Autoinducer 2 activity in *Escherichia coli* culture supernatants can be actively reduced despite maintenance of an active synthase, LuxS

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Production of the signalling molecule (autoinducer-2) synthesized by LuxS has been proposed to be pivotal to a universal mechanism of inter-species bacterial cell–cell communication (quorum sensing); however recently the function of LuxS has been noted to be integral to central metabolism since it contributes to the activated methyl cycle. This paper shows that when *Helicobacter pylori* LuxS is overproduced in *Escherichia coli*, it forms cross-linkable multimers. These multimers persist at comparable levels after 24 h of growth if glucose is omitted from the growth medium; however, the levels of extracellular autoinducer-2 decline (Glucose Retention of AI-2 Levels: GRAIL). Glycerol, maltose, galactose, ribose and L-arabinose could substitute for glucose, but lactose, D-arabinose, acetate, citrate and pyruvate could not. Mutations in (i) metabolic pathways (glycolytic enzymes *eno, pgk, pgm*; galactose epimerase; the Pta–AckA pathway), (ii) sugar transport (*pts* components, *rbs* operon, *mgl*, *trg*), and (iii) regulators involved in conventional catabolic repression (*creC, ccrA, cya, crp, spoT, cya, spoT, relA, spoT*, and the global carbon storage regulator *csrA*) did not prevent GRAIL. Although the basis of GRAIL remains uncertain, it is clear that the mechanism is distinct from conventional catabolite repression. Moreover, GRAIL is not due to inactivation of the enzymic activity of LuxS, since in *E. coli*, LuxS contained within stationary-phase cells grown in the absence of glucose maintains its activity in vitro.

INTRODUCTION

The co-ordinated expression of cellular components which ensure adaptation to different environments is pivotal to survival, and will depend upon integrating environmental cues with intercellular communication. Co-ordination of gene expression with population density, referred to as quorum sensing, has been subject to intense study over the past decade.

Different strategies have evolved to achieve effective quorum sensing. All comprise accumulation of signalling molecules in the extracellular milieu which modulates gene expression via cognate receptors and regulators (reviewed by Bassler, 1999; Swift et al., 2001; Withers et al., 2001). These processes are used to control a wide variety of phenotypes in many bacterial species including production of extracellular virulence factors by *Pseudomonas aeruginosa* (Winzer & Williams, 2001) and *Staphylococcus aureus* (McDowell et al., 2001; Novick & Muir, 1999), and bioluminescence by *Vibrio fischeri* (Nealson et al., 1970) and *Vibrio harveyi* (Bassler et al., 1994).

Many Gram-negative bacteria utilize N-acylhomoserine lactone (AHL) molecules as signals, whilst Gram-positive bacteria actively export peptide signalling molecules (Winzer & Williams, 2001; Withers et al., 2001). Recently the gene *luxS*, possessed by both Gram-positive and Gram-negative bacteria, was identified as a component of a quorum-sensing mechanism (Surette et al., 1999). LuxS is required for the production of a signalling molecule termed autoinducer-2 (AI-2). *V. harveyi* responds to the presence of AI-2 by producing bioluminescence via a phosphorylation cascade involving the periplasmic sensor LuxP, the inner-membrane protein LuxQ, the cytoplasmic signal integrator protein LuxU, and the response regulator LuxO (Bassler, 1999). This arrangement is mirrored in *Vibrio cholerae* (Miller et al., 2002). In the only other bacterium where the mechanism of response to AI-2 has been studied, *Salmonella typhimurium*, AI-2 uptake appears to require an ATP-binding cassette (ABC) transporter, Lsr (Taga et al., 2001), which shows similarity to ribose uptake systems.

Abbreviations: ABC, ATP-binding cassette; AI-2, autoinducer-2; DSP, dithioisuccinimidylpropionate; GRAIL, glucose retention of AI-2 levels; PTS, phosphotransferase system.
LuxS has been shown to convert S-ribosylhomocysteine to homocysteine and AI-2 in vitro (Schauer et al., 2001; Winzer et al., 2002a). Recent crystallographic analysis of four LuxS homologues (derived from Bacillus subtilis, Haemophilus influenzae, Deinococcus radiodurans and Helicobacter pylori) has shown that it is able to form a dimer with a Zn-binding active site compatible with this catalytic function (Hilgers & Ludwig, 2001; Lewis et al., 2001). The proposed reaction was previously shown to yield 4,5-dihydroxy-2,3-pentanedione as part of the cell’s central metabolism (Duerre & Miller, 1966; Duerre et al., 1971; Miller & Duerre, 1968), but this molecule is thought to cyclize spontaneously to form a furanone. Comparison of different furanones for AI-2 activity suggests that 4-hydroxy-5-methylfuranone (MHF) is the most active (Schauer et al., 2001; Winzer et al., 2002a), and this molecule has been identified by mass spectroscopy following LuxS-dependent in vitro synthesis of AI-2 and methanol extraction (Winzer et al., 2002a). The active chemical structure of AI-2 is however suggested to be a furanosyl borate diester, since this structure maps the electron-density images generated from X-ray crystallographic analysis of the periplasmic AI-2 sensor protein of V. harveyi, LuxP, following co-crystallization with AI-2 (Chen et al., 2002). Despite uncertainty concerning LuxS function and AI-2 structure, detection of extracellular AI-2 activity has revealed that a number of bacterial species produce functionally equivalent substances capable of inducing the production of bioluminescence by the V. harveyi biosensor, which are thus likely to share a conserved chemical composition. The presence of LuxS and/or AI-2 appears to influence bioluminescence in V. harveyi (Bassler et al., 1994), levels of an ABC transporter in Salmonella typhimurium (Taga et al., 2001), type III secretion in EHEC (Sperandio et al., 1999), the virulence factor VirB in Shigella flexneri (Day & Maurelli, 2001), protease production by Porphyromonas gingivalis (Burgess et al., 2002; Chung et al., 2001) and Streptococcus pyogenes (Lyon et al., 2001), in vivo fitness of Neisseria meningitidis (Winzer et al., 2002c), iron acquisition by Actinobacillus actinomycetemcomitans (Fong et al., 2001), and multiple, but moderate, effects upon the transcription of a number of genes in Escherichia coli (DeLisa et al., 2001). Clear phenotypes dependent upon the presence of LuxS have however remained elusive for some bacteria, e.g. H. pylori (Joyce et al., 2000; Forsyth & Cover, 2000) and Proteus mirabilis (Schneider, 2002).

Currently there is much discussion in the literature concerning the primary role of LuxS in different bacteria given that it has been demonstrated to act as a component of a quorum-sensing mechanism in V. harveyi to control bioluminescence, whilst having a distinct role within central metabolism as part of the activated methyl cycle to recycle S-adenosylhomocysteine which would otherwise have toxic effects on the cell (Bassler 2002; Winzer et al., 2002a,b). To date, few of the published investigations pertaining to the role for LuxS have taken account of the latter, and consequently the basis of the influence of LuxS on cell physiology is unclear.

We set out to investigate the link between the presence of catalytically active LuxS and measurable extracellular AI-2 activity. Since there is apparent conservation of both LuxS structure and AI-2 chemistry, we took advantage of the LuxS overproduction system we have developed. This system relies on the production in E. coli of high levels of soluble LuxS derived from the curved, Gram-negative bacterium H. pylori, which colonizes the gastric epithelium of humans, causing the development of peptic ulcer disease and gastric adenocarcinoma (Blaser et al, 1995; Cover & Blaser, 1996; Mobley, 1996). In agreement with recent crystallographic investigations we demonstrate that LuxS is dimeric in vivo, and show that in the E. coli background it directs the production of AI-2 influenced by carbohydrate availability. The carbohydrate dependence however merely affects extracellular AI-2 activity, since no detectable alteration in the ability of LuxS to synthesize AI-2 in vitro was observed. We also conclude that E. coli must possess a mechanism for degradation or uptake of AI-2. Taken together this work adds weight to the arguments of Winzer et al. (2002b), which propose that the primary role of LuxS is in central metabolism rather than quorum sensing (Bassler, 2002).

**METHODS**

**Bacterial strains and growth conditions.** E. coli strains used in this study are listed in Table 1, and were grown in Luria–Bertani (LB) medium or on LB agar plates at 37°C. Where required, carbenicillin (50 µg ml−1) or chloramphenicol (50 µg ml−1) were added. Construction of plasmid pProEX-luxS<sub>E</sub>, is described by Winzer et al. (2002a). V. harveyi biosensor strains were grown in AB medium (Greenberg et al., 1979) at 30°C. Where indicated glucose (0–4 %, w/v), lactose (0–6 %, w/v), maltose (2 %, w/v), potassium acetate (0–2 %, w/v), sodium citrate (0–2 %, w/v), glycerol (1 %, w/v), galactose (0–36 %, w/v), arabinose (0–4 %, w/v), pyruvic acid (0–2 %, w/v) or ribose (0–4 %, w/v) were added.

**DNA manipulation.** DNA was manipulated by standard methods (Sambrook et al., 1989). Restriction enzymes (Promega UK) were used according to the manufacturer’s instructions. For isolation of plasmid DNA from E. coli the Qiagen Mini and Midi kits were used. Genomic DNA was extracted from H. pylori according to Atherton (1997). Standard methods were used for preparation of competent cells, and for the electroporation of plasmids into E. coli (Sambrook et al., 1989).

Following PCR amplification (5 min 95°C; 30 cycles of 30 s 95°C/30 s 55°C/1 min 72°C; 5 min 72°C) of genomic DNA from H. pylori strain 26695 with primers luxSF (gcatgAAGCTTaaaccaatcaaacccc) and luxSR (gctaAGAATTCgcatccctaaaacgc), the ORF HP0105 plus 150 bp 5’ flanking DNA was cloned into pBluescript (creating pKH4) and the lower copy number pHG327 (creating pKH5). Clones were confirmed by restriction digestion and sequencing. 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). Plasmid pcrp encoding high levels of Crp was a gift from Steve Busby (Bell et al., 1990).

**Antibody generation.** E. coli strain DH5α(pKH4) was grown in LB+glucose overnight and cells were harvested into SDS-PAGE.


### Table 1. E. coli strains used in this study

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<tr>
<th>CGSG no.</th>
<th>Original strain name</th>
<th>Genotype*</th>
<th>Reference</th>
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<td>CA198</td>
<td>χ− galU106 relA1 spoT1</td>
<td>Shapiro (1966)</td>
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<td>5526</td>
<td>SA191</td>
<td>tss-83? pgm-191 his-87 relA1 χ− rpsL181(strR)</td>
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<td>58-161</td>
<td>bio-1(Unst) spoT1 metB1 creC510</td>
<td>Tatum (1945)</td>
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<td>4427</td>
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<td>relA1 spoT1 metB1 rnb-2 creC510</td>
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<td>1101</td>
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<td>DF576</td>
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<td>DF262</td>
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<td>χ− ΔptsI227 relA1 spoT1 thi-1 bglR7,p,ap</td>
<td>W. Epstein strain CHE30</td>
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<td>Lin225</td>
<td>thi-1 leuB6 lacZ4 gnrY44(AS) rpsL8 glpK31(p) ptsI24</td>
<td>Bermin &amp; Lin (1971)</td>
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<td>7519</td>
<td>LI2806</td>
<td>friuR11::Tn10 xyIA7 ilvA215 ΔargHI</td>
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<td>CA8439</td>
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<td>7200</td>
<td>SP850</td>
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<td>MC4100</td>
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<td>MCA100 rbsB61 rbsR22</td>
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<td>χ− rph-1</td>
<td>Guyer et al. (1981)</td>
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<td>thr-1 araC14 leuB6(Am) flhuA31 lacY1 tsx-78 χ− zde-253::Tn10 trg::Tn5 zde-230::Tn9 eda-50 hisG4(Oc) rbdD2 rpsL150(strR) sylA5 mtl-1 metF159(AM) thi-1</td>
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<td>Δ(nadA–galE)35 χ− recA1 relA1 spoT1 thi-1 rpsL104 or rpsL180(strR); this strain is ΔmglR</td>
<td>Mizuuchi &amp; Fukasawa (1969)</td>
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<td>Hammer-Jespersen &amp; Nygaard (1976)</td>
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<td>S0303</td>
<td>ΔcyrR0: deoR201</td>
<td>Hammer-Jespersen &amp; Munch-Petersen (1975)</td>
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<td>Romeo et al. (1993)</td>
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<td>5899</td>
<td>BJ4 L1</td>
<td>ΔrpoS</td>
<td>Frieda Jörgenson</td>
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*Genotypes relevant to this study are shown in bold.
sample buffer. Following separation through 15% SDS-PAGE, LuxS was excised from the gel and eluted into 50 mM ammonium bicarbonate/0-1% (w/v) SDS. Rabbit polyclonal antibodies were raised according to a protocol based on Harlow & Lane (1988). New Zealand White rabbits were immunized subcutaneously biweekly with between 50 and 400 µg mixed 50:50 with Freund’s complete adjuvant on the first immunization and subsequently with Freund’s incomplete adjuvant. A test bleed was carried out following the third immunization. A final fourth immunization was carried out before finally obtaining complete bleeds from the rabbits. Absorption of non-specific antibodies was carried out using a lysate of E. coli DH5α[pBluescript] at 37°C for 60 min.

**SDS-PAGE and Western blotting.** These were performed as described by Hardie et al. (1996), except phosphate-buffered saline with 0-5% (v/v) Tween 20 (PBST) replaced TBST. The primary antibody, Anti-LuxS, was used at dilutions of 1:2000. Western blots were developed using the enhanced chemiluminescence kit (ECL, Amersham) according to the manufacturer’s instructions. SDS-polyacrylamide gels were either stained with 0-1% (w/v) Coomassie blue/45/ (v/v) methanol/9% (v/v) acetic acid and destained in 20% (v/v) methanol/7% (v/v) acetic acid, or stained with silver stain (Wray et al., 1981).

**Analysis of AI-2 production and degradation.** AI-2 production was essentially analysed as described by Bassler et al. (1997) using 20 µl AI-2 extract and 180 µl 1:5000 diluted overnight cultures V. harveyi biosensor BB170 in AB medium. Changes in bio-luminescence upon addition of AI-2 were determined at 30°C every 60 min using an automated luminometer (VICTOR2, 1420 multi-label counter, Wallac). For a single experiment, the V. harveyi bioassay was performed at least in duplicate for each sample. Experiments were repeated at least three times.

**Protein cross-linking.** Protein cross-linking with formaldehyde and dithiobis(succinimidylpropionate) (DSP) was performed as described by Hardie et al. (1996).

**In vitro generation of AI-2.** Cells were harvested by centrifugation at 10,286 g, 15 min, 4°C and resuspended in 1/5 vol. 10 mM sodium phosphate pH 7 and frozen at −70°C. Following two passages through a French press (SIM AMINCO Spectronic Instruments) at 2000 p.s.i. (13-8 MPa), cell debris was removed by centrifugation at 2571 g for 10 min at 4°C, leaving the cell-free extract. To generate AI-2 in vitro, 10 µl 200 mg S-adenosylmethionine ml−1/10 mM sodium phosphate pH 7 was added to 1 ml cell-free extract and incubated at room temperature for 60 min. Control incubations were performed with the addition of 10 µl 10 mM sodium phosphate pH 7. All reactions were filtered through a 0-2 µm filter before addition to the V. harveyi biosensor BB170 bioassay to remove any contaminating bacteria, which was verified by viable counts following serial dilution of extracts. Control extracts to which no substrate (S-adenosylmethionine) was added were assayed in parallel to confirm that the AI-2 detected originated from catalysis by LuxS during the incubation.

**Purification of LuxS.** Cells were harvested from overnight cultures of E. coli DH5α(pKH4) by centrifugation at 10,286 g for 10 min at 4°C, and resuspended in 20 mM Tris/HCl pH 8-0. Following two passes through the French press (SIM AMINCO Spectronic Instruments) at 2000 p.s.i. (13-8 MPa), cell debris was removed by centrifugation at 2571 g for 10 min at 4°C, leaving the cell-free extract, which was filtered through a 0-2 µm filter before loading onto a monoQ ion-exchange column in 20 mM Tris/HCl pH 8-0. For elution, a gradient of 1-10% 1 M NaCl/20 mM Tris/HCl pH 8-0 was first applied and held until no further peaks of absorbance at 280 nm were detected. The first peak of absorbance at 280 nm eluting after reintegrating a gradient with the same buffer contained the majority of LuxS. Eluted LuxS was pooled and applied to a Superdex 200 column in 20 mM Tris/HCl pH 8-0. LuxS eluted at a purity of approximately 90%, as judged by eye from Coomassie- and silver-stained SDS-PAGE, at a predicted molecular mass of 40 kDa. Western blots confirmed the identity of the purified protein as LuxS. All column chromatography was achieved using the Pharmacia Biotech AKTA explorer.

**RESULTS**

The H. pylori LuxS functionally complements an E. coli luxS mutant

In order to study the requirements for AI-2 synthesis, and relate this to the functional status of LuxS, we took advantage of the high level of soluble LuxS generated in E. coli from a plasmid bearing the luxS homologue derived from H. pylori (HP0105). The ORF HP0105 plus 150 bp 5’ flanking DNA was cloned into pBluescript (creating pKH4) and the lower copy number pHG327 (creating pKH5), and introduced into the LuxS-deficient E. coli strain DH5α. Filtered spent supernatants from early stationary-phase cultures of DH5α(pKH4) and DH5α(pKH5) were assayed for the presence of LuxS-dependent autoinducers (AI-2) with the biosensor strain V. harveyi BB170 (Fig. 1a, b). HP0105 clearly directed the synthesis of autoinducers capable of inducing light production in the biosensor strain V. harveyi BB170.

Comparison of whole-cell profiles of E. coli DH5α(pKH4) with those of E. coli DH5α(pBluescript) revealed one major overproduced protein (Fig. 1c). The identity of the overproduced protein was confirmed as H. pylori LuxS by N-terminal sequencing, and it was purified by electroelution and used to inoculate a rabbit as described in Methods to generate a specific polyclonal antiserum. High levels of LuxS were produced even in the absence of the inducer of the plasmid-borne promoter, IPTG.

**Carbon source affects extracellular AI-2 levels**

Surette & Bassler (1998) reported that the presence of AI-2 activity in spent culture supernatants of E. coli and S. typhimurium depended on addition of glucose to the growth medium. Interestingly, addition of glucose to the growth medium affected the measurable AI-2 activity in supernatants harvested from stationary-phase cultures of E. coli DH5α(pKH4). Spent culture supernatants were harvested from E. coli strains DH5α(pBluescript), DH5α(pKH4) and MG1655(pBluescript) grown in either LB or LB + 0.4% glucose at the indicated times (Fig. 2). Similar levels of AI-2 were detectable by the V. harveyi BB170 bioassay in DH5α(pKH4) and MG1655(pBluescript) supernatants after 6-5 h growth in both the presence and the absence of glucose, but following longer periods of growth (24 h) detectable AI-2 persisted in the supernatants harvested from these strains grown with supplemented glucose, but not without. From this point onwards, the ability of glucose to sustain AI-2 activity in stationary-phase...
culture supernatants will be referred to as GRAIL (Glucose Retention of AI-2 Levels). As expected, supernatants harvested from *E. coli* DH5α (pBluescript) remained devoid of detectable AI-2 in all conditions (Fig. 2). Other carbon sources (see Methods) which had a similar effect to glucose included glycerol, maltose, galactose, ribose and L-arabinose. In contrast, lactose, D-arabinose, acetate, citrate and pyruvate did not sustain high levels of AI-2 activity in stationary-phase cultures despite maintaining high-level LuxS production (Table 2). In each case results obtained with *E. coli* DH5α (pKH4) were mirrored by those of *E. coli* MG1655 (pBluescript), indicating that the origin of LuxS (whether from *E. coli* or *H. pylori*) was of little consequence.

As expected, *E. coli* MG1655 bearing either pBluescript or pKH4 exhibited blue colonies on agar plates containing 40 μg X-Gal ml⁻¹ and 0·1 mM IPTG, indicating that the inability of lactose to mimic GRAIL was not due to an inactive β-galactosidase, which cleaves lactose into glucose and galactose.

**Alteration in extracellular AI-2 levels in response to carbon source is not mediated through modulation of LuxS**

Western blots revealed that LuxS levels were similar in *E. coli* DH5α (pKH4) cells grown in the presence or absence of 0·4 % glucose for 24 h. Between 5·3 h and 6·5 h of growth, LuxS levels were higher in the presence of 0·4 % glucose (Fig. 2c). However, after this point in the absence of glucose LuxS levels continued to rise slightly as AI-2 levels declined.

In contrast, in the presence of 0·4 % glucose the levels of LuxS declined slightly despite the retention of high AI-2 activity. Similar levels of LuxS were also detected by Western blots for *E. coli* DH5α (pKH4) grown for 24 h with all the other carbon sources described above (data not shown).

**The influence of exogenous glucose upon extracellular AI-2 activity is not affected by lesions in metabolic or regulatory systems known to involve glucose**

To determine whether GRAIL resulted from metabolism of the carbon source, the activity of extracellular AI-2 following growth of a range of *E. coli* mutants in LB or LB + 0·4 % glucose was compared. *E. coli* mutants were assessed whilst containing pKH4 or pBluescript, and compared to parent strains. Where data are presented (Table 3, Fig. 5) the legend specifies whether pKH4 was present for each particular strain. The complete glycolytic pathway is not required for GRAIL as *E. coli* mutants defective in the glycolytic enzymes (phosphoglycerate kinase, phosphoglycerate mutase and enolase, Fig. 4) display wild-type GRAIL (Table 3). As galactose is able to substitute for glucose, the effect of galactose epimerase (GalU) was analysed and it was found that GRAIL could be mediated in its absence by exogenous glucose or galactose, but not by lactose.

The presence of glucose in the growth medium prevents the simultaneous utilization of another sugar (e.g. lactose) by inhibiting the appearance of lactose-metabolizing enzymes
until glucose is depleted, resulting in diauxic growth (catabolite repression). Glucose acts on at least three levels to achieve this by (i) inhibiting uptake into the cell of inducer molecules necessary for the induction of repressed genes via effects on the phosphotransferase system (PTS), (ii) lowering the level of cAMP which would otherwise complex with its receptor protein (CRP) to influence gene transcription, (iii) generating high levels of catabolites as a result of its metabolism, which in turn cause repression of inducible enzyme synthesis predominantly via cAMP (Kolb et al., 1993). Each of these mechanisms was investigated for its role in GRAIL using mutants in E. coli defective for their function.

Since GRAIL was observed in E. coli strains bearing mutations in ptsI, ptsII (crr) and hrp and containing plasmid pKH4 or pBluescript, a functional PTS (Postma et al., 1996) was not required. A mechanism dependent upon cAMP and CRP was also ruled out since GRAIL was observed in crp and cya (adenylate cyclase) mutants of E. coli containing plasmid pKH4 or pBluescript.

The regulatory mechanisms believed to participate in cAMP-independent catabolite repression are numerous, and include (i) the global regulatory protein FruR (fructose repressor, which shows similarity to several periplasmic sugar receptors including those specific for ribose, arabinose and galactose), (ii) the stationary-phase-specific sigma factor σ7 (RpoS), (iii) the sensor kinase–response regulator CreBC which is presumed to be involved in regulating gene expression in response to environmental catabolites, and (iv) (p)ppGpp as increased levels correlate with the onset of starvation (otherwise known as the stringent response, and reliant upon relA and spoT: Cashel et al., 1996). Another global regulator, the carbon storage regulator (CsrA), impacts strongly upon the metabolic carbon flow by...

**Fig. 2.** Influence of glucose upon detectable autoinducer. E. coli MG1655(pBluescript) (circles) and also E. coli DH5α bearing pBluescript (diamonds) or pKH4 (squares) were grown in either LB (filled symbols with full lines) or LB + 0.4% glucose (open symbols with dotted lines). Throughout the growth curve samples were removed as indicated and the cell density (OD600) measured (shown in a). Duplicate samples were centrifuged; the culture supernatant was filtered and assayed for its ability to stimulate the production of light in the V. harveyi bioassay for AI-2 (b), whilst the cell pellet was resuspended in SDS-PAGE sample buffer and the presence of LuxS (indicated by the arrow) detected following Western blotting with anti-LuxSHp (c; shown for DH5α bearing pKH4).
repressing gluconeogenesis, glycogen biosynthesis and glycogen catabolism whilst activating glycolysis (Lin et al., 1997). Since GRAIL was observed in fraR, rpoS, creC, relA, spoT and csaR mutants of *E. coli* containing plasmid pKH4 or pBluescript, no role for these regulators in the control of AI-2 synthesised from *luxS* was indicated (Table 3; see Saier et al., 1996, for description of each mechanism of regulation).

Having found no role for most of the well-known metabolic pathways, and since the phosphotransacetylase–ATP:acetate phosphotransferase (Pta–AckA) pathway plays a critical catabolic role during aerobic growth on excess glucose or other glycolytic intermediates (Chang et al., 1999), we investigated the effect of mutants in this pathway upon GRAIL. Under aerobic conditions, when the carbon flux into cells exceeds the amphibolic capacity of the central metabolic pathways, e.g. the TCA cycle, *E. coli* cells adjust by moving acetyl-CoA through the Pta–AckA pathway, excreting acetate and generating ATP. Later, as they begin the transition to stationary phase, cells undergo the metabolic switch to acetyl-CoA synthetase (AcS) and resorb acetate (see Fig. 4, Pruss et al., 1994). Under conditions that result in mixed acid fermentation, cells also convey acetate-CoA through the Pta–AckA pathway to gain ATP via substrate-level phosphorylation. *E. coli* mutants in this pathway containing either pKH4 or pBluescript were however still able to demonstrate GRAIL (Table 3).

**Table 2.** Exogenous supplements which maintain detectable extracellular AI-2

*E. coli* strains DH5α(pBluescript), DH5α(pKH4) and MG1655-(pBluescript) were grown overnight in LB containing the supplement indicated. Cleared supernatants were analysed for the presence of AI-2 using the *V. harveyi* BB170 bioassay and scored + if early induction of bioluminescence was reported. Independent experiments were done on at least three occasions, and representative results are shown. DH5α(pKH4) and MG1655(pBluescript) gave the same results. Supplements were added at the following concentrations: glucose (0·4 %, w/v), lactose (0·6 %, w/v), maltose (2 %, w/v), potassium acetate (0·2 %, w/v), sodium citrate (0·2 %, w/v), glycerol (1 %, w/v), galactose (0·36 %, w/v), arabinose (0·4 %, w/v), pyruvic acid (0·2 %, w/v), ribose (0·4 %, w/v).

<table>
<thead>
<tr>
<th>Exogenous supplement</th>
<th>Induction of bioluminescence</th>
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<tr>
<td>DH5α(pBluescript)</td>
<td>DH5α(pKH4) or MG1655(pBluescript)</td>
</tr>
<tr>
<td>Glucose</td>
<td>–</td>
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<tr>
<td>Glycerol</td>
<td>–</td>
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<td>Maltose</td>
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<td>Galactose</td>
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<td>Ribose</td>
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<td>L-Arabinose</td>
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<td>Acetate</td>
<td>–</td>
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<tr>
<td>Citrate</td>
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<td>Pyruvate</td>
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**Table 3.** Mutation of glycolysis enzymes, selected sugar uptake mechanisms, and selected regulators does not diminish the effect of glucose-supplemented growth medium upon AI-2 production

*E. coli* strains mutated in the individual genes listed were grown in LB or LB + 0·4 % glucose overnight. Cleared supernatants were analysed for the presence of AI-2 using the *V. harveyi* BB170 bioassay and scored + if early induction of bioluminescence was reported. If the strain used was devoid of AI-2, and thus deficient in chromosomally encoded LuxS, the plasmid pKH4 was introduced to supply LuxS (as indicated by *). See Table 1 for strain names and references. As the results were the same for each mutant tested, they are shown collectively for each mutant class.

<table>
<thead>
<tr>
<th>Relevant mutation</th>
<th>Induction of bioluminescence</th>
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<tr>
<td></td>
<td>LB</td>
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<tr>
<td>Transport</td>
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<tr>
<td>ptsI*, ptsII, hpr, rbsR, rbsBR, rbsD, mgl*, trg</td>
<td></td>
</tr>
<tr>
<td>Metabolic conversion</td>
<td></td>
</tr>
<tr>
<td>galU, eno, pgk, pgm, ackA/pta</td>
<td></td>
</tr>
<tr>
<td>Regulators</td>
<td></td>
</tr>
<tr>
<td>creC, fruR, csrA, crp, cya/crp crp, rpoS, cyaR, deoR, tsx, napC</td>
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**LuxS is dimeric in vivo, and dimer formation is not dependent upon exogenous glucose**

Cross-linking of cellular proteins in vivo with formaldehyde and DSP revealed the presence of multimeric forms of *H. pylori* LuxS overproduced in *E. coli* (Fig. 3). DH5α(pBluescript) and DH5α(pKH4) were grown in LB with and without supplementation with 0·4 % glucose to stationary phase, protein cross-linking was performed, and complexes containing LuxS revealed by Western blotting. Patterns of cross-linked products were not affected by the presence or absence of glucose in the growth medium. Formaldehyde cross-linking yielded a number of high-molecular-mass complexes containing LuxS including two distinct complexes, one which migrated at the predicted dimeric size, and one at a size intermediate between monomeric and dimeric forms of LuxS (marked with an asterisk in Fig. 3). Following partial disruption of formaldehyde cross-links by boiling, monomeric and dimeric forms of LuxS remained. Similarly with DSP, multiple forms of multimeric LuxS were observed, the most prominent being the dimeric form, which was stable following disruption of cross-links in reducing conditions. The cross-linked product asterisked in Fig. 3(a) that was seen following cross-linking with formaldehyde was not observed when complexes were cross-linked with DSP. Interestingly, separation of whole-cell extracts by non-reducing SDS-PAGE followed by Western blotting revealed a similar...
pattern of multimeric proteins containing LuxS as was observed following cross-linking with DSP, suggesting that these multimers may be stabilized by disulphide bridges. The observation of more abundant high-molecular-mass complexes in non-reducing SDS-PAGE following addition of DSP confirms that additional cross-links were formed under these conditions, as does the presence of the dimer following DSP treatment and resolution using reducing SDS-PAGE.

**LuxS retains functionality in stationary-phase cells in the absence of glucose in the growth medium**

Having seen no alteration in the cross-linking pattern of LuxS in the presence and absence of glucose in the growth medium, we compared the functionality of LuxS in these conditions *in vitro*. Crude cell extracts of *E. coli* strains DH5α(pBluescript), DH5α(pKH4), DH5α(pProEX-luxS<sub>Ec</sub>), MG1655(pBluescript) and MG1655(pKH4) were prepared after 7 h and 24 h growth in LB alone, or LB + 0·0-4 % glucose. None of these cell extracts contained detectable levels of AI-2 activity. Incubation of these cell extracts in the presence of S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) or S-ribosylhomocysteine (SRH) resulted in the production of AI-2 as measured by bioluminescence of the *V. harveyi* BB170 biosensor from all 7 h extracts except that prepared from *E. coli* DH5α(pBluescript) (data not shown). As seen in previous experiments, following 24 h growth in the absence of glucose in the culture medium, reduced levels of AI-2 were detected in the culture supernatants of *E. coli* strains DH5α(pKH4), DH5α(pProEX-luxS<sub>Ec</sub>), MG1655(pBluescript), and MG1655(pKH4) compared with growth in the presence of glucose (Fig. 2, Table 4 and Table 5). Despite this, the ability of LuxS contained within cell-free extracts prepared from 24 h cultures of *E. coli* strains DH5α(pKH4), DH5α(pProEX-luxS<sub>Ec</sub>), MG1655(pBluescript) and MG1655(pKH4) to synthesize AI-2 *in vitro* was unaffected by the addition of 0·0-4 % glucose to growth medium (Tables 4 and 5, and data not shown). Control incubations containing the cell extracts, but lacking substrate, did not result in detectable levels of AI-2 activity. Western blots revealed production of LuxS (derived from either *E. coli* or *H. pylori*, as appropriate) to comparable levels in all strains whether glucose was present in the medium or not (data not shown). Control incubations containing the cell extracts, but lacking substrate, did not result in detectable levels of AI-2 activity. Western blots revealed production of LuxS (derived from either *E. coli* or *H. pylori*, as appropriate) to comparable levels in all strains whether glucose was present in the medium or not (data not shown). The *H. pylori* LuxS contained within crude cell-free extracts of *E. coli* directed the production of AI-2 *in vitro* using SAM, SAH or SRH as substrates; however following purification (see Methods, Fig. 1b) the addition of the Pf6 enzyme was required if SAH was used as a substrate (data not shown). These observations are similar to those reported for other LuxS homologues (Schauder *et al.*, 2001; Winzer *et al.*, 2002a), and thus
support our prediction that LuxS homologues derived from different sources possess similar properties.

**Is extracellular AI-2 imported via the ribose uptake system?**

LuxS remains capable of synthesizing AI-2 in stationary-phase *E. coli* cells grown in the absence of glucose; however since AI-2 is cleared from the culture supernatant under these conditions, it is possible that the cells become more efficient at taking up AI-2. Taga *et al.* (2001) reported that *S. typhimurium* use an ABC transporter to import AI-2 which resembles the ribose uptake transporter. *E. coli* possesses two machineries which function to take up ribose. The ribose transporter encoded by the genes *rbsA*, *rbsB*, *rbsC*, *rbsD*, *rbsK* and *rbsR* has the highest affinity for ribose, and has been extensively studied. Mutants interrupted in *rbsR* (encoding a regulatory protein that functions to repress transcription of the *rbs* operon in the absence of ribose: Mauzy & Hermodson, 1992), *rbsB* (encoding a periplasmatic-binding protein that recognizes ribose and interacts with RbsC: Park *et al.*, 1999), and *rbsD* (encodes a protein with no known function: Bell *et al.*, 1986) were obtained from the *E. coli* Genetic Stock Center. All mutant strains exhibited GRAIL in stationary phase. All strains were tested whilst bearing either pBluescript or pKH4, and where the strain contained an active, chromosomally encoded LuxS, the same pattern of AI-2 activity was recorded, although only the data in the presence of pKH4 are shown (Fig. 5). Furthermore, the presence or absence of an active chromosomally encoded LuxS was mirrored in parent strains.

The ribose- and galactose-binding proteins of *E. coli* (*RbsB* and *MglB* respectively) exhibit more similarity to each other (26% similarity/44% identity) than any other pair of sugar-binding proteins in *E. coli*, and both interact with the inner membrane translocator, Trg (Falke *et al.*, 1997). Although mutations in *trg* did not alter the manifestation of GRAIL (Fig. 5b), a complete deletion of the *mgl* operon resulted in lower, shorter-lived levels of AI-2 activity in the absence of exogenous glucose. For completeness, *E. coli* mutants of genes encoding nucleoside uptake components and regulators of such machineries (*cytR*, *deoR*, *tss*, *nupC*) were

### Table 4. LuxS is active *in vitro* in the absence of exogenous glucose

*E. coli* DH5α(pKH4) was grown overnight in LB or LB+0·4% glucose. The culture supernatant was filtered to remove cells (SN), whilst the cells were lysed in the French press, and the AI-2 generated using the *in vitro* assay with (SAM) or without (buffer) the addition of the substrate S-adenosylmethionine. All samples were assayed for the presence of AI-2 using the *V. harveyi* BB170 bioassay, and the bioluminescence is shown as the fold increase in induction compared to a DH5α(pKH4) negative control following incubation with *V. harveyi* BB170 for 4 h. The results shown are representative of three independent experiments; parallel assays with diluted samples indicated that the *V. harveyi* AI-2 detection assay was not saturated.

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Fold induction</th>
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<tr>
<td></td>
<td>LB</td>
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<tr>
<td>Buffer</td>
<td>1-00</td>
</tr>
<tr>
<td>SAM</td>
<td>142-00</td>
</tr>
<tr>
<td>SN</td>
<td>1-38</td>
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Fig. 4. Schematic depicting selected steps in metabolism and carbohydrate uptake. The main steps in glycolysis and other relevant metabolic pathways (Fraenkel, 1996) are indicated along with the genes encoding enzymes deficient in strains used in this study (pgk, phosphoglycerate kinase; pgm, phosphoglucomutase; eno, enolase; pta, phosphotransacetylase; ackA, acetate kinase; acs, acetyl-CoA synthetase; galU, galactose epimerase). The tricarboxylate (TCA) cycle is indicated as are the carbohydrates added to the growth medium during this study. Those carbohydrates which sustained AI-2 activity in stationary-phase cultures of DH5α(pKH4) are underlined. Pertinent carbohydrate uptake systems are also indicated (*lamb*, maltose uptake protein; *mgl*, galactose transport; *rbs*, ribose transport; *b1513*, proposed low-affinity ribose transport; *trg*, inner-membrane transport accepting sugars bound to MglB and RbsB; Pts, phosphotransferase system comprising ptsI, ptsII, hpr).
assayed, and none exhibited altered GRAIL (data not shown).

**DISCUSSION**

Our demonstration here that LuxS persists in stationary-phase cells, whilst extracellular AI-2 levels decline, mirrors data obtained with homologues in other species including *P. gingivalis* (Burgess et al., 2002), emphasizing the functional relatedness of this family of proteins. Our data also support recent crystallographic studies (Lewis et al., 2001) by using protein cross-linking approaches to reveal that LuxS associates into dimers *in vivo*. Analysis of AI-2 production throughout growth of *E. coli* strains MG1655 and DH5α(pKH4) has extended previous observations for *S. typhimurium* LT2 and *E. coli* AB1157 (Surette & Bassler, 1998), which reported that the sustained presence of active AI-2 in stationary-phase culture supernatants requires the presence of glucose in the growth medium (GRAIL). Our observation that LuxS presence, functionality and dimerization is independent of exogenous glucose suggests (i) that AI-2 is actively removed or degraded in the absence of glucose during stationary phase, and (ii) that if LuxS becomes incapable of producing AI-2 *in vivo* in stationary-phase *E. coli* cells grown in the absence of glucose, it is not a result of degradation or inactivation of LuxS. Rather, it is likely to be due to a lack of substrate availability. The absence of increased levels of AI-2 upon overproduction of LuxS in *E. coli* is in agreement with limited substrate availability within cells as suggested for *S. typhimurium* by Beeston & Surette (2002).

In accordance with the carbon source dependence of AI-2 detection in supernatants of *S. typhimurium* and *E. coli* strain AB1157 (Surette et al., 1999; Surette & Bassler, 1999), detectable AI-2 is found in 24 h cultures of *E. coli* MG1655 and *E. coli* DH5α(pKH4) in the presence of both PTS (glucose, maltose) and non-PTS (galactose and arabinose) sugars. However, in contrast to the data published for *S. typhimurium* (Surette & Bassler, 1999), addition of glycerol (which feeds into glycolysis at the level of glyceraldehyde 3-phosphate) mimicked GRAIL. Supplementary carbon sources that feed into the TCA cycle directly (pyruvate, citrate, acetate) did not result in extracellular AI-2 activity in stationary-phase cultures. This is particularly surprising since the addition of acetate to nutrient broth has been reported to induce the production of LuxS (Kirkpatrick et al., 2001). Mutations in the glycolytic enzymes which link glyceraldehyde 3-phosphate to pyruvate (encoded by *pgk, pgm* and *eno*) did not prevent GRAIL. Together, this evidence suggests that substrate availability, but not breakdown via the glycolytic pathway, determines AI-2 activity in culture supernatants.

Surprisingly, lactose (a disaccharide containing glucose and galactose, both of which can induce GRAIL) was unable to mimic glucose in cells which are β-galactosidase-positive and therefore capable of breaking it down into its constituent monosaccharides. This finding argues that glucose implements its effect via a specific uptake pathway. Mutations in the phosphotransferase proteins Hpr, PtsI and PtsII which constitute the specific route of glucose entry into cells did not however prevent its effect upon AI-2 activity, ruling this out as the basis for GRAIL. Supporting this is the ability of strains deficient in different mechanisms of achieving catabolite repression and non-PTS transported carbon sources to display GRAIL.

The potential loss in production of AI-2 during stationary phase in the absence of certain carbon sources is unlikely to be due to a conformational change in LuxS since cross-linking patterns were unaltered, which is suggestive of maintenance of quaternary structure. This is consistent with retention of LuxS activity *in vitro*, and limiting substrate availability. The multimeric forms of LuxS detected in these experiments are likely to be the dimers also discovered during crystallographic studies. The presence of dimers noted here in non-reducing conditions can also be explained following analysis of the crystal structures since they revealed that cysteine residues are located at the interface of adjacent molecules.

The loss of detectable AI-2 in stationary-phase culture supernatants grown in the absence of glucose may result from uptake of AI-2 by the cells, or inactivation of AI-2. An absence of extracellular degrading agents produced by *E. coli* DH5α(pKH4) is suggested by the inability of filtered stationary-phase culture supernatants grown without glucose to diminish AI-2 activity following incubation for up to 2 h at a range of temperatures (20–42°C) with AI-2-containing culture supernatant (data not shown). It is however possible that degradation requires the presence of cells. Surette et al. (1999) stated that *E. coli* DH5α does not

### Table 5. *E. coli* LuxS is active *in vitro* in the absence of exogenous glucose

<table>
<thead>
<tr>
<th>Sample</th>
<th>Induction of bioluminescence</th>
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<tr>
<td></td>
<td>DH5α(pProEx-LuxS&lt;sub&gt;Ec&lt;/sub&gt;)</td>
</tr>
<tr>
<td></td>
<td>LB</td>
</tr>
<tr>
<td>SN</td>
<td>–</td>
</tr>
<tr>
<td>Buffer</td>
<td>–</td>
</tr>
<tr>
<td>SRH</td>
<td>+</td>
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*E. coli* strains DH5α(pProEx-LuxS<sub>Ec</sub>) and MG1655 were grown overnight in LB or LB + 0.4% glucose. The culture supernatant was filtered to remove cells (SN), whilst the cells were lysed in the French press, and the AI-2 generated using the *in vitro* assay with either buffer alone (Buffer), or with the substrate S-ribosyl-homoserine (SRH). All samples were analysed for the presence of AI-2 using the *V. harveyi* BB170 bioassay and scored if early induction of bioluminescence was reported. The results shown are representative of three independent experiments; parallel assays with diluted samples indicated that the *V. harveyi* AI-2 detection assay was not saturated.
BB170 is shown for spent supernatants harvested from *E. coli* a mgl 517 (strains TR1-5 (strains CGSC 7585), BJ4 L1 (rpoS), CGSC 6300 (parent of 7460), CGSC 7460 (rbsD), following growth for 8 h (open bars) or 24 h (solid bars) in LB (LB) or LB supplemented with 0.4% glucose (LBGlu). Each strain was assayed carrying pKH4. (b) The fold induction (compared to following addition of *E. coli* DH5α spent supernatant) of bioluminescence by *V. harveyi* BB170 is shown for spent supernatants harvested from *E. coli* strains CGSC 6152 (rbsR), CGSC 7585 (rbsRB), CGSC 6300 (parent of 7460), CGSC 7460 (rbsD), following growth for 8 h (open bars) or 24 h (solid bars) in LB (LB) or LB supplemented with 0.4% glucose (LBGlu). Each strain was assayed carrying pKH4. The fold induction (compared to following addition of *E. coli* DH5α spent supernatant) of bioluminescence by *V. harveyi* BB170 is shown for spent supernatants harvested from *E. coli* strains TR1-5 (craA), BJ4 L1 (rpoS), CGSC 6388 (trg), CGSC 517 (mgl) following growth for 5 h (solid bars), 6 h (open bars), 7-5 h (gray bars) or 24 h (striped bars) in LB (LB) or LB supplemented with 0.4% glucose (LBGlu). Each strain was assayed carrying pKH4 to provide LuxS.

Regulation of AI-2 uptake is unlikely to result from conventional catabolite repression as mutations in *crp* (cyclic AMP receptor protein), rpoS (starvation-induced sigma factor), cya (adenylate cyclase), csaA (carbon storage regulator), fruR (fructose regulator: Saier et al., 1996), effectors of the stringent response (relA, spoT) and cec (the catabolite-repression-linked sensor kinase–response regulator) did not alter the ability of exogenous glucose to induce clearance of extracellular AI-2 activity (Kolb et al., 1993; Saier et al., 1996). Further studies are under way to identify potential AI-2 uptake mechanisms in *E. coli*, and determine whether they are regulated by the presence of glucose.

Further analysis is required to determine the basis of carbon source dependence of AI-2 activity. This paper has highlighted that there is likely to be a degradation or uptake mechanism for AI-2 in *E. coli*, which may facilitate its degradation AI-2 based on evidence presented in Surette & Bassler (1998); however the latter article does not contain results to this effect. Our results described here clearly show that *E. coli* DH5α is capable of removing AI-2 from the supernatant of stationary-phase cultures in the absence of glucose, indicating that *E. coli* DH5α cells either degrade or take up AI-2. Currently we can not distinguish between these two possibilities. Uptake of AI-2 may be a mechanism of monitoring the population density (quorum sensing), but is also consistent with a role in central metabolism (Winzer et al., 2002a, b). In the latter context, given its proposed structure as a furanosyl borate diester, AI-2 may be acting as a source of carbon or boron (Coulthurst et al., 2002; Winans, 2002; Winzer et al., 2002b). The production of the precursor of AI-2 (4,5-dihydroxy-2,3-pentanedione, DPD) is linked to the flux through the activated methyl cycle; however the production of AI-2 does not correlate with the activity of pfs in the absence of glucose (Beeston & Surette, 2002), suggesting that the production of DPD is a separate event from export of AI-2 activity. AI-2 activity in the culture supernatant is higher in carbohydrate-rich media; thus, like acetate, AI-2 can be produced and excreted in the presence of preferred carbon sources, and utilized when preferred nutrients are exhausted, which in turn suggests that uptake of AI-2 is another independent event, unlinked to synthesis and export.

The ABC transporter Lsr in *S. typhimurium* is proposed to import AI-2 (Taga et al., 2001). In *E. coli*, two transporters show similarity to the *S. typhimurium* Lsr transporter, the products of the *rbs* genes (24% similarity/42% identity for the periplasmic sugar-binding protein), and the products of the unstudied operon b1513 (77% similarity/82% identity for the periplasmic sugar-binding protein): Blattner et al. (1997); Iida et al. (1984). We show here that mutations in different *rbs* genes do not prevent uptake of AI-2 produced by LuxS. This suggests that the products of operon b1513, and not *rbs*, may perform a function analogous to Lsr in *E. coli*. Promotion of GRAIL by galactose in a strain mutated in the gene encoding galactose epimerase may indicate that it exerts its effect at the level of binding to the transport protein, MglB, in the periplasm, rather than following metabolism. In support of a link between sugar uptake and detectable AI-2 activity, mutation of the *mgl* operon encoding the galactose uptake system (which displays the highest level of similarity to ribose uptake systems: periplasmic sugar binding proteins RbsB and MglB are 26% identical/44% similar) resulted in lower, shorter-lived levels of AI-2 activity. The common denominator is however unlikely to be the inner membrane translocator, Trg, to which both RbsB and MglB bind (Falke et al., 1997) since mutation of *trg* did not alter GRAIL.
recycling into central metabolism (discussed by Winzer et al., 2002a, b, Winans, 2002: suggested by Lewis et al., 2001; Fong et al., 2001). It is perhaps interesting to speculate that a role in central metabolism is more important to some bacteria than a role in quorum sensing. V. harveyi luminescence is clearly a quorum-sensing phenomenon; however no obvious phenotypes could be attributed to loss of luxS in other organisms, suggesting that it may play a more subtle role in their life cycle. Pertinently, many of these studies were performed in nutrient-rich medium, where recycling of homocysteine is less important. This duel functionality dictates that studies to show the effects of luxS upon bacterial physiology must incorporate complementation with both the gene (to discount effects caused by second-site mutation) and purified AI-2 (to determine whether effects are likely to be mediated via quorum sensing).

ACKNOWLEDGEMENTS

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