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Assessment of the Roles of LuxS, *S*-Ribosyl Homocysteine, and Autoinducer 2 in Cell Attachment during Biofilm Formation by *Listeria monocytogenes* EGD-e

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LuxS is responsible for the production of autoinducer 2 (AI-2), which is involved in the quorum-sensing response of *Vibrio harveyi*. AI-2 is found in several other gram-negative and gram-positive bacteria and is therefore considered a good candidate for an interspecies communication signal molecule. In order to determine if this system is functional in the gastrointestinal pathogen *Listeria monocytogenes* EGD-e, an AI-2 bioassay was performed with culture supernatants. The results indicated that this bacterium produces AI-2 like molecules. A potential ortholog of *V. harveyi luxS*, lmo1288, was found by performing sequence similarity searches and complementation experiments with *Escherichia coli* DH5α, a *luxS* null strain. lmo1288 was found to be a functional *luxS* ortholog involved in AI-2 synthesis. Indeed, interruption of lmo1288 resulted in loss of the AI-2 signal. Although no significant differences were observed between Lux1 and EGD-e with regard to planktonic growth (at 10°C, 15°C, 25°C, and 42°C), swimming motility, and phospholipase and hemolytic activity, biofilm culture experiments showed that under batch conditions between 25% and 58% more Lux1 cells than EGD-e cells were attached to the surface depending on the incubation time. During biofilm growth in continuous conditions after 48 h of culture, Lux1 biofilms were 17 times denser than EGD-e biofilms. Finally, our results showed that Lux1 accumulates more *S*-adenosyl homocysteine (SAH) and *S*-ribosyl homocysteine (SRH) in culture supernatant than the parental strain accumulates and that SRH, but not SAH or AI-2, is able to modify the number of attached cells.

Listeria monocytogenes is a human and animal pathogen that is frequently found in feces, soil, and water and on vegetation (33). Due to its ubiquitous nature, its high stress tolerance, and its resistance to cleaning procedures, this bacterium is present in raw materials and in food plant environments, where it can contaminate food products during processing or packaging. Consequently, *L. monocytogenes* is associated with food-borne disease outbreaks, which are characterized by widespread distribution and high mortality rates.

Most of the time, in the food processing environment, *L. monocytogenes* forms biofilms on abiotic surfaces in association with other bacteria, such as *Pseudomonas* spp. (12). Biofilms allow bacteria to better resist environmental stresses, such as dehydration, and antimicrobial and sanitizing agents (19).

Inside a biofilm, some bacteria are known to communicate using signaling molecules, such as peptides or homoserine lactone derivatives, depending on whether they are gram positive or gram negative. As quorum-sensing mechanisms of the marine bacterium *Vibrio harveyi* have been deciphered, a new kind of molecule, autoinducer 2 (AI-2), has been added to the list of known signaling compounds (3). Bassler et al. have hypothesized that AI-2 or its derivatives could in fact be a universal signal involved in interspecies communication (1), whereas Joyce et al. suggested that in some species AI-2 serves as a barometer of cellular health (17). Although the biosynthesis pathway of AI-2 is not fully understood, recent data suggest that Pfs and LuxS enzymes catalyze the two-step conversion of *S*-adenosyl homocysteine (SAH), a very toxic by-product of methyl transfer reactions (28), into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is a very unstable molecule that can subsequently rearrange into various cyclic compounds, such as furanosyl borate diester (6) or (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (23), which is called AI-2 (Fig. 1). Recent reports have shown that, depending on the bacterium, AI-2 plays a role in motility (11, 34, 35), pathogenicity (36), and biofilm formation (4, 7, 22, 29, 41).

In this study, we demonstrated that *L. monocytogenes* is able to produce AI-2-like molecules via LuxS encoded by lmo1288, a functional ortholog of *luxS*. We obtained a *luxS* mutant strain that forms denser biofilms than the parental strain forms in batch or continuous-culture conditions. Finally, the results of addition of in vitro-synthesized SAH, *S*-ribosyl homocysteine (SRH), and AI-2 suggest that SRH, but not AI-2 or SAH, has an effect on biofilm formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Biofilm and planktonic cultures of *L. monocytogenes* EGD-e, which was isolated from a rabbit outbreak (25), and *L. monocytogenes* Lux1, a mutant derivative of *L. monocytogenes* EGD-e, were grown in tryptic soy broth (TSB) at 25°C. *Escherichia coli* DH5α [F^- ϕ80d*lac*ZΔ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*] (Invitrogen R&D Systems Europe, Oxon, United Kingdom) and *E. coli* EC101 (*E. coli* JM101 with *repA* from pWV01 integrated into the chromosome) (18) were grown aerobically at 37°C in TSB supplemented with 250 μg ml^{-1} erythromycin and 25 μg ml^{-1} kanamycin when necessary. *E. coli* BL21(DE3) (Novagen, VWR International S.A.S., Fontenay-sous-Bois,

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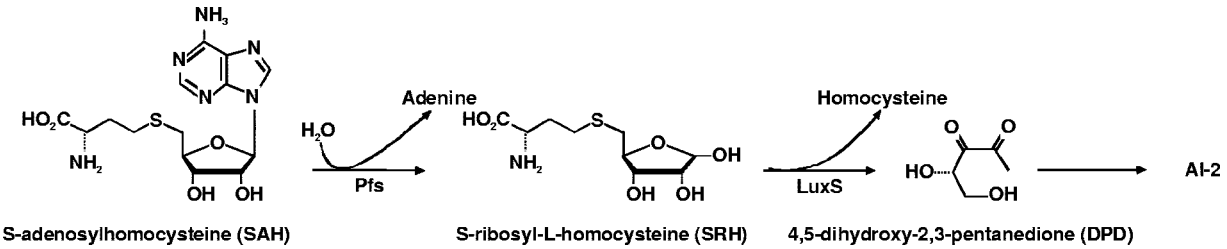

FIG. 1. Partial pathway for conversion of SAH to AI-2.

France) was grown aerobically on LB medium supplemented with 50 μg ml^{-1} kanamycin.

Vibrio harveyi BB170 (sensor 1^-, sensor 2^+) (37) and *V. harveyi* MM30 (autoinducer 1^+, autoinducer 2^-) (38) were kindly provided by B. Bassler (Princeton University) and were grown aerobically in AB medium at 30°C (2).

Cloning of *luxS* gene in *E. coli* DH5α. A 723-bp DNA fragment was generated by colony PCR from *L. monocytogenes* EGD-e using primers LUXS1 (5′-TGG CTAGCAATACCCGAAAT-3′) and LUXS2 (5′-CCATAAACCATCACGCCC TT-3′). The fragment containing the coding sequence from lmo1288, along with a 133-bp upstream region and a 122-bp downstream region, was cloned into the pCR2.1 TOPO vector (Invitrogen, Cergy-Pontoise, France) to obtain plasmid pSCB1. This vector was used to electroporate *E. coli* DH5α.

A 203-bp internal fragment of the coding sequence of *luxS* was also generated by colony PCR using primers LUXS3 (5′-AATGCCAGCGCTACACTCT-3′) and LUXS4 (5′-CTGTCTTCAAGGACGTACGT-3′) and was cloned into the pCR2.1 TOPO vector to obtain plasmid pSCB2. This vector was transferred into *E. coli* DH5α and was used as a negative control when necessary.

Construction of an *L. monocytogenes luxS*-negative strain. Several steps were necessary to generate the mutant strain. First, plasmid pSCB2 was digested with HindIII and XbaI, and the resulting 315-bp fragment containing an internal part of lmo1288 was ligated into pORI19 (18) to obtain pSCB3. This plasmid was routinely maintained in the recipient strain *E. coli* EC101 (18). pSCB3 was then transferred into *L. monocytogenes* EGD-e previously transformed with the thermosensitive helper plasmid pVE6007 (20). In order to select for integration mutants, *L. monocytogenes* EGD-e(pSCB3, pVE6007) was grown for 28 generations on brain heart infusion (BHI) medium (Difco Laboratories, Elancourt, France). A temperature upshift from 25°C to a nonpermissive temperature, 37°C, resulted in the loss of pVE6007. Plating on BHI agar supplemented with 5 μg erythromycin ml^{-1} and 25 μg lincomycin ml^{-1} selected for chromosomal integration of pSCB3 at the point of homology with lmo1288. The mutant selected was designated Lux1.

Complementation of the *luxS* mutation. A reversion mutant of *L. monocytogenes* Lux1 was obtained by excision of pSCB3 from the chromosome. Strain Lux1 was grown for 120 generations on BHI medium without erythromycin at 37°C. A total of 4,000 clones from this culture were tested for loss of the integrated plasmid pSCB3 by plating in duplicate onto BHI medium plates with or without erythromycin. Three clones were selected based on their sensitivity to the antibiotic. Excision of pSCB3 was confirmed by PCR and sequencing of the lmo1288 region. The three revertants were used as controls in order to confirm the phenotype differences between strains EGD-e and Lux1.

Swimming motility, hemolytic, and phospholipase assays. For swimming motility, tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) were inoculated with a sterile toothpick and incubated for 16 h at 25°C. Motility was then qualitatively assessed by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

Hemolytic and phospholipase activities were examined as previously described (27).

AI-2 bioassay. Cell-free culture supernatants of *V. harveyi* BB170, *L. monocytogenes* EGD-e, *L. monocytogenes* Lux1, *E. coli* DH5α, *E. coli* DH5α(pSCB1), or *E. coli* DH5α(pSCB2) were obtained by centrifugation (6,000 × *g*, 10 min, 25°C) and filtration (0.22-μm-pore-size SLGV R25 KS filter; Millipore, Saint Quentin en Yvelines, France) of 1-ml portions of 22-h cultures. AI-2 in the supernatants was detected using the biosensor *V. harveyi* MM30 as previously described (38), with some modifications. Briefly, *V. harveyi* MM30 was grown aerobically in AB medium for 16 h at 30°C. The culture was then diluted 1/5,000 (vol/vol) in fresh AB medium, and 90 μl of it was placed into each well of a 96-well microplate (Optiplate-96 white; PerkinElmer Life Sciences, Courtaboeuf, France). Ten microliters of each supernatant to be tested was then added to wells. For each experiment, cell-free culture supernatants of *E. coli* DH5α and *V. harveyi* BB170 were used as negative and positive controls, respectively. Luminescence was monitored hourly using a Fusion universal microplate analyzer (PerkinElmer Life Sciences, Courtaboeuf, France) at 30°C until an AI-2 signal was detected (generally 5 h after the beginning of the experiment). Each experiment was repeated four times with three different inocula.

Biofilm formation and quantitation. Discontinuous biofilm cultures were grown on polystyrene microplates (Nunc; Dominique Dutscher S.A., Brumath, France) as follows. An overnight culture of the bacterium in TSB was used to inoculate (1/100, vol/vol) fresh TSB and was grown at 25°C to an optical density at 600 nm of 0.05. One hundred microliters of the culture to be tested was then transferred to a microtiter plate well, and the plate was tightly sealed and incubated at 25°C for 5 to 72 h.

Cells attached to the well walls were quantified as previously described (9), with some modifications. After incubation, the medium was removed from each well, and the plates were washed twice using a microtiter plate washer (Cellwash; Thermolabsystems, Cergy Pontoise, France) with a 150 mM NaCl solution in order to remove loosely attached cells. The plates were then stained with a 0.05% (wt/vol) aqueous crystal violet solution for 45 min and washed four times. In order to quantitatively assess biofilm production, 100 μl of a destaining solution (96% [vol/vol] ethanol) was added to each well, and the optical density at 595 nm was determined. For each experiment, 40 replicates resulting from five different inocula were analyzed. Each microtiter plate included eight wells with sterile TSB in control wells.

Continuous-flow biofilm cultures were grown on AISI 304 stainless steel chips (Goodfellow SARL, Lille, France) in flow cells inoculated (1/100, vol/vol) with overnight cultures at 25°C. During growth, TSB was pumped through the flow cells at a constant flow rate (14 ml · h^{-1}) by using a peristaltic pump (model 205S; Watson Marlow, Calmouth, Cornwall, England).

ESEM. For environmental scanning electron microscopy (ESEM) observations, bacterial cells were grown on chemically defined medium MCDB 202 (CryoBiosystem, L'Aigle, France). Biofilms were grown for 36 h on AISI 304 stainless steel chips in MCDB 202 medium. The cells were fixed with glutaraldehyde and osmic vapors as previously described (5) and were observed using a Philips XL30 ESEM.

pET cloning of *pfs* and *luxS* genes. *pfs* (lmo1494) and *luxS* (lmo1288) coding sequences were amplified by PCR using primers PFSF3 (5′ TTAATACTCATG ACAATTGGTATTATCGG 3′) and PFSR3 (5′ TATTAACTCGAGAATTGT TTTAAGCAATTC 3′) and primers LUXSF3 (5′ TTAATACTCATGATGGC AGAAAAAATGAATG 3′) and LUXSR3 (5′ TATTAACTCGAGTTCACCA AACACATTTTTCC 3′), respectively. In both cases, the primers generated a BspHI site and a XhoI site (underlined) that were used to clone the BspHI/Xho1-digested PCR fragments into NcoI/XhoI-digested pET 28a(+) (Novagen, VWR International S.A.S., Fontenay-sous-Bois, France). The resulting plasmids, pSCB4 and pSCB5, contained the coding sequences of *pfs* and *luxS*, respectively, fused in frame with a downstream sequence encoding six histidine residues (His tag). They were controlled by sequencing and used to transform *E. coli* BL21(DE3) cells for the production of the recombinant proteins.

The recombinant LuxS protein contained an additional N terminus methionine and two additional residues (Leu and Glu) between the C terminus of the mature protein and the His tag, whereas the recombinant Pfs protein contained two additional residues (Leu and Glu) before the His tag.

Production and purification of the LuxS and Pfs proteins. Both LuxS and Pfs were produced using the same procedure. Briefly, *E. coli* BL21(DE3) transformed with pSCB4 and pSCB5 was grown aerobically on Luria-Bertani medium supplemented with kanamycin (50 μg/ml) at 37°C to an optical density at 600 nm of 1. The cultures were then cooled to 20°C and incubated aerobically for 15 h in the presence of 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) in order to induce synthesis of the recombinant proteins. The cells were cooled to 5°C and harvested by centrifugation, resuspended in cold 30 mM Tris-HCl (pH 8) binding

buffer (BB), and disrupted with a Constant cell disruption system (Cell-D; Constant System Ltd., Roquemaure, France). The crude extract was centrifuged at 26,000 × *g* for 15 min, and the supernatant was loaded into a 3-ml Ni-nitrilotriacetic acid column previously equilibrated with BB. After 20 column volume washes with BB, the proteins were eluted with BB supplemented with 400 mM imidazole. The eluates were dialyzed against BB and concentrated with an Amicon concentrator. The protein purification steps were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Aliquots of the protein solution were filter sterilized and stored at 4°C.

Protein concentrations were calculated by measuring the absorbance of a 6 M guanidium chloride protein solution at 280 nm using molar extinction coefficients of 11,520 M^{-1} cm^{-1} for Pfs and 16,620 M^{-1} cm^{-1} for LuxS.

In vitro synthesis of SRH and AI-2. DPD, the AI-2 precursor, was synthesized in 20 mM KH_2PO_4 (pH 7.0) buffer containing 1 mM SAH, 14.4 μM purified recombinant Pfs, and 8.6 μM purified recombinant LuxS. The reaction mixture was incubated for 14 h at 30°C. For SRH synthesis, the same procedure was used, except that LuxS was omitted from the reaction mixture. In both cases, after incubation, the enzymes were removed by ultrafiltration, and the reaction products were quantified as follows.

The in vitro reaction was monitored by measuring the amounts of homocysteine and AI-2 released during the reaction using Ellman's reagent (15) and the AI-2 bioassay, respectively. Under these experimental conditions the substrate (SAH) was totally converted into DPD and homocysteine when Pfs and LuxS were used.

AI-2, SAH, and SRH complementation. Complementation experiments were performed with 42-h-old *L. monocytogenes* EGD-e and Lux1 biofilms grown on microtiter plates. Biofilm cultures were complemented with 50 μM (final concentration) in vitro-synthesized SAH, SRH, or AI-2 and incubated for another 8 h before cell quantification. The amount of luminescence measured in the AI-2 bioassay in the presence of 50 μM synthetic AI-2 was similar to the amount of luminescence generated by a 50-h-old *L. monocytogenes* EGD-e biofilm supernatant.

SAH and SRH detection in cell-free biofilm supernatants. Fifty-hour-old biofilms were used in the experiments for detection of SAH and SRH in cell-free biofilm supernatants and were treated as follows. Biofilm supernatants were filter sterilized, tested for the absence of LDH activity (13), which indicated that no cell lysis occurred, and heat treated (100°C, 30 min) to inactivate AI-2. At the same time, the amount of attached cells was determined by measuring the optical density at 595 nm. To detect the presence of SAH, the supernatants were incubated for 1 h at 37°C with 38 μM Pfs and 56 μM LuxS (final concentrations). For SRH detection, only LuxS was added. After this, supernatants were tested for AI-2 activity using the AI-2 bioassay. The amount of luminescence measured in the bioassay was directly proportional to the amount of SAH or SRH converted into AI-2. The results were expressed in relative light units per optical density unit to take into account the differences in the numbers of attached cells in EGD-e and Lux1 biofilms.

Statistical analysis. Data were analyzed for statistical significance using the SigmaStat 3.0.1 software (SPSS France S.A., Paris-La-Défense, France) for the *t* test (Student-Newman-Keuls method).

RESULTS

***L. monocytogenes* produces an autoinducer 2-like molecule.** In order to understand cell-to-cell communication mechanisms in *L. monocytogenes*, we focused on the ability of this bacterium to produce AI-2 molecules. Therefore, cell-free culture supernatants of *L. monocytogenes* EGD-e were tested for the presence of AI-2 using *V. harveyi* MM30 as a biosensor. As shown in Fig. 2, cell-free culture supernatants of *L. monocytogenes* EGD-e induced luminescence of *V. harveyi* MM30, indicating that AI-2 like molecules were produced by strain EGD-e.

L. monocytogenes EGD-e AI-2 production was growth phase independent, as the same amount of induced luminescence (reported to the biomass) was detected for early-, mid-, and late-log-phase culture supernatants (data not shown).

Identification and isolation of the *luxS* gene and complementation of *E. coli* DH5α. lmo1288, a gene similar to *V. harveyi luxS*, was found in the genome of *L. monocytogenes* EGD-e by a BLAST search on the ListiList World Wide Web server (Institut Pasteur) (14, 24). This gene encodes a 155-amino-acid protein that is fully conserved in various *Listeria* strains (26) and exhibits 41% identity and 59% similarity with the *V. harveyi* LuxS protein. Sequence comparisons showed that Lmo1288 was closely related to LuxS of other species, such as *Staphylococcus aureus* Mu50 (78% identity and 87% similarity), *Helicobacter pylori* 26695 (68% identity and 82% similarity), *Clostridium perfringens* (51% identity and 68% similarity), and *Bacillus subtilis* (44% identity and 62% similarity). For example, all the metal ligands (H54, H58, and C126) of LuxS of *B. subtilis* are conserved in Lmo1288. Moreover the amino acids involved in hydrogen bonding and electrostatic interactions with the substrate or the product of LuxS of *B. subtilis* (S6, K35, E57, R65, D78, I79, S80, C84, Q125, and G127) are also conserved in Lmo1288 (16, 30, 31, 45). These results strongly suggest that Lmo1288 is a LuxS-like protein.

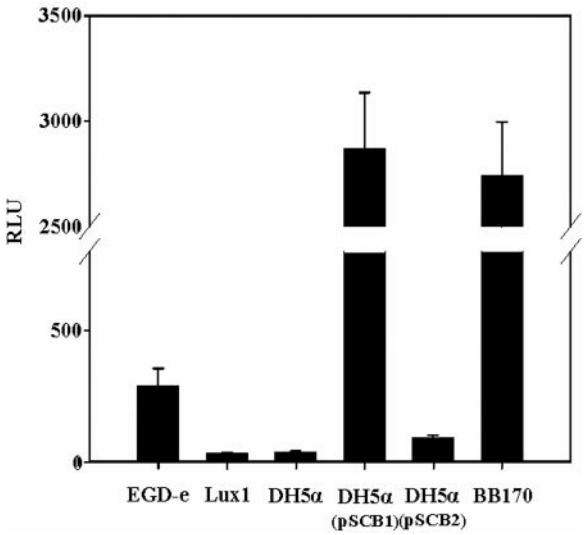

FIG. 2. Induction of *V. harveyi* MM30 luminescence by cell-free supernatants of planktonic cultures of *L. monocytogenes* EGD-e and Lux1, *E. coli* DH5α, DH5α(pSCB1), and DH5α(pSCB2), and *V. harveyi* BB170. Each bar indicates the mean from eight experiments, and the error bars indicate standard deviations. RLU, relative light units.

To confirm the function of lmo1288, we designed a pair of primers, LUXS1 and LUXS2, to amplify the 723-bp region containing the lmo1288 CDS, 133 bases upstream of the initiation codon and 122 bases downstream of the stop codon. The PCR fragment was cloned into the pCR2.1 TOPO vector, and its sequence was confirmed by sequencing. The resulting plasmid, pSCB1, was electroporated into *E. coli* DH5α, a *luxS*-negative strain (38), in order to perform complementation experiments. Cell-free culture supernatants of *E. coli* DH5α (negative control), *E. coli* DH5α(pSCB2) (negative control), *E. coli* DH5α(pSCB1), and *L. monocytogenes* EGD-e were tested for AI-2 activity. As shown in Fig. 2, *E. coli* DH5α(pSCB1) culture supernatants induced a 77-fold increase in luminescence compared to the luminescence induced by the *E. coli* DH5α supernatant. This result confirmed that lmo1288 encodes a LuxS-like protein involved in AI-2-like molecule synthesis. Therefore, we refer to the *L. monocytogenes* protein as $LuxS_{Lm}$ below.

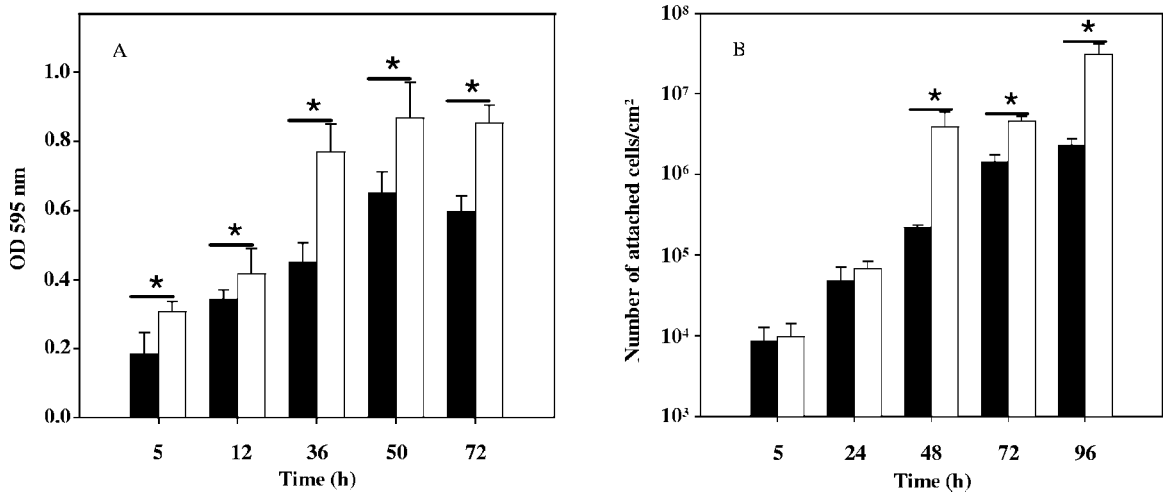

FIG. 3. Biofilm formation by *L. monocytogenes* EGD-e (solid bars) and *L. monocytogenes* Lux1 (open bars) in batch conditions (A) or in continuous-culture conditions (B) over time, as described in Materials and Methods. In panel A, each bar indicates the mean from five independent experiments with eight measurements for each point, and in panel B, each bar indicates the mean from three independent experiments with three measurements for each point; the error bars indicate standard deviations. Asterisks indicate statistically significant differences ($P < 0.001$). OD 595 nm, optical density at 595 nm.

Phenotypic characterization of the *L. monocytogenes* Lux1 mutant. To confirm the role of $LuxS_{Lm}$ in AI-2 synthesis, we generated mutant Lux1 from *L. monocytogenes* EGD-e by disruption of the *luxS* gene. This construction was controlled by PCR and Southern blotting (data not shown).

Lux1 cell-free culture supernatants did not generate any luminescence in our bioassay (Fig. 2). Furthermore, complementation of the *luxS* mutation by excision of pSCB3 from lmo1288 restored the ability to induce luminescence of *V. harveyi* (data not shown). This result confirmed the involvement of $LuxS_{Lm}$ in AI-2 synthesis.

To characterize the mutant phenotype, we compared planktonic growth of *L. monocytogenes* EGD-e and planktonic growth of Lux1 in TSB at 10°C, 15°C, 25°C, and 42°C. Irrespective of the conditions, the growth rates of the two strains were similar (data not shown), and there was no difference between the two strains in terms of swimming motility and hemolytic and phospholipase activities.

As recent reports have demonstrated the involvement of *luxS* in biofilm development of various oral pathogens (4, 21, 22, 43), we investigated the abilities of *L. monocytogenes* EGD-e and Lux1 to form biofilms in batch culture conditions (Fig. 3A). Statistically significant differences between the two strains ($P \leq 0.00021$ for 5 h and $P \leq 0.00001$ for 12 h, 36 h, 50 h, and 72 h, as determined by *t* tests) were observed, and interestingly, denser biofilms were obtained with Lux1. The difference was maximal after 36 h of incubation, when 58% more mutant cells than EGD-e cells were attached to the surface. Then the abilities of the two strains to form biofilms on stainless steel in continuous culture conditions were compared. Again, major differences in terms of the number of attached cells were observed. Indeed, the Lux1 strain formed denser biofilms than the EGD-e strain formed, with 10 to 17 times more attached cells. Interestingly, the differences were significant only after 48 h of incubation (Fig. 3B).

ESEM microscopic observations of Lux1 and EGD-e confirmed these results. As shown in Fig. 4, Lux1 formed thicker biofilms with large spaces separating cell patches, whereas EGD-e biofilms were thinner and more confluent. As reversion of the mutation in Lux1 restored the EGD-e biofilm phenotype (data not shown), we concluded that *luxS* was important for biofilm formation in *L. monocytogenes* EGD-e.

Complementation of Lux1 biofilms. To understand the role of *luxS* in biofilm formation, synthetic AI-2 was produced from SAH using purified His-tagged Pfs_{Lm} and $LuxS_{Lm}$ proteins and was used to complement supernatants of 42-h-old biofilms. To make sure that the right quantity of AI-2 was added in these experiments, throughout the AI-2 bioassay we measured the amount of luminescence generated by the supernatant of a 42-h-old EGD-e biofilm culture. Then we added to the supernatant of a 42-h-old Lux1 biofilm culture the amount of synthetic AI-2 necessary to generate the same luminescence. Surprisingly, as shown in Fig. 5, addition of AI-2 failed to restore the parental strain biofilm phenotype. As a *luxS* strain does not produce AI-2 but may accumulate SAH and SRH (Fig. 1), we investigated the effects of these molecules on Lux1 biofilm. As shown in Fig. 5, 50 μM SAH had no effect on Lux1 biofilm formation, whereas on average, addition of 50 μM SRH resulted in a 15% increase in the number of attached cells, irrespective of the strain tested.

Presence of SAH and SRH in biofilm culture supernatant. As the effect of SRH on *L. monocytogenes* biofilms was unexpected, we addressed the following questions. Do EGD-e and Lux1 biofilm culture supernatants contain any SRH? If so, is there any difference between EGD-e and Lux1 in terms of the SRH supernatant concentration, which could explain the Lux1 phenotype? To answer these questions, we used Lux1 and EGD-e supernatants that were heat treated to eliminate AI-2 activity, and we added $LuxS_{Lm}$ with or without Pfs_{Lm} in order to convert SAH and SRH into AI-2; the latter compound was detected using the AI-2 bioassay. At the same time, lactate dehydrogenase activity was monitored in each supernatant to

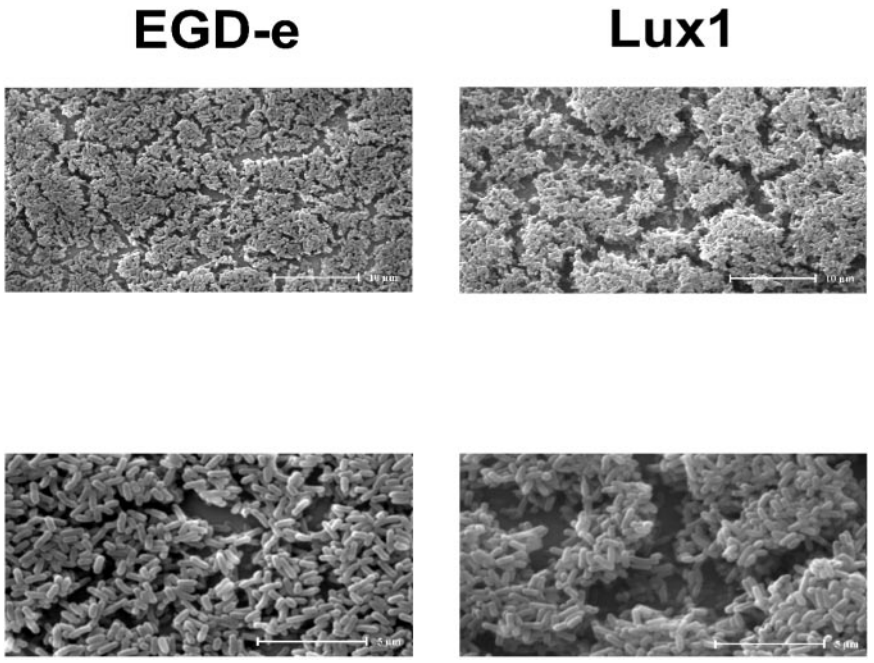

FIG. 4. ESEM images of 36-h-old *L. monocytogenes* EGD-e and Lux1 biofilms incubated at 25°C on stainless steel in batch conditions.

make sure that the presence of the molecules was not the result of cell lysis (data not shown). As shown in Fig. 6, it appeared that both EGD-e and Lux1 supernatants contained SAH and SRH but different quantities of these compounds. There was twice as much SRH in the Lux1 supernatant as in the supernatant of EGD-e. As Lux1 was unable to convert SRH into AI-2 and as SRH increased the number of attached cells, this result could partially explain the Lux1 phenotype.

For SAH, the only way to evaluate the amount in culture supernatants was to subtract the amount of luminescence obtained when only LuxS was added from the amount of luminescence obtained after addition of Pfs and LuxS to the supernatant. Both supernatants contained SAH, but there was almost twice as much SAH present in the Lux1 supernatant as in the supernatant of EGD-e.

Similar results were obtained with planktonic culture supernatants of *V. harveyi* BB170 and MM30 and *E. coli* DH5α. In each case, SAH and SRH were detected in supernatants, and proportionally, there was more SAH and SRH in the supernatants of the *luxS*-negative strains (*V. harveyi* MM30 and *E. coli* DH5α).

DISCUSSION

In this study, we demonstrated that *L. monocytogenes*, like several other gram-positive bacteria, is able to produce an AI-2 or an AI-2-like signal recognized by *V. harveyi* MM30. We looked for a *luxS* ortholog in the *L. monocytogenes* genomic database and found lmo1288, a putative gene encoding a 155-amino-acid protein. As most of the amino acids involved in the substrate binding site and in the metal chelating site of LuxS from *B. subtilis* perfectly match amino acids in Lmo1288, we

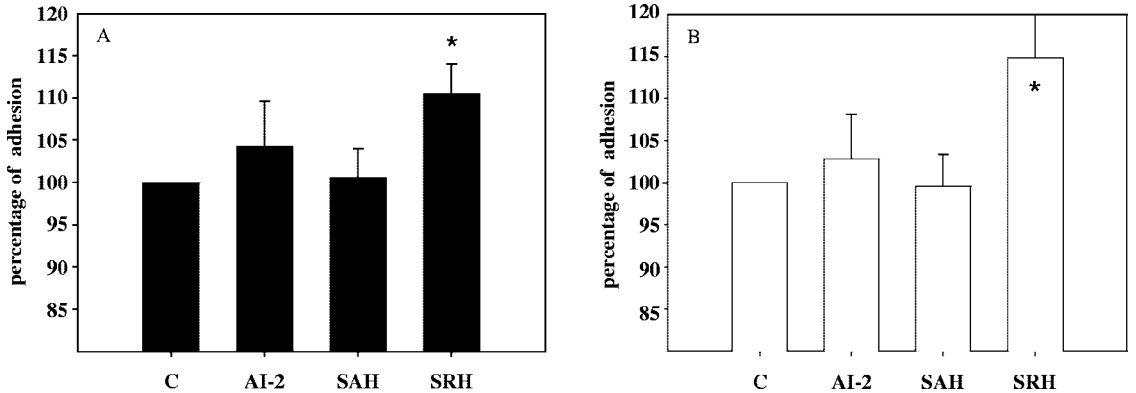

FIG. 5. Effect of addition of 50 μM synthetic AI-2, SAH, or SRH on 42-h-old biofilms. The results are expressed as quantities relative to that for the control experiment (C), in which nothing was added to the supernatant for strain EGD-e (A) and strain Lux1 (B). Asterisks indicate statistically significant differences from the value for the control ($P < 0.001$).

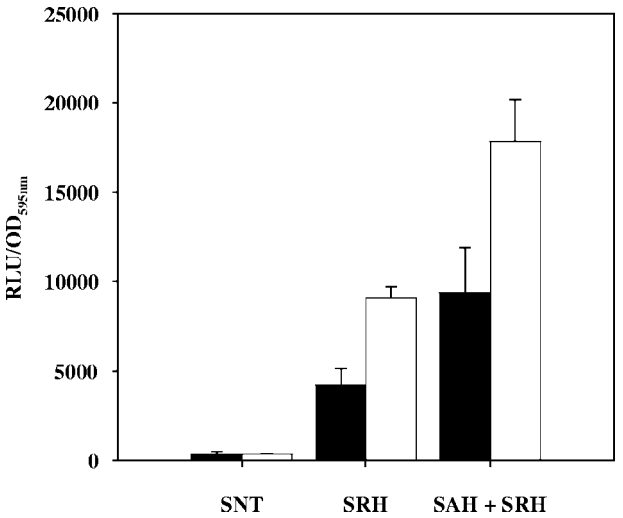

FIG. 6. Quantities of SRH and SAH present in heat-treated biofilm supernatants of EGD-e (solid bars) and Lux1 (open bars). SNT, heat-treated supernatants alone; SRH, heat-treated supernatants incubated with recombinant LuxS; SAH + SRH, heat-treated supernatants incubated with recombinant Pfs and LuxS. The amount of luminescence produced is adjusted for the number of cells present in the biofilm, as described in Materials and Methods. RLU, relative light units; OD_{595nm}, optical density at 595 nm.

postulated that Lmo1288 is an *S*-ribosylhomocysteinase (16, 31, 45).

Complementation experiments with *E. coli* DH5α and mutagenesis of lmo1288 from *L. monocytogenes* EGD-e confirmed the involvement of Lmo1288 in AI-2 biosynthesis. Moreover, overproduction, purification, and an activity assay of the protein clearly demonstrated that it is an *S*-rybosylhomocysteinase which catalyzes the bioconversion of SRH into homocysteine and DPD. As previously described, the latter compound undergoes chemical modifications to generate AI-2-like signals that are able to induce the luminescence of *V. harveyi* MM30.

The Lux1 strain does not differ from the parental strain in terms of the growth rates at various temperatures, motility, and phospholipase and hemolytic activities. As a *luxS* mutation sometimes affects biofilm formation (7, 43), we compared the abilities of the two strains to form biofilms under batch and continuous-culture conditions. Surprisingly, it appeared that Lux1 cells formed denser biofilms than cells of the parental strain formed. For example, in continuous-culture conditions, 17 times more Lux1 cells than EGD-e cells were attached to the support after 96 h of incubation. These results were confirmed by ESEM observations and are in accordance with those obtained by Cole et al. with a *luxS* mutant of *H. pylori* (7). In contrast, Wen and Burne, working with *Streptococcus mutans*, demonstrated that less biofilm was formed by a *luxS* mutant (41). Nevertheless, irrespective of the phenotype, it seems that *luxS* plays a key role in the formation of biofilms.

For some bacteria, the pleiotropic effect of a *luxS* mutation has been demonstrated (8, 35, 44). Considering the differences, we investigated the ability of synthetic AI-2 to restore the phenotype of the parental strain when it was added to Lux1 supernatant. Surprisingly, addition of AI-2 had no effect on *L. monocytogenes* biofilm formation. Therefore, we hypothesized that perhaps the accumulation of the precursors SAH and SRH may have an effect. Indeed, these molecules were found in biofilm culture supernatants of both strains, but larger quantities were observed for Lux1 than for EGD-e and only SRH was able to increase the number of attached cells. These results raised the question of the SAH detoxification pathway of *L. monocytogenes*. Indeed, during the activated methyl cycle (AMC) it is accepted that *S*-adenosylmethionine provides methyl groups for proteins, RNA, and DNA. Depending on the organism, the resulting SAH, a toxic compound, is then converted into homocysteine using a one-step reaction (SAH hydrolase) or a two-step reaction (Pfs and LuxS). The latter reaction, found in *L. monocytogenes*, generates DPD, which spontaneously rearranges to form AI-2-like molecules (40). Our observations suggest that SAH and SRH can accumulate outside the cell via an unknown mechanism and, moreover, that SRH modulates the ability of *L. monocytogenes* to form biofilms. The reason why such a mechanism exists is unclear, but it may be necessary to eliminate SAH when the detoxification pathway is overwhelmed or down regulated. Moreover, as there are different AI-2-like compounds (6, 23), it could be useful for the bacteria to be able to detect the presence of other bacteria using SRH, an AMC-linked molecule.

In conclusion, AI-2 has been demonstrated to be a mediator of quorum sensing in *V. harveyi*, *Vibrio cholerae*, *E. coli*, and *Salmonella enterica* serovar Typhimurium (3, 39, 42). However, in other bacteria, such as *Neisseria meningitidis* (10, 32), AI-2 does not trigger any global transcriptional response. Similarly, our results suggest that in *L. monocytogenes*, the physiological role of AI-2 may be limited to the detoxification of SAH and may not play a key role in cell-to-cell signaling. In contrast, SRH, the precursor of AI-2, plays an important role in biofilm formation in *L. monocytogenes* and may be involved in cell-to-cell communication. The mechanisms of excretion of AI-2, SAH, and SRH must be deciphered in order to better understand the roles of these AMC intermediates in biofilm formation in *L. monocytogenes*.

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