Heterologous Expression of sahH Reveals That Biofilm Formation Is Autoinducer-2-independent in Streptococcus sanguinis but Is Associated with an Intact Activated Methionine Cycle*1

Received for publication, May 7, 2012, and in revised form, August 27, 2012. Published, JBC Papers in Press, August 31, 2012, DOI 10.1074/jbc.M112.379230

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**Background:** AI-2 is a by-product of the LuxS-mediated reaction within the activated methionine cycle (AMC).

**Results:** The biofilm phenotype of a S. sanguinis luxS mutant was restored by SahH expression but not by AI-2 supplementation.

**Conclusion:** The luxS mutant biofilm phenotype is not caused by lacking AI-2 but by an AMC defect.

**Significance:** The SahH bypass is essential for further studies on AI-2 utilizing luxS mutants.

Numerous studies have claimed deleterious effects of LuxS mutation on many bacterial phenotypes, including bacterial biofilm formation. Genetic complementation mostly restored the observed mutant phenotypes to WT levels, leading to the postulation that quorum sensing via a family of molecules generically termed autoinducer-2 (AI-2) is essential for many phenotypes. Because LuxS mutation has dual effects, this hypothesis needs to be investigated in detail for each bacterial species. In this study we used S. sanguinis SK36 as a model biofilm bacterium and employed physiological characterization and transcriptome approaches on WT and luxS-deficient strains, in combination with chemical, luxS, and sahH complementation experiments. SahH enables a direct conversion of SAH to homocysteine and thereby restores the activated methionine cycle in a luxS-negative background without formation of the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione. With this strategy we were able to dissect the individual contribution of LuxS and AI-2 activity in detail. Our data revealed that S. sanguinis biofilm formation is independent from AI-2 substance pools and is rather supported by an intact activated methyl cycle. Of 216 differentially transcribed genes in the luxS mutant, 209 were restored by complementation with a gene encoding the S-adenosylhomocysteine hydrolase. Only nine genes, mainly involved in natural competence, were directly affected by the AI-2 quorum-sensing substance pool. Cumulatively, this suggested that biofilm formation in S. sanguinis is not under control of AI-2. Our study suggests that previously evaluated LuxS mutants in other species need to be revisited to resolve the precise contribution of AI-2 substance pools and the methionine pathways.

In 1979 it was demonstrated for the first time that a quorum sensing system worked across marine bacterial species (1). The mediator of this system, now known and commonly referred to as autoinducer-2 substance pool (AI-2),2 was identified in several nonmarine, pathogenic bacteria (2, 3). Subsequently, the gene responsible for the generation of AI-2, luxS, was found to be widely conserved throughout the bacterial kingdom (4) and identified in a multitude of Gram-positive and Gram-negative, pathogenic and apathogenic bacteria (5, 6). Thus, the idea of an AI-2/LuxS-mediated interspecies communication was intensively discussed and suggested to play an essential role in development of virulence, especially biofilm formation (7–11).

LuxS together with Pfs represents an integral part of the activated methyl cycle (AMC) (Fig. 1), which provides activated methyl groups for the methylation of DNA, RNA, proteins and other substrates (5, 9, 12, 13). The LuxS/Pfs pathway, which is present in Gamma-, Beta-, and Epsilonproteobacteria as well as in Firmicutes (5), is responsible for the recycling of the toxic intermediate S-adenosylhomocysteine (SAH) to homocysteine.

Within this two-step enzymatic reaction, a byproduct occurs: the AI-2 substance pool precursor 4,5-dihydroxy-2,3-pentanedione (DPD) (14). Thus, the deletion of luxS to study the role of AI-2, would have two consequences: (i) the depletion of DPD, which is the desired phenotype, and (ii) a defect within the AMC caused by the interrupted LuxS/Pfs pathway, which could have additional effects on the phenotype and lead to misinterpretations of the role of AI-2. To circumvent this pitfall, it is necessary to study the effect of DPD depletion without affecting the AMC. One approach to accomplish this strategy is to bypass the interrupted LuxS/Pfs pathway by the SAH hydrolase (SahH), an alternative single-step reaction that catalyzes the conversion of SAH directly to homocysteine without the formation of DPD (5, 9, 12). This pathway exists within the AMC of Gamma-, Beta-, and Alphaproteobacteria, Chlorobia, Cya-

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2 The abbreviations used are: AI-2, autoinducer-2; AMC, activated methionine cycle; BHI, brain heart infusion; CDM, chemical defined medium; DPD, 4,5-dihydroxy-2,3-pentanedione; SAH, S-adenosylhomocysteine; SahH, S-adenosylhomocysteine hydrolase; SAM, S-adenosyl-methionine; SRH, S-ribozyme; THY, Todd-Hewitt broth supplemented with 0.5% yeast extract.

*This work was supported by PathoGenoMic Plus Program Grant FKZ 0313801M and ERANet PathoGenoMics I Program Grant FKZ 0313936B from the German Federal Ministry of Education and Research.

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FIGURE 1. Schematic diagram of the AMC and the generation of the byproduct Al-2. Within the AMC the methyl donor SAM is generated from methionine by SAM synthetase (MetK, EC 2.5.1.6, SSA_1495) and recycled to methionine. SAM is needed to provide activated methyl groups for the methylation of DNA, RNA, proteins, and other substrates (X) by SAM-dependent transmethylyases (SSA_1812). The resulting toxic metabolite SAH is then converted in a single step directly to homocysteine via SahH (EC 3.3.1.1) or in a two-step conversion to S-ribosylhomocysteine (SRH) by a SAH nucletidase (Pfs, EC 3.2.2.9, SSA_1639) and then to homocysteine by a S-ribosylhomocysteine lyase (LuxS, EC 4.4.1.21, SSA_1853), depending on the organism. Subsequently, homocysteine is recycled to methionine by the methionine synthase MetH (EC 2.1.1.13) or MetE (EC 2.1.1.14, SSA_0416) (5, 9, 12). A byproduct of the reaction catalyzed by LuxS is DPD, the precursor of AI-2. After spontanaeous cyclization and hydration of DPD (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-TMF-borate), the first substance described as active AI-2 molecule (14, 66, 67), all genes indicated with a gene-ID ("SSA_XXXX") were found in the exclusively human pathogen S. sanguinis K86. EC numbers and gene-IDs were assigned using Kyoto Encyclopedia of Genes and Genomes.

nobacteria, Bacteriodes, and Actinobacteria, as well as in Archaea and Eukarya (5).

In the present study we investigated the complementation of the AMC in a luxS deletion mutant by heterologous expression of Pseudomonas aeruginosa SahH. The functionality of a SahH complemented interrupted AMC has been demonstrated only in Gram-negative bacteria (15, 16). Consequently, in our study we examined for the suitability of such heterologous complementation in a Gram-positive bacterium. This could be of broad interest because in several single and multispecies studies, especially on human pathogenic streptococci, a multitude of effects was observed, investigating luxS deletion mutants; in the exclusively human pathogen Streptococcus pyogenes, LuxS mutation had a pronounced effect on many virulence traits, including hemolytic and proteolytic activity as well as acid tolerance (17–19). Similar pleiotropic effects were observed in another important human pathogen, Streptococcus pneumoniae. A LuxS mutant was reduced in virulence with a defect in the ability to spread from initial colonization sites and to persist in nasopharyngeal tissue (20, 21). A down-regulation of natural competence and a severe defect in biofilm formation was also noted (22, 23).

The group of oral streptococci was intensively studied, because they mostly occur in biofilm structures in the oral cavity and have intensive contact to neighboring species, and thus, for them an interspecies communication via AI-2 could be crucial for survival. For example, it was postulated for Streptococcus mutans, the main cariogenic streptococcal species, that LuxS-dependent quorum sensing is involved in biofilm formation and acidic tolerance. Mutant-derived biofilms adopted a more granular appearance (24), and luxS-deficient bacteria displayed an increased sensitivity to acidic killing (25). Culture filtrates of Streptococcus gordoniae, Streptococcus sobrinus, and Streptococcus anginosus complemented the LuxS defect in S. mutans, whereas Streptococcus oralis, Streptococcus salivarius, and Streptococcus sanguinis filtrates had no effect (26). A reduced biofilm formation of strains with a defect in LuxS was further shown for S. anginosus (27), S. gordoniae (28, 29), and Streptococcus intermedius (30). In addition, a reduced mutualistic biofilm growth could be demonstrated for S. oralis luxS mutant in combination with Actinomyces naeslundii (8).

Complementation approaches in the mentioned species would be subject to the above described pitfall: Genetic complementation with homo- or heterologous luxS genes always restored all phenotypes; thus it was not possible to attribute all phenotypes to AI-2-based quorum sensing. The addition of external AI-2 did not always complement all observed effects, suggesting a central metabolic role of an intact AMC. This central role of LuxS in cellular metabolism was demonstrated for S. mutans by microarray analysis: up to 30% of the transcriptome was affected by the deletion of luxS; addition of AI-2 did not restore this effect (31, 32). These findings underscore the need of a tool for answering the question, if all the observed effects of LuxS deficiency were a result of the missing quorum sensing signal substance pool (AI-2) or a consequence of a defect in methionine metabolism (AMC).

In this study we focused on an inhabitant of the oral cavity. As a model we choose S. sanguinis, a member of the S. sanguinis group, and one of the causative agents of endocarditis (33–35).

We found a growth phase-dependent release of AI-2, which disappeared after deletion of luxS. The mutant was characterized by a changed in vitro biofilm phenotype compared with its WT strain. Therefore, in depth analyses of effects mediated by AI-2 and the lost LuxS activity were done, employing homologous luxS and heterologous sahH complementation analyses, chemical complementation, and DNA whole genome array based transcriptome experiments.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—Bacterial strains used in this study are listed in Table 1. S. sanguinis was grown on brain heart infusion (BHI; Oxoid, Wesel, Germany) agar or in BHI broth, aerobically supplemented with 5% CO2 at 37°C. Recombinant strains were cultured on BHI agar supplemented with erythromycin (5 µg ml-1) or kanamycin (300 µl ml-1), respectively. Vibrio harveyi was cultivated on LB (36, 37) agar or in autoinducer bioassay medium (1) at 30°C aerobically. Escherichia coli was grown on LB agar or in LB at 37°C aerobically. Recombinant E. coli strains were selected on LB agar supplemented with ampicillin (100 µg ml-1), erythromycin (200 µg ml-1), or kanamycin (30 µg ml-1), respectively.

Setup for Biofilm Cultures—Preparatory cultures of all streptococcal strains were grown in BHI aerobically with 5% CO2 at 37°C to stationary phase. Bacteria were washed with PBS (pH 7.4) and adjusted to an A600 of 0.6 to obtain 1 × 108 cells/ml. Subsequently, each bacterial suspension was diluted 1:10 in a chemical defined medium (CDM) (38) supplemented with 50 mM sucrose (39) and inoculated in 96-Transwell polystyrene
TABLE 1
Bacterial strains and plasmids used in this study

| Strains and plasmids | Relevant characteristics or distributions | Source or reference |
|----------------------|------------------------------------------|---------------------|
| E. coli DH5α          | Cloning host                             | Invitrogen (68)     |
| *Pseudomonas aeruginosa* V14447 | Wild type                               | Strain collection of the University Rostock (Germany) |
| S. sanguinis SK36     | luxS::ermAM                               | American Type Culture Collection BAA-1455 |
| S. sanguinis SK36 ΔluxS | luxS::ermAM; pbIB184Km–luxS::sang-TTBOB | This study          |
| S. sanguinis SK36 ΔluxS/luxS | luxS::ermAM; pbIB184Km–P<sub>luxS</sub>“sahH”-StrT- TTBOB | This study          |
| V. harveyi MM77       | luxS::ermAM                               | This study          |
| Plasmids             |                                         | Ref. 42             |
| pIB184km             | oriR (gram−); Strept-tag II<sup>R</sup>; Amp<sup>R</sup> | IBA BiocTagnology GmbH |
| pIB184km-P<sub>recA</sub>-sahH-StrT-TTBOB | oriR (gram−<sup>/+</sup>–<sup>/−</sup>) (Stuttle Vector); Ery<sup>R</sup> | Ref. 69             |
| pUC19                | oriR (gram−); Amp<sup>R</sup>            | Ref. 70             |
| pUC19-Sang-Fu-ery    | oriR (gram−<sup>/+</sup>–<sup>/−</sup>) (Stuttle Vector); K<sup>R</sup> | Ref. 71             |
| pASK IBA3-sahH       | oriR (gram−<sup>/+</sup>–<sup>/−</sup>) (Stuttle Vector); Amp<sup>R</sup> | This study          |
| pIB184km             | pASK IBA3 containing sahH from *P. aeruginosa* V14447 | This study          |
| pIB184km-P<sub>recA</sub>-sahH-StrT-TTBOB | pbIB184km containing an artificial transcription terminator, the promoter region of recA of *S. pyogenes* M2T2/44/RB4/119, sahH from pASK IBA3-sahH including Strept-tag II<sup>R</sup> | This study          |
| pIB184km-luxS-sang-TTBOB | pbIB184km-TTBOB containing luxS from S. sanguinis SK36 | This study          |

**Deletion of luxS** — The sequence of the luxS region in *S. sanguinis* SK36 (accession number NC_009009.1) was used to design primers for amplification of luxS flanking regions (~1000 bp in length). PCR was done with the Phusion high fidelity PCR system. The primers Sang<sub>up</sub>_for and Sang<sub>up</sub>_rev were used for amplification of the luxS upstream fragment. For the downstream fragment primers Sang<sub>down</sub>_for<sub>2</sub> and Sang<sub>down</sub>_rev were applied, respectively (supplemental Table S1).

The downstream fragment was digested with XbaI/SphI and ligated into suicide vector pUC19. The resulting plasmid was named pUC19_Sang_down. Subsequently, the upstream fragment was digested with BamHI/SphI and ligated into the digested pUC19_sang_down. The resulting plasmid was designated pUC19_Sang_Fu. Finally, the ermAM cassette was amplified using pAT19 as template and the primers ErmAM_for<sub>Spe</sub> and ErmAM_rev<sub>Spe</sub>. The amplified DNA was digested with SphI and ligated into digested pUC19_Sang_Fu. The resulting plasmid pUC19-Sang-Fu-ery was used for transformation of *S. sanguinis* SK36, thereby obtaining *S. sanguinis* SR ΔluxS. Double crossover events were selected on THY agar supplemented with 5 μg ml<sup>−1</sup> erythromycin. Transformants were checked for correct substitution of luxS with ermAM by PCR and Southern blotting (data not shown).

**Complementation of S. sanguinis SR ΔluxS with Heterologous sahH** — To control against polar effects, the artificial transcription terminator TT<sub>BOR</sub> was introduced into pbIB184km. In previous experiments, the functionality of TT<sub>BOR</sub> was demonstrated (data not shown). The sequence was synthesized by Eurogentec GmbH (Cologne, Germany): GCATGCaatcaaatataaaattggcacggacgctactacaagtagcgtccgtgccatattgttattattttatattttatattattACATGC. Underlining indicate the attached restriction sites for SphI/NspI and the complementary sequences forming the hairpin loop. TT<sub>BOR</sub> was digested with NspI, recognizing the sequence RCATGY and producing the SphI-compatible site within the plasmid.
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sticky ends. TT\textsubscript{BOB} was subcloned adjacent to the multiple cloning site of pAT19 via Sph\textsubscript{I} to receive the following fragment: 5’-EcoRI-multiple cloning site-Sph\textsubscript{I}-TT\textsubscript{BOB}-HindIII-3’. This modified multiple cloning site was amplified by PCR using the primers M13\_FP and M13\_RP-XhoI. The product was subsequently digested with BamHI/XhoI and ligated into the digested pBl184km, obtaining pBl184km-TTBOB.

\( P_{\text{precA}} \), a constitutively active promoter of \( S. pyogenes \) routinely used in our laboratory, was cloned into pBl184km-TT\textsubscript{BOB} using the primers Prec\_for-Ma-Sac and Prec\_rev-Sy-Bam, obtaining pBl184km-\( P_{\text{precA}}\)-TT\textsubscript{BOB}.

\( sahH \) was amplified by PCR using the primers SahH\_for-Pap-Not, SahH\_rev-Pap-Not (15), and chromosomal DNA of \( P. aeruginosa \) V14447. The product was subcloned into pBluescriptII KS\textsuperscript{+} for sequencing, subsequently amplified with Sah\_over_Eco\_for and Sah\_over_Pst\_rev, EcoRI/PstI-digested, and ligated into pASK-IBA3. Finally, \( sahH \) was amplified from pASK-IBA3-\( sahH \) by PCR using Sah\_pAT\_F_Bam and Sah-StT_TT_Sph. The resulting DNA fragment was BamHI/SphI-digested and cloned into pBl184km-\( P_{\text{precA}}\)-TT\textsubscript{BOB}. Detailed sequence information of all primers is contained in supplemental Table S1.

\( S. sanguinis \) SR \( \Delta \)luxS was finally transformed with pBl184km-\( P_{\text{precA}}\)-\( sahH\)-Str-T-TT\textsubscript{BOB} obtaining \( S. sanguinis \) SR \( \Delta \)luxS/\( sahH \). The presence of the active enzyme was demonstrated according to the quality control test procedure outlined in the product information sheet provided by Sigma (enzymatic assay for SahH, EC 3.3.1.1.; data not shown).

\textbf{Complementation of \( S. sanguinis \) SR \( \Delta \)luxS—luxS, including its natural promoter, was amplified by PCR using chromosomal DNA of \( S. sanguinis \) SK36 and employing primers Kom_Lux_sang_F plus Kom_Lux_Sang_R (supplemental Table S1). The plasmid pBl184km-TT\textsubscript{BOB} was Apal/SphI-digested and ligated with the digested luxS fragment. \( S. sanguinis \) SR \( \Delta \)luxS was then transformed with pBl184km-luxS-sang-T-TT\textsubscript{BOB} obtaining \( S. sanguinis \) SR \( \Delta \)luxS/luxS.

\textbf{Safranin Assay}—Bacterial cells were grown in uncoated polystyrene 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). After incubation for 24 h under anaerobic conditions at 37 °C, liquid medium was removed, and the wells were washed gently with PBS to remove nonadherent sedimented cells. For determination of biofilm mass, the wells were stained with 0.1% safranin for 15 min, washed with PBS, and air-dried. Crystal violet stain was removed with 1 ml of 1% SDS for 15 min of agitation. Before determination of \( A_{540} \) the solution was diluted 1:10 with 1% SDS.

\textbf{Autoinducer-2 Bioassay—\( S. sanguinis \) strains were cultured in uncoated polystyrene 24-well plates (Greiner Bio-One) as mentioned above. One-ml culture was removed every hour and centrifuged, and the sterile filtered (0.2-μm pore size; Sterifix, Braun) cell-free supernatant was stored at −20 °C until measurement. The detection of AI-2 was performed as previously described (6, 41) with slight modification. \( V. harveyi \) MM77 (42) was used as the reporter strain because this strain does not produce AI-1 nor AI-2. Thus, it should show no auto luminescence, which was confirmed by our test system (data not shown). Luminescence was measured using a Spectramax M2 (Molecular Devices). For quantification of AI-2 concentration in culture samples, artificial AI-2 (DPD; Omm Scientific) was added on every tested 96-well plate in a graded dilution of 0.025-50 μM, serving as positive control and to plot a standard curve.

\textbf{Scanning Electron Microscopy—}For a qualitative documentation of biofilm architecture, bacterial cells were cultured in uncoated polystyrene 24-well plates (Greiner Bio-One) as mentioned above. Each well contained an uncoated, sterile plastic coverslip (13-mm diameter; Nunc, Wiesbaden; Germany). After 24 h, biofilms on coverslips were fixed for 24 h in 2.5% glutaraldehyde, subsequently washed with 0.1 M sodium acetate buffer (pH 7.3) and dehydrated in a graded series of ethanol. Coverslips were subjected to critical point drying with CO\(_2\), sputter-coated with gold (thickness, ~10 nm), and examined with a Zeiss DSM 960A electron microscope.

\textbf{Transcriptome Analysis—}\( S. sanguinis \) was grown in CDM/sucrose under anaerobic conditions in uncoated CELLSTAR\textsuperscript{®} tissue culture flasks (75 cm\(^2\); 250 ml; Greiner Bio-One, Frickenhausen, Germany). For analysis of gene regulation depending on AI-2, DPD was added after 5.5 and 7 h of growth to gain a final concentration of 4.5 and 9 μM, respectively. Cells were harvested and washed with PBS after 8 h of growth, meaning at the end of transient growth phase and after the AI-2 release in \( S. sanguinis \) WT reached its maximum.

RNA preparation was carried out from 100 mg of cell material (wet weight) with the Fast RNA\textsuperscript{®} Pro Blue kit (MP Biomedicals, Solon, OH) following the manufacturer’s instructions. cDNA synthesis, labeling, hybridization, scanning, feature extraction, and quality control of the arrays were essentially done as previously described (43). Normalization and background correction was done with NimbleScan Software using the RMA (robust multiarray analysis (44)).

The resulting data (log2) were statistically analyzed with GeneSpring GX (version 11.0; Agilent Technologies, Waldbronn, Germany). One-way ANOVA (45) was used for the significance analysis of \( S. sanguinis \) SR \( \Delta \)luxS compared with \( S. sanguinis \) SK36 and \( S. sanguinis \) SR \( \Delta \)luxS/\( sahH \). A t test was performed for analysis of \( S. sanguinis \) SR \( \Delta \)luxS/\( sahH \) with and without the addition of DPD. In all cases a \( p \) value of \( p \leq 0.05 \) was used (multiple testing correction: Benjamini-Hochberg (46)).
Competence Assay—A transformation assay was performed as previously described by Merritt et al. (47) with slight modifications. *S. sanguinis* strains were cultured overnight in competence medium (Todd-Hewitt broth; supplemented with 0.4% BSA) and subsequently 1:30 diluted in fresh competence media with and without the addition of DPD (4.5 μM). After the culture reached an A<sub>600</sub> of ~0.2, DPD addition was repeated (9 μM). Simultaneously, linearized plasmid DNA (20 μg ml<sup>−1</sup>) was added to each culture, conferring a tetracycline resistance. Prior to the transformation, the transforming plasmid pFW2 (48) was BamHI-digested. Subsequently, the cultures were allowed to grow for an additional 2 h and were then plated on BHI agar supplemented with tetracycline (5 μg ml<sup>−1</sup>) as well as on nonselective BHI plates. Transformation efficiency was defined as the ratio of transformants/total viable cells (47).

Reproducibility and Statistics—Each experiment was performed on at least three independent occasions (biological replicates) with two or three replicates each (technical replicates). The statistical parameters (mean, standard deviation, and p values) and tests were determined employing the following software: GenSpringGX 11.0, SoftMax Pro 5.4, and Windows Excel. p values less than 0.05 were considered as significant.

Accession Numbers—The raw data and meta information of the DNA array-based transcriptome experiments have been deposited in GEO database (accession number GSE37007). The nucleotide sequence information of *sahH* from *P. aeruginosa* V14447 have been placed on NCBI database with the accession numbers JQ894861.

RESULTS

The AI-2 Release Is Growth Phase-dependent—*S. sanguinis* was cultured anaerobically in CDM/sucrose. These growth conditions proved to be optimal for biofilm formation (39). We quantified AI-2 under the selected conditions, monitored its release for 24 h, examined the A<sub>600</sub>, and determined the biofilm mass. The maximum amount of AI-2 (2.9 nM) was found in the early stationary growth phase in the *S. sanguinis* culture after the biofilm mass reached its maximum (Fig. 2A).

The Deletion of luxS Significantly Affects Biofilm Formation in *S. sanguinis*—Based on the published full genome sequence of *S. sanguinis* SK36, we generated the luxS deletion mutant *S. sanguinis* SR ΔluxS. The phenotype of the luxS mutant was investigated under anaerobic conditions in CDM/sucrose. Biofilm masses were analyzed by safranin staining after 24 h of growth. The *S. sanguinis* SR ΔluxS mutant displayed significantly altered biofilm structures compared with its parental WT strain (p < 0.001) (Fig. 3, left pair of columns). Identical results were obtained either using safranin or crystal violet staining procedures (Fig. 2B). To rule out a reduced biofilm mass simply as a consequence of diminished growth caused by
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FIGURE 3. Biofilm mass of S. sanguinis, SK36, S. sanguinis ΔluxS, S. sanguinis ΔluxSΔsahH, and S. sanguinis ΔluxS/sahH were grown for 24 h anaerobically in CDM/sucrose. Depicted is the absorption at 492 nm of safranin-stained biofilms. The error bars indicate standard deviation. Differences in biofilm mass of S. sanguinis SR ΔluxS was highly significant compared with its parental WT strain S. sanguinis SK36 (p = 9.31E-8; n = 11), after complementation of luxS (S. sanguinis SR ΔluxS/luxS; p = 2.79E-6; n = 11), and after transgenic complementation with sahH (S. sanguinis SR ΔluxS/sahH; p = 9.31E-8; n = 11). Significance was determined by two-tailed U test.

Concentrations of DPD were selected to match the physiological range, which was defined by quantification of AI-2 release over the time in the previous experiments of this study (Fig. 2A). The resulting biofilm mass of S. sanguinis SR ΔluxS was not influenced by the addition of 1, 5, and 25 μM DPD (Fig. 5A), although former studies and control experiments in our laboratory proved that active AI-2 substances can be formed from this precursor (31, 50) (standard curve with the artificial DPD (data not shown)).

Second, S. sanguinis SR ΔluxS was co-cultured with its parental WT strain in a Transwell test system, which enables the exchange of small molecules but prevents direct cell contact. Also, with this assay setup we found no significant differences in biofilm mass. AI-2 produced by the WT strain in the upper compartment did not elevate the biofilm mass of S. sanguinis SR ΔluxS in the lower compartment (Fig. 5B).

These two strategies revealed that neither external addition of AI-2 (DPD), nor WT supernatant were able to complement the attenuated biofilm architecture of S. sanguinis SR ΔluxS, thereby indicating that biofilm formation in S. sanguinis is independent of AI-2 substance pools. Consequently, the hampered biofilm forming capacity of the S. sanguinis SR ΔluxS mutant could be a result of a defect in methionine metabolism caused by the missing LuxS activity (Fig. 1).

Transgenic sahH Restored WT Biofilm Phenotype of S. sanguinis SR ΔluxS—Next we introduced transgenic sahH into S. sanguinis SR ΔluxS to bypass the Psf/LuxS pathway within the AMC. The resulting transgenic sahH strain S. sanguinis SR ΔluxS/sahH was tested for its AI-2 release. Moreover, the A600 and viability of the mutant and wild type strains were monitored for 24 h. The growth curves (Fig. 2, A and B) and viable cell counts (data not shown) of both strains were similar. These results corresponded well with the staining experiments, because safranin predominantly interacts with the intercellular matrix, and crystal violet is directed to the cellular components of a biofilm. Thus, the deletion of luxS caused a reduction of biofilm mass of S. sanguinis SR ΔluxS but did not affect the bacterial growth rates or viable cell count. The AI-2 concentrations in all cultures of the luxS deletion mutant were reduced, demonstrating that functional deletion of luxS in final consequence leads to deletion of DPD production and thus AI-2 substance pool depletion.

Plasmid Encoded luxS Restored WT Phenotype of S. sanguinis SR ΔluxS—With the S. sanguinis SR ΔluxS strain at hand, we now had the tools to investigate the individual contributions of an AI-2 deficiency and methionine deficiency for the observed phenotype of the S. sanguinis SR ΔluxS mutant.

First, the S. sanguinis SR ΔluxS strain was complemented with a plasmid encoding luxS of its parental WT strain, with the intention to restore both, the AI-2 substance pool formation and the complete methionine pathway (Fig. 1). The complemented strain S. sanguinis SR ΔluxS/luxS was investigated by AI-2 measurement, scanning electron microscopy, crystal violet, and safranin stain. The AI-2 release as well as the biofilm architecture was restored to WT level (Figs. 2C, 3, and 4), demonstrating that the observed effects in S. sanguinis SR ΔluxS were caused by a single gene deletion.

Biofilm Mass Production of S. sanguinis Is Independent from AI-2 Substance Pools—Several previous studies postulated AI-2 substances as key players in single and mixed species biofilm development (7, 8, 49). Thus, in the next set of experiments we studied the individual role of the AI-2 compound on S. sanguinis biofilm formation. The ability of AI-2 to restore the S. sanguinis SR ΔluxS biofilm back to WT level was investigated by two approaches. First, artificial AI-2 (DPD) was added directly to the growth medium of the S. sanguinis SR ΔluxS strain. Contrariwise in CDM/sucrose. Depicted is the absorption at 492 nm of safranin-stained biofilms. The error bars indicate standard deviation. Differences in biofilm mass of S. sanguinis SR ΔluxS was highly significant compared with its parental WT strain S. sanguinis SK36 (p = 9.31E-8; n = 11), after complementation of luxS (S. sanguinis SR ΔluxS/luxS; p = 2.79E-6; n = 11), and after transgenic complementation with sahH (S. sanguinis SR ΔluxS/sahH; p = 9.31E-8; n = 11). Significance was determined by two-tailed U test.

Concentrations of DPD were selected to match the physiological range, which was defined by quantification of AI-2 release over the time in the previous experiments of this study (Fig. 2A). The resulting biofilm mass of S. sanguinis SR ΔluxS was not influenced by the addition of 1, 5, and 25 μM DPD (Fig. 5A), although former studies and control experiments in our laboratory proved that active AI-2 substances can be formed from this precursor (31, 50) (standard curve with the artificial DPD (data not shown)).

Second, S. sanguinis SR ΔluxS was co-cultured with its parental WT strain in a Transwell test system, which enables the exchange of small molecules but prevents direct cell contact. Also, with this assay setup we found no significant differences in biofilm mass. AI-2 produced by the WT strain in the upper compartment did not elevate the biofilm mass of S. sanguinis SR ΔluxS in the lower compartment (Fig. 5B).

These two strategies revealed that neither external addition of AI-2 (DPD), nor WT supernatant were able to complement the attenuated biofilm architecture of S. sanguinis SR ΔluxS, thereby indicating that biofilm formation in S. sanguinis is independent of AI-2 substance pools. Consequently, the hampered biofilm forming capacity of the S. sanguinis SR ΔluxS mutant could be a result of a defect in methionine metabolism caused by the missing LuxS activity (Fig. 1).

Transgenic sahH Restored WT Biofilm Phenotype of S. sanguinis SR ΔluxS—Next we introduced transgenic sahH into S. sanguinis SR ΔluxS to bypass the Psf/LuxS pathway within the AMC. The resulting transgenic sahH strain S. sanguinis SR ΔluxS/sahH was tested for its AI-2 release. Moreover, the A600 and viability of the mutant and wild type strain culture, although no AI-2 release was detectable (Fig. 2D). After a 24-h culture period, the biofilm mass of the sahH-complemented strain was significantly different compared with S. sanguinis SR ΔluxS (p < 0.001). It formed biofilm masses similar to those of the parental WT and the luxS complemented SR ΔluxS/luxS strain (Fig. 3). Thus, transgenic sahH alone restored WT biofilm in a luxS mutant of S. sanguinis. This strongly suggests that a defect in methionine production in a luxS mutant background is the primary factor responsible for the attenuated biofilm phenotype. However, indirect and not readily predictable factors and mechanisms could also play a role.

Electron Microscopy of S. sanguinis SR ΔluxS/sahH—To verify the restoration of WT biofilm phenotype and to rule out a changed cell shape, S. sanguinis strains were investigated by scanning electron microscopy (Fig. 4). Moreover, this technique allowed a qualitative evaluation of biofilm architecture. The cells were grown for 24 h on uncoated, sterile plastic coverslips in CDM/sucrose and examined with a Zeiss DSM 960A electron microscope.

S. sanguinis SR ΔluxS/sahH (Fig. 4, J–L) and S. sanguinis SR ΔluxS/luxS (Fig. 4, G–I) formed solid biofilms, similar to the WT culture (Fig. 4, A–C). All three strains were able to cover the surface of carrier material completely, resulting in dense biofilms. Compared with the biofilms of the parental WT strain, the luxS, or the sahH complemented luxS mutants (Fig. 4, D–F), biofilms of S. sanguinis SR ΔluxS were reduced in com-
plexity and compactness. No differences in cell morphology or cell arrangement were detectable.

**Genome Wide Transcriptome Analysis Confirmed Metabolic Complementation by Transgenic SahH**—The previous experiments of this study demonstrated a recovery of natural biofilm masses and architecture in the *S. sanguinis* SR/H9004 strain by transgenic complementation with *sahH* but not by addition of AI-2.

In the next set of experiments, we investigated the genome wide influence of the *luxS* deletion and the transgenic *sahH* complementation by comparative transcriptome analysis employing a recently developed and validated array platform (43). The aim was to investigate whether the heterologous expression of *sahH* also complemented transcriptional changes of the *luxS* deletion.

The cells were cultured anaerobically in CDM/sucrose and harvested after 8 h of growth. At this time point AI-2 release of *S. sanguinis* WT had reached its maximum; therefore, we expected a maximum difference in gene expression. The data sets from *S. sanguinis* SR ΔluxS/sahH and *S. sanguinis* SK36 were compared with *S. sanguinis* SR ΔluxS.

Using a fold change cutoff level of ≥3, 216 genes were identified to be differentially transcribed. Regarding their metabolic function, they were grouped into five classes and 19 subclasses. Several genes are mentioned in multiple categories; however, they were not counted in multiples for the calculation of the total number of differentially transcribed genes. Gene expression changes were displayed in a color-coded manner in Table 2 (precise values are shown in supplemental Table S2). Among the genes with differential transcript abundance, we identified...
several involved in cell division processes, e.g., polA (SSA_0100), coding for a putative DNA polymerase I, gyrA (SSA_1220), coding for DNA gyrase A, or putative members of the fts regulon (ftsA (SSA_0655), H (SSA_0015), K (SSA_1626), and Z (SSA_0656)) and several ribosomal proteins. Furthermore, we found differential expression of regulators involved in stress response, catabolite control, and sugar uptake: csbD (SSA_1745, putative general stress response protein), ctsR (SSA_1000, putative galactose operon transcriptional repressor), or ccpA (SSA_1576), coding for the putative catabolite control protein A.

Taken together, the deletion of luxS influences important genes coding for proteins with central roles in cell growth and metabolism. Nevertheless, the majority of these genes were restored in their expression in the sahH complemented strain: 97% (209 genes) were regulated in the same direction in the WT and luxS mutant.
Table 3

| GeneID | Gene name/description | Fold change |
|--------|------------------------|-------------|
| SSA_0019 | pcsB/secreted antigen GbpB/SagA; peptidoglycan hydrolase:PcsB protein precursor, putative | 3.47 |
| SSA_0184 | comYA/competence protein ComYA, putative | 3.07 |
| SSA_0185 | comYB/competence protein ComYB, putative | 3.7 |
| SSA_0188 | Conserved hypothetical protein | 3.44 |
| SSA_0189 | Competence protein ComGF, putative | 3.19 |
| SSA_1398 | hylIII/membrane protein, hemolysin III-like, putative | 3.88 |
| SSA_1925 | serS/seryl-tRNA synthetase, putative | 4.41 |

And the transgenic complemented strain compared with the luxS mutant, indicating a functional complementation of the luxS deletion on transcriptional level.

AI-2 Regulates Competence—Next we investigated whether there are genes of *S. sanguinis* specifically regulated by AI-2. The array experiments were repeated by culturing the *S. sanguinis* ΔluxS/sahH strain with and without the addition of DPD. This expression analysis revealed nine differentially expressed genes with a fold change of ≥3 (p ≤ 0.05; Table 3). Among them we identified a putative hemolysin, a putative secreted peptidoglycan hydrolase, and three up-regulated genes coding for competence proteins (*comYA*, *comYB*, and *comGF*). This suggested that the main activity of AI-2 substance could be regulation of competence in *S. sanguinis*. This hypothesis was experimentally challenged and confirmed by testing the natural competence of the *S. sanguinis* WT, its luxS mutant, and the heterologous *sahH* complemented luxS mutant (Fig. 6). In the absence of external DPD, the transformation efficiency of the luxS mutant, as well as of the *sahH* complemented luxS mutant, was significantly reduced compared with the WT strain. The addition of DPD resulted in modest enhancement of competence of both strains, but without restoring WT level. These findings underscore the relevance of AI-2 in competence development in *S. sanguinis*.

**Discussion**

In the present study we addressed the issue of the genome wide consequences of a luxS mutation in *S. sanguinis*, its potential effects on oral streptococcal biofilm formation, and which molecular defects lead to the observed phenotype. The latter is difficult to study because deletion of LuxS has three potential consequences: autoinducer-2 substance pools are depleted, methionine produced via the AMC is not available, and finally, consequences: autoinducer-2 substance pools are depleted, methionine produced via the AMC is not available, and finally, LuxS activity within the AMC are crucial for AI-2 production and biofilm formation in this species. Genetic complementation with a plasmid-encoded autologous luxS gene fully restored AI-2 production and biofilm formation over the growth cycle. This was not unexpected and has been shown for many streptococcal species like *S. pneumoniae* and *Streptococcus gordonii* (23, 29).

The *S. sanguinis* LuxS mutant was significantly attenuated in biofilm formation, indicating that the complex consequences of LuxS activity within the AMC are crucial for AI-2 production and biofilm formation in this species. Genetic complementation with a plasmid-encoded autologous luxS gene fully restored AI-2 production and biofilm formation over the growth cycle. This was not unexpected and has been shown for many streptococcal species like *S. pneumoniae* and *Streptococcus gordonii* (23, 29).

Synthetic, autologous, and also heterologous AI-2 addition to many of the LuxS mutants has allowed a partial restoration of the phenotype and revealed the quorum sensing contribution of the AMC. For example, *S. mutans* LuxS mutants were successfully complemented by exposure to WT supernatants, supernatants from heterologous species, and also synthetic DPD (25, 26, 31). *S. anginosus*, *Streptococcus suis*, and *S. intermedius* LuxS mutants were also phenotypically restored by AI-2 addition (53–55).

In our study multiple approaches for applying AI-2 and WT supernatants to the *S. sanguinis* LuxS mutant did not restore the defect in biofilm formation. This argues against an AI-2-dependent quorum sensing-mediated control of biofilm forma-
Heterologous SahH Restored AMC in S. sanguinis luxS Mutant

tion in S. sanguinis and hints at the influence of other defects caused by the LuxS mutation. One possibility would be a restoration of the metabolic defects of LuxS mutation without affecting the production of AI-2 pool substances. The SahH (EC 3.3.1.1) catalyzes the conversion from SAH to homocysteine in a single step bypassing AI-2 substance pool formation (Fig. 1 and Refs. 5, 9, and 12). From experiments in Gram-negative species, it is known that this pathway can produce sufficient amounts of methionine (15, 16). Expression of SahH in the S. sanguinis LuxS mutant restored biofilm development back to WT levels without any detectable AI-2 production (Figs. 2D and 4). This evidence supports the notion of an AI-2-independent biofilm formation in S. sanguinis. In addition, this experiment demonstrated that SahH complementation also works in Gram-positive species and thus can serve as a tool for analysis of LuxS-mediated phenotypes in these bacteria as well.

The results described and discussed above lead to the issue of the relevant functions of AI-2 substances in the lifestyle of S. sanguinis. The addition of synthetic AI-2 to the SahH-complemented LuxS mutant and investigation of genome-wide expression in all recombinant strains allowed us to address this issue.

In a first series of experiments, we found that the inactivated Pfs/LuxS pathway in S. sanguinis influenced genes with central roles in cell growth and metabolism, a fact also observed in other species (9, 12). Of note, the general stress response was induced in the LuxS mutant. This could be due to the accumulation of S-ribosylhomocysteine or SAH caused by the interrupted Pfs/LuxS pathway. SAH is known to be a toxic intermediate (56, 57). In line with these results, the acid stress response was affected in LuxS mutants of S. mutans, S. pyogenes, and Lactobacillus spp., respectively (19, 25, 58).

In the related species S. mutans up to one-third of the whole transcriptome was altered by deletion of LuxS with many similar changes in gene transcript amounts as observed in our study (31). Another global study in S. mutans detected 60 genes altered in their transcript abundance (32). This supports a central metabolic role of LuxS and the AMC and leads to the question whether the metabolic changes in the LuxS mutant are due to direct or indirect effects. Apparently, LuxS mutation does not cause growth deficiencies. A rather moderately changed pattern of gene regulation observed in our study could explain this. Alternatively, an additional pathway for methionine production as described in Gram-negative could exist in Gram-positive species. For instance, in E. coli homocysteine can be produced via a pathway starting with oxalacetate (16).

SahH complementation in the S. sanguinis LuxS mutant background almost completely restored the transcriptional differences induced in the LuxS mutant (209 of 216 genes (97%)). Within the minor fraction of unrestored genes, we identified purL and purB, coding for a phosphoribosylformylglycinamidine synthase and an adenylosuccinate lyase, respectively. These enzymes are involved in purine metabolism (Kyoto Encyclopedia of Genes and Genomes). The differential expression in the WT and the sahH complemented strain could be explained by the generation of different byproducts occurring within the Pfs/LuxS and the SahH pathway; the conversion from SAH to homocysteine results in the generation of adenine or adenosine in the Pfs/LuxS pathway or SahH reaction, respectively (Fig. 1 and Refs. 5 and 12). This may cause an altered regulation of genes and enzymes involved in purine metabolism and thus could explain the altered transcription of purL and purB. Five more genes were not restored by the expression of sahH: clpL, ctpA, and three genes of unknown function (SSA 0141, SSA 1354, and SSA 2276). SSA 0141 encodes for a putative copper chaperone and belongs to the same operon as ctpA, a copper translocating P-type ATPase (Kyoto Encyclopedia of Genes and Genomes). ClpL is a chaperone not involved in protein degradation, because of the missing recognition tripeptide, which enables the interaction with ClpP (59). It is involved in stress tolerance and biofilm formation (60, 61).

Altogether, the expression of sahH restored the effects of the luxS deletion on transcriptome level except a minor fraction of genes. This should be considered in future studies of luxS mutants.

In a second series of experiments, the addition of AI-2 substance to a culture of the S. sanguinis LuxS mutant complemented with sahH uncovered the direct AI-2-dependent responsive network. Of note, only a small number of genes responded to this treatment. Mainly genes involved in genetic competence development are dependent on the presence of the AMC byproduct AI-2, in particular those involved in DNA binding and uptake. The transformation efficiency assay confirmed the prediction of reduced competence in the luxS mutant, as well as in the sahH-complemented strain. As expected, the addition of DPD elevated the level of competence in the luxS strain but surprisingly also demonstrated a measurable effect in the sahH-complemented strain. This suggests a level of complexity in which the defect within the AMC caused by missing LuxS activity in the luxS mutant also has a negative effect on competence over and above AMC-independent effects on competence that stem directly from the lack of AI-2. A further confounding factor may be copy number effects of the gene coding for SahH, which is located on a high copy number plasmid and which could have interfered with the transforming plasmid DNA. S. sanguinis WT and the luxS mutant did not possess any plasmid.

The deletion of luxS has a negative effect on natural competence. This has been demonstrated for S. mutans and is consistent with the findings of the present study (47). An influence on expression of late competence genes was also found in LuxS mutants of S. pneumoniae (22, 62); however, in those studies the direct influence of AI-2 was not investigated.

S. sanguinis is naturally competent, a feature that is controlled by stress response pathways and competence-stimulating peptides (63–65). Our data also suggest that also AI-2 substance potentially has an influence on natural competence of this species.

In summary we have shown that (i) S. sanguinis biofilm formation is independent of AI-2 based quorum sensing; (ii) genetic complementation of LuxS mutants with autologous or heterologous genes, as well as chemical complementation with AI-2 substances, is not sufficient to determine the exact biological roles of the AMC components; (iii) a heterologous complementation with SahH as a pathway short cut restored the metabolic deficiencies of LuxS mutation; and (iv) such a com-
plemented strain in combination with chemical complementation could be the tool of choice to determine the individual biological roles of AI-2-based quorum sensing and AMC metabolic functions. Based on the results presented here, also LuxS mutants in other Gram-positive species might be subjected to a second look at their methionine pool and AI-2-dependent pathways.

Acknowledgments—We thank G. Fulda and W. Labs from the Electron Microscopic Centre of the Medical Faculty of the University of Rostock for technical assistance.

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