



TURNING NOVEL DISCOVERIES INTO
INNOVATIVE HEALTHCARE SOLUTIONS

CSP: A Novel Anti-caries Peptide Technology

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1. Introduction

Bacteria in their natural ecosystems preferentially grow as polysaccharide-enclosed biofilms attached to surfaces. Biofilm formation is an example of group behavior that is coordinated through a quorum-sensing (QS) system. In the biofilm mode of growth, microorganisms exhibit increased resistance to antimicrobials, environmental stresses and host immune defense mechanisms (Costerton et al., 1999). The role of quorum-sensing systems and signals in biofilm formation has been characterized in several gram-negative and gram-positive bacteria. The quorum-sensing systems in gram-positive bacteria including streptococci typically use secreted peptides (often referred to as pheromones) as signal molecules and a two-component regulatory system to detect the peptide and trigger the required changes in gene expression.

Gram-positive bacteria such as streptococci use short peptides called “competence stimulating peptide” or CSP, a quorum sensing molecule, which requires a two-component (a histidine kinase sensor and a cognate response regulator) transduction system for detection and function (Li, *et al.* 2001). Although streptococci constitute 60-90% of the early colonizers of dental plaque, *Streptococcus mutans* is considered a primary causative agent of human dental caries (Emilson and Krasse, 1985). *S. mutans* secretes and utilizes CSP to initiate quorum sensing for genetic competence, biofilm formation, acid resistance, stress responses, and bacteriocin production (Syvitski et al., 2007).

S. mutans has evolved to be dependent upon a biofilm “lifestyle” for survival and persistence in dental plaque. Because of their role in biofilm formation and activating virulence factors in many bacteria including streptococci, quorum-sensing systems have emerged as an enticing target for biofilm infections. Thus, inhibition of the streptococcal CSP-mediated pathway appears a feasible method to interfere with biofilm formation, rendering streptococci more susceptible to antimicrobials and the host immune responses. One approach involves the use of synthetic analog of CSP as specific competitive inhibitor of the interaction between CSP and histidine kinase receptor, hindering the induction of biofilm formation. Future antimicrobials based on quorum sensing interference should have a high specificity against target organisms,

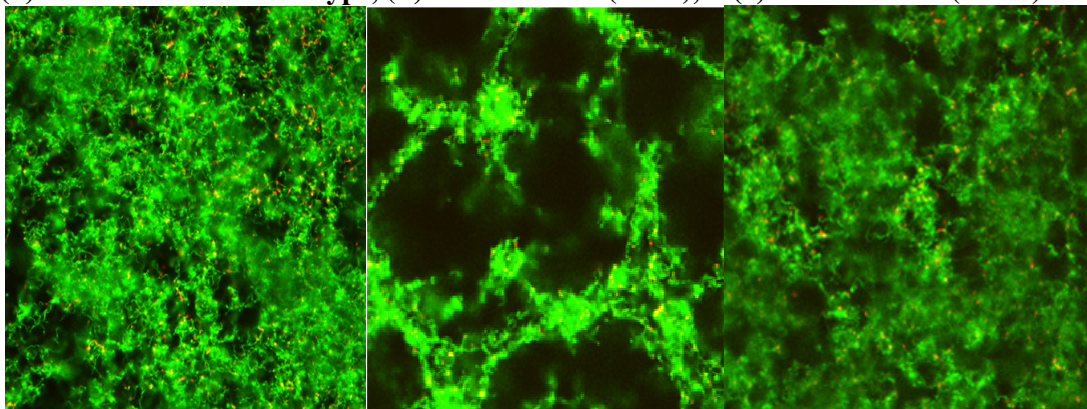
leaving beneficial commensal bacteria unharmed during therapy, with a side benefit of minimal likelihood of antibiotic resistance being transmitted between species.

2. *S. mutans* CSP

Using genomic analysis, Dr. Dennis Cvitkovitch and his colleagues (Li et al., 2001) identified a quorum-sensing peptide pheromone signaling system similar to those previously found in other streptococci. The genetic locus of this system comprises three genes, *comC*, *comD*, and *comE* that encode a precursor to the peptide competence factor, a histidine kinase, and a response regulator, respectively. They deduced the sequence of *comC* and its active pheromone product and chemically synthesized the corresponding 21 amino acid CSP. Further studies by Dr. Cvitkovitch's group to determine the role of the quorum-sensing system in *S. mutans* biofilm formation showed that inactivation of any of the individual genes (*comC*, *comD*, and *comE*) resulted in the formation of an abnormal biofilm (Li et al. 2002a). Also, the *comC* mutant, unable to produce a competence stimulating peptide (CSP), formed biofilms with altered architecture (**1b**), whereas the *comD* and *comE* mutants, which were defective in sensing and responding to the CSP, formed biofilm without reduced biomass. Furthermore, addition of synthetic CSP into the culture medium restored the wild-type biofilm architecture of *comC* mutants (**1c**→**1a**). Thus, it is evident that the CSP-mediated quorum-sensing system is involved in *S. mutans* biofilm formation.

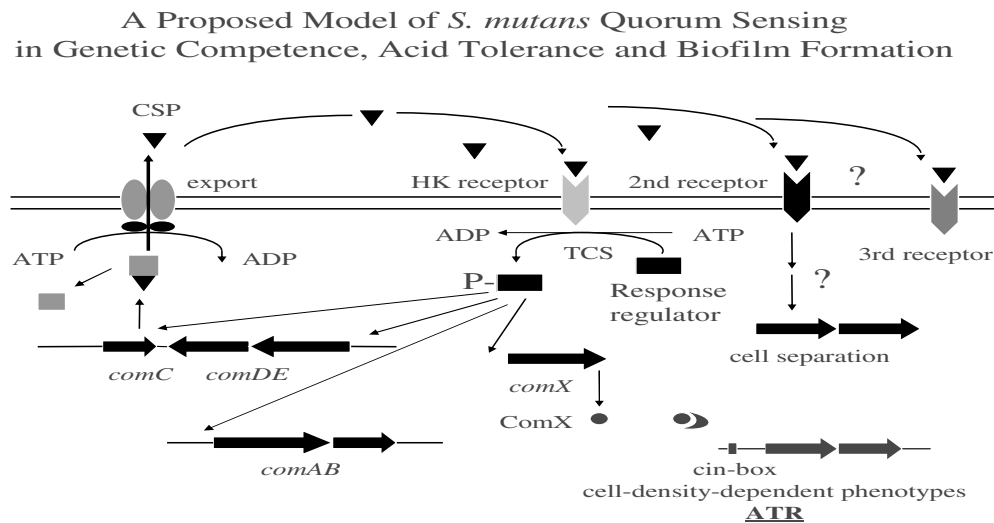
Fig. 2: Confocal Scanning Laser Microscopy image of biofilm formation by the *comC* mutants with and without CSP

(a) *S. mutans* NG8 Wild Type, (b) *comC* mutant (-CSP), (c) *comC* mutant (+CSP)



The quorum sensing system of *S. mutans* involves at least five gene products encoded by *cslAB* (*comAB*) and *comCDE* genes (Li et al., 2002b; **Fig. 2**). *comC*, *comD*, and *comE* genes respectively encode a competence-stimulating peptide (CSP) precursor, its histidine kinase (HK) sensor protein, and a cognate response regulator (RR). The other two genes, *cslA* and *cslB*, encode a CSP-specific secretion apparatus consisting of an ATP-binding cassette (ABC) transporter (ComA) and its accessory protein (ComB), which are presumably involved in the processing and export of the CSP.

Fig. 2: Hypothetical two-receptor model of *S. mutans* cell-cell signaling in genetic competence and biofilm formation.



Of thirteen (Ajdic et al, 2002) identified two-component signal transduction systems (TCSTS) in *S. mutans*, the ComCDE and HK/RR11 two-component systems are peptide (e.g. Competence Stimulating Peptide, CSP)-mediated quorum-sensing systems. These two TCSTS are well known to regulate virulence-associated traits such as genetic competence, biofilm formation, bacteriocin production and stress responses. Li et al. (2008) tested the hypothesis that inactivation of ComCDE, HK/RR11 alone or both would attenuate the virulence and cariogenicity of *S. mutans*. The test results showed that simultaneous inactivation of both signal transduction systems additively attenuated *S. mutans* virulence and cariogenicity and the double deletion mutant was defective in genetic competence, unable to grow at pH 5.0 and

formed an abnormal biofilm with reduced biomass. Furthermore, animal studies showed that this mutant had reduced capabilities for oral colonization, succession and initiation of dental caries. This study provides further evidence for the involvement ComCDE and HK/RR11 signal transduction systems in *S. mutans*-associated dental caries and also validates them as targets for developing anti-caries products. The study data also supports our approach of disrupting the CSP-mediated quorum sensing system using CSP analogue E2 for prevention and treatment of dental caries.

While investigating the stimulation of antimicrobial peptide (bacteriocin) production by *S. mutans* 21-amino acid CSP (21-CSP), Petersen et al. (2006) found a peptide similar to 21-CSP, but lacking the three C-terminal amino acid residues (18-CSP). In addition to isolating 18-CSP from *S. mutans* culture supernatant, they also synthesized both 21-CSP and 18-CSP. These peptides were synthesized by GenScript Corporation, New Jersey. They found 18-CSP more potent in inducing competence, biofilm formation, and antimicrobial activity than the 21-CSP. Furthermore, the results of their screening for the presence of CSP encoding genes in 14 *S. mutans* strains suggest that CSP is widely present and it may be synthesized in three variants, including 21-CSP, 18-CSP and 23-CSP (**Table 1**).

Table 1: Predicted sequence of mature CSPs

<i>S. mutans</i> strain	Predicted CSP	Amino Acid Sequence
Km-1	18-mer	SGSLSTFFRLFNRSFTQA
BM71	21-mer	SGSLSTFFRLFNRSFTQALGK
NCTC10449	23-mer	SGSLSTFFRLFNRSFTQALGKIR

3. CSP Analogs

CSP peptide analogs were designed by modifying their length, charge and hydrophobicity. This included deletions of either the 1st, 2nd, 3rd, 4th or 5th residue from each terminal (N- or C-) separately or substitution of charged internal residues with hydrophobic (valine or alanine) residues (**Table 2**). Both CSP and its analogs were synthesized by Mimotopes (Roseville, MN, USA). The qualitative analysis of these peptides was done by Electro-Spray Mass Spectrometry (ESMS) and purified to 90% by reverse phase-high performance liquid chromatography (RP-HPLC). The

peptides were readily solubilized in sterile double distilled reverse osmosis water and stored in aliquots at -20°C. Cvitkovitch, et al. (2006) demonstrated that peptide analogs synthesized based on the amino acid sequence of native *S. mutans* CSP were able to interfere with biofilm formation, acid resistance, and competence development; three of the key virulence factors in *S. mutans* pathogenesis. A number of analogs were screened and the most effective KBI-3221 analog was chosen for further studies.

Table 2: CSP and Peptide Analog Amino Acid Sequence ^a

Peptide Analog	Amino Acid Sequence	Modification
CSP	SGSLSTFFRLFNRSFTQALGK	
IH-1	ΔGSLSTFFRLFNRSFTQALGK	1st residue deleted from N
IH-2	SGSLSTFFRLFNRSFTQALGΔ	1st residue deleted from C
B1	SΔSLSTFFRLFNRSFTQALGK	2nd residue deleted from N
C1	SGΔLSTFFRLFNRSFTQALGK	3rd residue deleted from N
D1	SGSΔSTFFRLFNRSFTQALGK	4 th residue deleted from N
E1	SGSLΔTFFRLFNRSFTQALGK	5 th residue deleted from N
F1	SGSLSTFFRLFNRSFTQALΔK	2nd residue deleted from C
G1	SGSLSTFFRLFNRSFTQAAΔGK	3rd residue deleted from C
H1	SGSLSTFFRLFNRSFTQALGK	4 th residue deleted from C
A2	SGSLSTFFRLFNRSFTΔALGK	5 th residue deleted from C
B2	SGSLSTFFVLFNRSFTQALGK	1 st R replaced with V
C2	SGSLSTFFALFNRSFTQALGK	1 st R replaced with A
D2	SGSLSTFFRLFNVSFTQALGK	2 nd R replaced with V
E2 (KBI-3221)	SGSLSTFFRLFNASFTQALGK	2nd R replaced with A
F2	SGSLSTFFRLFNRSFTQALGV	K replaced with V
G2	SGSLSTFFRLFNRSFTQALGA	K replaced with A

^aKrastel *et al.*, 2007.

Syvitski et al. (2007) designed and synthesized a series of C- and N-terminal truncated peptides and peptides with amino acid substitutions to investigate their structure-activity relationships based on the three-dimensional structures of *S. mutans*

wild-type signaling peptide (CSP or UA159sp). The sequences of the peptides and their designations are shown in **Table 3**. All the peptides were chemically synthesized and purified by reverse-phase HPLC, and their identities were confirmed by mass spectrometry (GL Biochem Ltd., Shanghai, China). This study is the first study to recognize the importance of the signaling peptide C-terminal residues in streptococcal quorum sensing, and to provide an insight into the structure-activity relationship of signal peptides in streptococci.

Table 3: C- and N-terminal truncated and amino acid substituted CSP analogs

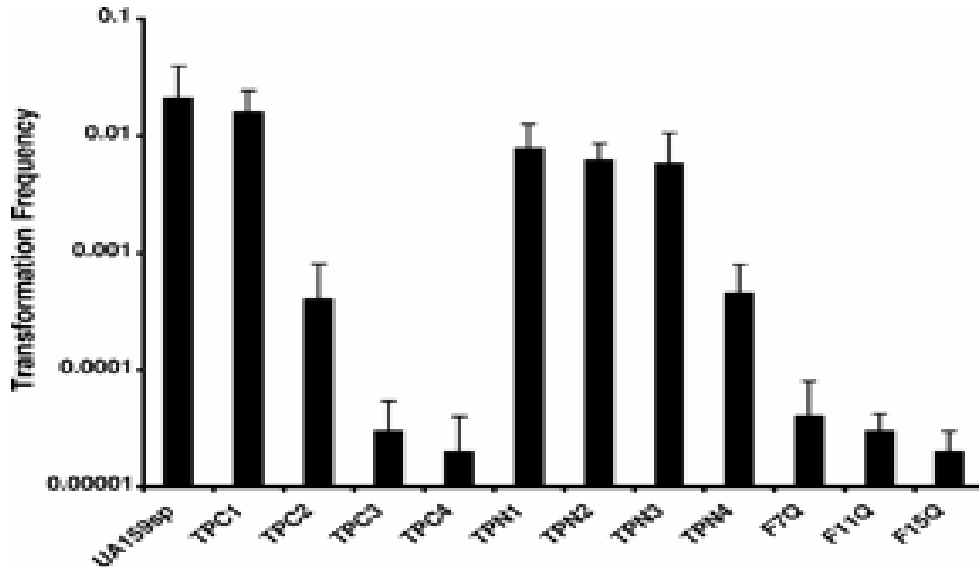
Peptide	Amino Acid Sequence
CSP (UA159sp)	SGSLSTFFRLFNRSFTQALGK
<i>Truncated Peptide</i>	
TPC1	SGSLSTFFRLFNRSFTQALG
TPC2	SGSLSTFFRLFNRSFTQAL
TPC3 (JH1005sp)	SGSLSTFFRLFNRSFTQA
TPC4	SGSLSTFFRLFNRSFTQ
TPN1	GSLSTFFRLFNRSFTQALGK
TPN2	SLSTFFRLFNRSFTQALGK
TPN3	LSTFFRLFNRSFTQALGK
TPN4	STFFRLFNRSFTQALGK
<i>Peptides with Substitutions^a</i>	
F7Q	SGSLST <u>Q</u> FRLFNRSFTQALGK
F11Q	SGSLSTFFRL <u>Q</u> NRSFTQALGK
F15Q	SGSLSTFFRLNRS <u>Q</u> TQALGK

^aAmino acid substitutions are underlined.

Furthermore, Syvitski et al. (2007) studied the effects of both truncated and substituted peptides on the induction of genetic competence using *S. mutans comC* null mutant that was unable to produce but still respond to CSP, enabling assays of peptide-dependent genetic transformation. This study revealed that deletion of even two amino acid residues from C terminus significantly affects the activity of CSP in inducing the genetic competence. Neither deletion of three or four amino acid

residues from the C terminus nor phenylalanine substitutions induced genetic competence (Fig. 3).

Fig. 3: Truncated and phenylalanine substituted peptides-dependent genetic competence.



4. CSP Fusion Peptides

Eckert *et al.* (2006) developed a new class of pathogen-selective molecules, called specifically targeted antimicrobial peptides (STAMPs) based on the fusion of a species-specific targeting peptide domain with a wide-spectrum antimicrobial peptide domain. In particular, they explored the possibility of CSP produced by *S. mutans* as a STAMP targeting domain to mediate *S. mutans*-specific delivery of an antimicrobial peptide domain. They generated fusion peptides by linking the antimicrobial peptide G2 (16 amino acids) derived from the wide-spectrum antimicrobial peptide novispirin G10 to 16-mer and 8-mer CSP peptides. In addition, He *et al.* (2007) generated fusion peptides by linking the antimicrobial peptide B-33 (S6L3-33, 11 amino acids) to 16-mer and 8-mer CSP peptides (**Table 4**). From these peptide libraries, they identified multiple peptides with robust killing activity against *S. mutans*.

Table 4: Peptide sequences (single-letter amino acid code) of CSP, CSP_{C16} containing STAMPs and STAMP components

Peptide	Amino acid sequence ^a	Molecular wt.
CSP	SGSLSTFFRLFNRSFTQALGK	2,364.9
CSP _{C16}	TFFRLFNRSFTQALGK	1933.3
G2	KNLRIIRKGIHIKKY ^b	1933.5
C16G2	TFFRLFNRSFTQALGK <u>GGG</u> KNLRIIRKGIHIKKY ^b	4079.0
CSP _{M8}	TFFRLFNR	1,100.6
M8G2	TFFRLFN <u>GGG</u> KNLRIIRKGIHIKKY ^b	3,246.9
S6L3-33 (B-33)	FKKFWKWFRRF	1,677.5
C16-33	TRRRLFNRSFTQALGK <u>SGGG</u> FKKFWKWFRRF	3,849.0
M8-33	TFFRLFN <u>SGGG</u> FKKFWKWFRRF	3,016.9

^alinker regions between targeting and killing peptides are underlined.

^bpeptide C-terminal amidation.

5. Antibacterial/Antibiofilm Activity

Antibacterial activity of CSP against *S. mutans* and other caries-associated streptococci has been demonstrated by several researches (Qi *et al.*, 2005; Petersen *et al.*, 2006; Eckert *et al.*, 2006; LoVetri, et al., in preparation). Peptide KBI-3221, an analog of CSP, has been shown to inhibit biofilm formation in caries-associated streptococci, including *S. mutans* (Krastel, et al., 2007; LoVetri et al., in preparation). It has also been shown to have inhibitory effect on mature biofilms of *S. mutans* and *S. sobrinus* (Krastel, et al., 2007).

5.1 Antibacterial Activity

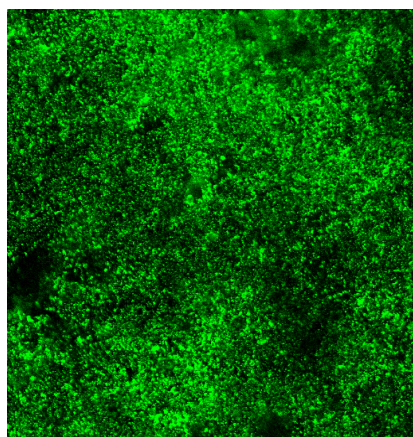
5.1.1 CSP peptide

The antibacterial activity for CSP has been determined using minimum inhibitory concentration (MIC) assay as described previously (Qi, *et al.*, 2005). The overnight culture of *S. mutans* was diluted to approximately 10⁵ CFU/ml (colony forming units per milliliter) in Todd Hewitt broth supplemented with yeast extract (0.3%) and hog gastric mucin (0.01%), pH 7.0 and aliquoted. CSP was added to cell suspensions and diluted two-fold serially. The plates were incubated anaerobically and optical density

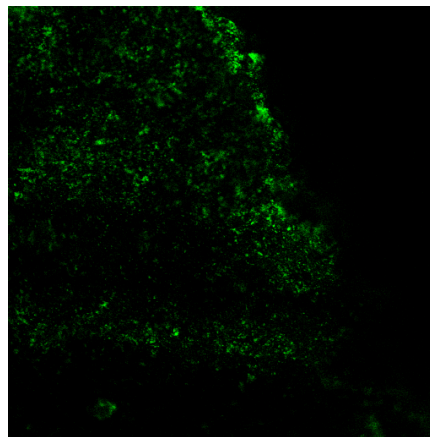
was measured at 600 nm to determine MIC. The MIC of CSP determined by Qi, *et al.* (2005) for *S. mutans* ranged from 10 to 20 μM .

The antibacterial activity of CSP was also visualized (**Fig. 4**) using a confocal scanning laser microscopy (CSLM). The CSLM was done after the biofilms were grown on hydroxyapatite disks for 24 h followed by a wash of the disk. The disks were then transferred to new culture tubes containing 500 μl of a 2% solution of wheat germ agglutinin-Alexa Fluor 488[®] conjugate diluted in PBS. This lectin conjugate binds to N-acetylglucosamine in biofilms of both gram-positive and gram-negative bacteria. Incubation of disks for 2 h (in dark) at 4°C was followed washes. The disks were air dried for 30 min (in dark) and the biofilms were observed using an Olympus IX-70 CSLM with an argon laser for excitation at 488 nm (green fluorescence). Images were captured and processed by using FluoView software and Image Pro-Plus software (Burton *et al.*, 2006).

Fig. 4: Confocal images of *S. mutans* growing on hydroxyapatite disks.



Untreated (Control)



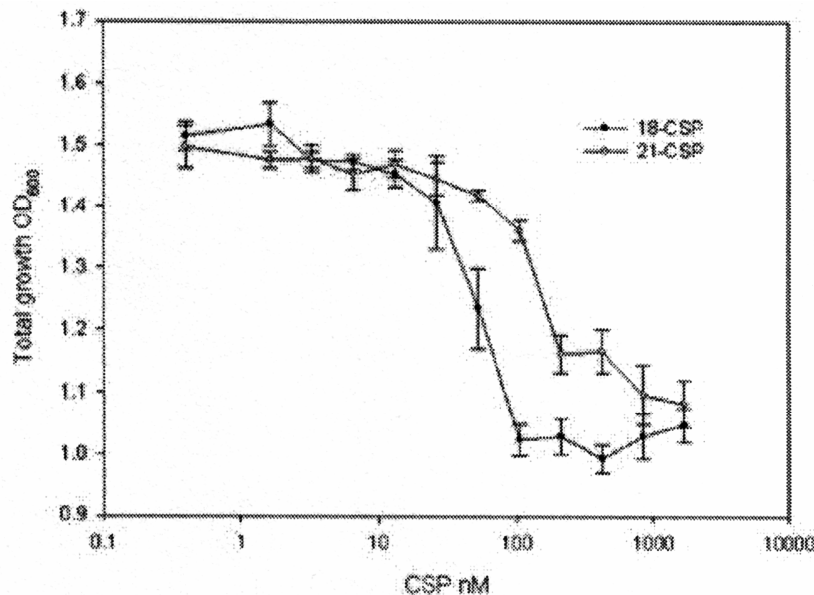
CSP-Treated

5.1.2 18- and 21-CSP peptides

Petersen *et al.* (2006) studied the antibacterial activity of both 18-mer and 21-mer CSP peptides against *S. mutans* using agar plate zone of inhibition assay and 96-well plate assay. They found 18-mer CSP more potent in inhibiting *S. mutans* growth than the 21-mer CSP (**Fig. 5**). However, the growth inhibition by 18-mer and 21-mer CSP

peptides reached comparable levels upon exposure to higher concentrations of 21-mer CSP.

Fig. 5: Inhibitory effect of 18-CSP and 21-CSP on *S. mutans* growth



5.2 Antibiofilm Activity

5.2.1 CSP analogs

Biofilms were assayed by crystal violet staining method, as described previously (Cvitkovitch, *et al.*, 2001). *S. mutans* were grown under anaerobic conditions in Todd Hewitt Broth supplemented with yeast extract (3%) and hog gastric mucin (0.01%) pH 7.0. The biofilm was grown in a 96-well microtiter plate (Corning Inc., New York) initiated by inoculation of overnight culture at 3.3% into semi-defined minimal (SDM) media. Biofilm was grown in the absence and presence of analog KBI-3221. Biofilm growth was determined by measuring the absorbance at 630 nm.

To determine if biofilm development was affected by the peptide analogs, an *in-vitro* static biofilm assay using 96 well polystyrene microtiter plates was performed. The growth of the biofilm was initiated by inoculating an overnight culture (1:30 dilution) into semi-defined minimal (SDM) medium supplemented with glucose and containing 5 µg/ml of peptide analogs. The biofilms were developed at 37°C in air with 5% CO₂ for 16 h. Biofilm biomass was quantified by measuring the absorbance of safranin stained biofilms at 490 nm. Biofilms grown in the presence of peptide analogs F1,G1, E2 (KBI-3221), F2 and G2 had a significantly reduced

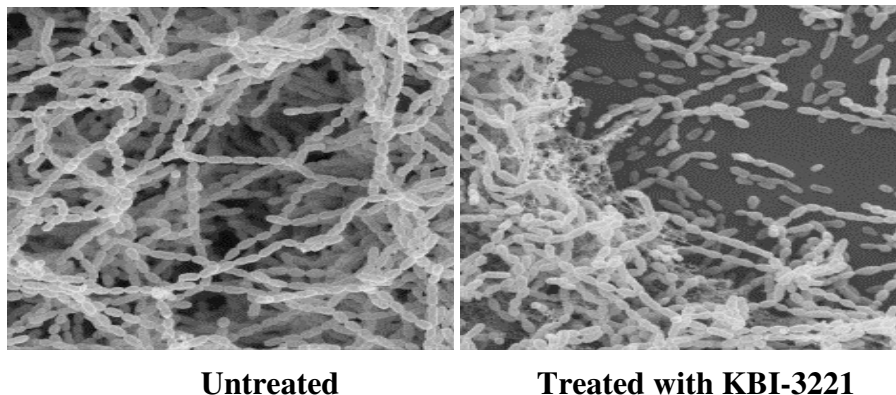
biomass ranging from 24 to 40% compared to the *S. mutans* biofilm grown in the presence of the control peptide (**Table 5**).

The antibiofilm effect of KBI-3221 was visualized by scanning electron microscopy (**Fig. 6**). The scanning electron micrographs show biofilm accumulation on the surface of glass discs. Biofilms formed during incubation with peptide KBI-3221 had an altered structure and the cells tended to form clumps with large channels between them (Magnification x 5,000).

Table 5: Effect of CSP analogs on biofilm formation of *S. mutans*

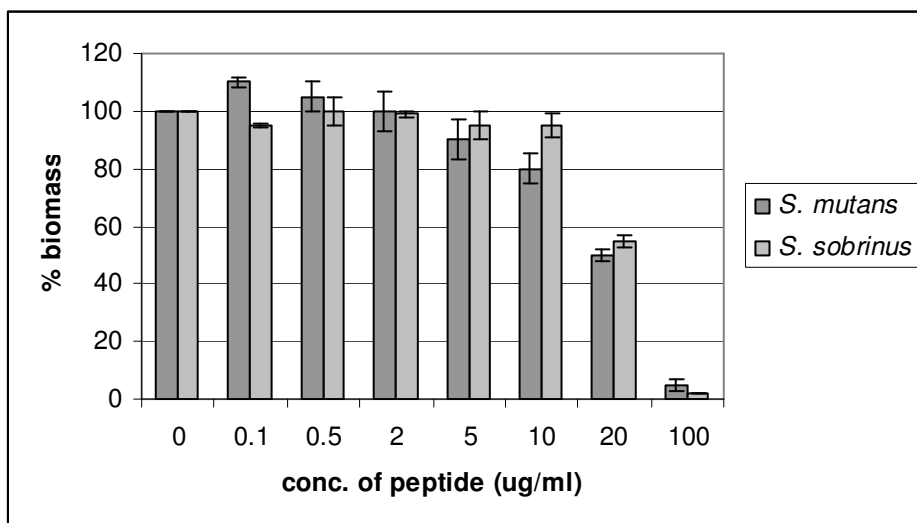
Peptide Analog	Biofilm formation
CSP (control)	No effect
IH-1	No effect
IH-2	No effect
B1	No effect
C1	No effect
D1	No effect
E1	No effect
F1	↓ 36.7% biomass
G1	↓ 24.4% biomass
H1	No effect
A2	No effect
B2	No effect
C2	No effect
D2	No effect
E2 (KBI-3221)	↓ 40.0% biomass
F2	↓ 38.7% biomass
G2	↓ 35.6% biomass

Fig. 6: Scanning Electron Micrograph of *S. mutans* growing in biofilm in the presence and absence of KBI-3221



Effect of KBI-3221 peptide on biofilm formation in *S. mutans* and *S. sobrinus* in the presence of 20 $\mu\text{g/ml}$ of KBI-3221 was determined and it showed approximately 50% decrease in biomass relative to the control (no added peptide). When tested at 100 $\mu\text{g/ml}$ KBI-3221 almost completely inhibited the formation of biofilm for both species (**Fig. 7**).

Fig 7: Peptide KBI-3221 inhibits biofilm development^a



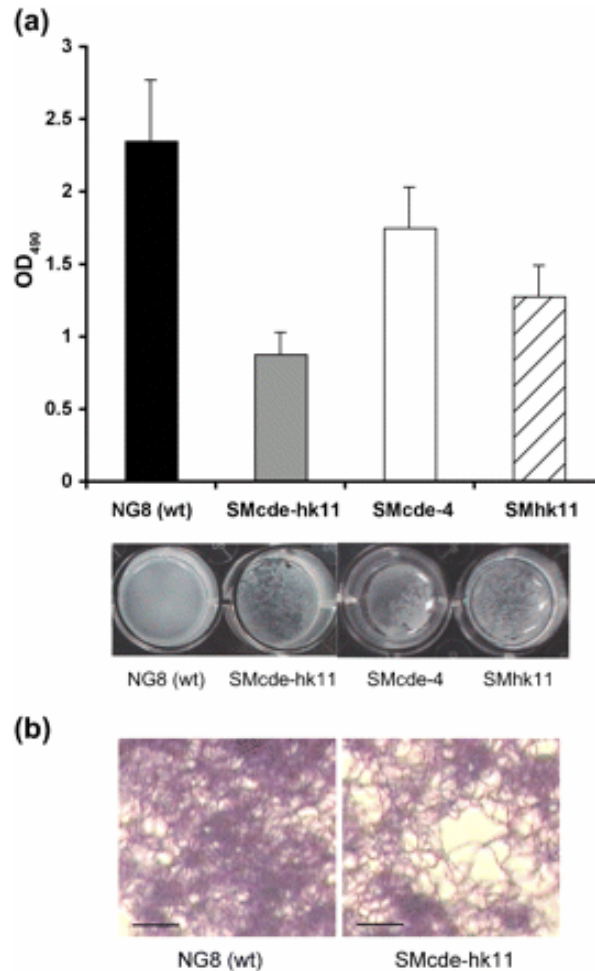
^aMean \pm SE of three independent experiments

5.2.2 Mutants in Biofilm Formation

Li et al. (2008) showed that mutations in either of the two-component signal transduction systems (TCSTS) ComCDE or HK/RR11 or both contributed to a reduced biofilm formation and a sponge-like architecture (**Fig. 8a & 8b**). Figure 8

shows biofilm formation by the mutants and parental strain on the surface of a polystyrene microtitre plate. (a) The biofilm formed by SMcde-hk11 shows reduced biomass with a sponge-like architecture. (b) Phase-contrast microscopy shows the SMcde-hk11 biofilm to be composed of cells in extremely long chains. Bars, 20 μ m

Fig 8: Biofilm Formation by TCSTS Mutants & Parental Strain^a



^aLi *et al.*, 2008

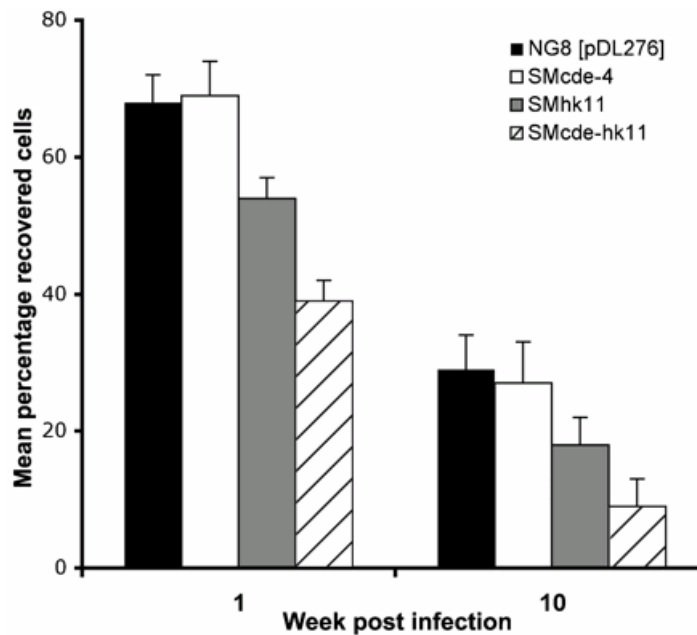
Animal studies showed that these TCSTS mutants had reduced capabilities for oral colonization, succession and initiation of dental caries (**Table 6**). Oral colonization by *S. mutans* NG8 [pDL276] and mutants SMcde-4, SMhk11 and SMcde-hk11 are shown in mono-infected animals during the first week and the last (tenth) week post-inoculation (**Fig. 9**).

Table 6: Caries Scores for *S.mutans* NG8[pDL276] and its mutants in a rat caries model^a

Group*	Mean caries score ± SD	Percentage of caries on smooth surface †	P value ‡
Mono-infection			
NG8 [pDL276]	56.4 ± 8.5	55	-
SMcde-4	36.7 ± 6.4	35	0.012
SMhk11	35.6 ± 7.2	24	0.015
SMcde-hk11	28.6 ± 5.8	13	0.002
Mixed infection			
SMcde-4/ NG8 [pDL276]	53.5 ± 6.8	56	0.48
SMhk11/ NG8 [pDL276]	52.4 ± 7.4	54	0.55
SMcde-hk11/ NG8 [pDL276]	54.7 ± 6.5	55	0.42

*n=8 animals per group. † (Number of smooth surface caries/total number of caries scored)×100. ‡ A comparison with the wild-type group and significant by Student's *t* test ($P<0.05$). ^aLi *et al.*, 2008

Fig 9: Oral Colonization by *S. mutans* TCSTS Mutants & Parental Strain^a



^aLi *et al.*, 2008

5.3 Broad-Spectrum Antibacterial/Antibiofilm Activity

5.3.1 CSP Peptide

The broad-spectrum antibacterial activity for CSP against *Streptococcus spp.* such as *S. mutans*, *S. sobrinus*, *S. oralis* and *S. sanguis* has been determined using minimum inhibitory concentration (MIC) assay as described under 5.1. The MIC values ranged from 0.65 to >160 μ M (Table 7).

Table 7: MIC of CSP for *Streptococcus spp.*

Species/strains	MIC (μ M)
<i>S. mutans</i> UA140	0.65-1.3 ^a
<i>S. mutans</i> UA159	10-20 ^a
<i>S. sobrinus</i> HNG909S	80 ^b
<i>S. oralis</i> NCTC 11472	160 ^b
<i>S. sanguis</i> SK120	> 160 ^b

^a Qi, *et al.*, 2005; ^bKane Laboratory experimental results

5.3.2 CSP Analogs

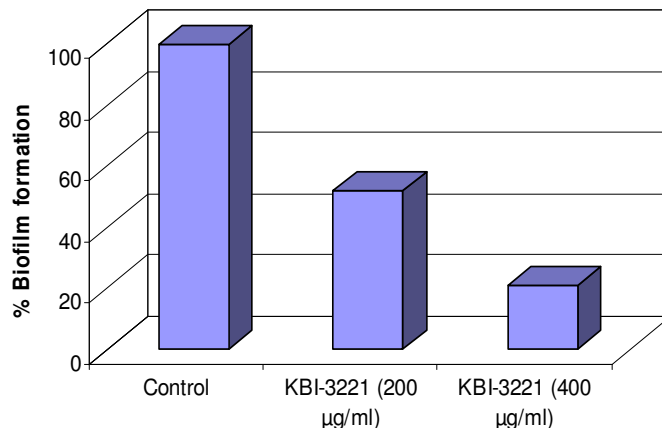
Biofilms were assayed by crystal violet staining method, as described 4.2. The *Streptococcus spp.* were grown under anaerobic conditions in Todd Hewitt Broth supplemented with yeast extract (3%) and hog gastric mucin (0.01%) pH 7.0. The broad spectrum of KBI-3221 (20-50 μ g/ml) for inhibiting biofilm can be seen in Table 8 where it was tested against other *Streptococcus spp.*

Table 8: Effect of KBI-3221 on biofilm-embedded *Streptococcus spp.*

Organism	% Biofilm inhibition in the presence of KBI-3221
<i>S. sobrinus</i>	43.04
<i>S. mitis</i>	39.42
<i>S. oralis</i>	68.42
<i>S. sanguis</i>	39.97
<i>S. gordonii</i>	62.06

KBI-3221 was also tested in an *in vitro* oral biofilm model as described by Guggenheim *et al.* (2001). A mixed-species biofilm was produced and biofilms were grown in 24-well polystyrene cell culture plates on sintered hydroxyapatite disks (9 mm; Clarkson Chemical Company, Williamsport, PA, USA) that had been preconditioned in pooled saliva. The disks were covered with growth medium and 200 µl of a mixed cell suspension prepared from equal volumes and densities of each strain. A tryptone-yeast-based broth medium with 67 mM Sørensen's buffer (mFUM, pH 7.2) was used for growth media. The carbohydrate concentration in mFUM was 0.3% (w/v) and consisted of glucose for the first 16 h and from then on a 1:1 (w/w) mixture of glucose and sucrose. Biofilms were incubated anaerobically at 37°C for a total of 64.5 h. Following the 16.5 h incubation, the disks were dipped for 1 min (gentle shaking) in test solutions (1 ml) and immediately transferred to a new equilibrated 24-well plate. This process was repeated every 4 h (three times/day) and disks were placed back into the 24-well plate. This same process was repeated the following day with a new plate being prepared to be used for final day of dipping and incubation. After a final dipping, the plate was incubated for 16 h followed by biofilm harvest and processing. The disks were washed then transferred to a 50 ml Falcon tube. Addition of 1.0 ml of physiological saline, followed by vortexing for 2 min, then sonication for 5 s. Cultures were diluted and plated. KBI-3221 at either 200 or 400 µg/ml concentrations was effective in decreasing the biofilm formation in the oral biofilm model (**Fig. 10**).

Fig. 10: Effect of KBI-3221 on oral biofilm formation in an *in vitro* oral biofilm model



5.3.3 CSP Fusion Peptides

Eckert et al. (2006) tested the broad-spectrum antibacterial activity of CSP fusion peptides (STAMPs) against streptococci using the MIC assay as described under 4.1. As shown in **Table 9**, M8G2 fusion peptide displayed MICs against *S. mutans* and other streptococci similar to those seen with C16G2. As shown in **Table 10**, a two to three-fold difference in MICs between S6L3-33 and the derived STAMPs against *S. mutans* and other oral streptococci was observed.

Table 9: MICs of STAMPs constructed with G2 and CSP for caries-associated *Streptococcus spp.*

MIC ± SD (µM) ^a						
Strain	CSP	CSP _{C16}	G2	C16G2	CSP _{M8}	M8G2
<i>S. mutans</i> UA159	50.8 ± 9.3	> 60	12.1 ± 4.5	3.0 ± 1.6	> 60	3.25 ± 1.9
<i>S. gordonii</i>	> 60	> 60	41.3 ± 14.0	23.5 ± 7.8	> 60	20.0 ± 5.0
<i>S. sanguinis</i>	> 60	> 60	33.6 ± 7.5	19.1 ± 4.0	> 60	15 ± 2.5

^a MIC's represent averages of 3 independent experiments with standard deviations.

Table 10: MICs of STAMPs constructed with S6L3-33 (=B-33) and CSP for caries-associated *Streptococcus spp.*

MIC \pm SD (μ M) for indicated strain ^a				
Peptide	<i>S. mutans</i> UA159	<i>S. mutans</i> <i>comD</i>	<i>S. sanguinis</i>	<i>S. gordonii</i>
S6L3-33	7.0 \pm 3.0	6.5 \pm 2.5	40.0 \pm 7.5	20 \pm 5.0
C16-33	2.5 \pm 2.1	2.2 \pm 0.5	13.3 \pm 5.8	14.6 \pm 5.0
M8-33	2.5 \pm 2.0	2.5 \pm 2.0	20 \pm 2.0	10 \pm 2.5

^a MIC's represent averages of at least 3 independent experiments with standard deviations

6. Mode of Action

Although the exact mechanism of CSP antimicrobial activity is not clearly known at this point, it may possibly be due to autolysin activation by excess CSP leading to the lysis of *S. mutans* cells (Petersen *et al.*, 2005; Shibata *et al.*, 2005). Qi *et al.* (2005) showed that addition of exogenous CSP beyond the levels necessary for competence inhibited the growth of *S. mutans*. The also demonstrated that further increases of CSP stopped *S. mutans* cell division leading to cell death.

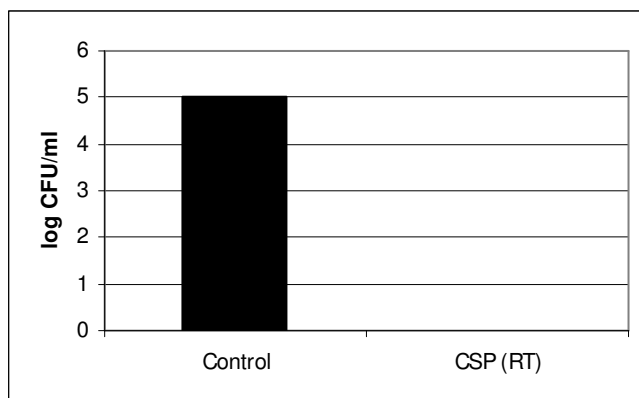
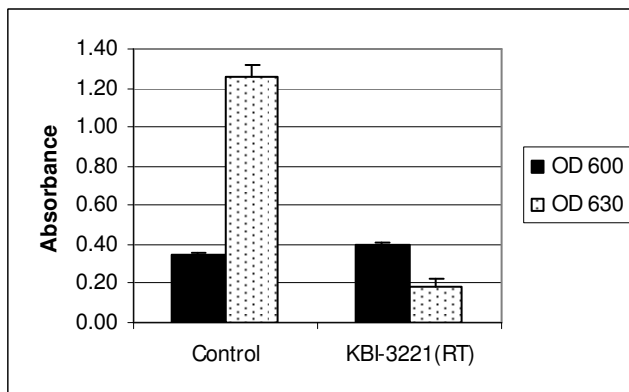
Goodman and Kay (2006) showed that CSP is specific for *S. mutans* and functions exclusively as a regulator of genes such as *gtfB* and *gtfC* and leads to repression of their expression. Glucosyltransferases (GTFs) are the products of these genes and are essential for efficient attachment of *S. mutan* to the surface of teeth. In the presence of their substrate (sucrose), GTFs catalyze the formation of long polymers of glucose called glucans, which are instrumental in the adherence of *S. mutans* to the surface of teeth. Once the attachment is complete, the *gtf* genes are repressed and sucrose is utilized as energy source. The concentration of CSP is not naturally produced in significant quantities during the initial attachment to the tooth but is otherwise present after the adhesion is completed to ensure that *gtf* genes are repressed. Thus, the CSP concentration that is beyond the levels necessary for initiating the quorum-sensing process can reduce the efficiency of *S. mutans* adherence to tooth and thereby it should reduce the dental caries. Moreover, since

early colonizing non-pathogenic oral bacteria rely on their own *gtf* genes for efficient adherence but are not affected by the presence of the *S. mutans* CSP, such bacteria will gain a competitive advantage over *S. mutans* if the ability of *S. mutans* to adhere to tooth surfaces is reduced.

The antibiofilm activity of an analog of CSP such as KBI-3221 is possibly due to the binding of peptide analogue in a weakened manner to *S. mutans* (Eckert *et al.*, 2006) or a conformational change caused by the weakened binding that changes the ability of the three to four C-terminal residues to activate the quorum sensing signaling system (Syvitski *et al.*, 2007). Li et al. 2008 study data also supports Syvitski et al. 2007 in that the disruption of the CSP-mediated quorum sensing system decreases biofilm and the initiation of caries in a rat model. Furthermore, an analog of CSP could be a specific competitive inhibitor of the interaction between CSP and histidine kinase receptor, hindering the induction of biofilm formation in *S. mutans* and other caries-associated streptococci.

7. Stability

A slightly modified biofilm assay of Li *et al.* (2001) was used to study the stability of CSP. The biofilms were developed in a 12-well microtiter plate (Costar 3513, Corning Inc., Corning, N.Y.) Growth of biofilm was initiated by inoculation of overnight culture at 1.0% into 0.25 dilute THYE + 0.01% hog gastric mucin media. Planktonic cells were removed and wells were rinsed and biofilm cells were resuspended, serially diluted and plated. The 96-well assay (described section 4.2) was used to determine the stability of KBI-3221. The stability at room temperature (RT) of the peptides was tested at 4 week intervals over a 1 year period (**Fig. 11a & 11b**). These graphs show that both peptides were stable after the 1 year period.

Fig. 11a: 1 Year stability of CSP using 12-well Assay**Fig. 11b: 1 Year stability of KBI-3221 using 96-well biofilm Assay**

8. CSP in Combination with Antimicrobials

The 12-well assay (described Section 6) was used to test CSP in combination with numerous compounds. Some of these are currently used in the oral care market and others which have shown potential against oral bacteria. The compounds tested were: chlorhexidine, triclosan, sodium fluoride, xylitol, citric acid, zinc citrate, nisin, lansoprazole, epigallocatechin gallate (EGCg), oleanolic acid, lactoferrin and chitosan. CSP has antimicrobial activity against the caries-associated streptococci and also acts synergistically with currently used oral antimicrobials in inhibiting the growth and proliferation of biofilm-embedded streptococci (LoVetri et al., in preparation).

9. CSP Analog in Combination with Antimicrobials

The 96-well assay (described Section 4.2.1) was used to test KBI-3221 in combination with numerous compounds. Some of these are currently used in the oral care market and others which have shown potential against oral bacteria. The compounds tested were: chlorhexidine, triclosan, sodium fluoride, xylitol, citric acid, zinc citrate, nisin, lansoprazole, epigallocatechin gallate (EGCg), oleanolic acid, lactoferrin and chitosan. KBI-3221 peptide inhibits biofilm formation in caries-associated streptococci and also acts synergistically with antimicrobials to inhibit biofilm formation in *S. mutans*, the primary etiological agent of dental caries (LoVetri et al., in preparation).

10. Concluding Remarks

Developing oral prophylactic strategies through interference with two-component systems or quorum-sensing of biofilm microorganisms represents an interesting future challenge (Scheie and Petersen, 2004). Unlike strategies that target microbial viability, such approaches may interfere with microbial adaptive pathways without killing the microorganism. Therefore, resistance development would probably represent a minor problem. A better understanding of genetic regulation of quorum-sensing system in oral biofilm formation is necessary to develop novel strategies for oral disease prevention and control based on the interference of two-component signal transduction systems or quorum sensing. Since the systems contain both conserved and variable components, both broad- and narrow-spectrum responses may be available. This could allow tailoring of prophylactic measures based on individual oral health status and risk assessment.

11. Desirable Features

- a) Safety: CSP or KBI-3221, an analog of the naturally occurring CSP peptide, is unlikely to pose any safety concerns
- b) Resistance: No known danger of bacteria developing resistance to a small peptide
- c) Activity: Micro-quantities of peptide are needed to prevent dental caries

d) Specificity: Since the antibacterial or antibiofilm activity of CSP and KBI-3221 is specific to oral streptococci, the beneficial oral bacteria are left unharmed during therapy.

12. Applications

CSP or KBI-3221 could be used alone or in combination with antimicrobial agents for:

- a) Toothpaste and Mouthwash
- b) Denture wash and Adhesives
- c) Chewing gum and Candies
- d) Bottled water
- e) Soft drinks and Sports drinks
- f) Dog biscuits

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