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Multilevel Control of Competence Development and Stress Tolerance in *Streptococcus mutans* UA159

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Genetic competence appears to be important in establishment of biofilms and tolerance of environmental insults. We report here that the development of competence is controlled at multiple levels in a complex network that includes two signal-transducing two-component systems (TCS). Using *Streptococcus mutans* strain UA159, we demonstrate that the histidine kinase CiaH, but not the response regulator CiaR, causes a dramatic decrease in biofilm formation and in transformation efficiency. Inactivation of *comE* or *comD* had no effect on stress tolerance, but transformability of the mutants was poor and was not restored by addition of competence-stimulating peptide (CSP). Horse serum (HS) or bovine serum albumin (BSA) had no impact on transformability of any strains. Interestingly, though, the presence of HS or BSA in combination with CSP was required for efficient induction of *comD*, *comX*, and *comYA*, and induction was dependent on ComDE and CiaH, but not CiaR. Inactivation of *comC*, encoding CSP, had no impact on transformation, and CiaH was shown to be required for optimal *comC* expression. This study reveals that *S. mutans* integrates multiple environmental signals through CiaHR and ComDE to coordinate induction of *com* genes and that CiaH can exert its influence through CiaR and as-yet-unidentified regulators. The results highlight critical differences in the role and regulation of CiaRH and *com* genes in different *S. mutans* isolates and between *S. mutans* and *Streptococcus pneumoniae*, indicating that substantial divergence in the role and regulation of TCS and competence genes has occurred in streptococci.

Streptococcus mutans, one of the principle causative agents of dental caries and a leading cause of infective endocarditis, has developed a variety of mechanisms to colonize tooth surfaces and to tolerate the stresses commonly experienced in the oral cavity. The abilities to form tenacious biofilms and to tolerate environmental insults are considered major virulence attributes of this organism (5, 7, 27, 28). Rapid and substantial variation in metabolizable energy sources and external pH are common in the human mouth and have a profound impact on oral biofilm ecology. Nutrient limitation and acidic conditions induce many phenotypic changes that can modulate virulence in ways that enhance the capacity of *S. mutans* to form and persist in biofilms (7, 17, 36, 61, 62). A number of recent reports have disclosed an intimate linkage between stress tolerance and efficient biofilm formation (32, 57, 64, 65). For example, mutations that affect cellular levels of the (p)ppGpp synthase RelA (33), ClpP protease (34), DnaK (24, 25, 29, 35), and the serine protease and chaperone HtrA (1, 11) in *S. mutans* concomitantly influence stress resistance and the ability of the cells to form biofilms. In addition, stress tolerance and biofilm formation are also dependent on quorum-sensing systems in *S. mutans*, including the LuxS-dependent autoinducer 2 (47, 64, 66) and the competence regulon, which governs genetic transformation (37, 39).

Genetic transformation of *S. mutans* requires the development of competence, a transient physiological state permitting uptake of DNA (4, 13). Many bacteria are naturally competent for genetic transformation. The occurrence of competence was first described for the pneumococcus (3) and was later demonstrated in *S. mutans* (50). Natural competence in streptococci arises at a specific stage of growth, typically during the early to mid-exponential phase of growth, and persists for a limited time, although there is substantial variation in the optimal conditions for competence development among different species (47, 59) and strains (35). Development of natural competence has been shown to be mediated by quorum sensing. The quorum-sensing signal for competence induction is a competence-stimulating peptide (CSP), which is derived from a CSP precursor (ComC) and exported by an ATP-binding cassette transporter, ComAB (2, 18, 20). CSP is sensed by a two-component regulatory system (TCS) comprising the sensor histidine kinase, ComD, and the response regulator (RR), ComE (4, 9, 19, 52). ComE controls the expression of *comX*, a gene encoding an alternative sigma factor specifically required for the synthesis of late competence proteins that are usually involved in DNA uptake and internalization (31, 42, 43). In previous studies using *S. mutans* strain NG8 (36, 39), mutants defective in the *comC*, *-D*, or *-E* genes of *S. mutans* were reported to have deficiencies in biofilm formation, acid tolerance, and competence development.

Recently, we demonstrated that HtrA, a surface-anchored serine protease, was required for efficient biofilm formation and optimal genetic transformation of *S. mutans* UA159 (1). Notably, expression of *htrA* was shown to be induced by the addition of CSP to growing cultures of *S. mutans* UA159. In continuing our studies of the regulation of *htrA*, we noted that the TCS CiaR/H, which appears widely disseminated in streptococci, was shown to regulate the expression of an *htrA* homologue in *Streptococcus pneumoniae* (22, 46, 59). Originally, the *cia* regulatory system was identified to be involved in *c*ompe-

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tence *i*nduction and *a*ltered cefotaxime susceptibility in *S. pneumoniae* (16), and it has also been reported that CiaRH may regulate cell wall biosynthesis (15). More recently, Merritt and coworkers showed that CiaH of *S. mutans* UA140 decreased genetic transformation, most likely by directly or indirectly modulating the levels of ComY in the cells (48, 57). These authors also revealed a role for CiaH in optimal stress tolerance.

Our understanding at the molecular level of competence development and its relationship to virulence expression by *S. mutans* is incomplete, although considerable progress has been made over recent years. Many pathways or mechanisms of genetic competence for *S. mutans* have been disclosed, based largely on data derived from *S. pneumoniae*. However, the mutans streptococci are genetically and phenotypically very diverse (1, 8, 37, 48) and differ substantially from *S. pneumoniae* in basic physiology, ecology, and pathogenesis. Moreover, the identification of additional competence-associated genes in streptococci (23, 48), along with the isolation of novel regulatory systems that can govern competence gene expression (15, 26, 38, 67), has revealed a complexity in regulation and function of the competence pathways that was not previously appreciated. The fact that many of the regulatory networks have now been shown to impact stress tolerance and biofilm formation in *S. mutans* suggests that the competence regulons in this organism may have evolved to govern multiple cellular functions in a complex signaling cascade, and perhaps many of these functions are unrelated to acquisition of DNA from the environment. Here, we present evidence that two TCS, CiaRH and ComDE, are linked in a competence network and that CiaH, which is believed to act on its cognate RR CiaR, may cross-regulate gene expression via at least one additional RR. We also demonstrate that, although serum proteins such as horse serum (HS) and bovine serum albumin (BSA) are not absolutely required for genetic transformation, these components dramatically influence the expression of competence genes in the presence of exogenous CSP through a TCS network. Collectively, the results show that genetic competence of *S. mutans* UA159 is regulated at multiple levels overlapping with critical cellular functions and reveal that there are substantial differences between strains of *S. mutans* in the role of various regulators and competence genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH10B was grown in Luria broth, and *S. mutans* strain UA159 and its derivatives were grown in brain heart infusion (BHI) broth (Difco). For selection of antibiotic-resistant colonies after genetic transformation, ampicillin (100 μg ml^{-1} for *E. coli*), erythromycin (300 μg ml^{-1} for *E. coli* or 10 μg ml^{-1} for *S. mutans*), kanamycin or spectinomycin (50 μg ml^{-1} for *E. coli* or 1 mg ml^{-1} for *S. mutans*), or tetracycline (10 μg ml^{-1} for *E. coli* and *S. mutans*) was added to the medium, when needed. For growth rate comparisons, cultures of *S. mutans* were initiated with a 1:100 dilution of overnight cultures, and the absorbance (optical density) at 600 nm (OD_{600}) was measured at routine time intervals. For biofilm formation assays, *S. mutans* strains were grown in the semidefined medium BM (40) supplemented with glucose or sucrose at a final concentration of 20 mM. Plasmid pDL278 (30), an *E. coli-Streptococcus* shuttle vector carrying a spectinomycin resistance (Sp^r) gene that confers resistance to spectinomycin in both organisms, was used to measure transformation efficiency. The strains and plasmids used in this study are listed in Table 1.

Construction of mutant strains. Primers used for deletion mutagenesis are listed in Table 2. To make deletions in *com* genes (*comD*, *-E*, and *-X*) and *cia* genes (*ciaH* and *-R*), 5′ and 3′ flanking regions of each gene were amplified from chromosomal DNA from *S. mutans* UA159, ligated together using BamHI sites designed in each primer set, and cloned into the pGEM-T Easy vector (Promega, Madison, WI). These plasmids were digested with BamHI, and a nonpolar (NPKm) or polar (ΩKm) kanamycin cassette or a polar erythromycin cassette (Em) digested with the same enzyme from pALH124, pVT924, and pUC18Em, respectively, was inserted (Table 1). The desired mutagenic plasmids were selected after PCR amplification using vector-originated m13 primers, isolated, and used to transform *S. mutans* UA159. Transformants were selected on BHI agar containing kanamycin (1 mg/ml), and double-crossover mutants of each gene were confirmed by PCR and sequencing. The mutant strains are listed in Table 1. The deletion mutant of *comC* was constructed by insertion of a tetracycline resistance gene released from pLN2 (6).

To construct a reporter gene fusion for measuring the transcription level of the *comC* gene, a 285-bp fragment containing the putative promoter region of *comC* was cloned into the EcoRI and BamHI sites in front of a promoterless chloramphenicol acetyltransferase (CAT) gene (*cat*) in pGEM3cat (1) to yield plasmid pGEMcat-P_{comC}. The P_{comC}-*cat* gene fusion was released using EcoRI and HindIII digestions, treated with Klenow fragment, and then cloned into integration vector pBGK2 (63, 64) at the SmaI site (63). The resulting CAT fusion vector (pBGK-P_{comC}-cat) was integrated into the *gtfA* locus of the chromosome of the wild-type and *ciaH* (SAB22) and *-RH* (SAB23) mutant strains in single copy to create strains SAB52 and SAB53, respectively. Double-crossover recombination of the reporter gene fusion into the *S. mutans* chromosome was confirmed by PCR amplification using primers internal to *gtfA*.

Growth kinetics. Growth of all strains in BHI medium was monitored using a Bioscreen C lab system (Helsinki, Finland) Microbiology Reader with multiwell disposable microtiter plates. The Bioscreen reader was equipped with Biolink software that allowed automatic recording and conversion of optical density readings into growth curves. An aliquot (3 μl) of cell suspension was inoculated into each well containing 300 μl of fresh medium. Inocula were adjusted to the same OD_{600} before dilution. Each sample was assayed at least in triplicate, and wells containing only medium were used as blank controls.

Biofilm assay. The ability to form stable biofilms was assessed by growing the cells in 96-well, flat-bottom microtiter plates (Costar 3595; Corning, Inc., Corning, N.Y.) as previously described (1). Briefly, overnight cultures of *S. mutans* UA159 and its derivatives were transferred to prewarmed BHI medium and grown at 37°C in a 5% CO_2, aerobic atmosphere to an OD_{600} of ≈0.5. The cultures were diluted 1:100 in fresh BM supplemented with glucose or sucrose, and 200-μl aliquots of the cell suspension were inoculated into the wells of the microtiter plates. Wells containing uninoculated growth medium were used as negative controls. Plates were incubated at 37°C in a 5% CO_2, aerobic atmosphere for 24 to 48 h. For biofilm quantification, the microtiter plates were slowly immersed in water and dumped out to remove the remaining planktonic and loosely bound cells. After performing this twice, the plates were blotted on paper towels and air dried. The adherent bacteria were stained with 50 μl of 0.1% crystal violet for 15 min at room temperature and then the plates were slowly immersed in water twice to rinse the wells. The bound dye was extracted from the stained cells by adding 200 μl of ethanol-acetone (8:2). Biofilm formation was then quantified by measuring the absorbance of the solution at 575 nm.

CAT assay. To measure induction of *comC*, CAT activity expressed from the *comC* promoter was measured by a spectrophotometric method (60) using the colorimetric substrate 5,5′-dinitro-bis-nitrobenzoic acid (Boehringer Mannheim, Indianapolis, Ind.). One unit of CAT activity was defined as the amount of enzyme needed to acetylate 1 nmol of chloramphenicol min^{-1}. Protein concentrations were determined by the bicinchoninic acid assay (Sigma).

Transformation assays. Transformation efficiency was measured as previously described with minor modifications (1). Briefly, overnight cultures of *S. mutans* strains were diluted 1:20 in BHI medium containing horse serum (10%, vol/vol). A 0.2-ml aliquot of the cultures was incubated at 37°C for 2 h in a 96-well microtiter plate (Costar 3595; Corning) and then treated with or without 5 μl of synthetic CSP solution (1 μmol ml^{-1}). After incubation for 30 min to allow induction of competence, the cultures were exposed to 500 ng of plasmid pDL278. After 3 h at 37°C, cultures were chilled on ice and transformants and total CFU were enumerated by plating cells on BHI agar plates with or without 1 mg ml^{-1} spectinomycin, respectively. Transformation efficiency was determined after 48 h of incubation and was expressed as the percentage of transformants among the total viable recipient cells.

Gene expression analysis. (i) Growth conditions. To measure the expression of the *htrA* gene, *S. mutans* UA159 and its derivatives (SAB7, SAB22, and SAB23) were grown in 10 ml of BHI to mid-exponential phase (OD_{600}, ≈0.5). To measure the expression of *com* and *cia* genes in relation to competence development, the wild-type strain was grown in 50 ml of BHI to early exponential

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
DH10B	F$^-$ *mcrA* (*mrr-hsdRMS-mcrBC*) Ø80*lacZ* M15 *lacX74 deoR recA1 endA1 araD139* (*ara-leu*)7697 *galU galK-rpsL* (Str^r) *nupG*	Gibco-BRL
S. mutans strains		
UA159	Wild type	
SAB4	Δ*comX*::Km^r	This study
SAB7	Δ*ciaR*::NPKm^r	This study
SJ233	Δ*comC*::Tc^r	This study
SAB19	Δ*comED*::Km^r	This study
SAB21	Δ*comD*::Km^r	This study
SAB22	Δ*ciaH*::Km^r	This study
SAB22Em	Δ*comC*::Em^r	This study
SAB23	Δ*ciaRH*::Km^r	This study
SAB23Em	Δ*ciaRH*::Em^r	This study
SAB39	Δ*comE*::NPKm^r	This study
SAB52	SAB22Em::P_{comC}-*cat*[a]	This study
SAB53	SAB23Em::P_{comC}-*cat*	This study
SJ232	UA159::P_{comC}-*cat*	This study
Plasmids		
pALH124	Vector harboring an NPKm^r cassette	Y. Y. Chen, Univ. of Florida
pBGK2	*Streptococcus* integration vector, Km^r	63, 64
pBGK-P_{comC}-cat	CAT fusion vector including the putative promoter of *comC*	This study
pDL278	*E. coli-Streptococcus* shuttle vector, Sp^r	30
pGEM-T Easy	PCR cloning vector	Promega
pGEM3	*E. coli* cloning vector	Promega
pGEM3cat	pGEM3 harboring a promoterless *cat*	1
pGEM3cat-P_{comC}	pGEM3 harboring the P_{comC}-*cat* gene fusion	This study
pLN2	Vector harboring a Tc^r cassette	6
pUC18Em	pUC18 harboring an Em^r cassette	Y. Y. Chen, Univ. of Florida
pVT924	Vector harboring a Km^r cassette	Y. Y. Chen, Univ. of Florida

[a] *comC* promoter fused to chloramphenicol acetyltransferase gene (*cat*).

phase (OD_{600}, ≈0.15) in the absence or presence of HS (10%, vol/vol), and CSP was added at a concentration of 10 μM. Samples (12 ml) were removed at 0, 10, 20, and 40 min after inoculation and transferred into 15-ml Falcon tubes (Becton Dickinson Labware, Franklin lakes, NJ) containing rifampin at a final concentration of 150 μg ml^{-1}, and RNA was extracted as follows.

(ii) Extraction of total RNA. Total RNA was extracted using a previously described protocol (1). Briefly, pellets obtained after centrifugation of bacterial cultures were mixed with 1 ml RNAprotect bacterial reagent (QIAGEN) by vortexing to stabilize RNA before cell lysis. After incubation for 10 min at room temperature and then centrifugation, the pellet was mixed with 300 μl of Tris-EDTA buffer and then transferred to a 2-ml screw-cap tube (Sarstedt, Newton, NC) containing 300 μl of saturated acid phenol, 10 μl of a 10% (wt/vol) solution of sodium dodecyl sulfate, and 0.3 ml of 0.1-mm glass beads (Biospec Products, Inc., Bartlesville, OK). The bacterial cells were disrupted by bead milling for 30 s, twice, with 2-min intervals on ice. The lysate was clarified in a microcentrifuge at maximum speed at 4°C for 10 min. Total RNA was isolated from the supernate and purified by the RNeasy mini kit (QIAGEN, Inc., Chatsworth, CA), including on-column DNase digestion with RNase-free DNase (QIAGEN). The purity and concentration of the RNA were determined by spectrophotometry and gel electrophoresis (58).

(iii) Real-time PCR. First-strand cDNA templates were created from 1 μg of RNA using the SuperScript first-strand synthesis system (Invitrogen Corp., Carlsbad, CA) according to the recommended procedure. The primers used for reverse transcription reactions and real-time PCR are shown in Table 3. Real-

TABLE 2. Primers used for construction of deletion mutants in this study[a]

5′-end amplicon		Gene deleted	3′-end amplicon	
Primer	Nucleotide sequence (5′→3′)		Primer	Nucleotide sequence (5′→3′)
ciaR-flanking-RV	GCCAATGAGTCTCTTCCATGA	*ciaRH*	ciaH-BamHI-C	CAAGGTATTGGATCCGAGGATAAGA
ciaR-BamHI-B	TATTGACATGGATCCTGACATTCTC	*ciaRH*	ciaH-D2	TTGCCAGAGACATTTGGAAAG
ciaH-A	TCGAAATATCCCAAGTCATGC	*ciaH*	ciaH-BamHI-C	CAAGGTATTGGATCCGAGGATAAGA
ciaH-BamHI-B	TATTGACATGGATCCTGACATTCTC	*ciaH*	ciaH-D1	TGAGAAAGACTTGCCAAATATGTTA
ciaR-flanking-RV	GCCAATGAGTCTCTTCCATGA	*ciaR*	ciaR-BamHI-C	TAGATAGACGGATCCCGTCTTCTAC
ciaR-BamHI-B	AGTGTAGGAGGATCCTTGAAGGAT	*ciaR*	ciaR-flanking-FW	CCACCCTTTTGTCGTGTTCT
comED-A3	GCAGGAGCTGGCTATCTTTTT	*comED*	comED-BamHI-C	AGTAAAATAGGATCCCCTGAGATGG
comED-BamHI-B3	CCTTGTTGTGGATCCTCATCTTCCA	*comED*	comED-D	CCCCTTCCCCATTTTTAGTT
comD-A	TTTTTGCGGTTAGCAAAATG	*comD*	comED-BamHI-C	AGTAAAATAGGATCCCCTGAGATGG
comD-BamHI-B	GAAAGTATCAGGATCCCTTCATTCA	*comD*	comED-D	CCCCTTCCCCATTTTTAGTT
comED-A3	GCAGGAGCTGGCTATCTTTTT	*comE*	comE-BamHI-C	AGTAAAATAGGATCCCCTGAGATGG
comED-BamHI-B3	CCTTGTTGTGGATCCTCATCTTCCA	*comE*	comE-D	CCCCTTCCCCATTTTTAGTT
Comm55	ACTTATCAGATGAGTTTGTCCATC	*comC*	Comm-BamHI-35	TAGGATCCGCTAACATTGGAATAAAACAAG
Comm-BamHI-53	AAGGATTCTGTTGATAGGCTTCCGCTTCC	*comC*	Comm-33	TGCTGTCAAGGGTATCTTGTCAGC

[a] In addition, primers used for amplification of the putative promoter of *comC* were as follows: PcomC RI5′, AAGAATTCAAATGCTTGTGTATTCATATG; PcomC-Bm3′, ATGATAGTGTTTTTTTCATGGATCCTCTCC.

TABLE 3. Primers used for real-time PCR in this study

Primer	Nucleotide sequence (5′→3′)	Primer	Nucleotide sequence (5′→3′)	Product size (bp)
ciaR-sense	GAAGCAGAGAGTGGCGTTTATG	ciaR-antisense	TGTCATCCAAACCTTCCTTAGC	145
comD-sense	TATGGTCTGCTGCCTGTTGC	comD-antisense	TGCTACTGCCCATTACAATTCC	97
comX-sense	CGTCAGCAAGAAAGTCAGAAAC	comX-antisense	ATACCGCCACTTGACAAACAG	89
comYA-sense	ATTATCTCTGAGGCATCGTCCG	comYA-antisense	ACCATTGCCCCTGTAAGACTTG	102
htrA-sense	AAGTTGTTAGACCCGCTCTTGG	htrA-antisense	ACCGCTTGTGACATCACTTGG	101

time PCRs were carried out as previously described (1) using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) and iQSYBR Green supermix (Bio-Rad). For confirmation of amplicon presence and purity, the real-time PCR products were run on a 1.0% Tris-acetate-EDTA gel and stained with ethidium bromide.

RESULTS

Phenotypic characterization of *ciaR* and *ciaH*. The *ciaR* and *ciaH* genes were disrupted by nonpolar (SAB7) and polar (SAB22) insertions, respectively (Table 1), because the two genes were shown to be cotranscribed by reverse transcription-PCR (data not shown). The nonpolar insertion into *ciaR* was confirmed to allow efficient read-through to *ciaH* by real-time PCR (data not shown). To evaluate effects of removal of the entire *ciaRH* operon, both *ciaR* and *ciaH* were deleted (SAB23). No obvious differences were observed in the growth rate of the *cia* mutants using BHI medium at pH 7.4 (data not shown). However, unlike the *ciaR* (SAB7) or *ciaHR* (SAB23) deletion mutants, the *ciaH* mutant (SAB22) showed a significant growth defect in BHI medium acidified to pH 6.4. When the medium was adjusted to pH 5.4, all three mutants presented growth defects (Fig. 1A). Different behaviors of the mutated strains were also seen in biofilm formation (Fig. 1B) and transformation efficiencies (Fig. 1C). The *ciaH* mutation exhibited a 77% reduction in biofilm formation in BM medium supplemented with glucose but caused an enhancement by about 23% in biofilm formation in BM-sucrose. Loss of CiaH almost completely abolished the ability to be transformed in the absence of treatment with exogenous CSP. The provision of exogenous CSP restored the ability of the strains to be transformed, albeit not to the level of the wild-type strain (Fig. 1C), suggesting that CiaH is not required for transduction of the CSP signal. Mutation of *ciaH* also resulted in up-regulation of *htrA* by about 100-fold (Fig. 1D), in contrast to what has been observed in pneumococci, where CiaHR positively regulates *htrA* expression. Unlike the *ciaH* mutant, deletion of *ciaR* caused only a slight reduction in biofilm formation in BM-glucose medium and resulted in enhanced transformability in the absence of CSP.

TCS ComE/D regulates genetic competence but is not involved in biofilm formation and acid tolerance in strain UA159. ComED has been shown to be essential for development of competence in *S. mutans* strain NG8 (39). In addition, disruption of *comDE* in NG8 resulted in a diminished capacity to form biofilms and to grow in acidified media. Deletion mutations were generated in the *comD* (SAB21), *comE* (SAB39), and *comED* (SAB19) genes of *S. mutans* UA159, and the ability of the strains to be transformed by plasmid DNA was evaluated. Because the *comE* and *comD* genes were shown to be cotranscribed by reverse transcription-PCR (data not shown), *comE* was disrupted by a nonpolar insertion and the other genes were disrupted by polar insertions. Polarity or the lack thereof was confirmed by real-time PCR (data not shown). None of the mutant strains displayed any obvious defects in biofilm formation as assessed by crystal violet staining of biofilms formed in BM medium supplemented with glucose or sucrose (data not shown). All *comE*, *comD*, and *comED* mutants presented growth curves similar to the parental strain in BHI medium adjusted to different pH values (7.4, 6.4, and 5.4) (data not shown). However, these mutations diminished the transformation frequency by severalfold in the absence of CSP and transformability could not be restored to the mutants by the addition of CSP (Fig. 2), unlike what was observed for the *ciaH* mutant. Therefore, unlike in strain NG8, the TCS ComED of *S. mutans* UA159 is not involved in acid tolerance or biofilm formation, although biofilm architecture was not examined in any detail in this study. However, the essential role of ComED in CSP sensing and development of competence for DNA uptake has been retained. The *comX* gene was also inactivated by insertion of an EZ::TN transposon containing a polar kanamycin resistance gene in the *comX* structural gene, as previously described (1) (Table 1). Mutation of *comX* also resulted in almost complete elimination of transformation compared to the wild-type strain (data not shown).

A serum-rich environment affects the expression of competence genes. As shown in Fig. 3A, CSP significantly increased the transformation frequency of UA159 regardless of whether the growth medium was supplemented with HS, which is typically added to enhance transformation of *S. mutans* (50, 51). However, HS had no effect on transformation, regardless of whether the cells were treated with exogenous CSP (Fig. 3A). No effects of HS on growth were observed, whereas the addition of CSP to the medium slowed the growth of *S. mutans* (Fig. 3B).

Interestingly, when the expression of competence genes was evaluated, no induction of *comED*, *comX*, or *comYA* was observed in the presence of either CSP or HS alone. However, in the presence of HS and CSP, *comED*, *comX*, and *comYA* were induced 4-, 20-, and 40-fold, respectively, at 40 min after exposure to exogenous CSP (Fig. 4A). Similar levels of induction of the same *com* genes were also noted in the presence of CSP when HS was replaced by high-purity (>99%) BSA (Fig. 4B). Notably, *ciaRH* showed no induction under any of the conditions tested (data not shown).

Both CiaH and ComD are required for induction of competence genes, and CiaH affects expression of *comED*. To begin to dissect the signal transduction pathways governing induction of *com* genes in *S. mutans* UA159 in response to exogenous peptides/proteins, the induction levels of *comX* and *comYA* were measured in strains carrying mutations in *comD* (SAB21)

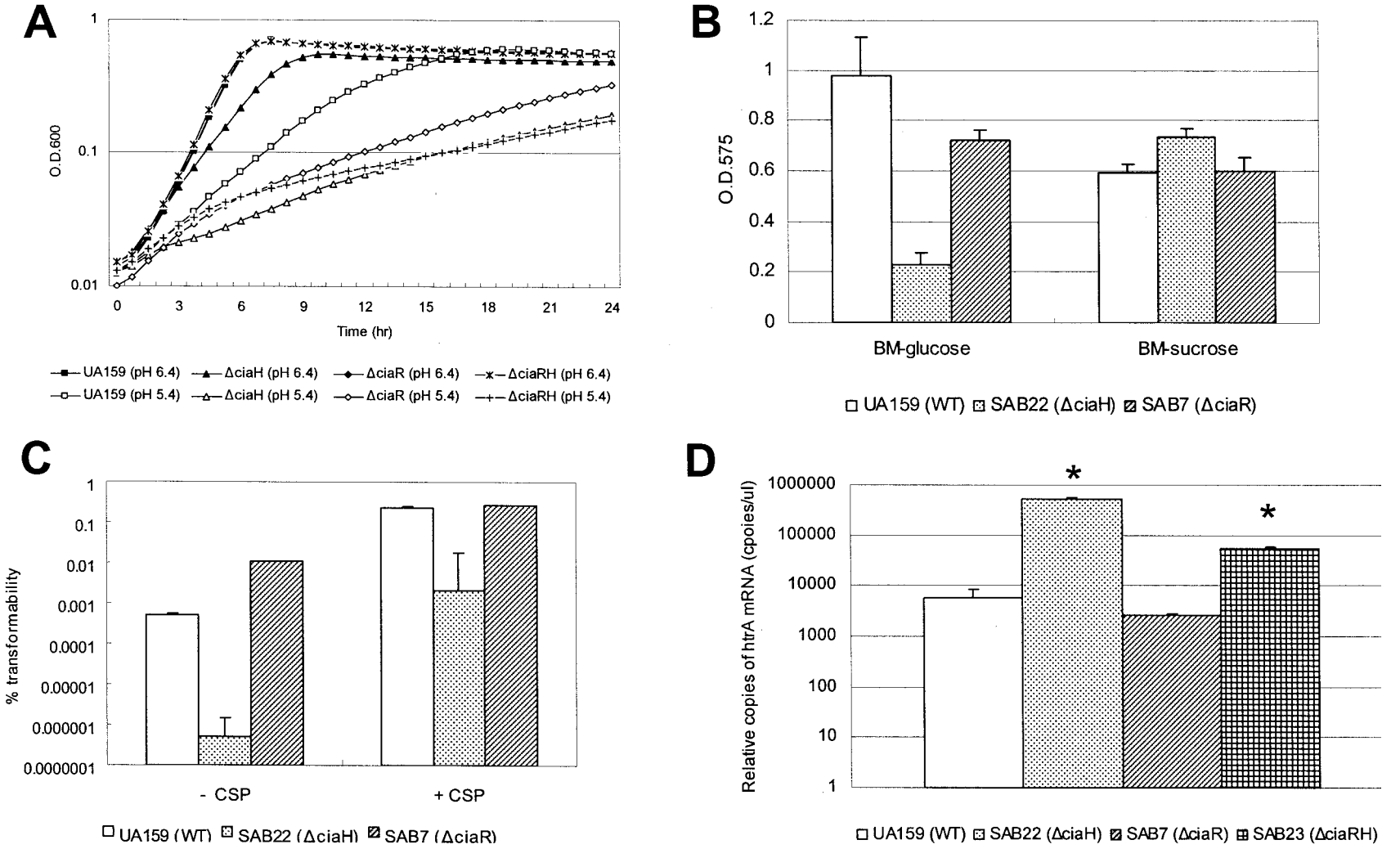

FIG. 1. Phenotypic characterization of the *ciaH*, *ciaR*, and *ciaRH* mutants. (A) Growth at pH 6.4 and 5.4. Growth in BHI medium adjusted to different pH values (7.4, 6.4, and 5.4) was monitored in a Bioscreen C system. The results at two different pHs (6.4 and 5.4) showing different growth patterns between the wild type and mutants are presented. Data points are averages of triplicate samples. (B) Biofilm formation. Strains were grown in BM medium supplemented with glucose (BM-glucose) or sucrose (BM-sucrose) at a final concentration of 20 mM for 24 h. Biofilms were assayed in polystyrene microtiter plates by staining with crystal violet and quantified by adding to an ethanol-acetone mix and reading the optical density at 575 nm. (C) Transformation frequency. Plasmid pDL278 (Sp^r) was transformed into wild-type and mutant strains grown in 200 μl BHI medium (OD_{600} = 0.15) with or without CSP. Transformation frequency was determined from the ratio of the number of transformants versus that of the total viable recipients, multiplied by 100. (D) Differential expression of *htrA* measured by real-time PCR. Data are representative of at least two separate experiments. The data shown in panels B, C, and D are means ± standard deviations (error bars) of at least three replications. *, $P < 0.001$; Student's *t* test.

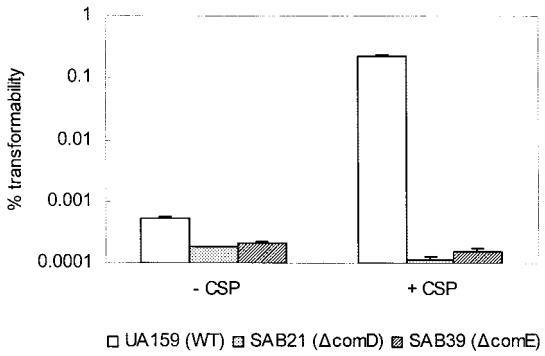

FIG. 2. Frequency of transformation of the *comD*, *comE*, and *comED* mutants. Data are representative of at least two separate experiments. The data shown are means ± standard deviations (error bars) of at least three replications. See the text for more details.

and *ciaH* (SAB22) in the presence of HS and CSP. Inactivation of either *comD* or *ciaH* resulted in almost complete loss of expression of the *comX* and *comYA* genes, suggesting that both ComD and CiaH are required for interaction with exogenous CSP signals (Fig. 5A). Interestingly, when the cognate RRs (*comE* and *ciaR*) were inactivated, the *comE* mutant (SAB39) abolished expression of *comX* and *comYA* to the same extent as the *comD* mutant (Fig. 5B). Importantly, loss of CiaR, the apparent cognate response regulator of CiaH, had no significant effect on *comD*, *-X*, or *-YA* expression levels, in sharp contrast to the profound impact that loss of CiaH has on expression of these genes. Also of note, inactivation of *ciaH* significantly reduced the expression of *comD* (Fig. 5C and D), but inactivation of *comD* did not affect the expression of *ciaRH* (data not shown). Finally, a nonpolar mutation in *comE* caused significant down-regulation of *comD* (Fig. 5D).

Mutation of *comC* had no effect on transformation of strain UA159. As described above, the exposure of cells to exogenous

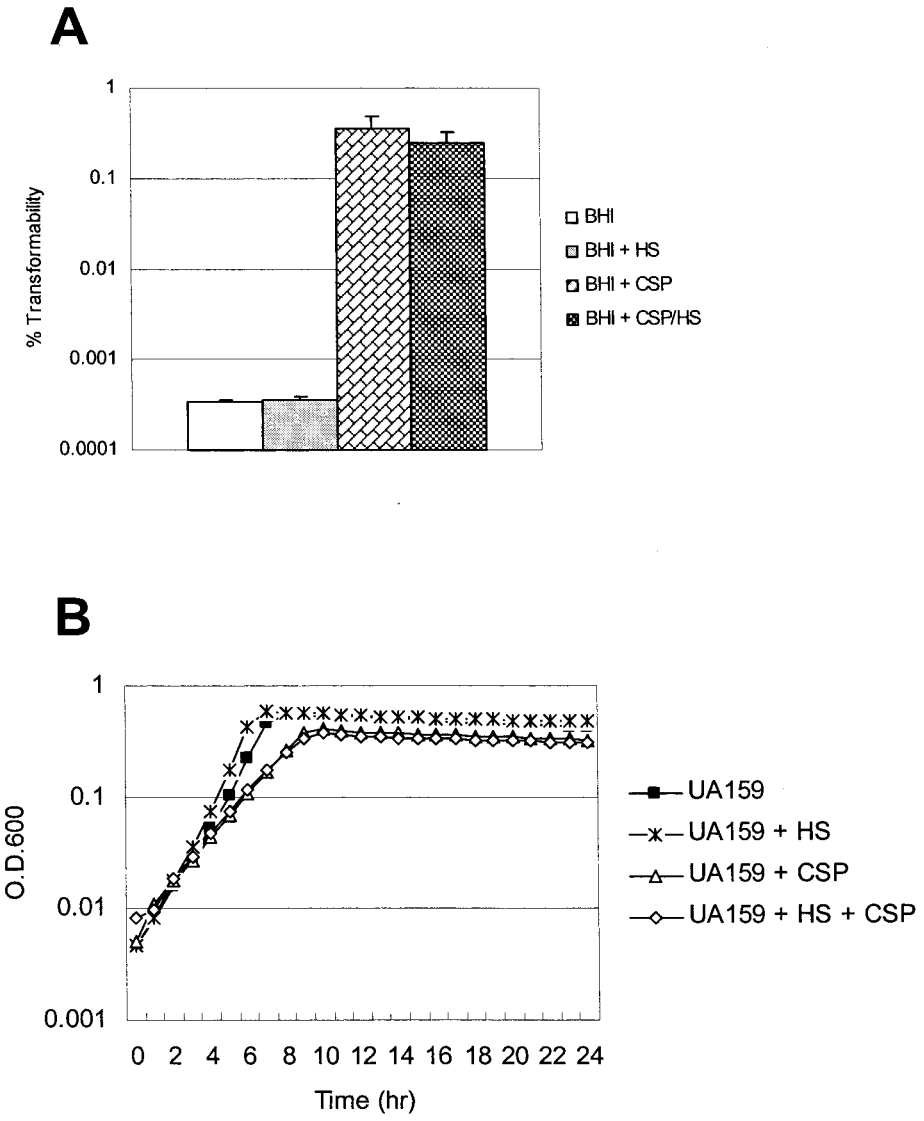

FIG. 3. Effects of HS and CSP on the frequency of transformation of UA159 (A) and growth (B). See the text for more details.

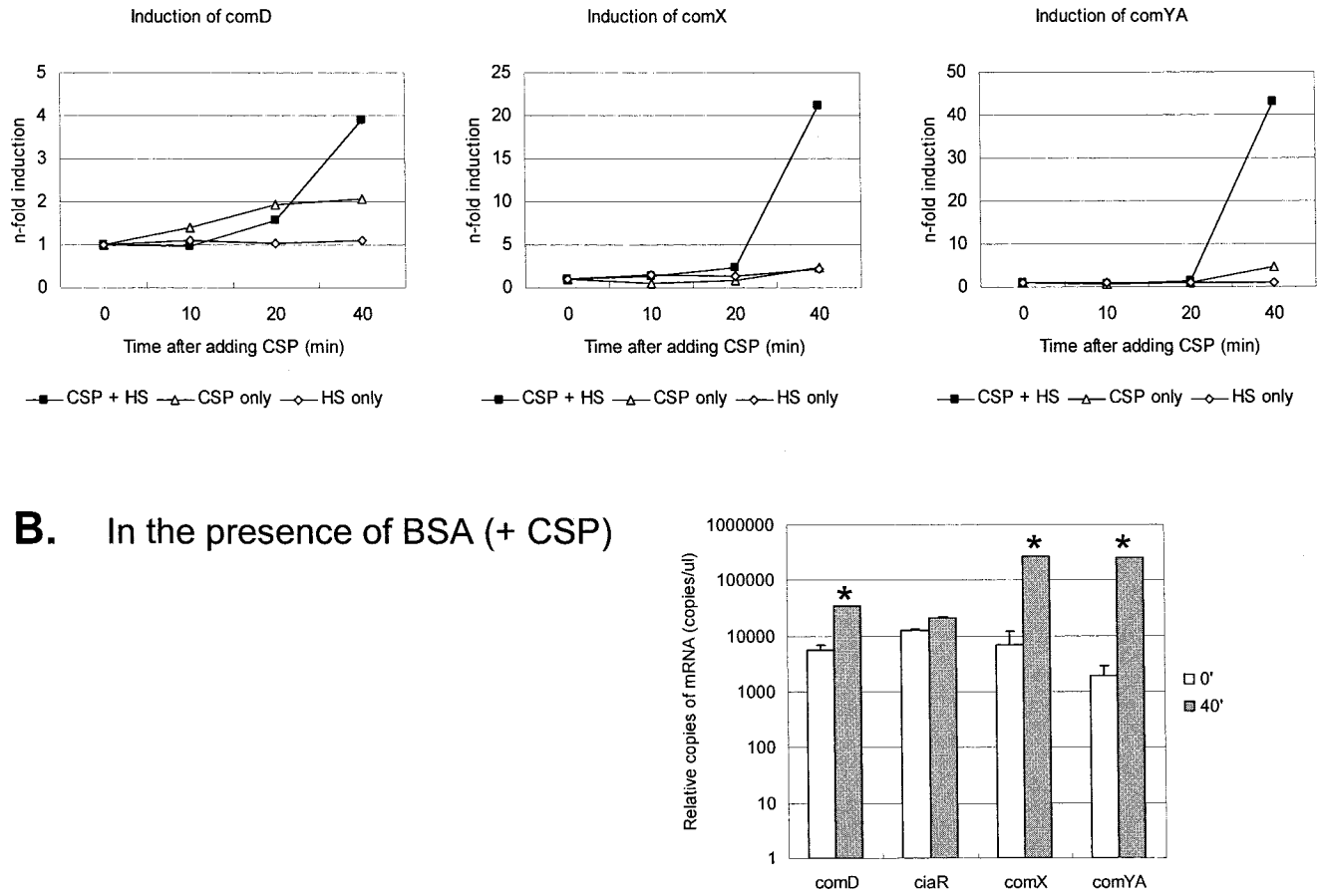

FIG. 4. Induction of the *ciaRH*, *comED*, *comX*, and *comYA* genes by CSP treatment in the absence or presence of HS (A) and in the presence of BSA (B) by using real-time PCR. *S. mutans* UA159 was grown in 50 ml BHI medium supplemented with HS (10%, vol/vol), and synthetic CSP was added at a concentration of 0.2 mM when the culture reached an OD_{600} of 0.15. A 12-ml sample was removed at 0, 10, 20, and 40 min after addition of CSP, and RNA was extracted for real-time PCR. BSA was used at the same concentration as the total protein measured in HS using a commercial Bradford reagent. Data shown in panel A are representative of three independent experiments. Data shown in panel B are means ± standard deviations from two independent experiments. *, $P < 0.01$; Student's *t* test. See the text for more details.

CSP affects the expression of competence genes in the presence of HS and dramatically enhances the efficiency of transformation. The precursor of CSP is encoded by *comC*, and the active, processed CSP signal has been shown to be sensed by histidine kinase in a quorum-dependent manner (19, 39, 41). However, when the *comC* gene of *S. mutans* UA159 was mutated to create strain SJ233, no effect on transformation was observed (data not shown). Also, real-time PCR assays showed no significant difference in the expression of *comAB*, *comED*, and *comX* in the *comC* mutant background, compared to the wild-type strain (data not shown). Inactivation of *comC* had no effect on biofilm formation on polystyrene microtiter plates in BM medium supplemented with glucose or sucrose (data not shown) but resulted in a slightly reduced growth rate in BHI medium (Fig. 6A). Interestingly, *comC* expression was significantly reduced in response to mutation of *ciaH*, as measured using a transcriptional fusion of the *comC* promoter region to *cat* (Fig. 6B).

DISCUSSION

The results presented herein add further support to the concept that the competence regulons of *S. mutans* are interconnected with critical virulence attributes and that competence pathways have evolved to efficiently detect environmental cues and quorum-sensing molecules to govern a complex regulatory network. In this study, two TCS, CiaRH and ComED, have been shown to coordinate multiple environmental signals to control the expression of *com* genes and development of competence. The involvement and interrelationship of these two regulatory systems in competence for natural genetic transformation has been studied in *S. pneumoniae* (2, 10) but has not been examined in detail in *S. mutans*. Clearly, CiaRH and ComED function in a hierarchical and cooperative fashion to optimize expression of selected competence genes. Our results also highlight critical differences between strains of *S. mutans* in terms of the importance and requirement for

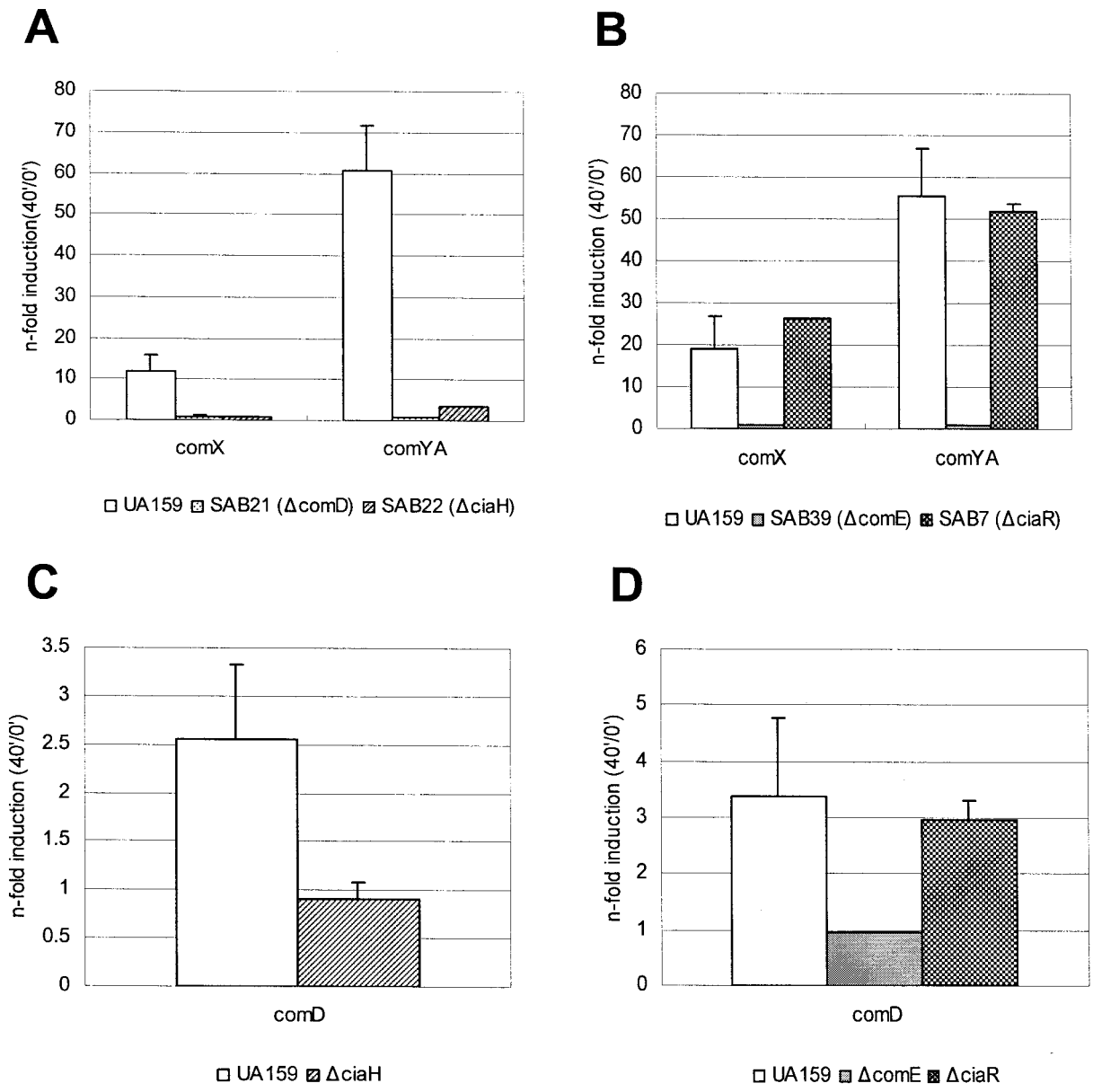

FIG. 5. Differential transcriptional profiles of *com* genes of wild-type and mutant strains of TCS in the presence of horse serum and CSP by real-time PCR. Differential expression is expressed as fold induction at 40 min after adding synthetic CSP, compared to 0 min. The data shown are means ± standard deviations (error bars) of two independent experiments. See the text for more details.

ComC and ComED in acquisition of exogenous DNA, as well as in biofilm formation and growth at low pH.

Interestingly, strains with mutations in *ciaH* and *ciaR* in *S. mutans* UA159 showed obviously different behaviors in various cellular functions, such as acid tolerance, biofilm formation, and competence development. Also, inactivation of *ciaH* caused up-regulation of *htrA* expression by about 2 logs, revealing a molecular connection between the stress response pathways, signal transduction, and competence development. Importantly, though, the behavior of the *ciaH* mutant was dramatically different than that of the *ciaR* mutant. These results strongly support the possibility that CiaH, which is believed to act on its cognate RR CiaR, can also regulate gene expression via at least one additional RR to control competence development, biofilm formation, and stress tolerance. Different behaviors of *ciaH* and *ciaR* mutants have previously been observed in strain UA140 of *S. mutans* with some minor differences (57) from our findings. The *ciaR* mutant of UA159 significantly reduced acid tolerance at pH 5.4, but not at pH 6.4. Also, the *ciaH* mutant of UA159 reduced sucrose-independent biofilm formation by about 80%, but in medium supplemented with sucrose, biofilm formation was enhanced by about 20%. The *ciaH* strain derived from UA140 displayed a defect in sucrose-dependent biofilm that was ascribed to poor adhesion to the substratum.

In our study, a double mutant of *ciaR* and *ciaH* was also

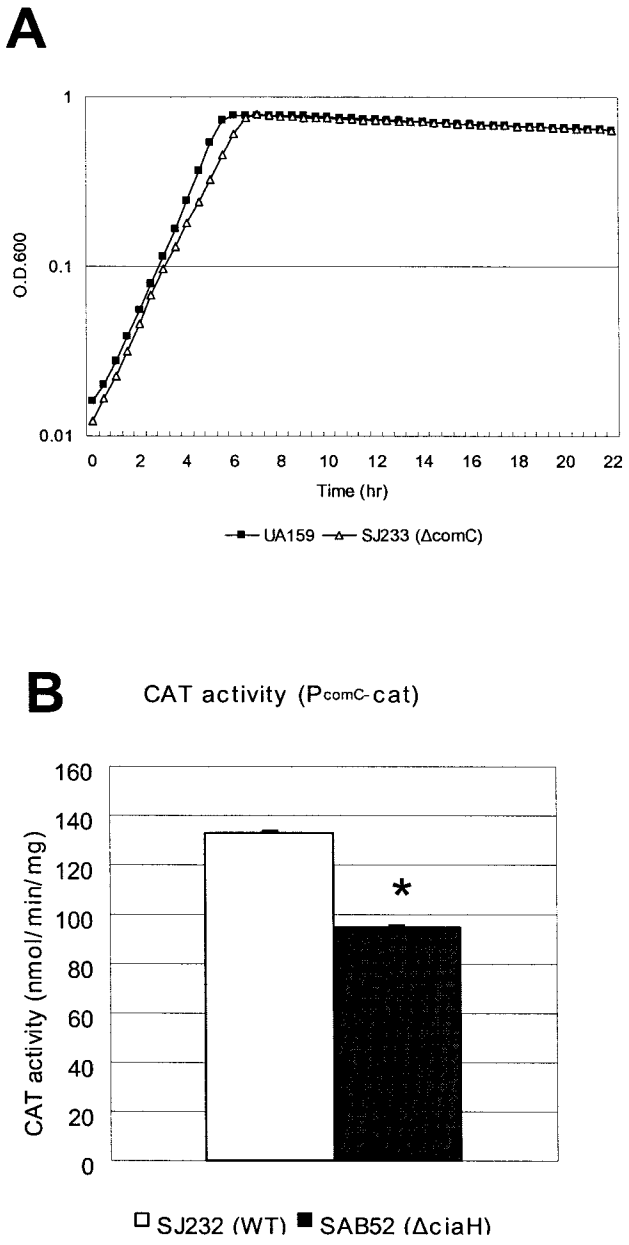

FIG. 6. Growth of the wild type and SJ233 (Δ*comC*) mutant generated with Bioscreen C (A) and the regulation of *comC* by CiaH as measured by CAT assay (B). The promoter fusions of *comC* with the *cat* gene were inserted in a single copy into the chromosome of the wild type (SJ232) and the *ciaH* mutant (SAB52). Data presented are means ± standard deviations (error bars) of two independent experiments. *, $P < 0.001$; Student's *t* test.

constructed. Surprisingly, this strain behaved more like a *ciaR* single mutant in terms of acid tolerance but did not behave uniformly like the CiaH-deficient strain in terms of biofilm formation, competence development, and expression of *htrA* (data not shown). The basis for this behavior is likely complex and could arise from the fact that loss of one response regulator could impact the utilization of remaining effectors. Nonetheless, a reasonable interpretation of our results is that CiaR has roles in regulation of acid tolerance and possibly other cellular physiology functions that are independent of the CiaH signaling cascade and arise as a result of cross talk between the Cia system and other TCS. This idea is clearly supported by our results showing effects of CiaH on expression of ComCDE. It is also noteworthy that inactivation of *ciaH* could have effects on the phenotypic behavior of *S. mutans* UA159 through changes in the level of expression of *htrA*. We previously showed that the levels of HtrA in the cell are tightly controlled and that overexpression of *htrA* on a plasmid had a profound impact on growth. Also consistent with this idea is that some phenotypes caused by deficiency of CiaRH in *S. pneumoniae* were apparently due to alterations in the levels of HtrA, a surface-localized serine protease (22). The effects on cell behavior could arise through changes in the efficiency of secretion of selected molecules or by alterations in the posttranslational modification of signaling molecules or sensing systems catalyzed by HtrA (11, 44, 54, 59), which has also been implicated in processing of the CSP precursor (59).

Our results also demonstrate that the ComED TCS, which has been shown to be essential for development of competence in *S. mutans* NG8 (39), is regulated by CiaR/H. Mutation of *ciaH* significantly reduced the expression of *comED*, which appears to be negatively regulated by CiaH in *S. pneumoniae* (14, 45), suggesting that the *cia* regulatory system may be located upstream of ComED in the regulatory cascade. Our results also support that expression of *comED* can be autoregulated by ComE, indicating that there are at least two points for control of ComE/D production. Unlike what we observed for CiaRH, ComED does not seem to control other critical virulence attributes beside competence in strain UA159. This finding contrasts with previous reports for *S. mutans* NG8 (36, 39), in which mutations in *comD* or *comE* were shown to significantly affect biofilm formation and acid tolerance. Clearly though, the role of *comCDE* in transformation in UA159 has been conserved, as it has for *S. mutans* NG8 (36, 39) and *S. pneumoniae* (9), as evidenced by diminished transformation frequencies in strains with mutations in *comE* or *comD*. ComD- or ComE-deficient strains of UA159 fail to respond to exogenous CSP with enhanced transformability, unlike the *ciaH* mutant. Thus, ComED in UA159 has retained the quorum-sensing function for competence development, but control of virulence attributes by these two TCS occurs primarily through CiaH.

The treatment of *S. mutans* with CSP dramatically increases induction of competence (1, 39), and many competence-associated genes have been known to be CSP responsive (53). However, our real-time PCR and transformation data clearly show that treatment with exogenous CSP, in the absence of horse serum or BSA, dramatically enhances the transformation frequency in the absence of induction of competence genes. Interestingly, mutation of *comC* in UA159 had no effect on transformation, in contrast to *S. mutans* NG8, in which inactivation of *comC* resulted in a competence-deficient phenotype (19, 39, 41). In addition, the *comC* mutant of UA159 had no effect on biofilm formation and a slightly reduced growth rate, which is also different from what was reported for strain NG8. These results suggest that *comC* is not absolutely required for genetic transformation of UA159, despite the fact that addition of excess synthetic CSP has a profound effect on the frequency of transformation. We also found that expression of

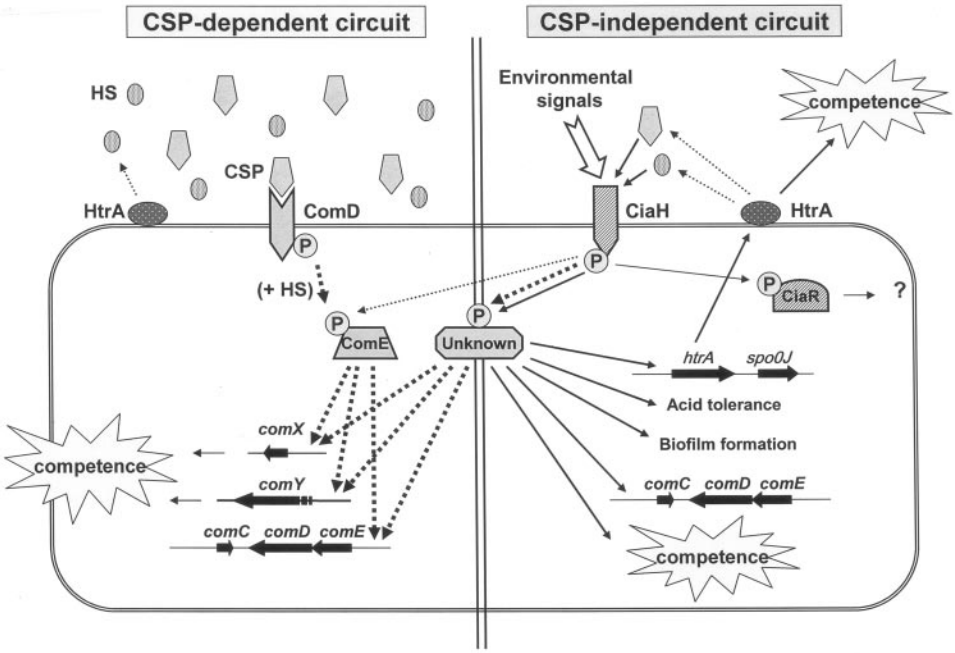

FIG. 7. Working model for competence development in *S. mutans* UA159. This model presents gene networks controlling competence development, acid tolerance, and biofilm formation through CiaRH and ComE/D TCS driven by exogenous signals. The network is separated into circuits that are CSP dependent and CSP independent. We hypothesize that ComED is the primary conduit for sensing of CSP, while CiaH integrates signals detected in serum proteins, such as HS. We also hypothesize the presence of a third RR which integrates signals derived from CiaH. Possible pathways driven by HS are shown by thick dashed lines. The model includes the possibility that ComE is a target for CiaH and that HtrA is involved in processing of CSP or HS (thin dotted lines). Thus, the model shows that CiaRH and ComED control competence development and *com* gene expression, as well as various virulence-related functions in a hierarchical and cooperative fashion, responding to CSP and environmental cues.

com genes, including *comED*, *-X*, and *-YA*, showed no significant differences between the wild type and the *comC* mutant over different cell densities (data not shown). Thus, there is likely a basal level of transcription of the genes for competence development that occurs independently of ComC peptide sensing. This is supported by our real-time PCR data showing constitutive expression of selected *com* genes in the absence of CSP, horse serum, or BSA. We have not been able to identify a second *comC* gene in the *S. mutans* genome, although recent studies with pneumococci indicate that this is a distinct possibility and also reveal that there are genetic polymorphisms in the competence quorum-sensing systems of streptococci (21, 55). Moreover, we also found that the expression of the *comC* gene is controlled by CiaH, which was shown to be involved in regulation of *comED*. Thus, the deficiency of transformation arising from mutation of *ciaH* may involve changes in the basal expression level of *comC* when the assays are performed in the absence of CSP and HS.

A particularly important discovery in this study is that induction of the competence genes with CSP treatment was achieved only in the presence of serum proteins, such as HS or BSA. In previous studies (12, 49, 56), serum proteins from horses, calves, and swine, as well as serum albumin, have been reported to stimulate the transformation of *S. mutans*; however, this does not appear to be the case for *S. mutans* UA159. On the other hand, the addition of CSP, the processed form of ComC, clearly induces a dramatic increase in transformation efficiency. However, CSP alone is not sufficient to signal major changes in competence gene expression. Therefore, our studies reveal that *S. mutans* requires two inputs to achieve marked changes in *com* gene expression, perhaps reflecting a need in vivo to integrate CSP-dependent quorum sensing with the detection of specific host molecules.

Based on our results and existing evidence, we present a working model of the way in which exogenous signals are integrated into gene networks controlling transformation, biofilm formation, and stress tolerance in *S. mutans* UA159 through CiaRH and ComED (Fig. 7). In this working model, the network is separated into two circuits, CSP dependent and CSP independent. CiaRH and ComED control competence development and *com* gene expression in a hierarchical and cooperative fashion, responding to CSP and a serum-rich environment. We hypothesize that ComED is the primary conduit for sensing of CSP, while CiaH integrates signals detected in serum components and possibly other peptide/protein-rich environments. We also hypothesize the presence of a third response regulator which integrates the signals derived from CiaH. The model does not exclude the possibility that ComE is also a target for CiaH, but evidence we have accumulated to date supports that the effects of inactivation of *ciaH* on stress resistance are, at least in large part, CiaR independent. Also, unlike in strain NG8, the ComCDE pathway is not linked to the stress tolerance and biofilm component of our model. We have not yet integrated the role of processing of the CSP precursor into our model, which has been suggested to be performed by ComA/B but is also possibly influenced by HtrA

levels. It should also be noted that we cannot exclude the existence of the secondary CSP(s).

Clearly, *S. mutans* has evolved to integrate quorum and environmental sensing through TCS, not only for control of the acquisition of exogenous DNA but also as regulatory systems that are critical to expression of key virulence attributes: formation of tenacious biofilms and tolerance of low pH. One reason for maintenance of these pathways for detection of these signals may be to integrate endogenously produced quorum signals, such as ComC, with detection of host molecules. For example, CiaH may modulate gene expression in response to the presence of peptides released from salivary proteins or from other bacteria in the complex biofilms on the tooth surface, or even another peptide quorum-sensing molecule produced by *S. mutans* that is mimicked by serum preparations. Similarly, CiaH could serve to detect serum-specific components present in gingival crevicular fluid or to which the organisms are exposed when they enter the circulation. Identification of the extracellular targets of CiaH and the RR that may be modified by CiaH will be essential to completely dissect the regulatory network so as to understand fully how these systems coordinate competence, biofilm formation, and stress tolerance.

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