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Regulation of Bacteriocin Production in *Streptococcus mutans* by the Quorum-Sensing System Required for Development of Genetic Competence

Jan R. van der Ploeg*

Institute for Oral Biology, Center for Dental-, Oral Medicine and Maxillofacial Surgery, University of Zürich, Zürich, Switzerland

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In *Streptococcus mutans*, competence for genetic transformation and biofilm formation are dependent on the two-component signal transduction system ComDE together with the inducer peptide pheromone competence-stimulating peptide (CSP) (encoded by *comC*). Here, it is shown that the same system is also required for expression of the *nlmAB* genes, which encode a two-peptide nonlantibiotic bacteriocin. Expression from a transcriptional *nlmAB′-lacZ* fusion was highest at high cell density and was increased up to 60-fold following addition of CSP, but it was abolished when the *comDE* genes were interrupted. Two more genes, encoding another putative bacteriocin and a putative bacteriocin immunity protein, were also regulated by this system. The regions upstream of these genes and of two further putative bacteriocin-encoding genes and a gene encoding a putative bacteriocin immunity protein contained a conserved 9-bp repeat element just upstream of the transcription start, which suggests that expression of these genes is also dependent on the ComCDE regulatory system. Mutations in the repeat element of the *nlmAB* promoter region led to a decrease in CSP-dependent expression of *nlmAB′-lacZ*. In agreement with these results, a *comDE* mutant and mutants unable to synthesize or export CSP did not produce bacteriocins. It is speculated that, at high cell density, bacteriocin production is induced to liberate DNA from competing streptococci.

The gram-positive bacterium *Streptococcus mutans* is a major cause of human dental caries due to its capacity to metabolize carbohydrates to generate lactic acid, even at low pH. Some of the virulence factors that determine colonization and survival of *S. mutans* in dental plaque, such as adherence, acid resistance, acidogenicity, and resistance to other stress conditions, have been well studied (4). Another determinant that could be important for colonization is the production of bacteriocins, ribosomally synthesized peptides or proteins with antibacterial activity. In a survey of 143 strains of *S. mutans*, 70% were shown to produce one or more bacteriocins in vitro (29). However, little information is available about the ecological significance of bacteriocins produced by *S. mutans*. It is not known whether bacteriocins are produced in dental plaque, although some studies have provided indirect evidence that this is the case (9).

Bacteriocins from gram-positive bacteria have been classified into three groups: class I bacteriocins contain the post-translationally modified bacteriocins or lantibiotics, class II comprise small nonmodified, heat-stable peptide bacteriocins, and class III bacteriocins contain large, heat-labile proteins (21). Class II bacteriocins can be further divided into two groups: those that resemble the antilisterial bacteriocin pediocin from *Pediococcus* species (class IIA) and those that are composed of two peptides (class IIB). The class II bacteriocins are synthesized as prepeptides that contain a leader peptide with two glycine residues at the cleavage site. The mature peptide is cleaved off from the leader peptide after these two glycine residues and released in the medium by an ABC transporter. The class II bacteriocins act on other organisms by insertion in the membrane, causing formation of pores that perturb the membrane potential. Bacteriocin-producing organisms are resistant to their own bacteriocins through the action of immunity proteins.

Most of the bacteriocins from *S. mutans* characterized up to now belong to the group I lantibiotics. Production of two of these, mutacin I and mutacin 1140, appears to be dependent on the culture conditions, since they could be detected only after growth on agar or agarose-containing medium (10, 27). A nonlantibiotic class IIB bacteriocin, mutacin IV, was purified from *S. mutans* strain UA140, which also produces the lantibiotic mutacin I (27). Whereas mutacin I could be produced only when cells were grown on solid medium, mutacin IV was produced in liquid culture exclusively. Mutacin IV is encoded by the *nlmA* and *nlmB* genes, which are probably organized in an operon.

Expression of class II bacteriocins generally requires an inducer peptide pheromone and a two-component signal transduction system (TCSTS) (20). The TCSTS is composed of a histidine protein kinase that serves to sense the presence of the peptide outside of the cell and transmits the signal over the membrane to a response regulator that activates transcription of the target genes. In many cases, these systems are autoregulated (20). Related two-component regulatory systems are required for competence development in streptococci. In *S. mutans*, development of competence is under the control of the *comCDE* system, where *comC* encodes the precursor of the pheromone competence-stimulating peptide (CSP) and *comD* and *comE* encode the histidine kinase sensor and response

* Mailing address: Institute for Oral Biology, Center for Dental-, Oral Medicine and Maxillofacial Surgery, University of Zürich, Plattenstrasse 11, CH-8032, Zürich, Switzerland. Phone: 41 446343329. Fax: 41 446344310. E-mail: jvdploeg@zzmk.unizh.ch.

TABLE 1. Primers used for construction of mutants and *lacZ* fusion strains

Primer	Sequence (5′-3′)	Construct(s) for which used
comDE1	TCTGGATCCGCAACCGACATCTC	*comDE* mutants, *comC* mutants
comDE2	GGACTCTAGACTCCAATCGCGGTATT	*comDE* mutants, *bsmA′-lacZ*
comDE3	GTCTTCCATGGTAGCTGCAATG	*comDE* mutants
comDE4	GATCCTGCAGTTACTGTCGTC	*comDE* mutants
comC1	TACCCTCGAGAATATCGGCCATC	*comC* mutants
comC3	CCGCCTGCAGTAGCCATATAACC	*comC* mutants
comC5	GTCCCATGGGTTTTTTTCATTTTATATCTCC	*comC* mutants, *bsmA′-lacZ*
ciaRH1	TGTAGGGATCCCCACTACAGATAC	*ciaRH* mutants
ciaRH2	GCTCAAGCTTTGCATGACATCAGC	*ciaRH* mutants
ciaRH3	GCTACCATGGAAGAGGTCAGAAAC	*ciaRH* mutants
ciaRH4	GAGCTGCAGACATTACTCCTTGTG	*ciaRH* mutants
comX1	GATCGGATCCTGATGTCGTAGGTTCG	*comX* mutants
comX2	GATCGAGCTCTTCACGAGTCCAC	*comX* mutants
comX3	CATGCCATGGGAGCGCTTGGTAGCA	*comX* mutants
comX4	GATCCTGCAGTGCAGCAGCATCAC	*comX* mutants
comA1	GATCGGATCCTCGTCATAGCCGTTA	*comA* mutants
comA2	GATCGAGCTCATGACCTGCTGGATA	*comA* mutants
comA3	GATCCCATGGTCTTGTCGAATCTGAA	*comA* mutants
comA4	GATCGAATTCCAGTACGCTGTGAA	*comA* mutants
nlmAB1	GATCGAATTCGATGTAATGGACAGCC	*nlmAB′-lacZ* (OMZ1008 and derivatives)
nlmAB2	AGTGTGGATCCACTACAGATCCAAC	*nlmAB′-lacZ* (OMZ1008 and derivatives)
immA2	AGAGGATCCTTGCGCAGCCAG	*immA′-lacZ*
immA9	GTAGGCATGCTGATTCTGCTGGCA	*immA′-lacZ*
immB9	GATCTCTAGATTGCACAAGCACGCTG	*immB′-lacZ*
immB10	GATCGCATGCAGCAGCTGAGAAG	*immB′-lacZ*
bsmK1	GATCGCATGCAACCTAGCCACTCTC	*bsmK′-lacZ*
bsmK2	GATCTCTAGATCCAGGGAGCGGAAA	*bsmK′-lacZ*
bsmE1	CTACTCTAGATATTGGCTGCTTTTACACT	*bsmE′-lacZ*
bsmE2	GATCGCATGCAACTTGCTGCACCTGA	*bsmE′-lacZ*
bsmI1	GATCTCTAGACGGCGAACTGCTGTA	*bsmHI′-lacZ*
bsmI3	GATCGCATGCTAACGTCTCTGTATCC	*bsmHI′-lacZ*
nlmAB10	CATGTCTAGAGGTGTAGAGGCTATTATGTAG	*nlmAB′-lacZ* (OMZ1033)
nlmAB11	GATCGCATGCTTCAAATGCCTGTGTA	*nlmAB′-lacZ* (OMZ1033)
nlmAB-EcorV-f	CATGGATATCTTAAGACAAAATAGCTACCA	*nlmAB′-lacZ* with EcoRV site (OMZ1034)
nlmAB-EcorV-r	CATGGATATCATTTTTGATGTTCTGAAACT	*nlmAB′-lacZ* with EcoRV site (OMZ1034)
nlmAB-PstI-f	ATAGCTGCAGTTTAGGATATTTTGCTCTATTTTG	*nlmAB′-lacZ* with PstI site (OMZ1035)
nlmAB-PstI-r	GTACCTGCAGCTATTTTGTCTTAAACGGTC	*nlmAB′-lacZ* with PstI site (OMZ1035)
nlmAB-ClaI-f	CATGATCGATATTTTGCTCTATTTTGAAAA	*nlmAB′-lacZ* with ClaI site (OMZ1036, OMZ1037)
nlmAB-ClaI-r	AGCTATCGATTTTTGATGTTCTGAAAC	*nlmAB′-lacZ* with deletion and ClaI site (OMZ1037)
nlmAB-ClaI-r2	GATCATCGATAATGGTAGCTATTTTGTCT	*nlmAB′-lacZ* with ClaI site (OMZ1036)

regulator, respectively (15). The precursor of CSP is processed and exported through an ATP-binding cassette transporter and an accessory protein, which are encoded by *comA* and *comB*, respectively (37).

In this study, it is shown that expression of *S. mutans nlmAB*, encoding mutacin IV, and that of other class IIB bacteriocins and associated genes are induced upon addition of CSP and that inactivation of the two-component regulatory system required for competence development abolishes the production of bacteriocins.

MATERIALS AND METHODS

Materials. Reagents for molecular biology were obtained from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA). CSP from *S. mutans* (NH_2-SGSLSTFFRLFNRSFTQALGK-COOH) was synthesized by ESGS (Evry, France). The primers used for generation of mutants and *lacZ* fusion strains are listed in Table 1 and were obtained from Microsynth (Balgach, Switzerland).

Strains and growth conditions. The strains of *S. mutans* that were used in this study are listed in Table 2. OMZ1001 is a derivative of strain UA159 (ATCC 700610), characterized by a high transformation efficiency. It was isolated in our laboratory. *S. mutans* OMZ1001 and OMZ67 (GS-5), *Streptococcus oralis* OMZ607 (12), and *Streptococcus gordonii* OMZ505 (ATCC 10558) were grown routinely in Todd-Hewitt broth supplemented with 0.3% yeast extract (THY) at 37°C under aerobic conditions or under anaerobic conditions (5% CO_2, 10% H_2, 85% N_2). When required, antibiotics were used at the following concentrations: erythromycin, 15 μg/ml; kanamycin, 750 μg/ml.

Escherichia coli strain DH5α was used as a host for plasmids and grown in LB medium. When required, antibiotics were added at the following final concentrations: erythromycin, 200 μg/ml; kanamycin, 50 μg/ml.

DNA manipulations. Plasmid DNA purification from *E. coli*, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation, and transformation of *E. coli* were carried out using standard methods (3). Chromosomal DNA from *S. mutans* was isolated by using the Genelute bacterial genomic DNA kit (Sigma, Buchs, Switzerland).

Construction of *lacZ* fusion strains. To generate a chromosomal *nlmAB′-lacZ* fusion, a fragment internal to the *nlmAB* genes (bp 205 to bp 614 [27]) was amplified by PCR using the primers nlmAB1 and nlmAB2 (Table 1) and chromosomal DNA from OMZ1001. The PCR fragment was digested with BamHI and EcoRI and cloned into the vector pSF151 (31) to give plasmid pOMZ46. Subsequently, a BamHI fragment harboring a promoterless *lacZ* gene (11) was inserted downstream of *nlmAB* in the same direction of transcription, which resulted in plasmid pOMZ47. The plasmid was then introduced into *S. mutans* OMZ1001 by transformation (23) followed by selection for kanamycin resistance. Since pSF151 and its derivatives cannot replicate in *S. mutans*, kanamycin-

TABLE 2. *S. mutans* strains used in this study

Strain designation	Genotype or characteristic(s)	Source or reference
OMZ1001	Derivative of UA159, high transformation frequency	Laboratory collection
OMZ67	GS-5	Laboratory collection
SMUHK1	*vicK*::*ermAM*	D. Cvitkovitch, University of Toronto
OMZ1002	As OMZ1001 but *comC*::*ermAM*	This study
OMZ1003	As OMZ1001 but *comDE*::*ermAM*	This study
OMZ1004	As OMZ1001 but *ciaRH*::*ermAM*	This study
OMZ1005	As OMZ1001 but *vicK*::*ermAM*	This study
OMZ1006	As OMZ1001 but *comX*::*ermAM*	This study
OMZ1007	As OMZ1001 but *comA*::*ermAM*	This study
OMZ1008	OMZ1001 with *nlmAB′-lacZ* generated with primers nlmAB1 and nlmAB2	This study
OMZ1009	As OMZ1008 but *comDE*::*ermAM*	This study
OMZ1010	As OMZ1008 but *comC*::*ermAM*	This study
OMZ1011	As OMZ1008 but *ciaRH*::*ermAM*	This study
OMZ1012	As OMZ1008 but *vicK*::*ermAM*	This study
OMZ1013	As OMZ1008 but *comX*::*ermAM*	This study
OMZ1014	As OMZ1008 but *comA*::*ermAM*	This study
OMZ1015	OMZ1001 with *bsmA′-lacZ*	This study
OMZ1016	As OMZ1015 but *comDE*::*ermAM*	This study
OMZ1017	OMZ1001 with *immA′-lacZ*	This study
OMZ1018	As OMZ1017 but *comDE*::*ermAM*	This study
OMZ1019	OMZ1001 with *immB′-lacZ*	This study
OMZ1020	As OMZ1019 but *comDE*::*ermAM*	This study
OMZ1021	As OMZ67 but *comC*::*ermAM*	This study
OMZ1022	As OMZ67 but *comDE*::*ermAM*	This study
OMZ1023	As OMZ67 but *ciaRH*::*ermAM*	This study
OMZ1024	As OMZ67 but *vicK*::*ermAM*	This study
OMZ1025	As OMZ67 but *comX*::*ermAM*	This study
OMZ1026	As OMZ67 but *comA*::*ermAM*	This study
OMZ1027	OMZ1001 with *bsmHI′-lacZ*	This study
OMZ1028	OMZ1001 with *bsmK′-lacZ*	This study
OMZ1029	OMZ1001 with *bsmE′-lacZ*	This study
OMZ1030	As OMZ1027 but *comDE*::*ermAM*	This study
OMZ1031	As OMZ1028 but *comDE*::*ermAM*	This study
OMZ1032	As OMZ1029 but *comDE*::*ermAM*	This study
OMZ1033	OMZ1001 with *pnlmAB′-lacZ* generated with primers nlmAB10 and nlmAB11	This study
OMZ1034	As OMZ1033 but with EcoRV site in repeat 1	This study
OMZ1035	As OMZ1033 but with PstI site in repeat 2	This study
OMZ1036	As OMZ1033 but with ClaI site in repeat 2	This study
OMZ1037	As OMZ1033 but with deletion of both repeats, ClaI site	This study

resistant colonies must have arisen from single crossover and contain a *lacZ* fusion to *nlmAB*. The resulting strain was designated OMZ1008.

For the construction of the *lacZ* fusions with the *bsmA*, *bsmE*, *bsmHI*, *bsmK*, *immB*, and *immA* genes of *S. mutans*, a derivative of plasmid pSF151 was prepared by digestion with XbaI and PstI, followed by filling in with T4 DNA polymerase and self-ligation, resulting in pOMZ119. A 3.1-kb BamHI fragment from pALH122 harboring the *lacZ* gene was cloned in the BamHI site of pOMZ119 to give pOMZ125. Fragments harboring part of the gene of interest were generated by PCR amplification and cloned into the SphI/XbaI sites of plasmid pOMZ125. The resulting plasmids were introduced into *S. mutans* OMZ1001 by transformation, again followed by selection for kanamycin resistance. For each strain, correct integration was confirmed by PCR analysis.

Transcriptional fusions between mutated *nlmAB* promoter regions and *lacZ* were generated as follows. PCR of the region upstream of and including the prospective mutation was carried out with a primer that contained an XbaI restriction site (nlmAB10) and a primer that introduced a second restriction site and the desired mutation (Table 1). In a second PCR, the region downstream of the mutation was amplified with a primer that introduced an SphI site (nlmAB11) and a primer that introduced the same restriction site and mutation as those in the first PCR. The two PCR products were digested with the enzymes that recognized the newly created restriction sites and ligated together into pOMZ125, which had been digested with SphI and XbaI. The resulting plasmids were subsequently transformed into *S. mutans*. Restriction analysis of PCR products was then used to confirm that the mutation was present at the expected location.

Construction of mutants. Mutants were constructed by first cloning fragments containing portions of the 5′ and the 3′ ends of the gene or genes of interest up- and downstream of the erythromycin resistance gene of the plasmid pFW15 (26). The resulting plasmids were then linearized and introduced into *S. mutans* by transformation and selection for erythromycin resistance. The mutants were verified by PCR analysis. As summarized in Table 2 this procedure was applied to the *S. mutans* strains OMZ1001 and OMZ67, as well as to various derivatives of OMZ1001 containing *lacZ* fusions.

Specifically, a mutant of *S. mutans* in which part of both the *comD* and the *comE* gene was replaced by the erythromycin resistance gene was constructed as follows. A fragment was amplified by PCR using the primers comDE3 and comDE4 and chromosomal DNA from *S. mutans* OMZ1001. The PCR product was then digested with NcoI and PstI and cloned into the vector pFW15 to give plasmid pOMZ43. A second fragment was obtained by PCR with primers comDE1 and comDE2. This fragment was first cloned in pBluescript KS as a BamHI/XbaI fragment, recovered by digestion with BamHI and SacI, and cloned into pOMZ43 to give plasmid pOMZ61. pOMZ61 was then linearized with BamHI and introduced into *S. mutans* by transformation. Erythromycin-resistant colonies were selected, and one of the colonies was purified for further use. Analogously, mutants were generated, which contained a deletion/erythromycin resistance insertion in *comC*, *ciaRH*, *comA*, and *comX*. The *vicK* mutants were

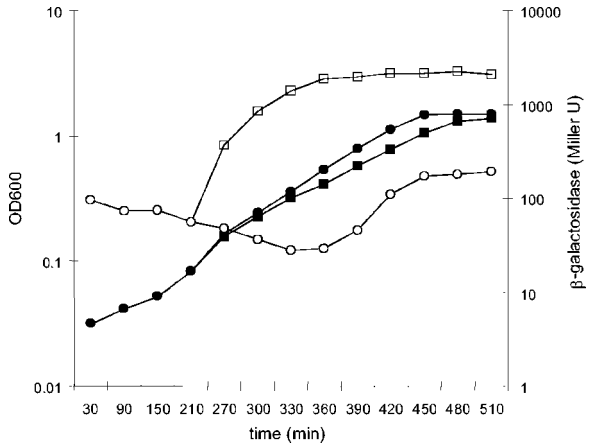

FIG. 1. Induction of *nlmAB′-lacZ* expression by CSP. An overnight culture of *S. mutans* strain OMZ1008 was diluted 100-fold in fresh medium and grown aerobically at 37°C. After 4 h of growth, the culture was split and CSP was added to a final concentration of 0.5 μg/ml to one portion of the culture. The optical density at 600 nm (closed symbols) and β-galactosidase activity (open symbols) were measured throughout growth. Circles: the culture did not receive CSP; squares, the culture received CSP. The figure shows the results of a representative experiment.

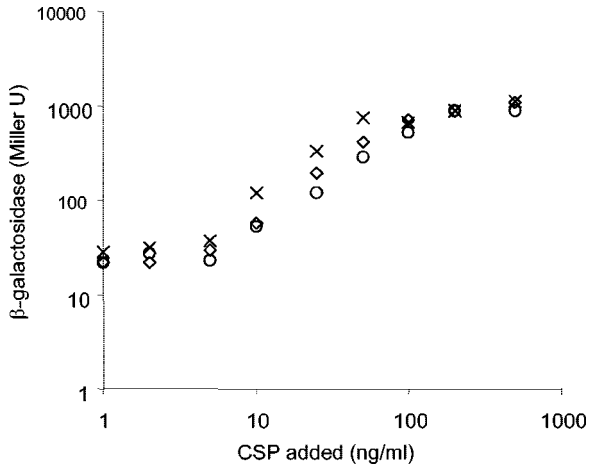

FIG. 2. Influence of the amount of CSP on expression of *nlmAB′-lacZ*. An overnight culture of *S. mutans* strain OMZ1008 was diluted 100-fold in fresh medium and grown aerobically at 37°C. After 4 h of growth, the culture was divided in several aliquots. Each aliquot of the culture received a different amount of CSP and was allowed to grow for another 2 hours. The activity of β-galactosidase was then measured in each fraction. The figure shows the results of three independent experiments (indicated by circles, diamonds, and crosses).

constructed by transformation of *S. mutans* with chromosomal DNA from strain SMUHK1, a *vicK* mutant obtained from D. Cvitkovitch.

β-Galactosidase and bacteriocin assays. β-Galactosidase was measured according to the method of Miller (17) with *o*-nitrophenylgalactoside as substrate.

To measure bacteriocin production, producer strains were grown overnight in THY broth, stab inoculated into THY agar, and grown anaerobically for 24 or 48 h. The indicator strains *S. oralis* OMZ607 and *S. gordonii* OMZ505 were grown overnight in THY broth, and 0.1 ml of the indicator culture was mixed with 4 ml of molten THY top agar and poured over the plate. The plates were incubated for 24 h at 37°C in an anaerobic chamber, and the diameter of the zone of inhibition around the producing strains was measured.

Determination of the transcription initiation site of *bsmA*. *S. mutans* OMZ1001 was grown to the early-exponential phase in THY at 37°C under aerobic conditions, CSP was added to a final concentration of 100 ng/ml, and growth was continued for 2 h. RNA was isolated by using the FastRNA Pro Blue kit (Qbiogene, Inc., Carlsbad, Calif.) and the FastPrep instrument (Qbiogene). For primer extension analysis, 20 pmol of primer bsmAPE (5′-GCTGTACCG CCTGCAGTAGCCATATAAC-3′), labeled at its 5′ end with 6-carboxyfluorescein, was annealed with 20 μg of RNA. cDNA was produced by using Superscript II (Invitrogen, Basel, Switzerland). The product was precipitated with ethanol and dissolved in 2 μl of water, and 1 μl was loaded on an ABI Prism sequence analyzer model 3100 (Applied Biosystems, Rotkreuz, Switzerland). Fragment sizes were calculated by applying Genescan DB-30 markers (Applied Biosystems) together with the sample. To verify the size of the reverse transcriptase reaction product, a PCR was carried out with the 6-carboxyfluorescein-labeled primer bsmAPE and a second primer, using chromosomal DNA from *S. mutans* OMZ1001 as template. The product, which should be 200 bp in size, was applied together with the Genescan DB-30 standard. The determined product size was 197 bp. Therefore, to correct for this difference, 3 bp was added to the size determined for the reverse transcription product.

RESULTS

Expression of β-galactosidase from a transcriptional *nlmAB′-lacZ* fusion. Expression of β-galactosidase from the transcriptional *nlmAB′-lacZ* fusion present in strain OMZ1008 was measured during aerobic growth in THY broth (Fig. 1). An initial decrease upon entry in the exponential growth phase and an increase upon entry in the stationary growth phase were observed. When *S. mutans* OMZ1008 was grown anaerobically for 48 h in solid THY medium, expression of β-galactosidase from the *nlmAB′-lacZ* fusion was increased approximately sixfold compared to cells that were grown anaerobically in liquid THY medium (253 ± 125 Miller units and 41 ± 6 Miller units, respectively). There was a small difference in expression between aerobically and anaerobically grown cells after 48 h (58 ± 10 and 41 ± 6 Miller units, respectively).

It was investigated whether addition of the competence-stimulating peptide had an effect on expression of *nlmAB′-lacZ*. Addition of CSP at a concentration of 0.5 μg/ml to an aerobic liquid culture led to a strong increase of β-galactosidase activity (Fig. 1). After 6 h of growth, the activity of β-galactosidase was about 60-fold higher in cells that were grown with CSP than in cells that were grown in its absence.

The amount of CSP added to the growth medium was varied from 1 ng/ml to 500 ng/ml, and β-galactosidase activity was measured (Fig. 2). Above 5 ng/ml, an increase in β-galactosidase activity was observed. This stimulation was linear until a CSP concentration of about 100 ng/ml. Thus, the expression of *nlmAB* is dependent on the amount of CSP present in the growth medium and NlmAB bacteriocin synthesis is therefore regulated as part of the *comCDE* quorum-sensing regulon.

Expression of *nlmAB′-lacZ* in mutants. To consolidate the above results and to investigate the effect of mutations in genes known to influence competence development, β-galactosidase expressed from the *nlmAB′-lacZ* fusion was measured in different mutants (Table 3). Activity in the *comC* and *comA* mutants was enhanced as in the wild-type strain, but the *comDE* mutant did not show an increase in activity of β-galactosidase after CSP addition. This confirms that the three-component *comCDE* system is necessary for expression of *nlmAB*.

In analogy to the situation in *Streptococcus pneumoniae* (13), the *comX* gene of *S. mutans* (SMU.1997) is thought to encode an alternative sigma factor required for transcription of the

TABLE 3. β-Galactosidase activity expressed from a transcriptional *nlmAB′-lacZ* fusion in wild-type and mutant strains

Strain	Mutation	β-Galactosidase activity from *nlmAB′-lacZ* (Miller U)[a]	
		−CSP	+CSP
OMZ1008	None	39 ± 9	1,182 ± 83
OMZ1010	*comC*	13 ± 3	943 ± 55
OMZ1009	*comDE*	15 ± 1	18 ± 4
OMZ1013	*comX*	37 ± 31	778 ± 103
OMZ1011	*ciaRH*	53 ± 28	1,001 ± 189
OMZ1014	*comA*	16 ± 1	1,021 ± 23
OMZ1012	*vicK*	230 ± 77	920 ± 185

[a] Wild-type and mutant strains were grown overnight in THY medium, diluted 50-fold in fresh medium, and grown aerobically at 37°C. After 3 hours of growth, cultures were split and CSP (100 ng/ml) was added to one of the samples. The cultures were grown for 2 more hours, and β-galactosidase was measured. The values are the means of at least two independent experiments, and the standard deviation is indicated.

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BsmA     --MNTQA--FEQFNVMDNEALSAVEGG
BsmC     --MNTQA--FEQFNVMDNEALSTVEGG
NlmA     --MDTQA--FEQFDVMDSQTLSTVEGG
BsmB     --MNTHV--LEQFDVMDSQVPSAIEGG
BsmE     --MNTKM--MEQFETMDAETLSHVTGG
BsmF     --MNTRT--LEQFDAMDVDMLAAVEGG
BsmH     --MEIKA--LDQFETMDTDMLAAVEGG
BsmI     --MNTQK--LNQFETMDTETLATIEGG
BsmG     MKTQTEI--WKRFEALDTADLAIIQGG
NlmB     --MELNV---NNYKSLTNDELSEVFGG
BsmK     --MNYSK---NHVKSLTETELMAIVGG
BsmL     --MEKQY---NNFKILNTDALENIQGG
ComC     --MKKTLSLKNDFKEIKTDELEIIIGG
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FIG. 3. Clustal W sequence alignment of the leader peptide sequences of putative type II bacteriocins and CSP. Asterisk, identical residue; colon, conserved residue; period, semiconserved residue.

late competence genes, whose expression is induced about 5 min later than that of the genes directly regulated by ComE (25). The *comX* gene of *S. mutans* was previously found to be necessary for competence (15). But the *comX* mutation had no effect on expression of β-galactosidase from the *nlmAB′-lacZ* fusion, indicating that the ComX protein is not required as an alternative sigma factor for transcription of *nlmAB*.

The *ciaRH* genes (SMU.1128 and SMU.1129) of *S. mutans* encode a two-component signal transduction system that is involved in competence development. Inactivation of *ciaH*, encoding the histidine kinase, led to a decrease of competence (28), whereas interruption of the complete *ciaRH* gene cluster resulted in an increase of competence (J. R. van der Ploeg, unpublished results). Interestingly, a *ciaH* deletion, but not a *ciaR* deletion, was found to abolish production of the lantibiotic bacteriocin mutacin but had no influence on production of mutacin IV (encoded by *nlmAB*) (28). The *ciaRH* mutant investigated in this study is defective in both *ciaR* and *ciaH*, yet this mutation had no apparent effect on expression of *nlmAB′-lacZ* (Table 3).

Another two-component signal transduction system, encoded by *vicRK*, has been shown to influence competence development (D. Cvitkovitch, personal communication). The *vicK* gene (SMU.1516) encodes a histidine kinase, whereas *vicR* (SMU.1517) encodes a response regulator. When a *vicK* mutation was introduced into OMZ1008, the level of *nlmAB′-lacZ* expression in uninduced cultures was higher than in uninduced cultures of the wild-type strain (Table 3), indicating that *vicK* acts negatively on *nlmAB* expression.

Several putative bacteriocins are regulated by CSP and the *comDE* system. Analysis of the *S. mutans* genome sequence (1) revealed 10 small open reading frames with high similarity to the leader peptides of NlmA and NlmB that could encode class II bacteriocins (Fig. 3 and 4). The putative bacteriocins, designated Bsm (bacteriocin *Streptococcus mutans*) ranged in size from 47 to 87 amino acids, had leader peptides from 22 to 25 amino acids (Table 4 and Fig. 3), and contained a double glycine motif that could be recognized by the ComAB processing and export system. Some of the genes encoding the putative bacteriocins were located in tandem, indicating that they might act cooperatively, as is typical for class IIB bacteriocins (Table 4 and Fig. 4). Most of the putative bacteriocins were predicted to be of hydrophobic nature. Out of these putative bacteriocins, eight are encoded within a region of about 20 kb in size, which also harbors the *comC*, *comD*, and *comE* genes (Fig. 4) (15) Two pairs of bacteriocin-encoding genes (*bsmFG* and *bsmHI*) appear to be separated by an insertion element (Fig. 4). One of the bacteriocin-encoding genes (*bsmA*, SMU.1914c) is located immediately upstream of the *comC* gene (SMU.1915c), but in the opposite direction of transcription.

DNA sequence analysis showed that the region upstream of the putative bacteriocin-encoding genes *bsmA*, *bsmB*, *bsmC*, and *nlmAB* was highly conserved. This region was also found to be present upstream of an open reading frame encoding a putative immunity protein (*immB*, SMU.925). The ImmB protein sequence was 26% identical to BlpL, a putative immunity protein from *S. pneumoniae* (6). The conserved region contains a 9-bp repeat, which is separated by 12 bp (Fig. 5). The sequence of the repeat element matched with the previously proposed consensus binding site sequence for response regulators from the AlgR/AgrA/LytR family (22). Putative bacteriocins encoded by tandemly organized *bsmH* and *bsmI* also contained a repeat element upstream of a possible extended −10 region, but the repeat did not completely match with the proposed consensus sequence (not shown).

The presence of this highly conserved region indicated that expression of these bacteriocin-encoding genes and associated genes is under coordinate control by the *comDE* two-component signal transduction system. In order to verify this hypothesis, chromosomally encoded transcriptional *lacZ* fusions to *bsmA* and *immB* were constructed and β-galactosidase activity was measured in a wild-type strain and in a *comDE* background with or without addition of CSP to the medium. Expression of the *bsmA′-lacZ* and *immB′-lacZ* fusions was found to be inducible by CSP and dependent on functional ComDE (Table 5), supporting the assumption that the five loci are coregulated. Expression of *bsmA′-lacZ* was highest in the stationary phase of growth (data not shown). A gene encoding a second putative bacteriocin immunity protein, designated *immA* (SMU.1913c), whose deduced protein sequence has 80% identity to that of *immB*, was detected downstream of

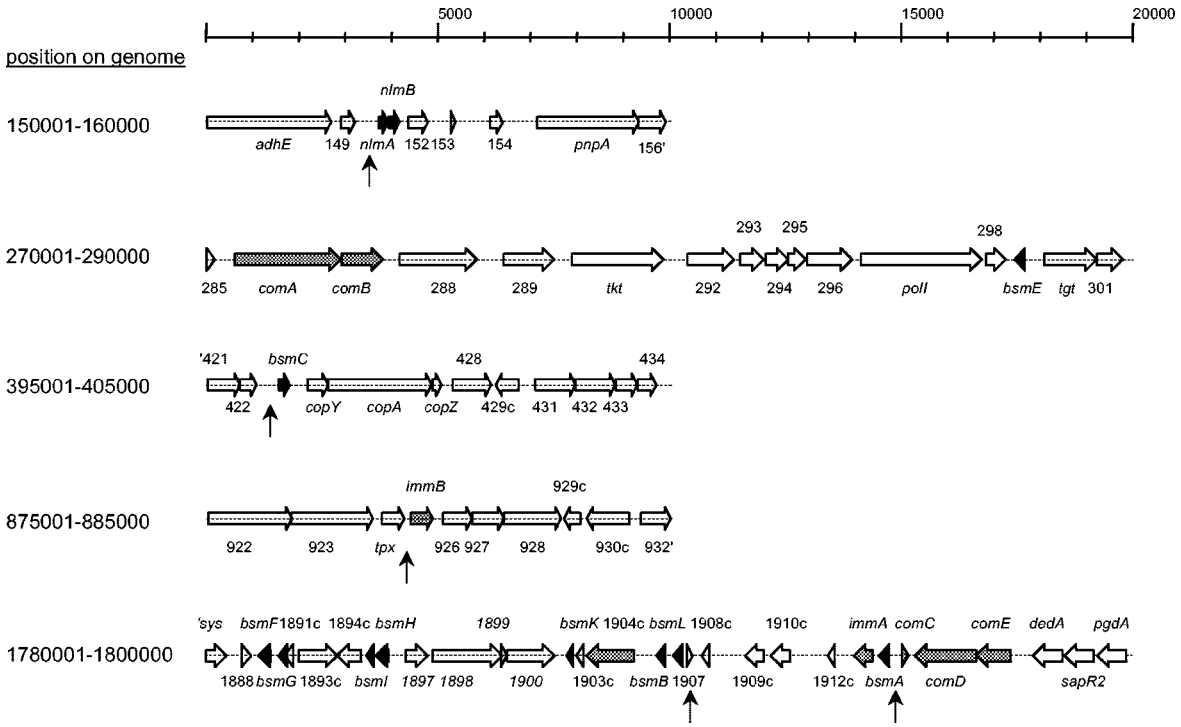

FIG. 4. Genetic organization of loci harboring the *bsm*, *imm*, and competence-related genes. Genes shown by black arrows and arrowheads encode putative bacteriocins, whereas genes associated with competence and bacteriocin production are shown by shaded arrows. The scale above the maps is in base pairs. Numbers above or below the genes correspond with the numbering by Ajdic et al. (1). Incomplete genes are denoted by a prime. Vertical arrows indicate the position of the conserved direct repeat involved in ComCDE-dependent expression.

bsmA. β-Galactosidase expressed from an *immA'-lacZ* fusion was also stimulated by CSP and dependent on *comDE* (Table 5). Thus, both *immA*, which is probably transcribed in one unit with *bsmA*, and *immB* are coexpressed with bacteriocins.

The transcription start site of the *bsmA* gene was mapped 42 bp downstream from the repeat element closest to the start codon of *bsmA* (data not shown) (Fig. 5). Upstream of the transcription start, a well-conserved extended −10 region was present.

To further confirm that the direct repeat is required for CSP-dependent expression, transcriptional *lacZ* fusions to genes that lacked this repeat (*bsmE*, *bsmHI*, and *bsmK*) were generated and β-galactosidase was measured (Table 5). Expression of the *bsmE'-lacZ* and the *bsmHI'-lacZ* fusions was not increased by addition of CSP and not dependent on *comDE*. The *bsmK'-lacZ* fusion exhibited a small increase in β-galactosidase activity upon addition of CSP and showed less-than-twofold-reduced levels of activity in a *comDE* background. It is possible that this dependence on *comCDE* results from expression from the *bsmBL* promoter, which is located about 2.3 kb upstream of *bsmK* and which contains a conserved direct repeat element (Fig. 4).

TABLE 4. Putative bacteriocins encoded by *S. mutans* UA159

Gene	Annotation	Size of prepeptide (amino acids)	Repeat element upstream[a]	Remark
bsmA	SMU.1914c	76	+	Adjacent to *comC*
bsmB	SMU.1906c	70	+	Adjacent to *bsmL*
bsmC	SMU.423	76	+	
nlmA	SMU.150	67	+	Adjacent to *nlmB*
bsmE	SMU.299c	72	−	
bsmF	SMU.1889c	87	−	Adjacent to *bsmG*
bsmG	SMU.1892c	61	−	Adjacent to *bsmF*
bsmH	SMU.1896c	83	−	Adjacent to *bsmI*
bsmI	SMU.1895c	53	−	Adjacent to *bsmH*
nlmB	SMU.151	71	+	Adjacent to *nlmA*
bsmK	SMU.1902c	47	−	
bsmL	SMU.1905c	62	+	Adjacent to *bsmB*

[a] Presence (+) or absence (−) of direct repeat element less than 1 kb upstream of the gene.

Mutational analysis of the *nlmAB* direct repeat. To substantiate the hypothesis that the direct repeat could function as a binding site for a transcriptional regulator (most likely ComE) and thus be required for CSP-dependent expression, chromosomally located transcriptional fusions between the *nlmAB* promoter region and *lacZ* were constructed. Mutations were introduced in the direct repeat element, and expression of these *nlmAB'-lacZ* fusions was measured in cultures that had received CSP (Fig. 6). Both mutations in repeat 2 led to approximately 10-fold-reduced levels of β-galactosidase activity, whereas the mutation in repeat 1 resulted in 40-fold-lower levels of β-galactosidase. Removal of both repeats and the region in between abolished expression nearly completely. These data clearly demonstrate that the direct repeat is important for full expression of *nlmAB*.

Competence mutants do not produce bacteriocins. The results presented above have shown that expression of several genes related to bacteriocin synthesis is controlled by the *comCDE* quorum-sensing system. It was investigated whether this could be correlated with in vivo bacteriocin production by

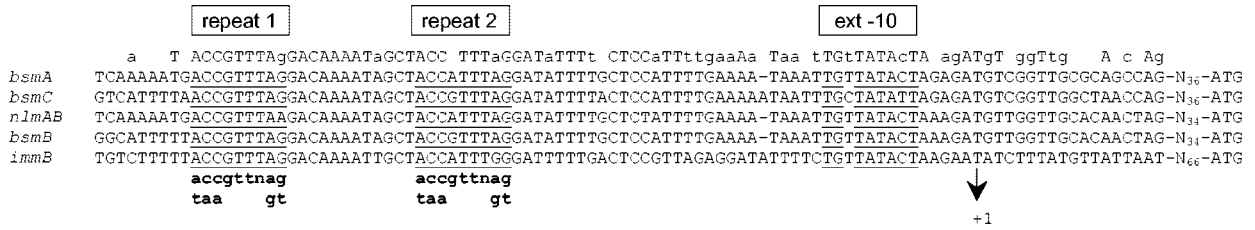

FIG. 5. Sequence alignment of upstream regions from four genes encoding putative bacteriocins and one gene encoding a putative bacteriocin immunity protein. The direct repeat elements and the extended −10 promoter regions are underlined. The consensus sequence is shown above the sequences (nucleotides present in each of the sequences are in uppercase, and nucleotides occurring in four of the sequences are in lowercase), whereas the general consensus binding site proposed for TCSTSs of the AlgR/AgrA/LytR family (22) is given below the sequences in boldface. The start of the *bsmA* transcript is indicated by an arrow. The distance in nucleotides between the end of the conserved region and the start codon is shown.

studying growth inhibition of selected indicator strains by *S. mutans* strains with mutations in the competence genes (Table 6). *S. mutans* strain OMZ1001 bacteriocins were assayed with *S. gordonii* OMZ505, whereas *S. oralis* OMZ607 served as an indicator for bacteriocins produced by *S. mutans* OMZ67. The zone of growth inhibition of the indicator strains triggered by the two wild-type strains was not affected by the presence of CSP. Apparently, the amount of CSP produced was sufficient to induce production of bacteriocins. While *comC* and *comDE* mutants did not produce bacteriocins in the absence of CSP, the *comC* mutant could be complemented by exogenous CSP. Both *comA* mutants failed to produce bacteriocins in the absence of CSP. Addition of CSP could complement the *comA* mutant of OMZ67 but not the *comA* mutant of OMZ1001. This suggests that in the latter strain the ComAB system is required for both processing and transport of both CSP and bacteriocins, whereas strain OMZ67 might have a second system for processing and export of bacteriocins. Other mutations tested had no effect on production of bacteriocins by either strain of *S. mutans*.

TABLE 5. β-Galactosidase activities expressed from transcriptional *lacZ* fusions in a wild-type background and in a *comDE* mutant[a]

Strain	*lacZ* fusion	Mutation	β-Galactosidase activity (Miller U)	
			−CSP	+CSP
OMZ1015	*bsmA*		221 ± 80	1,016 ± 218
OMZ1016	*bsmA*	*comDE*	18 ± 4	24 ± 6
OMZ1019	*immB*		10 ± 2	60 ± 3
OMZ1020	*immB*	*comDE*	10 ± 1	10 ± 2
OMZ1017	*immA*		35 ± 26	295 ± 74
OMZ1018	*immA*	*comDE*	9 ± 1	9 ± 1
OMZ1029	*bsmE*		100 ± 25	100 ± 25
OMZ1032	*bsmE*	*comDE*	97 ± 5	103 ± 14
OMZ1027	*bsmHI*		268 ± 37	237 ± 18
OMZ1030	*bsmHI*	*comDE*	347 ± 32	320 ± 19
OMZ1028	*bsmK*		346 ± 75	414 ± 92
OMZ1031	*bsmK*	*comDE*	238 ± 71	230 ± 81

[a] Strains were grown overnight in THY medium, diluted 50-fold in fresh medium, and grown aerobically at 37°C. After 3 hours of growth, cultures were split and CSP (final concentration, 100 ng/ml) was added to one of each of the samples. The cultures were grown for 2 more hours, and β-galactosidase was measured. The values are the mean values of at least two independent experiments.

DISCUSSION

Two-component signal transduction systems that respond to the presence of inducer peptide pheromones are known to regulate a variety of processes: bacteriocin production, competence for transformation, biofilm formation, and virulence (30). The results presented here and those from previous studies (14, 15) show that *S. mutans* uses one and the same TCSTS to control at least three of these at first sight unrelated processes. Mutants in *comA*, *comC*, and *comDE* are competence deficient (14), form biofilms that are different from those formed by wild-type strains (15), and do not produce bacteriocins (this study; 32, 36).

Unlike competence, which exhibits an optimum during a short interval of the exponential phase (data not shown) (19, 23), expression of *nlmAB′-lacZ* was lowest in this period but reached a maximum in the stationary phase. Since both activities are controlled by the same regulatory system, the question arises as to how this differential regulation is established. The key to the answer may lie in the fact that expression of genes required for competence development is governed by the accessory sigma factor ComX, whereas production of bacteriocins is not, as shown in the present study. In *S. pneumoniae*, the *comX* gene, under the regulatory control of the *comCDE* system, is transcribed only during the first 15 min after addition of CSP, but then transcription is shut off by an unknown mechanism (25). This short period of transcription of *comX*, together with the short half-life of the ComX protein, results in transient expression of the late competence genes. A similar situation probably applies to *S. mutans*, since expression of a *comX-lacZ* fusion was induced only during the first minutes after addition of CSP (2). In contrast, the *nlmAB′-lacZ* fusion exhibited an increase of β-galactosidase activity more than 2 h after addition of CSP.

Many of the bacteriocin-encoding genes are located in the vicinity of the *comCDE* genes. It is therefore likely that ComE binds to the direct repeat upstream of these genes to activate transcription of these genes, but it cannot be ruled out that ComE regulates the activity of a second transcription factor that then acts on the promoter regions of bacteriocins. In *S. pneumoniae*, the ComE protein binds to the promoter region of the *comCDE* and *comAB* (33), and it was expected to bind as well upstream of *comX* (13). However, no such binding sites

Strain	repeat 1	repeat 2	β–galactosidase (Miller U)
OMZ1033	TCAAAAATGACCGTTTAAGACAAAATAGCTACCATTTAGGATATTT		313 ± 131
OMZ1034	TCAAAAATGA**tatc**TTAAGACAAAATAGCTACCATTTAGGATATTT		8 ± 1
OMZ1035	TCAAAAATGACCGTTTAAGACAAAATAGCT**g**C**ag**TTTAGGATATTT		33 ± 9
OMZ1036	TCAAAAATGACCGTTTAAGACAAAATAGCTACCATT**atc**GATATTT		32 ± 4
OMZ1037	TCAAAAAT**c**----------GATATTT		6 ± 1

FIG. 6. Mutations in the direct repeat of the *nlmAB* promoter region and their effect on activity of β-galactosidase expressed from a transcriptional *nlmAB′-lacZ* fusion. Direct repeats are underlined. Residues that were mutated are shown in lowercase and boldface. In *S. mutans* strain OMZ1037, both repeats and the region in between the repeats were deleted. Activity of β-galactosidase was measured in late-exponential-phase cultures 3 h after addition of 100 ng/ml of CSP.

in front of the *S. mutans comAB*, *comX*, *comC*, and *comDE* genes could be detected.

As many as 12 putative bacteriocins are encoded on the chromosome of *S. mutans*. There could be even more, as pointed out by a recent survey (7). The finding of so many type II bacteriocins raises several questions: (i) towards which species are these bacteriocins active, (ii) under what conditions are they expressed, and (iii) is the production of bacteriocins relevant for colonization or survival of *S. mutans* in dental plaque?

Although there is no obvious sequence similarity among the mature peptides, all putative bacteriocins from *S. mutans* are hydrophobic, suggesting that they interact with the bacterial cytoplasmic membrane. Since purified NlmAB bacteriocin is active against a broad range of oral streptococci (27), it is likely that the others are also directed towards these organisms. Detailed analysis with purified peptides is necessary to obtain conclusive evidence about the spectrum of the microbes that are sensitive. Class IIB bacteriocins generally act much more efficiently than two-peptide systems. It is possible that different combinations of bacteriocins have different antimicrobial spectra. The 12 peptides encoded in the genome of *S. mutans* would thus allow for a large repertoire.

Consistent with this hypothesis is the observation that *S. mutans* OMZ1001 produces bacteriocins against *S. gordonii* OMZ505 but not against *S. oralis* OMZ607, whereas *S. mutans* strain OMZ67 inhibited growth of OMZ607 but not of OMZ505 (results not shown), assuming heterogeneity with respect to the bacteriocins produced by these strains.

TABLE 6. Growth inhibition by *S. mutans* wild-type and mutant strains[a]

Mutation	Bacteriocin production by mutants and wild-type strain:			
	OMZ1001		OMZ67	
	−CSP	+CSP	−CSP	+CSP
Wild type	+	+	+	+
comC	−	+	−	+
comDE	−	−	−	−
comX	+	+	+	+
ciaRH	+	+	+	+
comA	−	−	−	+
vicK	+	+	+	+

[a] Wild-type and mutant strains (Table 2) were stab inoculated in THY agar plates and grown anaerobically at 37°C. After 24 hours of growth, 0.5 μl of 1-mg/ml CSP was added at the position where *S. mutans* had been inoculated. After 48 hours, the indicator strain *S. gordonii* OMZ505 (for OMZ1001 and its derivatives) or OMZ607 (for OMZ67 and its derivatives) was added. The presence (+) or absence (−) of a zone of growth inhibition was evaluated after another 24 hours of incubation.

The second question concerning conditions of bacteriocin expression has been answered to some extent in this study. At least the six bacteriocins that contain the conserved upstream direct repeat element are likely to be regulated by the *comCDE* signal transduction system. The level of expression of *nlmAB* was found to be dependent on the concentration of CSP. It is not known how expression of the *comC* gene is regulated in *S. mutans*, but it seems likely that the concentration of CSP reaches its maximum in the stationary phase. Growth in biofilms is also expected to lead to high concentrations of CSP. Indeed, when strains were grown on plates, conditions comparable to growth, expression of *nlmAB′-lacZ* was higher than when strains were grown in liquid cultures. This correlates with the observation that addition of CSP to plates did not lead to a large increase of bacteriocin production by the wild-type strain, whereas β-galactosidase activities were strongly increased upon addition of CSP in liquid cultures. My results appear to contrast with those of Qi et al., who found higher expression of *nlmAB* in liquid cultures than on plates (27). Recently, it was shown that *S. mutans* GS-5, which is the same as strain OMZ67 used in this study, produces a two-peptide lantibiotic bacteriocin named Smb. Expression of the *smb* operon that encodes the bacteriocin structural proteins, immunity, processing, and transport were found to be dependent on the *comCDE* system (36). The promoter region of this operon contained a very similar direct repeat element as described here. Thus, the *comCDE* system appears to regulate nonlantibiotic as well as lantibiotic bacteriocin synthesis.

Derivatives of strain OMZ1001 and OMZ67 deficient in *comA*, *comC*, or *comDE* lost the ability to inhibit growth of *S. gordonii* OMZ505 and *S. oralis* OMZ607, respectively. This can likely be ascribed to the loss in expression of the bacteriocin genes that contain the direct repeat region. But these mutants may have kept the ability to produce one or more of the remaining bacteriocins. The observation that the *com* mutants did not inhibit growth of indicator strains suggests either that these remaining bacteriocins are not expressed under the conditions investigated or that they are expressed but active against other organisms. The latter explanation appears most likely, since the *bsmE*, *bsmK*, and *bsmHI* genes were expressed when *S. mutans* was grown in liquid medium.

The loss of growth inhibition by the *comA* mutant of OMZ1001 could not be complemented by addition of CSP. This suggests that, at least in this strain, processing and export of CSP and bacteriocins are carried out by the same transport

proteins. This is not completely unexpected, since the N-terminal sequences of the bacteriocins and that of CSP are similar in size and sequence (Fig. 3). The existence of a unique transporter for bacteriocins and for the inducing peptide has also been proposed for bacteriocin production by *Lactobacillus sakei* (5). The *comA* mutant of strain OMZ67 could be complemented by addition of CSP. It might well be that processing and export of the lantibiotic bacteriocin Smb produced by this strain require a specific protein. Indeed, a gene that could encode a bacteriocin transporter was present in the *smb* operon (36).

What is the link between competence, biofilm formation, and bacteriocin production? Several studies have shown that oral streptococci lacking one of the components of the *comCDE* system produce poorer or differently structured biofilms (15, 16). In *Streptococcus intermedius*, biofilm formation was stimulated by addition of CSP (24). This stimulation was detectable at CSP concentrations that were in the same range as the concentrations required for induction of expression of *nlmAB′-lacZ*. In addition, competence of *S. mutans* was found to be increased when grown in biofilms (14). Other studies have also suggested a connection between competence and bacteriocin production. In *Bacillus subtilis*, production of the dipeptide bacilysin was regulated by the system required for genetic competence (35). In *S. pneumoniae*, genes encoding bacteriocins are also induced by CSP and regulated by *comDE*, but they belong to the late-induced genes. The late-induced genes do not contain a direct repeat sequence that could act as a binding site for ComE but instead require ComX and have a conserved element (TACGAATA) just upstream of the transcription start site (13). This element could not be detected upstream of the genes encoding bacteriocins in *S. mutans*.

It has been shown recently that *S. pneumoniae* releases chromosomal DNA after addition of CSP and that this DNA release is dependent on ComD and ComE (18). Interestingly, the highest amounts of DNA were released in the stationary phase of growth, which coincides with the maximum in expression of bacteriocins in *S. mutans*. Although a speculative hypothesis, it is possible that bacteriocins produced during the stationary phase aid in release of DNA either from *S. mutans* itself or from competing streptococci by permeabilization of the cytoplasmic membrane. The DNA that is liberated could serve several functions. First, it might function as a component of a biofilm structure. It has recently been shown that addition of DNase inhibited biofilm formation of *S. intermedius* and *Pseudomonas aeruginosa* (24, 34), indicating that DNA contributes to biofilm development. Second, the DNA, and possibly other cellular constituents, might fulfill a nutritional requirement for starving cells (8). The "classical" role of competence development, uptake of DNA and subsequent recombination to increase the fitness, remains as a third possibility.

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