Published in final edited form as:

Mol Oral Microbiol. 2012 April ; 27(2): 83–94. doi:10.1111/j.2041-1014.2011.00633.x.

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 oral commensal streptococci, *S. gordonii* is considered beneficial in promoting biofilm homeostasis. CcpA is known as 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 relative 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 (Murphy *et al.*, 2006, Lemos *et al.*, 2005, Monds & O'Toole, 2009, Ahn *et al.*, 2006). Several studies document, for example, that bacteria residing in a biofilm show increased resistance to antibiotics when compared to 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).

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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 (Senadheera *et al.*, 2007, Bhagwat *et al.*, 2001, Li *et al.*, 2002). 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.*, 2002, Li *et al.*, 2001). Competence is also regarded as important part of the streptococcal general stress response since 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 CcpA (Browning *et al.*, 2004, Abranches *et al.*, 2008). CcpA (carbon control protein A) is the major regulator of carbon catabolite repression (CCR) in Gram-positive bacteria and also 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 (Browning *et al.*, 2004, Li *et al.*, 2002, 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. 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* (Jenkinson & Lamont, 2005, Jakubovics *et al.*, 2005, Chung *et al.*, 2000). 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 expression of several adhesin genes (Nobbs *et al.*, 2007a). SrtA mutants of *S. gordonii* and several other species are also impaired in biofilm formation (Guiton *et al.*, 2009, Yamaguchi *et al.*, 2006, Nobbs *et al.*, 2007a) and 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. Since 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

S. gordonii strain used in this study are listed in Table 1 and were routinely grown aerobically (5% CO₂) or in an anaerobic chamber (90% N₂, 5% CO₂, 5% H₂) at 37°C on BHI (Brain Heart Infusion; Difco, Sparks, MD) agar plates except when stated as grown in TYE medium (1% tryptone, 0.5% yeast extract, 0.3% K₂HPO₄) or TH medium (Todd-Hewitt 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., 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 before (Zheng *et al.*, 2011a).

Construction of a $\Delta ccpA$ 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 1h until the culture reached an $A_{600\text{ nm}}$ of 0.1. Cells were aliquoted into 1.5 ml tubes (1ml/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. The cells were further incubated for 2h, followed by a brief sonication to break-up cell chains and 10 times serial diluted in BHI. 10 μl of each sample was plated on kanamycin-containing BHI agar plates as well as on nonselective BHI plates. Transformation efficiency was calculated as the number of kanamycin-resistant CFUs relative to the number of CFUs on non-selective Todd-Hewitt agar.

Biofilm formation and quantification

Biofilm formation was measured using a modification of the crystal violet (CV) microtiter assay as reported (Ashby *et al.*, 2009). Briefly, microtitre 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 medium 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 of residual medium and cells. Cells were stained with 150 μl per well crystal violet (CV, 2.3% w/v; Accustain Crystal Violet Solution, Sigma Diagnostics; St. Louis, MO) for 15 min. The microtitre 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, Biorad; Hercules, CA). Collagen coated microtitre plates (BD BioCoat Cellware, Collagen Type IV) were from BD Biosciences.

RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated using the QIAGEN RNeasy kit, cDNA was synthesized using the SuperScript II™ Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative Real-Time RT-PCR (qRT-PCR) was performed to detect specific transcripts with the comparative threshold cycle (CT) method using the Bio-Rad MyiQ Cycler (Hercules, CA). Relative changes in gene expression were calculated using the ΔCT 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. 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 BHI with 2% glc. A similar growth defect was observed for the CcpA mutant when grown in TYE with 2% glc, but not in TYE with 0.2% glc (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 strain. 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. 2A). Supplementing BHI with 2% sucrose increased the attached biofilm mass (Fig. 2B), while no difference was observed for TH and TYE plus 2% scr.

Since collagen can serve as an attachment substrate during biofilm formation in some *in vivo* conditions (Abranches *et al.*, 2011), 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 expression of surface adhesin genes, wild type, CcpA mutant and the complemented strain were grown in TH medium and compared for adhesin gene expression. The major cell surface adhesins amylase binding protein AbpA and AbpB, the sialic acid binding protein Hsa, the antigen I/II family adhesin protein SspA and SspB, the fibronectin binding protein CshA and CshB as well as ScaA, mediating coaggregation with *Actinomyces naeslundii* were further 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 to 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 48h incubation on the Congo red plate. The CcpA mutant produced white colonies and the agar remained bright red. Contrary, 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 types and complemented strain, while the CcpA mutant remained white and opaque (Fig. 4B).

CcpA mutant has increased cell numbers in streptococcal chains

Static planktonic cultures of the CcpA mutant as resulted in precipitation of the cells, while the wild type and complemented strain 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 to 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 catalase. When grown aerobically, the CcpA mutant produced increased H₂O₂, which slows growth on BHI transformation plates (data not shown). Hence catalase was used to ensure that H₂O₂ 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 to 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 9 to 17-fold reduction in the expression of competence genes when compared to the wild-type. The complemented strain showed a two- to five-fold increase in *comC*, *comD* and *comYA* expression when compared to the wild-type, most likely as a result of an increased *ccpA* copy number due to its delivery by a multiple-copy shuttle plasmid.

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 to be 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 (CCR) in Gram-positive bacteria (Warner & Lolkema, 2003). During CCR, CcpA represses non-required metabolic pathways when a preferred carbohydrate source is present in the environment. This ensures that all necessary resources for optimized utilization 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 (Gorke & Stulke, 2008, Deutscher *et al.*, 2005). 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 (Koo *et al.*, 2010, Yamanaka *et al.*, 2011, Yildiz & Schoolnik, 1999). We could, however, exclude a defect of GtfG-dependent glucan synthesis as a major reason for the observed phenotype, since a GtfG mutant showed the same phenotype as the wild-type on Congo red BHI plates (data not shown). It is possible, however, that the here used Congo red assay is not detecting 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 to be connected as well 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 the 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 due to 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, Zheng *et al.*, 2011b, Novichkov *et al.*, 2010), 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 tested condition, expression of *abpA* was reduced about 2.5-fold in the CcpA mutant suggesting CcpA acts an activator of *abpB* expression, while Rogers and Scannapieco reported moderately increased expression in the CcpA mutant (Rogers & Scannapieco, 2001). 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 utilization important for ecological fitness, including biofilm formation (Abranches *et al.*, 2008). Yet *S. mutans* does not rely on CcpA as sole regulator of CCR 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 (PTS) permeases (Zeng & Burne, 2010). Initial studies with *S. gordonii* point to CcpA-independent control of CCR mediated through the EIIAB^{Man} PTS permease (Tong *et al.*, 2011).

We sought to understand the advantage for *S. gordonii* to have CCR controlled by an alternative mechanism and shift fitness or stress-related control 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 easy metabolizable carbohydrate sources 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 factor known to challenge the oral biofilm, including sudden pH and temperature shifts and sheer-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 CCR 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, *S. 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 H₂O₂ not responsive to CCR (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 streptococci 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.

Acknowledgments

Support through NIH/NIDCR grant 4R00DE018400 to J.K. and R01DE08590 to M.C.H. is gratefully acknowledged.

References

- Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* 2005; 43:5721–5732. [PubMed: 16272510]
- Abranches J, Miller JH, Martinez AR, Simpson-Haidaris PJ, Burne RA, Lemos JA. The collagen-binding protein Cnm is required for *Streptococcus mutans* adherence to and intracellular invasion of human coronary artery endothelial cells. *Infect Immun.* 2011; 79:2277–2284. [PubMed: 21422186]
- Abranches J, Nascimento MM, Zeng L, Browngardt CM, Wen ZT, Rivera MF, Burne RA. CcpA regulates central metabolism and virulence gene expression in *Streptococcus mutans*. *J Bacteriol.* 2008; 190:2340–2349. [PubMed: 18223086]
- Ahn SJ, Wen ZT, Burne RA. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. *Infect Immun.* 2006; 74:1631–1642. [PubMed: 16495534]
- Arciola CR, Campoccia D, Borrelli AM, Donati ME, Montanaro L. Congo red agar plate method: improved accuracy and new extended application to *Staphylococcus aureus*. *New Microbiol.* 2001; 24:355–363. [PubMed: 11718373]
- Ashby MT, Kreth J, Soundarajan M, Sivuili LS. Influence of a model human defensive peroxidase system on oral streptococcal antagonism. *Microbiology.* 2009; 155:3691–3700. [PubMed: 19684069]
- Bhagwat SP, Nary J, Burne RA. Effects of mutating putative two-component systems on biofilm formation by *Streptococcus mutans* UA159. *FEMS Microbiol Lett.* 2001; 205:225–230. [PubMed: 11750807]
- Browngardt CM, Wen ZT, Burne RA. RegM is required for optimal fructosyltransferase and glucosyltransferase gene expression in *Streptococcus mutans*. *FEMS Microbiol Lett.* 2004; 240:75–79. [PubMed: 15500982]
- Chung WO, Demuth DR, Lamont RJ. Identification of a *Porphyromonas gingivalis* receptor for the *Streptococcus gordonii* SspB protein. *Infect Immun.* 2000; 68:6758–6762. [PubMed: 11083792]
- Cisar JO, Sandberg AL, Abeygunawardana C, Reddy GP, Bush CA. Lectin recognition of host-like saccharide motifs in streptococcal cell wall polysaccharides. *Glycobiology.* 1995; 5:655–662. [PubMed: 8608267]
- Deutscher J, Herro R, Bourand A, Mijakovic I, Poncet S. P-Ser-HPr--a link between carbon metabolism and the virulence of some pathogenic bacteria. *Biochim Biophys Acta.* 2005; 1754:118–125. [PubMed: 16182622]
- Dong Y, Chen YY, Burne RA. Control of expression of the arginine deiminase operon of *Streptococcus gordonii* by CcpA and Flp. *J Bacteriol.* 2004; 186:2511–2514. [PubMed: 15060059]
- Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* 2010; 8:623–633. [PubMed: 20676145]
- Gorke B, Stulke J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol.* 2008; 6:613–624. [PubMed: 18628769]
- Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ. Contribution of autolysin and Sortase a during *Enterococcus faecalis* DNA-dependent biofilm development. *Infect Immun.* 2009; 77:3626–3638. [PubMed: 19528211]
- Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. *Cell Microbiol.* 2009; 11:1034–1043. [PubMed: 19374653]
- Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents.* 2010; 35:322–332. [PubMed: 20149602]
- Jakubovics NS, Brittan JL, Dutton LC, Jenkinson HF. Multiple adhesin proteins on the cell surface of *Streptococcus gordonii* are involved in adhesion to human fibronectin. *Microbiology.* 2009; 155:3572–3580. [PubMed: 19661180]

- Jakubovics NS, Kerrigan SW, Nobbs AH, Stromberg N, van Dolleweerd CJ, Cox DM, Kelly CG, Jenkinson HF. Functions of cell surface-anchored antigen I/II family and Hsa polypeptides in interactions of *Streptococcus gordonii* with host receptors. *Infect Immun*. 2005; 73:6629–6638. [PubMed: 16177339]
- Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. *Trends Microbiol*. 2005; 13:589–595. [PubMed: 16214341]
- Johnson BP, Jensen BJ, Ransom EM, Heinemann KA, Vannatta KM, Eglund KA, Eglund PG. Interspecies signaling between *Veillonella atypica* and *Streptococcus gordonii* requires the transcription factor CcpA. *J Bacteriol*. 2009; 191:5563–5565. [PubMed: 19542285]
- Kolenbrander PE, Palmer RJ Jr, Rickard AH, Jakubovics NS, Chalmers NI, Diaz PI. Bacterial interactions and successions during plaque development. *Periodontol 2000*. 2006; 42:47–79. [PubMed: 16930306]
- Koo H, Xiao J, Klein MI, Jeon JG. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol*. 2010; 192:3024–3032. [PubMed: 20233920]
- Kreth J, Hagerman E, Tam K, Merritt J, Wong DT, Wu BM, Myung NV, Shi W, Qi F. Quantitative analyses of *Streptococcus mutans* biofilms with quartz crystal microbalance, microjet impingement and confocal microscopy. *Biofilms*. 2004; 1:277–284. [PubMed: 16429589]
- Kreth J, Merritt J, Qi F. Bacterial and host interactions of oral streptococci. *DNA Cell Biol*. 2009; 28:397–403. [PubMed: 19435424]
- Lemos JA, Abranches J, Burne RA. Responses of cariogenic streptococci to environmental stresses. *Curr Issues Mol Biol*. 2005; 7:95–107. [PubMed: 15580782]
- Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiology*. 2008; 154:3247–3255. [PubMed: 18957579]
- Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J Bacteriol*. 2001; 183:897–908. [PubMed: 11208787]
- Li YH, Lau PC, Tang N, Svensater G, Ellen RP, Cvitkovitch DG. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J Bacteriol*. 2002; 184:6333–6342. [PubMed: 12399503]
- Monds RD, O'Toole GA. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol*. 2009; 17:73–87. [PubMed: 19162483]
- Murphy C, Carroll C, Jordan KN. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J Appl Microbiol*. 2006; 100:623–632. [PubMed: 16553716]
- Nobbs AH, Lamont RJ, Jenkinson HF. Streptococcus adherence and colonization. *Microbiol Mol Biol Rev*. 2009; 73:407–450. Table of Contents. [PubMed: 19721085]
- Nobbs AH, Vajna RM, Johnson JR, Zhang Y, Erlandsen SL, Oli MW, Kreth J, Brady LJ, Herzberg MC. Consequences of a sortase A mutation in *Streptococcus gordonii*. *Microbiology*. 2007a; 153:4088–4097. [PubMed: 18048922]
- Nobbs AH, Zhang Y, Khammanivong A, Herzberg MC. *Streptococcus gordonii* Hsa environmentally constrains competitive binding by *Streptococcus sanguinis* to saliva-coated hydroxyapatite. *J Bacteriol*. 2007b; 189:3106–3114. [PubMed: 17277052]
- Novichkov PS, Laikova ON, Novichkova ES, Gelfand MS, Arkin AP, Dubchak I, Rodionov DA. RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes. *Nucleic Acids Res*. 2010; 38:D111–118. [PubMed: 19884135]
- Otto M. Staphylococcal biofilms. *Curr Top Microbiol Immunol*. 2008; 322:207–228. [PubMed: 18453278]
- Periasamy S, Kolenbrander PE. Mutualistic biofilm communities develop with *Porphyromonas gingivalis* and initial, early, and late colonizers of enamel. *J Bacteriol*. 2009; 191:6804–6811. [PubMed: 19749049]
- Rogers JD, Haase EM, Brown AE, Douglas CW, Gwynn JP, Scannapieco FA. Identification and analysis of a gene (*abpA*) encoding a major amylase-binding protein in *Streptococcus gordonii*. *Microbiology*. 1998; 144(Pt 5):1223–1233. [PubMed: 9611797]

- Rogers JD, Scannapieco FA. RegG, a CcpA homolog, participates in regulation of amylase-binding protein A gene (*abpA*) expression in *Streptococcus gordonii*. *J Bacteriol.* 2001; 183:3521–3525. [PubMed: 11344161]
- Schachtele, CF.; Nobbs, A.; Zhang, Y.; Costalonga, M.; Herzberg, MC. Oral Streptococci: commensals and opportunistic pathogens. In: Hakenbeck, R.; Chhatwal, S., editors. *The molecular biology of Streptococci*. Horizon Scientific Press; Norfolk, UK: 2007. p. 411-462.
- Senadheera MD, Lee AW, Hung DC, Spatafora GA, Goodman SD, Cvitkovitch DG. The *Streptococcus mutans* *vicX* gene product modulates *gffB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. *J Bacteriol.* 2007; 189:1451–1458. [PubMed: 17114248]
- Suntharalingam P, Cvitkovitch DG. Quorum sensing in streptococcal biofilm formation. *Trends Microbiol.* 2005; 13:3–6. [PubMed: 15639624]
- Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, Engebretsen IL, Bayles KW, Horswill AR, Kielian T. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. *J Immunol.* 2011; 186:6585–6596. [PubMed: 21525381]
- Tong H, Zeng L, Burne RA. The EIIABMan phosphotransferase system permease regulates carbohydrate catabolite repression in *Streptococcus gordonii*. *Appl Environ Microbiol.* 2011; 77:1957–1965. [PubMed: 21239541]
- Warner JB, Lolkema JS. CcpA-dependent carbon catabolite repression in bacteria. *Microbiol Mol Biol Rev.* 2003; 67:475–490. [PubMed: 14665673]
- Wickstrom C, Herzberg MC, Beighton D, Svensater G. Proteolytic degradation of human salivary MUC5B by dental biofilms. *Microbiology.* 2009; 155:2866–2872. [PubMed: 19556293]
- Yamaguchi M, Terao Y, Ogawa T, Takahashi T, Hamada S, Kawabata S. Role of *Streptococcus sanguinis* sortase A in bacterial colonization. *Microbes Infect.* 2006; 8:2791–2796. [PubMed: 17045503]
- Yamanaka T, Yamane K, Furukawa T, Matsumoto-Mashimo C, Sugimori C, Nambu T, Obata N, Walker CB, Leung KP, Fukushima H. Comparison of the virulence of exopolysaccharide-producing *Prevotella intermedia* to exopolysaccharide non-producing periodontopathic organisms. *BMC Infect Dis.* 2011; 11:228. [PubMed: 21864411]
- Yildiz FH, Schoolnik GK. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc Natl Acad Sci U S A.* 1999; 96:4028–4033. [PubMed: 10097157]
- Zeng L, Burne RA. Seryl-phosphorylated HPr Regulates CcpA-Independent Carbon Catabolite Repression in Conjunction with PTS Permeases in *Streptococcus mutans*. *Mol Microbiol.* 2010
- Zeng L, Dong Y, Burne RA. Characterization of cis-acting sites controlling arginine deiminase gene expression in *Streptococcus gordonii*. *J Bacteriol.* 2006; 188:941–949. [PubMed: 16428398]
- Zhang Y, Lei Y, Khammanivong A, Herzberg MC. Identification of a novel two-component system in *Streptococcus gordonii* V288 involved in biofilm formation. *Infect Immun.* 2004; 72:3489–3494. [PubMed: 15155656]
- Zhang Y, Lei Y, Nobbs A, Khammanivong A, Herzberg MC. Inactivation of *Streptococcus gordonii* SspAB alters expression of multiple adhesin genes. *Infect Immun.* 2005; 73:3351–3357. [PubMed: 15908361]
- Zheng L, Chen Z, Itzek A, Ashby M, Kreth J. Catabolite control protein A controls hydrogen peroxide production and cell death in *Streptococcus sanguinis*. *J Bacteriol.* 2011a; 193:516–526. [PubMed: 21036992]
- Zheng L, Itzek A, Chen Z, Kreth J. Environmental influences on competitive hydrogen peroxide production in *Streptococcus gordonii*. *Appl Environ Microbiol.* 2011b; 77:4318–4328. [PubMed: 21571883]
- Zhou Q, Feng F, Wang L, Feng X, Yin X, Luo Q. Virulence Regulator PrfA is Essential for Biofilm Formation in *Listeria monocytogenes* but not in *Listeria innocua*. *Curr Microbiol.* 2011; 63:186–192. [PubMed: 21656247]

Fig. 1. Growth curves of *S. gordonii* wild type strain DL1 (squares), CcpA mutant (circles) and complemented strain (triangle) in BHI and BHI supplemented with 2% glucose. Presented are means and standard deviations of three independent experiments.

Fig. 2. Biofilm production of wild type, CcpA mutant and complemented strain on polystyrene microtiter plates. (a) Representative of microtiter plate wells from each experiment showing the respective biofilm formation of each *S. gordonii* strain. (b) Quantitative analysis of biofilm production measuring the absorption of destained biofilms at 570 nm in a crystal violet biofilm assay. Presented are means and standard deviations of three independent experiments. * indicates statistical significant difference in biofilm formation of the mutant when compared to the wild type under the respective test condition ($p < 0.05$).

Fig. 3.

Adhesin gene expression. Expression of adhesin genes in the wild type, CcpA mutant and complemented strain determined with Real-Time RT PCR. The relative cDNA abundance of the wild type was arbitrarily assigned a value of 1. Presented are means and standard deviations of three independent experiments. * indicates statistical significant differential expression of the respective gene between wild-type and mutant ($p < 0.05$).

Fig. 4. Exopolysaccharide production of wild type, CcpA mutant and complemented strain. (a) Growth phenotype on Congo red BHI plate supplemented with 3% sucrose. (b) Appearance of individual colonies on Congo red BHI plates supplemented with 3% sucrose.

Fig. 5. Cell aggregation phenotype of the CcpA mutant. (a) Cells of the wild type, CcpA mutant and complemented strain were grown overnight as static cultures in TH and 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 strain ($n = 30$ chains per strain).

Fig. 6. Transformation phenotype and competence gene expression. (a) Serial dilutions of wild type, CcpA mutant and complemented strain used in a transformation assay with 1 μ g chromosomal DNA encoding a kanamycin resistance cassette. Cells were spotted on 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 strain determined with Real-Time RT PCR. The relative cDNA abundance of the wild type was arbitrarily assigned a value of 1. Presented are means and standard deviations of three independent experiments.

Tab.1

Strains, plasmids and primers used in this study

Strain	Relevant characteristics	Reference
DL1	Wild-type <i>S. gordonii</i>	(Zheng <i>et al.</i> , 2011b)
DL1 <i>ccpA</i>	<i>ccpA</i> ; <i>Erm</i> ^r	
DL1 compl	DL1 <i>ccpA</i> ; <i>ccpA</i> complemented; <i>Spc</i> ^r	
Plasmids	Relevant characteristics	Reference
pDL278+ <i>ccpA</i> _{compl}	<i>ccpA</i> cloned into pDL278; <i>Spc</i> ^r	(Zheng <i>et al.</i> , 2011b)
Primers	Sequence	Reference
sspA-F	5'-TCCTGACAAACCTGAGACACC-3'	(Zhang <i>et al.</i> , 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'-CAAAACTCCGGAAAAACCA-3'	
abpB-R	5'-GGAGCTTGACTCGGTTCTTG-3'	
hsa-F	5'-CAGAGCTGCAAATCCAAACA-3'	
hsa-R	5'-GCCGAGATACTTGCGCTTAC-3'	
cshB-F	5'-CGTTGTTTCAGCAAGGATCAA-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'-TGAAAAAGAAAAACAAACAAATCT-3'	
comD-F	5'-TGTTACGAGCAGACTTCAGA-3'	
comD-R	5'-TCATTGGTTTCAGCGAAAATG-3'	
comYA-F	5'-GAAACTGCTAGAGCGGTGGT-3'	
comYA-R	5'-TCCTCGCTAACACCCAATTC-3'	