Functional Analysis of luxS in Staphylococcus aureus Reveals a Role in Metabolism but Not Quorum Sensing

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The function of AI-2 in many bacteria and the physiological role of LuxS, the enzyme responsible for its production, remain matters of debate. Here, we show that in Staphylococcus aureus the luxS gene forms a monocistronic transcriptional unit under the control of a σ^A-dependent promoter. The gene was transcribed throughout growth under a variety of conditions, including intracellular growth in MAC-T cells. AI-2 was produced in rich media under aerobic and anaerobic conditions, peaking during the transition to stationary phase, but was hardly detectable in a sulfur-limited defined medium. In the presence of glucose or under anaerobic conditions, cultures retained considerable AI-2 activity after entry into stationary phase. Inactivation of luxS in various S. aureus strains did not affect virulence-associated traits, such as production of hemolysins and extracellular proteases, biofilm formation, and the agr signaling system. Conversely, AI-2 production remained unchanged in an agr mutant. However, luxS mutants grown in a sulfur-limited defined medium exhibited a growth defect. When grown together with the wild type in mixed culture, luxS mutants of various S. aureus strains showed reduced ability to compete for growth under these conditions. In contrast, a complemented luxS mutant grew as well as the parent strain, suggesting that the observed growth defect was of an intracellular nature and had not been caused by either second-site mutations or the lack of a diffusible factor. However, the LuxS/AI-2 system does not appear to contribute to the overall fitness of S. aureus RN6390B during intracellular growth in epithelial cells: the wild type and a luxS mutant showed very similar growth patterns after their internalization by MAC-T cells.

Many bacteria, including pathogens and commensals, are known to communicate via diffusible signal molecules (26, 63). It is often assumed that these molecules are employed to regulate genes in concert with cell population density (quorum sensing). Bacteria of the genus Staphylococcus are known to possess an autoinducing peptide (AIP)-based signaling system, encoded by the agr locus, the function of which has been studied in detail in Staphylococcus aureus and Staphylococcus epidermidis (for reviews see references 36 and 49). In S. aureus, this system is involved in the regulation of many exoproteins, including exoenzymes, exotoxins, and surface proteins (49). Sequence analysis of completed genomes revealed that Staphylococcus spp., like many other bacteria, also contain a luxS gene and therefore may employ a second signaling system based on the furanone derivative, autoinducer 2 (AI-2).

The LuxS/AI-2 system has been analyzed in detail in Vibrio spp., in particular Vibrio harveyi and Vibrio cholerae, where it is involved in the regulation of bioluminescence and virulence-associated traits, respectively (24, 25, 34, 40) (for a review, see reference 76). Synthesis of AI-2 depends on the enzyme LuxS (55, 71), which generates the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) from S-ribosyl-L-homocysteine (SRH). DPD gives rise to several furanone derivatives (11, 41), often collectively referred to as AI-2. One of these, a furanosyl borate diester, is bound by the periplasmic binding protein LuxP in Vibrio spp. (11). The resulting complex then interacts with the histidine kinase LuxQ (45), triggering a complex response that involves a phosphorelay system and small regulatory RNAs (34). In Vibrio cholerae and V. harveyi, this system also integrates the signals from other autoinducers and their corresponding sensor kinases (24, 40).

The precise role of AI-2 in other bacteria remains a matter of debate. In many instances, a role for AI-2 in intra- and interspecies signaling has been proposed (5, 19, 30, 55, 76). However, in the vast majority of these studies, only indirect or incomplete evidence for AI-2-dependent signaling has been provided (see reference 66 for a summary), as these analyses have been complicated by the fact that LuxS also plays a metabolic role in the activated methyl cycle (AMC). Presently, outside of the genus Vibrio, the only definitive genes shown to be regulated by the AI-2 signal are those involved in its own uptake, phosphorylation, and (probably) degradation in Salmonella enterica serovar Typhimurium (64, 65) and Escherichia coli (68, 77), i.e., the lsr system, comprising an ABC transporter, an AI-2 kinase, and putative enzymes for the subsequent conversion of phosphorylated AI-2.

A role for LuxS in the AMC has been described in a number of publications (71, 72, 73). This cycle is responsible for the generation of the major methyl donor S-adenosyl-L-methionine (SAM) and the recycling of methionine from the toxic S-adenosyl-L-homocysteine (SAH), which is formed as a product of SAM-dependent methylation reactions (17). LuxS takes part in this cycle by salvaging the homocysteine moiety from
the cycle intermediate SRH. As a by-product of this reaction, DPD is formed (17, 55, 79). The only currently known biological role of DPD is that of being a direct AI-2 precursor. Indeed, it is intriguing that formation of this molecule is so closely coupled with the metabolic flux through the AMC (one molecule of DPD is formed per SAM-dependent methylation event), making it an ideal signal for metabolic activity and cell population density (7, 73, 76). However, it is also possible that in many bacteria the generation of DPD is not required for signaling but for metabolic purposes.

Two versions of the AMC exist (67, 73, 75). Eukaryotes and archaeabacteria, but also many eubacteria, use the enzyme SAH hydrolase to convert toxic SAH into homocysteine and adenosine (thus, they do not produce DPD/AI-2). Other eubacteria generate homocysteine in the combined reactions of Pfs (methylthioadenosine/S-adenosyl-L-homocysteine nucleosidase, which converts SAH to SRH and adenosine) and LuxS. Presently, there is only one bacterium known to possess both variants of the AMC, Bifidobacterium longum (73).

The fact that the vast majority of organisms contain a complete AMC suggests that its functions are important for metabolism and thus for overall fitness (71, 73). However, it has been argued that the Pfs enzyme is sufficient for the detoxification of SAH and that bacteria use the Pfs-LuxS variant of the AMC because it allows them to generate the AI-2 signal (76). Indeed, an E. coli pfs mutant shows a severe growth defect (10), even in complex media, whereas this has not been reported for luxS mutants in the same or other genetic backgrounds. On the other hand, the pfs and luxS genes, in agreement with their roles in methionine recycling, are often located next to genes involved in sulfur metabolism, in particular, those linked to de novo synthesis of cysteine and methionine (73).

The aim of this study was to undertake a functional analysis of the S. aureus luxS gene (luxS<sub>Sa</sub>) in the context of both quorum sensing and metabolism. Here, we present a detailed molecular analysis of the luxS<sub>Sa</sub> locus and the consequences of its inactivation under various growth conditions.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. E. coli strains DHA5 and JM109 were used in cloning experiments. S. aureus strains RN4220 was used as a gateway strain prior to propagation of plasmids or mutant markers into other S. aureus strains. Bacteria were routinely grown in Luria-Bertani (LB) broth or agar plates at 37°C, unless otherwise indicated. V. harveyi BB170 was grown in LB or AB medium (21). A chemically defined medium (CDM-S) was used for S. aureus growth experiments (see below). Anaerobic growth experiments were carried out in an anaerobic cabinet (Don Whitley Macs MG1000) containing a humidified atmosphere of nitrogen, carbon dioxide, and hydrogen (80:10:10). Intracellular growth of S. aureus RN6390B in MAC-T cells was monitored using the nYLA-luxABCDE reporter fusion nSB203 as described by Quazi et al. (54). Antibiotics were used at the following concentrations (for E. coli and S. aureus, respectively): tetracycline at 50 µg/ml and 10 µg/ml, chloramphenicol at 50 µg/ml and 10 µg/ml, and ampicillin at 50 µg/ml (E. coli only).

Development of a chemically defined medium (CDM-S). To study the growth of S. aureus strains under more nutrient (sulfur)-restricted conditions, a formulation based on that of Onoue and Mori (51) was used. Initially, all sulfur-containing salts of this medium were replaced with their chloride equivalents. Following concentrations (for E. coli and S. aureus, respectively): tetracycline at 50 µg/ml and 10 µg/ml, chloramphenicol at 50 µg/ml and 10 µg/ml, and ampicillin at 50 µg/ml (E. coli only).

The chemically defined medium (CDM-S) used in this study consisted of 100 mM K<sub>2</sub>HPO<sub>4</sub>, 23 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 37.4 mM NH<sub>4</sub>Cl, 2.72 mM trisodium citrate, 29.6 µM thiamine, 97.5 µM nicin, 200 mM biotin, 1.0 µM calcium pantothenate, 44.6 µM uracil, 33 µM guanine, 37 µM adenine, 45 µM cytosine, 158.6 µM thymine, and amino acids at a final concentration of 0.1 mg/ml corresponding to the following concentrations: 1.12 mM alanine, 464 µM arginine, 757 µM asparagine, 751 µM aspartic acid, 680 µM glutamic acid, 684 µM glutamine, 1.33 mM glycine, 137 µM histidine, 762 µM isoleucine, 762 µM leucine, 548 µM lysine, 670 µM methionine, 605 µM phenylalanine, 869 µM proline, 840 µM threonine, 490 µM tryptophan, 552 µM tyrosine, 951 µM serine, and 853 µM valine. Glucose (10 mM) was added as a carbon and energy source. Complete CDM-S also contained cysteine at 825 µM, although titration experiments revealed that near-maximal growth could be achieved with concentrations ranging from 40 to 80 µM. For experiments under sulfur-limited conditions, cysteine was added at the concentrations indicated below (5 and 8.75 µM, respectively), and methionine was limited to 1 µM. All chemicals were of the highest purity (Sigma Ultrapure) and were dissolved as recommended by the supplier. All solutions were filter sterilized prior to storage and again prior to use. The final pH of the medium was adjusted to 7.2 to 7.4 with 1 M NaOH. Strains were precultured in CDM-S to stationary phase and washed three times in sterile phosphate-buffered saline (PBS). The final OD<sub>600</sub> at 600 nm (OD<sub>600</sub>) of each cell suspension was adjusted to 0.1, and subsequent cultures were inoculated at 1 in 1,000.

Coculture experiments. Wild-type and lux<sub>Sa</sub> mutant strains were grown as separate cultures for 18 h in CDM-S (containing cysteine and methionine at concentrations of 8.75 µM), centrifuged, and washed twice in PBS. The cell pellets were individually resuspended in PBS and adjusted to an OD<sub>600</sub> of 0.25 ± 5%. The wild type was then inoculated into CDM-S at a fixed dilution of 1 in 200. The cocultured lux<sub>Sa</sub> strain was subsequently added to give starting ratios of 1:0.8, 1:1, and 1:1.2 (vol/vol). Retrospective calculation of the viable count of the starting inocula (in quadruplicate, with respect to each strain) enabled selection of a particular combination for further analysis, which gave a ratio of 1:1 ± 5%. The cultures were incubated at 37°C, and viable-count measurements were taken on selective and nonselective media in quadruplicate. The numbers of CFU in the population of lux<sub>Sa</sub> mutant cells were indicated by viable counts on selective media, enabling subtractive determination of the wild-type population from viable counts in nonselective media. The ratio of mutant to wild-type cells (the competitive index [CI]) was then calculated.

Measurement of AI-2 activity. Analysis of the AI-2 activities of culture supernatants was essentially carried out as previously reported (6, 71). However, the AB medium (21) was modified to also contain 25 mM of HEPES buffer, pH 7.8, to counteract changes in bioluminescence due to the addition of acidic culture supernatants. Briefly, samples were filtered sterilized and diluted 1:10 in the modified AB medium containing V. harveyi BB170 (inoculated 1:5,000 from overnight culture) to give a final volume of 200 µl. The culture was incubated at 37°C, and the luminescence and optical density were determined at 30-min intervals for at least 8 h using an automated combined spectrophotometer/luminometer (Anthis Labtech Lucky 1.0). AI-2-mediated alterations in bioluminescence were expressed as an index (I), (fold-change in bioluminescence (FI) over baseline bioluminescence prior to coculture) (66). The baseline bioluminescence was determined from cultures of each strain grown in the absence of the other strain (control cultures) and in the presence of a combination of equal concentrations of both strains (co-culture cultures). The index was calculated as the ratio of co-culture to control cultures, enabling selection of a particular combination for further analysis.

Synthesis of bacterial signal molecules. AI-2 was synthesized enzymatically as described previously, using the purified Lux<sub>Sa</sub> protein and SRH as a substrate (71). S. aureus AIP (group I) was synthesized as described by McDowell et al. (37).

DNA and RNA manipulation. Recombinant DNA techniques were performed using standard methods. Restriction digests were performed in accordance with the enzyme manufacturer’s instructions (Promega, Madison, WI). All PCR were performed with Taq DNA polymerase (Roche Diagnostics, Lewes, United Kingdom). Genomic and plasmid DNA extractions were carried out using the QIAGEN range of nucleic acid recovery kits. TA cloning was carried out using the pCR2.1 vector system (Invitrogen, Carlsbad, CA). DNA-sequencing reactions were performed using the Big Dye Terminator kit v. 3.1 and processed using a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

RNA was isolated and analyzed by Northern blot hybridization as described previously (70). The transcriptional start point of the lux<sub>Sa</sub> gene was confirmed by 5′ rapid amplification of cDNA ends (RACE) in strain RN6390B using the 5′ RACE System of Gibco BRL (now a division of Invitrogen) according to the manufacturer’s protocol.

Southern blot analysis. Staphylococcal DNA was digested with HindIII, separated by electrophoresis, and bound to Hybond N+ membranes by capillary transfer. The DNA and membranes were cross-linked by UV exposure (312 nm) for 15 min prior to use. The diagnostic priming method from PCR products or restriction fragments used as templates (Dig DNA-labeling kit; Roche Diagnostics). Membranes were incubated with probes overnight at 40°C in Dig Easy Hyb (Roche Diagnostics). Posthybridization washes were conducted in 2× SSC (0.3 M NaCl, 30 mM

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TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/notes</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>dh80ΔluxZ::M15 deoR phoA λ-</td>
<td>GIBCO BRL</td>
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</table>
| DH5α | endA1 recA1 gyrA96 thi-1 hsdRI7(rK- mK+)
Δ(lacZYA-argF)U169 F' | Promega |
| JM109 | endA1 recA1 gyrA96 thi hsdRI7 (rK- mK+)
Δ(lac-proAB) [F' traD36 proAB+] | Promega |
| V. harveyi | | |
| BB170 | AI-1 sensor negative/AI-2 sensor positive | 5 |
| *Staphylococcus aureus* | | |
| BB | Invasive bovine isolate | 16 |
| NCTC8325-4 | Laboratory strain; rsbU defective | 47 |
| Newman | Wild type | 18 |
| IK184 | Newman derivative; ΔrsbUVW sigB::erm' | 33 |
| RN4220 | Derived from NCTC8325-4; rRm+ | 47 |
| RN6390B | Standard laboratory strain | 50 |
| RN63911 | RN6390B derivative; agr locus replaced with tetM cassette | 50 |
| SA564 | Invasive isolate | 44 |
| SH1000 | SH1000 rB'U | 27 |
| AC1 | Carried isolate; QMC, Nottingham, United Kingdom | This study |
| AC5 | Carried isolate; QMC, Nottingham, United Kingdom | This study |
| AC7 | Lung isolate; QMC, Nottingham, United Kingdom | This study |
| AC11 | Lung isolate; QMC, Nottingham, United Kingdom | This study |
| AC15 | Exit wound isolate; QMC, Nottingham, United Kingdom | This study |
| AC16 | Blood isolate; QMC, Nottingham, United Kingdom | This study |
| AC22 | Sputum isolate; QMC, Nottingham, United Kingdom | This study |
| AC23 | Leg wound isolate; QMC, Nottingham, United Kingdom | This study |
| AC24 | Blood isolate; QMC, Nottingham, United Kingdom | This study |
| AC27 | Deep sepsis isolate; QMC, Nottingham United Kingdom | This study |
| BB luxS | BB; ΔluxS::Te' | This study |
| NCTC8325-4 luxS | NCTC8325-4; ΔluxS::Te' | This study |
| Newman luxS | Newman; ΔluxS::Te' | This study |
| IK184 luxS | IK184; ΔluxS::Te' ΔrsbUVW sigB::erm' | This study |
| RN4220 luxS | RN4220; ΔluxS::Te' | This study |
| RN6390B luxS | RN6390B; ΔluxS::Te' | This study |
| SA564 luxS | SA564; ΔluxS::Te' | This study |
| SH1000 luxS | SH1000; ΔluxS::Te' rB'U | This study |
| Plasmids | | |
| pCR2.1 TOPO | Commercial TA cloning vector; Ap' Kan' | Invitrogen |
| pET19-luxSsa | Expression vector encoding luxS of *S. aureus* for the production of His-tagged LuxS protein; Ap' | 71 |
| pND-ls2 | pCR2.1 bearing the luxS gene; Ap' Kan' | This study |
| pND-ls2::tet | Suicide vector derived from pND-ls2; for luxS mutagenesis; Ap' Kan' Te' | This study |
| pSB2030 | xyAI-gfp-luxABCDE reporter; Ap' Cm' constitutive expression of lux operon | 53 |
| pSB2035 | agrP3-gfp-luxABCDE reporter; used to monitor AIP-dependent RNAIII expression; Ap' Cm' | 53 |
| pS10 | Staphylococcal expression vector; Ap' Cm' | 43 |
| pS10-luxS | pS10 with luxSa, expressed from a constitutive promoter; Ap' Cm' | This study |
| pUF1 | luxS promoter test vector; contains luxS upstream region, lacking putative −10 and −35 regions; luxCDABE reporter; Ap' Cm' | This study |
| pUF2 | luxS promoter test vector; contains luxS upstream region, including putative −10 region; luxCDABE reporter; Ap' Cm' | This study |
| pUF3 | luxS promoter test vector; contains luxS upstream region, including putative −10 and −35 regions; luxCDABE reporter; Ap' Cm' | This study |
| pUF4 | luxS promoter test vector; contains luxS upstream region, including putative −10 and −35 regions, and additional 0.25 kb; a luxCDABE reporter; Ap' Cm' | This study |

a QMC, Queen’s Medical Centre.

Mutagenesis of the luxSa locus. A 2.4-kb region of the genome of RN4220 was amplified using the primer pair L2-F/L2-R (forward, 5'-ACCATGGATCCGTT GATCTTTT-3'; reverse, 5'-TTATATCCAGATCCGATTAGA-3'). This was cloned into the pCR2.1 TA vector system (Invitrogen) to give pND-ls2, and a 658-bp fragment containing the extended promoter region and 30% of the luxS coding sequence was excised with NdeI. A cassette conferring tetracyline resistance was amplified from pDG1515 (23) using the primer pair Tet-Nde-F/Tet-Nde-R (forward, 5'-AAGTCTTGCAATGGTGCATATGGTTCTCAATG-3'; reverse, 5'-ACCATGGATCCGTT GATCTTTT-3'). This was cloned into the pCR2.1 TA vector system (Invitrogen) to give pND-ls2, and a 658-bp fragment containing the extended promoter region and 30% of the luxS coding sequence was excised with NdeI. A cassette conferring tetracycline resistance was amplified from pDG1515 (23) using the primer pair Tet-Nde-F/Tet-Nde-R (forward, 5'-AAGTCTTGCAATGGTGCATATGGTTCTCAATG-3'; reverse, 5'-ACCATGGATCCGTT GATCTTTT-3').

Transformation and transduction. Electrocompetent *E. coli* and *S. aureus* were produced using established protocols (2). Bacteria were electroporated into a Bio-Rad Gene Pulser with Pulse Controller. *E. coli* was treated at 2.5 kV, with a resistance of 200 Ω at 25 μF capacitance. *S. aureus* was treated at 2 kV, with a resistance of 100 Ω at 25 μF capacitance. Transformations were performed using the general transducing phage dphi (48).

sodium citrate, pH 7.0) with 0.1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature, followed by 0.1× SSC at 68°C. Hybridized probe was detected using an anti-digoxigenin-alkaline phosphatase conjugate and the lumigenic substrate CDP Star (Boehringer Mannheim).

Transformation and transduction. Electrocompetent *E. coli* and *S. aureus* were produced using established protocols (2). Bacteria were electroporated in a Bio-Rad Gene Pulser with Pulse Controller. *E. coli* was treated at 2.5 kV, with a resistance of 200 Ω at 25 μF capacitance. *S. aureus* was treated at 2 kV, with a resistance of 100 Ω at 25 μF capacitance. Transformations were performed using the general transducing phage dphi (48).
construct, pND-hc::tet, is nonreplicative in *S. aureus* and was used as a suicide vector in strain RN4220. Several transformants were screened on the basis of tetracycline resistance. Subsequent PCR with the primer pair MCF-F/MCF-R (forward, 5'-ATCTCGGCCAACAGCTAGTAC-3'; reverse, 5'-AGCGCCGCA CTTCAGTACATTG-3') revealed a number of integrants, including single- and double-crossover mutants. A putative double-crossover strain was selected, and the presence of the tetracycline resistance marker (and absence of the suicide vector backbone) was confirmed by Southern analysis using a PCR product (forward, 5'-CTCGCAAGTGTAGTAAACACAACTTACAAC-3'; reverse, 5'-CCCCGCCCATCAGCCATTGCTGGTGTT-3') as a template for a luxS-specific probe and a 1.6-kb PstI fragment from pCR2.1 as a vector backbone probe template.

Construction of transcriptional luxS reporter fusions. Various luxABCDE reporter constructs were generated to confirm the luxS promoter region and identify the elements required for its activity. Forward-facing primers were designed to amplify increasing stretches of the region upstream of the luxS initiator codon (see Fig. 2), in combination with a common reverse primer (forward UF1, 5'-TGAATTCATTTAGAAACACAACTTAC-3'; UF2, 5'-GTAAGAA TTCAAGCCCTATTGTTACAATTAG-3'; UF3, 5'-AAACGAATTICAAATT GTAAACAATAGGCCC-3'; UF4, 5'-CGTCTAGATTTAGTACATGCTAGAT TTGG [EcoRI sites are underlined]; reverse UR1, 5'-ATTCCGCGGTGGTTC AATTTTCTCCATTAAAC-3' [the Small site is underlined]). These were cloned into a derivative of pSB2035 (53), from which the ampR3 promoter had been removed by SmaI/EcoRI double digestion. These constructs were transformed into RN6390B, and light outputs from the reporter strains were compared during growth in LB, CYGP, and RPMI 1640 (Gibco-BRL). Bioluminescence was measured using an automated combined spectrophotometer/luminometer (Amersham LifeTech, Buckinghamshire).

Complementation of luxS strains in trans. The *E. coli/S. aureus* shuttle vector pS10 (43) was used to express luxS in trans in recipient bacteria. This vector contains the constitutive S10 promoter and the T2 transcriptional terminator, pS10 (43) was used to express *S. aureus*; UF2, 5'-H11032 TTAACTTGATTGACTA-3' (the PstI site is underlined); reverse, 5'-H11032 TTAACTTGATTGACTA-3' (the PstI site is underlined). These were cloned into a derivative of pSB2035 (53), from which the ampR3 promoter had been removed by SmaI/EcoRI double digestion. These constructs were transformed into RN6390B, and light outputs from the reporter strains were compared during growth in LB, CYGP, and RPMI 1640 (Gibco-BRL). Bioluminescence was measured using an automated combined spectrophotometer/luminometer (Amersham LifeTech, Buckinghamshire).

Production of polyclonal antiserum raised against LuxSSa. The LuxSSa protein was generated using the Novagen pET19b/Es. coli BL2I DE3 expression system. Purified Hi-tagged LuxSSa (71) was combined with Freund's complete adjuvant, and 50 to 400 µg was used to subcutaneously inoculate New Zealand White rabbits. Booster doses were administered in Freund's incomplete adjuvant at 2, 4, and 6 weeks after the primary dose. Serum was collected 1 week after the final immunization.

Phenotypic assays. Biofilm formation was assessed using a procedure based on the method and conditions published by Cronin et al. (12). Bacteria were grown overnight in tryptic soy broth (TSB) containing 1% (wt/vol) glucose. Five-microliter aliquots of this culture were then transferred into the wells of a tissue culture-treated 24-well plate (Costar 3524; Corning Inc., Corning, NY), each containing 1 ml TSB with 1% (wt/vol) glucose and 3% (wt/vol) sodium chloride. The plates were incubated for 18 h to 48 h at 37°C under strictly anaerobic conditions (to enhance biofilm formation) (12) in a MG1000 cabinet (Don Whitley Scientific), and the culture supernatant was carefully removed. Five hundred microliters of 0.1% (wt/vol) safranin solution was added, and the plates were incubated at room temperature for 30 min. The safranin was then removed, and each well was washed twice with 1 ml filter-sterilized PBS. Adherent bacteria were clearly visible as a safranin-stained layer at the bottom of the well.

Hemolysis assays. Hemolysis assays were carried out as follows. Bacteria were grown to mid-exponential phase in LB, and 5 µl of the culture was inoculated onto LB-agar containing 5% (vol/vol) rabbit erythrocytes, which had been washed twice in ice-cold PBS. The plates were incubated at 37°C for 18 h and then placed at 4°C for at least 1 hour. Lysis of erythrocytes resulted in a zone of clearance around the inoculated spot of bacteria. The phenotypes observed were classified as positive (a halo of 2 to 3 mm around the region of bacterial growth) or negative (sometimes seen as a very narrow rim of 1 mm or less around the region of bacterial growth). A quantitative assay was also employed, which was based on a dilution series of bacterial culture supernatant incubated with rabbit erythrocytes in microtiter plates (14).

The protease activities of staphyloccocal supernatants were assessed as follows. Mid-exponential-phase bacterial cultures were centrifuged at 5,000 × g, and the supernatants were filter sterilized (pore diameter, 0.2 µm) (Millipore Acrodisc; Millipore, Bedford, MA). Two milliliters of this fraction was concentrated 100-fold using centrifugal filter devices with a 10-kDa nominal molecular mass cutoff (Amicon YM-10; Millipore). The protease activities of the samples were assessed using the Novex zymogram in-gel protease detection system (Invitrogen), with proteases forming zones of clearance in casein gels stained with bromophenol blue. The total protein profile of these preparations was analyzed after SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue. Cell wall proteins were isolated and analyzed as previously described (43).

RESULTS

*S. aureus* possesses a functional luxS gene and produces AI-2 under aerobic and anaerobic conditions. Analysis of the publicly available genome sequences of *S. aureus* (strains N315, Col, MW2, Mu50, MSSA476, and MRSA252) revealed a putative luxS gene. The predicted protein (LuxSSa) was over a stretch of 145 amino acids, 44% identical and 64% similar to the LuxS protein of *V. harveyi* (LuxSa). This suggested that *S. aureus* is capable of producing the signal molecule AI-2 in addition to the AIPs. However, homologues of the known *V. harveyi* AI-2 receptor and signal transduction genes, *luxP*, *luxQ*, *luxU*, and *luxO*, could not be identified, suggesting that LuxSa produced AI-2 was sensed, if at all, via a different pathway. In all available *S. aureus* genome sequences, LuxSa is located upstream of *hmrA* (which is involved in methicillin resistance, similar to amidases/aminocyclases/carboxypeptidases) and downstream of a conserved hypothetical gene with an unknown function (a predicted membrane protein). The LuxSa open reading frame lies on the complementary strand with respect to both of these flanking genes and thus does not appear to be part of an operon. PCR primers were generated, which allowed the identification of the LuxSa gene in a range of clinical and laboratory *S. aureus* isolates (see Materials and Methods for the strains tested). The gene was present in all isolates tested, which was further confirmed by Southern blot hybridization (data not shown).

Several *S. aureus* strains (RN6390B, Newman, and NCTC 8325-4) were grown in LB broth under aerobic and strictly anaerobic conditions, both with and without 50 mM glucose. Under these conditions, AI-2 activity in the culture supernatant was detectable from the mid-exponential phase onward, reaching maximum levels during the transition to, or the beginning of, stationary phase (Fig. 1 for *S. aureus* RN6390B and data not shown). During stationary phase, AI-2 levels decreased in aerobic LB cultures without glucose and were almost undetectable by 24 h (not shown). Similar data were obtained with CYGP medium (Fig. 2C, and data not shown). In the presence of glucose, however, AI-2 levels in aerobic cultures decreased only to approximately 60% of the peak value during the 2 h following the peak before rising slightly again during the further course of stationary phase. Interestingly, AI-2 profiles similar to this were also observed during anaerobic growth in the absence and, more dramatically, the presence of glucose (Fig. 1). In the latter case, AI-2 levels, after peaking initially at the beginning of stationary phase, transiently decreased before rising again to levels equal to or above those observed during the first peak.

Although maximal AI-2 levels were lower in anaerobic cultures, cell population densities were also reduced under these conditions (Fig. 1). Taking into account both parameters, an-
aerobic cells on average produced two to three times more extracellular AI-2.

Maximal AI-2 activities in aerobically grown LB cultures of S. aureus RN6390B, NCTC8325-4, and Newman were substantially lower (10- to 20-fold) than those observed for E. coli MG1655 cultures under the same conditions. Two clinical S. aureus isolates, AC23 and AC24, were also found to produce only low levels of AI-2 (10-fold activation of the bioassay) (data not shown).

**Characterization of the luxS transcript.** A putative transcriptional start point for the luxSsa gene was identified 97 base pairs upstream of the ATG start codon using 5’ RACE (Fig. 2A). Immediately upstream of the putative start point, a potential σ70-dependent promoter (TTGTAAN17-TACAAT) was identified on the basis of its similarity to the consensus sequence (TTGACAN16-19-TATAAT). This promoter contained an extended −10 region (i.e., an additional TGn motif immediately upstream of the −10 motif), which in some bacteria is known to increase promoter strength (4, 42). A putative −44 motif (AGTT), identified upstream of strongly expressed promoters in lactococci (46), was also present. The functionality of this promoter in the S. aureus strains RN4220 and RN6390B was confirmed using a series of promoter test vectors that were derived from the reporter plasmid pSB2035 (53). These plasmids contained various components of the luxSsa upstream region fused to a gfp-luxABCDE reporter cassette (Fig. 2A). Luciferase activity obtained from these constructs after their transfer into S. aureus reflected the relative abilities of the cloned fragments to stimulate transcription of the reporter genes.

Reporter constructs either lacking the entire promoter region (pUF1) or containing only the extended −10 region (pUF2) gave rise to only very low levels of luciferase activity, close to the detection limit (Fig. 2B). However, substantial activities were obtained with constructs containing both the −10 and −35 regions (pUF3 and pUF4, respectively). pUF3 contains the −10 and −35 regions and 5 base pairs upstream of the latter, whereas pUF4 contains the same sequence and an additional 0.25 kbp of upstream region, including the putative AGTT motif. Higher luciferase activities observed with pUF4 suggest that elements upstream of the −35 region contribute to the strength of the luxSsa promoter. Similar activity ratios were observed with the above-mentioned constructs whether S. aureus was grown in LB, CYGP, or RPMI medium (Fig. 2B) or when activities were determined at different time points during growth (not shown). Furthermore, the overall expression profiles for pUF3 and pUF4

![FIG. 1. AI-2 profiles of S. aureus RN6390B cultures under aerobic and anaerobic conditions. S. aureus RN6390B (squares) and the isogenic luxSsa mutant (circles) were grown in LB medium under aerobic (left) and anaerobic (right) conditions. Growth (top) and AI-2 contents of the supernatants (bottom) were monitored at the indicated intervals, the latter using the V. harveyi BB170-based bioassay (6). Cultures were grown in the presence (open symbols) and absence (closed symbols) of 0.5% glucose. The data shown represent the mean values of four parallel AI-2 bioassays; the experiment was repeated three times with similar results.

Aerobic | Anaerobic
---|---
Growth [OD600] | |
0.01 | 0.01
Time [h] | 0 | 0
AI-2 activity [fold induction] | 0 | 0
0 | 0
2 | 2
4 | 4
6 | 6
8 | 8
10 | 10
12 | 12
FIG. 1. AI-2 profiles of S. aureus RN6390B cultures under aerobic and anaerobic conditions. S. aureus RN6390B (squares) and the isogenic luxSsa mutant (circles) were grown in LB medium under aerobic (left) and anaerobic (right) conditions. Growth (top) and AI-2 contents of the supernatants (bottom) were monitored at the indicated intervals, the latter using the V. harveyi BB170-based bioassay (6). Cultures were grown in the presence (open symbols) and absence (closed symbols) of 0.5% glucose. The data shown represent the mean values of four parallel AI-2 bioassays; the experiment was repeated three times with similar results.
during growth were remarkably similar under all conditions tested (not shown). Taken together, these results suggest that (i) \( \text{luxS}_{Sa} \) is constitutively expressed and (ii) the region upstream of the \( \sigma^{35} \) region, although contributing to promoter strength, does not bear further regulatory elements.

A \( \text{luxS}_{Sa} \) transcript size of approximately 600 nucleotides was determined by Northern blot analysis, indicating a monocistronic transcriptional unit (Fig. 2C). For \( S. aureus \) RN6390B, \( \text{luxS}_{Sa} \) transcript profiles were also monitored during growth in CYGP medium (Fig. 2C). The gene was transcribed continuously
LuxS is required for AI-2 production by *Staphylococcus aureus*. An insertion/deletion *luxSsa* mutant was produced in strain RN4220, replacing the promoter region and approximately 50% of the *luxSsa* coding region with a tetracycline resistance cassette (see Materials and Methods for details). The generalized transducing phage λ11 was used to transfer this mutation into strains RN6390B, Newman, NCTC8325-4, BB, SH1000, SA564, and IK184. AI-2 production was completely abolished in these strains, confirming that *luxSsa* is a key determinant in the AI-2 production pathway in *S. aureus* (Fig. 1 and data not shown).

Complementation of the *luxSsa* mutation in *trans* was carried out by cloning the *luxSsa* structural gene into the staphylococcal expression vector pS10 (43). In the resulting plasmid, pS10-luxS, *luxSsa* is under the control of a constitutive promoter. The *E. coli* strain DH5a, as well as the *S. aureus* strains RN6390B luxS and Newman luxS, were transformed with either pS10 alone or pS10-luxS. Western blot analysis with rabbit polyclonal LuxSSa antiserum and AI-2 bioassays confirmed that LuxSSa and AI-2 production was restored in the complemented strains (Fig. 3). In all backgrounds, the constitutive expression of the plasmid-borne gene led to production of LuxSSa at levels exceeding that observed in the wild type. However, in the complemented mutants, AI-2 production was comparable to that of the parent strains, suggesting that in both backgrounds production was limited by substrate availability rather than LuxS activity. No immunoreactive LuxSSa protein was observed in mutant strains transformed with pS10 alone.

There is no cross talk between the *agr* and LuxS/AI-2 signaling systems. Experiments were designed to establish whether *agr*-dependent AIP signaling influences the potential LuxS/AI-2 quorum-sensing system or vice versa. First, the AI-2 production profiles for *S. aureus* RN6390B (the *agr*-positive parental strain) and its derivative, *S. aureus* RN6911 (lacking the *agr* locus), were monitored during growth in LB broth. No significant differences were observed (Fig. 4A). In addition, the reporter construct pSB2035 (53) was used to monitor the activity of the *agr* promoter in *S. aureus* strains RN6390B and Newman and their respective *luxS* mutants (the plasmid contains a luxABCDE cassette under the control of the *agr* P3 promoter). The expression profile of this reporter was the same in the wild type and mutants (Fig. 4B and data not shown).
shown), suggesting that luxS<sub>Sa</sub> inactivation did not influence agr-dependent gene expression under the conditions tested.

The effects of exogenously added synthetic signal molecules on <i>agr</i> P3 and luxS<sub>Sa</sub> reporter genes were also tested. RN6390B had previously been characterized as an <i>agr</i> group 1 strain (28), and our <i>agrD</i> sequence analysis of the Newman strain revealed that this strain also produces the AIP-1 peptide (GenBank accession number AY580334). In both strains, addition of 1 μM AIP-1 stimulated the <i>agr</i> P3 promoter but had no significant effect on the activity of the luxS<sub>Sa</sub> promoter carried by the reporter plasmid pUF4 (data not shown). Furthermore, addition of in vitro-synthesized AI-2 (equivalent to 7.2 μM DPD) failed to induce any effect on either the <i>agr</i> P3 promoter or the luxS<sub>Sa</sub> promoter (data not shown). The expression profiles of the latter promoter were identical in RN6390B and the isogenic luxS<sub>Sa</sub> mutant, further demonstrating that AI-2 does not exert a feedback effect on its own production, i.e., AI-2 production is not autoinducible in <i>S. aureus</i>.

**Phenotypic analysis of luxS<sub>Sa</sub> mutants.** To determine whether mutation of luxS<sub>Sa</sub> influenced the expression of staphylococcal virulence determinants, a number of different assays were performed. Hemolysis production was studied using both rabbit blood agar plates and a semiquantitative dilution assay, and biofilm formation on polystyrene was quantified after safranin staining (see Materials and Methods for details). The Novex in-gel zymogram system was employed to analyze the production of extracellular proteases, whereas protein profiles of cell wall preparations and spent culture supernatants were compared after separation by one-dimensional SDS-polyacrylamide gel electrophoresis. Strains analyzed in this way included RN6390B, Newman, NCTC8325-4, BB, SH1000, SA564, and IK184 and their isogenic luxS<sub>Sa</sub> mutants.

Phenotypic differences were initially observed between wild-type and luxS<sub>Sa</sub> mutant strains in the RN6390B and NCTC 8325-4 backgrounds, with a general reduction of protease production, hemolysis, and biofilm formation upon loss of luxS<sub>Sa</sub>. However, the parental phenotype was not restored by addition of in vitro-synthesized AI-2 or transformation with the complementation vector, pS10-luxS. Most importantly, rederivation of luxS<sub>Sa</sub> mutants in these backgrounds by phi1 phage transduction failed to consistently generate the range of luxS<sub>Sa</sub> mutant phenotypes described above, leading to the conclusion that these had arisen as a result of second-site mutations and were therefore not directly attributable to the loss of luxS<sub>Sa</sub>.

Our inability to restore the putative luxS<sub>Sa</sub> phenotypes observed in distinct genetic backgrounds by complementation with a functional copy of the gene, or addition of in vitro-synthesized AI-2, and the apparent occurrence of second-site mutations led us to consider phenotypes associated with the metabolic burden of luxS<sub>Sa</sub> inactivation, rather than quorum sensing per se. For all subsequent experiments, only those luxS mutant isolates of <i>S. aureus</i> RN6390B and Newman were used that did not display the above-mentioned defects in hemolysin production, biofilm formation, and protease production and hence appeared to be free of second-site mutations.

**luxS<sub>Sa</sub> strains are outgrown by wild-type <i>S. aureus</i> in chemically defined medium.** Metabolic roles for LuxS in the recycling of methionine and the detoxification of SAH through the active methyl cycle have been described (17, 67, 71, 72, 73). However, the relative contribution of the activated methyl cycle to methionine biosynthesis in different bacteria is not known. It is speculated that a disruption of the activated methyl cycle will increase the need for methionine uptake from the environment or, if not available, de novo synthesis. In the latter case, the metabolic burden imposed on the cells might influence growth or fitness. However, in complex media, such as LB, CYGP medium or TSB, there was no detectable growth defect associated with the mutation of the luxS<sub>Sa</sub> locus in <i>S. aureus</i> (Fig. 1 and data not shown). Therefore, the effect of luxS<sub>Sa</sub> inactivation on <i>S. aureus</i> growth was examined in a more restricted and chemically defined medium (CDM-S) under conditions of sulfur limitation (5 μM cysteine was provided as a sulfur source; see Materials and Methods for medium composition). Experiments using <i>S. aureus</i> Newman and the corresponding luxS<sub>Sa</sub> mutant indicated that the mutant grew less well in this medium than the wild type (Fig. 5A). Strain RN6390B was not amenable to this type of analysis, as it had an increased tendency to clump when grown in CDM-S. Next, it was necessary to establish whether the observed growth defect was luxS<sub>Sa</sub> dependent or whether it was the result of second-site mutations. Furthermore, the possibility that growth was dependent on AI-2 production had to be investigated. The presence of the tetracycline resistance marker in luxS<sub>Sa</sub> mutant strains enabled the determination of relative and absolute population sizes in competition experiments, where <i>S. aureus</i> luxS<sub>Sa</sub> mutants were grown in coculture together with their respective parent strains. If the growth phenotype observed for luxS<sub>Sa</sub> mutants in CDM-S medium were relieved under coculture conditions, this would suggest one of two possibilities: either a LuxS<sub>Sa</sub>-dependent quorum-sensing mechanism is involved in growth regulation or a growth factor that is required for maximal growth in CDM-S medium is produced and released by the parent strain (but not the luxS<sub>Sa</sub> mutant). Alternatively, if the growth defect of luxS<sub>Sa</sub> strains was not relieved under coculture conditions, it would suggest that the observed effect was of an intracellular (metabolic) nature.

In CDM-S, the CI (the ratio of mutant to wild type in the culture after 24 h of incubation following inoculation at a 1:1 ratio) decreased considerably for all strains tested (Newman, BB, and SA564) (Fig. 5B), again indicating a reduced capability of the luxS<sub>Sa</sub> mutants to grow under these conditions. In contrast, in LB medium, <i>S. aureus</i> Newman and Newman luxS showed CIs of approximately 1. To eliminate the possibility that secondary-site mutations were responsible for the observed growth defects in CDM-S, the coculture studies were repeated using the complemented Newman strains described below. Initial experiments included either the Newman wild-type and Newman luxS strains containing pS10 only (vector control) or Newman wild type containing pS10 and Newman luxS containing pS10-luxS (Fig. 5B). Conditions under which both strains contained the control vector resulted in a lower CI (0.25) than conditions under which the wild-type strain contained pS10 and the luxS<sub>Sa</sub> mutant strain contained pS10-luxS (CI of 0.7). This suggested that complementation of the luxS<sub>Sa</sub> mutation in trans did indeed increase the ability of the Newman luxS mutant to compete with the wild type in CDM-S. Due to the strong constitutive promoter in pS10-luxS, large quantities of the LuxS<sub>Sa</sub> protein were expressed in the complemented strain (Fig. 3). This suggested that the additional metabolic burden of LuxS<sub>Sa</sub> overproduction influenced the growth
of the complemented luxS<sub>sa</sub> mutant. Therefore, the complementation studies were repeated using Newman luxS<sub>pS10-luxS</sub> and Newman pS10-luxS in coculture experiments. The CI returned to 1 under these conditions, suggesting that the two strains grew equally well in sulfur-limited CDM-S (Fig. 5).

**S. aureus** and its corresponding luxS<sub>sa</sub> mutant show similar growth profiles after the invasion of MAC-T cells. Several human pathogens with mutations in sulfur metabolism genes have been shown to be attenuated (35, 62). Furthermore up-regulation of sulfur acquisition genes upon host cell contact has been reported (22), suggesting that sulfur availability could be a growth-limiting factor during infection. As mutation of luxS<sub>sa</sub> affected growth in CDM-S but not in complex media, we investigated whether the mutant and wild type displayed any differences during intracellular growth in epithelial cells. The ability of **S. aureus** cells to multiply in MAC-T cells was assessed using the method of Qazi et al. (54), which utilizes the reporter plasmid pSB2030 containing a gfp-luxABCDE reporter operon under the control of the xylA promoter. In **S. aureus**, this operon is expressed in a growth-phase-dependent manner, so that cell growth is directly correlated with an increase in luxABCDE-dependent bioluminescence. Growth rates, and thus expression, of the reporter in **S. aureus** RN6390B and RN6390B luxS<sub>sa</sub> were virtually identical when the strains were grown in LB medium (not shown). When the respective strains were grown in MAC-T cells, the increases in reporter-dependent bioluminescence were again very similar (Fig. 6A), suggesting that inactivation of luxS<sub>sa</sub> had no obvious effect on intracellular growth. However, luxS<sub>sa</sub> was expressed under these conditions, as demonstrated by the expression of the transcriptional luxS<sub>sa</sub>-luxABCDE fusion present on the reporter plasmid pUF4 (Fig. 6B). Interestingly, the bioluminescence output from this construct mirrored that of pSB2030, suggesting that like the latter, it is expressed at a constant level under these conditions and is thus suited to monitor intracellular growth.

**DISCUSSION**

In bacteria of the genus *Vibrio*, and probably also in closely related species, the LuxS/AI-2 system forms part of a complex, multilayered quorum-sensing system (24, 25, 26, 31, 34, 40). Diffusible molecules with AI-2 activity are also produced by a diverse range of other bacteria, among them many pathogens, but their involvement in quorum-sensing-related processes remains a matter of debate (5, 30, 60, 72, 76). This is mainly due to the dual function of LuxS, which is involved in both metabolic and signaling processes (i.e., the salvage of homocysteine from SAH and the generation of the AI-2 precursor molecule DPD). It is clear, however, that luxS<sub>p</sub> inactivation in several pathogens affects functions important for virulence, such as the production of exoenzymes and toxins (*Campylobacter jejuni*, *Clostridium perfringens*, *Porphyromonas gingivalis*, *Streptococcus*).
**FIG. 6.** Growth of *S. aureus luxS* mutants in MAC-T cells (A) and intracellular expression of *luxS* (B). (A) Invasion and intracellular growth of *S. aureus* RN6390B in MAC-T cells were studied using a constitutively expressed *xylA-luxABCDE* fusion (pSB2030) as described by Oazi et al. (54). The intracellular growth of the parent strain (open squares) and *luxS*mutant (closed squares) was quantified by measuring the bioluminescence (in relative light units) of the respective cell cultures. Mean values and standard deviations of 24 parallel assays are shown; the experiment was repeated three times with similar results. (B) Intracellular expression of *luxS* was established using *S. aureus* RN6390B carrying plasmid pSB2030 (open squares; as a positive control), the transcriptional *luxS-luxABCDE* reporter pUF4 (closed squares) (Fig. 2), and pUF1 (open circles; as a negative control). Mean values and standard deviations of 12 parallel assays are shown; the experiment was repeated three times with similar results.

*pneumoniae*, *Streptococcus* *mutans*, *Streptococcus* *pyogenes*, and *Vibrio* *vulnificus*), motility (*enterohemorrhagic E. coli*, *enteropathogenic E. coli*, *Helicobacter pylori*, and *C. jejuni*), and biofilm formation (*Klebsiella pneumoniae*, *Porphyromonas gingivalis*, *S. enterica*, *Streptococcus gordonii*, *S. mutans*, and *H. pylori*) (reviewed in references 26, 30, 66, 73, and 76). Some *luxS* mutants (*Neisseria meningitidis*, *Salmonella enterica*, *Streptococcus pneumoniae*, and *Vibrio vulnificus*) have also been shown to be attenuated in various experimental-infection model systems (9, 29, 31, 61, 74), whereas others showed increased virulence (13). This paper analyzes the *S. aureus luxS* system in the contexts of both quorum-sensing and metabolism.

AI-2 formation by *S. aureus* was strictly *luxS*-dependent and peaked during the transition to stationary phase under a range of different conditions, including anaerobiosis. After entry into stationary phase, AI-2 activity was significantly reduced in the absence of glucose, suggesting either uptake or degradation. However, none of the published *S. aureus* genomes contains potential homologues for either the *lr* AI-2 uptake system present in a diverse range of bacteria (including several gram-positive *Bacillus* spp.) or the LuxP/LuxQ AI-2 signal transduction system found in *Vibrio* spp. Thus, if AI-2 is deployed as an extracellular signal by *S. aureus*, it must be sensed via a different mechanism. The monocistronic *luxS* gene is under the control of a σ70-dependent promoter and was continuously expressed under all conditions tested. This implies that LuxS is required throughout growth. Accordingly, the gene was also expressed when *S. aureus*, after its escape from the endosome, grew intracellularly in MAC-T cells. Constitutive expression has also been reported for *S. enterica* serovar Typhimurium (7), whereas in *Streptococcus bovis* *luxS*, transcript levels have been reported to vary considerably depending on the growth phase (1).

Inactivation of the LuxS/AI-2 system had no effect on the well-established *agr*-based AIP signaling system, and vice versa, but initial investigations suggested that virulence-associated traits, such as extracellular proteases, hemolysin production, and biofilm formation, might be down-regulated. Similar observations have been published for several other pathogenic or commensal bacteria. However, detailed analysis, including the construction of *luxS* mutants in different *S. aureus* strains, investigation of several independently obtained *luxS* mutants for each background, and plasmid-based complementation studies, as well as the use of defined preparations of synthetic AI-2, revealed that these phenotypes were neither directly nor consistently associated with the inactivation of *luxS* or the lack of extracellular AI-2. Most likely, they were the result of second-site mutations.

Interestingly, *luxS* mutants of certain bacteria have been described as being impaired in the ability to form monospecies or mixed-species biofilms. These include *P. gingivalis*, *S. gordonii*, *S. mutans*, *S. enterica* subspecies, and *K. pneumoniae* (3, 8, 20, 38, 39, 52, 69, 78). Although the phenotype has generally been attributed to the loss of AI-2-dependent signaling, this conclusion is based on indirect evidence, i.e., the phenotype of *luxS* mutants, addition of AI-2-containing spent culture supernatants, or coculture of the wild type and mutant. Furthermore, genetic complementation of the *luxS* mutation has often not been undertaken (reviewed in reference 66). Significantly, none of the above-mentioned studies attempted to use a defined preparation of AI-2 to restore the biofilm characteristics of the wild type. Very recently, however, De Keersmaecker et
al. (15) demonstrated that AI-2 derived from synthetic DPD could not restore biofilm formation by an *S. enterica* serovar Typhimurium luxS mutant, whereas introduction of luxS under the control of its own promoter complemented the defect. Similarly, addition of synthetic AI-2 failed to restore type III secretion and motility defects in enterohemorrhagic *E. coli* (60), phenotypes previously attributed to luxS/AI-2-based quorum sensing by Sperandio et al. (57, 58, 59). Addition of in vitro-synthesized AI-2 also failed to induce significant changes in the *N. meningitidis* proteome (56), although luxS mutants had previously been shown to be attenuated (74). Taken together, these reports once again highlight the fact that a number of criteria have to be met before a signaling function can be assigned to a secreted metabolite (71, 72). Currently, the only definitively confirmed function of AI-2 outside of the genus *Vibrio* is the induction of its own uptake and modification via the *lsr* system in *E. coli* and *S. enterica* serovar Typhimurium (64, 65, 68, 77).

An intriguing question raised in the current LuxS/AI-2 debate is concerned with the conservation of SAH recycling pathways (76, 71, 72) and their impact on bacterial metabolism. It is not known why some bacteria use the SAH hydrolase and others the Pfs/LuxS pathway to convert SAH back to homocysteine. Furthermore, we have only limited information on how disruption of these pathways impacts on cellular metabolism and thus bacterial fitness. We addressed these issues by analyzing the growth of luxS mutants in a defined, sulfur-limited medium. The rationale for this approach was as follows. From a metabolic point of view, the combined reactions catalyzed by Pfs and LuxS serve at least two purposes. First, SAH needs to be detoxified. Second, the homocysteine moiety of SAH is salvaged and, in organisms with a complete AMC, reused for methionine and thus SAM synthesis. Salvage pathways are expected to be particularly important when the supply of major nutrients is severely limited. In the case of the AMC, luxS mutants might not be able to make the most economical use of the available sulfur source, and under conditions of sulfur limitation, this might manifest itself in an imbalanced metabolism and thus a growth defect.

Indeed, in sulfur-limited CDM-S medium, *S. aureus* luxS mutants were less competitive than their respective parent strains (the strains used in this study could not use sulfate and had to rely on the provided supplement of cysteine and methionine [see reference 32 for a discussion of staphylococcal sulfur requirements]), supporting the notion that luxS plays an important role in sulfur metabolism under these conditions. However, the alternative explanation, that this phenotype was due to a lack of AI-2-dependent signaling, had to be considered. Furthermore, Sperandio et al. (60) provided evidence, at least in *E. coli*, for the existence of yet another LuxS-dependent signal molecule, AI-3. To clarify whether the observed growth defect was indeed caused by the lack of a signal molecule, such as AI-2 or AI-3, or any other diffusible factor or whether it was indeed of intracellular nature, parent strains and luxS mutants were grown in mixed culture and the competitive index was determined. Even under coculture conditions, where all cells share the same diffusible signals, extracellular metabolites, and nutrients, a growth defect for the *S. aureus* luxS mutants was still observed. Most importantly, the phenomenon was clearly luxS-dependent (i.e., in the presence of a plasmid-borne copy of luxS, the parent strain and mutant grew equally well).

Indirect evidence obtained from other pathogens suggests that access to sulfur sources might be a limiting factor during infection for some bacteria (e.g., up-regulation of sulfur acquisition genes upon host cell contact for *N. meningitidis* (22), although this clearly depends on a range of different factors, such as the specific site of the infection or the particular requirements of the infecting organism. In a model for intracellular growth and survival, no significant differences were observed for *S. aureus* RN6390B wild type and the corresponding luxS mutant after their invasion of MAC-T cells, suggesting that, although the gene is expressed in the wild type, the metabolism of luxS mutants is not perturbed under these conditions. This could be due to a sufficient intracellular supply of methionine, as reported for *Mycobacterium tuberculosis* (75).

However, an imbalanced sulfur metabolism is probably not the only metabolic cause behind the various luxS phenotypes described. For *S. enterica* serovar Typhimurium, De Keersmaecker et al. (15) showed that the addition of neither cysteine, methionine, SAM, nor DPD restored biofilm formation by a luxS mutant. Thus, it is unlikely that a lack of sulfur amino acids or extracellular DPD was responsible for this phenotype. The accumulation of a toxic metabolite might have been responsible for the effect observed. Also, changes in key metabolite levels might have contributed to the deregulation of methionine metabolism. For instance, the down-regulation of *metE* in *S. enterica* serovar Typhimurium luxS mutants (64) is likely to be caused by a reduced homocysteine pool (73). Alternatively, the notion that DPD might be required intracellularly and for functions unrelated to signaling (although nevertheless yielding molecules with AI-2 activity as by-products) might form the basis of a useful working hypothesis. Bacteria may be able to effectively metabolize DPD and its derivatives under certain conditions and prevent their release into the environment.

Future work will need to address the different functions of the AMC in more detail. A link between methionine metabolism and the Pfs-LuxS pathway is suggested by the genetic organization of these genes in a number of bacterial genomes (73). However, the relative contribution of the Pfs/LuxS salvage pathway to methionine synthesis has yet to be established. In some bacteria, or under certain conditions, these contributions might be too small to yield an obvious phenotype in growth experiments. However, they are still likely to confer a long-term fitness advantage. Also of major interest are the metabolic fates of DPD and its derivatives. How much of the DPD formed in the AMC is released from the cell (in the form of diffusible furanone derivatives), why does this occur, and what are the metabolic intermediates that DPD and its derivatives are converted into?

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