Autolysis, DNA uptake and recombination: role in *Streptococcus mutans* competence and biofilm formation

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INTRODUCTION

Bacteria in natural environments are most often found attached to surfaces, embedded in an extracellular matrix. The composition of the extracellular matrix includes polysaccharides, proteins and DNA. This mode of growth is associated with differential expression of several genes, including often regulatory genes such as those involved in bacterial cell-to-cell communication. In the biofilm mode, bacteria exhibit increased resistance to antimicrobials and to host defense systems (3).

Streptococcus mutans is among the bacteria commonly found in biofilms on the tooth surface. This Gram-positive, facultative anaerobic bacterium is commonly found in the human oral cavity and is associated with tooth decay. *S. mutans* was first described by Clarke in 1924. The current classification of *S. mutans* is presented in figure 1. *S. mutans* growth and metabolism leads to changes in local environmental conditions, such as Eh, pH, co-aggregation, and substrate availability. *S. mutans,* along with *Streptococcus sobrinus*, *is* thought to play a major role in tooth decay, metabolizing sucrose to lactic acid. The acidic environment created in the mouth by this process may favor demineralization of the tooth enamel that may ultimately lead to tooth decay. *S. mutans* is equipped with variety of receptors that help for better adhesion to the surface of teeth. In addition *S. mutans* utilize sucrose to produce a sticky, extracellular, dextran-based polysaccharide that contributes to adhesion to other cells and dental plaque. Conversely, other sugars such as glucose, fructose, and lactose can be metabolized by *S. mutans* to produce lactic acid as an end product. It is the combination of plaque and acid that leads to dental decay.

Figure 1. *S. mutans* classification:

Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Cocci
Order:	Lactobacillales
Family:	Streptococcaceae
Genus:	*Streptococcus*
Species:	**mutans** [1]

Cell-to-cell communication via auto inducers, also known as quorum sensing, is a mechanism that enables bacteria to sense and respond to changes in the density of the bacteria in a given environment. Typically, if there are only a few cells in an area, nothing will happen, if there

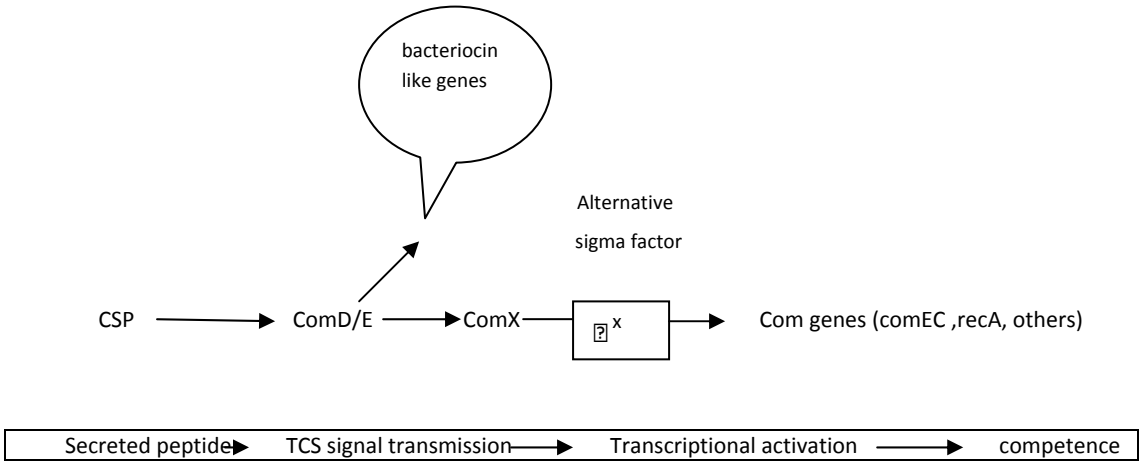
are a lot of cells, the secreted chemicals may reach a threshold concentration, causing the cells to perform a specific action. In *S. mutans*, a cell-to-cell communication system induced by the competence signaling peptide (CSP) is involved in biofilm formation (figure 3). The CSP signal induces global changes in gene expression, including genes associated with extracellular DNA binding, uptake, and DNA recombination, resulting in competence for genetic transformation. DNA binding is required for *S. mutans* transformation, as it allows the extracellular DNA to be transported into the cell, most probably through a water channel formed by ComEC. The chain of events following DNA uptake includes incorporation of the incoming DNA within the bacterial chromosome via homologous recombination. Both *comEC* and *recA* expression is increased in CSP-induced competent cells (6).

RecA has a conserved function in homologous recombination, and plays a central role in this step (2) . RecA is a 38 kilo Dalton *Escherichia coli* protein essential for the repair and maintenance of DNA. RecA has a structural and functional homologue in virtually every species and serves as an archetype for homologous DNA repair proteins. The homologous protein in *Homo sapiens* is called *RAD51*. The association of RecA with DNA is based on its central role in homologous recombination. The RecA protein binds strongly and in long clusters to ssDNA to form a nucleoprotein filament. The protein has more than one DNA binding site, and thus can hold a single strand and a double strand together. This feature makes it possible to catalyze a DNA synapsis between a DNA double helix and a homologous region of single stranded DNA. The reaction initiates the exchange of strands between two recombining DNA double helices. After the synapsis event, in the heteroduplex region a process called *branch migration* begins. In branch migration an unpaired region of one of the single strands displaces a paired region of the other single strand, moving the branch point without changing the total number of base pairs. Spontaneous branch migration can occur, however as it generally proceeds equally in both directions it is unlikely to complete recombination efficiently. The RecA protein catalyzes unidirectional branch migration and by doing so makes it possible to complete recombination, producing a region of heteroduplex DNA that is thousands of base pairs long. Since it is a DNA-dependent ATPase, RecA contains an additional site for binding and hydrolyzing ATP. RecA associates more tightly with DNA when it has ATP bound than when it has ADP bound. [(1)]

The gene *comC* encodes the auto inducer molecule, competence-stimulating peptide (CSP). CSP is synthesized as a precursor containing a Gly-Gly leader sequence. This competence-

stimulating peptide (CSP) is exported and matured by ComA-ComB, an ABC exporter that cuts off a leader peptide after a Gly-Gly motif in the 45-residue pre-CSP encoded by *comC*. Upstream of *comC* are the *comDE* genes. Homology searches have identified their products as a histidine kinase (ComD) and a response regulator (ComE), which together constitute a two component regulatory system. ComDE is responsible for sensing CSP, and initiate a regulatory response. Signal transduction pathways of this kind are common in bacteria, where they play a central role in adapting bacterial growth to changing environmental conditions. Histidine kinases are usually situated in the plasma membrane, serving as environmental sensors. They modulate the activity of their cognate cytoplasmic response regulators by a phosphor transfer mechanism, which in most cases activate transcription of the appropriate target genes (1). A model for the CSP regulatory cascade in streptococci is shown in figure 2.

Figure 2. ComX is a competence-specific sigma factor responsible for expression of the late competence genes, such as comEC and recA. CSP regulates the expression of com genes by activating transcription of the comDE genes.

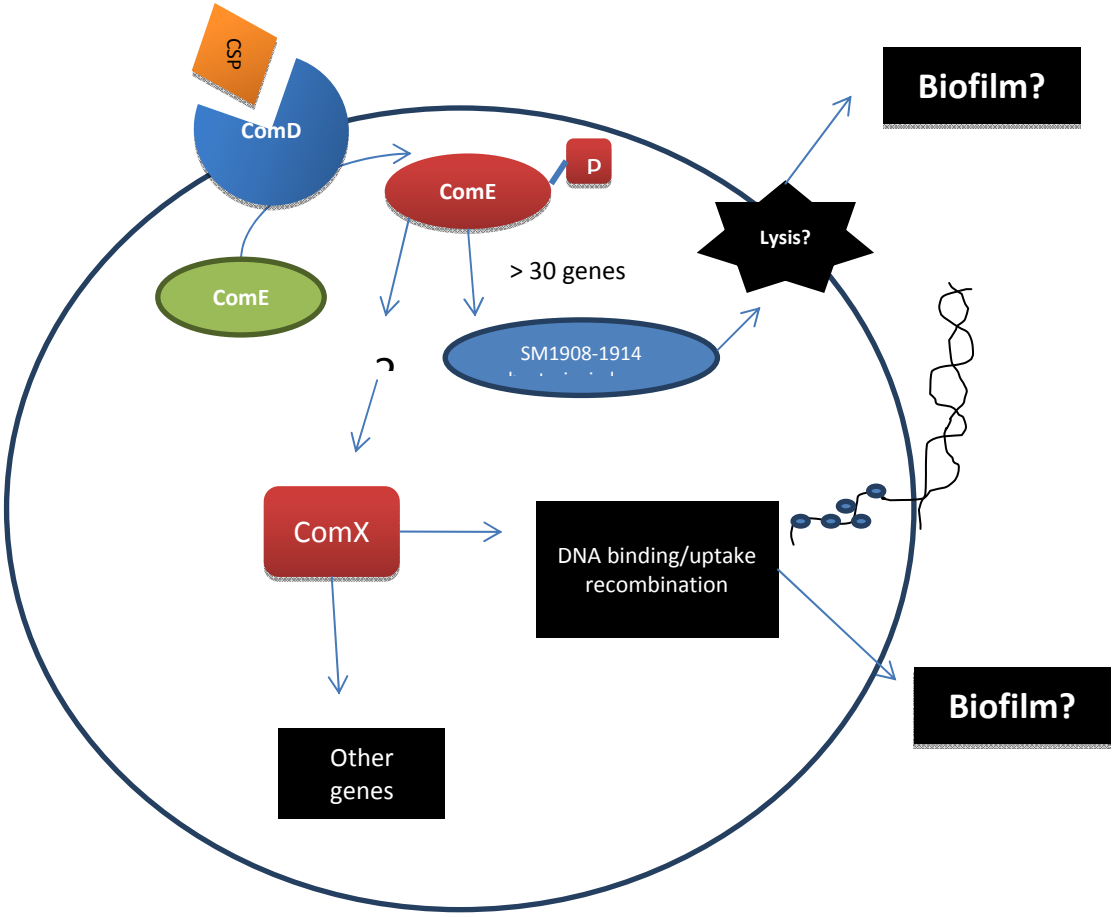


The mechanism by which CSP signaling enhances biofilm formation is rather unknown. Previous results indicate, however, that the extracellular DNA may play an important role (8). Part of the CSP-induced competent cells undergoes autolysis, resulting in the release of DNA that may be used as a matrix component of *S. mutans* biofilms. According to this hypothesis, isogenic deletion mutants that are deficient in DNA binding form less biofilm than the wild type strain. Mutants deficient in DNA binding are, however, also deficient in DNA uptake. It is possible, therefore, that the DNA entering the cells may play a role in the increased biofilm formation observed in CSP-induced competent cells. More recently, the CSP-induced autolysis was shown to be mediated by the bacteriocin mutacin V, which expression is also up regulated in the presence of CSP (7). Since autolysis is involved in the increased DNA release in CSP induced cells, it is possible that this mechanism may also be involved in biofilm formation.

AIM

The aim of this study was to investigate mechanisms by which CSP communication may enhance biofilm formation, including DNA uptake, recombination, and autolysis.

Figure 3. *S. mutans* competence model and association with biofilm formation. The sensor histidine kinase ComD is activated by an accumulation of the CSP pheromone and activates its cognate response regulator ComE by phosphorylation. Activated ComE then directly regulates the expression of more than 30 genes, including the alternate sigma factor ComX, and the SMU.1908 to SMU.1914 in the bacteriocin locus. Lysis may be associated with increased biofilm formation. Expression of genes involved in DNA binding, DNA uptake, and recombination are directly controlled by ComX, and may also be involved in biofilm formation.



MATERIALS AND METHODS

Bacterial strains

The following *S. mutans* strains were used in this study:

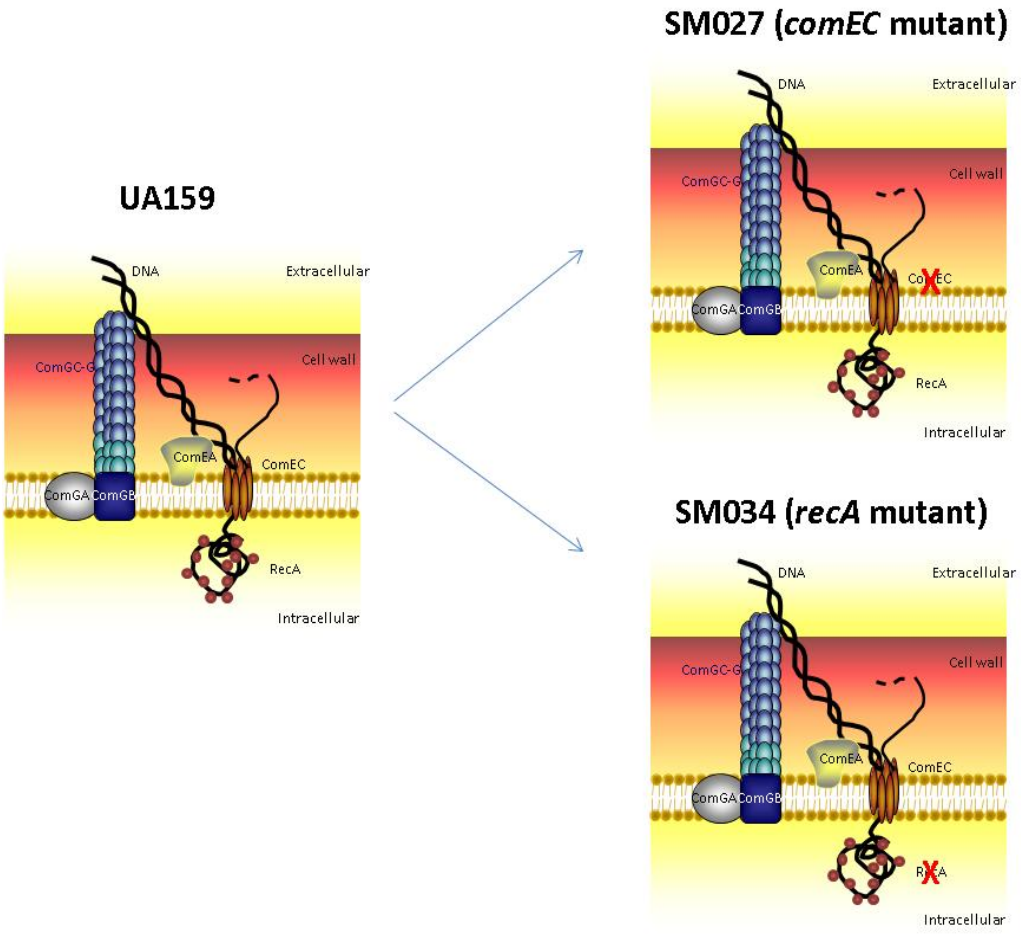
- *S. mutans* UA 159, wild type.
- *S. mutans* UA 159 isogenic mutants:

  -SM027, a comEC deletion mutant (figure 4).

  -SM034, a recA deletion mutant (figure 4).

  -SM049 SMU.1908 to SMU.1914 deletion mutant.

Figure 4. *S. mutans* UA159 wild type, and two of the isogenic mutants used in this study, SM027 (*comEC* mutant), and SM034 (recA mutant).



The functions that have been attributed to comEC, recA, and SMU.1908-1914 are summarized in table 1.

Table 1. Functional attributes of the deleted genes in the isogenic mutants

Encoded protein		**Function**	***S. mutans* competence**	***S. mutans* biofilm formation**
ComEC		Forms an aqueous transport channel through which DNA enters the cell (Ref Draskovic and Dubnau 2005) Needed for DNA uptake but not for binding to the cell surface	Not tested	Not tested
RecA		DNA strand-exchange Needed for transformational recombination	(9)	Less biofilm (4,9)
SMU.1908-SMU.1914 proteins	SMU.1908c	Hypothetical protein	Not tested	Not tested
	SMU.1909c	Hypothetical protein		
	SMU.1910c	Hypothetical protein		
	SMU.1912c	Hypothetical protein		
	SMU.1913c	Bacteriocin immunity protein		
	SMU.1914c	Mutacin V Autolysis in response to CSP	Not stimulated by CSP (4,7,9)	Not stimulated by CSP (7)

Media and synthetic CSP

- Trypticase soy broth (TSB)
- Synthetic Competence-Stimulating Peptide (CSP):

The 18-CSP (NH2-SGSLSTFFRLFNRSFTQA-COOH) was synthesized by GenScript (GenScript Corporation, NJ), with a purity grade of > 95%. The lyophilized peptide was reconstituted in distilled water and stored in small aliquots at −20°C. The 18-CSP was used at a final concentration of 360 nM.

Preparation of pre-culture:
The bacteria were grown in Trypticase soy broth (TSB). Three colonies of each strain in 5 mL TSB was grown overnight (18-24h) at 37˚C in 5% CO_2 aerobic atmosphere.

We incubated 200µL overnight culture in 20mL TSB at 37˚C in 5% CO_2 aerobic atmosphere (2-4h) until the optical density at 600 nm (OD_{600}) was 0,2.

Culture of overnight grown cells (800µL) was added to 100µL glycerol and the prepared stocks were stored at -80 ˚C.

Biofilm assay:

Before each experiment, polystyrene discs were placed in the wells of two 24-well polystyrene microtiter plates (figure 5).

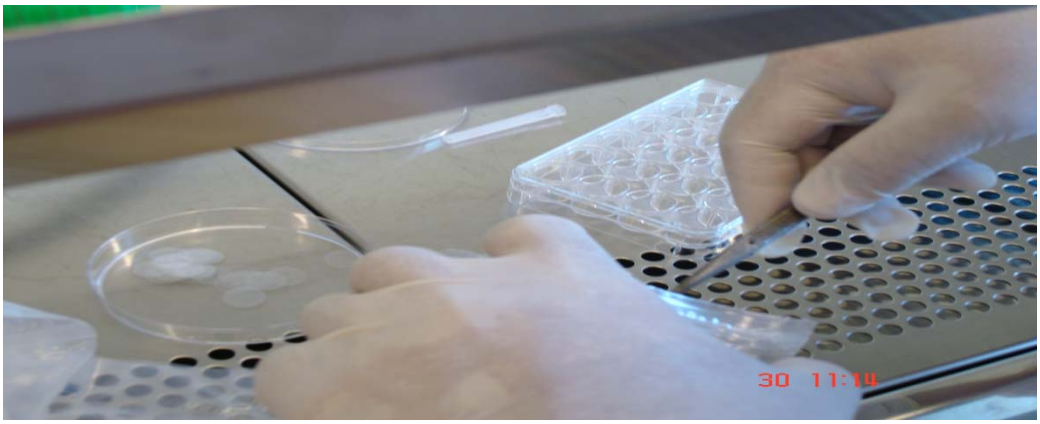


Figure 5. Polystyrene discs were placed in 24-well microtiter plates.

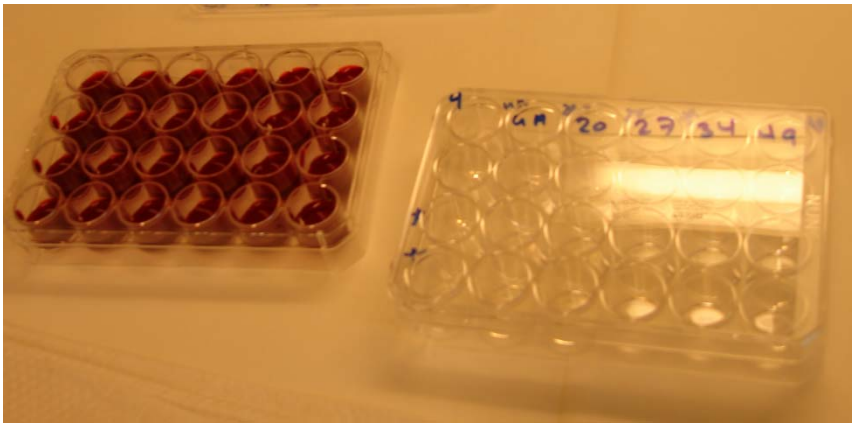
Pre-culture stocks were diluted 1:50 in TSB (200µL pre-culture in 20mL TSB), and were incubated at 37 ˚C in 5% CO_2 aerobic atmosphere for 30 min.
We then added 2µL CSP to wells C1 to D6, and added 500µL of the diluted cultures to each well. The plates were incubated at 37 C˚ for 1h in air and then for 18-24h in 37 ˚C in 5% CO_2 aerobic atmosphere.

Table 2.Content of each wells in the 24 wells microtiter plates.

	UA159	SM020	SM027	SM034	SM049
TSB	500µL	500µL	500µL	500µL	500µL
TSB	500µL	500µL	500µL	500µL	500µL
TSB	2µL CSP+500µL	2µL CSP+500µL	2µL CSP+500µL	2µL CSP+500µL	2µL CSP+500µL
TSB	2µL CSP+500µL	2µL CSP+500µL	2µL CSP+500µL	2µL CSP+500µL	2µL CSP+500µL

After incubation, we added 500µL Safranin to each well and the biofilms on the discs were stained for 10 min (figure 6).

Figure 6. Microtiter plate after the addition of safranin.



Each disc was removed with tweezers and rinsed with sterile distilled water (figure 7). The discs were then placed in a new 24 well microtiter plate (figure 8). The discs were allowed to dry for one day at room temperature.

Figure7. Rinsing of dics

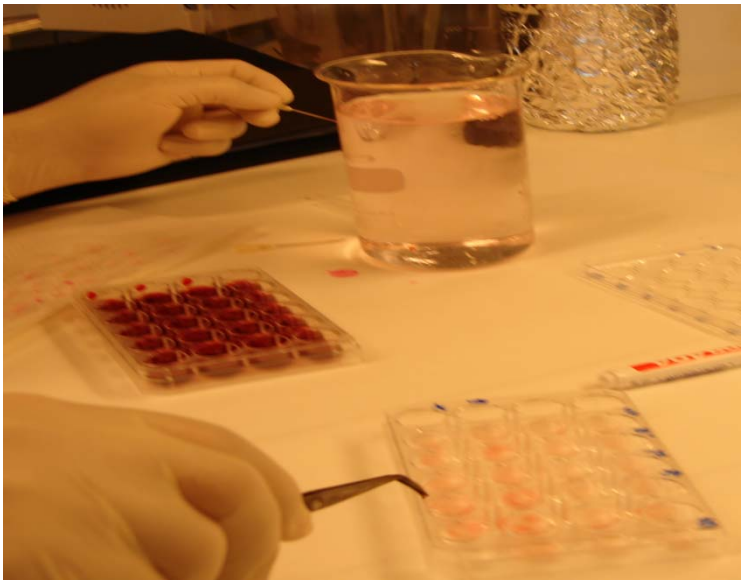
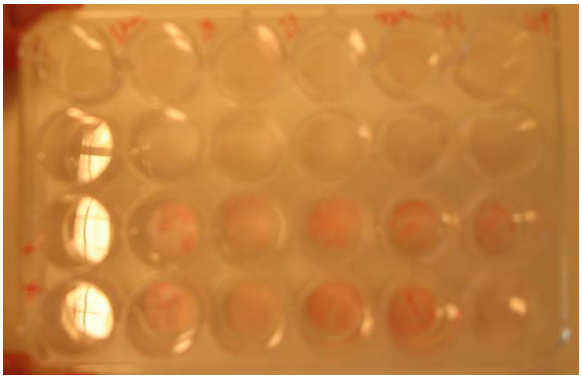


Figure 8. Transfer of stained discs to a new microtiter plate.



We then added 250µL acetic acid to each well and waited for 5 min. After that, 200µL were transferred in a 96- well plate and the results were read in a spectrophotometer at OD530 (figure 9 and 10).

Figure 9. Microtiter plate with the safranin from biofilm discs suspended in acetic acid.

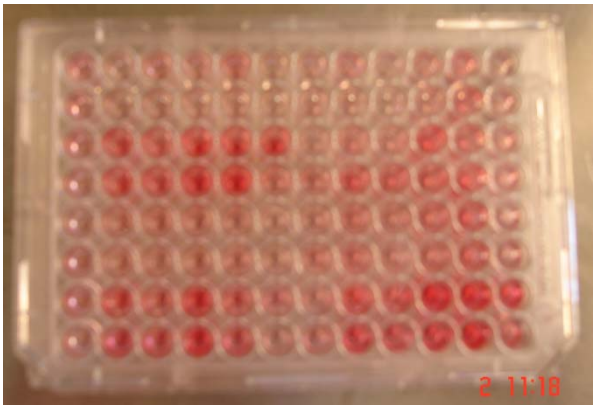


Figure 10. Spectrophotometer used to quantitate biofilms.

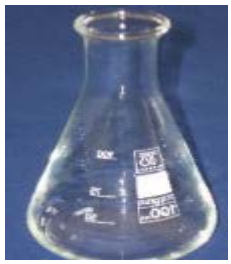
Transformation assay

(a) Preparation of agar plates.

- Todd–Hewitt broth (THB)
- Bacto agar
- Erythromycin
- sterile distilled water

Sterile distilled water (1000mL) was mixed with 30g THB and 15g Bacto Agar (figure 11). Then the mixture was divided in two and autoclaved. Both mixtures were allowed to cool down within in a water bath at 55˚C for 45-60 min. Then one was selected for addition of erythromycin at a final concentration of 1µg mL^{-1} (figure 11).

Figure 11. THB agar preparation

500mL sterile distilled water	500mL sterile distilled water
15g THB	15g THB
7,5g Bacto agar	7,5g Bacto agar
	
Without Erythromycin	With 1µg mL^{-1} Erythromycin

Plates containing approximately 20ml THB, with or without erythromycin were prepared (figure 12). A gas flame was used to avoid bubbles to appear on the agar. Then the agar plates were left to solidify in a sterile cabinet.

Figure 12. THB agar plate.

(b) Transformation with pVA838

Pre-cultures were diluted 1:50 in TSB (120µL pre-culture in 5mL TSB), and were incubated at 37 ˚C in 5% CO_2 aerobic atmosphere for 30 minutes.

One milliliter of each culture was transferred to two Eppendorf tubes, and 2.5µL CSP (stock 175 ng/µL) was added to one of the tubes. Four microliters pVA838 (stock 0.1µg/µL) was added to all samples. All tubes were mixed gently by inverting 6X. The tubes were incubated in air for one hour and in 5% CO_2 aerobic atmosphere for two hours.

Fifty Eppendorf tubes with 900µL sterile distilled water were used to prepare ten-fold dilutions of the bacterial cultures (figure 13 and 14).

Figure 13. Bacterial dilution before plating.

Figure 14. Sterile cabinet used during the transformation assay.

Hundred microliters of each dilution were plated (table 3). All the plates were placed in the incubator at 37 ˚C in 5% CO_2 aerobic atmosphere for approximately 48 hours.

Table 3. Overview of the dilutions used on plates with or without erythromycin.

With Erythromycin					Without Erythromycin	
	100	10-1	10-2	10-3	10-4	10-5
UA159	Without CSP	Without CSP	With CSP	With CSP	With CSP Without CSP	With CSP Without CSP
SM027	With CSP Without CSP				With CSP Without CSP	With CSP Without CSP
SM034	Without CSP	Without CSP	With CSP	With CSP	With CSP Without CSP	With CSP Without CSP
SM049	Without CSP	With CSP Without CSP	With CSP	With CSP	With CSP Without CSP	With CSP Without CSP

Colony forming units (CFU) on each plate were counted using a colony counter (figure 15).

Figure 15. Colony counting on each plate.

SEM assay

In the scanning electron microscope (SEM) the sample surfaces are scanned with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms in the sample producing signals with information about the surface topography, composition and other properties of the samples, such as electrical conductivity.

The biofilm in the discs were formed as described above, rinsed with distilled water, and fixed with 2.5 % glutaraldehyde for at least 24h in refrigerator to preserve and stabilize bacterial structures.

To avoid collapse and shrinkage, the fixed samples were dehydrated by critical point drying. Dehydration involved replacement of water in the cells with organic solvents (used 30,40,50,60,70,80,90, 100% ethanol each for 10 min). The discs were then mounted on aluminum plates (Figure 16) and the solvents were removed with a liquid carbon dioxide at 32˚C in 50-90 atmospheric pressure for 30 min to 1 hour (Figure 17). The carbon dioxide was finally removed while in a supercritical state, so that no gas-liquid interface would be present within the sample during drying. The dry specimen was mounted on a stub and sputter coated with palladium alloy (Figure 18) before examination in the SEM (Figure 19).

Figure 16. Discs mounted on aluminum plates to be placed in the CO2 chamber for critical point drying.

Figure 17. chamber with flowing CO2 under high atmospheric pressure and 32˚C for critical point drying.

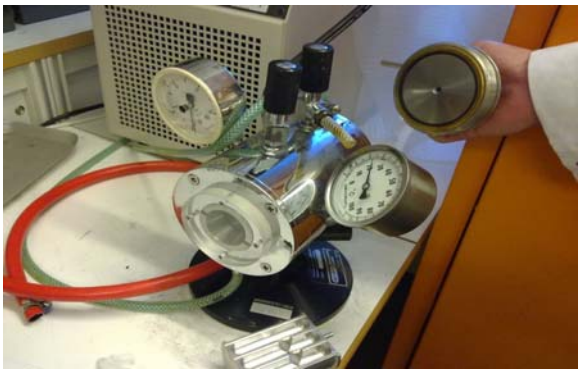
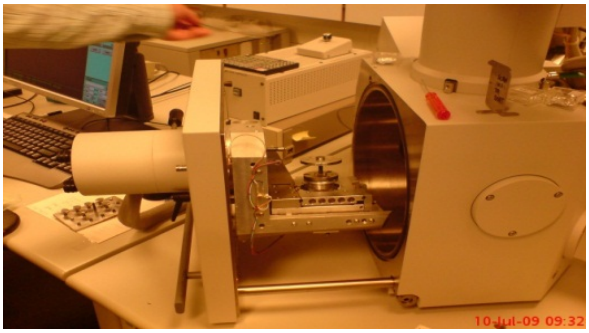


Figure 18. Palladium sputter coater

Figure 19.SEM opened sample chamber



RESULTS

CSP stimulated transformation in the wild type and in the bacteriocin locus deletion mutant. The transformation results from two independent experiments are shown in Table 4. In the wild type and in the bacteriocin deletion locus mutant (SM049), transformation was significantly increased in the presence of CSP. The transformation efficiency in the wild type was increased by more than 500 fold in the presence of CSP, compared to the transformation levels without CSP addition. In SM049 the transformation levels in the presence of CSP were 3500 fold and 14000 fold higher than in the absence of exogenous CSP in experiment I and II, respectively. Such variation may be related to differences in total colony forming units (CFU) observed between the wild type and SM049, and between the two independent experiments.

ComEC was essential for transformation. Transformation was completely abolished in the SM027 mutant, in the presence or absence of CSP (table 4).

RecA was required for optimal plasmid transformation. Transformation was abolished or reduced in the SM034 mutant, compared with the wild type. In experiment I, no transformants were detected, whereas in experiment II transformants were observed, but at significant lower levels than in the wild type (table 4).

Viability at early growth phase was reduced in the presence of CSP. Streptococcal viability measured as total CFU was reduced in the presence of CSP both in the wild type and in the isogenic mutants. The effect was more pronounced in experiment II, in which a reduction in CFU varying between 1.7 and 3.6 fold was observed (table 4).

Table.4. *S. mutans* transformation

Strain	CSP [a]	Transformants mL^{-1}		Total CFU mL^{-1} [b]		Efficiency %	
		I	II	I	II	I	II
UA159	-	2470	21200	102×10^6	148×10^6	0,002	0,014
	+	460000	3110000	53×10^6	43×10^6	1,15	7,2
SM027	-	0	0	111×10^6	116×10^6	0	0
	+	0	0	76×10^6	70×10^6	0	0
SM034	-	0	340	18×10^6	48×10^6	0	0,0007
	+	0	73000	14×10^6	19×10^6	0	0,4
SM049	-	4	2320	26×10^6	58×10^6	0,0001	0,004
	+	348000	2250000	24×10^6	16×10^6	1,4	14

I and II shows the results of two independent experiments

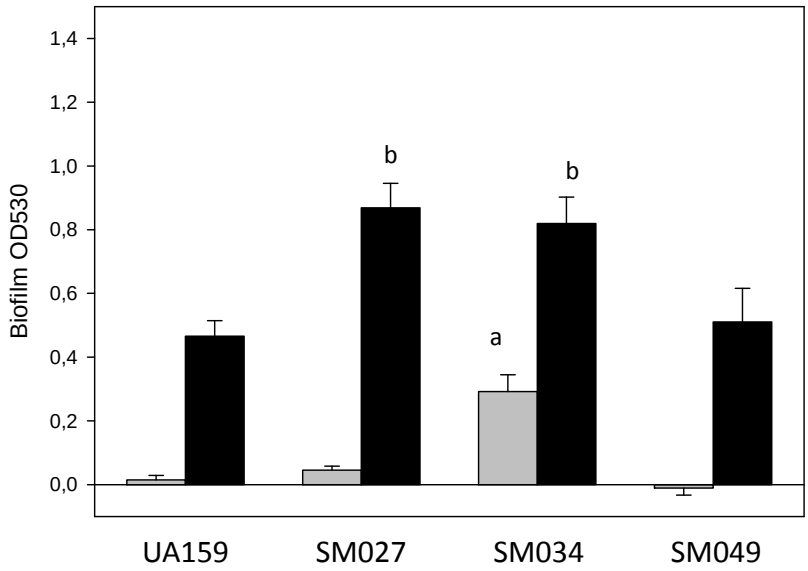
[a]CFU- colony forming units

[b]CSP-competence stimulating peptide

CSP stimulated biofilm formation to levels similar or higher than the wild type. CSP stimulated biofilm formation in the wild type and isogenic mutants (figure 20). The difference in biofilm levels in the presence or absence of CSP was similar in the wild type, and in the SM034 and SM049 mutants. This difference was, however, approximately two fold higher in the SM027 comEC deletion mutant.

Compared with the wild type, the SM034 recA mutant formed approximately two fold more biofilm both with and without CSP. In the presence of CSP, the comEC deletion mutant exhibited a two fold increase in biofilm formation compared with the wild type. Biofilm formation was similar in the wild type and SM049 mutant.

Figure 20. Biofilm formation by the wild type UA159 or deletion mutants SM027 (comEC), SM034 (recA), and SM049 (bacteriocin locus SMU. 1908-1914) in the absence (grey bars) or in the presence (black bars) of CSP. The results are means and standard errors from three independent experiments with four parallels each.



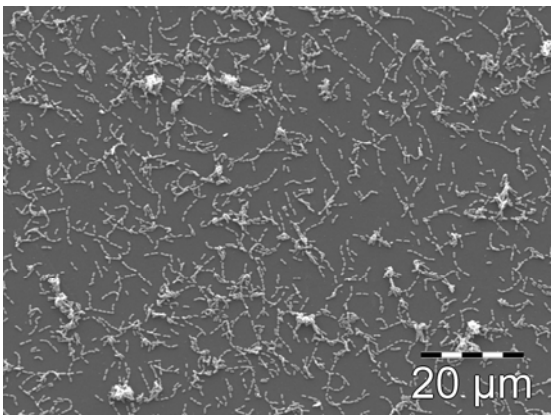
[a] In the absence of CSP, significantly different from the wild type (P<0,5).

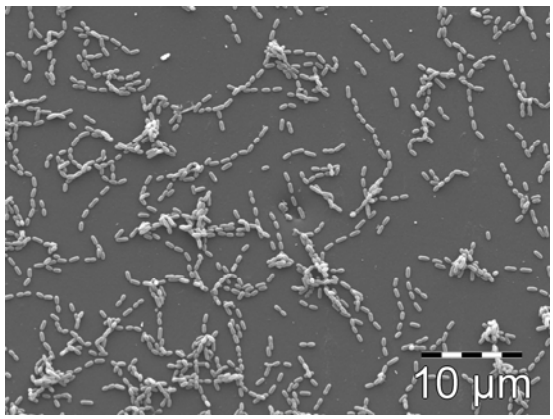
[b] In the presence of CSP, significantly different from the wild type (P<0,5).

Scanning electron microscopy confirmed the CSP stimulatory effect on biofilm formation (Figure 20). The presence of bacteria with altered cell shape was observed in the presence of CSP in both the wild type and isogenic mutants.

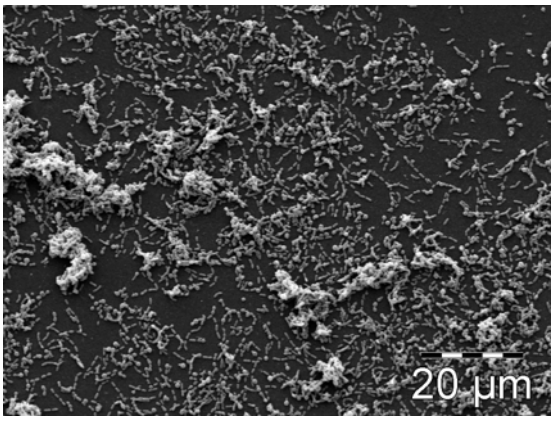
Figure 21. SEM images of the wild type and isogenic mutants in the presence or absence of CSP.

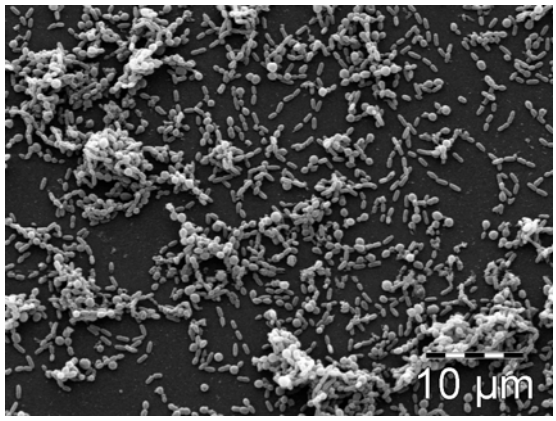
UA159 without CSP



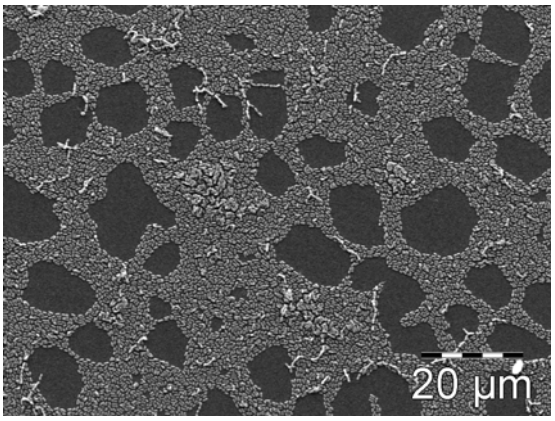


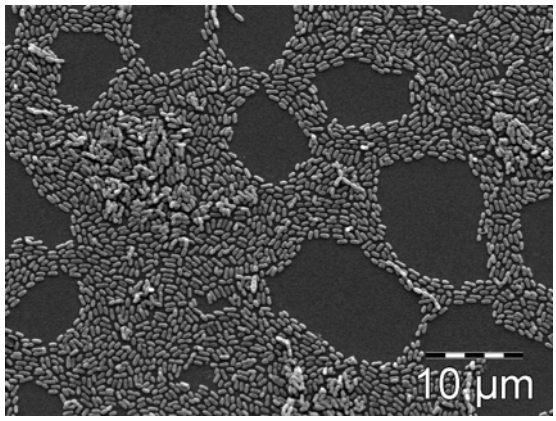
UA159 with CSP



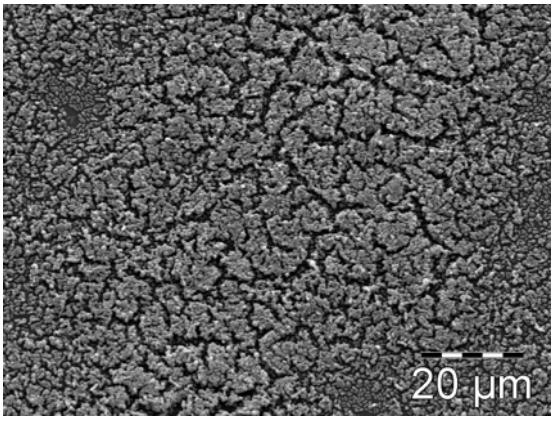


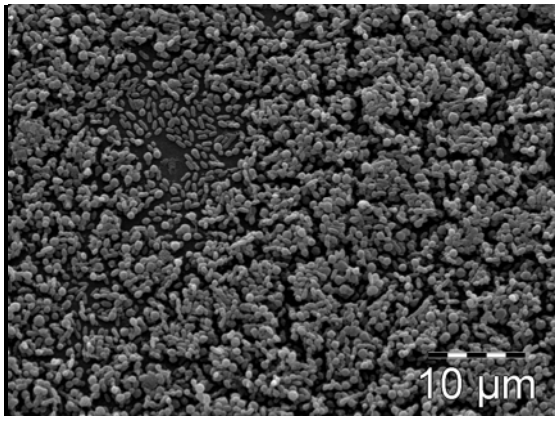
SM027 comEC without CSP



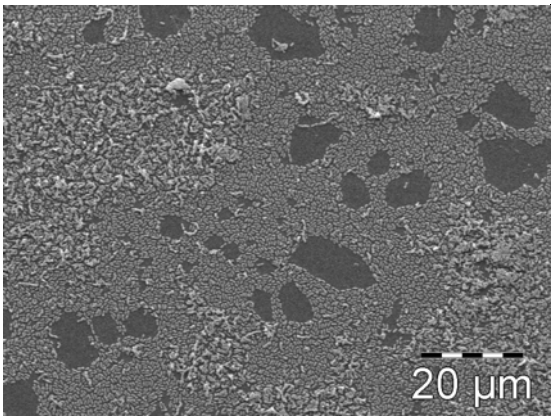


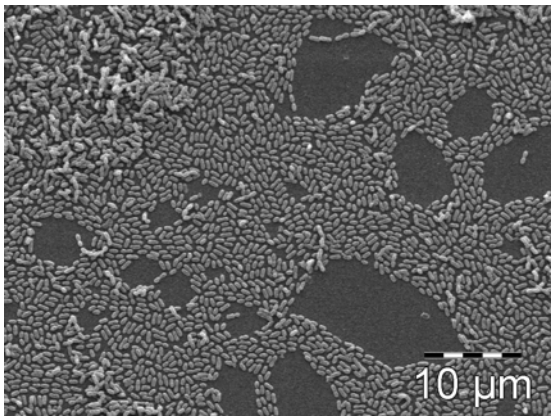
SM027 comEC with CSP





SM034 recA without CSP





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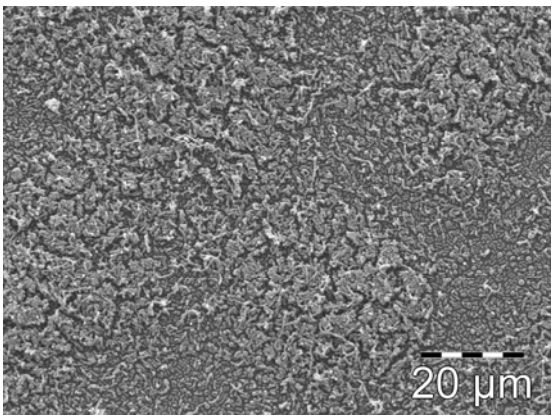
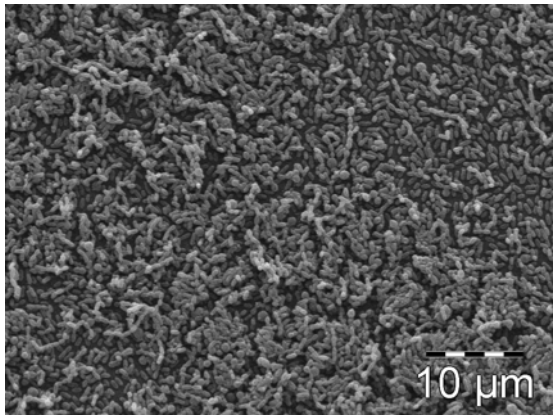
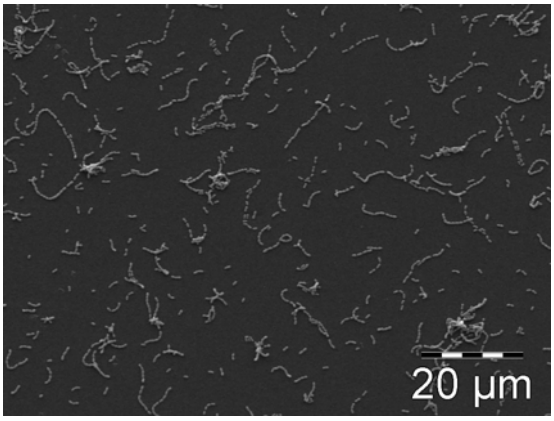
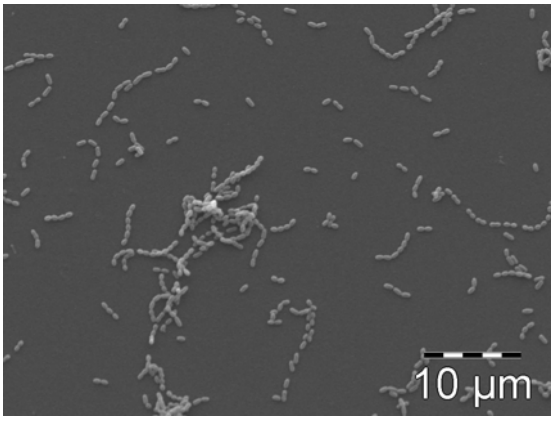




SM049 SMU.1908-SMU.1914 without CSP





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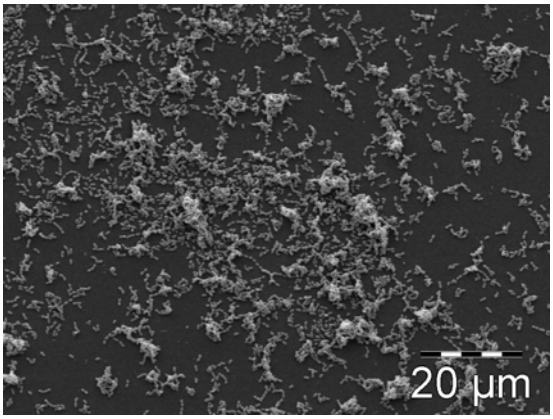

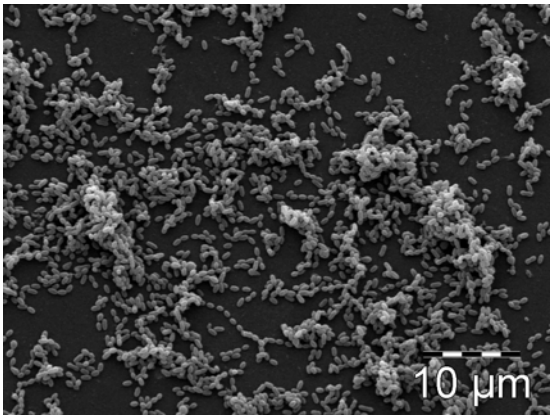

DISCUSSION AND CONCLUSION

The competence regulatory system has been associated with streptococcal biofilm formation in several species, including S. mutans, S. intermedius, and S. pneumoniae. In S. mutans, the increase in biofilm formation in the presence of CSP is abolished in a comX deletion mutant, indicating that genes regulated by ComX may be involved in biofilm formation. Possible candidates are proteins involved in DNA binding or DNA processing. This is supported by previous studies showing that inactivation of the pseudopilus thought to be required for DNA binding results in mutants with reduced biofilm formation upon CSP stimulation. Our results indicated that DNA uptake or recombination may not be involved in the CSP enhancing biofilm effect. Biofilm in the presence of CSP was not reduced in mutants deficient in the DNA transport channel ComEC or the recombination protein RecA.

ComEC is a putative DNA transport channel conserved in streptococci and in Bacillus subtilis. In S. pneumoniae and in B. subtilis, comEC inactivation results in mutants completely deficient in transformation. Our results support the role of ComEC in transformation, since comEC was also found to be essential for S. mutans transformation. It was interesting to find out that in the presence of CSP, the comEC deletion mutant formed more biofilm formation than the wild type. The reason for such an increase is not known, but it may be related to the ability of comEC mutants to bind extracellular DNA, an important component of biofilm matrixes. In S. pneumoniae, the inactivation of comEC results in decreased DNA uptake, but increased DNA binding by the mutants. Increased DNA binding would thus possibly contribute to increase the adherence of cells into biofilms.

Transformation using the streptococcal replicative plasmid pVA838 was reduced in the S. mutans recA mutant. Such a reduction has also been observed in S. gordonii recA deletion mutants, in which transformation frequencies with both plasmid and chromosomal markers were reduced (7,11). This is in contrast with S. pneumoniae, in which abolishment of transformation has been reported in recA deletion mutants (5). The mechanism by which RecA may be involved in replicative plasmid transformation is rather unknown, but it may involve a role in the reformation of double complementary strands in the recipient cells (11). The recA mutant formed more biofilm than the wild type, in the presence or absence of CSP, suggesting a CSP-independent role. The involvement of RecA in biofilm formation has also been found in a recent study, but in contrast to our results, the recA mutant showed a reduction in biofilm formation (4). It is possible that the growth of biofilms under different environmental conditions may explain such differences. The lack of recA expression has been associated, for instance, with the appearance of morphological distinct colonies in a P. aeruginosa biofilm model. The reason for the selection of a morphologically distinct population is possibly associated with the inability of recA mutants to repair damages in their DNA. RecA is, however, often a multifunctional protein with roles not only in DNA repair, but also with enzymatic and regulatory properties, involved in the SOS response, growth, and adhesion. Studies on the role of RecA in streptococci have been mostly limited, however, to homologous recombination. In S. mutans recA deficient mutants are more sensitive to acid killing. (10). It is noteworthy, however, that in acid adapted cells RecA has apparently no functional role, suggesting the presence of an alternative acid-inducible DNA repair system.

In the SM049 mutant, a locus comprising six genes in a bacteriocin related genetic island in the S. mutans UA159 genome was deleted. Four of the genes have been annotated as hypothetical proteins (SMU.1908c, SMU.1909c, SMU.1910c, SMU. 1912c). The other two genes encode the bacteriocin immunity protein SMU.1913c, and mutacin V (SMU.1914c). Recently, mutacin V was found to play a central role in the lysis of cells observed in the presence of CSP. Moreover, deletion of the mutacin V gene resulted in a mutant in which transformation and biofilm were not increased in the presence of CSP. It was surprising, therefore, to find out that in our study inactivation of the six gene locus (SMU1908c to SMU.1914c) resulted in a mutant that in the presence of CSP exhibited increased transformation and biofilm formation, to levels higher or similar to the wild type. It seems, therefore, that lysis in the presence of CSP may involve other mechanisms than the altruistic mechanism recently attributed to mutacin V (7).

We conclude that the stimulation of biofilm formation in the presence of CSP is not mediated by the ComEC DNA channel, RecA, or the bacteriocin locus SMU.1908-1914. Our findings support, therefore, the model in which DNA binding to the bacterial cell surface may play a role in the association of the competence system with biofilm formation. DNA binding mechanisms may thus represent a target for the control of biofilm formation, not only in S. mutans, but possibly also in other species with similar DNA binding mechanisms.

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