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Examination of the *hdrRM* regulon yields insight into the competence system of *Streptococcus mutans*

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SUMMARY

Previous studies have identified the *hdrRM* operon as a novel regulatory system induced by conditions of high cell density. Little is known about the genes under the control of this system, but a variety of important phenotypes are associated with either *hdrR* overexpression or mutation of *hdrM*. To characterize the regulatory function of the HdrRM system in *Streptococcus mutans* we used a microarray approach to compare the transcriptional profiles of an *hdrR* overexpression strain with an *hdrM* mutant. Both strains exhibited almost identical profiles, which included all of the known late competence genes as well as a variety of competence-induced bacteriocins. Through a combination of real-time reverse transcription–polymerase chain reaction (PCR), reporter gene analysis and random amplification of complementary DNA ends PCR, we confirmed the role of *comX* as a central intermediate regulator of numerous genes in the *hdrRM* regulon. Through these studies, we also identified novel *comX*-regulated genes required for natural competence. Taken together, our results suggest that the primary function of the HdrRM system is to regulate the late competence genes together with various bacteriocins. This occurs independently of the ComCDE system, even though both systems regulate nearly identical genes. This suggests that *S. mutans* has multiple parallel input sensory systems that control the same output response: the induction of natural competence and concurrent production of bacteriocins.

INTRODUCTION

Streptococcus mutans is a typical member of the human oral biofilm and is a major contributor to the development of dental caries (Bowden, 1990; van Houte, 1993; Zambon & Kasprzak, 1995; Nobre dos Santos *et al.*, 2002; Barsamian-Wunsch *et al.*, 2004; Munson *et al.*, 2004; Thenisch *et al.*, 2006). *S. mutans* has several abilities that are thought to facilitate its ability to promote disease: aciduricity, acidogenicity, biofilm formation, natural competence and bacteriocin production (Kuramitsu, 2003; Banas, 2004; Napimoga *et al.*, 2005). Several of these abilities were demonstrated to be under the control of the recently characterized two-gene regulatory system referred to as the *hdrRM* operon (Merritt *et al.*, 2007).

The *hdrRM* operon was first identified as a result of its induction under conditions of extremely high cell density (Merritt *et al.*, 2007). Based upon the predictd amino acid sequences of both open reading frames, it was predicted that *hdrR* encodes a transcription regulator of the LytTR family (Nikolskaya & Galperin,

2002) and *hdrM* encodes a putative membrane protein. A mutation of *hdrM* resulted in biofilm structural deformations, altered bacteriocin production, and greatly increased natural competence, whereas both polar and non-polar mutations of *hdrR* resulted in wild-type phenotypes (Merritt *et al.*, 2007). Hence, a single mutation of *hdrR* was not associated with any noticeable phenotypes, but a double mutation of *hdrR* and *hdrM* could fully suppress the *hdrM* phenotypes. Despite the lack of phenotypes associated with a single mutation of *hdrR*, the data suggested that *hdrR* is a crucial mediator of the phenotypes in the *hdrM* genetic pathway.

To learn more about the functionality of the HdrRM regulatory system, we chose to further investigate the mechanism of the increased competence phenotype. This phenotype was also of particular interest because it was the only example of a defined mutation causing increased competence in *S. mutans*. Natural competence in species of *Streptococcus* has been shown to occur through the *comCDE* two-component signal transduction system (Cvitkovitch, 2001; Johnsborg & Havarstein, 2009). The gene product of the *comC* gene encodes a small peptide signal that is recognized by the ComD membrane sensor, which activates the transcription factor activity of the ComE response regulator (Havarstein *et al.*, 1996; Pestova *et al.*, 1996; Cheng *et al.*, 1997; Li *et al.*, 2002). Activated ComE then induces the expression of a competence-specific sigma factor *comX* (Luo & Morrison, 2003; Aspiras *et al.*, 2004; Perry *et al.*, 2009), which directs the transcription of all of the genes required for the uptake of exogenous DNA from the environment (Lunsford & London, 1996; Lee & Morrison, 1999; Li *et al.*, 2002). In *S. mutans*, the *comCDE* system is also a central regulator of multiple competence-induced bacteriocins (Kreth *et al.*, 2005, 2006, 2007). Surprisingly, we found that a mutation of *hdrM* abrogated the requirement of the *comCDE* system to activate transcription of the competence system and related bacteriocin genes (Okinaga *et al.*, 2010). A similar result was also observed in an *hdrR* overexpression strain, which suggested that *hdrR* was indeed the likely mediator of the transcriptional effects seen in the *hdrM* background. Furthermore, overexpressing *hdrM* together with *hdrR* could suppress the *hdrR* overexpression competence and bacteriocin phenotypes. These data all suggested a role for HdrM as a negative regulator of HdrR activity.

In this study, we aimed to further our understanding of the regulatory role of the HdrRM system in the cell by examining its regulon. Using a microarray approach, we compared the transcriptional effects of *hdrR* overexpression with that of an *hdrM* mutation. We found nearly identical responses from both constructs, which suggests that *hdrR* and *hdrM* are not major components of other pathways or regulatory systems. Further analysis of a subset of the genes identified by microarray implicates ComX as a major regulator of numerous genes within the *hdrRM* regulon. In addition, we were also able to identify three previously uncharacterized genes required for *S. mutans* natural transformation. Taken together, our results indicate that the competence system is a principal output of the HdrRM regulatory system with ComX as its primary regulator. This suggests that the major function of HdrRM is highly analogous to that of the *comCDE* two-component system.

METHODS

Bacterial strains and culture conditions

All bacterial strains are listed in Table S1. *S. mutans* UA140 and its derivatives were grown in brain–heart infusion broth (BHI, Difco, Franklin Lakes, NJ) and on agar plates. For the selection of antibiotic-resistant colonies, BHI plates were supplemented with either 800 $\mu g\ ml^{-1}$ kanamycin (Sigma, St Louis, MO), 1000 $\mu g\ ml^{-1}$ spectinomycin (Sigma), or 15 $\mu g\ ml^{-1}$ tetracycline (Sigma). *S. mutans* strains were grown anaerobically (80% N_2, 10% CO_2 and 5% H_2) at 37°C. *Escherichia coli* cells were grown in Luria–Bertani (LB, Difco) medium with aeration at 37°C. *E. coli* strains carrying plasmids were grown in LB medium containing 100 $\mu g\ ml^{-1}$ spectinomycin or 100 $\mu g\ ml^{-1}$ kanamycin.

Construction of the *hdrR* overexpression strain and *comX* mutant

Construction of the *hdrR* overexpression plasmid has been described previously (Okinaga *et al.*, 2010). In this construct, *hdrR* has been placed under the control of the highly expressed constitutive *ldh* promoter. The *comX* mutant was created by insertion duplication mutagenesis and has been previously described (Li *et al.*, 2002). The *comX* mutation was moved into UA140 by transforming genomic DNA.

RNA extraction and real-time reverse transcription–polymerase chain reaction analysis

Overnight cultures of UA140 and its derivatives were diluted 1 : 30 in 300 ml BHI + 0.4% weight/volume bovine serum albumin and harvested when the optical density at 600 nm OD_{600} reached 0.3. Cells were centrifuged at 4°C and then stored at −80°C. RNA was extracted from cell pellets using a previously described method (Niu *et al.*, 2008). RNA quality was then confirmed by the presence of clearly defined ribosomal RNA bands separated by agarose gel electrophoresis. Total RNA (300 ng) was used for complementary DNA (cDNA) synthesis using Stratascript reverse transcriptase (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's protocol. For real-time reverse transcription–polymerase chain reaction (RT-PCR), primers were designed using PRIMER EXPRESS 3.0 software, the reactions were prepared using Bio-Rad iTaq SYBR Green Supermix with ROX reference dye (Bio-Rad, Hercules, CA) and an Applied Biosystems 7300 was used for detection (Applied Biosystems, Foster City, CA). Relative changes in gene expression were calculated using the $\Delta\Delta C_T$ method described previously (Niu *et al.*, 2008). Total cDNA abundance between samples was normalized using primers specific to the *gyrA* gene. All primers used for real-time RT-PCR are listed in Table S2.

Microarray

The microarray protocol has been previously described (Ajdic & Pham, 2007). Briefly, RNA extraction was performed using the Ambion RiboPure™-Bacteria RNA purification kit (Ambion, Austin, TX) followed by further purification with the Qiagen RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). Then, 15 μg of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and fragmented with Roche DNase I (Roche, Basel, Switzerland). Hybridization, washing and scanning of the custom *S. mutans* GeneChip expression microarrays (Affymetrix, Santa Clara, CA) were performed according to the procedures described by Affymetrix. Microarray data processing and analysis employed the GENECHIP operating software (GCOS) version 1.4. Samples with a signal detection *P*-value <0.01 were analysed for expression differences. Three independent samples were analysed for both the *hdrM* mutant and *hdrR* overexpression strains.

Construction of luciferase reporter gene fusions

The upstream promoter region of *coiA*, SMU.836, SMU.1055, SMU.1967 were generated by PCR using Accuprime Pfx (Invitrogen) and the primers SMU.644 F/R, SMU.836 F/R, SMU.1055 F/R, and SMU.1967 F/R (Table S2). The resulting fragments were digested using *Bam*HI and *Xho*I and cloned into the vector pFW5-luc (Podbielski *et al.*, 1996) digested with the same enzymes. The resulting plasmids were confirmed with restriction analysis, PCR and sequencing. To construct cin-box deletion luciferase reporter gene fusions, each luciferase reporter gene fusion plasmid was used as a template for inverse PCR using the following primers previously phosphorylated with T4 polynucleotide kinase (NEB, Ipswich, MA): SMU.644ip F/R, SMU.836ip F/R, SMU.1055ip F/R and SMU.1967ip F/R (Table S2). The PCR fragments were then ligated and transformed into *E. coli*. The expected deletions were confirmed by sequencing the resulting plasmids. All reporter plasmids were transformed into the *hdrM* mutant strain (ZX-m) and integrated onto the chromosome via single crossover homologous recombination. Transformants were selected on BHI plates containing spectinomycin and confirmed by PCR.

Luciferase assay

Luciferase assays were performed as described previously (Loimaranta *et al.*, 1998). Briefly, 25 μl 1 mM D-luciferin (Sigma) solution was added to 0.1 ml cell culture. Luciferase activity was measured using a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). Overnight cultures of three independent reporter strains were diluted 1 : 20 and grown to OD_{600} 0.3–0.4 before measuring luciferase activity.

Determination of transcription start sites

The FirstChoice RLM random amplification of cDNA ends (RACE) kit (Ambion) was used to determine transcription start sites according the manufacturer's protocol. To summarize, RNA was isolated from an *hdrR* overexpression strain of *S. mutans* grown in BHI medium to an optical density of OD_{600} 0.3.

Ten micrograms of total RNA was treated with calf intestinal phosphatase in a total volume of 20 μl for 1 h and extracted with pH 4.5 phenol (EMD, Darmstadt, Germany) and chloroform (Mallinckrodt, Phillipsburg, NJ) and finally resuspended in 11 μl RNase-free water. The calf intestinal phosphatase-treated RNA (5 μl)was further treated with tobacco acid pyrophosphatase in a total volume of 10 μl for 1 h. Reaction mixture (2 μl) was used directly in a ligation reaction containing T4 RNA ligase and the 5′ RACE adaptor in a total volume of 10 μl for 1 h. Ligated RNA (2 μl) was subjected to reverse transcription using random decamers and Moloney murine leukemia virus reverse transcriptase or Affinityscript reverse transcriptase (Stratagene), followed by nested PCR. For the first-round PCR, the 5′ RACE outer primer and the gene-specific outer reverse primers (Table S2) were used with the reverse transcription reaction products as the template. Products from the first-round PCR were then subjected to a second-round of PCR amplification using the 5′ RACE inner primer and the gene-specific inner reverse primers (Table S2). The resulting PCR products were then cloned into the pGEM-Teasy vector (Promega, Madison, WI) and sequenced using M13 primers.

Construction of SMU.431, .507, .539, .769, .836, .1055, and .1904 mutants

We constructed the SMU.431, .507, .539, .769, .836, .1055, and .1904 deletion mutants via an overlapping extension PCR approach. To generate the constructs, two fragments corresponding to approximately 1 kb of the upstream and downstream sequences of these genes was generated by PCR using Accuprime Pfx (Invitrogen) with the primer pairs SMU.431 Up F/R and SMU.431 Dn F/R, SMU.507 Up F/R and SMU.507 Dn F/R, SMU.539 Up F/R and SMU.539 Dn F/R, SMU.769 Up F/R and SMU.769 Dn F/R, SMU.836 Up F/R and SMU.836 Dn F/R, SMU.1055 Up F/R and SMU.1055 Dn F/R, and SMU.1904 Up F/R and SMU.1904 Dn F/R (Table S2). Each of the Up R and Dn F primers incorporated 18 bases that are complementary to the erythromycin-resistance cassette *ermAM* (Martin *et al.*, 1987). The *ermAM* gene was amplified by PCR using the primers ermAM F/R. All PCR amplicons were purified with the Qiagen PCR Purification kit and the corresponding upstream and downstream amplicons were mixed in a 1 : 1 : 1 ratio with the *ermAM* PCR product. The amplicon mixture was then used as a template for a second PCR using the appropriate upstream forward and downstream reverse primers. The resulting PCR products were then transformed into UA140. In addition, the deletion constructs for SMU.539 and SMU.836 were also transformed into the *hdrR* overexpression strain.

Transformation assays

Determination of transformation efficiency was performed using a previously described methodology (Li *et al.*, 2001; Merritt *et al.*, 2005). UA140 and its derivatives were diluted 1 : 30 from overnight cultures and grown to OD_{600} 0.2–0.3 in BHI + 0.4% weight/volume bovine serum albumin before adding transforming genomic DNA containing a tetracycline marker at a final concentration of 10 μg ml^{-1}. The cultures were subsequently allowed to grow for an additional 2 h and then briefly sonicated to disperse cell chains before plating on tetracycline-containing BHI agar plates as well as on non-selective BHI plates. Transformation was scored based on the number of colony-forming units on selective plates and the number of total viable cells was determined by the number of colony-forming units on non-selective plates. Transformation efficiency was calculated by comparing the ratio of transformants to total viable cells.

RESULTS

Determination of the *hdrRM* regulon

Our previous studies of the *hdrRM* operon found bacteriocin production, biofilm, and natural competence phenotypes associated with a mutation of *hdrM* (Merritt *et al.*, 2007). Subsequent studies demonstrated that overexpressing *hdrR* could induce similar phenotypes as seen in the *hdrM* background (Okinaga *et al.*, 2010). Additionally, several lines of evidence indicated that HdrR and HdrM function together in the same regulatory pathways. Given the wide array of phenotypes associated with the *hdrRM* operon, we were curious to determine the genes under the control of the HdrRM system so as to better characterize its regulatory role in the cell. To this end, we employed a microarray approach to examine the *hdrRM* regulon. It was unknown whether HdrR and HdrM exclusively function together in the same

pathways or if they also individually participate with other systems, so we decided to assay the transcriptomes of both the *hdrR* overexpression strain and the *hdrM* mutant. A portion of these results is listed in Table 1. In general, we found almost identical transcriptional responses from both strains, although the degree of regulation was generally more severe in the *hdrR* overexpression strain. This strain has a greater competence phenotype than the *hdrM* mutant (Okinaga *et al.*, 2010). In total, we found 103 genes that were affected two-fold or greater in one or both of the microarray data sets, with numerous genes exhibiting >10-fold expression differences (Table S3). After comparing the results from the *hdrR* overexpression strain and the *hdrM* mutant, we only found eight genes that had opposite responses. Of these, most were only minimally increased or decreased in expression. The largest difference was from SMU.1805, which was >2.5-fold increased in the *hdrR* overexpression strain and approximately three-fold decreased in the *hdrM* mutant (Table S3). Given the high degree of similarity between the *hdrR* overexpression and *hdrM* mutant data sets, it seems likely that under the conditions of our assay both *hdrR* and *hdrM* primarily function together in the same regulatory pathways and are not major components of other regulatory systems. By comparing the two data sets, it is also evident that the majority of changes were the result of increased expression. Of the 103 genes in the two microarray data sets, 31 exhibited lower expression in either or both strains. Most of these genes had less than three-fold reduced expression, with the greatest difference being slightly more than four-fold less expressed (SMU.1806 in the *hdrM* mutant) (Table S3). Therefore, unlike the more highly expressed genes, those with reduced expression only exhibited minor differences in transcription.

Table 1 Selected genes from the microarray data set[1]

Gene name/ID	*hdrR* OE	*hdrM* mutant	Functional class
SMU.423	2.71 (0.21)	3.91 (0.15)	Putative bacteriocin
comFA	51.16 (7.59)	22.19 (2.31)	Late competence; DNA helicase
SMU.505	4.34 (0.83)	1.79 (0.19)	Adenine-specific DNA methylase
SMU.507	3.18 (0.26)	2.41 (0.1)	Transcriptional regulator
SMU.539c	56.36 (10.74)	29.75 (7.07)	Putative prepilin peptidase
comEA	42.29 (2.93)	7.9 (1.35)	Late competence; DNA uptake
coiA	27.5 (4.7)	9.23 (1.14)	Late competence; DNA recombination
SMU.769	7.18 (1.0)	12.54 (2.22)	Conserved hypothetical protein
SMU.836	20.78 (3.08)	5.18 (0.54)	Hypothetical protein
SMU.925	2.99 (0.45)	3.18 (0.26)	Bacteriocin immunity protein
dprA	35.94 (1.42)	8.81 (0.95)	Late competence; DNA uptake
radC	27.99 (3.47)	11.37 (1.41)	Late competence protein
SMU.1904	3.49 (0.24)	1.88 (0.26)	Hypothetical protein
SMU.1967c	3.25 (0.0)	1.87 (0.13)	Putative single-stranded binding protein
comYA	26.7 (2.87)	4.56 (0.99)	Late competence; DNA uptake
comX	24.83 (1.0)	11.31 (0.0)	Late competence; sigma factor
cinA	3.25 (0.23)	1.79 (0.19)	Late competence protein

[1]Only one gene per operon is listed. Standard deviations are listed in parentheses.

The microarray data for both the *hdrR* overexpression strain and the *hdrM* mutant indicated that there was little or no change in expression from *comC* (SMU.1915), *comED* (SMU.1916–SMU.1917), or the putative competence stimulating peptide (CSP) transporter *cslAB* (SMU.1897–SMU.1900) (Petersen & Scheie, 2000; Hale *et al.*, 2005), despite the greatly increased competence phenotypes of both strains. However, both strains did exhibit large increases in expression from the downstream competence regulator *comX* (SMU.1997c) as well as the late competence genes, such as the *comY* operon (SMU.1980c–SMU.1987c), *comEA* (SMU.625), *coiA* (SMU.644) and *dprA* (SMU.1001). To further confirm the validity of the microarray data, we extracted RNA from the *hdrR* overexpression strain and performed real-time RT-PCR to assay the expression levels of *comYA*, *comEA*, *coiA* and *dprA*. As each of these genes has been demonstrated to be under the control of ComX in other species, we also tested gene expression in the *hdrR* overexpression/*comX*$^-$ background to determine whether the increased expression was *comX*-dependent. As shown in Fig. 1, each of these genes exhibited much higher expression in the *hdrR* overexpression background (30- to 80-fold increased over wild-type), which was consistent with the microarray results. In each case, differential regulation in the *hdrR*

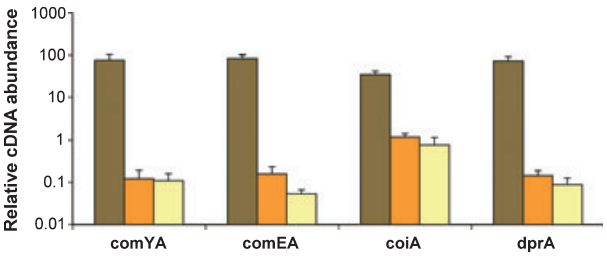

Figure 1 Increased late competence gene expression in the *hdrR* overexpression strain is *comX*-dependent. The transcript abundance of *comYA*, *comEA*, *coiA* and *dprA* is presented relative to their corresponding wild-type UA140 values, which were arbitrarily assigned a value of 1 and are not shown in the graph. Bars shaded in brown are the values from the *hdrR* overexpression strain, while the orange bars represent the *hdrR* overexpression/*comX*$^-$ strain and the yellow bars are from the *comX* single mutant strain. These data are the average of three independent experiments.

overexpression strain was dependent upon *comX*, which suggested that the increased expression of *comYA*, *comEA*, *coiA* and *dprA* was likely mediated by ComX. Data from the *comX* single mutant strain also suggested that *comYA*, *comEA* and *dprA* transcription was heavily reliant upon *comX*, as their expression levels were considerably lower than in the wild-type (Fig. 1).

ComX is a principal output of the *hdrRM* regulatory system

In *Streptococcus pneumoniae*, it has been demonstrated that genes under the control of ComX have a consensus sequence termed the cin-box in their promoters (Campbell *et al.*, 1998), which is recognized by ComX (Lee & Morrison, 1999). As it appeared that at least some of the microarray data could be attributed to increased *comX* expression, we examined each of the genes in the microarray data sets for the presence of potential cin-boxes. Upon closer inspection of the upstream intergenic regions of these genes, we found at least 30 sequences that matched to six of eight bases of the *S. pneumoniae* consensus (TACGAATA) with the vast majority matching to seven of eight or having perfect matches. Within this group of genes, few were more lowly expressed in the *hdrR* overexpression or *hdrM* mutant strains. This implicated ComX as a potential regulator of a large portion of the genes identified by microarray. For this reason, we used real-time RT-PCR to measure gene expression of a subset of putative cin-box-containing genes in the *hdrR* overexpression and *hdrR* overexpression/*comX*$^-$ backgrounds and compared the results to the *comX* single mutant strain. Similar to what we had observed for *comY*, *comEA*, *coiA* and *dprA*, the results all suggested that the increased expression in the *hdrR* overexpression background was dependent upon *comX*. In the *hdrR* overexpression strain, the degree of regulation varied widely within this group of genes (>7- to >40-fold increased), but all similarly exhibited substantially lower expression in the *hdrR* overexpression/*comX*$^-$ background (Fig. 2). It was also apparent that the expression of SMU.1967 was likely to be competence-specific

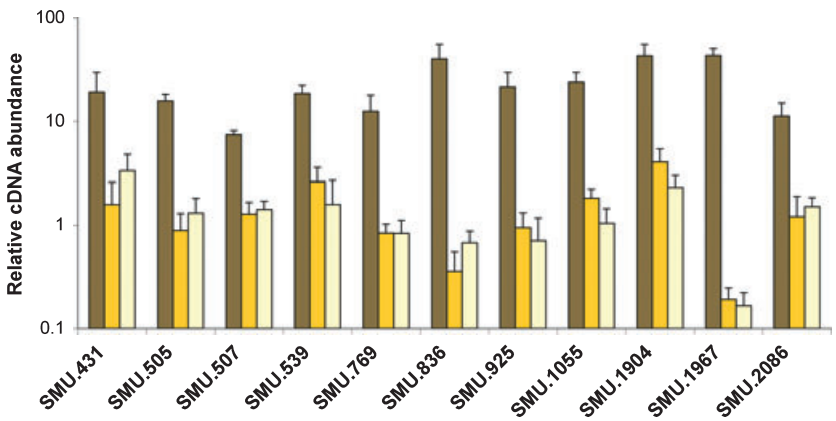

Figure 2 Increased expression of putative cin-box-containing genes requires *comX* in the *hdrR* overexpression background. The transcript abundance of SMU.431, .505, .507, .539, .769, .836, .925, .1055, .1904, .1967 and .2086 is presented relative to their corresponding wild-type UA140 values, which were arbitrarily assigned a value of 1 and are not shown in the graph. Bars shaded in brown are the values from the *hdrR* overexpression strain, while the orange bars represent the *hdrR* overexpression/*comX*$^-$ strain and the yellow bars are from the *comX* single mutant strain. These data are the average of three independent experiments.

because its expression was critically dependent upon *comX*, similar to the results seen with *comY*, *comEA* and *dprA*. For these genes, ComX may be their exclusive regulator during competence, whereas the other genes we tested by real-time RT-PCR all seemed to be inducible by ComX, but not solely dependent upon it for expression. Our results therefore point to two categories of competence-induced genes: those that are upregulated during competence and those that are largely competence-specific.

Cin-box sequences are required for transcription

Our real-time RT-PCR data suggested a major role for ComX as an intermediate regulator of numerous genes within the *hdrRM* regulon, which was consistent with our observation of frequent cin-box-like sequences in these genes. Although it had yet to be determined in *S. mutans* whether the cin-box functions in gene transcription, in *S. pneumoniae* the cin-box has been demonstrated to function as an alternative – 10 sequence that is crucial for ComX-dependent gene expression (Campbell *et al.*, 1998; Lee & Morrison, 1999; Luo & Morrison, 2003; Luo *et al.*, 2003). In addition, it has been reported that cin-boxes are located close to translation start sites (i.e. short messenger RNA leader regions) and are just downstream of variable stretches of thymidine residues centered in the – 25 region (Campbell *et al.*, 1998). In many cases, the *comX*-regulated genes we had identified also possessed cin-box regions that are highly analogous to those of *S. pneumoniae*. For example, the competence-specific genes (*comY*, *comEA*, *dprA* and SMU.1967) all have putative cin-box sequences 18–26 bases upstream from their start codons and are preceded by stretches of thymidine residues (Fig. 3). However, unlike *S. pneumoniae*, in these genes the largest concentration of thymidine residues is found adjacent to the cin-box. Other competence-induced genes such as SMU.539, SMU.769; and *cinA* (SMU.2086) also share this arrangement (Fig. 3). Given their high degree of similarity to ComX-dependent promoters from *S. pneumoniae*, it seemed likely that ComX directly controls these genes. However, among the genes we tested by real-time RT-PCR, we also found multiple genes with apparent cin-boxes, but the surrounding cin-box regions were less similar to those reported for *S. pneumoniae* (Fig. 3). Either they had unusually long leader sequences, few thymidine residues, or combinations of both features. Given the short size of the putative cin-box sequences, we were curious to determine whether the cin-box-like sequences we had identified were truly required for gene expression or were coincidental. To this end, we created several luciferase transcription fusion reporter strains to genes with promoter regions containing varying levels of similarity to the typical *S. pneumoniae* ComX-dependent promoter. To determine the importance of the putative cin-boxes, we created two reporter constructs for each gene: one with a wild-type sequence and another that was devoid of the putative cin-box. As shown in Fig. 4(A), each of the reporters exhibited >30- to >40-fold reductions in activity as the result of the deletion of the putative cin-boxes. Despite the

comYA	AAATAAAAAACCTAATTTTTTCCTGGTTTTTTCGAATA… (18)…ATG
comEA	TTAGGATTTTTACTAAACTTCTTTTTTTTTTACGAATA… (19)…ATG
dprA	TAATGAAACTCTATCAATTGATAGAGTTTTTTCGAATA… (22)…ATG
coiA	TTGAAAAAGACATCAATTTTTGTTGGGATTTACGAATA…(135)…ATG
SMU.431	AAAAGAATCAAAGCAGAAAGTATTTCGTTTTCCGAATA…(100)…ATG
SMU.504	TCTCCTTTTCTTTTTCTGTAACAATAATATTACGAATA…(219)…ATG
SMU.507	TATTCTTAAGTGTTTTTACATACAAATACTTAAGAATA… (53)…ATG
SMU.539	AAATAAAAAGCAATTATTTTGTTTTCTTTTAACGAATA… (7)…ATG
SMU.769	TAAGCAAAACGATAAATTCTAAAATTTTTTTACGAATA… (20)…ATG
SMU.836	AGTTCCTTTTTCAAAATTTCACATCCTATTTCCGAATA… (39)…ATG
SMU.925	GACTCCGTTAGAGGATATTTTCTGTTATACTAAGAATA… (28)…ATG
SMU.1055	TATTTTTTGAAAAGAAATGAATGAATTGAACACGAATA… (69)…ATG
SMU.1904	AATTCTTTGGAATAGTATTATTCTATTGTTTTGGGAATA…(106)…ATG
SMU.1967	TTAATTTTAGCATTGTTTTTCTTTCTTTTTTTGCGAATA… (26)…ATG
SMU.2086	GGAATTTGTGCTATAATATGGTCAATCTTTTACGAATA… (19)…ATG

Figure 3 Alignment of cin-box regions from various *comX*-regulated genes. The putative cin-boxes are underlined. The number of nucleotides between the cin-boxes and the start codons is listed in parentheses.

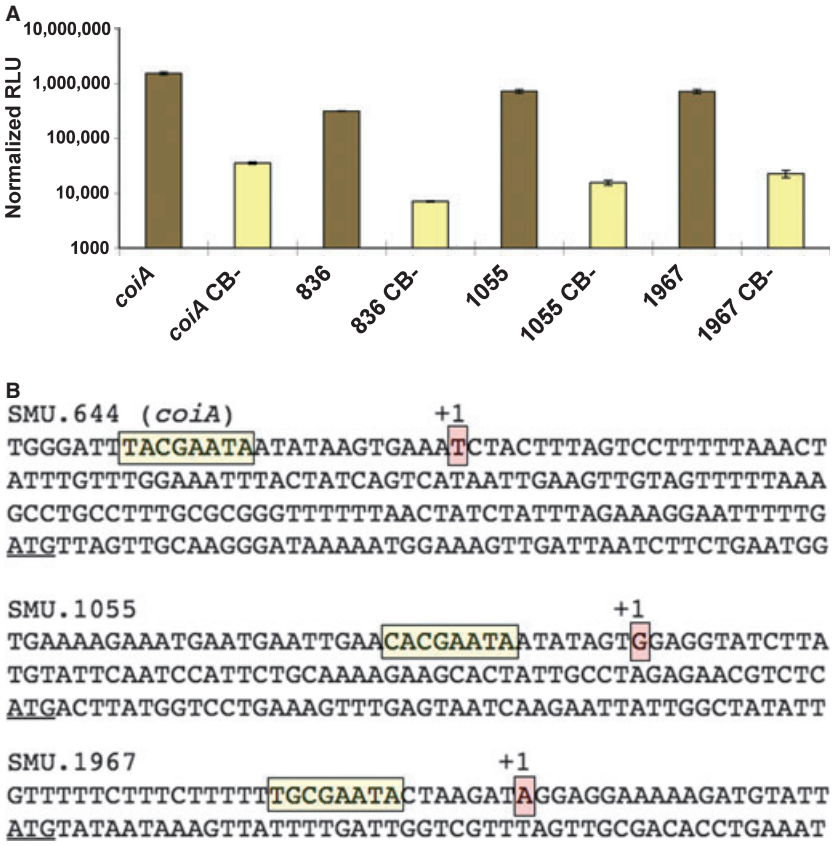

Figure 4 Cin-boxes direct transcription of genes in the *hdrRM* regulon. (A) Luciferase transcription fusion reporters were constructed for *coiA*, SMU.836, SMU.1055 and SMU.1967. Bars shaded in brown represent the reporter activity of the wild-type reporter strains, whereas the yellow bars represent the reporter activity of the same strains devoid of putative cin-box sequences (CB–). (B) Random amplification of cDNA ends polymerase chain reaction (RACE PCR) was used to determine the transcription start sites of *coiA*, SMU.1055 and SMU.1967. The start codons are underlined, the transcription start sites are shaded in red and the cin-boxes are shaded in yellow. The luciferase reporter data are presented as the average of three independent experiments, while at least three separate RACE PCR clones were sequenced with identical results.

differences in the surrounding regions of the putative cin-boxes, each reporter was critically reliant upon the deleted sequences for activity. As further evidence that these sequences were likely to be cin-boxes, we were also interested to determine the transcription start sites for each of these same genes. As previously mentioned, in *S. pneumoniae*, the cin-box functions as an alternative – 10 sequence recognized by ComX (Campbell *et al.*, 1998; Lee & Morrison, 1999; Luo & Morrison, 2003; Luo *et al.*, 2003). Therefore, if the same were true in *S. mutans*, we would predict that the transcription start sites would begin in the proximity of the putative cin-boxes. We employed RACE PCR to determine each start site. As shown in Fig. 4(B), we were able to determine the start sites for *coiA*, SMU.1055, and SMU.1967. In all three strains, the transcription start sites were indeed located just downstream of the predicted cin-boxes. In the case of SMU.1055 and SMU.1967, the spacing between the transcription start and the cin-box was quite similar to a typical – 10 sequence. In *coiA*, the spacing was about five bases longer than for SMU.1055 and SMU.1967. Repeated attempts to map the start site for SMU.836 were unsuccessful, as we were only able to detect RACE PCR products that started well within the SMU.836 open reading frame (data not shown). A similar result was also obtained using another *S. mutans* strain UA159. Therefore, it may be that SMU.836 messenger RNA is either unstable or processed, which may render it difficult to detect the 5′ end of the transcript. From the results obtained with *coiA*, SMU.1055, and SMU.1967, it seems likely

that their predicted cin-boxes are actually used for ComX-dependent transcription, because none of the genes possess obvious sigma-70 type promoters near to their transcription start sites. These results also suggested that the other genes in the microarray data that we had observed as having cin-box-like sequences were also probably directly inducible by ComX in an *hdrR* overexpression or *hdrM* mutant background. Given that we identified at least 30 cin-box-like sequences, it is possible that ComX could be directly responsible for >25% of the gene expression changes seen in our microarray study. Certainly, this number could be even higher if the indirect effects of ComX-dependent transcription are considered as well.

Uncharacterized ComX-regulated genes required for natural competence

Given the central role of ComX in mediating the gene expression changes we detected by microarray, we were next curious to determine whether any uncharacterized ComX-regulated genes we tested by real-time RT-PCR could also have a role in natural competence. Consequently, we created deletions of SMU.431, SMU.507, SMU.539, SMU.769, SMU.836, SMU.1055 and SMU.1904. As shown in Fig. 5(A), the SMU.431, SMU.507, SMU.769, and SMU.1904 mutants all exhibited lower transformation rates from the wild-type, but only by two-fold or less. These genes probably have little or no role in natural transformation. In contrast, the SMU.539, SMU.836 and SMU.1055 mutations strongly affected transformation. The SMU.1055 mutant yielded slightly <10% of the transformants of the wild-type, whereas the SMU.539 and SMU.836 mutants failed to yield any transformants (Fig. 5A). Given the extremely poor transformability of the SMU.539 and SMU.836, mutant strains, we were curious to test these mutations in the *hdrR* overexpression background because *hdrR* overexpression results in a greatly increased transformation efficiency phenotype. Interestingly, even in the *hdrR* overexpression strain both mutations reduced the transformation efficiency below our detection limit (Fig. 5B), which suggests that these two genes may play a critical role in transformation. Of the seven uncharacterized ComX-regulated genes we tested, four are dispensable for transformation in *S. mutans* and three are required.

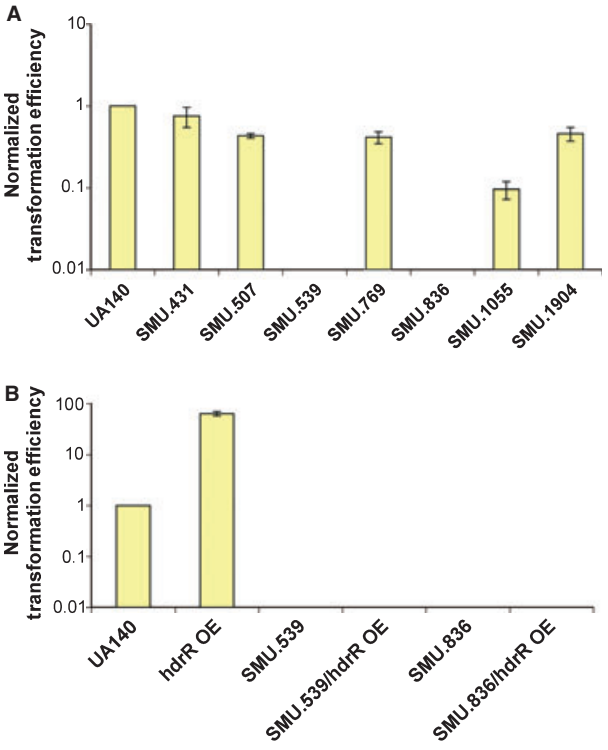

Figure 5 Transformation efficiency of uncharacterized *comX*-regulated genes. (A) The transformation efficiencies of the SMU.431, .507, .539, .769, .836, .1055 and .1904 mutants are presented relative to the wild-type UA140 value (2.87×10^{-6}), which was arbitrarily assigned a value of one. (B) The transformation efficiencies of the SMU.539 and SMU.836 mutants are compared with the same strains carrying the *hdrR* overexpression construct (hdrR OE). All values are presented relative to UA140. These data are the average of three independent experiments.

DISCUSSION

The *hdrRM* regulatory system is a newly described two-gene operon that can control a variety of important phenotypes in *S. mutans*. Little is known about the genes that are subject to regulation by this system, which prompted our investigation of the *hdrRM* regulon. Previously, we demonstrated that *hdrR* and *hdrM* function in the same regulatory pathways and both the *hdrR* overexpression and the *hdrM* mutant strains share the same phenotypes. Furthermore, it was not known whether the two genes function independently in other pathways in addition to their previously described functions. For this reason, we performed a microarray study of both the *hdrR* overexpression strain and the *hdrM* mutant and compared the results. Under our assay conditions, we found a major overlap between the two microarray data sets

with only several minor differences between them. Thus, our current data suggest that the two genes mainly function in the same regulatory pathways, but we cannot exclude the possibility that other separate functions may exist for *hdrR* and *hdrM* under different growth conditions. For most of the expression changes, we saw a greater effect in the *hdrR* overexpression background, which is consistent with its stronger competence phenotype. When examining the data, we found numerous genes that appeared to be under the control of ComX because of the presence of cin-box-like sequences in their upstream intergenic regions. By testing a subset of these genes with real-time RT-PCR, we found evidence that implicated *comX* as a major component of the transcriptional effects seen in the *hdrR* overexpression background. Several of these genes were also tested with 5′ RACE PCR and found to start transcription just downstream of the putative cin-boxes. This suggested that ComX was likely to be a direct regulator of many genes contained within the *hdrRM* regulon. The potent induction of *comX* and the late competence genes in both the *hdrR* overexpression strain and *hdrM* mutant suggests that the *hdrRM* regulatory system plays a major role in controlling the competence machinery. However, consistent with our previous results, we saw no induction of *comC* (data not shown). Similarly, the microarray results determined that the putative CSP transporter *cslAB* is not affected in the *hdrM* background and is only slightly increased in the *hdrR* overexpression strain. A similar result was also obtained for the *comED* operon (Table S3). In contrast, *comC*, *cslAB*, and *comED* are all readily inducible by CSP (Perry *et al.*, 2009), which supports our previous suggestion that increased late competence gene expression in the *hdrR* overexpression and *hdrM* mutant strains occurs independently of CSP signaling (Okinaga *et al.*, 2010). The strong induction of the competence genes in these strains also gives insight into the genes that are subject to regulation by the competence system of *S. mutans*.

In addition to the well-characterized late competence genes/operons, such as *comYA*, *comEA*, *coiA*, *dprA* and *comFA*, we also observed large gene expression increases in our microarray data for numerous uncharacterized genes, some of which are likely components of the competence machinery. For example, the competence-specific gene SMU.1967 was >40-fold more highly expressed in the *hdrR* overexpression background and is a likely ortholog of the single-stranded binding protein *cilA* in *S. pneumoniae*. Mutants of *cilA* exhibit greatly reduced competence (Campbell *et al.*, 1998) and both *S. pneumoniae* and *S. mutans* contain two genes encoding single-stranded binding (SSB) proteins in their genomes. In *S. mutans*, the other *ssb* gene is SMU.1859. SMU.1859 was highly expressed and exhibited almost no differences in expression in both microarray data sets (data not shown). Presumably, this gene encodes the primary housekeeping SSB because it is located in an operon encoding two ribosomal proteins. In contrast, our real-time RT-PCR and RACE PCR data suggest that SMU.1967 (*cilA*) is exclusively regulated by ComX during competence and therefore, probably performs a competence-specific function in the cell. SMU.539 was found to be stringently required for natural transformation, because we never detected any transformants in the mutant. A BLASTP search of the SMU.539 gene product suggests that this gene is likely to be an ortholog of CilC in *S. pneumoniae* (Campbell *et al.*, 1998) and ComC in *Bacillus subtilis* (Chung & Dubnau, 1995). In *B. subtilis*, it has been demonstrated that this gene encodes a type IV prepilin peptidase required for processing the ComGC protein (Chung & Dubnau, 1995). The *comGC* gene is an ortholog of the *S. mutans comYC* gene, which similarly encodes a putative prepilin-type leader sequence. Given that the *comY* genes have each been shown to be essential for transformation in *S. mutans* (Merritt *et al.*, 2005), the SMU.539 competence deficiency is likely to be at least partially attributable to an inability to properly process ComYC and possibly ComYD as well. Like SMU.539, we also found a severe natural competence defect in the SMU.836 mutant. A BLASTP search of the putative sequence of SMU.836 suggests that this gene is likely to be a murein hydrolase because of the presence of a cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain as well as having strong sequence homology to other characterized autolysins. Interestingly, this gene does not appear to have a characterized ortholog in *S. pneumoniae*, although the major pneumococcal autolysin *lytA* is inducible by CSP (Peterson *et al.*, 2004). However, *lytA* is apparently required for DNA release during competence, rather than DNA uptake (Tomasz *et al.*, 1988; Moscoso & Claverys, 2004).

Therefore, there does not seem to be a strong correlation between the functions of these two genes during competence. In contrast, a protein referred to as Tpc in the naturally competent organism *Neisseria gonorrhoeae* could potentially perform a similar function as SMU.836. Disruption of the *tpc* gene resulted in a drastically reduced natural competence phenotype and a functional analysis of Tpc suggested that it is either directly or indirectly involved in murein hydrolytic functions (Fussenegger *et al.*, 1996). It was speculated that Tpc might be required to open the peptidoglycan layer to facilitate the entrance of extracellular DNA and/or the extrusion of the competence machinery. It is conceivable that SMU.836 could play a similar role in *S. mutans* transformation. SMU.1055 is an ortholog of the pneumococcal *radC* gene. While the exact function of *radC* is still unknown, in *S. pneumoniae* this gene is highly induced during late competence, yet fully dispensable for transformation (Peterson *et al.*, 2004). Curiously, our data implicate a role for the *radC* gene in natural competence because the mutant strain exhibited markedly lower rates of transformation. However, whether this occurs as a direct result of interactions with the transformation apparatus, transforming DNA, or through some other indirect route remains to be determined. Our RACE PCR data do suggest that SMU.1055 is directly transcribed by ComX so this gene likely plays an important role during competence.

In addition to controlling competence in *S. mutans*, CSP signaling through the ComCDE system plays a major role in regulating the expression of multiple bacteriocins (Hale *et al.*, 2005; Kreth *et al.*, 2005, 2006, 2007; van der Ploeg, 2005; Yonezawa & Kuramitsu, 2005; Perry *et al.*, 2009). In both the *hdrR* overexpression and *hdrM* mutant backgrounds, we found increased expression of a variety of CSP-inducible bacteriocin-related genes, such as SMU.423, SMU.925, SMU.1905 and SMU.1906. Likewise, our previous studies demonstrated that either *hdrR* overexpression or an *hdrM* mutation could abrogate the requirement for *comC* and *comED* to produce the bacteriocin mutacin IV (SMU.150 and SMU.151) on agar plates (Okinaga *et al.*, 2010). Hence, the HdrRM system exerts control over all of the known CSP-inducible bacteriocin-related genes, except for SMU.1914 (referred to as *nlmC* and *cipB*), which does not exist in UA140. Taken together, our results indicate that that the HdrRM regulon is remarkably similar to that of the ComCDE system. Both our current and previous data suggest that the HdrRM system acts in parallel with ComCDE. Consequently, *S. mutans* likely encodes at least two independent competence regulatory systems that both control DNA uptake and bacteriocin production. This suggests that the coordination of competence and bacteriocin production must be a critical ability for the persistence of *S. mutans* in the oral cavity.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Table S1. Bacterial strains and plasmids used in this study in pdf format.

Table S2. Primers used in this study in pdf format.

Table S3. Complete microarray data set in xls format.

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REFERENCES

Ajdic, D. and Pham, V.T. (2007) Global transcriptional analysis of *Streptococcus mutans* sugar transporters using microarrays. *J Bacteriol* **189**: 5049–5059.

Aspiras, M.B., Ellen, R.P. and Cvitkovitch, D.G. (2004) ComX activity of *Streptococcus mutans* growing in biofilms. *FEMS Microbiol Lett* **238**: 167–174.

Banas, J.A. (2004) Virulence properties of *Streptococcus mutans*. *Front Biosci* **9**: 1267–1277.

Barsamian-Wunsch, P., Park, J.H., Watson, M.R., Tinanoff, N. and Minah, G.E. (2004) Microbiological screening for cariogenic bacteria in children 9 to 36 months of age. *Pediatr Dent* **26**: 231–239.

Bowden, G.H. (1990) Microbiology of root surface caries in humans. *J Dent Res* **69**: 1205–1210.

Campbell, E.A., Choi, S.Y. and Masure, H.R. (1998) A competence regulon in *Streptococcus pneumoniae*

revealed by genomic analysis. *Mol Microbiol* **27**: 929–939.

Cheng, Q., Campbell, E.A., Naughton, A.M., Johnson, S. and Masure, H.R. (1997) The com locus controls genetic transformation in *Streptococcus pneumoniae*. *Mol Microbiol* **23**: 683–692.

Chung, Y.S. and Dubnau, D. (1995) ComC is required for the processing and translocation of comGC, a pilin-like competence protein of *Bacillus subtilis*. *Mol Microbiol* **15**: 543–551.

Cvitkovitch, D.G. (2001) Genetic competence and transformation in oral streptococci. *Crit Rev Oral Biol Med* **12**: 217–243.

Fussenegger, M., Kahrs, A.F., Facius, D. and Meyer, T.F. (1996) Tetrapac (tpc), a novel genotype of *Neisseria gonorrhoeae* affecting epithelial cell invasion, natural transformation competence and cell separation. *Mol Microbiol* **19**: 1357–1372.

Hale, J.D., Heng, N.C., Jack, R.W. and Tagg, J.R. (2005) Identification of *nlmTE*, the locus encoding the ABC transport system required for export of non-antibiotic mutacins in *Streptococcus mutans*. *J Bacteriol* **187**: 5036–5039.

Havarstein, L.S., Gaustad, P., Nes, I.F. and Morrison, D.A. (1996) Identification of the streptococcal competence-pheromone receptor. *Mol Microbiol* **21**: 863–869.

van Houte, J. (1993) Microbiological predictors of caries risk. *Adv Dent Res* **7**: 87–96.

Johnsborg, O. and Havarstein, L.S. (2009) Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. *FEMS Microbiol Rev* **33**: 627–642.

Kreth, J., Merritt, J., Shi, W. and Qi, F. (2005) Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol Microbiol* **57**: 392–404.

Kreth, J., Merritt, J., Zhu, L., Shi, W. and Qi, F. (2006) Cell density- and ComE-dependent expression of a group of mutacin and mutacin-like genes in *Streptococcus mutans*. *FEMS Microbiol Lett* **265**: 11–17.

Kreth, J., Hung, D.C., Merritt, J. *et al.* (2007) The response regulator ComE in *Streptococcus mutans* functions both as a transcription activator of mutacin production and repressor of CSP biosynthesis. *Microbiology* **153**: 1799–1807.

Kuramitsu, H.K. (2003) Molecular genetic analysis of the virulence of oral bacterial pathogens: an historical perspective. *Crit Rev Oral Biol Med* **14**: 331–344.

Lee, M.S. and Morrison, D.A. (1999) Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. *J Bacteriol* **181**: 5004–5016.

Li, Y.H., Lau, P.C., Lee, J.H., Ellen, R.P. and Cvitkovitch, D.G. (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J Bacteriol* **183**: 897–908.

Li, Y.H., Tang, N., Aspiras, M.B. *et al.* (2002) A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J Bacteriol* **184**: 2699–2708.

Loimaranta, V., Tenovuo, J., Koivisto, L. and Karp, M. (1998) Generation of bioluminescent *Streptococcus mutans* and its usage in rapid analysis of the efficacy of antimicrobial compounds. *Antimicrob Agents Chemother* **42**: 1906–1910.

Lunsford, R.D. and London, J. (1996) Natural genetic transformation in *Streptococcus gordonii*: *comX* imparts spontaneous competence on strain Wicky. *J Bacteriol* **178**: 5831–5835.

Luo, P. and Morrison, D.A. (2003) Transient association of an alternative sigma factor, ComX, with RNA polymerase during the period of competence for genetic transformation in *Streptococcus pneumoniae*. *J Bacteriol* **185**: 349–358.

Luo, P., Li, H. and Morrison, D.A. (2003) ComX is a unique link between multiple quorum sensing outputs and competence in *Streptococcus pneumoniae*. *Mol Microbiol* **50**: 623–633.

Martin, B., Alloing, G., Mejean, V. and Claverys, J.P. (1987) Constitutive expression of erythromycin resistance mediated by the *ermAM* determinant of plasmid pAM beta 1 results from deletion of 5′ leader peptide sequences. *Plasmid* **18**: 250–253.

Merritt, J., Qi, F. and Shi, W. (2005) A unique nine-gene *comY* operon in *Streptococcus mutans*. *Microbiology* **151**: 157–166.

Merritt, J., Zheng, L., Shi, W. and Qi, F. (2007) Genetic characterization of the *hdrRM* operon: a novel high-cell-density-responsive regulator in *Streptococcus mutans*. *Microbiology* **153**: 2765–2773.

Moscoso, M. and Claverys, J.P. (2004) Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. *Mol Microbiol* **54**: 783–794.

Munson, M.A., Banerjee, A., Watson, T.F. and Wade, W.G. (2004) Molecular analysis of the microflora associated with dental caries. *J Clin Microbiol* **42**: 3023–3029.

Napimoga, M.H., Hofling, J.F., Klein, M.I., Kamiya, R.U. and Goncalves, R.B. (2005) Transmission, diversity and virulence factors of *Streptococcus mutans* genotypes. *J Oral Sci* **47**: 59–64.

Nikolskaya, A.N. and Galperin, M.Y. (2002) A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. *Nucleic Acids Res* **30**: 2453–2459.

Niu, G., Okinaga, T., Zhu, L., Banas, J., Qi, F. and Merritt, J. (2008) Characterization of irvR, a novel regulator of the irvA-dependent pathway required for genetic competence and dextran-dependent aggregation in *Streptococcus mutans*. *J Bacteriol* **190**: 7268–7274.

Nobre dos Santos, M., Melo dos Santos, L., Francisco, S.B. and Cury, J.A. (2002) Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. *Caries Res* **36**: 347–352.

Okinaga, T., Niu, G., Xie, Z., Qi, F. and Merritt, J. (2010) The *hdrRM* operon of *Streptococcus mutans* encodes a novel regulatory system for coordinated competence development and bacteriocin production. *J Bacteriol* **192**: 1844–52.

Perry, J.A., Jones, M.B., Peterson, S.N., Cvitkovitch, D.G. and Levesque, C.M. (2009) Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence. *Mol Microbiol* **72**: 905–917.

Pestova, E.V., Havarstein, L.S. and Morrison, D.A. (1996) Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol Microbiol* **21**: 853–862.

Petersen, F.C. and Scheie, A.A. (2000) Genetic transformation in *Streptococcus mutans* requires a peptide secretion-like apparatus. *Oral Microbiol Immunol* **15**: 329–334.

Peterson, S.N., Sung, C.K., Cline, R. *et al.* (2004) Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol* **51**: 1051–1070.

van der Ploeg, J.R. (2005) Regulation of bacteriocin production in *Streptococcus mutans* by the quorum-sensing system required for development of genetic competence. *J Bacteriol* **187**: 3980–3989.

Podbielski, A., Spellerberg, B., Woischnik, M., Pohl, B. and Lutticken, R. (1996) Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* **177**: 137–147.

Thenisch, N.L., Bachmann, L.M., Imfeld, T., Leisebach Minder, T. and Steurer, J. (2006) Are mutans streptococci detected in preschool children a reliable predictive factor for dental caries risk? A systematic review. *Caries Res* **40**: 366–374.

Tomasz, A., Moreillon, P. and Pozzi, G. (1988) Insertional inactivation of the major autolysin gene of *Streptococcus pneumoniae*. *J Bacteriol* **170**: 5931–5934.

Yonezawa, H. and Kuramitsu, H.K. (2005) Genetic analysis of a unique bacteriocin, Smb, produced by *Streptococcus mutans* GS5. *Antimicrob Agents Chemother* **49**: 541–548.

Zambon, J.J. and Kasprzak, S.A. (1995) The microbiology and histopathology of human root caries. *Am J Dent* **8**: 323–328.