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A novel function for the competence inducting peptide, XIP, as a cell death effector of *Streptococcous 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 (CDM), 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 10μM XIP resulted in the 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 (Cody *et al.*, 2003; Didelot & Maiden, 2010; Feil *et al.*, 1999; Kroll *et al.*, 1988; Seifert *et al.*, 1988). 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; Aspiras *et al.*, 2004). In *S. mutans*, competence does not develop in the absence of ComX, as it is critical for the

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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; 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. mutan*s cultures exposed to high CSP concentrations (2–4 μM) cause growth arrest and eventually undergo cell death by lysis (Perry *et al.* 2009, Qi *et al.* 2005). In this work, we asked whether synthetic XIP can elicit a similar response to cause cell death of *S. mutans*. Our viability assays revealed that supplementing 10μM XIP killed approximately 82% of the population. We further report that in addition to the *comR/S,* 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*. (2011) and Desai *et al.*, (2012). Further, using tandem mass spectrometry, we successfully detected the 7 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

S. mutans UA159 (Ajdic *et al.*, 2002) and its mutant strains in *comC* (ΔSMcomC), *comD* (ΔSMcomD), *comE* (ΔSMcomE) (Li *et al.*, 2001a) and *comX* (ΔSMcomX) (Li *et al.*, 2002) were used in this study. The *comS* (ΔSMcomS) and the *comR* (ΔSMcomR) mutants were constructed using a non-polar ligation PCR mutagenesis method described previously (Lau *et al.*, 2002). *S. mutans* strains were grown at 37°C with 5% CO_2 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 10μg/mL and 1mg/mL, respectively. Synthetic XIP (sXIP) and synthetic CSP (sCSP) peptides were

synthesized using F-MOC chemistry (Advanced Protein Technology Centre, Hospital for Sick Kids, Toronto, Canada). Stock concentrations of 1μM of sXIP and 0.4mM 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 (1x PBS). The resuspended culture was diluted 1:50 using prewarmed THYE or CDM and grown to an OD_{600} ~0.1. Next, 1μg/mL of the donor plasmid DNA (pDL277; $spec^R$) (LeBlanc *et al.*, 1992) was added to 1mL aliquots of the culture in the presence or absence of CSP (0.4μM) or XIP (10μM) 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 one hundred.

Cell Viability Assays

Overnight cultures in THYE were pelleted, washed, resuspended in sterile 1X PBS, and diluted 1:50 using warm THYE or CDM. Each suspension was supplemented with either 2μM CSP or 10μM XIP. Cultures without peptides or containing 1%DMSO were used as controls. All cultures were grown to an OD_{600}~0.8, at which point, the cells were gently sonicated on ice and used for viability assays. Cell 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 1X PBS, and diluted 1:20 using warm CDM. The subcultures were allowed to grow to an OD_{600} of 0.4, after which they were split into two where one was exposed to 1% DMSO and the other to 10μM XIP. Cultures were further incubated and samples were taken at varying time points (0h, 1h, 2h, 3h, 4h and 5h) 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 10μM XIP and used to inoculate polystyrene plates. After 24h incubation, the biofilms were dried and strained 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 OD_{600} of 0.4–0.5 in the presence or absence of 0.4μM CSP or 10μM XIP, respectively. For growth in CDM, overnight cells were washed, resuspended in 1xPBS 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: *comR* (For: CGTTTAGGAGTGACGCTTGG, Rev: TGTTGGTCGCCATAGGTTG), *comS* (For: TTTTGATGGGTCTTGACTGG, Rev: TTTATTACTGTGCCGTGTTAGC) and *comX* (For: ACTGTTTGTCAAGTCGCGG Rev: TGCTCTCCTGCTACCAAGCG). Expression was normalized to that of 16SrRNA, and statistical analyses were performed on four independent experiments using Student's t-test (P <0.05).

XIP detection and quantification

Overnight cultures in CDM were diluted 100-fold and grown for 48h at 37°C in 5% CO_2 air mixture. Cell-free supernatants were obtained by centrifugation and filter sterilized using a 0.45μm syringe filter. Samples were lyophilized and, once dry, reconstituted in 2 mL of 5% MeOH/H_2O (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 250x 4.60 mm Phenomenex Luna 5μ C18(2) 100Å 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, equilibration 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 to 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) tandem mass spectrometry. 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 CFSs. 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.

RESULTS

Transformation frequencies (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 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). Since 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 2(a) and (b)). We were able to detect XIP at concentrations ranging from 95 ng/mL to 750 ng/mL (or 109nM to 857nM), and consistent with the loss of transformability ΔSMcomS, XIP was absent in their cell free supernatants (Fig. 2(c)). 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, ΔSMcomR 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. 2(c)). 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 10μM XIP in CDM (Fig. 3a). Since 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 10μM 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 healthy cultures at varying time points (Fig. 3c). Furthermore, *S. mutans* was not able to form biofilms in the presence of XIP (Fig. 3d). This drastic effect on biofilm development may be attributed to XIP's drastic effect on the viability of cells. These results suggest an important role for XIP as a novel killing peptide that can be targeted to kill *S. mutans*.

Similar to lysis by XIP, CSP-induced cell death was also largely diminished in the absence of *comR/S* or *comX* (Fig. 3), suggesting that the CSP-induced killing pathway previously described requires the presence of *comR/S* and *comX* for optimal killing.

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. Due 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 hierarchal 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. 4(a)). In THYE without added CSP, *comR/S*, and *comX* expression was not significantly affected by loss of *comD/E* relative to wild-type (Fig. 5(a)). However, with CSP, expression of *comS* was significantly decreased over 100-fold in both mutants ($p<0.001$), relative to wild-type (Fig. 5(b)). Addition of CSP also decreased *comX* expression by nearly 30-fold in ΔSMcomD and ΔSMcomE strains, respectively, compared with the parent (Fig. 5(b)). 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. 5(b)), 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 3-fold 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 (Claverys *et al.*, 2007; Steinmoen *et al.*, 2002; 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 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 10μM. 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 (Lemme *et al.*, 2011; Perry *et al.*, 2009; Dufour *et al.*, 2011), here we argue that competence-associated cell death in *S. mutans*, is instead, largely due to activity downstream of ComX. This is also supported by the fact that *nlmC* (synonyms: *cipB, bsmA*) encoding mutacin V also modulates *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 towards 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 *Streptococci*, located within a conserved genomic context (Mashburn-Warren *et al.*, 2010). Similar peptides specific for *Streptococcus agalactiae*, *Streptococcus porcinus*, and *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 *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* (Figure 6b).

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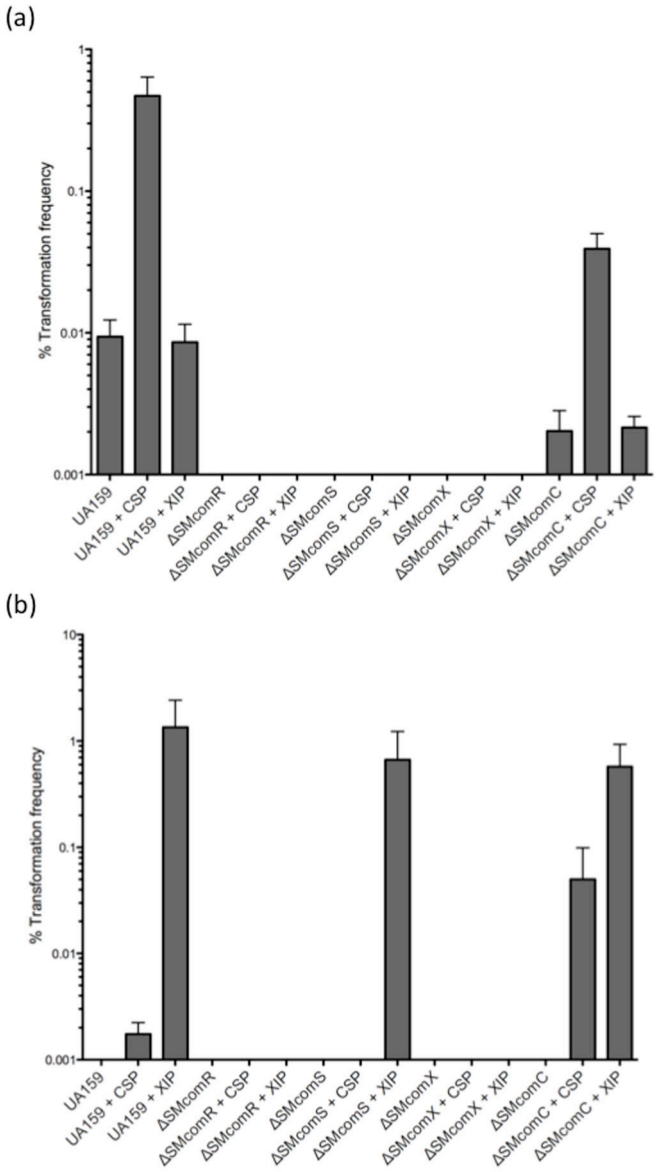

Fig. 1. Transformation frequency (TF) of *S. mutans* strains in THYE and CDM growth media

S. mutans UA159 and mutant strains were subcultured to an OD_{600} of 0.1 in either THYE (a) or CDM (b). Plasmid DNA pDL277 ($spec^r$) was added alone or with 0.4μM sCSP or 10μM sXIP for transformation experiments. Results show the mean TF of three independent experiments ± standard error.

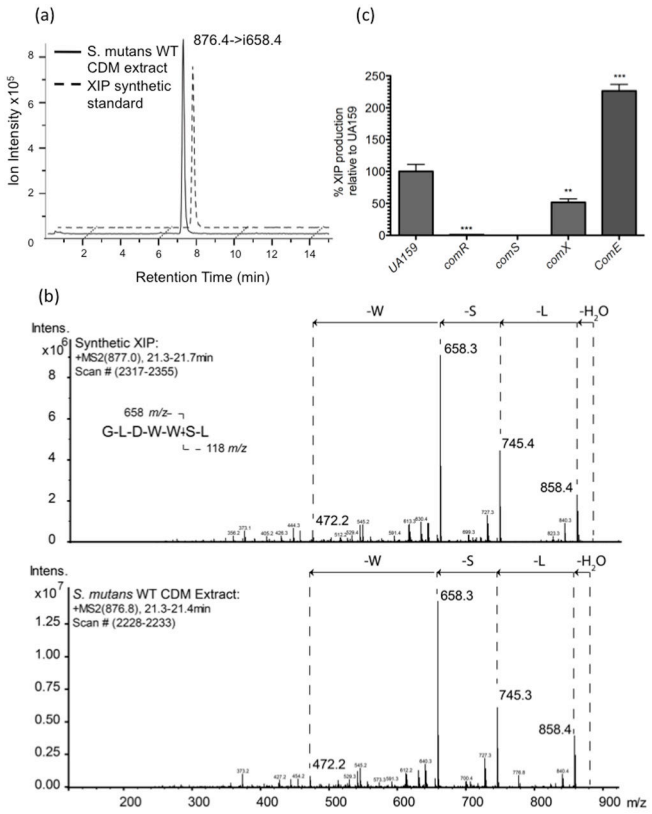

Fig. 2. Secretion of XIP by *S. mutans* UA159 and *comX*-, *comR*- and *comS*-mutant strains

(a) Chromatograms resulting from selected-reaction monitoring (SRM) LC-MS experiments used for extraction and quantification of XIP. A transition corresponding to the loss of S and L residues from the XIP peptide parent ion at 876.4m/z, generating a daughter ion at 658.4 m/z was monitored. (b) A comparison of MS2 fragmentation pattern of authentic standard XIP peptide (top panel) and wild-type *S. mutans* sample (bottom panel). Specific amino acid and water losses are annotated. (c). XIP levels in the supernatant of each strain in stationary phase were quantified using HPLC-ESI-MS/MS. Results shown represent those from at least 3 separate experiments, error bars indicate standard deviation. Statistical analyses were performed using a Student's T-test: *p<0.05.

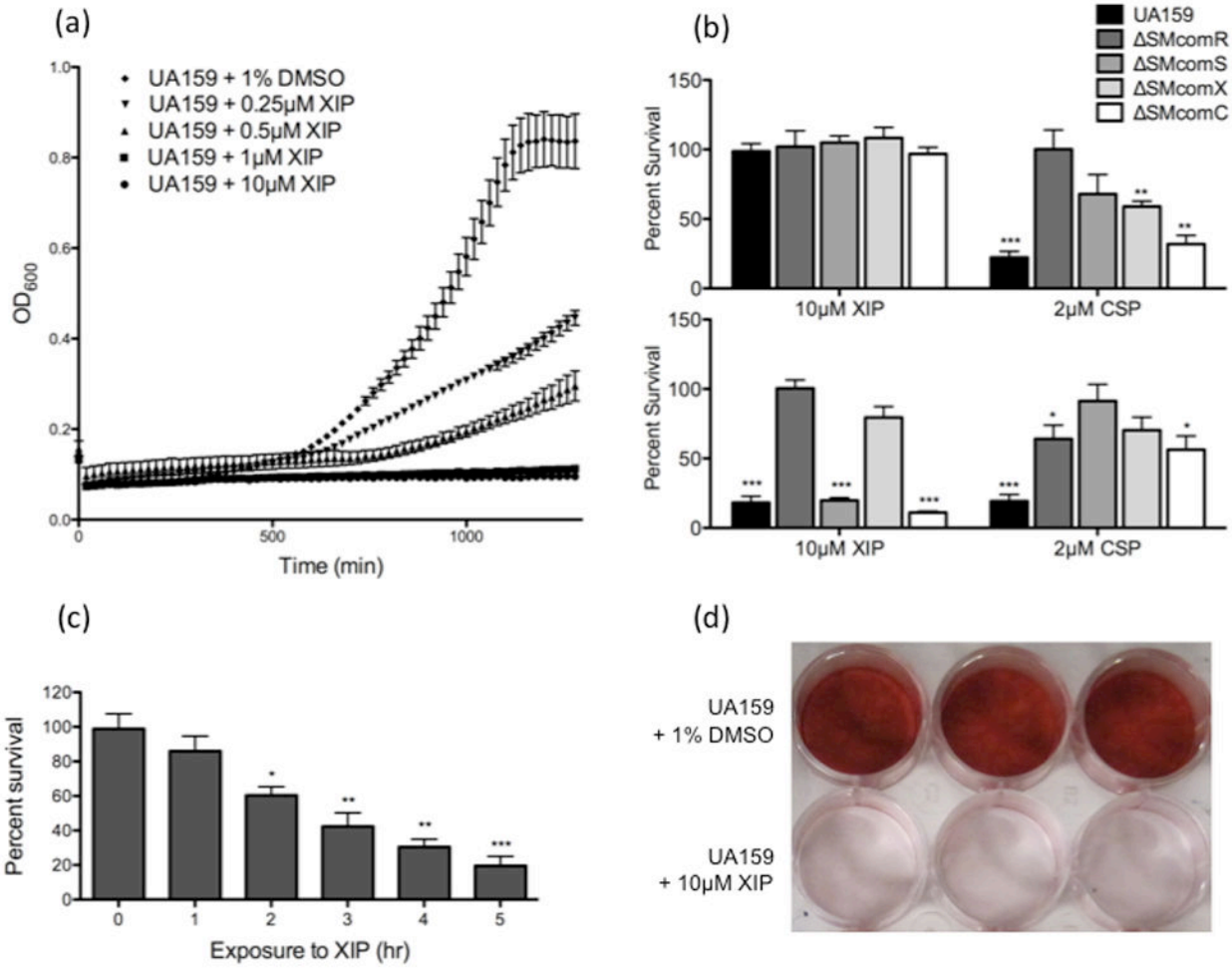

Fig. 3. Effect of CSP and XIP on cell survival of *S. mutans*

(a). The effect of XIP at varying concentrations (10μM, 1μM, 0.5μM, 0.25μM, 1% DMSO) on the growth of *S. mutans* UA159 was examined by monitoring OD_{600} using the Bioscreen C automated growth reader (b). *S. mutans* UA159, as well as *comR*, *comS*, *comX* and *comC* knockout mutant strains were grown in the presence or absence of either XIP (10μM) or CSP (2μM) to OD_{600}~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 10μM XIP for 0, 1, 2, 3, 4 and 5h. 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). *S. mutans* biofilms were grown on polystyrene plates overnight in the presence or absence of 10μM XIP, and stained with 0.1% Safranin Red. The image presented here is a representative of four independent experiments.

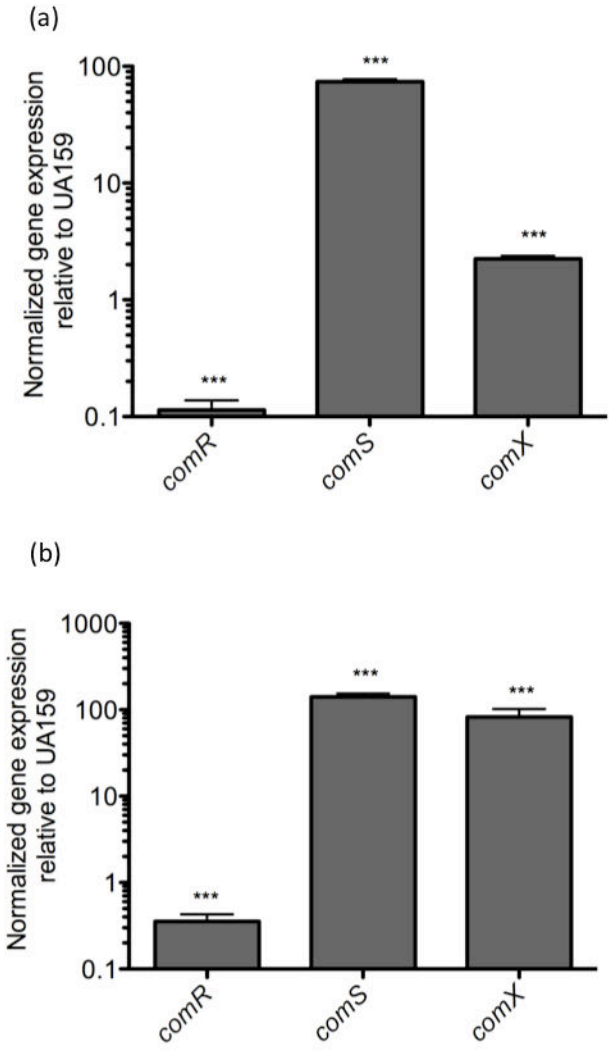

Fig. 4. Gene expression of *comR*, *comS* and *comX* in response to CSP and XIP

Real-time analysis of gene expression of *comR*, *comS* and *comX* were preformed using mid-logarithmic UA159 cells grown in THYE (a) and CDM (b) in the presence or absence of either 0.4μM CSP (a) or 10μM XIP (b). Gene expression was normalized to UA159 with no peptide added. Results shown here are the average of at least three independent experiments ± standard error. Statistical analyses were performed using Student's T-test: ***p<0.001

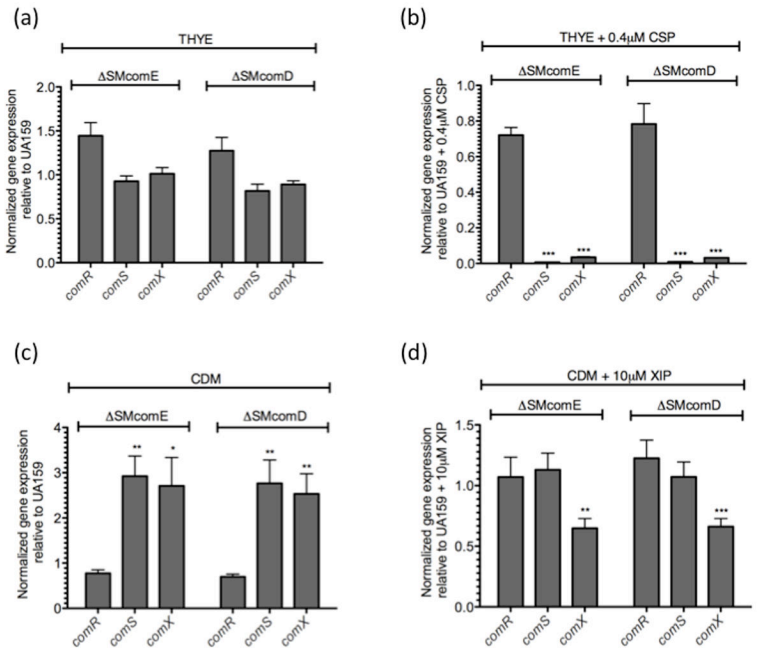

Fig. 5. Differential regulation of *comRS* in THYE and CDM media grown in the presence or absence of CSP and XIP, respectively

Real-time analysis of *comR*-, *comS*- and *comX*-specific expression in *comD*- and *comE*-knockout mutants grown in THYE (a) and CDM (c) media, as well as in the presence of either 0.4μM CSP (b) or 10μM XIP (d). Gene expression was normalized with 16SrRNA and fold-expression was calculated relative to that of UA159 held at a user-defined value of 1.0. Results shown are the mean expression values of at least three independent experiments ± standard error. Statistical analyses were performed using Student's T-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

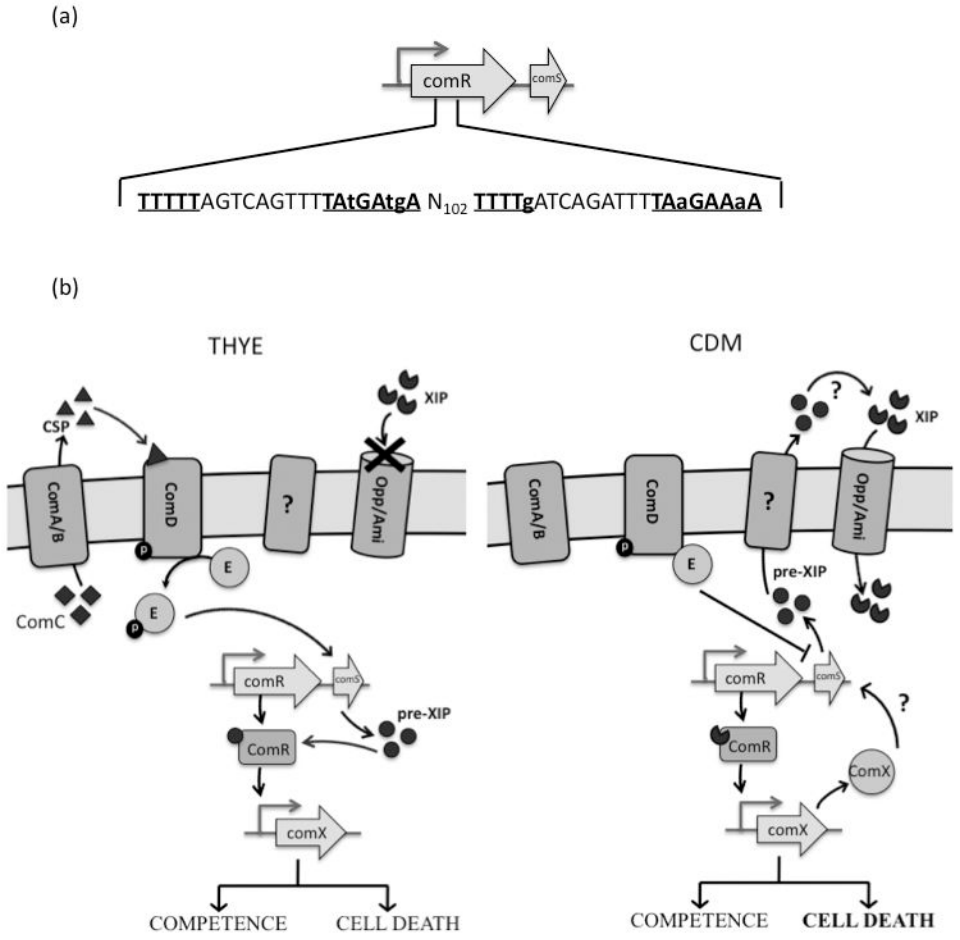

Fig. 6. Model for competence and cell killing pathways of *S. mutans* in THYE versus CDM

(a). Two putative ComX binding sites within the *comR* gene, located 102bp apart are underlined and shown in bold font (b). In complex medium (THYE), CSP is sensed by the ComDE system, which stimulates the expression of *comS*, leading to an intracellular accumulation of the ComS peptide or its cleavage products, which in turn, can activate ComR to induce *comX* expression. In CDM, the ComDE system represses *comS* expression. This repression is released by an unknown mechanism to upregulate *comS*, whose product is then secreted, processed and imported by the Opp/Ami transporter to activate *comX* transcription, as first described by Mashburn-Warren *et al* (2010). ComX positively regulates XIP production, which may contribute to the persistence of the competent state in CDM, as described by Desai *et al* (2012).