Natural Transformation of Oral Streptococci by use of Synthetic Pheromones

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Abstract

The discovery that *Streptococcus pneumoniae* uses a competence-stimulating peptide (CSP) to induce competence for natural transformation, and that other species of the mitis and the anginosus streptococcal groups use a similar system, has expanded the tools to explore gene function and regulatory pathways in streptococci. Two other classes of pheromones have been discovered since then, comprising the bacteriocin-inducing peptide class found in *Streptococcus mutans* (also named CSP, although different from the former) and the SigX-inducing peptides (XIP), in the mutans, salivarius, bovis, and pyogenes groups of streptococci. The three classes of peptide pheromones can be ordered from peptide synthesis services at affordable prices, and used in transformation assays to obtain competent cultures consistently at levels usually higher than those achieved during spontaneous competence. In this chapter, we present protocols for natural transformation of oral streptococci that are based on the use of synthetic pheromones, with examples of conditions optimized for transformation of *S. mutans* and *Streptococcus mitis*.

Key words: oral streptococcus; *Streptococcus mutans*, *Streptococcus mitis*, competence, natural transformation, competence-stimulating peptide (CSP), SigX-inducing peptide (XIP).

1. Introduction

Natural genetic transformation is widespread in oral streptococci, including the mutans, the salivarius, the mitis, and the anginosus groups [1, 2, 3]. The mechanisms depend on streptococci entering competence, a physiological state triggered by pheromones, and characterized by the induction in expression of several gene clusters, including those essential for DNA uptake and recombination.

Streptococcal competence pheromones can be divided into three classes: (1) competence stimulating peptides (CSPs), (2) SigX-inducing peptides (XIPs) , and (3) bacteriocin-inducing peptides with competence-inducing activity. The first class, used by the mitis and anginosus groups, is sensed by a two-component system comprising a histidine kinase (ComD) that binds the extracellular cognate CSPs, leading to the phosphorylation of the response regulator ComE [4, 5]. The XIP class is used by most other streptococci, and is transported inside the cells by Opp oligopeptide permeases, leading to the activation of intracellular Rgg-type regulators [6, 7, 8, 9]. The last class, comprising bacteriocin-inducing peptides, is only known in *S. mutans*. Although primarily involved in bacteriocin induction, these peptides, also named CSPs, do induce competence, but in contrast to the CSP-class in the mitis and anginosus groups, they act indirectly by stimulating the XIP-class [10, 11]. All classes of pheromones lead, ultimately, to the induction of the alternative sigma factor SigX, which then induces the expression of competence genes required for DNA uptake and recombination.

Spontaneous competence development under laboratory conditions is in practice not always reliable, and subtle changes in factors such as medium batch, medium composition, pH, and growth temperatures, may dramatically affect competence levels [12, 13]. The introduction of synthetic pheromones in protocols for natural transformation has overcome many of these

difficulties, by bypassing stringent conditions for production and secretion of natural pheromones, and by prolonging the transient nature of the competent state in streptococci [5]. The higher transformation efficiencies and reproducible results usually achieved with synthetic pheromones can be further optimized by the choice of growth medium and DNA donor, to reach levels that allow the practical use of markerless strategies for direct genome editing, as recently demonstrated for *S. mutans* and *S. pneumoniae* (*see* Chapter 14) [14].

This chapter describes transformation protocols for oral streptococci based on competence stimulated by synthetic pheromones. We present examples of optimized assays for *S. mutans* UA159 (XIP and bacteriocin-inducing classes of pheromones) and the *S. mitis* type strain CCUG 31611^{T} (CSP class of pheromone), and general protocols with CSP that have been used for other strains of the mitis group, as well as for the anginosus group. These can be used as starting points for transformation of other streptococcal strains and species for which optimal protocols have not yet been established.

2. Materials

2.1 Competence Induction Using Synthetic Peptides

1. Agar plates: Todd-Hewitt Broth (THB) 30 g/L (Difco Laboratories, Detroit, MI, USA). Add 15 g/L of agar to the medium (VWR Chemicals, Radnor, PA, USA), and autoclave at 121 °C for 15 min. For selective agar plates, the appropriate antibiotic(s) should be added to the medium once it has cooled to below 60 °C, and the media should be stored under conditions appropriate for the antibiotics (see Note 1).

2. Liquid media: For transformation of *S. mutans*: Tryptone Soya Broth (Thermo Scientific, Waltham, MA, USA)) 30 g/L is used for stimulation with CSP. For stimulation

with XIP, chemically defined medium (CDM) [15], prepared from concentrated stock solutions with 1 % glucose is used for growth, stock preparation of streptococcal pre-cultures and transformation assays; for transformation of the anginosus group: THB-HS broth comprising Todd Hewitt Broth (THB; Difco Laboratories) supplemented with 2–10 % (v/v) heat-inactivated horse serum (HS); for transformation of *Streptococcus mitis*: C + Y_{YB} [16], a chemically defined medium derived from the previously described C + Y medium [17], but with an increased concentration of yeast extract and bovine serum albumin; for other oral streptococci: THY (see Notes 2 – 4).

3. Synthetic peptides: Synthetic pheromones specific for the strain or species used may be ordered from peptide synthesis services. Examples of pheromones that have been used in transformation of streptococci are shown in Table 1. A stock solution of CSP is prepared by resuspending the lyophilized peptide in distilled water to a concentration of 10 mM, and storing it at −20 °C. Working solutions of 10 or 100 μM are aliquoted and stored at −20 °C. Lyophilized XIP (GenScript, Piscataway, NJ, USA) is reconstituted with 20 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MI, USA) to which 1 mL distilled water is added to give a final concentration of 10 mM (stored at −20 °C). Working stocks are prepared at 100 μM by dilution in distilled water (see Note 5). For the CSP and XIP sequences, see Subheading 3.3, step 4.

4. DNA donor: Amplicons and replicative plasmid used in the experiments are listed in Table 2. For maximal recovery of transformants, use purified DNA at saturation levels (see Notes 6 and 9).

5. DNaseI (Roche, DNaseI recombinant, 10 U/μL): DNaseI is used to degrade DNA not taken up by the cells. This step is usually omitted if the aim is to obtain maximal number of transformants.

3. Methods

Compared with protocols based on spontaneous competence, the use of synthetic pheromones leads often to higher transformation efficiencies, a better control over the time of competence development, and may extend transformation to a wider range of strains. The peptides are stable, and offer a straightforward approach for transformation of strains in which the pheromone sequence is known.

We present two protocols for transformation of *S. mutans,* the first optimized for transformation by XIP using CDM as the growth medium [14]. The high efficiency of this method is particularly useful for direct markerless construction of mutants (*see* Chapter 14). The second protocol involves the use of synthetic CSP (bacteriocin-inducing class) in rich medium. Although only a fraction of the *S. mutans* population becomes competent in rich medium, compared with stimulation by XIP in CDM, transformation can reach levels sufficiently high for marker-based constructions [11, 18]. The advantage of using rich medium such as TSB is that there is no need for the availability of an extensive list of ingredients such as that used for CDM preparation and it is much simpler to prepare.

Genetic manipulation of *S. mitis* by natural transformation is known to be challenging. Here, we present a procedure for *S. mitis* transformation in THB-HS broth and an optimized protocol using a semi-defined medium known to support endogenous competence.

In the mitis group, CSPs often vary among the different species and strains. Thus, it may be necessary to characterize the CSP-encoding gene in strains for which the CSP sequence is unknown. Strategies that have been used for CSP gene identification based on PCR amplification and sequencing are therefore presented.

3.1. Transformation efficiency /kinetics protocol using synthetic XIP in CDM in *S. mutans*

Given the influence of (i) as yet undefined environmental conditions, (ii) the transient nature of competence, and (iii) strain to strain variations in transformation efficiencies, one may choose to run kinetic experiments before establishing routine transformation protocols. The following protocol has been used to determine the kinetics of competence development in *S. mutans* UA159 using synthetic XIP and PCR amplicon with antibiotic marker as donor DNA (*see* **Fig. 1**).

1. Stock cultures are stored at -80°C or -20°C in 30% glycerol.
2. Plate the desired bacteria on THB agar plates. Incubate at 37°C overnight in 5% CO_2.
3. Preparation of pre-cultures: Inoculate 3 to 10 colonies using a sterile transfer loop in 5 mL of CDM. Incubate at 37°C in 5% CO_2 for 3 to 4 h or until absorbance at 600 nm (A_{600})=~0.5. Store the cells in 15% glycerol at -70°C or use it directly in the transformation experiments (*see* **Note 7**).
4. Dilute the pre-culture culture 1:10 in CDM (A_{600}=~ 0.05) and incubate at 37°C in air. Thaw the specific CSP on ice while waiting for the next step.
5. After 1.5-2 h incubation (A_{600}=~0.08 – 0.1 or CFU=~7 x 10^6/mL), add XIP (*see* **Table 1**) to the culture to a final concentration of 1 µM, and continue incubating at 37°C in air (*see* **Note 8)**.

6. Add 50 to 100 ng donor DNA to a 100 µL aliquot of cells at different time points (*see* **Fig. 1**). Mix it by gently tapping the base of the microfuge tube or gently stir with a micropipette tip. Incubate the culture at 37°C for 20 min (*see* **Notes 9 - 11**).
7. Add 200 µL of warm CDM containing 20 U/mL DNaseI (Roche, DNaseI recombinant, 10U/µL) to each aliquot of the competent cells, and proceed with incubation at 37°C in air for another 40 min.
8. Spread 20 to 200 µL from serial dilutions of the transformation mix on selective or non-selective THB agar plates. Wait for the liquid to dry on the agar. Invert the plates and incubate them at 37°C in 5% CO_2 for 24 – 48 h.
9. Count the colonies in each plate and calculate the transformation efficiency using the formula below:

$$\text{Transformation efficiency (\%)} = \frac{\text{CFU transformants} \times 100}{\text{Total CFU}}$$

10. Select at least three colonies (putative transformants) for further characterization. The colonies should be individually transferred to separate tubes with fresh TSB containing the appropriate antibiotic and incubated at 37°C in 5% CO_2 for 24 h.
11. Screen the colonies to verify whether the DNA construct was correctly incorporated in the mutant. Methods based on PCR using primers designed to provide amplicons that distinguish the mutants from the wild-type may be used for this purpose. Carry out the next two steps if the mutants will be used in downstream applications.
12. Plate the selected bacteria in the presence of the antibiotic and incubate them at 37°C in 5% CO_2 for 24-48 h. Ensure at least two passages in antibiotic-containing media (*see* **Note 12**).

13. Grow the selected bacteria in THB until exponential phase is reached, and store the culture in 15% glycerol at -20°C or -70°C.

3.2. Transformation protocols for downstream applications using synthetic pheromones

These are simplified protocols that we have used in experiments in which determination of the kinetics of competence are not the focus, or in which acceptable efficiency levels are obtained without the need for further optimization steps.

3.2.1. Streptococcus mutans

3.2.1.1. Transformation using synthetic CSPs in rich medium

This protocol has been used for transformation of different *S. mutans* strains using DNA with antibiotic marker (*see* **Note 13**).

1. Follow steps 1 and 2 described in Subheading **3.1**.
2. Inoculate 3-10 colonies using a sterile transfer loop in 5 mL of TSB. Incubate at 37°C in 5% CO_2 for 3 to 4 h (A_{600}=~0.5). Store the cells in 15% glycerol at -80°C or use it directly in the transformation experiments.
3. Dilute the pre-culture 1:10 in TSB and prepare 500 µL to 1000 µL aliquots in 1.5 mL Eppendorf tubes. Add 18-CSP to a final concentration of 50 nM and donor DNA, and incubate at 37°C in air for 2.5 to 3 h (*see* **Notes 4 and 9**).
4. Follow the steps 8-13 described in Subheading **3.1**.

3.2.2. Streptococcus mitis

Here we present two protocols: one optimized protocol in semi-defined medium, and other in THB-HS medium. By following the protocol in semi-defined medium using a 7-kb PCR

amplicon as donor DNA, we achieved 30-40% transformation efficiency while in THB-HS the efficiency was only ~0.01%. (*see* **Fig. 2**) (*see* **Note 4**).

3.2.2.1. Transformation Using Synthetic CSP in C+Y_{YB} medium

This protocol has been optimized for transformation of the *S. mitis* type strain CCUG 31611 (corresponds to NCTC 12261). Pre-culture preparation:

1. Plate the desired bacteria in a blood plate without antibiotics. Incubate at 37°C overnight in 5% CO_2.
2. Pre-culture preparation: Inoculate 3 to 10 colonies using a sterile transfer loop in 5 mL of TSB. Incubate at 37°C in 5% CO_2 for 3 to 4 h (A_{600}=~0.5). Store the cells in 15% glycerol at -80°C or use it directly in the transformation experiments (*see* **Note 7**).

Transformation:

1. Inoculate 10 µL of the pre-culture in 900 µL C+Y_{YB}. The volumes can be scaled up but the 1:100 dilution should be maintained.
2. Incubate at 37°C in 5% CO_2 for 2 to 3 h (A_{600}=~0.04).
3. Add CSP at a final concentration of 300 nM and donor DNA. Saturated concentrations for DNA are in the range of 100 to 150 ng per mL for PCR amplicons and above 10 µg for genomic DNA and above 1 µg for plasmid DNA (pVA838).
4. Incubate at 37°C in air for 3 h (see **Note 8**).
5. Follow the steps 8-13 as described in Subheading **3.1** for *S. mutans*.

3.2.2.2. Transformation using synthetic CSP in rich medium

Pre-culture preparation:

1. Inoculate 5 ml THY with ~10 µL of the stock culture using a sterile transfer loop, and incubate at 37°C overnight in 5% CO_2 microaerophilic atmosphere.

2. Dilute the ON culture 1:100 in THY and incubate at 37°C in a 5% CO_2 atmosphere for 4 to 5 h (A_{600}=~0.3). Store the cells in 10% glycerol at -70°C, or use it directly in the transformation experiments.

Transformation:

1. Inoculate 100 µL of the pre-culture in 900 µL THB-HS. The volumes can be scaled up but the 1:10 dilution should be maintained.
2. Add CSP at a final concentration of 200 nM and transforming DNA at the concentration described above. Incubate at 37°C in air for 3-4 h (s*ee* **Note 8**).
3. Follow the steps 7 - 12 described in Subheading **3.1**.

3.2.3. The Anginosus Group: Streptococcus intermedius, Streptococcus anginosus, and Streptococcus constellatus

This protocol, slightly modified from Gaustad and Morrison [19], has allowed transformation of *S. intermedius*, *S. anginosus*, and *S. constellatus* (*see* **Notes 4 and 15**). This protocol uses THB-HS, but TSB can also be used [20].

1. Stock cultures are stored at -80°C or -20°C in 30% glycerol.
2. Inoculate 5 mL THB-HS with ~10 µL of the stock culture using a sterile transfer loop and incubate at 37°C overnight in a 5% CO_2 (in air) atmosphere.
3. Dilute the overnight culture 1:10 in THB-HS and prepare 500 µL to 1000 µL aliquots in 1.5 mL Eppendorf tubes. Incubate at 37°C in air for 1-1.5 h. Thaw the specific CSP on ice while waiting for the next step. (*see* **Note 16**)
4. Add the CSP and DNA donor to the culture to a final concentration of 20-50 nM and allow growing at 37°C in air for 1 h, followed by 37°C in 5% CO_2 for 1-3 h.
5. Follow the steps 8 to 13 described in Subheading **3.1**.

3.2.4. Other oral streptococci

The protocol used for the anginosus group (Subheading **3.2.3**) may also be applicable to *S. gordonii*, *S. sanguinis*, and other oral streptococci [19]. The following modified assay that allows *S. gordonii* stocks to be stored in frozen aliquots, and directly applied in competence experiments has been suggested [21]:

Pre-culture preparation:

1. Add 10 μg/mL synthetic CSP to overnight cultures grown at 37°C in THY supplemented with chloramphenicol (5 μg/mL).
2. Freeze the cells in 100 μL aliquots following the addition of 10% glycerol.

Transformation:

1. Add 900 μL THY to 100 μL of the frozen cell aliquot.
2. Add transforming DNA, and incubate the cells at 37°C for 3 h.
3. Follow the steps 8 - 13 described in Subheading **3.1**.

3.3. Synthetic CSPs: sequence identification

Verify whether the pheromone for the strain you will be working with has been previously identified. Some of the CSPs and XIPs that have been used for transformation of oral streptococci are presented in **Table 1**. For updated information on other strains, search gene/protein databases such as Entrez. If the pheromone in your chosen strain has not yet been identified, you may use PCR to amplify and sequence the gene for the competence pheromone. This information is then used to predict the pheromone amino acid sequence for your strain.

1. In *S. mutans*, the *comC* and *comS* genes encoding the CSP and XIP pheromones, respectively, are highly conserved [22]. Thus, the 18-CSP and the XIP for *S. mutans* UA159 shown in **Table 2** should function for most, if not all, other *S. mutans* strains.

In the mitis group, there is a large variation of CSP pheromones, particularly among *S. mitis* strains, whereas in the anginosus group of streptococci different species may produce the same pheromone. In these two groups, the CSP-encoding (*comC*) gene has been identified by using primers annealing to conserved Arg-tRNA and Glu-tRNA genes flanking the *comCDE* operon [4]. In this case, the primer pair t-Arg 5'-GGCGGTGTCTTAACCCCTTGACCAACGGACC and t—Glu 5'-CATAGCTCAGCTGGATAGAGCATTCGCCTTC is expected to amplify a fragment of approximately 2.5 to 2.6 kb [4]. For sequencing, the final reaction volume of 25 µL should contain 200-300 ng chromosomal DNA, *Taq* Polymerase or a high-fidelity DNA polymerase such as *Pfu* at the recommended concentration, 0.2 mM dNTP, 1× PCR buffer containing standard $MgCl_2$ concentration, and 0.1 µM of each forward and reverse primer. The following cycle parameters for amplication of *comC* in *S. mutans* may be used: 94°C for 3 min; 36 cycles of 94°C for 30s, 60°C for 30s and 72°C for 30s; and a final polymerization step of 72°C for 7 min. Adjust the PCR conditions for amplification of the *comCDE* operon in the other oral streptococci by replacing the 30s extension time at 72°C with 2 min during the 36 cycles (*see* **Note 17**).

2. Verify the presence of the amplified product by electrophoresis in a 0.7% (w/v) agarose gel, and determine the *comC* sequence in the amplified product, which by using the primers indicated above is within ~350 bp from the 5'-end in other streptococci.
3. To predict the CSP sequence you may align the sequence of your amplified product with previously identified *comC* genes. Most often, the deduced CSP peptide sequence, when translated from your PCR product, is preceded by a double glycine cleavage site. Alignment of ComC with peptides of known cleavage sites may help

define the mature peptide sequence [4]. Many peptide synthesis services are currently available, making the acquisition of the peptides convenient and affordable.

4. Notes

1. Brain Heart Infusion (BHI), TSB, or blood agar plates may also be used. The following antibiotic concentrations were used: kanamycin (Kan) 500 µg/mL, and erythromycin (Erm) 10 µg/mL.
2. Competence for natural transformation persists for a longer time in CDM using XIP when compared to TSB with CSP (*see* **Fig. 1**). Other media that have been used in *S. mutans* CSP transformation assays include BHI-HS [23] and THY-HS [24]. For markerless constructions we recommend to transform *S. mutans* in CDM with XIP as detailed in Chapter **14**.
3. We have had a previous experience in which a particular TSB batch resulted in very poor streptococcal transformation efficiencies, a problem that was solved by simply changing the batch of TSB used.
4. In choosing the medium for transformation, one important factor to consider is the mechanism used by streptococci to sense the different pheromones. For instance, when using XIP, a peptide-free medium is advantageous because other peptides present in the medium compete with XIP for transport into the cells.
5. Crude desalted XIP of >80% purity is usually highly active.
6. Specific commercial DNA purification kits can be used for isolation of plasmids, PCR amplified fragments or genomic DNA, following the recommended DNA isolation protocols. Lysis procedures for oral streptococci should take into consideration the rigidity of the streptococcal cell wall. For *S. mutans*, we usually incubate the cells (up to 10^9 cells) for 20 min in the presence of 100 U/mL mutanolysin and 20 mg/mL

lysozyme. In trying alternative methods, the purity of the isolated DNA, which may impact on transformation efficiency, should be considered.

7. It is our experience that pre-cultures prepared from fresh colonies give higher transformation efficiencies, although we have not assessed it systematically (see Subheading **3.1**).
8. Although streptococcal transformation protocols usually recommend growth of the cells in 5% CO_2, we usually obtain higher transformation efficiency levels when the cells are simply grown in air.
9. The transient nature of competence should be considered in kinetic studies. In *S. mutans*, the competent state may often last for more than 4 h in CDM, but in TSB transformation is finished around this time (*see* **Fig. 1**). In *S. mitis*, for instance, competence lasts for about 1 hour in $C+Y_{YB}$ (data not shown). The onset of competence may also vary, with *S. gordonii* exhibiting an almost immediate response to CSP [25], whereas in *S. mutans* a delay in competence induction response is observed [23,26]. This is because the CSP in *S. mutans* acts first by stimulating bacteriocin response before competence is triggered.
10. Saturating levels of amplicon DNA donor for *S. mutans* were experimentally determined and a final concentration of 75 ng/mL was shown to saturate the reaction [14]. Transformation efficiency determined in two experiments using saturation amounts of amplicon DNA donor (no addition of DNase) shows that efficiencies from 20% to 50% are often achieved (*see* **Fig. 3**).
11. The use of positive and negative controls is highly recommended, particularly during the construction of new mutants. A negative control will provide information on the selective activity of the antibiotics. Positive controls are usually DNA donor with an antibiotic resistance marker that is known to transform the target strain. The routine use

of the same positive control allows comparison of the transformation efficiency between different experiments.

12. The repeated passage in media with the relevant antibiotic is conducted to avoid carry over of non-transformed bacteria. This step is particularly pertinent when using selection markers conferring resistance to antibiotics that are bacteriostatic (i.e., those that inhibit the growth of the cells without killing them). For selection of markerless mutants, we refer to Chapter **14**.
13. The synthetic 18-CSP (SGSLSTFFRLFNRSFTQA), analogous to the peptide found in the supernatant of *S. mutans,* induces maximal competence at 20 nM [27], but it may be used at concentrations as high as 1000 nM without compromising transformation efficiency. The synthetic 21-CSP (SGSLSTFFRLFNRSFTQALGK) predicted from the *comC* sequence has often been used, but a delay in competence may be observed because this form of the peptide needs further processing by *S. mutans* into the 18-CSP active form.
14. *S. mitis* CCUG 31611^{T} synthetic CSP (EIRQTHNIFFNFFKRR) has shown to induce maximal number of transformants by using a final concentration of 200-300 nM (data not shown).
15. We have used this protocol to obtain consistent transformation levels of the *S. intermedius*, *S. anginosus*, and *S. constellatus* type strains, and *S. intermedius* CCUG 28205.
16. The type strains of *S. intermedius*, *S. anginosus*, and *S. contellatus* respond to the same pheromone (*see* **Table 1**). However, analyses of recent genome sequence for these species reveal that this is not always the case. This information should be checked before deciding which pheromone is going to be used.

17. The PCR strategy to identify the CSPs will depend on the presence of the flanking regions annealing to the specified primers. Different sequences or gene arrangements may, therefore, escape detection. Note that among the transformable oral streptococci, it is only in *S. mutans* that the *comC* gene is not flanked by the Arg-tRNA and Glu-tRNA genes.

Acknowledgments

This work was partially supported by the National Science Foundation, grant no. MCB-1020863, by the Faculty of Dentistry, University of Oslo, and by the Norwegian surveillance system for antibiotic resistance in microbes (*Norsk overvåkingssystem for antibiotikaresistens hos mikrober* – NORM).

References

1. Federle MJ, Morrison DA (2012) One if by land, two if by sea: signalling to the ranks with CSP and XIP. Mol Microbiol 86:241-245
2. Fontaine L, Wahl A, Flechard M, Mignolet J, Hols P (2015) Regulation of competence for natural transformation in streptococci. Infection, genetics and evolution J Mol Epidemiol Evol Genet Infect Dis 33:343-360
3. Johnsborg O, Eldholm V, Håvarstein LS (2007) Natural genetic transformation: prevalence, mechanisms and function. Res Microbiol 158:767-778
4. Håvarstein LS, Hakenbeck R, Gaustad P (1997) Natural competence in the genus *Streptococcus*: evidence that streptococci can change pherotype by interspecies recombinational exchanges. J Bacteriol 179:6589-6594

5. Morrison DA (1997) Streptococcal competence for genetic transformation: regulation by peptide pheromones. Microb Drug Resist 3:27-37

6. Khan R, Rukke HV, Ricomini Filho AP, Fimland G, Arntzen MO, Thiede B, Petersen FC (2012) Extracellular identification of a processed type II ComR/ComS pheromone of *Streptococcus mutans*. J Bacteriol 194:3781-3788

7. Mashburn-Warren L, Morrison DA, Federle MJ (2010) A novel double-tryptophan peptide pheromone controls competence in *Streptococcus* spp. via an Rgg regulator. Mol Microbiol 78589-606

8. Fontaine L, Boutry C, de Frahan MH, Delplace B, Fremaux C, Horvath P, Boyaval P, Hols P (2010) A novel pheromone quorum-sensing system controls the development of natural competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. J Bacteriol 192:1444-1454

9. Monnet V, Juillard V, Gardan R (2016) Peptide conversations in Gram-positive bacteria. Crit Rev Microbiol 42:339-351

10. Lemme A, Grobe L, Reck M, Tomasch J, Wagner-Dobler I (2011) Subpopulation-Specific transcriptome analysis of competence stimulating peptide induced *Streptococcus mutans*. J Bacteriol 193:1863-1877

11. Son M, Shields RC, Ahn SJ, Burne RA, Hagen SJ (2015) Bidirectional signaling in the competence regulatory pathway of *Streptococcus mutans*. FEMS Microbiol Lett 362:doi: 10.1093/femsle/fnv159

12. Chen JD, Morrison DA (1987) Modulation of competence for genetic transformation in *Streptococcus pneumoniae*. J Gen Microbiol 133:1959-1967

13. Son M, Ghoreishi D, Ahn SJ, Burne RA, Hagen SJ (2015) Sharply tuned pH response of genetic competence regulation in *Streptococcus mutans*: a microfluidic study of the environmental sensitivity of *comX*. Appl Environ Microbiol 81:5622-5631

14. Morrison DA, Khan R, Junges R, Amdal HA, Petersen FC (2015) Genome editing by natural genetic transformation in *Streptococcus mutans*. J Microbiol Methods 119:134-141

15. Chang JC, LaSarre B, Jimenez JC, Aggarwal C, Federle MJ (2011) Two group A streptococcal peptide pheromones act through opposing Rgg regulators to control biofilm development. PLoS Pathogens 7:e1002190

16. Stevens KE, Chang D, Zwack EE, Sebert ME (2011) Competence in *Streptococcus pneumoniae* is regulated by the rate of ribosomal decoding errors. mBio 2:doi: 10.1128/mBio.00071-11.

17. Lacks S, Hotchkiss RD (1960) A study of the genetic material determining an enzyme in Pneumococcus. Biochim Biophys Acta 39:508-518

18. Reck M, Tomasch J, Wagner-Dobler I (2015) The alternative sigma factor SigX controls bacteriocin synthesis and competence, the two quorum sensing regulated traits in *Streptococcus mutans*. PLoS Genet 11:e1005353

19. Gaustad P, Morrison DA (1998) Induction of transformation in streptococci by synthetic competence stimulating peptides. Methods Cell Sci 20:65-70

20. Petersen FC, Pecharki D, Scheie AA (2004) Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. J Bacteriol 186:6327-6331

21. Warren TK, Lund SA, Jones KF, Hruby DE (2007) Comparison of transformation protocols in *Streptococcus gordonii* and evaluation of native promoter strength using a multiple-copy plasmid. Can J Microbiol 53:417-426

22. Palmer SR, Miller JH, Abranches J, Zeng L, Lefebure T, Richards VP, Lemos JA, Stanhope MJ, Burne RA (2013) Phenotypic heterogeneity of genomically-diverse isolates of *Streptococcus mutans*. PLoS One 8:e61358

23. Ahn SJ, Wen ZT, Burne RA (2006) Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect Immun 74:1631-1642

24. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 183:897-908

25. Vickerman MM, Iobst S, Jesionowski AM, Gill SR (2007) Genome-wide transcriptional changes in *Streptococcus gordonii* in response to competence signaling peptide. J Bacteriol 189:7799-7807

26. Kreth J, Merritt J, Shi W, Qi F (2005) Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. Mol Microbiol 57:392-404

27. Petersen FC, Fimland G, Scheie AA (2006) Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in *Streptococcus mutans*. Mol Microbiol 61:1322-1334

Table 1. Sequence of CSP and XIP from selected strains of oral streptococci for which the synthetic pheromones have been shown to induce competence.

Group	Strain	CSP sequence[a]	XIP sequence
Mutans	*S. mutans* UA159[G]	SGSLSTFFRLFNRSFTQA (18-CSP) SGSLSTFFRLFNRSFTQALGK (21-CSP)	GLDWWSL
Salivarius	*S. salivarius* JIM8777/CCHSS3[G]	-	PYFTGCL
	S. vestibularis ACTC 49124/FO396	-	PFFMIYY
Mitis	*S. mitis* CCUG 31611[TG]	EIRQTHNIFFNFFKRR	-
	S. gordonii Challis CH1[G]	DVRSNKIRLWWENIFFNKK	-
	S. gordonii NCTC 7865[T],	DIRHRINNSIWRDIFLKRK	-
	S. sanguinis SK36[G]	DLRGVPNPWGWIFGR	-
	S. oligofermentans LMG 21535[T]	DSRNIFLKIKFKKK	-
	S. cristatus ACTC 51100[G]	DLRNIFLKIKFKKK	-
	S. infantis ATCC 700779[G]	DKRLTYFITNLFPKRKK	-
	S. oralis COL19[G]	EMRLPKILRDFIFPRKK	-
Anginosus	*S. anginosus* NCTC 10713[T]	DSRIRMGFDFSKLFGK	-
	S. constellatus NCTC 11325[T]	DSRIRMGFDFSKLFGK	-
	S. intermedius NCTC 11324[T]	DSRIRMGFDFSKLFGK	-

[a] In *S. mutans* the CSP belongs to the class of bacteriocin-inducing peptides, and differs from the CSP class identified in the mitis and anginosus groups.

T; type strain

G; genome sequence available

Table 2. List of strains, primers, plasmids and amplicons used in this study.

Strain	**Description**
S. mitis CCUG31611^{T}	Wild-type *S. mitis* biovar 1 type strain, corresponds to NCTC 12261^{T}
S. mutans UA159	Wild-type *S. mutans* UA159
MI074	CCUG31611, but ΔSM12261_0092::Kan; Kan^{R}
SM045	UA159, but Δ*dexA*::*kan*; Kan^{R}
Primers	Sequence (5' to 3')
FP906	ATTCACCCCAAAAAGTGCTG
FP907	ATAATATGCGGACGCTGAGG
FP1163	CATCTTGATAGCGTGGCTCA
FP1166	TTGAATTGAGACGGATTGGA
Plasmid	**Marker**
pVA838	Erm^{R}
Amplicon	**Description**
aRJ02	FP906/FP907 – 6.3 kb – Kan^{R}, from SM045
aRJ21	FP1163/FP1166 – 6.9 kb - Kan^{R}, from MI074

Figures

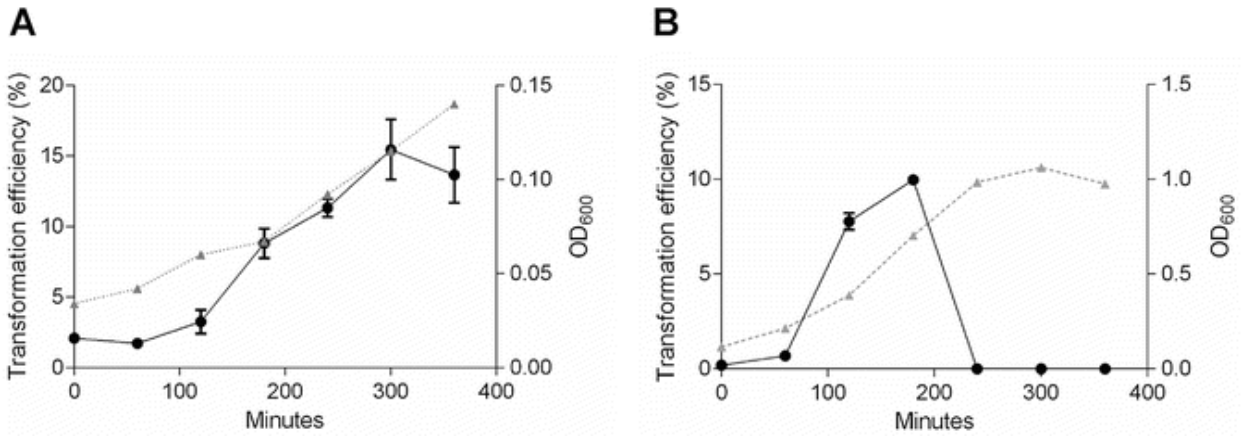

Fig. 1. Kinetics of *Streptococcus mutans* UA159 competence development in the presence of **(A)** synthetic XIP during growth in CDM and **(B**) synthetic CSP in TSB. Transformation used the 7-kb PCR amplicon (aRJ02) as donor DNA. The dots represent transformation efficiency values and the triangles are the corresponding absorbance values at 600 nm (A_{600}) of the growing culture corresponding to the time at which DNA was added, and are averages of three replicates. XIP or CSP was added at time "0". For each time point, DNase I was added after 20 min DNA exposure, and incubation proceeded for 40 min before plating the culture on non-selective and selective antibiotic plates. Bars represent standard error of the mean (SEM).

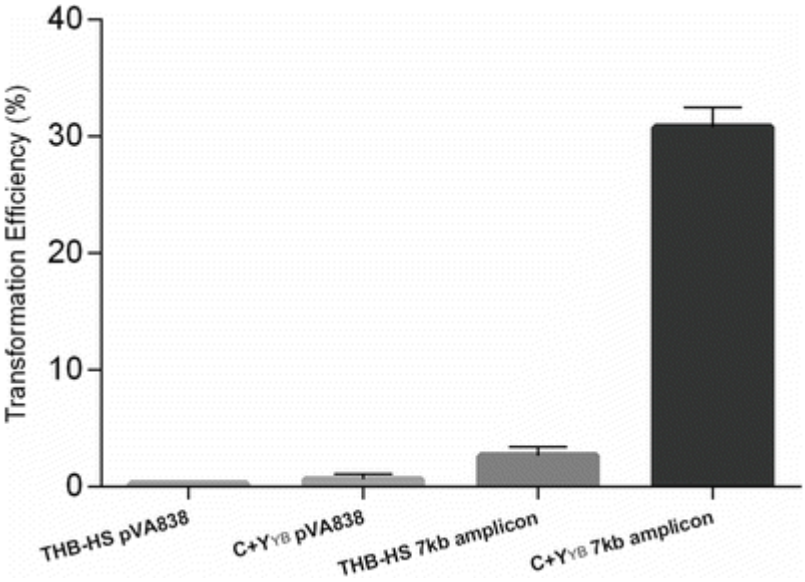

Fig. 2. *S. mitis* transformation in THB-HS or C+Y_{YB} using pVA838 (1 µg/mL) or a 7 kb amplicon (aRJ21) (150 ng/mL) as donor DNA, and CSP at a final concentration of 300 nM. Average of 3 replicates. Error bars represent standard error.