In *Helicobacter pylori* auto-inducer-2, but not LuxS/MccAB catalysed reverse transsulphuration, regulates motility through modulation of flagellar gene transcription

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**Abstract**

**Background:** LuxS may function as a metabolic enzyme or as the synthase of a quorum sensing signalling molecule, auto-inducer-2 (AI-2); hence, the mechanism underlying phenotypic changes upon luxS inactivation is not always clear. In *Helicobacter pylori*, we have recently shown that, rather than functioning in recycling methionine as in most bacteria, LuxS (along with newly-characterised MccA and MccB), synthesises cysteine via reverse transsulphuration. In this study, we investigated whether and how LuxS controls motility of *H. pylori*, specifically if it has its effects via luxS-required cysteine metabolism or via AI-2 synthesis only.

**Results:** We report that disruption of luxS renders *H. pylori* non-motile in soft agar and by microscopy, whereas disruption of mccA\(_{Hp}\) or mccB\(_{Hp}\) (other genes in the cysteine provision pathway) does not, implying that the lost phenotype is not due to disrupted cysteine provision. The motility defect of the ΔluxS\(_{Hp}\) mutant was complemented genetically by luxS\(_{Hp}\) and also by addition of *in vitro* synthesised AI-2 or 4, 5-dihydroxy-2, 3-pentanedione (DPD, the precursor of AI-2). In contrast, exogenously added cysteine could not restore motility to the ΔluxS\(_{Hp}\) mutant, confirming that AI-2 synthesis, but not the metabolic effect of LuxS was important. Microscopy showed reduced number and length of flagella in the ΔluxS\(_{Hp}\) mutant. Immunoblotting identified decreased levels of FlaA and FlgE but not FlaB in the ΔluxS\(_{Hp}\) mutant, and RT-PCR showed that the expression of flaA, flgE, motA, motB, flhA and fliI but not flaB was reduced. Addition of DPD but not cysteine to the ΔluxS\(_{Hp}\) mutant restored flagellar gene transcription, and the number and length of flagella.

**Conclusions:** Our data show that as well as being a metabolic enzyme, *H. pylori* LuxS has an alternative role in regulation of motility by modulating flagellar transcripts and flagellar biosynthesis through production of the signalling molecule AI-2.

**Background**

Many bacteria release extra-cellular signalling molecules (auto-inducers) to perform intercellular communication. It is generally assumed that auto-inducers are employed to regulate aspects of bacterial behaviour in response to cell population density (so-called quorum sensing). This includes changes in the expression of genes crucial for bacterial survival or virulence [1,2]. Auto-inducer-2 (AI-2) production is widespread among bacterial species; its formation is catalysed by the enzyme LuxS [3]. Many Gram-positive and Gram-negative bacterial species possess LuxS, and in some it has been shown to catalyse AI-2 production and to control quorum sensing (QS). Good examples include *Vibrio harveyi* and *Vibrio cholera*, where AI-2 has been shown to regulate density-dependent bioluminescence and virulence factor production, respectively [4,5]. luxS inactivation has also been shown to cause phenotypic alterations such as biofilm formation, changes in motility, toxin production, and...
reduced colonisation in various experimental infection models [3,6].

In addition to its QS role, LuxS catalyses one of the steps of the activated methyl cycle (AMC). The AMC is a central metabolic pathway that generates the S-adenosylmethionine (SAM) required by methyltransferases allowing the widespread methylation of proteins and DNA needed for cell function. It recycles the toxic product of these reactions, S-adenosylhomocysteine (SAH), to help provide the cell with sulphur-containing amino acids [7]. As part of the AMC, the Pfs enzyme, 5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase converts SAH to S-ribosylhomocysteine (SRH) which is subsequently converted to homocysteine by LuxS. The precursor of AI-2, 4, 5-dihydroxy-2, 3-pentanedione (DPD) is generated as a by-product of this reaction. Through a process of dehydration and spontaneous cyclisation, some or all of the DPD is rearranged into a cocktail of chemically related molecules known as AI-2, including 4-hydroxy-5-methyl-3 (2H) furanone, (2R, 4S) -2-methyl-2, 3, 3, 4-tetrahydroxy-tetrahydrofuran and furanosyl borate diester. These have been shown to function as signals of communication between bacteria [3,8,9]. In some organisms, the AMC is different. For example, in Pseudomonas aeruginosa, LuxS and Pfs are replaced by a single enzyme (SAH hydrolase) which converts SAH to homocysteine in a one step reaction without the concomitant production of DPD [7].

Helicobacter pylori, a Gram-negative bacterium which causes peptic ulceration, gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma, contains a luxS homologue and produces AI-2 [10-12]. luxSHp (HP010526695; JHP0097J99) is positioned next to housekeeping genes mccAHp (HP010726695; JHP0099J99) and mccBHp (HP010626695; JHP0098J99) on the H. pylori chromosome, in a putative operon [13-15]. Data from our laboratory have demonstrated that the AMC of H. pylori is incomplete, and that LuxS Hp, MccA Hp and MccB Hp constitute the sole cysteine biosynthetic pathway in this bacterium via a reverse transsulphuration pathway (RTSP) [15].

To date, the mechanisms underlying phenotypic changes exhibited as a result of luxS Hp inactivation remain elusive. Two ΔluxS Hp mutants have been shown to form biofilms more efficiently than the parent strain, indicating a possible but counterintuitive role of luxS Hp in biofilm reduction [16]. A subsequent study demonstrated that ΔluxS Hp mutants in two strains lost growth-phase-dependent regulation of the gene encoding the major flagellin FlaA, and that cell culture supernatant containing AI-2 could increase flaA transcription [17]. Studies by two independent groups looked at fitness of ΔluxS Hp mutants in vivo using mouse and gerbil models, respectively [18,19]. The motility of ΔluxS Hp mutants was diminished and bacterial fitness reduced in co-infection experiments. Restoration of luxS Hp by genetic complementation partially restored these phenotypes [18,19]. The authors argued that the decreased fitness in the ΔluxS Hp mutant was most likely due to the disruption of the cycle of SRH consumption and homocysteine synthesis and that AI-2 seemed unlikely to be a QS signal molecule [18]. More recently however, Rader et al. reported that luxS Hp disruption affected flagellar morphology in the absence of one of the transcriptional regulators (g28, flgS or flgM), and that this could be complemented upon the addition of DPD. They reported that loss of luxS Hp caused decreased transcription of the flagellar regulator flaA, and that expression of flaA was induced by DPD [20]. This complementation through the addition of exogenous DPD resurrected the possibility of LuxS-dependent signalling in H. pylori.

There are several possible mechanisms whereby a motility defect could be associated with loss of luxS Hp. Firstly, reduced flagellar structural gene transcription and related protein synthesis would lead to loss of flagella. Secondly, normal flagella structures may be synthesised in the ΔluxS mutant but lack of a functional motor may prevent rotation. Thirdly, both motor and flagellum may be functional, but unable to respond to tactic signals, leading to aimless movement.

In this study, we set out to distinguish between the mechanisms underlying the alteration in motility of ΔluxS Hp mutants, and to clarify whether this originated from a disruption of metabolism or QS. To do this, electron microscopy was employed to examine flagellar assembly and the levels of individual components of flagella were assessed at a transcriptional and translational level. Our demonstration here of the lack of motility defects in mutants disrupted in components of the RTSP other than LuxS, coupled to the inability of cysteine to complement the motility defect of the ΔluxS Hp mutant, shows that disruption of cysteine biosynthesis is not the mechanism underlying the reduction in motility. In contrast, we show that exogenously added AI-2 (or DPD) influences motility via regulating flagellar gene transcription (and thus the number and length of flagella). This supports the existence of an additional role for LuxS in H. pylori as a signalling molecule synthase.

Methods

Strains and growth culture conditions

All strains used in this study are listed in Table 1. DH5α was used in the production of proteins needed for AI-2 biosynthesis and cloning [21]. V. harveyi BB170 was used in the bioluminescence bioassay as a reporter strain [22]. E. coli strains were routinely grown in Luria-Bertani (LB) (Bacto) broth or on agar plates at 37°C.
**Table 1 Strains and plasmids used in this study**

| Strains/Plasmids | Description | Reference |
|------------------|-------------|-----------|
| **Strains**      |             |           |
| **Vibrio harveyi** |             |           |
| BB170            | luxN: Tn5 AI-1 sensor negative; AI-2 sensor positive | [43] |
| **Escherichia coli** |             |           |
| DH5α             | endA1 recA1 gyrA96 thi-1 hsdR17(ω, mcrA) relA1 supE44∆lacZYA-argF U169 FΔ80lacZΔM15 deoA phoA A1 | [21] |
| DH5α LuxS        | DH5α containing the plasmid pProEx-luxS<sub>EC</sub> | [8] |
| DH5α Pfs         | DH5α containing the plasmid pProEx HT mtan | [8] |
| **Helicobacter pylori** |             |           |
| J99 (ATCC700824) | Wild-type motile strain | [44] |
| J99 ΔluxS        | J99 derivative; ΔluxS:: km; Km<sup>+</sup> | [15] |
| J99ΔluxS-F       | J99 derivative; ΔluxS:: km-sacB; Km<sup>+</sup> Suc<sup>−</sup> | This study |
| J99 ΔluxS<sup>+</sup> | J99ΔluxS-F derivative; ΔluxS:: km-sacB replaced with original luxS locus; Suc<sup>+</sup> Km<sup>+</sup> | This study |
| J99 ΔmccA        | J99 derivative; ΔmccA:: km; Km<sup>+</sup> | [15] |
| J99 ΔmccB        | J99 derivative; ΔmccB:: km; Km<sup>+</sup> | [15] |
| J99 ΔflhB        | J99 derivative; ΔHP0770 Lys<sup>+</sup> to Glu<sup>−</sup>; Km<sup>+</sup>; non-motile | [24] |
| CCUG 17874*      | Wild-type strain | [29] |
| 17874 ΔflaA      | 17874 derivative; ΔflaA:: cat; Cm<sup>+</sup> | Paul O'Toole |
| 17874 ΔflgE      | 17874 derivative; ΔflgE:: km; Km<sup>+</sup> | [30] |
| **Plasmids**     |             |           |
| pGEMT            | Commercial TA cloning vector; Amp<sup>+</sup> | Promega |
| pGEMTluxSXN396   | pGEM-T with inserted 26695 luxS; ΔluxS:: km-sacB; Suc<sup>+</sup> Km<sup>+</sup> | [17] |
| pGEMTluxS        | pGEM-T with inserted full-length luxS fragment | This study |
| pProEx-luxS<sub>EC</sub> | pProEX HT containing the luxS gene of E. coli MG1655 | [8] |
| pProEx HT mtan   | pProEX HT containing the pfs gene of E. coli | [8] |

* CCUG 17874 is identical to the type strain NCTC 11637, isolated by B. J. Marshall at Royal Perth Hospital, May 1982 [29].

V. harveyi was grown in LB or AB medium [23] at 30°C, also under normal atmospheric conditions. H. pylori strains were routinely grown and maintained on Columbia blood agar plates (No.2, with 5% [v/v] horse blood; Oxoid) or grown in Brucella broth (Bacto) containing 7% (v/v) fetal bovine serum (Gibco). H. pylori J99 was incubated at 37 °C for 24 h to 72 h as required in a MG500 VAIN-cabinet (Don Whitley Scientific) in an atmosphere of 5% CO<sub>2</sub>, 86% N<sub>2</sub>, and 5% O<sub>2</sub> (all v/v). For motility experiments the method of Wand et al. [24] was used to achieve motile cultures for analysis, see below. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 30 μg/ml.

**Molecular biology methods**

Preparation of plasmid DNA, DNA ligation, gel electrophoresis and transformation of E. coli strains were performed in accordance with standard methods [25]. All PCRs were performed with Taq DNA polymerase (Roche Diagnostics, Lewes, UK). TA cloning was carried out using the pGEM-T vector system (Promega, Madison, WI). Plasmid DNA was extracted using the QIAquick spin miniprep kit (QIAGEN, UK). DNA fragments were purified from agarose gel using a QIAquick gel extraction kit (QIAquick, UK) according to the manufacturer’s instructions.

H. pylori genomic DNA was isolated as described previously [26]. DNA sequencing was conducted using standard fluorescent dye terminator chemistries, and analysis performed using the Applied Biosystems 3730 DNA Analyzer system (Geneservice, Cambridge, UK, Applied Biosystems Inc, Foster City, CA.). Results were analysed using the Bioedit software suite [27].

**Construction of the complemented ΔluxS<sup>+</sup> strain**

H. pylori J99 wild-type was transformed with the plasmid pGEMTluxSXN396 containing a km-sacB construct encoding kanamycin resistance (Km<sup>+</sup>) and (5%) sucrose sensitivity (Suc<sup>−</sup>) [17]. Disruption of the chromosomal luxS gene was accomplished by natural transformation, allelic exchange, and screening for kanamycin-resistance as previously described [15], resulting in the J99 ΔluxS mutant strain. The presence of the km-sacB cassette was verified by amplifying fragments of H. pylori chromosomal DNA.
using primers luxS-F/luxS-R (forward, 5’-GTG GCT TTA GCG GGA TGT ‘TTT<3’; reverse, 5’-GCGA ACA AAT CCC CGC TG<3’) and DNA sequencing. The J99 ΔluxS was then transformed with plasmid pGEMTluxS (encoding wild-type luxS), and transformants in which km-sacB had been replaced with the introduced original luxS locus were selected for sucrose resistance on medium containing 5% sucrose and screened for kanamycin sensitivity. The presence of the original luxS gene was verified by amplifying fragments on H. pylori chromosomal DNA using primers luxS-F/luxS-R and DNA sequencing.

Bacterial growth curves and V. harveyi bioluminescence assay
Bacterial broth cultures were started from a blood agar plate culture, diluted to an OD600 nm of 0.05 in fresh BB medium, and grown at 37°C in a VAIN-cabinet with shaking. OD600 nm measurements were taken at the 6 h, 24 h, 48 h and 72 h time points, and at the same time cell suspensions were harvested and filtered through a 0.2 μm pore size filter. The AI-2 activity in cell free supernatants (CFS) was tested as previously described using the V. harveyi reporter strain BB170 [9,22]. Briefly, an overnight V. harveyi culture was diluted 1:2500 in fresh AB medium [23]. CFS samples were diluted 1:10 in the AB medium containing BB170 into the 96 well bioluminescence plates to give a final volume of 200 μl and were incubated at 30°C. The bioluminescence and optical density were determined at 30 min intervals for at least 8 h using a luminometer (Anthos Labtech LUCY 1.0). AI-2 activity alterations in bioluminescence were expressed as induction (n-fold) over the negative control.

Motility assay
Plate motility assay of H. pylori was performed in Brucella broth medium (BD Biosciences), supplemented with 7% (v/v) fetal bovine serum (Gibco), 0.35%-0.45% (w/v) agar (No.1, Oxoid) and the indicator, 40 μg/ml triphenyl tetrazolium chloride (Sigma, UK). Inclusion of this indicator made it easier to see the small recombinant colonies. Plates were seeded with 5 μl H. pylori liquid culture (forming a circle with 3 mm diameter) standardised to an OD600 nm of 1.0 and were incubated at 37°C for up to 7 days under the conditions described above. The motility halos were recorded using a digital camera and the area of each halo was measured using a GS-800 Calibrated Densitometer (Biorad).

Plate motility bioassay using chemically defined media (CDM)
The liquid chemically defined media were prepared as previously described [15,28]. 60 ml of sterile chemically defined media were added to 40 ml of molten 1% Oxoid No. 1 agar base to make 0.4% semi-solid chemically defined agar. Cysteine supplemented plates (CSP) were made by adding cysteine to the molten agar, shortly before it set. The final concentration of cysteine was 1.0 mM, which was non-limiting for H. pylori growth. The centre of each plate was seeded with one-day incubated H. pylori cells and was incubated for 5 days under the conditions described above. The motility halos were recorded using a digital camera and the area of each halo was measured using a GS-800 Calibrated Densitometer (Biorad).

Motility assay with AI-2 complementation
AI-2 was synthesised enzymatically as described previously using purified proteins LuxS_E.coli and Pfs_E.coli [8]. For complementation of the ΔluxS_Hp motility phenotype, soft motility agar plates (0.4% w/v) were made as previously described. Bioluminescence activity of the AI-2 product was quantified using the V. harveyi bioassay and compared to CFS from H. pylori wild-type broth culture standardised to an OD600 nm of 1.0 at the time point in the growth curve that maximal AI-2 activity was measured. 1/400 diluted in vitro synthesised AI-2 product shows the same level of bioluminescence as seen in the H. pylori wild-type CFS in the V. harveyi bioassay. Therefore, in the complementation experiment AI-2 was added to motility plates to a final concentration of 0.25% (v/v). 24 h H. pylori cultures were seeded individually onto the centre of each motility plate and incubated for 5 days. The area of outward migration was recorded with a digital camera and measured using a GS-800 Calibrated Densitometer (Biorad).

Tissue culture and bacterial co-culture
All chemicals were obtained from Gibco, UK. AGS cells were grown in nutrient mixture Ham’s F-12 supplemented with L-glutamine (200 mM) and fetal bovine serum (Gibco) (10% v/v) in a 37°C incubator containing 5% CO2. After cells had grown to confluency, a 1 in 5 or 1 in 8 dilution was added to a 75 cm2 flask containing fresh media mix and incubated in the same conditions as before to allow cells to re-grow to confluency.

AGS cells were counted using the trypan blue dye method. Cells were seeded at a density of 1 x 10^5 cells/ml into 6 well plates and grown to 80% confluency.
The cell-media mix was removed and replaced with 2 ml fresh F-12 media. Plates were inoculated with 24 h *H. pylori* liquid cultures standardised to an OD_{600 nm} of 0.1 and incubated for one day in a microaerobic environment. Bacterial cells were then analysed using a phase-contrast Nikon Eclipse E600 microscope and electron microscopy.

**Electron microscopy (EM)**

*H. pylori* cells were pre-grown as described above for motility analysis. 15 μl of culture was allowed to settle on a carbon formvar grid (Agar Scientific) for 1 min. The suspension was removed and the grid washed by addition of 15 μl of Phosphate Buffered Saline (PBS) for an additional minute. This was removed and the cells stained with 0.5% Phospho-tungstic acid (PTA) pH 7.0 for 1 min. Grids were examined and pictures taken using a JEOL JEM1010 Transmission Electron Microscope. We quantified changes, rounding to the nearest 5% and quote means ± SD. Essentially, three groups of *H. pylori* cell samples prepared on different dates were examined. Each group of samples contained wild-type, ΔluxS and ΔluxS' cells treated and not treated with DPD. For each group, 100 *H. pylori* cells from each culture sample were examined.

**Cysteine and DPD complementation experiments**

Cysteine from Sigma products was dissolved in distilled water according to the manufacturer’s recommendation. Synthetic DPD was purchased from Omm Scientific Inc. DPD (AI-2) activity was quantified with the bioluminescence bioassay and compared to wild-type *H. pylori* grown to an OD_{600 nm} of 1.0, at which maximal AI-2 activity was obtained. To test for complementation of motility, DPD (at a physiological concentration of 150 μM) and non-limiting cysteine (1.0 mM) were added individually to bacteria-AGS cell co-cultures. DPD was added after 10 h of incubation and once again after 18 h of incubation. Cysteine was added from the beginning of incubation. Bacterial motility and cells were observed and visualized by phase-contrast microscope and EM, respectively. For gene transcription studies, DPD (150 μM) and cysteine (1.0 mM) were also added (in the same way) individually to *H. pylori* liquid cultures of different genotypes. After 24 h, RNA was extracted and the transcript levels of genes of interest were measured.

**Protein electrophoresis and western blotting**

*H. pylori* wild-type, its ΔluxS_Hp mutant, the complemented ΔluxS_Hp mutant and controls (*H. pylori* wild-type 17874 [29], and derived mutants ΔflaA (a kind gift from Paul O’Toole) and ΔfgeE [30]) were grown in Brucella broth at 37°C for up to 24 h, at which point high levels of AI-2 activity were detected. To exclude global differences in protein production between strains, we corrected our loading for numbers of bacteria rather than for total protein levels. To do this, 24 h liquid (Brucella broth) culture of each strain was adjusted to OD_{600 nm} of 1.0. A 500 μl cell sample of each strain was then centrifuged at 5500 rpm for 1 min. Culture supernatants were removed and cell pellets were fully resuspended in 1 ml sterile PBS. 100 μl protein sample was collected. The same volume of 2 x sample buffer was added and boiled for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting were carried out as described previously under standard conditions [25]. The gel contained 10% acrylamide. 4 μl protein stock from each strain sample was loaded into each well of the SDS-PAGE gel. For immunoblotting, proteins were transferred from SDS-PAGE gels to nitrocellulose paper by the methanol Tris-glycine system described by Towbin et al. [31]. To see whether similar amounts of protein were loaded using our methodology, membranes were inspected following Ponceau red staining prior to immunoblotting; protein levels appeared similar on each membrane by inspection. The blots were incubated with rabbit polyclonal antibodies against *H. pylori* flagellin and hook protein (a generous gift from Paul O’Toole) [32]. Bound antibodies were detected using secondary anti-rabbit IgG alkaline phosphatase conjugate antibody (Sigma, UK). The blots were developed using the BCIP/NBT substrate system (Dako, UK). The quantitative scan of the protein bands was performed using a GS-800 Calibrated Densitometer (Biorad). The reflective density (RD) of each protein band was measured using the Quantity One 4.6.5 software (Biorad).

**RNA extraction and transcription analysis**

RNA was isolated from *H. pylori* cells grown in BB medium for 24 h. Cultures were treated with RNA protection reagent (QIAGEN, UK) and RNA was extracted using RNeasy mini kit (QIAGEN, UK). Contaminating genomic DNA was removed using a DNA free kit (Ambion). Synthesis of cDNA was performed using Ominiscript RT kit (QIAGEN, UK) and random hexamers (Roche, Germany). Quantitation of transcripts of selected genes of interest was accomplished by quantitative reverse transcription-PCRs (qRT-PCRs) using Rotor-gene 3000. Primers utilised in RT-PCRs are listed in Table 2. All RT-PCR reaction mixtures contained 12.5 μl of SYBR Green Mix (QIAGEN, UK), 5 μl of gene specific primers, 2 μl cDNA template (cDNA was diluted 10-fold prior to adding into the RT-PCR reactions) and RNase free water to a final volume of 25 μl. The amplification program was 95°C for 15 min, followed by 35 cycles of 95°C for 15 sec, 56°C for 60 sec, and 72°C for 30 sec. All samples, including the
controls (16S rRNA and no-template), were run in triplicate. Transcript levels of each gene were normalised to the 16S rRNA in each sample. The relative quantity of transcription of each gene was obtained using Pfaffl’s analytical methodology.

Statistics
Statistical analysis was by Student’s t test.

Results
The H. pylori ΔluxS mutant lost the ability to produce AI-2 while the wild-type, ΔmccAHp, and ΔmccBHp mutants did not

Our previous study has demonstrated that luxS Hp, mccA Hp, and mccB Hp genes comprise a reverse transphosphorylation pathway in H. pylori, which is the sole cysteine biosynthesis pathway [15]. We then wanted to determine whether these mutants in a motile strain of H. pylori, J99, would be useful in differentiating whether H. pylori motility was affected by luxS associated AI-2 production or by cysteine provision. Firstly, we needed to establish whether mutations in mcc Hp genes in our candidate motile strain J99 changed expression of luxS Hp and AI-2 biosynthesis. To do this, H. pylori J99 wild-type and derived ΔmccA Hp, ΔmccB Hp, and ΔluxS Hp mutants were grown in Brucella broth containing serum (10% v/v). Once they reached logarithmic growth phase, AI-2 activity in the culture supernatant was measured using the V. harveyi AI-2 bioassay previously described [4,8]. As expected, the wild-type produced AI-2 in a growth dependent manner, with AI-2 accumulating during the late logarithmic phase, and reaching maximal levels in the stationary phase. During stationary phase, AI-2 levels decreased and were almost undetectable by 72 h. Similar data were obtained with ΔmccA Hp and ΔmccB Hp mutants, despite the fact that the ΔmccB Hp mutant grew slightly less well than the other mutants and the wild-type. The ΔluxS Hp mutant, unlike the wild-type and the other two mutants, yielded almost undetectable levels of bioluminescence at each time point, indicating that the production of AI-2 is luxS Hp-dependent and that insertion of a kanamycin cassette (aphA3) into mccHp and mccB Hp did not affect expression of the downstream gene luxS Hp (Figure 1A).

Deletion of luxS Hp abolishes motility while the ΔmccA Hp and ΔmccB Hp mutants remained motile

To investigate whether motility of H. pylori was affected by cysteine biosynthesis, we first compared the motility of H. pylori wild-type with ΔluxS Hp, ΔmccA Hp and ΔmccB Hp mutants. To do this, a 24 h liquid culture of each strain was spotted onto each quarter of a semi-solid agar plate and incubated for up to 7 days. The resulting motility halo areas were quantified after 3, 5 and 7 days of incubation. Halo areas that surrounded the wild-type, ΔmccA Hp and ΔmccB Hp strains kept increasing during continuous incubation, although the ΔmccA Hp strain was slightly delayed in comparison to the others. After 7 days of culture, the ΔluxS Hp mutant remained almost non-motile and produced a significantly (p < 0.001) reduced motility halo compared to wild-type, ΔmccA Hp and ΔmccB Hp strains in 3 independent repeat experiments (Figure 1B). After 7 days, the wild-type, ΔmccA Hp and ΔmccB Hp mutants produced halos of (mean ± SD) 8.5 ± 0.6 mm, n = 4; 5.6 ± 0.9 mm, n = 4; and 7.8 ± 0.6 mm, n = 4 increases in diameter, respectively, all significantly greater than the ΔluxS Hp mutant which produced a halo size of 1.1 ± 0.1 mm, n = 4. These results revealed that the reduction in motility was likely a result peculiar to luxS Hp mutation rather than due to disruption of cysteine biosynthesis.

Genetic complementation or exogenous AI-2 can restore the motility defect of the ΔluxS Hp mutant, but exogenous cysteine addition cannot

To rule out the possibility that second site mutations in the ΔluxS Hp mutant were inhibiting motility, genetic complementation was performed to create the ΔluxS Hp strain (see Materials and Methods). The non-motile ΔflhB mutant was used as a negative control [24]. 24 h cultures of wild-type, ΔluxS Hp, ΔluxS Hp+ and ΔflhB Hp strains grown in Brucella broth were individually spotted onto normal motility plates. After 5 days of incubation, the mean halo diameter of the ΔluxS Hp+ strain was 6.9 ± 0.2 mm, n = 4, which was slightly larger than that of the wild-type (4.7 ± 0.7 mm, n = 4). The ΔluxS Hp+ strain was approximately 75% and 45% of the wild-type halo size when grown in the presence of cysteine or D-cysteine, respectively.
and ΔflhB Hp mutants showed non-motile phenotypes (Figure 2A).

To examine whether AI-2 can influence the motility of H. pylori, we assessed the motility of the wild-type, ΔluxS Hp and ΔflhB mutants on AI-2 supplemented plates (ASP). The ASP was prepared using 0.4% soft agar containing in vitro synthesised AI-2 (0.25% v/v). The buffer control plate (BCP) was also produced using 0.4% soft agar into which was added the buffer control solution (0.25% v/v) produced in parallel to in vitro AI-2 synthesis (buffer containing no AI-2). After 5 days of incubation, the halo size of the wild-type on ASP increased by 11.2 ± 0.7 mm, n = 4, compared with a 5.4 ± 0.2 mm, n = 4 increase on the non-supplemented

Figure 1 The ΔluxS Hp mutant of H. pylori strain J99 lacks AI-2 and is non-motile unlike other mutants deficient in cysteine biosynthesis. (A) AI-2 production in J99 wild-type (black column), ΔluxS Hp (red column), ΔmccB Hp (blue column) and ΔmccA Hp (white column) mutants was measured. Strains were grown in Brucella broth, and aliquots were removed at 24 h, 30 h, 48 h and 72 h to assess the optical density (wild-type, square; ΔluxS Hp, diamond; ΔmccB Hp, circle; ΔmccA Hp, triangle) and the amount of AI-2 in the filtered culture supernatant using the V. harveyi bioassay. AI-2 activity is shown as a relative bioluminescence (corrected by OD600nm of H. pylori) in the presence of H. pylori culture supernatants over the negative control (Brucella broth alone). A diluted in vitro synthesised AI-2 sample was utilised as a qualitative positive control [8]. Bioluminescence induced by wild-type, ΔmccB Hp, and ΔmccA Hp strains was significantly greater than that induced by the ΔluxS Hp mutant, as determined by paired Student’s t-test (p < 0.001). The lines represent the growth (OD, righthand axis) and the bars represent the AI-2 activity (bioluminescence, lefthand axis). (B) 5 μl of liquid culture (24 h) of the wild-type, ΔluxS Hp, ΔmccB Hp and ΔmccA Hp mutants was seeded on each quarter of a soft agar plate. After 3, 5 and 7 days of incubation, the motility halo of each strain was recorded using a digital camera. All experiments were done in triplicate: a representative experiment is shown and the mean results are presented in the text.
plate (compare Figure. 2A or the right panel of Figure. 2B with the left panel of Figure. 2B). Whilst the ΔluxSHp mutant was non-motile on the BCP, the halo increased by 4.6 ± 0.4 mm, n = 4 on ASP (Figure 2B). The control strain ΔflhBHp mutant remained non-motile on the ASP (Figure. 2B).

Having established an influence on motility for one of the chemicals reliant on LuxS Hp function (AI-2), we sought to establish whether another (cysteine) would have a similar influence. Our previous studies revealed that exogenous cysteine rescues growth defects of mutants unable to complete cysteine biosynthesis via the RTSP of H. pylori (ΔluxS Hp, ΔmccA Hp and ΔmccB Hp mutants) in chemically defined broth [15]. Chemical complementation of motility was thus performed using chemically defined plates supplemented with 1.0 mM cysteine. Methionine was added to these plates as the sulphur source since all known H. pylori strains are methionine auxotrophs. After 5 days of incubation, wild-type H. pylori and ΔmccA Hp and ΔmccB Hp mutants formed motility halos of 4.9 ± 0.3 mm, n = 4; 3.6 ± 0.6 mm, n = 4; and 4.3 ± 0.9 mm, n = 4 increases in diameter, respectively. The ΔluxS Hp mutant remained non-motile (Figure. 2C).

Taken together, these data indicate that the motility defect of the ΔluxS Hp mutant was restored either
genetically or chemically with Al-2, but not with exogenous cysteine. This suggests that luxS and Al-2 play a role in enhancing bacterial motility, rather than an intact cysteine biosynthesis pathway, implying a likely role of luxS in signalling.

ΔluxS<sub>Hp</sub> mutants have altered flagella morphology and motility patterns

Motility plates effectively indicate motility phenotypes of the population, but do not give any indication of the structure of the motility organelles (flagella), or the motility pattern of individual cells. To characterise the phenotypes underlying the decreased ability of the ΔluxS<sub>Hp</sub> mutant to swarm in soft agar, we examined motility of individual bacterial cells using phase-contrast microscopy and also the flagellar morphology of the cells using electron microscopy. Cells tested included wild-type, ΔluxS<sub>Hp</sub> and ΔluxS<sub>Hp</sub> <sup>+</sup>, all grown in the presence and absence of DPD and cysteine. All cells were grown in co-culture with human gastric adenocarcinoma (AGS) cells for 24 h before testing, as previous experiments in our laboratory have shown that this gives highly reproducible results in H. pylori motility experiments.

Phase-contrast microscopy revealed that > 40% of wild-type and ΔluxS<sub>Hp</sub> <sup>+</sup> cells were motile; whereas less than 2% of ΔluxS<sub>Hp</sub> cells were motile. When grown with exogenous DPD, motile cells again made up > 40% of the population for wild-type and ΔluxS<sub>Hp</sub> <sup>+</sup> cells, but now also made up > 40% of the population for ΔluxS<sub>Hp</sub> cells. Cultures of the ΔluxS<sub>Hp</sub> grown with exogenous cysteine consistently contained less than 2% motile cells. To exclude the possibility that the restoration of motility of ΔluxS<sub>Hp</sub> cells was due to an effect of DPD on AGS cells rather than on H. pylori, we set up a control sample in which the wild-type and ΔluxS<sub>Hp</sub> mutant were co-cultured individually with AGS cells that had been treated with DPD overnight. DPD was washed off with the media before co-culturing. As expected, both wild-type and ΔluxS<sub>Hp</sub> cells in these control cultures showed very similar motility phenotypes to those co-cultured with normal AGS cells, indicating that DPD is a functional signalling molecule to H. pylori cells rather than it working through affecting eukaryotic cells. Moreover, the approximate speed of motile ΔluxS<sub>Hp</sub> cells was visibly lower compared to the wild-type, ΔluxS<sup>+</sup> and all cell samples plus DPD.

Electron microscopic images (Figure. 3) showed that all samples tested (wild-type, ΔluxS<sub>Hp</sub> and ΔluxS<sub>Hp</sub> <sup>+</sup>, grown in the presence or absence of DPD) produced a flagellar filament of some kind in the majority of bacterial cells, but those of the ΔluxS<sub>Hp</sub> strain were consistently short and usually fewer in number. In our experiments, nearly all of the wild-type cells tested had flagella (95% ± 3%, n = 3) and most of these had multiple flagella, which were usually at one pole and typically 3-4 in number (90% ± 3%, n = 3) (Figure. 3A). In contrast, fewer ΔluxS<sub>Hp</sub> cells tested had flagella (70% ± 5%, n = 3) and these were typically shorter and also fewer in number (30% ± 5%, n = 3) of cells had only one or two short flagella (Figure. 3B). The complemented ΔluxS<sub>Hp</sub> <sup>+</sup> cells were similar to wild-type, with nearly all cells possessing 3-4 normal long flagella at least one pole (95% ± 3%, n = 3) (Figure. 3C). Addition of DPD to ΔluxS<sub>Hp</sub> cells also converted them to a wild-type morphology, with the vast majority producing 3-4 wild-type length flagella usually present at a single pole (95% ± 3%, n = 3) (Figure. 3E). Addition of DPD to wild-type cells had little significant effect with nearly all remaining flagellate as before (95% ± 3%, n = 3) although more cells were seen with a flagellum at both poles (Figure. 3D). Addition of DPD to the ΔluxS<sub>Hp</sub> <sup>+</sup> cells had a similar effect, with more cells with flagella at both poles (Figure. 3F).

Mutation of luxS<sub>Hp</sub> resulted in the decreased production of flagellar proteins FlaA and FlgE

The reduced number and length of flagella in ΔluxS<sub>Hp</sub> cells observed by electron microscopy could emanate from a number of different changes in the proteome. As previous work had suggested possible involvement of major flagella proteins, we investigated these first by immunoblotting whole cell lysates. Cell lysates were adjusted so that protein from equivalent numbers of bacteria was loaded (see Materials and Methods), and probed with anti-flagellin (FlaA and FlaB) and anti-FlgE (hook protein) antiserum (Figure. 4). In practice, FlaB levels were very similar between all wild-type and mutant strains and were not shown to vary in our subsequent transcription analysis. Our main aim here was to compare ratios of flagella proteins between wild-types and mutants, so we expressed results of other flagella proteins (FlaA and FlgE) relative to FlaB levels within each strain. H. pylori wild-type 17874, and derived mutants (ΔflaA and ΔflgE) were used as positive and negative controls, respectively. In our experiments, four repetitions were included, when the reflective density (RD) of each protein band was measured using Quantity One 4.6.5 software (Biorad).

We found that all strains tested produced FlaB at approximately the same level (Figure. 4). The reflective density of the FlaB bands of the wild-type, ΔluxS<sub>Hp</sub> mutant and the complemented ΔluxS<sub>Hp</sub> <sup>+</sup> mutant were (means ± SD) 0.210 ± 2.0E-03 RD, n = 4; 0.204 ± 5.8E-04 RD, n = 4; and 0.207 ± 5.8E-04 RD, n = 4, respectively. We expressed all other results (FlaA and FlgE) relative to FlaB in each strain. Mutagenesis of LuxS<sub>Hp</sub> reduced the expression of FlaA relative to FlaB (from
mean 1.60 in the wild-type to 1.23 in the ΔluxSHp mutant, \( p < 0.01 \), and complementation increased the ratio back to wild-type levels (mean 1.70 in the ΔluxH+ mutant, \( p < 0.01 \)).

Next, we examined FlgE expression, and a similar trend was found (wild-type FlgE:FlaB ratio mean 0.74; ΔluxSHp mutant 0.51; complemented ΔluxH+ mutant 0.77; \( p < 0.01 \) for differences between ΔluxSHp mutant and wild-type and complemented stains). These data show that FlaA and FlgE synthesis was reduced relative to FlaB in the ΔluxSHp mutant and these changes were restored by genetic complementation.

AI-2 regulates the transcription of flagellar genes

Previous reports have provided evidence that luxSHp-dependent QS may occur to modulate motility via transcriptional regulation of flaA or flhA [20]. We utilised quantitative RT-PCR (qRT-PCR) to screen for alterations in transcription of these and other genes involved in flagellar assembly to extend our understanding of the regulatory mechanisms that might be involved. To exclude an effect of cysteine biosynthesis, exogenous addition of cysteine was also undertaken. The concentration of cysteine was non-limiting to H. pylori growth.

16S rRNA transcription was used for normalization and ureA served as a non-flagella linked gene control (Figure 5D).

The flagellar genes tested included several from different regulatory hierarchy positions in flagellar synthesis [33]: class 1 genes flhA (encodes flagellar regulator component), motA and motB (encode flagellar motor proteins); class 2 genes flaB (encodes hook-proximal minor flagellin) and flgE (encodes flagellar hook protein); and class 3 gene flaA (encodes major flagellin). flil (encodes membrane-associated export ATPase of the flagellar basal body) was also examined (Figure 5).

For class 1 genes tested, flhA showed a consistent pattern of 1.75 fold reduced transcription (\( p < 0.001 \)), and both motA and motB showed a consistent pattern of 2 fold (\( p < 0.001 \)) reduced transcription in the ΔluxSHp mutant compared to the wild-type (Figure 5A).

For class 2 genes tested, flgE was 1.5 fold (\( p < 0.001 \))
down-regulated in the ΔluxS Hp mutant; while flaB did not exhibit any significant change. flaA was the only class 3 gene tested, which was 3.5 fold (p < 0.001) down-regulated in the ΔluxS Hp mutant compared to the wild-type (Figure 5B). Additionally, the transcript of fliI was also significantly (1.5 fold, p < 0.001) decreased in the mutant (Figure 5C).

The reduced transcription of flhA, motA, motB, flgE, flaA and fliI was restored genetically by the complementation of the mutant with the wild-type luxS Hp gene. Also, 150 μM DPD was sufficient to restore the transcription of these genes in the ΔluxS Hp mutant to levels similar to the wild-type (Figure 5E). Although Figure 5E shows that 50 μM and 150 μM DPD induced almost the same level of bioluminescence as the wild-type, we chose to use 150 μM DPD in the complementation experiment because this concentration was shown to be more reproducible (it has the smaller error bar). In wild-type cells, addition of DPD markedly increased transcription of motA, motB, flaA and flaB, whilst flhA, flgE and fliI only showed a marginal increase. Exogenous addition of cysteine to the ΔluxS Hp mutant did not significantly increase transcription of any of the genes studied; suggesting that addition of cysteine was not able to restore the transcription of flagellar genes (data not shown). Consistent with the analysis of protein levels, these RT-PCR data indicate that luxS Hp disruption has a greater effect upon transcription of flaA than of flaB. Taken together, these data suggest that the effect of LuxS in cysteine metabolism does not regulate expression of flagellar genes, and that the effects on flagellar gene transcription are likely through AI-2 production.

**Discussion**

The function of luxS Hp is controversial due to putative roles both in signalling and metabolism. Disruption of cysteine biosynthesis by independent mutations that had
no influence on AI-2 production did not alter motility. In contrast, the motility defect of a ΔluxSHp mutant of *H. pylori* was genetically complemented by luxS Hp, or chemically complemented by the addition of exogenous AI-2 but not by exogenous cysteine. The processes underlying the loss of motility of the ΔluxS Hp mutant were manifested by fewer and shorter flagella that presumably derived from the altered flagella protein production and the modulated expression of a number of genes linked with flagella assembly and function.

Previous studies have shown that mutations of luxSHp in *H. pylori* diminished motility on soft agar. The altered motility phenotype was restored completely by genetic complementation with luxS Hp or significantly restored by metabolic complementation with wild-type CFS [18-20]. In contrast to our study, in Osaki et al. and Rader et al.’s studies complementation of luxS Hp was performed by placing luxS Hp at a second site in the chromosome rather than at the original locus [19,20]. Like these previous reports, our study shows that abolished motility of J99 ΔluxS Hp mutation was restored entirely by complementation with the luxS Hp gene and significantly by in vitro synthesised AI-2. The previous studies, with complete complementation of motility with luxS Hp through insertion at a new chromosomal locus, argue against polar effects of luxS Hp mutagenesis on other genes which influence motility. Our study, with complementation with luxS Hp through creating a revertant results in similar levels of LuxS Hp to wild-type and thus better shows that the phenotypes attributed to the mutant were not due to secondary mutations elsewhere in the chromosome.

Furthermore, having demonstrated that MccA Hp and MccB Hp function consecutively to convert the product of LuxS Hp (homocysteine) into cysteine as part of the RTSP [15], we reasoned that inactivation of any of these three enzymes would have a similar influence upon cysteine biosynthesis, whilst only the ΔluxS Hp mutant would be devoid of AI-2. Thus, if the reduced motility of the ΔluxS Hp mutant derived from disrupted cysteine biosynthesis, mutants in mccA Hp and mccB Hp would have a similar motility defect. Therefore, we performed an experiment to exclude the possibility that the effect on motility was due to non-specific secondary metabolic effects of LuxS Hp. To do this, wild-type, ΔluxS Hp, ΔmccA Hp, and ΔmccB Hp strains were inoculated on the same motility plate, allowing the production of AI-2 and the biosynthesis of cysteine to be isolated from each other. As expected, only the ΔluxS Hp mutant was non-motile. This, for the first time, suggests that motility of *H. pylori* cannot be affected by disrupting the cysteine provision pathway, but can be blocked by the loss of luxS Hp itself. By using a chemically defined medium, we confirmed the provision of cysteine had no effect on motility of *H. pylori*.

Earlier publications have suggested that AI-2 may not act as a signal in some bacteria but instead may simply be a by-product of the important AMC pathway [9]. In support of this, in some bacteria, production of AI-2 does appear to be associated with metabolic rather than regulatory phenomena [34]. However, data from our motility bioassays using both motility plates and microscopy demonstrate that in *H. pylori* AI-2 (or DPD) controls motility. In our experiments, the shorter flagella observed in the mutant could result from the observed alteration in the FlaA:FlaB ratio as previously described [35,36]. However, proving this would require extensive immuno-EM analysis with anti-FlaA and anti-FlaB antisera, which is beyond the scope of this work. As flaA has been confirmed to be essential for motility in *H. pylori* while flaB is a structural subunit of the flagellar filament which increases motility [35,36], the change of the ratio between flagellins FlaA and FlaB may be one factor resulting in the abolished motility of the ΔluxS Hp mutant. Also, LuxS Hp/AI-2 appears to affect the position of flagella, suggesting that LuxS Hp/AI-2 may affect genes involved in the formation of flagella at the cell poles.

The reduced expression of flagellar motor genes (motA and motB) which control flagellar rotation may be a further factor contributing to slower motility of the ΔluxS Hp mutant although it could also be caused by the lower flagellar number requiring fewer motor units to encircle each flagellar base. Thus it is likely that the flagella in the ΔluxS Hp strain are too short and too few to form effective flagellar propellers to produce *Helicobacter* movement. This is in contrast to a previous report where truncated flagella were only reported in G27 strains that also lacked one of the transcriptional regulators (σ28, flgS or flgM) and where wild-type length flagella were reported for the ΔluxS Hp mutant alone [20]. However, surprisingly in that report, the addition of DPD to the double mutants lengthened the flagellar filaments.

Mutants defective in flhA were previously described as being defective in flagellar apparatus assembly and in motility. Recently Rust and coworkers (2009) reported that the anti-sigma factor for σ28, FlgM, interacts with FlhA at the base of the *Helicobacter* flagellum and this interaction modulates the expression of flagellar genes by σ28 [37]. The decrease in flaA expression, seen in our ΔluxS Hp mutant could explain the change in flagellar length but not via a FlgM-dependent pathway as seen by Rader et al. [20], as Rust and coworkers report that FlgM levels were wild-type in a ΔflhA mutant in *Helicobacter* strains N6 and 88-3887 [37].

Both Rust and co-workers [37] and Neihus and co-workers [33] show that FlaB is not regulated by the same regulatory pathway as FlaA, and as FlaB levels in
our ΔluxS1hp mutant concur with this, the short flagella we observe in the ΔluxS1hp mutant are likely to be predominantly composed of FlaB (normally hook-proximal) flagellins. These may be extended, to give functional length propellers by synthesis and assembly of FlaA in wild-type filaments and in filaments from luxS1hp-complemented ΔluxS1hp bacteria or ΔluxS1hp bacteria+DPD which have longer flagella.

FlaB and FlgE are both part of the regulon that is controlled by the FlgS/FlgR two component system and the sigma factor $\sigma^{54}$ (RpoN) [33]. Interestingly, though no significant change in FlaB was found, FlgE production as well as its gene expression was affected by loss of LuxS/Al-2. This suggests that luxS inactivation might affect transcription of the same class of flagellar genes differently. One possibility is that the FlgR/FlgS-$\sigma^{54}$ regulatory complex might have different effects on the same class of genes when affected by loss of LuxS; another possibility is that there may be additional regulation from the other regulator genes, for example fliH.

Flagellar assembly uses a secretion apparatus similar to type III secretion systems. This is dependent upon export chaperones that protect and transport structural subunits using the membrane-associated export ATPase, FliI [38,39]. Therefore, the decreased transcription of $fliI$ might be another factor in blocking motility via shortened filament length in the ΔluxS1hp mutant as Helicobacter fliI mutants are non-motile and synthesise reduced amounts of flagellin (FlaA, FlaB) and hook protein (FlgE) subunits [38].

In our experiments, the motility defect, down-regulated flagellar gene expression and reduced synthesis of flagellar proteins in the ΔluxS1hp mutant were due to loss of Al-2 only, and not to the metabolic effect of luxS1hp on biosynthesis of cysteine. These results suggest that LuxS/Al-2 is likely to be a functional signalling system contributing to control motility in H. pylori. However, it is still uncertain whether Al-2 functions as a true QS signal in H. pylori, in part because there are no genes encoding proteins that can be confidently identified as components of an Al-2 sensory and regulatory apparatus in H. pylori [13,40]. Also, we cannot exclude the possibility that Al-2 acts through other undefined effects and not as a signalling molecule, although as it is known to have similar effects through signalling in other bacteria, this appears unlikely.

Campylobacter jejuni also possesses a luxS homologue and produces Al-2. Inactivation of luxS in a C. jejuni strain (81-176) also resulted in reduced motility and affected transcription of some genes [41]. However, despite its effect on signalling, Al-2 does not function as a QS molecule in C. jejuni (NCTC 11168) during exponential growth in vitro when a high level of Al-2 is produced [42]. Thus, so far there is no good evidence to ascertain whether Al-2 functions as a true QS signal in this species. In H. pylori, Lee et al. and Osaki et al. looked at fitness of ΔluxS1hp mutants in vivo using mouse and gerbil models, respectively [18,19]. The authors did not favour a QS or even a signalling explanation for the reduced fitness mechanisms but both speculated that it might be caused by metabolic disturbances upon loss of luxS1hp [18,19]. However, it could potentially be explained by reduced signalling leading to reduced motility, and given the ecological niche of H. pylori there would be logic to a signalling (perhaps even QS) system increasing motility. For example, we speculate that if a microcolony of H. pylori in a particular area of the stomach reached a critical size it would be potentially advantageous for flagellar biogenesis to be enhanced so that highly motile bacteria could disseminate to new regions of the stomach. If this hypothesis was confirmed, it would have important implications for H. pylori virulence and for the spread of infection within and between people.

Conclusions
Our study suggests that as well as being a metabolic enzyme in the reverse trans sulphuration pathway, H. pylori LuxS has a second role in regulation of motility by modulating flagellar transcripts and flagellar biosynthesis. This is achieved through production of the signalling molecule Al-2, rather than the metabolic effect of LuxS in cysteine biosynthesis.

List of abbreviations
AMC: activated methyl cycle; AI-2: auto-inducer-2; CFS: cell free supernatant; DPD: 4, 5-dihydroxy-2, 3-pentanedione; QS: quorum sensing; RD: reflective density; RTSP: reverse trans sulphuration pathway.

Authors’ contributions
JCA and KRH contributed to the design and supervision of the study. FS participated in the design of experiments, carried out the study, analysed data and drafted the manuscript. LH and RES contributed to the work of microscopy and flagellar morphology, and wrote the related section of the manuscript. ND contributed to the construction of the ΔluxS mutant. JTL and TLC designed and generated the plasmids needed for the construction of the complemented ΔluxS+ mutant. KRH, RES, TLC, LH and ND gave useful comments to the manuscript. JCA and FS coordinated the manuscript to the final version. All authors read and approved the final manuscript.

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