Genetic characterization of the *hdrRM* operon: a novel high-cell-density-responsive regulator in *Streptococcus mutans*

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Many species of bacteria can adhere to surfaces and grow as sessile communities. The continued accumulation of bacteria can eventually lead to the extremely high-cell-density environment characteristic of many biofilms or cell colonies. This is the normal habitat of the cariogenic species *Streptococcus mutans*, which normally resides in the high-cell-density, multispecies community commonly referred to as dental plaque. Previous work has demonstrated that the transcription of two separate bacteriocins can be activated by the high-cell-density conditions created through the centrifugation and incubation of cell pellets. In this study, we identified an uncharacterized two-gene operon that was induced >10-fold by conditions of high cell density. The genes of the operon encode a putative transcription regulator and a membrane protein, which were renamed as *hdrR* and *hdrM*, respectively. A transcription fusion to the *hdrRM* operon confirmed its induction by high cell density. Mutation of *hdrM* abolished bacteriocin production, greatly increased natural competence, reduced the growth rate, and severely affected biofilm formation. Interestingly, no obvious phenotypes were observed from a non-polar mutation of *hdrR* or mutations affecting the entire operon. These data suggest that the *hdrRM* operon may constitute a novel regulatory system responsible for mediating a cellular response to a high-cell-density environment.

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INTRODUCTION

In the wild, many species of bacteria possess the capacity to attach to solid surfaces and grow as extremely densely packed communities. For example, supragingival dental plaques generally contain 10^{10} – 10^{11} c.f.u. ml⁻¹, while subgingival plaques and human faeces both yield an astonishing 10^{11} – 10^{12} c.f.u. ml⁻¹ (Evaldson *et al.*, 1982). Therefore, these species commonly exist in an environment that can be hundreds to thousands of times denser than a typical laboratory broth culture. The cariogenic species *Streptococcus mutans* is a regular inhabitant of dental plaque, and thus routinely grows within an exceptionally high-cell-density environment. Interestingly, we have previously observed that the expression of at least two bacteriocins (mutacins) of *S. mutans* is largely dependent upon a cell density similar to that attained in the dental

plaque or within a cell colony. While both bacteriocins normally exhibit little or no expression in broth cultures, we have demonstrated that the centrifugation and incubation of pelleted exponential-phase cultures can strongly induce the expression of both bacteriocins (Kreth *et al.*, 2005; Merritt *et al.*, 2005b). Thus we hypothesized that bacteriocin expression may be controlled by density-dependent regulators that are responsive to extremely high cell density. Using this approach, we identified a previously uncharacterized two-gene operon consisting of a putative transcription regulator and membrane protein. A transcription fusion to this operon confirmed its inducibility during high-density incubation, and phenotypic analysis suggested a role in the control of various density-dependent phenomena such as bacteriocin production, genetic competence and biofilm formation. These results suggest that this operon could be a mediator of the response of the cell to a high-cell-density environment.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. All *S. mutans* strains were grown in either brain heart infusion (BHI) medium or Todd–Hewitt (TH) medium (Difco). For the selection of antibiotic-resistant colonies, TH

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Abbreviations: CLSM, confocal laser scanning microscopy; DIC, differential interference contrast.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference
Strains		
<i>E. coli</i> DH5 α	Cloning strain	
<i>S. mutans</i> UA140	Wild-type <i>S. mutans</i> ; Em ^s Sp ^s	Qi <i>et al.</i> (2000)
140IFD	<i>S. mutans</i> in-frame deletion recipient strain; Em ^r	Merritt <i>et al.</i> (2007)
<i>S. sobrinus</i> OMZ176	Wild-type <i>S. sobrinus</i> ; Em ^s Sp ^s	Caufield <i>et al.</i> (1990)
LZ89ins	UA140::pLZ89-i; <i>hdrR</i> ; Em ^r Sp ^s	This work
LZ89del	UA140 Δ <i>hdrR</i> ; Em ^s Sp ^r	This work
LZ89ifd	140IFD Δ <i>hdrR</i> ; Em ^r Sp ^s	This work
LZ90	UA140::pLZ90-i; <i>hdrM</i> ; Em ^r Sp ^s	This work
LZ8990	UA140 Δ <i>hdrRM</i> ; Em ^s Sp ^r	This work
LZ89-luc	UA140::pLZ1689-luc; Em ^s Sp ^r	This work
KO1689-luc	LZ89ifd::pLZ1689-luc; Em ^r Sp ^r	This work
KO1690-luc	LZ90::pLZ1689-luc; Em ^r Sp ^r	This work
Plasmids		
pCR2.1	PCR cloning vector; Ap ^r Km ^r	Invitrogen
pGEM-T Easy	PCR cloning vector; Ap ^r	Promega
pJY4164	Streptococcal suicide vector; Em ^r	Achen <i>et al.</i> (1986)
pFW5-luc	Streptococcal luciferase reporter vector; Sp ^r	Podbielski <i>et al.</i> (1999)
pIFD-Sm	<i>S. mutans</i> in-frame deletion cloning vector; Sp ^r	Merritt <i>et al.</i> (2007)
pLZ1689-i	pJY4164 + 238 bp internal fragment of <i>hdrR</i> ; Em ^r	This work
pLZ1689-d	pJY4164 + allelic replacement of <i>hdrR</i> ; Em ^r Sp ^r	This work
pLZ1689-ifd	pIFD-Sm + in-frame deletion of <i>hdrR</i> ; Sp ^r	This work
pLZ1690-i	pJY4164 + 256 bp internal fragment of <i>hdrM</i> ; Em ^r	This work
pLZ8990-d	pJY4164 + allelic replacement of <i>hdrRM</i> ; Em ^r	This work
pLZ1689-luc	pFW5-luc + Φ (<i>hdrRM</i> _P -luc); Sp ^r	This work

*Em, erythromycin; Sp, spectinomycin; Ap, ampicillin; Km, kanamycin.

plates were supplemented with 15 μ g erythromycin ml⁻¹ (Sigma), 800 μ g spectinomycin ml⁻¹ (Sigma) or 800 μ g kanamycin ml⁻¹ (Sigma). All *S. mutans* strains were grown anaerobically (80 % N₂, 10 % CO₂, 10 % H₂) at 37 °C. *Escherichia coli* cells were grown in LB medium (Fisher) with aeration at 37 °C. *E. coli* strains carrying plasmids were grown in LB medium containing 250 μ g erythromycin ml⁻¹ or 150 μ g spectinomycin ml⁻¹.

Real-time RT-PCR. Total RNA (3 μ g) was used for cDNA synthesis using Stratascript reverse transcriptase (Stratagene) according to the manufacturer's protocol. For real-time RT-PCR, primers were designed according to sequence data provided by the Los Alamos National Laboratory Oral Pathogens Sequence Database (<http://www.oralgen.lanl.gov/oralgen/bacteria/smut/>), and SYBR green nucleic acid stain (Bio-Rad) was used for fluorescence detection with the iCycler Real-Time PCR system (Bio-Rad) according to the manufacturer's protocols. Total cDNA abundance between samples was normalized using primers specific to the 16S RNA gene. After normalization, fold induction was calculated as the ratio of target gene cDNA abundance between the pelleted and dispersed samples. Primer sequences used for target gene amplification and normalization are listed in Table 2.

Construction of *hdrR* and *hdrM* insertion duplication mutants. Internal DNA fragments of *hdrR* and *hdrM* were amplified by PCR using the primer pairs 1689F/1689R and 1690F/1690R (Table 2). Primer sequences were designed using sequence data obtained from the Los Alamos National Laboratory Oral Pathogens Sequence Database. The PCR fragments were then cloned into the vector pCR2.1 (Invitrogen), digested with *Xba*I/*Sph*I, and ligated to

compatible sites on the suicide vector pJY4164 (Achen *et al.*, 1986) to generate pLZ1689-i and pLZ1690-i. The resulting constructs were confirmed by restriction analysis and PCR before integration onto the chromosome of UA140 via single-crossover homologous recombination. Antibiotic-resistant isolates were tested by PCR to confirm the genotypes, and representative clones of both *hdrR* and *hdrM* mutants were frozen and renamed LZ89ins and LZ90, respectively.

Construction of the *hdrR* deletion mutant. Two fragments corresponding to approximately 1 kb upstream and downstream of *hdrR* were generated by PCR using PfuUltra polymerase (Stratagene) and the primer pairs 1689upF/1689upR and 1689downF/1689downR (Table 2). The resulting fragments were cloned into the vector pCR2.1, cut with *Hind*III/*Bam*HI (upstream fragment) and *Kpn*I/*Sac*I (downstream fragment), and ligated to compatible sites in pJY4164. The resulting plasmid was digested with *Bam*HI, blunted with Klenow (NEB), and ligated with a spectinomycin resistance cassette (*aad*9) removed from pFW5 (Podbielski *et al.*, 1996) using *Sma*I/*Hinc*II to generate the plasmid pLZ1689-d. After confirmation of the resulting plasmid by restriction analysis and PCR, the entire insert was PCR-amplified using PfuUltra and transformed into *S. mutans* (Li *et al.*, 2001). Antibiotic-resistant isolates were tested with PCR using several combinations of primer sets to confirm the genotype. A confirmed clone was renamed LZ89del and frozen for future use.

Construction of the *hdrR* markerless in-frame deletion mutant. To generate the markerless in-frame deletion construct, a cloning strategy similar to that described above was used initially to clone the same 1 kb *hdrR* upstream and downstream fragments into pJY4164. This construct (pJY4164-upstream-downstream) was digested with

Table 2. Primers used in this study

Primer	Sequence (5'→3')	Purpose
16s RT F	GATAATTGATTGAAAGATGCA	Real-time RT-PCR normalization
16s RT R	ATCCCTACTGCTGCCTCCC	Real-time RT-PCR normalization
hrcA RT F	GCTTTTCGTTTAGGGGATCTT	<i>hrcA</i> real-time RT-PCR
hrcA RT R	CGTTGCTTTTTAGGCTCCAC	<i>hrcA</i> real-time RT-PCR
sodA RT F	GCTTTTGGCTGATGTGGAA	<i>sodA</i> real-time RT-PCR
sodA RT R	TCCCAGAAAAGAGCGTGATT	<i>sodA</i> real-time RT-PCR
843 RT F	AATGGCCCTTCTTGGTTTAGG	SMU.929c/SMu843 real-time RT-PCR
843 RT R	TAAGCACCATACGCAGCAAC	SMU.929c/SMu843 real-time RT-PCR
1689 RT F	AGAAATTTACCTGATTATTTTCAGCA	SMU.1854/SMu1689 real-time RT-PCR
1689 RT R	TTGGGGGTAGAGGAGAAAGAC	SMU.1854/SMu1689 real-time RT-PCR
1805 RT F	CCCCTAGCTCTTGGTTTCG	SMU.1988c/SMu1805 real-time RT-PCR
1805 RT R	TGTCCTTGTTCACTCTTTTGCTT	SMU.1988c/SMu1805 real-time RT-PCR
1886 RT F	TTGGTGTGTCTGTTAGTGACC	SMU.2078c/SMu1886 real-time RT-PCR
1886 RT R	CGATCAAAGCCAAACTCTCC	SMU.2078c/SMu1886 real-time RT-PCR
1689 F	CCCTCTAGACCCGGTGTTATGGAGATTCA	<i>hdrR</i> insertion duplication
1689 R	CCCGCATGCTCAAGGCTATTGGCAATCTG	<i>hdrR</i> insertion duplication
1690 F	CCCTCTAGATTTTGGTACGGTCTGCTTGG	<i>hdrM</i> insertion duplication
1690 R	GGGGCATGCTGTTTAAATAACGCCGATAATGA	<i>hdrM</i> insertion duplication
1689 up F	GATAAGCTTGAGCAATGAGTGCTATTG	<i>hdrR</i> and <i>hdrRM</i> deletion; <i>hdrR</i> IFD; <i>hdrRM</i> reporter
1689 up R	GATGGATCCTCATCAAAATGTATCTTGTCTCCA	<i>hdrR</i> and <i>hdrRM</i> deletion; <i>hdrR</i> IFD; <i>hdrRM</i> reporter
1689 down F	GATGGTACCAGTCTTTCTCCTCTACCCC	<i>hdrR</i> allelic replacement
1689 down R	GATGAGCTCGCCTTCTTCTCTCAAGAGA	<i>hdrR</i> allelic replacement
1690 down F	AACTCACTCCCCCTTTGCTT	<i>hdrRM</i> allelic replacement
1690 down R	TGACACGTTTTGTGGCAGTT	<i>hdrRM</i> allelic replacement

HindIII/SacI to remove the entire insert, blunted with Klenow, and ligated to an *SmaI*-digested pIFD-Sm vector (Merritt *et al.*, 2007). The resulting plasmid (pLZ1689-*ifd*) was confirmed by PCR and restriction analysis and transformed into the in-frame deletion recipient strain IFD140. The counter-selection procedure was performed as described previously (Merritt *et al.*, 2007). Ten antibiotic-sensitive isolates were randomly selected and screened by PCR to confirm the presence of the expected markerless deletion. A confirmed clone was renamed LZ89*ifd* and frozen for later use.

Construction of the *hdrR* and *hdrM* double-deletion mutant.

The *hdrR* upstream fragment was amplified with PfuUltra and cloned into pCR2.1. This insert was removed from pCR2.1 with *HindIII/BamHI*, while the spectinomycin cassette was cut from pFW5 using *BamHI/SmaI*. These two fragments were ligated to pJY4164 digested with *HindIII/SmaI*. The resulting construct (pJY4164-upstream-*aad9*) was checked by restriction analysis and PCR for the proper configuration. Next, the *hdrM* downstream fragment was amplified by PCR using PfuUltra and the primer pair 1690downF/1690downR (Table 2) and cloned into the pGEM-T Easy vector (Promega). The downstream fragment was then released from pGEM-T Easy with *EcoRI*, blunted with Klenow, and ligated to pJY4164-upstream-*aad9* cut with *SmaI*. Clones were analysed for the proper configuration by PCR and restriction analysis, and a confirmed isolate was renamed pLZ8990-d. This vector was subsequently linearized and transformed into UA140. Antibiotic-resistant clones were screened with PCR to confirm the expected mutation. A confirmed mutant was renamed LZ8990 and saved for future analysis.

Plate assay for mutacin production. To assay for mutacin I production, the wild-type UA140 and various mutant strains were first grown overnight in liquid cultures under standard anaerobic conditions. A 5 µl volume of the overnight culture was spotted onto TH plates and incubated anaerobically at 37 °C overnight. The

following day, the plates were overlaid with a soft agar suspension of the indicator strain (*Streptococcus sobrinus* strain OMZ176) and incubated anaerobically for an additional 16 h. Zones of inhibition were indicative of mutacin I production.

Construction of luciferase operon fusions. To construct a luciferase transcriptional reporter fusion to the *hdrRM* operon, a fragment containing the promoter region was amplified using the primer pair 1689upF/1689upR (Table 2). This fragment consisted of ~1 kb of sequence upstream of the *hdrR* start codon as well as a small portion (~50 bp) of coding sequence. The resulting fragment was cloned into pCR2.1, and later digested from pCR2.1 using *SpeI/XhoI* and ligated to the *NheI/XhoI* sites on the luciferase reporter plasmid pFW5-luc (Podbielski *et al.*, 1999) to create pLZ1689-luc. This plasmid was transformed and integrated onto the chromosome of wild-type UA140 via single-crossover homologous recombination to generate the luciferase reporter strain LZ89-luc. The reporter plasmid was also transformed into the *hdrR* and *hdrM* mutant strains to create the mutant reporter strains KO1689-luc and KO1690-luc, respectively. Antibiotic-resistant isolates were screened with PCR for the expected insertions, and multiple clones were assayed for similar reporter activities.

Luciferase assays. Luciferase assays were performed similarly to ones described previously (Merritt *et al.*, 2005a). A 50 µl volume of 1 mM D-luciferin (Sigma) suspended in 0.1 M pH 6 citrate buffer was added to 200 µl cell culture. All samples were measured using a TD 20/20 luminometer (Turner Biosystems). For all luciferase assays, luminescence values were normalized relative to sample optical densities, and luciferase values were determined from the mean of three independent samples taken for each time point. To determine the influence of high cell density upon *hdrRM* expression, overnight cultures of LZ89-luc were diluted 1:30 into fresh TH broth and incubated anaerobically as batch cultures for 2.5 h (OD₆₀₀ 0.08–0.09).

The cultures were then divided into 1 ml aliquots in microcentrifuge tubes and further incubated anaerobically as planktonic cells or as cell pellets. Pelleted cells were centrifuged at 16 000 g for 1 min before incubation. After incubation, both planktonic and pelleted cells were resuspended by vortexing before the luciferase activities and optical densities were measured. Luciferase data were expressed as a percentage of the activity measured at the start of the assay. The initial luciferase activity was arbitrarily defined as 100%. To determine the effect of high cell density upon the *hdrR* and *hdrM* mutants, the reporter strains LZ89-luc, KO1689-luc and KO1690-luc were all assayed as described above with the following modifications. After the initial incubation for 2.5 h (OD₆₀₀ 0.08–0.09), cells from each reporter strain were divided into planktonic cells and cell pellets, and incubated for an additional 2.5 h. After the incubation period, luciferase and optical density measurements were made for each sample. The luciferase activity data were expressed as a percentage of the activity relative to the wild-type planktonic sample. The activity of the wild-type planktonic sample was arbitrarily defined as 100%.

Transformation assays. Determination of natural transformation efficiency was performed using a method similar to that described previously (Li *et al.*, 2001; Merritt *et al.*, 2005c). UA140 and the various *hdrR* and *hdrM* mutants were grown anaerobically to OD₆₀₀ ~0.2–0.3 in TH broth supplemented with 0.4%, w/v, BSA. *S. mutans* genomic DNA containing a kanamycin marker was then added to each culture at a final concentration of ~1.2 µg ml⁻¹. The cultures were then incubated for an additional 3 h before plating. Transformation efficiency was determined by calculating the ratio of c.f.u. on selective plates to that on non-selective plates.

Biofilm imaging. Fluorescent images obtained from confocal laser scanning microscopy (CLSM) were produced as described previously (Kreth *et al.*, 2004). Biofilms were grown as static cultures in modified Lab-Tek II chamber slides (Nalge Nunc International) containing a total volume of 700 µl of chemically defined medium (CDM) supplemented with 1%, w/v, sucrose. In some cases, 1%, w/v, glucose was substituted for sucrose to measure sucrose-independent surface attachment and growth. Biofilm inoculation was performed by first growing overnight cultures of wild-type UA140 and the *hdrR* and *hdrM* mutants in CDM. The following day, stationary-phase cultures were gently sonicated to disperse the cells using a water sonicator (Misonix), and optical density adjustments were made to equalize the overnight cultures before diluting each sample 1:100 in the chamber wells. The chambers were then incubated anaerobically for 24 h at 37 °C to allow biofilm development to reach maturity in each sample. After the incubation period, the spent medium was removed and replaced with 500 µl fresh CDM plus 1 µl CellTracker Orange CMTMR (Molecular Probes), followed by further incubation at 37 °C for 1 h. After incubation, the wells were washed three times with PBS before imaging. CLSM was performed using an LSM 5 PASCAL confocal laser scanning microscope with LSM 5 PASCAL software (Carl Zeiss).

RESULTS

Identification of the SMU.1854/SMu1689 operon through hybridization of high-cell-density cultures

A preliminary microarray hybridization (not shown) identified six genes that were upregulated in cell pellets as compared with low-density conditions. We performed real-time RT-PCR on these genes to establish density-dependent regulation (Table 3). Of these, the most strongly affected genes were found to be the stress regulator *hrcA* (~9 × induction) and a conserved hypothetical ORF (~11 × induction) annotated as SMU.1854 (NCBI database)/SMu1689 (Los Alamos database) (Table 3). Sequence analysis of this ORF yielded strong homologies to the DNA-binding domains of putative LytTR family regulators from various Gram-positive species. This ORF also appeared to be the first gene of a two-gene operon, since its stop codon overlaps with a putative start codon of a downstream ORF. The downstream ORF also appeared to be the end of the operon, since the next three putative ORFs are transcribed from the opposite DNA strand. Bioinformatic analysis using the Los Alamos National Laboratory Oral Pathogen Sequence Database predicted that the second gene of the putative operon encodes a membrane protein with two transmembrane segments (SMU.1855, NCBI/SMu1690, Los Alamos). The operon structure was also reminiscent of the membrane sensor/response regulator arrangement typical of two-component system operons. However, there was no identifiable kinase domain homology within the predicted amino acid sequence of the putative membrane protein.

Transcriptional analysis of the SMU.1854/SMu1689 operon

A luciferase transcription fusion to the SMU.1854/SMu1689 operon was constructed in order to confirm the results of our initial experiment as well as characterize the expression profile of the operon. We began by performing RT-PCR to confirm the boundaries of the operon. As suspected, there was no detectable read-through upstream of SMU.1854/SMu1689, and the downstream ORF appeared to form part of a bicistronic message (data

Table 3. Real-time RT-PCR of selected genes identified by microarray analysis

Gene name/ID*	Fold induction	Function/class
<i>hrcA</i>	8.8	Transcriptional regulator of the stress response
<i>sodA</i>	3.35	Superoxide dismutase
SMU.929c/SMu843	3.14	Conserved; membrane protein
SMU.1854/SMu1689	11.1	LytTR family transcriptional regulator
SMU.1988c/SMu1805	2.45	Conserved; putative DNA-binding protein
SMU.2078c/SMu1886	3.4	Conserved; putative Holliday junction resolvase

*IDs are listed with the NCBI designation first and the Los Alamos designation second.

not shown). Therefore, an ~1 kb upstream fragment of SMU.1854/SMu1689 was cloned into the luciferase reporter vector pFW5-luc to create the transcription reporter strain LZ89-luc. To test the response of LZ89-luc to high cell density, the reporter was first grown to a very low optical density ($OD_{600} < 0.1$) and divided into dispersed and pelleted samples. Optical density and luciferase measurements were made every 30 min until the dispersed samples were well into stationary phase about 5 h later. As shown in Fig. 1(a), the dispersed samples exhibited increasing reporter activity at the beginning of the assay and plateaued about 90 min later ($OD_{600} \sim 0.2$). The pelleted cells also exhibited a similar increase in luciferase activity in the early time points of the assay (Fig. 1a). However, these samples continued to increase in activity until reaching a much higher maximal activity ~210 min (t test, $P=0.0006$) after the start of the assay (Fig. 1a). Based on the response of this operon to high cell density, we subsequently renamed SMU.1854/SMu1689 as ‘high density responsive regulator’ (*hdrR*) and SMU.1855/SMu1690 as ‘high density responsive membrane protein’ (*hdrM*).

The *hdrRM* operon regulates its own promoter activity

We were next interested to determine whether the *hdrRM* operon would be able to respond similarly to high cell

density in the absence of either *hdrR* or *hdrM*. Therefore, as described in Methods, we transferred the *hdrRM* operon reporter construct into the *hdrR* markerless in-frame deletion mutant (LZ89ifd) and the *hdrM* insertion duplication mutant (LZ90). These strains were grown similarly to those in the previous growth-curve experiment, and luciferase activity was measured at the 210 min time point, when both the dispersed and pelleted samples yielded maximal reporter activity (Fig. 1a). As shown in Fig. 1(b), the mutant reporter strains showed virtually no response to high cell density, whereas the wild-type reporter exhibited the expected increase in activity after centrifugation. In addition, the weak activity of the mutant reporter strains suggested that *hdrR* and *hdrM* are both required for induction of the *hdrRM* operon under both low- and high-cell-density conditions.

The *hdrRM* operon regulates the production of mutacin I

Previously, we reported that the expression of the bacteriocin mutacin I could be induced by growth at a high cell density (Merritt *et al.*, 2005b). Therefore, we were interested to determine whether the *hdrRM* operon was required for the production of mutacin I. Initially, we tested the insertion-duplication mutants of both *hdrR* and *hdrM*, LZ89ins and LZ90, respectively. With these mutants,

Fig. 1. Transcription profile of the SMU.1854/SMu1689 operon. (a) As described in Methods, the assay started after LZ89-luc was grown for 2.5 h and separated into dispersed and pelleted cells. Optical density and luciferase measurements were made every 30 min for a total of 300 min. The normalized activity measured for the dispersed and pelleted samples at the start of the assay was arbitrarily defined as 100 %. Shown here are the means of three independent samples taken for each time point in one representative experiment. Dark-grey bars, dispersed samples; light-grey bars, pelleted samples. The experiment was repeated twice with similar results. (b) The experiment was performed similarly to that described above, except that measurements were obtained at the 210 min time point, and both KO1689-luc (*hdrR* in-frame deletion) and KO1690-luc (*hdrM* insertion mutant) were assayed as well. The normalized activity of the dispersed sample at this time point was arbitrarily defined as 100 %. Shown here are the means of three independent samples in one representative experiment. Dark-grey bars, dispersed samples; light-grey bars, pelleted samples. The experiment was repeated twice with similar results.

we consistently found that only LZ90 exhibited mutacin I deficiency (Fig. 2). This was surprising, given that LZ89ins was likely to have had a polar effect upon *hdrM*. To rule out any unexpected artefacts that may have occurred with the *hdrR* insertion, we also tested mutacin I production in both a markerless in-frame deletion mutant (LZ89ifd) and a polar allelic replacement of *hdrR* (LZ89del). As shown in Fig. 2, these *hdrR* mutant strains were also able to produce mutacin I. Thus, the two polar mutations and the non-polar mutation of *hdrR* all had no effect upon mutacin I. Finally, we deleted the entire operon and found that this strain (LZ8990) was similarly proficient at mutacin I production (Fig. 2). Consequently, it appeared that only the *hdrM* mutation could abolish mutacin I production and this phenotype could be rescued by mutation of *hdrR*.

The *hdrRM* operon regulates natural genetic competence

Since *S. mutans* has been shown to exhibit greatly increased levels of natural genetic competence during biofilm growth (Li *et al.*, 2001), we were next interested to determine whether the *hdrRM* operon had any influence on competence development. Based on the results with the previous mutacin I assay, we decided to measure the natural competence ability of the *hdrR* and *hdrM* mutants, as well as the double-deletion strain. Surprisingly, we found that LZ90 exhibited a transformation frequency that was 80-fold higher than those of the wild-type UA140 and other mutant strains (data not shown). As for the previous mutacin I results, only a mutation in *hdrM* could produce the phenotype, whereas deletion of both genes in the operon restored competence to normal levels. This

Fig. 2. Mutacin I production assay of the various *hdrRM* operon mutants. Wild-type UA140, LZ89ins (*hdrR* insertion), LZ89ifd (*hdrR* in-frame deletion), LZ89del (*hdrR* allelic replacement), LZ90 (*hdrM* insertion) and LZ8990 (*hdrRM* operon deletion) were all spotted and overlaid with the mutacin I-sensitive strain *S. sobrinus* OMZ176.

suggested that the *hdrRM* operon acts as a negative regulator of natural competence.

The *hdrM* mutant has a reduced growth rate

While performing the transformation assays, we noticed that LZ90 consistently took longer to reach the optimal optical density for natural competence induction. Based on this observation, we decided to compare the growth curves of LZ89ifd, LZ90 and LZ8990 with that of the wild-type UA140. As shown in Fig. 3, UA140, LZ89ifd, and LZ8990 all had growth profiles that were largely indistinguishable, whereas LZ90 had a noticeably longer doubling time and a lower terminal optical density than all the other strains. Once again, the double-deletion strain seemed to compensate for the *hdrM* phenotype.

Biofilm formation is severely impaired in the *hdrM* mutant strain

As mentioned in the Introduction, dental plaque biofilms are known to reach extremely high cell densities. Given the induction of *hdrRM* at a similarly high cell density, it was of great interest to determine whether the operon was required for biofilm formation as well. Biofilms of UA140, LZ89ifd, LZ90 and LZ8990 were grown as described in Methods and imaged using CLSM. For each biofilm, two images were taken: a low-magnification differential interference contrast (DIC) image for the gross external features of the biofilm, and a high-magnification fluorescent image for analysis of the interior biofilm architecture. In Fig. 4(a, c, g), the DIC images demonstrated that both LZ89ifd and LZ8990 had an overall biofilm structure that was very similar to that of UA140. The biofilms were very smooth, confluent layers that covered the entire surface with a few large aggregates present in each. In contrast, LZ90 appeared sparsely populated on the surface and failed

Fig. 3. Growth curves of the *hdrRM* operon mutants. Three independent samples for each strain were diluted 1 : 30 and grown to stationary phase. Shown here are the means for one representative experiment. The experiment was repeated twice with similar results.

Fig. 4. CLSM biofilm images of the *hdrRM* operon mutants. Biofilms were grown as static cultures as described in Methods. DIC images were all taken at a total magnification of $\times 100$, while the fluorescent images were taken at a total magnification of $\times 400$. Fluorescent images are shown with a representative section taken from a 3D reconstruction. (a) DIC image of UA140; (b) fluorescent image of UA140 (maximum height 30 μm); (c) DIC image of LZ89ifd; (d) fluorescent image of LZ89ifd (maximum height 30 μm); (e) DIC image of LZ90; (f) fluorescent image of LZ90 (maximum height 55 μm); (g) DIC image of LZ8990; (h) fluorescent image of LZ8990 (maximum height 30 μm). Bars: (a) 180 μm , (b) 51 μm .

to achieve noticeable confluence, and a sizeable portion of the biofilm appeared to be localized within extremely large, densely packed aggregates (Fig. 4e). At high magnification, the differences in the LZ90 biofilm became even more apparent. The horizontal section of the LZ90 fluorescent image was taken very near to the bottom of the biofilm, because much of the surface contained only a very thin layer of biofilm that could not be seen in the higher layers of the image (Fig. 4f). However, the vertical sections of the LZ90 biofilm were in stark contrast. As shown in Fig. 4(f), the aggregates formed in the LZ90 biofilm were extremely tall: about 55 versus 30 μm for each of the other biofilms. Thus, the *hdrM* mutant formed a very weak biofilm over much of the surface, while in some localized areas there were clusters of biofilm that were almost double the height attained by the other strains. Interestingly, this phenotype was reproducible for both sucrose-dependent and sucrose-independent biofilms (data not shown). In addition, the fluorescent images of LZ89ifd suggested that it formed a more densely packed homogeneous biofilm than did UA140, whereas LZ8990 had a biofilm architecture somewhere between those of UA140 and LZ89ifd (Fig. 4b, d, h). Since both LZ90ifd and LZ8990 formed biofilms of approximately the same height and overall external structure, these mutations did not seem to have much effect upon basic biofilm-forming ability, but they did seem to produce some minor differences in biofilm architecture.

DISCUSSION

In this report, we describe the identification of a novel high-cell-density-responsive operon (*hdrRM*) that is required for several important virulence factors of *S. mutans*.

An interesting feature of this operon is the consistency of phenotypes associated with the *hdrM* mutation and not the *hdrR* or *hdrRM* mutations. The CiaRH two-component system of *S. mutans* also exhibits similar behaviour. Inactivation of the CiaH membrane sensor has been shown to create numerous phenotypes related to acid tolerance, growth rate, natural competence, bacteriocin production and biofilm formation. However, inactivation of either the CiaR response regulator or the whole CiaRH operon causes no observable phenotype (Ahn *et al.*, 2006; Qi *et al.*, 2004). Our genetic data also suggest a couple of features of the *hdrRM* operon. First, both *hdrR* and *hdrM* are likely to function in the same pathway(s), since the polar *hdrR* mutations and the double mutation of *hdrRM* all exhibited wild-type behaviour for each of the phenotypes observed in the *hdrM* mutant. For example, bacteriocin production, natural competence, growth rate and biofilm formation were all affected in the *hdrM* mutant, while each of the mutations that affected both *hdrR* and *hdrM* behaved similarly to the wild-type. Secondly, in addition to the *hdrRM* whole-operon mutants, wild-type behaviour was also observed with the *hdrR* in-frame deletion mutant.

Therefore, it can be hypothesized that the activity of HdrR is regulated, possibly by HdrM or via some intermediate. In this scenario, the phenotypes caused by the *hdrM* mutation could be attributed to the production of an unregulated HdrR. However, it is currently unclear whether this regulation is positive or negative. Since no identifiable kinase domain homology was observable in the HdrM sequence, the mechanism of signal transduction may be distinct from the typical phosphorelay scheme used in two-component systems.

Another interesting feature of this operon is the connection with natural competence. While there are numerous examples of mutations that lower the natural competence ability of *S. mutans* (Abranches *et al.*, 2006; Ahn *et al.*, 2005; Ahn & Burne, 2006; Hussain *et al.*, 2006; Merritt *et al.*, 2005c; Qi *et al.*, 2004; Rolerson *et al.*, 2006; Senadheera *et al.*, 2005, 2007; Wang & Kuramitsu, 2006; Wen *et al.*, 2005), to our knowledge, there is only one other report of a mutation that increases natural competence (Tao *et al.*, 1993). This mutation was generated by chemical mutagenesis and its identity has, so far, not been published. Therefore, the *hdrM* competence phenotype is very unusual within the *S. mutans* literature. Since the genetic pathway leading to natural competence has been well characterized in *S. mutans* and other streptococci (Martin *et al.*, 2006), it will be very interesting to determine whether the *hdrM* mutation affected the expression of the known competence regulators or had an alternative mechanism to affect the expression of the DNA-uptake complex. Also, as shown in Fig. 3, the growth defect of the *hdrM* mutant was first noticeable as the cells achieved $\text{OD}_{600} \sim 0.2$. This is the period of the growth curve at which *S. mutans* initiates its natural competence programme (Perry *et al.*, 1983; Shah & Caufield, 1993). Furthermore, as shown in Fig. 1(a), dispersed cells of the *hdrRM* luciferase reporter achieved maximum expression about 90 min after the start of the assay. At this time point, the OD_{600} was also ~ 0.2 . Therefore, it is conceivable that there is a regulatory connection between the *hdrM* phenotypes associated with growth and natural competence. This result would not be surprising, given that competence escape in *Bacillus subtilis* is linked to cell growth (Hahn *et al.*, 1995; Haijema *et al.*, 2001). In addition, it has been demonstrated that natural competence in *S. mutans* is greatly enhanced within the biofilm (Li *et al.*, 2001). So the biofilm phenotype may also be connected to the growth and natural-competence phenotypes. Experiments are currently in progress to elucidate the pathway(s) leading to these phenotypes. More extensive high-cell-density microarray studies are also currently in progress.

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