ComX activity of *Streptococcus mutans* growing in biofilms

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Received 26 May 2004; received in revised form 26 May 2004; accepted 15 July 2004

First published online 28 July 2004

Abstract

In many streptococci, including *Streptococcus mutans*, genetic competence is regulated by a quorum sensing system mediated by a competence stimulating peptide (CSP) pheromone, encoded by the *comC* gene. In *Streptococcus pneumoniae*, a central component of this system is ComX, which acts as an alternative sigma factor to activate competence genes involved in DNA uptake and processing. The quorum sensing system responsible for genetic competence induction in *S. mutans* has been linked to biofilm formation and the acid tolerance response. To examine the response of *comX* to CSP in *S. mutans*, a transcriptional fusion of the *comX* promoter (*pcomX*) with *lacZ* was constructed to generate reporter vector *pcomX::pALH122* (replicative vector) and transformed into *S. mutans* UA159 *comC**, which is unable to produce endogenous CSP. CSP was added and *pcomX::lacZ* relative expression index (REI) examined, revealing a 2-fold increase in maximal β-gal activity 5 and 10 min after CSP addition. The effect of endogenous CSP on *pcomX::lacZ* expression was also examined by measuring REI in cells grown as a biofilm; peak *pcomX* activity was observed at 3 h. To determine the temporal pattern of transformation frequency, *pMA2*, a Sp r shuttle vector, was transformed into biofilm-grown cells, with maximal transformation frequency observed at 3 h. Confocal microscopy was performed to examine *pcomX* activity using a similarly constructed green fluorescent protein reporter vector, *pcomX::gfp*, in a 4-h biofilm, revealing active *pcomX* activity in high cell density areas within the biofilm population. These results demonstrated a positive correlation between *pcomX* activity, natural transformation and competence development in biofilms.

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Keywords: Competence; Biofilm; *Streptococcus mutans*

1. Introduction

Genetic competence is a physiologic state that enables bacteria to internalize and incorporate foreign DNA into the chromosome. DNA uptake by a bacterium enhances its ability to survive and adapt in the natural environment [1–3]. Genetic competence in many streptococci is governed by a quorum sensing competence stimulating peptide (CSP)-dependent signal transduction system which regulates a range of bacterial adaptive responses to stimuli resulting from environmental changes [4–6]. Quorum sensing is initiated by a CSP, a cytoplasmic membrane integrated histidine kinase sensor and an intracellular response regulator [7,8]. As an effector molecule, the response regulator is involved in the up or downregulation of specific genes, including *comX*.

*Streptococcus mutans* is a major contributor to human dental caries [9]. Under conditions of high carbohydrate intake by the host, acid production by these bacteria can lead to demineralization of the subjacent tooth surface. Bacterial cells are known to alter expression of certain genes in the transition from the planktonic (free living) state to the biofilm environment,
and this would appear to be the case with *S. mutans* in dental plaque [10].

CSP activates expression of several competence genes including those encoding proteins of the DNA processing pathway [11,12]. At least six gene products encoded by *comAB* [12], *comCDE* [13] and *comX* [14] are known to be involved in cell–cell signaling in streptococci. In *S. mutans*, the role of these genes in competence induction and the central role played by CSP in their regulation has been demonstrated [15,13]. *ComA* and *comB* encode a CSP-specific export apparatus comprised of an ATP-binding cassette (ABC) transporter (*ComA*) and its accessory protein (*ComB*), which are involved in the processing and secretion of CSP. *ComC*, *comD* and *comE*, respectively encode the CSP precursor, its histidine kinase sensor protein and the cognate response regulator. *ComC* and *comED* are adjacent located on the chromosome and appear to be divergently transcribed, while *comA* and *comB* are situated in a different area of the chromosome [13].

In *Streptococcus pneumoniae*, ComE activates a number of early competence genes including ComX, an alternate sigma factor that initiates transcription of late competence genes required for DNA uptake and recombination [14]. ComX dependent genes including *ssb*, *dala*, *cel*, *celAB*, *caAB*, and *cina-rec* possess a unique consensus sequence (TACGATAATA) in their promoter regions. [1,16,17–20]. Similarly, the homolog of the comX alternate sigma factor in *S. mutans* is postulated to direct transcription by RNA polymerase of several competence related genes as a result of the CSP-induced signal cascade.

The β-galactosidase (*lacZ*) gene has been extensively used to assay transcriptional activity of several genes in *S. pneumoniae* [21], including measurement of temporal activity of the *pcomX* promoter (*pcomX*) during competence induction [14]. *LacZ* has also been used successfully in *S. mutans* to measure gene expression in controlled conditions [22–24].

The GFP (green fluorescent protein) gene has also recently been used successfully as a reporter gene in *S. mutans* to assay transcriptional activity. As GFP requires no extraneous substrates or cofactors, it has an advantage over other reporter systems in experimental models where diffusion may limit access to a substrate [25,26].

In this study, we examined the effect of CSP on *pcomX* activity in *S. mutans* using *lacZ* and *gfp* gene reporters. The expression of *pcomX*: *lacZ* transcriptional fusions was examined in *S. mutans* UA159 and its *comC* mutant (SMCC3) defective in endogenous CSP production. For the *gfp* reporter, plasmid-based expression of *pcomX*: *gfp* transcriptional fusions was examined in biofilms of *S. mutans* UA159. Finally, the relationship between *comX* expression and transformation frequency in the natural growth cycle was explored in *S. mutans* UA159 planktonic and biofilm cultures using *pcomX*: *lacZ*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

*S. mutans* UA159 and *comC* mutant strains were maintained on Todd–Hewitt agar plates supplemented with 0.3% yeast extract (THYE) (BBL; Becton–Dickinson, Cockeysville, MD) supplemented with erythromycin (10 µg/ml) or spectinomycin (1200 µg/ml) as required. Strains were grown at 37 °C overnight under 5% CO2. A summary of strains and plasmids used is shown in Table 1.

2.2. Construction of strain SMCC3

Strain SMCC3 harboring *pcomX*: *lacZ* was constructed by introduction of a spectinomycin gene into the *comC* locus via allelic exchange. Primer pairs *comC*-F, 5’-GATGATTGGCCGGAACGCTATCAAC-3’ and *comC*-B, 5’-GCTCTAGAGCTCAGAATGCATACAAATGGACGGTTAGGAC-3’ were used to amplify the PCR-Script-*comC* plasmid outward from the flanking regions of *comC*. This generated a linear plasmid containing flanking *S. mutans* DNA without the intact *comC* open reading frame. A spectinomycin resistance cassette (primer pairs *Spe*-F 5’-GCTTGAAGACTCAATATAACGTAACGTGACTGGC-3’ containing an *XbaI* site and *Spe*-B 5’-GACTTAGATGTTGCTGGATAGGACGAG-3’ containing a *SpeI* site) was amplified from pDL277 [27] and ligated to the PCR-Script-*comC* vector via *XbaI* and *SpeI* restriction sites. The new construct pComC2-KO was transformed into Epicurian *E. coli* XL-1 gold cells (Stratagene, La Jolla, CA) for propagation. Transformants were isolated on Luria–Bertani–spectinomycin (100 µg/ml) agar plates. Purified plasmids were digested with *SalI*, transformed into *S. mutans* UA159 and transformants were selected on THYE–spectinomycin (1200 µg/ml) agar.

2.3. Construction of *pcomX*: *lacZ* reporter and *pcomX*: *gfp* vectors

For amplification of the *S. mutans* UA159 *pcomX* region, oligonucleotides were synthesized based upon the nucleotide sequence of *pcomX* and the intergenic region approximately 500 bp 5’ upstream of the promoter region. The *pcomX* amplicon was then cloned into the *S. mutans*–*E. coli* replicative vector pALH122 [23] containing a promoterless *lacZ* reporter gene. Primer pairs (with restriction sites italicized) for cloning into pALH122 were 5’-TCCCCCAGGAGGACTATTACGATGACCTCC-3’ (PrF1 containing a *SmaI* site)
and 5’-TCCCCGGGGGACTATTAGAATGATTGT-3’ (PrB1 containing a SmaI site). This ampiclon was cloned into the SmaI site of pALH122 generated pcmX::pALH122. The insert within pcmX::pALH122 was sequenced to confirm the orientation of the PCR amplified promoter region of the pcmX::lacZ insert. For the transformation frequency assays, pcmC [28] was used as transforming DNA.

To construct the pcmX::gfp reporter vector, a 200 bp region comprising pcmX and sequences 5’ proximal was amplified with appropriately incorporated restriction sites for cloning. Primer pairs were 5’-CGGAATTCCGATCTCCTAAATGT (PrF5 containing an EcoRI site) and 5’-CCCAAGCTTGGGCTATTACGATACCCTCC (PrB6 containing HindIII). The ampiclon was subcloned into the EcoRI/HindIII site of pPROBE-OT [29] upstream of the promoterless gfp cassette to generate pcmX::pPROBE-OT. An ampiclon consisting of the pcmX::gfp cassette was then PCR amplified from pcmX::PROBE-OT’ using primer pairs PrF5 and 5’-ACATGCATGCTCCTGATGCATG (PrB7, incorporating a Sph I site). The amplified pcmX::gfp cassette was cloned into the EcoRI/SphI site of pDL277[27] to generate pcmX::pDL277 and transformed into UA159 to generate strain SMCOM4.

2.4. β-Gal assay in planktonic and biofilm phase

pcmX transcriptional activity in planktonic cells was measured by a slight modification of the β-gal assay using 4-methyl-umbelliferyl β-d-galactopyranoside (MUG) (Molecular Probes, Eugene, OR) as the β-galactosidase substrate [23]. Ten ml cultures grown overnight at 37 °C TYE supplemented with 0.5% glucose (TYG) were pelleted in prewarmed medium and grown to early log phase (OD600 0.15–0.2) and to inoculate 90 ml prewarmed TYG and cells were grown to early log phase. For strain SMCC3 with a comC− background, exogenous CSP (1 µg/ml) was added. For each sampling time, cells were centrifuged briefly, resuspended in TES (10 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.0) and mechanically lysed in a beadbeater (BIO101 FastPrep 120) for 20 s, speed 6. Ten microliter lysate was added to 80 µl AB buffer (60 mM K2HPO4, 40 mM KH2PO4, 100 mM NaCl) in triplicate in microtiter wells (COSTAR black side, clear bottom microtiter plate). Two hundred microliter AB buffer was then added to each well to slow or terminate the substrate reaction to minimum levels for standardization. Fluorescence intensity was measured in a fluorescent plate reader (BioTek FL600 Microplate Fluorescence Reader) at 355 nm excitation, 460 nm emission. Data for each replicate was expressed as mean fluorescence index (MFI), or mean fluorescent units (MFU) per total amount of protein (µg/ml) in 10 µl lysate assayed. A relative expression index (REI) for each sampling time was standardized by dividing mean values of lacZ reporter strains by mean values of wild type controls. Experiments were performed with triplicate independent cultures with the standard error of the mean calculated for triplicate relative expression indices.

### Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)a</th>
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<tbody>
<tr>
<td>S. mutans</td>
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<tr>
<td>UA159</td>
<td>Wt; Sp′ Em′</td>
<td>J. Ferretti, OU-ACGT</td>
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<tr>
<td>SMCC3</td>
<td>UA159::pcomC2-KO; comC′ Sp′ Em′</td>
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<td>SMCOM6</td>
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<tr>
<td>E. coli XL1-Blue</td>
<td>Cloning host</td>
<td>Stratagene</td>
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**Strains and plasmids**

**PCR-script**

- **PCR cloning vector; Amp r**
- **Streptococcus–E. coli**
- **Plasmids**
  - **pcomX::pDL277**
  - **pALH122**
  - **pcomX::pALH122**
  - **pDL277**
  - **pMA2**
  - **pPROBE-OT**
  - **pcmX::pPROBE**
  - **pcmX::pDL277**

**Plasmids**

- **PCR-cloning vector; Amp r**
- **Streptococcus–E. coli**
- **pcomX::gfp**
- **Sp cassette; Sp r**
- **Shuttle vector containing Sp cassette; Sp r**
- **Shuttle vector used as transforming DNA; Sp r**
- **Shuttle vector containing gfp cassette; Sp r**
- **Shuttle vector containing lacZ cassette; Em r**
- **lacZ gene; Amp r**
- **Sp r**
- **Spr Ems**
- **Amp r Spr**

**Reference or source**

- **Stratagene**
- **This study**
- **J. Ferretti, OU-ACGT**
Analysis of β-gal reporter activity of pcomX in biofilm cells of S. mutans UA159 was similar to the measurement of β-gal activity in planktonic phase cells except for the following minor differences. Two hundred microliter of cultures grown overnight in THYE were transferred to 20 ml of 1:4 diluted biofilm medium (THYE with 5 mM glucose) in a standard size petri dish. Cells were grown overnight to allow for biofilm formation. Biofilm medium was then decanted carefully to preserve intact biofilms and the medium was replaced with fresh TYE. Biofilm cells were allowed to grow for 2 h to attain genetic competence. The supernatant was removed, and the competent biofilms cells were scraped and harvested from the petri dish substratum using a rubber scraper before resuspension in TES. The harvested biofilms were then processed for the β-gal assay as previously described.

2.5. Transformation assay

One microgram pMA2/ml [28] was added as the transforming DNA to determine the transformation frequency of the S. mutans UA159 biofilm. Plasmid DNA was added at the same time points used to assay pcomX activity following induction of competence after 2 h biofilm growth. Biofilms were scraped and harvested from the plate, dispersed by brief sonication and resuspended in 2 ml 10 mM KPO4 buffer (PB, pH 7.2). Cells were serially diluted and spread on THYE plates containing appropriate antibiotics (1200 μg/ml spec). After a 48-h incubation, transformation frequency was determined and expressed as the percentage of transformants over total viable recipient cells.

2.6. Confocal scanning laser microscopy observation of pcomX::gfp activity

pComX transcriptional activity was visualized using SMCOM4 biofilms. Thirty microliter of an overnight THYE culture were transferred to 3 ml biofilm medium (1:4 diluted THYE with 5 mM glucose final concentration) in 35 × 10 mm petri dishes. After an overnight incubation, the medium was removed carefully so as not to disrupt the biofilm. Fresh THYE was added and the biofilm incubated for 4 h. Spent medium was decanted and 3 ml 50 mM phosphate buffer was carefully added to the biofilm to enhance fluorescence [21]. The biofilms were observed directly by CSLM with a 100× water immersion lens (LSM 510, Zeiss, Germany).

3. Results

3.1. Effect of exogenous CSP on pcomX expression through β-gal reporter activity

One microliter exogenous CSP was added at time point 0 to planktonic cultures and pcomX transcriptional activity assayed via lacZ reporter expression 5 min before CSP addition and 5, 10 and 15 min after addition of CSP. Data are presented as MFU/total protein (μg/ml) in 10 μl lysate (Fig. 1). β-gal activity for control strain SMCOM1 was stably maintained for all time points assayed (data not shown). The REI was calculated by dividing mean values of strain SMCOM2 (pcomX::lacZ) with mean fluorescent values of strain SMCOM1 (pALH122). Five min and 10 min after CSP addition, REI of strain SMCOM2 increased by about 2-fold, decreasing to near time point 0 and −5 levels 15 min post-CSP addition. (Fig. 1) The mean at 5 and 10 min is almost 25% higher than that of the 0 and −5 time point levels.

3.2. Effect of endogenous CSP on β-gal activity in S. mutans UA159 during growth in biofilms

β-Gal activity was also assayed during growth of S. mutans UA159 during early biofilm development. Biofilms of strain SMCOM1 were grown to competence for 2 h before being pooled and harvested for assays. PcomX activity was determined for the first 10 min followed by samples assayed after 1, 2, 3 and 4 h (Fig. 2(a)). β-gal activity exhibited a significant increase (p = 0.002) of nearly 1.5-fold in REI at 1 h relative to the first 10-min time point. Relative β-gal activity was linear for 3 h post-CSP addition with maximal activity observed at 3 h. The 3 h activity was 3-fold higher than the 0.1 h start point (p < 0.001). Expression reached a plateau after 4 h.

![Fig. 1: Effect of exogenous CSP on pcomX activity in strain SMCOM2. Results are expressed as the relative expression index (REI) and are derived by dividing mean fluorescence values of strain SMCOM2 by mean fluorescence values of control strain SMCOM1. Standard error of the mean of REI was calculated from triplicate independent cultures.](image-url)
3.3. Transformation frequencies of \textit{S. mutans} \textit{UA159} biofilms relative to \textit{pcomX::lacZ} activity

To determine if natural competence development in biofilms reflects a similar temporal trend to expression of \textit{pcomX::lacZ} in strain SMCOM5, transformation frequencies were determined at the same time points used to assay β-gal activity. Biofilms were grown for 2 h to induce competence induction at which point 1 μg/ml pMA2 was transformed into the cells. Transformation frequencies were determined for several time points and plotted (Fig. 2(b)). A greater than 2-fold increase was seen in transformation frequency after the 10-min time point, with a maxima at 3 h with at least a 10-fold and 5-fold increase in transformation frequency relative to the 1 and 2-h time points, respectively. At the fourth hour, a nearly 5-fold reduction in transformation frequency relative to the third hour was observed, suggesting that transformability was maximal at 3 h after induction of competence. This time point coincided with the peak of β-gal activity observed after 3 h in biofilms grown to competence, suggesting a positive correlation between \textit{pcomX} upregulation and increased uptake and expression of transforming DNA in \textit{S. mutans} \textit{UA159} biofilms. A pronounced decrease in transformation frequency relative to the slight increase in \textit{pcomX} expression at 4 h could be due to the continued presence of saturating levels of accumulated CSP. Other physiological mechanisms involved in DNA uptake could be attenuated to conserve cellular energy as the bacterium senses that density dependent uptake of foreign DNA is no longer necessary.

3.4. CSLM of GFP expression of strain SMCOM4 in biofilms

Biofilms of strain SMCOM4 grown on a polystyrene surface were observed under CSLM in situ after 4-h incubation in fresh medium. Control strain SMCOM3 revealed no GFP expression (data not shown). Upregulation of \textit{pcomX} was evidenced by the sporadic expression of GFP visible in small subsets of the population (Fig. 3). Superimposition of the images from CSLM (Fig. 3(b)) over those from differential interference contrast (DIC) microscopy (Fig. 3(a)) revealed variations in fluorescence intensity among cell clusters (Fig. 3(c)). The overall patterns of GFP expression suggested a random distribution of ComX upregulation with peak expression within scattered microclusters of cells.
4. Discussion

ComX is an alternative sigma factor that activates late competence genes which drive processing and integration of foreign DNA to the streptococcal chromosome. It is upregulated in response to CSP-induced activation of the competence cascade. The working model for the CSP regulon in *S. mutans* suggests that when CSP reaches a critical density outside the cell, adjacent cells will detect CSP through a sensor kinase-response regulator involving ComD and ComE, respectively. In its active form, phosphorylated ComE is then believed to induce expression of *comX* [15,13]. The use of a transcriptional fusion of *pcomX* to a *lacZ* gene allowed us to confirm the response of *comX* promoter driven transcription to the presence of active CSP in *S. mutans* and to correlate this with the competent state in biofilms.

We observed an initial peak of *pcomX* transcription at the 5- and 10-min time points following exogenous CSP addition followed by attenuation after 15 min. It has been previously shown that in *S. pneumoniae* naive cells exposed to exogenous CSP there was a peak in *pcomX* mRNA levels after 5 min that continued to the 12-min time point, after which there was a steady reduction to near baseline levels after 15 min [14,19]. Luo and Morrison [30] also demonstrated through Western blot assay using anti-ComX antibody that the ComX protein in *S. pneumoniae* is maximally present at about 10–20 min after CSP addition. The steady reduction in *pcomX* transcriptional activity after 10 min in *S. mutans* UA159 planktonic cultures suggests that maximal transcriptional activity within a short time window of under 10 min appears to be present in both species after competence induction by exogenous CSP. The brief period of *pcomX* transcriptional activity may reflect a conservation of cellular activity so that the minimal requirements for activation of the competence genes are maintained. However, the extended competence window present in biofilms indicates that CSP may mediate many more processes in a biofilm, acting as a biofilm “switch” instead of merely a 10-min event observed during growth in liquid media (planktonic phase).

Approximately 0.03% (3/1000) of biofilm cells were shown to be competent using the natural transformation assay. Using CSLM, a similar low percentage of cells exhibited GFP fluorescence. Significantly, only a small percentage of cells appear to be competent at any given time and take up DNA even under the CSP saturating conditions used. These data suggest that the time of peak transformation frequency observed in tandem with maximal competence induction likely represent parallel events in the competence regulatory cycle and that they occur in a scattered population of biofilm cells. Nevertheless, after a 4-h incubation in fresh medium, the biofilm cells would have produced sufficient amounts of CSP to upregulate *pcomX* transcription. Upregulation of competence genes may be localized to discrete micro-niches within the biofilm where signal transduction mechanisms related to quorum sensing are most active. These subpopulations may represent foci of optimal cell density, metabolic state, growth rate, substrate availability and end product accumulation.

The general trend of a gradual increase in *comX* transcription in the biofilm reflects the stable nature of bacterial communities living as a biofilm. It is possible that an eventual build-up of cell density and its effect on subsequent CSP production and localized *pcomX* activation occurs in a more concerted pattern. The longer time during which *pcomX* expression in biofilms approaches maximal activity when compared with cells in planktonic culture possibly reflects the longer time it takes to achieve the stabilized critical density necessary for competence development in the biofilm.

Fig. 3. CSLM analysis of strain SMCOM4 containing a comX:: gfp reporter plasmid and grown as a 4 h biofilm. A DIC image of a single field of view (a); the same field of view by CSLM fluorescence detection (b); and overlay of DIC image from panel a on CSLM image in panel b (c). Panel c reveals GFP fluorescence in microclusters of high cell density in the biofilm population.
Nevertheless, after a 4-h incubation in fresh medium, biofilm cells in the population should likely have produced sufficient amounts of CSP to upregulate comX transcription. *S. mutans* in vitro biofilms, once well established and stable, are also a highly transformable system with a hyperactive competence induction system, acquiring DNA readily from the local environment [13]. Accumulation of extracellular substrates or macromolecules possibly mitigates immediate dispersion of signal peptides, such as CSP, to neighboring cells, leading to slower competence induction among cells in the biofilm. Future studies of comX expression in *S. mutans* biofilms will address the response of the competent biofilm as a whole to controlled environmental and metabolic changes such as pH and temperature, which can influence biofilm behavior and transformation efficiency.

Acknowledgements

Our work was supported by a Grant from the National Institute of Dental and Craniofacial Research R01DE013230-03, Grant MT-15431 from The Canadian Institutes of Health Research. D.G. Cvitkovitch is supported by a Canada Research Chair. R.P. Ellen is a member of the CIHR group in Matrix Dynamics.

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