RESEARCH LETTER

A novel function for the competence inducing peptide, XIP, as a cell death effector of Streptococcus mutans

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Abstract
In Streptococcus mutans, ComX, an alternative sigma factor, drives the transcription of the ‘late-competence genes’ required for genetic transformation. ComX activity is modulated by inputs from two signaling pathways, ComDE and ComRS, that respond to the competence-stimulating peptide (CSP) and the SigX-inducing peptide (XIP), respectively. In particular, the comRS, encoding the ComR regulatory protein and the ComS precursor to XIP, functions as the proximal regulatory system for ComX activation. Here, we investigated the individual and combinatorial effects of CSP and XIP on genetic transformation and cell killing of S. mutans. Our transformation results confirm the recent reports by Mashburn-Warren et al. and Desai et al. that XIP functions optimally in a chemically defined medium, whereas its activity is inhibited when cells are grown in complex medium. Using tandem mass spectrometry (MS/MS) fragmentation, a drastic reduction in XIP levels in ComX-deficient cultures were observed, suggesting a ComX-mediated positive feedback mechanism for XIP synthesis. Our evaluation of cell viability in the presence of 10 μM XIP resulted in killing nearly 82% of the population. The killing activity was shown to be dependent on the presence of comR/S and comX. These results suggest a novel role for XIP as a compelling effector of cell death. This is the first report that demonstrates a role for XIP in cell killing.

Introduction
The acquisition of novel, heritable DNA by genetically competent bacteria not only propagates antibiotic resistance and virulence determinants, but also shapes bacterial genomes contributing to rapid evolutionary changes (Kroll et al., 1988; Seifert et al., 1988; Feil et al., 1999; Cody et al., 2003; Didelot & Maiden, 2010).

In Streptococcus mutans, an oral resident associated with dental caries, competence development relies on multiple input systems that relay environmental signals to ultimately modulate the transcription of comX, encoding an alternate sigma factor, ComX (SigX) (Li et al., 2001, 2002; Aspiras et al., 2004). In S. mutans, competence does not develop in the absence of ComX, as it is critical for the expression of genes involved in DNA uptake and recombination (Aspiras et al., 2004). Expression of comX was first shown to be regulated by the ComDE two component signaling system comprising of a sensor kinase and a response regulator, respectively, which responds to accumulation of the competence-stimulating peptide (CSP) (Li et al., 2001, 2002; Aspiras et al., 2004). Recently, Mashburn-Warren et al. (2010) identified the ComR regulatory protein of the ComRS signaling pathway as the proximal regulator necessary for comX expression. ComR, in conjunction with its cognate signal peptide, XIP (SigX inducing peptide), modulates comX transcription in S. mutans (Mashburn-Warren et al., 2010). The XIP precursor encoded by comS is consequently exported, processed to its mature form, and then internalized via the Opp/Ami transporter to interact with ComR for comX regulation (Mashburn-Warren et al., 2010; Desai et al., 2012). The loss of ComR abolishes comX expression and competence development, which cannot be restored by the addition of CSP. Furthermore, XIP does not require a functional comE gene to induce...
the expression of *comX* (Mashburn-Warren *et al.*, 2010). These observations highlight the central role of ComRS in the regulation of *comX*.

Previously, it has been demonstrated that *S. mutans* cultures exposed to high CSP concentrations (2–4 μM) cause growth arrest and eventually undergo cell death by lysis (Qi *et al.*, 2005; Perry *et al.*, 2009). In this work, we asked whether synthetic XIP (sXIP) can elicit a similar response to cause cell death of *S. mutans*. Our viability assays revealed that supplementing 10 μM XIP killed approximately 82% of the population. We further report that in addition to the *comRS*, the presence of *comX* is vital for optimal killing. Moreover, we also report the effects of XIP on genetic transformation, which support findings by Mashburn-Warren *et al.* (2010) and Desai *et al.* (2012). Further, using tandem mass spectrometry (MS/MS), we successfully detected the seven amino acid XIP peptide (GLDWWSL) in the wild-type UA159 supernatant, but not in that of the ComS-deficient mutant. While these results concur with those recently reported by Khan *et al.* (2012), we further show that supernatant XIP levels are drastically reduced in ComX-deficient cultures, suggesting a positive role for ComX in ComS/XIP production, export, or processing. Taken together, in addition to its widely discussed role in competence, our work reveals a novel role for XIP as a potent effector of cell death in *S. mutans*, which may be potentially used for the development of therapeutic strategies to prevent dental caries.

**Materials and methods**

**Bacterial strains and growth conditions**

*Streptococcus mutans* UA159 (Ajdic *et al.*, 2002) and its mutant strains in *comC* (ΔASMcomC), *comD* (ΔASMcomD), *comE* (ΔASMcomE) (Li *et al.*, 2001), and *comX* (ΔASMcomX) (Li *et al.*, 2002) were used in this study. The *comS* (ΔASMcomS) and the *comR* (ΔASMcomR) mutants were constructed using a nonpolar ligation PCR mutagenesis method described previously (Lau *et al.*, 2002). *Streptococcus mutans* strains were grown at 37 °C with 5% CO2 in either Todd-Hewitt broth (Becton Dickinson, MD) containing 0.3% yeast extract (Difco Laboratories) (THYE), or chemically defined medium (CDM) described previously (Mashburn-Warren *et al.*, 2010). Erythromycin and spectinomycin were used as needed at concentrations of 10 μg mL−1 and 1 mg mL−1, respectively. sXIP and synthetic CSP (sCSP) peptides were synthesized using F-MOC chemistry (Advanced Protein Technology Centre, Hospital for Sick Kids, Toronto, ON, Canada). Stock concentrations of 1 μM of sXIP and 0.4 mM sCSP were prepared in DMSO and water, respectively. Growth kinetics were monitored using an automated growth reader (BioScreen C; Labsystems, Finland) as previously described (Senadheera *et al.*, 2007).

**Transformation frequency (TF) assays**

Overnight cells grown in THYE were pelleted, washed, and resuspended in phosphate buffered saline (1× PBS). The resuspended culture was diluted 1 : 50 using prewarmed THYE or CDM and grown to an OD600 nm ~ 0.1. Next, 1 μg mL−1 of the donor plasmid DNA (pDL277; specR) (LeBlanc *et al.*, 1992) was added to 1-mL aliquots of the culture in the presence or absence of CSP (0.4 μM) or XIP (10 μM), and samples were incubated for 90 min. For XIP, control cultures containing 1% DMSO were utilized. After incubation, cultures were serially diluted and plated on THYE plates with and without antibiotics. TF was calculated as transformant colony-forming units (CFUs) divided by the total number of viable CFUs, times 100.

**Cell viability assays**

Overnight cultures in THYE were pelleted, washed, resuspended in sterile 1× PBS, and diluted 1 : 50 using warm THYE or CDM. Each suspension was supplemented with either 2 μM CSP or 10 μM XIP. Cultures without peptides or containing 1% DMSO were used as controls. All cultures were grown to an OD600 nm ~ 0.8, at which point, the cells were gently sonicated on ice and used for viability assays. Cells were serially diluted, plated on THYE agar, and CFUs counted. Results standardized using cellular dry weight. These standardized values were then used to calculate the percentage survival by dividing the standardized number of viable cells after treatment by the standardized total number of cells without peptide, times 100.

**Time-course killing analyses**

Overnight cultures of UA159 in THYE were pelleted, washed, resuspended in sterile 1× PBS, and diluted 1 : 20 using warm CDM. The subcultures were allowed to grow to an OD600 nm of 0.4, after which they were split into two where one was exposed to 1% DMSO and the other to 10 μM XIP. Cultures were further incubated, and samples were taken at varying time points (0, 1, 2, 3, 4, and 5 h) after exposure to XIP, gently sonicated, serially diluted, and plated on THYE plates for CFU determination. Results were standardized using cellular dry weight. Percentage viability was calculated as the number of viable cells after treatment divided by the total number of cells without peptide, times 100.
Biofilm formation assays
Overnight cultures in THYE were pelleted, washed, resuspended in sterile 1x PBS, and diluted 1:100 using warm CDM. Each suspension was supplemented with either 1% DMSO or 10 μM XIP and used to inoculate polystyrene plates. After 24-h incubation, the biofilms were dried and stained with 0.1% Safranin Red.

Quantitative real-time PCR (qRT-PCR) analyses
Overnight cultures of UA159 and its derivatives were diluted 20x in fresh THYE or CDM and grown to an OD600 nm of 0.4–0.5 in the presence or absence of 0.4 μM CSP or 10 μM XIP, respectively. For growth in CDM, overnight cells were washed and resuspended in 1x PBS prior to inoculation and harvesting. Controls included THYE without added peptide, as well as CDM with 1% DMSO. RNA isolation, DNase treatment, cDNA synthesis, qRT-PCR, and expression analyses were carried out as previously described (Senadheera et al., 2005). Primers used for qRT-PCR are as follows: comR (For: CGTTTAGGAGTGAGCGCTTGG, Rev: TGTTGGTCGCCATAGGTTG), comS (For: TTTTGATGGGTCTTGACTGG, Rev: TTTATCTGTGCCGTGTAGC) and comX (For: ACTGTGTGTCAAGTCGGG Rev: TGCTCTCTGCTACCGAG). Expression was normalized to that of 16SrRNA gene, and statistical analyses were performed on four independent experiments using Student’s t-test.

XIP detection and quantification
Overnight cultures in CDM were diluted 100-fold and grown for 48 h at 37 °C in 5% CO2 air mixture. Cell-free supernatants were obtained by centrifugation and filter sterilized using a 0.45-μm syringe filter. Samples were lyophilized and, once dry, reconstituted in 2 mL of 5% MeOH/H2O (v/v) prior to analysis by HPLC-ESI-MS/MS (Dionex UltiMate 3000 HPLC system with variable UV detection in line to a Bruker amaZon X ion-trap mass spectrometer operating in positive ionization mode with auto MS/MS enabled). Analytical scale analysis was performed on a 250 × 4.60 mm Phenomenex Luna 5μ C18 (2) 100 Å column (Serial no. 516161-20) with a flow rate of 1 mL min−1 and the following program consisting of solvents A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid): 0–2 min, equilibrium at 5% B; 2–18 min, linear gradient to 100% B; 18–20 min, constant 100% B, 20–20.5 min, linear decrease to 5% B; 20.5–23 min re-equilibration at 5% B. The identity of XIP in culture supernatants was confirmed by comparison with the retention time and MS/MS fragmentation of sXIP. To quantify XIP levels, a directed LC-MS/MS experiment was performed using selected-reaction monitoring (SRM) MS/MS. The SRM m/z transition 876.4 → 658.4 was monitored, corresponding to a –SL loss from the GLDWWSL parent ion, generating a GLDWW daughter ion. Resulting peak areas were integrated, and final concentrations calculated from a linear calibration curve created using CDM spiked with sXIP and processed in an identical way to cell free supernatants. Peak areas from each sample were standardized to optical densities of 48-h culture samples prior to centrifugation for HPLC-MS analysis. Results were obtained for four independent experiments, and statistics were conducted using the Student’s t-test.

Fig. 1. TF of Streptococcus mutans strains in THYE and CDM growth media. Streptococcus mutans UA159 and mutant strains were subcultured to an OD600nm of 0.1 in either THYE (a) or CDM (b). Plasmid DNA pDL277 (spec+) was added alone or with 0.4 μM sCSP or 10 μM sXIP for transformation experiments. Results show the mean TF of three independent experiments ± standard error.
Results

TF with CSP and/or XIP in CDM and complex media

The initial finding that XIP induces genetic transformation via ComX was reported by Mashburn-Warren et al. (2010) using cells grown in CDM. Recent work by Desai et al. (2012) reported that the induction of comX by XIP was largely inhibited when grown in rich nutrient Todd Hewitt Broth (THB), a medium commonly used to study CSP-induced competence. In accordance with these reports, our TF assays show that XIP is optimally functional in CDM in eliciting transformation and its activity is inhibited when cells are grown in complex medium (i.e., THYE) (Fig. 1). In contrast, we observed that CSP was largely ineffective at inducing competence in CDM and that it was optimally functional in complex medium (Fig. 1). As CSP and XIP were shown not to function optimally in the same growth medium, we did not obtain significant combinatorial effects in either THYE or CDM (data not shown).

Measurement of XIP in the culture supernatants of S. mutans UA159, comR/S, comE, and comX knockout mutants

To elucidate the role of known S. mutans competence genes in the regulation of XIP production, its processing, and/or secretion, we used HPLC-ESI-MS/MS to monitor extracellular XIP levels in comR/S, comE, and comX-deficient mutants. We were able to successfully identify the presence of XIP in the wild-type supernatant by comparison of the retention time and of the fragmentation patterns to the sXIP standard (Fig 2a and b). We were able to detect XIP at concentrations ranging from 95 to 750 ng mL\(^{-1}\) (or 109–857 nM), and consistent with the loss of transformability \(\Delta\)SMcomS, XIP was absent in their cell-free supernatants (Fig. 2c). These results are in accordance with that of Khan et al. (2012) who also reported their inability to detect mature XIP in culture supernatants of the ComS mutant. As expected of a positive regulator of comS expression, \(\Delta\)SMcomR also displayed highly reduced levels of XIP. Our further
quantification of XIP in the ComX and ComE mutants suggested a significant decrease ($P < 0.05$) of this peptide in the ΔSMcomX supernatant, whereas it was significantly increased in the ΔSMcomE supernatant (Fig. 2c). These results suggested that while ComX positively influenced the production, processing and/or secretion of XIP, the ComDE two-component system negatively affected one or more of these processes in *S. mutans*.

**Effect of XIP on cell viability of *S. mutans* UA159 grown in CDM**

While investigating the effects of sXIP on genetic transformation, we noted that growth of UA159 was drastically impaired by the addition of 10 μM XIP in CDM (Fig. 3a). As this indicated a likely effect on cell death, we performed cell viability assays to determine whether XIP could act as a death effector of *S. mutans*. In the presence of 10 μM XIP in CDM, we observed only an 18% survival rate relative to the no-peptide control, suggesting that XIP can function as a potent killing peptide under these conditions (Fig. 3b). XIP was unable to induce killing in ΔSMcomR and was also largely ineffective in ΔSMcomX, demonstrating the importance of both comR and comX in the killing activity of XIP. Furthermore, in ΔSMcomS and ΔSMcomC grown in CDM exposed to XIP, we noted 80% and 89% killing, respectively (Fig. 3b). In contrast to CDM, XIP was not able to induce killing when *S. mutans* strains were grown in THYE. To confirm the effect of XIP on cell viability, time-course killing analyses were performed, which demonstrated a negative effect of XIP on the CFU counts of

![Graph](image1)

**Fig. 3.** Effect of CSP and XIP on cell survival of *Streptococcus mutans*. (a) The effect of XIP at varying concentrations (10, 1, 0.5, 0.25 μM, 1% DMSO) on the growth of *S. mutans* UA159 was examined by monitoring OD$_{600}$nm using the Bioscreen C automated growth reader (b) *Streptococcus mutans* UA159, as well as comR, comS, comX, and comC knockout mutant strains, were grown in the presence or absence of either XIP (10 μM) or CSP (2 μM) to OD$_{600}$nm = 0.8 in THYE (top panel) or CDM (bottom panel). Viable CFUs were counted, and results were standardized to the dry weight and presented as the percentage of the CFUs obtained in the absence of the peptide. Results shown here are the average of four independent experiments ± standard error. Statistical analyses were performed using Student’s t-test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (c) Actively growing cultures of UA159 were exposed to 10 μM XIP for 0, 1, 2, 3, 4, and 5 h. Following sonication, viable CFUs were counted, and results were standardized to the dry weight and presented as the percentage of the CFUs obtained in the absence of the peptide. Results shown here are the average of four independent experiments ± standard error. Statistical analyses were performed using Student’s t-test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (d) *Streptococcus mutans* biofilms were grown on polystyrene plates overnight in the presence or absence of 10 μM XIP and stained with 0.1% Safranin Red. The image presented here is a representative of four independent experiments.
Regulation of comR/S, and comX expression by XIP, CSP, and the ComDE system

Our transformation and viability results, as well as that obtained by Mashburn-Warren et al. (2010) and Desai et al. (2012), strongly suggest that the ComCDE system may regulate comX transcription through ComRS, although this was not directly tested. Hence, we examined comR/S and comX transcription in UA159, ΔSMcomD, and ΔSMcomE strains grown with and without CSP or XIP. Owing to the poor activity of CSP in CDM and no activity of XIP in THYE, experiments with CSP were performed in THYE, whereas those with XIP were conducted from cells grown in CDM. Supporting a hierarchical position of the ComCDE system upstream of ComRS, we observed that addition of CSP increased comS and comX expression by 73.9-fold and 2.3-fold, respectively (Fig. 4a). In THYE without added CSP, comR/S and comX expression was not significantly affected by loss of comD/E relative to wild type (Fig. 5a). However, with CSP, expression of comS was significantly decreased over 100-fold in both mutants (P < 0.001), relative to wild type (Fig. 5b). Addition of CSP also decreased comX expression by nearly 30-fold in ΔSMcomD and ΔSMcomE strains, respectively, compared with the parent (Fig. 5b). These results suggested that in complex medium, comS expression can be modulated by adding CSP and that comS induction by the CSP is ComDE dependent.

In wild type, addition of sXIP increased expression of comX and comS by 83-fold and 141-fold, respectively (Fig. 5b), thus confirming the autoregulatory loop described by Mashburn-Warren et al., 2010. In ΔSMcomD and ΔSMcomE grown in CDM, comS and comX genes were upregulated almost threefold without added peptide, likely suggesting that ComDE may repress their expression in CDM medium (Fig. 5c). This finding was also supported by the high levels of XIP detected in the ΔSMcomE culture supernatant. Further, upon addition of sXIP to ΔSMcomD and ΔSMcomE mutants, no change in comR and comS expression was observed (Fig. 5d), suggesting that the ComDE system does not affect XIP signaling, once the ComRS system is activated.

Discussion

Competence has been observed in a number of bacteria to occur in conjunction with lysis of a subpopulation of cells (Steinmoen et al., 2002; Claverys et al., 2007; Perry et al., 2009; Lemme et al., 2011). The lysed subpopulation is thought to contribute to the genetic pool used for DNA uptake by the competent cells. Herein, we have demonstrated a role for the XIP competence peptide as a potent modulator of cell death in S. mutans. Our viability assays show XIP can kill nearly 82% of the population when supplied at a concentration of 10 μM. To our knowledge, this is the first report that demonstrates a function for XIP as an effector of cell death.

We further report that XIP-mediated killing works via the ComR/S system and ComX, which positions the ComR/S and ComX in a more centralized position in the killing pathway of S. mutans. Although previous reports...
have attributed CSP-induced lysis to an imbalance between the ComE-regulated mutacin V and its immunity protein ImmB (Perry et al., 2009; Dufour et al., 2011; Lemme et al., 2011), here we argue that competence-associated cell death in \( S. mutans \), is instead, largely owing to activity downstream of ComX. This is also supported by the fact that \( nlmC \) (synonyms: \( cipB \) and \( bsmA \)) encoding mutacin V also modulates \( \text{comX} \) activity, which in turn, may contribute to its killing activity (Dufour et al., 2011). We are currently examining genes downstream of ComX stimulated by XIP that may function as killing effectors using global transcriptome analysis.

Although the killing activity of CSP harbors specificity toward its parent strain (Qi et al., 2005), the spectrum of activity of XIP has yet to be determined. XIP contains a double-tryptophan (WW) motif conserved among short hydrophobic peptides of the pyogenic and bovis groups of \( \text{Streptococcus} \), located within a conserved genomic context (Mashburn-Warren et al., 2010). Similar peptides specific for \( \text{Streptococcus agalactiae} \), \( \text{Streptococcus porcinus} \), and \( \text{Streptococcus parauberis} \) have been shown to bear no effect on competence or growth of \( S. mutans \), suggesting that these peptides may be specific to their parental strain (Desai et al., 2012). XIP therefore may be exploited for targeted killing of \( S. mutans \).

Our transformation and cell viability results with CSP and/or XIP in both THYE and CDM media showed that these peptides do not function optimally under the same conditions. Our transformation results are in agreement with Desai et al. (2012) who reported that titration of THB into UA159 cultures in CDM inhibited XIP-induced transformability. While they demonstrated some level of activity of XIP in 100% THB, our results showed complete inhibition of XIP in THYE. It is likely that the yeast extract in THYE is largely responsible for the inhibition observed.

Despite the observation that XIP is inactive in THYE-grown cells, the \( \text{comS} \) gene is still required for
transformation despite growth in a complex medium. Hence, it is possible that the ComS peptide may also function intracellularly without its export and subsequent import into the cell. We have also taken into consideration that conditions tested in complex medium may not be optimal for the expression of the XIP exporter, which can likely result in the accumulation of ComS inside the cell, making it vulnerable to intracellular cleavage.

Our expression analysis combined with LC-MS/MS in CDM demonstrates a negative-regulatory role for the ComDE system in XIP production. Kreth et al. (2007) reported that ComDE repressed comC expression prior to CSP stimulation. It is possible that ComDE may prevent premature expression of comS, thereby delaying competence induction in CDM to the latter stages of growth. As observed by Desai et al. (2012), competence in CDM is first observed in mid-logarithmic cells of S. mutans and continues well into the stationary phase.

We further observe that the amount of XIP was significantly reduced in ΔSMcomX, suggesting a ComX-mediated positive feedback mechanism for XIP synthesis. Putative ComX binding sites were located within the comR gene, upstream of comS, suggesting that ComX may directly regulate comS expression (Fig. 6a). This positive autoregulation of XIP production may contribute to the persistence of the competent state in CDM. Based on previous works and our findings presented here, we propose a growth condition–dependent model for genetic competence in S. mutans (Fig. 6b).

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