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CcpA regulates biofilm formation and competence in *Streptococcus gordonii*

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SUMMARY

***Streptococcus gordonii* is an important member of the oral biofilm community. As an oral commensal streptococcus, *S. gordonii* is considered beneficial in promoting biofilm homeostasis. CcpA is known as the central regulator of carbon catabolite repression in Gram-positive bacteria and is also involved in the control of virulence gene expression. To further establish the role of CcpA as central regulator in *S. gordonii*, the effect of CcpA on biofilm formation and natural competence of *S. gordonii* was investigated. These phenotypic traits have been suggested to be important to oral streptococci in coping with environmental stress. Here we demonstrate that a CcpA mutant was severely impaired in its biofilm-forming ability, showed a defect in extracellular polysaccharide production and reduced competence. The data suggest that CcpA is involved in the regulation of biofilm formation and competence development in *S. gordonii*.**

INTRODUCTION

Streptococcus gordonii is an important member of the oral microbiota (Schachtele *et al.*, 2007). It can be found in relatively high abundance on different intraoral sites (Aas *et al.*, 2005). As one of the pioneer oral streptococcal species, *S. gordonii* is involved in the development of the multispecies oral biofilm and promotes biofilm homeostasis (Kolenbrander *et al.*, 2006).

Biofilm formation is a regulated developmental process in microorganisms and provides protection against environmental stresses (Lemos *et al.*, 2005; Ahn *et al.*, 2006; Murphy *et al.*, 2006; Monds & O'Toole, 2009). Several studies document, for example, that bacteria residing in a biofilm show increased resistance to antibiotics when compared with planktonic counterparts (Hoiby *et al.*, 2010). Similarly, bacteria in biofilms show increased resistance to immune cells like macrophages and the innate immune response (Thurlow *et al.*, 2011). In parallel, biofilm structures provide protection against shear stress (Kreth *et al.*, 2004) produced during mastication and swallowing, or by the flow of saliva.

Biofilm development involves several global regulators, suggesting a coordinated process dependent on internal and external signals. For example, multiple sensory two-component signal transduction systems (TCSS) in *Streptococcus mutans* have been implicated in the control of biofilm formation (Bhagwat *et al.*, 2001; Li *et al.*, 2002; Senadheera *et al.*, 2007). The TCSS are important for cellular adaptation

processes to diverse environmental stresses. For example, the best-studied TCSS ComDE in *S. mutans* regulates the development of competence for the uptake of extracellular DNA under biofilm conditions (Li *et al.*, 2001, 2002). Competence is also regarded as an important part of the streptococcal general stress response because the acquisition of new genetic material might aid in the adaptation to a challenging environment (Suntharalingam & Cvitkovitch, 2005; Ahn *et al.*, 2006). During biofilm development, *S. mutans* also coordinates internal signals using global transcriptional regulators like carbon control protein A (CcpA; Browngardt *et al.*, 2004; Abranches *et al.*, 2008). CcpA is the major regulator of carbon catabolite repression in Gram-positive bacteria and is involved in the regulation of virulence properties (Warner & Lolkema, 2003; Gorke & Stulke, 2008). Mutations in the respective genes for TCSS and CcpA all lead to altered biofilm phenotypes in *S. mutans* and other oral streptococci (Li *et al.*, 2002; Browngardt *et al.*, 2004; Zhang *et al.*, 2004; Zheng *et al.*, 2011a).

Adherence capabilities of *S. gordonii* are crucial during initial biofilm development. Adherence is mediated through specific cell-surface exposed proteins called adhesins. The adhesins mediate the interactions with host components found in saliva and the mucosal surface (Nobbs *et al.*, 2009). Additionally, adhesins are responsible for biofilm community development. For example, the major adhesins SspA and SspB of the antigen I/II protein family facilitate the binding of *S. gordonii* to mucosal surfaces via host-cell exposed glycoprotein 340 (gp340) and to other members of the oral biofilm community, such as *Porphyromonas gingivalis* (Chung *et al.*, 2000; Jakubovics *et al.*, 2005; Jenkinson & Lamont, 2005). Interestingly, adhesin gene expression also appears to be coordinated. Unable to anchor LPXTG-linked surface adhesins in a Sortase A (SrtA) mutant, *S. gordonii* alters the expression of several adhesin genes (Nobbs *et al.*, 2007a). SrtA mutants of *S. gordonii* and several other species are also impaired in biofilm formation (Yamaguchi *et al.*, 2006; Nobbs *et al.*, 2007a; Guiton *et al.*, 2009) and are therefore less tolerant of environmental stress, suggesting a link between adhesin gene regulation, biofilm formation and stress resistance.

In this report we demonstrate the CcpA-dependent regulation of *S. gordonii* biofilm formation. The deletion of CcpA in *S. gordonii* influenced the expression of several adhesin genes and the TCSS regulated development of natural competence for DNA uptake. Furthermore, deletion of CcpA affected the production of extracellular polysaccharides. As biofilm formation, natural competence and extracellular polysaccharide production are important in environmental stress tolerance, our results indicate an important role of CcpA in stress resistance and fitness of *S. gordonii*.

METHODS

Bacterial strains and media

The S. gordonii strains used in this study are listed in Table 1 and were routinely grown aerobically (5% CO_2) or in an anaerobic chamber (90% N_2, 5% CO_2, 5% H_2) at 37°C on Brain–Heart infusion (BHI; Difco, Sparks, MD) agar plates except when stated as grown in TYE medium (1% tryptone, 0.5% yeast extract, 0.3% K_2HPO_4) or Todd–Hewitt medium (TH medium; Difco). The carbohydrates glucose and sucrose were filter-sterilized 20% stock solutions and used as supplements when indicated.

Growth curves of *S. gordonii* and its derivatives were generated using a BIOSCREEN C ANALYZER, version 2.4 (Oy Growth Curves AB Ltd., Helsinki, Finland), which kinetically and simultaneously measured the development of turbidity in multiple cultures. The pre-cultures were supplemented with antibiotics for the mutant and complemented strain. The growth curves were measured without antibiotics in the respective growth medium.

DNA manipulations

Standard recombinant DNA techniques were used as described previously (Zheng *et al.*, 2011a).

Construction of a Δ*ccpA* mutant and *in trans* complemented strain

The construction of both strains was described previously (Zheng *et al.*, 2011b).

Transformation efficiency assay

Fresh overnight cultures were diluted 30 times in TH medium and incubated at 37°C for 1 h until the cul-

Table 1 Strains, plasmids and primers used in this study

	Relevant characteristics	Reference
Strain		
DL1	Wild-type *Streptococcus gordonii*	Zheng *et al.* (2011b)
DL1 *ccpA*	*ccpA*; Erm^r	
DL1 compl	DL1 *ccpA*; *ccpA* complemented; Spc^r	
Plasmids		
pDL278 + $ccpA_{compl}$	*ccpA* cloned into pDL278; Spc^r	Zheng *et al.* (2011b)
Primers	Sequence	
sspA-F	5′-TCCTGACAAACCTGAGACACC-3′	Zhang *et al.* (2005)
sspA-R	5′-TTTAACTTTCAGAGCTTAGTTGCTTTC-3′	
sspB-F	5′-TCCTGACAAACCTGAGACACC-3′	
sspb-R	5′-CATCAAAGATGAAACAAGTCTAAGC-3′	
scaA-F	5′-CACCGAAGAAGAAGGCACTC-3′	
scaA-R	5′-TGTCTCCATCTTCGCCTTTT-3′	
apbB-F	5′-CAAAAACTCCGGAAAAACCA-3′	
abpB-R	5′-GGAGCTTGACTCGGTTCTTG-3′	
hsa-F	5′-CAGAGCTGCAAATCCAAACA-3′	
hsa-R	5′-GCCGAGATACTTGCGCTTAC-3′	
cshB-F	5′-CGTTGTTCAGCAAGGATCAA-3′	
cshB-R	5′-GCCGTTCTGTTGTCCAGTAG-3′	
apbA-F	5′-TGATGCAGTTGAAGGTGGAA-3′	
abpA-R	5′-TAGCTGCACCAACACGTTTC-3′	
cshA-F	5′-CAGACGATGCAACCCCTATT-3′	
cshA-R	5′-TAACGGTCAAGGTCACCACA-3′	
16S rRNA-F	5′-AAGCAACGCGAAGAACCTTA-3′	
16s rRNA-R	5′-GTCTCGCTAGAGTGCCCAAC-3′	
comC-F	5′-AAAGAATATATTTTCCCACCATAATC-3′	This study
comC-R	5′-TGAAAAAGAAAAACAAACAAAATCT-3′	
comD-F	5′-TGTTCACGAGCAGACTTCAGA-3′	
comD-R	5′-TCATTGGTTCAGCGAAAATG-3′	
comYA-F	5′-GAAACTGCTAGAGCGGTGGT-3′	
comYA-R	5′-TCCTCGCTAACACCCAATTC-3′	

ture reached an absorbance at 600 nm of 0.1. Aliquots of cells were placed in 1.5-ml tubes (1 ml per tube) and 1 μg chromosomal DNA was added. The chromosomal DNA was isolated from strain DL1 carrying a kanamycin-resistance cassette inserted upstream of the *spxB* promoter. The cassette did not disrupt any gene and had no effect on pyruvate oxidase activity (Zheng *et al.*, 2011b). DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific-NanoDrop, Wilmington, DE). The cells were further incubated for 2 h, followed by a brief sonication to break-up cell chains and 10 times serial diluted in BHI. Then, 10 μl of each sample was plated on kanamycin-containing BHI agar plates as well as on non-selective BHI 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.

Biofilm formation and quantification

Biofilm formation was measured using a modification of the crystal violet microtiter assay as reported by Ashby *et al.* (2009). Briefly, microtiter plates (Falcon Microtest™ 96; Becton Dickinson, Franklin Lakes, NJ) were inoculated from overnight BHI-grown cultures of *S. gordonii* diluted 1 : 60 in fresh TYE, TH and BHI media supplemented with 0.2 or 2% sucrose (final volume 150 μl). To form biofilms, cells were grown for 16 h as static cultures at 37°C. The medium was removed by inverting the dish and shaking off the residual medium and cells. Cells were stained with 150 μl per well crystal violet (2.3% weight/volume, Ac-

custain Crystal Violet Solution; Sigma Diagnostics, St. Louis, MO) for 15 min. The microtiter dish was subsequently washed twice with water and air-dried. Biofilm formation was quantified by solubilization of the CV staining using 150 μl 95% ethanol per well. The absorbance of the resulting solution (100 μl) was measured at 570 nm with a microplate reader (Model 680; Bio-Rad, Hercules, CA). Collagen-coated microtiter plates (BD BioCoat Cellware, Collagen Type IV) were from BD Biosciences (Sparks, MD).

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

RNA was isolated using the QIAGEN RNeasy kit, cDNA was synthesized using the SuperScript II™ Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time reverase transcription polymerase chain reaction (qRT-PCR) was performed to detect specific transcripts with the comparative threshold cycle (C_T) method using the Bio-Rad MyiQ Cycler (Hercules, CA). Relative changes in gene expression were calculated using the ΔC_T method described previously (Zheng *et al.*, 2011a). The 16S rRNA gene was used as the housekeeping reference gene. All primers used for qRT-PCR are listed in Table 1.

Detection of exopolysaccharide production

The Congo red method was performed as described previously (Arciola *et al.*, 2001). BHI plates with 3% sucrose were incubated aerobically at 37°C for 48 h. Plates were inspected with an Olympus BX51 microscope using an UPlan FL N 10× lens and photographed with an Olympus DP72 digital camera and cellSens 1.3 software (Hitschfel Instruments, Inc.; St. Louis, MO). Entire images were adjusted for size, brightness and contrast with photo-processing software GIMP 2.6.10. (http://www.gimp.org/).

Statistical analysis

Statistical analysis of data was performed with the QuickCalcs online calculators (http://www.graphpad.com/quickcalcs/index.cfm) using the *t*-test software to compare the means of two groups. The data were considered significantly different if the two-tailed *P*-value was <0.05.

RESULTS

Deletion of *ccpA* causes a general growth defect

To learn if a CcpA mutation causes a general growth defect in *S. gordonii*, growth was monitored for strain DL1, an isogenic CcpA mutant and a complemented strain expressing *ccpA in trans* from shuttle plasmid pDL278. As presented in Fig. 1, the CcpA mutant showed a decreased growth rate in BHI and in BHI with 2% glucose. A similar growth defect was observed for the CcpA mutant when grown in TYE with 2% glucose, but not in TYE with 0.2% glucose (data not shown). Complementation with an *in trans* expressed *ccpA* did restore wild-type growth rates.

The CcpA mutant forms unstable biofilms

To learn if different growth media influence CcpA-dependent biofilm formation, a standardized biofilm microtiter plate assay was used to compare wild-type, CcpA mutant and complemented strains. Biofilm formation was tested with cells grown in TYE, TH and BHI medium supplemented with 0.2 or 2% sucrose. Under all tested conditions, the CcpA mutant showed a reduced biofilm-forming ability, which was restored to wild-type levels in the complemented strain (Fig. 2 A). Supplementing BHI with 2% sucrose increased the attached biofilm mass (Fig. 2B), whereas no dif-

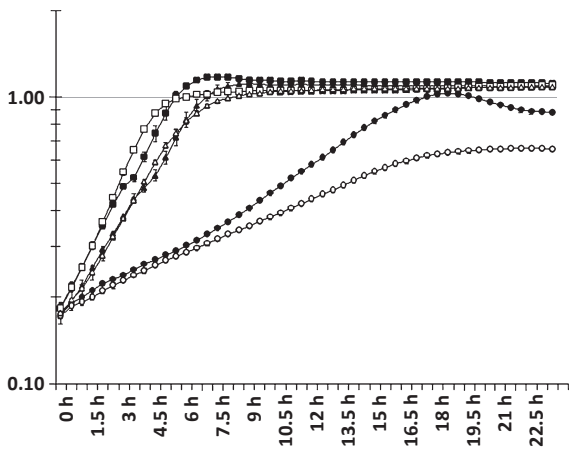

Figure 1 Growth curves of *Streptococcus gordonii* wild-type strain DL1 (squares), CcpA mutant (circles) and complemented strain (triangle) in brain–heart infusion (BHI) and BHI supplemented with 2% glucose. Means and standard deviations of three independent experiments are presented.

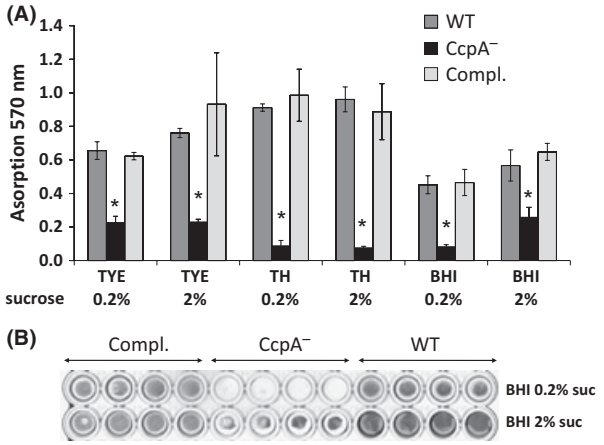

Figure 2 Biofilm production of wild-type, CcpA mutant and complemented strains on polystyrene microtiter plates. (A) Representative of microtiter plate wells from each experiment showing the respective biofilm formation of each *Streptococcus gordonii* strain. (B) Quantitative analysis of biofilm production measuring the absorption of destained biofilms at 570 nm in a crystal violet biofilm assay. Means and standard deviations of three independent experiments are presented. Asterisk indicates statistically significant difference in biofilm formation of the mutant when compared with the wild-type under the respective test conditions ($P < 0.05$).

ference was observed for TH and TYE plus 2% sucrose.

Collagen can serve as an attachment substrate during biofilm formation in some *in vivo* conditions (Abranches *et al.*, 2011) so we evaluated biofilm formation using collagen-coated microtiter plates. The biofilm phenotype for the CcpA mutant was similar on collagen-coated and uncoated microtiter plates (data not shown).

Altered adhesin gene expression in the CcpA mutant

Biofilm formation by *S. gordonii* depends on the expression and abundance of surface adhesins, which enable attachment to the substratum (Nobbs *et al.*, 2007a). To learn whether CcpA affected the expression of surface adhesin genes, wild-type, CcpA mutant and the complemented strains were grown in TH medium and compared for adhesin gene expression. The major cell surface adhesins amylase binding proteins AbpA and AbpB, the sialic acid binding protein Hsa, the antigen I/II family adhesin proteins SspA and SspB, the fibronectin binding proteins CshA and CshB and ScaA, which mediate coaggregation with *Actinomyces naeslundii*, were further

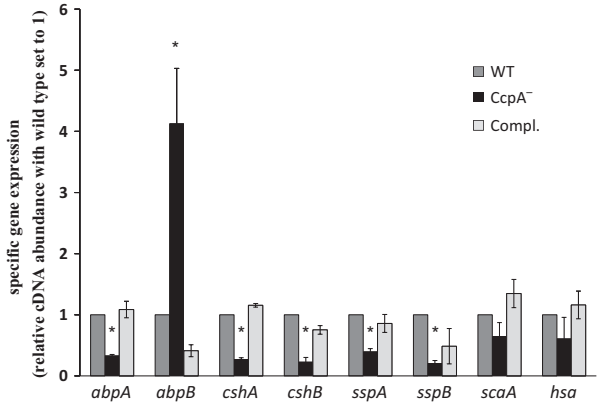

Figure 3 Adhesin gene expression. Expression of adhesin genes in the wild-type, CcpA mutant and complemented strains determined with real-time reverse transcription–polymerase chain reaction. The relative cDNA abundance of the wild-type was arbitrarily assigned a value of 1. Means and standard deviations of three independent experiments are presented. Asterisk indicates statistically significant differential expression of the respective gene between wild-type and mutant ($P < 0.05$).

examined (Zhang *et al.*, 2005; Jakubovics *et al.*, 2009). The *abpA*, *cshA*, *cshB*, *sspA*, *sspB*, *scaA* and *hsa* genes showed reduced expression in the CcpA mutant. The *abpB* gene was an exception, increasing in expression about four-fold when compared with the wild-type (Fig. 3).

Exopolysaccharide production is CcpA dependent

Exopolysaccharide production is important for biofilm formation (Otto, 2008). The ability of *S. gordonii* wild-type, CcpA mutant and complemented strain to produce exopolysaccharides was tested. Detection of exopolysaccharides was accomplished using Congo red BHI plates supplemented with 3% sucrose, a method described for the detection of slime produced by *Staphylococci* (Arciola *et al.*, 2001). An obvious difference in the appearance of the CcpA mutant was visible after 48 h of incubation on the Congo red plate. The CcpA mutant produced white colonies and the agar remained bright red. In contrast, the wild-type and complemented mutant formed grey colonies and the agar turned brownish (Fig. 4A). Higher magnification of the colonies confirmed the difference in the phenotype, showing dark round spots in single colonies of the wild-type and complemented strains, whereas the CcpA mutant remained white and opaque (Fig. 4B).

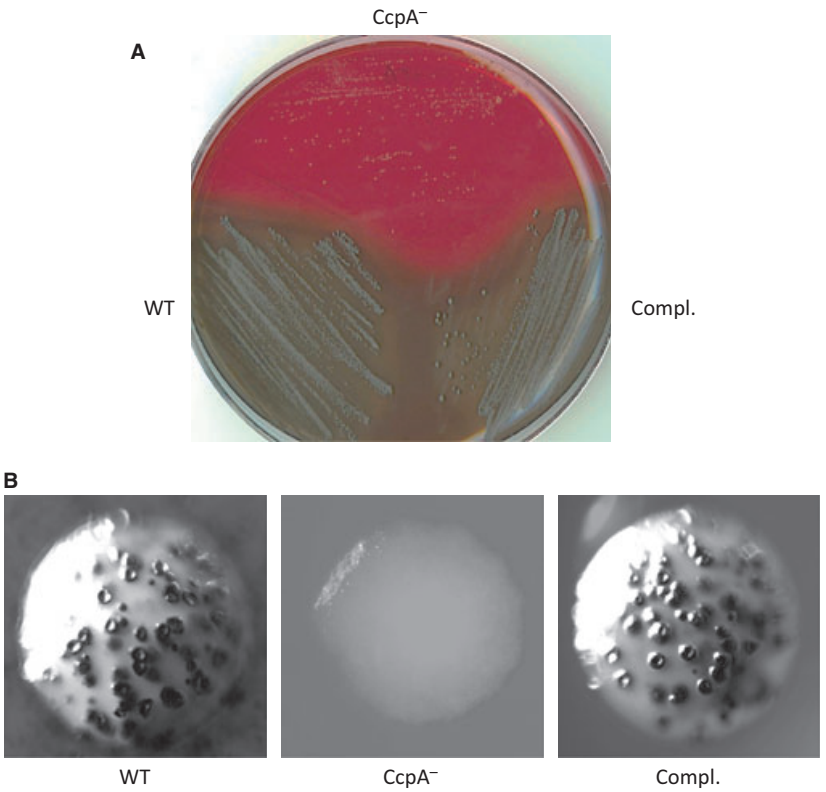

Figure 4 Exopolysaccharide production of wild-type, CcpA mutant and complemented strain. (A) Growth phenotype on Congo red brain–heart infusion (BHI) plate supplemented with 3% sucrose. (B) Appearance of individual colonies on Congo red BHI plates supplemented with 3% sucrose.

CcpA mutant has increased cell numbers in streptococcal chains

Static planktonic cultures of the CcpA mutant resulted in precipitation of the cells, whereas the wild-type and complemented strains remained in suspension (Fig. 5A). The CcpA mutant showed greater chain length and more tangling than the wild-type or complemented strains when viewed by light microscopy (Fig. 5B). The CcpA mutant showed a four-fold greater number of cells per chain, averaging 35 cells in comparison with eight for the wild-type and six for the complemented strain (Fig. 5C).

Impaired competence development in the CcpA mutant

To determine whether mutation of CcpA affects development of competence, the transformation efficiency was tested in the presence of 100 μg ml^{-1} catalase. When grown aerobically, the CcpA mutant produced increased H_2O_2, which slows growth on BHI transformation plates (data not shown). Hence catalase was used to ensure that H_2O_2 did not interfere with the transformation. The transformation efficiencies for the wild-type and the complemented strain were $5 \pm 0.84 \times 10^{-4}$ and $2 \pm 0.14 \times 10^{-4}$, respectively. When compared with the wild-type and the CcpA complemented strain, the CcpA mutant did not yield any transformants in the presence of catalase (Fig. 6A). To test if impaired transformability of the CcpA mutant is caused by a decrease in *com* gene expression, the abundance of *comC*, *comD* and *comYA* transcripts was measured (Fig. 6B). The CcpA mutant showed a nine-fold to 17-fold reduction in the expression of competence genes when compared with the wild-type. The complemented strain showed a two-fold to five-fold increase in *comC*, *comD* and *comYA* expression when compared with the wild-type, most likely as a result of an increased *ccpA* copy number because of its delivery by a multiple-copy shuttle plasmid.

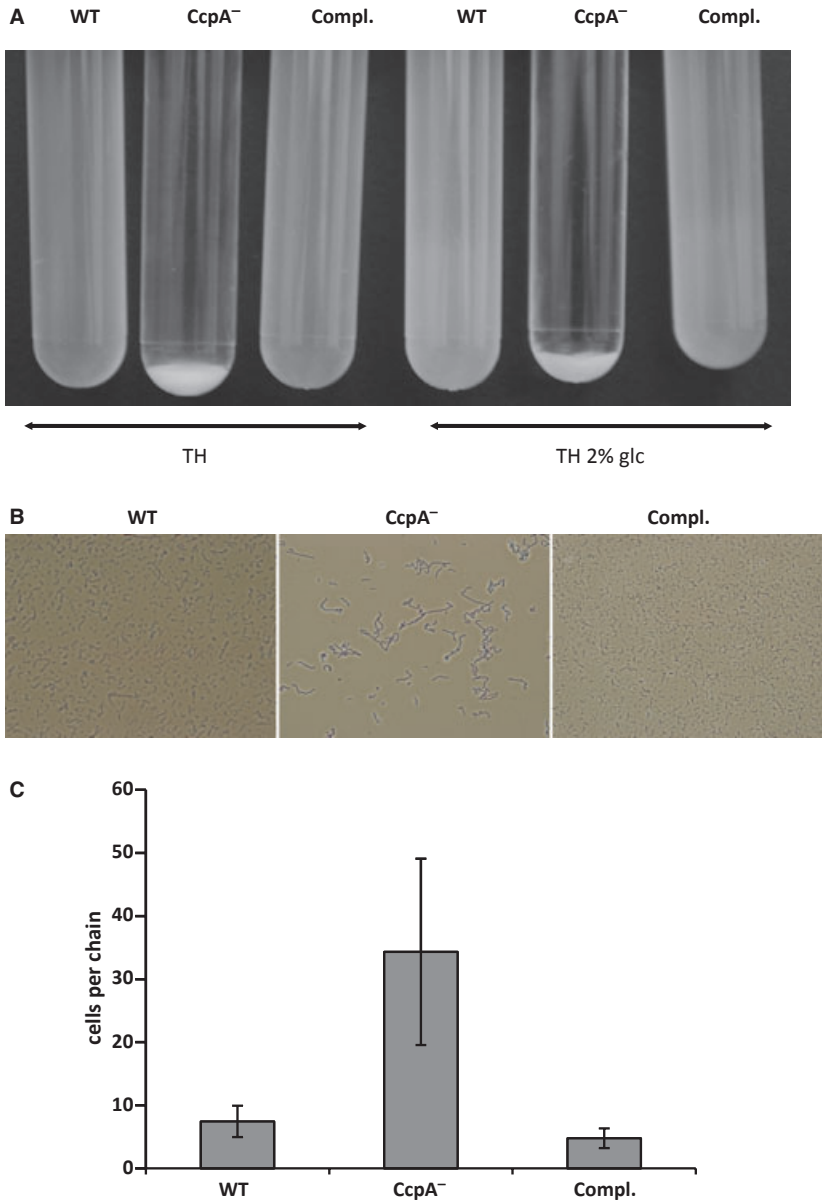

Figure 5 Cell aggregation phenotype of the CcpA mutant. (A) Cells of the wild-type, CcpA mutant and complemented strains were grown overnight as static cultures in Todd–Hewitt (TH) and in TH supplemented with 2% sucrose. (B) Microphotograph of cell chains. (C) Quantification of cells per chain determined by counting the number of individual cells forming a chain in the wild-type, CcpA mutant and complemented strains (n = 30 chains per strain).

DISCUSSION

Biofilm formation provides increased protection against environmental stresses like antibiotics, shear stress and the invasion of competitors into an existing community (Hall-Stoodley & Stoodley, 2009). To maintain biofilm homeostasis, individual members of the biofilm must be able to react to changes in the environment. Here we identified the pleiotropic transcription factor CcpA as being important in regulating biofilm formation in *S. gordonii*. A member of the LacI family of transcriptional regulators, CcpA is well established as global regulator of sugar metabolism and carbon catabolite repression in Gram-positive bacteria (Warner & Lolkema, 2003). During carbon catabolite repression, CcpA represses the metabolic

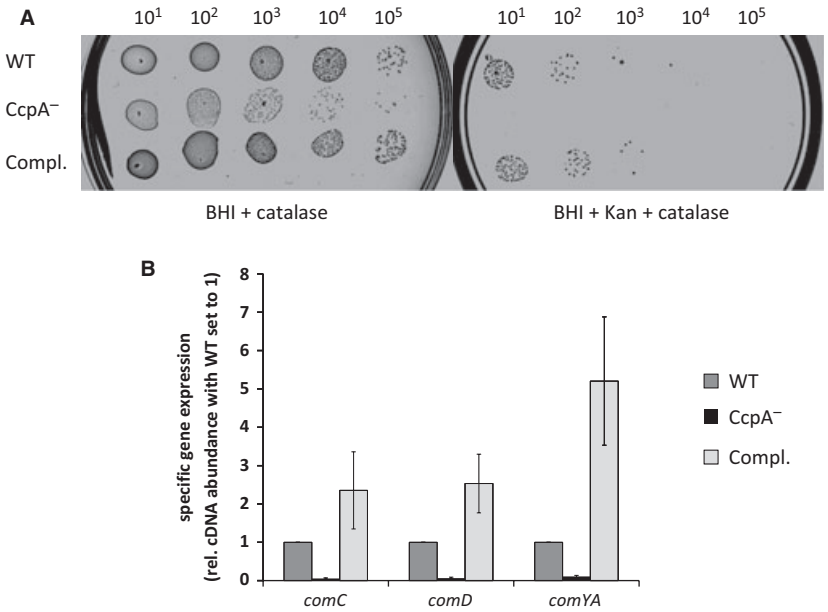

Figure 6 Transformation phenotype and competence gene expression. (A) Serial dilutions of wild-type, CcpA mutant and complemented strains used in a transformation assay with 1 μg chromosomal DNA encoding a kanamycin-resistance cassette. Cells were spotted on brain–heart infusion (BHI) + catalase as control and on BHI + catalase + Kan to demonstrate the transformation phenotype. (B) Expression of competence genes *comC*, *comD* and *comYA* in the wild-type, CcpA mutant and complemented strains determined with real-time reverse transcription–polymerase chain reaction. The relative cDNA abundance of the wild-type was arbitrarily assigned a value of 1. Means and standard deviations of three independent experiments are presented.

pathways that are not required when a preferred carbohydrate source is present in the environment. This ensures that all necessary resources for optimized use of the preferred carbohydrate are available. The activity of CcpA is modulated by the phosphorylated cofactor HPr(Ser46-p). The phosphorylation of HPr(Ser46-P) is catalyzed by the metabolite-controlled bi-functional HPrK/P kinase/phosphorylase, which is dependent on the intracellular concentration of fructose 1,6-bisphosphate (Deutscher *et al.*, 2005; Gorke & Stulke, 2008). CcpA is therefore able to integrate information about the metabolic status of the cell into an appropriate transcriptional response.

We characterized the CcpA-dependent regulation of biofilm development in *S. gordonii* in view of the observed biofilm phenotype. The defect in biofilm formation by the CcpA mutant could have resulted from reduced exopolysaccharide production. Exopolysaccharides are an essential part of the biofilm matrix (Flemming & Wingender, 2010) and failure to produce exopolysaccharides results in a biofilm formation defect in several bacterial species (Yildiz & Schoolnik, 1999; Koo *et al.*, 2010; Yamanaka *et al.*, 2011). We could, however, exclude a defect of GtfG-dependent glucan synthesis as a major reason for the observed phenotype, because a GtfG mutant showed the same phenotype as the wild-type on Congo red BHI plates (data not shown). It is possible, however, that Congo red assay used here does not detect GtfG synthesized glucans or is not sensitive enough. Therefore the possibility exist that important non-glucan exopolysaccharides are impaired in their production, for example Gal/GalNAc-containing cell wall polysaccharides. They are important in interspecies co-aggregation with *Actinomyces* spp., but might also mediate surface attachment (Cisar *et al.*, 1995).

Biofilm formation and natural competence development seem also to be connected in oral streptococci (Li *et al.*, 2001). We observed a decrease in competence development in the CcpA mutant. The *comCDE* operon, which encodes the precursor of the competence-stimulating peptide CSP, and its dedicated two-component system, ComDE are decreased in expression, suggesting that the early steps in competence development are inactive. Subsequently, the late competence gene *comYA*, encoding a component required for DNA uptake, is not transcribed and

the cells are not able to take up extracellular DNA. No CcpA binding site is present in the promoter regulating *comCDE* expression. This suggests that the observed effect is indirect. Possibly CcpA regulates the expression of other regulators involved in the expression control of competence, also indirectly influencing biofilm development.

The initial step in biofilm formation is the adhesion of planktonic cells to the substratum initiated by surface exposed adhesins. We detected a decrease in adhesin gene expression in planktonic cells, which could explain the observed biofilm phenotype because of failure of initial adhesion. The effect of CcpA on adhesin gene expression is most likely indirect. A search for CcpA binding sites in the promoter regions of the respective adhesin genes did not reveal canonical *cre* sites. CcpA and biofilm formation could be linked through seryl-phosphorylated HPr. In the Gram-positive *Listeria monocytogenes*, seryl-phosphorylated HPr negatively regulates a major regulator of biofilm formation PrfA (Zhou *et al.*, 2011). Disruption of CcpA in *L. monocytogenes* also inhibits PrfA activity, possibly through elevated seryl-phosphorylated HPr levels (Deutscher *et al.*, 2005). Future research will further dissect a possible role of seryl-phosphorylated HPr in biofilm formation of *S. gordonii*. The observed growth defect of the CcpA mutant might also play an important role in the observed biofilm phenotype.

CcpA regulates other genes of interest, including the pyruvate-oxidase-encoding *spxB*, the arginine deiminase gene *arcA* and the amylase gene *amyB* (Dong *et al.*, 2004; Johnson *et al.*, 2009). Direct regulation via CcpA binding to the promoter of *arcA* and *spxB* was confirmed *in vitro*. Additionally, the RegPrecise database predicts CcpA binding sites in all three genes (Zeng *et al.*, 2006; Novichkov *et al.*, 2010; Zheng *et al.*, 2011b), suggesting that CcpA directly regulates *amyB*. We did not detect CcpA-mediated repression of the *abpA* gene, encoding the well-characterized amylase binding protein A (Rogers *et al.*, 1998). Under the conditions tested, expression of *abpA* was reduced about 2.5-fold in the CcpA mutant, suggesting that CcpA acts as an activator of *abpB* expression, while Rogers & Scannapieco (2001) reported moderately increased expression in the CcpA mutant. Slight differences in the experimental setup and strain-specific regulation might explain this discrepancy.

The role of CcpA in global gene regulation is well established in *S. mutans* (Abranches *et al.*, 2008), a cariogenic niche competitor of *S. gordonii*. Consistent with a role in stress tolerance in *S. gordonii*, CcpA in *S. mutans* controls traits other than carbohydrate use that are important for ecological fitness, including biofilm formation (Abranches *et al.*, 2008). Yet *S. mutans* does not rely on CcpA as sole regulator of carbon catabolite repression and a second CcpA-independent network exists to regulate gene expression in response to carbohydrate availability. This network is controlled by the seryl-phosphorylated HPr and specific phosphoenolpyruvate-dependent carbohydrate : phosphotransferase system permeases (Zeng & Burne, 2010). Initial studies with *S. gordonii* point to CcpA-independent control of carbon catabolite repression mediated through the $EIIAB^{Man}$ phosphotransferase system permease (Tong *et al.*, 2011).

We sought to understand the advantage for *S. gordonii* to have carbon catabolite repression controlled by an alternative mechanism and fitness or stress-related control shifted towards CcpA. Although *S. gordonii* is exposed to environmental stresses in the oral cavity including saliva flow and pH fluctuations, the bacteria in the oral cavity do not starve for an extended period of time and the oral cavity is not considered an oligotrophic environment (Lemos & Burne, 2008). In fact, *S. gordonii* can grow with saliva (Periasamy & Kolenbrander, 2009) or MUC5B (Wickstrom *et al.*, 2009) as sole nutritional source in a multispecies environment. Cells can adapt to growth with moderate carbohydrate availability by metabolizing glycoproteins in saliva, although an ideal supply of nutrients including easily metabolized carbohydrate sources supplied during food intake would be preferred. In these situations requiring adaptation, the major stressor for the cells might not be the availability of nutrients, but other factors known to challenge the oral biofilm, including sudden pH and temperature shifts and shear stress dislodging cells from the protective biofilm environment (Kreth *et al.*, 2009). If the sudden availability of preferred carbohydrates like glucose, fructose or mannose were to interfere with the ability of *S. gordonii* to form a biofilm, develop competence or respond in general to stress, its ecological competitiveness would be severely impaired. A CcpA-independent carbon catabolite repression mechanism would facilitate metabolic adaptation by *S. gordonii* to the preferred carbohydrate source,

while CcpA-dependent control of stress enables cells to remain competitive in the biofilm. Interestingly, the most abundant streptococci in the early benign biofilm, *Streptococcus sanguinis* (Nobbs *et al.*, 2007b) uses CcpA to control *spxB* in a carbohydrate-independent manner, expressing the pyruvate oxidase responsible for the production of growth inhibiting H_2O_2 not responsive to carbon catabolite repression (Zheng *et al.*, 2011a). Hence, CcpA has additional carbohydrate-independent regulatory functions in oral streptococci. Additional analysis is required to fully understand the regulatory role of CcpA and the $EIIAB^{Man}$ phosphotransferase system permease in CCR and the general stress response.

In conclusion, we demonstrated that the pleiotropic transcriptional regulator CcpA controls major stress and fitness-related phenotypes in *S. gordonii*. Considering that *S. gordonii* is a commensal streptococcus promoting biofilm homeostasis, detailed knowledge of the CcpA regulon could reveal candidate molecular targets affecting stress tolerance of *S. gordonii*. Given that it is an interspecies competitor of cariogenic *S. mutans*, *S. gordonii* and CcpA may present novel targets for caries prevention.

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