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LuxS: its role in central metabolism and the *in vitro* synthesis of 4-hydroxy-5-methyl-3(*2H*)-furanone

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Many bacteria produce extracellular molecules which function in cell-to-cell communication. One of these molecules, autoinducer 2 (AI-2), was first described as an extracellular signal produced by *Vibrio harveyi* to control luciferase expression. Subsequently, a number of bacteria have been shown to possess AI-2 activity in their culture supernatants, and bear the *luxS* gene product, which is required for AI-2 synthesis. In *Porphyromonas gingivalis*, *luxS* and *pfs*, encoding a 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTA/SAH'ase), form an operon, suggesting that *S*-adenosylhomocysteine (SAH) or 5′-methylthioadenosine (MTA) serves as a substrate for AI-2 production. Cell-free extracts of *Escherichia coli* MG1655, but not DH5α (which carries a *luxS* frame-shift mutation) were capable of generating AI-2 activity upon addition of SAH, but not MTA. *S*-Ribosylhomocysteine (RH) derived from SAH also served as a substrate in *E. coli* MG1655 extracts. RH-supplemented cell-free extracts of *Pseudomonas aeruginosa*, a bacterium that lacks *luxS*, only generated AI-2 activity following the introduction of a plasmid containing the *Por. gingivalis pfs-luxS* operon. In addition, defined *in vitro* systems consisting of the purified LuxS proteins from *Por. gingivalis*, *E. coli*, *Neisseria meningitidis* or *Staphylococcus aureus* converted RH to homocysteine and a compound that exhibits AI-2 activity. 4-Hydroxy-5-methyl-3(*2H*)-furanone was identified by mass spectrometry analysis as a major product formed in this *in vitro* reaction. In *E. coli* MG1655, expression of T3SH [the bacteriophage T3 *S*-adenosylmethionine (SAM) hydrolase] significantly reduced AI-2 activity in culture supernatants, suggesting that AI-2 production is limited by the amount of SAH produced in SAM-dependent transmethylase reactions. The authors suggest that the LuxS protein has an important metabolic function in the recycling of SAH. They also show that *Ps. aeruginosa* is capable of removing AI-2 activity, implying that this molecule may act as a nutrient. In many bacteria AI-2 may in fact represent not a signal molecule but a metabolite which is released early and metabolized in the later stages of growth.

Keywords: AI-2, *S*-adenosylhomocysteine, *S*-ribosylhomocysteine, *S*-ribosylhomocysteine-cleavage enzyme, quorum sensing

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Abbreviations: AI-2, autoinducer-2; DMHF, 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone; MTA, 5′-methylthioadenosine; MTA/SAH'ase, 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase; MHF, 4-hydroxy-5-methyl-3(*2H*)-furanone; RH, *S*-ribosylhomocysteine; RLSS, rat liver *S*-adenosylmethionine synthetase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; T3SH, bacteriophage T3 SAM hydrolase.

INTRODUCTION

Many bacteria have evolved signalling systems that allow gene expression to be coordinated in a multicellular fashion. Production of signal molecules, also called 'autoinducers', enables individual cells to sense when the minimal number of bacteria has been achieved for a concerted response to be initiated ('quorum sensing'). Quorum-sensing-controlled multicellular behaviour includes a variety of physiological and morphological processes, e.g. bioluminescence, antibiotic biosynthesis, biofilm differentiation, plasmid conjugal transfer, competence for DNA uptake, sporulation, and the production of virulence determinants in animal, fish and plant pathogens (for reviews see Bassler, 1999; Greenberg, 1999; Williams *et al*., 2000; Withers *et al*. 2001).

Several chemically distinct families of autoinducers have been identified. *N*-Acyl-L-homoserine lactones (AHLs) are exclusively produced by Gram-negative bacteria, usually employing enzymes belonging to the LuxI protein family. Acylated acyl carrier protein (acyl-ACP) or acyl-CoA provides the acyl chain of AHLs while *S*-adenosylmethionine (SAM) provides the homoserine lactone moiety (Moré *et al*., 1996; Jiang *et al*., 1998). AHL molecules accumulate during the growth of a bacterial population and activate transcriptional regulators of the LuxR family once a critical concentration has been reached. Besides their role as signal molecules, some AHLs may also act as virulence factors (Telford *et al*., 1998; Gardiner *et al*., 2001).

In contrast, many Gram-positive bacteria employ post-translationally modified peptides as signal molecules. These peptides, which are created from larger precursors, are usually secreted via ATP-binding cassette (ABC) transporters. Some of these peptides interact with membrane-bound sensor kinases that transduce a signal across the membrane; others are transported into the cell by oligopeptide permeases, where they then interact with intracellular receptors (for reviews see Kleerebezem *et al*., 1997; Lazazzera & Grossman, 1998; Novick, 1999; Novick & Muir, 1999).

Recently, a new family of autoinducer synthases has been described which is present in both Gram-negative and Gram-positive bacteria (Surette *et al*., 1999). In *Vibrio harveyi*, the LuxS protein is required for the production of a signal molecule of unknown structure, autoinducer 2 (AI-2), which exerts its activity via a complex phospho-relay system. AI-2 activity has been discovered in spent culture supernatants of many organisms, including *Escherichia coli*, *Helicobacter pylori*, *Neisseria meningitidis*, *Porphyromonas gingivalis*, *Streptococcus pyogenes*, *Shigella flexneri* and *Salmonella typhimurium* (Bassler *et al*., 1997; Surette & Bassler, 1998; Sperandio *et al*., 1999; Forsyth & Cover, 2000; Joyce *et al*., 2000; Burgess *et al*., 2002; Day & Maurelli, 2001; Lyon *et al*., 2001; Winzer *et al*., 2002). Furthermore, highly conserved LuxS homologues have been identified in a large number of pathogenic and non-pathogenic bacteria by database analysis (Surette *et al*., 1999; Bassler, 1999). However, little is known about the regulation of AI-2 production or turnover. Some bacteria, e.g. *Sal. typhimurium* and *E. coli*, produce extracellular AI-2 during early exponential growth, but later apparently degrade the autoinducer in the absence of glucose (Surette & Bassler, 1998, 1999). In the presence of glucose, AI-2 activity remains in the culture supernatant, suggesting that degradation is subject to catabolite repression. AI-2 levels have also been shown to be affected by environmental parameters like osmolarity and pH (Surette & Bassler, 1999). In chemostat experiments, pulses of glucose, iron or NaCl induced the up-regulation of AI-2 production by *E. coli* K-12, whereas others, e.g. heat shock, ethanol or H_2O_2, decreased AI-2 activity (DeLisa *et al*., 2001a). These data suggest that AI-2 production and degradation are tightly linked with the physiological and metabolic state of the cells.

The discoveries outlined above led to the suggestion that AI-2 may be employed for interspecies communication (Bassler, 1999). However, apart from bioluminescence in *V. harveyi*, no obvious phenotype has been associated with the extracellular accumulation of this molecule in other bacteria, despite the fact that *luxS* mutants have been constructed in *E. coli*, *H. pylori*, *N. meningitidis*, *Por. gingivalis*, *Sal. typhimurium*, *Sh. flexneri* and *Strep. pyogenes* (Sperandio *et al*., 1999; Forsyth & Cover, 2000; Joyce *et al*., 2000; Burgess *et al*., 2002; Day & Maurelli, 2001; Lyon *et al*., 2001; Winzer *et al*., 2002). Although a DNA-microarray-based approach suggested that several hundred genes were up- or down-regulated in an *E. coli* O157:H7 *luxS* mutant (Spernandio *et al*., 2001), these changes have not yet been linked to a lack of AI-2 activity, and may in fact be explained by additional functions of the *luxS* gene product. Similarly, the significant transcriptional changes observed with an *E. coli luxS* mutant after the addition of conditioned medium from wild-type cultures (DeLisa *et al*., 2001b) may result from molecules different from AI-2. So far, a signal-transduction pathway responding to AI-2 has only been identified in *V. harveyi*, and genome analysis has not revealed homologous pathways in unrelated species. This raises the question whether AI-2 is indeed a general signal molecule used by many bacteria. If this is the case, then phenotypes abolished in *luxS* mutants should be restored by exogenous AI-2. Therefore, we sought to investigate the biosynthesis and chemical nature of AI-2.

In this paper, we describe the production of AI-2 *in vitro* from *S*-ribosylhomocysteine and present evidence to suggest that AI-2 is similar in structure to 4-hydroxy-5-methyl-3(2*H*)-furanone. The LuxS protein appears to be identical to the *S*-ribosylhomocysteine cleavage enzyme described by J. A. Duerre & C. H. Miller more then 30 years ago (Duerre & Miller, 1966; Miller & Duerre, 1968). Whilst the current manuscript was in preparation, Schauder *et al*. (2001) also identified the pathway of AI-2 synthesis, and suggested that this pathway is employed by a variety of bacteria to produce AI-2 for intra- and interspecies communication. However, the work pre-

sented in this study suggests that the AI-2 molecule may not act as a signal molecule for the majority of bacteria, but is the by-product of an important metabolic pathway, which is secreted during exponential growth, and degraded at a later stage.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for cloning experiments. *E. coli* and *Pseudomonas aeruginosa* were grown at 37 °C in Luria–Bertani (LB) medium or on LB agar plates. *V. harveyi* BB170 was grown at 30 °C in LB medium or AB medium (Greenberg *et al.*, 1979). Where required, kanamycin (50 μg ml^{-1}), ampicillin (75 μg ml^{-1}) or carbenicillin 200 (μg ml^{-1}) was added for *V. harveyi*, *E. coli* and *Ps. aeruginosa*, respectively.

DNA manipulation. DNA was manipulated by standard methods (Sambrook *et al.*, 1989). Restriction enzymes (Promega) were used according to the manufacturer's instructions. For isolation of plasmid DNA from *E. coli* the Qiagen Mini and Midi kits (Qiagen) were used.

Transformation. Standard methods were used for the preparation of competent cells, and for the electroporation of plasmids into *E. coli* (Sambrook *et al.*, 1989) and *Ps. aeruginosa* (Smith & Iglewski, 1989).

DNA sequencing and sequence analysis. Automated non-radioactive sequencing reactions were carried out using the BigDye terminator cycle sequencing kit in conjunction with a 373A automated sequencer (Perkin Elmer Applied Biosystems). Sequence analysis and database searches were performed with the Genetics Computer Group software packages and the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/). Metabolic pathways were compared using the 'Kyoto Encyclopedia of Genes and Genomes' (http://star.scl.kyoto-u.ac.jp/kegg/). For sequence comparisons the programs Gap (complete protein sequences) or BESTFIT (for truncated protein sequences) were used.

Preparation of crude extracts and purification of 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTA/SAH'ase) and LuxS proteins. For the small-scale preparation of cell-free extracts of *E. coli* and *Ps. aeruginosa*, cells were washed once with 50 mM Tris/HCl buffer, pH 7·8. Cells were suspended in the same buffer containing 100 μg lysozyme ml^{-1} and incubated at room temperature for 15 min, prior to cell disruption by sonication. Disrupted cells were then centrifuged (10000 ***g***) at 4 °C for 30 min to remove cell debris. The clear supernatant is referred to as cell-free extract. LuxS of *Por. gingivalis* was provided as an N-terminal MalE-fusion protein (MalE-$LuxS_{Pg}$) (Burgess *et al.*, 2002). All other proteins contained N-terminal His-tag fusions. MTA/SAH'ase from *E. coli* (Cornell & Riscoe, 1998) was overproduced using pProEX HT (Life Technologies) as an expression vector (K. A. Cornell, unpublished). The *luxS* genes of *E. coli* MG1655 [amplified with primers LuxSECF (5′-TTTACCATGGCAATGCCGTTGTTAGATAGC-3′) and LuxSECR (5′-TAGTCTCGAGACTAGATGTGCAGTTCCTGC-3′)] and *N. meningitidis* MC58 [amplified with primers LuxSNMF (5′-TTTACCATGGCAATGCCCCTACTAGACAGTTTC-3′) and LuxSNMR (5′-TTTTCTCGAGTTAGGCGTTCAGCAGCCCTTC-3′)] were cloned into the *Nco*I/*Xho*I sites of pProEX HT (Life Technologies) to create pProEx-$luxS_{Ec}$ and pProEx-$luxS_{Nm}$, respectively. MTA/SAH'ase, MalE-$LuxS_{Pg}$, $LuxS_{Ec}$ and $LuxS_{Nm}$ were overproduced in *E. coli* DH5α, to avoid contamination with the native *E. coli* LuxS protein. The *Staphylococcus aureus* RN6390B *luxS* gene [amplified with primers LuxSSAF (5′-AATGCTCGAGATGAATGTTGAAAGTTTTAATTTAG-3′) and LuxSSAR (5′-TTAACTCGAGTGACTAAGATTTATTTTCCTGTACCG-3′)] was cloned into the *Xho*I site of pET19b (Novagen) to create pET19-$luxS_{Sa}$ and overexpressed in *E. coli* BL21 DE3. For large-scale purification, cells were harvested from 100 ml

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype	Reference
Strains		
Ps. aeruginosa PAO1	Wild-type strain	Holloway collection
E. coli DH5α	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac–proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Yanisch-Perron *et al.* (1985)
E. coli MG1655	Prototrophic *E. coli* K-12, F^- λ^- *rph*	ATCC 47076
V. harveyi BB170	AI-1 sensor negative/AI-2 sensor positive	Bassler *et al.* (1997)
Plasmids		
pUCP18	As pUC18, but additional 1·8 kb stabilizing fragment for maintenance	Schweizer (1991)
pUCP18-*pfs*-$luxS_{Pg}$	pUCP18 containing the *Por. gingivalis pfs-luxS* operon	This study
pProEX HT	Prokaryotic protein expression vector	Life Technologies
pProEX HTmtan	pProEX HT containing the *pfs* gene of *E. coli*	K. A. Cornell, unpublished
pProEx-$luxS_{Ec}$	pProEX HT containing the *luxS* gene of *E. coli* MG1655	This study
pProEx-$luxS_{Nm}$	pProEX HT containing the *luxS* gene of *N. meningitidis* MC58	This study
pET19b	Prokaryotic protein expression vector	Novagen
pET19b-$luxS_{Sa}$	pET19b containing the *luxS* gene of *Staph. aureus* RN6390B	This study
pNB3	A 545 bp fragment containing *luxS* of *Por. gingivalis* ligated into pMal-c2 (New England Biolabs)	Burgess *et al.* (2002)
pBAD24	Contains arabinose-inducible *pBAD* promoter	Guzman *et al.* (1995)
pBAD-RLSS	RLSS cDNA in pBAD24	Posnick & Samson (1999)
pBAD-T3SAH	T3SH gene in pBAD24	Posnick & Samson (1999)

cultures grown in LB medium and lysed using BugBuster protein extraction reagent (Novagen) according to the manufacturer's instructions. The soluble His-tagged proteins were purified using the NTA-purification kit from Novagen.

Production of AI-2 *in vitro*. If not stated otherwise, 2 mM *S*-adenosylhomocysteine (SAH, purchased from Sigma) in 50 mM Tris/HCl buffer, pH 7·8, was converted enzymically to *S*-ribosylhomocysteine (RH) through incubation with MTA/SAH'ase (100 μg ml^{-1}) at 37 °C for 1 h. This reaction is nearly quantitative and irreversible (Duerre, 1962), and resulted in conversion rates of greater then 98 %. The MTA/SAH'ase protein was removed by filtration through Centricon YM-10 or Microcon YM-10 units (Millipore). Subsequently, purified LuxS (500 μg ml^{-1}) was added, and the reaction mixture incubated for a further 1–4 h. SAH solutions were bubbled with helium before the addition of enzyme, and the reaction mixtures were incubated in an anaerobic cabinet to prevent oxidation of RH and the reaction products. AI-2 was also synthesized by incubating SAH or RH with cell-free extracts (2·5 mg protein ml^{-1}) prepared from *E. coli* and *Ps. aeruginosa* cells. For the partial purification of AI-2 activity, reaction mixtures were dried by rotary evaporation or, alternatively, freeze-dried. AI-2 activity was extracted from the solids of a 5 ml reaction mixture using 0·5 ml methanol.

Analysis of AI-2 production and degradation. AI-2 production was analysed essentially as described by Bassler *et al*. (1997), using 20 μl AI-2 extract and 180 μl 1:5000 diluted overnight cultured *V. harveyi* BB170 in AB medium. Changes in bioluminescence upon addition of AI-2 were determined at 30 °C every 30 min using a combined, automated luminometer-spectrometer (the Anthos Labtech Lucy1). For quantitative analysis, serial dilutions were performed to determine the AI-2 concentrations which gave half-maximal induction of light production. AI-2 activity was defined as the fold increase in light production in comparison with medium or buffer controls. For a single experiment, the *V. harveyi* bioassay was performed at least in duplicate for each sample. Experiments were repeated at least three times.

Solutions containing AI-2 synthesized *in vitro* and *Ps. aeruginosa* culture supernatants were extracted twice with chloroform prior to the analysis of AI-2, which remained in the water phase. This step was introduced to terminate *in vitro* reactions and to remove toxic compounds (e.g. pyocyanin from *Ps. aeruginosa* assays) which may otherwise interfere with the assay. For AI-2 degradation experiments, 30 μl *in vitro*-synthesized AI-2 was mixed with 90 μl washed cells (adjusted to OD_{600} 2·0) or freshly inoculated cultures and incubated at 37 °C for 1–4 h. The cell suspensions were then extracted twice with chloroform, and the aqueous phase analysed for AI-2 activity. Furanones tested for AI-2 activity [2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), dihydro-2(3*H*)-furanone, dihydro-5-methyl-2(3*H*)-furanone, 5-methyl-2(3*H*)-furanone, 2,4(3*H*, 5*H*)-furandione, α-methyl-γ-butyrolactone, (*S*)-β-hydroxy-γ-butyrolactone, 2(5*H*)-furanone, (*R*)-(−)-dihydro-5-(hydroxymethyl)-2(3*H*)-furanone and (*S*)-(−)-dihydro-5-(hydroxymethyl)-2(3*H*)-furanone] were purchased from Fluka, Sigma or Acros Organics. 4-Hydroxy-5-methyl-3(2*H*)-furanone (MHF) was obtained as a free sample from Givaudan.

Analysis of *in vitro* reactions. Conversion of SAH to RH, and its degradation during the LuxS reaction were monitored by thin-layer chromatography (TLC) as described by Duerre & Miller (1966). TLC plates were developed in 80 % (v/v) 1-propanol or ethanol/water/acetic acid (65:34:1, by vol.). Ninhydrin (0·2 %, w/v) in acetone was used for the detection of homocysteine residues. Homocysteine levels in solutions were quantified using Ellman's reagent (Sigma) according to the method described in the thiol and sulfide quantitation kit from Molecular Probes. Fifty microlitres of the reaction mixture was combined with 350 μl 5 mM sodium acetate buffer, 50 mM NaCl, 0·5 mM EDTA, pH 4·7, and then added to 690 μl 40 mM sodium phosphate buffer, 2 mM EDTA, pH 7·6. Ten microlitres of Ellman's reagent [5,5′-dithio-bis-(nitrobenzoic acid); 40 mg ml^{-1} in DMSO] was added and the A_{412} of the mixture was measured, using the molar absorption coefficient for 2-nitro-5-thiobenzoate (13 600 M^{-1} cm^{-1}), which is generated from Ellman's reagent following reaction with the free thiol group of homocysteine. Alternatively, homocysteine was modified using *N*-ethylmaleimide and then incubated with 3 M NaOH to yield a pink product, which can be quantified at 520 nm as described by Duerre & Miller (1966). The maleimide derivative of homocysteine was also analysed by HPLC in comparison with a commercial homocysteine standard (Sigma). A Genosys AQ 4μ column (Jones Chromatography) in conjunction with the Photodiode array system 996 (Waters) was employed to separate the compounds present in the reaction mixtures using a gradient of acetonitrile in water. Data were collected and analysed using the Millennium 3.2 software (Waters).

Electrospray MS analysis was used to identify SAH and RH in reaction mixtures. Proteins were removed by filtration through Centricon YM-10 or Microcon YM-10 units (Millipore), and the reaction mixes were diluted 1:1 with methanol and analysed by direct injection. Methanol extracts containing *in vitro*-synthesized AI-2 were analysed by GC-MS. The GC-MS was operated in full-scan mode and spectra were collected from m/z 40 to 200.

RESULTS

SAH is a precursor in AI-2 synthesis

Whilst analysing the function of LuxS in *Por. gingivalis*, we noticed that in this organism *luxS* is located directly downstream of a putative *pfs* homologue, encoding MTA/SAH'ase (Burgess *et al*., 2002). RT-PCR analysis revealed that *pfs* and *luxS* form an operon, a finding also recently reported by Chung *et al*. (2001). Whilst the *luxS* gene could be inactivated, this was not possible for *pfs*, suggesting an essential function (Burgess *et al*., 2002). Since it was conceivable that *pfs* is required for AI-2-dependent signalling, we were interested to determine whether a similar gene arrangement is present in other bacteria. Analysis of the finished and unfinished genomes of more then 40 bacterial species revealed that in most species, *luxS* is not linked to any specific gene class. However, in two species *luxS* is situated adjacent to genes involved in homocysteine and methionine metabolism. In *Borrelia burgdorferi*, *pfs* and *luxS* are located downstream of *metK*. Furthermore, in *Clostridium difficile*, the *metH* gene is separated from *luxS* by only one open reading frame. *metH* and *metK* encode N^5-methyltetrahydrofolate-homocysteine methyltransferase and SAM synthetase, respectively. Fig. 1 shows that these enzymes, together with MTA/SAH'ase, are part of a metabolic cycle, the function of which is the production of the methyl-group donor SAM and the conversion of SAH, the product of SAM-dependent transmethylase reactions, back to homo-

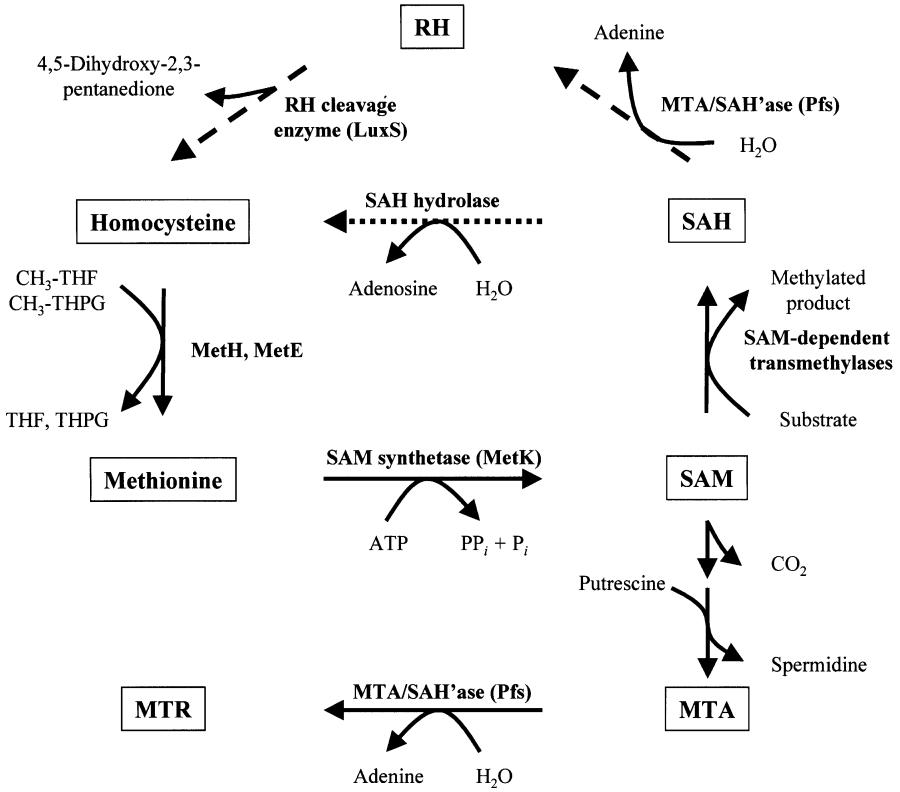

Fig. 1. *S*-Adenosylmethionine (SAM) metabolism in bacteria. Methionine is converted to SAM in a reaction catalysed by SAM synthetase (methionine adenosyltransferase). In SAM-dependent methyltansferase reactions SAM is converted to *S*-adenosylhomocysteine (SAH). In some bacteria, e.g. *Ps. aeruginosa*, SAH hydrolase catalyses the hydrolysis of SAH to homocysteine and adenosine (dotted arrow). Other bacteria, e.g. *E. coli*, produce homocysteine in two steps, catalysed by methylthioadenosine/SAH nucleosidase (MTA/SAH'ase) and *S*-ribosylhomocysteine (RH) cleavage enzyme (dashed arrows). Methionine is then recycled from homocysteine. Homocysteine is also formed from oxaloacetate in seven steps as part of the *de novo* methionine-biosynthesis pathway (not shown). In *E. coli*, two enzymes catalyse the transfer of the methyl group of N^5-methyltetrahydropteroryl glutamate (CH_3-THPG) to the sulphur of homocysteine to form methionine. One of these, the cobalamin-dependent methionine synthase (MetH) can also use N^5-methyltetrahydrofolate (CH_3-THF) as a methyl donor, whereas the cobalamin-independent methionine synthase (MetE) is restricted to CH_3-THPG. SAM is also involved in the production of polyamines, thereby yielding MTA. MTA is converted to methylthioribose (MTR) and adenine by MTA/SAH'ase, the same enzyme that hydrolyses SAH to RH and adenine. The further metabolism of MTR is not known in *E. coli*, but in *Klebsiella pneumoniae* MTR is converted back to methionine. Arrows in the figure indicate the direction of metabolic conversions *in vivo*. For a review of SAM metabolism see Greene (1996).

cysteine and methionine. However, SAM is also a substrate in polyamine biosynthesis, where it is converted in two steps to methylthioadenosine (MTA). Interestingly, both SAH and MTA are substrates of the MTA/SAH'ase, suggesting that either may serve as a precursor in AI-2 synthesis.

To test this hypothesis, MTA and SAH were added to cell-free extracts of *E. coli* MG1655, *E. coli* DH5α (which contains a *luxS* frame-shift mutation; Surette *et al.*, 1999) and *E. coli* DH5α harbouring the *Por. gingivalis pfs-luxS* operon [*E. coli* DH5α(pUCP18*pfs-luxS*$_{Pg}$)]. AI-2 production was monitored using the *V. harveyi* bioassay. AI-2 production was only observed for *E. coli* MG1655 and *E. coli* DH5α(pUCP18*pfs-luxS*$_{Pg}$) in the presence of SAH, but not MTA (Table 2). This indicated that SAH is a precursor of AI-2 synthesis, although it may not be the direct substrate of LuxS. Since SAH is converted to *S*-ribosylhomocysteine (RH) and adenine in an irreversible reaction catalysed by MTA/SAH'ase in *E. coli* (Fig. 1), these substrates were analysed as well. Using the purified MTA/SAH'ase from *E. coli*, SAH was almost quantitatively converted to RH and adenine (see Methods) prior to the addition of cell-free extracts and a very similar profile of AI-2 production to that seen with SAH was obtained (Table 2). As addition of adenine to crude extracts did not result in AI-2 production, RH is likely to be the precursor of AI-2 synthesis.

To determine whether only LuxS and MTA/SAH'ase are required to generate AI-2 from SAH, pUCP18*pfs-luxS*$_{Pg}$ was introduced into *Ps. aeruginosa*, a bacterium that does not contain *pfs* or *luxS* homologues. However, culture supernatants of *Ps. aeruginosa* PAO(pUCP18*pfs-luxS*$_{Pg}$) contained no AI-2 activity and no activity was detected after adding SAH to cell-free extracts (Table 2). As *Ps. aeruginosa* contains an SAH hydrolase (Fig. 1), SAH levels may have been too low to sustain production of detectable levels of AI-2. Therefore, enzymically

Table 2. AI-2 production by cell-free extracts of *E. coli* and *Ps. aeruginosa*

AI-2 activities for a single experiment are shown, although the experiment was repeated three times with similar results. Replicate assays agreed within 20%. AI-2 activity is compared with background activities (crude extracts without substrate).

Cell-free extract	Substrate (2 mM)	AI-2 activity (fold induction)
E. coli		
MG1655	SAH	9500
MG1655	RH	13300
MG1655	MTA	1
MG1655	Adenine	3
DH5α	SAH	1
DH5α	RH	2
DH5α(pUCP18*pfs-luxS*$_{Pg}$)	SAH	10400
DH5α(pUCP18*pfs-luxS*$_{Pg}$)	RH	11100
DH5α(pUCP18*pfs-luxS*$_{Pg}$)	MTA	3
DH5α(pUCP18*pfs-luxS*$_{Pg}$)	Adenine	2
Ps. aeruginosa		
PAO(pUCP18)	SAH	2
PAO(pUCP18)	RH	1
PAO(pUCP18*pfs-luxS*$_{Pg}$)	SAH	9
PAO(pUCP18*pfs-luxS*$_{Pg}$)	RH	340

prepared RH was added (after the removal of MTA/SAH'ase) to cell-free extracts of *Ps. aeruginosa* carrying either pUCP18*pfs-luxS*$_{Pg}$ or pUCP18. Significant AI-2 activity was detected in cell-free extracts containing the *Por. gingivalis* LuxS protein (Table 2), suggesting that RH is the immediate precursor of the LuxS-catalysed reaction.

LuxS converts RH to AI-2 and homocysteine

The results obtained with the cell-free extracts suggested that LuxS may be identical to the RH cleavage enzyme described by Miller & Duerre (1968). Therefore, a defined *in vitro* assay was developed, using the purified MTA/SAH'ase from *E. coli*, and the purified LuxS proteins of *Por. gingivalis* (MalE-LuxS$_{Pg}$, a chimeric protein with MalE N-terminally fused to LuxS$_{Pg}$), *N. meningitidis* MC58 (LuxS$_{Nm}$), *Staph. aureus* RN6390B (LuxS$_{Sa}$) or *E. coli* MG1655 (LuxS$_{Ec}$). Each enzyme, with the exception of LuxS$_{Sa}$, was purified from *E. coli* DH5α to avoid contamination with LuxS$_{Ec}$. SAH was first converted to RH by the addition of MTA/SAH'ase. Both TLC and MS analysis indicated that the conversion to RH was greater then 98% (data not shown). The MTA/SAH'ase protein was then removed by filtration, and the resulting RH solution incubated with the LuxS proteins. In control experiments, RH was replaced by adenine, ribose and MTA. Production of AI-2 activity was only observed in the presence of RH and a LuxS protein (Table 3), indicating that these are sufficient to produce AI-2 activity.

According to Duerre & Miller (1966) and Miller & Duerre (1968), homocysteine is one of the products formed from RH in a reaction catalysed by the RH-cleavage enzyme. Indeed, a compound reacting with the SH-group-specific Ellman's reagent was formed in all assays in the presence of LuxS. This compound was tentatively identified as homocysteine by comparing HPLC elution profiles of the reaction mixtures and a commercial standard following *N*-ethylmaleimide modification (data not shown). TLC analysis and electrospray MS analysis (negative mode) indicated that a large quantity of RH was still present in the reaction mixtures, even after extending the incubation time from 2 to 4 h (data not shown). Quantification of SH groups

Table 3. *In vitro* production of AI-2

AI-2 activities for a single experiment are shown, although the experiment was repeated three times with similar results. Replicate assays agreed within 22%. AI-2 activity is with background activities (enzyme without substrate).

Enzyme	Substrate (2 mM)	AI-2 activity (fold induction)
MalE-LuxS$_{Pg}$	SAH	4
MalE-LuxS$_{Pg}$	RH	37300
MalE-LuxS$_{Pg}$	MTA	2
MalE-LuxS$_{Pg}$	Adenine	3
MalE-LuxS$_{Pg}$	Ribose	2
LuxS$_{Nm}$	SAH	4
LuxS$_{Nm}$	RH	45400
LuxS$_{Ec}$	SAH	3
LuxS$_{Ec}$	RH	11800
LuxS$_{Sa}$	SAH	4
LuxS$_{Sa}$	RH	15600

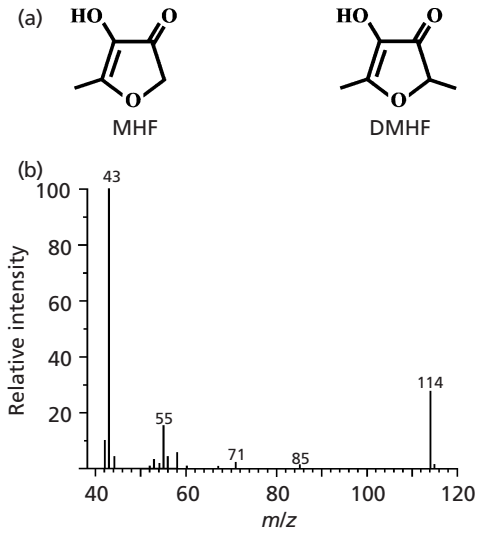

Fig. 2. (a) Chemical structures of 4-hydroxy-5-methyl-3(*2H*)-furanone (MHF) and 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone (DMHF); (b) mass spectrum of *in vitro*-synthesized MHF. The mass spectrum for MHF shows the parent peak at *m/z* 114 and characteristic peaks at *m/z* 85, 71, 55 and 43.

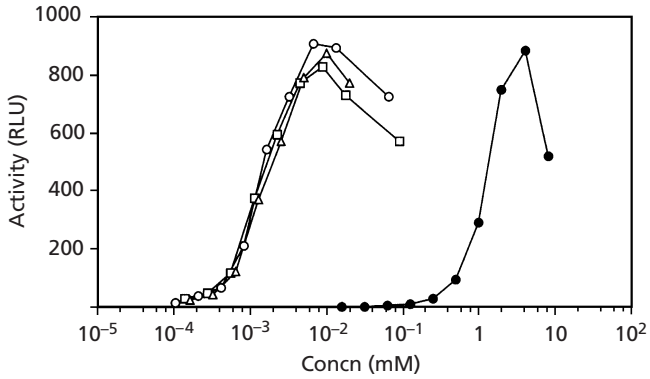

Fig. 3. Activities of *in vitro*-synthesized AI-2 and synthetic MHF. AI-2 was synthesized *in vitro* using 10 mM sodium phosphate buffer, pH 7·5, containing 0·5 mg $LuxS_{Ec}$ ml^{-1} and 2 mM RH. The reaction mixture was incubated at 37 °C and stopped after 15 min (○), 30 min (□) and 60 min (△). Serial dilutions of these reactions and synthetic MHF (●) were analysed for activity in the *V. harveyi* bioassay. Activity is expressed in relative light units (RLU). The concentration of *in vitro*-synthesized AI-2 was estimated from the homocysteine levels determined after 15, 30 and 60 min incubation by assuming a 1:1 ratio. The data shown in this figure represent the mean values of four bioassays; the experiment was repeated three times with similar results.

using Ellman's reagent suggested that between 62 % and 68 % of the RH had been converted by MalE-$LuxS_{Pg}$ and $LuxS_{Nm}$ after 2 h incubation (data not shown).

4-Hydroxy-5-methyl-3(*2H*)-furanone is a product of the LuxS *in vitro* reaction

Apart from homocysteine, 4,5-dihydroxy-2,3-pentanedione has been described as an immediate product of the reaction catalysed by the RH cleavage enzyme (Duerre *et al*., 1971). However, this compound is very unstable and is probably the precursor of a carbohydrate-like compound described in an earlier publication (Miller & Duerre, 1968), which derives from the ribose unit of RH. We therefore sought to determine the identity of the carbohydrate-like compound, which may represent AI-2. Preliminary experiments indicated that AI-2 activity from freeze-dried culture supernatants could be extracted by methanol (not shown). Therefore, methanol extracts were prepared from 5 ml reactions after 2 h incubation, containing 2 mM RH and either $LuxS_{Nm}$, $LuxS_{Sa}$ or MalE-$LuxS_{Pg,}$ at a concentration of 0·5 mg ml^{-1}. The extracts had a fruity-caramel-like odour and contained a substantial amount of AI-2 activity. These were analysed by GC/MS in comparison with extracts from control reactions lacking either the enzymes or the substrate. The GC profiles of reactions and controls revealed only one major difference. This corresponds to a compound with a GC retention time of 15·5 min (eluting from the column just before the C_{11} alkane standard, undecane), and was identified as 4-hydroxy-5-methyl-3(2*H*)-furanone (MHF) by mass spectrometry (Fig. 2), in comparison with the fragmentation pattern for MHF described in the literature (Farine *et al*., 1993). The same GC retention time and fragmentation pattern was also observed for a synthetic MHF standard (data not shown). The identification of MHF was not unexpected since this compound was known to be one of products formed in aqueous solutions by the reactive 4,5-dihydroxy-2,3-pentanedione (Nedvidek *et al*., 1992; Blank & Fay, 1996).

To establish whether synthetic MHF and *in vitro*-synthesized AI-2 exhibit identical activities in the *V. harveyi* bioassay, serial dilutions of MHF and AI-2 were analysed (Fig. 3). The concentration of *in vitro*-synthesized AI-2 was estimated by assuming a 1:1 ratio between homocysteine and AI-2 formation. Synthetic MHF fully activated the bioassay at a concentration of 4 mM. Interestingly, half-maximal activation for synthetic MHF was observed at a concentration of approximately 1·1 mM, whereas *in vitro*-synthesized AI-2 showed a comparable activation at a 1000-fold lower concentration. The results presented in Fig. 3 were obtained using the $LuxS_{Ec}$ protein, but half-maximal activation at concentrations between 1 and 3 μM were also observed with AI-2 synthesized using $LuxS_{Sa}$ and $LuxS_{Nm}$. Thus, it appears that MHF, the major product formed in the reaction catalysed by LuxS, is different from AI-2. Incubation of MHF under the conditions described for the *in vitro* reaction did not increase its activity in the bioassay (data not shown), suggesting that AI-2 is not a degradation product of MHF.

In addition to MHF, various commercially obtainable furanones were tested for AI-2 activity: 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), dihydro-2(3*H*)-furanone, dihydro-5-methyl-2(3*H*)-furanone, 5-methyl-2(3*H*)-furanone, 2,4(3*H*,5*H*)-furandione, α-methyl-γ-

butyrolactone, (*S*)-*β*-hydroxy-*γ*-butyrolactone, 2(5*H*)-furanone, (*R*)-(−)-dihydro-5-(hydroxymethyl)-2(3*H*)-furanone and (*S*)-(−)-dihydro-5-(hydroxymethyl)-2(3*H*)-furanone. Apart from MHF, only DMHF (which is structurally very similar to MHF; Fig. 2a) exhibited activity in the *V. harveyi* bioassay, although activation was maximal (90-fold) only at high concentrations (approx. 2 mM). Therefore, our results suggest that AI-2 has a 3(2*H*)-furanone structure and is very similar to MHF.

In vivo manipulation of SAM levels influences AI-2 production

If LuxS acts primarily as a metabolic enzyme involved in the recycling of SAH, the level of AI-2 in culture supernatants depends on the net flux of metabolites through the methionine–SAM–SAH–homocysteine cycle depicted in Fig. 1. The amount of SAH produced, and thus the amount of RH, will correlate with the number of SAM-dependent methylations carried out by the cells. Thus, it can be predicted that lowering the cellular concentration of SAM will lead to a decrease in SAH and hence AI-2 levels. To test this hypothesis, culture supernatants of *E. coli* MG1655 carrying pBAD24, pBAD-T3SH (encoding bacteriophage T3 SAM hydrolase) or pBAD-RLSS (encoding rat liver SAM synthetase, a highly conserved homologue of bacterial SAM synthetases) were analysed for AI-2 production (plasmids gratefully received from L. D. Samson, Massachusetts Institute of Technology). In *E. coli*, the expression of T3 SAM hydrolase (T3SH) and rat liver SAM synthetase (RLSS) genes using these plasmids has been shown to decrease and increase the SAM pool size, respectively, without having marked effects on cell growth (Posnick & Samson, 1999). Culture supernatants were taken from cultures growing in LB medium in the absence of glucose after 4 h, and from overnight cultures (14 h) grown in the presence of 10 mM glucose. These conditions were chosen as glucose represses the *pBAD* promoter and has been suggested to prevent the loss of AI-2 activity during late-exponential phase (Surette & Bassler, 1998). To induce the *pBAD* promoter, 10 mM L-arabinose was added. A significant reduction of AI-2 levels was observed in cultures producing the SAH hydrolase (Fig. 4). In contrast, AI-2 levels in cultures overexpressing RLSS were moderately increased in comparison with the control (pBAD24 vector only).

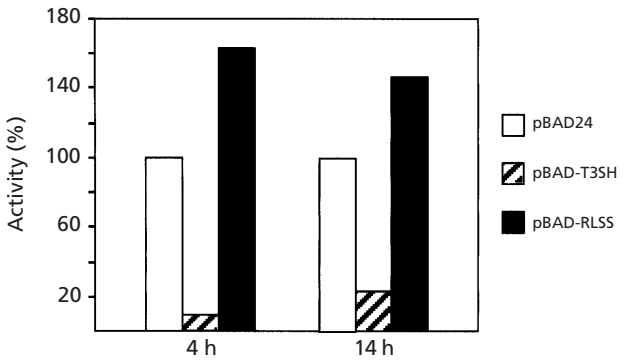

Fig. 4. *In vivo* manipulation of SAM levels influences AI-2 production: expression of T3SH, a SAM hydrolase, drastically reduces AI-2 levels in *E. coli* MG1655, whereas expression of RLSS, a SAM synthetase, increases AI-2 activity. Samples were taken after 4 h from cultures grown in the absence of glucose (OD_{600} approximately 1·2) and after 14 h (OD_{600} approximately 3·6) from cultures grown in the presence of 10 mM glucose. Expression of T3SH and RLSS was induced by the addition of 10 mM L-arabinose 1 h after inoculation. AI-2 activities observed with the control (pBAD24 vector only), were defined as 100 %. This corresponded to a 300-fold and 790-fold induction of bioluminescence in the *V. harveyi* bioassay for the 4 h and 14 h sample, respectively. AI-2 activities for a single experiment are shown, although the experiment was repeated three times with similar results. Replicate assays agreed within 18 %.

Table 4. Inactivation of AI-2 activity by *Ps. aeruginosa*

In vitro-synthesized AI-2 in 50 mM Tris/HCl buffer, pH 7·8, was incubated with LB medium or cell suspensions for 2 h. The remaining AI-2 activities for a single experiment are shown, although the experiment was repeated three times with similar results and replicate assays agreed within 20 %. AI-2 activity is compared with the background activity observed for the control (50 mM Tris/HCl buffer, pH 7·8, incubated with LB medium).

Incubation mixture	AI-2 activity (fold induction)
LB medium/AI-2	570
E. coli DH5α/AI-2	610
E. coli DH5α/Tris buffer	2
Ps. aeruginosa/AI-2	3
Heat-inactivated *Ps. aeruginosa*/AI-2	590
Ps. aeruginosa/Tris buffer	2

AI-2 is inactivated by *Ps. aeruginosa*

AI-2 and MHF may represent metabolites which are excreted early during growth, but taken up and utilized during a later stage. Bacteria not producing AI-2, such as *Ps. aeruginosa*, may still be capable of inactivating or degrading these compounds. *Ps. aeruginosa* and *E. coli* DH5α (described not to be capable of AI-2 degradation by Surette *et al.*, 1999) were grown for 13 h in LB medium, then harvested and washed with the same medium prior to incubation with *in vitro*-synthesized AI-2. Within 2 h, washed cells of *Ps. aeruginosa* were capable of completely removing AI-2 activity (Table 4). Similarly, loss of activity was observed when AI-2 was added to growing cultures of *Ps. aeruginosa* (data not shown). However, the loss of AI-2 activity was not observed following incubation with heat-inactivated *Ps. aeruginosa* cells, *E. coli* DH5α, or fresh culture medium.

DISCUSSION

In this paper we have identified the pathway of AI-2 synthesis. LuxS is the sole requirement for the production of AI-2 activity and homocysteine from RH *in vitro*. We have demonstrated that the LuxS proteins of *E. coli* MG1655, *N. meningitidis*, *Por. gingivalis* and *Staph. aureus* catalyse identical reactions. We propose that LuxS is identical to the RH-cleavage enzyme identified first in *E. coli* by Duerre & Miller (1966) and Miller & Duerre (1968), which catalyses the conversion of RH to homocysteine and a reactive compound, identified as 4,5-dihydroxy-2,3,-pentanedione (Duerre *et al*., 1971). During the preparation of this manuscript Schauder *et al*. (2001) reported similar findings using the LuxS proteins of *E. coli*, *V. harveyi*, *V. cholerae* and *Enterococcus faecalis* and suggested that this pathway is used by bacteria to produce AI-2, a compound which may have a role in interspecies signalling. However, Schauder *et al*. (2001) were unable to elucidate the chemical structure of the product formed from 4,5-dihydroxy-2,3-pentanedione which exhibits AI-2 activity. 4,5-Dihydroxy-2,3-pentanedione is not stable in aqueous solutions and is known to form furanone derivatives via cyclization and dehydration (Nedvidek *et al*., 1992; Blank & Fay, 1996; for a review see Slaughter, 1999). We have demonstrated that the furanone MHF is formed *in vitro* using RH as a substrate and one of three different LuxS homologues derived from both Gram-negative and Gram-positive bacteria. Furthermore, MHF and a structurally related furanone, DMHF, were found to possess AI-2 activity. Schauder *et al*. (2001) have also demonstrated that furanones, including MHF, DMHF and 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (EMHF), exhibit AI-2 activity in the *V. harveyi* bioassay. Taken together, these results strongly suggest that AI-2 has a 3(2*H*)-furanone structure. The results presented in this study indicate that MHF is the main product formed from 4,5-dihydroxy-2,3-pentanedione. However, it is not yet clear whether the formation of MHF occurs spontaneously or is catalysed by the LuxS protein.

These findings raised the question as to whether MHF is AI-2. Although MHF appears to be the major product formed from the ribose moiety of RH, synthetic MHF showed half-maximal activation in the *V. harveyi* bioassay only at the relatively high concentration of approximately 1 mM. In contrast, half-maximal activation was observed with only 1–3 μM of the *in vitro*-synthesized AI-2, suggesting that this compound is different from MHF. Although we could not identify any substantial concentration of other furanones, MHF may not represent the only product formed from 4,5-dihydroxy-2,3-pentanedione *in vivo* or in our *in vitro* system. Also, MHF itself is unstable under certain conditions (Whitfield & Mottram, 1999, 2001) and may have formed additional compounds. The calculation of AI-2 concentrations obtained *in vitro* is based on the amount of homocysteine produced and assumes a 1:1 ratio between homocysteine and AI-2. However, the true AI-2 concentrations may have been much lower and therefore not detected by GC-MS analysis. Incubation of synthetic MHF under the conditions used for *in vitro* synthesis of AI-2 did not increase the activity in the bioassay, suggesting that AI-2 is not a degradation product of MHF. Thus, our data suggest that AI-2 is either a minor (but highly active) side product formed from 4,5-dihydroxy-2,3-pentanedione, or a precursor in MHF formation. We are currently optimizing the *in vitro* production of larger quantities of AI-2 to establish unequivocally its chemical nature.

In contrast to our study, Schauder *et al*. (2001) reported that MHF concentrations of approximately 100 μM were required for half-maximal activation in the *V. harveyi* bioassay, whereas AI-2 was active at a 1000–10000-fold lower concentration (10–100 nM). This could be the result of a discrepancy in data interpretation. The concentrations stated by Schauder *et al*. (2001) clearly coincide with the midpoint of graphs representing bioassay activity as a function of AI-2 or MHF concentration. However, the respective activity data have been presented using a logarithmic scale. Therefore, the midpoint of these graphs does not represent half-maximal activity, but a much lower value.

Whether AI-2 is a furanone or not, direct evidence for AI-2 as a quorum-sensing signal molecule has only been obtained for *V. harveyi* (Bassler *et al*., 1994). The main function of the LuxS enzyme is clearly a metabolic one. All living organisms, with the possible exception of a few bacterial pathogens, produce SAM, a central metabolite, which functions as the major methyl donor. SAH is the co-product of SAM-dependent methyltransferase reactions (Fig. 1), which methylate DNA and RNA, as well as certain metabolites and proteins. The subsequent conversion of SAH is important, as SAH is a potent feedback inhibitor of the SAM-dependent methyltransferases (Gordon *et al*., 1987). Furthermore, it is advantageous to recycle the 'building blocks' of SAH. Eukaryotes and a number of bacteria remove SAH in a single step, catalysed by the SAH hydrolase, producing adenosine and homocysteine (Walker & Duerre, 1975; Fig. 1). Other bacteria employ MTA/SAH'ase and the RH cleavage enzyme (LuxS) for the conversion of SAH to homocysteine (Walker & Duerre, 1975; Shimizu *et al*., 1984). The importance of these reactions is demonstrated by the fact that the genomes of nearly all organisms analysed to date contain homologues for one of the two pathways. Even more remarkable is the fact that to date there is not a single example where bacteria contain both a SAH hydrolase and a LuxS protein (Fig. 5). This further supports the idea that LuxS represents the RH cleavage enzyme and fulfils primarily a metabolic function, together with MTA/SAH'ase, which is analogous to the SAH hydrolase reaction.

The metabolic flux from methionine to SAM, SAH, and finally homocysteine (Fig. 1) appears to be tightly controlled as, for instance, very low concentrations of SAM result in a cell division defect in *E. coli* (Newman *et al*., 1998). The amount of SAH produced correlates with the number of SAM-dependent methylations car-

Fig. 5. Distribution of SAH-degrading pathways in various bacteria. The figure is based on biochemical evidence (Walker & Duerre, 1975; Shimizu *et al.*, 1984) and the presence of putative homologues identified by genome analysis. Only completed genomes have been considered. For gapped BLAST alignments, the LuxS, MTA/SAH'ase and SAH hydrolase proteins of *V. harveyi*, *E. coli* and *Rhodobacter capsulatus*, respectively, have been used. Only hits with an 'expect value' (*E*) of lower than 10^{-12} have been considered. Positive results are marked with black boxes. For clarity, only the genera are given for the following species: *Acinetobacter calcoaceticus*, *Agrobacterium tumefaciens*, *Alcaligenes faecalis*, *Aquifex aeolicus*, *Arthrobacter globiformis*, *Bacillus cereus*, *Bacillus halodurans*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Campylobacter jejuni*, *Caulobacter crescentus*, *Chlamydia muridarum*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Citrobacter freundii*, *Citrobacter intermedius*, *Clostridium acetobutylicum*, *Deinococcus radiodurans*, *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus influenzae*, *Helicobacter pylori*, *Mesorhizobium loti*, *Micrococcus luteus*, *Micromonospora faeni*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Mycoplasma pulmonis*, *Neisseria meningitidis*, *Nocardia asteroides*, *Nonomuraea angiospora* (formerly *Micropolyspora angiospora*), *Pasteurella multocida*, *Porphyromonas gingivalis*, *Proteus inconstans*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Rickettsia prowazekii*, *Rhodobacter capsulatus*, *Rhodobacter*

ried out in the cell. Thus, it can be predicted that lowering the cellular concentration of SAM will lead to a decrease in SAH levels and hence AI-2 production. We found that this is indeed the case by expressing the SAM-hydrolysing T3SH enzyme in *E. coli* MG1655. Furthermore, overexpression of the SAM synthetase RLSS increased AI-2 levels moderately. It is possible that increasing SAM levels did not result in a substantial increase in SAM-dependent methylase reactions, and thus SAH, due to the conditions employed in this study.

Our findings raise the interesting question why some bacteria employ two enzymic steps to achieve what other bacteria and eukaryotes can accomplish in a single step. It has been suggested that one of the reasons for employing two steps is that it leads to the production of the signal molecule AI-2 (Schauder *et al*., 2001). However, an alternative explanation is that one of the products formed via the MTA/SAH'ase and LuxS reactions may fulfil an additional function not connected with signalling. It is, however, remarkable that the synthesis of AI-2, which is clearly used as a signal molecule by *V. harveyi*, relies on the degradation of a metabolite, whereas other signal molecules, for instance the *N*-acylhomoserine lactones, are synthesized by dedicated synthases.

In a number of publications authors have claimed to present evidence for AI-2-based quorum sensing after detecting AI-2 activity in culture supernatants or discovering phenotypic differences between wild-type strains and *luxS* mutants (Surette & Bassler, 1998; Sperandio *et al*., 1999, 2001; Forsyth & Cover, 2000; Joyce *et al*., 2000; Burgess *et al*., 2002; Day & Maurelli, 2001; Lyon *et al*., 2001). Their work had been motivated by initial studies showing that the production of AI-2 is *luxS* dependent (Surette *et al*., 1999), and because it had been suggested that AI-2 may represent an universal signal molecule, used for intra- as well as interspecies communication (Bassler, 1999). However, a second, metabolic, function of the LuxS protein had not been taken into account, and therefore few attempts have been made to restore wild-type phenotypes in *luxS* mutants through the addition of exogenous AI-2. Using a DNA-microarray-based approach, Sperandio *et al*. (2001) identified more than 400 genes in *E. coli* O157:H7 which showed significant changes in expression in a *luxS* mutant. They concluded that AI-2-based quorum sensing is a global regulatory mechanism in this organism, although only two phenotypes (growth rate and halo size on motility plates) were analysed for restoration after the addition of conditioned medium from wild-type cultures. However, with the expression pattern for so many genes changed, conditioned medium of wild-type and *luxS* mutant is very likely to differ not only with regard to AI-2 but also in many other respects, e.g. the concentration of various fermentation products and secondary metabolites. Therefore, restoration of wild-type characteristics could depend on compounds different from AI-2, but also changed in the *luxS* mutant due to the disruption of the metabolic cycle described in Fig. 1. The same is true for the *E. coli* DNA microarray studies performed by DeLisa *et al*. (2001b) and the *virB* expression studies of Day & Maurelli (2001) in *Sh. flexneri*. DeLisa *et al*. (2001b) identified significant transcriptional changes in 242 genes by comparing expression profiles of *E. coli luxS* mutants incubated for 20 min in the presence of conditioned medium from either wild-type or *luxS* mutant. Although the conditioned medium was derived from cultures grown to an OD_{600} of 3, but added to cells derived from cultures grown to an OD_{600} of 1·0, no data have been presented to demonstrate that the changes observed were specific for the *luxS* mutant and AI-2 and did not occur with wild-type cells. We therefore conclude that convincing evidence for AI-2-based quorum sensing in the organisms mentioned above has not yet been presented, and that many phenotypes linked to *luxS* may have their origin in the disruption of the metabolic cycle outlined in Fig. 1. Furthermore, with the exception of other *Vibrio* species, genome analysis reveals no evidence for a signal-transduction cascade similar to the one described for the *V. harveyi* quorum-sensing system. Thus, these results again raise the question whether AI-2 functions as a signal molecule for the majority of species or whether it is just a 'by-product' of the LuxS reaction.

Despite the evidence for the significance of SAH-degrading pathways, not all *luxS* mutants show significant phenotypic changes under laboratory conditions (Forsyth & Cover, 2000; Joyce *et al*., 2000). However, the presence of these pathways in nearly all organisms as well as the high degree of conservation within the SAH hydrolase, MTA/SAH'ase and LuxS protein families indicates the importance of these metabolic conversions in the natural environment. The inactivation of *luxS* is likely to manifest itself in various phenotypes, depending on the metabolic capabilities of the species studied and the growth conditions used. For instance, methionine-auxotrophic bacteria will be expected to differ from prototrophic species, and the artificial growth conditions used in the laboratory may or may not reveal a reduction in fitness. This view is supported by the observation that a *N. meningitidis luxS* mutant, which shows no obvious phenotype under laboratory conditions, is clearly attenuated in the infant rat model, even in mixed-inoculum experiments, where AI-2 produced by the wild-type is expected to overcome a potential quorum-sensing defect (Winzer *et al*., 2002). Thus, the attenuation observed for the *N. meningitidis luxS* mutant is most likely the result of metabolic problems

sphaeroides, *Roseobacter denitrificans*, *Salmonella enterica* serovar Typhimurium, *Sinorhizobium meliloti*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptomyces hygroscopicus*, *Streptomyces kentuckensis* (formerly *Streptoverticillium kentuckense*), *Streptomyces yerevanensis* (formerly *Microellobospora violacea*), *Streptosporangium roseum*, *Synechocystis* sp., *Thermotoga maritima*, *Treponema pallidum*, *Ureaplasma urealyticum*, *Vibrio cholerae*, *Xylella fastidiosa.*

arising from the incapability to degrade RH, rather than a quorum-sensing defect. With the discovery of the AI-2 biosynthesis pathway and the availability of *in vitro*-synthesized AI-2, we will now be able to establish whether any phenotypes observed with a *luxS* mutant are caused by a metabolic or a signalling defect. The latter should be overcome by the addition of exogenous AI-2.

E. coli and *Sal. typhimurium* have both been reported to degrade AI-2 activity in the absence of glucose (Surette & Bassler, 1998, 1999), and the removal of AI-2 activity has also been described for *Strep. pyogenes* and *N. meningitidis* (Lyon *et al.*, 2001; Winzer *et al.*, 2002). Thus, it appears that AI-2 is released during exponential growth, and degraded, depending on bacterial species and culture conditions, during late exponential, transition to stationary or stationary phase. But why should bacteria remove AI-2 molecules? From a signalling perspective, such degradation could be part of a 'switch-off' mechanism, which resets the system. From a metabolic perspective, it would be advantageous to avoid the loss of 'expensive' compounds, which could be channelled back into the central metabolism. With regard to the LuxS reaction, assuming a 1:1 ratio between homocysteine and AI-2 production and a complete release of AI-2, bacteria would lose one 'ribose-equivalent' unit per methyl-group transfer. This would be a high price to pay. Thus, in the absence of glucose, *E. coli* and *Sal. typhimurium* may make use of AI-2 (or MHF, the final product), inducing a system which is otherwise controlled in a fashion resembling catabolite repression. Interestingly, MHF and DMHF have both been identified as DNA-damaging agents, and MHF is also mutagenic to *Sal. typhimurium* (Hiramoto *et al.*, 1996; Yamashita *et al.*, 1998), which could explain the exclusion of AI-2 from the cells during exponential growth. At a later stage, controlled uptake and degradation of AI-2 (and MHF) may prevent build-up of toxic intracellular levels. Bacteria such as *Ps. aeruginosa*, which do not produce AI-2 but are capable of degrading it, may also metabolize the compound and use it as a carbon or energy source. However, degradation by these bacteria could also serve to disrupt communication between other bacteria, similar to the degradation of *N*-acylhomoserine lactones by *Variovorax paradoxus* (Leadbetter & Greenberg, 2000) or *Bacillus* sp. 240B1 (Dong *et. al.* 2000).

Where used for interspecies communication, the AI-2 system could only detect the 'quorum' of its producers. But such a system is difficult to envisage, as different species may produce the molecule at different rates while others may degrade the molecule at the same time. Furthermore, furanones present in the environment may interfere with such a system. DMHF, for instance, has been discovered in fruits like tomatoes, strawberries and pineapples, as well as in yeast fermentation products, and is also produced by lactic acid bacteria (Slaughter, 1999; Hayashida *et al.*, 2001). Thus it appears that the use of AI-2 molecules for intra- or interspecies communication will only make sense in certain habitats, such as that occupied by *V. harveyi*.

In summary, we propose that in many species AI-2 does not function as a signal molecule but is simply a metabolite, which is excreted early during growth but can be taken up and metabolized at a later stage. Some bacteria, notably *V. harveyi* and possibly related species, may have evolved signalling systems which use AI-2 as a 'quorum-sensing molecule'. Competing bacteria, even those which do not produce AI-2, may co-metabolize the molecule and use it as a source of carbon.

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