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The *hdrRM* Operon of *Streptococcus mutans* Encodes a Novel Regulatory System for Coordinated Competence Development and Bacteriocin Production[▽]

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The *Streptococcus mutans hdrRM* operon encodes a novel two-gene regulatory system induced by high cell density. Previous studies identified *hdrM* as the only known negative regulator of competence development in *S. mutans*. In the present study, we demonstrated that the HdrRM system bypasses the prototypical competence gene regulators ComC and ComDE in the transcriptional regulation of the competence-specific sigma factor *comX* and the late competence genes. Similarly, the HdrRM system can abrogate the requirement for ComE to produce the bacteriocin mutacin IV. To further probe the regulatory mechanism of *hdrRM*, we created an *hdrR* overexpression strain and showed that it could reproduce each of the *hdrM* competence and mutacin phenotypes, indicating that HdrM acts as a negative regulator of HdrR activity. Using a mutacin IV-luciferase reporter, we also demonstrated that the *hdrRM* system utilizes the same promoter elements recognized by ComE and thus appears to comprise a novel regulatory pathway parallel to ComCDE.

Streptococcus mutans is a gram-positive oral commensal species often associated with the development of dental caries (tooth decay) (2, 3, 22, 25, 30, 32, 36). Similar to numerous other species of *Streptococcus*, *S. mutans* is naturally competent and thus actively internalizes exogenous DNA from the environment, which can lead to genetic transformation (6). Furthermore, the regulatory machinery of the competence system has been shown to affect a variety of virulence factors in *S. mutans* such as acid tolerance, biofilm formation, and bacteriocin (mutacin) production (11, 13, 14, 35).

Among the streptococci, *Streptococcus pneumoniae* has the most thoroughly characterized competence system, and consequently, its regulatory scheme has become the prototypical model for competence gene regulation among other naturally competent *Streptococcus* species. Competence initiation begins with the product of the gene *comC*, which is a secreted cell signaling peptide referred to as the competence-stimulating peptide (CSP) (5, 14, 17, 26). When the optimal cell density, and thus CSP concentration, has been achieved, CSP is sensed by a two-component regulatory system composed of the ComD sensor kinase and the ComE response regulator (5, 8, 26). Upon binding CSP, ComD phosphorylates ComE, which subsequently stimulates its transcription factor activity, resulting in the expression of a competence-specific sigma factor, *comX* (12, 14, 17). Finally, ComX is ultimately responsible for transcribing the late competence genes, which comprise all of the machinery required for exogenous DNA internalization and recombination (12, 18, 20). In *S. mutans*, evidence suggests that the competence cascade seems to function quite similarly, except that the *comC* and *comED* genes are not absolutely essential for transformability (1). However, they are essential for the expression of numerous bacteriocins (7, 9, 11, 34), which supports the suggestion that bacteriocin production and competence development are functionally linked in *S. mutans* (10).

Recently, we reported the identification of a previously uncharacterized two-gene operon (*hdrRM*) that encodes a predicted transcription regulator (HdrR) and membrane protein (HdrM). The operon was found to be induced by conditions of extremely high cell density, while *hdrM* was shown to be a potent negative regulator of genetic competence (21). The connection to competence development was of particular interest, as *hdrM* is currently the only reported negative regulator of competence in *S. mutans*. Surprisingly, this and other phenotypes were only observable with a mutation in *hdrM*; an in-frame deletion of *hdrR* and an *hdrRM* double deletion both produced wild-type behavior. This suggested that *hdrR* is likely part of the pathway regulated by *hdrM*, since the *hdrM* phenotype was only observable with *hdrR* present. In the present study, we further investigated the competence phenotype in order to examine the mechanism of regulation by the HdrRM system. We show that in *S. mutans*, the HdrRM system appears to regulate competence and mutacin IV production similarly to ComDE, but both systems apparently function in response to distinct stimuli. Furthermore, HdrM appears to function as a negative regulator of HdrR, which functions as a positive regulator of competence and mutacin gene expression. These results suggest that the mechanisms governing competence development in *S. mutans* are potentially much more elaborate than suggested by the *S. pneumoniae* model.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *S. mutans* UA140 and its derivatives were grown in brain heart infusion broth (BHI; Difco) and on BHI agar plates. When testing for mutacin IV production, cells were grown on Todd-Hewitt (TH; Difco) agar plates. For the selection of antibiotic-resistant colonies, BHI plates were supplemented with 800 μg ml^{-1} kanamycin (Sigma), 15 μg ml^{-1} erythro-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
E. coli DH5α	Cloning strain	
UA140	Wild-type *S. mutans*	28
NY101	Wild-type *S. sanguinis*	33
TO-m	UA140::pHdrM-i *hdrM* Sp^r	This work
TO-c	UA140Δ*comC* Km^r	This work
TO-e	UA140::pComD-KO *comE* Em^r	14
TO-x	UA140::pComX-KO *comX* Em^r	14
TO-yad	UA140Δ*comYA-D* Em^r	20
TO-mc	UA140::pHdrM-i Δ*comC* Spe^r Km^r	This work
TO-me	UA140::pHdrM-i/pComD-KO Spe^r Em^r	This work
TO-mx	UA140::pHdrM-i/pComX-KO Spe^r Em^r	This work
TO-myad	UA140::pHdrM-i Δ*comYA-D* Spe^r Kan^r	This work
TO-Roe	UA140/pHdrRoe Sp^r	This work
TO-Roe−	UA140/pHdrRoe− Sp^r	This work
TO-RMoe	UA140/pHdrRMoe Sp^r	This work
TO-eRoe	UA140::pComD-KO/pHdrRoe Em^r Sp^r	This work
TO-nLuc	UA140::pNlmA-luc2 Sp^r	This work
TO-nLucRoe	UA140::pNlmA-luc2/pHdrRoe Sp^r Km^r	This work
TO-nLucRMoe	UA140::pNlmA-luc2/pHdrRMoe Sp^r Km^r	This work
TO-nLucE	UA140::pNlmA-luc2/pComD-KO Sp^r Km^r Em^r	This work
TO-nLucERoe	UA140::pNlmA-luc2/pComD-KO/pHdrRoe Sp^r Km^r Em^r	This work
TO-nLucERMoe	UA140::pNlmA-luc2/pComD-KO/pHdrRMoe Sp^r Km^r Em^r	This work
TO-nLucDR	UA140::pNlmA-DR-luc Sp^r Km^r	This work
TO-nLucDRRoe	UA140::pNlmA-DR-luc/pHdrRoe Sp^r Km^r	This work
TO-nLucDRRMoe	UA140::pNlmA-DR-luc/pHdrRMoe Sp^r Km^r	This work
Plasmids		
pFW5	Suicide vector; Sp^r	27
pDL278	*E. coli-Streptococcus* shuttle vector; Sp^r	4
pBS-Kan	pBluescript with *aphAIII* in MCS;[b] Ap^r Km^r	G. Niu, unpublished data
pHdrM-i	pFW5 + internal fragment of *hdrM*; Sp^r	This work
pHdrRoe	pDL278::ϕ(ldh_p-*hdrR*); Sp^r	This work
pHdrRoe-	pDL278::ϕ(*ldhp*[no RBS]-*hdrR*); Sp^r	This work
pHdrRMoe	pDL278::ϕ(*ldhp-hdrR*)/ϕ(*ldhp-hdrM*); Sp^r	This work
pNlmA-luc	pFW5::ϕ(*nlmAp-luc*); Sp^r	This work
pNlmA-luc2	pFW5::ϕ(*nlmAp-luc*); Sp^r Km^r	This work
pNlmA-DR-luc	pFW5::ϕ(*nlmAp*[ΔDR]-luc); Sp^r Km^r	This work
pComC-del	pFW5 + allelic replacement of *comC*; Sp^r	This work

[a] Ap^r, ampicillin resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance.
[b] MCS, multiple cloning site.

mycin (MP Biomedicals), 15 μg ml^{-1} tetracycline (Sigma), or 1,000 μg ml^{-1} spectinomycin (Sigma). *S. mutans* strains were grown anaerobically (85% N_2, 10% CO_2, 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 μg ml^{-1} spectinomycin or 100 μg ml^{-1} kanamycin.

Construction of *hdrM* mutants. The primers used in this study are described in Table 2. An internal DNA fragment of *hdrM* was amplified by PCR using the primer pair 1690 F and 1690 R. The PCR fragment was then digested with XhoI and HindIII and cloned into the vector pFW5 digested with the same enzymes. The resulting construct was confirmed by restriction analysis and PCR before integration into the chromosome of UA140 via single-crossover homologous recombination.

Construction of the *comC* mutant. Two fragments corresponding to approximately 1 kb upstream and downstream of *comC* were generated by PCR with Accuprime *Pfx* high-fidelity polymerase (Invitrogen) and the primers comC up F, comC up R, comC dn F, and comC dn R. The resulting fragments were cloned sequentially into the vector pFW5. The upstream fragment was cut with PstI and NcoI, and the downstream fragment was cut with HindIII and XhoI. Both fragments were ligated to identical sites in pFW5, which occur on either side of a spectinomycin resistance cassette. After confirmation of the resulting plasmid by restriction analysis and PCR, the linearized plasmid was transformed to *S. mutans* UA140.

Construction of the *comE*, *comX*, and *comY* mutants. The *comE*, *comX*, and *comY* mutants were previously constructed (14, 20). These mutations were moved into UA140 and its derivatives by transforming genomic DNA.

Construction of overexpression strains. To generate the *hdrR* overexpression strain, the entire predicted *hdrR* open reading frame (ORF) and the highly expressed constitutive lactate dehydrogenase (*ldh*) promoter were generated by PCR using Accuprime *Pfx* and the primers hdrR OE F and hdrR OE R (*hdrR* ORF), as well as ldh-p F and ldh-p R (*ldh* promoter). Next, the *ldh* amplicon was digested with BamHI and SpeI and the *hdrR* amplicon was digested with SpeI and HindIII. Both fragments were ligated together with the shuttle vector pDL278 digested with BamHI and HindIII. To create the ribosome binding site (RBS)-lacking *hdrR* overexpression strain, a similar strategy was used, except that the *ldh* promoter fragment was amplified using the primers ldh-p F and ldh-RBS R. The overexpression plasmids were confirmed by DNA sequencing to confirm sequence integrity and the expected fusion of the *hdrR* ORF. Confirmed plasmids were then transformed to *S. mutans* UA140. To generate the *hdrRM* overexpression plasmid, we first amplified the *ldh* promoter with ldh-p F2 and ldh-p R and the *hdrM* ORF with hdrM OE F and hdrM OE R. Next, both amplicons were digested with HindIII and SpeI and ligated together. The ligation mixture was amplified by PCR using Accuprime *Pfx* and subsequently digested with HindIII. The digested fragment was then ligated to the *hdrR* overexpression plasmid pldh-hdrR digested with HindIII. The *hdrRM* overexpression plasmid was confirmed by DNA sequencing to confirm sequence integrity and the in-frame fusion of the *hdrM* ORF.

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)	Purpose
1690 F	CCCCTCGAGTTTTGGTACGGTCTGCTTGG	*hdrM* mutation
1690 R	CCCAAGCTTTGTTTAAAATAACGCCGATAATGA	*hdrM* mutation
comC up F	GGCCTGCAGCTGTACCCATTCAGAAATCTC	*comC* mutation
comC up R	GCCCCATGGAAGTCATTTTTTAATGATAGTGTTTTTTTC	*comC* mutation
comC dn F	GGGAAGCTTTGGGAAAATAAGATAGGCTAACAT	*comC* mutation
comC dn R	CCCCTCGAGCCTCTTTCTCAGTGTGTTC	*comC* mutation
hdrR RT F	TCAGCAAATTGGCAAGAGTCA	*hdrR* qPCR[a]
hdrR RT R	GGAGAAAGACTTATTGATGGCAGAA	*hdrR* qPCR
comC RT F	GACTGATGAATTAGAGATTATCATTGG	*comC* qPCR
comC RT R	TTTCCCAAAGCTTGTGTAAAACT	*comC* qPCR
comE RT F	GAGTTCTCCCACCGCATTGA	*comE* qPCR
comE RT R	ACCATTCTTCTGGCTGTTTTCC	*comE* qPCR
comX RT F	TTATTTCGTGATAGTTTGCTTGCAT	*comX* qPCR
comX RT R	CAAGCGCTCAAACAGCTCTTG	*comX* qPCR
comYA RT F	CTTTTTTCTGGACGTCACGATTT	*comYA* qPCR
comYA RT R	TCGCCCCTTGATTTCATTTAAA	*comYA* qPCR
ldhp F	GCCGGATCCCCGAGCAACAATAACACTC	*hdrR*/*M* overexpression
ldhp R	CGCACTAGTAACATCTCCTTATAATTTATTAAGTATATATTCTAT	*hdrR*/*M* overexpression
ldh-RBS R	CCCACTAGTTATAATTTATTAAGTATATATTCTATACATTTTCATTCTAAC	*hdrR* overexpression
ldhp F2	GCCAAGCTTCCGAGCAACAATAACACTC	*HdrM* overexpression
hdrR oe F	GCCACTAGTATGGAGACAAGATACATTTTTGATG	*hdrR* overexpression
hdrR oe R	CCCAAGCTTTCATAGTAAACTCCTTTTTTTCATAAGT	*hdrR* overexpression
hdrM oe F	CCCACTAGTATGAAAAAAAATTATTTTTGGTACGGT	*hdrM* overexpression
hdrM oe R	CCCAAGCTTTTAATATTGAATGTTTAGAGATCCCATAG	*hdrM* overexpression
nlmA DR F	AAAATAAATTGTTATACTAAAGATGTTGGTTG	*nlmA* reporter
nlmA DR R	TATTTTGTCTTAAACGGTCATTTTTGA	*nlmA* reporter

[a] qPCR, quantitative PCR.

Construction of *nlmA*-luciferase reporter gene fusions. The *nlmA-luc* plasmid pFW5::ϕ(*nlmA*p-*luc*) was constructed previously (10). In order to use this construct with our *hdrR* and *hdrRM* overexpression constructs, it was necessary to first add a kanamycin resistance marker to the reporter plasmid. The kanamycin resistance cassette was excised from the vector pGNaa3 (24) with PvuII and ligated to a SmaI site in pNlmA-luc to create the plasmid pNlmA-luc2. In order to construct an *nlmA-luc* reporter plasmid containing a deletion of a single ComE binding site direct repeat, pNlmA-luc2 was used as a template for inverse PCR using the primers nlmA DR F and nlmA DR R, both of which had previously been phosphorylated with T4 polynucleotide kinase (NEB). The PCR mixture was then treated with DpnI to remove the template, and the reaction product was ligated and transformed into *E. coli*. The plasmids were confirmed by DNA sequencing to confirm sequence integrity and the expected deletion of the direct repeat. The plasmids were transformed into UA140 and integrated into the *nlmA* promoter region via single-crossover recombination. These strains were used as recipients for the *hdrR* and *hdrRM* overexpression constructs.

Luciferase assays. Luciferase assays were performed using previously described methods (16, 19). Briefly, 25 μl 1 mM D-luciferin (Sigma) suspended in 100 mM (pH 6) citrate buffer was added to 100 μl of cell culture. Luciferase activity was measured using a TD 20/20 luminometer (Turner Biosystems). Overnight cultures of reporter strains were diluted 1:20 and grown to an optical density at 600 nm (OD_{600}) of 0.7 to 0.8 before measurement of luciferase activity.

Transformation assays. Determination of transformation efficiency was performed using a previously described methodology (14, 20). UA140 and its derivatives were diluted 1:30 from overnight cultures and grown to an OD_{600} of 0.2 to 0.3 in BHI plus 0.4% (wt/vol) bovine serum albumin (BSA) before the addition of DNA. Transforming genomic DNA containing a tetracycline marker was added at a final concentration of 10 μg ml^{-1} for each reaction, and the cultures were subsequently allowed to grow for an additional 2 h. Reaction mixtures assaying the effect of CSP also included 1 μg ml^{-1} CSP with the transforming DNA. After the incubation period, cultures were briefly sonicated to disperse cell chains and plated on tetracycline-containing BHI agar plates, as well as on nonselective BHI plates. Successful transformation was scored based on acquired tetracycline resistance following transformation, and the total number of viable cells was determined by counting the colonies growing on nonselective plates. Transformation efficiency was determined by calculating the ratio of transformants to the total number of viable cells.

RNA extraction and transcriptional analysis. Overnight cultures of UA140 and its derivatives were diluted 1:30 in 300 ml BHI plus 0.4% (wt/vol) BSA and collected at three separate OD_{600} values, 0.2 to 0.3, 0.5 to 0.6, and 0.8 to 0.9. Cells were harvested by centrifugation at 4°C and then stored at −80°C. RNA was extracted from cell pellets using a previously described methodology (24). Total RNA (300 ng) was used for cDNA synthesis using Stratascript reverse transcriptase (Stratagene) according to the manufacturer's protocol. For real-time reverse transcription (RT)-PCR, primers were designed using Primer Express 3.0 software (ABI), the reaction mixtures were prepared using Applied Biosystems SYBR green PCR mastermix, and an Applied Biosystems 7300 was used for detection. Relative changes in gene expression were calculated using the $\Delta\Delta C_T$ method described previously (24). Total cDNA abundance between samples was normalized using primers specific to the 16S rRNA gene.

Plate assay for mutacin production. To assay for mutacin IV production, wild-type UA140 and its derivatives were first grown overnight in liquid cultures under standard anaerobic conditions. A 5-μl volume of each overnight culture was spotted onto TH agar plates and incubated anaerobically at 37°C overnight.

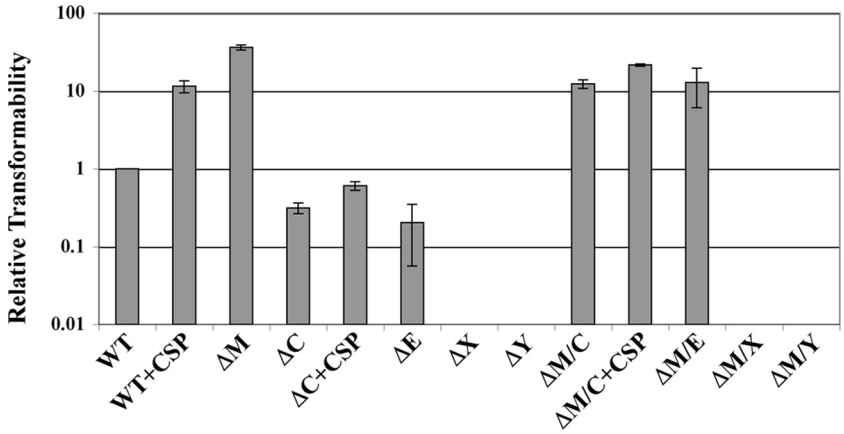

FIG. 1. Transformation efficiencies of various *hdrM* mutants. Transformation efficiency was measured as described in Materials and Methods, and all values were normalized to the wild-type UA140 value (3.14×10^{-6}). The strains are identified as follows: WT (wild-type UA140), WT+CSP (UA140 with CSP), ΔM (*hdrM* mutant), ΔC (*comC* mutant), ΔC+CSP (*comC* mutant with CSP), ΔE (*comE* mutant), ΔX (*comX* mutant), ΔY (*comY* mutant), ΔM/C (*hdrM comC* double mutant), ΔM/C+CSP (*hdrM comC* double mutant with CSP), ΔM/E (*hdrM comE* double mutant), ΔM/X (*hdrM comX* double mutant), and ΔM/Y (*hdrM comY* double mutant). The results presented here are the average of three independent experiments. Each experiment measured three independent clones for each sample.

The following day, the plates were overlaid with a soft-agar suspension of the indicator strain (*Streptococcus sanguinis* strain NY101) and incubated anaerobically for an additional 16 h. Zones of inhibition were indicative of mutacin IV production.

RESULTS

Epistatic analysis of the competence cascade in the *hdrM* background. In our previous studies, we determined that a mutation of *hdrM* caused several phenotypes, including greatly increased genetic competence (21). This phenotype was highly unusual, as the only other reported comparable competence phenotype came from an uncharacterized mutation created by chemical mutagenesis (29). Thus, we were interested in determining the connection between *hdrM* and competence. Since the major components of the competence cascade were already well established, we reasoned that the competence phenotype of the *hdrM* mutant must require the function of the *comC*, *comED*, *comX*, and/or *comY* genes. Since the *comY* genes are late competence genes known to be absolutely essential for DNA uptake (20), this operon was assumed to be the minimal gene set required for the *hdrM* competence phenotype. Thus, we created double mutations of *hdrM* with each of these components of the competence cascade and assayed transformation efficiency. For comparison, we also measured transformation in the presence of exogenously added synthetic CSP. As shown in Fig. 1, CSP addition increased the transformation efficiency of wild-type UA140 >10-fold, which was considerably less than the >35-fold higher transformation efficiency of the *hdrM* mutant. As expected, both the *comC* and *comED* mutants had noticeably lower rates of transformation, while no transformants were detected in the *comX* and *comY* backgrounds. In contrast, in the *hdrM* background, both the *comC* and *comED* mutants were highly transformable and, in fact, exhibited transformation efficiencies >10-fold higher than the wild-type level (Fig. 1). As expected, CSP was able to increase the transformation efficiency of the *comC* (CSP gene) mutant. Interestingly, CSP could also further increase the elevated transformation efficiency of the *hdrM comC* double mutant. Thus, the classical competence pathway and the *hdrM* mutant pathway appeared to have additive effects upon competence, which suggested they are likely parallel pathways. However, *hdrM* was not able to rescue the severe competence defects of the *comX* and *comY* mutants, which indicated that the *hdrM* phenotype likely functioned through *comX*, since it is upstream of *comY*.

HdrM controls competence via transcriptional regulation of *comX*. The results described above suggested that the *hdrM* mutation affected competence downstream of the ComCDE system, possibly through transcriptional regulation of *comX* or *comY*. To test this, we measured the transcription of the competence genes in the *hdrM* background to determine whether the competence phenotype could be attributed to altered transcription. Moreover, we had previously determined that *comY* operon expression correlated with transformation efficiency (20), so there was reason to suspect that, at a minimum, the *comY* operon would exhibit altered transcription. Given that competence development is transient during early log phase (15), we further reasoned that it was possible that competence gene transcription was not necessarily higher than the wild-type level but actually lasted longer. Therefore, in order to reconcile these two possible transcriptional effects, we measured the transcription of each competence gene at three different growth phases to determine whether the *hdrM* mutation simply induced greater competence gene expression or created an alteration in the temporal pattern of gene expression. Samples were taken at the early (OD_{600}, 0.2 to 0.3), mid (OD_{600}, 0.5 to 0.6), and late (OD_{600}, 0.8 to 0.9) log phases. As shown in Fig. 2A and B, *comC* gene expression and *comE* gene expression were both quite similar between the wild type and the *hdrM* mutant throughout the different growth phases, although during the early log phase we had observed a slight but reproducible increase in *comE* gene expression in the *hdrM* mutant. In contrast, we observed a dramatic increase in *comX* and *comY* gene expression in the *hdrM* background. At the peak expression of competence in the early log phase, *comX* expression was >35-fold higher in the *hdrM* background (Fig. 2C),

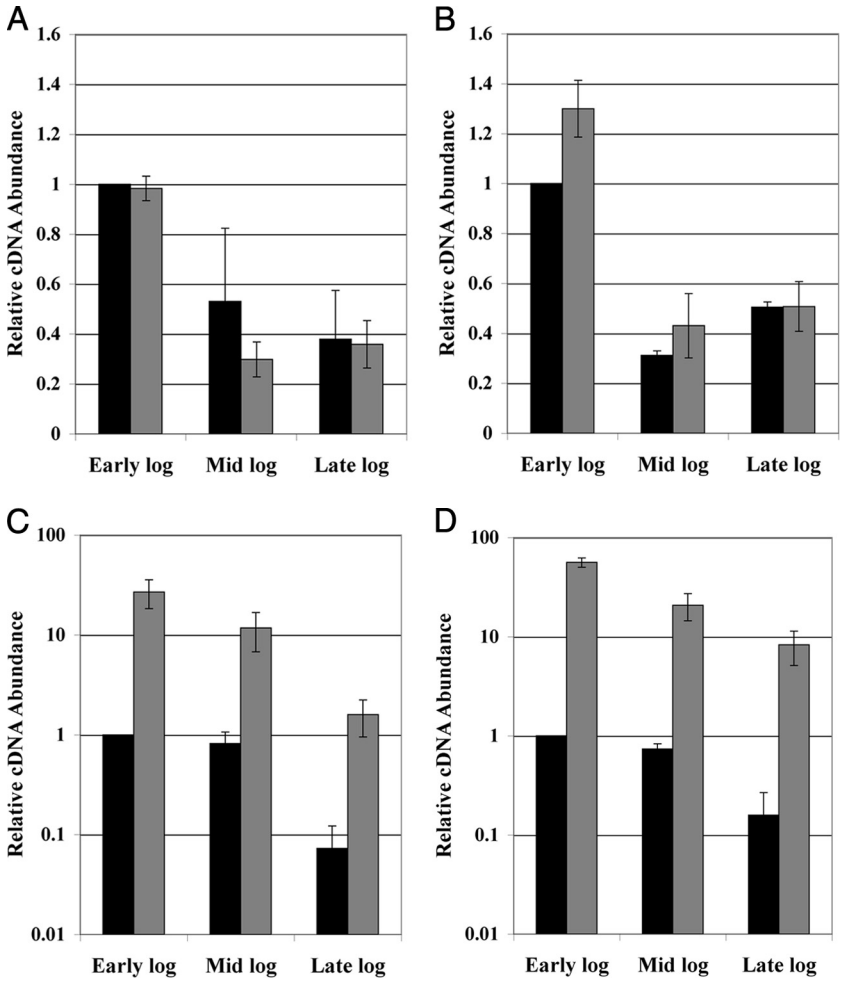

FIG. 2. Transcription of competence genes in the *hdrM* background. Wild-type UA140 and the *hdrM* mutant were tested for the expression levels of (A) *comC*, (B) *comE*, (C) *comX*, and (D) *comY* during the early, mid, and late log phases. Data are presented relative to the transcript abundance of the wild-type early log phase sample, which was arbitrarily assigned a value of 1. Black bars represent wild-type UA140, whereas the *hdrM* mutant is represented by dark gray bars. These results are the average of three independent experiments. Each experiment measured three independent clones for each sample. All experiments measuring transcription via real-time RT-PCR were normalized by using the 16S rRNA gene as a housekeeping control.

whereas *comY* was >50-fold increased (Fig. 2D). Expression of both the *comX* and *comY* genes remained higher than the wild-type level at all three time points, although the effect was consistently larger for *comY*. Interestingly, despite the tremendous increase in *comX* and *comY* gene expression in the *hdrM* mutant, these genes seemingly retained a wild-type pattern of gene expression (Fig. 2C and D). These results suggested that in the *hdrM* background, the transcription of *comX* and *comY* is increased over that in the wild type but the temporal pattern of gene expression is largely unaffected.

The *hdrM* mutation increases mutacin IV production independently of ComE. In *S. mutans*, multiple bacteriocins (mutacins) have been demonstrated to be under the control of the competence regulon (7, 9, 11, 31, 34). In addition, the *comE* gene has been recently shown to be an essential regulator for a variety of mutacins and uncharacterized mutacin-like genes (11). Given the apparent link between mutacin production and competence, we were curious as to whether a mutation of *hdrM* could rescue the mutacin IV deficiency seen in the *comE* background. As shown in Fig. 3, we performed a deferred antago-

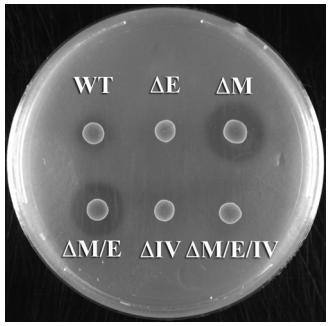

FIG. 3. Analysis of mutacin IV (*nlmA*) production in the *hdrM* background. The production of mutacin IV was tested by the deferred antagonism assay as described in Materials and Methods. The development of a growth inhibition halo is indicative of the presence of mutacin IV inhibiting the sensitive strain *S. sanguinis* NY101. *S. mutans* strains are identified as follows: WT (wild-type UA140), ΔE (*comE* mutant), ΔM (*hdrM* mutant), ΔM/E (*hdrM comE* double mutant), ΔIV (*nlmA* mutant), and ΔM/E/IV (*hdrM comE nlmA* triple mutant). This experiment was performed three times with similar results.

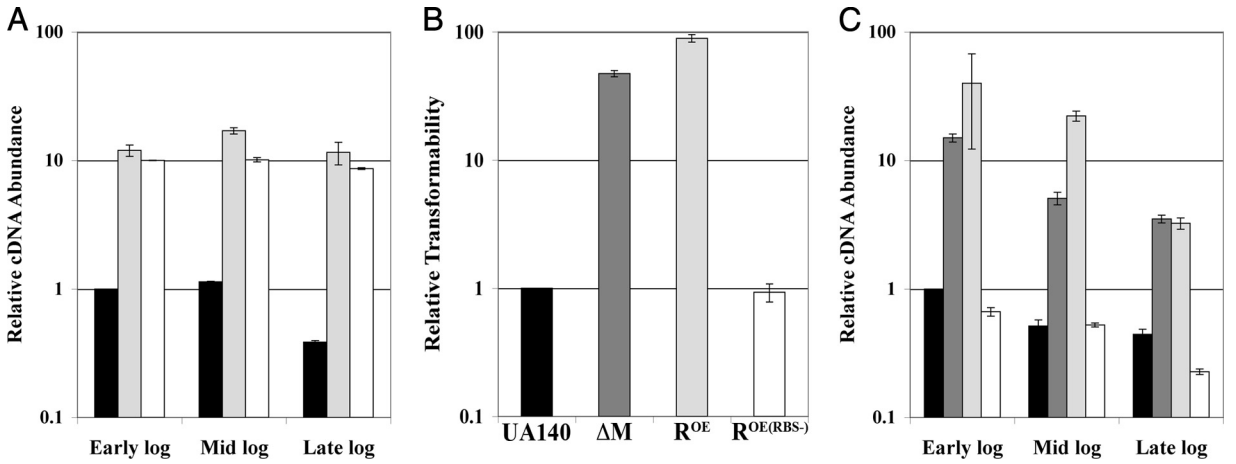

FIG. 4. Analysis of the *hdrR* overexpression strain. (A) The expression of *hdrR* was measured by real-time RT-PCR during the early, mid, and late log phases, and results are presented relative to the early log phase transcript abundance of the wild type. Wild-type UA140 is represented by black bars, the translational fusion *hdrR* overexpression strain is represented by light gray bars, and the RBS-negative *hdrR* overexpression strain is represented by solid white bars. (B) Comparison of the transformation efficiencies of wild-type UA140 (black), the *hdrM* mutant (dark gray), the translational fusion *hdrR* overexpression strain (light gray), and the RBS-negative *hdrR* overexpression strain (white). Values are presented relative to the wild type, which was arbitrarily assigned a value of 1. (C) Comparison of the expression of *comX* among wild-type UA140 (black), the *hdrM* mutant (dark gray), the translational fusion *hdrR* overexpression strain (light gray), and the RBS-negative *hdrR* overexpression strain (white). Samples were measured by real-time RT-PCR during the early, mid, and late log phases, and results are presented relative to the early log phase transcript abundance of the wild type. The data in panels A to C are the average of three independent experiments. Each experiment measured three independent clones for each sample.

nism assay to measure the growth inhibition of the mutacin IV-sensitive strain *S. sanguinis* NY101. As expected, no evidence of mutacin IV production was seen in the *comE* mutant. In contrast, both the *hdrM comE* double mutant and the *hdrM* single mutant produced more mutacin IV than the wild type, suggesting a regulatory mechanism similar to the competence phenotype. The *hdrM comE nlmA* triple mutant was also tested to ensure that the mutacin phenotype could be attributed to mutacin IV rather than another induced bacteriocin. In addition, we performed the same assay utilizing a *comC* mutant and obtained identical results (data not shown). Thus, mutacin IV, like genetic competence, is likely to have an additional *hdrM*-regulated pathway governing its expression that is independent of ComCDE.

Overexpression of *hdrR* reproduces *hdrM* phenotypes. As mentioned previously, genetic evidence suggested that *hdrR* was involved in the same pathway as *hdrM*; however, a deletion of *hdrR* yielded none of the phenotypes seen in the *hdrM* mutant (21). From these results, we hypothesized that *hdrM* may act as a negative regulator of *hdrR* and thus overexpressing *hdrR* might generate the *hdrM* phenotype. To this end, we created an HdrR overexpression construct by creating a translational fusion to the highly expressed constitutive lactate dehydrogenase (*ldh*) gene. As a negative control, we also created another construct containing the *ldh* promoter but lacking an RBS (see Materials and Methods). Both constructs were then transformed into the wild-type UA140 strain. We first tested the expression of *hdrR* from our overexpression strains and confirmed that both constructs transcribed *hdrR* constitutively during growth (Fig. 4A). Next, we tested the transformation efficiency of these strains against the wild type and the *hdrM* mutant. Surprisingly, we found that the translational fusion *hdrR* overexpression strain not only reproduced the increased-competence phenotype of the *hdrM* mutant but also had almost twofold higher competency than the *hdrM* mutant strain (Fig. 4B). As expected, the RBS-negative *hdrR* overexpression strained was virtually indistinguishable from the wild type. Since we had previously determined that *comX* was the likely mediator of the *hdrM* competence phenotype, we also tested *comX* gene expression in the *hdrR* overexpression backgrounds. As shown in Fig. 4C, in the RBS-positive strain, the transcription of *comX* was greatly increased throughout the growth period and its expression was generally higher than in the *hdrM* mutant, whereas the RBS-negative strain never showed *comX* expression above wild-type levels. This result mirrored the transformation results. As a final independent confirmation of these results, we tested mutacin IV production in the *hdrR* overexpression strains. Similar to what was found in the *hdrM* mutant, the translational fusion *hdrR* overexpression strain produced more mutacin IV than the wild type and was able to rescue the mutacin IV deficiency of a *comE* mutant (Fig. 5). In contrast, no rescue was observed in the RBS-negative overexpression strain. These results suggested that HdrR is likely a positive regulator of both competence and mutacin IV gene expression.

HdrR activity is regulated by HdrM and functions similarly to ComE. From our analysis of *hdrR* and *hdrM*, it seemed likely that both genes function together to regulate competence development and bacteriocin production. Also, from our *hdrR* overexpression data, we speculated that *hdrM* somehow serves to antagonize the function of *hdrR*. In order to test this further, we created another overexpression construct containing separate *ldh* translational fusions to both *hdrR* and *hdrM*. Next, we assayed the transformation efficiency of this strain and determined that the addition of *hdrM* to the overexpression construct reduced the transformation efficiency to nearly wild-type

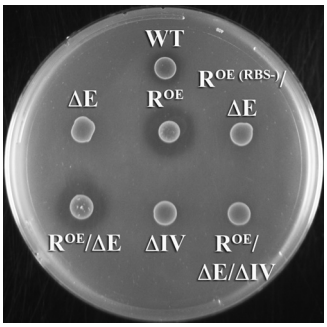

FIG. 5. Analysis of mutacin IV production in the *hdrR* overexpression strain. The production of mutacin IV was detected with the deferred antagonism assay using *S. sanguinis* NY101. *S. mutans* strains are identified as follows: WT (wild-type UA140), ΔE (*comE* mutant), R^{OE} (translational fusion *hdrR* overexpression strain), $R^{OE(RBS-)}$/ΔE (RBS-negative *hdrR* overexpression strain in the *comE* background), R^{OE}/ΔE (translational fusion *hdrR* overexpression strain in the *comE* background), ΔIV (*nlmA* mutant), and R^{OE}/ΔE/ΔIV (translational fusion *hdrR* overexpression strain in the *comE nlmA* background). This experiment was performed three times with similar results.

levels (Fig. 6A). Thus, overexpression of *hdrM* together with *hdrR* was indeed able to counteract the competence phenotype associated with *hdrR* overexpression.

Next, we decided to take a similar approach to test whether this was true for mutacin IV regulation as well. For this purpose, we created luciferase reporter constructs for the mutacin IV structural gene *nlmA*. As shown in Fig. 6B, the *hdrR* overexpression strain increased *nlmA-luc* reporter activity, which agreed well with the mutacin IV phenotype of this strain. However, overexpressing *hdrR* and *hdrM* together reduced the reporter activity to nearly wild-type levels. The same result was repeatable in the *comE* background. Since the ComE binding site for competence-regulated bacteriocins has been previously determined (9, 11), we also decided to test whether the mutacin IV phenotype of the *hdrR* overexpression strain functioned through the ComE recognition sequence. We hypothesized that this may be the case since *hdrR* appeared to regulate the same genes as *comE*. Additionally, both *hdrR* and *comE* belong to the LytTR family of transcription regulators and it has been proposed that members of this family recognize a conserved consensus pattern of imperfect direct repeats separated by 12- to 13-bp spacers (23). This is also the same motif recognized by ComE in competence and mutacin gene promoters of *S. mutans* (9). When the *hdrR* overexpression mutant was tested in an *nlmA-luc* reporter containing a deletion of just one of these two direct repeats, we observed a severe reduction in *nlmA-luc* reporter activity (Fig. 6B). This suggested that HdrR or possibly a downstream regulator recognizes the same *nlmA* promoter elements as ComE. Since a similar LytTR-type recognition sequence does not exist in the *S. mutans comX* promoter, it is unknown whether potential ComE or HdrR binding sites exist within the *comX* promoter.

DISCUSSION

In this study, we investigated the mechanism by which the novel HdrRM system regulates competence and mutacin production in *S. mutans*. Through a combination of epistatic analysis and gene expression studies, we determined that the *hdrM* mutation affects competence through the transcription of *comX* and is independent of the *comC* and *comED* genes. The *hdrM* mutation also affected the production of mutacin IV, which has been previously demonstrated to require ComE (10, 11, 21). Similar to competence, the *hdrM* mutation was able to increase mutacin IV production, even in the *comE* background. In addition, each of these phenotypes was reproducible when HdrR was overproduced in a wild-type background. This provides an explanation for our previous observation that an *hdrR* deletion does not result in the same phenotypes as an *hdrM* mutation (21). Furthermore, the phenotypes of the *hdrR* overexpression strain could be reverted by overexpressing *hdrM* together with *hdrR*. This suggests that *hdrM* is probably a negative regulator of *hdrR* activity. Finally, using the mutacin

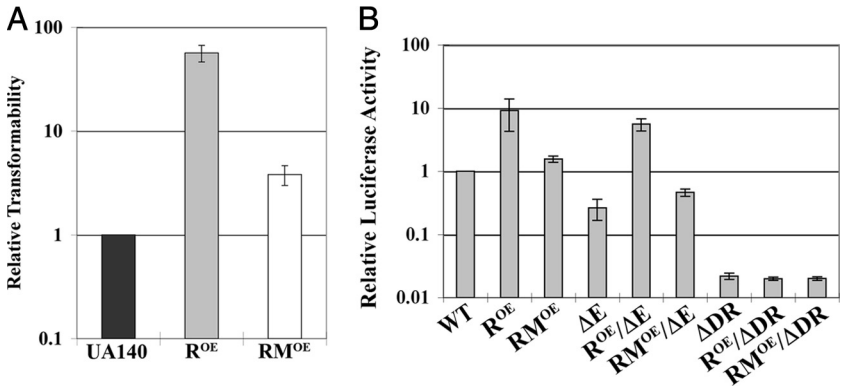

FIG. 6. Investigation of the effect of *hdrRM* operon overexpression upon competence and mutacin IV production. (A) Comparison of transformation efficiencies among wild-type UA140 (black), the *hdrR* overexpression strain (gray), and the *hdrRM* overexpression strain (white). (B) Luciferase activities of the *nlmA-luc* reporter strain in the following backgrounds: WT (wild-type UA140), R^{OE} (*hdrR* overexpression strain), RM^{OE} (*hdrRM* overexpression strain), ΔE (*comE* mutant), R^{OE}/ΔE (*hdrR* overexpression strain in the *comE* background), RM^{OE}/ΔE (*hdrRM* overexpression strain in the *comE* background), ΔDR [UA140 in *nlmA*(Δdirect repeat)-*luc*], R^{OE}/ΔDR [*hdrR* overexpression strain in *nlmA*(Δdirect repeat)-*luc*], and RM^{OE}/ΔDR [*hdrRM* overexpression strain in *nlmA*(Δdirect repeat)-*luc*]. The competence and luciferase assays were normalized to the wild-type UA140 values. All results are the average of three independent experiments. Each experiment measured three independent clones for each sample.

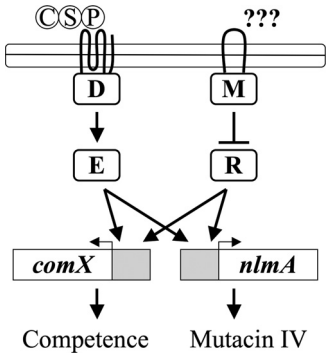

FIG. 7. Model of the *hdrRM*-regulated competence pathway. HdrM (M) responds to unknown environmental signals which regulate the antagonism of HdrR (R) transcription factor activity. When the environmental conditions are such that HdrM is unable to antagonize HdrR, this will allow HdrR to activate the transcription of *comX* for natural competence and *nlmA* for mutacin IV production. This pathway functions in parallel with ComD (D) and ComE (E), which respond to the presence of CSP.

reporter *nlmA-luc*, we demonstrated that HdrR likely recognizes the same direct repeats in the mutacin IV promoter as ComE but does not function through ComE.

Based upon these results, we propose a model in which the *S. mutans* competence regulon has at least two independent upstream regulatory systems, the ComCDE system and the HdrRM system (Fig. 7). The two pathways appear to be parallel, as the competence and mutacin IV phenotypes were fully independent of *comC* and *comED* in the *hdrM* mutant or *hdrR* overexpression background. Yet, the two systems strongly overlap in their ability to regulate the competence regulon. Both pathways converge on *comX* in order to regulate competence, and they both regulate mutacin IV through conserved direct repeats in the *nlmA* promoter. However, based upon our genetic and transcriptional data, it appears that the two systems utilize different mechanisms to achieve similar results. Unlike the ComDE two-component system, in the HdrRM system, the transcription regulator (HdrR) does not require the membrane protein (HdrM) for functionality. Rather, we propose that HdrM serves to antagonize the function of HdrR (Fig. 7). Thus, in the *hdrM* background, the ability to antagonize HdrR would be lost, which could account for the increased expression of *comX* and *nlmA*. This model is also consistent with our previous observation that a double mutation of *hdrR* and *hdrM* can suppress the *hdrM* phenotype (21). Our present data suggest that HdrR is the mediator of this phenotype. We have yet to determine whether the antagonism of HdrR occurs directly between HdrM and HdrR or through some other mechanism, but these studies are under way.

An obvious question arising from this study is why *S. mutans* might require multiple parallel regulatory systems for competence and mutacin production. Part of the answer may be the natural life cycle of *S. mutans* in the oral cavity. For example, current data suggest that the ComCDE system is the primary competence regulatory system in planktonic cultures of *S. mutans*. However, as a primarily biofilm-dwelling organism, *S. mutans* also routinely proliferates in an environment that contains a cell density 2 to 3 orders of magnitude greater than that of a planktonic culture. Under these vastly different conditions, an entirely separate set of stimuli may activate another regulatory system, such as HdrRM, to stimulate competence. In fact, both mutacin IV and the *hdrRM* operon have been previously shown to be inducible by conditions of extremely high cell density, similar to that obtained in a biofilm or plate colony (10, 21). Furthermore, we speculate that the standard transformation assay probably does not contain the proper stimulus required to counteract the antagonistic effect of *hdrM*, which would explain why a deletion of *hdrR* produces a wild-type competence phenotype. Under these conditions, the negative regulatory function of *hdrM* might predominate, which would then be the functional equivalent of an *hdrR* mutation (i.e., no HdrR transcription factor activity). However, if under these same conditions HdrR were forced to be active through a mutation of *hdrM* or through constitutive overexpression, then it would induce competence together with the ComCDE system. Presumably, the additive effect of both pathways working simultaneously would result in greatly increased competence. These results imply that the competence system can be regulated by a variety of stimuli sensed by distinct regulatory systems. Considering the substantial expenditure of energy and genetic information devoted to controlling the competence regulon, as well as producing the competence machinery and competence-regulated bacteriocins, it seems that competence must play a significant role in the survival of the species in the oral cavity.

From the data presented in the present study, two inferences can be made regarding the functionality of the *hdrRM* system. First, it seems that the antagonist function of HdrM is likely to be sensitive to the concentration of HdrR. Even though overexpressing *hdrM* together with *hdrR* strongly inhibited the competence and *nlmA* gene expression phenotypes seen in the *hdrR* overexpression strain, the phenotypes were never fully restored to wild-type levels (Fig. 6A and B). In the *hdrRM* overexpression construct, both genes were expressed as separate translational fusions to the same *ldh* promoter sequence. Thus, HdrR and HdrM protein levels should be nearly equivalent. Further support for this notion may be contained within the *hdrR* gene itself. Upstream of the annotated *hdrR* start codon, there is no Shine-Dalgarno-type RBS, nor is there a potential alternative translation start site with an RBS sequence. In fact, we examined five other strains of *S. mutans* and found the same result (data not shown). In contrast, the *hdrM* ORF is preceded by an obvious RBS sequence. Given the apparent lack of an RBS in *hdrR*, it is possible that fewer HdrR molecules are translated relative to HdrM. Future studies will determine if translational efficiency is indeed a component required for the proper regulation of the system. Second, we speculate that HdrR functions similarly to ComE, albeit under different growth conditions (Fig. 7). Conspicuously, both HdrR and ComE belong to the LytTR family of transcription regulators, which recognize a conserved but flexible consensus sequence (23). Consistent with this prediction, both ComE and HdrR regulate competence and mutacin production and require the same consensus LytTR family DNA binding motif in the *nlmA* promoter. Interestingly, we identified five other putative LytTR family transcription regulators within the genome of *S. mutans* in addition to *hdrR* and *comE*. An alignment of each of these putative proteins with HdrR suggests that the homology between four of these proteins

(SMU.294, SMU.433, SMU.576c, and SMU.1070c) and HdrR is even greater than that between HdrR and ComE. Therefore, it will be interesting to determine whether any of these proteins are similarly able to influence the transcription of the competence regulon. These genes may well comprise additional uncharacterized competence regulatory systems. It would also be interesting to determine whether *S. mutans* is unique in its ability to control competence through multiple regulatory systems or other species such as *S. pneumoniae* contain similar parallel competence regulatory pathways yet to be discovered.

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