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The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule

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Summary

Many bacteria control gene expression in response to cell population density, and this phenomenon is called quorum sensing. In Gram-negative bacteria, quorum sensing typically involves the production, release and detection of acylated homoserine lactone signalling molecules called autoinducers. *Vibrio harveyi*, a Gram-negative bioluminescent marine bacterium, regulates light production in response to two distinct autoinducers (AI-1 and AI-2). AI-1 is a homoserine lactone. The structure of AI-2 is not known. We have suggested previously that *V. harveyi* uses AI-1 for intraspecies communication and AI-2 for interspecies communication. Consistent with this idea, we have shown that many species of Gram-negative and Gram-positive bacteria produce AI-2 and, in every case, production of AI-2 is dependent on the function encoded by the *luxS* gene. We show here that LuxS is the AI-2 synthase and that AI-2 is produced from *S*-adenosylmethionine in three enzymatic steps. The substrate for LuxS is *S*-ribosylhomocysteine, which is cleaved to form two products, one of which is homocysteine, and the other is AI-2. In this report, we also provide evidence that the biosynthetic pathway and biochemical intermediates in AI-2 biosynthesis are identical in *Escherichia coli*, *Salmonella typhimurium*, *V. harveyi*, *Vibrio cholerae* and *Enterococcus faecalis*. This result suggests that, unlike quorum sensing via the family of related homoserine lactone autoinducers, AI-2 is a unique, 'universal' signal that could be used by a variety of bacteria for communication among and between species.

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Introduction

Quorum sensing or the regulation of gene expression in response to cell population density is a process that bacteria use to co-ordinate the gene expression of the community. Presumably, the ability to control behaviour on a collective scale enables bacteria to behave like multicellular organisms. Quorum sensing involves the production of extracellular signalling molecules called autoinducers. As a population of autoinducer-producing bacteria grows, the external concentration of autoinducer increases. When a threshold autoinducer concentration is reached, the bacteria detect the autoinducer and initiate a signal transduction cascade that culminates in a change in the behaviour of the population (Nealson and Hastings, 1979; Kleerebezem *et al*., 1997; Lazazzera and Grossman, 1998; Bassler, 1999; de Kievit and Iglewski, 2000). In Gram-negative bacteria, quorum sensing typically involves an acylated homoserine lactone (HSL) autoinducer whose synthesis is dependent on a 'LuxI' autoinducer synthase and a cognate 'LuxR' autoinducer binding/transcriptional activator protein (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984; de Kievit and Iglewski, 2000). Binding of an HSL autoinducer by a LuxR protein results in the transcriptional activation of specific target genes. This nomenclature refers to the bioluminescent marine bacterium *Vibrio fischeri*, the first bacterium in which a LuxI–LuxR signalling cascade was identified. *V. fischeri* regulates the expression of luciferase with this quorum-sensing circuit (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984; 1987).

In contrast to other Gram-negative quorum-sensing bacteria, *V. harveyi* regulates quorum sensing via an elaborate two-component phosphorylation cascade (Bassler, 1999). A model of the *V. harveyi* quorum-sensing circuit is presented in Fig. 1. This bacterium produces and responds to two autoinducers, AI-1 and AI-2, to control the transcription of the luciferase structural operon *luxCDABEGH* (Bassler *et al*., 1993; 1994a,b). AI-1 is the homoserine lactone *N*-(3-hydroxybutanoyl)-L-homoserine lactone (Cao and Meighen, 1989), and its synthesis is dependent on the *luxLM* locus (Bassler *et al*., 1993).

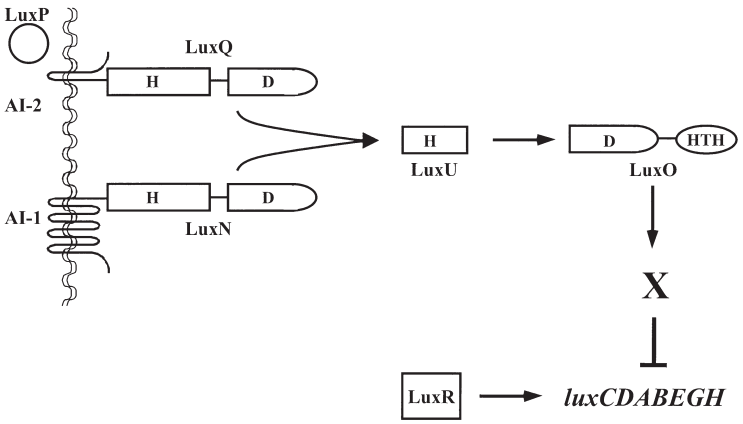

Fig. 1. Model for quorum sensing in *V. harveyi*. *V. harveyi* regulates light production using two parallel quorum-sensing circuits. A complex two-component signal transduction system is responsible for detection of the autoinducers and information relay to the luciferase structural operon (*luxCDABEGH*). The proposed functions of the proteins are described in the text. H, D and HTH denote histidine, aspartate and helix–turn–helix respectively.

These genes share no homology to the *luxI* family of autoinducer synthases; however, a common biosynthetic mechanism is probably used (Bassler *et al*., 1993; Hanzelka *et al*., 1999). The structure and biosynthesis of AI-2 have never been reported and are the focus of this paper. Each autoinducer, AI-1 and AI-2, is detected by its cognate sensor LuxN and LuxPQ respectively. LuxN and LuxQ are two-component hybrid kinases (Bassler *et al*., 1993; Bassler *et al*., 1994a; Freeman *et al*., 2000). LuxP, which we hypothesize interacts with LuxQ to transduce the AI-2 signal, is homologous to the ribose-binding protein of *E. coli* (Bassler *et al*., 1994a). LuxN and LuxPQ transduce the signal to a shared phosphotransferase protein called LuxU, and LuxU, in turn, conveys the signal to a response regulator protein called LuxO (Freeman and Bassler, 1999a). LuxO indirectly represses the *luxCDABEGH* operon at low cell density (Bassler *et al*., 1994b; Freeman and Bassler, 1999b). Specifically, LuxO acts by activating the expression of a putative repressor protein denoted X (Lilley and Bassler, 2000). A transcriptional activator called LuxR is also required for the expression of *luxCDABEGH* in *V. harveyi* (Martin *et al*., 1989; Showalter *et al*., 1990). The *V. harveyi* LuxR protein is not similar to the LuxR protein of *V. fischeri*.

We wanted to understand what specific function each of the quorum-sensing systems has in *V. harveyi*. Early *V. harveyi* studies revealed that wild-type *V. harveyi* induces light production in response to substances produced by other free-living marine *Vibrios* (Greenberg *et al*., 1979). We wondered whether these heterologous signals were communicated to *V. harveyi* through the LuxN circuit, the LuxPQ circuit or through both quorum-sensing circuits. To test these possibilities, we developed a bioassay specifically to examine signalling through LuxN and LuxPQ. We constructed *V. harveyi* reporter strains capable of producing light exclusively in response to AI-1 or AI-2 (Bassler *et al*., 1997). These strains were used to show that many other species of bacteria produce stimulatory substances that mimic the action of AI-2, but only rarely could species of bacteria be identified that produce an AI-1-like activity (Bassler *et al*., 1997; Surette and Bassler, 1998). This result led to our hypothesis that *V. harveyi* uses the AI-1/LuxN system for intraspecies communication and the AI-2/LuxPQ system for interspecies communication. We propose that *V. harveyi* uses these two systems to monitor both its own cell density and also that of other species of bacteria. If *V. harveyi* can regulate gene expression differentially in response to AI-1 and AI-2, *V. harveyi* could behave differently under conditions when it exists in pure culture versus conditions when it exists in consortia (Bassler *et al*., 1997; Bassler, 1999). Consistent with this notion, we have evidence that LuxN and LuxQ each interact with additional downstream regulators that enable *V. harveyi* to possess autoinducer-specific outputs (B. L. Bassler, unpublished).

To investigate the mechanism of AI-2 signalling, we made mutants and cloned the gene responsible for AI-2 production from *V. harveyi*, *E. coli* and *S. typhimurium*. The gene we identified in all three species of bacteria is highly conserved, and we named it *luxS* (Surette *et al*., 1999). Database analysis showed that conserved *luxS* homologues exist in over 30 species of both Gram-negative and Gram-positive bacteria (Bassler, 1999; Surette *et al*., 1999). Before our analysis, no function had been ascribed to any *luxS* homologue. We propose that these genes define a new family of proteins involved in autoinducer production (Surette *et al*., 1999). In support of this hypothesis, we and others have now shown that most of the species of bacteria possessing a *luxS* gene produce AI-2 activity, and *luxS* mutants have been constructed in *V. harveyi*, *E. coli*, *S. typhimurium*, *V. cholerae*, *Helicobacter pylori* and *Streptococcus pyogenes*. In each case, mutation of *luxS* eliminated AI-2 production (Sperandio *et al*., 1999; Surette *et al*., 1999; Joyce *et al*., 2000; Lyon *et al*., 2001). The functions that are controlled by this class of signalling molecule have not been identified in many bacteria. However, there are reports indicating that AI-2 regulates pathogenicity in *E. coli*, *S. pyogenes* and *Vibrio*

vulnificus (Sperandio *et al*., 1999; Kim *et al*., 2000; Lyon *et al*., 2001).

AI-2 signalling appears to be widespread, and a number of clinically important pathogens produce this activity. It is therefore of interest to determine both the structure of the AI-2 molecule(s) and the mechanism of its biosynthesis. This knowledge would be of value both for furthering our understanding of intra- and interspecies communication among bacteria and also for developing novel antimicrobial drugs that specifically interfere with AI-2 biosynthesis or reception. In this report, we present the complete AI-2 biosynthetic pathway and demonstrate that AI-2 is derived from *S*-adenosylmethionine. We also show that there are two specific enzymes involved in the process, Pfs and LuxS. LuxS is the autoinducer synthase and is responsible for catalysis of the final step in AI-2 biosynthesis. Our analysis of AI-2 biosynthesis in several Gram-negative and Gram-positive bacterial species shows that an identical pathway is used in each case. These results, along with preliminary structural analyses of AI-2, suggest that many bacteria could use an identical 'universal' molecule for intercellular communication.

Results

A genomic approach to the analysis of the function of LuxS

The strategy for determining the biosynthetic pathway for AI-2 was inspired by our analysis of the genomic locations of the various *luxS* genes. We find that the *luxS* genes do not consistently reside in any one particular location in the chromosome; they are not typically found in close proximity to any specific gene(s), nor do they exist in operons. However, we noticed that, in *Borrelia burgdorferi*, the *luxS* gene is the third gene in a three-gene operon with the two genes *metK* and *pfs*. We therefore wondered whether LuxS might act in a biochemical pathway that also included MetK and Pfs.

MetK and Pfs function in the synthesis and utilization of *S*-adenosylmethionine (SAM). The role of MetK is to convert methionine to SAM, which plays an important role in one-carbon metabolism. Subsequently, SAM acts as a methyl donor in the biosynthesis and/or modification of DNA, RNA and a variety of cell proteins. In a separate pathway, SAM is used as an aminopropyl donor in the formation of polyamines. Pfs acts in the catabolism of the SAM-derived by-products of these processes (Della Ragione *et al*., 1985; Cornell *et al*., 1996). These two SAM utilization pathways are shown in Fig. 2 (Greene, 1996).

When SAM is used as a methyl donor, several SAM-dependent methyltransferases act on SAM to transfer the methyl group from SAM to its substrates (Fig. 2A). This step produces *S*-adenosylhomocysteine (SAH). SAH functions as a potent inhibitor of SAM-dependent methyltransferases. Therefore, bacteria rapidly degrade SAH via the enzyme called Pfs. Pfs is a nucleosidase that removes adenine from SAH and produces *S*-ribosylhomocysteine (SRH) in the process. In a final step, SRH is reported to be converted to homocysteine and 4,5-dihydroxy-2,3-pentanedione (Miller and Duerre, 1968; Duerre and Walker, 1977; Greene, 1996). The enzyme responsible for this step has never been identified, cloned or purified. Furthermore, no role is known for 4,5-dihydroxy-2,3-pentanedione. The homocysteine that is produced in this step can re-enter the pathway. It is methylated to generate methionine, which can be used by MetK to produce SAM (Greene, 1996). In polyamine biosynthesis, SAM undergoes sequential decarboxylation and cleavage reactions (Fig. 2B). These two reactions yield methylthioadenosine (MTA). MTA, like SAH, is an inhibitor of critical cellular processes, and similar to SAH, MTA is degraded by the enzyme Pfs. In this case, Pfs removes adenine from MTA to form methylthioribose (MTR). The further metabolism of MTR is not known (Duerre and Walker, 1977; Schlenk, 1983). We hypothesize that LuxS could act in either of these pathways, as Pfs is involved in both of them.

Production of AI-2 from LuxS and dialysed cell-free S. typhimurium *extracts*

To test whether LuxS is a member of one of the pathways shown in Fig. 2, we assayed whether AI-2 could be synthesized from dialysed cell-free extracts prepared from *luxS*$^+$ and *luxS*$^-$ *S. typhimurium* if SAM, SAH, SRH or MTA was added.

The *S. typhimurium luxS* null strain SS007 carrying either the arabinose-inducible plasmid pBAD18 containing the cloned *luxS* gene or the pBAD18 vector alone was grown to mid-exponential phase in LB with added arabinose to induce expression from the vector. The cells from the two cultures were harvested, lysed by French press and dialysed to remove small soluble molecules as described in *Experimental procedures*. To test the different substrates, SAM, SAH, SRH and MTA were added at 1 mM concentration to cell extracts containing 1 mg ml^{-1} cell protein in 10 mM sodium phosphate buffer, pH 7.5. The mixtures were incubated at 37°C for 1 h. After incubation, protein was removed from the mixtures by filtration, and serial dilutions of the filtrates were tested for AI-2 activity in the *V. harveyi* BB170 bioassay. The results are presented in Fig. 3 as the normalized fold induction of the reporter strain over control assays in which only the reaction buffer was added. These values were calculated from the dilution of each filtrate that resulted in half-maximal induction of the reporter strain.

Figure 3 shows that the addition of any of the test

substrates to *S. typhimurium* SS007 dialysed extracts containing only the pBAD18 vector did not result in the production of AI-2 (white bars). However, when the cell-free dialysed extracts had been prepared from *S. typhimurium* SS007 expressing *luxS* from the plasmid, significant AI-2 was produced after the addition of SAM, SAH or SRH (black bars). Specifically, quantities of AI-2 sufficient to induce the *V. harveyi* reporter strain 200-fold were produced after incubation of the extracts with SAM, and 50 to 100 times more AI-2 was produced when either SAH or SRH was added to the reaction (9000-fold and 18 000-fold induction respectively). These results demonstrate that, in the presence of LuxS, AI-2 can be synthesized from SAM or the products of the SAM utilization pathway shown in Fig. 2A. Importantly, Fig. 3 shows that, when MTA was tested as a substrate in these experiments, no AI-2 activity was produced. Therefore, LuxS does not act in the SAM catabolism pathway shown in Fig. 2B. Other compounds tested as substrates in these experiments included adenine, adenosine, D-ribose, *S*-adenosyl-L-cysteine, D,L-homocysteine and D,L-homocysteine thiolactone. Incubation of any of these compounds with the dialysed extracts containing LuxS did not result in AI-2 activity (data not shown).

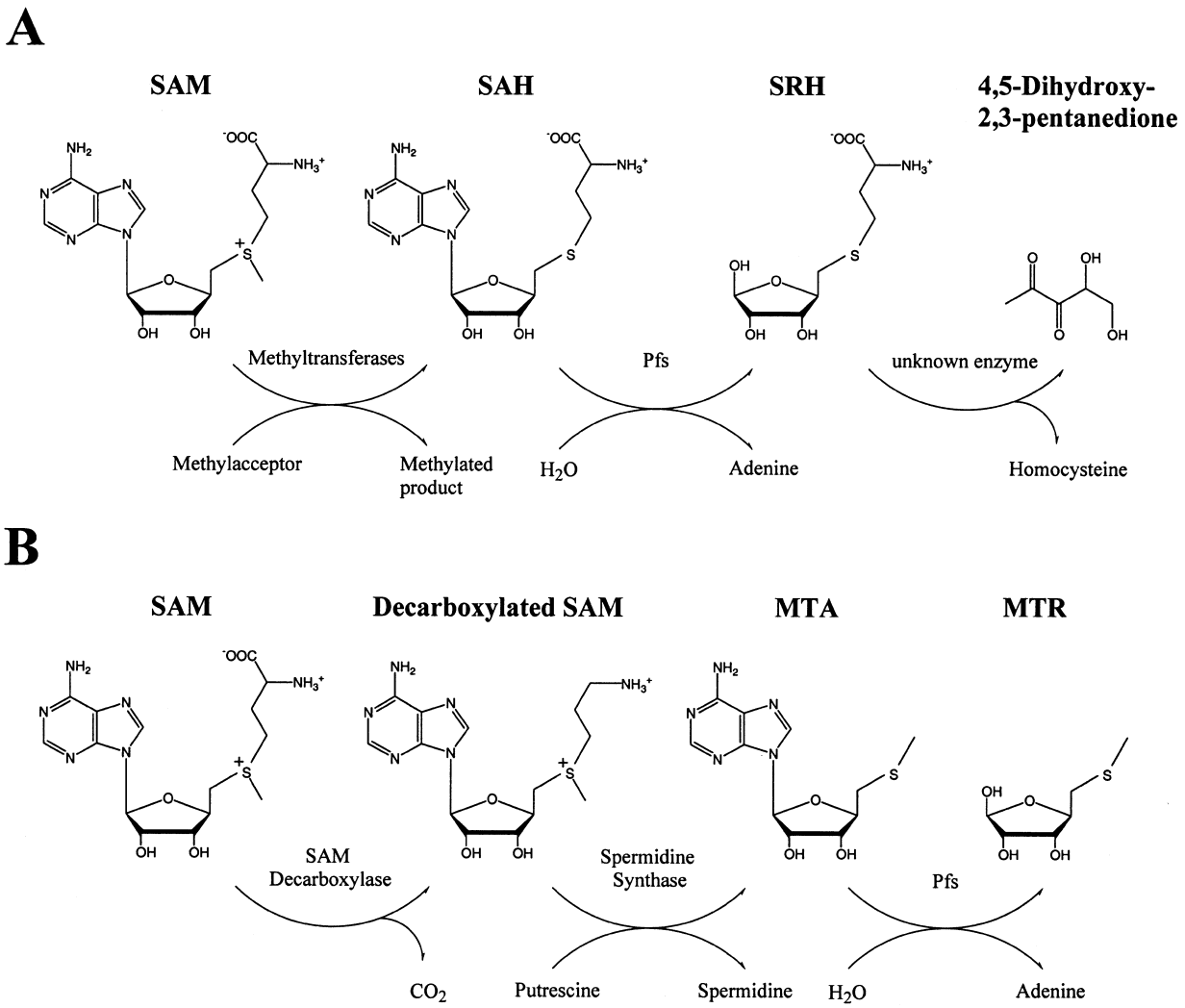

Fig. 2. *S*-adenosylmethionine (SAM) utilization pathways in bacteria. In bacteria, SAM is used as a methyl donor (A) and in polyamine biosynthesis (B).
A. A number of methyltransferase enzymes catalyse the transfer of the methyl group from SAM to particular cellular substrates. *S*-adenosylhomocysteine (SAH) is formed in this process. The Pfs nucleosidase subsequently cleaves adenine from SAH forming S-ribosylhomocysteine (SRH). SRH is converted to homocysteine and 4,5-dihydroxy-2,3-pentanedione by an unknown enzyme.
B. In two sequential reactions, SAM is decarboxylated and cleaved, and the aminopropyl moiety is used in the synthesis of polyamines such as spermidine. Methylthioadenosine (MTA) is formed by these reactions. The Pfs nucleosidase catalyses the hydrolysis of adenine from MTA to form methylthioribose (MTR). The subsequent steps in the utilization of MTR are not known.

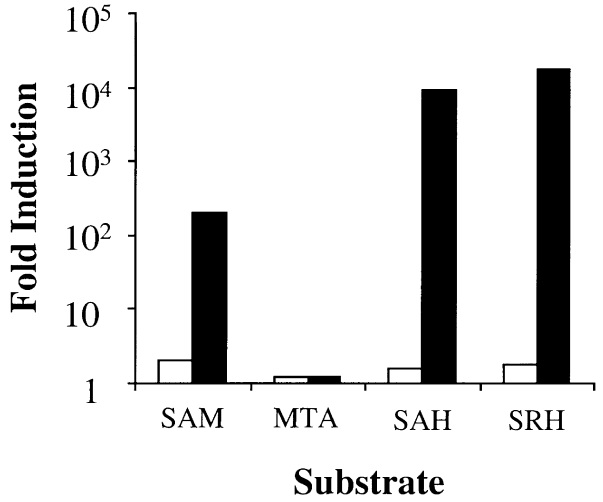

Fig. 3. Production of AI-2 from cell-free extracts. Cell-free extracts were prepared from an *S. typhimurium luxS* null strain harbouring the pBAD18 vector (white bars) or *luxS* cloned on pBAD18 (black bars). The extracts were dialysed to remove small molecules and, subsequently, potential substrates for AI-2 biosynthesis were added to the extracts at a concentration of 1 mM. The mixtures were incubated for 1 h at 37°C, after which they were filtered to remove protein. The filtrates were tested at various dilutions for AI-2 activity in the *V. harveyi* BB170 bioassay. The graph shows the fold stimulation of the reporter over that of the controls when assay buffer was added.

In vitro *production of AI-2 from purified proteins*

A simple interpretation of the results shown in Fig. 3 is that LuxS is the unidentified enzyme involved in the pathway shown in Fig. 2A, and LuxS acts on SRH to produce AI-2 and homocysteine. However, it is possible that LuxS does not act immediately after Pfs. Instead, after the formation of SRH, additional unknown enzymes could be required before or after the action of LuxS in the formation of AI-2. To confirm the required role for Pfs in AI-2 biosynthesis, to show that LuxS acts immediately after Pfs and to show that LuxS is the final enzyme required in the AI-2 biosynthetic pathway, we cloned and purified the Pfs and LuxS enzymes. We show in Table 1 that we can produce AI-2 *in vitro* using only these purified proteins and their substrates.

In this series of experiments, combinations of potential substrates and purified enzymes were prepared and incubated for 1 h at 37°C, followed by filtration to remove the proteins. Tenfold serial dilutions of the filtrates were tested in the *V. harveyi* BB170 bioassay, and the fold induction of the reporter strain was determined as described in the previous experiment. The control experiments in Table 1 show that the putative substrates and products of the pathway, 1 mM SAH, SRH, adenine, ribose and homocysteine, have no activity because all these controls resulted in only fourfold induction of light production in the reporter. Likewise, incubation of the enzymes Pfs and LuxS followed by filtration and addition of the filtrate to the assay did not stimulate light production in the reporter (fivefold induction). In contrast, incubation of SAH with the purified Pfs and LuxS enzymes resulted in AI-2 activity equivalent to 86 000-fold induction of the reporter. Table 1 shows that both the Pfs and the LuxS enzymes are required for high-level AI-2 production from SAH, because incubation of either enzyme alone with SAH did not result in significant AI-2 production (150-fold and 200-fold respectively). We suspect that the low level of activity we observe when SAH is incubated with either of the proteins alone arises from contamination of our Pfs protein preparation with traces of LuxS protein and vice versa. The proteins we used in these assays are only about 95% pure. In support of this interpretation, we showed in the experiments using dialysed cell-free extracts from the *luxS*$^-$ strain that, when zero LuxS protein is present, no production of AI-2 occurs (Fig. 3). Controls from these experiments are in the one- to twofold stimulation range.

The above results demonstrate that SAH is the only substrate required for the production of AI-2 from the enzymes Pfs and LuxS. Our hypothesis is that AI-2 is produced in two steps. First, Pfs acts on SAH to produce adenine and SRH and, secondly, LuxS acts on SRH to produce AI-2 and homocysteine (Fig. 2A). We have already noted that the data in Table 1 show that the addition of SAH directly to LuxS protein does not result in AI-2 production, which is further proof that LuxS must act after the conversion of SAH to SRH. To confirm the order of the biosynthetic pathway, we performed the *in vitro* AI-2 biosynthesis in two sequential steps. First, we incubated SAH with purified Pfs protein, then filtered the reaction mixture to remove the Pfs enzyme and, subsequently, incubated the filtrate with LuxS protein. Table 1 shows that performing the synthesis in this order resulted in the *in vitro* production of AI-2 sufficient to induce the reporter strain 83 000-fold. In contrast, performing this experiment in the opposite order

Table 1. *In vitro* AI-2 production from purified proteins and substrates.

Incubation mixture		
Substrate	Protein	Normalized fold induction
SAH	–	4
SRH	–	4
Adenine	–	4
Ribose	–	4
Homocysteine	–	4
–	Pfs + LuxS	5
SAH	Pfs + LuxS	86 000
SAH	Pfs	150
SAH	LuxS	200
SAH	Pfs/filter/LuxS[a]	83 000
SAH	LuxS/filter/Pfs[a]	150
SRH	Pfs	70
SRH	LuxS	27 000

a. The symbol '/' indicates that the reactions were performed sequentially and filtered in between to remove protein.

by first incubating LuxS with SAH, filtering the mixture and then incubating the filtrate with Pfs did not result in AI-2 production (150-fold induction). These results show that Pfs must act on SAH before the enzymatic action of LuxS.

Our data indicate that the substrate for LuxS is SRH and that AI-2 is produced directly from LuxS and SRH. To verify this assumption, we prepared SRH by boiling SAH in 1 M HCl (Palmer and Abeles, 1979). We then tested whether SRH could be used as a substrate in these *in vitro* reactions. Incubation of SRH with Pfs did not result in significant activity (70-fold induction), whereas incubation of SRH with LuxS *in vitro* resulted in AI-2 activity sufficient to stimulate the reporter strain 27 000-fold. This level of AI-2 activity is very significant, but lower than the activity resulting from the SAH reaction with Pfs and LuxS. It must be emphasized that the boiling procedure to chemically convert SAH into SRH is very crude and evidently results in the formation of SRH and a number of side-products. Mass spectroscopy was used to confirm that this procedure resulted predominantly in SRH, but other species were formed as well (data not shown). Therefore, lower activity occurred in this assay, presumably because of our inability to fully convert the SAH to SRH.

According to the pathway, *in vitro* production of AI-2 from Pfs and LuxS occurs with the concomitant production of homocysteine. We checked for the production of homocysteine using the colorimetric Ellman's assay. This assay enables us to quantify the free sulphydryl group that appears when homocysteine is formed. Ellman's test was performed after all the experiments shown in Table 1. A positive Ellman's reaction only occurred in the samples in which AI-2 was produced.

Mass spectral analyses of the intermediates in the AI-2 biosynthetic pathway

Electrospray mass spectral analyses performed on the filtered products of each of the reaction mixtures presented in Table 1 provide further evidence for the order of our hypothesized biosynthetic scheme. Figure 4 shows some of the negative-mode mass spectra. Figure 4A shows the mass spectrum for SAH after 1 h incubation under our reaction conditions. The SAH peak is at the expected position of m/z 383. There is also a peak at m/z 134, which could be caused by contamination of the commercial preparation of SAH with adenine and/or homocysteine, both having the same mass. According to the SAM utilization pathway in Fig. 2A, the addition of Pfs to SAH converts the SAH to SRH and adenine (Della Ragione *et al*., 1985; Cornell *et al*., 1996). Figure 4B shows that this is the case. Specifically, Fig. 4B shows that, when Pfs is incubated with SAH, the m/z 383 SAH peak disappears, and a new peak appears at m/z 266 corresponding to SRH. Electrospray analysis of this same sample in the positive mode allowed us to observe a similar increase in the m/z 134 peak corresponding to adenine (not shown). Furthermore, our biosynthetic pathway indicates that LuxS cannot act on SAH, as it must first be converted to SRH. The data in Fig. 4C show that LuxS does not act on SAH; the m/z 383 SAH peak remains, and no additional products are formed. Finally, simultaneous incubation of LuxS and Pfs with SAH results in a large reduction in both the m/z 383 (SAH) and the m/z 266 (SRH) peaks (Fig. 4D). Loss of peak intensity of SRH at m/z 266 was accompanied by an increase in AI-2 activity. Positive mode electrospray analysis demonstrated that adenine is produced during this process, and homocysteine was identified as a reaction product by high-performance liquid chromatography (HPLC) analysis coupled to electrospray mass spectroscopy after derivatization with Ellman's reagent (not shown). Furthermore, the addition of Ellman's reagent to our *in vitro* AI-2 preparations did not affect the AI-2 activity, providing further evidence that the free thiol group is not part of the AI-2 molecule, but rather is on homocysteine.

We recognize that the mass spectra presented in Fig. 4B and D appear more complex than those presented in Fig. 4A and C. This seeming complexity results from the production of a lower concentration of SRH in the samples in Fig. 4B and D than the input concentration of SAH in the samples in Fig. 4A and C. Therefore, in spectra containing SRH, the background peaks appear larger.

Unfortunately, electrospray mass spectral analysis of this sample and others like it did not allow us to identify a peak for AI-2. Electron impact ionization is often superior for the detection of low-molecular-weight species such as sugars. However, even with this mass spectral analysis, we were unable to detect a molecular ion for AI-2. The reason for our inability to identify a molecular ion for AI-2 appears to be the low abundance of this molecule formed during *in vitro* biosynthesis, even though bacterial luminescence assays suggested that the amount of AI-2 appeared to be quite large. In fact, the high sensitivity of this assay provided the ability to detect biologically small amounts of AI-2, yet offered no method for determining a concentration for AI-2. We believe that the probable reactive nature of the 4,5-dihydroxy-2,3-pentanedione molecule contributes to the low abundance of AI-2 in our samples. The dione moiety of 4,5-dihydroxy-2,3-pentanedione is quite electrophilic and is known to react with a variety of nucleophilic species, such as amino, sulphydryl and carboxyl groups, that are present in our cell extracts and on our purified proteins (Hofmann, 1998).

Structural predictions for AI-2 and the activity of AI-2 analogues

Based on the *in vitro* biosynthesis data and the previously

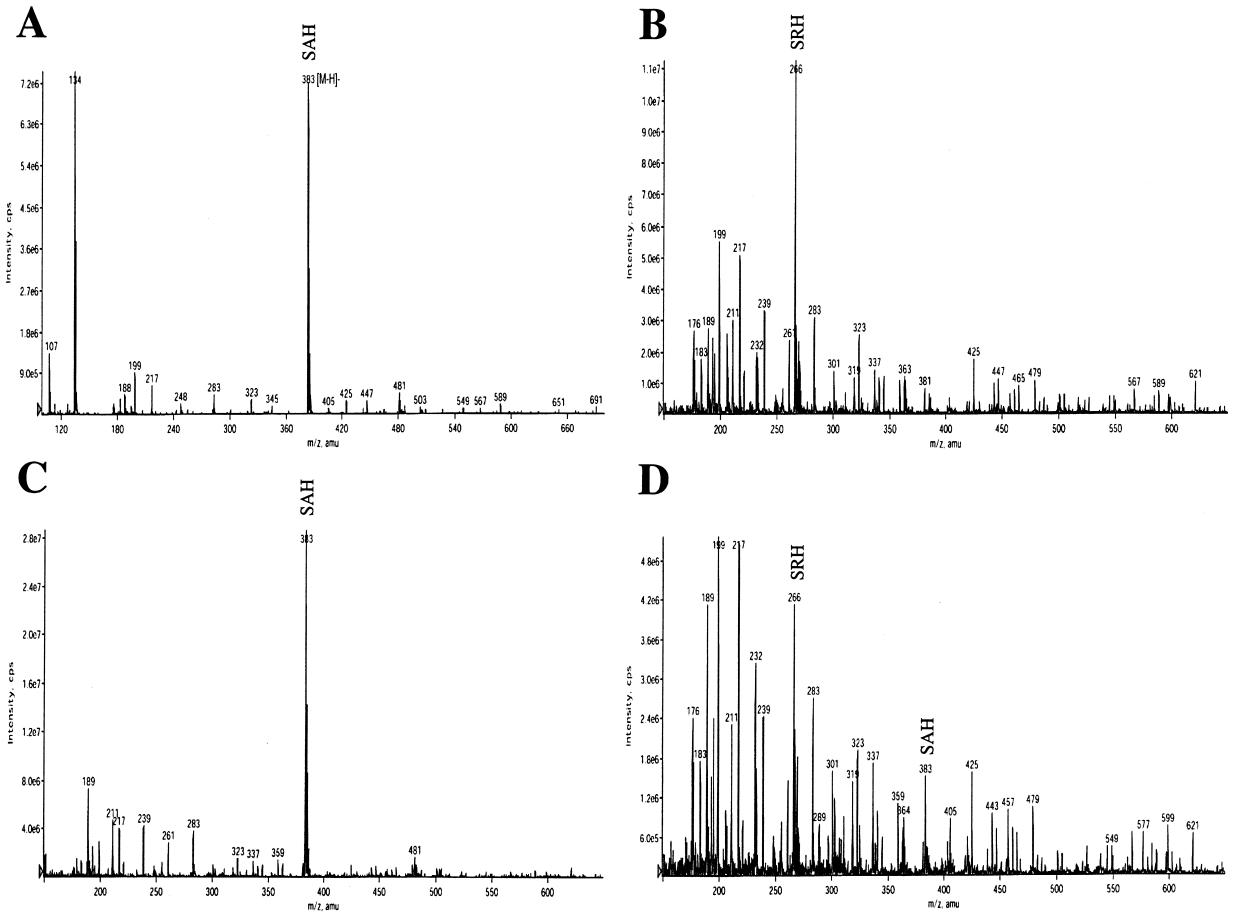

Fig. 4. Mass spectral analyses of *in vitro* AI-2 biosynthesis reactions. Representative mass spectra are presented for *in vitro* AI-2 biosynthesis reactions and controls.
A. The spectrum for SAH incubated for 1 h at 37°C under our *in vitro* assay conditions.
B. The spectrum resulting from an identical 1 h incubation of SAH with purified Pfs enzyme.
C. The spectrum after incubation of SAH with purified LuxS enzyme.
D. The spectrum resulting from simultaneous incubation of SAH with Pfs and LuxS.
All reactions were carried out in 10 mM sodium phosphate buffer (pH 7.5), and proteins were used at 1 mg ml^{-1}. All reactions were filtered to remove proteins before mass spectral analysis.

reported production of 4,5-dihydroxy-2,3-pentanedione from *S*-ribosylhomocysteine, we investigated several candidates for AI-2. As mentioned, we reasoned that the 2,3 pentanedione moiety of 4,5-dihydroxy-2,3-pentanedione would be highly susceptible to either intra- or intermolecular attack by the adjacent hydroxyl groups or water respectively. In fact, 4,5-dihydroxy-2,3-pentanedione has been suggested to be an unstable intermediate in the formation of 4-hydroxy-5-methyl-furanone (MHF) in the Mallaird reaction (Hofmann and Schieberle, 1998). We therefore presume that, after formation by LuxS, 4,5-dihydroxy-2,3-pentanedione spontaneously cyclizes to become a furanone. We tested MHF as well as two other one-carbon analogues of MHF for their ability to stimulate reporter activity in the *V. harveyi* BB170 bioassay. These molecules are shown in Fig. 5A, and the corresponding activity titration curves are shown in Fig. 5B.

Figure 5B shows that *in vitro*-synthesized AI-2 activates the reporter to produce the half-maximal level of light at ≈ 100 nM. Our estimation of the concentration of AI-2 in our *in vitro* preparation is calculated using the Ellman's assay. If we assume that a 1:1 production of AI-2 and homocysteine occurs in the *in vitro* reactions, then the Ellman's test allows us to estimate the amount of AI-2 produced in our reactions. Approximately 0.4 mM AI-2 is produced under the following conditions: 1 mM SAH, 1 mg ml^{-1} Pfs and LuxS for 15 min at 37°C. It should be noted that this is our best estimate of the concentration of AI-2 in our samples. If it turns out that 4,5-dihydroxy-2,3-pentanedione is not completely converted to AI-2 in the *in vitro* reaction, then the concentration calculated here is an overestimation of the level of AI-2 in our samples.

We find that the compound MHF has activity, but that a 1000-fold higher concentration than that for AI-2 (≈ 100 μM) is required to stimulate the reporter. Even higher concentrations of the compounds 4-hydroxy-2,

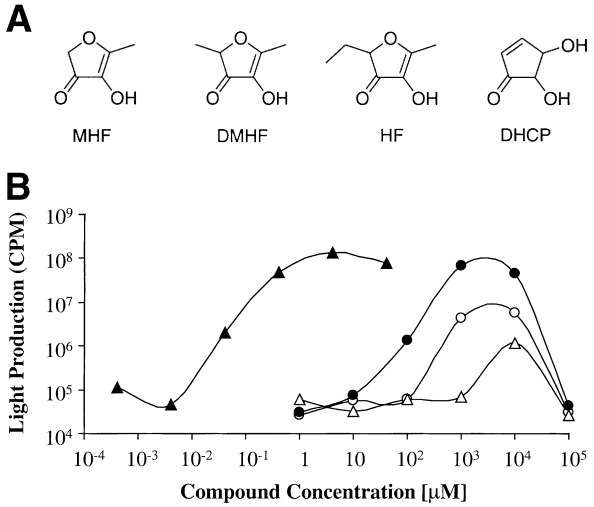

Fig. 5. Structures and activities of AI-2 analogues.
A. The structures of some compounds tested for AI-2 activity.
B. Titration curves showing the activity of *in vitro*-prepared AI-2 and for the compounds shown in (A). A range of concentrations of AI-2 and the candidate compounds was tested for stimulation of light production of the *V. harveyi* BB170 reporter strain. The compounds and their corresponding symbols are as follows: AI-2, black triangles; MHF, black circles; DMHF, white circles; and HF, white triangles. No curve is shown for DHCP because it had no activity.

5-dimethyl-furanone (DMHF) and homofuraneol (4-hydroxy-2-ethyl-5-methyl-furanone) (HF) are required to observe activity. Finally, an alternative reaction mechanism for LuxS suggested to us that a product formed via an intramolecular aldol reaction, 4,5-dihydroxy-2-cyclopentan-1-one (DHCP), might be another candidate for AI-2. The latter compound was synthesized but was found to be completely inactive in the reporter assay (data not shown). The structure of DHCP is presented in Fig. 5A. It is notable that, of the AI-2 analogues tested, only MHF is capable of full induction of the reporter strain. None of the AI-2 analogues enhanced the growth of the reporter strain and, in fact, all the AI-2 analogues (including MHF) were toxic at high concentrations. None of the molecules tested in the above experiments resulted in activity in our analogous AI-1 reporter strain *V. harveyi* BB886, showing that the activities we observed are specific for the *V. harveyi* AI-2 detection system LuxPQ (not shown).

In addition to studying the *E. coli* LuxS enzyme, we performed experiments using purified LuxS proteins from *S. typhimurium*, *V. harveyi*, *V. cholerae* and *E. faecalis* to determine the specific activities of *in vitro*-produced AI-2 from these bacterial species. Specifically, incubation mixtures were prepared that contained SRH and each of the five LuxS enzymes we had purified. Every LuxS enzyme we tested resulted in AI-2 with half-maximal activity in the range of 10–100 nM. Only the AI-2 activity profile for the *E. coli* LuxS reactions is shown in Fig. 5B because the activity–titration curves we obtained for all five LuxS enzymes are essentially indistinguishable.

Taken together, all our results suggest that AI-2 is a five-carbon furanone that results from the spontaneous cyclization of 4,5-dihydroxy-2,3-pentanedione, the product of the LuxS-catalysed cleavage of the ribosyl moiety from SRH. Furthermore, the results presented in Fig. 5 indicate that the AI-2 detection apparatus, LuxPQ, is highly sensitive to substituents at position 2 of the furanone ring. We postulate that the cyclization of 4,5-dihydroxy-2,3-pentanedione to give cyclic products such as MHF may proceed through intermediates with highly reactive substituents that could react with a variety of species in the *in vitro* and *in vivo* AI-2 biosynthesis reactions. Therefore, the yield of AI-2 after the formation of 4,5-dihydroxy-2,3-pentanedione or an equivalent species derived from the ribose moiety of *S*-ribosylhomocysteine may be low. Furthermore, MHF itself is unstable under certain conditions in the presence of amino acids and forms large-molecular-weight species by condensation with itself in acid-catalysed reactions (Hofmann, 1998). We did not detect any highly coloured species, yet these known reactions suggest that even MHF itself may be converted to other species under biologically relevant conditions. These features have prevented the absolute determination of the structure of enzymatically produced AI-2.

Discussion

We have reported previously that numerous species of bacteria produce a novel signalling molecule that we have termed AI-2 and also that, in every case, its production is dependent on the *luxS* gene (Bassler *et al.*, 1997; Bassler, 1999; Surette *et al.*, 1999). In an effort to determine the structure of AI-2, we developed a method for the *in vitro* production of the molecule using purified proteins. We have not yet succeeded in an absolute assignment of the AI-2 structure. However, our studies have allowed us to determine unequivocally the biosynthetic pathway for AI-2 production, to show that LuxS is the AI-2 synthase and, finally, to predict that AI-2 is a furanone.

Our biosynthetic studies show that AI-2 is produced from SAM (Figs 2A and 3). SAM is converted to SAH by the action of numerous methyltransferases. Subsequently, the Pfs nucleosidase cleaves adenine from SAH to produce SRH. Figure 3 demonstrates that LuxS acts on SRH to make AI-2. The results shown in Table 1 with the different candidate substrates and purified Pfs and LuxS proteins enabled us to determine the sequence of enzymatic reactions and to show that LuxS acts on the product of the Pfs reaction, namely SRH. By performing the enzymatic reactions sequentially with purified proteins (Table 1) in combination with mass spectral analyses of these reaction mixtures (Fig. 4), we were able to identify the intermediates in the biosynthetic pathway for AI-2. Together, all these results confirm that the pathway shown

in Fig. 2A results in AI-2 production. This pathway has always been considered to be a pathway for salvaging adenine and homocysteine, but we show here that it is in fact also the AI-2 biosynthetic pathway.

It is known that 4,5-dihydroxy-2,3-pentanedione is produced in this pathway (Miller and Duerre, 1968; Duerre and Walker, 1977; Greene, 1996). Our results demonstrate that synthesis of this molecule must depend on LuxS. However, we argue that 4,5-dihydroxy-2,3-pentanedione is not AI-2, but that 4,5-dihydroxy-2,3-pentanedione cyclizes into a furanone ring for several reasons. First, in its original identification, 4,5-dihydroxy-2,3-pentanedione was suggested as an intermediate in the SAM utilization pathway, but its further modification/cyclization/catabolism could not be established (Miller and Duerre, 1968). Secondly, we have not observed evidence of the formation of 4,5-dihydroxy-2,3-pentanedione in the mass spectral or nuclear magnetic resonance (NMR) analyses of our *in vitro*-synthesized AI-2. Thirdly, 4,5-dihydroxy-2,3-pentanedione is a highly reactive molecule that readily undergoes nucleophilic attack and is not expected to be stable in solution. We therefore suggest that AI-2 is a furanone similar to those with activity shown in Fig. 5. The spontaneous cyclization of 4,5-dihydroxy-2,3-pentanedione probably results in a mixture of furanones. We favour the idea that one of these compounds is AI-2. However, it is possible that the combinatorial action of two or more molecules is required for full AI-2 activity.

Based on the evidence presented in this manuscript regarding the biosynthetic pathway for AI-2 coupled with preliminary structural studies of the molecule, we suggest the following chemistry for the LuxS reaction. The cleavage of homocysteine from *S*-ribosylhomocysteine is probably catalysed by the initial oxidation of C 3′ to a ketone. We suggest this by analogy with *S*-adenosylhomocysteinase, the enzyme that catalyses the same bond cleavage in eukaryotes. *S*-adenosylhomocysteinase cleaves SAH to adenosine and homocysteine. In this case, the oxidation reaction is catalysed by an enzyme-bound NAD^+. The oxidation at C 3′ activates the C 4′ proton for abstraction and expulsion of the homocysteine at C 5′ to form a stable enone moiety. The reduced form of the cofactor NADH then reduces the C 3′ ketone to complete the reaction cycle. These reactions are described fully by Palmer and Abeles (1979). We could not detect an enzyme-bound NAD^+ or equivalent FAD^+ by UV absorbance measurement, and thus suspect that the enzyme contains a bound metal atom that is probably the redox active cofactor in LuxS.

Furanones have been implicated as signalling molecules in both bacteria and eukaryotes. The seaweed *Delisea pulchra* produces halogenated furanones that inhibit swarming motility and therefore colonization of the plant by the bacterium *Serratia liquefaciens* (Givskov *et al*., 1996; 1997; 1998). It should be noted that this is a model system, and *S. liquefaciens* and *D. pulchra* do not encounter one another naturally. These studies are interesting because swarming in *S. liquefaciens* is regulated by an HSL quorum-sensing mechanism. The *D. pulchra* halogenated furanones are antagonists of the true autoinducer pheromone. Furanones are structurally related to HSLs. The halogenated furanones produced by *D. pulchra* interact specifically with and inactivate the LuxR transcriptional activator required for LuxI/LuxR-type HSL quorum sensing (Manefield *et al*., 1999). These reports raise the possibility that some unidentified quorum-sensing bacterium that is a true pathogen of *D. pulchra* could be thwarted by a similar mechanism. Furthermore, if AI-2 is a furanone, the possibility also exists that bacteria that produce AI-2 could use it to enhance or thwart the HSL-controlled behaviours of other species of quorum-sensing bacteria co-existing in the environment. The furanone MHF (Fig. 5) is one component of the sex pheromone of the male cockroach *Eurycolis floriona* (Farine *et al*., 1993). Female cockroaches are attracted to its odour. Additionally, furanones have long been used as flavourings and additives in the food industry because they have pleasing aromas (Slaughter, 1999). For example, the pleasant smell of strawberries, pineapples and caramel results from furanones. This fact suggests that furanones act as pheromones for humans as well.

Furanones have been suggested to possess some ideal characteristics for signalling molecules. For example, they are commonly occurring molecules and can be derived from SAM via LuxS as we have shown and also from sugars and amino acids. Depending on the ring substituents, furanones can be water soluble, lipid soluble or volatile. Lastly, for each furanone, several stereoisomers are possible, which could be critical for signalling specificity (Slaughter, 1999).

Our previous genetic analysis of the quorum-sensing circuit of *V. harveyi* has shown that the sensory apparatus for the detection of AI-2 is composed of the LuxPQ proteins (Bassler *et al*., 1994a). As mentioned in the *Introduction* and depicted in Fig. 1, LuxP is a soluble periplasmic protein homologous to the ribose-binding protein of *E. coli* and *S. typhimurium*. We have proposed that LuxP is the primary sensor for AI-2, and that LuxP, in a complex with AI-2, interacts with the two-component sensor kinase LuxQ to initiate the AI-2 signal transduction cascade (Bassler *et al*., 1994a). In this report, we show that AI-2 is derived from the ribose moiety of SRH and that AI-2 is most probably a furanone with structural similarity to ribose. This finding is fitting, given that a ribose-like binding protein is required for its detection at least in *V. harveyi*. The detection apparatuses for AI-2 in other *luxS*-containing bacteria are not known.

Our finding that SAM and Pfs are involved in AI-2 production is especially intriguing because SAM and Pfs also have required roles in HSL autoinducer biosynthesis (Hanzelka and Greenberg, 1996; More *et al*., 1996; Schaefer *et al*., 1996; Val and Cronan, 1998; Parsek *et al*., 1999). In HSL biosynthesis, the LuxI enzymes are the HSL autoinducer synthases. The LuxI enzymes drive the formation of an amide bond linking the acyl side-chain of a specific acyl-acyl carrier protein (acyl-ACP) to SAM. Subsequently, lactonization of the ligated intermediate results in the formation of the HSL autoinducers. MTA is released in this process. As shown in Fig. 2B, Pfs cleaves adenine from MTA to form MTR. In *V. harveyi*, LuxLM fulfils the role of LuxI. There is evidence suggesting that, although LuxLM has no homology to the LuxI enzymes, LuxLM makes the HSL autoinducer AI-1 by a mechanism identical to that of LuxI (Hanzelka *et al*., 1999). Our results show that bacteria such as *V. harveyi* that produce both an HSL autoinducer and AI-2 have evolved an economical mechanism for linking AI-1 and AI-2 biosynthesis. Synthesis of both signals relies on the essential metabolite SAM and on Pfs, an enzyme required for removal of the products of SAM utilization. This facet of the biosynthesis implies a critical role for signal production as well. Using essential components of central metabolism as substrates, the bacteria ensure that a continual supply of material will be available for signal generation. Additionally, because the intermediates SAH and MTA in the signal biosynthetic pathways are toxic, the bacteria also ensure that the signal-producing reactions will be driven to completion. Several other bacteria have been shown to be similar to *V. harveyi* in that they produce both an HSL-type AI-1 molecule as well as AI-2, indicating that joint control of the production of multiple autoinducers could be quite common.

It is striking that, in bacteria, elimination of SAH, the toxic product formed from SAM utilization, occurs in two sequential enzymatic steps. This seems especially inefficient because eukaryotes manage this process in a single step. Specifically, eukaryotes cleave SAH directly into two usable products, adenosine and homocysteine (Palmer and Abeles, 1979). Therefore, in the eukaryotic process, the only enzyme required for detoxification is *S*-adenosylhomocysteinase. We hypothesize that the two-step process in *luxS*-containing bacteria is critical because detoxification/recycling is not the only purpose of the pathway. The process is also required for AI-2 production. It could be that bacteria that do not have *luxS* and *pfs* will instead possess a gene encoding a function analogous to *S*-adenosylhomocysteinase of eukaryotes.

In this report, we have presented our findings for experiments using purified Pfs and LuxS from *E. coli*. However, we have performed similar experiments with LuxS purified from *V. harveyi*, *S. typhimurium*, *V. cholerae* and the Gram-positive bacterium *E. faecalis*. In each case, we have obtained results identical to those presented here. Specifically, SRH is the substrate for LuxS in every case tested, and AI-2 and homocysteine are produced. Furthermore, as described previously, Ellman's test allowed us to estimate how much AI-2 was made in our *in vitro* reactions. Specific amounts of AI-2 produced by *E. coli*, *V. harveyi*, *S. typhimurium*, *V. cholerae* and *E. faecalis* were compared in our bioassay. We observes no difference in the specific activities of the AI-2s produced from LuxS from these species. We therefore conclude that a common biosynthetic pathway exists in all these organisms and presumably in all other *luxS*-containing bacteria. We also speculate that AI-2 is not a family of related molecules, but that bacterial species possessing *luxS* probably produce an identical molecule. This result is in contrast to the LuxI family of HSL autoinducer synthases that act on specific acyl-ACP substrates to produce specific acylated-HSL autoinducers in Gram-negative bacteria, and to the machinery responsible for specific oligopeptide autoinducer synthesis in Gram-positive bacteria. When we complete our AI-2 structural analysis, if we confirm that the AI-2s of different species of bacteria are identical, this result could be understood in the following way. Bacteria might need a signal that is specific and unrecognizable to other species for intraspecies signalling, but they might need a universally recognized and utilized signal for interspecies signalling. Our results with AI-2 suggest that this is the case.

Our analysis of the biosynthetic pathway and preliminary structural studies suggest that AI-2 is a very simple small molecule. Our previous genetic and genomic studies revealed that the *luxS* gene is present in over 30 bacterial species, indicating that the production and use of this signal are widespread in the bacterial kingdom (Bassler *et al*., 1997; Bassler, 1999; Surette *et al*., 1999). Several recent reports suggest that AI-2 is important for pathogenicity in some bacteria (Sperandio *et al*., 1999; Kim *et al*., 2000; Lyon *et al*., 2000). To date, *luxS* has not been identified in higher organisms, which indicates that targeting Pfs, LuxS and/or AI-2 as the basis for the design of novel antibacterial therapies could be worthwhile. We show in Fig. 5 that several furanones have some agonist activity in our bioassay. We have now begun an analysis of naturally occurring and synthetic molecules related to furanones in the hope of identifying some with antagonist activity that could be tested for antibacterial action.

Finally, our evidence suggests that LuxS catalyses the cleavage of homocysteine from SRH and that a subsequent cyclization reaction produces the active AI-2 species. At present, our successful determination of the AI-2 structure awaits our purification of the AI-2 molecule from the other reactants and products of our *in vitro*

reaction mixture in sufficient quantities for NMR analyses. We are currently working on this chemical analysis.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are: *V. harveyi* BB120 (wild type); *V. harveyi* BB170 (*V. harveyi* BB120 *luxN*::Tn*5*); *V. harveyi* BB886 (*V. harveyi* BB120 *luxP*::Tn*5*); *S. typhimurium* 14028 (wild type); *S. typhimurium* SS007 (*S. typhimurium* 14028 *luxS*::T-POP); *E. coli* MG1655 (wild type). Luria broth (LB) contained 10 g l^{-1} Bacto tryptone (Difco), 5 g l^{-1} yeast extract (Difco) and 10 g l^{-1} NaCl (Sambrook *et al*., 1989). The recipe for autoinducer bioassay (AB) medium has been reported previously (Greenberg *et al*., 1979). Antibiotics were used at the following concentrations: tetracycline (tet) 10 mg l^{-1}; ampicillin (amp) 100 mg l^{-1}. IPTG was used at 0.5 mM.

AI-2 bioassays

The AI-2 bioassay that uses the *V. harveyi* reporter strain BB170 has been reported previously (Bassler *et al*., 1997). Briefly, the *V. harveyi* reporter strain was grown for 13 h at 30°C with aeration in AB medium, diluted 1:5000 into fresh medium, and 90 μl of the diluted cells was added to microtitre wells containing 10 μl of the different substances to be tested for AI-2 activity. The microtitre dishes were shaken in a rotary shaker at 175 r.p.m. at 30°C. Every 30 min, light production was measured using a Wallac model 1450 Microbeta Plus liquid scintillation counter. Serial dilutions of *in vitro*-synthesized AI-2 were assayed to identify a sample dilution resulting in approximately half-maximal stimulation of the reporter strain. This AI-2 activity is reported as the fold induction of the reporter strain over background when buffer or medium alone was added to the reporter. All assays were repeated at least three times, and the values agreed within 20%.

Construction of the S. typhimurium luxS *null strain SS007*

Random mutagenesis of *S. typhimurium* 14028 was carried out with a derivative of Tn*10* called T-POP, and insertion mutants were selected on LB plates containing tet (Rappleye and Roth, 1997). A total of 5000 *S. typhimurium* insertion mutants was tested for AI-2 production using the *V. harveyi* bioassay. One T-POP insertion resulted in a strain (SS007) that produced no AI-2. The T-POP insertion site was identified by polymerase chain reaction (PCR) amplification and sequencing of the chromosomal DNA at the insertion junction using a described PCR method (Caetano-Anolles, 1993; Surette *et al*., 1999).

Cloning of luxS *and preparation of dialysed cell-free extracts*

Primers that flanked the *luxS* gene and incorporated restriction sites were designed and used to amplify the *E. coli* O157:H7 *luxS* gene. The primers used were 5′-GTG AAGCTTGTTTACTGACTAGATGTGC-3′ and 5′-CCGAATT CCCGGAGGTGGCTAAATGCC-3′, which introduced *Hin*dIII and EcoRI sites respectively. The PCR product was purified, digested with *Hin*dIII and EcoRI and cloned into similarly digested pBAD18 (Guzman *et al*., 1995) to make pMS234. The clone was sequenced to ensure that no errors had been introduced during PCR. Cultures of the *S. typhimurium luxS* mutant strain SS007 carrying either the pBAD18 vector or pMS234 were grown with aeration overnight at 37°C in LB broth containing 1 mg l^{-1} amp. Subsequently, fresh LB medium containing 1 mg l^{-1} amp and 0.1% L-arabinose was inoculated at a 1:100 dilution with the overnight cultures. Arabinose was included to induce expression from the vector. The cultures were grown with aeration at 37°C for 3.5 h, after which the cells were harvested by centrifugation at 10 000 r.p.m. for 10 min. The cell pellets were suspended at 1:200 volume in 50 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT) and lysed by two passages through a French pressure cell. Cell debris was removed by centrifugation at 10 000 r.p.m. for 10 min. The supernatant was collected and subjected to centrifugation at 100 000 r.p.m. for 1 h, after which the cytoplasmic fraction was dialysed against 50 mM sodium phosphate buffer (pH 7.5) using Sephadex G-25M mini-columns (Amersham Pharmacia) according to the manufacturer's recommendations.

Overexpression and purification of LuxS and Pfs

The *pfs* and *luxS* genes were cloned and overexpressed as glutathione-*S*-transferase (GST) fusions. Primers that contained the genomic DNA sequences flanking *luxS* and *pfs* and that incorporated restriction sites were used to amplify the *E. coli* MG1655 genes. For amplification of the *pfs* gene, the primers used were 5′-GTGGATCCATGAAAATCGGCATC ATTG-3′ and 5′-TGAATTCTGACTTAGCCATGTGCAAG-3′. For amplification of the *luxS* gene, the primers used were 5′-GTGAATTCATGCCGTTGTTAGATAGCT-3′ and 5′-ATTC TCGAGATAGTTTACTGACTAGATG-3′. Both PCR products were purified, digested and cloned into the GST gene fusion vector pGEX-4T-1 (Amersham Pharmacia). The restriction sites used were *Bam*HI and *Eco*RI for *pfs* and *Eco*RI and *Xho*I for *luxS*. These constructs were maintained in *E. coli* strain BL21 (Novagen) for overexpression. Cultures of these two strains were grown at 37°C with aeration to an OD_{600} of 1.0. IPTG was subsequently added to a final concentration of 0.5 mM, and the cultures were incubated with aeration for an additional 3 h. The cells were harvested and lysed as described above. The fusion proteins were purified on glutathione agarose matrix columns by washing with 10 volumes of 25 mM sodium phosphate, pH 7.5, 140 mM NaCl and 0.5 mM $MgCl_2$, followed by elution with 5 volumes of 50 mM sodium phosphate, pH 7.5, 10 mM reduced glutathione. The purified fusion proteins were concentrated in Centriprep-10 concentrators (Amicon) and dialysed against 50 mM sodium phosphate buffer (pH 7.5) using Sephadex G-25M mini-columns as described above. The sizes of the GST fusion proteins were confirmed by SDS–PAGE.

In vitro *production of AI-2*

Unless otherwise indicated, *in vitro* AI-2 synthesis reactions were carried out for 1 h at 37°C. The reaction mixtures

contained 1 mM substrate, 10 mM sodium phosphate buffer (pH 7.5) and 1 mg ml^{-1} of the specified cell extract or purified GST fusion protein. After incubation, reactions were filtered through Biomax-5 ultrafree centrifugation filters (Millipore) to remove protein from the reaction products.

Mass spectral analyses

Mass spectral analysis of filtered *in vitro* AI-2 biosynthesis reactions was performed on a Perkin-Elmer API 100 instrument. Both negative- and positive-mode ionization was performed. Mass ranging from 50 to 800 m/z were collected and analysed. In some cases, high-resolution spectra were collected in an attempt to differentiate between adenine and homocysteine because they share the same nominal molecular weight.

Ellman's test for free sulphydryl groups

In vitro AI-2 biosynthesis reactions were carried out for 15 min, filtered and diluted 20-fold in 100 mM sodium phosphate buffer (pH 7.2), 0.1 mM EDTA. The diluted assay mixtures (200 μl) were mixed with 100 μl of a 5 mM solution of Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid)] in the same buffer. The absorption of the mixtures was measured at 412 nm. The concentration of 2-nitro-5-thiobenzoate (TNB) formed in the Ellman's reaction was determined using the molar absorption coefficient (14 150 M^{-1} cm^{-1}). One mole of TNB mole^{-1} target sulphydryl group is formed in this reaction. Freshly prepared homocysteine stock solutions of known concentrations were used to confirm the accuracy of this method. A short (15 min) incubation time was used in experiments in which we quantified the homocysteine because homocysteine is unstable at 37°C.

Preparation of SRH from SAH

SRH was prepared from SAH by hydrolysing the *N*-glycosidic bond of adenosine under acidic conditions by a procedure adapted from that of Palmer and Abeles (1979). Briefly, SAH was dissolved in 1 M HCl at a concentration of 10 mg ml^{-1}. This solution was incubated in a boiling water bath for 20 min. Subsequently, an equal volume of 1 M NaOH was added to adjust the pH to 7.5. A 1 M stock solution of sodium phosphate buffer (pH 7.5) was added to give a final concentration of 100 mM.

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