Coordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species

Jens Kreth[1], **Justin Merritt**[1], **Wenyuan Shi**[1,2], and **Fengxia Qi**[1,*]

1 *Department of Oral Biology and Medicine, UCLA School of Dentistry, P.O. Box 951668, Los Angeles, CA 90095-1668, USA.*

2 *UCLA Molecular Biology Institute, Los Angeles, CA 90095, USA.*

Abstract

It is important to ensure DNA availability when bacterial cells develop competence. Previous studies in *Streptococcus pneumoniae* demonstrated that the competence-stimulating peptide (CSP) induced autolysin production and cell lysis of its own non-competent cells, suggesting a possible active mechanism to secure a homologous DNA pool for uptake and recombination. In this study, we found that in *Streptococcus mutans* CSP induced coordinated expression of competence and mutacin production genes. This mutacin (mutacin IV) is a non-lantibiotic bacteriocin which kills closely related Streptococcal species such as *S. gordonii*. In mixed cultures of *S. mutans* and *S. gordonii* harbouring a shuttle plasmid, plasmid DNA transfer from *S. gordonii* to *S. mutans* was observed in a CSP and mutacin IV-dependent manner. Further analysis demonstrated an increased DNA release from *S. gordonii* upon addition of the partially purified mutacin IV extract. On the basis of these findings, we propose that *Streptococcus mutans*, which resides in a multispecies oral bio-film, may utilize the competence-induced bacteriocin production to acquire transforming DNA from other species living in the same ecological niche. This hypothesis is also consistent with a well-known phenomenon that a large genomic diversity exists among different *S. mutans* strains. This diversity may have resulted from extensive horizontal gene transfer.

Introduction

Competence is a physiological state in which bacteria develop a capacity to take up exogenous DNA (Dubnau, 1991a). Competence development is an elaborate process involving multiple protein components and sophisticated regulatory networks (Dubnau, 1991b; Morrison, 1997; Morrison and Lee, 2000; Hamoen *et al*., 2003; Dagkessamanskaia *et al*., 2004). It is important to ensure that a DNA pool is available when the cells become competent. In a recent study by Steinmoen *et al*. it was shown that development of competence in *Streptococcus pneumoniae* triggers cell lysis and DNA release from a subfraction of the cell population (Steinmoen *et al*., 2002). Further studies demonstrated that competence-induced cells of *S. pneumoniae* lyse competence-deficient cells of the same species during cocultivation (Steinmoen *et al*., 2003). This competence-induced cell lysis has been suggested as a means to provide the competent cells with a donor DNA pool from its own species.

Streptococcus mutans is an oral member of the *Streptococcus* genus. It lives in a multispecies biofilm commonly known as the dental plaque with over 500 other bacterial species (Moore and Moore, 1994; Kroes *et al*., 1999; Paster *et al*., 2001). In addition to having a natural competence system similar to that of *S. pneumoniae* (Li *et al*., 2001), *S. mutans* also produces

a plethora of bacteriocins, which are small peptide antibiotics that kill closely related species (Hamada and Ooshima, 1975; Caufield *et al*., 1985; Baca *et al*., 1990; Bondi *et al*., 1991; Baba and Schneewind, 1998). Our laboratory has been interested in understanding the mechanism of bacteriocin gene regulation in *S. mutans* and its function in interspecies interaction. In this study, we found that the production of one of the bacteriocins, mutacin IV (Qi *et al*., 2001), was linked to the competence development system in *S. mutans*. Further analysis of this interesting connection has led to the hypothesis that *S. mutans* may be able to acquire DNA from other streptococcal species that live in close proximity.

Results

High level mutacin IV gene expression was induced in the pellet of early log phase cells

In previous studies we routinely observed higher mutacin IV production during the transition between late log and early stationary phase (Qi *et al*., 2001). To further investigate the mechanism of mutacin IV gene regulation, we constructed mutacin IV promoter (*nlmA*) fusions to the luciferase (*luc*) and green fluorescent protein (*gfp*) reporters in strain UA140 (Kreth *et al*., 2004). To quantify *nlmA* gene expression as a function of time, an overnight culture of UA140::Ø(*nlmAp-luc*) was diluted 1:20 into fresh Brain Heart Infusion (BHI) broth and incubated anaerobically as a batch culture. Starting at the early log phase ($OD_{600} \sim 0.1$), culture samples were taken at designated time intervals and luciferase activity was measured. As shown in Fig. 1A, after normalization to cell density, the expression level of *nlmAp-luc* was low throughout log phase (time 0–270 min). However, the expression started to increase at the late log phase (at 330 min) and reached its peak at the transition from the log to the stationary phase (at 390 min). The peak level activity remained for ~60 min and then started to decline. At the peak level, *nlmA-luc* activity was ~fivefold higher than that at the log phase. A similar pattern was observed when cells were incubated in TH broth (data not shown). These results suggest that mutacin IV gene expression is temporally regulated at the transcriptional level.

To distinguish whether the observed regulation is due to a higher cell density at the early stationary phase, or it is part of a stationary phase phenomenon, we simulated a high cell density environment by centrifuging cells growing in early log phase ($OD_{600} \sim 0.1$) when the expression of *nlmA* was normally low, creating a cell pellet. As a control, a portion of the same culture was kept in planktonic phase. The planktonic culture and the cell pellet were then incubated at 37°C as static cultures, and samples were taken at designated time points to measure luciferase activity. As shown in Fig. 1B, luciferase activity in the centrifuged culture increased ~fivefold after 30 min of incubation compared with cells in planktonic phase. By 60 min after centrifugation, luciferase activity increased nearly 20-fold, reaching a relative light unit (RLU)/OD_{600} of ~2500. This high activity remained nearly unchanged until the end of the experiment (120 min). In contrast, luciferase activity of the planktonic culture remained low and unchanged through the entire experimental period (120 min). The OD_{600} of the pelleted culture was ~0.2 after 120 min of incubation while that of the planktonic culture was ~0.4. These results suggest that either high cell density or nutrient limitation or both in the cell pellet may have triggered the high level mutacin IV gene expression.

We also performed experiments to directly visualize the high-level *nlmA* gene expression in the cell pellet. Strain UA140::Ø(*nlmAp–gfp*), which carried a *nlmAp–gfp* fusion, was subjected to a similar pelleting assay. After 2 h of incubation, cells from both planktonic and pelleted cultures were examined under a fluorescence microscope. As shown in Fig. 1C, cells from the pelleted culture displayed bright green fluorescence, even in some single cells. In contrast, cells from the planktonic culture showed only weak fluorescence in cell aggregates (Fig. 1D). These results further confirmed the high-level mutacin IV gene expression in the cell pellet as observed with the luciferase reporter fusion.

The ComDE two-component system regulated mutacin IV gene expression

To explore the molecular mechanism of the high-level mutacin IV gene expression in the cell pellet, we examined the involvement of quorum-sensing systems in mutacin IV gene expression. Two major quorum-sensing systems have been described in *S. mutans.* The LuxS system is an interspecies quorum-sensing system, which has been shown to be involved in biofilm formation and stress tolerance of *S. mutans* (Merritt *et al*., 2003; Wen and Burne, 2004). The ComDE system is a intraspecies quorum-sensing system that has been known to be required for competence development (Li *et al*., 2001). In addition to controlling competence, the ComDE system was also shown to be involved in biofilm formation of *S. mutans* (Li *et al*., 2002; Yoshida and Kuramitsu, 2002). To determine whether mutacin IV gene expression was regulated by either of the two systems, we transferred previously constructed *luxS* and *comD* null mutations into the luciferase reporter strain UA140::Φ(*nlmAp-luc*) and measured luciferase activity in planktonic and pelleted cultures. While the *nlmAp-luc* gene expression was slightly increased in the Δ*luxS* background (Fig. 2A) possibly because of inactivation of mutacin I gene expression (Merritt *et al.* manuscript submitted), it was severely reduced (~10-fold) in the Δ*comD* background (Fig. 2A). Furthermore, luciferase activity of the *comD* mutant in the pelleted culture remained the same as in the planktonic culture, indicating that the *comD* mutation abolished the high-level mutacin IV gene expression in the cell pellet.

To confirm whether the effect of a *comD* mutation on mutacin IV gene expression was indeed mediated by the same ComDE signalling pathway as for competence gene regulation, we transferred *comE*, *comC* and *comX* mutations into strain UA140::Φ(*nlmA-luc*). ComE is the cognate response regulator for ComD, ComC is the structural gene for the signaling peptide CSP (Competence Stimulating Peptide), and ComX is an alternative sigma factor. According to the model for competence regulation (Pestova *et al*., 1996), the binding of CSP to ComD triggers autophosphorylation of ComD, which then transfers the phosphate group to ComE. Phosphorylated ComE activates transcription of ComX, which then enables transcription of the downstream genes for DNA uptake and recombination (Lee and Morrison, 1999; Luo and Morrison, 2003). When *nlmAp-luc* gene expression was measured in the *comC* null background, we found that the *nlmAp-luc* expression was dramatically reduced to the same level as in the *comD* background in both planktonic and pelleted cultures (Fig. 2A). Interestingly, in the *comE* background, there was no detectable *nlmAp-luc* activity in either the planktonic or pelleted cultures. In contrast, in the *comX* background, *nlmAp-luc* gene expression was not affected. These data indicate that *nlmA* is regulated by the ComDE system; however, this regulation is not mediated by ComX.

Addition of synthetic CSP triggered nlmA *gene expression*

To further confirm the involvement of *comDE* in mutacin IV gene expression, we chemically synthesized the 21-amino acid CSP peptide. Using the UA140::Φ(*nlmAp-luc*) reporter strain, we first determined if *nlmAp-luc* gene expression responded to CSP addition in a dose-dependent manner. CSP was added to the planktonic culture in concentrations ranging from 0.01 to 2 μg ml^{-1} for 2 h, and luciferase activity was measured. We found that *nlmAp-luc* gene expression responded to increasing amounts of CSP in a dose-dependent fashion up to 1 μg ml^{-1} (Fig. 3A). This concentration agrees well with previous reports in which 1 μg ml^{-1} CSP was used to induce competence or biofilm formation in a *comC* null mutant (Li *et al*., 2001; Yoshida and Kuramitsu, 2002). The time of induction was also measured and found to peak at ~3 h (data not shown). Using 1 μg ml^{-1} CSP and 3 h of induction time, we then measured the response of *nlmAp-luc* expression in the *comE* and *comC* backgrounds in early log phase cells (OD$_{600}$ ~ 0.1). As shown in Fig. 3B, CSP addition to the wild-type UA140::Φ(*nlmAp-luc*) strain resulted in ~eightfold higher luciferase activity compared with the control. As expected, a similar response was also observed in the *comC* background. In contrast, in the

comE background, *nlmAp-luc* expression did not respond to CSP addition. These results further confirmed the involvement of ComE in mutacin IV gene regulation. Addition of CSP to the planktonic culture of strain UA140::Φ(*nlmAp–gfp*), which was normally not fluorescent (Fig. 1D), also induced fluorescence (Fig. 3C). This was in sharp contrast to strain UA140::Φ (*mutAp–gfp*) carrying a *gfp* fusion to the mutacin I promoter *mutA* (Fig. 3D), which did not become fluorescent upon CSP addition. This demonstrated that CSP-dependent induction of mutacin gene expression was specific to mutacin IV.

Mutacin IV gene expression and competence development were temporally coordinated

Results presented above suggested that both mutacin IV production and competence development are regulated by the same ComCDE quorum-sensing system. To determine whether the two events were also coordinated temporally, or their regulation by the same quorum-sensing system was merely coincidental, we measured simultaneously *nlmAp-luc* expression and transformation efficiency in response to CSP. BHI medium was chosen for this experiment because competence did not develop naturally in this medium (J. Merritt *et al.* unpubl. obs.). Strain UA140::Φ(*nlmAp-luc*) cells were grown to OD_{600} of ~0.05 (early log) in BHI, and CSP was added. At 30 min before each designated time point (30, 60, 120, 180 and 240 min), plasmid pVA838 DNA was added to the cell culture. The culture was first measured for luciferase activity and cell density, and then plated on erythromycin plates to select for transformants. As shown in Fig. 4A, *nlmAp-luc* gene expression started to increase (~ fivefold) at 30 min after CSP addition, peaked at 3 h, and plateaued at 4 h. Compared with *nlmAp-luc* gene expression, competence started to increase at 2 h after CSP addition, reached its peak level at 3 h, and dropped to a basal level at 4 h (Fig. 4B, S2). In comparison, luciferase activity in cells without CSP addition remained very low throughout the experiment (Fig. 4A), and no transformants could be detected in these cultures at any time point (Fig. 4B, S1). The 2 h delay in competence after CSP addition was possibly due to the delayed response of *comX* expression as observed by Aspiras *et al.* in *S. mutans* UA159 cells (Aspiras *et al.*, 2004). Taken together, these results demonstrate that both mutacin gene expression and competence development in BHI respond to CSP addition, and that the temporal pattern of mutacin IV gene expression and competence development overlap. This may suggest that mutacin IV production and competence development are coordinated.

S. mutans *was transformed by plasmid DNA harbored in* S. gordonii *during co-cultivation*

The interesting (and initially surprising) finding of the coordination between mutacin IV production and competence development led to the hypothesis that ComCDE-controlled mutacin IV production may be used to acquire DNA from sensitive streptococcal species living in close proximity during competence of *S. mutans* in its natural habitat, the dental plaque. To test this hypothesis, we performed a mixed culture transformation assay. We used a member of the mitis group streptococci, *S. gordonii*, as the potential donor because it naturally coexists with *S. mutans* in the same niche and is sensitive to mutacin IV (Qi *et al.*, 2001). To monitor DNA transfer, we transformed *S. gordonii* with the shuttle plasmid pVA838 which carries an erythromycin (Erm) resistance gene (Macrina *et al.*, 1982). To determine the role of mutacin in possible DNA release, we constructed three mutant strains from the parental strain UA140, which produces two mutacins, the lantibiotic mutacin I and the non-lantibiotic mutacin IV (Qi *et al.*, 2001). UA140I⁻IV⁺ is defective in the lantibiotic mutacin I production (Qi *et al.*, 2000) but proficient in mutacin IV production; UA140I⁺IV⁻ is negative in mutacin IV production but positive in mutacin I production; and, UA140I⁻IV⁻ is defective in both mutacin I and mutacin IV production.

The wild-type (I⁺IV⁺) and the mutant strains were mixed with *S. gordonii*/pVA838 separately, and the mixed cultures were pelleted with and without the addition of 1 μg ml⁻¹CSP. After 4 h of coincubation the cell pellet was plated on Tet/Kan and Erm plates to select for the mutacin

defective strains transformed with pVA838, or on Spc and Erm plates to select for wild-type *S. mutans* UA140 strain transformed with pVA838. Without the addition of CSP (Fig. 5A), the wild-type and the mutacin IV positive strain UA140I⁻IV⁺ showed a three- to fivefold higher transformation frequency than the mutacin IV negative strains UA140I⁺IV⁻ and UA140I⁻IV⁻. However, these four strains gave rise to similar transformation frequencies when pure plasmid DNA was used for transformation, which suggested that competence was not affected by any of the mutacin mutations. In addition, monocultures of *S. mutans* did not show any colonies on Erm plates when no plasmid was added (data not shown), demonstrating that spontaneous Erm resistance did not occur. These results suggested that the *S. mutans* Erm-resistant colonies obtained from the mixed cultures with *S. gordonii*/pVA838 likely came from transformants with pVA838 that was probably released from *S. gordonii*/pVA838. To further confirm this, Erm-resistant *S. mutans* colonies were randomly selected from the plate, and a colony polymerase chain reaction (PCR) was performed using a pair of primers designed specifically for the *erm* gene in pVA838 (see *Experimental procedures*). The analysed 20 colonies all produced positive results (data not shown). Taken together, these data indicated that mutacin IV production was, at least partially, responsible for the increased DNA release from *S. gordonii*/pVA838, which could then result in increased number of transformants in *S. mutans*.

Addition of CSP to the mixed culture further increased the transformation frequency in all strains compared to the non-CSP treated cells, but the overall pattern remained similar (Fig. 5B). This is likely due to the fact that CSP addition increased the overall transformability of *S. mutans*. It is also worth noting that the transformation frequency observed in this experiment was typical for *S. mutans* transformed with plasmid DNA, while transformation frequency using chromosomal DNA from the same species was usually much higher (Li *et al*., 2001;Merritt *et al*., 2005).

Partially purified mutacin IV caused DNA release from S. gordonii

Aiming to provide more direct evidence on mutacin IV-induced DNA release from *S. gordonii*, we partially purified mutacin IV (referred to as crude extract) from the mutacin I negative, mutacin IV positive strain UA140I⁻IV⁺ using established procedures (Qi *et al*., 2001) (see *Experimental procedures*). To exclude the possibility that other components in the crude extracts may affect DNA release, crude extract was also obtained from the double mutacin-negative strain UA140I⁻IV⁻. Different dilutions of the crude extract from both strains were tested for their activity against *S. gordonii* in an overlay assay (Fig. 6A). Crude extract from the mutacin IV positive strain UA140I⁻IV⁺ displayed inhibitory activity (as shown by a clear zone) even after 64-fold dilution (Fig. 6A, #7), while the crude extract from the mutacin IV negative strain UA140I⁻IV⁻ showed only weak activity up to a fourfold dilution (Fig. 6A, #3). To test DNA release, equal amounts of the crude extracts were mixed with *S. gordonii*/pVA838, and the mixtures were incubated at room temperature. As a control, similarly diluted 5 M urea solution (used to resuspend the crude extract) was also mixed with *S. gordonii*/pVA838. At designated time points aliquots of samples were removed, filtered, and the DNA precipitated with yeast tRNA as carrier. Real-time PCR was used to quantify plasmid pVA838 DNA in the precipitates. As shown in Fig. 6B, 1 min after crude extract addition, four times more plasmid DNA was detected from the culture supernatant treated with UA140I⁻IV⁺ crude extract compared with that treated with the control extract. This pattern remained constant throughout the course of the experiment (32 min). Interestingly, compared with the urea control, cultures treated with the control extract (UA140I⁻IV⁻) still exhibited higher levels of DNA release from all time points. This may indicate that some minor, uncharacterized bacteriocins were still produced from the double mutacin minus strain, which may have caused the additional DNA release from *S. gordonii*. The finding that the control extract

(UA140I⁻IV⁻) also exhibited weak activity against *S. gordonii* (Fig. 6A) seemed to add further support to this notion.

Prevalence of mutacin IV gene in S. mutans *clinical isolates*

Results presented above demonstrated that there was a link between mutacin IV production and competence development, and that these coordinated events may serve a purpose of acquiring DNA from other streptococcal species. To probe whether this is a rather common phenomenon among *S. mutans* isolates or just a specific case for some strains like UA140, we used PCR to determine the prevalence of the mutacin IV gene in the *S. mutans* strain population. Three sets of primers were designed: two for the two mutacins produced by UA140 (mutacin I and mutacin IV) and one for 16S DNA of all *S. mutans* isolates (see *Experimental procedures*). The three sets of primers were mixed in a 'cocktail' and used to amplify chromosomal DNA from *S. mutans* strains (e.g. UA140, UA159 and clinical isolates), or from preserved saliva samples from which *S. mutans* had been detected by using monoclonal antibodies (Shi *et al*., 1998). Among all *S. mutans* positive samples (70 total), mutacin IV gene was detected in ~50% of the samples. In a small percentage (~5%) of samples, both mutacin I and IV genes were detected (data not shown). These results demonstrate that at least half of the *S. mutans* clinical strains harbour the mutacin IV gene in their genomes.

Discussion

In this study we demonstrated that mutacin IV gene expression is dramatically increased in the cell pellet, and that this regulation is mediated by the ComCDE system in a pathway independent of the ComX regulatory pathway (Figs 1 and 2). Further studies suggested that mutacin IV gene expression and competence development may be coordinated temporally in response to CSP (Figs 3 and 4). This raised an interesting question of whether this coordinated mutacin production and competence development could be used as a means for *S. mutans* to acquire transforming DNA from neighbouring streptococcal species. Mixed culture assays with *S. gordonii* demonstrated that increased transformation of *S. mutans* by plasmid DNA originally harboured in *S. gordonii* was dependent on the presence of the mutacin IV gene, suggesting a role of mutacin IV in DNA release from *S. gordonii* (Fig. 5). Further investigation using partially purified mutacin IV demonstrated increased DNA release upon addition of mutacin IV crude extract to the *S. gordonii* culture (Fig. 6). Based on these results, we propose the following model for the coordinated mutacin IV production and competence development (Fig. 7): high cell density or nutrient limitation or both triggers high-level *comC* gene expression, resulting in high-level production of CSP. CSP binding to the ComDE two-component system activates transcription of a set of genes including the *comX* gene and *nlmA*. While expression of the late competence genes such as *comY* is dependent on ComX, the *nlmA* gene expression is independent of ComX. The consequence of this complex regulatory pathway (Fig. 7) results in a co-ordinated regulation between competence and bacteriocin production. High levels of mutacin IV production would kill neighbouring streptococcal species such as *S. gordonii* causing DNA release from these species. Released DNA is then internalized by the ComYA-G complex (Merritt *et al*., 2005), whose expression is dependent on ComX. It is worth noting that the temporal correlation between *nlmA* gene expression and competence is not strict; there is a 90 min delay of competence after *nlmA* gene expression is turned on (Fig. 4). We speculate that this delay is probably due to a signal relay in the competence regulatory pathway to turn on the late competence genes for DNA uptake and recombination, while no such a signal relay is required to turn on the mutacin IV gene expression. This is apparently different from the findings in *S. pneumoniae* where autolysis and competence strictly correlate (Steinmoen *et al*., 2002). However, given the reason that mutacin IV has to be produced, secreted, bound to the sensitive cells to kill before donor DNA

is released, it would make perfect sense for it to be produced first before the competence system becomes fully functional.

Mutacin-mediated interspecies DNA acquisition may not be limited to UA140. Inspection of the clinical isolates of *S. mutans* revealed that ~50% contain the mutacin IV gene in their chromosome. These mutacin IV genes are likely to be controlled by CSP as their promoter sequences could also be amplified by primers designed using the UA159 sequence (data not shown). In fact, we have tested a number of clinical isolates on a plate assay and showed that all displayed larger inhibition zones against the mitis group streptococci after CSP addition to the plate (J. Kreth *et al.* unpubl.). Furthermore, mutacin IV may not be the only mutacin controlled by the ComCDE system. In a recent report by Yanezawa and Kuramitsu (Yonezawa and Kuramitsu, 2005), a two-component lantibiotic mutacin called Smb was found to be regulated by CSP in the strain GS-5. As this Smb is active against a group C streptococcus, it would be interesting to see if this mutacin is capable of causing DNA release from the target cells during competence.

The association of bacteriocin production with competence may not be limited to *S. mutans*. In *Bacillus subtilis*, it was found that *comS*, a small peptide required for competence, was embedded within the second open reading frame of the *srfA* operon-encoding enzymes for the biosynthesis of a surfactin. This organization was suggested as a way for *B. subtilis* to lyse microorganisms for the release of genetic material during competence (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995). In a recent microarray study in *S. pneumoniae*, Rimini *et al.* and Peterson *et al.* found that some genes encoding bacteriocin-like peptides were induced by CSP, although these findings were not verified by genetic studies (Rimini *et al.*, 2000; Peterson *et al.*, 2004). Although some of the bacteriocin-like peptides (e.g. genes *blpA*, *blpY* and *blpZ*) were later found to be regulated by a different pheromone, BlpR, which does not cross react with the ComDE system (Reichmann and Hakenbeck, 2000; de Saizieu *et al.*, 2000), a recent study by Knutsen *et al.* reported that an unknown ABC transporter is co-regulated by both the ComCDE and the BlpR system (Knutsen *et al.*, 2004). Whether this unknown ABC transporter is a link between the two quorum-sensing systems is yet to be determined. Nonetheless, findings from this study provide the first genetic evidence for a coordinated production of a bacteriocin and the development of competence as a possible mechanism for DNA acquisition from other species. This mechanism may serve as a basis for acquiring DNA from other species, which may provide the opportunity for horizontal gene transfer in multispecies microbial communities. We speculate that as more bacteriocin and competence systems are characterized in other bacterial species, this mechanism may prove to be a prevalent phenomenon among naturally competent microorganisms that inhabit multispecies environments.

Experimental procedures

Bacterial strains and growth conditions

The mutacin I and mutacin VI positive *S. mutans* strain UA140 (Qi *et al.*, 2001) was used for this study. The *luxS*, *comC, comD, comE* and *comX1* mutant strains were constructed by transferring the individual mutations to UA140::Φ(*nlmAp-luc*) from previously constructed *S. mutans* strains, JM03 (*luxS*) (Merritt *et al.*, 2003), JMcC-luc Δ*comC* Kan[r] (Merritt *et al.*, 2005), SMCD1 ComD[−] Erm[r] (Li *et al.*, 2001), SMCE1 ComE[−] Erm[r] (Li *et al.*, 2001) and SMComX1 Δ*comX1*::PcEm Erm[r] (Li *et al.*, 2002) respectively. Each individual mutation was originally generated by double crossover integration to avoid polar effects on the downstream genes. Strains SMCD1, SMCE1 and SMComX1 were kindly provided by Dr D. Cvitkovitch (University of Toronto, Canada). Transformants were selected on Km (800 μg ml^{-1} or Erm (15 μg ml^{-1}) plates, and the mutations confirmed by PCR. Plasmid and chromosomal DNA was transformed into *S. mutans* following previous protocols (Shah and Caufield, 1993). To construct a DNA donor, the mutacin-sensitive *Streptococcus gordonii* Challis (Kilian *et al.*,

1989; Jenkinson and Easingwood, 1990) was transformed with the shuttle plasmid pVA838 (Macrina *et al*., 1982), which carries an Erm resistance marker. The presence of the plasmid was confirmed by PCR using a pair of primers specific for the Erm gene. All UA140 variants and *S. gordonii* were grown in BHI medium (Difco; Sparks, MD) or on BHI agar plates anaerobically (nitrogen 85%, carbon dioxide 5%, hydrogen 10%) at 37°C. *E. coli* Top10 (Invitrogen; Carlsbad, CA) was used for cloning and plasmid amplification.

DNA manipulations

Restriction enzymes were obtained from Promega (Madison, WIS), Fisher (Pittsburgh, PA) or NEB (Beverly, MA). Ligations were performed using the Quick LigationTM Kit from NEB. PCR products were cloned into the TOPO TA CLONING® Kit from Invitrogen. All plasmids were extracted and purified from *E. coli* with the QIAGEN (Valencia, CA) Miniprep Kit. DNA extracted from agarose gels (1%) was purified with the QIAGEN QIAquick® Gel Extraction Kit. PCR was performed with a MyCycler™ thermocycler (Bio-Rad; Hercules, CA) using a protocol supplied with the thermocycler. Taq-DNA Polymerase was obtained from Promega and dNTPs from Fisher.

Construction of reporter gene fusion to the mutacin IV promoter

The backbone vector for the construction of all reporter gene fusions was pFW5, which contains a spectinomycin resistance marker (*aad9*) that works in both Gram-negative and Gram-positive bacteria (Podbielski *et al*., 1996). To construct the *gfp* gene fusion, the promoterless *gfp–mut2* gene with its own ribosome-binding site was excised from vector pKEN (Cormack *et al*., 1996) with *Bam*HI and *Hin*dIII, and inserted into pFW5 at the *Bam*HI and *Hin*dIII sites to generate plasmid pFW5::*gfp*. To insert the *nlmAp* in front of the *gfp* reporter, the promoter region was amplified by PCR from chromosomal DNA of strain UA140 using primers nlmApo-F (5′-taatgcatgctgtagatgttgagcc-3′) and nlmApo-R (5′-ccggatccat tacatcaaattgttcaaatgcc-3′). Primer nlmApo-R had a *Bam*HI site incorporated at its 5′ end (underlined sequence). The PCR product was subcloned into pCR®2.1-Topo® (Invitrogen) to generate pCR2.1::*nlmAp*. Correct fragment insertion and promoter sequence was confirmed by sequencing. The plasmid pCR2.1::*nlmAp* was digested with *Bam*HI and *Eco*RV, and the DNA fragment containing the *nlmA* promoter was purified. The *nlmA* promoter region was subsequently inserted upstream of the *gfp* gene in pFW5::*gfp* at the *Bam*HI and an upstream *Stu*I site compatible with *Eco*RV to form plasmid pFW5::Ø(*nlmAp–gfp*). For the construction of the firefly luciferase (*luc*) reporter, plasmid pFW5-*luc* (Elsner *et al*., 2002) was digested with *Sal*I and *Bam*HI. The *nlmA* promoter was excised from pCR2.1::*nlmAp* with *Bam*HI and *Xho*I, and ligated into the *Bam*HI site and the *Xho*I compatible *Sal*I site to form plasmid pFW5::Ø(*nlmAp-luc*). For the construction of the reporter strains UA140::Ø(*nlmAp-luc*) and UA140::Ø(*nlmAp–gfp*), plasmids pFW5::Ø(*nlmAp-luc*) and pFW5::Ø(*nlmAp–gfp*) were transformed into UA140 and selected with spectinomycin (800 μg ml^{-1}). Integration of each reporter into the chromosome was confirmed by PCR. Confirmed reporter strains had both reporter-gene activity as well as wild-type levels of mutacin production.

Construction of mutacin defective strains

Streptococcus mutans strain UA140 produces two mutacins, the lantibiotic mutacin I and the non-lantibiotic mutacin IV (Qi *et al*., 2001). To study the role of each mutacin in causing DNA release from *S. gordonii*, we constructed three derivative strains defective in either mutacin I (UA140I⁻IV⁺), mutacin IV (UA140I⁺V⁻), or both (UA140I⁻IV⁻). To construct UA140I⁻IV⁺, the tetracycline (Tet) resistance gene *tetM* from Tn*916* (Flannagan *et al*., 1994) was amplified by PCR and cloned into pCR2.1 cloning vector (Invitrogen). A DNA fragment encompassing 1 kb upstream and downstream of *mutC* (Qi *et al*., 2000) was amplified by PCR using primers BF1 (5′-TATTTGAAACTCCCAGAAAACTTACAATG-3′) and DR1

(5′-TCCTTTCTTCTTCATACTCTTACC-5′) and cloned into pCR2.1 to form pCRBCD. To delete *mutC*, an inverse PCR was performed by using two primers BR1 (5′-ggcggc*aggc ct*TTACTTTGTCTCATTACCCAC-3′) and DF1 (5′-ggcggc*ag gcct*AACTGAACGGAGAAATAATTATGG-3′), both of which had a *Stu* I restriction site incorporated at their 5′ ends. The *tetM* gene cassette was released from pCR2.1 by cutting with *Stu* I restriction enzyme, whose recognition sequence was also incorporated into the primers for amplifying *tetM*, and inserted into pCRBCD at the same restriction site. The resulting plasmid was digested with *Pst* I and *Sph* I and transformed into UA140. The deletion construct was integrated into the chromosome by double cross-over homologous recombination. The transformants were selected on Tet plates (10 μg ml^{-1}). Ten transformants were randomly selected and tested for mutacin I production by the deferred antagonism assay (Hamada and Ooshima, 1975) using *S. sobrinus* OMZ176 as the indicator, which is sensitive only to mutacin I. The mutacin I negative isolates were further confirmed by PCR. To construct UA140I$^+$V$^-$, a DNA fragment encompassing *nlmA* and *nlmB* was PCR amplified by using primers Adh dn1 (5′-CCAGCTAATCCACGTCAGC-3′) and 135 μp1 (5′-CAAGAAAAGATGAGTAAGATGGC-3′), and cloned into pCR2.1 to form pCRnlmAB. An inverse PCR reaction was performed using pCRnlmAB as template and primers nlmA up1 (5′-ggcatcgat*T*GTGTAGGCACTTGGG GAC-3′) and nlmB dn1 (5′-ggcatcgat*G*ATATAATGGTGGG CAATGTC-3′). Both primers had a *Cla* I restriction site incorporated at their 5′ ends. The PCR product was digested with *Cla* I and ligated with a *Cla* I digested kanamycin (Km) resistance gene cassette (*aph*III, Trieu-Cuot and Courvalin, 1983) that was constructed from previous studies (Qi *et al*., 2004). The plasmid was linearized by *Sph* I digestion and transformed into UA140. The deletion was confirmed by PCR. As no indicator strain was sensitive only to mutacin IV, the defect in mutacin IV production was further confirmed by mutacin isolation under conditions that mutacin I was not produced (Qi *et al*., 2001). To construct the double-mutant strain UA140I$^-$IV$^-$, chromosome DNA was isolated from strain UA140I$^+$IV$^-$ and transformed into strain UA140I$^-$IV$^+$. The transformed strains were selected on Tet plus Km plates, tested for the lack of mutacin production by using the indicator strain *S. sangunis* NY101, which was sensitive to both mutacins (Qi *et al*., 2001). Mutacin defective isolates were further confirmed by PCR.

Luciferase assay

Luciferase assays were performed as previously described (Loimaranta *et al*., 1998). Briefly, 25 μl of 1 mM D-luciferin (Sigma; St. Louis, MO) suspended in 100 mM citrate buffer, pH 6, was added to 100 μl of the cell culture. To ensure sufficient levels of intracellular ATP pool, cells were recharged with 1% glucose for 10 min prior to luciferin addition. Luciferase activity was measured by using a TD 20/20 luminometer (Turner Biosystems; Sunnyvale, CA). Usually, three parallel cultures were measured at each time point and the average value was taken. Each experiment was repeated at least two times.

Cell pelleting assay

To create an environment mimicking the cell density in the dental plaque (10^{11} cells g^{-1}wet weight), anaerobically grown log-phase cells were spun down at ~16 000 *g* for 1.5 min in a microcentrifuge. The cell density in the pellet was found to be close to the cell density in the dental plaque. To study the effect of cell density on mutacin IV gene expression, overnight cultures were diluted 1:20 in fresh BHI and grown at 37°C anaerobically to an OD$_{600}$ of ~0.2 (early log phase). The culture was divided into 1 ml aliquots in 1.5 ml tubes and half of the samples were spun down. With the supernatant remaining in the tubes, both planktonic and spin-down cultures were further incubated at 37°C anaerobically for a designated amount of time. Both planktonic and pelleted cells were resuspended by vortexing before luciferase activity and OD$_{600}$ were measured. In general, three parallel cultures for each treatment were used, and the experiment was repeated at least three times.

CSP-dependent transformation assay

To determine the coordination between competence development and mutacin IV (*nlmA*) gene expression, we simultaneously measured luciferase activity and transformation efficiency in strain UA140::Φ(*nlmAp-luc*) in response to the CSP. A fresh overnight culture was diluted to OD_{600} of ~0.025 in BHI and incubated anaerobically until OD_{600} of ~0.05. The culture was divided into 1 ml aliquots in 1.5 ml tubes with and without the addition of CSP (1 μg ml^{-1}) and incubated anaerobically at 37°C. At 30 min prior to each sampling time, 0.5 μg of pVA838 plasmid DNA was added. Luciferase activity (RLU) and OD_{600} were measured at each designated time point followed by plating of 700 μl of the samples on Erm plates.

Interspecies transformation assay

To determine the possible *S. mutans* competence-induced DNA release from *S. gordonii*, the shuttle vector pVA838 (Erm^R) was transformed into *S. gordonii* Challis. *S. mutans* UA140::Φ(*nlmAp-luc*) (wild-type) and mutacin-negative derivative strains and *S. gordonii*/pVA838 were grown overnight in BHI. The overnight cultures were centrifuged and washed twice to eliminate possible released plasmid DNA during stationary phase, and the cell pellets were resuspended in fresh BHI to an OD_{600} of ~0.1. Both cultures were allowed to grow to OD_{600} of 0.2 and mixed in a 5:1 ratio of *S. mutans: S. gordonii*/pVA838. The mixed culture was pelleted and incubated at 37°C for 4 h. The cell pellet was plated on BHI plates containing both Spc and Erm to detect transformants. Colonies on this plate could come from *S. mutans* transformed with the shuttle plasmid, or *S. gordonii* transformed with chromosomal DNA from *S. mutans*. Different colony morphologies would allow us to distinguish the two species. As a control, 0.5 μg of the shuttle plasmid DNA isolated from *E. coli* was added to *S. mutans* single species cultures to normalize variations in transformation efficiency of different isogenic strains.

Mutacin IV induced DNA release assay by real-time PCR

To test if mutacin IV is responsible for the release of plasmid DNA from *S. gordonii* cells, mutacin IV was partially purified (referred to as crude extract) as described previously (Qi *et al*., 2000). To measure mutacin IV-induced DNA release, an overnight culture of *S. gordonii*/pVA838 was washed twice in BHI and adjusted to OD_{600} of 0.4 in TE containing 10 mM EDTA to inhibit DNase activity. To 1.75 ml of the cell suspension, 250 μl of crude extract from either UA140I$^-$IV$^+$ or UA140I$^-$IV$^-$ was added. As a negative control, 250 μl of 5 M urea was added (crude mutacin extract was suspended in 5 M urea). The cells were incubated at 37°C and 600 μl of the cell suspension was removed after 1, 16 and 32 min. The suspension was centrifuged at 16 000 *g* for 5 min and 500 μl of supernatant was immediately removed, filter-sterilized, and stored on ice until further processing. The DNA was precipitated by ethanol precipitation using yeast tRNA as carriers and used as template for real-time PCR. A pair of *erm*-cassette specific primers was used to detect the presence of the shuttle plasmid, and real-time PCR was performed as previously described (Merritt *et al*., 2005).

Confocal Laser Scanning Microscopy

For microscopy of planktonic, pelleted and CSP-treated cells, an overnight culture in BHI was diluted 1:20 and incubated at 37°C anaerobically to OD_{600} of ~0.2. The cultures were treated with CSP or centrifuged as described in previous sections. Before microscopy, the cells were washed with PBS and incubated for 10 min at room temperature to promote folding of the *gfp* fluorophore (Hansen *et al*., 2001). Images were obtained with a 40 × 1.4 Plan-Neofluar oil objective.

Detection of mutacin IV in S. mutans *clinical isolates*

To see the prevalence of the mutacin genes in *S. mutans* isolates, three sets of primers were designed. M1F (5′-ATAG TAAAGTGGGTAGTTTC-3′) and M1R (5′-GCCTAATGG TTTTCTGTAC-3′) would amplify a 270 bp fragment from the mutacin I gene (Qi *et al*., 2000), Orf1 (5′-ATGGATACACAGG CATTTGAAC-3′) and Orf2 (5′-TTTAGTGTGGAAAAACTACA GATCC-3′) would amplify a 450 bp fragment from the mutacin IV gene (Qi *et al*., 2001), and SmF205 (5′-TTGATTGAAA GATGCAAGC-3′) and BacR335 (5′-GCTGCCTCCCGTA GGTG-3′) would amplify a 130 bp fragment of the *S. mutans* 16S DNA. Chromosomal DNA was isolated by using the bead beater. After testing the specificity and sensitivity of each pair of primers with *S. mutans* UA140, which produces both mutacin I and IV (Qi *et al*., 2001), and closely related species such as *S. sobrinus* OMZ176 and *S. sanguinis* NY101, a primer cocktail was made, in which all primers (25 μM each) were mixed in equal portions. For PCR, Taq polymerase was used under standard conditions with the primer cocktail at a final concentration of 1 μM. PCR was performed for one cycle at 94° C/3 min, 50°/1 min, and 72°C/30 s, and 25 cycles of 94°C/1 min, 50°/1 min and 72°C/30 s.

Chemical synthesis of CSP

The mature peptide of CSP (SGSLSTFFRLFNRSFTQALGK) was synthesized by automated 9-fluorenylmethoxy carbonyl chemistry (Applied Biosystems 431 A Peptide Synthesizer) and confirmed with reverse-phase high-performance liquid chromatography and mass spectrometry profiles. Purified CSP was dissolved in dionized water, aliquoted (5 mg ml^{-1}) and stored at −20°C.

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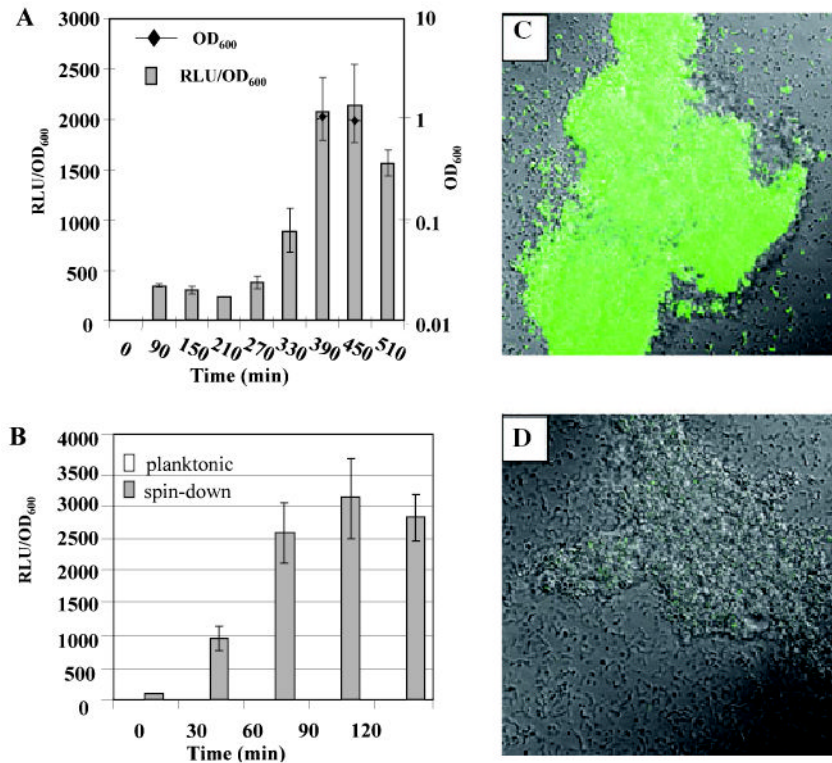**Fig. 1.**
A. Time-course of *nlmAp-luc* gene expression in strain UA140::Φ(*nlmAp-luc*). The experiments were repeated two times with variations of less than 20% in between experiments. Presented here is one of the experiments. Values are averages of triplicate samples.
B. *nlmAp-luc* gene expression in planktonic and pelleted cultures over time. Experiments were repeated three times. Presented here are the average values of the three independent experiments. RLU, relative light unit.
C. Confocal micrograph of strain UA140::Φ(*nlmAp–gfp*) cells in pelleted culture.
D. Confocal micrograph of strain UA140::Φ(*nlmAp–gfp*) cells in planktonic culture.
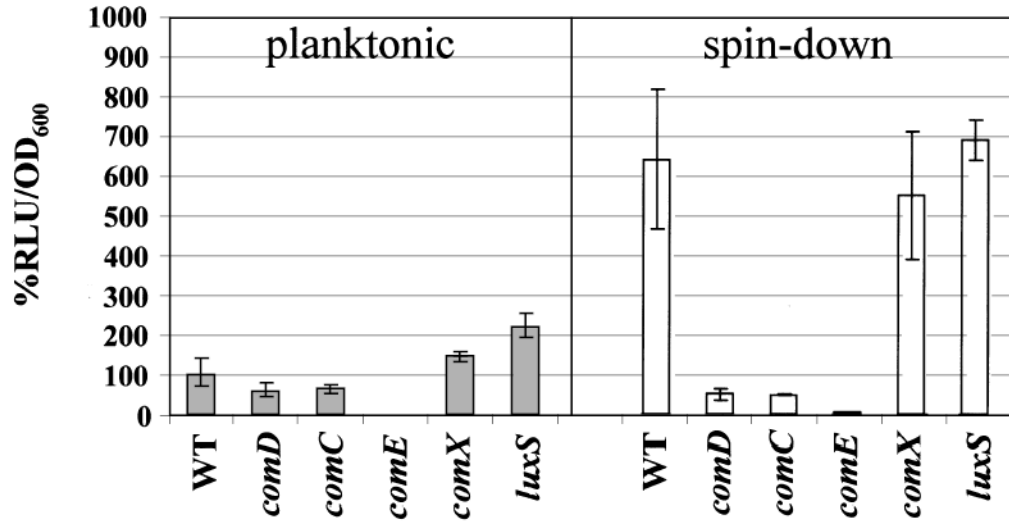
Fig. 2.
nlmAp-luc gene expression in various mutant backgrounds in planktonic and pelleted cultures. Luciferase activity (RLU) was normalized with cell density (OD_{600}) and expressed as a percentage of the wild-type level in planktonic culture, which was arbitrarily assigned as 100%. Values shown here represent the mean of at least two independent experiments performed in triplicate.
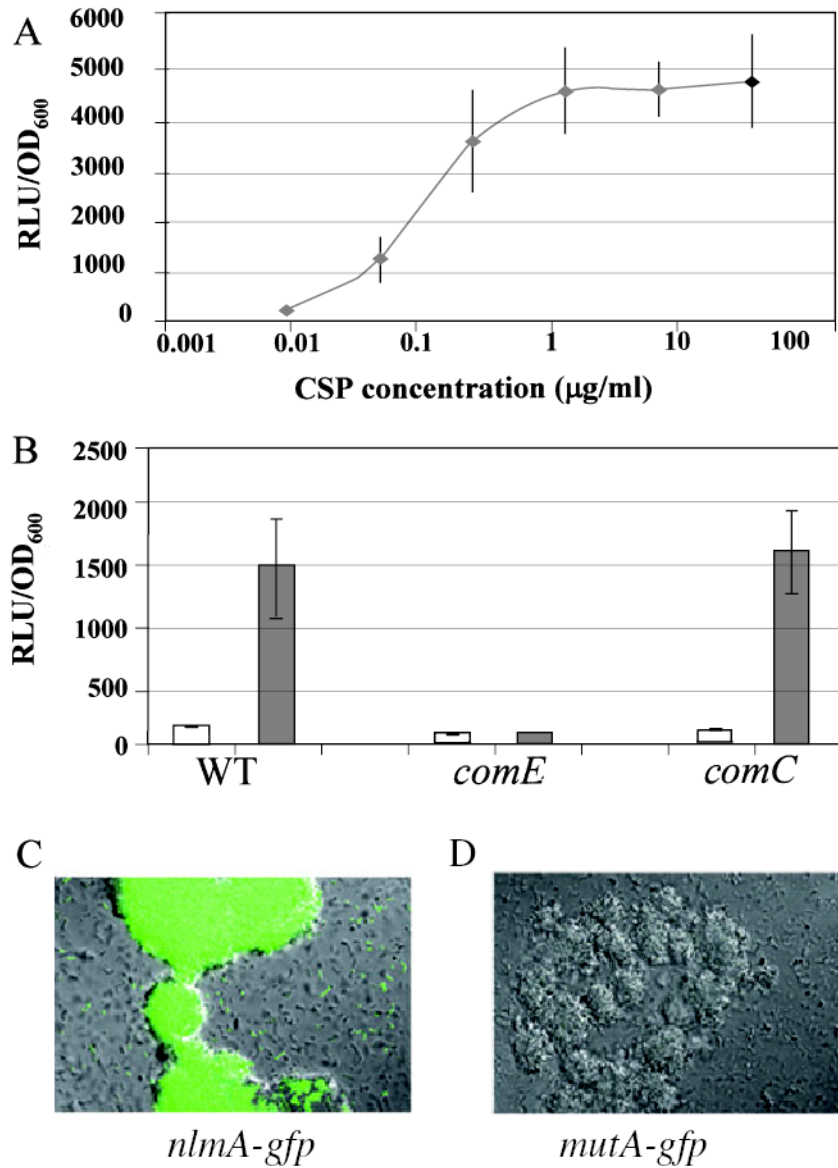
Fig. 3.
Response of mutacin IV gene expression to CSP.
A. Response of *nlmAp-luc* gene expression to different concentrations of CSP.
B. Expression of *nlmAp-luc* gene expression in the wild-type, *comE* null, and *comC* null background in response to CSP. The experiment was repeated three times, and values are averages of the three experiments with triplicate samples of each.
C. Confocal image of *nlmAp–gfp* gene expression in response to CSP addition.
D. Confocal image of *mutpA-gfp* gene expression in response to CSP addition.
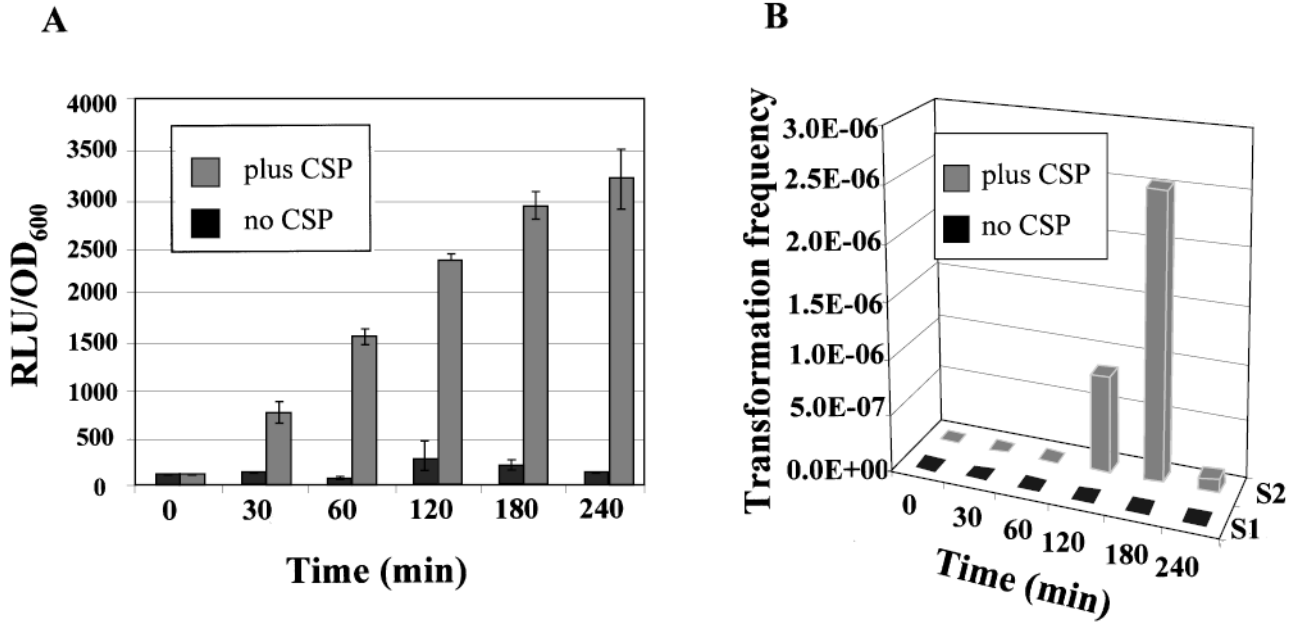
Fig. 4.
Coordination of competence development and mutacin IV gene expression.
A. Time-course of *nlmAp-luc* gene expression in response to CSP addition.
B. Transformation frequency of the same culture as (A). S1, no CSP; S2, with CSP.
Transformation frequency was calculated as the ratio of transformants versus the number of total viable cells per ml in the transforming culture. The data shown are averages of two independent experiments assayed in triplicate. Although no error bars could be shown in (B) because of the 3-D projection, the variation between experiments is less than 20%.
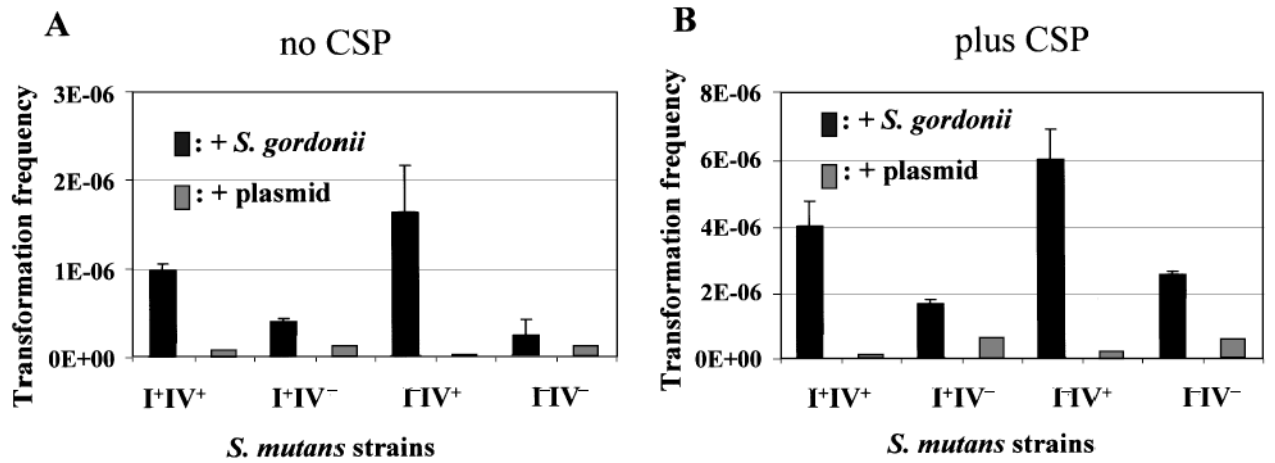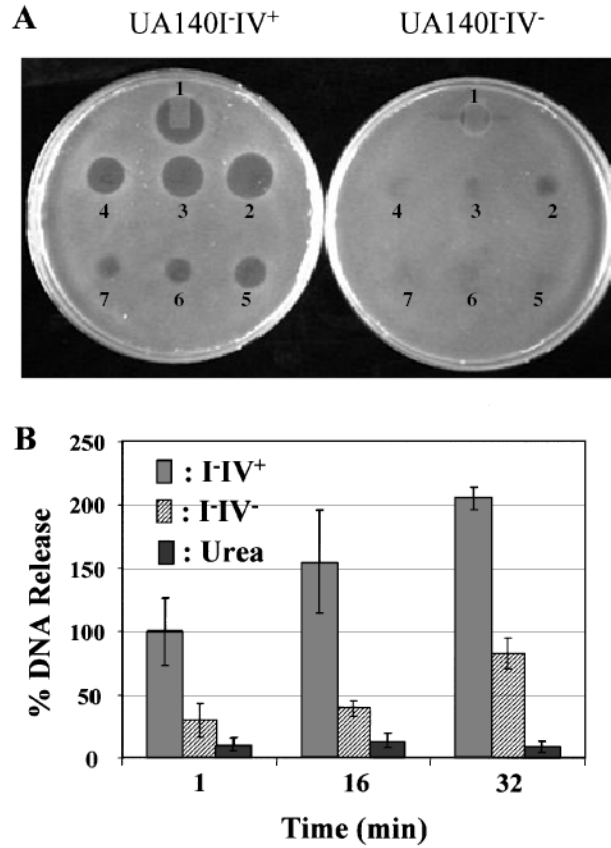
Fig. 5.
Transformation assay of *S. mutans* in mixed cultures with *S. gordonii*/pVA838 in the absence (A) and presence (B) of CSP. Transformation frequency was calculated as in Fig. 4. Data presented here are averages of two independent experiments assayed in triplicate.

Fig. 6.
Quantification of DNA release from *S. gordonii*/pVA838 mediated by partially purified mutacin IV.

A. Inhibitory activity of the partially purified mutacin IV (crude extract) from strain UA140I⁻IV⁺ and the control extract from strain UA140I⁻IV⁻ against *S. gordonii*. Each spot contained 10 μl of twofold serially diluted extract (e.g. #1, undiluted; #2, twofold diluted; #3, fourfold diluted, etc.).

B. Real-time PCR quantification of DNA release from *S. gordonii*/pVA838 treated with crude extracts from UA140I⁻IV⁺ and UA140I⁻IV⁻. The amount of DNA released upon treatment with UA140I⁻IV⁺ crude extract at 1 min was arbitrarily assigned as 100%, which was used to normalize all samples. The values shown here represent the mean of at least two independent experiments done in triplicate.
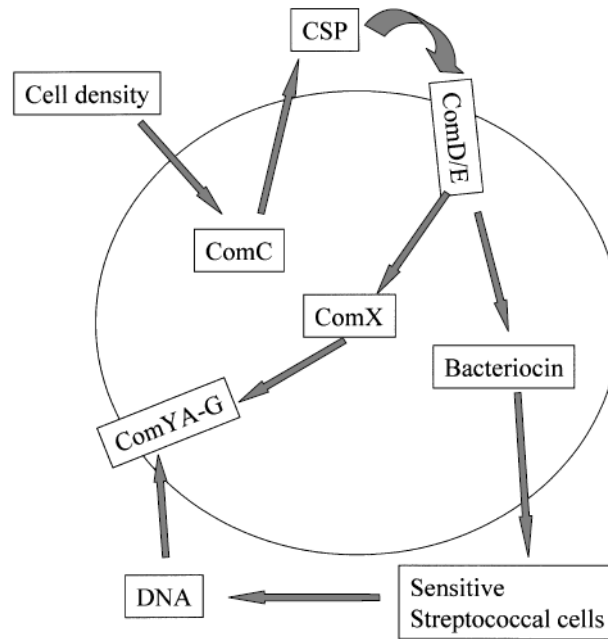
Fig. 7.
Model for coordinated mutacin IV production and competence in DNA release and uptake from other streptococcal species (see text for details).