ciaR impacts biofilm formation by regulating an arginine biosynthesis pathway in *Streptococcus sanguinis* SK36

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*Streptococcus sanguinis* is an early colonizer of the tooth surface and competes with oral pathogens such as *Streptococcus mutans* to maintain oral health. However, little is known about its mechanism of biofilm formation. Here, we show that mutation of the *ciaR* gene, encoding the response regulator of the CiaRH two-component system in *S. sanguinis* SK36, produced a fragile biofilm. Cell aggregation, *gtfP* gene expression and water-insoluble glucan production were all reduced, which suggested polysaccharide production was decreased in ΔciaR. RNA sequencing and qRT-PCR revealed that arginine biosynthesis genes (*argR, argB, argC, argG, argH* and *argJ*) and two arginine/histidine permease genes (SSA_1568 and SSA_1569) were upregulated in ΔciaR. In contrast to ΔciaR, most of strains constructed to contain deletions in each of these genes produced more biofilm and water-insoluble glucan than SK36. A ΔciaRΔargB double mutant was completely restored for the *gtfP* gene expression, glucan production and biofilm formation ability that was lost in ΔciaR, indicating that argB was essential for ciaR to regulate biofilm formation. We conclude that by promoting the expression of arginine biosynthetic genes, especially argB gene, the ciaR mutation reduced polysaccharide production, resulting in the formation of a fragile biofilm in *Streptococcus sanguinis*.

Biofilms are microbial communities embedded in a self-produced matrix of extracellular polymeric substances of bacterial origin. They are variously composed of polysaccharides, proteins, nucleic acids and lipids, which mediate cell adhesion to solid surfaces and form cohesive, three-dimensional polymer networks. Clinically, biofilms are a significant risk factor in medical-device related infections and are highly associated with chronic infections, such as infective endocarditis, periodontal disease and cystic fibrosis.

In the oral cavity, biofilm in the form of dental plaque is a highly organized, multi-species network initiated by the colonization of oral streptococci. *Streptococcus sanguinis*, an indigenous gram-positive bacterium, has long been recognized as a pioneering colonizer, aiding in the attachment of succeeding organisms, and a key player in plaque biofilm development. Several studies have examined the importance of *S. sanguinis* in early colonization. It has been demonstrated that *S. sanguinis* can compete with pathogenic bacterial species associated with oral diseases. One of the most well-studied examples is the antagonism between *S. sanguinis* and *Streptococcus mutans*, a predominant contributor to dental caries formation. However, there are only a few studies focusing on the mechanism of biofilm formation by *S. sanguinis*.

Arginine is reported to be detrimental for biofilm formation in *S. mutans*. Treatment with 15 mg/mL of L-arginine (a clinically effective concentration) decreased the proportion of *S. mutans*, increased the proportion of *Streptococcus gordonii*, and maintained *Actinomyces naeslundii* proportion within biofilms. Moreover, L-arginine treatment reduced the amount of insoluble extracellular polysaccharide production, which significantly altered the architecture of the biofilm in *S. mutans*. In addition, metabolism of arginine by bacteria possessing the arginine deiminase system results in increased pH, which protects against caries caused by *S. mutans* and other acidic bacteria.

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The two-component regulatory system (TCS) CiaRH has been shown to affect β-lactam resistance, cell lysis, genetic competence and virulence in Streptococcus pneumoniae21–24. In S. mutans, CiaRH controls bacteriocin production, genetic competence, and tolerance to environmental stresses25–27. In addition, the biofilm formation of a ciaR mutant was defective in S. mutans, but the reason for this effect was unclear27.

CiaR is a response regulator of the CiaHR two-component system. For S. pneumoniae and S. mutans, activated CiaR acts as a repressor of DNA uptake by reducing the concentration of the competence stimulating peptide (CSP), a small (16 to 19-residue) unmodified peptide pheromone28–30. CiaR represses conC (the gene responsible for CSP biosynthesis) at the transcriptional level through single ssRNAs in S. pneumoniae29 and promotes the transcription of htrA, whose protein product directly digests CSP30,31. The genetic competence system can also influence biofilm formation as CSP-induced cell death was shown to contribute to the release of chromosomal DNA into the extracellular matrix of S. mutans biofilms32–34.

Amino acid alignments suggest that SSA_0959 encodes CiaR and SSA_0960 encodes CiaH in S. sanguinis SK36. Secondary structures of CiaH and CiaR were predicted by SMART (http://smart.embl.de)35. CiaH has two transmembrane domains, a phosphoacceptor domain, and a histidine kinase-like ATPase domain, which presumably senses a stimulus and transfers a signal to the response regulator (Fig. S1). CiaR contains a CheY-homologous receiver domain and a transcriptional regulatory domain, which likely receives the signal and mediates the cellular response (Fig. S1).

In this study, we showed that mutation of ciaR in S. sanguinis SK36 resulted in a fragile biofilm. By testing the concentration of water-insoluble glucan (WIG), the expression of the gtfP gene and the binding ability of polysaccharide-specific fluorescent dye, we confirmed that polysaccharide production was decreased in the ΔciaR mutant, which resulted in biofilm formation deficiency. These phenotypes of ΔciaR could be restored by ΔciaRΔargB double mutation, indicating that argB was essential for ciaR to regulate biofilm formation. We conclude that this decrease in GtfP-mediated polysaccharide production was responsible for the observed deficiency of this mutant for biofilm formation.

Results

Mutation of ciaR decreases biofilm formation in S. sanguinis SK36. Because S. sanguinis is a pioneering colonizer and a key player in plaque biofilm development28,39, we were interested in examining the mechanism of biofilm formation in S. sanguinis. In our previous work, a comprehensive mutant library of S. sanguinis SK36 was generated by high-throughput PCR36,37. By using a microtiter dish biofilm assay, we tested the biofilm formation ability of all mutants predicted to be two-component system regulators. Our initial screening indicated that deletion of the ciaR gene (SSA_0959) resulted in a decreased biofilm phenotype compared to the wild-type SK36 (Fig. 1A). This phenotype was restored in the ΔciaR-complemented strain, ΔciaR/ciaR (Fig. 1A). The wild-type strain (WT) and ΔciaR were statically cultured in biofilm media (BM) for 24 hours in a 4-well chamber then stained by SYTO9/propidium iodide (PI), which marked live/dead cells, respectively. This resulted in ΔciaR forming a loose biofilm, which was easily broken (Fig. 1B). These biofilms were subsequently observed by confocal laser scanning microscopy (CLSM) and quantified using a COMSTAT script in Matlab software38. Although containing more biomass by this analysis, the biofilm of ΔciaR was also much thicker and as a result had a lower ratio of biomass/average thickness (Fig. 1C and D). In other words, the cell density inside the ΔciaR biofilm was less than that of WT. The differential interference contrast (DIC) images and the roughness coefficient illustrated that the biofilm of ΔciaR had an irregular structure (Fig. 1C and D). Heat maps of biofilm thickness were generated using the COMSTAT script, which showed the distribution of biomass in biofilms. The biomass of WT was uniformly distributed, but there were many large gaps within the ΔciaR biofilm (Fig. 1C). These results indicate that the biofilm structure was impacted by ciaR mutation.

Extracellular polysaccharide is one of the most important components of many biofilm matrixes2,39. The phenotype of cell auto-aggregation has been linked with over-production of extracellular polysaccharide in other bacterial species40,41. When WT and ΔciaR strains were incubated in BM at 200 rpm for 24 hours, cell aggregation was observed only in the WT culture, suggesting that polysaccharide concentration might be reduced in ΔciaR (Fig. 2A). The extracellular polysaccharide of WT and ΔciaR was stained by Hippeastrum hybrid lectin (HHA)-FITC (EY Labs, USA) and measured by flow cytometry42. Compared with ΔciaR, a subpopulation of WT cells had increased staining and, presumably, a higher concentration of polysaccharide, which might promote auto-aggregation and biofilm formation (Fig. 2B).

Growth curves of WT and ΔciaR were generated in BM with a microtiter plate reader. ΔciaR had a higher value of OD600 and no significant change of colony forming units (CFU) (Fig. 2C). Because cell aggregation might impact OD600, the growth curves and CFU were also measured in BHI with continuous shaking. Similar to the result in BM medium, the final OD600 values of ΔciaR were higher than WT in BHI (Fig. S2). Cell cultures were sonicated to reduce auto-aggregation and then CFUs were determined. The CFUs from the ΔciaR culture were even higher than that of WT. Taken together, the results suggest that the deficiency of biofilm formation seen in ΔciaR was not caused by impaired cell growth (Fig. S2).

Extracellular DNA (eDNA) is also an essential component of the biofilm matrix in many species2,39. The amount of dead cells and eDNA in ΔciaR was less than that of WT, suggesting that ciaR might impact biofilm formation by reducing eDNA levels. To examine this possibility, the biofilms of WT and ΔciaR were cultured in BM for 24 hours and then treated with DNase I (100 U/mL; QUIGEN) for 1 hour. The biofilm assay was repeated. The addition of DNase I did not affect the biofilm biomass of WT or ΔciaR (Fig. 2D). The same result was observed if DNase I was added at the beginning (not shown). These results suggest that the ciaR mutation did not reduce biofilm formation by decreasing eDNA production.

Taken together, the data suggest that ΔciaR may impact biofilm formation by inhibiting polysaccharide production. To further address the mechanism, RNA sequencing was done to explore the change of gene expression in ΔciaR.
ΔciaR activates the arginine biosynthesis pathway. RNA sequencing data were generated and analyzed (Supplementary Dataset). There were 309 genes up-regulated (fold change ≥ 1.5, qvalue ≤ 0.01) and 190 genes down-regulated (fold change ≤ 0.667, qvalue ≤ 0.01). These regulated genes were analyzed by DAVID gene functional classification tools to identify enriched functional groups. Mutants deleted for the enriched differentially expressed genes were selected and examined for their biofilm formation using our biofilm formation assay. The impact of ciaR mutation on biofilm formation (A) The biofilms of WT, ΔciaR and ΔciaRciaR were cultured in a 96-well plate in BM media for 24 hours. Biomass was measured by crystal violet staining. (B) Biofilms of WT and ΔciaR cultured in 4-well chambers after being washed with PBS buffer. (C) Biofilms grown in 4-well chambers were stained by SYTO9 and propidium iodide. Fluorescence (left) and differential interference microscopy images (middle) were obtained by confocal laser scanning microscopy. Fluorescence images were analyzed by COMSTAT script, and heat maps of biofilm thickness were generated, which showed the distribution of biomass in biofilms (right). (D) The fluorescence images were analyzed by COMSTAT. Biofilm biomass, average thickness, propidium iodide signal and roughness coefficient were quantified, respectively. All the data in Fig. 1D were compared with their WT control. *P ≤ 0.05, **P ≤ 0.01, Student's t-test. Means and standard deviations from triplicate experiments are shown.

Figure 2. Polysaccharide production and cell growth of WT and ΔciaR. (A) Strains were incubated in BM at a shaking speed of 200 rpm for 24 hours. The arrow points a cellular auto-aggregate. (B) Strains were incubated in BM at a shaking speed of 200 rpm for 24 hours. Extracellular polysaccharide was stained by HHA-FITC and measured by flow cytometry. Arrow indicates a tail of increased staining in the WT strain. (C) WT and ΔciaR were cultured in BM. Every 30 minutes, cells were shaken for 3 minutes and OD600 was read using a platereader (Top). CFU values were determined after 16 hours of incubation (bottom). (D) Biofilms were cultured in BM for 24 hours and then treated with 100 U/mL of DNase I for 1 hour. Microtiter dish biofilm assay was performed after DNase I treatment. Means and standard deviations from triplicate experiments are shown. ND: no significant difference, Student's t-test.
comprehensive gene deletion mutant library and a high-throughput biofilm assay. Based on the biofilm deficiency in the \(\Delta ciaR\) mutant, if a gene was up-regulated in \(\Delta ciaR\) and the mutant deleted for this gene had more biofilm, this gene was judged as positively related to \(\Delta ciaR\) biofilm formation. If a gene was down-regulated in \(\Delta ciaR\) and the mutant deleted for this gene had more biofilm, it was negatively related. Most of the pathways enriched in

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**Figure 3.** Differentially expressed genes in \(\Delta ciaR\). (A) Genes with fold change \(\geq 1.5\) or \(\leq 0.67\) and qvalue \(\leq 0.01\) in \(\Delta ciaR\) RNA sequencing data were analyzed by DAVID gene functional classification tools. The enriched pathways were shown. (B) Transcript levels of arg genes in \(\Delta ciaR\) and \(\Delta ciaR/ciaR\). (C) qRT-PCR was performed to examine the expression of arg genes in \(\Delta ciaR\). Means and standard deviations from triplicate experiments are shown. *P ≤ 0.05, **P ≤ 0.01, Student's t-test.
DAVID analysis contained only a few genes that were positively related to ΔciaR biofilm formation. The exception was the arginine biosynthesis pathway.

We found that all of the arg genes associated with arginine biosynthesis were up-regulated in ΔciaR (Fig. 3B) and biofilm formation by all but one of these arginine biosynthesis-related mutants was increased (Fig. S3), which led us to hypothesize that ciaR mutation inhibited biofilm formation by activating arginine biosynthesis. qRT-PCR confirmed that the expression of arginine biosynthesis genes (argR, argB, argC, argG, argH and argJ) and two arginine/histidine permease genes (SSA_1568 and SSA_1569) was significantly increased in ΔciaR (Fig. 3C).

ΔciaR reduces biofilm formation by activating the arginine biosynthesis pathway. To further test the hypothesis, eight double mutants (ΔciaR combined with ΔargR, ΔargB, ΔargC, ΔargG, ΔargH, ΔargJ, ΔSSA_1568 and ΔSSA_1569) were constructed. Firstly, the growth of these strains, along with that of the parent strains, was examined in BHI with continuous shaking. The growth rates and maximum optical densities of ΔargR, ΔargC, ΔargG, ΔargH, ΔargJ, ΔSSA_1568 and ΔSSA_1569 were all less than WT (Fig. S4A–G). It has been reported that alkali generation through ammonia production, especially from arginine, is essential for maintaining pH homeostasis in the oral cavity. The deletion of arginine biosynthesis genes might decrease arginine production and as a result affect cell growth. The OD$_{560}$ of ΔargR, ΔargC, ΔargG, ΔargH, ΔSSA_1568 and ΔSSA_1569 was less than or similar to that of ΔciaR (Fig. S4A–D and F–G). These results suggested that the increased biofilm levels observed in these arg mutants was not caused by a change in cell growth. The ΔargB mutant exhibited severe auto-aggregation even when cultured in BHI medium with continuous shaking (Fig. S4I).

To ensure the measurement of biofilm formation was not impacted by differences in growth, we tested the biofilm biomass by a microtiter dish biofilm assay and at the same time quantified the total protein concentration of each sample (including the protein of cells in the supernatant). Biofilm formation was defined as biomass (OD$_{560}$) divided by total protein concentration (μg/mL). There was no significant difference of biofilm formation ability between ΔargB and ΔciaRΔargB (Fig. 4A).

The biofilm structures of ΔargB and ΔciaRΔargB were measured by CLSM. As mentioned above, the mutation of ciaR reduced cell density of biofilm (biomass / average thickness ratio) and resulted in the biofilm with an irregular structure. In contrast, deletion of argB significantly increased cell density (Fig. 1D). The cell density and biomass distribution of ΔciaRΔargB was similar to that of WT, indicating that mutation of argB could restore the biofilm formation ability of ΔciaR to WT levels (Fig. 1C and D).

We next examined whether the effect of the ciaR mutation on polysaccharide production could be related to arginine biosynthesis. The generation of WIG is positively related with the concentration of polysaccharide and controlled by GtfP, the only glucosyltransferase present in S. sanguinis. In ΔciaR, WIG production was half that of WT (Fig. 4B). This phenotype was restored in the complemented mutant, ΔciaRΔciaR. In RNA sequencing experiment, the expression of gtfP in ΔciaR was about one-fifth that of WT; however, because the reads of gtfP were low, it did not show a significant difference. Quantitative RT-PCR (qRT-PCR) was performed to confirm that gtfP was down-regulated in ΔciaR and restored in ΔciaRΔciaR (Fig. 4C).

The concentration of WIG in ΔargB was increased and the ΔciaRΔargB mutant produced WT levels of WIG (Fig. 4B). Interestingly, the gtfP gene was over-expressed in ΔargB whereas the ΔciaRΔargB double mutant expressed gtfP at WT levels (Fig. 4C). These data are consistent with a model whereby CiaR promotes biofilm formation by suppressing argB transcription, which in turn, increases gtfP expression and concomitant polysaccharide production.

Compared with WT, ΔargR, ΔargC, ΔargG, ΔargH, ΔargJ and ΔSSA_1568 produce more biofilm (Fig. 4A). Moreover, ΔargR, ΔargC, ΔargG, ΔSSA_1568 and ΔSSA_1569 produced more WIG (Fig. S5). Mutation of ciaR was defined as one factor impacting biofilm formation, and the mutation of arg gene was defined as another, then two-way ANOVA was utilized to analyze whether ciaR is influenced by arg genes for biofilm formation. As shown in Table S1, the p values of column factors were all less than 0.0001, illustrating that the mutation of ciaR could affect biofilm formation. The p values of the row factors ΔargR, ΔargB, ΔargC ΔargG ΔargH ΔargJ and ΔSSA_1568 were less than 0.05, suggesting these mutations could also modulate biofilm formation. The ΔargR, ΔargB, ΔargC and ΔargG mutations significantly interacted with ciaR mutation to alter biofilm formation, but ΔargH, ΔargJ, ΔSSA_1568 and ΔSSA_1569 altered biofilm formation independent from ciaR, which suggests that not only argB, but also argR, argC and argG are regulated by ciaR to modulate biofilm formation.

GtfP utilizes sucrose but not glucose for polysaccharide production. When biofilms were examined in cells cultured in BM + 1% glucose instead of 1% sucrose, gtfP mutation had no effect on biofilm formation (Fig. S6). The biofilm biomass of ΔargR and ΔargC mutants was almost the same as that of WT (Fig. S6). The biomass of the ΔargB and ΔargG mutants was even lower than WT (Fig. S6). Compared with that in BM + 1% sucrose, the biofilm formation ability of ΔargR, ΔargB, ΔargC and ΔargG were all decreased in BM + 1% glucose (Fig. S6). These data also suggested that arg genes affect biofilm formation by impacting polysaccharide production. There have been some reports demonstrating reduction of biofilm formation by addition of arginine. We added exogenous arginine to BM medium to test its effect on biofilm biomass using a microtiter dish biofilm assay. Addition of L-arginine to 7.5 mg/mL reduced WIG production and also inhibited the biofilm formation of WT (Fig. 4B and D). Moreover, the expression of gtfP in WT was decreased with the addition of 7.5 mg/mL of L-arginine (Fig. 4C). These data are consistent with the hypothesis that ciaR affects biofilm formation by regulating arginine biosynthesis.

L-ornithine is a precursor for L-arginine production. We added different concentrations of L-ornithine to BM, which decreased the biofilm forming ability of WT (Fig. S7). Arc is an ornithine carbamoyltransferase, which is necessary for the conversion of ornithine to arginine. Additional L-ornithine did not impact the biofilm formation of Δarc. These results are also consistent with our conclusion that arginine biosynthesis is inhibitory for biofilm formation.
ciaR affects biofilm formation of *S. sanguinis* in flow conditions. Oral biofilms on tooth surfaces are constantly exposed to flow conditions. To model the biofilm formation ability of ΔciaR in the oral cavity, a flow cell system was established. Two fluorescent proteins, mTFP1 (green) and mCherry (red), were expressed from the plasmids pVMTeal and pVMCherry, respectively. Strains expressing mTFP1 or mCherry were cultured in BM with 10 μg/mL erythromycin in the channels of a microfluidics chip with a flow speed of 0.1 μL/min. Images were obtained using fluorescence microscopy at different time points. Four days later, biofilms in the chip were observed by CLSM. Images were analyzed by COMSTAT.

Perhaps because ΔciaR cells adhered less well, the biofilm biomass of ΔciaR was much less than that of WT in the flow cell system, which was different from the biofilm biomass tested in the 4-well chamber but similar to the result in the 96-well plate (Fig. 5A). Surprisingly, when ΔciaR was co-cultured with WT, the biomass of ΔciaR was similar to WT (Fig. 5B). CLSM images showed that ΔciaR grew on the surface of the biofilm formed by WT (Fig. 5C). One explanation for this finding is that ΔciaR might be lacking in polysaccharide for attachment and utilize the polysaccharide produced by WT for localization. In this model, reduced polysaccharide production decreases the attachment of ΔciaR in monoculture and as a result, decreases the abundance of ΔciaR in flow conditions.

Arginine affects biofilm formation in *S. sanguinis* by affecting GtfP-mediated polysaccharide production rather than alkali generation. As mentioned above, arginine is utilized by the arginine deiminase system for alkali generation and plays an important role in pH homeostasis in *S. sanguinis*. When WT and ΔciaR were cultured overnight in BHI medium, the pH of both strains dropped to 5.36 ± 0.23. In the
regulate biofilm formation by repressing the expression of genetic competence genes. \(\Delta\text{ciaR}\) was unaffected for biofilm formation (Fig. 6E). Taken together, the results indicate that arginine affects biofilm formation in S. sanguinis by affecting GtfP-mediated polysaccharide production rather than alkali generation.

\(\text{ciaR}\) is a genetic competence inhibitor in S. sanguinis. Previous studies have demonstrated that \(\text{ciaR}\) is a genetic competence inhibitor in Streptococcus pneumoniae and Streptococcus mutans. In our RNA sequencing data, most of the \(\text{com}\) genes (\(\text{comC}, \text{comD}, \text{comE}, \text{comFA}, \text{comFC}, \text{comX}, \text{comYA-D}\)) were up-regulated (Table 1) while \(\text{comGF}\) was down-regulated in \(\Delta\text{ciaR}\) (Supplementary Dataset). The data above suggest that \(\text{ciaR}\) is a repressor of the genetic competence system in S. sanguinis. Due to the up-regulation of \(\text{comC}\) transcript levels in \(\Delta\text{ciaR}\), we expected that \(\Delta\text{ciaR}\) produced more CSP than WT because the \(\text{comC}\) gene encodes the CSP precursor in S. sanguinis. To compare the concentration of CSP in WT and \(\Delta\text{ciaR}\), we measured the transformation efficiency of \(\Delta\text{comC}\) supplemented with either the supernatant or cell lysate from WT and \(\Delta\text{ciaR}\). Compared with WT, the addition of the supernatant or cell lysate from \(\Delta\text{ciaR}\) elevated the transformation efficiency of \(\Delta\text{comC}\) to a greater extent, suggesting a higher concentration of CSP produced by the \(\text{ciaR}\) mutant (Fig. 6A and B).

The over-expression of genetic competence genes in \(\Delta\text{ciaR}\) does not lead to the fragile biofilm phenotype. Three experiments were done to explore whether \(\Delta\text{ciaR}\) was able to alter biofilm formation by producing more CSP. First, a \(\Delta\text{ciaR}\Delta\text{comC}\) double mutant was constructed. The addition of the \(\text{comC}\) mutation did not affect the biofilm formation ability of \(\Delta\text{ciaR}\) (Fig. 6C). Second, although a very high concentration of exogenous CSP (10 \(\mu\)g/mL) could slightly decrease biofilm biomass at the early stage of biofilm formation (8 hours, data not shown), in our experimental conditions, the addition of exogenous CSP did not affect biofilm biomass (Fig. 6D). Third, three \(\text{com}\) gene mutants, \(\Delta\text{comC}, \Delta\text{comD}\) and \(\Delta\text{comE}\), all of which eliminate competence, were unaffected for biofilm formation (Fig. 6E). Taken together, the results indicate that \(\text{ciaR}\) does not regulate biofilm formation by repressing the expression of genetic competence genes.

Discussion

Our results suggest that the \(\Delta\text{ciaR}\) mutation disrupts normal biofilm formation in S. sanguinis predominantly if not entirely by increasing expression of the arginine biosynthetic pathway resulting in increased arginine levels, which reduces transcription of the gtfP gene encoding glucosyltransferase, which reduces production of water-insoluble glucan (WIG)—the major component of S. sanguinis biofilms. (In experiments not shown, soluble glucan was produced at levels nearly 50 times lower than WIG, confirming that WIG is the predominant glucan in S. sanguinis biofilms.) There are data supporting each part of this model. The \(\Delta\text{ciaR}\) mutation significantly reduces gtfP expression and significantly increases arg gene expression (Figs 3 and 4). Exogenous arginine also reduces gtfP expression, biofilm formation, and WIG production (Figs 4 and S5). Exogenous ornithine, an arginine precursor, also reduces biofilm formation, but not in a mutant incapable of converting ornithine to
have identified genes directly regulated by CiaR in SK36. Thus, SK36 and tested gene expression by microarray. In their study, genes responsible for arginine biosynthesis were

Transcript levels of genetic competence-related genes in Table 1.

arginine (Fig. S7). The ΔciaR strain produces significantly less biofilm in the standard assay than its wild-type parent, while most of the arg mutants produce more, and all of the ΔciaR arg double mutants produce WT levels of biofilm (Figs 1, 4 and S3), consistent with the model of ciaR affecting biofilm formation through its effect on arg gene expression. Nearly identical results are observed if WIG production is measured rather than biofilm formation (Figs 4 and S2). The arg mutants do not show increased biofilm formation in the absence of sucrose, which is the substrate for GtIP (Fig. S6). We also ruled out some alternative explanations for our results. In multiple experiments, we found no support for either the ΔciaR mutation or the arg mutations affecting biofilm formation through effects on growth (Figs 2, S2 and S4). We also ruled out the possibility that the ΔciaR mutation affected biofilm formation through its effect on competence induction, which could otherwise have been a reasonable explanation (Fig. 6). Related to this final result, Rodriguez et al. added CSP to a ΔcomC mutant of S. sanguinis SK36 and tested gene expression by microarray. In their study, genes responsible for arginine biosynthesis were not regulated by the addition of CSP. Thus, ciaR appears to control genetic competence and arginine biosynthesis independently.

In one sense, the effect of arginine on biofilm formation should not have been that surprising, in that there have been many studies showing the effect of exogenous L-arginine on polysaccharide production and biofilm formation in S. pneumoniae and S. mutans. In those studies, however, the mechanism by which this occurred was not determined. Further, we are unaware of any previous studies that have examined the relationship between arginine biosynthesis and biofilm formation.

One remaining question concerns the ΔargB mutant. Although this mutant was like most of the other arg biosynthetic mutants in many respects, including demonstrating increased expression in ΔciaR (Fig. 3B,C), increased WIG production (Figs 4 and S5), and increased biofilm formation in sucrose compared to glucose (Fig. S6), the ΔargB strain was unique in that it severely aggregated in both the planktonic (Fig. S4) and biofilm (Fig. 1C) states. This reason for this difference is not clear, although one possibility is that accumulation of N-acetyl-glutamate, the substrate of the ArgB enzyme (acetylglutamate kinase), is responsible for this unique phenotype. Further studies will be required to answer this question.

Another question concerns the mechanism by which CiaR controls arg gene expression. Previous studies have identified genes directly regulated by CiaR in S. pneumoniae and Bacillus anthracis. To predict genes directly regulated by CiaR in S. sanguinis, we collected 200 bp of sequence upstream of each ORF to establish a promoter database for S. sanguinis SK36. Three CiaR binding sequences reported in S. pneumoniae (NTTNAG-N5-TTTAAN, NTTNAG-N5-TTTTAN and NTTNAG-N5-ATTAAN) were searched against the database and the results are shown in Table S2. There were 17 genes predicted to have a CiaR binding sequence, including htrA and comE (Table S2). The expression of most of these genes was significantly changed in ΔciaR

| Synonym | Name | Annotation | ΔciaR | ΔciaR/ciaR |
|---------|------|------------|-------|------------|
| SSA_2394 | comC | competence stimulating peptide | 24.46 | 0.00E+00 | 0.84 1.00E+00 |
| SSA_2379 | comD | signal transduction protein | 35.15 | 0.00E+00 | 0.93 7.73E-01 |
| SSA_2378 | comE | two-component system LytR/AlgR family transcriptional regulator | 12.76 | 0.00E+00 | 0.74 8.54E-01 |
| SSA_0715 | comEA | DNA uptake protein | 93.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_1497 | comEB | dCMP deaminase | 1.24 | 5.07E-06 | 1.07 5.70E-02 |
| SSA_0716 | comEC | competence protein | 78.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_1836 | comFA | superfamily II ATP-dependent DNA/RNA helicase | 78.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_1835 | comFB | competence protein | 94.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_0189 | comGF | competence protein ComGF | 81.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_0016 | comX | ComX1, transcriptional regulator of competence-specific genes | 56.25 | 0.00E+00 | 0.51 1.20E-02 |
| SSA_0184 | comYA | competence protein ComYA | 226.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_0185 | comYB | competence protein ComYB | 143.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_0186 | comYC | competence protein ComYC | 62.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_0187 | comYD | competence protein ComYD | 76.00 | 0.00E+00 | 1.00 1.00E+00 |
| SSA_2246 | comA | competence damage-inducible protein A | 11.05 | 0.00E+00 | 1.00 2.84E-01 |
| SSA_0749 | comB | competence protein | 8.00 | 0.00E+00 | 1.00 1.00E+00 |

Table 1. Transcript levels of genetic competence-related genes in ΔciaR and ΔciaR/ciaR.
In addition, previous studies have shown that CiaR directly binds to the promoter of htrA and controls its expression in S. pneumoniae, which suggests that CiaR of S. sanguinis may have a similar binding site to that of S. pneumoniae. The arginine biosynthetic genes are distributed among three different operons in S. sanguinis SK36 (Fig. S9). None of these three operons was predicted to contain a CiaR binding site, which suggests that the arginine biosynthetic genes are regulated by ciaR indirectly (Table S2). Some other transcriptional regulators like mga listed in Table S2 were also controlled by ciaR, which might explain the effect of ciaR on arg gene transcription. The mechanism by which ciaR regulates the expression of arg genes will require further study.

**Materials and Methods**

**Bacterial strains, growth and antibiotics.** Strains and plasmids used in this study are listed in Table S3. Unless otherwise stated, strains were grown in brain heart infusion broth (BHI; Difco Inc., Detroit, MI) media overnight and then diluted 100-fold into biofilm media (BM) and incubated under microaerobic conditions (6% O₂, 7.2% CO₂, 7.2% H₂ and 79.6% N₂) at 37°C C using an Anoxomat® system (Spiral Biotech, Norwood, MA). BM was supplemented with 1% sucrose for the growth of static biofilms and the measurement of bacterial growth. Kanamycin was added to a concentration of 500 μg/ml for mutant cultures.

**Mutant construction and complementation.** For double mutant construction, three sets of primers were used to independently PCR amplify the 1-kb sequence of the upstream fragment of ciaR, the downstream fragment of ciaR and the erm gene for erythromycin resistance. The three fragments were combined through a second round of PCR. The final recombinant PCR product was transformed into S. sanguinis SK36 single mutants. Double mutants were selected by erythromycin resistance and confirmed by PCR analysis. For
complementation of the ciaR mutant, a similar PCR-based method was employed. Briefly, three DNA fragments were independently amplified, the 1-kb sequence upstream plus the coding sequence of ciaR, the erythromycin resistance cassette (from the plasmid pVA838) and the 1-kb sequence downstream of ciaR. Overlapping PCR was done to generate the final recombinant PCR product. It was then introduced into ΔciaR to replace the kanamycin resistance cassette with the ciaR ORF and the erythromycin resistance cassette. An erythromycin resistant and kanamycin sensitive transformant was selected and confirmed by PCR analysis.

**Microtiter dish biofilm assay.** Overnight cultures were diluted 1:100 into BM in a 96-well microtiter plate (Falcon 3911). After incubation at 37 °C for 24 hours under microaerobic conditions, the planktonic cells were removed and biofilms were washed once with distilled water, and stained by the addition of 0.4% crystal violet (CV) for 30 minutes at room temperature. CV was then removed with a pipette and biofilms were washed twice with distilled water, solubilized in 30% acetic acid and measured at A560 as described previously. Three replicates were examined and data were analyzed by Student’s t-test.

**Static biofilm assay.** Static biofilms were grown in 4-chambered glass coverslip wells (Chambered #1.5 German Coverglass System, Nunc) in BM at 37 °C under micro-aerobic conditions for 24 hours. The supernatant was discarded, and biofilms were washed by PBS and stained stained with a live/dead staining kit (Molecular Probes, Invitrogen) in darkness for 10 minutes. The fluorescent and differential interference microscopy (DIC) images were acquired with a Zeiss LSM710 confocal laser scanning microscope (Zeiss, Germany) and quantified by COMSTAT in Matlab software. Three images of each sample were quantified to calculate the means and standard deviations. The signal of SYTO9 (green) showed live cells; the signal of PI (red) was dead cells and eDNA; the biomass / average thickness ratio represented cell density inside of biofilms.

**Biofilms in a flow cell system.** Two fluorescent proteins, mTFP1 (green) and mCherry (red), were expressed from the plasmids pVMTeal and pVMcherry, respectively. BM with 10 μg/mL erythromycin was pre-incubated in a jar under microaerobic conditions 3 days prior to the experiment. Strains were cultured in BHI under microaerobic conditions overnight and then pumped into the channels of a microfluidic chip (straight channel chips, productor cord: 01-0176-0142-01, ChipShop) with a flow speed of 0.1 mL/minute for 1 minute by using an NE-1200 Twelve Channel Programmable Syringe Pump (Newera, USA). Flow was stopped for 30 minutes to allow cells to attach. The syringes with bacterial cultures were then discarded and new syringes with pre-prepared BM were linked with the chip. The pump was turned on again with a speed of 0.1 mL/minute for 1 minute to wash away unattached cells and remaining BHI. The flow speed was then changed to 0.1 mL/minute for biofilm formation. Images were obtained with a Zeiss Axiover 200 M fluorescent microscope (Zeiss, Germany) at different time points. Four days later, biofilms in the chip were observed with a Zeiss LSM710 confocal laser scanning microscope. The images were analyzed by COMSTAT. The strength of the fluorescence signal represented biofilm biomass. Ten images from each sample were quantified to calculate the means and standard deviations.

**Growth curve measurement.** Strains were cultured in 96-well plates with continuous shaking, and growth was monitored every 15 minutes at 600 nm with a Synergy H1 Hybrid Reader (BioTek, USA). The microaerobic condition (6% O₂, 6% CO₂) was maintained by injection of CO₂ and N₂ to maintain CO₂/O₂ set concentrations at 37 °C. Samples were collected, treated with RNA protect bacteria reagent (Qiagen, Valencia, CA) for 5 min to stabilize RNA and stored at −80 °C. Cells were lysed by mechanical disruption using FastPrep lysing matrix B (Qbiogene, Irvine, CA). Total RNA was treated with DNase I (Qiagen) and prepared using RNA easy mini kits (Qiagen) according to the manufacturer’s instructions. Ribo-Zero Zero Magnetic Kit for Bacteria (Illumina) was used to deplete ribosomal RNA from 2 μg of total RNA. NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs) was used for the following RNA sequencing library preparation according to the manufacturer’s protocol. Library sequencing was performed by the Nucleic Acids Research Facilities at Virginia Commonwealth University using an Illumina HiSeq 2000 instrument. The raw RNA sequencing data are available in the NCBI Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo/query) under the accession number: GSE99864. Reads obtained from RNA sequencing were aligned against the S. sanguinis SK36 genome using Rockhopper v. 2.03. Analyses were run using default parameter settings. Significance was determined by a q-value adjusted for a false discovery rate of 1%. Transcriptome profiles were analyzed for enriched pathways and functionally related genes using DAVID v. 6.8 Beta. Four replicates were examined for data analysis.

**Competence assay.** Competence of S. sanguinis strains was determined by transformation with pJFP96, a suicide plasmid containing the spectinomycin resistance gene (aadA) with ~1 kb upstream and downstream of the SSA_0169 as described previously. Briefly, overnight cultures were diluted 200-fold into Todd Hewitt broth containing 2.5% horse serum (Fisher Scientific, Pittsburgh, PA) and incubated microaerobically for 3 hours. Culture aliquots of 300 μL were transferred into prewarmed microfuge tubes containing 50 ng of pJFP96 and incubated for 1 h at 37 °C. Cells were serially diluted, plated on BHI agar plates with and without spectinomycin (100 μg/ml) and grown microaerobically for 2 days at 37 °C. The transformation efficiency was defined as the ratio of spectinomycin-resistant colonies to total CFU. Three replicates were analyzed to calculate the means and standard deviations.

**High-throughput biofilm assay.** Biofilms were tested by a protocol similar to the microtiter dish biofilm assay. The differences were: OD₆₀₀ was tested before CV staining; CV and water were injected into 96 wells
at the CV staining step and washing step respectively by using a Caliper Sciclone G3 liquid handling robot (PerkinElmer, USA) with an injection speed of 100μL/second. All of the mutants used in the experiment were generated by Ge et al.27.

**Polysaccharide production assay.** Cells were cultured in BM under microaerobic conditions at 37°C for 24 hours at 200 rpm, harvested by centrifugation and then diluted to a density of 10^6/mL. Extracellular polysaccharide was stained by HHA-FITC (EY Labs, USA) as previously described22. HHA-FITC was used at a final concentration of 100–200 mg/mL for 1 hour in darkness and fluorescence signal was measured using a Guava® EasyCyte Flow Cytometer.

WIG was measured as previously described15. Biofilms were grown in BM for 24 hours in 24-well plates. The supernatant was then removed and biofilms were resuspended in 1 mL of distilled water. One-half mL of cell suspension was prepared for the determination of total protein concentration. Another 500μL of bacterial suspension was centrifuged. The sediment was dissolved in the same volume of 1 N NaOH for 3 hours, and centrifuged. The supernatants were precipitated by 3 volumes of isopropanol overnight at −20°C. The precipitates obtained by centrifugation were then air dried, and dissolved in 100μL of 1 N NaOH. The amount of glucans in each fraction was quantified by the phenol-sulfuric acid method as previously described60. Glucose was used as a reference carbohydrate to generate a standard curve. The concentration of WIG was normalized by total protein concentration in the biofilm. Four replicates were examined to calculate the means and standard deviations.

**qRT-PCR assay.** RNA extraction was performed as described above for the RNA sequencing assay. Reverse transcription followed the standard procedure provided with the SuperScript™ III Reverse Transcriptase Kit (Qiagen). The CDNA was used as the template, combined with 2X SYBR Green PCR Master Mix (Qiagen) and the q-PCR primers showed in Table S4. Gene expression in ΔciaR is relative to that in WT. Three replicates were analyzed to calculate the means and standard deviations.

**The measurement of protein concentration.** Cells were harvested and resuspended in lysis buffer (Tris pH 7.4 50 mM, NaCl 150 mM, glycerol 10%, NP-40 1%, SDS 0.1%). Cell suspensions were incubated on ice for 30 min and then lysed by mechanical disruption using FastPrep lysing matrix B (Qbiogene, Irvine, CA). The protein concentration of cell lysate was measured by following the standard protocol of Pierce® BCA Protein Assay Kit (Thermo Scientific). Four replicates were analyzed to calculate the means and standard deviations.

**DAVID gene function classification.** All the genes whose expression was significantly altered in ΔciaR were input into DAVID database (https://david.ncifcrf.gov/summary.jsp)61. The KEGG_pathway option was chosen for functional annotation clustering.

**Statistical analysis.** All data were obtained from at least three biological replicates. Student's t-test was applied to analyze data on CFU, biofilm assay, qRT-PCR, WIG and competence assay. The data of biofilm formation in Fig. 4A were analyzed by two-way ANOVA. In flow cell experiments, ten images from fluorescence microscopy were analyzed to calculate the means and standard deviations. The RNA sequencing data were analyzed by Rockhopper software62.

**Data Availability.** The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Accession code.** GSE99864.

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Author Contributions
B.Z. and P.X. conceived and designed this study. B.Z. carried out all of biofilm, polysaccharide and competence assays with assistance of X.G., Y.L. and F.E., X.G., B.Z., V.S., X.K. and P.X. performed RNA-seq and the sequence data analysis. B.Z., T.K. and P.X. analyzed the data and wrote this manuscript. All authors reviewed and discussed the manuscript.

Additional Information
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