Regulation of \textit{ciaXRH} Operon Expression and Identification of the CiaR Regulon in \textit{Streptococcus mutans} \textsuperscript{†}

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The \textit{ciaRH} operon in \textit{Streptococcus mutans} contains 3 contiguous genes, \textit{ciaXRH}. Unlike the CiaRH system in other streptococci, only the \textit{ciaH}-null mutant displays defective phenotypes, while the \textit{ciaR}-null mutant behaves like the wild type. The objective of this study was to determine the mechanism of this unusual property. We demonstrate that the \textit{ciaH} mutation caused a \textgreater{}20-fold increase in \textit{ciaR} transcript synthesis. A \textit{ciaRH} double deletion reversed the \textit{ciaH} phenotype, suggesting that overexpressed \textit{ciaR} might be responsible for the observed \textit{ciaH} phenotypes. When \textit{ciaR} was forced to be overexpressed by a transcriptional fusion to the \textit{ldh} promoter in the wild-type background, the same \textit{ciaH} phenotypes were restored, confirming the involvement of overexpressed \textit{ciaR} in the \textit{ciaH} phenotypes. The \textit{ciaH} mutation and \textit{ciaR} overexpression also caused transcriptional alterations in 100 genes, with 15 genes upregulated \textgreater{}5-fold. Bioinformatics analysis identified a putative CiaR regulon consisting of 8 genes/operons, including the \textit{ciaXRH} operon itself, all of which were upregulated. \textit{In vitro} footprinting on 4 of the 8 promoters revealed a protected region of 26 to 28 bp encompassing two direct repeats, NTTAAG-n5-WTTAAG, 10 bp upstream of the \textit{−10} region, indicating direct binding of the CiaR protein to these promoters. Taken together, we conclude that overexpressed CiaR, as a result of either \textit{ciaH} deletion or forced expression from a constitutive promoter, is a mediator in the CiaH-regulated phenotypes.

Bacterial two-component signal transduction systems (TCS) play important roles in bacterial environmental adaptation, production of virulence factors, self-defense, and biofilm formation (5–8, 11, 17, 26). A typical TCS consists of a membrane-bound histidine kinase (HK) sensor and a cytoplasmic response regulator (RR). Upon receiving a signal, the HK undergoes autophosphorylation. The phosphorylated kinase then transfers the phosphate group to its cognate RR, which then activates or represses its target genes by binding to their promoters (24).

The sequenced oral pathogen \textit{Streptococcus mutans} UA159 contains 14 pairs of TCS and one orphan response regulator (3, 16). Of these, the CiaRH TCS has been shown to be a global regulator for multiple stress responses such as biofilm formation, acid tolerance, bacteriocin production, and genetic competence (1, 3, 22). CiaRH is also widely distributed among other streptococcal species and has been shown to play an essential role in stress resistance and pathogenesis (4, 12, 23). Recently, studies from our group demonstrated that the \textit{S. mutans} CiaRH TCS is unique among the CiaRH systems in other streptococci in that it actually contains a third component, CiaX, which is encoded by the first gene of the operon (10). We further showed that the \textit{ciaXRH} operon expression is autoregulatory and that the operon expression is repressed by calcium through CiaX (10). Interestingly, when the activity of a \textit{ciaH-luc} (luciferase) reporter was measured, deletion of \textit{ciaH} affected only the calcium-dependent repression of operon expression, rather than abolishing the regulation of the operon altogether. This suggested that the \textit{ciaXRH} operon is likely to be regulated differently from the typical autoregulatory TCS.

Another unique feature of the \textit{S. mutans} CiaRH TCS is the lack of phenotypes associated with a \textit{ciaR} mutation. Currently, only mutations in the \textit{ciaH} sensor kinase have been demonstrated to affect multiple cellular functions (1, 22). Based on these findings, it was suggested that CiaR functions independently of the CiaH signaling cascade, possibly as a result of a cross talk between the Cia system and another TCS (1). In this study, we sought to explain this unusual finding by examining the regulatory mechanism employed by the \textit{S. mutans} CiaRH system. We show that CiaR is in fact in the same signaling pathway as CiaH; however, unlike a typical TCS, deletion of \textit{ciaH} causes overexpression of \textit{ciaR}. Overexpressed CiaR then acts as a positive regulator for the expression of the \textit{ciaXRH} operon as well as a variety of other downstream targets and a negative regulator for late competence genes and competence-associated bacteriocin-like genes (14). Through microarray and bioinformatic analyses, a CiaR regulon was identified, consisting of at least 8 genes/operons that are all positively regulated by CiaR. \textit{In vitro} DNA footprinting confirmed the putative CiaR binding site identified through bioinformatics.

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analysis. From these results, we were able to construct a unique regulatory scheme of the S. mutans CiaRH TCS.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** Escherichia coli DH5α was grown in Luria broth, and S. mutans UA140 and its derivatives were grown in brain heart infusion (BHI) broth (Difco). For the selection of antibiotic-resistant colonies after genetic transformation, erythromycin (300 µg/l for E. coli or 10 µg/ml for S. mutans) or spectinomycin (100 µg/ml for E. coli or 1 mg/ml for S. mutans) was added to the medium. Plasmid pDL278 (15), an E. coli-Streptococcus shuttle vector carrying a spectinomycin resistance (spc) gene, was used to overexpress ciaR. For the preparation of cells used in the microarray, overnight cultures of wild-type (WT) and mutant strains were diluted 1:30 in fresh BHI medium containing 0.8% bovine serum albumin (BSA). The cells were grown as a static culture at 37°C with 5% CO2 and collected by centrifugation when the optical density at 600 nm (OD_{600}) of the culture reached 0.3. Cell pellets were stored at −80°C until use.

**Construction of mutant strains.** The S. mutans UA140 ciaH mutant was constructed previously (10). The construction of the ciaRH deletion is described as follows. An approximately 600-bp fragment upstream of ciaH and a 350-bp fragment downstream of ciaR were amplified using primer pairs h5F-up-F and ciaRH-up-R and ciaRH-dn-F and ciaRH-dn-R (Table 1). The upstream and downstream fragments were digested with XbaI and XhoI, respectively, and ligated to a kanamycin resistance cassette released from the vector pBS-kan digested with XbaI and Xhol. The ligation mixture was directly transformed into S. mutans UA140, and transformants were selected on kanamycin plates (50 µg/ml). To construct the overexpression mutant, the lactate dehydrogenase (ldh) promoter (19) was used to drive the expression of ciaH. The promoter region of ldh was amplified with primers ldh-F and ldh-R (Table 1), and the full-length ciaH was PCR amplified with primers ciaH-F and ciaH-R (Table 1). Next, the ldh promoter fragment was digested with SacI and BamHI, the ciaH fragment was digested with BamHI and HindIII, and the shuttle plasmid pDL278 was digested with SacI and HindIII. After gel purification, all three fragments were mixed and ligated. The ligation mixture was transformed into E. coli, and the correct recombinant plasmid, pDL-OEciaH, was confirmed by restriction enzyme digestion and sequencing. pDL-OEciaRH was then transformed into S. mutans UA140, and the transformants were selected on spectinomycin plates and confirmed by real-time PCR analysis of the ciaRH gene expression.

**Transformation assay.** Genetic competence was determined by a transformation efficiency assay with transforming genomic DNA. Overnight cultures of S. mutans strains were diluted 1:20 in BHI medium containing horse serum (0.4%/vol/vol) and grown to an optical density at 600 nm of ~0.3. A 0.3-ml aliquot of the culture was distributed into Eppendorf tubes, and genomic DNA (10 µg/ml) isolated from UA140 derivative strain carrying a tetracycline resistance marker was added to each culture. After 2 h at 37°C, the cultures were briefly sonicated (Misonix) to break the cell chains and plated on antibiotic-containing BHI agar plates, as wells as on nonselective BHI agar for transformation efficiency measurement. Transformation efficiency was determined after 48 h of incubation and expressed as the percentage of transformants among the total viable recipient cells.

**Microarray analysis.** (i) RNA extraction. Total RNA was extracted using the FastPrep system (MPBio). Frozen samples were thawed on ice and resuspended in 1.0 ml Trizol. The cell suspension was then transferred to a tube containing prechilled Lysing Matrix B. Cells were subjected to two homogenizations (at a speed of 6.0 M/s; time, 30 s) at a 5-min interval. The mixture was centrifuged at 30,000 × g for 10 min, and the supernatant was transferred to a new 2.0-ml tube. RNA was purified using the RiboPure-CTM kit. From these results, we were able to construct a unique regulatory scheme of the S. mutans CiaRH TCS.

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(ii) Microarray. For cDNA synthesis, 15 µg of total RNA was mixed with 1.25 µg random hexamers and heated at 70°C for 10 min for denaturation before being added to the reverse transcription reaction mixture. Reverse transcription was performed in a total volume of 60 µl, and the reverse transcription reaction mixture contained 12 µl 5× Superscript II buffer (Invitrogen, Carlsbad, CA), 6 µl 100 mM dithiothreitol (DTT), 3 µl 10 mM deoxynucleoside triphosphate (dNTP), 1.5 µl RNaseOUT RNase inhibitor, and 7.5 µl Superscript II reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min, and then the reaction was terminated by incubation of the mixture at 70°C for 10 min. RNA in the mixture was then hydrolyzed by adding 15 µl 1 N NaOH and incubating the mixture at 65°C for 30 min. The solution was neutralized with 1 N HCl, and the cDNA was then purified using a Qiagen MinElute PCR purification kit. The concentration of cDNA was determined by measuring the absorbance at 260 nm. A 3-µg sample of cDNA was labeled in a 35-µl mixture containing 3.5 µl Roche 10× DNano buffer and 7.5 µl diluted Roche DNano (0.06 U/µl). The fragmentation reaction mixtures were prepared on ice and then incubated at 37°C for 10 min before the reaction was terminated by incubation of the mixture at 98°C for 10 min. For hybridization, 32 µl of the labeled cDNA was labeled with the BioArray terminal labeling kit (Enzo, New York, NY) in a total volume of 50 µl containing 10 µl 5× buffer, 5 µl 10× CoCl2, and 2 µl terminal deoxynucleotidyl transferase. The reaction mixture was incubated at 37°C for 60 min. Labeled cDNA was hybridized to a standard 49-format custom GeneChip microarray (Affymetrix, Santa Clara, CA) containing each of the predicted open reading frames (ORFs) of S. mutans UA159 as well as both strands of the intergenic regions. The hybridization and washing procedures were performed similarly as recommended in the GeneChip Expression Analysis Technical Manual and as previously described (2). The GeneChips were scanned at 570 nm using an Affymetrix 7G laser scanner.

(iii) Data analysis. Analysis of signal intensities was performed using the GeneChip operating system software (GCOS), version 1.4, and gene expression data were compared using the GCOS batch analysis function. Normalization procedures were performed directly by the software using a script designed by Affymetrix and provided with the S. mutans custom array. Four data sets were generated for each ciaH mutant and ciaRH overexpression strain. The 4 data sets were standardized by batch mean, and the common overexpressing mutant was used to generate the P values with gcr4 as a reference. A gene expression change of ≥2-fold and a P value of <0.05 were used as cutoff values to generate the gene list presented in Table 2.

**Real-time RT-PCR.** Real-time reverse transcriptase PCR (RT-PCR) was performed to validate the results generated from the microarray analysis. Primers were designed using Applied Biosystems Primer Express 3.0 software, which suggested an optimal candidate set of primers suitable for threshold cycle (ΔC_{T}) analysis. The primer sequences are listed in Table 1. Cells were cultured under the same conditions as those described for the microarray. Three hundred nanograms of total RNA was reverse transcribed using the manufacturer’s protocol for Affinityscript reverse transcriptase (Stratagene, La Jolla, CA). Real-time PCR was performed using an Applied Biosystems 7300 PCR system, and the reaction mixtures were prepared using Applied Biosystems SYBR Green PCR Master Mix. Changes in gene expression were calculated automatically in the Applied Biosystems 7300 System software using the ΔΔC_{T} method and are briefly described as follows: ΔC_{T} = C_{T} (target) − C_{T} (housekeeping gene); ΔΔC_{T} = ΔC_{T} (target) − ΔC_{T} (housekeeping gene); and all samples included a no-RT control to assess genomic DNA contamination in the reactions.

**Construction of transcriptional fusion by 5′ RACE.** To locate the transcription start site of the ciaH transcript, 5′ random amplification of cDNA end (5′ RACE) experiments were performed using the FirstChoice RLM RACE kit (Ambion) according to the manufacturer’s protocol. For the first-round PCR, the 5′ RACE outer primer and the gene-specific inner reverse primers (Table 1) were used with the RT reaction products as the template. PCR mixtures 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 1). The resulting PCR products were then cloned into the pGEM-T Easy vector (Promega) and sequenced using M13 primers.

**Cloning and purification of CiaR.** The ciaH coding sequence was amplified by PCR using Hercule DNA polymerase (Stratagene) and chromosomal DNA derived from S. mutans UA159 with gene-specific primers oG9426 and oG9427 (Table 1). Subsequently, the amplicons were digested with BamHI and EcoRI and ligated into the expression vector pCRT7/NT (Invitrogen) to generate a fusion protein with an enterokinase cleavage site, Xpress epitope, and an amino-terminal 6-histidine tag. The resulting plasmid was introduced into E. coli strain Top10, selected for resistance to ampicillin, and sequenced.

For overexpression, the plasmids containing His-CiaR were transformed into E. coli BL21 (DE3) pLysS cells (Invitrogen) and grown in 1 liter of LB with 100 µg/ml ampicillin at 37°C with shaking. When the cells reached an OD_{600} of 0.3, the temperature was lowered to 0°C, and IPTG (100 µM IPTG, 0.5 mM IPTG, pro- pyl-p-β-thiogalactopyranoside) for 3 h. The cells were harvested by centrifugation (5,000 × g, 15 min, 4°C), frozen at −20°C overnight, defrosted, and resuspended in column buffer (50 mM NaH_{2}PO_{4}, 0.5 M NaCl, pH 8.0) before 1 mg/ml of lysozyme was added. After incubation on ice for 30 min, 0.5% Sarkosyl was added.
added, cells were lysed by sonication and any unlysed cells along with other debris were removed by centrifugation (10,000 × g, 20 min, 4°C). The cleared cell lysates were diluted in column buffer to a final Sarkosyl concentration of 0.05%, lysate was diluted in column buffer to a final concentration of 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 6.25 mM MgCl2, and 10% glycerol) in a reaction volume of 50 μl. Fifty microliters of a 5 mM CaCl2-10 mM MgCl2 solution was added to the mixture prior to the Dnase I treatment, for 10 min at room temperature. Each substrate was digested with 0.5 units of Dnase I (Promega) for 1 min at room temperature. The digestion was terminated by adding 90 μl of the stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml salmon sperm DNA) followed by a phenol chloroform extraction. The

binding site of ciaX was randomized with mutations in both direct repeat I (DRI) and DRII. The template DNA for the negative control was generated by subjecting oSG579 and oSG598 (Invitrogen) to a single PCR cycle. These oligonucleotides are 85 bp long, incorporate the mutations in DRI and DRII, and are complementary at the last 30 bp of the 3' ends. This double-stranded DNA was used as the template for PCR with oSG576 and oSG577 to amplify a 131-bp fragment. The scrambled 
ciaX promoter was chosen as the negative control because its wild-type form contains a binding site that most closely matches the putative consensus sequence. The labeled DNA for each substrate was also used as the template for sequencing using the SequiTherm Excel II (Epicentre Biotechnologies) DNA sequencing kit.

For the footprinting assays, each labeled fragment was incubated at room temperature for 30 min with 0 to 2,000 nM His-CiaR in the reaction buffer (25 mM Tris-HCl, pH 7.5, 25 mM KCl, 6.25 mM MgCl2, and 10% glycerol) in a reaction volume of 50 μl. Fifty microliters of a 5 mM CaCl2-10 mM MgCl2 solution was added to the mixture prior to the Dnase I treatment, for 10 min at room temperature. Each substrate was digested with 0.5 units of Dnase I (Promega) for 1 min at room temperature. The digestion was terminated by adding 90 μl of the stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml salmon sperm DNA) followed by a phenol chloroform extraction. The
### TABLE 2. Differentially expressed genes in ciaH and OEciaR mutants

| SMU no. | ciaH mutant vs WT | OEciaR mutant vs WT | Annotation | Functional class/other properties |
|---------|------------------|-------------------|------------|----------------------------------|
|         | Expression change |                   |            |                                  |
|         | Mean  | SD     | P value | Mean  | SD     | P value |                          |

- **Expression change**
  - **ciaH mutant vs WT**
  - **OEciaR mutant vs WT**

| Gene Description                                      | Functional Class/Other Properties                          |
|--------------------------------------------------------|------------------------------------------------------------|
| Conserved hypothetical protein                         | Plasmid stabilization system                               |
| Malate permease                                        | Energy metabolism; fermentation                            |
| Malate permease                                        | Transport and binding                                      |
| Oxalate decarboxylase                                  | Unknown                                                    |
| Glutathione reductase                                  | Cellular processes; adaptations to atypical conditions     |
| Alcohol-acetaldehyde dehydrogenase                     | Energy metabolism; fermentation                            |
| Alcohol-acetaldehyde dehydrogenase                     | Cell envelope                                              |
| nlnA, mutacin IV subunit A                             | Bacteriocin                                                |
| nlnB, mutacin IV subunit B                             | Bacteriocin                                                |
| Hypothetical protein                                   | Membrane protein                                           |
| Hypothetical protein                                   | Membrane protein                                           |
| Hypothetical protein                                   | Small peptide                                              |
| Hypothetical protein                                   | Small peptide                                              |
| Mutacin VI                                             | Bacteriocin                                                |
| ABC transporter, permease, and solute binding protein   | Membrane-spanning permease                                 |
| Response regulator                                     | DNA transformation                                          |
| Putative histidine kinase                              | Competence                                                 |
| Hypothetical protein                                   | Membrane protein                                           |
| Cell wall protein precursor                            | Cell envelope                                              |
| Hypothetical protein                                   | Membrane protein                                           |
| Competence protein                                     | DNA transformation                                          |
| Competence protein                                     | DNA transformation                                          |
| Hypothetical protein                                   | Membrane protein                                           |
| Hypothetical protein                                   | Membrane protein                                           |
| GTP pyrophosphokinase family protein                   | Unassigned                                                 |
| Ferrichrome ABC transporter (permease)                 | Membrane-spanning permease                                 |
| ABC transporter, permease protein; possible ferrichrome transport system | Transport and binding                                      |
| Inorganic ion ABC transporter, ATP-binding protein; possible ferrichrome transport system | ATP-binding protein                                         |
| ABC transporter, ferrichrome-binding protein            | Substrate-binding protein                                   |
| Hypothetical protein                                   | Membrane protein                                           |
| Hypothetical protein                                   | Small peptide                                              |
| DNA processing protein, Smf family                     | DNA transformation                                          |
| DNA processing protein, Smf family                     | DNA transformation                                          |
| Peptidyl-prolyl cis-trans isomerase; cyclophilin       | Cell envelope; surface structures; cellular processes; cell adhesion; energy metabolism; sugars |
| ABC transporter, ATP-binding protein homolog            | Transport and binding proteins: ABC superfamily; ATP-binding protein |
| Putative ABC transporter, permease protein              | Transport and binding                                      |
| ABC transporter, ATP-binding protein                   | Transport and binding                                      |
| ABC transporter, ATP-binding protein                   | Transport and binding                                      |
| Peptidyl-prolyl cis-trans isomerase; cyclophilin       | Protein folding and stabilization                          |
| Conserved hypothetical protein                         | BAAT/acyl-CoA thioester hydrolase, Cterminal               |
| Hypothetical protein                                   | Bacteriocin-like                                           |
| Serine protease HtrA                                    | Degradation of proteins, peptides, and glycopeptides      |
| Chromosome segregation protein, ParB-like nuclease domain | Cell division                                             |
| Histidine kinase sensor CalH                           | Two-component system                                       |
| Response regulator CiaR                                 | Two-component system                                       |
| Calcium binding peptide                                 | Calcium binding peptide                                     |
| Mannitol PTS EII                                       | Signal transduction; PTS                                    |
| Conserved hypothetical protein (possible dihydroorotase family) | Unknown                                                   |
| Hypothetical protein                                   | Small peptide                                              |

Continued on following page
The aqueous layer was transferred to 0.5 ml 70% ethyl alcohol (EtOH) with 20 μg of salmon sperm DNA and allowed to precipitate overnight at –20°C. The precipitated DNA was washed with 70% EtOH, dried, and redissolved in the sequencing stop/loading buffer (95% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). The samples were then denatured at 95°C for 4 min, placed on ice, and centrifuged. The footprinting and sequencing reaction mixtures were separated on 6% polyacrylamide gels and then subsequently dried and analyzed using ImageQuant (Molecular Dynamics) software.

| SMU no. | ciaH mutant vs WT | OECiaR mutant vs WT | Annotation | Functional class/other properties |
|---------|-------------------|---------------------|------------|----------------------------------|
|         | Mean   | SD     | P value | Mean   | SD     | P value |          |                          |
| 1396c   | 3.74   | 0.87   | 0.01    | 4.68   | 0.74   | 0.00    | Glucan-binding protein C, GbpC | Stress response                          |
| 1409c   | 7.06   | 1.24   | 0.05    | 1.90   | 0.35   | 0.02    | Aldose 1-epimerase            | Energy metabolism; glycocalyx/glucosynthesis |
| 1400c   | 1.25   | 0.25   | 0.00    | 1.64   | 0.07   | 0.00    | Phospho-β-2-galactosidase     | Energy metabolism; sugars                |
| 1401c   | 2.13   | 0.41   | 0.01    | 1.76   | 0.17   | 0.00    | PTS system, lactose-specific component HBC | Signal transduction; PTS |
| 1492c   | 2.22   | 0.11   | 0.00    | 1.38   | 0.27   | 0.14    | PTS system, cellulose-specific IIA component | Signal transduction; PTS |
| 1493c   | 2.99   | 0.72   | 0.01    | 2.02   | 0.31   | 0.01    | Tagatose-1,6-bisphosphate aldolase | Energy metabolism; sugars |
| 1494c   | 2.67   | 0.60   | 0.01    | 1.91   | 0.29   | 0.01    | Tagatose-6-phosphate kinase    | Energy metabolism; sugars                |
| 1495c   | 2.24   | 0.55   | 0.02    | 1.53   | 0.36   | 0.10    | Galactose-6-phosphate isomerase | Energy metabolism; sugars |
| 1496c   | 2.27   | 0.70   | 0.04    | 1.55   | 0.36   | 0.09    | Galactose-6-phosphate isomerase | Energy metabolism; sugars |
| 1657c   | 0.48   | 0.05   | 0.00    | 0.38   | 0.04   | 0.00    | Nitrogen regulatory protein PII | Amino acid biosynthesis; glutamate family |
| 1658c   | 0.54   | 0.01   | 0.00    | 0.42   | 0.01   | 0.00    | Ammonium transporter, NgA protein | Transport and binding proteins; cations and iron-carrying compounds |
| 1660c   | 0.48   | 0.02   | 0.00    | 0.43   | 0.04   | 0.00    | Branched chain amino acid ABC transporter, permease | Transport and binding |
| 1707c   | 0.51   | 0.16   | 0.00    | 0.29   | 0.08   | 0.00    | spoIV-related rRNA/rRNA methylase | Protein synthesis; RNA and rRNA base modification |

TABLE 2—Continued

* OECiaR, ciaH overexpression strain. The t test was used to calculate P values. All data were calculated based on 4 data sets generated from independent cultures. A cutoff of ≥2-fold change and a P value of ≤0.05 were used to generate the data set. Entries in bold are those that did not meet the criteria but were included as a comparison with the other data set. All genes presented have hybridization signals significantly higher than the noise (P ≤ 0.0001) and are designated as P (present) by the microarray data analysis software GCOS, version 1.4. Numbers in parentheses are ratios obtained by RT-PCR. CoA, coenzyme A; PTS, phosphotransferase.
results

Deletion of ciaR suppressed the ciaH phenotype. During our previous studies of S. mutans strain UA140, we noticed that a mutation of ciaH resulted in defects in competence, acid tolerance, and bacteriocin production, while a ciaR mutant behaved similarly to the wild type (22). The same phenomenon was subsequently reported in the strain UA159 as well (1). This unexpected result was suggested to arise from cross talk between different TCS (1). To examine this further, we made a ciaRH double mutation and tested its phenotype. As shown in Fig. 1, the ciaRH double mutation reversed the competence deficiency of the ciaH mutant, resulting in a transformation efficiency similar to that of the wild type. The double mutation also suppressed the mutacin I bacteriocin deficiency and acid tolerance phenotype of the ciaH mutant (reference 22 and data not shown). These data suggested that ciaH is likely to be in the same signaling pathway as ciaH; however, unlike a typical two-component system, deletion of ciaR alone does not affect the normal function of the cell.

Deletion of ciaH caused overexpression of ciaR. Based on the data presented above, we hypothesized that ciaR may function as a repressor for competence development, mutacin production, and acid tolerance. Interestingly, our previous studies showed that the ciaXRH operon is autoregulatory; however, unlike other autoregulatory TCS operons, deletion of ciaH abolished only its transcriptional repression by calcium (10). Thus, we reasoned that the ciaXRH operon is likely regulated by an uncharacterized mechanism, which may help explain why mutations of ciaH and ciaR did not result in similar phenotypes. To identify the mechanism, we used real-time RT-PCR to quantify the ciaR transcript in the ciaH mutant background. To our surprise, the ciaH mutation increased ciaR gene expression ~25-fold (Fig. 2). This result suggested that (i) CiaH is a negative regulator of ciaR and (ii) overexpressed ciaR might be responsible for the observed phenotypes of the ciaH mutant.

Overexpression of ciaR in the wild-type background resulted in the same phenotype as that of the ciaH mutant. To further test whether overexpression of ciaR itself is sufficient to cause the functional changes observed in the ciaH mutant, we cloned the ciaR gene under the control of the constitutively expressed ldh promoter on the shuttle plasmid pDL278. The ciaR overexpression strain was tested for transformability, mutacin production, and acid tolerance. As shown in Fig. 1, the transformation efficiency in the ciaR overexpression strain was not only reduced compared to that of the wild type but also 2 times lower than that of the ciaH mutant strain. This is probably due to the higher ciaR gene expression in the ciaR-overexpressing strain (2-fold) than in the ciaH mutant strain (data not shown).

Similarly, mutacin production was completely abolished in the ciaR overexpression strain, and acid tolerance in the ciaR overexpression strain was even weaker than that in the ciaH mutant strain (data not shown), possibly for the same reason as stated above. As expected, overexpressing ciaR in the ciaRH double mutation background resulted in the same phenotypes (data not shown). Taken together, these results suggested that overexpressed ciaR is an essential mediator of ciaH mutant phenotypes.

The ciaH deletion and ciaR overexpression affected the same set of genes. To further determine the mechanism by which CiaR mediates gene regulation in the CiaRH signaling pathway, we performed microarray analysis of the wild-type, ciaH mutant, and ciaR overexpression strains grown to an OD600 of 0.3 when natural competence is at its peak level (20). From 4 data sets of each mutant and a cutoff of ≥2-fold expression change and a P value of ≤0.05, a list of 100 genes was generated (Table 2). Among these, 45 genes were upregulated and 55 were downregulated in the ciaH mutant and ciaR overexpression backgrounds compared with the wild type. Comparing the ciaH data set with the ciaR overexpression data set revealed nearly identical gene lists, except for 4 genes, SMU.225c, SMU.528c, SMU.539c, and SMU.574c, which showed a 2- to 3-fold downregulation in the ciaR overexpression strain but no change in the ciaH mutant strain. Three of the four genes also showed a large standard deviation and P values in the ciaH data set, making the assignment difficult to ascertain. In addition, 36 genes did not meet the cutoff in one data set or the other, but the trend of change is consistent in the two data sets.

To confirm the microarray results, real-time RT-PCR was performed to quantify relative gene expression for a subset of the genes in the microarray data set. RNA was isolated from independent cultures grown under the same conditions as in the microarray, and gene expression was measured in both the ciaH deletion and the ciaR overexpression backgrounds. All tested
genes showed a trend of expression consistent with that in the microarray data sets (numbers in parentheses in Table 2), suggesting that the trends in gene expression as measured by microarray are likely to be reflective of the results obtained by RT-PCR.

Identification of a CiaR regulon. To further investigate the regulatory function of CiaR, we sought to identify the genes directly regulated by CiaR. During previous studies, we identified a putative CiaR binding site upstream of \( ciaX \), based upon the CiaR binding site consensus identified in \( Strep\text{t}oc\text{o}c\text{cus pneumo}n\text{iae} \) (9). The consensus sequence is an NTTAAG-3'-WTTAAG direct repeat that is located ~10 bp upstream of the −10 region. Therefore, MotifSearch was used to scan the \( S.\ muta\text{n}ts \) genome for the same sequence pattern. After a list of “hits” was generated, each sequence was manually analyzed for its location (upstream of an open reading frame [ORF]) and the presence of a putative −10 sequence (~10 bp downstream of the second direct repeat [DRII] WTTAAG motif). From these analyses, a total of 8 promoters were identified, including the \( ciaX \) promoter (Fig. 3A). The promoters for SMU.40, SMU.139, SMU.648, SMU.739, SMU.1093, SMU.2164, and SMU.1131c (\( ciaX \)) have a perfect match to the consensus sequence, and all contain a fairly strong −10 sequence to 10 bp downstream of DRII. The promoter for SMU.239 is almost identical but has a 1-bp deletion in the spacer. SMU.40, SMU.139, SMU.239, SMU.739, SMU.1093, and \( ciaX \) are all highly upregulated in both the \( ciaH \) mutant and \( ciaR \) overexpression strains (18- to 95-fold, Table 2). In contrast, the expression of SMU.2164 was only moderately upregulated (2- to 3-fold), while the expression of SMU.648 was barely affected. Inspection of the genomic organization of SMU.648 revealed that while it may have its own promoter, its transcription might also be influenced by read-through from the upstream gene SMU.647.

DNA footprint of the CiaR regulon promoters. To further confirm that CiaR indeed binds to the putative CiaR binding site of the CiaR regulon promoters identified by bioinformatics, DNase I footprinting assays were performed on selected promoters (\( ciaX \), SMU.139, SMU.239, and SMU.739) using purified CiaR protein. As shown in Fig. 3B to E, CiaR clearly binds to each of the aforementioned promoter regions. In each case a 26- to 28-bp region was protected with the consensus sequence located nearly in the middle of the protected region. More importantly, scrambling the consensus CiaR binding site in \( ciaX \) from the wild-type sequence (\( ATTAGTCTCTTTAAG \)) to the mutant derivative (\( GAAAT\text{TCTCTGATATT} \)) effectively abolished CiaR-DNA protection (Fig. 3E). This further supported the role of the direct repeats as the target for CiaR binding. In addition, RACE PCR was performed to identify the transcription start site of the \( ciaX \) promoter. Transcription starts at the A residue 7 bp downstream of the putative −10 sequence (Fig. 3A), further confirming the authenticity of the \( ciaX \) promoter region (data not shown). Taken together, these results indicate that the 8 genes operons identified by bioinformatic analysis are likely to be directly controlled by CiaR.

**DISCUSSION**

In this study we sought to address an unresolved aspect of the \( ciaXRH \) TCS in \( S.\ muta\text{n}ts \): why does a deletion of the \( ciaH \) sensor kinase gene cause multiple phenotypes, while a deletion of the response regulator \( ciaR \) has no effect? To this end, we made a \( ciaRH \) double mutation and showed that \( ciaR \) is essential for expression of the \( ciaH \) phenotypes (Fig. 1), suggesting that CiaR is indeed in the same signaling pathway as CiaH. We further showed that these \( ciaH \) mutant phenotypes are caused by the resulting overexpression of \( ciaR \) (Fig. 2). Similarly, overexpressing \( ciaR \) in a wild-type or \( ciaR \) double mutant background could reproduce the \( ciaH \) phenotypes (Fig. 1), indicating that the overexpression of \( ciaR \) alone is sufficient for the observed \( ciaH \) phenotypes. Microarray analysis of the \( ciaH \) mutant and \( ciaR \) overexpression strains identified 100 genes whose expression is altered ≥2-fold, 96 of which showed similar changes in the two mutants (Table 2). Bioinformatics and DNA footprinting analysis identified 8 genes operon as the CiaR regulon (Fig. 3), 6 of which are the most highly upregulated among all affected genes. Based on these observations, we propose the following model for the mode of regulation by the CiaRH TCS upon its own promoter and that of the CiaR regulon (Fig. 4). When \( ciaH \) is absent, expression of \( ciaR \) and the \( ciaXRH \) operon is increased. This increase in \( ciaR \) gene expression creates a positive feedback loop on the gene expression of the \( ciaXRH \) operon itself, as well as on the expression of the CiaR regulon genes. Increased expression of the CiaR regulon genes eventually leads to repression of the late competence genes and the mutacin biosynthesis genes (see below), resulting in diminished competence development and mutacin production, two of the \( ciaH \) phenotypes. In the presence of \( ciaH \), the activity of CiaR is negated, resulting in lower-level expression of the CiaR regulon and the development of competence and mutacin production. This model suggests that CiaR activates the transcription of the CiaR regulon genes in the absence of CiaH and that CiaH somehow negatively affects the function of CiaR. This notion is further supported by the following observations. (i) The expression of most of the CiaR regulon genes is increased >10-fold in the \( ciaH \) mutant over that in the wild type (Table 2). This makes it unlikely for CiaR to serve as a repressor for the CiaR regulon genes in the absence of CiaH, for that would result in downregulation instead of upregulation of these genes in the \( ciaH \) mutant or \( ciaR \) overexpression strains. (ii) Inspection of the promoter region of all CiaR regulon genes found a fairly strong −10 sequence (~4 bp out of the 6-bp consensus sequence) but very poor −35 region (~3 bp out of the 6-bp consensus sequence) (Fig. 3A). This indicates that without the assistance of CiaR, RNA polymerase would bind poorly to the promoter region, which explained the large increase in transcription of the CiaR regulon promoters when \( ciaR \) was overexpressed as a result of either a \( ciaH \) mutation or transcription from the \( ldh \) promoter. Whether CiaH affects CiaR due to phosphorylation or dephosphorylation of CiaR has yet to be determined.

As mentioned above, among the significantly downregulated (5- to 10-fold) genes in the \( ciaH \) mutant and \( ciaR \) overexpression strains are numerous late competence genes, including the 7 genes in the \( comY \) operon (SMU.1980c to SMU.1987c), \( comEA \) and \( comEC \) (SMU.625 and SMU.626), \( comFA \) and \( comFC \) (SMU.498 and SMU.499), and \( dprA \) (SMU.1001). Based on our previous studies, the transcription of the \( comY \) operon correlates with the level of competence (20). Thus, the
competence deficiencies observed in the ciaH mutant and the ciaR overexpression strains are likely attributable to downregulation of the late competence genes. In addition, comX is moderately downregulated in the ciaH mutant (1.6-fold) and ciaR overexpression strain (1.75-fold) detected by both microarray and RT-PCR assays (data not shown). Whether this moderate effect is responsible for the 5- to 10-fold downregulation of the late competence genes cannot be determined at this time. None of the genes upstream of comX in the competence regulation cascade (comC, comD, and comE) was affected, suggesting that regulation of competence by the ciaRH system is at the later steps. Inspection of comX and other late competence genes did not find any putative CiaR binding site in their promoter regions, suggesting that CiaRH regulation of competence is likely an indirect effect rather than an effect of direct regulation by CiaR. The same finding was also reported for S. pneumoniae (18).

While the competence phenotype can be attributed to the downregulation of the late competence genes in the ciaH mutant and ciaR overexpression strains, the genes responsible for the mutacin I phenotype cannot be discerned from the microarray data set. This is due to the fact that the microarray chip is designed based on the sequence of strain UA159, which does not harbor the mutacin I gene cluster. Another reason is that mutacin I production requires high cell density such as that of colonies grown on a plate (21). Indeed, when RT-PCR was used to measure mutA (structural gene for mutacin I) gene expression in cultures grown on plates, mutA gene expression was downregulated over 10-fold in both the ciaH mutant and ciaR overexpression strains (data not shown). Thus, overexpressed CiaR is also responsible for downregulation of the mutacin I gene expression in the ciaH mutant strain. Like the late competence genes, neither the mutA nor the mutR (positive regulator for mutA) promoter appears to contain the CiaR binding site, suggesting that CiaR regulation of mutacin I production is likely to be indirect.

The mediators for acid tolerance cannot be determined because many of the genes detected in the microarray have not been characterized with regard to their functions in S. mutans. Of particular interest are those that were upregulated >20-fold, such as SMU.40, the SMU.139 operon, SMU.239, SMU.739, and the SMU.1093 operon. These genes all belong to the putative CiaR regulon (Fig. 3) and thereby are assumed to be directly regulated by CiaR. SMU.40 encodes a 53-amino-acid (aa) peptide with a relE-like toxin domain. It is localized in the same operon as SMU.41, encoding a 33-aa peptide, in strain UA159. This organization is typical of the toxin-antitoxin pairs used by bacteria for plasmid maintenance. SMU.139 en-

FIG. 3. (A) Alignment of the putative CiaR binding sites of the putative CiaR regulon promoters. Underlined sequences are the direct repeats (DRI and DRII), and bold letters denote the putative −10 sequence. Shaded regions in SMU.139, SMU.239, SMU.739, and ciaX are protected by CiaR as determined in the DNA footprint assay (B to E). Lines above the sequence indicate the −35 position. (B to E) DNA footprints of the promoters of SMU.139 (B), SMU.239 (C), SMU.739 (D), and ciaX (E) under different concentrations of recombinant CiaR protein. The dashed line labeled FP indicates the protected region, the dashed line labeled CS indicates the consensus CiaR binding sequences, and the solid line labeled −10 indicates the putative −10 sequence.
codes a putative oxalate decarboxylase protein whose function in \textit{S. mutans} has not been characterized. SMU.139 appears to be cotranscribed with two downstream genes, SMU.140, encoding a putative glutathione reductase, and SMU.141, encoding a membrane protein with unknown function. SMU.239c encodes a membrane protein with a VanZ-like transporter domain. SMU.739 encodes a large hypothetical protein predicted to be extracellular and cell wall associated. SMU.1093 and SMU.1094 encode ABC transporters with unknown function. Experiments are under way to determine whether any of these genes are involved in the \textit{ciaH} mutant phenotypes.

It is interesting that among the 100 differentially regulated genes only 5 encode transcription regulators (\textit{ciaR}, \textit{ciaH}, SMU.927, SMU.928, and SMU.345c). In addition to the \textit{ciaH} operon itself, SMU.927 and SMU.928 encode another TCS, which is also upregulated (~2-fold) in the \textit{ciaH} mutation and \textit{ciaR} overexpression strains (Table 2). SMU.927 or SMU.928 resides in the same operon as SMU.926, which encodes a putative GTP pyrophosphokinase family protein. In a previous report, Biswas et al. deleted the histidine kinase gene, \textit{hxlR}, the CiaR regulon also includes 5 small RNAs (sRNAs) (9). Bioinformatics analysis of the \textit{S. mutans} UA159 genomic sequence did indeed identify 3 putative small RNAs (IG53, IG358, and IG1234) that are homologous to the ones identified in \textit{S. pneumoniae}; however, none of these putative sRNAs exhibited significant levels of signal above the background in our microarray (data not shown). Thus, whether the CiaR regulon of \textit{S. mutans} includes small RNAs remains an open question.

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