triangles) and LF119 ($IR_{shp0316}$::P32-*cat*; circles).

of *comX* transcription (Fig. 3A). As expected from P_{comX} activity, competence experiments revealed that strain LF117 was no longer transformable (Table 2), supporting its role in natural transformation.

Since most Rgg regulator genes are located close to their associated pheromone-encoding genes (25, 26), a closer examination of the *ster_0317* early competence locus was performed. We identified a 75-bp ORF that was not annotated in the public database. It encodes a 24-amino-acid hydrophobic peptide and was therefore called Shp0316 (Shp for small hydrophobic peptide) (Fig. 2B). The putative signaling function of Shp0316 was tested by monitoring the P_{comX} activity and transformation rate of the Shp0316$^-$ strain (LF118) (Table 1

and Fig. 2B), which was constructed using the same strategy described above for strain LF117. The results in Fig. 3B showed that the deletion of *shp0316* impaired *comX* induction in CDM. Indeed, a maximum RLU/OD_{600} of 230 was measured in LF118, which is dramatically reduced compared to the control strain CB001 and similar to the Ster_0316$^-$ and AmiA1-AmiF$^-$ reporter strains. In addition, no transformant could be obtained for strain LF118 in natural competence experiments (Table 2). These results demonstrate that Shp0316 is essential for early competence development and acts at the *comX* transcriptional level in *S. thermophilus*.

The essential role of *shp0316* in *comX* induction and the presence of an ECom box in its promoter sequence suggest that its transcription depends on a positive feedback loop. In this context, the Rgg regulator Ster_0316 could play a role in this regulation pathway either directly, by binding to the ECom box of the $P_{shp0316}$ promoter sequence, or indirectly by stimulating the transcription of a second regulator. To assess the importance of the putative Ster_0316 binding site for *comX* induction, we replaced the corresponding ECom box and T tract ($IR_{shp0316}$) with the P32-*cat* cassette in the reporter strain CB001 (LF119) (Table 1 and Fig. 2B). In this construct, the putative −10 box of $P_{shp0316}$ and *shp0316* translation signals were maintained, but *shp0316* transcription was under the control of the upstream constitutive P32 promoter. The consequences of this promoter exchange were studied by comparing the P_{comX} activities of the reporter strains CB001 and LF119 ($IR_{shp0316}$::P32-*cat*) grown in CDM (Fig. 3C) and by performing competence experiments. Interestingly, the growth rates of the CB001 and LF119 strains were similar, but the kinetics of P_{comX} induction dramatically changed. Expression was no longer induced during growth, but rather, constitutively increased from the beginning of growth until the cells entered the stationary phase. The maximum RLU/OD_{600} measured was lower than that of the control strain CB001 but high enough to develop normal competence (Fig. 3C and Table 2). This clearly shows that the ECom box of $P_{shp0316}$ plays a critical role in the kinetics of *comX* induction, supporting the hypothesis that Shp0316 is part of an autoamplification loop governing the early competence state.

Altogether, our results strongly suggest that Ster_0316 and Shp0316 are part of an Rgg regulator/signaling peptide system controlling early competence induction in *S. thermophilus* LMD-9.

Shp0316 is the precursor of the competence-stimulating peptide in *S. thermophilus*. To fulfill its putative signaling function, peptide Shp0316 must be secreted and probably matured in order to be sensed by *S. thermophilus* cells. For example, the peptides internalized by oligopeptide transporters and involved in the regulation of conjugation in *Enterococcus faecalis* (33) and virulence in *Bacillus cereus* (52) are synthesized as longer precursor peptides in the cytoplasm. Then, they undergo several maturation steps leading to the release of the active pheromone in the extracellular medium (5, 43). Shp316 (MKTLKIFVLFSLLIAILPYFAGCL) displays all the characteristics of a signal sequence from a lipoprotein: a size between 20 and 25 amino acids, a positively charged N-terminal end, a central hydrophobic core, and a conserved −2, −1 cleavage site followed by a +1 Cys residue (lipobox) (24) (Fig. 4A). Interestingly, all mature conjugation peptides of *E. faecalis* identified to date correspond to the C-terminal part of lipoprotein signal sequences (10).

To test the hypothesis that Shp0316 is matured and released in the medium in order to act as a competence pheromone, experiments with supplementation of the competence defect of the Shp0316$^-$ strain (LF118) were carried out. Based on the sizes and sequences of known conjugation-inducing peptides (10) and of the virulence-signaling peptide PapR (43), a heptapeptide corresponding to the C-terminal part of Shp0316 was synthesized ($Shp0316_{18-24}$) (Fig. 4A and B) and tested for its ability to induce P_{comX} activity and natural transformation. Different concentrations of $Shp0316_{18-24}$ (0 nM, 10 nM, 100 nM, 500 nM, 1 μM, and 2.5 μM) were added to the early log growth phase of LF118 cells grown under CDM conditions at 37°C. Luciferase activity was next monitored for 5 h before competence experiments were performed. The results presented in Fig. 4C show that the luminescence driven by P_{comX}-*luxAB* from strain LF118 (Shp0316$^-$) displayed a dose response to the amount of $Shp0316_{18-24}$, which is typical of pheromone-regulated systems. Indeed, $Shp0316_{18-24}$ concentrations lower than 100 nM had no inducing effect on P_{comX}-*luxAB* activity, while the response was almost linear between 100 nM and 1 μM. At concentrations higher than 1 μM, the luciferase activity of LF118 reached a plateau, indicating saturation of $Shp0316_{18-24}$ induction. In each case, the maximum RLU/OD_{600} was measured before the mid-log growth phase. The measured transformation rates and P_{comX}-*luxAB* activity of strain LF118 displayed a similar dose response to the $Shp0316_{18-24}$ concentration (Fig. 4D). Transformation rates in the presence of 500 nM or higher concentrations of $Shp0316_{18-24}$ were even higher than those of the control strain CB001 grown in the absence of inducing peptide (Table 2 and Fig. 4D). Altogether, our results demonstrate that Shp0316 is the precursor of the competence-stimulating peptide in *S. thermophilus* LMD-9.

Longer Shp0316 derivative forms were synthesized in order to test the importance of the N-terminal residues in the signaling activity of Shp0316 (peptides $Shp0316_{17-24}$, $Shp0316_{16-24}$, and $Shp0316_{15-24}$) (Fig. 4B). The maximum peptide length tested was fixed at 10 amino acids, since importation by the Ami/Opp systems of Gram-positive species was shown to be optimal in this peptide size range (13). Since lipoprotein signal sequences are cleaved upstream of the +1 Cys residue, we also assessed the role of the two last C-terminal amino acids by synthesizing peptides $Shp0316_{18-23}$, $Shp0316_{18-22}$, and $Shp0316_{18-20}$ (Fig. 4B). The inducing potential of 1 μM of each peptide was evaluated in strain LF118 (Shp0316$^-$) as described above. The transformation rates obtained (Fig. 4B) show that (i) the N-terminal residues of Shp0316, or at least amino acids 15 to 17, are not critical for the signaling activity of Shp0316, while (ii) the C-terminal Leu residue is specifically involved in this function. The latter conclusion was further confirmed, since the removal of Leu_{24} from the active Shp0316 derivative forms displaying a longer N-terminal part abolished their inducing properties (peptides $Shp0316_{17-23}$, $Shp0316_{16-23}$, and $Shp0316_{15-23}$) (Fig. 4B).

To provide evidence that the Ami system and the Ster_0316 regulator are obligate intermediates between Shp0316 signaling and *comX* induction during early competence, we performed induction experiments with the nontransformable

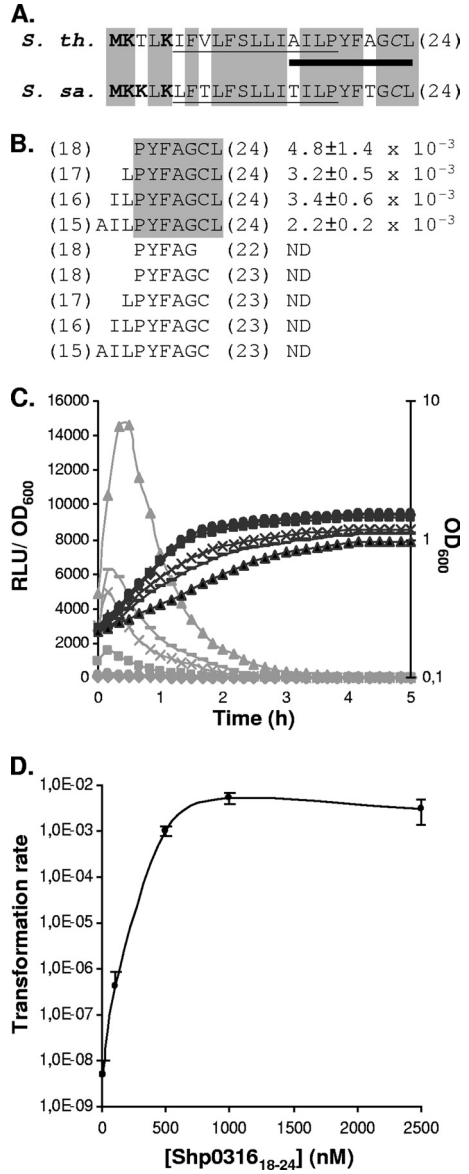

FIG. 4. Role of the C-terminal region of Shp0316 in the activation of the *comX* promoter and transformation rate of LF118 (Shp0316$^-$). (A) Alignment of Shp0316 of *S. thermophilus* (*S. th.*) LMD-9 and its orthologue identified in the *S. salivarius* SK126 (*S. sa.*) genome. Identical amino acids are shown in gray, the positively charged amino acids are shown in boldface, the hydrophobic region is underlined, and the conserved Cys residue of a putative lipobox is in italics. The sequence used to generate the synthetic peptides is indicated with a thick black line. (B) Sequences of the different synthetic peptides derived from the C-terminal region of Shp0316 (left) and mean transformation rate ± standard deviations (triplicates) of strain LF118 (Shp0316$^-$) in the presence of 1 μM of each synthetic peptide (right). The numbers in parentheses indicate amino acid positions relative to the Shp0316 sequence. The heptapeptide required for activation is indicated in gray. ND, transformation rate below the detection limit ($<1 \times 10^{-8}$). (C) Growth (OD_{600}; black) and luciferase activities (RLU/OD_{600}; gray) of strain CB001 (control strain; triangles) and strain LF118 (Shp0316$^-$) in response to increasing concentrations of $Shp0316_{18\text{-}24}$ (nM): 0 (diamonds), 10 (+), 100 (circles), 500 (squares), 1,000 (X), and 2,500 (bars). The RLU/OD_{600} obtained with 0 and 10 nM $Shp0316_{18\text{-}24}$ constantly decreased during growth, and the corresponding curves overlap. A 1.5-fold increase in luciferase activity in the presence of 100 nM $Shp0316_{18\text{-}24}$ was measured directly after peptide addition. (D) Transformation rate of strain LF118 (Shp0316$^-$) in response to increasing concentrations of $Shp0316_{18\text{-}24}$ (nM): 0, 10, 100, 500, 1,000, and 2,500. Mean values ± standard deviations were calculated from triplicates. The substrate DNA was plasmid pGIUD0855ery (1 μg). The transformation rates at 0 mM and 10 mM were arbitrarily fixed below the detection limit ($<1 \times 10^{-8}$).

AmiA1-AmiF$^-$ and Ster_0316$^-$ reporter strains and the poorly transformable AmiA3$^-$ reporter strain (LF120) (Table 1); the latter displayed a 100-fold reduced transformation rate compared to the control strain CB001 (Table 2), as reported previously by Gardan et al. (20). Peptide $Shp0316_{18\text{-}24}$ was added to CDM at a final concentration of 1 μM. As expected, addition of $Shp0316_{18\text{-}24}$ could not restore the P_{comX}-*luxAB* activity and competence deficiency of AmiA1-AmiF$^-$ and Ster_0316$^-$ strains (maximum RLU/OD_{600}, 210 and 250, respectively) (Table 2), which strongly supports their respective hypothetical roles in Shp0316 importation and *comX* transcription. In contrast, the competence level of strain AmiA3$^-$ was partially restored in the presence of 1 μM $Shp0316_{18\text{-}24}$, since the maximum RLU/OD_{600} and the transformation rate increased 1.3-fold and 4-fold, respectively (Table 2). In this strain, a high extracellular $Shp0316_{18\text{-}24}$ concentration could eventually facilitate its recognition by AmiA1, which was also shown to play a role in competence induction (20).

Mature Shp0316 supplements the competence deficiency of *S. thermophilus* and *S. salivarius* strains that are poorly transformable or not transformable. The ability of *S. thermophilus* to naturally turn on high competence levels under CDM growth conditions seems restricted to strain LMD-9 (reference 20 and L. Fontaine, unpublished results). Since all of the key early competence genes identified to date are in the genome of the poorly transformable LMG18311 and CNRZ1066 strains, we assumed that differences in competence efficiency between *S. thermophilus* strains could be due to different levels of Shp0316 production, maturation, and/or recognition. To test this hypothesis, we performed competence experiments with strains LMG18311 and CNRZ1066 induced with 1 μM $Shp0316_{18\text{-}24}$. The results presented in Table 2 show that the extracellular addition of the heptapeptide restores the competence defect of these strains to levels similar to those of LMD-9 grown in CDM.

From our work and recent results of Gardan et al. (20), it is clear that evolution has selected different regulation mechanisms to govern competence in the *salivarius* and *mitis*/*mutans* groups of streptococci. In this context, analysis of the recently available draft genome sequence of *S. salivarius* SK126 (GenBank accession no. NZ_ACLO00000000) allowed the identification of a locus with a strong sequence identity to the *ster_0316-shp0316* genes of *S. thermophilus*. The orthologue of Shp0316 from SK126 displays 79% identity to Shp0316 from LMD-9 but with only a single amino acid difference (A21T) in the C-terminal heptapeptide (Fig. 4A). To provide evidence that *S. salivarius* has a similar competence regulation pathway, we performed transformation experiments with *S. salivarius* ATTC 25975, JIM8777 (12), and JIM8780 (12). *S. salivarius* was grown at 37°C in CDM containing 1 μg of pG$^+$host9 plasmid DNA. Induction was performed with 1 μM $Shp0316_{18\text{-}24}$ from LMD-9, as described for *S. thermophilus*.

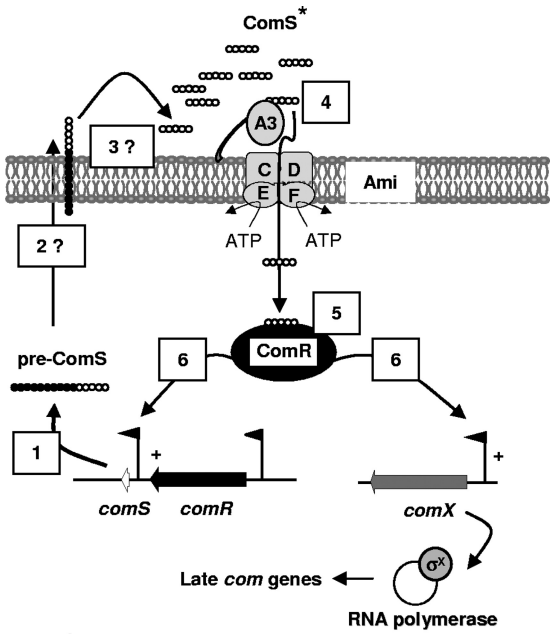

FIG. 5. Model of quorum-sensing activation of *comX* of *S. thermophilus* LMD-9 in CDM. (1) Under CDM growth conditions, the transcription of *comS* (*shp0316*) is triggered and the precursor pre-ComS is produced. (2) Pre-ComS is secreted, probably through the general secretory system. (3) Pre-ComS undergoes one or more maturation steps, and its mature form, ComS*, accumulates in the extracellular medium. (4) At a critical concentration, ComS* is recognized by the oligopeptide-binding protein AmiA3 and internalized by the oligopeptide Ami transporter (AmiCDEF). (5) Intracellular ComS* interacts with and activates the transcriptional regulator ComR (Ster_0316). (6) ComR binds to its operator sequence (ComR box; ECom box) located upstream of the −10 box of *comS* (*shp0316*) and *comX*, resulting in the autoamplification loop and activation of the late competence genes, respectively. Black arrowheads, promoters; +, activation by ComR.

The *S. salivarius* strains were poorly transformable or not transformable in the absence of the inducing peptide, but addition of $Shp0316_{18-24}$ activated competence development of the three strains with a transformation rate for strain JIM8777 similar to that of *S. thermophilus* CNRZ1066 (Table 2).

DISCUSSION

The presence of late competence genes in all streptococcal genomes sequenced to date strongly suggests that natural DNA transformation has been positively selected through evolution of these microorganisms, whatever their niche. However, the regulatory mechanisms governing *comX* induction in early competence development seem to have evolved independently in species from the *S. mitis*/*mutans* and *salivarius* groups. These systems mainly differ in the mechanisms of competence pheromone recognition and information transfer for *comX* induction. Indeed, the results obtained in our study strongly suggest the following model for early competence induction in *S. thermophilus* and *S. salivarius* (Fig. 5): under CDM growth conditions, peptide Shp0316 (renamed ComS for competence signal) is produced, matured, secreted, and mainly sensed by the AmiA3 substrate-binding protein when a critical extracellular concentration is reached. The mature Shp0316 form, ComS*, would then be imported by the Ami transporter and interact with the cytoplasmic Rgg regulator Ster_0316 (renamed ComR for competence regulator), resulting in its activation. Subsequent binding to the ECom box could finally promote *comX* transcription and the activation of the ComS-mediated positive feedback loop.

This model of competence development in *S. thermophilus* has similarities to the peptide-mediated circuits governing conjugation in *Enterococcus faecalis* (33, 51), virulence in the *B. cereus* group (52), and Phr signaling in *Bacillus subtilis* (45). Precursors of the conjugation pheromone, PapR (43), and the competence-stimulating factor, CSF (31), that, respectively, regulate these systems undergo several maturation steps. For example, the release of active conjugation octapeptides from signal sequences of lipoproteins requires two sequential processing steps, potentially catalyzed by the signal peptidase II Lsp (10) and the pheromone-specific peptidase Eep (5). In the case of cCF10, a still unknown additional peptidase is required to remove the C-terminal residue, yielding a heptapeptide pheromone (5). The composition of ComS and the presence of a putative lipobox initially suggest that it could be exported through the type II Sec-dependent secretion pathway, as suggested for lipoprotein precursors of conjugation-signaling peptides. However, the signal peptidase II does not seem to be involved in ComS maturation, since we showed that the C-terminal Leu residue is essential for ComS-inducing properties. However, the Eep homologue of *S. thermophilus* and *S. salivarius* may be responsible for the release of the C-terminal active ComS from the precursor, which is predicted to form one transmembrane segment (from amino acids 4 to 18) with a surface-exposed C terminus (TMHMM prediction [http://www.cbs.dtu.dk/services/TMHMM]). Additionally, we could not exclude the possibility that ComS is further matured by peptidases after its internalization. Whatever the actors involved in the maturation step(s), the active form of ComS shows some flexibility in its N terminus, similar to PapR (43). Indeed, the addition of 3 amino acids to the N terminus of $ComS_{18-24}$ did not affect its inducing properties.

ComR belongs to the Rgg family of pleiotropic transcriptional activators. This family is well represented among enterococci, lactococci, and streptococci, where they regulate various functions, such as production of secreted proteins (RopB of *Streptococcus pyogenes*) (6), production of mutacin II (MutR of *S. mutans*) (47) and lactocin S (LasX of *Lactococcus lactis*) (48), production of acid (GadR of *L. lactis*) (50), and oxygen tolerance (RggC of *S. thermophilus*) (16). The Rgg family is characterized by a well-conserved N-terminal XRE helix-turn-helix (HTH) DNA binding motif and a more variable C-terminal domain (36). ComR displays the highest level of identity with its orthologue in *S. salivarius* (93% identity) and with SMU.3181c of *S. mutans* (42% identity) and a lower level of identity (ranging from 34% to 28%) with regulators present in other streptococcal genomes. Prediction of the secondary and tertiary structures of ComR identified the quorum-sensing regulators PlcR of *Bacillus thuringiensis* and PrgX of *E. faecalis* as the best homologues (LOMETS predictions [http://zhang.bioinformatics.ku.edu/LOMETS]). The members of this superfamily of regulators are characterized by a C-terminal regulatory domain composed of 11 α-helices involved in

dimerization and specific pheromone interaction. Binding of their cognate signaling peptide induces a conformational change in the regulatory domain, which rearranges the DNA-binding domain (11, 51). The C-terminal domains of the PrgX, PlcR, MutR, and Rap proteins are believed to derive from a common ancestor domain to which additional domains, like HTH or phosphatase domains, have been added, yielding proteins with new regulatory functions (11). Since PlcR is known to bind to inverted repeats similar to those of ComR (30% identity) (44), it is likely that ComS-activated ComR directly binds to the *in silico*-identified ECom box. The activator-versus-repressor function of ComR is supported by three observations: (i) P_{comX} is no longer induced in the ComR$^-$ strain, (ii) ECom boxes are localized upstream of putative −10 boxes, and (iii) most regulators of the Rgg family are activators (6, 44, 47, 50), except PrgX (51) and LasX, with the latter displaying both repressor and activator functions (48).

Remarkably, we observed that competence development in *S. thermophilus* LMD-9 has a clear deleterious effect on growth under CDM conditions. In all competence-deficient LMD-9 derivative strains, both the growth rate and yield were improved. This effect was not observed in the poorly transformable strain LMG18311, where *comX* deletion had no impact on growth (data not shown). The negative effect of a high level of competence could be the consequence of the energy cost associated with induction of late competence genes. Alternatively, it is possible that a subpopulation of cells undergoes lysis when competence is induced, as reported for *S. pneumoniae* (53) and *S. mutans* (41). In this context, most early competence genes identified in our transcriptomic study are related to bacteriocin production, a process shown to be responsible for competence-induced lysis in streptococci (41). Interestingly, most *S. thermophilus* strains are unable to spontaneously turn on competence under CDM conditions while keeping the ability to respond to the addition of $ComS_{18-24}$ or to develop competence in coculture experiments with LMD-9 (unpublished results). This could indicate "cheating" behavior that is generally observed when the benefits of a particular function are balanced by energy costs that are unfavorable for the population fitness (e.g., bacteriocin production and immunity and protease$^+$ and protease$^-$ strains of *L. lactis*) (23, 28). An alternative explanation could be that the environmental conditions that trigger ComS production in most *S. thermophilus* strains are different from those in strain LMD-9.

Multilocus sequence typing (MLST) studies performed on *S. thermophilus*, *S. salivarius*, and *Streptococcus vestibularis* revealed that these species have recently diverged from a common ancestor (12). This is further supported by our cross-induction experiments, which showed that ComS-mediated communication is still possible between *S. salivarius* and *S. thermophilus*. An interesting perspective to gain further insight into the mechanisms involved in speciation within the *salivarius* group would be to investigate the diversity of the *comRS* locus in order to evaluate the presence or absence of distinct competence pherotypes. Finally, the identification of ComRS as a key quorum-sensing system activating competence instead of a classical phosphorelay three-component quorum-sensing system raised the question of the recruitment through evolution of similar regulation systems to control competence in other streptococci and related species.

ACKNOWLEDGMENTS

This research was carried out with financial support from Danisco and FNRS. C.B. holds a doctoral fellowship from FRIA. L.F. is a postdoctoral researcher at FNRS. P.H. is a research associate at FNRS.

We are grateful to E. Maguin for providing the pG$^+$host9 vector, E. Guédon for providing plasmid pJIM4900, and P. Renault for providing *S. salivarius* JIM8777 and JIM8780. We are grateful to E. J. Smit for the generous gift of custom Agilent microarrays of *S. thermophilus* LMG18311. We warmly thank P. Goffin for critically reading the manuscript.

REFERENCES

1. **Ahn, S. J., Z. T. Wen, and R. A. Burne.** 2006. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect. Immun. **74:**1631–1642.
2. **Alloing, G., B. Martin, C. Granadel, and J. P. Claverys.** 1998. Development of competence in *Streptococcus pneumonaie*: pheromone autoinduction and control of quorum sensing by the oligopeptide permease. Mol. Microbiol. **29:**75–83.
3. **Bachmann, H., M. Kleerebezem, and J. E. Hylckama Vlieg.** 2008. High-throughput identification and validation of in situ-expressed genes of *Lactococcus lactis*. Appl. Environ. Microbiol. **74:**4727–4736.
4. **Blomqvist, T., H. Steinmoen, and L. S. Havarstein.** 2006. Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. Appl. Environ. Microbiol. **72:**6751–6756.
5. **Chandler, J. R., and G. M. Dunny.** 2008. Characterization of the sequence specificity determinants required for processing and control of sex pheromone by the intramembrane protease Eep and the plasmid-encoded protein PrgY. J. Bacteriol. **190:**1172–1183.
6. **Chaussee, M. A., E. A. Callegari, and M. S. Chaussee.** 2004. Rgg regulates growth phase-dependent expression of proteins associated with secondary metabolism and stress in *Streptococcus pyogenes*. J. Bacteriol. **186:**7091–7099.
7. **Claverys, J. P., B. Martin, and L. S. Havarstein.** 2007. Competence-induced fratricide in streptococci. Mol. Microbiol. **64:**1423–1433.
8. **Claverys, J. P., M. Prudhomme, and B. Martin.** 2006. Induction of competence regulons as a general response to stress in gram-positive bacteria. Annu. Rev. Microbiol. **60:**451–475.
9. **Claverys, J. P., M. Prudhomme, I. Mortier-Barriere, and B. Martin.** 2000. Adaptation to the environment: *Streptococcus pneumoniae*, a paradigm for recombination-mediated genetic plasticity? Mol. Microbiol. **35:**251–259.
10. **Clewell, D. B., F. Y. An, S. E. Flannagan, M. Antiporta, and G. M. Dunny.** 2000. Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. Mol. Microbiol. **35:**246–247.
11. **Declerck, N., L. Bouillaut, D. Chaix, N. Rugani, L. Slamti, F. Hoh, D. Lereclus, and S. T. Arold.** 2007. Structure of PlcR: insights into virulence regulation and evolution of quorum sensing in Gram-positive bacteria. Proc. Natl. Acad. Sci. U. S. A. **104:**18490–18495.
12. **Delorme, C., C. Poyart, S. D. Ehrlich, and P. Renault.** 2007. Extent of horizontal gene transfer in evolution of streptococci of the *salivarius* group. J. Bacteriol. **189:**1330–1341.
13. **Detmers, F. J., F. C. Lanfermeijer, R. Abele, R. W. Jack, R. Tampe, W. N. Konings, and B. Poolman.** 2000. Combinatorial peptide libraries reveal the ligand-binding mechanism of the oligopeptide receptor OppA of *Lactococcus lactis*. Proc. Natl. Acad. Sci. U. S. A. **97:**12487–12492.
14. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
15. **Ferain, T., J. N. Hobbs, Jr., J. Richardson, N. Bernard, D. Garmyn, P. Hols, N. E. Allen, and J. Delcour.** 1996. Knockout of the two *ldh* genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. J. Bacteriol. **178:**5431–5437.
16. **Fernandez, A., F. Borges, B. Gintz, B. Decaris, and N. Leblond-Bourget.** 2006. The *rggC* locus, with a frameshift mutation, is involved in oxidative stress response by *Streptococcus thermophilus*. Arch. Microbiol. **186:**161–169.
17. **Finkel, S. E., and R. Kolter.** 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J. Bacteriol. **183:**6288–6293.
18. **Fontaine, L., C. Boutry, E. Guedon, A. Guillot, M. Ibrahim, B. Grossiord, and P. Hols.** 2007. Quorum-sensing regulation of the production of Blp bacteriocins in *Streptococcus thermophilus*. J. Bacteriol. **189:**7195–7205.
19. **Fontaine, L., and P. Hols.** 2008. The inhibitory spectrum of thermophilin 9 from *Streptococcus thermophilus* LMD-9 depends on the production of multiple peptides and the activity of BlpG(St), a thiol-disulfide oxidase. Appl. Environ. Microbiol. **74:**1102–1110.
20. **Gardan, R., C. Besset, A. Guillot, C. Gitton, and V. Monnet.** 2009. The oligopeptide transport system is essential for the development of natural competence in *Streptococcus thermophilus* strain LMD-9. J. Bacteriol. **191:**4647–4655.
21. **Heng, N. C., J. R. Tagg, and G. R. Tompkins.** 2007. Competence-dependent

bacteriocin production by *Streptococcus gordonii* DL1 (Challis). J. Bacteriol. **189:**1468–1472.

22. **Hols, P., F. Hancy, L. Fontaine, B. Grossiord, D. Prozzi, N. Leblond-Bourget, B. Decaris, A. Bolotin, C. Delorme, E. S. Dusko, E. Guedon, V. Monnet, P. Renault, and M. Kleerebezem.** 2005. New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. FEMS Microbiol. Rev. **29:**435–463.
23. **Hugenholtz, J., R. Splint, W. N. Konings, and H. Veldkamp.** 1987. Selection of protease-positive and protease-negative variants of *Streptococcus cremoris*. Appl. Environ. Microbiol. **53:**309–314.
24. **Hutchings, M. I., T. Palmer, D. J. Harrington, and I. C. Sutcliffe.** 2009. Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold 'em, knowing when to fold 'em. Trends Microbiol. **17:**13–21.
25. **Ibrahim, M., A. Guillot, F. Wessner, F. Algaron, C. Besset, P. Courtin, R. Gardan, and V. Monnet.** 2007. Control of the transcription of a short gene encoding a cyclic peptide in *Streptococcus thermophilus*: a new quorum-sensing system? J. Bacteriol. **189:**8844–8854.
26. **Ibrahim, M., P. Nicolas, P. Bessieres, A. Bolotin, V. Monnet, and R. Gardan.** 2007. A genome-wide survey of short coding sequences in streptococci. Microbiology **153:**3631–3644.
27. **Johnsborg, O., and L. S. Havarstein.** 2009. Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. FEMS Microbiol. Rev. **33:**627–642.
28. **Kerr, B., M. A. Riley, M. W. Feldman, and B. J. Bohannan.** 2002. Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature **418:**171–174.
29. **Kreth, J., J. Merritt, W. Shi, and F. Qi.** 2005. Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. Mol. Microbiol. **57:**392–404.
30. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **73:**1126–1135.
31. **Lanigan-Gerdes, S., A. N. Dooley, K. F. Faull, and B. A. Lazazzera.** 2007. Identification of subtilisin, Epr and Vpr as enzymes that produce CSF, an extracellular signalling peptide of *Bacillus subtilis*. Mol. Microbiol. **65:**1321–1333.
32. **Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts.** 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. **177:**7011–7018.
33. **Leonard, B. A., A. Podbielski, P. J. Hedberg, and G. M. Dunny.** 1996. *Enterococcus faecalis* pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. Proc. Natl. Acad. Sci. U. S. A. **93:**260–264.
34. **Letort, C., and V. Juillard.** 2001. Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*. J. Appl. Microbiol. **91:**1023–1029.
35. **Li, Y. H., X. L. Tian, G. Layton, C. Norgaard, and G. Sisson.** 2008. Additive attenuation of virulence and cariogenic potential of *Streptococcus mutans* by simultaneous inactivation of the ComCDE quorum-sensing system and HK/RR11 two-component regulatory system. Microbiology **154:**3256–3265.
36. **Loughman, J. A., and M. G. Caparon.** 2007. Contribution of invariant residues to the function of Rgg family transcription regulators. J. Bacteriol. **189:**650–655.
37. **Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. **178:**931–935.
38. **Martin, B., Y. Quentin, G. Fichant, and J. P. Claverys.** 2006. Independent evolution of competence regulatory cascades in streptococci? Trends Microbiol. **14:**339–345.
39. **Monnet, V.** 2003. Bacterial oligopeptide-binding proteins. Cell Mol. Life Sci. **60:**2100–2114.
40. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
41. **Perry, J. A., M. B. Jones, S. N. Peterson, D. G. Cvitkovitch, and C. M. Levesque.** 2009. Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence. Mol. Microbiol. **72:**905–917.
42. **Peterson, S. N., C. K. Sung, R. Cline, B. V. Desai, E. C. Snesrud, P. Luo, J. Walling, H. Li, M. Mintz, G. Tsegaye, P. C. Burr, Y. Do, S. Ahn, J. Gilbert, R. D. Fleischmann, and D. A. Morrison.** 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol. Microbiol. **51:**1051–1070.
43. **Pomerantsev, A. P., O. M. Pomerantseva, A. S. Camp, R. Mukkamala, S. Goldman, and S. H. Leppla.** 2009. PapR peptide maturation: role of the NprB protease in *Bacillus cereus* 569 PlcR/PapR global gene regulation. FEMS Immunol. Med. Microbiol. **55:**361–377.
44. **Pomerantsev, A. P., O. M. Pomerantseva, and S. H. Leppla.** 2004. A spontaneous translational fusion of *Bacillus cereus* PlcR and PapR activates transcription of PlcR-dependent genes in *Bacillus anthracis* via binding with a specific palindromic sequence. Infect. Immun. **72:**5814–5823.
45. **Pottathil, M., and B. A. Lazazzera.** 2003. The extracellular Phr peptide-Rap phosphatase signaling circuit of *Bacillus subtilis*. Front. Biosci. **8:**d32–d45.
46. **Prudhomme, M., L. Attaiech, G. Sanchez, B. Martin, and J. P. Claverys.** 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science **313:**89–92.
47. **Qi, F., P. Chen, and P. W. Caufield.** 1999. Functional analyses of the promoters in the lantibiotic mutacin II biosynthetic locus in *Streptococcus mutans*. Appl. Environ. Microbiol. **65:**652–658.
48. **Rawlinson, E. L., I. F. Nes, and M. Skaugen.** 2005. Identification of the DNA-binding site of the Rgg-like regulator LasX within the lactocin S promoter region. Microbiology **151:**813–823.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold spring Harbor Laboratory, Cold Spring Harbor, N. Y.
50. **Sanders, J. W., K. Leenhouts, J. Burghoorn, J. R. Brands, G. Venema, and J. Kok.** 1998. A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. Mol. Microbiol. **27:**299–310.
51. **Shi, K., C. K. Brown, Z. Y. Gu, B. K. Kozlowicz, G. M. Dunny, D. H. Ohlendorf, and C. A. Earhart.** 2005. Structure of peptide sex pheromone receptor PrgX and PrgX/pheromone complexes and regulation of conjugation in *Enterococcus faecalis*. Proc. Natl. Acad. Sci. U. S. A. **102:**18596–18601.
52. **Slamti, L., and D. Lereclus.** 2002. A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. EMBO J. **21:**4550–4559.
53. **Steinmoen, H., E. Knutsen, and L. S. Havarstein.** 2002. Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. Proc. Natl. Acad. Sci. U. S. A. **99:**7681–7686.
54. **van Kranenburg, R., J. D. Marugg, I. I. van Swam, N. J. Willem, and W. M. de Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. **24:**387–397.