Institute of Medical Microbiology and Hygiene
University Hospital Ulm
Head of Institute: Prof. Dr. Steffen Stenger



Cre-recombinase based genetic manipulation and the regulation of hemolysis in *Streptococcus anginosus*

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Richard Bauer
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Current dean:

Prof. Dr. Thomas Wirth

Thesis Advisory Committee:

- First supervisor: Prof. Dr. Barbara Spellerberg

- Second supervisor: Prof. Dr. Christian Sinzger

- Third supervisor: Dr. Shaynoor Dramsi

External Reviewer:

Prof. Dr. Marcus Fulde

Prof. Dr. Holger Rohde

Day doctorate awarded:

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Abbreviations

aa	Amino acid
ABC	ATP binding cassette
Amp^R	Ampicillin resistance
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
bp	Base pair
CcpA	Catabolite control protein A
CCR	Carbon catabolite repression
CF	Cystic fibrosis
cfb	CAMP-factor gene
CFU	Colony Forming Unit
ClpP	ATP-dependent Clp protease proteolytic subunit
Cm^R	Chloramphenicol resistance
com	competence
ComX	Competence-specific sigma factor
CovR	Control of virulence Regulator
CPS	Capsular polysaccharide
cre	Catabolite responsive element
C-source	Carbon-source
CSP	Competence stimulating peptide
DTT	Dithiothreitol
EI	Enzyme I
EII	Enzyme II
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid

EGFP	Enhanced Green Fluorescent Protein
EMSA	Electrophoretic mobility shift assay
Ery^R	Erythromycin resistance
FBP	Fructose-1,6-bisphosphate
FBS	Fetal bovine serum
G6P	Glucose-6-phosphate
Gal	Galactose
GAS	Group A streptococcus (*S. pyogenes*)
GBS	Group B streptococcus
genomosubsp.	genomosubspecies
Glc	Glucose
gnd	6-phosphogluconate dehydrogenase gene
Hly+	Hemolysis positive
His	Histidine
HGT	Horizontal gene transfer
HPr	Histidine-containing Protein
HPr-His-P	Histidine phosphorylated Histidine-containing Protein
HPrK	HPr kinase/phosphorylase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth
Lmb	Laminin-binding protein
L. monocytogenes	*Listeria monocytogenes*
lox	Locus of crossing-over
M	Molecular weight marker
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MEP	Mobile Element Protein
MFI	Mean fluorescence intensity

MLSA	Multilocus sequence analysis
NMR	Nuclear magnetic resonance
OD	Optical density
OE-PCR	Overlap extension PCR
ori	Origin of replication
PCR	Polymerase chain reaction
PBS	Phosphate-Buffered Saline
PEG	Polyethylene glycol
PMNLs	Polymorphonuclear leukocytes
poly(dI-dC)	Poly(deoxyinosinic-deoxycytidylic) acid
PRD	PTS-regulatory domain
PTS	Phosphoenolpyruvate-carbohydrate phosphotransferase system
rec	Recombinase
RitR	Repressor of iron transport
RT	Reverse transcriptase
SAG	*Streptococcus anginosus* group
sag	Streptolysin S associated gene
S. anginosus	*Streptococcus anginosus*
S. aureus	*Staphylococcus aureus*
SB	Sheep blood
S. constellatus	*Streptococcus constellatus*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. dysgalactiae	*Streptococcus dysgalactiae*
S. intermedius	*Streptococcus intermedius*
SLS	Streptolysin S
S. milleri	*Streptococcus milleri*
S. mitis	*Streptococcus mitis*

S. mutans	*Streptococcus mutans*
S. oralis	*Streptococcus oralis*
spc	Spectinomycin resistance gene
Spc^R	Spectinomycin resistance
spe	Streptococcal pyrogenic exotoxin genes
speC	Streptococcal pyrogenic exotoxin C gene
speG	Streptococcal pyrogenic exotoxin G gene
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes* (GAS)
S. sanguinis	*Streptococcus sanguinis*
subsp.	subspecies
TBE	TRIS borate-EDTA Buffer
TCS	Two-component system
THY	Todd Hewitt Broth with 5% yeast extract
TOMM	Thiazole/oxazole-modified microcins
tufA	Translation elongation factor EF-Tu 1 gene
WT	Wild type
XIP	SigmaX inducing peptide

1 Introduction

Streptococcus anginosus belongs to the *Streptococcus anginosus* group (SAG) that also includes the closely related species *Streptococcus constellatus* and *Streptococcus intermedius*. The bacteria of this group share some characteristics, including a small colony phenotype on blood agar plates (< 0.5 mm) and a specific butterscotch-like smell [30], but they show an inconsistent hemolysis behavior with all three hemolytic phenotypes (α-, β- and γ-hemolysis) being present in different strains. Nevertheless, the SAG was assigned to the viridans group of streptococci [49]. Bacteria of the SAG can be isolated as commensals of mucosal membranes in the oral cavity, the urogenital tract and the gastrointestinal tract [213]. However, difficulties to culture and identify the SAG correctly lead to an underestimation of the pathogenic potential of these bacteria in older investigations [163,174]. Nowadays, an increasing number of epidemiological data highlight the clinical importance of these bacteria and they show that the SAG species have to be considered as etiological agents of a variety of different infections [101,157,163].

1.1 Phylogeny of the *Streptococcus anginosus* group

The species in the SAG passed through several taxonomic changes. They were previously denominated as *Streptococcus MG-intermedius* and *Streptococcus anginosus-constellatus* [50] or as the *'Streptococcus milleri'* group, although this designation was not included in the approved lists of bacterial names [176]. Additionally it was proposed that the whole group constitutes a single species [36,205]. Meanwhile, the SAG was demonstrated to consist of three distinct species (*S. anginosus*, *S. constellatus*, *S. intermedius*) which is the currently accepted taxonomic status [22,91,212,214]. Combining multilocus sequence analysis (MLSA), the sequence of the 16S rRNA gene and phenotypic traits further demonstrated the presence of seven distinct clusters within the SAG (*S. intermedius*, *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharyngis*, *S. constellatus* subsp. *viborgensis*, *S. anginosus* subsp. *anginosus*, *S. anginosus* subsp. *whileyi*, *S.*

anginosus genomosubsp. *AJ1*) [70,91,214]. While the first complete genome sequences of SAG members published in 2013 substantiate the presence of these clusters in the SAG [141], the MLSA of clinical *S. anginosus* isolates collected in Vellore (India) revealed an additional cluster in *S. anginosus* which was proposed to be denominated as *S. anginosus* genomosubsp. *vellorensis* and contains the *S. anginosus* type strain SK52 [14]. This demonstrates that the taxonomy of the SAG will most likely encounter further changes and the current view of the taxonomic status needs continuing verification which will be facilitated by the increasing amounts of genome data that can be generated by next-generation sequencing techniques.

1.2 Clinical importance of the *Streptococcus anginosus* group

SAG species can frequently be isolated from invasive infections, including sepsis and abscesses of the central nervous system as well as the gastrointestinal tract [101,104,163]. Besides, members of the SAG are reported as emerging pathogens in cystic fibrosis (CF) patients [70,147].

In a population-based laboratory surveillance study for invasive pyogenic streptococcal infections the incidence rate of SAG infections (8.65/100,000 population) exceeded the combined incidence rate of group A and B streptococcal infections (7.40/100,000 population) [104]. Besides, Siegman-Igra et al. reported an annual incidence of 8.8/10,000 hospital admissions due to SAG infections in a tertiary care university hospital in Israel, highlighting the clinical relevance of the group [175]. In a retrospective cohort study the pyogenic potential of the SAG was further pointed out. Among 263 patients suffering from SAG infections, 60 % were classified as pyogenic, defined as an abscess, empyema or deep space surgical site infection. It was thereby reported that *S. intermedius* was more often associated with pyogenic infections than the rest of the SAG bacteria [33,101]. Patients with pyogenic infections showed lower frequencies of bacteremia (8 %) than patients without pyogenic infections (68 %) and a population based surveillance of SAG blood stream infections reported an annual incidence of 3.7/100,000 population [105]. The SAG are thereby also able to cause infective endocarditis and they were reported to be responsible for 9 % of endocarditis cases caused by streptococci [98]. Studies investigating the

etiologic agent of invasive infections caused by β-hemolytic group C and G streptococci further revealed that 17-19 % were due to SAG infections [27,157]. It has to be assumed that these percentages underestimate the SAG presence as these studies focused on β-hemolytic isolates and the majority of SAG strains is non-β-hemolytic [212].

An association of the different species of the SAG with their clinical manifestations has been proposed by some authors. *S. anginosus* showed a higher prevalence in blood cultures, whereas *S. constellatus* and *S. intermedius* were the dominant species in pyogenic infections [33,101,213]. However, these reports are still controversial and the results could not be verified by similar studies [94,175].

The antimicrobial resistance profile of SAG isolates is still not a major concern in clinical therapies [17,65,110,140,224]. But it has to be highlighted that the first vancomycin resistant *S. anginosus* isolate was described in 2014 [181] emphasizing further surveillance of the antibiotic resistance development in the SAG.

1.3 Phenotypic characteristics and identification of the *Streptococcus anginosus* group

Beside the above mentioned common characteristics (small colony size and specific odor), members of the SAG show an inconsistent hemolysis behavior and express a variety of different Lancefield antigens [49,212]. The Lancefield antigens A, C, G and F as well as nongroupable isolates are present in SAG. These antigens can also be expressed in other streptococci and although the F-antigen was proposed to be limited to SAG in the past, the detection of *Streptococcus sinensis* interacting with F-antiserum finally demonstrated that the Lancefield typing is not really helpful for the identification of SAG [26,221]. Phenotypical traits and several biochemical assays have been proposed to identify the SAG to the species level and these characteristics were implemented into commercial identification tests [15,187,212,214]. The majority of these assays provide reliable results for the identification of the SAG to the species level although some false-negative results have been reported [9,61,71]. The developments in microbiology diagnostics further aimed to accelerate species identification by direct bacterial profiling. The widely used matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) thereby provides an accurate and rapid identification of a range of bacteria and fungi but the results of this method concerning the identification of the SAG is still controversial. Friedrichs et al. reported that MALDI-TOF MS is a reliable method for the identification of viridans streptococci including SAG [58]. However, it was also demonstrated that only 22 % of *S. intermedius* isolates were identified to the species level and the discrimination of the SAG to the subspecies level is still a major obstacle [9,222], although it has to be highlighted that the identification to the subspecies level is actually of minor interest for diagnostic issues. For scientific purposes the correct subspecies designation could emphasize the scientific statement and thus the use of MLSA and whole genome sequencing approaches should be applied as gold standard as these methods currently allow the best discrimination of the SAG to the subspecies and even to the genomosubspecies level [14,91,141].

1.4 Virulence related factors in the *Streptococcus anginosus* group

The manifestation of a disease relies on virulence factors that operate in multiple events such as colonization, internalization and invasion of host tissues as well as dissemination and evasion of the host defense. But it has to be kept in mind that described virulence traits often include proteins known to be involved in metabolism or housekeeping functions [146]. Owing to the underestimation of the pathogenic potential of the members of the SAG in the past, the knowledge about virulence factors in these species is scarce [11] and studies so far have mainly focused on specific virulence traits.

Adhesion

The prerequisite for the colonization of the host is the adhesion of the bacterium to biological material. As a member of the oral microbiome, *S. anginosus* was investigated for its potential to adhere to the tooth surface and to tooth-restorative materials [216,223]. The molecules conferring the binding to these materials were not described, but it was demonstrated that the mode of adhesion differed between

Streptococcus mutans and *S. anginosus* as inhibitors of *S. mutans* adsorption did not interfere with the binding of *S. anginosus* to saliva-coated hydroxyapatite beads [193]. The binding of bacteria to extracellular matrix proteins in general is not only crucial for the colonization of a host, but also important for invasion and manifestation of a clinical infection. In this context, *S. anginosus* was reported to be able to bind to fibronectin, fibrinogen and laminin [4]. Clinical SAG isolates showed thereby a greater fibronectin binding capability than other SAG strains and the incubation of SAG strains with fibronectin increased the ability of the bacterium to bind to hydroxyapatite [216,217].

In a whole genome analysis of SAG isolates numerous potential adhesins were identified [141]. Several of these proteins contain a cell surface anchor motif (LPxTG) and collagen-binding domains although collagen binding of *S. anginosus* was demonstrated to be only minor [4]. Additional adhesion proteins, including a fibronectin binding protein (Fbp54), a laminin binding protein (Lmb), an Internalin A homolog, a glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), the pullulanase PulA, the pneumococcal surface adhesin A (PsaA) as well as an enolase, were predicted with the genome analysis that could explain the genetic basis of the binding of SAG to extracellular matrix proteins [141]. All of these predicted adhesins in SAG are functionally characterized in different bacteria, but they were not studied in the SAG at the molecular level so far.

Invasion and evasion of host defense

Concordantly, factors involved in invasion or evasion from the host defense are poorly investigated and only a few *in vivo* analyses have been performed.

SAG isolates were reported to produce infective vegetations in an endocarditis infection model of Wistar rats [100] and the high potential of SAG to cause pyogenic infections insinuates that SAG strains have evolved mechanisms to inhibit their killing by polymorphonuclear leukocytes (PMNLs). This was demonstrated by Wanahita et al. who reported that, although SAG strains were associated in higher numbers with PMNLs, they were killed less effectively by these cells compared to the prototypical abscess forming bacterium *Staphylococcus aureus* [206].

Whole genome analysis of different SAG strains further proposed four virulence related loci that are conserved in all SAG genomes. These loci were previously reported to be involved in invasion and evasion from host defense in other streptococci [141]. This included a homolog of the streptococcus invasion locus of *Streptococcus pyogenes* (GAS), UDP-glucose pyrophosphorlyase protein (HasC) involved in hyaluronic acid synthesis in GAS and parts of the hemolysin operon *cyl* of GBS. The forth locus is a well-investigated streptococcal virulence factor, the capsular polysaccharide (CPS) biosynthesis operon. Capsule production was already reported in the SAG and it was demonstrated that encapsulated *S. constellatus* strains showed an increased abscess formation in a murine infection model and a higher resistance to phagocytic killing compared to unencapsulated isolates [95]. Recently, the *cps* locus in the SAG was analyzed verifying that the members of the SAG contain a *cps* biosynthesis operon that shows high homology to the *cps* operon of *Streptococcus pneumoniae* and other oral streptococci [177,202]. However, a detailed analysis of the capsule production in SAG was not performed so far.

Recently, GAS related pyrogenic virulence factors (*speC*, *speG*) were detected in *S. anginosus* isolates from India [14]. These genes encode streptococcal pyrogenic exotoxins which include superantigens [155] and they stimulate the release of high levels of pro-inflammatory cytokines which can lead to the streptococcal toxic shock syndrome, a life-threatening condition accompanied with multi-organ failure and high mortality rates [78]. The high identity of the *spe* genes in *S. anginosus* and GAS, together with the lineage independent presence of these genes in some *S. anginosus* strains, strongly indicates recent horizontal gene transfer events. The publication of Babbar et al. thereby highlighted that some *S. anginosus* strains acquired pyrogenic virulence related genes that might increase their pathogenic potential [14] and thus emphasizes the importance of the SAG as emerging pathogens.

Virulence regulation

Bacteria need to sense their environmental conditions in order to be able to adapt their gene expression. A common bacterial mechanism to sense the environment are two-component systems (TCS) that are known to play a key role in virulence [80]. Whole-genome analysis in SAG revealed the presence of 14 different TCS in this

group [141]. Eight of these 14 TCS have orthologs in *S. pneumoniae* that have been linked to virulence [194]. These include the well-investigated VicRK, CiaRH, ComDE and BlpRH TCS which are involved in quorum sensing, competence and virulence in *S. pneumoniae* [18,87,137,194]. Additionally, a control of virulence regulator (CovR) homolog was detected in the SAG genomes [141]. CovR is a prominent transcription factor that controls the capsule production and virulence gene expression in GAS, GBS, *Streptococcus dysgalactiae* and *S. mutans* [45,68,103,109,182].
Overall, the pathogenicity factors of the SAG members are poorly investigated on the molecular level although the knowledge about virulence factors in these species starts to grow as the members of the SAG are increasingly recognized as emerging pathogens that need further attention.

1.5 Streptolysin S and Streptolysin S related gene clusters

One exception of the scarce knowledge about virulence factors in the SAG is the hemolysin responsible for the β-hemolytic phenotype of the *S. anginosus* type strain SK52. The genetic locus of the hemolysin was identified by random mutagenesis using the pGhost9::IS*S1* vector system [10,189]. The locus is comprised of ten genes and shows high homologies to the Streptolysin S (SLS) operon of GAS (Figure 1) [25,139].

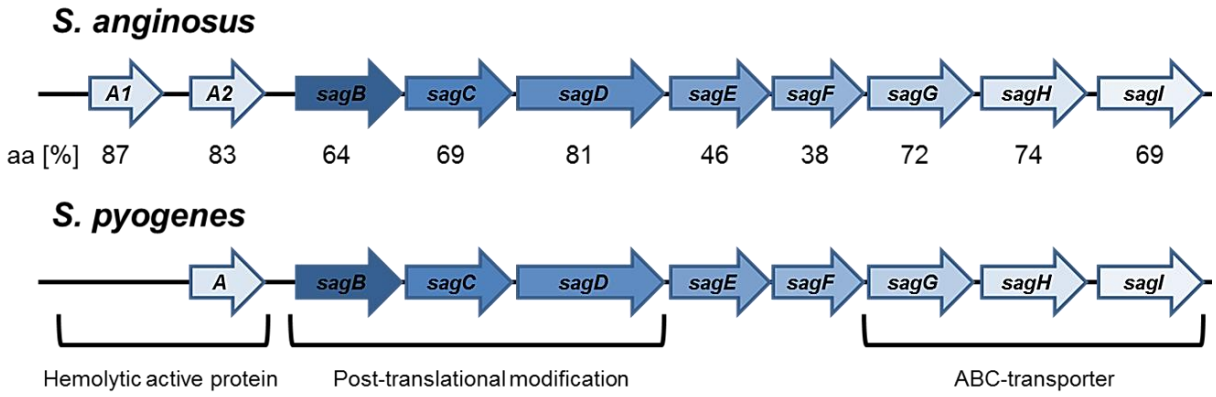

Figure 1: Schematic representation of the SLS operon of *S. pyogenes* and the homologous SLS operon in *S. anginosus* SK52. Depicted are the identities [%] on the amino acid (aa) level of the respective encoded proteins.

The hemolysin operon in GAS is comprised of nine genes and these genes are sufficient to produce the SLS [139]. The *sag* (Streptolysin S associated gene) operon shows similarities to operons of class I bacteriocins and thus contains genes encoding for a post-translational modification machinery as well as a transporter [124]. The first gene in the operon is *sagA* encoding the precursor of the active hemolysin. It encodes a 53 aa protein that contains a double glycine leader cleavage site. SagBCD is responsible for the conversion of the prepeptide SagA to SLS [108]. This complex introduces heterocycles into SagA which are essential for the activity of the hemolysin [39,130]. The *sagGHI* genes encode an ABC type transporter which is a common mechanism for bacteriocin export [39,164]. The role of the latter two genes *sagE* and *sagF* is still ambiguous. While SagF is predicted to be membrane associated, SagE is a membrane spanning peptidase that was proposed to confer immunity functions [39,108]. Strikingly, SagE only provided immunity to endogenous production of SLS and not to the exogenous exposure to SLS, as a GAS *sagE* mutant showed normal growth in the β-hemolytic zone of wild-type GAS colonies [39].

Such biosynthetic gene clusters, that encode a precursor peptide and a modification machinery that introduces heterocycles (thiazole, oxazole and methyloxazole) into the precursor, are widely distributed among bacteria and are designated thiazole/oxazole-modified microcins (TOMM) [72]. Members of the TOMM can be found in other streptococci including *Streptococcus dysgalactiae* subsp. *equisimilis*, *Streptococcus iniae* and *Streptococcus equi* [54,59,86]. However, these microcins are also encoded in more distantly related Gram-positive bacteria, in some Gram-negatives as well as in archaea [108] and several members of the family possess antibacterial activity [142,156,166,226].

The SLS in GAS is a major virulence factor that was already discovered in 1938 [196]. Occasionally, non-hemolytic GAS isolates are described which were associated with infections, but 99% of clinical GAS isolates are hemolytic and thus it is consent that the hemolysin in GAS plays an important role in the pathogenicity of this bacterium [90,203,228]. The SLS enables GAS to systemically disseminate by activating the degradation of the intercellular junctions, allowing GAS to perform paracellular invasion [186]. Additionally, the SLS contributes to soft-tissue damage and a non-hemolytic GAS mutant shows a reduced virulence in a murine soft-tissue

infection model [23,39]. The hemolysin is further implicated in evasion of the innate immune system by inhibiting neutrophil recruitment, by destroying neutrophils and by activating an inflammatory programmed cell death pathway in macrophages [52,64,132].

The identified SLS homolog in SAG is present in some strains of *S. constellatus* as well as *S. anginosus* but in *S. intermedius*, encoding the human specific cytolysin intermedilysin, it was not detected so far [136,212]. The hemolysin in SAG shares some features with the SLS in GAS. Operons encoding the two hemolysins show high homologies but it has to be highlighted that, although *S. constellatus* strains encode one *sagA* gene like GAS, *S. anginosus* strains were reported two contain either one copy of the gene or two *sagA* copies, like in the case of the *S. anginosus* type strain SK52 [10,190]. Another common feature of the SLS of SAG and GAS is their temperature dependent hemolytic activity that is only present at temperatures above 22 °C [12,28]. However, the two hemolysins also show remarkable differences. While the SLS of GAS is able to lyse a variety of different cell types, including macrophages and neutrophils [64,84,132,191], the hemolysin of SAG was demonstrated to be a broad-range hemolysin able to lyse erythrocytes of different origin, but it does not show cytolytic activity against macrophages or granulocytes [12]. Additionally, the SLS of SAG was reported to confer cytolytic activity against HepG2 cells, a human hepatoma cell line [190].

The mechanism how the SLS of GAS is able to lyse erythrocytes remained controversial. Some reports suggested that the SLS induces hemolysis by a colloid-osmotic process, e.g. by pore formation directly in the cell membrane [28,47]. The possibility to inhibit this process by polyethylene glycol (PEG) of defined size further strengthened this hypothesis. The use of PEG in hemolysis assays of SAG SLS, in contrast, did not allow the prediction of a defined pore size generated by the hemolysin in the cell membrane [10]. Recently, Higashi et al. provided evidence that SLS leads to erythrocyte lysis through binding to Anion exchanger 1 (band 3) which mediates the exchange of chloride and bicarbonate. The binding of SLS to this anion channel leads to influx of chloride into the erythrocyte, followed by influx of water and subsequent lysis of the erythrocyte [79]. Thus the SLS of GAS could exert its activity by directly targeting membrane channels, just like other peptide toxins [48].

The expression of the hemolysin in GAS is influenced by a diverse array of different regulators (CovR, CodY, RofA, Mga) reflecting the importance of this virulence factor in GAS [21,77,97,115]. Additionally, GAS was reported to regulate hemolysis in connection to its carbohydrate metabolism, similarly to other streptococcal species [173,192,197,208]. In *S. anginosus*, the expression of the SLS was also demonstrated to be dependent on carbon availability, as the SLS expression was inhibited in the presence of high glucose concentrations in the growth medium [12]. Thus, carbon catabolite control seems to be a common mechanism for the regulation of hemolysin expression and it was demonstrated that one major factor of this mechanism, the catabolite control protein A (CcpA), directly regulates the expression of the Intermedilysin in *S. intermedius* and of the SLS in GAS [99,197].

1.6 Catabolite repression in Gram-positive bacteria

The majority of bacteria can use a variety of different substances as carbon source. They can either be co-metabolized or the bacteria can preferentially take up and consume the carbon source which is most easily accessible and allows the highest energy conservation. To be able to perform this hierarchical metabolism, the bacteria evolved complex regulatory mechanisms which are summarized as carbon catabolite repression (CCR) [114]. Only a few pathogenic bacteria, like Chlamydiae that are highly adapted to host niches, seem to lack these complex regulatory pathways [138]. The phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) is the key component in CCR of Gram-negative as well as Gram-positive bacteria [43]. It comprises a phosphorelay system that mediates the import and simultaneous phosphorylation of sugars. The PTS systems are composed of a sugar unspecific part that all PTS systems in the cell share and a sugar specific part. The phosphorylation relay starts with the phosphorylation of the Enzyme I (EI) by phosphoenolpyruvate and subsequent transfer of the phosphoryl group to the Histidine-containing Protein (HPr) which is phosphorylated at a histidine residue (HPr-His-P). The HPr then transfers the phosphoryl group to the substrate specific Enzyme IIA (EIIA) followed by the phosphorylation of the Enzyme IIB (EIIB) and transport of the sugar into the cell

by Enzyme IIC (EIIC). The EII complex can either be a multidomain protein consisting of EIIABC or it can be a multiprotein complex composed of the single EII components that can either be located in the cytosol or imbedded in the bacterial membrane [152]. Although the PTS is involved in CCR in Gram-negative and Gram-positive species, the regulatory pathways differ in both groups. The phosphorylation state of the EIIA of the PTS mainly confers CCR in Gram-negative species and the phosphorylation state of the HPr is important in CCR of Gram-positive species [66]. However, the final result of the CCR is similar, as the expression of genes that are needed for the consumption of alternative carbon sources is reduced in the presence of a preferred sugar.

In Gram-positive species, the CCR mechanisms can be divided in global and operon specific mechanisms (Figure 2). The operon specific mechanisms include the terms inducer exclusion and induction prevention. The inducer exclusion describes the mechanism that an uptake of an operon-specific inducer is inhibited. A prominent example is the galactose exclusion in *Lactobacillus brevis*. In the presence of Glucose serine-phosphorylated HPr is formed and binds to the galactose permease thereby inhibiting the transport of galactose into the cell. As galactose is needed for the activation of the galactose operon, the expression of this operon is blocked (inducer exclusion, Figure 2A) [44].

The term induction prevention addresses the activity of operon-specific transcription factors that contain PTS-regulatory domains (PRD) [184]. One of the best studied induction prevention mechanisms occurs in the levanase operon (*levDEFG* and *sacC*) of *Bacillus subtilis* which encodes a fructose-specific PTS and a levanase that hydrolysis fructose polymers in the environment [117]. The expression of the operon is regulated by the activator LevR which itself is regulated by HPr and the fructose-specific EIIB (LevE) [29,40,183]. LevR contains two PRDs and the phosphorylation state of these domains determines the LevR activity. In the presence of preferentially metabolized sugars the concentration of HPr-His-P is low accompanied by a scarce PRD1 phosphorylation which results in an inactive conformation of LevR (induction prevention, Figure 2B) [118].

The global regulation of CCR in Gram-positive bacteria is primarily mediated by CcpA in conjunction with the HPr, the HPr kinase/phosphorylase (HPrK) and the intermediates of the glycolytic pathway fructose-1,6-bisphosphate (FBP) and glucose-

6-phosphate (G6P, Figure 2C) [195,207]. CcpA is a pleiotropic transcription factor that binds to the promoter regions of target genes, thereby mainly reducing the expression of the genes. To be able to bind to DNA, CcpA needs to be activated by a serine-phosphorylated HPr [167]. In the phosphorelay of the PTS HPr is phosphorylated at a histidine at the position 15. However, this protein contains a second phosphorylation site (serine-46) which exhibits regulatory functions. The availability of glucose and other preferred PTS sugars leads to increased FBP levels in the cell. High levels of FBP activate the kinase activity of the HPrK which phosphorylates the HPr at serine-46 which in turn can activate the DNA binding capability of CcpA [42]. CcpA binds to so called catabolite responsive elements (*cre*). The sequence of the *cre* sites are well investigated in different species and it was shown that they consist of highly degenerate pseudo-palindromes [131,185,219,230]. The binding of CcpA to *cre* is further stimulated by the presence of high levels of FBP and G6P allowing a delicate control of the CcpA activity [168].

CCR is a genome-wide regulatory process that was reported to affect the expression of 5-10 % of the genes in different bacteria [134,219,227]. The majority of these genes is repressed by CcpA and encodes proteins involved in the metabolism of alternative carbon sources. However, CcpA also activates the expression of genes involved in glucose metabolism [195].

Furthermore, CcpA represents a link between virulence and metabolism as it is involved in the regulation of virulence gene expression in various bacterial pathogens [160]. Examples include the control of capsule expression in different bacteria [62,169,218], the repression of the toxic shock syndrome toxin-1 in *S. aureus* and the regulation of the toxin production of *Bacillus anthracis* [31,170]. In *Clostridium difficile*, CcpA controls the expression of the alternative sigma factor TcdR which in turn is responsible for the expression of high toxin levels and it represses phospholipase C and perfringolysin expression in *Clostridium perfringens* which contribute to gas gangrene formation [8,127].

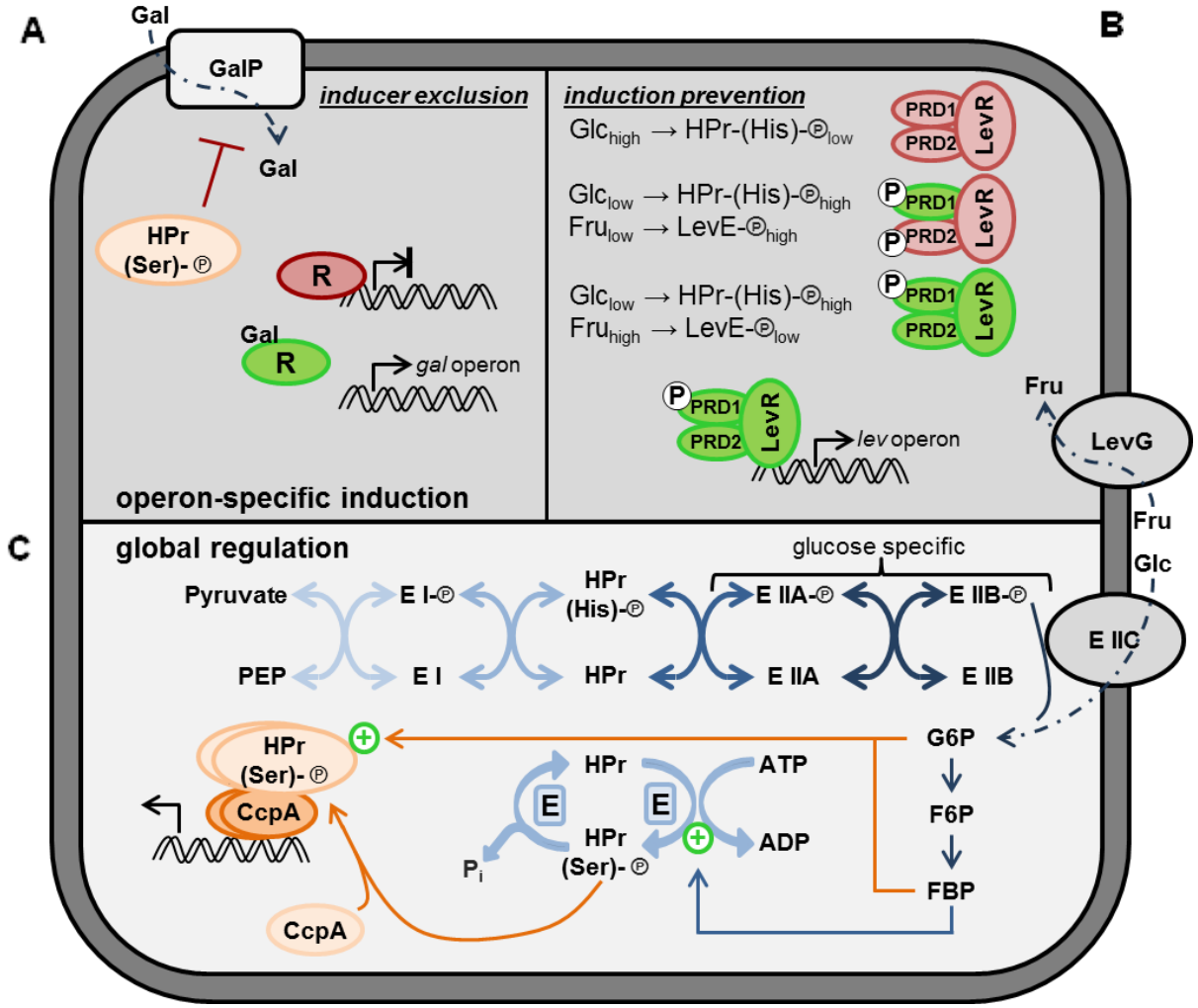

Figure 2: Schematic representation of carbon catabolite repression mechanisms in Gram-positive bacteria. **A:** Inducer exclusion: Serine-phosphorylated HPr [HPr(Ser)-Ⓟ] inhibits GalP (Galactose Permease) activity; In the absence of Gal (Galactose) the expression of the *gal* operon is blocked. **B:** Induction prevention: In the absence of Glc (Glucose), histidine-phosphorylated HPr [HPr-(His)-Ⓟ] is present. This leads to the phosphorylation of the PRD1 (PTS-regulatory domain) of LevR. High Fru (Fructose) concentrations in the environment lead to low levels of phosphorylated LevE (fructose-specific enzyme IIB) combined with low levels of PRD2 phosphorylation. LevR is active if PRD1 is phosphorylated and PRD2 is non-phosphorylated which results in *lev* operon transcription and further Fru import by LevG (Fructose transporter). **C:** Global regulation: Glucose specific PTS is depicted. In the presence of high fructose-1,6-bisphosphate (FBP) and high ATP concentrations in the cell the HPr kinase/phosphorylase (E) phosphorylates the HPr at a serine residue. HPr(Ser)-Ⓟ binds and activates the catabolite control protein A (CcpA) which increases the DNA binding capability of the regulator. This interaction is further supported by glucose-6-phosphate (G6P) and FBP.

In the genus streptococcus, numerous reports have demonstrated that CcpA controls different virulence traits. A *S. pneumoniae ccpA* mutant is attenuated in murine nasopharyngeal and lung infection models [89]. CcpA controls the acid production in *S. mutans* and thus is involved in the control of the cariogenic potential of this oral bacterium [1]. Furthermore, CcpA plays an important role in the pathogenicity of GAS. A *ccpA* mutant shows a hypervirulent phenotype in murine skin and systemic infections and CcpA seems to activate the transcription of the multiple gene regulator of GAS (Mga) that in turn controls genes needed for adherence, internalization and host immune evasion [5,81,99]. Additionally, CcpA directly regulates the expression of the SLS in GAS by binding to a *cre* site overlapping the SLS promoter [99]. CcpA was also investigated in the SAG and it was demonstrated that it regulates the intermedilysin expression in *S. intermedius* [197].

1.7 Competence system in the genus *Streptococcus*

Horizontal gene transfer (HGT) is one of the major driving forces for the evolution of the genomes in prokaryotes [7,37,151,215] and it has been proposed that over 60 % of the pan-genomes in streptococci have been subjected to HGT [159]. Bacteria can acquire new genetic material by transduction, conjugation and natural transformation. The latter is the only mechanism that does not need a DNA donor and thus is believed to be the simplest way to acquire new genetic material. Natural transformation is widespread among different phyla and was demonstrated for some archaea, Gram-negative as well as Gram-positive bacteria [92,112,171].

The physiological state of a bacterial cell where it is able to take up DNA from the environment is designated competence, a process that is tightly controlled in Gram-positive bacteria [35]. In streptococci, two different competence regulation mechanisms have evolved independently. Species of the salivarius, pyogenic, mutans and bovis group rely on the ComRS system whereas the SAG and the mitis group species use the ComCDE system for competence regulation [57,119]. The competence development in both groups is dependent on the alternative sigma factor ComX. In the early phase of the competence induction, a secreted peptide

pheromone is sensed which leads to the upregulation of the *comX* expression. Subsequently, ComX induces the expression of the late phase genes which built up the transformasome responsible for the DNA uptake [93].

Although homologs of ComX and genes encoding the transformasome are ubiquitously distributed in streptococcal genomes, it remained puzzling for a long time why only a subset of the streptococcal species showed natural transformation [34]. This has changed with the identification of ComR as a ComX regulator in *Streptococcus salivarius* and *Streptococcus thermophilus* in 2010 [55]. ComR is a stand-alone regulator that recognizes the sigmaX inducing peptide (XIP) encoded in the *comS* gene [55,125]. The discovery of the ComRS as competence regulator led to the expansion of the number of streptococcal species that are transformable and to date at least one species from the salivarius, pyogenic, mutans and bovis group were experimentally verified to be transformable using the XIP [41,55,122,123,135].

In contrast to the ComRS, the ComCDE system for competence development is well-investigated in the mitis group of streptococci (Figure 3). Natural transformation was already discovered at the beginning of the 20th century in *S. pneumoniae* [69] but it took further 35 years to show that competence could be induced in non-competent streptococci by a factor that was contained in sterile filtered supernatant of competent bacteria [145]. The factor that lead to competence induction was demonstrated to be heat sensitive and later turned out to be the competence stimulating peptide (CSP) encoded in *comC* [73]. The competence state in *S. pneumoniae* develops at a specific cell density, a mechanism that is tightly controlled by the competence regulation operon *comCDE* [35,148]. Besides ComC, the precursor of the CSP, the operon encodes the TCS ComD/E. The ComC is exported and processed by ComA/B, an ABC transporter that cuts the ComC prepeptide after a double glycine motif [74,85]. The extracellular CSP is recognized by the membrane-bound histidine kinase ComD that, upon activation, phosphorylates the response regulator ComE. The phosphorylated ComE then activates the expression of the so called early *com* genes that include the *comCDE* and *comAB* operons as well as *comX* and *comW* [120,121]. The alternative sigma factor ComX activates the expression of genes that are responsible for the DNA uptake [107,113,150] whereas ComW was reported as an activator and stabilizer of ComX [188,199]. The competence state develops

simultaneously in the cells and it is only maintained for around 15-20 min [161,199]. This short timeframe requires a delicate competence shut-off mechanism. The cells therefore evolved several competence exit strategies. It was demonstrated that unphosphorylated ComE accumulates to high levels after competence induction and it antagonizes the transcriptional activation of the phosphorylated ComE [121]. Additionally, the phosphorylated ComE is sequestered by one of the late competence gene products (DprA) leading to the reduction of the early gene expression [129,210]. Finally, a protein that antagonizes ComX activity was assumed, although the reported data concerning the ComW protection of ComX from proteolysis by ClpP are not consistent [188,210].

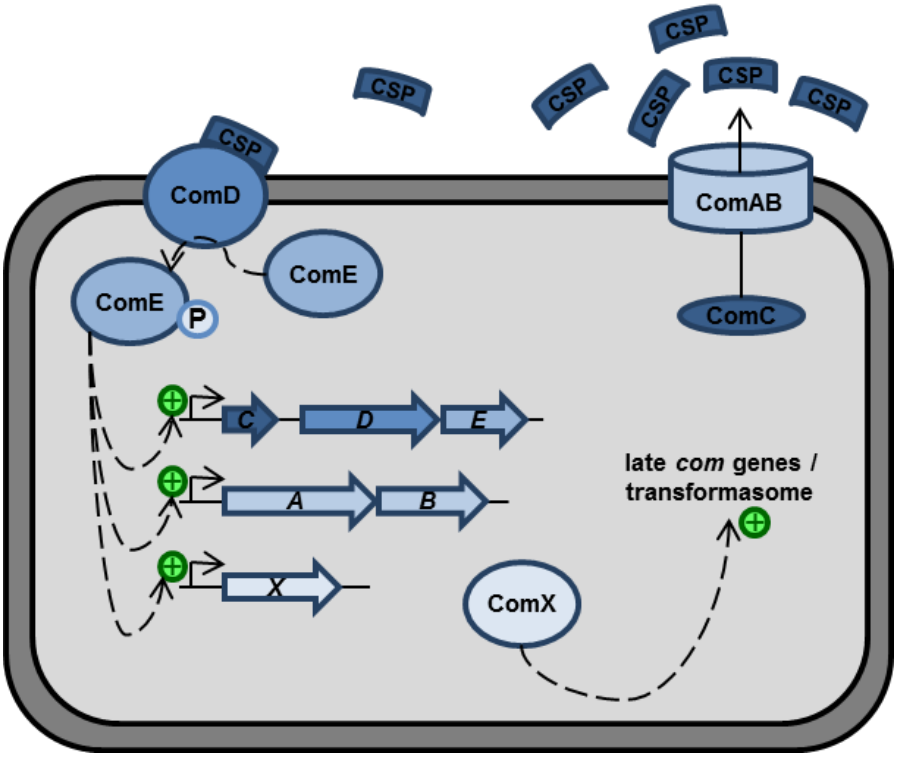

Figure 3: Schematic representation of the competence regulation in the mitis group of streptococci. ComC, the precursor of the CSP, is processed and exported by ComAB. High levels of CSP lead to activation of ComD which results in ComE phosphorylation. Phosphorylated ComE activates the transcription of the early *com* genes (*comCDE*, *comAB*, *comX*). The alternative sigma factor ComX induces the expression of the late *com* genes that built up the transformasome.

While the competence system in the mitis group and especially in *S. pneumoniae* has been characterized in detail as described above, the knowledge about the system in SAG is scarce. The SAG bacteria encode the ComCDE system to control the competence development and genes belonging to the competence regulon have been detected in the SAG genome [119,141]. Members of the SAG were demonstrated to encode identical CSPs which would allow interspecies communication in this group. However, the *comCDE* locus was not investigated thoroughly in the SAG and it is possible that different CSP pherotypes exist as it was shown that horizontal gene transfer has contributed to the diversification of the competence locus in streptococci [76,211].

1.8 Aim of the work

The aim of this work was to investigate the regulation of the SLS expression in *S. anginosus*. Due to the lack of suitable genetic tools in the SAG it was necessary to establish a site-directed mutagenesis procedure that allows fast and reliable generation of deletion mutants in *S. anginosus*. The competence system of *S. anginosus* was analyzed to be able to exploit this system for the mutagenesis technique. Using the newly established method would then allow to generate targeted deletions in the *S. anginosus* genome to be able to decipher the regulation of the SLS expression at a molecular level. Regulator mutants of *S. anginosus* should be analyzed in respect to their hemolytic behavior and the binding sites of identified regulators should be characterized which should finally result in the first description of a transcriptional regulator in the emerging pathogen *S. anginosus*.

2 Material and Methods

2.1 Material

2.1.1 Bacterial strains

The bacterial strains used in this study are summarized in Table 1.

Table 1: Strains. The bacterial strains and their definitions together with their source are summarized.

Strain	Definition	Source
S. anginosus		
BSU 458	*S. anginosus* type strain SK52, ATCC33397, Hly+	ATCC
BSU 554	BSU 458 derivative, carrying pBSU409	[63]
BSU 556	BSU 458 derivative, carrying pBSU409*::cfbprom*	[13]
BSU 805	BSU 458 derivative, carrying pBSU409*::sagprom*	[10]
BSU 886	BSU 458 derivative; carrying pBSU409*::sagprom_creA_del*	This study
BSU 902	BSU 458*::pG$^+$host5_covR*	This study
BSU 913	BSU 458 derivative; carrying pBSU409*::sagprom_creB_del*	This study
BSU 916	BSU 458 derivative; carrying pBSU409*::sagprom_creC_mut*	This study
BSU 924	BSU 458 derivative; Δ*sagA1*	This study
BSU 926	BSU 458 derivative; Δ*ccpA*	This study
BSU 928	BSU 458 derivative; Δ*ccpA*, carrying pBSU409*::sagprom*	This study
BSU 947	BSU 458::ccpA	This study
BSU 948	BSU 928 derivative; Δ*ccpA::ccpA*	This study
BSU 982	BSU 458 derivative; Δ*covR*	This study
BSU 994	BSU 458 derivative; Δ*sagBCD*	This study
BSU 1210	clinical isolate	Ulm collection
BSU 1211	clinical isolate	Ulm collection
BSU 1214	clinical isolate	Ulm collection
BSU 1215	clinical isolate	Ulm collection
BSU 1216	clinical isolate	Ulm collection
BSU 1217	clinical isolate	Ulm collection

BSU 1227	clinical isolate	Ulm collection
BSU 1289	clinical isolate	Ulm collection
BSU 1303	clinical isolate	Ulm collection
BSU 1306	clinical isolate	Ulm collection
BSU 1307	clinical isolate	Ulm collection
BSU 1308	clinical isolate	Ulm collection
BSU 1310	clinical isolate	Ulm collection
BSU 1312	clinical isolate	Ulm collection
BSU 1313	clinical isolate	Ulm collection
BSU 1317	clinical isolate	Ulm collection
BSU 1318	clinical isolate	Ulm collection
BSU 1319	clinical isolate	Ulm collection
BSU 1323	clinical isolate	Ulm collection
BSU 1324	clinical isolate	Ulm collection
BSU 1326	clinical isolate	Ulm collection
BSU 1327	clinical isolate	Ulm collection
BSU 1328	clinical isolate	Ulm collection
BSU 1329	clinical isolate	Ulm collection
BSU 1330	clinical isolate	Ulm collection
BSU 1331	clinical isolate	Ulm collection
BSU 1332	clinical isolate	Ulm collection
BSU 1334	clinical isolate	Ulm collection
BSU 1336	clinical isolate	Ulm collection
BSU 1338	clinical isolate	Ulm collection
BSU 1339	clinical isolate	Ulm collection
BSU 1344	clinical isolate	Ulm collection
BSU 1345	clinical isolate	Ulm collection
BSU 1346	clinical isolate	Ulm collection
BSU 1351	clinical isolate	Ulm collection
BSU 1354	clinical isolate	Ulm collection
BSU 1355	clinical isolate	Ulm collection
BSU 1358	clinical isolate	Ulm collection

BSU 1361	clinical isolate	Ulm collection
BSU 1362	clinical isolate	Ulm collection
BSU 1363	clinical isolate	Ulm collection
BSU 1364	clinical isolate	Ulm collection
BSU 1366	clinical isolate	Ulm collection
BSU 1369	clinical isolate	Ulm collection
BSU 1372	clinical isolate	Ulm collection
BSU 1373	clinical isolate	Ulm collection
BSU 1374	clinical isolate	Ulm collection
BSU 1375	clinical isolate	Ulm collection
BSU 1376	clinical isolate	Ulm collection
BSU 1379	clinical isolate	Ulm collection
BSU 1381	clinical isolate	Ulm collection
BSU 1382	clinical isolate	Ulm collection
BSU 1384	clinical isolate	Ulm collection
BSU 1386	clinical isolate	Ulm collection
BSU 1387	clinical isolate	Ulm collection
BSU 1388	clinical isolate	Ulm collection
BSU 1389	clinical isolate	Ulm collection
BSU 1390	clinical isolate	Ulm collection
BSU 1391	clinical isolate	Ulm collection
BSU 1392	clinical isolate	Ulm collection
BSU 1395	clinical isolate	Ulm collection
BSU 1396	clinical isolate	Ulm collection
BSU 1397	clinical isolate	Ulm collection
BSU 1398	clinical isolate	Ulm collection
BSU 1399	clinical isolate	Ulm collection
BSU 1400	clinical isolate	Ulm collection
BSU 1401	clinical isolate	Ulm collection
BSU 1402	clinical isolate	Ulm collection
BSU 1403	clinical isolate	Ulm collection
BSU 1404	clinical isolate	Ulm collection

BSU 1405	clinical isolate	Ulm collection
BSU 1406	clinical isolate	Ulm collection
BSU 1413	clinical isolate	Ulm collection
BSU 1414	clinical isolate	Ulm collection
BSU 1415	clinical isolate	Ulm collection
BSU 1417	clinical isolate	Ulm collection
E. coli		
BL21(DE3)	*E. coli* BL21 derivative with DE3 λ prophage carrying the T7 RNA polymerase gene and *lacI*^{q}	Novagen
DH5α	*endA1 hsdR17 supE44* DlacU169(f80lacZDM15) *recA1 gyrA96 thi-1 relA1*	Boehringer
EC101	*E. coli* JM101 derivative with *repA* from pWV01 integrated into the chromosome	[106]
BSU 957	BL21 derivative, carrying pET21a-*ccpA*-N-His	This study

2.1.2 Plasmids

The plasmids used in this study are summarized in Table 2.

Table 2: Plasmids. The plasmids, their characteristics and their source are listed.

Plasmid	Characteristics	Source
pAT18	*lacZα*, ori pUC, ori pAmβ1, Ery^R	[201]
pAT28	*lacZα*, ori pUC, ori pAmβ1, Spc^R	[200]
pET21a	pET21a; *lacI*, ori F1, ori pBR322, T7 promoter and terminator, Amp^R	Novagen
pGA14-Spc	Replication functions of pWVO1, Ery^R, Spc^R	[178]
pG$^+$host5	Replication functions of pWVO1, ori pBR322; Ery^R	[24]
pSET5s_P$_{tufA}$-cre	Replication function of pG$^+$host3 and pUC19, Cre-recombinase gene under control of *tufA* promoter, Cm^R	[102]
pBSU409	pAT28 derivative carrying *egfp*	[63]
pBSU803	pBSU409*::sagprom*; pBSU409 derivative, *egfp* under the control of the SLS promoter	[10]
pBSU846	pG$^+$host5_*covR*; pG$^+$host5 derivative carrying integration sequence of *covR*	This study
pBSU876	pBSU409*::sagprom_creC_mut*; pBSU803 derivative carrying mutations in *creC*	This study

pBSU880	pBSU409*::sagprom_creB_del*; pBSU803 derivative carrying a deletion of *creB*	This study
pBSU881	pBSU409*::sagprom_creA_del*; pBSU803 derivative carrying a deletion of *creA*	This study
pBSU910	pG^+host5-*ccpA*Δ*100nt*; pG^+host5 derivative carrying up- and downstream sequences of *ccpA*	This study
pBSU919	pAT18-cre-rec_{tufA}; pAT18 derivative carrying Cre-recombinase gene under the control of *tufA* promoter, Ery^R	This study
pBSU956	pET21a-*ccpA*-N-His; pET21a derivative carrying *ccpA*	This study

2.1.3 Oligonucleotides

The oligonucleotides used in this study are summarized in Table 3.

Table 3: Oligonucleotides. The oligonucleotides and their 5'-3' sequences are listed.

Name	Sequence
ccpA_pGh_F1_fwd_SalI	CCGATTGTCGACGGTCATTCTAGTACCTTC
ccpA_pGh_F1_rev	CAGAAACTTCCTTGTTATGGAATGACAACTCCAACAGTC
ccpA_pGh_F2_fwd	TAACAAGGAAGTTTCTGTTG
ccpA_pGh_F2_rev_EcoRI	GGCGGCGAATTCTACCTGCGAATCATC
ccpA_integration_rev	CCGAGACACTAGTATCTCAG
ccpA_integration_fwd	TCAAGAGGACAGTAAGAAC
ccpA_comp_F1_rev	GGGCGGTAATCCAAGCGGTC
ccpA_comp_F2_fwd	CTTGGATTACCGCCCAAATG
ccpA_comp_F2_rev	GTTTGCTTCTAAGCCACAAAGGTATAGAGCC
ccpA_comp_F3_fwd	CTTAGAAGCAAACTTAAGAGTGTG
ccpA_comp_F3_rev	CGATACAAATTCCCCGTAGGC
ccpA_comp_F4_fwd_2	CGGGGAATTTGTATCGGGGTATAATAGAAGAC
ccpA_comp_F4_rev	GTCCCGCACAGACAACCAC
sagprom_fwd	GGGCCCGAATTCGGTTGGATTTGATAGTAATGTACG
sagprom_rev	GGGCCCGGATCCGAAGAAAATTTTAACATAGTTTG
cre2_F1_rev(creA)	CCTTTGTCATGTTTTATTCACTCAGATGATAATAATTCTG
cre2_F2_fwd	GTGAATAAAACATGACAAAG
cre1_F1_rev(creB)	CACAAATATAACCATCATAGCATTTGAACACAC
cre1_F2_fwd	GATGGTTATATTTGTGAAATAGG

cre3_mut_fwd(creC)	CGCGAAGGATCCTTTTTTATATAATGTG
cre3_mut_rev	TATATAAAAAAGGATCCTTCGCGTAAAC
ccpA_N_His6_fwd	GAAGACATATGCACCACCACCACCACCACAACACAGACGATACAGTAACC
ccpA_N_His6_rev	GTCGGCCTCGAGTTACTTTCTTGTTGAACG
IRD700_cre_fwd(creC)	IRDye700-GTTTACGCGAAAGCGCTTTTTTTATATA
cre_rev(creC)	TATATAAAAAAAGCGCTTTCGCGTAAAC
cre_mut_fwd(creC_mut)	GTTTACGCGAAGGATCCTTTTTTATATA
cre_mut_rev(creC_mut)	TATATAAAAAAGGATCCTTCGCGTAAAC
creB_fwd	TGCTATAAGAACGCGCTTTTTATTTTGTTTTAGATGGT
creB_rev	ACCATCTAAAACAAAATAAAAAGCGCGTTCTTATAGCA
pAT28-2	CTCTTCGCTATTACGCCAGCT
pAT28-3	GTTGTGTGGAATTGTGAGCGG
pAT28-EGFP4	CCTTGAAGAAGATGGTGCGC
tufA_fwd_EcoRI	CCCGAATTCGTGAAAATAGTCAATATA
cre_rec_rev_XbaI	GCCTGCAGTCTAGACGATAAGCTTCTAATC
sagA1_del_F1_fwd	CTTTACTTGTCGCACGGAAC
sagA1_del_F1_rev	GCATACATTATACGAACGGTACATAGTTTGTCCTCCTTATAAAATG
sagA1_del_F2_fwd	TATAATGTATGCTATACGAACGGTAAATAATTTTTCTTAACTG
sagA1_del_F2_rev	ATTGCCGTGCTTCGTCTC
lox66_spec_rev	TACCGTTCGTATAATGTATGCTATACGAAGTTATATGCCTGCAGGTCGATTTTCG
lox71_spec_fwd	TACCGTTCGTATAGCATACATTATACGAAGTTATTTAAATGGCATTGGTACCC
response_fwd	AAATCCCTTGTCCAATCA
sagB_control_rev	CAGTTGCTTCCCCACTAAC
sagA0_fwd	GTTCAACAACTGGATCCGTA
sagBCD_F1_rev	GCATACATTATACGAACGGTAAATGAACTTTCTAACTAC
sagBCD_F2_fwd	TATAATGTATGCTATACGAACGGTAATGAATTTGTACACCCTATG
sagBCD_F2_rev	CCCATTAGAAGATGATTGAG
sag_pre_control_fwd	GATTCCCTAACTTCTATC
sagBCD_int_test_rev	AAGGTAAGACACGCAAGG
comC_fwd	AGAGCATTCGCCTTCTAAGC
comC_rev	GCAAATTGTAGCAATCATACT
RT_csrR_operon_fwd	TTCGTCAAGCCCTCTACTTC

RT_csrR_operon_rev	GCAAAGCGTTGGATTTCCTC
csrR_fwd_SalI	AGATGCGTCGACGTCTGCGAGAGCTAATC
csrR_rev_EcoRI	CTCCTTGAATTCTCCACACGCGTTCTAAC
csrR_Integration_fwd	AGTGGCGTTGGCTGTTCAAG
csrR_Integration_rev	TAGGGAAATACGCCCATTAG
covR_inverse_fwd	CCGTGCGTGGTGTTGGTTATG
covR_inverse_rev	GCCCCGCTATATCAATTTTAC
covR_test_rev	TCAGTAATTCTCGCTTAGTG
covR_F1_fwd	CGTAATCCAGATCTTGCAAAC
covR_F1_rev	GCATACATTATACGAACGGTACCGAAGCAGGCTTTATCACAC
covR_F2_fwd	TATAATGTATGCTATACGAACGGTAATCGGAATCTTCGGATTG
covR_lox_int_test_fwd	CTATTTGGCGTGCTGGTTG
covR_lox_int_test_rev	CTTGCTTGCAAATTGTAATAG

2.1.4 Instruments

Instrument	Supplier
Absorbance Plate reader Tecan infinite M Nano	Tecan Trading AG, Männedorf, Switzerland
Centrifuge	
Microfuge 3S-R	Heraeus Sepatech GmbH, Hanau, Germany
Variofuge 3.0R	Heraeus Sepatech GmbH, Hanau, Germany
Heraeus Fresco 17	Thermo Fisher Scientific, Waltham, Massachusetts
Flow cytometer: FACS-Calibur II	Beckton Dickinson, Heidelberg, Germany
Laminar flow work bench	
MSC-Advantage	Thermo Fisher Scientific, Waltham, Massachusetts
HERA safe	Heraeus Instruments, Hanau, Germany

Freezers	
-20 °C (Bosch economic)	Robert Bosch GmbH, Stuttgart, Germany
-20 °C (CNP 4758 20A/001)	Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany
-70 °C (Herafreeze HFU B Series)	Heraeus Sepatech GmbH, Osterode, Germany
-70 °C (V86-500.1)	Ewald Innovationstechnik GmbH, Rodenberg, Germany
Gel Documentation System Bio Doc Analyse	Biometra GmbH, Goettingen Germany
Gel dryer Model 543	Bio-Rad Laboratories Inc., Hercules, California
Heating block	
Thermomixer 5436	Eppendorf AG, Hamburg, Germany
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Incubator	
Hereaus BBD 6220	Thermo Fisher Scientific, Waltham, Massachusetts
Certomat® HK	Sartorius AG, Goettingen, Germany
Magnetic stirrer: MR3002	Heidolph, Leverkusen, Germany
Mikrowave seaWAVE®-real time-microbiology	SHARP, Hamburg, Germany
Multipette	Eppendorf AG, Hamburg, Germany
Odyssey Clx near-infrared fluorescence imaging system	LI-COR Corporate Offices, Lincoln, Nebraska
Pipetteboy	IBS Integra Biosciences, Fernwald, Germany
Pipettes: 10 µl, 100 µl 200 µl, 1000µl	Eppendorf AG, Hamburg, Germany
Photometer	
Ultraspec 3000	AMERSHAM, Pharmacia Biotech, NewYork

UV-1800 Spectrophotometer	Shimazu Corporation, Kyoto, Japan
pHenomenal™ pH-Meter	VWR International, Radnor, Pennsylvania
Ribolyser (cell disrupter, FastPrep FP120)	Qbiogene Inc., Carlsbad, California
SDS gel system	
PowerPac™ Basic	Bio-Rad Laboratories Inc., Hercules, California
MiniProtein® Tetrasystem	Bio-Rad Laboratories Inc., Hercules, California
Trans-Blot® Turbo™ Transfersystem	Bio-Rad Laboratories Inc., Hercules, California
Shaker Certomat®	Sartorius AG, Goettingen, Germany
Sonifier 450	Branson Ultrasonics, Danbury, Connecticut
Thermocycler	
TRIO-Thermoblock	Biometra GmbH, Goettingen, Germany
TProfessional Thermocycler	Biometra GmbH, Goettingen, Germany
Personal cycler	Biometra GmbH, Goettingen, Germany
T3 Thermocycler	Biometra GmbH, Goettingen, Germany
Vortexer	
REAX 2000	Heidolph, Nürnberg, Germany
Vortex-Gene2	VWR International GmbH, Darmstadt, Germany
Water bath Julabo 5	Julabo GmbH, Seelbach, Germany
Weighing scales	
Kern 470	Kern und Sohn GmbH, Balingen-Frommern, Germany
AG 204	Mettler, Toledo, Spain

2.1.5 Consumables

Consumable	Supplier
Amicon®-Ultra Centrifugal Filters Ultracel®-30K	Merck Millipore, Cork. Ireland
Eppendorf tubes (0.5 ml and 1.5ml)	Eppendorf AG, Hamburg, Germany
Falcon® (15 ml and 50 ml)	Corning Science, Tamaulipas, Mexico
Falcon® polystyrene round-bottom tube 5 ml	Corning Science, Tamaulipas, Mexico
Glass beads (150-212 µm)	Sigma-Aldrich, St. Louis, Missouri
Glass beads (3mm)	Merck KGaA, Darmstadt, Germany
Glass bottles 50 ml, 100 ml, 1000 ml	Schott, Mainz, Germany
Inoculation loops	Greiner Bio-One GmbH, Frickenhausen, Germany
Inoculation needles	Greiner Bio-One GmbH, Frickenhausen, Germany
Inoculation tube 13 ml	Sarstedt AG & Co., Nümbrecht, Germany
Microbank®	Pro-Lab diagnostics, Richmond Hill, Canada
Micro-Touch® Nitra-Tex® gloves	Ansell Healthcare Europe, Brussels, Belgium
Petri dishes	Sarstedt AG & Co., Nümbrecht, Germany
Pipette tips (10 µl; 100µl; 200 µl; 1000 µl)	Nerbe plus GmbH, Winsen, Germany
Polystyrene cuvettes	Sarstedt AG & Co., Nümbrecht, Germany
Ribolyser tubes (2 ml) with caps	Sarstedt AG & Co., Nümbrecht, Germany
Serological pipettes 5 ml, 10 ml, 25ml	Corning Science, Tamaulipas, Mexico
96-well plate conical bottom	Greiner Bio-One GmbH, Frickenhausen, Germany

96-well plate flat bottom	Thermo Fisher Scientific, Waltham, Massachusetts

2.1.6 Chemicals and reagents

Chemical	Supplier
Acrylamide/Bis Solution	Serva Electrophoresis GmbH, Heidelberg, Germany
Agarose	Sigma-Aldrich, St. Louis, Missouri
Ammonium persulfate	Sigma-Aldrich, St. Louis, Missouri
Ampicillin	Merck KGaA, Darmstadt, Germany
Antifect® N liquid	Schülke und Mayr GmbH, Norderstedt, Germany
Anti-Mouse IgG-Alkaline Phosphatase	Sigma-Aldrich, St. Louis, Missouri
Bacto™ Agar	Becton Dickinson GmbH, Heidelberg, Germany
Bacto™ Tryptone	Becton Dickinson GmbH, Heidelberg, Germany
Bacto™ Yeast Extract	Becton Dickinson GmbH, Heidelberg, Germany
Bovine serum albumin	Sigma-Aldrich, St. Louis, Missouri
Calcium Chloride	Sigma-Aldrich, St. Louis, Missouri
CDP-Star	Roche Diagnostics GmbH, Mannheim, Germany
Chloramphenicol	Sigma-Aldrich, St. Louis, Missouri
Deoxyribonucleotides (dNTPs)	Roche Diagnostics GmbH, Mannheim, Germany
Di-potassium hydrogen phosphate	Merck KGaA, Darmstadt, Germany
Dithiothreitol (DTT)	Merck KGaA, Darmstadt, Germany
DNA-Ladder 1kb Plus	Invitrogen Corporation, Carlsbad, California

Double-distilled water	Universität Ulm-Klinikum Apotheke, Ulm, Germany
Distilled water	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
Erythromycin	Serva Electrophoresis GmbH, Heidelberg, Germany
Ethanol	Sigma-Aldrich, St. Louis, Missouri
Ethidiumbromide	AppliChem GmbH, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Fluka Biochemika, Steinheim, Germany
FACS-Clean	Becton Dickinson GmbH, Heidelberg, Germany
FACS-Rinse	Becton Dickinson GmbH, Heidelberg, Germany
Fetal bovine serum Superior	Biochrom AG, Berlin, Germany
Fructose	Sigma-Aldrich, St. Louis, Missouri
Galactose	Carl Roth GmbH, Karlsruhe, Germany
GIBCO® Dulbecco's Phosphate-Buffered Saline (DPBS) 1x	Invitrogen GmbH, Darmstadt, Germany
Glucose	Sigma-Aldrich, St. Louis, Missouri
Glycerol	Sigma-Aldrich, St. Louis, Missouri
Glycine	Labochem international, Einhausen, Germany
Hydrochloric acid	Merck KGaA, Darmstadt, Germany
Hydrogen peroxide 30%	Otto Fischar GmbH & Co.KG, Saarbrücken, Germany
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth GmbH, Karlsruhe, Germany
Loading buffer 6x	Fermentas GmbH, St. Leon-Rot, Germany
Magnesium chloride	Sigma-Aldrich, St. Louis, Missouri

Magnesium sulphate	Sigma-Aldrich, St. Louis, Missouri
Maltose	Sigma-Aldrich, St. Louis, Missouri
β-Mercaptoethanol	Sigma-Aldrich, St. Louis, Missouri
Methanol	Sigma-Aldrich, St. Louis, Missouri
Milk powder	Carl Roth GmbH, Karlsruhe, Germany
Penta-His^{TM} Antibody	Qiagen, Hilden, Germany
Phosphoric acid	Merck KGaA, Darmstadt, Germany
Poly(deoxyinosinic-deoxycytidylic) acid (poly [dI-dC])	Sigma-Aldrich, St. Louis, Missouri
Potassium chloride	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck KGaA, Darmstadt, Germany
Prestained SDS-PAGE Standards, Low Range	Bio-Rad Laboratories Inc., Hercules, California
RNAprotect® Bacterial Reagent	Qiagen, Hilden, Germany
Roti®-Blue	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride	AppliChem GmbH, Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, St. Louis, Missouri
Sodium hydroxide (NaOH)	Merck KGaA, Darmstadt, Germany
Sodium phosphate-dibasic	Sigma-Aldrich, St. Louis, Missouri
Spectinomycin	Sigma-Aldrich, St. Louis, Missouri
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, St. Louis, Missouri
TODD-Hewitt Broth	Oxoid GmbH, Wesel, Germany
TRIS borate-EDTA Buffer (TBE)	Sigma-Aldrich, St. Louis, Missouri
Trizma® base	Sigma-Aldrich, St. Louis, Missouri
Tryptone soya agar with sheep blood	Oxoid GmbH, Wesel, Germany
Tween® 20	Sigma-Aldrich, St. Louis, Missouri

2.1.7 Solutions

Lysogeny Broth Miller (LB-Miller)

10 g l^{-1} NaCl

10 g l^{-1} Trypton

5 g l^{-1} yeast extract

Todd Hewitt Broth with 5% yeast extract (THY)

36.4 g l^{-1} Todd Hewitt Broth

5 g l^{-1} yeast extract

Induction Buffer pH 7.0

100 mM KH_2PO_4

2 mM $MgSO_4$,

30 mM maltose

Potassium phosphate buffer 0.1 M, pH 7.0

10.71 g l^{-1} K_2HPO_4

5.24 g l^{-1} KH_2PO_4

Tris-buffered saline (TBS) pH 7.3

8 g l^{-1} NaCl

1.21 g l^{-1} Trizma® base

Transfer buffer

25 mM Na_2HPO_4

Blocking buffer

1x TBS

3 % BSA

Alkaline Phosphate Buffer

12.11 g l^{-1} Trizma® base

5.84 g l^{-1} NaCl

5 mM $MgCl_2$

2.1.8 Enzymes

Enzyme	Supplier
DNase I	Roche Diagnostics GmbH, Mannheim, Germany
Expand Long DNA Polymerase Mix	Roche Diagnostics GmbH, Mannheim, Germany
Ligase (T4 DNA)	Thermo Fisher Scientific, Waltham, Massachusetts
Lysozym	Roche Diagnostics GmbH, Mannheim, Germany
Mutanolysin	Sigma-Aldrich, St. Louis, Missouri
Proteinase K	Sigma-Aldrich, St. Louis, Missouri
Restriction endonucleases BamHI, EcoRI, MluI, SalI, SspI, XbaI	Roche Diagnostics GmbH, Mannheim, Germany
Taq DNA Polymerase	Roche Diagnostics GmbH, Mannheim, Germany

2.1.9 Kits

Kit	Supplier
Expand Long Template PCR system	Roche Diagnostics GmbH, Mannheim, Germany
GenElute™ Bacterial Genomic DNA Kits	Sigma-Aldrich, St. Louis, Missouri
MinElute® Reaction Cleanup Kit	Qiagen, Hilden, Germany
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel GmbH&Co. KG, Düren, Germany
NucleoSpin® Plasmid	Macherey-Nagel GmbH&Co. KG, Düren, Germany
Protino® Ni-TED 1000 Packed Columns	Macherey-Nagel GmbH&Co. KG, Düren, Germany
Qiaprep® Spin Miniprep kit	Qiagen, Hilden, Germany

Qiagen® OneStep RT-PCR Kit	Qiagen, Hilden, Germany
Qubit™ dsDNA BR system	Thermo Fisher Scientific, Waltham, Massachusetts
Qubit® RNA HS system	Thermo Fisher Scientific, Waltham, Massachusetts
Quick Start™ Bradford Protein Assay	Bio-Rad Laboratories Inc., Hercules, California
Rapid DNA Ligation Kit	Thermo Fisher Scientific, Waltham, Massachusetts
RNeasy® Mini Kit	Qiagen, Hilden, Germany

2.1.10 Software

Software	Provider
BPROM - Prediction of bacterial promoters	http://www.softberry.com/berry.phtml
Cell Quest Pro	BD Biosciences, Heidelberg, Germany
CLC Main Workbench v7.0	http://www.clcbio.com
Image Studio Version 5.2	LI-COR Corporate Offices, Lincoln, Nebraska
GenBank database	http://www.ncbi.nlm.nih.gov/
Kyoto Encyclopedia of Genes and Genomes	https://www.kegg.jp/
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/

2.2 Methods

2.2.1 Bacterial strains and growth conditions

The *S. anginosus* and *Escherichia coli* strains used in this study are summarized in Table 1. *E. coli* strains were incubated aerobically at 37 °C in LB-Miller medium. *E. coli* DH5α served as host for pAT28 and pGA14-Spc plasmids and *E. coli* EC101

was used as cloning host for pAT18, pG$^+$host5 and pSET5s_P_{tufA}-cre plasmids. Heterologous expression of proteins was performed in *E. coli* BL21 with plasmid pET21. The growth medium was supplemented with the appropriate antibiotic if necessary (spectinomycin 100 µg ml^{-1}, erythromycin 400 µg ml^{-1}, ampicillin 100 µg ml^{-1}). *S. anginosus* was grown in THY broth or incubated on sheep blood agar pales (TSA+SB) at 37 °C and 5 % CO_2 in the presence of appropriate antibiotics. *S. anginosus* strains carrying pAT28 or pGA14-Spc derivatives were incubated on THY agar plates supplemented with 120 µg ml^{-1} spectinomycin and strains carrying pAT18 were grown on sheep blood agar plates overlaid with a final concentration of 5 µg ml^{-1} erythromycin. *S. anginosus* harboring p$^+$Ghost5 plasmid replicating at 30 °C was incubated in the presence of 5 µg ml^{-1} erythromycin and strains with chromosomal integration of pG$^+$host5 were incubated with 1 µg ml^{-1} erythromycin. For long-term storage, the bacteria were frozen at -70 °C in Microbank® vials.

2.2.2 General DNA techniques

Genomic DNA and plasmid DNA were isolated in standard procedures according to the instructions of the Kit manufacturers. The DNA concentration of samples was determined using the Qubit^{TM} dsDNA BR system. Polymerase Chain Reaction (PCR) was performed with *Taq*-Polymerase or Expand Long Template PCR system following the protocols of the manufacturers. Briefly, the PCR with *Taq*-Polymerase was conducted with an initial denaturation step of 3 min at 94 °C, 30 amplification cycles of 1 min 94 °C, 30 s 45-62 °C, 1-3 min 72 °C and a final elongation step of 7 min at 72 °C. The Expand Long Template PCR system was performed with an initial 2 min denaturation step at 94 °C, 10 amplification cycles of 10 s 94°C, 45-60 °C 30 s, 68 °C 2-3 min followed by 20 amplification cycles of 15 s at 94 °C, 45-60 °C 30 s, 68 °C 2-3 min and a final elongation of 7 min at 68 °C. The elongation time in the second amplification cycle was increased by 20 s per cycle. The annealing temperature as well as the elongation time varied depending on the predicted primer annealing temperature and the size of the PCR product. The primers used in this study are listed in Table 3.

Inverse PCR was performed to analyze the downstream region of the *covR** gene. The genomic DNA of the *S. anginosus* SK52 strain was digested with different restriction endonucleases and the resulting DNA fragments were ligated. Subsequently, the ligated DNA products were used as a template in a PCR with outward directed primers that bind directly downstream of the restriction endonuclease recognition sequence. The obtained PCR products were then sequenced by GATC Biotech AG (Konstanz, Germany).

Construction of plasmids was performed following standard procedures. Briefly, PCR was conducted to obtain the insert flanked by restriction endonuclease recognition sequences. The desired plasmid and the insert were digested with the respective restriction endonuclease and ligated using the Rapid DNA Ligation Kit. Ligation mixtures were transformed in *E. coli* using the heat shock procedure and the transformants were selected on LB-Miller agar containing the appropriate antibiotic. The construction of the desired plasmid derivatives was verified by restriction endonuclease digestion, PCR and sequencing. Standard electroporation based transformation of *S. anginosus* was performed as described by Ricci et al. [158]. This method was applied to transform plasmids into *S. anginosus* as long as it is not stated differently.

A reverse transcriptase PCR (RT-PCR) was performed to investigate the operon structure of the *covR** gene. Therefore, the RNA was extracted from *S. anginosus* SK52 with the Qiagen RNeasy® Mini Kit according to the protocol of the manufacturer with the following modifications. After growth of the bacteria to an optical density at 600 nm (OD_{600nm}) of 0.5 in 100 ml THY broth, the bacterial cells were pelleted and resuspended in 1 ml THY. 500 µl aliquots of the bacterial suspension were mixed with 1 ml RNAprotect® Bacterial Reagent followed by vortexing and an incubation of 5 min at room temperature. After centrifugation the pellets were resuspended in 350 µl RLT Buffer supplemented with 10 µl β-Mercaptoethanol. Subsequently, the mixture was transferred to Lysing Matrix B tubes and the bacterial cells were lysed with a Ribolyser. After centrifugation, the supernatant was transferred to QIAshredder columns and the protocol of the manufacturer was continued. The isolated RNA was subjected to a DNase digestion and the RNA concentration was determined using the

Qubit® RNA HS system. A total of 2 ng RNA was further used in the RT-PCR with the Qiagen® OneStep RT-PCR Kit following the instructions of the manufacturer.

2.2.3 CSP based transformation of *S. anginosus*

The CSP based transformation of *S. anginosus* strains was carried out as described previously [19]. Briefly, the CSP-1 (DSRIRMGFDFSKLFGK) and CSP-2 (DRRDPRGMIGIGKKLFG) were synthesized by the Core facility functional peptidomics of the University of Ulm (UPEP, Ulm, Germany). The CSP induced transformation kinetics was investigated with the plasmid pAT28. Therefore, THY broth was mixed with the respective CSP (final concentration 500 ng ml^{-1}) and inoculated with the respective *S. anginosus* strain to an OD_{600nm} of 0.02. The mixture was split into 1 ml aliquots and incubated at 37° C. In 10 min intervals the plasmid (final concentration 1 µg ml^{-1}) was added to the aliquots followed by a 1 h regeneration time at 37°C. Afterwards the bacteria were plated on THY agar plates with and without spectinomycin to be able to calculate the transformation efficiency (transformation efficiency [%] = [Colony Forming units (CFU) transformants / total CFU] *100). The same procedure was performed to analyze the uptake of linear DNA (sagA1-lox_Spc). For the optimization of the transformation procedure the same set up was used with varying DNA and CSP concentrations. Additionally, parts of the experiments were performed with THY broth supplemented with 10 % Fetal Bovine Serum (FBS).

2.2.4 Site-directed mutagenesis in *S. anginosus*

In the course of the study integration as well as deletion mutants of *S. anginosus* were constructed. Integration mutagenesis was performed in case of *covR** using the temperature sensitive pG^{+}host5 system. Therefore a 500 bp fragment of *covR** was ligated into pG^{+}host5 resulting in pG^{+}host5_*covR*. The plasmid was transformed into *S. anginosus* SK52 and the transformants were incubated directly at 39 °C. Integration mutants were screened on sheep blood agar plates containing 1 µg ml^{-1} erythromycin and the desired integration was verified by PCR and sequencing.

For the generation of deletion mutants in *S. anginosus* SK52, two different procedures were applied. The temperature sensitive pG$^+$host5 vector system was used for the generation of the *S. anginosus* Δ*ccpA* strain [24]. The up- and downstream region of the target were amplified by PCR and fused in an overlap-extension PCR (OE-PCR). The resulting DNA fragment was cloned into the pG$^+$host5 plasmid leading to the formation of pG$^+$host5-*ccpA*Δ*100nt*. The deletion vector was transformed into the *S. anginosus* SK52 with the CSP based transformation procedure and the regeneration of the transformation mixture was performed at 39 °C for 3 h to directly obtain *S. anginosus* clones that contain the chromosomally integrated plasmid. The transformants were selected on sheep blood agar plates supplemented with 1 µg ml^{-1} erythromycin and incubated for 2 days at 37 °C. After the verification by PCR that the erythromycin resistant clones integrated the plasmid at the desired locus, the induction of the loss of the chromosomally integrated plasmid was performed by incubation of the bacteria at 30 °C for 8 h. Single colonies were tested for their erythromycin susceptibility followed by the verification of the desired 100 bp deletion by PCR and sequencing.

The second procedure relies on the CSP based transformation of linear DNA fragments as described previously [19]. A linear DNA fragment consisting of the upstream region of the target, a spectinomycin resistance gene (*spc*) flanked by two *lox* sites and the downstream region of the target were constructed by OE-PCR. This DNA fragment is then transformed with the CSP based procedure into the *S. anginosus* SK52 strain leading to the integration of the construct and the loss of the target sequence. The resulting transformants were selected on THY agar plates supplemented with 120 µg ml^{-1} spectinomycin and the desired integration of the deletion construct was verified by PCR. Subsequently, the Cre-recombinase was introduced into the transformants. Therefore, the pAT18-cre-rec$_{tufA}$ plasmid was constructed by amplification of the recombinase gene and the *tufA* promoter from the pSET5s_P$_{tufA}$-cre vector and subsequent ligation of the PCR product in the plasmid pAT18. The pAT18-cre-rec$_{tufA}$ plasmid was introduced into the *S. anginosus* clones harboring the deletion fragment integrated in the desired locus. The regeneration of the transformation mixture was performed at 37 °C for 4 h which allows the expression of the recombinase. The Cre-recombinase recognizes the *lox*66 and *lox71*

site and excises the *spc* resulting in the formation of the *lox72* site. After verification of the *spc* excision by PCR and sequencing, the pAT18-cre-rec_{tufA} plasmid was removed by incubating the bacteria for 16 h in THY broth without antibiotic pressure. Finally, the erythromycin sensitive clones were analyzed by PCR for the loss of the plasmid.

2.2.5 Construction of complementation strains

Chromosomal complementation of the *ccpA* gene at its native locus was performed in this study. A linear DNA fragment was constructed by OE-PCR that consists of the upstream region of *ccpA*, the *ccpA* gene harboring a silent point mutation, an erythromycin resistance gene and the downstream sequence of *ccpA*. The complementation fragment was transformed into the *S. anginosus* Δ*ccpA* strain by CSP based transformation to construct *S. anginosus* Δ*ccpA::ccpA*. As additional control, the same complementation fragment was introduced into the *S. anginosus* wild type strain resulting in the formation of *S. anginosus::ccpA*. The transformation mixtures were plated on sheep blood agar supplemented with 1 µg ml^{-1} erythromycin and the integration and correct complementation of the *ccpA* gene was verified by PCR and sequencing with primers binding up- and downstream of the integration site.

2.2.6 Promoter reporter assay

The SLS promoter activity was determined using an EGFP reporter system as previously described [10]. Briefly, the *S. anginosus* SK52 strain carrying derivatives of pBSU409 were grown at 37 °C in THY medium supplemented with glucose, fructose or galactose with a concentration of 0 mM, 5 mM, 12.5 mM and 25 mM. After 16 h growth, the relative mean fluorescence intensity (MFI) of the bacteria was determined using a fluorescence-activated cell sorting machine. The influence of CcpA on the SLS promoter activity was investigated by transformation of the pBSU409*::sagprom* plasmid into *S. anginosus* Δ*ccpA* and subsequent measurement of the EGFP expression as described above.

To be able to characterize the influence of the predicted *cre* sites on the SLS promoter activity, we constructed pBSU409 derivatives carrying mutated SLS

promoters. The *creA* and *creB* site were deleted by OE-PCR and the resulting PCR products were cloned into pBSU409 leading to the generation pBSU409*::sagprom_creA_del* and pBSU409*::sagprom_creB_del*. The *creC* site was mutated by OE-PCR and the mutated SLS promoter was subsequently cloned into pBSU409 resulting in pBSU409*::sagprom_creC_mut*. The plasmid harboring the mutated SLS promoters were transformed into the *S. anginosus* type strain and the SLS promoter activity was determined in the presence of different glucose concentrations in the growth medium as described above. The *S. anginosus* type strain carrying pBSU409 was used as negative control and the *S. anginosus* pBSU409*::cfbprom* carrying the CAMP-factor promoter of *S. agalactiae* served as positive control during the course of the experiment [63]. The graphs in the study depict the MFI of the different strains normalized to the obtained values of the positive control measured in the presence of indicated supplementations.

2.2.7 Hemolysis assay

The hemolysis assay was performed to quantify the hemolytic activity of *S. anginosus* against human erythrocytes. Healthy adult volunteers gave written informed consent to donate blood for the use in the study and the ethics committee of the University of Ulm approved this procedure (Certificate No. 23/13). The methods were carried out in accordance with the regulations. Peripheral blood was collected and the hemolysis assay was performed as previously described with some modifications [10]. Bacterial cultures were grown for 16 h in THY medium supplemented with 0 mM, 5 mM, 12.5 mM and 25 mM glucose and the cells were treated as previously described. A two-times serial dilution of the cell suspension was performed and 100 µl of each dilution was mixed with 100 µl of a 0.5 % erythrocyte solution. The mixture was incubated at 37 °C for 1 h in a 96-well plate with conical bottom. The cells were centrifuged and 100 µl of the supernatant was transferred to a 96-well plate with flat bottom followed by the measurement of the absorption at 540 nm which corresponds to the measurement of free hemoglobin. As a negative control served PBS and ddH_2O was used as a positive control.

For the visualization of the hemolytic activity of *S. anginosus* Δ*sagA1* and *S. anginosus* Δ*sagBCD* strain a hemolytic droplet assay was performed. A total of 10 µl of a bacterial culture (OD_{600nm} 0.5) was spotted on a sheep blood agar plate and the hemolytic phenotype was assessed after 24 h incubation at 37 °C in 5 % CO_2 atmosphere.

2.2.8 Expression and purification of His-tagged CcpA

The pET21a system and *E. coli* BL21(DE3) were used to express the CcpA protein of *S. anginosus* SK52. The *ccpA* gene was amplified from chromosomal DNA with primers introducing restriction endonuclease recognition sequences on both ends of the PCR product. The gene was cloned into pET21a leading to the generation of pET21a-*ccpA*-N-His. The N-terminal His-tagged CcpA was produced by inducing the expression in the mid-logarithmic growth phase of *E. coli* BL21 with 0.1 mM isopropyl-β-D-thiogalactopyranoside. After 8 h growth at 30 °C the cells were centrifuged and the bacterial pellet was stored at -20 °C till further use. For the purification of CcpA the Protino®-Ni-TED 1000 Packed Columns Kit was used following the instructions for the purification under native conditions. Directly after the purification, the eluent containing the heterologously expressed CcpA was subjected to a buffer exchange. The elution buffer was exchanged to TKED buffer (100 mM Tris-HCl, 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol) [5] using the Amicon® Ultra Centrifugal filters. The protein concentration was determined with the Quick Start™ Bradford Protein Assay and the purity of the sample was verified using coomassie-stained gels of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot using an Anti-His-tag antibody.

2.2.9 Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed to verify the binding of CcpA to a *cre* site in the SLS promoter. Double stranded DNA probes were therefore generated by annealing sense and antisense oligonucleotides. Annealing was carried out with equal molar amounts of sense and antisense oligonucleotides in Annealing Buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, pH 8.0). The reaction was

performed in a PCR machine (5 min 95 °C, 70 cycles of 1 min reducing the temperature by 1 °C per cycle) and the oligonucleotides were purchased from metabion international AG (Planegg, Germany). The IRD700_cre_fwd oligonucleotide was ordered with the fluorescent dye IRDye700 which was attached to the 5' end of the sequence. The EMSA was carried out with increasing amounts of CcpA (0-8 µg) and a constant amount of the labeled oligonucleotide probe (5 pmol). After 30 min incubation of each reaction at room temperature in EMSA binding buffer (20 mM Tris-HCl, 1.5 mM dithiothreitol, 1 mM EDTA, 75 mM NaCl, 1 µg poly(dI-dC), 5 % glycerol, pH 8.0) in the absence or presence of specific competitor DNA, the samples were run on a native 6 % polyacrylamide gel in 0.5x TBE buffer. The migration of the DNA-protein complexes and the free DNA probe in the gel was visualized with the Odyssey Clx near-infrared fluorescence imaging system. The pictures were analyzed using the Image Studio Version 5.2 with the DNA Gel Setup and the 700 nm channel with a scan resolution of 42 µm and a focus offset of 2 mm.

2.2.10 Bioinformatical and statistical analysis

Nucleotide and protein sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). The Basic Local Alignment Search Tool at the NCBI server (http://www.ncbi.nlm.nih.gov/Blast/) was used to identify homologous sequences [6]. The further alignment of the homologous protein sequences was performed with CLC Main Workbench v7.0 (http://www.clcbio.com) and the information about metabolic pathways was extracted from the Kyoto Encyclopedia of Genes and Genomes (https://www.kegg.jp/). Experimental data is represented as the mean value ± standard deviation and the nonparametric Mann-Whitney U test was performed considering p-values smaller than 0.05 as significant.

3 Results

3.1 Cre-*lox* based genetic engineering in *S. anginosus*

3.1.1 Competence system in *S. anginosus*

Natural transformation is a widespread mechanism in bacteria that allows the uptake of extracellular DNA [46,126]. It was discovered at the beginning of the 20th century in *S. pneumoniae* [69] and it is controlled through the competence system [57]. The *com* regulon in the SAG has been detected in several genome projects [119,141] but a detailed analysis has not been performed so far.

To characterize the *com* genes of *S. anginosus*, we used the sequence of 22 genes that were reported to be essential for the transformation of *S. pneumoniae* [150] and screened for their presence in the genome of the *S. anginosus* type strain SK52. Homologs for 19 of the 22 genes could be identified in *S. anginosus* SK52 (Table 4) including all "early" genes of the regulatory cascade except for *comW* and *comC*. The absence of *comW* in *S. anginosus* SK52 prompted us to screen all available *S. anginosus* genomes in the NCBI database for the presence of a *comW* homolog. Three strains (*S. anginosus* 1_2_62CV, *S. anginosus* SK1138 and *S. anginosus* ChDC B695) with yet unfinished genomes contain a *comW* homolog with an identity of 44 % on the amino acid level. This gene is located in a different genomic region compared to the *comW* of *S. pneumoniae* and the up- and downstream genes do not share homologies. In both species the gene is located next to a transposase gene. We also failed to detect an annotated homolog of SP1808 in *S. anginosus* but a sequence showing high similarities is located in an intergenic region (ANG1_0791; ANG1_0792). The absence of a *comC* homolog in *S. anginosus* was expected as this gene encodes the CSP which is known to be diverse to limit interspecies crosstalk. To analyze the occurrence of yet unknown CSP pherotypes in the SAG, we used the published *comC* sequence of *S. anginosus* SK52 [76] and searched for homologous genes in the published SAG genomes. This led to the identification of a new CSP pherotype which is encoded in *S. anginosus* ssp. *whileyi* strains. The CSP of the type strain *S. anginosus* SK52 was designated CSP-1 and the CSP of *S. anginosus* ssp. *whileyi* as CSP-2, respectively. The presence of two CSP variants in *S. anginosus*

strains in the NCBI database prompted us to screen a collection of clinical *S. anginosus* isolates for the presence of further unknown pherotypes. A total of 76 isolates were analyzed. The majority of strains encoded CSP-1 (47/76) and CSP-2 (18/76) variants. One strain possessed a *comC* gene identical to *comC* of *Streptococcus sanguinis* SK36 and one isolate encoded a CSP that was already described for *Streptococcus milleri* NCTC10708 (CSP-3, Figure 4) [76].

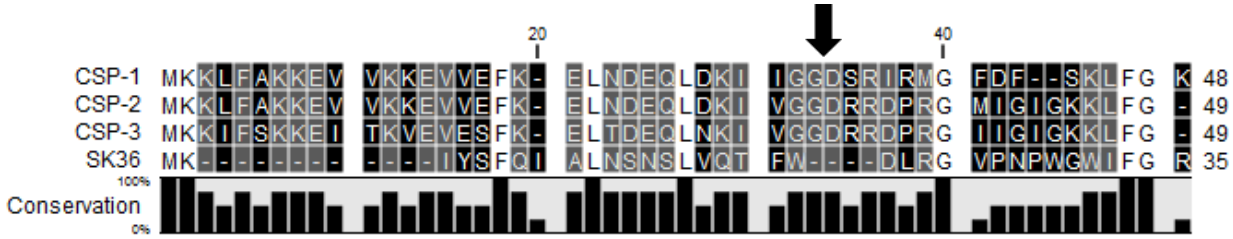

Figure 4: Alignment of ComC. The ComC protein sequences were aligned using CLC Main Workbench. Black arrow indicates peptidase cleavage site (double glycine leader). SK36: ComC sequence of *S. sanguinis* SK36 and *S. anginosus* BSU1313.

During the amplification of the *comC* genomic region, nine strains showed an increased length of the PCR product. Sequencing of the entire 2000 bp PCR product revealed that these strains contain a mobile element protein (ISL3 transposase family) inserted in the *comD* gene (Figure 5).

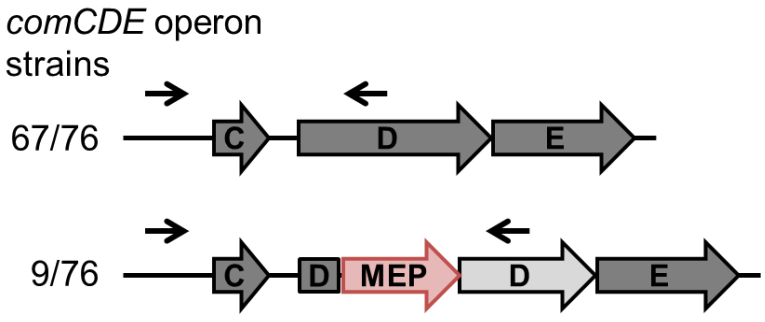

Figure 5: Schematic organization of the *comCDE* operon of *S. anginosus*. Numbers of strains with depicted organization are indicated. MEP: Mobile Element Protein. Black arrows indicate primers used for the screening of the *comC* gene.

Table 4: *Com* regulon in *S. anginosus* SK52. Genes important for transformation in *S. pneumoniae* TIGR4 were used to detect homologous genes in *S. anginosus SK52*. The coverage [%], identity [%] and positives [%] on the amino acid level are shown using tblastn from NCBI. a: *comC* no similarity between *S. anginosus* SK52 (ANG1_1284, *comC*) and *S. pneumoniae* TIGR4 (SP2237, *comC*) [19].

S. anginosus SK52 No.	Coverage [%]	Identity [%]	Positives [%]	*S. pneumoniae* TIGR4 No.	*S. pneumoniae* gene	Function
Genes early induced by CSP in *S. pneumoniae*						
ANG1_1859	96	55	77	SP0014	*comX1*	competence-specific sigma factor
ANG1_0253	96	53	67	SP0014	*comX1*	competence-specific sigma factor
no hit				SP0018	*comW*	hypothetical protein, ComX Cofactor
ANG1_0682	99	66	82	SP0042	*comA*	Transport ATP binding protein
ANG1_0683	99	34	59	SP0043	*comB*	Transport protein ComB
ANG1_1859	96	55	77	SP2006	*comX2*	competence-specific sigma factor
ANG1_0253	96	53	67	SP2006	*comX2*	competence-specific sigma factor
ANG1_1282	98	70	86	SP2235	*comE*	response regulator
ANG1_1283	77	48	69	SP2236	*comD*	putative sensor histidine kinase
no hit[a]				SP2237	*comC*	Competence stimulating peptide
Genes late induced by CSP in *S. pneumoniae*						
ANG1_0384	100	53	69	SP0954	*cilE, celA, comEA*	competence protein ComEA
ANG1_0383	100	53	71	SP0955	*celB, comEC*	competence protein ComEC
ANG1_0666	100	51	68	SP0978	*CPIP104, coiA*	competence protein CoiA
ANG1_827	99	76	87	SP1266	*dprA, dalA, cilB*	DNA processing protein DprA
not annotated	96	56	76	SP1808	*cclA, cilC*	Type IV prepilin peptidase
ANG1_0129	100	83	93	SP1908	*ssbB, cilA*	single strand binding protein
ANG1_1790	91	89	94	SP1940	*recA*	RecA protein
ANG1_0157	72	42	62	SP2047	*cglG*	conserved domain protein
ANG1_0160	99	55	69	SP2050	*cglD*	competence protein; *comGD*
ANG1_0161	93	67	76	SP2051	*cglC*	competence protein; *comGC*
ANG1_0162	99	67	84	SP2052	*cglB*	competence protein; *comGB*
ANG1_0163	100	75	84	SP2053	*cglA, CPIP186; cilD*	competence protein; *comGA*
ANG1_0799	99	48	63	SP2207	*cflB*	competence protein; *comFB*
ANG1_0800	98	72	86	SP2208	*cflA, CPIP901*	Helicase; *comFA*

3.1.2 CSP-induced transformation kinetics in *S. anginosus*

To evaluate the transformation kinetic after CSP stimulation, we tested the uptake of plasmid pAT28 after competence stimulation with CSP-1 and CSP-2 in the type strain *S. anginosus* SK52 encoding CSP-1 and *S. anginosus* BSU1216 encoding CSP-2 (Figure 6). *S. anginosus* SK52 showed a rapid competence development, reaching a maximum transformation efficiency of 0.24 % ± 0.08 % 40 min after CSP-1 stimulation followed by a fast decline in competence. The competence development could not be stimulated by the addition of the CSP-2. In contrast to this observation, the *S. anginosus* BSU1216 strain which does not encode CSP-1, showed transformants after CSP-2 as well as CSP-1 stimulation.

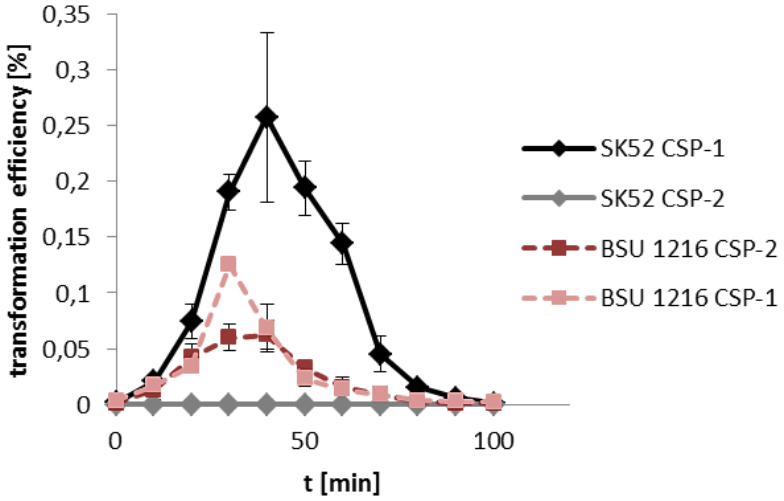

Figure 6: Development of competence in *S. anginosus* strain SK52 (encoding CSP-1) and *S. anginosus* strain BSU1216 (encoding CSP-2) following CSP-1 and CSP-2 stimulation. Transformation efficiency was determined for the uptake of plasmid pAT28. The mean values and standard deviations of five independent experiments are shown [19]. (Modified with permission by © 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.; modifications: only part A of the published figure is illustrated; the figure was colorized)

3.1.3 Combining CSP and Cre-recombinase for markerless gene deletion

The generation of deletion mutants in streptococci is currently performed by homologous recombination leading to an exchange of the target gene with an antibiotic resistance marker [179,189]. These methods are cumbersome and comprise the risk for undesired polar effects due to an introduction of the antibiotic resistance gene into the genome. To circumvent this risk, we established a method for the construction of markerless deletion mutants in *S. anginosus* based on the Cre-recombinase system which has been successfully used in other streptococcal species [16,56,102,209].

The method relies on the generation of a linear DNA fragment that consists of 500 bp fragments of the up- and downstream region of the target gene embracing a spectinomycin resistance gene (*spc*) flanked by two *lox* sites (Figure 7). The transformation of this DNA fragment was performed with the CSP leading to the uptake of the DNA and a double cross-over event that result in the incorporation of the *spc* into the desired genetic locus and the deletion of the target sequence. In a second transformation, the Cre-recombinase encoded on plasmid pAT18-cre-rec_{tufA} was introduced into the strain. The Cre-recombinase recognizes the *lox66* and *lox71* sites flanking the *spc* and removes the resistance gene leading to the formation of *lox72*. This *lox* site is a poor substrate for the Cre-recombinase [2] allowing the formation of multiple deletions in the same strain. In the last step, the pAT18-cre-rec_{tufA} is removed by culturing the deletion strain in the absence of antibiotics and subsequent screening for erythromycin sensitive clones.

The deletion procedure relies on the transformation of linear DNA into *S. anginosus*. So far, only the transformation of plasmid DNA was tested using the CSP (3.1.2). To demonstrate that *S. anginosus* SK52 can be transformed with linear DNA fragments, the transformation efficiency of the deletion construct sagA1-lox_Spc was investigated (Figure 8). Already at the beginning of the experiment, the cells showed relatively high transformation capabilities with a maximum at around 30 min after CSP-1 stimulation. The fast decline in transformation efficiency observed at 50 min after CSP stimulation is comparable to plasmid transformation but the transformation efficiency for linear DNA is decreased by one log in comparison to plasmid DNA.

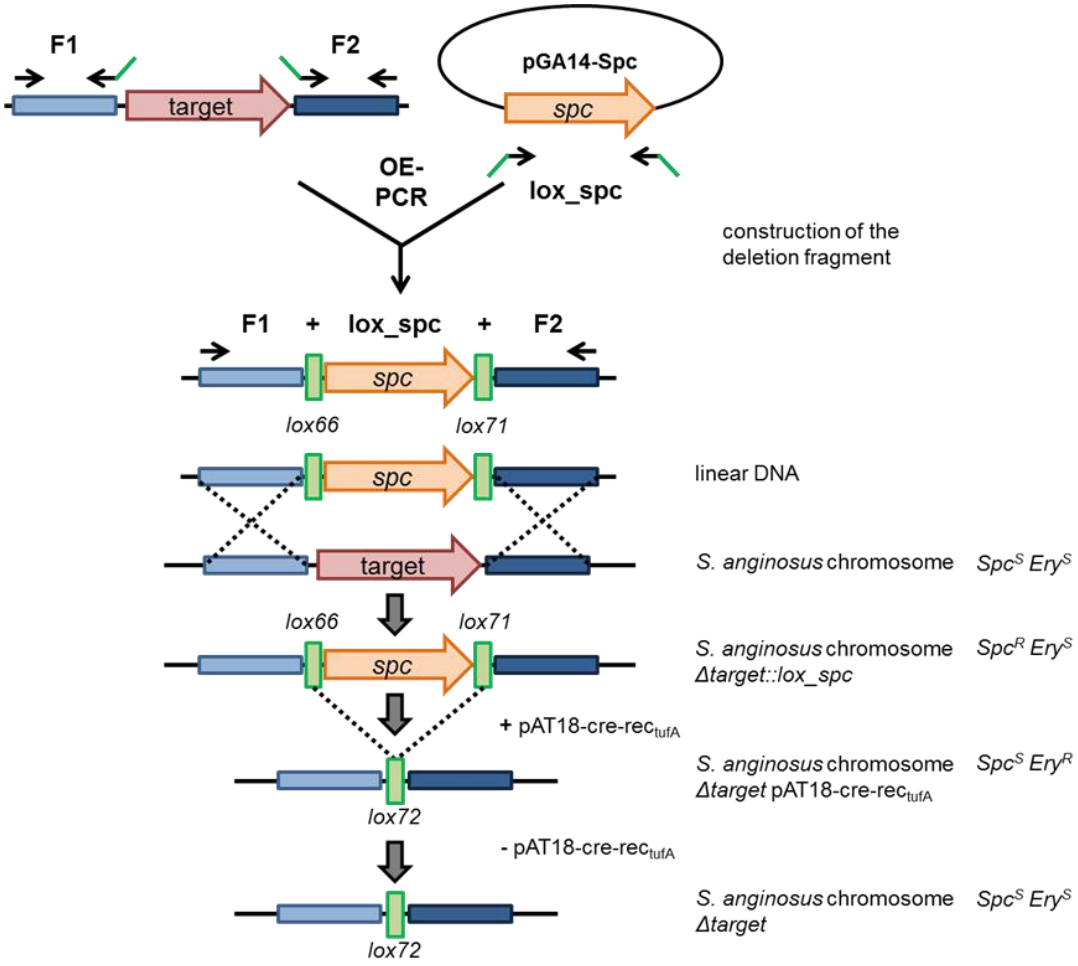

Figure 7: Schematic representation of the creation of markerless gene deletions in *S. anginosus*. In a first step, the up- and downstream regions of the target are amplified and fused to the *spc* gene flanked by *lox66* and *lox71* using OE-PCR. The linear deletion construct was transformed in *S. anginosus* using CSP. Spectinomycin resistant clones were selected and the integration of the deletion construct was verified by PCR using primers binding up- and downstream of the integration site. The pAT18-cre-rec$_{tufA}$ plasmid was transformed into spectinomycin resistant clones leading to the excision of the deletion construct and the formation of the *lox72*. Removal of the pAT18-cre-rec$_{tufA}$ plasmid was achieved by 16 h incubation in THY broth without antibiotics. Erythromycin sensitive clones were selected and analyzed for the loss of the plasmid and the deletion was verified sequencing the target locus with primers binding up- and downstream of the integration site [19]. (Modified with permission by © 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.; modifications: the figure was colorized)

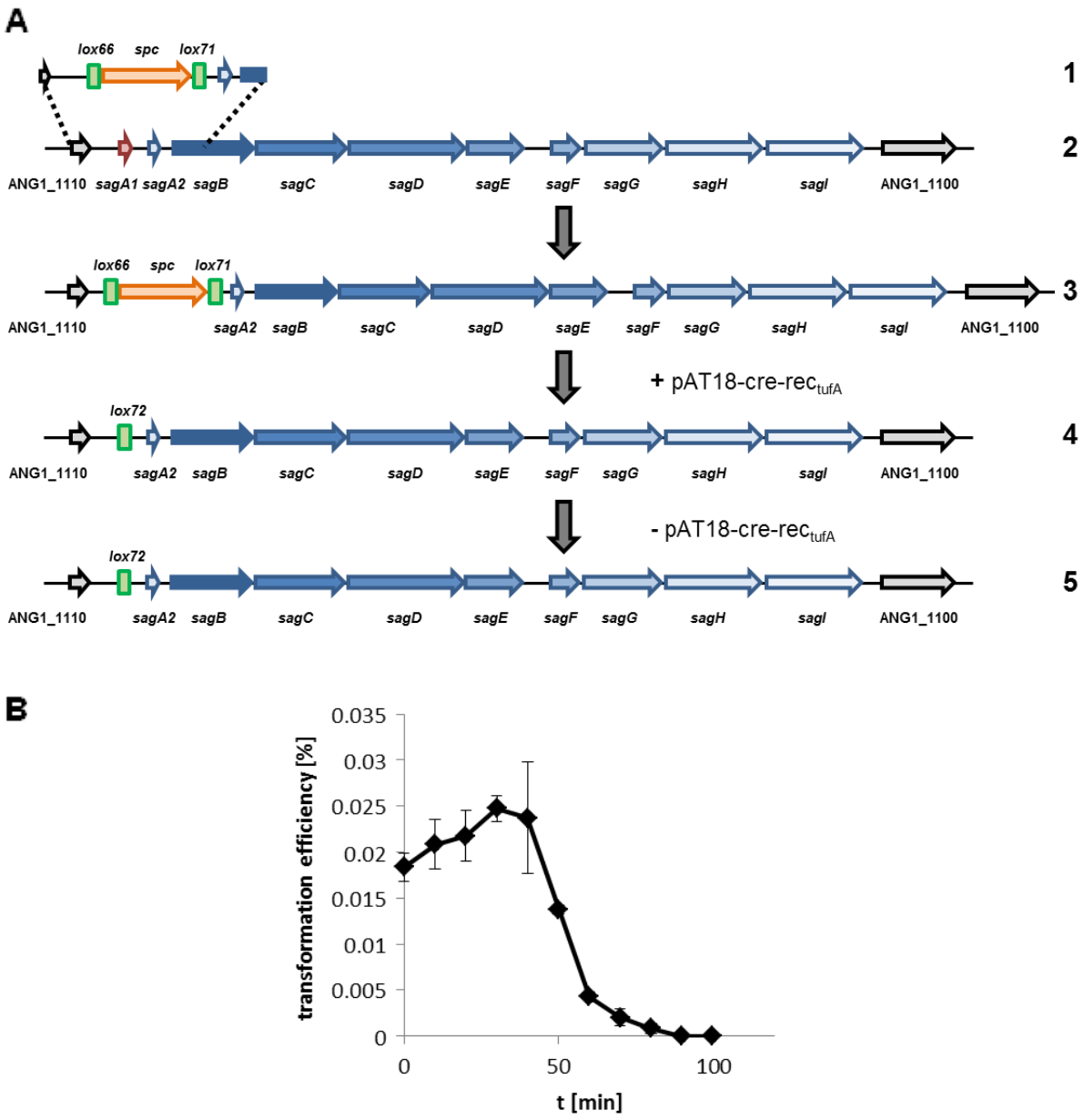

Figure 8A: Schematic representation of single steps during creation of *sagA1* deletion in *S. anginosus* SK52. The SLS operon is illustrated with the respective gene designations. 1: linear deletion construct sagA1-lox_Spc; 2: *S. anginosus* chromosome; 3: *S. anginosus* Δ*sagA1::lox_spc*; 4: *S. anginosus* Δ*sagA1* pAT18-cre-rec$_{tufA}$; 5: *S. anginosus* Δ*sagA1*.
B: Transformation efficiency of *S. anginosus* strain SK52 using CSP-1 and the linear deletion construct sagA1-lox_Spc depicted in A. The mean values and standard deviations of four independent experiments are shown [19]. (Modified with permission by © 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.; modifications: only parts of the published figures are illustrated; the figure was colorized)

To demonstrate the applicability of the deletion procedure we deleted *sagA1* and *sagBCD* in *S. anginosus* SK52. These genes are part of the SLS operon responsible for the β-hemolysin production. SagA1 encodes the hemolytic prepeptide and the proteins encoded in *sagBCD* are presumed to be responsible for the posttranslational modification of SagA. These two loci were selected to demonstrate that the method can be used to generate deletions of various sizes including small deletions (*sagA1*, 158 bp) and multi-gene deletions (*sagBCD*, 3347 bp). The single steps during the generation of the deletion mutants were controlled via PCR with primers binding up- and downstream of the integration sites (Figure 9A) and the final deletion strain was verified by sequencing the target locus. The phenotype of the resulting deletion strains was investigated in a hemolytic droplet assay demonstrating that the *S. anginosus* Δ*sagBCD* strain is non-hemolytic and the *S. anginosus* Δ*sagA1* strain shows a reduced hemolysis as described previously (Figure 9B) [189].

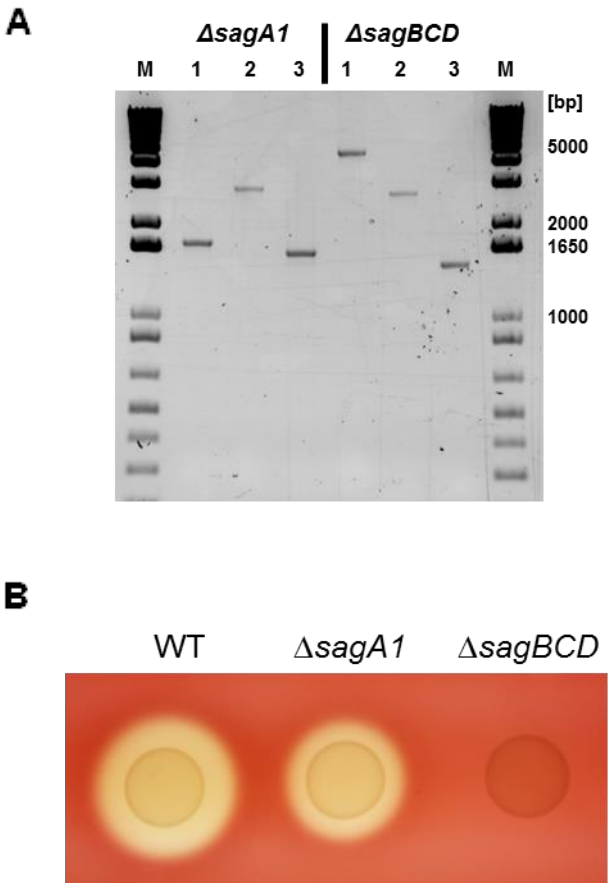

Figure 9A: PCR analysis of deletions mutants of *S. anginosus* SK52. Chromosomal DNA of the respective strains was used as template for the PCR reactions. Primer binding up- and downstream of the target locus were used to verify *sagA1* and *sagBCD* deletion. The 1 kb Plus DNA Ladder (Invitrogen) served as molecular weight marker (M). 1: *S. anginosus* SK52, 2: *S. anginosus* Δ*x::lox_spc*, 3: *S. anginosus* Δ*x*.
B: Analysis of the hemolytic activity of *S. anginosus* SK52 (WT), *S. anginosus* Δ*sagA1* and *S. anginosus* Δ*sagBCD* using a hemolytic droplet assay. A 10 µl droplet of the indicated strain (OD_{600nm} 0.5) was incubated for 24 h on sheep blood agar and the hemolytic zone was visually assessed [19]. (Modified with permission by © 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.; modifications: the figure was colorized)

3.1.4 Optimization of transformation efficiencies in *S. anginosus*

The transformation efficiencies obtained for type strain *S. anginosus* SK52 are high and suitable for the generation of deletion mutants with the above described procedure. But the transformation of clinical *S. anginosus* isolates turned out to be difficult. Only 14 of 44 tested clinical isolates were transformable with the previously described transformation method. Therefore, we aimed to optimize the transformation of *S. anginosus* SK52 to apply the changed transformation conditions to clinical *S. anginosus* strains. Increasing the DNA concentration up to 10 µg ml^{-1} in the transformation procedure resulted in the highest transformation efficiency (Figure 10A).

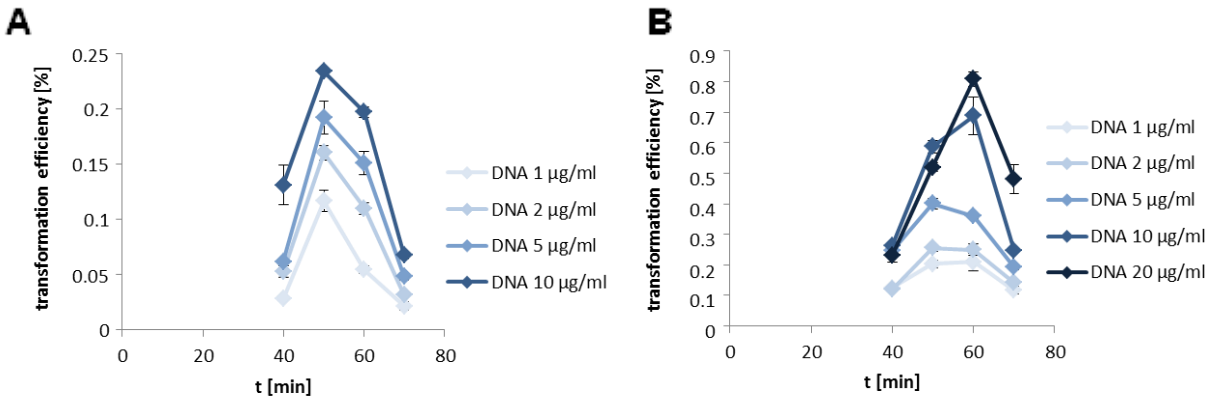

Figure 10: Transformation efficiency of *S. anginosus* strain SK52 using CSP-1 (500 ng ml^{-1}) and plasmid pAT28 with the indicated DNA concentrations. **A:** Cells were grown in THY medium. **B:** Cells were grown in THY supplemented with 10 % FBS. The mean values and standard deviations of three independent experiments are shown.

Exchanging the growth medium to THY supplemented with 10 % FBS further increased the transformation efficiency to a maximum of 0.81 % ± 0.024 (Figure 10B). To investigate the influence of the CSP concentration on the transformation, the assay was carried out using different CSP amounts (Figure 11).

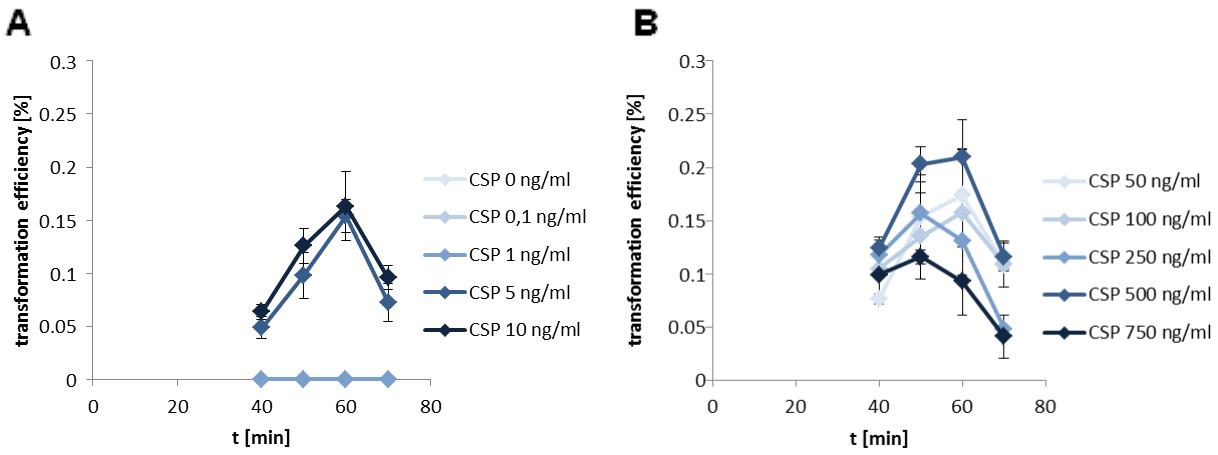

Figure 11: Transformation efficiency of *S. anginosus* strain SK52 grown in THY supplemented with 10 % FBS using plasmid pAT28 (1 µg ml^{-1}) and CSP-1 with the indicated concentrations. **A:** CSP-1 0-10 ng ml^{-1}. **B:** CSP-1 50-750 ng ml^{-1}. The mean values and standard deviations of three independent experiments are shown.

Cells grown in the presence of up to 1 ng ml^{-1} CSP did not show competence development. The highest transformation efficiency was obtained with 500 ng ml^{-1} CSP although the differences to the other tested concentrations are not significant.

The overall best results showed the transformation condition that involves the growth of cells in THY supplemented with 10 % FBS. High DNA amounts are thereby favorable and the optimal CSP concentration was 500 ng ml^{-1}. These conditions were applied to four clinical isolates BSU1211 (CSP-1), BSU1215 (CSP-2), BSU1308 (CSP-1) and BSU1312 (CSP-2) which showed no transformants using the standard transformation protocol (2.2.3). With the optimized protocol the BSU1211 strain showed transformants with a maximum transformation efficiency of 0.010 ± 0.0003. The three other strains were still not transformable with the applied method.

3.2 CcpA regulates the SLS expression in *S. anginosus*

3.2.1 SLS expression is under the control of catabolite repression

It was previously reported that the SLS expression of *S. anginosus* was reduced with increasing glucose supplementations in the growth medium, the molecular mechanisms of this effect were however not clear [12]. The glucose effect was observed using a promoter reporter system [63] that measures the EGFP expression which is under the control of the SLS promoter. The reduction of the SLS promoter activity with increasing glucose concentrations indicates a catabolite repression mechanism that controls the SLS expression. Classical CCR is mediated by the activity of CcpA but additional alternative CCR mechanisms are described that are independent of CcpA [38,198,229].

To further investigate the potential CCR at the SLS promoter, two additional sugars were tested in the reporter system (Figure 12). The sugars were selected according to their uptake mechanism and metabolism. Fructose is transported by a fructose specific PTS allowing the analysis of the effect of the glucose specific PTS on the SLS expression. The expression pattern, observed for cells grown with increasing fructose supplementation in the growth medium, resembles the EGFP expression measured with glucose indicating that proteins of the glucose specific PTS are not involved in controlling the SLS promoter activity. The other tested sugar, galactose, was chosen as this sugar is predicted to be taken up by a PTS and a non-PTS (permease) transporter and it was reported to lead to only low levels of FBP in the cell [143,144]. Increasing galactose supplementation in the growth medium did not result in reduced SLS promoter activity further indicating a CcpA dependent CCR that controls the SLS expression.

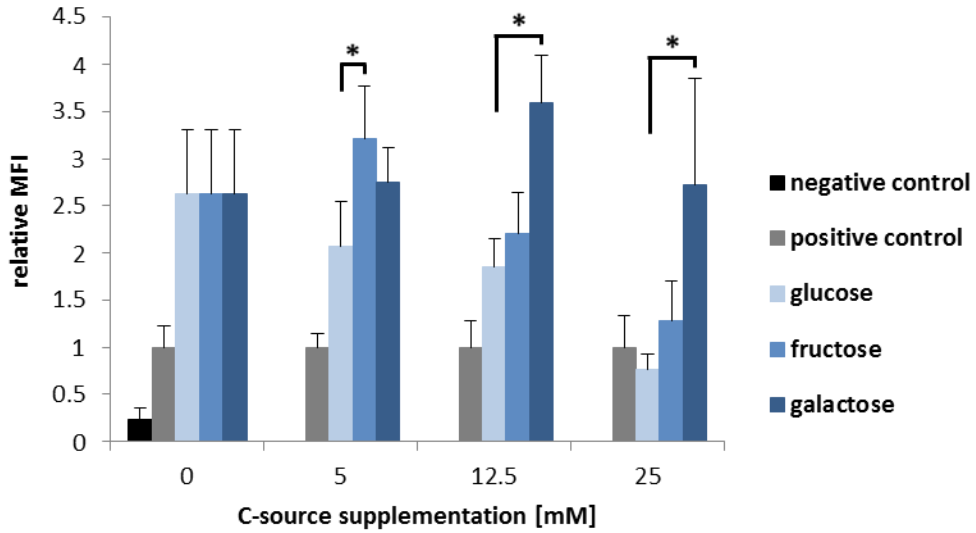

Figure 12: Effect of C-source supplementation on SLS promoter activity. The activity of the SLS promoter was determined using an EGFP reporter plasmid. The relative mean fluorescence intensity (MFI) of *S. anginosus* pBSU409*::sagprom* grown in THY medium supplemented with the indicated sugar concentrations is shown in comparison to the positive control. Negative control: *S. anginosus* pBSU409; positive control: *S. anginosus* pBSU409*::cfbprom*. The mean values and standard deviations of five independent experiments are shown. Mann-Whitney-U test was performed to illustrate significant differences to cells grown in presence of indicated glucose concentrations ($p < 0.05$) [20]. (Modified with permission by © CC BY 4.0, http://creativecommons.org/licenses/by/4.0/; modifications: the figure was colorized)

3.2.2 CcpA negatively affects SLS expression

To directly elucidate the effect of CcpA on the regulation of the SLS expression, we generated a *S. anginosus* Δ*ccpA* strain. This strain was constructed with the help of the CSP-1 and the temperature sensitive pG$^+$host5 vector system. It contains a 100 bp deletion in the *ccpA* gene that leads to a frameshift and a premature stop codon (Figure 13A). This strain was transformed with the reporter plasmid pBSU409*::sagprom* and the SLS promoter activity was analyzed in respect to different glucose supplementations in the growth medium (Figure 13B).

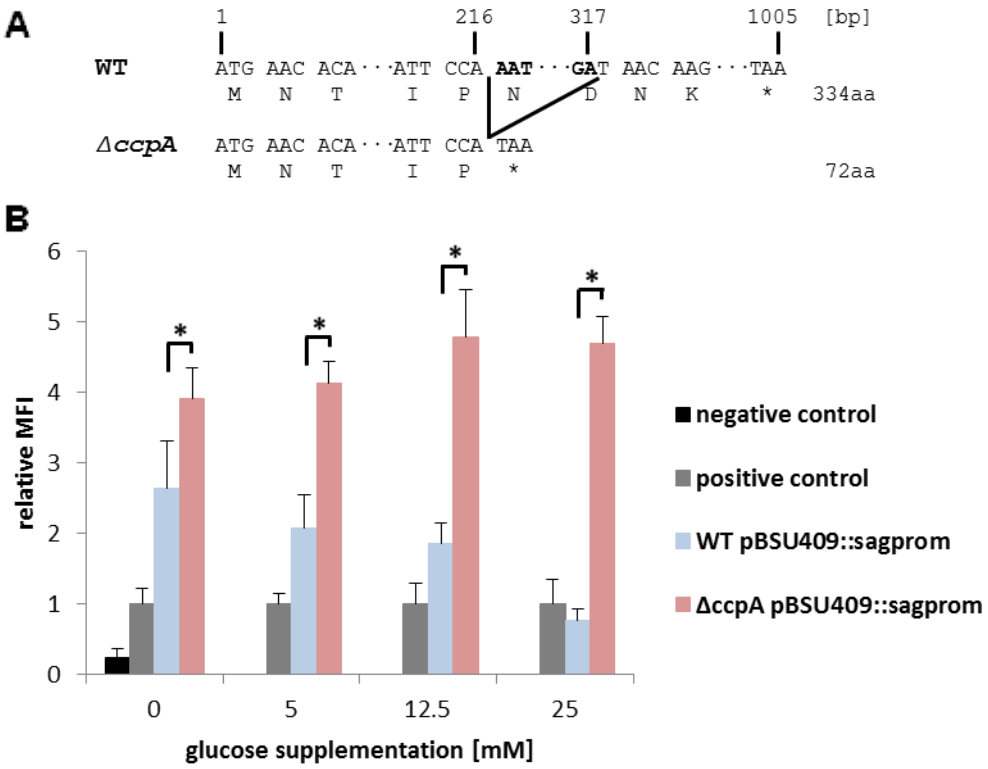

Figure 13A: Schematic representation of the 100 bp deletion in the *S. anginosus* Δ*ccpA* strain. The site of deletion is marked with black lines and the deleted base pairs in bold. The 100 bp deletion results in a frameshift and a premature stop codon (*).
B: Effect of glucose supplementation on SLS promoter activity. The activity of the SLS promoter was determined using an EGFP reporter plasmid. The relative mean fluorescence intensity (MFI) of cells grown in THY medium supplemented with the indicated glucose concentrations is shown in comparison to the positive control. Negative control: *S. anginosus* pBSU409; positive control: *S. anginosus* pBSU409*::cfbprom*; WT: *S. anginosus* type strain; ΔccpA: *S. anginosus ΔccpA* strain. The mean values and standard deviations of five independent experiments are shown. Mann-Whitney-U test was performed to illustrate significant difference to *S. anginosus* pBSU409*::sagprom* ($p < 0.05$) [20]. (Modified with permission by © CC BY 4.0, http://creativecommons.org/licenses/by/4.0/; modifications: the figure was colorized)

The reduced SLS promoter activity with increasing glucose concentrations in the *S. anginosus* wild type was absent in *S. anginosus* Δ*ccpA*. The deletion strain showed a constant EGFP expression over all tested glucose concentrations.

3.2.3 *S. anginosus* Δ*ccpA* maintains hemolytic activity in the presence of glucose

A functional hemolytic assay was performed to investigate the effect of the constant SLS expression of the *S. anginosus* Δ*ccpA* on the hemolytic activity in presence of high glucose concentrations. Two additional strains were constructed that served as controls in the assay. The *ccpA* complementation strain *S. anginosus* Δ*ccpA::ccpA* was generated by CSP-1 transformation of *S. anginosus* Δ*ccpA* with a linear DNA fragment that contained the *ccpA* gene with a silent point mutation, an erythromycin resistance gene and the up- and downstream region of *ccpA* (Figure 14). To exclude a possible effect of the erythromycin gene on the hemolytic behavior of the cells the *S. anginosus::ccpA* strain served as additional control.

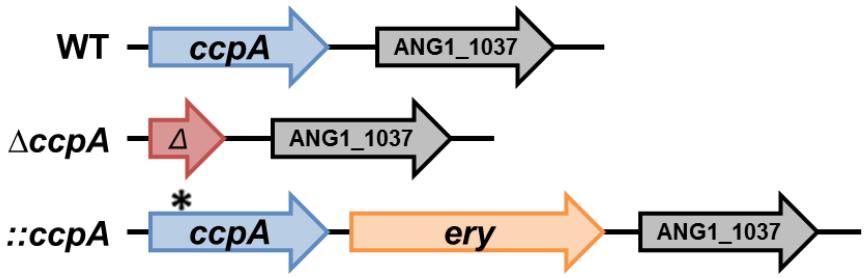

Figure 14: Schematic representation of the *ccpA* complementation strains. A linear DNA fragment containing the upstream region of *ccpA*, the *ccpA* gene carrying a silent point mutation, the erythromycin resistance gene (*ery*) and parts of ANG1_1037 was transformed with CSP-1 in *S. anginosus* Δ*ccpA* to construct *S. anginosus* Δ*ccpA::ccpA* (*::ccpA*). To exclude an effect of the integration of *ery* on the phenotype of the complemented strain, the same DNA fragment was transformed in *S. anginosus* type strain SK52 (WT) to generate *S. anginosus::ccpA* (*::ccpA*). *: silent point mutation.

The *S. anginosus* type strain was able to lyse human erythrocytes if cells were grown in the presence of up to 12.5 mM glucose (Figure 15). Cells that were incubated in medium with 25 mM glucose supplementation were non-hemolytic. The *S. anginosus* Δ*ccpA* strain in contrast performed regular β-hemolysis in presence of all tested glucose concentrations. Both complementation strains showed the same hemolytic behavior like the parental strain verifying that CcpA is responsible for the observed hemolytic difference of the *S. anginosus* Δ*ccpA* strain.

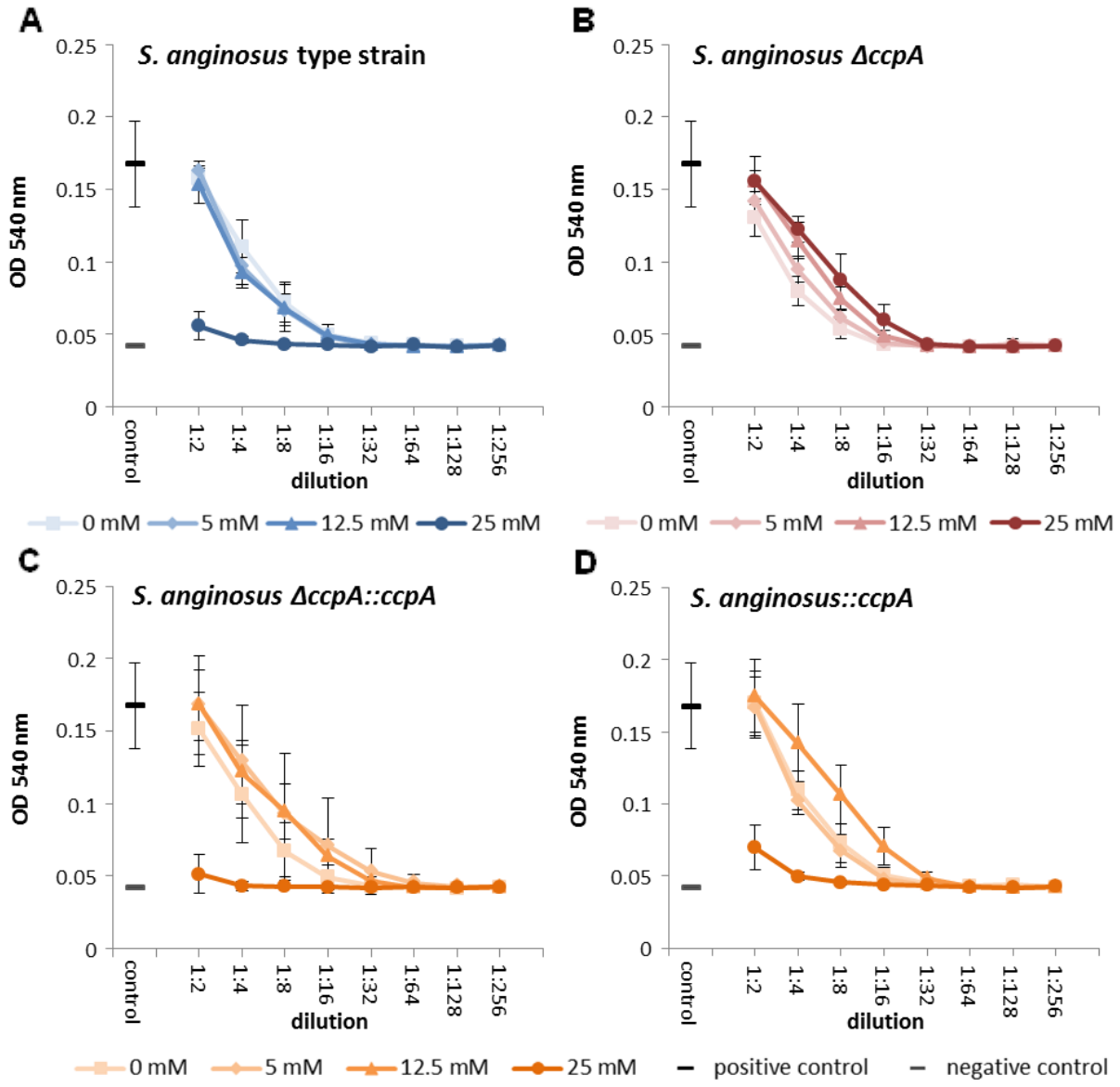

Figure 15: Hemolytic activity of *S. anginosus* strains for human erythrocytes. Bacteria were grown in THY medium supplemented with the indicated glucose concentrations. The hemolytic behavior of the *S. anginosus* type strain **(A)**, the *S. anginosus ΔccpA* **(B)**, the *S. anginosus ΔccpA::ccpA* **(C)** and the *S. anginosus::ccpA* **(D)** is illustrated for different bacterial cell dilutions. Positive control: ddH_2O; negative control: assay buffer. The mean values and standard deviations of five independent experiments are shown [20]. (Modified with permission by © CC BY 4.0, http://creativecommons.org/licenses/by/4.0/; modifications: the figure was colorized)

3.2.4 Mutation of *cre* abolishes glucose dependent SLS repression

Binding of CcpA to its target sequence (*cre*) is reported in different Gram-positive bacteria and consensus sequences of *cre* are published (http://regprecise.lbl.gov/RegPrecise/collection_tf.jsp?collection_id=163). Additionally, a second CcpA binding site was identified in *Streptococcus suis* (*cre2*) [219]. We used this information to screen the promoter of the SLS operon for the presence of potential CcpA binding sites to further investigate if CcpA directly regulates the SLS expression by binding to the SLS promoter. Three potential *cre* sites were identified in the SLS promoter allowing a maximum of three mismatches to the consensus sequences (Figure 16).

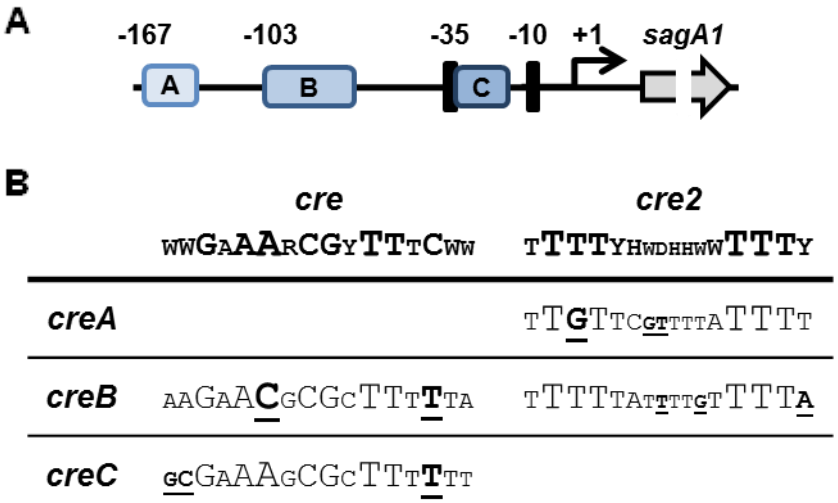

Figure 16A: The locations of putative *cre* sites are illustrated in respect to the transcription start site (arrow) of the SLS promoter. A: *creA*, B: *creB*, C: *creC*.
B: Comparison of the *in silico* predicted *cre* in the SLS promoter and the published consensus *cre* sites. The size of the single nucleotides corresponds to their conservation. The *creB* site consists of a *cre* homolog overlapping a potential *cre2* site. Mismatches to the consensus sequences are underlined and bold [20]. (Modified with permission by © CC BY 4.0, http://creativecommons.org/licenses/by/4.0/; modifications: the figure was colorized)

CreA is located 167 bp upstream of the transcription start site and shows homologies to the published *cre2* site. One predicted CcpA binding site (*creB*) is composed of a putative *cre* site overlapping a potential *cre2* site and the third *cre* site (*creC*) overlaps the promoter of the SLS operon.

To investigate the influence of the predicted *cre* sites on the expression of the SLS promoter in the presence of glucose, we mutated these sites in the promoter and performed a promoter reporter assay using three pBSU409*::sagprom* derivatives. The *creA* and *creB* site were separately deleted in the promoter (Figure 17A). The deletion of *creC* would destroy the bacterial promoter, thus this site was mutated in four potentially conserved nucleotides that should be important for the binding of CcpA to this sequence. The altered promoters were cloned in front of *egfp* in the reporter plasmid and transformed into the *S. anginosus* type strain SK52 to measure the promoter activity of cells grown in the presence of different glucose concentrations (Figure 17B). Deletion of *creA* resulted in a similar expression pattern like in the strain carrying the wild type promoter that showed a reduction of promoter activity with increasing glucose supplementations. The strain with the *creB* deletion showed a reduced promoter activity without glucose supplementation compared to the strain that contains the wild type promoter and further demonstrated a constant *egfp* expression for all tested glucose concentrations. In comparison to the wild type promoter, the *egfp* expression was higher for cells growing in presence of 25 mM glucose supplementation. Differing from the previous attainments, the mutation of *creC* shows a constantly high promoter activity irrespective of the glucose supplementations. This expression pattern closely resembles the promoter activity measured in the *S. anginosus* Δ*ccpA* strain.

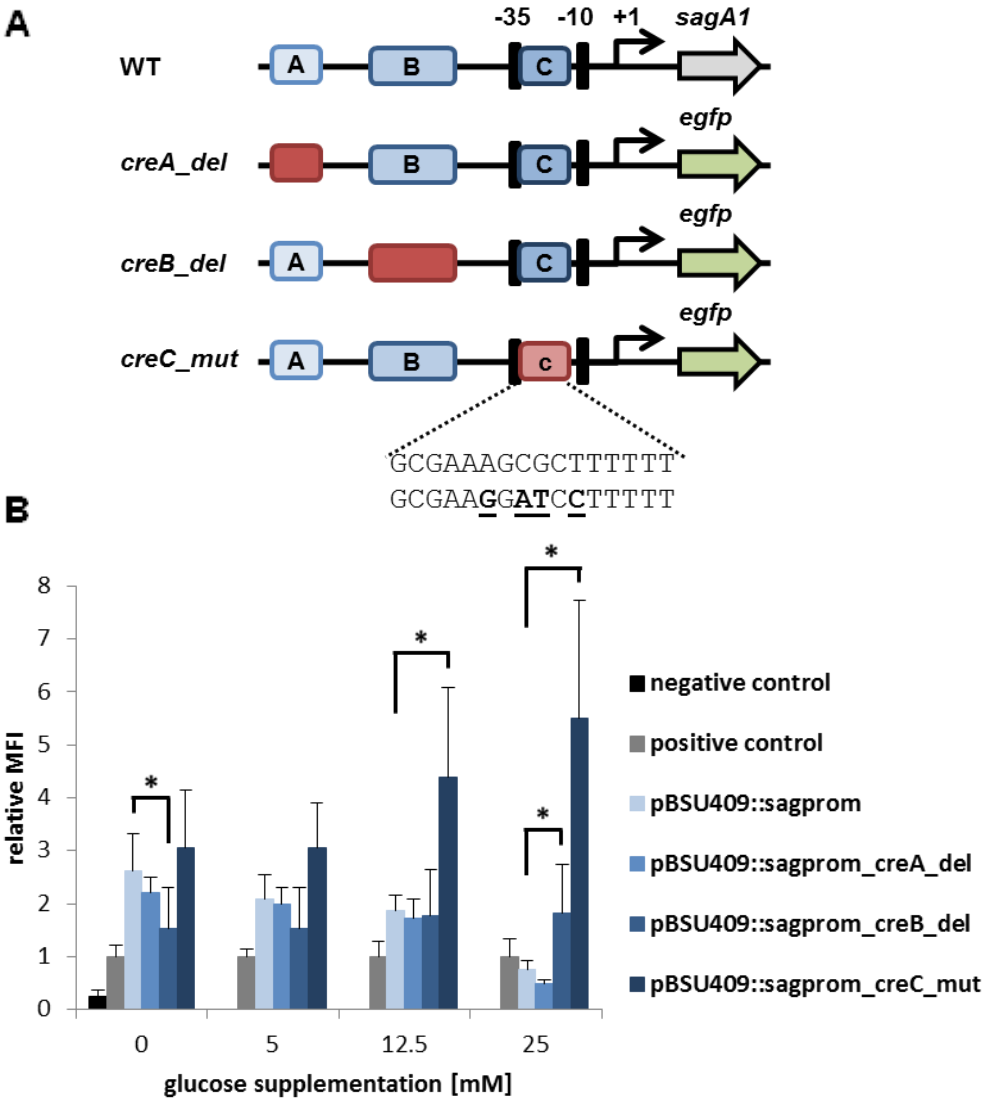

Figure 17: Schematic representation of reporter plasmids with mutated SLS promoters used to investigate the *in silico* predicted *cre* sites. WT: promoter of SLS; *creA_del*: pBSU409*::sagprom_creA_del* (pBSU881); *creB_del*: pBSU409*::sagprom_creB_del* (pBSU880); *creC_mut*: pBSU409*::sagprom_creC_mut* (pBSU876). c: The sequence of the predicted *cre* site (upper row) and the mutations of the sequence (bold and underlined) in pBSU409*::sagprom_creC_mut* (lower row) are shown.
B: Effect of glucose supplementation on the activity of mutated SLS promoters in the *S. anginosus* type strain SK52. The relative mean fluorescence intensity of cells grown in THY medium supplemented with the indicated glucose concentrations is shown in comparison to the positive control. Positive control: *S. anginosus* pBSU409*::cfbprom*; Negative control: *S. anginosus* pBSU409. The mean values and standard deviations of five independent experiments are shown. Mann-Whitney-U test was performed to illustrate significant difference to *S. anginosus* pBSU409*::sagprom* ($p < 0.05$) [20]. (Modified with permission by © CC BY 4.0, http://creativecommons.org/licenses/by/4.0/; modifications: the figure was colorized)

3.2.5 CcpA binds to *creC in vitro*

To verify that CcpA binds to the *in silico* predicted *creC* site in the SLS promoter, we performed EMSA experiments. We used heterologously expressed His-tagged CcpA in the absence of HPr as several *in vitro* studies reported that CcpA is able to bind to *cre* sites in the absence of its activator [1,5,197,219]. Increasing the amounts of CcpA in the bandshift reaction induced a shift of the labeled *creC* DNA fragment (GTTTAC**GCGAAAGCGCTTTTTT**TATATA) demonstrating that CcpA is able to bind to this sequence (Figure 18). The specificity of binding was tested using 500-fold molar excess of unlabeled *creC* as well as unlabeled mutated *creC* (GTTTAC**GCGAAGGATCCTTTTTT**TATATA). The addition of excess unlabeled *creC* was able to compete for CcpA binding and shows no shift of the labeled *creC*. In contrast to that the mutated *creC* sequence was unable to inhibit the binding of CcpA to *creC*. The observation that the deletion of *creB* in the promoter reporter assay resulted in a different expression pattern compared to the wild type promoter prompted us to test also the binding of CcpA to this sequence. The use of 500-fold excess of unlabeled *creB* had no effect on the CcpA binding to the *creC* sequence further demonstrating the specificity of CcpA binding to *creC*. Hence we were able to verify that CcpA can directly regulate the SLS expression by binding to the *creC* site that we identified in the SLS promoter.

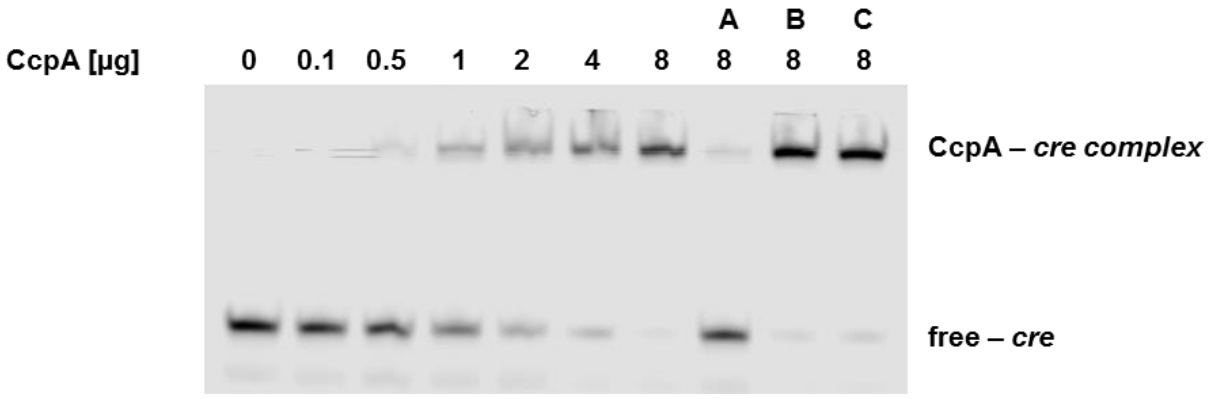

Figure 18: EMSA of *creC* using His-tagged CcpA. Increasing amounts of purified CcpA (0-8 µg) were used and tested for binding to labeled *creC* (GTTTAC**GCGAAAGCGCTTTTTT**TATATA). The specificity of binding was assayed using 500-fold molar excess of unlabeled *creC* (A), *creC_mut* (B; GTTTAC**GCGAAGGATCCTTTTTT**TATATA) and *creB* (C; TGCTAT**AAGAACGCGCTTTTTATTTTGTTTTA**GATGGT) [20].

3.3 Role of a CovR homolog in the regulation of SLS in *S. anginosus*

3.3.1 *In silico* analysis and genetic organization of the CovR homolog

The publication of the complete genome sequence of some *S. anginosus* strains in 2013 provided evidence for a CovR homolog (CovR*) in SAG [141]. CovR is the response regulator of the well-known TCS CovSR that is involved in the regulation of virulence gene expression in GAS and GBS [53,103,128]. Additionally, CovR was reported as regulator of the SLS operon in GAS [60]. Thus, we hypothesized a role of the CovR* in the regulation of hemolysis in *S. anginosus*.

The proposed CovR* shows similarities to other streptococcal CovR proteins and consists of an N-terminal signal receiver domain and a C-terminal OmpR-like winged helix-turn-helix DNA binding domain. But a further blast search revealed that CovR* is more closely related to a response regulator that can be found in *S. pneumoniae*, *Streptococcus oralis* and *Streptococcus mitis* (Figure 19A). CovR of GBS and GAS are well studied and it was reported that the phosphorylation of CovR at positions D53 and T65 are important for the regulation of the CovR activity [82,111]. The CovR* of *S. anginosus* is highly conserved in different strains (Figure 19B) but it lacks these phosphorylation sites (Figure 19C).

A

	identity	similarity
GBS	45 %	67 %
GAS	48 %	69 %
S. mutans	47 %	67 %
S. pneumoniae	69 %	82 %
S. oralis	72 %	82 %
S. mitis	72 %	83 %

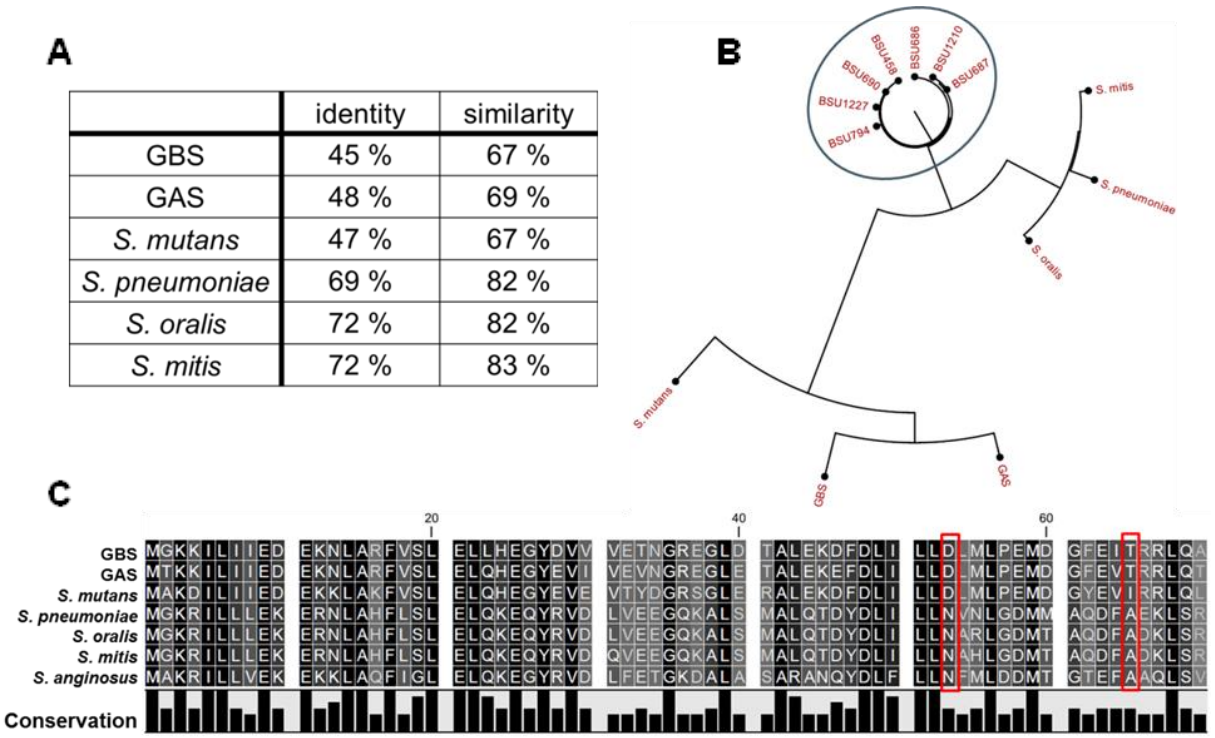

Figure 19A: Comparison (amino acid level) of CovR* of *S. anginosus* to CovR of GBS, GAS and *S. mutans* and a regulator of *S. pneumoniae*, *S. oralis* and *S. mitis*.
B: Circular phylogram showing the conservation of CovR* in different *S. anginosus* strains (blue circle). CovR* was amplified and sequenced with primers binding up- and downstream of the gene.
C: Alignment of CovR* of *S. anginosus*, CovR of GBS, GAS and *S. mutans* and a regulator of *S. pneumoniae*, *S. oralis* and *S. mitis*. The first 70 amino acids are shown. Red boxes highlight the phosphorylation sites of GBS and GAS which are important for the regulation of CovR activity.

Instead of being organized in one operon with *covS*, *covR** of *S. anginosus* is located directly downstream of the 6-phosphogluconate dehydrogenase gene (*gnd*) and a homolog of the sensor histidine kinase/phosphatase CovS could not be detected in a genome wide BLAST search (Figure 20A). CovR* of the *S. anginosus* type strain is the last gene of a contig in the NCBI database. Therefore, the downstream gene was assumed to be a homolog to the *covR** downstream genes located in the genome of completely sequenced *S. anginosus* strains like *S. anginosus* C1051. All sequenced *S. anginosus* strains contain a gene encoding an enzyme IIBCA component of the glucose-specific PTS downstream of *covR**. Due to the close proximity of *covR** to *gnd*, a RT-PCR was performed confirming that the two genes are organized in one operon (Figure 20B).

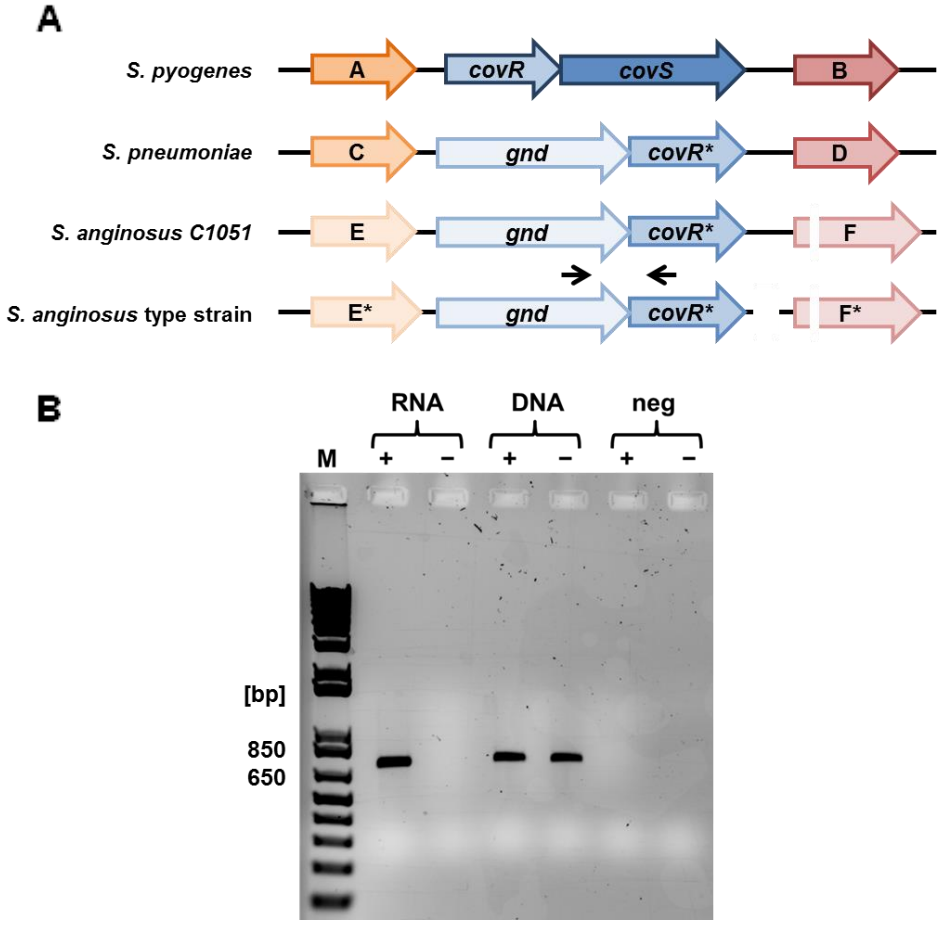

Figure 20A: Organization of *covR* encoding genomic region in *S. pyogenes* M1, *S. pneumoniae* R6, *S. anginosus* C1051 and *S. anginosus* type strain SK52. *gnd*: 6-phosphogluconate dehydrogenase; A: Spy_0334; B: Spy_0338; C: spr_0334; D: *cpbF*; E: SAIN_1372; F: PTS glucose-specific EIICBA component; E* homolog of E; F* homolog of F. Arrows indicate positions of primer used in the RT-PCR experiment.
B: Agarose gel of RT-PCR using template as illustrated (neg: H_2O) proving the organization of *covR** and *gnd* in one operon. +: with reverse transcription step, −: without reverse transcription step.

3.3.2 A *covR** integration mutant shows increased hemolysis

CovR has been shown to control hemolysis genes in GAS and GBS [60,103]. To elucidate the effect of CovR* on the hemolytic activity of *S. anginosus* SK52, we constructed a *covR** integration mutant (*S. anginosus::pG*$^{+}$*host5_covR**) and tested it in a hemolytic assay with cells that were grown in the presence of different glucose concentrations in the growth medium (Figure 21).

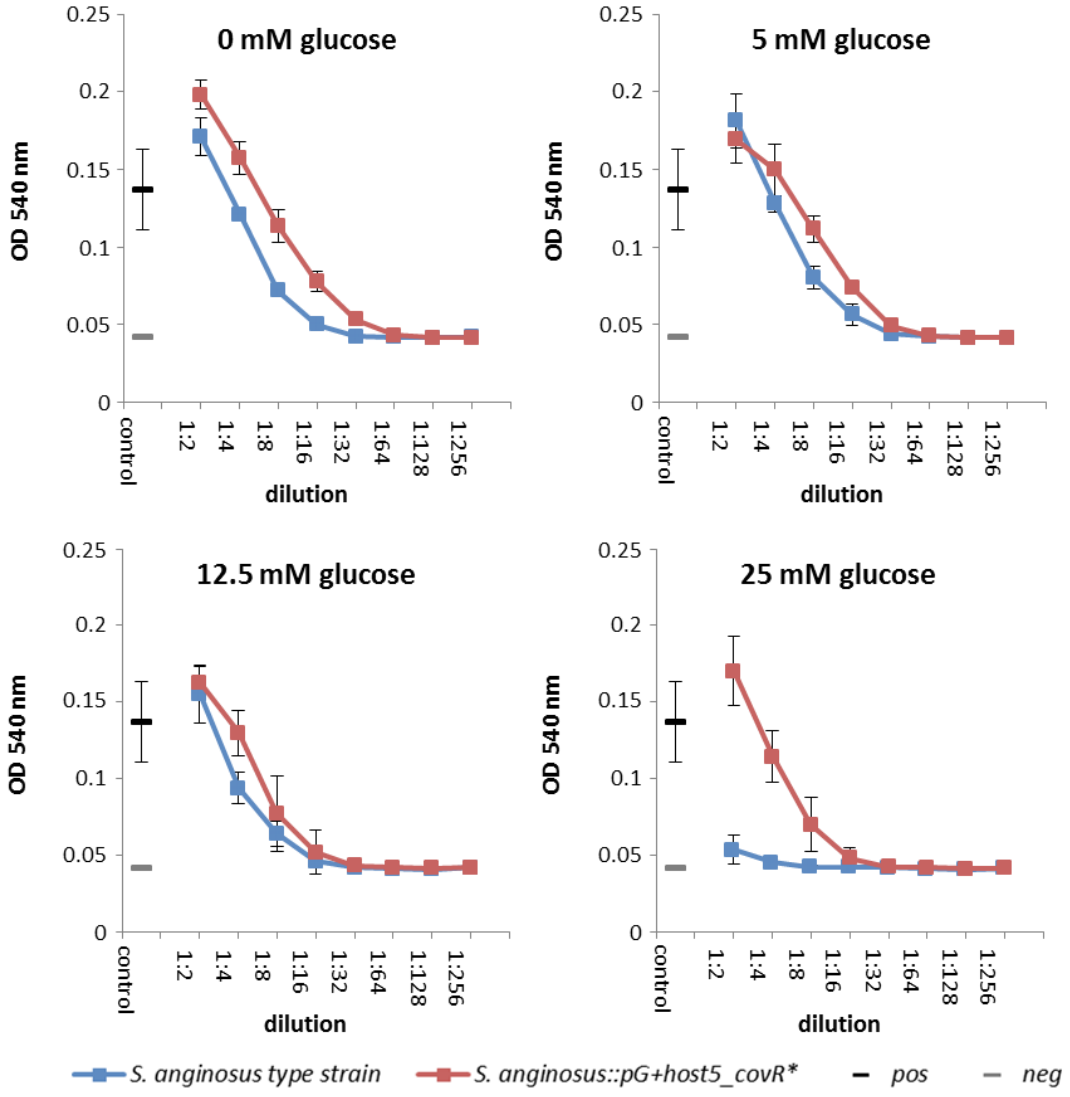

Figure 21: Hemolytic activity of the *S. anginosus* type strain and *S. anginosus::pG*$^{+}$*host5_covR** for human erythrocytes. Bacteria were grown in THY medium supplemented with indicated glucose concentrations. The hemolytic behavior of the strains is depicted for different cell dilutions. Positive control: ddH_2O; negative control: assay buffer. Mean values and standard deviations of three independent experiments are shown.

The hemolytic activity of the *S. anginosus::pG*$^{+}$*host5_covR** strain was slightly increased compared to the parental strain if cells have been grown in medium supplemented with 0, 5 and 12.5 mM glucose. In the presence of 25 mM glucose, the *covR* integration mutant was still able to lyse the erythrocytes whereas the type strain showed no hemolytic activity at all.

3.3.3 Markerless *covR** deletion mutant shows type strain hemolytic behavior

To exclude possible polar effects of the pG^{+}host5 integration into the *covR** locus on the hemolytic behavior of the strain, a markerless *covR** deletion mutant was constructed using the Cre-lox based genetic manipulation procedure [19]. Therefore, the downstream region of the *covR** locus needs to be amplified to be able to generate the deletion. This region is not available in the NCBI database, thus we assumed that the genome of the *S. anginosus* type strain is organized like the other completely sequenced *S. anginosus* strains. According to the available genetic information of other *S. anginosus* strains, primers were designed that bind downstream of their *covR** in the gene encoding a PTS glucose-specific EIICBA component. Several attempts to amplify this region in the *S. anginosus* type strain failed (data not shown). Thus, an inverse PCR was performed verifying that the *S. anginosus* type strain SK52 contains a different genetic organization downstream of *covR** (Figure 22).

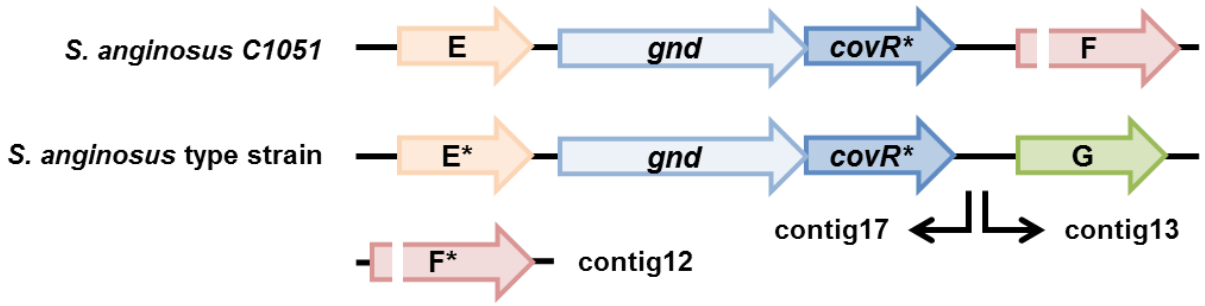

Figure 22: Organization of *covR** encoding genomic region in *S. anginosus* C1051 and *S. anginosus* type strain SK52. *gnd*: 6-phosphogluconate dehydrogenase; E: SAIN_1372; F: PTS glucose-specific EIICBA component; E* homolog of E; G: ANG1_1764, hypothetical protein. F* homolog of F; Accession numbers: contig17 BAST01000017.1; contig13 BAST01000013.1; contig12 BAST01000012.

With this knowledge, a *covR** deletion mutant was constructed and its hemolytic activity was tested (Figure 23). In contrast to the *S. anginosus::pG*$^{+}$*host5_covR** integration mutant, the clean deletion mutant *S. anginosus* Δ*covR** showed a similar behavior like the *S. anginosus* type strain in the hemolysis assay with no hemolytic activity of cells grown in the presence of 25 mM glucose supplementation.

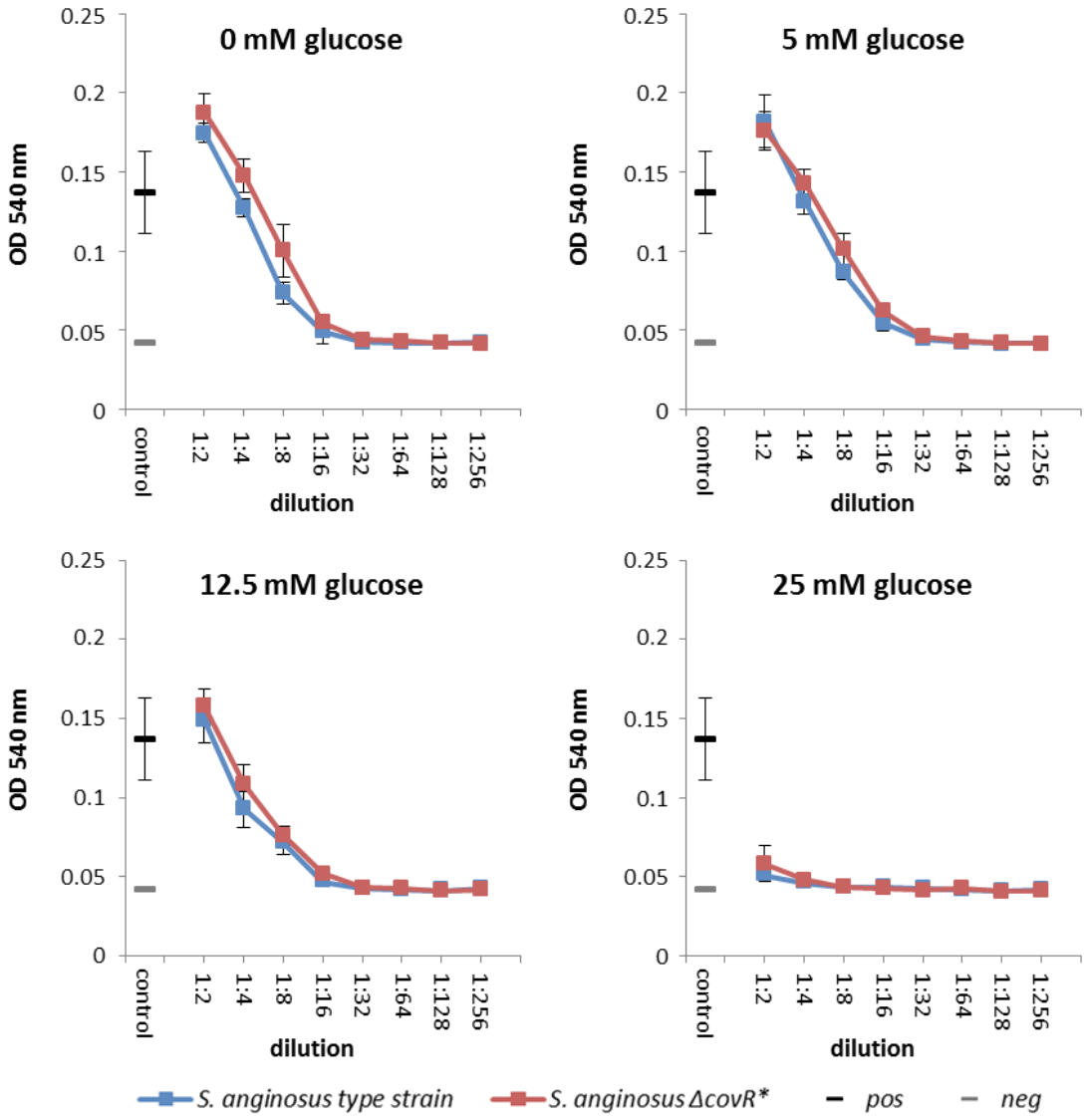

Figure 23: Hemolytic activity of the *S. anginosus* type strain and *S. anginosus* Δ*covR** for human erythrocytes. Bacteria were grown in THY medium supplemented with indicated glucose concentrations. The hemolytic behavior of the strains is depicted for different cell dilutions. Positive control: ddH_2O; negative control: assay buffer. Mean values and standard deviations of three independent experiments are shown.

4 Discussion

S. anginosus is considered a human commensal but an increasing number of reports in recent years highlighted the clinical relevance of this species [94,101,104,105,157,163,175]. Despite the rise of epidemiological knowledge about *S. anginosus*, the knowledge about the virulence gene repertoire is scarce [11]. One obstacle is the lack of genetic manipulation techniques that would allow fast and reliable investigations of molecular pathogenicity mechanisms in *S. anginosus*. The identification of the *com* regulatory system in different viridans streptococci revolutionized the scope for genetic manipulations in these species and the presence of competence genes in the genome of several *S. anginosus* strains was already reported [76,119,141] although an analysis of the transformation machinery has not been performed so far. In *S. pneumoniae*, the presence of 22 genes is required for transformation [150]. We used the sequence of these genes to find homologs in the genome of the *S. anginosus* type strain. While 19 genes were annotated in *S. anginosus*, three homologs were missing (SP1808, *comW* and *comC*). SP1808 encoding a type IV prepilin peptidase is not annotated in the genome but a sequence with high identity is located in an intergenic region indicating the presence of this gene in *S. anginosus*. The second gene for which a homolog could not be detected in *S. anginosus* SK52 is *comW*. ComW is reported as stabilizer and activator of ComX and thus as crucial for competence development in *S. pneumoniae* [188,199]. The absence of this gene in *S. anginosus* SK52 prompted us to screen all the available *S. anginosus* genomes in the NCBI database for the presence of a *comW* homolog leading to the identification of three *S. anginosus* isolates that contain a homolog. The protein encoded in this gene shares only an identity of 44 % and a similarity of 63 % on the amino acid level to ComW of *S. pneumoniae*. Therefore, it is tempting to speculate that the identified homologs share the same function as ComW. This would need further investigations by either deleting these genes in the strains or by introducing this homologous gene into *S. anginosus* SK52 followed by the analysis of the transformation behavior of the manipulated strains. Nevertheless, the absence of a ComW homolog in *S. anginosus* SK52 demonstrates that this species, in contrast to *S. pneumoniae*, does not need ComW for competence development. This is line with

the observation that *Streptococcus gordonii* shows competence development [75] although this species does also not encode a ComW homolog [119].

The absence of a homolog of the *S. pneumoniae comC* in *S. anginosus* was certainly expected as diversity of the CSP encoded in *comC* is crucial to limit interspecies crosstalk of the competence system [76,211]. We therefore used the published *comC* sequence of *S. anginosus* SK52 [76] to screen for yet unknown CSP pherotypes in SAG. Genetic variation in *comC* within the same species is common in different viridans streptococci [3,76,154] and we were able to identify two major CSP pherotypes in *S. anginosus*. According to the designation in *S. pneumoniae*, we termed the pherotypes CSP-1 and CSP-2 [154]. The *S. anginosus* BSU1417 isolate encoded a third CSP variant that is identical to the CSP described in *S. milleri* NCTC10708 [76], which is the former designation for the *S. anginosus*. All three ComC variants contain the double-glycine leader motif that allows the proteolytic cleavage of the prepeptide by ComAB which results in the formation of the CSP [74]. One *S. anginosus* isolate contains a *comC* identical to *comC* of *S. sanguinis* SK36. The ComC prepeptide of this strain lacks the double-glycine motif, but it was reported that the C-terminal region (DLRGVPNPWGWIFGR) is sufficient to induce competence [76,162]. The remaining nine *S. anginosus* isolates contain a Mobile Element Protein inserted in the *comD* gene. This protein belongs to the ISL3 transposase family which seems to be common in *S. anginosus* as one completely sequenced *S. anginosus* strain J4206 contains six copies of this gene located in different genetic loci. The integration of the transposase in the *comD* gene probably results in the disruption of the competence regulation. However, this needs to be verified by further investigations.

Although the CSP was already used to transform *S. anginosus* SK52 [189], an analysis of the transformation kinetics has so far not been performed. Therefore, we investigated the uptake of plasmid DNA of cells grown in the presence of the CSP. After CSP stimulation, the bacteria showed rapid competence development indicating a *S. pneumoniae*-like regulation cascade [161,199] that controls the competence in *S. anginosus*. The slower competence development using plasmid DNA in *S. anginosus* SK52, with a maximum transformation efficiency after ~ 40 min compared to *S. pneumoniae* (~ 20 min), could potentially be explained by the slower

growth behavior of *S. anginosus*. The uptake of linear DNA, in contrast, could already be observed in the beginning of the transformation experiment. One conceivable reason for this could be the lower complexity of the linear DNA in comparison to plasmid DNA which was reported as a factor that can affect transformation efficiencies [93,165]. The presence of two major CSP pherotypes further prompted us to test the cross-reactivity of the variants in the competence development. The *S. anginosus* SK52 developed competence only after stimulation with its cognate CSP-1, whereas the *S. anginosus* BSU1216, encoding CSP-2, showed considerable transformation with both CSP pherotypes. At first sight, this was an unexpected observation as the diversity of *comC* should limit crosstalk [76,211], but it was already reported that some *S. pneumoniae* strains can also recognize other than the self-expressed CSP variants [154,225].

By employing the gained knowledge about the competence system in *S. anginosus*, we aimed to establish a fast and reliable genetic manipulation technique that would facilitate the investigation of pathogenicity mechanisms in this species. Thereby we focused on the generation of markerless gene deletions as gene deletions in the SAG are currently constructed via allelic exchange of the target gene with an antibiotic resistance marker [88,189,202]. This method comprises the risk of undesired polar effects on the transcription of up- and downstream genes and the introduction of multiple gene deletions in the same strain can be limited by the availability of suitable antibiotic resistance markers. Therefore, we decided to establish a Cre-recombinase based genetic engineering technique that circumvents these potential problems by removing the antibiotic resistance gene from the bacterial genome. Additionally, this method does not comprise time consuming cloning procedures which are necessary in case of temperature sensitive vectors that are used in different Gram-positive bacteria for the generation of markerless gene deletions [24]. The mutation technique further relies on the transformation of linear DNA with the CSP and subsequent removal of the resistance gene via the Cre-recombinase. Thereby the mutated *loxP* sites *lox66* and *lox71* are fused to one *lox72* site [2]. This *lox* site is a poor substrate for the Cre-recombinase and thus allows the generation of multiple deletions in the same strain. To confirm that this novel mutation technique can be applied for gene deletions of various sizes, we deleted *sagA1* (158 bp) and *sagBCD* (3347 bp) from

the β-hemolysin gene cluster of *S. anginosus* SK52. The mutant strains possessed the expected hemolytic phenotype on blood agar plates with the *sagA1* mutant showing a reduced hemolysis as this strain encodes two SagA homologs [10,189].

Transformation of clinical bacterial isolates can be challenging [133,154,220]. Therefore, we aimed to optimize the transformation conditions to be able to transform clinical *S. anginosus* isolates that proved to be untransformable with the current method. The growth of the bacteria in medium containing serum was reported to enhance transformation frequency [149,153]. The use of 10 % FBS in the growth medium, together with an increase of the DNA concentration in the transformation procedure, yielded a maximal transformation efficiency of 0.81 % in the *S. anginosus* type strain. The concentration of the CSP had only a minor effect on the transformation frequency. Cells were transformable in the presence of a low level of CSP (5 ng ml^{-1}) which correlates to a concentration of 2.6 nM. This CSP concentration is in the range that was previously reported to induce genetic competence in different streptococci [73,165]. The highest transformation efficiency was observed using 500 ng ml^{-1} CSP although the difference to other concentrations was only minor. It has to be pointed out that the transformation efficiencies measured during the investigation of the transformation kinetics (3.1.2) were higher than during the optimization of the procedure (3.1.4) using the same transformation set up. The only deviation was that we used a new batch of CSP in the optimization which could be the reason for the observed difference. We applied the optimized conditions to four clinical *S. anginosus* isolates that could not be transformed with the standard procedure. Using the optimized method, one of these isolates (*S. anginosus* BSU1211) could be transformed with the pAT28 plasmid. This demonstrates that further optimization is needed and further attempts on improvements of the procedure will concentrate on the bacterial number and growth phase that were used in the transformation which could probably lead to further enhanced transformation efficiencies [35,165]. However, we were able to show that the competence system in *S. anginosus* can be used for genetic modifications that will facilitate to investigate this species on the molecular level.

Molecular pathogenicity mechanisms in *S. anginosus* are widely unknown [11]. One exception is the hemolysin that shows high homologies to SLS of GAS [10,12,189].

It was previously reported that the expression of the SLS in *S. anginosus* was reduced in the presence of increased glucose concentrations in the growth medium [12]. This expression pattern indicates a CCR mechanism controlling the SLS expression. To further investigate this phenomenon, we performed an EGFP promoter reporter assay with different sugars. We decided to use fructose as this sugar is transported by a fructose-specific PTS into the cell. Thus, through the use of fructose, we can exclude that the parts of the glucose-specific PTS regulate the SLS expression as it was reported that components of PTS systems can regulate gene expression independently of CcpA [198,229]. The SLS expression measured with increasing fructose supplementation in the growth medium resembled the expression pattern observed with the addition of glucose. This indicates that components of the glucose-specific PTS are not involved in the observed concentration dependent reduction of EGFP expression and further points towards a CCR mechanism that is regulated by CcpA. Additionally, we tested galactose in the reporter assay. This sugar was chosen as *S. anginosus* encodes the Leloir as well as the tagatose 6-phosphate pathway for galactose metabolism. The Leloir pathway starts with an unphosphorylated galactose that is transported via a permease and is further metabolized to FBP. For the tagatose 6-phosphate pathway, the galactose needs to be transported via a PTS, as galactose 6-phosphate is the first metabolite in this pathway. The presence of both galactose metabolism pathways in the genome of *S. anginosus* indicates that this bacterium transports galactose by PTS and non-PTS (permease) transporters like *S. pneumoniae*. For *S. pneumoniae*, it was shown by *in vivo* ^{13}C-NMR and ^{31}P-NMR that cells grown in presence of galactose accumulated only low amounts of FBP in comparison to cells grown in the presence of glucose [143,144]. Thus galactose should not be able to activate CcpA in a comparable manner like glucose, as high FBS levels are needed to activate the kinase activity of the HPr kinase/phosphorylase that controls the HPr serine phosphorylation accompanied with the CcpA activation [42]. Therefore, we should not observe a markedly reduced EGFP expression with increasing galactose supplementation in the growth medium and this we could demonstrate with the reporter system. The use of fructose and galactose in summary pointed towards a CCR mechanism at the SLS operon that is dependent on CcpA.

To further corroborate the CcpA dependent CCR, we constructed a *ccpA* deletion mutant by pG$^+$host5 mutagenesis [24]. The temperature sensitive plasmid pG$^+$host5 containing the deletion sequence for *ccpA* was thereby transformed with CSP-1 into *S. anginosus*. The vector was directly forced to integrate into the genome by growing the transformation mixture at 39 °C. This procedure finally allowed the construction of a 100 bp deletion in the *ccpA* gene that resulted in a frameshift and a premature stop codon. The *S. anginosus* Δ*ccpA* strain was then investigated in the reporter assay showing that the glucose dependent reduction of the promoter activity observed in the parental *S. anginosus* SK52 strain is absent in the *ccpA* mutant. Thus, we were able to demonstrate that CcpA regulates the SLS expression by inhibiting transcription at high glucose levels. During the reporter plasmid analysis we observed an increase of the relative MFI for mutant cells grown in the absence of glucose supplementation (Figure 13B). This could be explained by the use of the THY growth medium that already contains about 11 mM glucose.

We further analyzed the effect of the observed expression patterns in a functional hemolysis assay with human erythrocytes which showed that, in contrast to the parental *S. anginosus* SK52 strain, the *ccpA* mutant was able to lyse the erythrocytes also in the presence of high glucose concentrations. To verify that this observation results from the *ccpA* mutation, we constructed the complementation strain *S. anginosus* Δ*ccpA::ccpA*. We chose to use a chromosomal complementation as the introduction of the *ccpA* gene into the mutant by a plasmid would lead to an increased copy number of *ccpA* in the cell. As *ccpA* is a major regulator, this could have pleiotropic effects on the global gene expression pattern. The chromosomal complementation could be achieved by CSP driven transformation of a linear DNA fragment containing the *ccpA* gene and an erythromycin resistance gene. This complemented strain showed a similar behavior like the *S. anginosus* type strain and showed hemolytic activity up to 12.5 mM glucose supplementation, but was non-hemolytic in presence of 25 mM glucose supplementation. To exclude an effect of the introduced erythromycin resistance gene on the expression of *ccpA*, we additionally generated a *S. anginosus::ccpA* strain by transformation of the complementation construct into the *S. anginosus* type strain. This strain also demonstrated a type strain like behavior, finally confirming the role of CcpA in the regulation of the hemolysis.

The expression of up to 10 % of genes is affected by CcpA dependent CCR in different bacteria [134,219,227]. CcpA thereby mainly regulates genes involved in metabolism, but it also regulates the expression of other transcription factors [5,8,134]. This implies that the observed regulation of the SLS expression by CcpA could be direct by binding of CcpA to the SLS promoter or indirect by CcpA regulating the expression of an alternative transcription factor that in turn regulates the SLS expression. To investigate these two possibilities, we screened the SLS promoter for the presence of potential CcpA binding sites. This led to the identification of three potential *cre* sites in the SLS promoter with high homologies to the published consensus *cre* sites [116,219]. The predicted CcpA binding site *creC* overlaps the bacterial promoter and, as the location of the *cre* site in respect to the transcriptional start site determines the effect of the regulation, the position of *creC* would indicate that CcpA is a strong repressor of the SLS operon [116]. We decided to determine the effect of the putative *cre* sites on the glucose dependent reduction of the SLS promoter activity by mutating these sites in the reporter plasmid pBSU409*::sagprom*. The *creA* and *creB* site were separately deleted and the *creC* was mutated in four potentially conserved nucleotides as the deletion of this site would probably destroy the SLS promoter. The deletion of *creA* demonstrated a promoter activity with increasing glucose supplementation that closely resembles the expression pattern measured for the wild type promoter. Thus, this predicted *cre* site can be excluded as possible CcpA binding site. The deletion of *creB*, in contrast, resulted in an altered promoter activity. Without glucose supplementation, the relative MFI was decreased in comparison to *S. anginosus* pBSU*::sagprom* and it was constant for all tested glucose supplementations which resulted in a higher promoter activity for cells grown with 25 mM glucose supplementation in comparison to the strain carrying the wild type promoter. The most noticeable promoter activity alteration could be observed for the mutation of *creC*. This strain did not show the glucose dependent reduction of EGFP expression with increasing glucose concentrations in the growth medium. The expression pattern of this strain resembles the pattern measured in the *S. anginosus* Δ*ccpA* strain. Accordingly, the *creC* site was identified as the most promising CcpA binding site in the SLS promoter of *S. anginosus* SK52.

The final verification of direct regulation of the SLS operon by CcpA was performed by EMSA. It was previously reported that CcpA is able to bind to *cre* sites in the absence of serine phosphorylated HPr and FBP *in vitro* [1,5,197,219]. We therefore used heterologously expressed His-tagged CcpA alone and demonstrated that CcpA is able to induce a shift of the *creC* site in a concentration dependent manner. The binding could be inhibited using an excess of unlabeled *creC* and the specificity of CcpA-*creC* complex formation was tested with an unlabeled mutated *creC* sequence which was not able to compete for CcpA binding. Previous studies reported that more than one *cre* site can be present in the promoter of a CcpA regulated gene [116,219]. As we observed in the promoter reporter assay that the strain carrying the promoter with the deletion of the *creB* site showed an increased relative MFI at 25 mM glucose supplementation compared to the *S. anginosus* pBSU409*::sagprom* strain, we decided to test if CcpA is able to bind to this *creB* sequence. We therefore used an excess of unlabeled *creB* in the EMSA, but this sequence could not inhibit the binding of CcpA to the *creC* site, demonstrating that, under the conditions tested, CcpA does not bind to *creB*. It has to be noted that we did not incorporate serine phosphorylated HPr in our EMSA which was reported to increase the affinity of CcpA to the *cre* sites [67,167] and thus we cannot rule out that CcpA might be able to bind to the *creB* sequence *in vivo*. However, we were able to demonstrate that CcpA regulates the SLS expression and binds to a *cre* site in the SLS promoter. This is the first report of a regulator controlling a potential virulence factor in *S. anginosus* and it further highlights the role of CcpA in the regulation of the interplay between carbohydrate metabolism and virulence in Gram-positive species [160,173]. Efficient acquisition and metabolism of nutrients is required for growth in free-living as well as in host-associated bacteria. However, bacteria living in hostile environments have to cope with fluctuations in the availability of carbon sources. To deal with these diverse host environments, the bacteria have evolved virulence factors that facilitate the nutrient acquisition. Thus, the link of pathogenesis and access to nutrition explains why pathogenic bacteria concurrently use regulators of the metabolism to regulate expression of virulence determinants [160,172,180]. CcpA is a well investigated regulator in this context and it was demonstrated that the SLS of GAS is under the control of CcpA mediated CCR [99]. It was proposed that the SLS plays a role in the

secondary metabolism during infection. As a cytolysin, it may contribute to the nutrient release from host cells that can promote the growth of the bacterium. However, the tissue damage leads to host immune response and thus the SLS expression in GAS is tightly controlled by CcpA as well as by other regulatory systems (CovR, CodY, RofA, Mga) [21,77,97,115].

Although the knowledge about pathogenicity mechanisms in *S. anginosus* is scarce [11], the publication of the first complete *S. anginosus* genome in 2013 allows the prediction of potential virulence factors in this species [141]. Olson and colleagues proposed in this publication that the *S. anginosus* C238 strain encodes a *covR** homolog. CovR is the response regulator of the two component system CovSR and was originally identified in GAS [51,109]. It regulates the expression of virulence related genes in GAS, GBS and *S. dysgalactiae* as well as in *S. mutans* [45,68,103,182]. Additionally, it was reported that *covR* directly regulates the SLS expression in GAS and a *covSR* mutant in GBS exhibited an increased hemolytic phenotype [60,103]. Therefore we hypothesized that the *covR** homolog in *S. anginosus* could also be involved in the regulation of the hemolysin expression in this species.

We therefore constructed the *S. anginosus covR** integration mutant using the pG$^+$host5 vector system. We decided to test the hemolytic activity of this *S. anginosus::pG$^+$host5_covR** strain grown in presence of different glucose concentrations as the up- and the potential downstream gene of *covR** are supposed to be involved in glucose metabolism and regulators often control the expression of genes which are located in close proximity in the genome. The upstream gene encodes a 6-phosphogluconate dehydrogenase gene (*gnd*) which is part of the pentose phosphate pathway. For the downstream gene, we assumed that the *S. anginosus* type strain genome is organized like the available completely sequenced *S. anginosus* genomes. This was necessary as the *covR** homolog is located at the end of contig17 of *S. anginosus* SK52 that is published in NCBI. The predicted downstream gene was a PTS glucose-specific EIICBA component, further suggesting a role of *covR** in the regulation of metabolism that in turn might affect hemolysis as already observed for the *S. anginosus* Δ*ccpA* strain. The *S. anginosus::pG$^+$host5_covR** strain showed a slightly increased hemolytic activity

for cells that were grown in the presence of up to 12.5 mM glucose supplementation and the hemolytic activity was retained, in contrast to the parental strain, for cells grown in medium with 25 mM glucose supplementation. This resembled the hemolytic phenotype of the *S. anginosus* Δ*ccpA* strain.

The insertion of the pG$^+$host5 plasmid into the *covR** locus could have polar effects on the expression of up- and downstream genes. As we established the Cre-lox based mutagenesis tool that allows the markerless deletion of genes, we constructed a *S. anginosus covR** deletion strain and tested this strain in the hemolysis assay. For the generation of the *S. anginosus* Δ*covR** strain, we needed to amplify the downstream region of *covR**. It thereby turned out that the predicted downstream gene (PTS glucose-specific EIICBA component) is not located at the supposed position in the *S. anginosus SK52* genome. Thus, we performed an inverse PCR verifying that the *S. anginosus* type strain comprises a different genetic organization at this genomic locus. The downstream gene of *covR** in *S. anginosus* SK52 is ANG1_1764, a hypothetical protein without predicted function. We used this knowledge and constructed the *covR** deletion strain. This *S. anginosus* Δ*covR** strain showed, in contrast to the *S. anginosus::pG$^+$host5_covR**, a similar hemolytic behavior like the parental *S. anginosus* SK52 strain. Two possible explanations could be the reason for the observed hemolytic difference of the deletion strain in comparison to the integration strain. It would be conceivable that the integration of the pG$^+$host5 vector altered the expression level of *gnd* leading to a change in the carbon flux in the cell to a dominant pentose phosphate pathway. This would result in a decrease of ATP production and FBS concentration in the cell that could influence the CcpA activation. On the other hand, possible undesired multiple integrations of pG$^+$host5 in the *S. anginosus::pG$^+$host5_covR** are conceivable which could have effects on the hemolytic activity. However, this demonstrates that the construction of markerless gene deletions with the Cre-lox system can prevent possible constraints.

In the meanwhile, we further investigated the *covR** homolog in *S. anginosus*. We showed that *covR** is conserved in different clinical *S. anginosus* isolates, but it lacks the phosphorylation sites that were reported for CovR in different streptococcal species [82,83,96,111]. Instead of being transcribed in one operon with *covS*, the *covR** in *S. anginosus* is located in an operon with *gnd* and a CovS homolog could

not be detected in the *S. anginosus* genome. It has to be mentioned that *S. mutans* also lacks a *covS* homolog and the CovR in this species was reported as an orphan regulator [32]. The *covR* locus in *S. mutans* and GAS show high similarities not only on the amino acid level of CovR but also the *covR* surrounding genes in both species are similar, favoring the description of the *S. mutans* gene as *covR*. In *S. anginosus* the lack of CovS, the missing phosphorylation sites and the organization of *covR** in an operon with *gnd* strongly indicate that the proposed *covR** homolog in *S. anginosus* should not be denominated as *covR*. This, once again, is illustrated by the presence of a gene in *S. pneumoniae*, *S. mitis* and *S. oralis* that shows a high identity on the amino acid level to the proposed CovR* in *S. anginosus*. This gene was reported as *ritR* (repressor of iron transport) in *S. pneumoniae* and thus we suggest that the proposed *covR** homolog in *S. anginosus* should be declared as a *ritR* homolog. The *ritR* in *S. pneumoniae* is also cotranscribed with *gnd*, further strengthening this nomination [204].

The increasing amount of publicly available genome data can help to understand and predict pathogenicity mechanisms but the described data above highlight that the experimental verification of predicted gene functions is still crucial. It finally turned out that the predicted CovR* homolog is a RitR homolog in *S. anginosus* and is not involved in the regulation of hemolysis under the tested conditions. However, as a described regulator of iron transport and as RitR was shown to be required for lung pathogenicity in *S. pneumoniae* [194], it is worth to further investigate its role in the virulence of *S. anginosus*.

5 Summary

While *S. anginosus* is considered a human commensal of mucosal membranes, increasing number of reports showed their isolation from invasive infections highlighting the clinical relevance of this species. We aimed to establish a fast and reliable genetic manipulation technique to be able to investigate *S. anginosus* on the genetic level which would help to increase our scarce knowledge about the pathogenicity mechanisms in this species. Therefore, we analyzed the competence system of *S. anginosus* demonstrating that two major CSP pherotypes evolved in different strains. The CSP can be used to efficiently transform the *S. anginosus* type strain with plasmid as well as linear DNA, allowing the exchange of target genes with antibiotic resistance markers that can be removed using the Cre-lox system. This genetic manipulation technique facilitates the introduction of gene deletions in *S. anginosus* as it does not rely on time consuming cloning procedures allowing the construction of *S. anginosus* mutant strains within two weeks. We used the knowledge about the competence system to investigate the role of CcpA and a proposed CovR* homolog on the regulation of hemolysin expression. While the proposed CovR* homolog turned out to be a RitR homolog and was not involved in the regulation of hemolysis under the tested conditions, the CcpA was verified to directly regulate the SLS expression. We were able to demonstrate that different sugars had a different influence on the SLS promoter activity in an EGFP based reporter system, indicating a CCR mediated by CcpA and we further confirmed this by the generation of a *ccpA* deletion mutant. The findings could be reproduced using a functional hemolytic assay and we verified the effect of CcpA on the SLS expression by constructing a *ccpA* complementation strain. Analysis of the SLS promoter finally led to the identification of a CcpA binding site that was verified by the promoter reporter assay using mutated promoter sequences as well as electrophoretic mobility shift assays. Thus, we were able to demonstrate for the first time the regulation of a potential virulence factor in the emerging pathogen *S. anginosus* and we established a Cre-lox based genetic engineering technique that will facilitate the investigation of pathogenicity mechanisms in this species.

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7 List of Publications

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8 Acknowledgements

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