molecular oral microbiology

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Characterization of competence and biofilm development of a *Streptococcus sanguinis* endocarditis isolate

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SUMMARY

Streptococcus sanguinis **is an oral commensal bacterium and endogenous pathogen in the blood, which is generally naturally competent to take up extracellular DNA. Regarded as a stress response, competence development enables *S. sanguinis* to acquire new genetic material. The sequenced reference strain SK36 encodes and expresses the genes required for competence (*com*) and uptake of DNA. Isolated from blood cultures of a confirmed case of infective endocarditis, strain 133-79 encodes all necessary *com* genes but is not transformable under conditions permissive for competence development in SK36. Using synthetic competence-stimulating peptides (sCSP) based on sequences of SK36 and 133-79 *comC*, both strains developed competence at similar frequencies in cross-transformation experiments. Furthermore, downstream response pathways are similar in strains SK36 and 133-79 because platelet aggregation and biofilm formation appeared unaffected by CSP. Collectively, the data indicate that strains SK36 and 133-79 respond to CSP similarly, strongly suggesting that endogenous production or release of CSP from 133-79 is impaired.**

INTRODUCTION

Oral streptococci are constantly challenged by environmental stress. Saliva flow and food intake cause fluctuations in nutrient supply, pH, temperature and shear stress. The biofilm community adapts to dynamic conditions, therefore, by growth to high cell density and species diversity (Kreth *et al.*, 2009). Under biofilm growth conditions, competence development is an important mechanism of stress adaptation (Li *et al.*, 2002). Competence in streptococci seems to be a transient physiological state during which DNA is taken up from the environment (Morrison, 1997). DNA uptake as a consequence of competence development leads to horizontal gene transfer, genomic plasticity and the acquisition of new genetic traits.

The molecular details of competence development, including the identification of competence-regulating genes, were initially analysed in *Streptococcus pneumoniae* (for a recent review, see Johnsborg & Havarstein, 2009). In *S. pneumoniae*, competence development requires the production of the species-specific competence-stimulating peptide (CSP) (gene *comC*), which is secreted into the environment by the

ComAB transport proteins (Hui & Morrison, 1991). Environmental CSP is sensed by the two-component system ComDE; at a critical CSP threshold concentration a cellular response is triggered (Havarstein *et al.*, 1995; Pestova *et al.*, 1996). Accumulation of CSP results in quorum sensing, whereby early competence genes, including *comCDE*, *comAB* and *comX*, are transcribed and amplified in an autoregulatory mechanism (Morrison & Lee, 2000). ComX, an alternative sigma subunit of the DNA polymerase, then initiates transcription of delayed competence genes required for DNA uptake, processing and chromosomal integration (Lee & Morrison, 1999; Luo *et al.*, 2003; Luo & Morrison, 2003). Associated with competence development in streptococci, CSP controls the expression of other genes not involved in horizontal gene transfer, including bacteriocin production (Kreth *et al.*, 2005) and biofilm development (Li *et al.*, 2002). The competence state has been renamed the X-state, reflecting the general stress adaptation mechanisms responding to CSP accumulation and high cell density (Claverys *et al.*, 2006).

In this report, we investigate competence development leading to DNA uptake in two different strains of *Streptococcus sanguinis*, which is an oral commensal bacterium and pioneer colonizer during oral biofilm formation (Xu *et al.*, 2007). Associated with oral health, *S. sanguinis* is considered harmless in the oral cavity; healthy individuals have significantly higher numbers than individuals with caries who have virtually no detectable *S. sanguinis* (Becker *et al.*, 2002). Indeed, *S. sanguinis* and the cariogenic *Streptococcus mutans* are mutually antagonistic; acquisition of *S. mutans* is delayed when *S. sanguinis* is present in high abundance in human subjects (Caufield *et al.*, 2000). Upon entry into circulating blood, *S. sanguinis* bacteremia can cause severe infections in at-risk patients, such as infective endocarditis (Moreillon & Que, 2004). Infective endocarditis caused by oral streptococci remains associated with high morbidity and considerable mortality despite the use of antibiotics (Westphal *et al.*, 2009). A non-pathogenic commensal in the oral cavity and pathogen in the blood, the confounding lifestyles of *S. sanguinis* may be explained by environmental factors that elicit strain-specific differences in cell-to-cell signaling and related phenotypic characteristics.

In general, *S. sanguinis* is naturally competent to take up extracellular DNA (Turner *et al.*, 2009). The sequenced strain SK36, expresses the *com* genes required for competence development (*comCDE*) and uptake of DNA (*comYA–comYD*) (Xu *et al.*, 2007). In contrast, strain 133-79, isolated from blood cultures of a confirmed case of infective endocarditis (Herzberg *et al.*, 1990), does not become competent in conditions permissive for competence development in SK36. In this study, we compared both strains for competence gene expression and DNA uptake in response to CSP administration. We provide experimental evidence that 133-79 encodes the *com*-genes and responds to exogenous CSP, but might not produce sufficient CSP by itself to signal for competence.

METHODS

Bacterial strains, growth conditions and chemicals

Streptococcus sanguinis strains SK36 (Xu *et al.*, 2007) and 133-79 (Herzberg *et al.*, 1990) were routinely grown in Bacto™ Todd–Hewitt (TH) broth (BD, Sparks, MD). Liquid cultures were incubated without agitation at 37°C in ambient air, agar plates were incubated in candle jars at 37°C. Trypticase™ soy agar plates with 5% sheep blood (TSA II) were purchased from BD and incubated in candle jars at 37°C. For selective cultures, kanamycin was added to a final concentration of 400 μg ml^{-1}. Synthetic competence-stimulating peptides, sCSPs, (for sequence see Fig. 1) were custom-synthesized by ChemPep Inc. (Miami, FL). As assessed with high-performance liquid chromatography, peptide purity was determined to be 98% for $sCSP_{SK36}$ and 98.8% for $sCSP_{133\text{-}79}$. The peptides were dissolved in molecular grade water (1 mg ml^{-1}), divided into aliquots and stored at −20°C until further use.

Transformation assays

Transformation was performed essentially as described previously (Paik *et al.*, 2005) with modifications. Briefly, overnight cultures were grown in TH broth containing 2.5% heat-inactivated horse serum (PAA Laboratories, Dartmouth, MA). The next day, cells were freshly inoculated in the same medium (1 : 25) and incubated until the cell density reached an absorption at 600 nm (A_{600}) of 0.08. The cultures were split into 1-ml subsamples and 8 μg ml^{-1} chromosomal DNA carrying a kanamycin-resistance

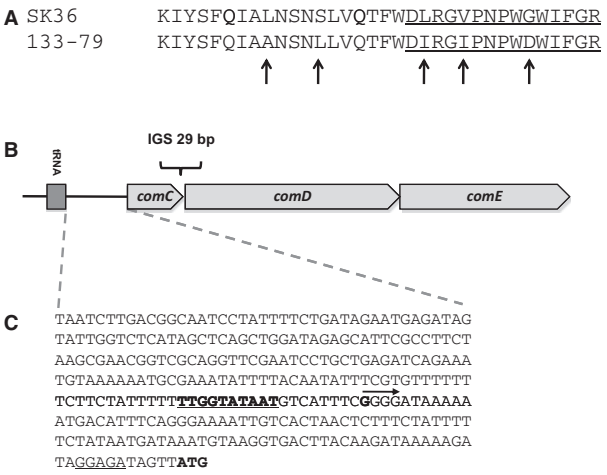

Figure 1 (A) Alignment of competence-stimulating peptides (CSP) from SK36 and 133-79 strains. Arrows indicate amino acid exchanges and underlined sequence marks the mature peptide. (B) *comCDE* operon organization. (C) Promoter sequence of strain SK36 and 133-79 with putative regulatory elements. Underlined = ribosome-binding-site; bold and underlined = putative extended −10 region; bold = start codon; bold with arrow = transcriptional start.

determinant (gene *aphA*) was added. The chromosomal DNA was isolated from a previously constructed SK36 strain (genotype *pknB::aphA*, Kan^R; Kreth, unpublished data). An assay for competence monitored uptake of chromosomal DNA and chromosomal integration via homologous recombination. DNA concentration was measured using a NanoDrop spectrophotometer. At the times indicated, sCSP was added to a final concentration of 0.5 μg ml^{-1}. Incubation continued for 2 h at 37°C and cells were plated on selective TH plates. Transformation efficiency was calculated as the number of kanamycin-resistant colony-forming units (CFUs) relative to the number of CFUs on non-selective TH agar.

RNA isolation, cDNA synthesis and real-time polymerase chain reaction

To isolate RNA, overnight cultures (1.6 ml) grown in TH medium were inoculated into 40 ml fresh TH medium in a 50-ml conical tube. Growth was monitored spectrophotometrically at A_{600}. Mid-log phase cells (A_{600} ~0.4) were induced with homologous or heterologous sCSP (0.5 μg ml^{-1} final concentration) and incubated for 2 h to ensure a high yield of RNA for downstream application. Cells were collected by centrifugation at 11,000 ***g*** for 15 min at 4°C and resuspended in 778 μl RLT buffer (QIAGEN RNeasy kit; QIAGEN, Valencia, CA) and 222 μl nuclease-free water. Cells were immediately disrupted twice for 1 min each time using Lysing Matrix B (MP Biomedicals; Solon, OH) and a FastPrep FP210 Homogenizer (Thermo Scientific, Waltham, MA). RNA was purified using a QIAGEN RNeasy kit following the manufacturer's protocol. Isolated RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove traces of chromosomal DNA. After the treatment, RNA samples were cleaned with the QIAGEN RNeasy kit. The concentration and quality of RNA samples were confirmed using NanoDrop spectrophotometer measurements and gel electrophoresis.

Complementary DNA (cDNA) was synthesized from 2 μg RNA using the SuperScript II™ Reverse Transcriptase and Random Primers (Invitrogen) according to the manufacturer's instructions.

Specific transcripts were quantified with the comparative threshold cycle (C_t) method using the Bio-Rad MyiQ real-time reverse transcription–polymerase chain reaction (RT-PCR) detection system (Bio-Rad, Hercules, CA). The RT-PCR protocol included one cycle of 95°C for 90 s, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For real-time RT-PCR, oligonucleotide primers were designed with Beacon Designer 7 software (PREMIER Biosoft, Palo Alto, CA), which selects primers optimized for SYBR® green-based PCR analysis. Primer sequences are listed in Table 1. Note that all primers could be used in both strains with equal efficiency, except for *comD* and *comC*, which required specific primers for each strain. Real-time PCR was performed using 1× SYBR green master mix (Bio-Rad) according to the manufacturer's instructions. Changes of gene expression were calculated using the C_t method as follows: $C_t = C_t$ (target) − C_t (housekeeping gene). The fold changes are calculated as $2^{-\Delta Ct}$. The *gyrA* gene was used as the housekeeping reference gene.

Biofilm formation and quantification

Biofilm formation was measured using a modification of the crystal violet (CV) microtiter assay as reported previously (Ashby *et al.*, 2009).

Determination of transcription start sites

The FirstChoice RLM random amplification of cDNA ends (RACE) kit (Ambion, Austin, TX) was used to

Table 1 Real-time polymerase chain reaction (RT) and random amplification of complementary DNA ends (RACE; RA) primers

Primer	Sequence (5′ to 3′)
RT 133-79 comC F	TGAAAATCTATTCTTTTCAAATTGC
RT 133-79 comC R	CAATCCCATGGATTTGGAAT
RT SK36 comC F	CCATGGATTTGGAACACCTC
RT comX R	CCAATCCAACATCATCAAGAGTTC
RT comX F	CCGCCAGCCTTATGAAGAAG
RT 133-79 comD F	GCTATTGAGAGTGCCATTGACAG
RT 133-79 comD R	CTCGGGCTGATAGAGTTCTTCC
RT SK36 comD F	GGAGATTCAGCTTTAAGGAGTGTC
RT SK36 comD R	ACAACTTGATTGGAAGGCGTTC
RT comYA R	AAGCGGCGTTCATCACCAATTCTC
RT comYA F	TGATTAGGCAGGCTCGGCAAGAAG
RT gyrA F	GCCGTGAGCGAATTGTCGTAA
RT gyrA R	CGAACAGCAGTGATACCGTCAATG
RA IP 133-79	GGATGAACAATTAATCCTATTATTTCAA
RA IP SK36	GAATGAATAATTAATCCAATTATTTCAA
RA OP 133-79	GAAATGAGATTTCAAATGAAAACACAT
RA OP SK36	GAATTGTGCTAGAAAATGAAAATATGT

determine transcription start sites essentially as described earlier (Okinaga *et al.*, 2010). RACE specific primers are listed in Table 1.

Sequencing

GenBank sequence accession numbers were GU943505, GU943506, GU943507, GU943508, GU943509 and GU943510.

Statistical methods

Statistical analysis of two datasets was performed with QuickCalcs online calculators (http://www.graphpad.com/quickcalcs/index.cfm) using the *t*-test software to calculate mean and SD. Statistically significant differences were set at a P value of <0.05.

RESULTS

Competence phenotype of *S. sanguinis* strain 133-79

Strain SK36 contains all the genes required for competence development, uptake and incorporation of DNA into the chromosome (Xu *et al.*, 2007). When tested for competence with chromosomal DNA encoding a kanamycin resistance cassette, the natural transformation efficiency of strain SK36 was about 10^{-7}. In contrast, strain 133-79 was not transformable when tested without the addition of sCSP, suggesting that strain 133-79 is unable to develop natural competence under laboratory conditions.

Comparison of competence genes in *S. sanguinis* strains SK36 and 133-79

To determine whether strain 133-79 contains the genes required for competence development and DNA uptake, the chromosomal loci for *comCDE*, *comX* and *comYA–comYD* were sequenced and compared with the annotated sequence of strain SK36 (Xu *et al.*, 2007). The *comCDE* operon, *comX* and the *comYA–comYD* operon are present in 133-79. Each SK36 and 133-79 gene was more than 95% identical at the nucleotide level, with the exception of *comC*, showing only 85% identity (data not shown). By aligning the full-length peptide sequences, SK36 and 133-79 CSPs showed five exchanges. Three conservative replacements of leucine and valine with isoleucine as well as leucine with alanine, a polar to non-polar exchange from serine to leucine and a non-polar to polar exchange from glycine to aspartate (Fig. 1A). Three of the exchanges occurred in the peptide sequence defined to be the mature form.

The *comCDE* promoter sequences of SK36 and 133-79 are identical. To better characterize the promoter region of the *comCDE* operon (Fig. 1B), the transcriptional start site was determined by RACE PCR in both strains. The start was determined to be 105 base pairs (bp) upstream of the start codon in both strains. A putative extended −10 region is located 8 bp upstream of the transcription start. No obvious −35 region could be located (Fig. 1C). When compared with SK36, the 133-79 competence gene sequence similarities suggest the ability to develop competence.

Strain-specific CSP cross-induces competence development

To learn whether the *comC* gene of strain 133-79 encodes a non-functional CSP, the CSPs of SK36 and 133-79 were synthesized as $sCSP_{SK36}$ and $sCSP_{133\text{-}79}$ using the published peptide sequence as reference (Gaustad & Havardstein, 1997), respectively. Addition of strain-specific sCSPs induced competence in the homologous strains. The $sCSP_{SK36}$ increased strain SK36 transformation efficiency about

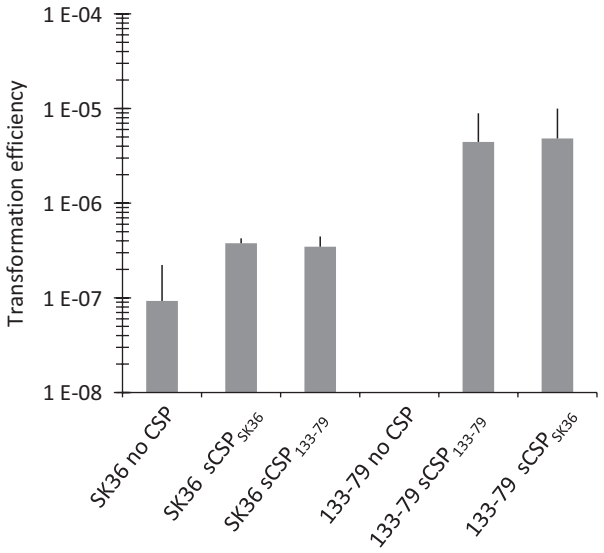

Figure 2 Transformation efficiency of SK36 and 133-79. Cells were grown to an absorbance at 600 nm of 0.08 in Todd–Hewitt (TH) medium plus 2.5% heat-inactivated horse serum. Then, 8 μg ml^{-1} chromosomal DNA (KanR) and synthetic competence-stimulating peptides (sCSP; 0.5 μg ml^{-1}) were added, incubation was continued for 2 h at 37°C, and the cells were plated on selective TH plates. Efficiency was calculated as the number of kan-resistant colony-fomring units (CFUs) relative to the number of CFUs on non-selective TH plates. Data presented are the means and standard deviations of three independent experiments.

four-fold (Fig. 2). When compared with SK36 induced with $sCSP_{SK36}$, addition of $sCSP_{133\text{-}79}$ to strain 133-79 increased transformation efficiency 10-fold more. Using the heterologous sCSPs, cross-induction resulted in similar transformation efficiency suggesting that $sCSP_{SK36}$ and $sCSP_{133\text{-}79}$ are equally effective for SK36 or 133-79 (Fig. 2).

Effect of sCSP concentration and growth phase on competence

To test for concentration dependence, strain 133-79 and SK36 were incubated with increasing sCSP concentrations and transformability was tested. Both strains formed similar numbers of transformants per μg DNA when increasing concentrations of strain-specific sCSP were added to the medium (Fig. 3A).

Next, we tested whether competence development in 133-79 and SK36 differs as a function of stage of growth and cell density. SK36 is naturally competent during early stages of growth (Fig. 3B), consistent with previous reports for *S. pneumoniae* (Morrison, 1997). Strain 133-79 did not develop competence during growth at any time or cell density.

Cross-induction of competence gene expression

SK36 responded to homologous sCSP by upregulating the expression of *comX* about 10-fold, *comYA* about seven-fold and *comD* about 20-fold when compared with the non-induced controls (Fig. 4A–C, respectively). Similar to strain SK36, 133-79,

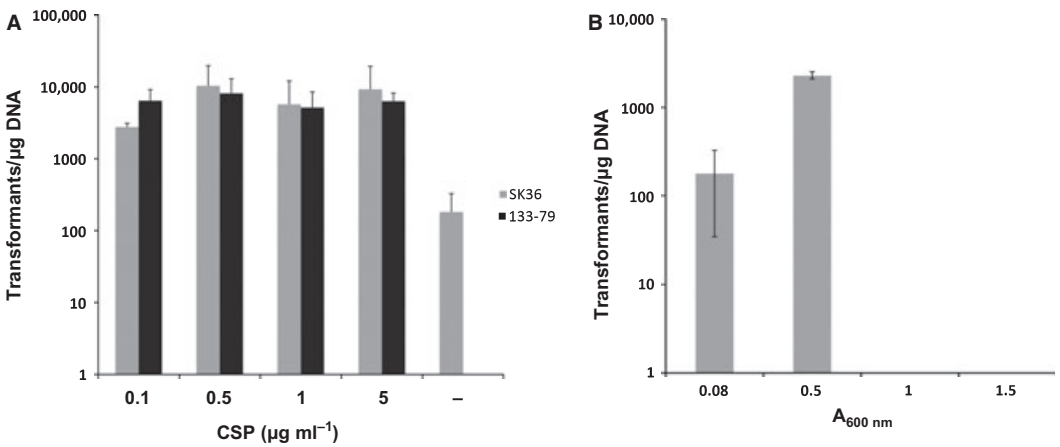

Figure 3 Influence of various synthetic competence-stimulating peptides (sCSP) concentrations and the growth phase on transformability. (A) Cells were grown to an absorbance at 600 nm (A_{600}) of 0.08 in Todd–Hewitt (TH) medium plus 2.5% heat-inactivated horse serum. Then, 8 μg ml^{-1} chromosomal DNA (KanR) and different concentrations of strain-specific sCSP were added, incubation was continued for 2 h at 37°C, and the cells were plated on selective TH plates. (B) To test for growth-phase-dependent transformability, cells were grown to different cell densities in TH medium plus 2.5% heat-inactivated horse serum. Then, 8 μg ml^{-1} chromosomal DNA (KanR) was added, incubation was continued for 2 h at 37°C, and the cells were plated on selective TH plates. Data presented are the means and standard deviations of two independent experiments performed in duplicate.

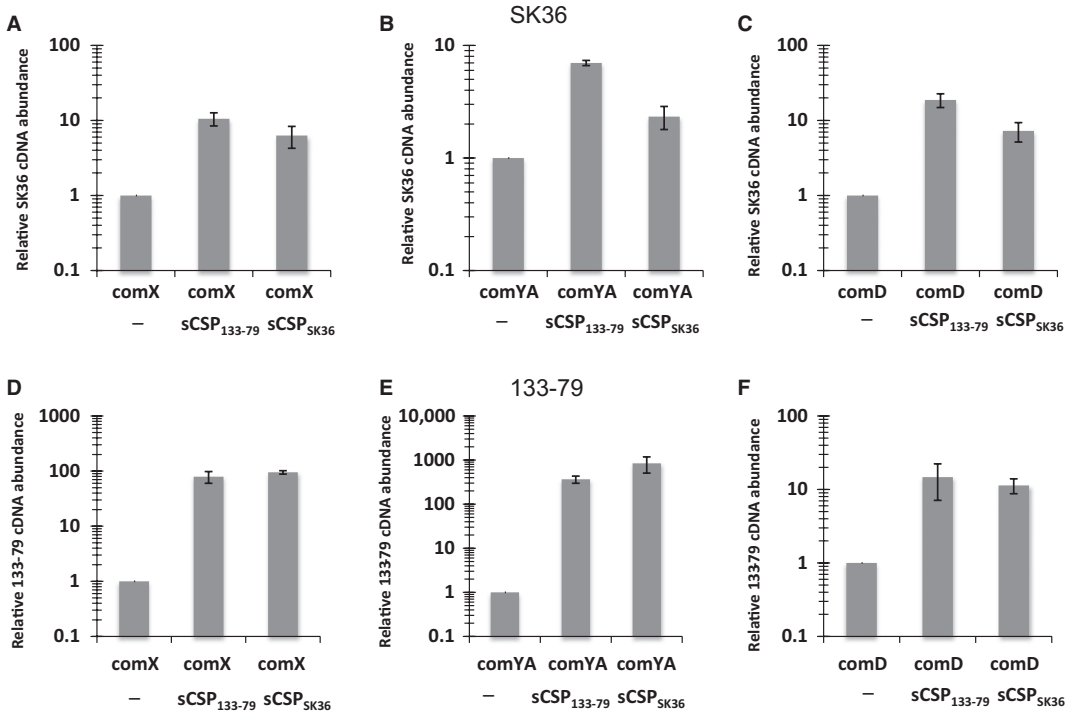

Figure 4 Real-time reverse transcription polymerase chain reaction analysis of early and late competence genes in SK36 (A–C) and 133-79 (D–F). Cells were induced with their own or heterologous competence-stimulating peptides. The expression levels are presented relative to the non-induced controls, which were arbitrarily assigned a value of 1. Strains were induced in mid-log phase with 0.5 μg ml^{-1} CSP. Data presented are the means and standard deviations of three independent experiments performed in duplicate from different days.

responded to homologous sCSP by inducing expression of *comX* about 100-fold, *comYA* about 500-fold, and *comD* about 20-fold (Fig. 4D–F, respectively). After addition of heterologous sCSPs, expression of competence genes also increased in both strains (Fig. 4A–C for SK36 and 4D–F for 133-79, respectively). The transcriptional responses of SK36 and 133-79 to sCSPs were consistent with the results obtained for transformation efficiency (Fig. 2).

Table 2 Comparison of primer efficiencies

	Strain	C_t ratio
C_t *gyrA*/C_t *comC*	SK36	0.99 ± 0.04
	133-79	0.97 ± 0.04
C_t *gyrA*/C_t *comD*	SK36	1.07 ± 0.01
	133-79	1.07 ± 0.02
C_t *gyrA*/C_t *comYA*	SK36	1.05 ± 0.02
	133-79	1.01 ± 0.05
C_t *gyrA*/C_t *comX*	SK36	1.13 ± 0.02
	133-79	1.09 ± 0.02

Comparison of competence gene expression in SK36 and 133-79

To compare the two strains for the expression levels of the competence genes, we first determined that the real-time RT-PCR primers amplify the selected genes in both strains with equal efficiency. The C_t values of *gyrA* in both strains were similar. When compared, SK36 and 133-79 showed similar ratios of competence genes to *gyrA* (Table 2). Non-induced SK36 expressed *comC*, *comD*, *comX* and *comYA* at higher levels than non-induced 133-79. Addition of $sCSP_{133-79}$ increased expression of *comX* and *comYA* in 133-79 above levels in non-induced SK36, whereas *comC* and *comD* were induced in 133-79 at lower levels than in non-induced SK36 (Fig. 5A–D).

Effect of cell density on the expression of competence genes

The relative expression of *comX* and *comYA* was compared in high cell-density cultures of 133-79 and

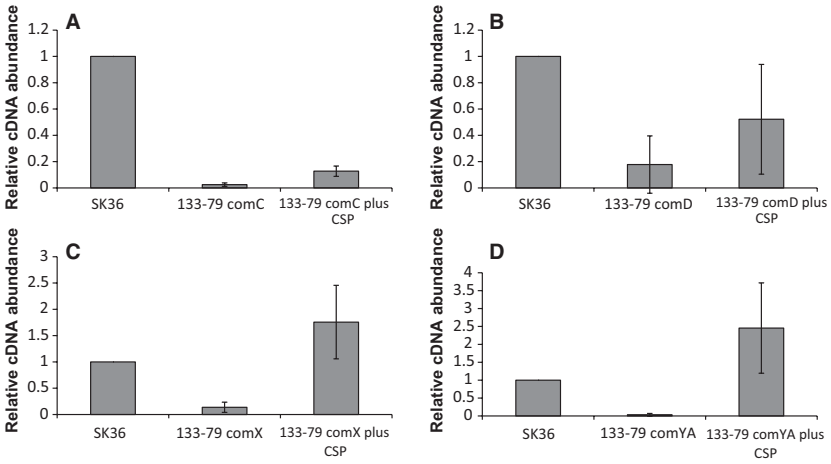

Figure 5 Comparative real-time reverse transcription polymerase chain reaction analysis of early and late competence genes in SK36 and 133-79. The expression levels of 133-79 are presented relative to that of SK36, which was arbitrarily assigned a value of 1. Data presented are the means and standard deviations of three independent experiments performed in duplicate from different days.

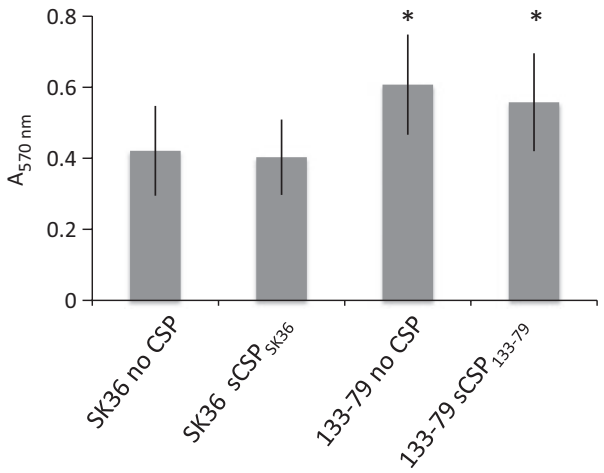

Figure 6 Quantification of biofilms. Biofilm mass of attached cells was quantified with a crystal violet microtiter well assay. Data presented are the means and standard deviations of two independent experiments carried out in multiple replicates from different days. Asterisks indicate statistical significance ($P = 0.0001$).

SK36. Cells were grown on blood agar plates for 5 h, harvested from the plates, and RNA was isolated. The expression of both genes was similar in 133-79 and SK36 (data not shown).

Increased biofilm formation by strain 133-79

We next tested biofilm development of both strains with and without addition of sCSP. The sCSPs showed no influence on static biofilm formation by either strain on polystyrene surfaces. Strain 133-79, however, formed significantly denser biofilms than SK36 ($P = 0.0001$) (Fig. 6).

DISCUSSION

Competence development is part of a CSP-mediated response mechanism to stress. The uptake and integration of DNA into the chromosome via homologous recombination might select for new, beneficial genetic traits and is the main source of horizontal gene transfer in streptococci (Havarstein, 2010). Competence development mutants show a reduced ability to take up or integrate DNA into the chromosome, form biofilms and produce bacteriocins (Li *et al.*, 2002; Kreth *et al.*, 2005). These mutants might, therefore, be less competitive in a multispecies community as found in the oral biofilm. *Streptococcus sanguinis* is naturally competent, represented by strain SK36. To our surprise, we found an endocarditis isolate 133-79 to be non-transformable under laboratory conditions. We sought to understand why the two strains differed in competence.

The competence genes are functional in 133-79. To establish this conclusion, we sequenced the respective *com* genes in 133-79 and found them to be intact and almost identical on the nucleotide level when compared with SK36, confirming the ability of 133-79 to develop competence. When added exogenously, homologous (133-79) or heterologous (SK36) sCSPs induced expression of competence genes and transformability in strain 133-79.

As 133-79 could respond to sCSPs, production or export of endogenous CSP was strongly suggested to be impaired in strain 133-79. The *comCDE* genes are encoded in an operon (Fig. 1B). The expression of *comC* is considerably lower in 133-79 than SK36, suggesting a much lower production of CSP.

Because *comCDE* expression is regulated in an autoregulatory circuit, CSP concentrations below a signaling threshold could explain the non-competent phenotype observed for 133-79.

The export of CSP in several streptococci requires the dedicated export system ComAB (Hui & Morrison, 1991; Claverys *et al.*, 2009). For *S. pneumoniae*, CSP export is limiting in competence development (Martin *et al.*, 2000). Interestingly, SK36 does not encode ComAB homologues, suggesting that an alternative CSP-export mechanism is used by *S. sanguinis* (Xu *et al.*, 2007). The CSPs of SK36 and 133-79 lack the typical double glycine motif for ComAB transport, which is found in CSPs of other sequenced streptococci. As the dedicated transport system has not been identified in *S. sanguinis*, we are unable to test if the low expression of *comCDE* is caused by impaired transport of CSP.

We therefore concentrated on regulation of the *comCDE* operon. Investigation of the promoter region showed no difference between SK36 and 133-79. No obvious −35 site could be identified. A search for Lys-RT type regulator binding sites did not show a ComE binding site upstream of the extended −10 region as describe for the *S. pneumoniae* ComE binding site (Ween *et al.*, 1999). Further experimental proof is required to show whether ComE interacts with the *comCDE* promoter and which region is covered by the bound protein. The promoter region was also searched for the presence of a *comX* box using the recently described sequences for *S. mutans* as template (Okinaga *et al.*, 2010). No *comX* binding site was identified. To consider whether the transcription start of the *comCDE* operon differs in SK36 *and* 133-79, RACE PCR was performed with CSP-induced and non-induced cells of both strains. The start site appeared the same in induced cells and uninduced controls. We conclude therefore that the regulation of *comCDE* operon expression is the same in both strains.

In strain 133-79, the most likely explanation for the lack of autonomous competence development is impaired export of CSP and little or no CSP is available for auto-induction. Deletion of the *comC* gene in SK36 results in the same non-transformable phenotype, which could be rescued to wild-type transformability when sCSP was added (T. Kitten, VCU School of Dentistry; personal communication). These data further support the conclusion that *S. sanguinis* has CSP-inducible competence development.

The transcriptional response of *S. gordonii* to CSP has been reported to change the expression of several surface proteins potentially involved in host cell interactions (Vickerman *et al.*, 2007). Homologues of these surface proteins are found in *S. sanguinis*. We, therefore, tested whether CSP auto-induction changes surface proteins on *S. sanguinis* sufficiently to alter the ability to trigger platelet aggregation (reviewed in Herzberg, 1996). Autologous sCSP induction of SK36 does not affect the platelet aggregation response (data not shown). We have also tested whether CSP addition affects biofilm formation by *S. sanguinis*. Although strain 133-79 forms denser biofilms than SK36, this function was unaffected by CSP. Hence, we found no evidence that CSP could contribute to virulence by affecting biofilm development or platelet aggregation in *S. sanguinis*.

In summary, the non-transformable *S. sanguinis* 133-79 has the ability to develop competence when exogenous CSP is supplied. Why *S. sanguinis* 133-79 is naturally non-transformable appears to reflect differences in release or processing of CSP when compared with SK36, although there is no obvious advantage of this phenotype based on the similar platelet aggregation and biofilm formation traits. From an ecological standpoint, if strain 133-79 was originally part of a multispecies community including CSP-producing *S. sanguinis*, a classification as 'cheater' might be possible. Strain 133-79 would take advantage of the heterologous CSP produced by other strains of *S. sanguinis* in the oral biofilm. It could therefore respond with full competence development, but without bearing the costly production of an excreted peptide. More detailed competition experiments in the future will answer this question.

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