THE ROLE OF LuxS IN METABOLISM AND SIGNALLING IN *HELICOBACTER PYLORI*

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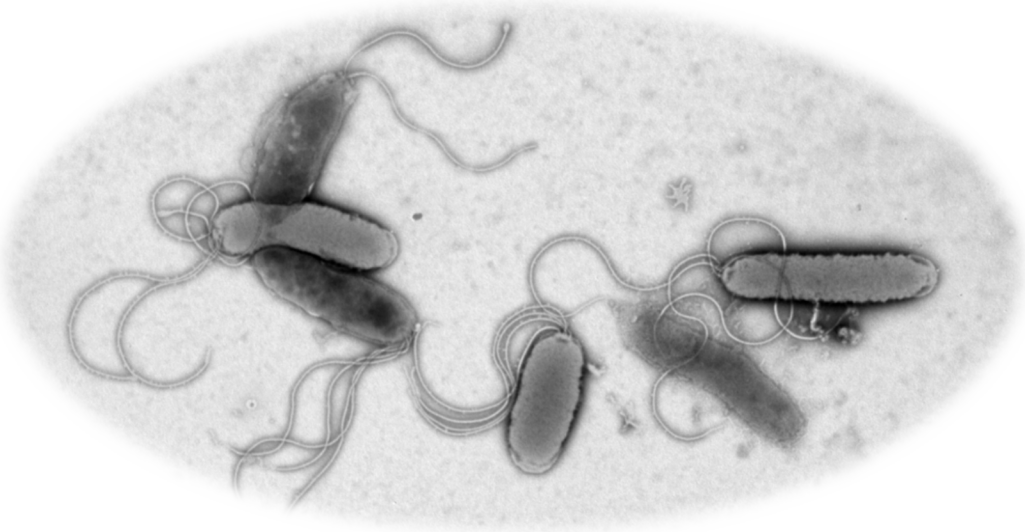

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DECLARATION

I declare that the work presented in this thesis is my own, unless otherwise stated.

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SUMMARY

In many bacteria, LuxS functions as a quorum sensing (QS) molecule synthase. Its product, auto-inducer-2 (AI-2) plays a role in regulation of various bacterial activities in concert with cell population density. LuxS also has a second more central metabolic function in the activated methyl cycle (AMC) which generates the *S*-adenosylmethionine (*S*AM) required by methyltransferases and recycles the product via methionine. *Helicobacter pylori* lacks an enzyme catalysing homocysteine to methionine conversion, rendering the AMC incomplete and thus making any metabolic role of $LuxS_{Hp}$ uncertain. Consequently, the mechanism underlying phenotypic changes upon *luxS* inactivation is not always clear.

The aims of this project were to define the metabolic role of LuxS in *H. pylori*; to assess whether $LuxS_{Hp}$ effects on bacterial motility were through metabolic effects or via production of the signalling molecule AI-2; and to explore the mechanism underlying motility phenotypic changes upon $luxS_{Hp}$ inactivation.

$luxS_{Hp}$ is located next to genes annotated as $cysK_{Hp}$ and $metB_{Hp}$, which are involved in cysteine and methionine metabolism in other bacteria. This study showed that isogenic mutants in $luxS_{Hp}$, $cysK_{Hp}$ and $metB_{Hp}$ could not grow without added cysteine (whereas wild-type could), suggesting roles in cysteine synthesis. Together with data from metabolite analyses, it confirmed that *cysK-metB-luxS* encode the capacity to generate cysteine from products of the incomplete AMC of *H. pylori* in a process of reverse transsulphuration. Consequently, the misnamed genes $cysK_{Hp}$ and $metB_{Hp}$ were recommended to be renamed $mccA_{Hp}$ (methionine-to-cysteine-conversion gene A) and $mccB_{Hp}$, respectively.

Data presented in this thesis also showed that disruption of *luxS* in *H. pylori* renders it non-motile, whereas disruption of *mccA* or *mccB* does not, implying that the loss of phenotype is not due to disruption of cysteine provision. The motility defect of the Δ*luxS*$_{Hp}$ mutant could be genetically complemented with *luxS*$_{Hp}$ and also by addition of *in vitro* synthesised AI-2, but not by addition of cysteine. Microscopy and immuno-blotting further revealed that the motility defect of the Δ*luxS*$_{Hp}$ mutant likely resulted from a reduction in the number and length of flagella due to loss of AI-2. This is supported by data obtained from quantitative RT-PCR (qRT-PCR).

In conclusion, this study looked into the metabolic capacity of a three-gene cluster in *H. pylori,* including *luxS.* It showed that LuxS$_{Hp}$ has a previously undescribed metabolic function in a cysteine provision pathway through a process of reverse transsulphuration. It also defined the precise steps in this pathway, and re-defined the roles of and renamed the two previously misnamed genes in the *luxS*$_{Hp}$ cluster. It then addressed the controversial topic of the role of LuxS in bacteria: apart from being a central metabolic enzyme, is it a QS signalling molecule synthase? This study distinguished between the mechanisms underlying the alteration in motility of *H. pylori* Δ*luxS* mutants, and clarified whether this originated from a disruption of cysteine metabolism or signalling. Results showed that LuxS and its product, AI-2, influence motility via regulating flagellar gene transcription, suggesting the existence of an additional role for LuxS in *H. pylori* as a signalling molecule synthase.

PUBLICATIONS

Neil C. Doherty*, Feifei Shen*, Nigel M. Halliday, David A. Barrett, Kim R. Hardie, Klaus Winzer, and John C. Atherton. **In *Helicobacter pylori,* LuxS is a key enzyme in cysteine provision through a reverse transsulphuration pathway.** *Joint first author. *Journal of Bacteriology* 192: 1184-1192. (2010).

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PRESENTATIONS (ORAL)

***Title*: Motility of *Helicobacter pylori* is dependent on *luxS*-required production of AI-2 but not the effect of *luxS* on sulphur metabolism.**
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Title 1*: The metabolic effect of *luxS* and the functional analysis of *metB* and *cysK* reveal the only cysteine biosynthesis pathway in *Helicobacter pylori.
***Title 2*: Motility of *Helicobacter pylori* is dependent on *luxS*-required production of AI-2, rather than the effect of *luxS* on sulphur metabolism.**
15th International Workshop on *Campylobacter, Helicobacter* and Related Organisms, Niigata, Japan, Sept 2-5, 2009.

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Title*: *luxS* is needed for both metabolism and signalling in *Helicobacter pylori.
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ABBREVIATIONS

A/E lesion	Attaching/Effacing lesion
AHL	*N*-acyl homoserine lactone (auto-inducer 1, or AI-1)
AMC	Activated methyl cycle
AI-2	Auto-inducer-2
AI-3	Auto-inducer-3
AIP	Auto-inducing peptide
Amp	Ampicillin
ASP	AI-2 supplemented plate
agr	Accessory gene regulation
BaBA	Blood-group-antigen-binding adhesion
BB	Brucella broth
BCP	Buffer control plate
CagA	Cytotoxin-associated antigen A
cag-PAI	*cag*-pathogenicity island
CBL	Cystathionine-β-lyase
CBS	Cystathionine-β-synthase
CDM	Chemically defined medium
CFS	Cell free supernatant
CGL	Cystathionine-γ-lyase
CGS	Cystathionine-γ-synthase
CSP	Cysteine supplemented plates
Cys	Cysteine
CTT	Cystathionine
DNA	Deoxyribonucleic acid
dNTPs	dATP, dCTP, dTTP,dGTP mixture
DPD	4,5-dihydroxy-2,3-pentanedione
DupA	Duodenal ulcer promoting A
EDTA	Ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *Escherichia coli*
EM	Electron microscopy

EPEC	enteropathogenic *Escherichia coli*
FBS	Fetal bovine serum
HC	Homocysteine
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Internal standard
Km	Kanamycin
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
Ler	LEE-encoded regulator
MALT	Mucosa-associated lymphoid tissue
NSAIDs	Nonsteroidal anti-inflammatory drugs
*O*AH	*O*-acetylhomoserine (*O*AH)
*O*AS	*O*-acetylserine
OD	Optical density
*O*SH	*O*-succinylhomoserine
PBS	Phosphate buffered saline
PBST	PBS with Tween 20
PCR	Polymerase chain reaction
Per	Plasmid-encoded regulator
Pfs	5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase
PQS	*Pseudomonas* quinolone signal
QS	Quorum sensing
QseA(B,C)	Quorum sensing *E. coli* regulator A (B,C)
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reation
RTSP	Reverse transsulphuration pathway
*S*AH	*S*-adenosyl-homocysteine
SACBP	Sulphate assimilatory cysteine biosynthesis pathway
*S*AM	*S*-adenosyl-methionine
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis

SPI-1	Salmonella pathogenicity island 1
*S*RH	*S*-ribosyl-homocysteine
SSC	Buffer, Sodium chloride and sodium citrate
TAE	Tris-acetate-EDTA
TE	Trisi-EDTA buffer (10mM Tris/HCl, 1mM EDTA, pH 8.0)
TSP	Transsulphuration pathway
TEMED	*N*, *N*, *N*', *N*',-tetramethylethylenediamine
TSS	Toxic shock syndrome
TTSS	Type III secretion system
VacA	Vacuolating cytotoxin A
X-Gal	5-bromo-4-chloro-3 indolyl (-D-galactoside)

CHAPTER 1. INTRODUCTION

1 Introduction

1.1 *Helicobacter pylori* pathogenesis

1.1.1 General information

Spiral bacteria were identified on the human gastric mucosa as early as 1896, but it was not until 1982 that *Helicobacter pylori* was isolated by Barry Marshall and Robin Warren from the stomach of gastritis patients, and research into its implications for human health was initiated.

H. pylori, a Gram-negative plylum is now recognised as one of the most prevalent bacterial pathogens in humans. Phylogeographic studies indicate that humans have been colonized by *H. pylori* since over 58,000 years ago (Blaser *et al.*, 2008, Cover & Blaser, 2009). The bacteria that inhabit the gastric epithelium under the mucus layer can survive in the harsh environment of the stomach (McGee & Mobley, 1999). Most people infected with *H. pylori* have no important clinical sequelae. In a small proportion, however, upper gastrointestinal diseases including peptic ulcerations, and more rarely, gastric adenocarcinoma or gastric mucosa-associated lymphoid tissue (MALT) lymphoma arise as a consequence of infections (Atherton, 2006, Cover & Blaser, 2009, Kusters *et al.*, 2006). *H. pylori* infection is present in almost all patients with duodenal ulcers and 50-90% of patients with gastric ulcers, though gastric and duodenal ulcerations can be caused by other factors, mainly the use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin (Atherton, 2006).

The discovery of *H. pylori* has attracted a great deal of attention in the fields of medicine including bacterial pathogenesis, but much of the biology of this bacterium is still poorly understood. In the last two decades, many studies on *H. pylori* have

focused on identifying genes and the functions of their protein products which allow this bacterium to colonize and cause disease in humans.

1.1.2 Prevalence of *H. pylori*

H. pylori is present in human populations throughout the world. Approximately a half of the world's population is infected by *H. pylori*, with a prevalence rate of 20-25% in most developed countries and more than 90% in some developing countries (Atherton, 2006, Dunn *et al.*, 1997, Pounder & Ng, 1995, Santos *et al.*, 2005). In most of North America and Western Europe, prevalence of *H. pylori* increases with age throughout childhood and middle adulthood. In most areas of Africa, Latin America, Eastern Europe, India, and the Middle East, prevalence increases rapidly through childhood to approximate 80% by young adulthood, and then remains high throughout adulthood. This can be explained by an age cohort phenomenon (Fischbach *et al.*, 2002, Pounder & Ng, 1995, Salih & Ipek, 2007). In Japan and parts of China, the association with age is changing to become more similar to that seen in Europe and the USA (Pounder & Ng, 1995, Salih & Ipek, 2007).

In both developed countries and developing countries, *H. pylori* infections begin in childhood in most people and can cause gastric diseases, for instance, peptic ulcers or gastric carcinoma later in life. However, in developed countries, although infections also occur mainly in childhood, children are much less likely to acquire the infection now compared to in the past. This might be due to the improved hygiene or increased antibiotic use in childhood (Fischbach *et al.*, 2002, Pounder & Ng, 1995). Consequently, more sixty year olds are infected than thirty year olds because it was much more common for the sixty year olds to become infected during their childhood.

Taking available data together, higher prevalence of *H. pylori* is associated with increasing age, non-Caucasian ethnicity, lower family income, lower education level, higher size of the family, poorer hygiene, low socio-economic conditions in childhood, higher numbers of siblings and attendance of day-care centres in childhood (Atherton, 2006, Moayyedi *et al.*, 2002, Neri *et al.*, 1996). Generally, *H. pylori* acquisition is thought to be related to overcrowding and poor hygiene in childhood. The lower prevalence in developed countries is thought mostly to be due to better living conditions.

1.1.3 Pathogenicity factors

H. pylori causes gastritis, which in itself does not cause any major adverse effects, but is associated with an increased risk of noncardia gastric adenocarcinoma, gastric lymphoma, and peptic ulcer (Cover & Blaser, 2009). Though *H. pylori* persists lifelong in the human stomach, only approximately 15% of infections result in peptic ulceration and only 0.5%-2% in gastric adenocarcinoma (Atherton, 2006). Development of disease after infection is determined by several factors including the virulence of the infecting *H pylori* strain, the susceptibility of the host and environmental co-factors. The virulence of the infecting *H. pylori* strain is the major disease determinant (Atherton, 2006, Cover & Blaser, 2009). *H. pylori* virulence factors include the vacuolating cytotoxin (VacA), the cytotoxin-associated antigen (CagA), the duodenal ulcer promoting protein (DupA) and the blood-group-antigen-binding adhesin (BabA). These virulence products of *H. pylori* may damage epithelial cells directly or stimulate these cells to produce proinflammatory cytokines, inducing inflammation (Atherton, 2006, Cover & Blaser, 2009, Prinz *et al.*, 2003).

1.2 Quorum sensing

1.2.1 General overview

Bacteria were originally thought to have no means of communication with each other. However, all bacteria produce extracellular molecules. Some are the products of metabolism, some play a role in the ecology of the organism (such as toxins), and some are utilised by quorum sensing (QS) systems as a tool for communication. QS is an inter-bacterial communication behaviour utilising small diffusible hormone-like molecules as auto-inducers to regulate aspects of bacterial activity in concert with cell population density, including modulation of cell population density, response to changes of cellular phenotypes, interaction with hosts and expression of genes crucial for bacterial survival or virulence (Hardie & Heurlier, 2008, Sperandio *et al.*, 2003, Vendeville *et al.*, 2005).

As a novel means of genetic regulation in bacteria, QS has attracted a great deal of attention in recent years. This type of bacterial cell-to-cell signalling which occurs through QS is similar to eukaryotic cell-to-cell signalling which occurs through hormones. The QS system may represent a 'language' that bacteria use to communicate between bacterial cells, and possibly between bacteria and host cells. As the bacterial population density increases, the concentration of QS signalling molecules increases. Once a threshold concentration has been attained, QS auto-inducers bind the corresponding receptors such that the auto-inducer-receptor complex activates or represses QS target genes, including those required for synthesis of QS signalling molecules (Bassler, 2002, Camilli & Bassler, 2006, Sperandio *et al*., 2003, Winzer *et al.*, 2002b, Ng & Bassler, 2009).

So far, different types of QS systems in various bacterial species have been determined and analysed. Examples include but are not restricted to *Vibrio fischeri*, *Vibrio harveyi*, *Vibrio cholerae*, enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7, enteropathogenic *Escherichia coli* (EPEC), *Porphyromonas gingivalis, Pseudomonas aeruginosa, Staphylococcus aureus* and *S. enterica* Serovar Typhimurium (Camilli & Bassler, 2006, Vendeville *et al*., 2005, Williams, 2007).

1.2.2 Quorum sensing in *Vibrio fischeri* and *Vibrio harveyi*

Vibrio fischeri

Bioluminescence was first described in *V. fischeri*, a marine bacterium commensally living in the light organs of the Hawaiian Bobtail Squid. The initial explanation for bioluminescence was that the culture media contained an inhibitor of luminescence, but later it was hypothesised that the luminescence was initiated by the accumulation of auto-inducers (Eberhard, 1972, Nealson & Hastings, 1979, Nealson *et al.*, 1970). Bioluminescence only occurs when *V. fischeri* is at high cell density, when the signalling molecules (auto-inducers) accumulate to a sufficient concentration that *V. fischeri* is able to detect them (Bassler, 2002). This mechanism of cell density sensing was termed QS. Analysis of QS in *V. fischeri* was first carried out by Engebrecht *et al*. (1983). This leads to the basic model for QS in *V. fischeri* and later this was applied to other species of bacteria.

V. fischeri possesses a classic QS system in which signalling molecules called *N*-acyl-L-homoserine lactones (AHLs) are produced by a biosynthesis protein LuxI. The LuxI/AHL QS system plays an important role in the regulation of expression of bioluminescence genes and in bacterial colonization. As the bacterial cell density increases, signalling AHLs diffuses freely in and out of the cell, and they accumulate

and bind to the cytoplasmic receptor protein LuxR. LuxR also acts as the transcriptional activator of the luciferase operon *luxICDABE*. The AHL/LuxR complex recognises a consensus binding sequence (*lux* box) upstream of the *luxICDABE* operon and activates its transcription so that the bacteria emit light (Figure 1.1). AHLs are produced by the synthesis protein LuxI, and expression of *luxI* is also activated by the AHL/LuxR complex, this consequently forms an auto-inducing positive feedback loop. Therefore, when QS circuit engages, the signalling molecules are accumulated in the surrounding environment, and the population of cells switches progressively from low-cell-density mode to high-cell-density QS mode (Ng & Bassler, 2009).

AHLs are primarily involved in intraspecies communication. The production of AHL is widespread in Gram-negative bacteria isolated, from marine and fresh water, plants and animals, and including a variety of pathogens. Many of these Gram-negative bacteria utilise QS systems similar to that of LuxI/LuxR system from *V. fischeri*, for example, *Aeromonas hydrophila*, *Agrobacterium vitiae*, *Burkholderia cenocepacia*, *Burkholderia pseudomallei*, *Chromobacterium violaceum*, *Pseudomonas aureofaciens*, *Pseudomonas syringae*, *Rhizobium leguminosarum*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (Williams, 2007). Many of these bacteria produce multiple AHLs and possess more than one AHL synthase which are usually members of the LuxI protein class. The AHL receptor gene of these AHL producers is usually a member of the LuxR family, which is in most cases located adjacent to the AHL synthase gene (Williams, 2007).

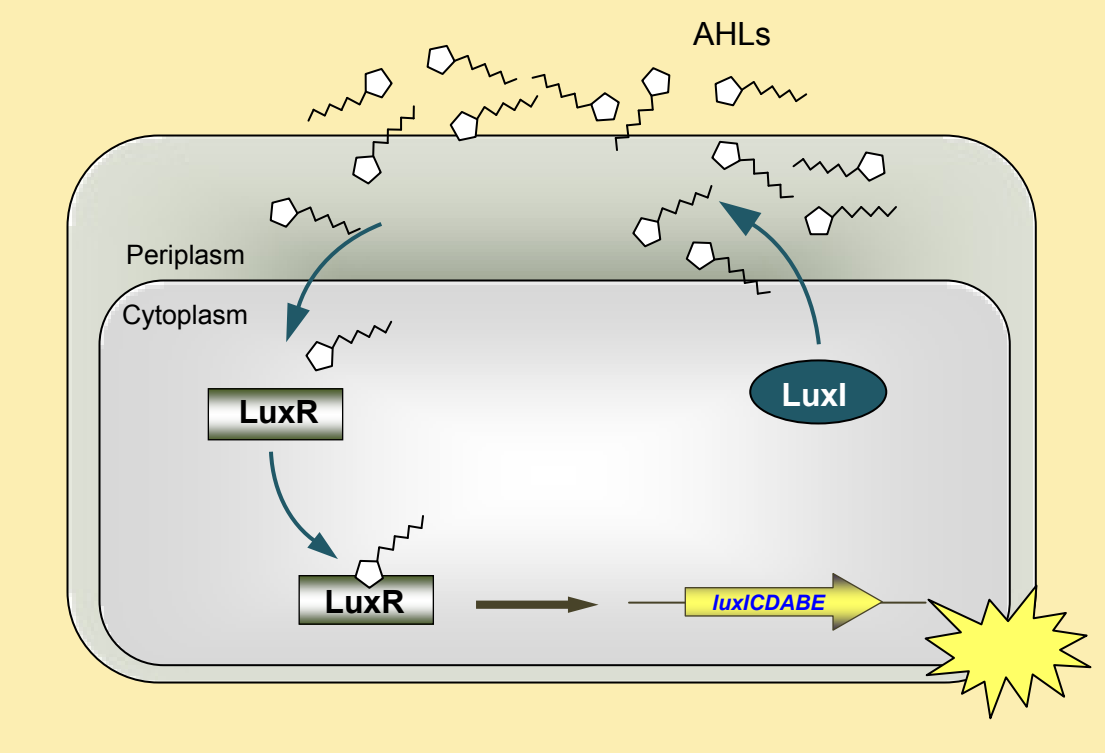


Figure 1.1 The LuxI/LuxR quorum sensing system of *V. fischeri*

Two proteins LuxI and LuxR control quorum sensing in *V. fischeri* and most Gram-negative bacteria. The LuxI proteins are the auto-inducer synthases that catalyse the formation of a specific auto-inducer molecule AHL. The auto-inducer freely diffuses through the cell membrane and accumulates at high cell density. At high auto-inducer concentration, the LuxR-like proteins bind their cognate auto-inducers. The LuxR-AHL complexes recognise the luciferase promoter and activate transcription of *luxICDABE*, forming a positive feedback on LuxI.

Vibrio harveyi

Studies of interspecies communication or QS originated with the free-living bioluminescent marine bacterium *Vibrio harveyi*, in which a new signalling molecule auto-inducer-2 (AI-2) was identified (Bassler *et al.*, 1993). Different from *V. fischeri* whose QS system is composed of LuxI/AHL (AI-1) and a regulator LuxR, *V. harveyi* produces at least three distinct auto-inducers to control bioluminescence, biofilm formation, Type III Secretion (TTS), and protease production (Henke & Bassler, 2004, Mok *et al.*, 2003). One involves the production of AI-1 by the LuxLM protein. Two additional auto-inducer molecules called AI-2 and CAI-1(Figure 1.3 c and d, respectively). AI-2 is an interconverting molecule derived from the precursor 4, 5-dihydroxy-2, 3-pentanedione (DPD), which is synthesised by the enzyme LuxS (Vendeville *et al*., 2005). CAI-1 is presumed to be related to CAI-1 produced by *V. cholera*. CAI-1 has not been purified from *V. harveyi*, but in *V. cholera* it has been identified as (*S*)-3-hydroxytridecan-4-one. CAI-1 is synthesised by the CqsA synthase, and *cqsA* shows sequence homology to aminotransferases (Henke & Bassler, 2004). In *V. harveyi*, AI-1 is detected by the LuxN histidine kinase. AI-2 is detected by the periplasmic protein LuxP in complex with the LuxQ histidine kinase. CAI-1 is detected by the CqsS histidine kinase. LuxN, LuxQ, and CqsS are bi-functional two-component enzymes that possess both kinase and phosphatase activities. The three systems work with an activator LuxR which differs from the *V. fischeri* LuxR protein (Henke & Bassler, 2004, Ng & Bassler, 2009).

At low cell density, LuxN, LuxQ and CqsS act as kinases and transfer phosphate to a shared integrator protein LuxU. LuxU transmits the signal in the form of phosphate to the response regulator protein LuxO (Schauder & Bassler, 2001). LuxO belongs to the NtrC family of response regulators and requires phosphorylation to act as a

transcriptional activator. Together with the alternative sigma facter σ^{54}, phospho-LuxO activates the transcription of genes encoding five small regulatory RNAs (sRNAs) called Qrr1–5. The main target of the Qrr sRNAs is the mRNA which encodes the QS master transcriptional regulator LuxR. At low cell density, LuxR protein is not produced. As a result, the luciferase structural operon *luxCDABE* is not transcribed so that no light is produced (Figure 1.2a) (Lenz *et al.*, 2004, Tu & Bassler, 2007, Ng & Bassler, 2009).

At high cell density, *V. harveyi* AI-1, AI-2 and CAI-1 accumulate to a critical concentration and are detected by protein sensors LuxN, LuxPQ and CqsS, respectively. During this stage, LuxN, LuxPQ, and CqsS receptors function as phosphatases because binding of auto-inducers to the receptors switches them from kinases to phosphatases. Phosphate flow through the signal transduction pathway is reversed, resulting in dephosphorylation and inactivation of LuxO. Consequently, dephospho-LuxO inactivates and terminates the expression of Qrr sRNAs and LuxR protein is produced (Ng & Bassler, 2009). In *V. harveyi* LuxR is demonstrated to be a dual functional protein (both an activator and a repressor). In addition to the luciferase operon, LuxR regulates at least another 50 genes including those encoding the type III secretion system and metalloproteases (Pompeani *et al.*, 2008, Ng & Bassler, 2009).

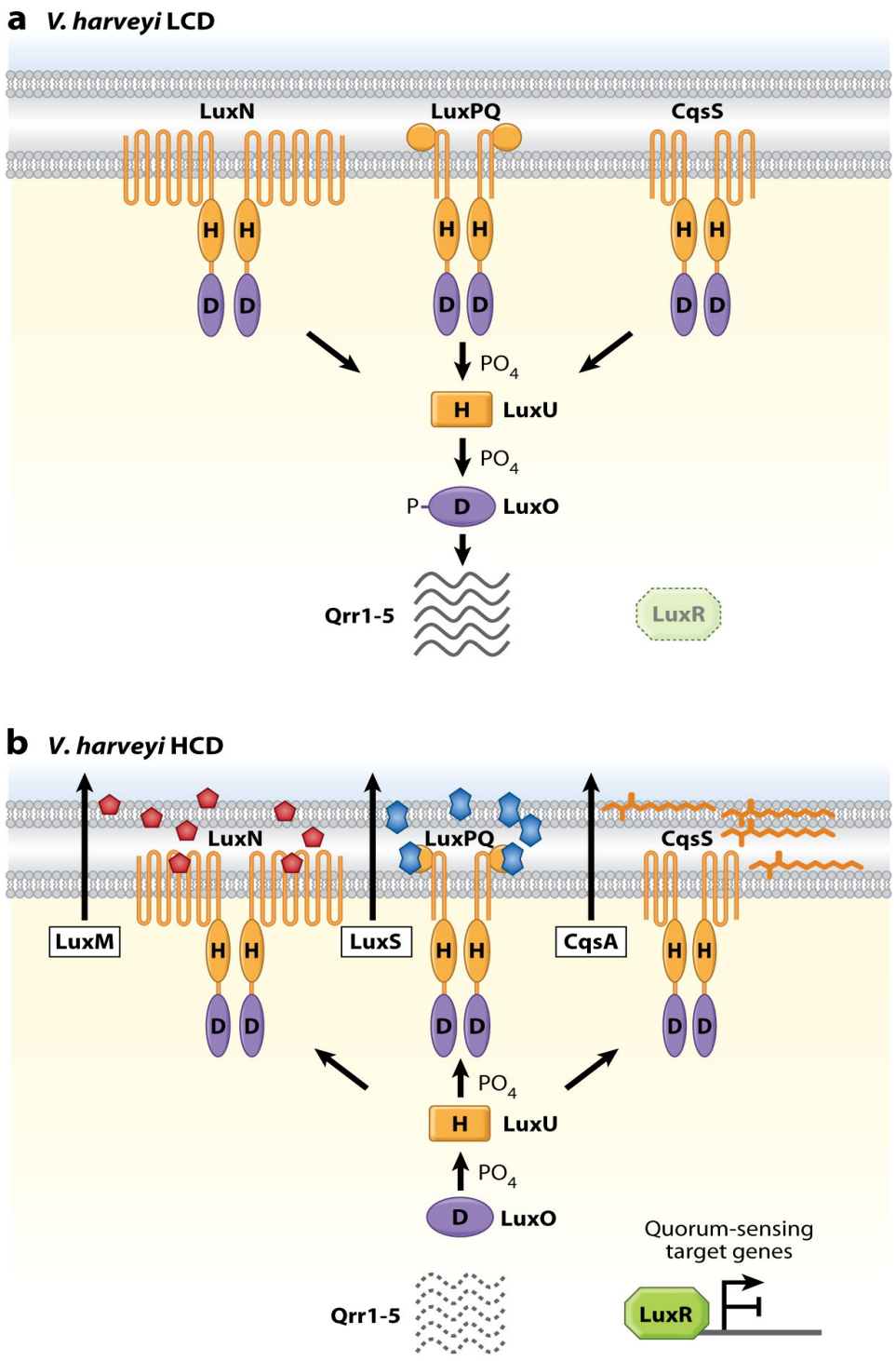


Figure 1.2 The hybrid quorum sensing circuit of *V. harveyi*[*]

(a) At low cell density, auto-inducer levels are low and the LuxN, LuxPQ, and CqsS receptors function as kinases. LuxO is phosphorylated, the Qrr1–5 sRNAs are transcribed, and LuxR protein is not produced. (b) At high cell density, auto-inducer levels are high and the LuxN, LuxPQ, and CqsS receptors function as phosphatases.

[*] Figure 1.2 is from Ng and Bassler, 2009.

LuxO is unphosphorylated, Qrr1–5 sRNAs are not transcribed, and LuxR protein is produced. Solid and dotted lines denote regulatory factors that are produced and not produced, respectively (Ng & Bassler, 2009). Details of the signalling mechanism are given in the text.

1.2.3 Categorisation of quorum sensing systems and auto-inducers

1.2.3.1 General information

Different types of QS systems are widely utilised in either Gram-positive or Gram-negative bacteria or both. Single or multiple auto-inducers may be utilised to coordinate cell actions. Figure 1.3 shows structures of different bacterial auto-inducers. Essentially, Gram-positive bacteria primarily use modified as auto-inducing oligopeptides (AIPs) in QS controlled gene expression systems. Acyl homoserine lactones (AHLs) are a major class of auto-inducer signal used by Gram-negative proteobacteria for intraspecies QS. AI-2 signal is shared by both Gram-negative and Gram-positive bacteria (Ng & Bassler, 2009). In addition, *Pseudomonas aeruginosa* possesses the alkyl quinolone signal (PQS) based system. It controls the expression of numerous virulence factors (Pesci *et al.*, 1999).

AHLs are composed of homoserine lactone rings carrying acyl chains of C_4 to C_{18} in length (Fuqua *et al.*, 2001). Bacterial cell membranes are permeable to most AHLs. It is observed that most short-chain AHL auto-inducers are freely permeable to the Gram-negative cell membrane, similar to what has been demonstrated for the *V. fischeri* AHL, and long-chain AHLs are actively transported (Pearson *et al.*, 1999). However, in contrast to this, AIPs, AI-2s and some AHLs are impermeable to biological membranes, and secretion of these molecules is usually mediated by specialised transporters. In addition, modifications to the initially synthesised

molecules, such as processing and cyclisation (for example DPD), are often associated with secretion (Ng & Bassler, 2009).

There are issues of diffusibility and membrane penetrating ability of many QS signalling molecules that may influence their solubility and the possibility of active transport mechanisms in movement of these molecules out of and into bacterial cells. This can be due to molecular characteristics of different auto-inducing signals, which include the hydophobicity, polarity and amphipathic properties. Therefore, some auto-inducing signals may need particular transport apparatus to be inserted into and transported out through the lipid membrane. For example, in the case of the *P. aeruginosa* auto-inducer *N*-(3-oxododecanoyl)-$_L$-homoserine lactone, there is evidence suggesting that the MexAB-OprM multidrug efflux pump is involved in export of the particular AHL signal (Pearson *et al*., 1999, Maseda *et al.*, 2004).

1.2.3.2 Gram-negative bacteria use homoserine lactones as words

Cell-to-cell communication via the QS system encoded by *V. fischeri luxI-luxR* homologues is the standard mechanism by which Gram-negative bacteria 'talk' to each other. In these bacteria, acylated homoserine lactones (AHLs, or AI-1) are the signalling molecules which are synthesised by LuxI-like synthase. A LuxR-like protein is responsible for recognition of the corresponding AHL auto-inducers and subsequent activation of expression of target genes (Williams, 2007)..

A. tumefaciens, a pathogen that causes crown gall tumors in plants employs the *tra* QS system to control the process of virulence (White & Winans, 2007). The regulatory proteins TraI/TraR play the same role in *A. tumefaciens* as LuxI/LuxR in *V. fischeri*, in which the circuits stimulate expression of target genes and induce

subsequent QS activities. *P. aeruginosa*, the most common Gram-negative bacterium found in nosocomial infections, has two LuxI/LuxR homologue pairs, which are named LasI/LasR and RhlI/RhlR (Gambello & Iglewski, 1991, Ochsner *et al.*, 1994, Latifi *et al.*, 1995). The LasI/LasR and RhlI/RhlR QS systems control the expression of partially overlapping sets of genes. Many of the regulated genes are implicated in virulence and biofilm formation of this opportunistic pathogen (Smith & Iglewski, 2003). The *las* system induces the transcription of virulence factors at high cell density. The *rhl* system further activates genes that have been under LasI/LasR control and activates other target genes. LasI/LasR also activates the transcription of *rhlI*/*rhlR* (Latifi *et al.*, 1996, Latifi *et al.*, 1995). A third LuxR homologue termed OscR which was shown to regulate the transcription of both *lasI* and *rhlI* was identified afterward (Chugani *et al.*, 2001).

Following identification of some of the AHL synthases, subtle differences between the structures of some AHL subtypes were analysed. It is generally known that *N*-(3-hydroxybutanoyl)-$_L$-homoserine lactone is used by *V. fischeri* and *V. harveyi*; *N*-(3-oxooctanoyl)-$_L$-homoserine lactone is utilised by *A. tumefaciens*; in *P. aeruginosa*, at least two different types of AHLs exist. They are *N*-(3-oxododecanoyl)-$_L$-homoserine lactone and *N*-butyryl-$_L$- homoserine lactone (Figure 1.3a) (Camilli & Bassler, 2006, Williams, 2007).

1.2.3.3 Gram-positive bacteria talk using oligopeptides

Gram-positive bacteria also possess QS mechanisms, by which the control of a variety of functions, including virulence, genetic competence and production of antimicrobial components can be modulated in a co-ordinated and population density- or growth phase-dependent manner (Ng & Bassler, 2009). In contrast to

Gram-negative bacteria, signals of intercellular communication in Gram-positive bacteria are usually auto-inducing oligopeptides secreted via an ATP-Binding Cassette (ABC) transporter complex and sensed at a critical concentration by corresponding membrane located receptors (Kleerebezem *et al.*, 1997, Schauder & Bassler, 2001, Ng & Bassler, 2009). The detector of the auto-inducing peptides is a sensor kinase, which transfers the phosphoryl signals to the response regulator, inducing the expression of population density-controlled target genes (Sturme *et al.*, 2002). Occasionally, the auto-inducing oligopeptide has dual functions, working both as a signalling pheromone involved in QS and also as an antimicrobial peptide (Sturme *et al*., 2002).

A number of Gram-positive QS systems have been or are being studied. One of the most intensively analysed systems is the *agr* system in *S. aureus* (Williams, 2007). *S. aureus* utilises an accessory gene regulator (*agr*) QS system to enable cell-to-cell communication and modulate the expression of colonization and virulence factors (Abdelnour *et al.*, 1993). In *S. aureus* the biosynthesis of AIP requires AgrD, the peptide precursor of AIP, and the integral membrane endopeptidase AgrB (Yarwood & Schlievert, 2003, Ng & Bassler, 2009). Other examples of AIP-based QS systems include the ComD/ComE system of *Streptococcus pneumoniae* that controls competence development, and the ComP/ComA system of *Bacillus subtilis* that controls competence and sporulation (Ng & Bassler, 2009). Structures of AIPs produced in these bacterial QS systems are shown in Figure 1.3b.

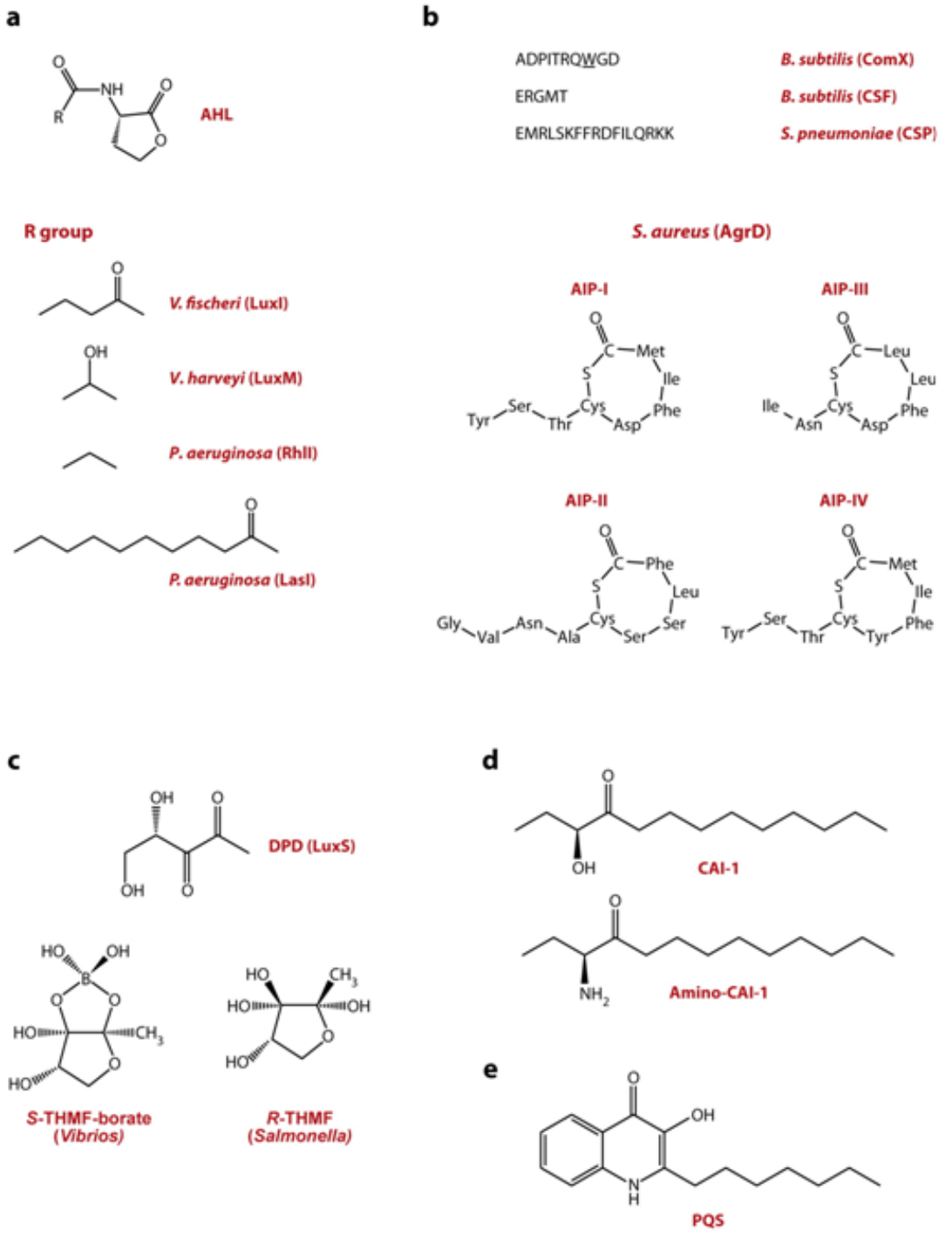


Figure 1.3 Different categories of auto-inducers[†]

Structures of different bacterial auto-inducers. (a) Acyl homoserine lactone auto-inducers produced by Gram-negative bacteria. (b) Amino acid sequences of three different auto-inducing peptides (AIPs) produced by Gram-positive bacteria. The underlined tryptophan in *B. subtilis* ComX is isoprenylated; and the four different AIPs produced by *S. aureus.* (c) DPD, the precursor of AI-2. AI-2 exists as *S*-THMF-borate by the presence of boron, and

[†] Figure 1.3 is from the paper Ng and Bassler, 2009.

exists as *R*-THMF by the absence of boron. (d) Structures of *V. cholerae* CAI-1 and Amino-CAI-1. (e) Structure of the quinolone signal PQS auto-inducer of *P. aeruginosa* (Ng & Bassler, 2009).

1.2.3.4 The universal LuxS-dependent language in multilingual bacteria

Many bacteria utilise multiple languages. *V. harveyi* is such a species in which it employs typical characteristics of both LuxLM/AI-1 system and LuxS/AI-2 system to communicate cell-to-cell. Like *V. harveyi*, some bacteria that contain *luxS* homologues also possess another QS circuit, for example, *Bacillus subtilis* (Lombardia *et al.*, 2006). *luxS* homologues and the dependent production of AI-2 have been found in around half of all bacterial genomes sequenced, including *V. cholerae*, *E. coli, Salmonella typhi, S. Typhimurium*, *B. subtilis, Neisseria meningitidis, Yersinia pestis, Campylobacter jejuni, Mycobacterium tuberculosis, S. aureus, Clostridium perfringens*, *Klebsiella pneumoniae* and *H. pylori* (Sun *et al.*, 2004, Tomb *et al.*, 1997, Vendeville *et al*., 2005). This indicates that the synthase LuxS and AI-2 signalling may define the most widely utilised bacterial language known to date. Some of the examples of *luxS*/AI-2-related QS effects in bacteria are listed in Table 1.1.

AI-2 is involved not only in intraspecies signalling, but also has been proposed to serve as a 'universal' signal forming the basis of interspecies communication (Surette *et al.*, 1999). The precursor of the molecule AI-2, 4, 5-dihydroxy-2, 3-pentanedione (DPD) (Figure 1.3c) is generated as a by-product in the conversion of *S*-ribosylhomocysteine to homocysteine by the enzyme LuxS. The molecule DPD is unstable and can spontaneously cyclise to a range of furanone derivatives (Winzer *et al.*, 2002a). The chemical structure of AI-2 was analysed by co-crystallising this

ligand with its receptor, *V. harveyi* LuxP. The crystallographic analysis reveals that the term AI-2 embraces a group of furanonsyl borate diesters (Chen *et al.*, 2002, Schauder *et al*., 2001, Winzer *et al.*, 2003). Two of these derivatives, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate) and (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) are recognised by specific periplasmic binding receptors in *V. harveyi* and *S. Typhimurium* (Figure 1.3c) (in which AI-2-binding protein is LsrB but not LuxP), respectively (Chen *et al*., 2002, Miller *et al.*, 2004). Another derivative, 4-hydroxy-5-methyl-3(2*H*)-furanone (MHF) as well as two other one-carbon analogues of MHF (structures are not shown) which have been successfully synthesised by LuxS *in vitro*, is capable of inducing bioluminescence in *V. harveyi* (Schauder *et al*., 2001, Vendeville *et al*., 2005).

1.2.3.5 The *Pseudomonas* quinolone signal

It was reported that *P. aeruginosa* produced a third intercellular signal in addition to the two homoserine lactone-type auto-inducers. The third *P. aeruginosa* signalling molecule was identified as 2-heptyl-3-hydroxy-4-quinolone that has been designated as the *Pseudomonas* quinolone signal (PQS) (Figure 1.3e) (Pesci *et al*., 1999). This unique cell-to-cell signal controls multiple virulence factors in the QS cascade, where it appears to be a regulatory link between the *las* and *rhl* QS systems (McKnight *et al.*, 2000, Diggle *et al.*, 2003). However, it has been proposed that the *rhl* system can be activated independently of the *las* system, and that PQS system controls this activation (Diggle *et al*., 2003). This was further supported by a more recent paper, where the authors provided evidence that *rhl* system is able to overcome the absence of the *las* system by activating specific LasR-controlled functions, including

production of *N*-(3-oxododecanoyl)-$_L$-homoserine lactone and PQS (Dekimpe & Deziel, 2009).

Table 1.1 LuxS regulated quorum sensing effects in varieties of bacterial species

Bacterial species	LuxS-dependent QS effects	References
V. harveyi	Bioluminescence	(Bassler *et al.*, 1997)
V. cholerae	Expression of virulence factors	(Miller *et al.*, 2002)
Vibrio alginolyticus	Expression of virulence factors, motility and flagella biogenesis	(Ye *et al.*, 2008, Tian *et al.*, 2008)
Vibrio vulnificus	Expression of virulence factors and in *vivo* survival	(Kim *et al.*, 2003)
Staphylococcus epidermidis	Regulation of biofilm formation and virulence	(Xu *et al.*, 2006, Li *et al.*, 2008b)
Serratia marcescens	Regulation of virulence and production of carbapenem and prodigiosin	(Coulthurst *et al.*, 2004)
EHEC *E. coli* and EPEC *E. coli*	Regulation of TTSS, motility, flagellar biogenesis and expression of virulence factors	(Sperandio *et al.*, 1999, Sperandio *et al.*, 2002)
C. jejuni	Biofilm formation, growth characteristics and motility	(Reeser *et al.*, 2007, He *et al.*, 2008)
Salmonella enterica	Biofilm formation and expression of virulent genes	(Choi *et al.*, 2007)
P. gingivalis	Modulation of protease and haemagglutinin activities, regulation of genes encoding hemin and iron acquisition	(Burgess *et al.*, 2002, James *et al.*, 2006)
Shigella flexneri	Modulation of *virB* expression	(Day & Maurelli, 2001)
Borrelia burgdorferi	Expression of virulence factors of lyme disease spirochetes	(Stevenson & Babb, 2002)
Streptococcus anginosus	Biofilm formation	(Petersen *et al.*, 2006)
Streptococcus mutans	Acid and oxidative stress tolerance and biofilm reduction	(Merritt *et al.*, 2003, Wen & Burne, 2004)
Streptococcus gordonii	Biofilm formation and control of carbohydrate metabolism	(McNab *et al.*, 2003)
Streptococcus pneumoniae	Expression of virulence factors	(Stroeher *et al.*, 2003)
Streptococcus pyogenes	Control of growth and virulence factor expression	(Lyon *et al.*, 2001, Marouni & Sela, 2003)
N. meningitidis	Full meningococcal virulence	(Winzer *et al.*, 2002c)
C. perfringens	Toxin production	(Ohtani *et al.*, 2003)
Actinobacillus actinomycetemcomitans	Biofilm formation, modulate of virulence and the uptake of iron	(Fong *et al.*, 2001)
Actinobacillus pleuropneumoniae	Biofilm formation and virulence regulation	(Li *et al.*, 2008a)
Y. pestis	Biofilm formation and protein expression	(Bobrov *et al.*, 2007)
H. pylori	Biofilm formation, flagellar gene expression and motility	(Cole *et al.*, 2004, Lee *et al.*, 2006, Rader *et al.*, 2007)

Sperandio *et al*. (2003) reported that EHEC senses another LuxS-dependent auto-inducer, auto-inducer-3 (AI-3) which serves as the QS signal for virulence genes. The authors stated that AI-3 is chemically distinct from AI-2 and able to regulate the transcription of genes encoding the locus of enterocyte effacement (LEE) in a type III secretion system (TTSS) in EHEC, whereas AI-2 does not possess such a function. In contrast, AI-2 induces bioluminescence in *V. harveyi* but AI-3 does not appear to have this capacity (Sperandio *et al*., 2003). Additionally, the mammalian (host) hormones epinephrine and norepinephrine may substitute for AI-3 in regulation of EHEC virulence factors when AI-3 is absent and have been suggested to cross talk with the AI-3 system (Sperandio *et al*., 2003).

Later on, Walters, *et al*. (2006) reported that AI-3 synthesis is not dependent on LuxS in *E. coli*, and that the AI-3 production defect in the Δ*luxS* mutant might be due to disruption of the AI-3 synthetic pathway caused by LuxS inactivation. Commensal *E. coli*, as well as several other intestinal bacterial species, including *Klebsiella pneumoniae*, *Shigella* sp., *Salmonella* sp., and *Enterobacter cloacae* was found to produce both AI-2 and AI-3, suggesting that AI-3 may serve as another interspecies QS signal (Walters & Sperandio, 2006, Ryan & Dow, 2008). Nevertheless, to date no evidence has been shown further to support related theories.

1.2.4 Why study quorum sensing?

For many pathogens, the outcome of the interaction between host cells and bacteria is strongly affected by the bacterial population density. For example, production of bacterial virulence factors only at a critical cell population density prevents the mammalian host building up an effective defence against bacterial attack (Winzer & Williams, 2001). QS-deficient biofilms are more unstable compared to those of wild-

type biofilms, and have lower colonization capacities in many bacteria, suggesting that QS might promote cell resistance by protecting the biofilm (Henzter M & Givskov M, 2003). In *S. aureus*, the QS gene *agr* controls virulence and is required for invasive infection in the lung (Heyer *et al.*, 2002). In *E. coli* O157:H7 (EHEC), colonization can be induced by QS signals produced by nonpathogenic *E. coli* in normal intestinal flora (Sperandio *et al*., 1999). This indicates that regulation of intestinal colonization factors by QS may play an important role in the pathogenesis of disease.

Disruption of the signalling pathway (QS gene mutagenesis) has been shown to attenuate virulence in many pathogenic bacteria. Current known contributions of cell density dependent-QS to the regulation of bacterial virulence include biofilm formation (surface-associated communities), protease production, spore formation, conjugal transfer of plasmid DNA and transformation competence (Vendeville *et al*., 2005, Williams, 2007, Ryan & Dow, 2008). All these suggest that interference with QS represents a possible strategy for the control of infections, and that QS systems of bacteria could be a potential target for the design of novel antibiotic drugs (Donabedian, 2003, Hentzer & Givskov, 2003, Hentzer *et al.*, 2003). The QS antibiotic targets could be the QS receptors (e.g. the LuxR family for AHLs, the species-dependent receptors for AI-2) or the signalling molecules (e.g. AHLs, AIPs, AI-2). Disruption of QS by plants and bacteria could be another target. The principle is that some species of plants and bacteria produce chemicals that disrupt the QS of other species. Manefield *et al*. (1999) found halogenated furanone compounds produced by the macroalga *Delisea pulchra* inhibit AHL-dependent gene expression through accelerated LuxR turnover. Other examples include: AIPs of strains of *S. aureus* inhibit the QS of other strains (Miller & Bassler, 2001); and the soil bacterium

B. subtilis secretes the enzyme AiiA which inactivates the AHL auto-inducer of *E. carotovora* (Dong *et al.*, 2000).

Another approach of developing new antibiotic drugs is to design QS antagonists. The proposal is interference with either the biosynthesis or transmission of QS signals by the so called QS antagonists (Finch *et al.*, 1998). The QS molecular antagonists or QS blockers are synthetic non-native compounds which compete with native auto-inducers and intercept the interaction between the native signals and the response regulators (Manefield *et al*., 1999, Manefield *et al.*, 2001, Rasmussen *et al.*, 2000, Givskov *et al.*, 1996). For example, the structural analog of the *P. aeruginosa* auto-inducer, *N*-3-oxo-dodecanoyl homoserine lactone, competes with its real auto-inducer binding to the receptor LasR and results in varied agonist activities (Passador *et al.*, 1996). Kim *et al*. (2008) found that the furanone derivatives as QS antagonists could remarkably inhibit both *P. aeruginosa* QS signalling and biofilm formation. Also, an AI-2 analogue, ascorbic acid results in AI-2 assay inhibition and was evaluated to control growth, sporulation, and enterotoxin production in *Clostridium perfringens* (Novak & Fratamico, 2004).

In summary, QS is essential for intra- and inter-bacterial communication which leads to aspects of bacterial behaviours including gene regulation, keeping colonies intact (biofilms) and allowing resident bacteria to enhance survival of the group. The QS system involving enzymes, transporters and auto-inducer-receptor interactions may present classic antibiotic targets for drug discovery. QS signalling process can be utilised to develop inhibitor drugs that have novel mechanisms of anti-bacterial action and might be more effective against bacterial strains that are currently antibiotic-resistant.

1.3 The role of LuxS in bacteria

1.3.1 Metabolic role of LuxS in the activated methyl cycle (AMC)

The LuxS/AI-2-dependent QS is the only known mechanism shared by both Gram-negative and Gram-positive bacteria, suggesting that AI-2 can be a 'universal' language for interspecies communication (Schauder *et al*., 2001, Henke & Bassler, 2004, Vendeville *et al*., 2005). On the other hand, LuxS has an alternative important role in cells, in which it works as an metabolic component in the activated methyl cycle (AMC) (Winzer *et al*., 2003). The metabolic function of LuxS may provide an explanation for the widespread presence of *luxS* and AI-2 in bacteria.

In all organisms, methylation reactions are a crucial means of detoxification and nucleic acid stabilisation. However, methyl transfer reactions are relatively difficult to perform due to the inherent stability of the methyl group hence its use as a stabilising adduct (Zappia *et al.*, 1969). To address this biochemical problem, methyl groups with a higher transfer potential generated via the AMC are utilised by organisms. Methyl groups enter the AMC by the conversion of homocysteine to methionine (Zappia *et al*., 1969, Duerre & Walker, 1977). As a part of the AMC, methionine is then converted to *S*-adenosylmethionine (*S*AM) in a reaction catalysed by *S*AM synthetase (MetK), in which the methyl group in methionine is activated by formation of *S*AM. In the following methyltransferase reactions, *S*AM is converted to *S*-adenosylhomocysteine (*S*AH), a potentially toxic methyl-transferase inhibitor which is then subsequently detoxified by Pfs and then the substrate of LuxS, *S*-ribosylhomocysteine (*S*RH) is generated. In the following step, *S*RH is cleaved by LuxS to homocysteine, a key metabolite in re-synthesis of methionine and then SAM. The precursor of AI-2, 4, 5-dihydroxy-2, 3-pentanedione (DPD) is generated as a by-

product during the conversion of *S*RH to homocysteine (Figure 1.4) (Duerre & Walker, 1977). Through a process of dehydration and spontaneous cyclisation, some or all of the DPD molecules are rearranged to a furanosyl borate derivative (as AI-2) and function as signalling molecules in micro-organisms (Winzer *et al*., 2002a, Winzer *et al*., 2003). There are two versions of the AMC. Many organisms, in particular eukaryotes, use a single enzyme, *S*AH-hydrolase, instead of the combined Pfs-LuxS to convert toxic *S*AH to homocysteine, hence no DPD (AI-2) is produced (Figure 1.4) (Duerre & Walker, 1977). The Pfs-LuxS reactions are conserved in the majority of bacteria.

If either *S*AH-hydrolase or Pfs is sufficient for the detoxification of *S*AH in the two different versions of AMC, why is the Pfs-LuxS variant of AMC employed in some micro-organisms? Two hypotheses may explain this: i) the Pfs-LuxS coupled reaction allows AI-2 signals to be generated and perform as a QS factor; and ii) LuxS is required for the completion of the conversion of *S*AH to homocysteine, acting as a mechanism for recycling of metabolic precursors (Winzer *et al*., 2003). The latter hypothesis is built on a bio-energetic perspective. Since all intermediates through the AMC are reduced sulphur compounds; and sulphur is a highly reactive element, which is energetically costly to incorporate into biological systems, processes enabling the recycling of these intermediates are energetically advantageous to the cells (Winzer *et al*., 2003).

In conclusion, the AMC pathway mainly functions in the following aspects: generation of the major methyl donor, *S*AM, production of AI-2 and the detoxification and re-utilisation of *S*AH (Winzer & Williams, 2003, Winzer *et al*., 2003). The metabolic roles of LuxS in the AMC include i) to act as an enzyme to

catalyse the conversion of *S*RH to homocysteine; and ii) to produce AI-2. The function of LuxS to generate homocysteine plays an important role in bacterial metabolism due to homocysteine's three main fates: to be remethylated to methionine, to enter the cysteine biosynthesis pathway, and to be released into the extracellular environment (Medina *et al.*, 2001).

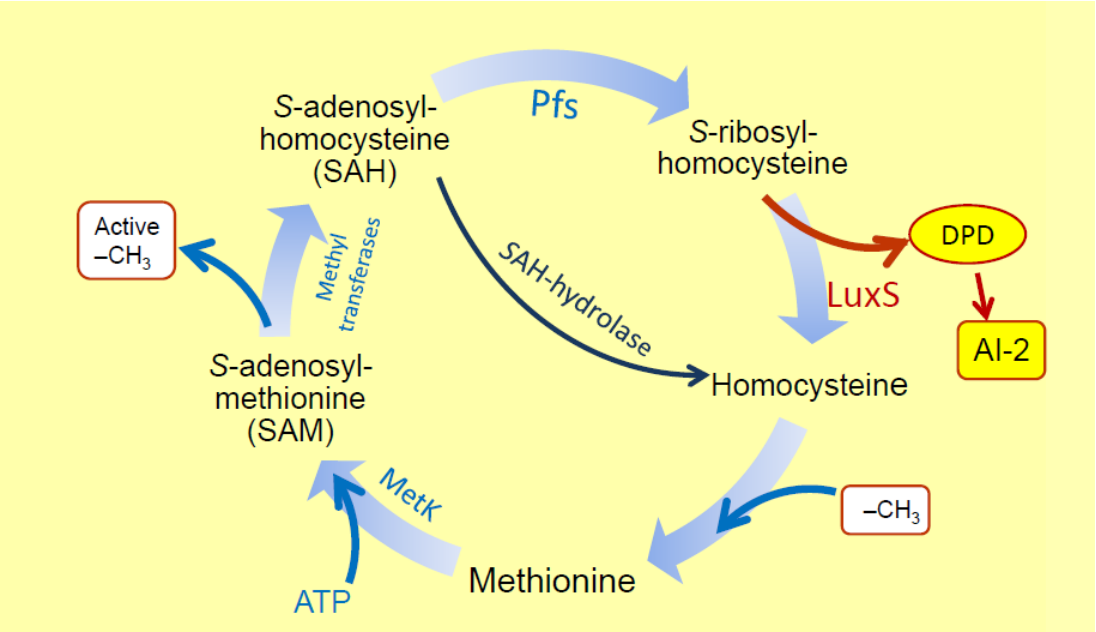


Figure 1.4 The Activated Methyl Cycle utilised by microorganisms

The activated methyl cycle (AMC) regenerates the active methyl donor *S*AM from the toxic methyl transferase product *S*AH. *S*AH is metabolised in one of two routes, depending on the organism; many bacteria, including *H. pylori*, generate homocysteine from *S*AH in a two step manner: a nucleosidase, Pfs, converts *S*AH to *S*RH, then LuxS converts this to homocysteine and the precursor of AI-2, DPD. The alternative route produces homocysteine directly from *S*AH in a one step reaction requiring *S*AH hydrolase.

1.3.2 LuxS-dependent quorum sensing in bacteria

1.3.2.1 *E. coli* (EHEC) O157:H7and *E.coli* (EPEC)

Currently, there are five recognised classes of enterovirulent *E. coli* that cause gastroenteritis in humans. Among these are strains designated *E. coli* O157:H7 and EPEC. EHEC O157:H7 is a normal inhabitant of the intestines and the agent responsible for outbreaks of bloody diarrhoea and haemolytic-uraemic syndrome throughout the world (Kaper, 1998). EPEC is a major cause of diarrhoea in infants by adhering to intestinal epithelial cells (Sherman *et al.*, 1989).

Both EHEC and EPEC form a characteristic histopathological feature known as the 'attaching and effacing' (A/E) lesion on the surface of intestinal epithelial cells. The genes necessary for formation of AE lesion are encoded on the locus of enterocyte effacement (LEE) Pathogenicity Island (McDaniel *et al.*, 1995, Elliott *et al.*, 1998). The LEE region mainly encodes a type III secretion system (TTSS), which is essential for signal transduction in host cells and A/E lesion development. The majority of the LEE genes are organised into five polycistronic operons, LEE (1-4) and *tir* (LEE5) (McDaniel *et al*., 1995, Elliott *et al*., 1998).

Two transcriptional regulators, QseA (quorum sensing *E. coli* regulator A) and Ler (LEE-encoded regulator) participate in LEE operon regulation in EHEC and EPEC. EPEC has an additional plasmid-encoded regulator, Per, which is also regulated indirectly by QS (Sircili *et al.*, 2004). In EHEC, QS was initially proposed to directly activate expression of the LEE1 and LEE2 operons and indirectly influences expression of LEE3 and the *tir* operon via Ler (Sperandio *et al.*, 2001). In EPEC, QS was proposed to directly activate expression of the LEE1 and LEE2 operons and

indirectly activate expression of the LEE3, LEE4 and *tir* operons via Ler. This model was refined afterwards and QS now is suggested to function through QseA in both strains (Figure 1.5) (Sperandio *et al*., 1999, Sircili *et al*., 2004).

Mutations of *luxS* in both EHEC and EPEC show decreased TTSS gene expression and decreased flagellation and motility (Sperandio *et al*., 1999). The loss of *qseA* in EPEC causes increased flagellin production and motility, whereas *qseA* in EHEC has no effect on the flagellar regulon (Sperandio *et al*., 1999). Instead, a novel QseB-QseC complex has been suggested to regulate flagellar synthesis and motility in EHEC. Also, a previous microassay analysis suggested that around 10% of all genes in EHEC are affected by *luxS*-dependent QS regulation, indicating that *luxS*/AI-2 is a global regulatory system in EHEC (Sperandio *et al*., 2001, Sperandio *et al*., 2002).

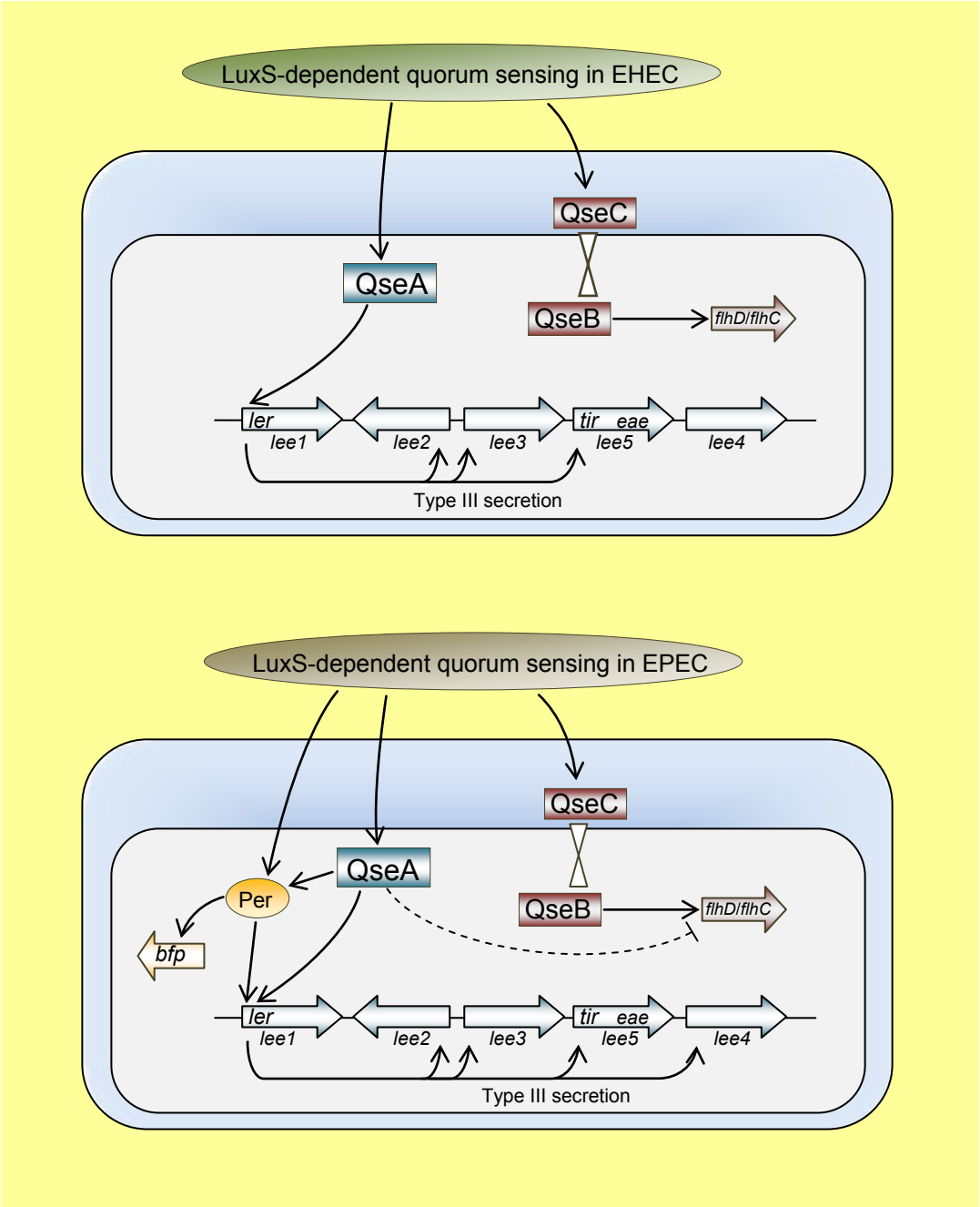


Figure 1.5 Model for LuxS-dependent QS in enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) *Echerichia coli*

The histidine kinase QseC (quorum sensing *E. coli* regulator C), together with its cognate response regulator QseB, activates the flagellar master operon *flhD*/*flhC*. Regulation of type III secretion is mediated through QseA and, indirectly, through Ler. Together, the two proteins activate the expression of the five important *lee* operons (*lee*1-5), encoding components of the type III secretion apparatus as well as an adhesin (intimin, Eae), the intimin adhesin receptor Tir, and other proteins. Transcription of *ler* (LEE-encoded regulator) (the first gene in the *lee1* operon) is activated by QseA. In EPEC, the plasmid-

encoded Per is also thought to mediate quorum-sensing (QseA)-dependent regulation of *ler* and *bfp* (bundle forming pilus).

1.3.2.2 *Salmonella typhimurium*

Salmonella enterica subspecies I, serovar Typhimurium (*S. Typhimurium*), is a leading cause of food-borne gastroenteritis (Baumler *et al.*, 1998). Like many Gram-negative bacteria, *S. Typhimurium* also utilise *luxS*/AI-2-dependent QS to regulate expression of a range of genes (Surette & Bassler, 1998).

In *S. Typhimurium*, AI-2 production is tightly correlated with *pfs* transcription, which could be explained by a raised level of Pfs giving a response to increase the need for *S*AH detoxification, in turn resulting in a higher level of *S*RH and LuxS activity (Beeston & Surette, 2002). This indicates that AI-2 is more likely to be a metabolic component instead of a QS molecule in the *S. Typhimurium* system. At earlier stage, the only genes known to be regulated by AI-2 in *S. Typhimurium* were a seven-gene *lsr* operon, which encodes the ATP binding cassette (ABC) transporter (Taga *et al.*, 2001). Mutations in the *lsr* operon render *S. Typhimurium* unable to eliminate AI-2 from the extracellular environment, indicating a role of the Lsr transporter in taking up AI-2 and the use of AI-2 as a metabolite, and that metabolic changes in the Δ*luxS* mutant consequently affect gene regulation (Taga *et al*., 2001).

In *S. Typhimurium*, a number of virulence proteins are translocated into host cells via a type III secretion system encoded by genes in *Salmonella* pathogenicity island 1 (SPI-1) (Zhou & Galan, 2001, Hapfelmeier *et al.*, 2005, Choi *et al*., 2007). In transcriptional assays, a *S. Typhimurium* strain lacking *luxS* showed reduced transcription of a regulator gene, *invF* and InvF-regulated genes from SPI-1. The

decreased expression of genes from SPI-1 in the Δ *luxS* mutant was restored genetically and also by addition of synthetic AI-2 (Choi *et al*., 2007).

Salmonella swims by using flagella made from two different flagellar subunits, either FliC (phase 1) or FljB (phase 2) (McClelland *et al.*, 2001). Karavolos *et al*. (2008) demonstrated that a *S. Typhimurium luxS* mutant polarises flagellar phase variation bias toward expression of the more immunogenic phase 1 flagellin. Interestingly, this effect in the Δ*luxS* mutant can be restored genetically, but not by addition of either AI-2 or other diffusible signals, indicating that the contributions of *luxS* to flagellar phase variation might be signal- or QS- independent and further implying that it is secondary to metabolic changes rather than QS (Karavolos *et al*., 2008).

1.3.2.3 *Porphyromonas gingivalis*

The Gram-negative bacterium, *P. gingivalis*, is an etiologic agent of severe periodontal diseases, including gingivitis and periodontitis which can lead to tooth loss and has been associated with diseases like cardiovascular disease and adverse pregnancy outcomes (Lamont & Jenkinson, 1998).

As reported in previous studies, *P. gingivalis* possesses a *luxS*-dependent QS system (Chung *et al.*, 2001). LuxS, along with its AI-2 product in *P. gingivalis*, exhibits a function of modulating protease and haemagglutinin activities (Burgess *et al*., 2002). Mutation of *luxS* in *P. gingivalis* leads to reductions in expression of some potential virulence determinants, including two extracellular cysteine proteases and haemagglutinating activity. However, it seems that *luxS* in *P. gingivalis* has no influence on proven virulence factors (Burgess *et al*., 2002).

A *P. gingivalis* Δ*luxS* mutant showed reduced transcription of a *tonB* homologue, which is responsible for energising transport of iron from the outer-membrane into the cytoplasm. The regulation of genes involved in such iron metabolism implies that AI-2 might be used as a signal in *P. gingivalis* for labeling nutrient limitation (James *et al*., 2006). Also, data from a microarray analysis suggests that *luxS* might be involved in promoting survival of *P. gingivalis* in the host by regulating its response to host-induced stresses, including raised temperature, hydrogen peroxide (H_2O_2) and high pH (Yuan *et al.*, 2005).

1.4 The role of *luxS* in *H. pylori*

1.4.1 The incomplete activated methyl cycle (AMC) in *H. pylori*

The activated methyl cycle (AMC) exists in many organisms, but *H. pylori* is not predicted to have a complete AMC since it lacks a gene encoding the homologue to enzymes that direct the production of methionine from homocysteine (Figure 1.6). This has been corroborated phenotypically, with all *H. pylori* strains tested being auxotrophic for methionine (Mendz & Hazell, 1995, Nedenskov, 1994). As a metabolic component in the AMC, the main metabolic role of LuxS is to convert *S*RH to generate homocysteine, to remove toxic *S*AH with the enzyme Pfs and to produce the signalling molecule AI-2 (Winzer *et al*., 2002a, Winzer *et al*., 2003). These functions of LuxS are predicted to exist in *H. pylori*. The main difference is that in *H. pylori* homocysteine is not converted to methionine and so cannot be recycled back to *S*AM hence it is not a source for further methylation reactions.

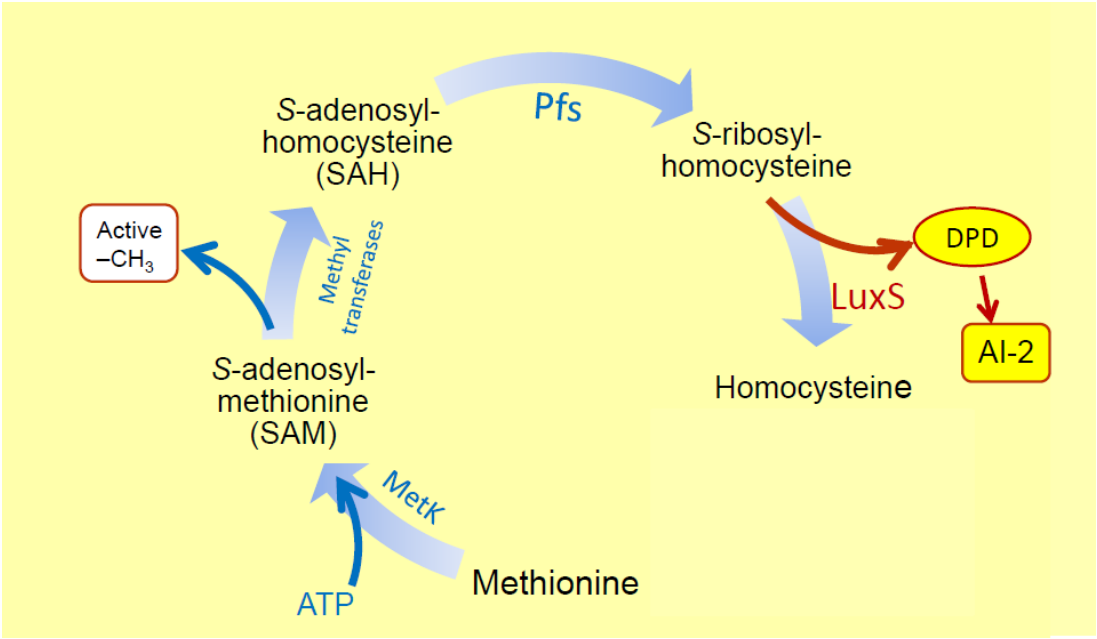


Figure 1.6 The incomplete activated methyl cycle in *H. pylori*

H. pylori has a *luxS* homologue and possesses an integral homocysteine and DPD (AI-2) biosynthesis pathway but it is not able to convert homocysteine to methionine because it does not contain a MetH/MetE homologue gene.

1.4.2 LuxS-dependent quorum sensing in *H. pylori*

luxS homologues and production of AI-2 exist widely in both Gram-positive and Gram-negative bacteria. However, it remains unclear whether LuxS is just a metabolic enzyme, or also performs as a QS factor in microorganisms.

The first work on LuxS in *H. pylori* was by Forsyth and Cover (2000) who found that *H. pylori* possesses the *luxS* gene and releases AI-2 at mid log phase, which induces bioluminescence in the *V. harveyi* reporter strain. They also expressed *luxS* from *H. pylori* in *E. coli* DH5α[‡] and restored AI-2 production in this strain using the heterologous gene. The report from Joyce *et al*. (2000) came out at almost exactly

[‡] *E. coli* DH5α is a *luxS*-negative strain

the same time as Forsyth's paper, and is very similar to Forsyth's, but they described maximal AI-2 production during early log phase. They found that *luxS* mutagenesis had no effect on virulence factor or protein expression profiles, as determined by two-dimensional gel electrophoresis (Joyce *et al.*, 2000). However, in neither study, was a phenotype convincingly attributed to the disruption of QS through $luxS_{Hp}$ inactivation (Forsyth & Cover, 2000, Joyce *et al*., 2000).

In later work, two Δ*luxS* mutants (SD3, SD14) of *H. pylori* were reported to form biofilms more efficiently than the parent strain, indicating a possible role of $luxS_{Hp}$ in biofilm reduction (Cole *et al*., 2004). Next, Δ*luxS* mutants in J99 and 22695 strains are reported to lose growth phase dependent regulation of expression of the flagellar gene, *flaA* (Loh *et al.*, 2004). Subsequent studies by two independent groups looked at fitness of Δ*luxS* mutants *in vivo* using mouse and gerbil models, respectively. They showed that mutated *luxS* in *H. pylori* strains SS1 and TK1402 diminished motility on soft agar and reduced fitness/competitiveness in co-infection experiments (Lee *et al*., 2006, Osaki *et al.*, 2006). Nevertheless, Lee *et al*. (2006) reported that a Δ*luxS* mutant constructed in *H. pylori* strain X47 did not show any of the defects described above, indicating that after mutagenesis motility might be altered in a strain dependent manner (Lee *et al*., 2006). More recently, Rader *et al*. (2007) found reduced motility of a *H. pylori* G27 Δ*luxS* strain on soft agar and that loss of *luxS* altered flagellar morphogenesis in a strain background lacking flagellar associated genes *flgM*, *flgS* and *fliA*. They also reported that loss of *luxS* down-regulated the transcription of the master flagellar regulator gene *flhA*, which was complementable by addition of DPD, implying a likely role of $luxS_{Hp}$/AI-2 in QS (Rader *et al*., 2007).

1.5 Sulphur amino acid metabolism in bacteria

1.5.1 General routes for the metabolism of methionine and cysteine

The metabolism of sulphur in organisms involves three different aspects: synthesis of the sulphur-containing amino acids (cysteine and methionine), along with the sulphur-containing coenzyme groups; catabolism and equilibration of the sulphur metabolite pool; and methionine recycling (since methionine is the first residue of all proteins) (Cooper, 1983).

Methionine and cysteine serve as initiators of protein synthesis and play a central role in cellular functions in all forms of life (Cooper, 1983, Griffith, 1987). Cysteine and methionine availability influences a range of cellular processes essential to bacterial survival and growth in the host (Cooper, 1983). Two different methionine biosynthetic pathways exist in organisms. One assimilates inorganic sulphur into cysteine, providing the sulphur source for methionine biosynthesis via the transsulphuration pathway (Droux, 2004). This pathway is unique to microorganisms and plants, rendering the enzyme an attractive target for the development of antimicrobials and herbicides (Droux, 2004, Cherest *et al.*, 1993). The other pathway bypasses the transsulphuration reactions via direct sulphhydrylation of *O*-succinyl (-acyl)-homoserine to homocysteine, and then to methionine (e.g. in *P. aeruginosa*, *P. putida* and *Corynebacterium glutamicum*) (Figure 1.7) (Foglino *et al.*, 1995, Lee & Hwang, 2003, Vermeij & Kertesz, 1999).

For cysteine biosynthesis, some organisms utilise a mechanism by which sulphur is reduced and incorporated into organic compounds, called the sulphate assimilatory cysteine biosynthesis pathway (SACBP) (eg. in *S. Typhimurium*, *E. coli*, *Lactococcus lactis*, *M. tuberculosis*, *Azospirillum brasilense*, plants and fungi) (Kredich &

Tomkins, 1966, Droux, 2004, Sperandio *et al.*, 2005, Wheeler *et al.*, 2005, Ramirez *et al.*, 2006). This pathway involves transport and reduction of inorganic sulphate to sulphide in one branch and the synthesis of *O*-acetyl-serine (*O*AS) in another. Some organisms synthesise cysteine via an alternative reverse transsulphuration pathway (RTSP) which generates homocysteine through the central metabolic AMC, followed by its cleavage with specific enzymes via the intermediary formation of cystathionine to yield cysteine (eg. mammals, *B. subtilis*, *P. aeruginosa*, and *M. tuberculosis*) (Figure 1.7) (Gunther *et al.*, 1979, Griffith, 1987, Vermeij & Kertesz, 1999, Wheeler *et al*., 2005, Hullo *et al.*, 2007).

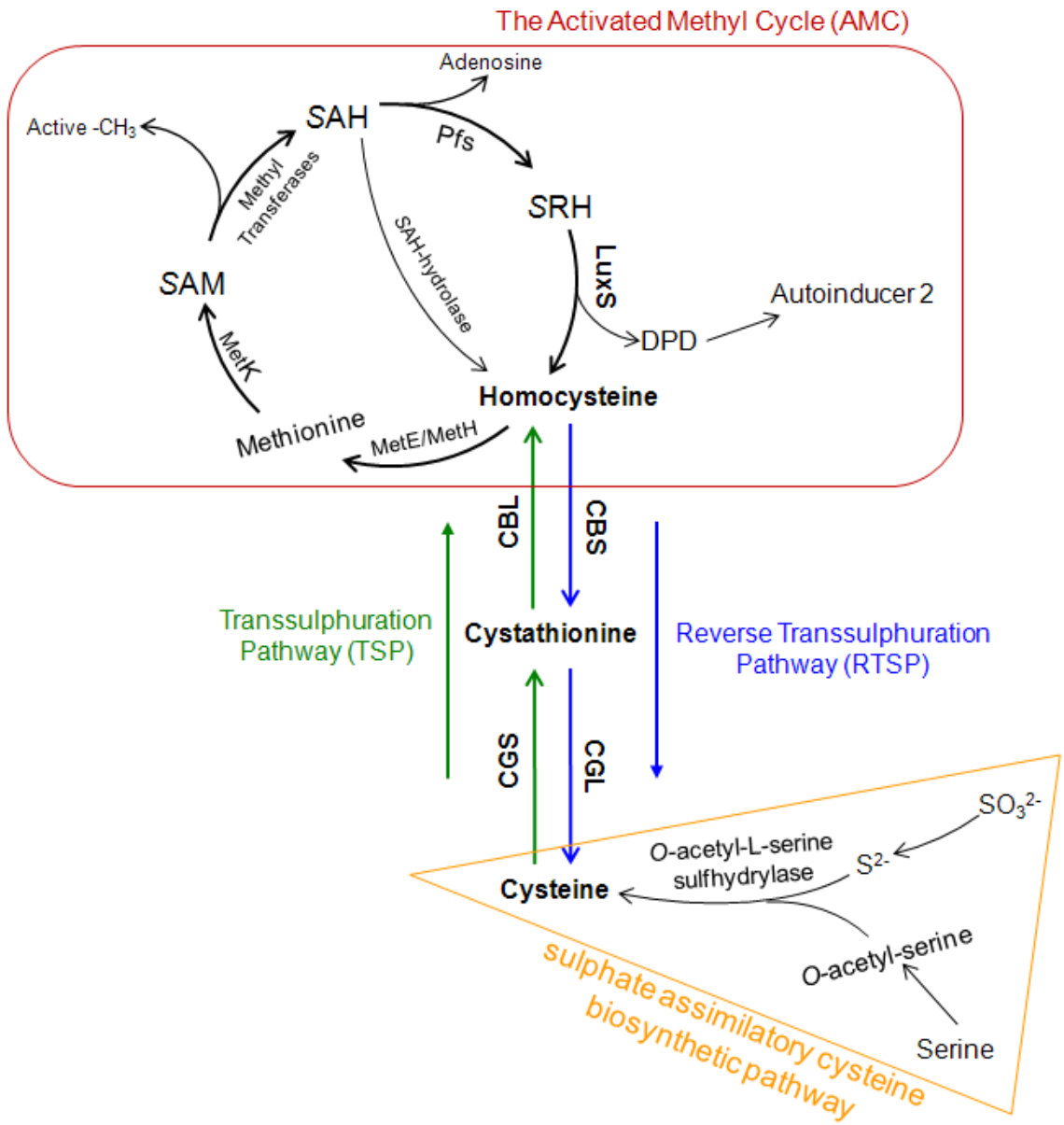


Figure 1.7 Bacterial methionine and cysteine interconversion pathways

Many bacteria synthesise cysteine through the sulphate assimilatory cysteine biosynthetic pathway which uses inorganic sulphur as substrate (pathway bounded by an orange triangle). The reactions of the transsulphuration (shown in green) and reverse transsulphuration (shown in blue) pathways connect the AMC and the SACBP. The intermediate product in cysteine interconversion, homocysteine, is generated by the AMC. Key interconversions are described in the text. CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; CGS, cystathionine γ-synthase; DPD, 4,5-dihydroxy-2,3-pentanedione; LuxS, *S*-ribosylhomocysteinase/auto-inducer-2 synthase; MetE/MetH, Methionine synthase; MetK, *S*-adenosylmethionine synthase; Pfs, 5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase; *S*AH, *S*-adenosylhomocysteine; *S*AM, *S*-adenosylmethionine ; *S*RH, *S*-ribosylhomocysteine.

1.5.2 The transsulphuration and reverse transsulphuration pathways and their main enzymes

The transsulphuration and reverse transsulphuration pathways (the TSP and RTSP) are part of the main routes of biosynthesis and consumption of sulphur-containing amino acids in organisms (Messerschmidt *et al.*, 2003). In the TSP, cysteine is the sulphur donor and is transformed into homocysteine via the intermediate, cystathionine. The conversion of cysteine to cystathionine and the cleavage of cystathionine to yield homocysteine are catalysed respectively by enzymes cystathionine γ-synthase (CGS) and cystathionine β-lyase (CBL) (Lee & Hwang, 2003, Sperandio *et al*., 2005, Lee *et al.*, 2007). In the RTSP, cystathionine is generated by cystathionine β-synthase (CBS) using homocysteine as substrate, and is then cleaved to cysteine by the enzyme, cystathionine γ-lyase (CGL) (Gunther *et al*., 1979, Chang & Vining, 2002, Sperandio *et al*., 2005, Wheeler *et al*., 2005, Hullo *et al*., 2007). Homocysteine is a key metabolite in both pathways due to its role either as a substrate to be re-methylated to methionine or as an intermediate involved in the cysteine biosynthesis via the RTSP, joining the AMC and other sulphur metabolism together (Figure 1.7).

CGS, CBL; CBS and CGL are 4 classic enzymes that act in the TSP or RTSP. CGS, CGL and CBL belong to a protein family, including enzymes involved in cysteine and methionine metabolism. The following are members: CGS, CGL, CBL, methionine γ-lyase (MGL), *O*-acetylhomoserine (*O*AH) / *O*-acetylserine (*O*AS) sulfhydrylase, *O*-succinylhomoserine (*O*SH) sulphhydrylase. All these enzymes use PLP (pyridoxal-5'-phosphate) as a co-enzyme (NCBI database http://www.ncbi.nlm.nih.gov/sites/entrez). Members of this group are widely distributed among all forms of life.

CBS belongs to a second family. This subgroup includes CBS, cysteine synthase and *O*-phosphoserine sulfhydrylase. CBS is a unique haem-containing enzyme that catalyses a pyridoxal 5'-phosphate (PLP)-dependent condensation of serine and homocysteine to give cystathionine. Cysteine synthase on the other hand catalyses the final step of cysteine biosynthesis. The *O*-phosphoserine sulphhydrylase is an enzyme which is able to produce cysteine directly from sulphide (NCBI database http://www.ncbi.nlm.nih.gov/sites/entrez).

The four enzymes CGS, CBL; CBS and CGL involvd in the TSP and RTSP are very similar to each other. Therefore, functions of these enzymes in transsulphuration in a specific organism are very difficult to predict. In most cases, the combination of CGS-CBL participates in the TSP, forward to the formation of methionine, while the combination of CBS-CGL usually acts the other way round to synthesise cysteine (Gunther *et al*., 1979, Wheeler *et al*., 2005, Hullo *et al*., 2007, Lee *et al*., 2007).

1.5.3 Sulphur metabolism and related genes in bacteria

1.5.3.1 Cysteine recycling in *E. coli*

E. coli utilises the sulphate assimilatory cysteine biosynthetic pathway (SACBP) to synthesise cysteine. The reaction catalysed by serine acetyltransferase (encoded by *cysE*) condenses an acetyl group from acetyl CoA on the hydroxyl group of serine, forming *O*-acetyl-serine (Kredich & Tomkins, 1966). Sulphur, reduced as S^{2-}, reacts with *O*-acetyl-serine (catalysed by *O*-acetyl-serine sulphhydrylase) to give cysteine. In the following step, $CGS_{E.\ coli}$ (encoded by *metB*) converts cysteine to cystathionine using *O*-succinyl homoserine as one of the substrates. Cystathionine is then cleaved by $CBL_{E.\ coli}$ (encoded by *metC*) to homocysteine and subsequently via methylation

to methionine. The conversion of homocysteine to methionine in *E. coli* is catalysed by methionine synthase encoded by *metH*. Cysteine is recycled by sulphate which is produced in consumption of methionine (Kredich & Tomkins, 1966, Sekowska *et al.*, 2000).

Generally, *E coli* synthesises cysteine directly via the normal sulphate assimilation route and consumes cysteine via the TSP. Although the bacteria grow well in the presence of methionine as the sulphur source, they do not contain the RTSP (Figure 1.8) (Sekowska *et al*., 2000).

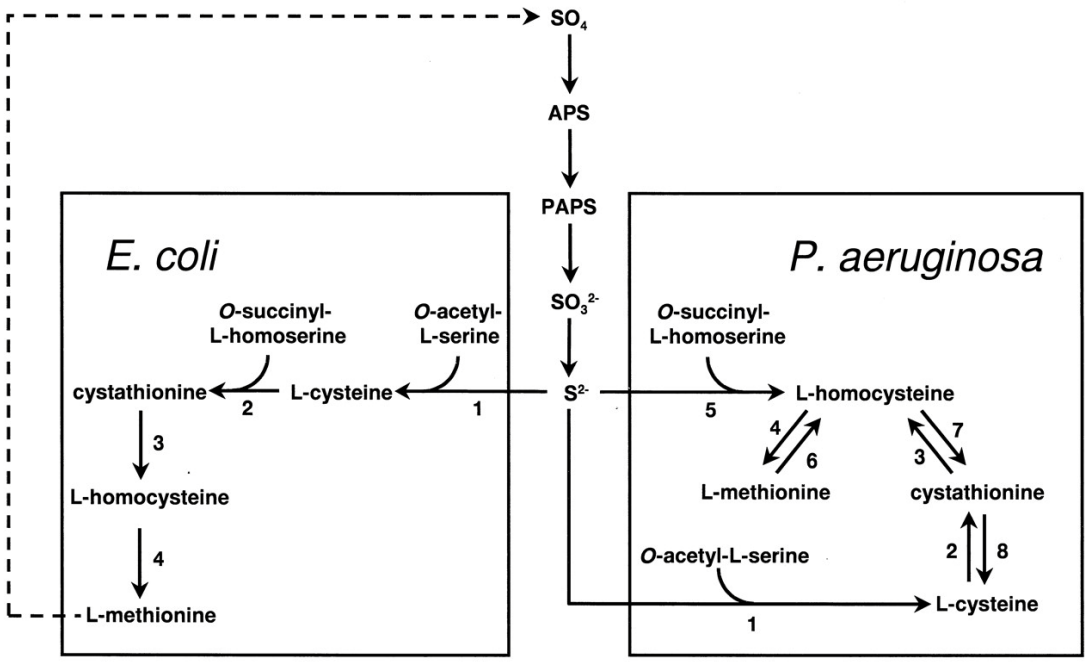


Figure 1.8 Pathways of cysteine and methionine metabolism in *E. coli* and *P. aeruginosa*[§]

E. coli utilises an ACSP to synthesise cysteine and a TSP to synthesise methionine. *P. aeruginosa* possesses all known routes of methionine or cysteine metabolic pathways. Details of interconversions are described in the text. Enzymes involved: 1, *O*-acetyl-L-serine sulphhydrylase; 2, cystathionine γ-synthase; 3, cystathionine β-lyase; 4, methionine synthase; 5, *O*-succinyl-L-homoserine sulphhydrylase; 6, *S*AM synthase-

[§] Figure 1.8 is from the paper Vermeij & Kertesz, 1999.

methyltransferases-*S*AH hydrolase pathway; 7, cystathionine β-synthase; 8, cystathionine γ-lyase (Vermeij & Kertesz, 1999).

1.5.3.2 Methionine and cysteine biosynthesis in *P. aeruginosa*

P. aeruginosa possesses the pathway of direct sulphhydrylation of *O*-succinylhomoserine to synthesise methionine and also contains substantial levels of *O*-acetylserine sulphhydrylase (cysteine synthase) activity (Foglino *et al*., 1995). Both the TSP and RTSP are involved in *P. aeruginosa* (Figure 1.8) (Foglino *et al*., 1995, Gunther *et al*., 1979). When cysteine is present as the sole sulphur source, enzymes involved in the TSP, CGS and CBL, are strongly upregulated during growth; when the bacterium grows with multiple sulphur sources, the two enzymes are expressed at low levels (Foglino *et al*., 1995). *P. aeruginosa* is able to grow with methionine as the sole sulphur source due to the presence of the RTSP (Gunther *et al*., 1979). Interestingly, the pathway between homocysteine and cysteine, with cystathionine produced as the intermediate, are shown to be necessary for rapid *P. aeruginosa* growth (Foglino *et al*., 1995, Gunther *et al*., 1979).

1.5.3.3 Methionine and cysteine biosynthesis in *M. tuberculosis*

Sulphur metabolic pathways in *M. tuberculosis* are one of those which have been well-studied (Figure 1.9). In *M. tuberculosis*, CBS and CBL are encoded by *cysM* and *metC*, respectively. The four genes *metZ*, *metB*, *metC*, and *metA*, encoding enzymes of the appropriate protein family in the genome of *M. tuberculosis* were annotated without a gene to encode CGL (Cole *et al.*, 1998, Hacham *et al.*, 2003, Wheeler *et al*., 2005). However, one of these genes, *metB* has been shown to encode a bifunctional enzyme (with both CGS and CGL activities), indicating that *metB* is involved in the production of cystathionine from cysteine in the TSP and also the

conversion of cystathionine to cysteine in the RTSP (Wheeler *et al*., 2005). Like *P. aeruginosa*, this bacterium contains almost all known routes for biosynthesis and utilisation of methionine and cysteine (Wheeler *et al*., 2005). However, in *M. tuberculosis*, CGS activity is not always needed to incorporate the sulphur of cysteine into methionine because sulphide can be recycled by *O*-succinyl-homoserine sulphhydrylase (*metZ*) to give homocysteine directly, thus by-passing the *metB* encoded pathway (Wheeler *et al*., 2005).

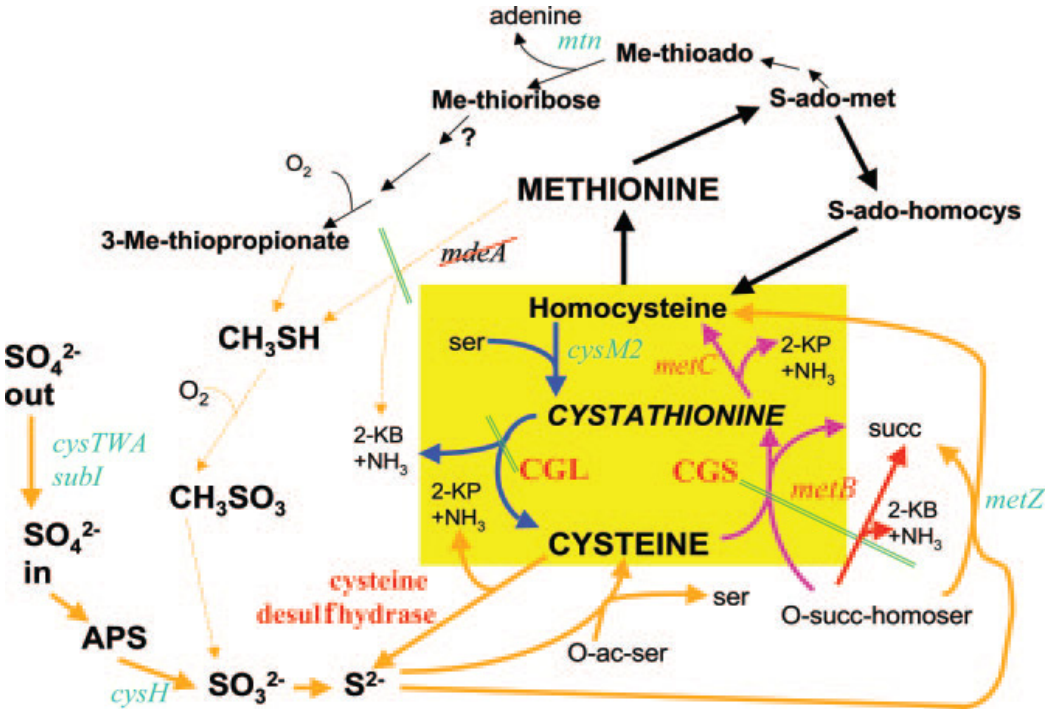


Figure 1.9 Biosynthesis and interconversions of sulphur amino acids in the *M. tuberculosis* complex[**]

All known pathways and related genes or proteins are shown in this figure. A color code is used to denote the different pathways as follows: *orange*, sulphur recycling, with inorganic sulphur as product or substrate; *magenta*, the transsulphuration pathway from cysteine to methionine; *blue*, the reverse transsulphuration pathway from methionine to

[**] Figure 1.9 is from the paper Wheeler *et al*., 2005.

cysteine; *black*, the activated methyl cycle and other relevant pathways and reactions shared by more than one pathway. Genes and enzyme names are as follows: *red*, shown functionally; *turquoise*, present, discussed in this report; *black with red strikethrough*, shown to be absent. Cysteine desulphhydrase was not annotated in the *M. tuberculosis* genome (Wheeler *et al*., 2005).

1.6 Sulphur-containing amino acid metabolism in *H. pylori*

Most microorganisms bear the central metabolic pathway, the activated methyl cycle (AMC), in which LuxS converts *S*RH to homocysteine, providing the substrate, homocysteine, either to form cysteine through the RTSP, or to re-generate methionine, completing the recycling of the AMC (Duerre & Walker, 1977, Winzer *et al*., 2003, Hullo *et al*., 2007). Interestingly, the pathway of re-formation of methionine does not exist in *H. pylori* due to a lack of the gene encoding methionine synthase (Tomb *et al*., 1997, Doig *et al.*, 1999).

In *H. pylori*, *luxS* is next to two housekeeping genes *metB* and *cysK*, and is located downstream on the putative operon *cysK-metB-luxS* (Tomb *et al*., 1997). The fuctions of *cysK* and *metB* in *H. pylori* have not been identified clearly to date. However, both *metB* and *cysK* are involved in cysteine or methionine metabolism in many other bacteria (Thompson *et al.*, 1982, Thanbichler *et al.*, 1999, Vermeij & Kertesz, 1999, Wheeler *et al*., 2005). According to the genome data of Genbank, $metB_{Hp}$ is predicted to encode a CGS; while *cysK* is predicted to encode a cysteine-synthase or CBS-like protein. Both are required for the operation of a classic route for the interconversion of homocysteine and cysteine (Figure 1.7).

H. pylori lacks the enzyme to incoporate sulphide into sulphate, consequently it is not able to synthesise cysteine directly via the sulphate assimilatory cysteine biosynthesis pathway (Kong *et al.*, 2008). On the other hand, homocysteine accumulates with continuing methylation from *S*AM in the incomplete AMC. Accordingly, the RTSP, a sulphur-metabolic pathway generating cysteine from homocyseine via the intermediate product, cystathionine, is very likely to be the main (or can be the only) cysteine provision pathway utilised by *H. pylori*, involving the conserved genes $metB_{Hp}$ and $cysK_{Hp}$ (Figure 1.10) (Hullo *et al*., 2007).

H. pylori is predicted to lack general machineries for sulphur metabolism, implying that genes associated with sulphur metabolism in *H. pylori* may be very limited (Tomb *et al*., 1997, Doig *et al*., 1999). Therefore, enzymes encoded by *luxS*, *metB* and *cysK* are likely to catalyse the most important sulphur metabolic pathway in *H. pylori*. By studying the metabolic function of $luxS_{Hp}$, $cysK_{Hp}$ and $metB_{Hp}$, it is likely to find a cysteine provision pathway encoded by the gene cluster $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$, and importantly, the effect of $luxS_{Hp}$ in metabolism distinct from the other effect in QS signalling.

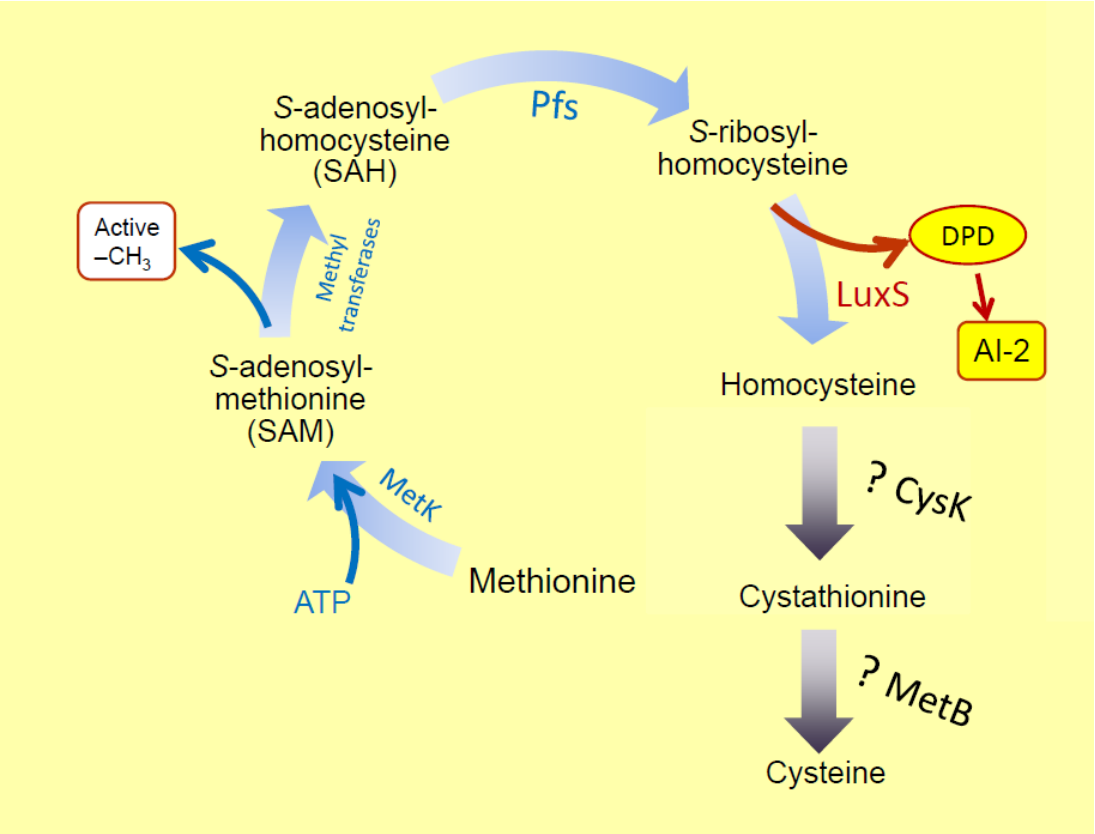


Figure 1.10 The incomplete activated methyl cycle (AMC) of *H. pylori* and our hypothesis on cysteine provision

H. pylori does not contain a MetH/MetE homologue gene, thus is not able to convert homocysteine to methionine. Homocysteine subsequently accumulates with continuing methylation from *S*AM in the incomplete AMC. $LuxS_{Hp}$ is an enzyme in the conversion of *S*RH to homocysteine, which is linked to two housekeeping genes $cysK_{Hp}$ and $metB_{Hp}$. Both are involved in cysteine and methionine metabolism in many other bacteria. This leads to a hypothesis: *H. pylori* is able to convert homocysteine to cysteine via the intermediate cystathionine through the RTSP.

1.7 Objectives

luxS homologues and the production of AI-2 exist widely in bacteria. Nevertheless, the role of LuxS remains unclear in many microorganisms due to its dual function both in amino acid metabolism and QS signalling. Some previous publications suggest that AI-2 may not act as a QS molecule in many bacteria, but may just be a by-product of central metabolic AMC, secreted during exponential phase and degraded at later stage. In other words, production of AI-2 may only be associated with metabolic purpose, and not with regulatory significance (Doherty *et al.*, 2006).

H. pylori is one of the controversial cases. Although *H. pylori* possesses *luxS* and releases AI-2 that is able to induce bioluminescence in the *V. harveyi* reporter strain, evidence for the phenotypic changes that are attributed to disruption of *luxS* is limited. Like many other microorganisms, *H. pylori* possesses the central metabolic pathway AMC (but incomplete), in which $LuxS_{Hp}$ is responsible for producing homocysteine, the key intermediate metabolite for biosynthesis of methionine or cysteine, and also AI-2. The dual products by $LuxS_{Hp}$ lead to a few questions: Is $LuxS_{Hp}$ a pure metabolic enzyme in amino acid metabolism? Is AI-2 only a metabolite generated as a by-product in the incomplete AMC, or also a QS signal utilised by *H. pylori*? If productions of both homocysteine and AI-2 are disrupted by loss of $luxS_{Hp}$, are corresponding phenotypic alterations induced by inactivation of $luxS_{Hp}$ due to loss of AI-2 or the disrupted amino acid metabolism, or both?

To answer these questions, firstly, it is needed to clarify the role of $LuxS_{Hp}$ in metabolism. To do this, the relative contribution of $luxS_{Hp}$ with linked $metB_{Hp}$ and $cysK_{Hp}$ to biosynthesis of sulphur-containing amino acids had to be analysed. Drawing known information on $luxS_{Hp}$ and genome data of $cysK_{Hp}$ and $metB_{Hp}$, it

was hypothesised that proteins encoded by the operon *cysK*$_{Hp}$-*metB*$_{Hp}$-*luxS*$_{Hp}$ catalyse a metabolic pathway for cysteine biosynthesis. In this study, amino acid requirements of *H. pylori* wild-types and all mutants were examined in different chemically defined media (CDM). Next, metabolite pools of cells of wild-types and each mutant were analysed, by which the only cysteine biosynthetic pathway in *H. pylori* was confirmed, and the precise steps in this pathway were defined. For details see Chapter 3 and 4.

To investigate whether LuxS$_{Hp}$ is also a functional QS signal synthase, varieties of experiments were performed, including motility bioassays, microscopy, gene transcriptional testing and microarrays. Importantly, due to the alternative role of LuxS$_{Hp}$ in proposed cysteine metabolism, careful studies were carried out to exclude the possibility that the contribution of LuxS$_{Hp}$ to QS was in fact due to non-specific metabolic effects of LuxS$_{Hp}$. To do this, specific conditions were set up, in which the *luxS*$_{Hp}$-required cysteine metabolic pathway and the production of AI-2 were isolated from each other. For details see Chapter 5 and 6.

In summary, the aims of this project were to:

1) Identify the cysteine biosynthetic pathway in *H. pylori*;
2) Clarify the metabolic role of the operon *cysK*$_{Hp}$-*metB*$_{Hp}$-*luxS*$_{Hp}$ in the cysteine biosynthetic pathway;
3) Confirm whether motility of *H. pylori* is *luxS*/AI-2 dependent;
4) Investigate whether the *luxS*/AI-2 system controls motility of *H. pylori* via AI-2 signals only, or also via *luxS*-required cysteine metabolism;
5) Explore the mechanism underlying motility phenotypic changes upon *luxS*$_{Hp}$ inactivation.

CHAPTER 2.
GENERAL MATERIALS AND METHODS

2 General Materials and Methods

2.1 Materials

2.1.1 Commercial materials and reagents

All commercial media were purchased from Bacto, UK and Oxoid Ltd, UK, and prepared according to the manufacturer's recommendations. Specialist media for individual methods are described in following chapters. All chemicals were purchased from Sigma Chemical Company, UK. All restriction digestion reagents and PCR reagents were purchased from New England Biolabs Inc., UK and Promega Ltd, UK. All chemicals used to make chemically defined media (CDM) were of high purity (cell culture tested), and obtained from Sigma-Aldrich Co.

2.1.2 Strains, plasmids and growth culture conditions

The strains and plasmids used in this study are listed in Table 2.1. *E. coli* strains DH5α and DS941 (Summers & Sherratt, 1988, Yanisch-Perron *et al.*, 1985) were used in cloning/subcloning experiments. *E. coli* was routinely propagated in Luria-Bertani (LB) broth or on LB-agar plates at 37^{o}C, under normal atmospheric conditions. *V. harveyi* BB170 was utilised as a reporter strain in the bioluminescence bioassay. *Vibrio harveyi* was grown in either LB or AB medium (Greenberg *et al.*, 1979) at 30^{o}C, also under normal atmospheric conditions. *H. pylori* strains were routinely subcultured on horse blood agar (Oxoid) every 2-3 days, and were grown in a MACS VA500 Microaerobic Workstation (Don Whitley Scientific) using a humidified atmosphere consisting of 6% O_2, 3% H_2, 5% CO_2 and 86% N_2. Antibiotic selection was carried out at 100µgml^{-1} with ampicillin (or carbenicillin), and 50µgml^{-1} for kanamycin.

For *H. pylori* metabolic supplementation experiments, the complete chemically defined medium (cCDM) of Reynolds and Penn was formulated as previously described (Reynolds & Penn, 1994), with variations as indicated in the text (Chapter 4.2.1). CDM batch culture comparative growth studies were carried out as described previously (Doherty *et al.*, 2009).

Table 2.1 Strains and plasmids used in this study

Strains/Plasmids	Genotype/notes	Source
Strains		
Escherichia coli		
DH5α	*endA*1 *recA*1 *gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *relA1 supE44* Δ(*lacZYA-argF*) *U169 F*$^-$ Φ80d*lacZ*Δ*M15 deoA phoA* λ$^-$	(Yanisch-Perron *et al*., 1985)
DS941	*thr*-1 *leu*-6 *thi*-1 *lacY*1 *galK*2 *ara*-14 *xyl*-5 *mtl*-1 *proA*2 *his*-4 *argE3 str*-31 *tsx*-33 *supE*44 *rec*+ *recF lacIq lacZ* Δ*M15*	(Summers & Sherratt, 1988)
Vibrio harveyi		
BB170	*luxN* : : Tn5 AI-1 sensor negative; AI-2 sensor positive	(Surette & Bassler, 1998)
Helicobacter pylori		
J99 (ATCC700824)	Wild-type strain	(Alm *et al.*, 1999)
J99Δ*luxS*	J99; Δ*luxS* : :Km^r	This study
J99Δ*luxS*-F	J99 derivative; Δ*luxS* : : *km-sacB*; Km^r Suc^s	This study
J99 Δ*luxS*$^+$	J99Δ*luxS*-F derivative; Δ*luxS* : : *km-sacB* replaced with original *luxS* locus; Suc^r Km^s	This study
J99Δ*metB*	J99; Δ*metB*: :Km^r	This study
J99Δ*cysK*	J99; Δ*cysK*: : Km^r	This study
J99 Δ*flhB*	J99 derivative; ΔHP0770 Lys^{13} to Glu^{347}; Km^r; non-motile	(Wand *et al.*, 2006)

NCTC 11637 (ATCC43504)	Wild-type strain	(Marshall *et al.*, 1984)
11637Δ*luxS*	11637; Δ*luxS* : :Km^r	This study
11637Δ*metB*	11637; Δ*metB*: :Km^r	This study
11637Δ*cysK*	11637; Δ*cysK* : :Km^r	This study
CCUG 17874[††]	Wild-type strain	(Marshall *et al*., 1984)
17874 Δ*flaA*	17874 derivative; Δ*flaA* : : *cat*; Cm^r	Paul O'Toole
17874 Δ*flgE*	17874 derivative; Δ*flgE* : : *km*; Km^r	(O'Toole *et al.*, 1994)
Plasmids		
pGEM-T	Commercial TA cloning vector; Ap^r	Promega, Madison, WI
pMWA2	Source of apolar *aphA3* cassette; Ap^r Km^r	(Wand *et al*., 2006)
pLUXS	pGEM-T::*luxS*$_{Hp}$::*aphA*3; Km^r	Theo Verboom
pMETB	pGEM-T bearing the *metB*$_{Hp}$ gene; Ap^r	This study
pCYSK	pGEM-T bearing the *cysK*$_{Hp}$ gene; Ap^r	This study
pGEMTluxSXN396	pGEM-T with inserted 26695 *luxS*;	(Loh *et al*., 2004)
pMF1	pMETB deletion-insertion derivative; Ap^r Km^r	This study
pCF2	pCYSK deletion-insertion derivative; Ap^r Km^r	This study
pProEx-*luxS*$_{EC}$	pProEX HT containing the *luxS* gene of *E. coli* MG1655	(Winzer *et al*., 2002a)
pProEx HT mtan	pProEX HT containing the *pfs* gene of *E. coli*	(Winzer *et al*., 2002a)

[††] CCUG 17874 is identical to the type strain NCTC 11637, isolated by B. J. Marshall at Royal Perth Hospital, May 1982. Marshall, B. J., H. Royce, D. I. Annear, C. S. Goodwin, J. W. Pearman, J. R. Warren & J. A. Armstrong, (1984) Original isolation of *Campylobacter pyloridis* from human gastric mucosa. *Microbios Lett.* **25**: 83-88.

2.2 General Methods

2.2.1 DNA preparation and DNA manipulation techniques

2.2.1.1 General molecular biology methods

Preparation of plasmid DNA, DNA ligation, gel electrophoresis and transformation of *E. coli* strains were performed in accordance with standard methods (Sambrook *et al.*, 1989). 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). DNA-sequencing reactions were performed on PCR purified fragments or plasmids by Geneservice (Cambridge, UK). 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 instruction. *H. pylori* genomic DNA was isolated as described previously (Letley *et al.*, 2003). DNA sequencing was conducted using standard fluorescent dye terminator chemistries, and analysis performed using the Applied Biosystems 3730 DNA Analyser system (Geneservice, Cambridge, UK, Applied Biosystems Inc, Foster City, CA.). Results were analysed using the Bioedit software suite (Hall, 1999).

2.2.1.2 Polymerase chain reaction (PCR)

A 25µl reaction mixture was set up for each DNA template containing: 2.5µl 10 × PCR buffer (Promega, UK), 2.5µl dNTPs (Promega, UK), 0.1µl Taq DNA polymerase (Promega, UK), 0.1µl each primer, 0.2µl template DNA, and 19.7µl distilled water. PCR reaction mixture without template was used as the negative control. Amplification conditions were; 95°C for 30s, 54°C for 30s, and 72°C for 2 min, for 30 cycles in total. Thermal cycling for amplification began with an initial denaturation for 5 min at 95°C and ended with a final elongation for 10 min at 72°C.

2.2.1.3 Restriction digestion

Reaction mixtures contained buffer, restriction enzymes, template and distilled water. All digestion reactions were carried out by incubating reaction mixtures at 37°C for 1 h. The percentage volume of enzymes in the reaction mixture was less than 10%.

2.2.1.4 T4 DNA ligation

Reactions were performed in a final volume of 10µl containing 1µl T4 ligase (or 1/10 diluted T4 ligase), 1µl T4 buffer, vector (1 unit), PCR amplified DNA or digested DNA fragment (3 units) and dH_2O. The mixture was incubated at 4°C overnight. Prior to use in transformation reactions, salt was removed by dialysis against water using 13mm, 0.025µm pore-size nitrocellulose filters for 40 min.

2.2.1.5 Electrophoresis of DNA

DNA fragments were separated by electrophoresis using 1% agarose gels prepared in 1 × Tris-acetate-EDTA (TAE) buffer (40nM Tris-acetate, 2mM EDTA, pH8.5), stained with ethidium bromide (0.5µg/ml). Diluted 7.5µl 1kb DNA ladder (500ng/µl, Promega) or diluted 7.5µl 2-log DNA ladder (NEB, UK) were used as the size markers. Gels were run at 80V to 100V in 1 × TAE buffer. DNA bands were visualised and photographed under ultra-violet (UV) light (Uvidoc).

2.2.1.6 Preparation of *H. pylori* chromosomal DNA

H. pylori was harvested from blood agar, and resuspended into 1ml of phosphate buffered saline (PBS). The suspension was then centrifuged at 13000 rpm for 5 min. Cell pellets were resuspended in 100µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). 500µl GES buffer (5M guanidine thiocyanate, 0.1M EDTA, 0.5% (w/v) sarkosyl) was added and the mixture was incubated at room temperature for 5 min, and then placed on ice. 250µl of ice cold ammonium acetate (7.5M) (Sigma, UK)

was added, gently mixed and chilled on ice for 5 min. 650µl of chloroform was then added and mixed gently. All the samples were then centrifuged at 13000 rpm for 5 min. The aqueous top layer containing DNA was removed into a fresh 1.5ml Eppendorf using wide end tips, and another 650µl of chloroform was added and centrifuged as before. The aqueous top layer was transferred again to a fresh 1.5ml Eppendorf using wide end tips and 0.54 × volume of isopropanol was added for DNA precipitation from the aqueous phase. The mixture was centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 70% 500µl ethanol. After air drying, the pellet was resuspended in 100µl-200µl nuclease free water.

2.2.2 Transformation methods

2.2.2.1 $CaCl_2$ method

This method was utilised to transform plasmid to *E. coli* DH5α. 5ml overnight culture of DH5α was used to inoculate 50ml fresh LB medium, and cultured at 37°C for 1-2 h till OD_{550} was 0.5 (+/-10%) The culture samples was divided into two aliquots and centrifuged at 4.5 × 1000 rpm for 10 min. The pellet from each aliquot was resuspended in 20ml 0.1M $CaCl_2$, and placed on ice for 30 min. Each aliquot was centrifuged at the same speed for 10 min. The supernatant was discarded. The pellet was resuspended in 2ml 0.1M $CaCl_2$ and chilled on ice for 10 min. 1-5µl DNA ligation was added to 100µl of cells. Cells were placed on ice for 40 min and heat shocked at 42°C for 1 min, and then placed on ice for further 10 min. Cells were spread on selective LB agar (containing 80µM X-Gal and 500µM IPTG).

2.2.2.2 Electroporation

Electroporation was used to transform plasmids into *E. coli* DS941. 20ml DS941 was incubated in LB broth overnight at 37°C whilst shaking. The overnight culture was

diluted 1:100 in 2L LB broth at 37°C whilst shaking until the OD_{600nm} reached 0.5 (+/-10%). Cells were pelleted by centrifugation for 35 min at 4.0 × 1000 rpm at 4°C. Cells were resuspended in 1L sterile distilled ice-cold water (4 × 250ml), and pelleted as before. Cells were washed twice with ice-cold water, and then pelleted and resuspended in 10% glycerol (4 × 20ml and 4 × 1ml), and divided into 55µl aliquots. Cells were frozen rapidly in liquid N_2, and stored at -80°C for use in electroporation. 5µl DNA ligation was prepared by dialysis against distilled water for 20-30 min using 0.025µm pore size nitrocellulose filters. Electro-competent cells were transformed with 2µl DNA ligation reaction mix. Transformation was carried out in a chilled electroporation cuvette, using a BioRad Gene Pulser. Electroporation voltage was 1.8kV/cm (0.1cm electrode gap), at a setting of 200Ω and 25µF. The cuvette was placed into the sample chamber and the pulse was applied. Then 1000µl of LB broth was added into the cuvette immediately. The cells were then incubated for 60 min at 37°C. Cells (100-200µl) was plated on selective LB agar and incubated overnight at 37°C.

2.2.2.3 Natural transformation of *H. pylori* strains

The *H. pylori* strains (J99 and 11637) were grown for 24 h on blood agar plates and were passed onto fresh blood plates and grew for another 24 h. The cells were harvested and suspended in 1000ml isosensitest-FBS (90 % (w/v) isosensitest, 10% (v/v) FBS) then pelleted by centrifugation at 13,000 rpm for 5 min. The cells were resuspended in 30-50µl fresh isosensitest-FBS and placed as a spot in the centre of a blood agar plate and incubated at 37°C for 6 h. 3-5µl of plasmid DNA (heat sterilised at 85°C for 10 min and allowed to cool to room temperature) was added to the centre of the *H. pylori* growth and incubated overnight. The cell mass was spread out over the surface of the plate and incubated at 37°C for 1 day and then transferred onto a

blood plates containing antibiotics (kanamycin 30 μg/ml, cloramphenicol 30μg/ml, sucrose 5% [w/v]). Transformants were selected by their ability to survive in the presence of antibiotic. The allelic double-crossover event and the orientation of the resistant cassette were verified by nucleotide sequencing across the points of insertion using the *s*ame primers.

2.2.3 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.3.1 Protein samples preparation

Bacterial cells were taken from agar plate into 1ml PBS by a swab (for broth culture, cells were normalised by resuspending in PBS to give a standard OD_{600nm} of 1.0). Samples were sonicated for 5 to 10 sec three times and kept on ice. Samples were then centrifuged for 30 sec by a high speed.100μl supernatants were taken and added with 100μl 2 × PAGE sample buffer (20% [v/v] glycerol, 125mM Tris-HCL, pH 6.8, 4% SDS, 0.01mg/ml bromophenol blue, 5% β-mercaptoethanol) and then inactivated by 100°C boiling water.

2.2.3.2 SDS-PAGE gel electrophoresis

A 12.5% resolving gel (4.2ml Acrylamide/Bis, 1.25ml RGB, 100μl 10% SDS, 4ml dH_2O, 5μl TEMED, and 75μl 10% Ammonium Persulphate) and the stacking gel (750μl Acrylamide/Bis, 1.5ml SGB, 60μl 10% SDS, 3ml dH_2O, 6μl TEMED, and 150μl 10% Ammonium Persulphate) was routinely used. The combs in stacking gel were removed and the wells were washed with dH_2O. The protein-PAGE gel was placed in the gel tank (Invitrogen) and 900ml 1 × SDS-PAGE running buffer (14.4g Glycine, 3g Tris, 1g SDS in 1L dH_2O). 10μl to 20μl of protein samples were gently loaded to each well. Pre-stained broad range protein marker (Biolabs) was utilised to eliminate the molecular weight of the protein tested. The gels were run at 180V for

40 min to 1 h till the dye comes out of the gels. The stacking gel was removed and the gels stained or proteins were transferred to nitrocellulose membrane by the methanol Tris-glycine system described by Towbin *et al* (1992).

2.2.4 Cysteine and DPD complementation experiment

Cysteine was Sigma products and dissolved according to the manufacturer's recommendation. Synthetic DPD was purchased from Omm Scientific Inc. DPD activity was quantified with the bioluminescence bioassay and compared to wild-type *H. pylori* grown to OD_{600nm} of 1.0, at which maximal AI-2 activity was obtained. To test for complementation of motility, DPD (150µM, after 10 h and once again after 18 h of incubation) and cysteine (1.0mM, at the beginning of incubation) were added individually to bacteria-AGS cells co-cultures. Bacterial motility and cell morphogenesis were observed and visualized by the phase-contrast microscope and EM, respectively. For analyses of flagella biosynthesis and RNA expression, DPD and cysteine were added (in the same way) individually to cell cultures of different *H. pylori* genotypes. After 24 h, cell culture samples were analysed using protein electrophoresis and Western blotting, and RNA was extracted and the transcript levels of genes of interest were measured.

2.2.5 Statistical methods used

The statistical analysis methods used in this study included Student's *t* distribution test and standard deviation.

CHAPTER 3.
MUTAGENESIS OF *luxS*, *cysK* AND *metB* ON *H. PYLORI cysK-metB-luxS* GENE CLUSTER

The work in this chapter has been published in the Journal of Bacteriology.

3 Mutagenesis of *luxS*, *cysK* and *metB* on *H. pylori* *cysK-metB-luxS* gene cluster

3.1 Introduction

Helicobacter pylori is a Gram-negative bacterium which causes peptic ulceration, gastric adenocarcinoma, and gastric lymphoma (Atherton, 2006). All *H. pylori* strains possess a homologue of *luxS*, best known as a quorum sensing (QS) molecule synthase. *luxS* homologues have been found in many bacterial genomes sequenced (Sun *et al*., 2004, Tomb *et al*., 1997, Vendeville *et al*., 2005, Hardie & Heurlier, 2008). Some of the molecules formed in the reaction catalysed by LuxS are collectively termed auto-inducer-2 (AI-2) and have been shown to act as signalling molecules. They have been described as mediating a variety of effects in different bacteria, including virulence. In *H. pylori*, disruption of *luxS* has been shown to increase biofilm formation (Cole *et al*., 2004) and to reduce *in vivo* fitness and infectivity (Lee *et al*., 2006, Osaki *et al*., 2006). Several motility-associated $luxS_{Hp}$ phenotypes have also been reported, including loss of growth phase-dependent *flaA* regulation (Loh *et al*., 2004), reduced motility on soft agar plates, and reduced transcription of the flagellar regulator gene *flhA* (Osaki *et al*., 2006, Rader *et al*., 2007).

In most bacteria, the enzyme encoded by *luxS* has an alternative (possibly primary and sometimes sole) function: it is an essential metabolic component of the activated methyl cycle (AMC). LuxS in the AMC catalyses cleavage of *S*-ribosylhomocysteine (*S*RH) to yield homocysteine and a by-product, 4, 5-dihydroxy-2, 3-pentanedione (DPD) (Figure 1.6) (Schauder *et al*., 2001, Winzer *et al*., 2002a, Winzer *et al*., 2003,

Vendeville *et al*., 2005). DPD is the precursor of the family of related, interconverting molecules "AI-2" (Camilli & Bassler, 2006, Vendeville *et al*., 2005). The AMC in *H. pylori* is incomplete as the bacterium lacks MetB and MetH homologues to re-generate methionine from homocysteine (see Chapter 4.1 for detail information of the AMC in *H. pylori*).

In searching for a possible metabolic role for $LuxS_{Hp}$, a hypothesis was formed: it could be part of a *de novo* cysteine biosynthesis pathway that uses methionine as a reduced sulphur source. The reasoning for this was based on the observation that *H. pylori* has an absolute requirement for methionine (Nedenskov, 1994, Reynolds & Penn, 1994, Mendz & Hazell, 1995), in agreement with its lack of *metE* and *metH*. Also, all complete *H. pylori* genomes available to date (including 26695, J99, HPAG1, G27, B128, Shi470 and 98-10) contain homologues of two genes, annotated as *cysK* and *metB*, which in other bacteria (e.g. *B. subtilis* and *M. tuberculosis*) are involved in the generation of cysteine and interconversion of cysteine and methionine, respectively (BLAST).

In all sequenced *H. pylori* strains, *cysK* and *metB* are located immediately upstream of *luxS*, forming a $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$ gene cluster. According to the genome data of Genbank, *H. pylori metB* is predicted to encode a cystathionine γ-synthase (CGS)-like enzyme, while *cysK* is predicted to encode a cysteine-synthase or a cystathionine β-synthase (CBS)-like enzyme. Recently, the *metB* gene from *H. pylori* strain SS1 was purified and characterised and has been suggested to encode a functional CGS, but its physiological role was not established (Kong *et al*., 2008).

When pondering the fate of homocysteine, which is not converted to methionine in *H. pylori* due to a lack of MetE and MetH, coupled with the observation that the gene responsible for its production, *luxS*, is linked with two other genes predicted to be involved in methionine and cysteine metabolism, it was hypothesised that *H. pylori* is able to utilise homocysteine by converting it to cysteine via the reverse transsulphuration pathway (RTSP), involving the associated genes *metB* and *cysK* (also see Chapter 4.1 for more details).

To define more specifically the primary metabolic roles of *cysK*, *metB*, and *luxS* homologues in the putative *H. pylori* RTSP, first of all, Δ*luxS*, Δ*metB* and Δ*cysK* mutants were constructed using an insertion-deletion strategy in two different *H. pylori* strtains J99 and NCTC11637 to avoid strain specific effects. As $luxS_{Hp}$ comprises the last of the genes in the operon $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$, to avoid polar effects of disrupting either $cysK_{Hp}$ or $metB_{Hp}$, a variant of *aphA3* which lacks both the promoter and terminator elements was used in producing mutants. Subsequently, the *V. harveyi* AI-2 assay (Bassler *et al*., 1997, Winzer *et al*., 2002a) was used to demonstrate that disruption of either $cysK_{Hp}$ or $metB_{Hp}$ does not block expression of downstream genes.

3.2 Materials and Methods

3.2.1 Construction of mutations

3.2.1.1 Construction of *H. pylori* Δ*luxS* strains

(This work was done by Neil Doherty)

All mutants were made following an insertion-deletion strategy. The *H. pylori* Δ*luxS* mutant strains were made by transforming J99 and NCTC11637 with pGEMT::*luxS*::*aphA*3 (a generous gift from Leo Smeets and Theo Verboom).

3.2.1.2 Construction of *H. pylori* Δ*metB* strains

A 2.1kb region of the genome of *metB* was amplified from *H. pylori* J99 genomic DNA using the primer pair *metB*-F/*metB*-R (forward, 5'>CTT GAT AAT CCC GCA GCC TAC TA<3'; reverse, 5'>ACC CCC ACT TCA GAC CAC TCA G<3'). Amplified product included full *metB* along with approximately 500bp of upstream and downstream flanking sequence. The fragment was cloned into the pGEM-T [TA] vector system (Promega) to produce the plasmid pMETB. A deletion of *metB* was generated by inverse PCR with the primer pair *metB*-IF/*metB*-IR (forward, 5'>CGT GAA TTC CGG CTA AAC CAG<3', *EcoR*I site is underlined; reverse, 5'>TGA ACA GGA TCC GTT AGA AGA TT<3', *BamH*I site is underlined) using pMETB as template. An 860bp fragment cut from pMWA2 encoding kanamycin resistant cassette *aphA3* (Wand *et al*., 2006) that contains the corresponding restriction site of *EcoR*I or *BamH*I on each end was cloned into pMETB inverse PCR product to form the plasmid pMF1. pMF1 was transformed into recipient wild-type *H. pylori* strains J99 and 11637. Putative double-crossover strains were selected, and the presence of the kanamycin resistant gene was verified by amplifying the mutated fragments on *H. pylori* chromosomal DNA using primers *metB*-F/*metB*-R and DNA sequencing.

3.2.1.3 Construction of *H. pylori* Δ*cysK* strains

Primers *cysK*-F/*cysK*-R (forward, 5'>CAC CAT TGA CA AAT CCT TCC<3'; reverse, 5'>TTT GGT GTT GGG CTT GAT AG<3') were designed to amplify a 1.7kb *cysK* fragment with approximate 500bp of upstream and downstream flanking sequence; the product was cloned into vector pGEM-T to generate a new plasmid pCYSK. A mutation of *cysK* was produced by inverse PCR mutagenesis using pCYSK as template with the primer pair *cysK*-IF/*cysK*-IR (forward, 5'>GCT GTT TTT CTG TGC TGA ATT CTT<3', *EcoR*I site is underlined; reverse, 5'>GTG GAT CCG AGG GTT CTA TTT TGA<3', *BamH*I site is underlined). The apolar *aphA3* cassette were digested from pMWA2 and cloned into the inverse PCR product to produce a plasmid pCF2. pCF2 was purified and utilised to disrupt the *cysK* locus in *H. pylori* wild-type strain J99 and 11637 by natural transformation and selection of kanamycin resistant colonies. The resulting *cysK* :: *km* (Δ*cysK*) strains were verified by PCR and a sequence of the genomic locus.

3.2.2 AI-2 *in vitro* biosynthesis

3.2.2.1 Purification of the proteins LuxS and Pfs

2ml of LB cultures of DH5αLuxS and DH5αPfs (with 2µl carbenicillin, 50mg/ml) were grown overnight. 0.5ml of the overnight cultures of DH5αLuxS and DH5αPfs were respectively used to inoculate 50ml fresh LB containing 50µl carbenicillin solution, grown at 37°C and 200 rpm for 2 h. 100µl of 0.5M IPTG added and incubation continued for another 3 h. Cell pellets were obtained by centrifugation and resuspended in 2ml bugbuster (Novagen), with 2µl benzonase (Novagen) added. Suspension was shaken gently for 20 min at room temperature and then centrifuged at 10,000g for 20 min at 4°C. Clear lysate was decanted off and collected ready for protein purification. For each protein preparation, approximately 2ml of the NI-NTA

His bind resin was loaded into small disposable chromatography column, and allowed to settle. Resin was washed with 5 × 1.5ml of bind buffer. Lysate was added to resin then washed with 5 × 3ml bind buffer followed by 5×3ml wash buffer. The column was then eluted with 5 × 1ml portions of the elute buffer, collecting individual 1ml fractions. Purified protein usually elutes in the 2nd or 3rd fraction, which is then stored at -80°C with the addition of 100µl 50% glycerol solution. Protein solutions could be used directly in following steps.

3.2.2.2 Staining and drying of protein gels

Gels (containing LuxS and Pfs protein bands, respectively) were stained with Coomassie blue staining solution (0.25g Coomassie brilliant blue, 45 % (v/v) methanol, 10 % (v/v) glacial acetic acid) for 20 min and destained in Coomassie blue destaining solution (20% [v/v] methanol, 7% [v/v] glacial acetic acid). After staining, gels were rinsed in dH_2O and placed in gel-drying solution (Novex) with two DryEase™ Mini-Cellophane sheets (Novex) for 10 min. The gel was sandwiched between the Mini-Cellophane sheets and air dried.

3.2.2.3 *In vitro* synthesis of AI-2

Sodium phosphate buffer (10mM, pH=7.7) was deoxygenated by sparging with helium (or nitrogen) for approximate 10 min. 1.7ml buffer was added to 3.08mg dehydrate *S*AH with 100µl of purified Pfs solution and incubated at 37°C for 2 h. 900µl of the incubated solution was added to 1ml fresh buffer containing 100µl of LuxS solution, and incubated for another 2 h. 1.1ml buffer was added to the remaining 900µl as a negative control.

3.2.3 Bacterial growth curves and *V. harveyi* bioluminescence assay

Bacterial broth cultures were started from a blood agar plate culture, diluted to an OD_{600nm} of 0.05 in fresh BB medium, and grown at 37°C in a VAIN-cabinet with shaking. OD_{600nm} 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 0.2µm pore size filter. The AI-2 activity in cell free supernatants (CFS) was tested as previously described using the *V. harveyi* reporter strain BB170 (Winzer *et al*., 2003, DeKeersmaecker & Vanderleyden, 2003). Briefly, an overnight *V. harveyi* culture was diluted 1:2500 in fresh AB medium (Greenberg *et al*., 1979). Cell free supernatant (CFS) samples were diluted 1:10 in the AB medium containing BB170 into the 96 well bioluminescence plates to give a final volume of 200µl 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.

3.3 Results

3.3.1 The mutagenesis of $MetB_{Hp}$ and $CysK_{Hp}$

Genome sequence analysis of *H. pylori* (strains J99 and 26696) revealed putative genes *luxS*, *metB* and *cysK* (Alm *et al*., 1999, Tomb *et al*., 1997). $luxS_{Hp}$ is located immediately downstream of $metB_{Hp}$ and $cysK_{Hp.}$ A Shine-Dalgarno sequence AGGAGA is generally located 10 basepairs upstream of the start codon ATG (methionine codon) of the putative operon $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$, and such sequence is not found between each two genes of the three on the putative operon (http://genolist.pasteur.fr/PyloriGene/). Thus, co-transcription within the $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$ genes cluster is predicted. The putative promoter includes putative -35 (TTAACG) and -10 (TAAAAT) boxes located upstream of $cysK_{Hp}$ (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). The transcriptional start site is predicted to locate 74 basepairs upstream of the start codon of the putative operon. The size of transcripts could be assessed by northern blot and actual transcription start site(s) by primer extension analysis. The encoded proteins are predicted to comprise 308 (CysK), 380 (MetB) and 145 (LuxS) amino acids, respectively.

To characterise the function of each of these genes, *luxS*, *metB* and *cysK* mutations were constructed in two *H. pylori* strains (J99 and 11637). Each mutant was created using a deletion/insertion approach, using the *aphA3* cassette (kanamycin resistance) as a selectable marker (Wand *et al*., 2006). The general approach to this mutagenesis strategy is shown in Figure 3.1 (the diagram of $cysK_{Hp}$ mutagenesis is not shown), and details are described in Chapter 3.2 Materials and Methods. Briefly, the $metB_{Hp}$ and $cysK_{Hp}$ PCR products were respectively cloned into vector pGEM-T to generate the new plasmids pMETB and pCYSK. Using pMETB and pCYSK as templates,

mutations of *metB*$_{Hp}$ and *cysK*$_{Hp}$ were produced by inverse PCR with primer pairs *metB*-IF/*metB*-IR and *cysK*-IF/*cysK*-IR, respectively. The mutagenesis of *metB*$_{Hp}$ and *cysK*$_{Hp}$ were confirmed by PCR (*metB*-F/*metB*-R and *cysK*-F/*cysK*-R) and DNA sequencing. To avoid polar effects of disrupting either *cysK*$_{Hp}$ or *metB*$_{Hp}$, the apolar kanamycin cassette *aphA3* which lacks both promoter and terminator sequences was used. The cassette ensures that transcription of the disrupted gene is initiated and terminated by the native promoter and terminator, respectively (see Chapter 3.3.2 for details). PCR products of each step for both *metB*$_{Hp}$ and *cysK*$_{Hp}$ mutangenesis are shown in Figure 3.2-3.8.

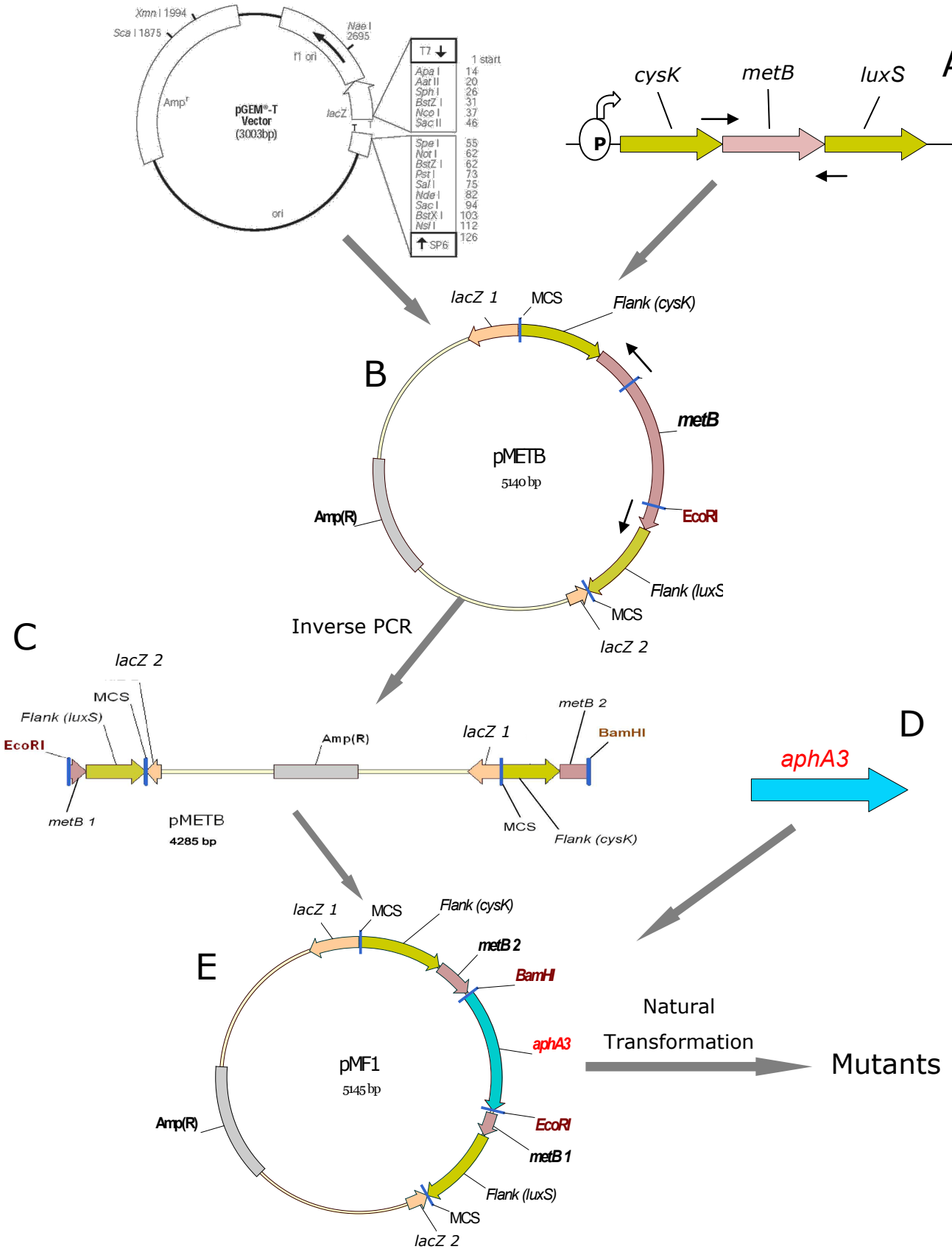


Figure 3.1 Flow diagram of *metB*$_{Hp}$ mutagenesis

For details described in text, see Chapter 3.2 Materials and Method. MCS=multiple cloning site

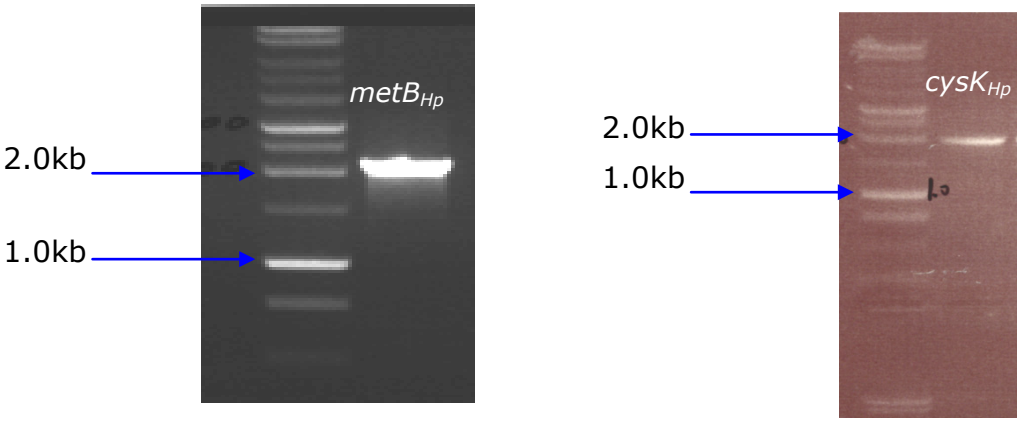


Figure 3.2 The *metB*$_{Hp}$ and *cysK*$_{Hp}$ PCR product

The gels show the products generated in figure 3.1 Step A. The PCR products *metB*$_{Hp}$ and *cysK*$_{Hp}$ were amplified from J99 wild-type genomic DNA. The size of the original *metB*$_{Hp}$ and *cysK*$_{Hp}$ were 1140bp and 918bp, respectively. 500bp of additional DNA sequence was included at both ends of the original *metB*$_{Hp}$ (and also cysK$_{Hp}$) sequence to make a final fragment containing the entire *metB*$_{Hp}$ (and cysK$_{Hp}$) gene plus flanking sequences. Primers *metB*$_{Hp}$-F/*metB*$_{Hp}$-R and cysK$_{Hp}$-F/cysK$_{Hp}$-R were used in the amplification. The size of the *metB*$_{Hp}$ PCR product was 2.1kb, and the size of the cysK$_{Hp}$ product was 1.9kb. Marker: Promega 1kb DNA ladder.

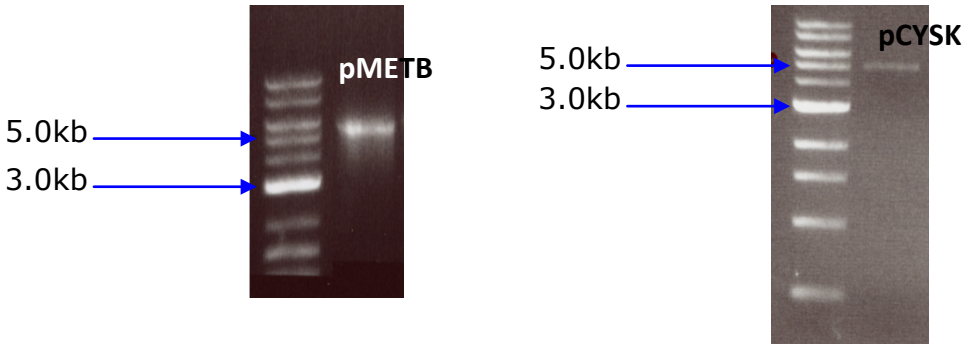


Figure 3.3 Digested products of plasmids pMETB and pCYSK

The gels confirm the products generated in figure 3.1 Step B. Plasmids pMETB and pCYSK were generated by cloning *metB*$_{Hp}$ and *cysK*$_{Hp}$ into the vector pGEM-T. Both plasmids were digested by the enzyme HindIII (Promega, UK). The size of the digest products of pMETB and pCYSK are 5.1kb and 4.9kb, respectively. This step was to confirm the successful cloning of *metB*$_{Hp}$ and *cysK*$_{Hp}$ into pGEM-T. Marker: Promega 1kb DNA ladder.

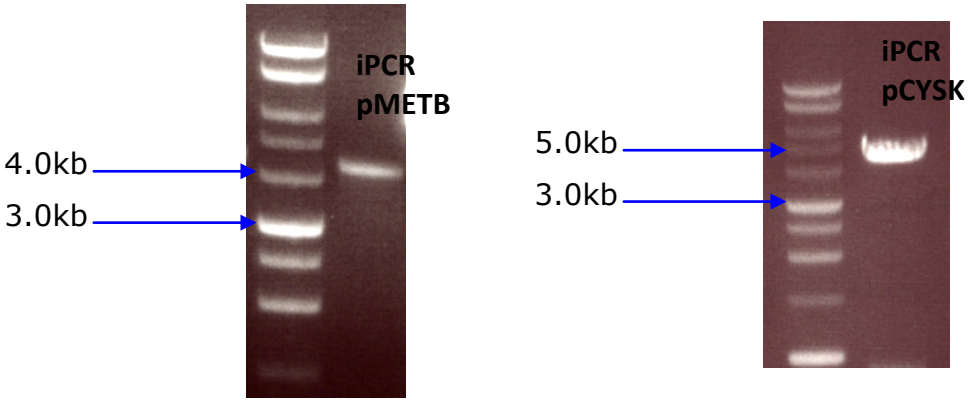


Figure 3.4 Inverse PCR products generated from plasmids pMETB and pCYSK

The gels show the inverse PCR products generated in figure 3.1 Step C. Mutations of *metB*$_{Hp}$ and *cysK*$_{Hp}$ were produced by inverse PCR mutagenesis using pMETB and pCYSK as templates with the primer pairs *metB*-IF/*metB*-IR and *cysK*-IF/*cysK*-IR, respectively. 860bp *metB*$_{Hp}$ sequence was deleted from the plasmid pMETB, and 227bp *cysK*$_{Hp}$ was deleted from the plasmid pCYSK. By inverse PCR, restriction sites of *EcoR*I and *BamH*I were produced respectively on each end of each inverse PCR product. The pMETB inverse PCR product is 4.3kb. The pCYSK inverse PCR product is 4.6kb. Marker: Promega 1kb DNA ladder. Marker: Promega 1kb DNA ladder.

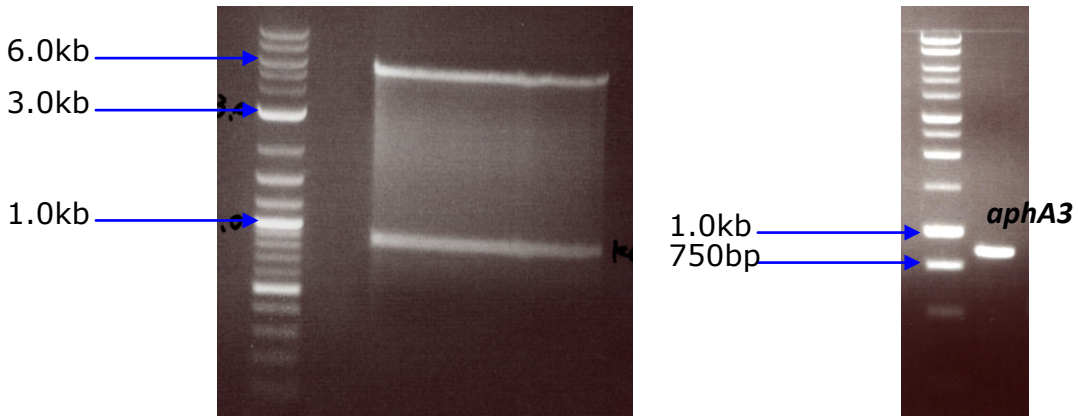


Figure 3.5 Digested product of the plasmid pMWA2 and gel purified *aphA3*

The two gels show the generation of the apolar cassette *aphA3* (figure 3.1 D). The apolar kanamycin resistance gene *aphA3* containing the corresponding restriction site of *EcoR*I or *BamH*I on each end was cut by restriction digestion from the plasmid pMWA2 (Wand *et al.*, 2006) (the gel shown on the left). The apolar *aphA3* product was purified from the

electrophoresis gel. The gel-purified *aphA3* product (860kb) was confirmed by electrophoresis (gel on the right). Marker: Promega 1kb DNA ladder.

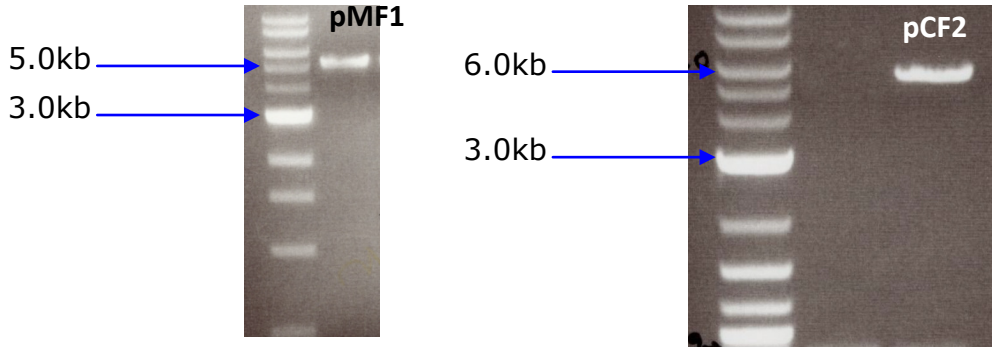


Figure 3.6 Digested products of pMF1 and pCF2

The two gels confirm the generation of pMF1 and pCF2 (figure 3.1 E).The plasmids pMF1 and pCF2 were generated by ligating *aphA3* with the pMETB and pCYSK inverse PCR products. The size of pMF1 and pCF2 are 5.1kb and 5.7kb, respectively. Putative pMF1 and pCF2 were digested by the enzyme HindIII (Promega, UK), and the size was confirmed by electrophoresis gel. Marker: Promega 1kb DNA ladder.

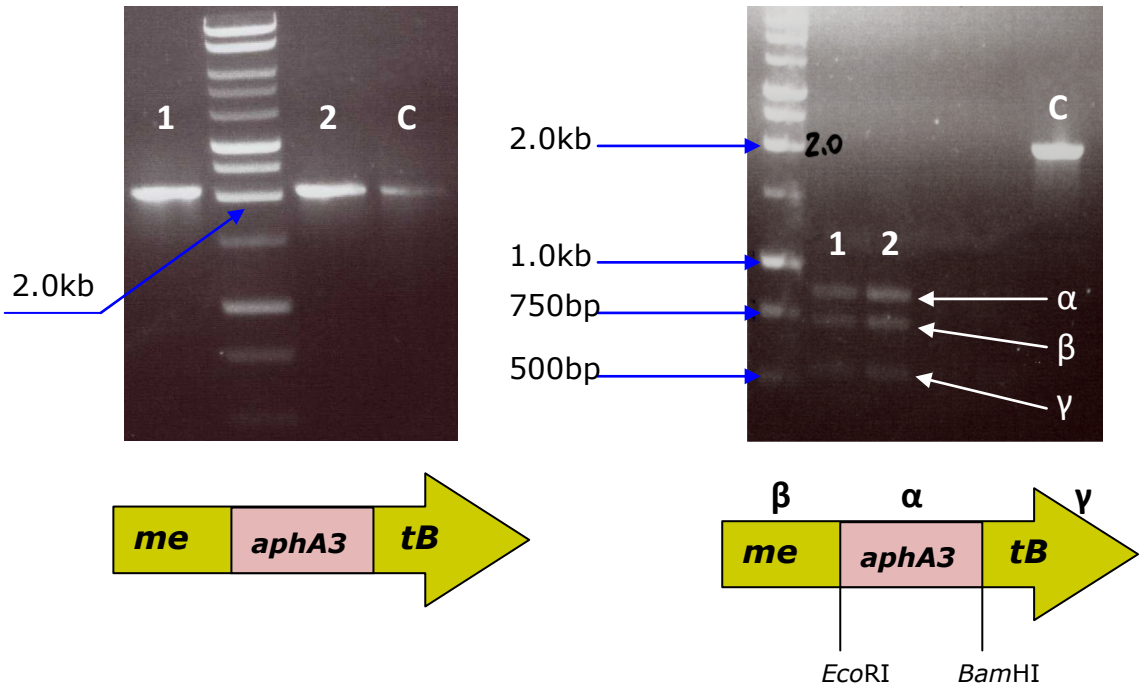


Figure 3.7 Confirmation of successful *metB*$_{Hp}$ mutangenesis in *H. pylori* J99 and 11637 strains

Mutagenesis of *metB*$_{Hp}$ was carried out in two *H. pylori* backgrounds, J99 and 11637. The mutated *metB*$_{Hp}$ was amplified with the original *metB*$_{Hp}$-F/*metB*$_{Hp}$-R primers using the chromosomal DNA of J99 Δ*metB*$_{Hp}$ (1) and 11637 Δ*metB*$_{Hp}$ (2) as templates (gel on the left). The size of the disrupted *metB*$_{Hp}$ is 2.1kb, which is almost the same as the wild-type *metB*$_{Hp}$. Therefore, restriction digestion was performed with enzymes *Eco*RI and *Bam*HI using mutated J99 *metB*$_{Hp}$ (1) and mutated 11637 *metB*$_{Hp}$ (2) as templates (gel shown on the right). Band 'α' was *aphA3* (860bp). Band 'β' (740bp) and 'γ' (540bp) were the 5' and 3' parts of *metB*$_{Hp}$ respectively. C: *metB*$_{Hp}$ PCR product amplified from 11637 wild-type genomic DNA was used as the sample control. Marker: Promega 1kb DNA ladder.

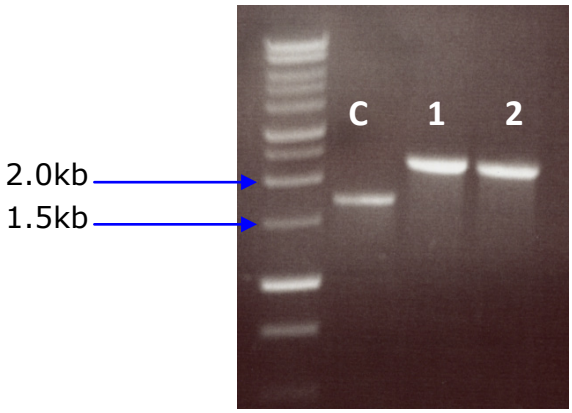


Figure 3.8 Confirmation of *cysK*$_{Hp}$ mutagenesis in *H. pylori* J99 and 11637 strains

Δ*cysK*$_{Hp}$ mutants were constructed in *H. pylori* J99 (1) and 11637 (2) strains. The mutated *cysK*$_{Hp}$ (2.3kb) was amplified with the original *cysK*$_{Hp}$-F/*cysK*$_{Hp}$-R primers using the chromosomal DNA of J99 Δ*cysK*$_{Hp}$ and 11637 Δ*cysK*$_{Hp}$ as templates. C: *cysK*$_{Hp}$ PCR product amplified from 11637 wild-type genomic DNA was used as the sample control (1.8kb). Marker: Promega 1kb DNA ladder.

3.3.2 Apolar disruption of *metB*$_{Hp}$ and *cysK*$_{Hp}$ did not affect expression of the downstream gene *luxS*$_{Hp}$

3.3.2.1 Auto-inducer-2 (AI-2) *in vitro* synthesis

The *in vitro* synthesised AI-2 product is used widely in this study. For instance, it was utilised as a positive control in the AI-2 activity bioassay and as a supplemented chemical in the complementation experiment of the plate motility assay (see Chapter 5). AI-2 was synthesised in the laboratory using the method reported previously, which exploits the two-step pathway catalysed by enzymes Pfs and LuxS (Figure 3.9B) (Winzer *et al*., 2002a). As already described in Matherials and Methods, proteins Pfs and LuxS were produced by purifying from the overnight cultures of DH5α$_{Pfs}$ and DH5α$_{LuxS}$ (Figure 3.9A). The dehydrated *S*AH was then added in sodium phosphate buffer as the substrate. After 2 h of incubation, the purified Pfs converted *S*AH to *S*RH, the substrate in the LuxS catalysed reation. The newly added LuxS solution subsequently converted *S*RH to homocysteine and the precursor of AI-2, DPD, after incubated at 37°C for another 2 h. The product of the synthetic pathway, AI-2 (or DPD) was examined using the *V. harveyi* BB170 bioluminescence assay.

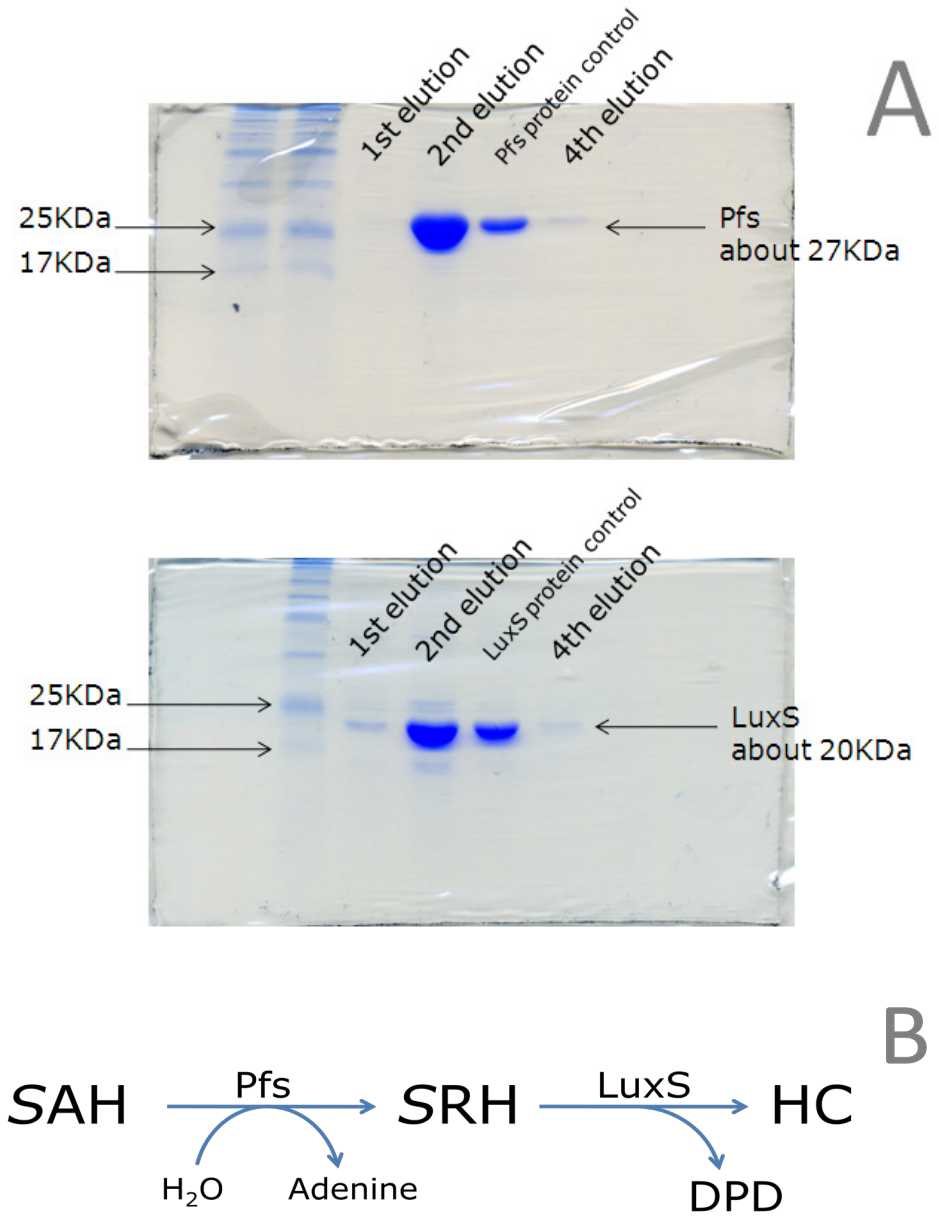


Figure 3.9 Purified enzymes Pfs and LuxS and the Pfs-LuxS catalysed AI-2 *in vitro* synthetic pathway

(A) The Coomassie blue stained SDS-PAGE gels of the purified Pfs and LuxS proteins, respectively. Both proteins were purified from the whole cell lysate. During the step of elution, columns containing resin were eluted with 5×1ml portions of the elute buffer, collecting individual 1ml fractions. Purified Pfs and LuxS products usually elute in the 2nd fraction. (B) The pathway of AI-2 synthesis was catalysed by Pfs-LuxS using *S*AH as the substrate. *S*AH, *S*-adenosylhomocysteine; *S*RH, *S*-ribosylhomocysteine; HC, homocysteine; Pfs, 5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase; LuxS, *S*-ribosylhomocysteinase/auto-inducer-2 synthase; DPD, 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2.

3.3.2.2 Bioluminescence assay for auto-inducer-2 activities in *H. pylori* wild-types and mutants

luxS$_{Hp}$, *metB*$_{Hp}$ and *cysK*$_{Hp}$ lie on the same operon. The integrity of downstream genes after mutagenesis of an upstream gene is essential to identify the function of each gene on the operon (Figure 3.10A). Therefore, the normal function of the downstream gene *luxS*$_{Hp}$ was tested in all mutants using the *V. harveyi* AI-2 detection assay described previously (Bassler *et al*., 1997, Winzer *et al*., 2002a). Before testing, *H. pylori* wild-type strains J99 and 11637 and all mutant strains were grown in Brucella broth containing serum (10% v/v). This study shows that *H. pylori* wild-type strains produce AI-2 in a growth dependent manner, in which AI-2 accumulated during late log phase, reaching maximum levels in early stationary phase (being reported in Chapter 5.3.2, Figure 5.4). Consequently, AI-2 activity in culture supernatants after 24 h of incubation was measured. Data presented here show that Δ*metB*$_{Hp}$ and Δ*cysK*$_{Hp}$ mutants exhibited AI-2 activity, suggesting that insertion of *aphA3* into *metB*$_{Hp}$ and *cysK*$_{Hp}$ did not affect expression of the downstream gene *luxS*$_{Hp}$. As expected, the Δ*luxS*$_{Hp}$ mutants, unlike the wild-type and the other two mutants, yielded significantly (p<0.001) reduced levels of bioluminescence, indicating that insertion of *aphA3* into the *luxS*$_{Hp}$ gene abrogated AI-2 production completely (Figure 3.10B).

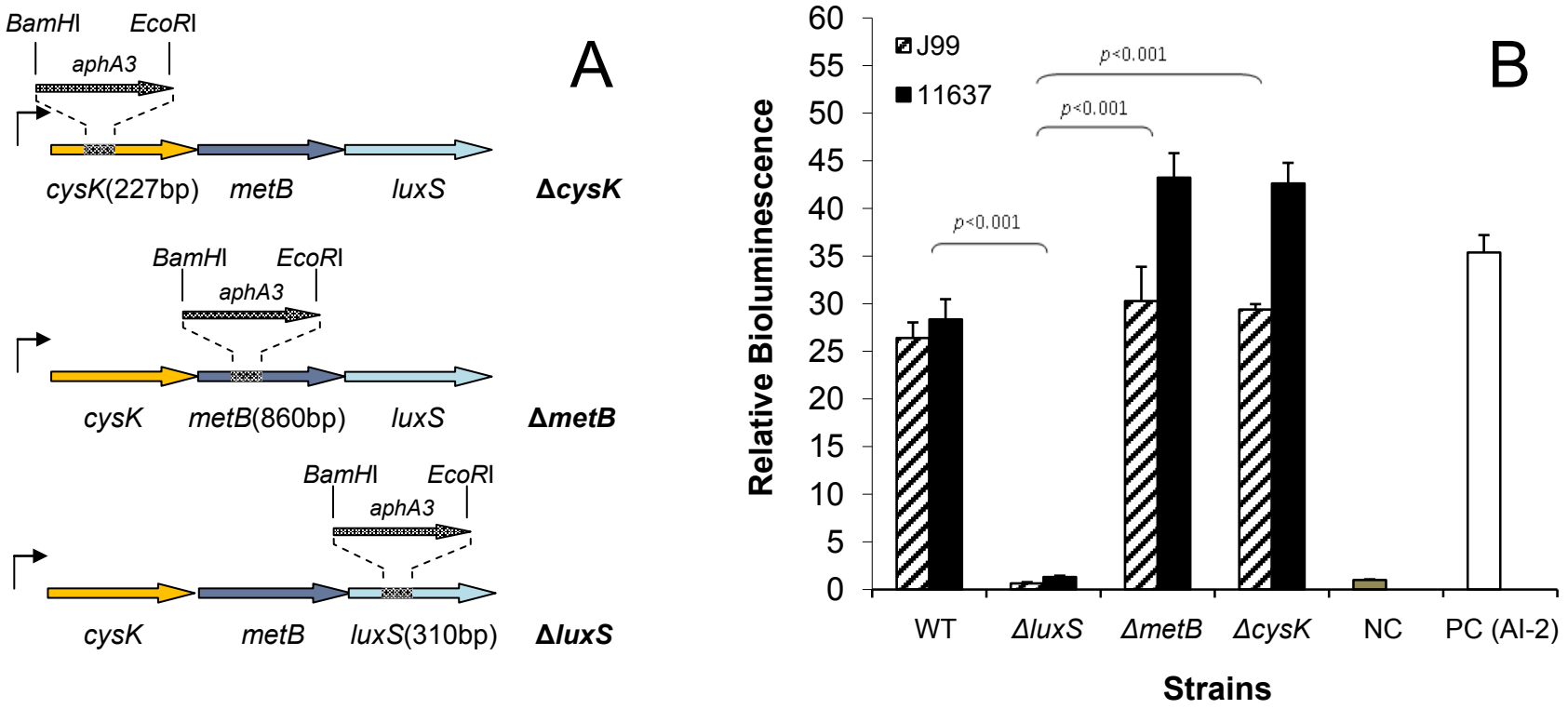


Figure 3.10 Disruption of *metB* and *cysK* has no polar effect on *luxS* function

(A) Schematic representation of the operon *cysK-metB-luxS* and gene regions ofΔ *luxS*, Δ*metB* and Δ*cysK* mutants. The promoter of this operon is located upstream of *cysK*. The mutant strains are J99/11637Δ *luxS*::km (a), J99/11637 Δ*metB*::km (b), J99/11637 Δ*cysK*::km (c). Arrows with a gray pattern indicate the kanamycin resistance gene, *aphA3*. The restriction sites are labelled on each end of *aphA3*. (B) Cell free supernatants of J99 and 11637 wild-type (WT) and mutants after 24 h incubation were examined for the ability to induce bioluminescence in the *V. harveyi* reporter strain BB170. AI-2 activity is shown as a ratio of bioluminescence (corrected by OD_{600nm} of *H. pylori* growth) in the presence of *H. pylori* cell free culture supernatants (1/10 diluted) over the negative control (NC). A diluted *in vitro* synthesised AI-2 sample was utilised as a qualitative positive control (PC). Bioluminescence induced by WT, Δ$metB_{Hp}$, and Δ$cysK_{Hp}$ strains was significantly greater than that induced by the Δ$luxS_{Hp}$ mutant, as determined by paired Student's *t*-test ($p<0.001$). Error bars indicate the standard deviation within triplicate samples.

3.4 Discussion

H. pylori presents an interesting case: although lacking homologues for the identified AI-2 receptors, the bacterium is known to respond to exogenously added, synthetic AI-2 (Rader *et al*., 2007), pointing toward a role of LuxS in cell-cell signalling. On the other hand, Lee and coworkers (2006) proposed that the reduced *in vivo* fitness observed for an *H. pylori* SS1 Δ*luxS* mutant was caused by metabolic disturbances, possibly by a disruption of the metabolic flux through the AMC or other LuxS-dependent pathways (Lee *et al*., 2006). Interestingly, previous physiological work (Mendz & Hazell, 1995, Nedenskov, 1994, Reynolds & Penn, 1994) and genome analyses have revealed that the AMC in *H. pylori* is incomplete, since the organism is a methionine auxotroph that lacks the genes required for methylation of homocysteine (*metE* or *metH*) (Doig *et al*., 1999, Old *et al.*, 1988, Thanbichler *et al*., 1999). This made us question whether LuxS in *H. pylori* had any metabolic roles apart from generating AI-2 and if so, what these roles could be.

Intriguingly, in all currently sequenced *H. pylori* strains, *luxS* is part of a three-gene cluster that in addition to *luxS* contains two other genes with putative functions in methionine and cysteine metabolism (Figure 3.10A). These genes, currently annotated as *metB* and *cysK*, until now have been proposed to encode cystathionine β-synthase and cysteine synthase, respectively (Doig *et al*., 1999). The gene cluster (*cysK*, *metB*, and *luxS*) is not only conserved among *H. pylori* strains and *H. acinonychis* but also is present in several Gram-positive bacteria (Winzer *et al*., 2003), suggesting that *metB* and *cysK* are not evolutionary relics of now inactive metabolic pathways but still fulfil a metabolic function linked to that of *luxS*.

The hypothesis that the *cysK*$_{Hp}$-*metB*$_{Hp}$-*luxS*$_{Hp}$ gene cluster is responsible for cysteine formation was based on several considerations. First, all *H. pylori* strains studied by Reynolds and Penn (1994) and the vast majority of strains characterised by Nedenskov (1994) were able to grow without cysteine (Nedenskov, 1994, Reynolds & Penn, 1994). Since the organism is not capable of using sulphate as a sulphur source (Doig *et al*., 1999), a hitherto-unidentified pathway must exist that makes use of the reduced sulphur present in methionine to generate cysteine. Second, the metabolic fate of the homocysteine generated in the LuxS reaction is unclear. High concentrations of homocysteine are toxic in some organisms (Roe *et al.*, 2002, Tuite *et al.*, 2005). Third, only two enzymatic steps are required to convert homocysteine to cysteine in the so-called reverse transsulphuration pathway (RTSP) present in some bacteria (for example, in *B. subtilis* and *P. putida*), where part of the AMC, including LuxS, is known to contribute to methionine-to-cysteine conversion by generating the required homocysteine (Hullo *et al*., 2007, Vermeij & Kertesz, 1999).

To determine the functions of the putative homologues of *cysK*, *metB*, and *luxS* in *H. pylori* (which appear to lie in a single operon), firstly a panel of mutant strains deficient in each one of these gene products were constructed in two different *H. pylori* strain backgrounds (J99 and 11637). The use of two different srtains is designed to avoid strain specific effects. To ensure that polar effects were not incurred with the Δ*metB*$_{Hp}$ and Δ*cysK*$_{Hp}$ mutants, a variant of *aphA3* lacking both the promoter and terminator elements which has been used successfully in previous studies (Wand *et al*., 2006) was inserted into the target genes.

Several previous analyses already established the Gram-positive origin of *luxS*, but did not consider the remaining genes of the *cysK-metB-luxS* cluster (Lerat & Moran,

2004). Interestingly, similar clusters exist in a number of Gram-positive species (Winzer *et al*., 2003), for instance *Oceanobacillus iheyensis*, *Clostridium perfringens*, and *Clostridium botulinum*. Intriguingly, in some *Bacillus* species, notably *B. subtilis*, *Bacillus cereus*, and *Bacillus anthracis*, similar clusters contain the *pfs* gene instead of *luxS*. More significantly, the genes in the *B. subtilis* cluster that show similarity to $cysK_{Hp}$ and $metB_{Hp}$ have recently been shown to encode functional cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL), respectively, and have therefore been renamed *mccA* and *mccB* (methionine-to-cysteine-conversion genes) (Hullo *et al*., 2007). Indeed, MccA and MccB bear closer resemblance to these gene products than to other putative CysK and MetB homologues found in *B. subtilis*. These findings lend further support to the suggestion that these gene clusters form functional units that contribute to the generation of cysteine via reverse transsulphuration. It thus appears that during the course of evolution, the common ancestor of *H. pylori* and *H. acinonychis* has lost its native *luxS* gene and instead acquired a Gram-positive homologue together with the ability to generate cysteine.

To unequivocally establish the nature of the reactions that link homocysteine with cysteine in *H. pylori* will require purification and detailed characterization of $CysK_{Hp}$ and $MetB_{Hp}$. The latter enzyme has already been studied, but not with respect to its physiological function. Kong *et al*. (2008) cloned the respective gene from *H. pylori* SS1 and purified the recombinant protein. However, the enzymatic activity of $MetB_{Hp}$ was measured only using an unphysiological side reaction that is observed for some enzymes *in vitro* in the presence of *O*-acetylserine and in the absence of other substrates (Kong *et al*., 2008). Thus, the true physiological role of $MetB_{Hp}$ remained unclear. With regard to $CysK_{Hp}$, a similar enzyme from *Lactobacillus casei*, also annotated CysK, has recently been proposed to act as a cystathionine β-synthase,

converting homocysteine to cystathionine (Irmler *et al.*, 2008). However, to date, $CysK_{Hp}$ has been thought to be a cysteine synthase that generates cysteine using sulphide (Doig *et al.*, 1999). To define the true physiological role role of the *cysK*$_{Hp}$-*metB*$_{Hp}$-*luxS*$_{Hp}$ gene cluster, and to clarify more accurately the metabolic function of each gene on this three-gene cluster of *H. pylori*, metabolic complementaion using chemically defined media was performed and the results were confirmed using a metabolomic approach. Details of these works are reported in the following chapter.

CHAPTER 4.

H. PYLORI LuxS, CysK AND MetB ARE KEY ENZYMES IN CYSTEINE BIOSYNTHESIS THROUGH A PROCESS OF REVERSE TRANSSULPHURATION

The work in this chapter has been published in the Journal of Bacteriology.

4 *H. pylori* LuxS, CysK and MetB are key enzymes in cysteine biosynthesis through a process of reverse transsulphuration

4.1 Introduction

As stated previously, *luxS* homologues have been found in around half of all bacterial genomes sequenced (Sun *et al*., 2004, Tomb *et al*., 1997, Vendeville *et al*., 2005). The enzyme encoded by *luxS* is now generally suggested to be involved in two processes: LuxS functions as an integral metabolic component of the AMC, a pathway that generates methyl groups with a higher transfer potential (Winzer *et al*., 2002a, Winzer *et al*., 2003). Its alternative function is in the production of the quorum sensing (QS) molecule auto-inducer 2 (AI-2), a by-product of the LuxS catalysed reaction (Winzer *et al*., 2002a, Winzer *et al*., 2003).

The AMC is a key metabolic pathway involving the interconversion of the amino acids methionine and homocysteine, generating *S*-adenosylmethionine (*S*AM) as an intermediate product (the full cycle is shown in Figure 4.1). *S*AM bears a methyl group with a relatively high transfer potential, and is used by numerous methyltransferases to carry out various cellular processes ranging from DNA methylation to detoxification of reactive metabolites. The product of the methyltransferase reaction is the toxic *S*-adenosyl-homocysteine (*S*AH). In the complete AMC, methionine is re-generated from *S*AH via homocysteine, ready for another round of methylation/transmethylation. *S*AH is metabolised in one of two ways; many bacteria, including *H. pylori*, generate homocysteine from *S*AH in a two step manner; a nucleosidase, Pfs, converts *S*AH to *S*-ribosylhomocysteine (*S*RH),

then LuxS converts this to homocysteine and DPD, the precursor of the molecule AI-2. The alternative route is slightly different, with homocysteine formed directly from *S*AH in a one step reaction requiring *S*AH hydrolase (Figure 1.4) (Duerre & Walker, 1977, Winzer *et al*., 2003, Winzer *et al*., 2002a). Therefore, no DPD is produced.

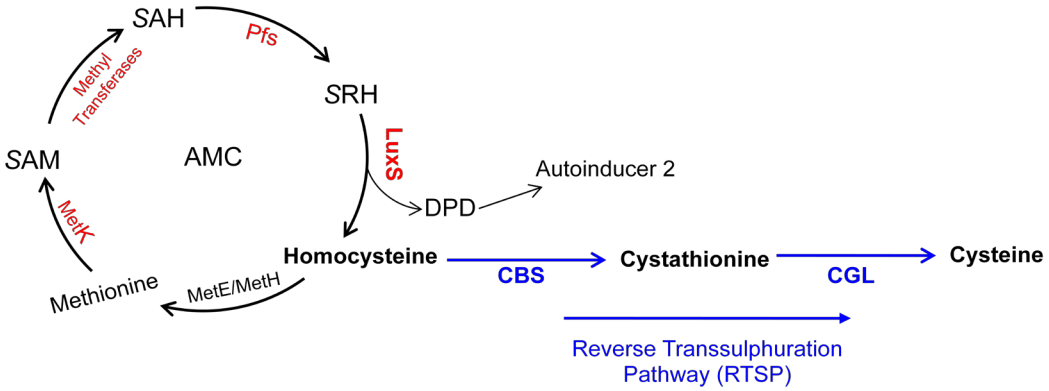


Figure 4.1 Bacterial cysteine provision pathway

Enzymes with previously described homologues in *H. pylori* are shown in red. Enzymes that do not have homologues in *H. pylori* are shown in black (MetE/MetH). The reverse transsulphuration pathway which is a major focus of this chapter is shown in blue. Data presented in this chapter show that $CysK_{Hp}$ is the candidate CBS and $MetB_{Hp}$ the candidate CGL in the reverse transsulphuration pathway. The two genes, *cysK*$_{Hp}$ and *metB*$_{Hp}$ are recommended to be renamed *mccA*$_{Hp}$ (methionine-to-cysteine-conversion gene A) and *mccB*$_{Hp}$, respectively. CBS; Cystathionine β-synthase, CGL; Cystathionine γ-lyase, DPD; 4,5-dihydroxy-2,3-pentanedione, LuxS; *S*-ribosylhomocysteinase/auto-inducer-2 synthase, MetE/MetH; Methionine synthase, MetK; *S*-adenosylmethionine synthase, Pfs; 5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase, *S*AM; *S*-adenosylmethionine, *S*AH; *S*-adenosylhomocysteine, *S*RH; *S*-ribosylhomocysteine.

Though *H. pylori* has a *luxS* homologue and possesses a functional homocysteine and DPD biosynthesis pathway (Forsyth & Cover, 2000, Joyce *et al*., 2000), it is not predicted to have a complete AMC due to its lack of a gene homologous to the genes encoding methyltransferase MetE or MetH required for the final step in the classical

AMC, the conversion of homocysteine to methionine (Tomb *et al*., 1997, Doig *et al*., 1999). This suggests that *H. pylori* is unable to carry out *de novo* biosynthesis of methionine (Figure 4.1). This is supported by the observation that all *H. pylori* strains tested are auxotrophic for methionine (Nedenskov, 1994, Mendz & Hazell, 1995). Interestingly, it has also been shown that many *H. pylori* strains do not require cysteine for growth, indicating that *H. pylori* possesses a cysteine provision pathway itself (Nedenskov, 1994, Mendz & Hazell, 1995). This presents a paradox, as genomic studies show that homologues to the genes which encode the components required for uptake of sulphate and conversion to sulphide are absent, suggesting that the classic route of the sulphate assimilatory cysteine biosynthetic pathway (SACBP)[‡‡] is not present in *H. pylori* (Tomb *et al*., 1997, Doig *et al*., 1999).

The above facts lead to a hypothesis that the *H. pylori luxS* gene is part of a *de novo* cysteine biosynthesis pathway that uses methionine as a reduced sulphur source. The reasons are as follows: the organism has an absolute requirement for methionine, but can synthesise cysteine without utilising oxidised sulphur compounds such as sulphate (Doig *et al*., 1999). Also, in the incomplete AMC, homocysteine presumably accumulates with each successive series of methylation from SAM. Consequently it must possess a pathway to consume homocysteine. The gene responsible for the production of homocysteine, *luxS*, is linked to other genes which are predicted to be involved in methionine and cysteine metabolism. Therefore, it was hypothesised that *H. pylori* is able to utilise homocysteine by converting it to cysteine via the reverse transsulphuration pathway (RTSP), involving the conserved genes $metB_{Hp}$ and $cysK_{Hp}$. In the classic route of the RTSP, the presence of the

[‡‡] The route of the sulphate assimilatory cysteine biosynthetic pathway (SACBP) is shown in figure 1.7.

enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL), assisted with their coenzyme pyridoxal 5'-phosphate (PLP), are required for the conversion of homocysteine to yield cysteine, in which CBS generates the intermediate cystathionine from homocysteine, and CGL in turn cleaves cystathionine to cysteine (Figure 4.1) (Cooper, 1983, Chang & Vining, 2002, Hullo *et al*., 2007). It was hypothesised that *cysK*$_{Hp}$ and *metB*$_{Hp}$ may encode enzymes possessing CBS and CGL activities.

To test these hypotheses, nutritional requirements of *H. pylori* wild-types and mutants defective in genes *luxS*, *metB* and *cysK* were analysed. The metabolic roles of the three genes were established by growing all strains in chemically defined media (CDM) containing different combinations of sulphur-containing amino acids. The results were confirmed by analysing metabolite pools of cells of each strain. Data in this chapter confirm the role of LuxS$_{Hp}$ in generation of homocysteine and DPD; and show that CysK$_{Hp}$ and MetB$_{Hp}$ are required for its subsequent conversion to cysteine (Δ *luxS*$_{Hp}$, Δ *metB*$_{Hp}$ and Δ *cysK*$_{Hp}$ mutants all becoming auxotrophic for cysteine), suggesting a primary metabolic role of LuxS$_{Hp}$, MetB$_{Hp}$ and CysK$_{Hp}$ in the RTSP.

4.2 Materials and Methods

4.2.1 Development of chemically defined media (CDM)

To study the growth of *H. pylori* strains under nutrient (sulphur) restricted conditions, formulated media based on Reynolds and Penn's previous report were utilised (Reynolds & Penn, 1994). Different medium formulations were generated by varying amino acid content and other chemical components. As absolute nutritional requirements vary in different strains, a medium formulation containing the 17 non-sulphur containing amino acids at non-limiting concentrations was produced. The chemically defined media (CDM) produced in this study comprised 0.5% (w/v) BSA, 271μM adenine, 3μg/ml lipoic acid, 11mM glucose, 24mM $NaHCO_3$, 7.2μM $FeSO_4 \cdot 7H_2O$, 423μM $Ca(NO_3)_2 \cdot 4H_2O$, 5.0mM KCl, 405μM $MgSO_4 \cdot 7H_2O$, 103mM NaCl, 6.0mM Na_2HPO_4, 1% (v/v) 100 × vitamin solution and 50% (v/v) 2 × amino acids solution. The 100 × vitamin solution consisted of 82μM biotin, 2mM choline chloride, 227μM folic acid, 19mM *myo*-inositol, 819μM niacinamide, 7mM *p*-aminobenzoic acid, 525μM calcium pantothenate, 486μM pyridoxine hydrochloride, 53μM riboflavin, 296μM thiamine hydrochloride and 10nM cyanocobalamine (vitamin B12). Each amino acid solution was made individually at 10mg/ml first, then mixed to a final volume of 50mls to give a 2 × solution corresponding to the following concentrations: 1.0mM glycine, 1.0mM alanine, 1.0mM serine, 530μM tryptophan, 1.0mM proline, 1.05mM aspartic acid, 1.01mM glutamic acid, 1.13mM asparagine, 1.01mM methionine, 1.08mM histidine, 2.2mM phenylalanine, 4.0mM valine, 4.0mM threonine, 4.12mM leucine, 4.10mM glutamine, 4.88mM isoleucine, 3.96mM lysine, 6.0mM arginine. Glucose was added as a carbon and energy source. Methionine is the unique sulphur source of the primary CDM. Complete CDM (cCDM) was generated by adding cysteine at a non-limiting concentration of 1.0mM. To produce different sulphur-containing conditions for metabolic complementation

experiments, 3 other variants of this medium were also made: CDM-Cys lacks cysteine (Cys), CDM+HC lacks cysteine, but contains homocysteine (HC) and CDM+CTT lacks cysteine, but contains cystathionine (CTT). Homocysteine (HC) and cystathionine (CTT) were added individually at the same molar concentrations as cysteine (1.0mM). All chemicals were highly purified Sigma products and were dissolved according to the manufacturer's recommendation. All solutions were filter sterilised before use. The pH of the final media was adjusted to 7.4-7.6 with [1M] HCl / [1M] NaOH. All strains were pre-cultured for 24 h and inoculated to each CDM with OD_{600nm} adjusted to 0.05.

4.2.2 Metabolic complementation using CDM

Four different fresh CDM were prepared as described in Chapter 4.2.1 (Doherty *et al*, 2010) on the day of doing the experiments. Metabolic complementation was performed using a $25cm^2$ tissue culture flask containing 5ml of each CDM. *H. pylori* 11637 and J99 wild-types and all mutants were grown on blood agar plates for 24 h before the experiment. To make the concentrated stock for each strain, bacterial cells of each strain were removed from each blood agar plate using a swab and suspended in CDM-Cys medium. The concentrated strain stocks were used to inoculate each flask containing CDM (in triplicate for each strain). The initial OD_{600nm} of each culture sample was 0.05. All inoculated flasks were incubated (with lids opened partially) on shakers with a speed of 450 rpm in a MACS VA500 Microaerobic Workstation (Don Whitley Scientific) using a humidified atmosphere consisting of 6% O_2, 3% H_2, 5% CO_2 and 86% N_2. OD_{600nm} of each sample was measured at the 6 h, 24 h, 48 h and 72 h time points.

4.2.3 Sample preparation and relative quantification of candidate metabolites

(In this experiment, sample preparation was completed by the author and quantification of metabolites was done by Mr Nigel Halliday, School of Molecular Medical Sciences, University of Nottingham)

To make cell pellets for metabolimic analysis, bacteria were grown in complete CDM (cCDM) for 24 h using the same protocol shown in Chapter 4.2.2. All subsequent manipulations were performed on ice or in chilled equipment, and all plastic ware was pre-chilled prior to use. 3ml of culture at an OD_{600nm} at 1.0 was taken as a standard sampling volume (variances from this OD were corrected for by using larger or smaller volumes). The required volume of cultures was quenched in 5ml ice-cold PBS in a 15ml Falcon tube. Samples were centrifuged at 2000 rpm for 10 min. The supernatants were discarded and cell pellets were washed in another 5ml ice-cold PBS. After the same spin stage was repeated, the supernatants were carefully removed and the tubes were spun briefly once more to bring the remaining liquid off the sides of the tubes. The residual liquid was removed and the dry cell pellets were stored at -80°C. At stage of quantification of metabolites, Methionine, *S*-adenosyl methionine (SAM), *S*-adenosyl homocysteine (SAH), *S*-ribosyl homocysteine (SRH), homocysteine (HC), cystathionine (CTT) and cysteine in cell pellets were analysed by quantitative liquid chromatography tandem electrospray ionization mass spectrometry (LC-MS-MS) (Singh *et al.*, 2006, Heurlier *et al.*, 2009). Briefly, 90µl water containing two internal standards (13CD3-Methionine and B-Hleu) was added to the freeze-dried sample of extracted metabolites. This was followed by the addition of 40µl iso-butanol/pyridine mixture (3:1 v/v), 10µl iso-butylchloroformate and 200µl dichloromethane/tert-butylmethyl-ether (1:2 v/v) with mixing at each stage. 150µl of upper organic phase was removed, and evaporated to

dryness under a stream of nitrogen gas. The derivatised metabolites were then redissolved in 100µl LC mobile phase (80% v/v methanol in 10mM ammonium formate) prior to LC-MS-MS analysis. LC separation was done on an Agilent 1100 system (Agilent, Waldbron, Germany) using a mobile phase (80% v/v methanol in 10 mM ammonium formate) at a flow rate of 200µl/min using a LUNA ODS C18(2) column (150 × 2.0 mm, 5µm particle size) (Phenomenex, Macclesfield, UK), maintained at 30°C. The MS system was a Waters Micromass Quattro Ultima triple quadrupole (Waters, Manchester, UK), used in positive ion multiple reaction monitoring scan mode to monitor specific precursor/product ions for each metabolite. The concentrations of individual metabolites were calculated from calibration lines using the peak area ratio to internal standard. Integrated values for each peak were normalized by dividing by the area of the corresponding internal standard peak in order to express the concentrations of metabolites in arbitrary units (Singh *et al*., 2006, Heurlier *et al*., 2009).

4.3 Results

4.3.1 $LuxS_{Hp}$, $CysK_{Hp}$ and $MetB_{Hp}$ are required for *de novo* cysteine biosynthesis in *H. pylori*

In order to determine the effects of mutagenesis of $cysK_{Hp}$, $metB_{Hp}$ and $luxS_{Hp}$, various chemically defined media (CDM) were composed based on the complete CDM (cCDM) of Reynolds and Penn (Reynolds & Penn, 1994). This is a versatile system which allows precise control of the composition of the medium. cCDM contained both of the sulphur containing amino acids, methionine and cysteine. 4 variants of this medium were also produced, all of which lacked cysteine. One had no additional source of sulphur (hereafter referred to CDM), the others contained either homocysteine (CDM+HC), or cystathionine (CDM+CTT) (see Chapter 4.2 Materials and Methods for concentrations). Using these media, all strains (the wild-type and $\Delta cysK_{Hp}$, $\Delta metB_{Hp}$ and $\Delta luxS_{Hp}$ mutants of both *H. pylori* strain 11637 and J99) were found to grow well in cCDM (Figure 4.2 for 11637 and J99). Upon omission of cysteine (CDM), the wild-type strains still grew, although less well than in the complete medium (Figure 4.2 A-a and B-a). In contrast, all three mutants were auxotrophic for cysteine, with no growth detected (Figure 4.2 A-bcd and B-bcd). These observations confirmed that wild-type *H. pylori* is able to grow in the absence of cysteine, and so possesses a cysteine *de novo* biosynthesis pathway. They also revealed that $cysK_{Hp}$, $metB_{Hp}$ and $luxS_{Hp}$ are all part of this pathway.

4.3.2 Metabolic complementation of growth defects demonstrates the steps in the cysteine biosnthesis pathway catalysed by $LuxS_{Hp}$, $CysK_{Hp}$ and $MetB_{Hp}$

Next, all steps in the proposed *H. pylori* reverse transsulphuration pathway (RTSP) (Figure 4.1) catalysed by $LuxS_{Hp}$, $CysK_{Hp}$ and $MetB_{Hp}$ were examined. These experiments gave the same results for mutants constructed in the 11637 and J99 background; results are presented in Figure 4.2 A-bcd and B-bcd.

For Δ*luxS*$_{Hp}$ mutants, adding homocysteine to the CDM lacking cysteine improved growth of the Δ*luxS*$_{Hp}$ strain, and cystathionine (CTT) restored growth to levels seen with cCDM (CDM with cysteine) (Figure 4.2 A-b and B-b). This implies that, as expected, $LuxS_{Hp}$ is required for the conversion of *S*-ribosylhomocysteine (*S*RH) to homocysteine.

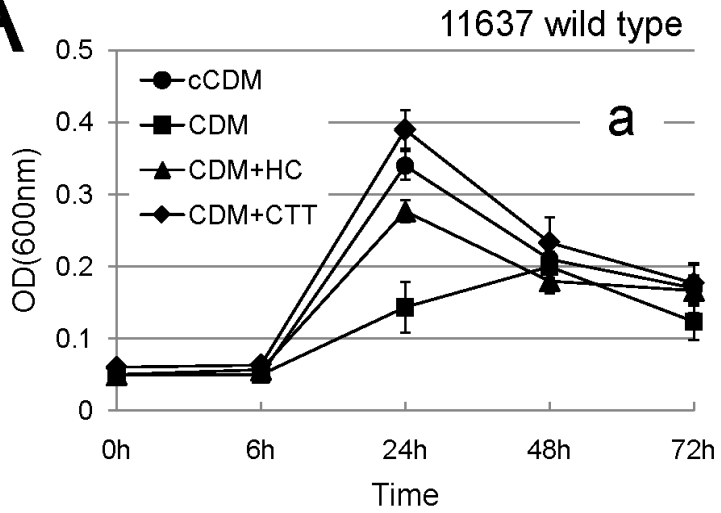
Δ*cysK*$_{Hp}$ mutants could grow in cCDM (CDM containing cysteine) and in CDM lacking cysteine but containing cystathionine (CDM+CTT), but not in CDM lacking cysteine (CDM) or CDM lacking cysteine but containing homocysteine (CDM+HC) (Figure 4.2 A-c and B-c), suggesting that $CysK_{Hp}$ catalyses the conversion of homocysteine to cystathionine.

Finally, Δ *metB*$_{Hp}$ mutants could only grow in cCDM, indicating an absolute requirement for cysteine (Figure 4.2 A-d and B-d). This would be consistent with $MetB_{Hp}$ catalysing the final step from cystathionine to cysteine, although further experimentation is needed to confirm this (see Chapter 4.3.3 metabolomic analysis).

In contrast to results for the wild-type, none of the mutants could grow in CDM lacking cysteine but containing potassium sulphide (CDM+S) (data not shown). Thus, it seems that $CysK_{Hp}$ is not acting like CysK homologues in other bacteria (which use sulphide to generate cysteine), in agreement with the reported absence of the assimilatory sulphate reduction pathway in *H. pylori* (Doig *et al*., 1999).

Taking these observations together, the hypothesis formulated was that *H. pylori* generates cysteine from methionine via components of the AMC, followed by reverse transsulphuration, with $LuxS_{Hp}$ converting *S*RH to homocysteine, and $CysK_{Hp}$ converting homocysteine to cystathionine which is finally converted to cysteine by $MetB_{Hp}$.

A


11637 wild type
a
cCDM
CDM
CDM+HC
CDM+CTT
OD(600nm)
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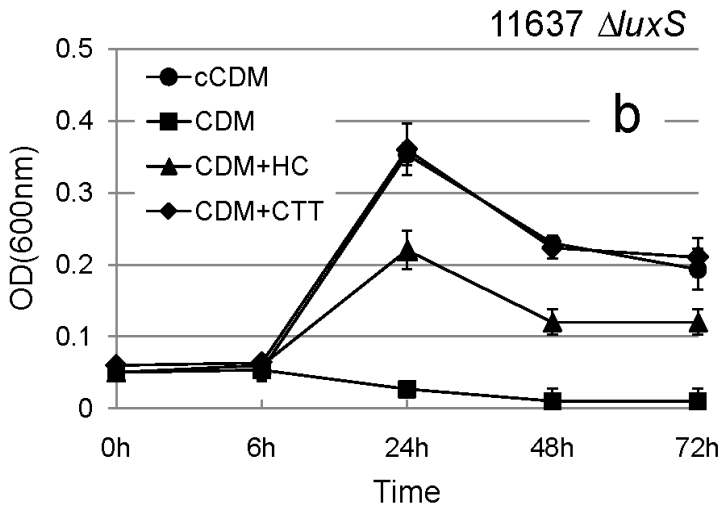
0.4
0.3
0.2
0.1
0
0h
6h
24h
48h
72h
Time


11637 ΔluxS
b
cCDM
CDM
CDM+HC
CDM+CTT
OD(600nm)
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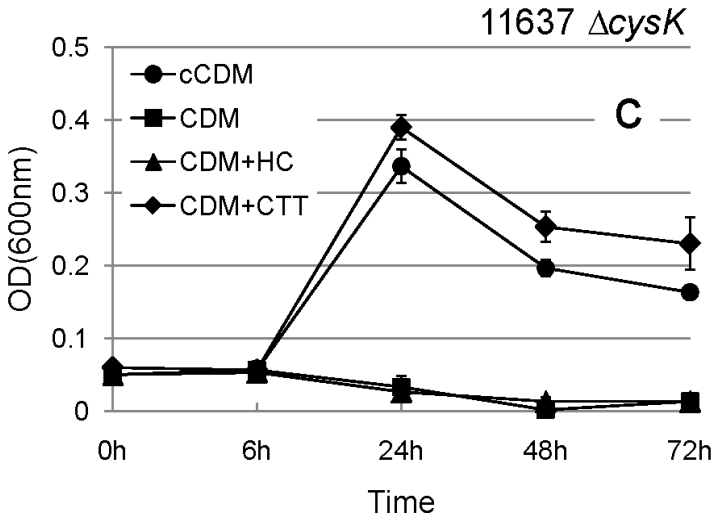
0.4
0.3
0.2
0.1
0
0h
6h
24h
48h
72h
Time


11637 ΔcysK
c
cCDM
CDM
CDM+HC
CDM+CTT
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0h
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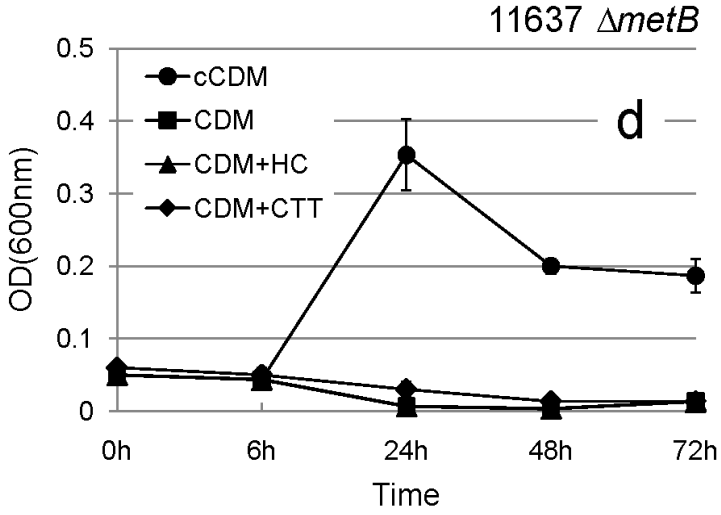
72h
Time


11637 ΔmetB
d
cCDM
CDM
CDM+HC
CDM+CTT
OD(600nm)
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0.3
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0
0h
6h
24h
48h
72h
Time

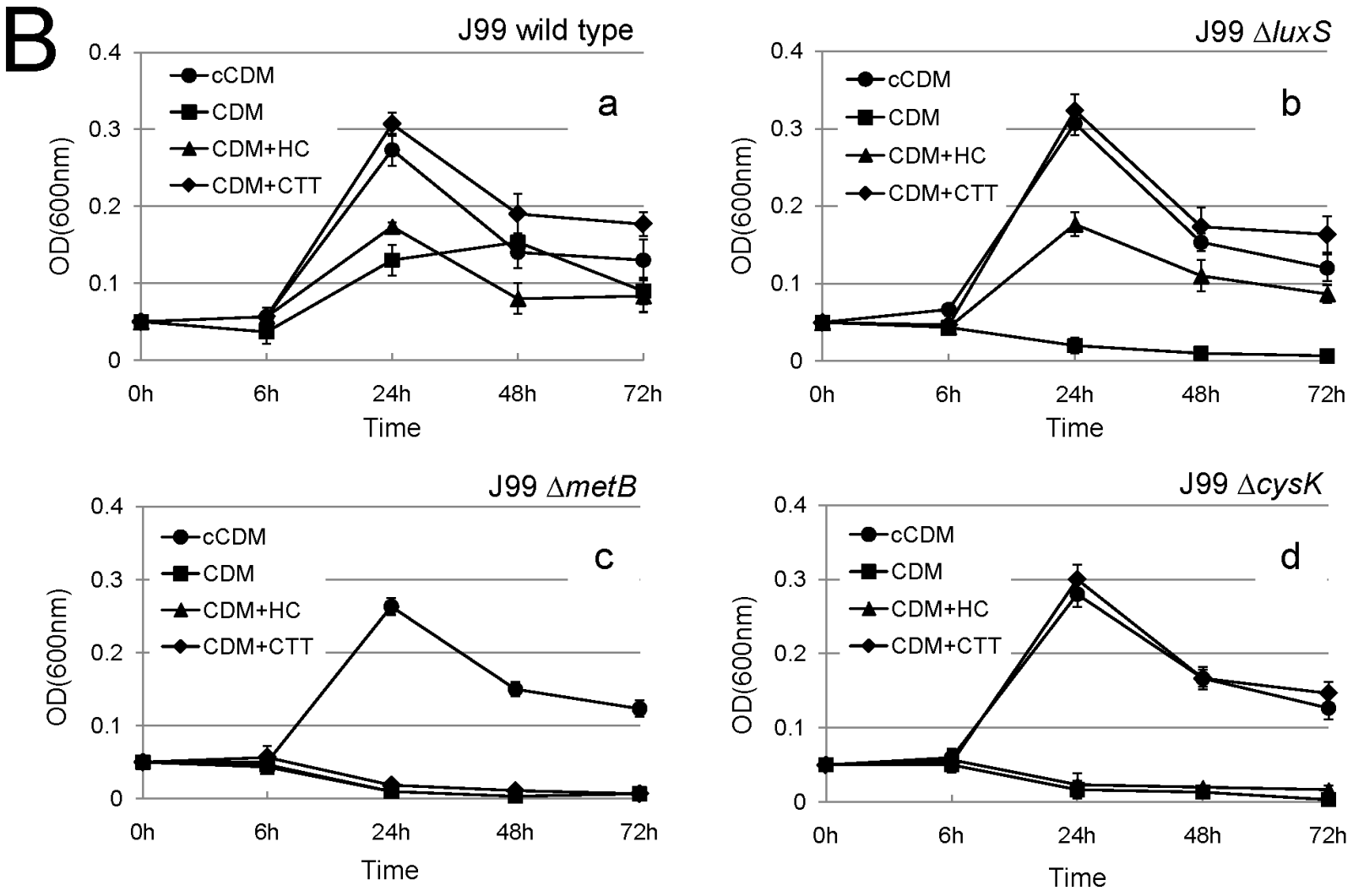

B
J99 wild type
a
J99 ΔluxS
b
J99 ΔmetB
c
J99 ΔcysK
d
cCDM
CDM
CDM+HC
CDM+CTT
OD(600nm)
0.4
0.3
0.2
0.1
0
0h
6h
24h
48h
72h
Time

Figure 4.2 Growth of *H. pylori* 11637 and J99 strains in chemically defined media (CDM)

Methionine is present in each CDM as a sulphur source. OD_{600nm} were measured at the indicated time intervals in cultures. The mean OD_{600nm} values of triplicate culture samples are shown on each growth curve; error bars indicate standard deviation. Growth of *H. pylori* strains 11637 and J99 wild-type (a), Δ*luxS* (b), Δ*metB* (c) and Δ*cysK* (d) in complete CDM (cCDM, circles), CDM lacking cysteine (CDM, squares), CDM lacking cysteine but containing homocysteine (CDM+HC, triangles) and CDM lacking cysteine but containing cystathionine (CDM+CTT, diamonds). The mean OD_{600nm} values of triplicate culture samples are shown. Error bars indicate standard deviations from the means.

4.3.3 Analysis of key metabolite pools confirms the proposed pathway of cysteine biosynthesis and specifically identifies reaction substrates

(In this experiment, sample preparation was completed by the author and quantification of metabolites was done by Mr Nigel Halliday, School of Molecular Medical Sciences, University of Nottingham)

In metabolic complementation experiments, both wild-type strains were tested but no mutant strains were able to grow without cysteine, indicating that disruption of any gene on the operon *cysK*$_{Hp}$-*metB*$_{Hp}$-*luxS*$_{Hp}$, as expected, disrupted the cysteine biosynthesis pathway in *H. pylori*. Accordingly, further experiments were designed both to investigate cysteine provision in more detail and to obtain more lines of evidence to clarify the role of *luxS*, *metB* and *cysK* in *H. pylori* metabolism. Metabolomic analysis was used to measure pool sizes of relevant metabolites to provide further evidence for the uptake and consumption of all components through the predicted cysteine biosynthesis pathway in *H. pylori*.

Cell extracts of *H. pylori* wild-type and all mutants were grown in cCDM for 24 h and then analysed. In accordance with known data, metabolites through the proposed RTSP in *H. pylori* include methionine, *S*AM, *S*AH, *S*RH, homocysteine (HC), cystathionine (CTT) and finally cysteine (Cys). In most cases, the levels of individual metabolites found were corrected for those found in the wild-type, and expressed as fold changes relative to this (Figure 4.3).

As expected, significantly (p<0.01) increased *S*RH level was detected in the Δ*luxS*$_{Hp}$ mutant. However, *S*RH was only detected in the *luxS* strain, and was below the detection threshold in the wild-type, Δ *cysK*$_{Hp}$ and Δ *metB*$_{Hp}$ strains. Therefore, the

relative *S*RH level was expressed as 1 in the Δ*luxS* strain, and as 0 in all other strains. The other main differences between the metabolite profiles of the Δ$luxS_{Hp}$ mutant relative to the wild-type were that HC and CTT were virtually undetectable in the mutant.

When analysing the metabolites present in the Δ$cysK_{Hp}$ mutant, approximately 2 fold more *S*AH ($p<0.01$) was found in the mutant compared with the wild-type. Most strikingly, the level of HC were 11 to 12 fold greater than in the wild-type, suggesting that this metabolite was accumulating in this strain. Like the Δ$luxS_{Hp}$ mutant, the Δ$cysK_{Hp}$ mutant seemed to produce minimal levels of CTT.

Finally, in the Δ$metB_{Hp}$ strain, around 2 fold more *S*AH ($p<0.01$), and 2-3 fold more HC ($p<0.01$) was found compared with the wild-type. Most importantly, CTT was present in around a 5 fold excess relative to the wild-type, indicating that this metabolite was accumulating in this strain.

These results are consistent with data obtained from the metabolic complementation experiments, in which it was demonstrated that $LuxS_{Hp}$ converts *S*RH to HC; $CysK_{Hp}$ cleaves HC to CTT; and $MetB_{Hp}$ acts in the conversion of CTT to Cys, the final step in the cysteine biosynthesis pathway.

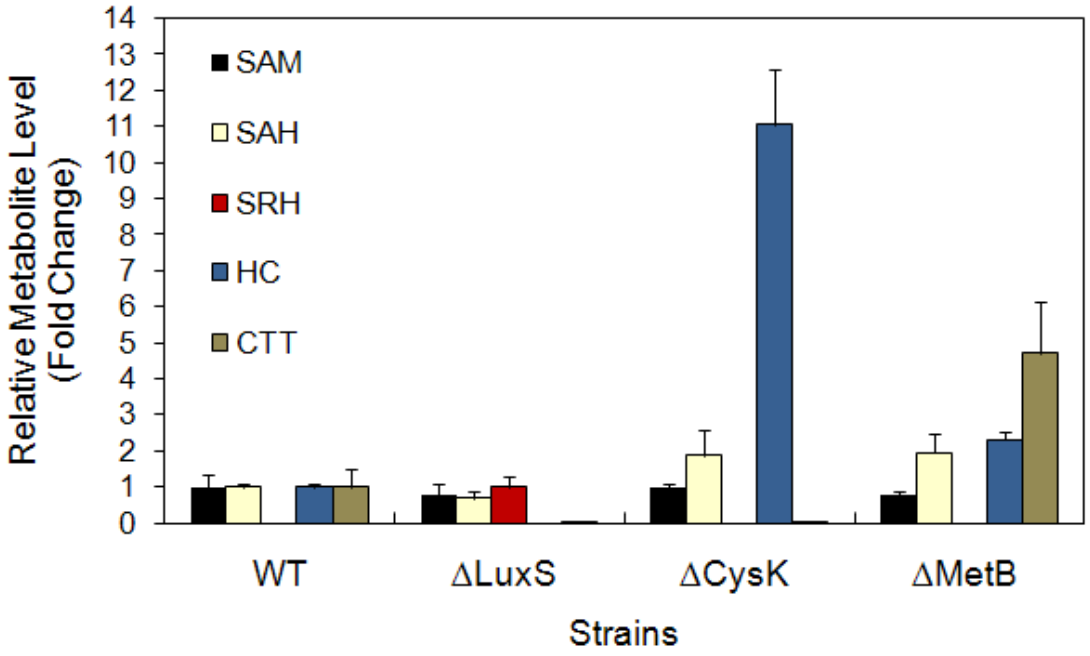


Figure 4.3 Metabolomic assays for all metabolites through the proposed reverse transsulphuration pathway in *H. pylori*

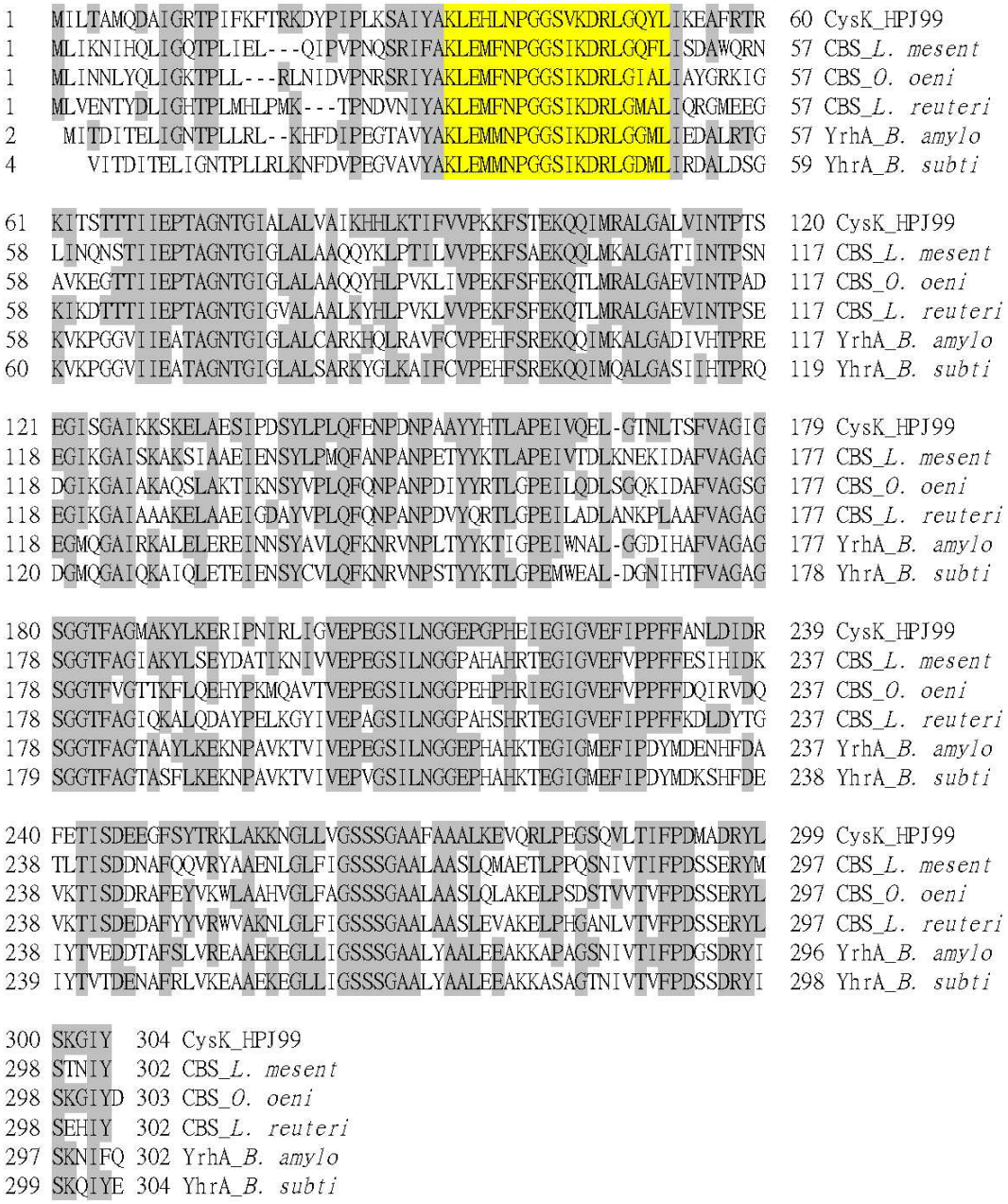
Cell extracts of *H. pylori* 11637 wild-type and mutant strains grown in complete CDM in triplicate were taken after 24 h of incubation. All metabolites involved in the conversion of methionine to cysteine via the RTSP were quantified individually. The Y axis shows the fold change of each metabolite normalised to the level of the wild-type (WT). Levels of metabolites that were below the detection threshold were expressed as 0. *S*RH, was only detected in theΔ *luxS*$_{Hp}$ strain, and was below the detection threshold in the wild-type, Δ*cysK*$_{Hp}$ and Δ*metB*$_{Hp}$ strains. Therefore, the relative *S*RH level was expressed as 1 in the Δ*luxS*$_{Hp}$ strain, and as 0 in all other strains. Values are mean activities of triplicate cell extract samples of each *H. pylori* strain. Error bars indicate standard deviation.

4.3.4 $CysK_{Hp}$ and $MetB_{Hp}$, could be enzymes with cystathionine β-synthase and cystathionine γ-lyase activities

The conversion of homocysteine to cysteine through the RTSP in *H. pylori* is performed via the intermediate cystathionine, followed by its subsequent cleavage to cysteine. Sufficient evidence has been provided to show that this reaction in *H. pylori* requires CysK and MetB. According to genome data of Genbank, *H. pylori cysK* is annotated to be a cysteine-synthase, while *metB* is annotated to be a CGS. The role of *H. pylori metB* to encode a CSG was also reported in a structural study (Kong *et al*., 2008). However, data presented in this chapter strongly suggest that $CysK_{Hp}$ produces cystathionine in the conversion of homocysteine to cysteine, which is supposed to be an enzyme with CBS activity, while $MetB_{Hp}$ cleaves cystathionine to cysteine and has CGL activity.

Protein sequences of *H. pylori* CysK (CBS) and MetB (CGL) were compared with those of enzymes similarly annotated from other microorganisms (Figure 4.4). In BLAST searches of Genbank, *H. pylori* CysK shares 56% identity with CBS from *Leuconostoc mesenteroides* and *Lactobacillus reuteri*, 55% identity with CBS from *Oenococcus oeni* and 54% identity with CBSs encoded by *yrhA* homologues from *Bacillus amyloliquefaciens* and *B. subtilis* (Figure 4.4A) (Liu *et al.*, 2008). *H. pylori* MetB shows 59% identity with CGL (encoded by *yhrB*) from *B. subtilis*, 57% identity with CGL from *Lactobacillus reuteri* and 55% identity with CGL from *Clostridium botulinum* (Figure 4.4B) (Liu *et al*., 2008). The sequence of pyridoxal phosphate binding motif of *H. pylori* CysK is over 75% identical to the motifs of CBSs from other microoganisms, while the same motif of *H. pylori* MetB is over 85% identical to the motifs of CGLs from other microorganisms.

A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1 MLINNLYQLIGKTPLL---RLNIDVPNRSRIYAKLEMFNPGGSIKDRLGIALIAYGRKIG 57 CBS_O. oeni
1 MLVENTYDLIGHTPLMHLPMK---TPNDVNIYAKLEMFNPGGSIKDRLGMALIQRGMEEG 57 CBS_L. reuteri
2 MITDITELIGNTPLLRL--KHFDIPEGTAVYAKLEMMNPGGSIKDRLGGMLIEDALRTG 57 YrhA_B. amylo
4 VITDITELIGNTPLLRLKNFDVPEGVAVYAKLEMMNPGGSIKDRLGDMLIRDALDSG 59 YhrA_B. subti
61 KITSTTTIIEPTAGNTGIALALVAIKHHLKTIFVVPKKFSTEKQQIMRALGALVINTPTS 120 CysK_HPJ99
58 LINQNSTIIEPTAGNTGIGLALAAQQYKLPTILVVPEKFSAEKQQLMKALGATIINTPSN 117 CBS_L. mesent
58 AVKEGTTIIEPTAGNTGIGLALAAQQYHLPVKLIVPEKFSFEKQTLMRALGAEVINTPAD 117 CBS_O. oeni
58 KIKDTTTIIEPTAGNTGIGVALAALKYHLPVKLVVPEKFSFEKQTLMRALGAEVINTPSE 117 CBS_L. reuteri
58 KVKPGGVIIEATAGNTGIGLALCARKHQLRAVFCVPEHFSREKQQIMKALGADIVHTPRE 117 YrhA_B. amylo
60 KVKPGGVIIEATAGNTGIGLALSARKYGLKAIFCVPEHFSREKQQIMQALGASIIHTPRQ 119 YhrA_B. subti
121 EGISGAIKKSKELAESIPDSYLPLQFENPDNPAAYYHTLAPEIVQEL-GTNLTSFVAGIG 179 CysK_HPJ99
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118 EGIKGAIAAAKELAAEIGDAYVPLQFQNPANPDVYQRTLGPEILADLANKPLAAFVAGAG 177 CBS_L. reuteri
118 EGMQGAIRKALELEREINNSYAVLQFKNRVNPLTYYKTIGPEIWNAL-GGDIHAFVAGAG 177 YrhA_B. amylo
120 DGMQGAIQKAIQLETEIENSYCVLQFKNRVNPSTYYKTLGPEMWEAL-DGNIHTFVAGAG 178 YhrA_B. subti
180 SGGTFAGMAKYLKERIPNIRLIGVEPEGSILNGGEPGPHEIEGIGVEFIPPFFANLDIDR 239 CysK_HPJ99
178 SGGTFAGIAKYLSEYDATIKNIVVEPEGSILNGGPAHAHRTEGIGVEFVPPFFESIHIDK 237 CBS_L. mesent
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238 VKTISDDRAFEYVKWLAAHVGLFAGSSSGAALAASLQLAKELPSDSTVVTVFPDSSERYL 297 CBS_O. oeni
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B

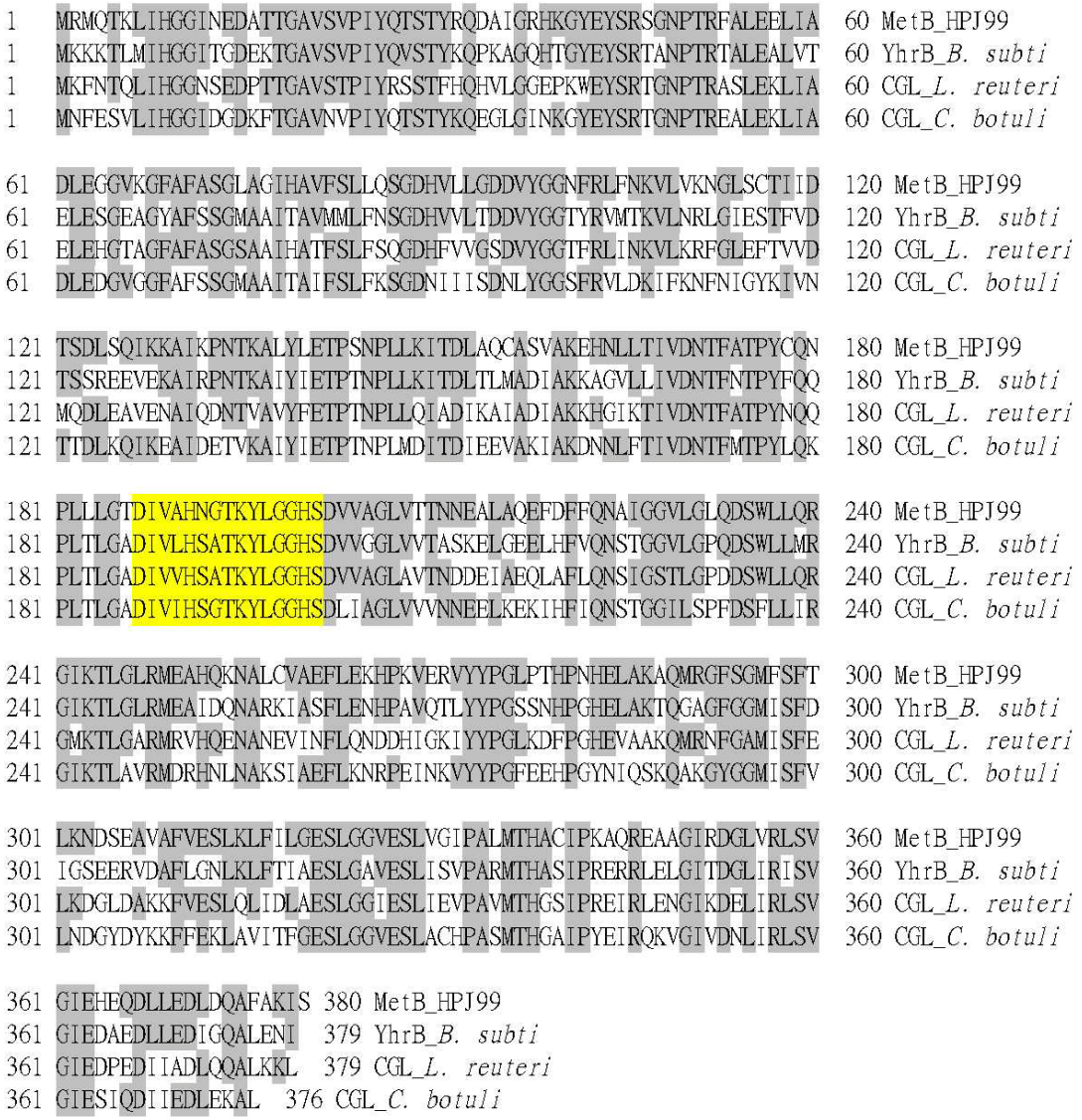


Figure 4.4 Comparison of aligments of *H. pylori* CysK and MetB with Genbank CBS and CGL

(A) Alignment of *H. pylori* J99 CysK and Genbank sequences of CBS from different microorganisms. (B) Alignment of *H. pylori* J99 MetB and Genbank sequences of CGL from some other microorganisms. The sequences with dark background represent identical amino acids in all enzymes analysed. The sequences with yellow background show the pyridoxal phosphate binding motifs in CBS and CGL from each microorganism.

4.4 Discussion

LuxS is now generally understood to carry out at least two functions, one in metabolism and one in cell-cell signalling (Bassler *et al*., 1997, Winzer *et al*., 2002b, Vendeville *et al*., 2005, Rezzonico & Duffy, 2008). Bacterial signalling systems based on LuxS-generated AI-2 are known to regulate gene expression in several members of *Enterobacteriaceae* (Li *et al.*, 2007, Xavier *et al.*, 2007) and notably *Vibrio* species. In this chapter, metabolic functions of $cysK_{Hp}$, $metB_{Hp}$ and $luxS_{Hp}$ in the cysteine biosynthetic pathway have been newly discovered and identified by analysing growth of different *H. pylori* genotypes in chemically defined media and quantifying metabolite pools in cell pellets, by which we confirm the primary metabolic role of $LuxS_{Hp}$ in the imcomplete AMC and in cysteine provision.

The results of this chapter are straightforward and in full agreement with the hypothesis (described in both chapters 3.1 and 4.1). First, it demonstrates that all genes of the *cysK-metB-luxS* gene cluster in *H. pylori* (in both 11637 and J99, to avoid strain specific effects) are required for cysteine prototrophy: mutation of any one of these genes ($\Delta luxS_{Hp}$, $\Delta metB_{Hp}$ and $\Delta cysK_{Hp}$) rendered the respective strain auxotrophic for cysteine (Figure 4.2A-b and B-b). Chemical complementation restored the growth of all mutants and also indicated a sequential order for the involved enzymatic activities, which is $LuxS_{Hp}$-$CysK_{Hp}$-$MetB_{Hp}$. The results suggested that the true physiological function of the $cysK_{Hp}$ gene product lies in the conversion of homocysteine to cystathionine; the latter is then used by $MetB_{Hp}$ to generate cysteine. This interpretation was further corroborated and extended by metabolite profiling. In full agreement with the proposed metabolic functions, the metabolite pools of $\Delta luxS_{Hp}$ mutants showed accumulation of *S*RH and depletion of both homocysteine and cystathionine, whereas $\Delta cysK_{Hp}$ mutants accumulated

homocysteine and were depleted for cystathionine. Interestingly, *S*AH were elevated 2 fold compared to the wild-type, which may indicate a weak feedback inhibition effect of homocysteine on Pfs, an enzyme catalysing the reaction immediately prior to the LuxS reaction in the AMC. Δ*metB*$_{Hp}$ mutants were the only strains that accumulated cystathionine. Again, an increase in the levels of *S*AH was seen, suggesting that either higher levels of homocysteine can inhibit *pfs*, or that both homocysteine and cystathionine may lead to its inhibition. All of these data provide the evidence that an RTSP is operational in *H. pylori*.

Data presented in this chapter indicate that compared with the bacteria which have mutiple pathways for metabolism of cystiene and methionine, *H. pylori* possesses a simpler route for degradation of methionine and biosynthesis of cysteine, which is the RTSP only. This is consistent with the analysis of the numerous *H. pylori* genomes available to date (e.g. KEGG http://www.genome.jp/kegg/ and PyloriGene http://genolist.pasteur.fr/PyloriGene/). Unlike *H. pylori*, many other organisms (e.g. *S. Typhimurium*, *E. coli*, *Lactococcus lactis*, *Azospirillum brasilense*, plants and fungi) utilise an alternative sulphate assimilatory cysteine biosynthesis pathway (SACBP) to synthesise cysteine, which involoves transport and reduction of inorganic sulphate to sulphide in one branch and the synthesis of *O*-acetyl-serine (*O*AS) in the other (Droux, 2004, Kredich & Tomkins, 1966, Sperandio *et al*., 2005, Ramirez *et al*., 2006). The SACBP cannot exist in *H. pylori* due to its lack of the enzyme needed to produce sulphide from sulphate (Hullo *et al*., 2007). Moreover, the TSP cannot be a principal supply route since there is no machinery to provide the second substrate, *O*-succinyl- (or *O*-acetyl-) homoserine (*O*AH / *O*SH) for the formation of cystathionine from cysteine in *H. pylori* (Tomb *et al*., 1997, Cooper,

1983). Consistently, all *H. pylori* strains tested are auxotrophic for methionine, but none are auxotrophic for cysteine (Nedenskov, 1994, Mendz & Hazell, 1995).

As mentioned in the last chapter, the enzyme $MetB_{Hp}$ has already been studied, but not with respect to its physiological function (Kong *et al*., 2008). Thus, the true physiological role of $MetB_{Hp}$ remained unclear until the presence of the experimental data here, by which $MetB_{Hp}$ appears to be an enzyme with cystathionine γ-lyase (CGL) activity. To date, $CysK_{Hp}$ has been thought to act as a cysteine synthase that uses sulphide to generate cysteine (Doig *et al*., 1999). However, the finding that sulphide cannot support growth of *H. pylori* in the absence of cysteine, at least under conditions in the experiments presented here, suggests that $CysK_{Hp}$ is solely dedicated to homocysteine conversion. Thus, $CysK_{Hp}$ is proposed to be an enzyme with cystathionine β-synthase (CBS) activity.

CBS and CGL both belong to the superfamily of pyridoxal-5'-phosphate (PLP) dependent enzymes. CBS lies in the β family (tryptophan synthase class) of PLP dependent enzymes, and CGL is in the γ-subdivision of the α family (aspartate aminotransferse class). Interestingly, similarity searches (BLAST) of the $metB_{Hp}$ and $cysK_{Hp}$ gene products (sequentially located upstream of $luxS_{Hp}$) reveals that these gene products also fall within the PLP dependent enzyme superfamily. $MetB_{Hp}$ falls into the γ-subdivision of the α family, and is annotated as a CGL. $CysK_{Hp}$ belongs to the β family, and is annotated as either a CBS or an *O*-acetyl-serine sulfhydrylase (*O*AS-sulfhydrylase).

The multiple sequence alignments of CBSs and CGLs have shown identities and similarities between the sequences of $cysK_{Hp}$ and $metB_{Hp}$ products and sequences of

enzymes with the same activities in some other bacteria including *L. mesenteroides*, *L. reuteri*, *O. oeni*, *C. botulinum*, *B. amyloliquefaciens* and *B. subtilis* (Figure 4.4). As stated previously (in Chapter 3.4), the genes cluster in *B. subtilis* that show similarity to $cysK_{Hp}$ and $metB_{Hp}$ have recently been shown to encode functional CBS and CGL, respectively, and have therefore been renamed MccA and MccB (methionine-to-cysteine-conversion genes) (Hullo *et al*., 2007). Accordingly, here in this study, $cysK_{Hp}$ is recommended to be renamed $mccA_{Hp}$ (methionine-to-cysteine-conversion gene A) and $metB_{Hp}$ is recommended to be renamed $mccB_{Hp}$.

The third gene on the operon $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$, *luxS*, has been the subject of much recent controversy in the bacterial literature. A range of microorganisms and pathogens including *H. pylori* have been shown to possess *luxS* homologues, and to produce AI-2, but appear to lack an AI-2 sensory apparatus (i.e. the LuxP/Q and the Lsr system) (Rezzonico & Duffy, 2008), in agreement with suggestions that in most species *luxS* probably fulfils a primarily metabolic role in the AMC (Rezzonico & Duffy, 2008, Winzer *et al*., 2002a, Winzer *et al*., 2002b, Winzer *et al*., 2003). The relevance of the established cysteine biosynthesis pathway for the lifestyle, fitness, and virulence of *H. pylori* is not clear. The reduced *in vivo* fitness observed for the *H. pylori* SS1 Δ*luxS* mutant (Lee *et al*., 2006) may indeed be linked to its inability to convert methionine to cysteine, although a role of AI-2 cannot be excluded.

H. pylori and other members of this genus have lost the ability to reduce sulphate (Doherty *et al.*, 2010, Doig *et al*., 1999). However, it can be assumed that they are well adapted to their specific habitats, which therefore must provide sufficient reduced sulphur sources for these organisms to thrive. Presumably, the conversion of homocysteine to cysteine removes a potentially toxic metabolite and at the same time

reduces the (energetically costly) need for cysteine uptake. On the other hand, a similar effect would have been achieved if the organism had maintained a functional AMC. In the absence of exogenous cysteine, growth of *H. pylori* will be limited by its capacity to generate this compound from homocysteine. Homocysteine availability, in turn, is determined by the number of methylation reactions carried out by the cells (i.e., the number of *S*AM molecules that can be converted to *S*AH, and then further to homocysteine via the action of Pfs and LuxS, see Figure 4.1). Under these conditions, generation of the quorum sensing products comprising AI-2, at least in theory, is directly proportional to growth of the population and the resulting AI-2 concentration should thus provide a very accurate measure of population density. It is now important to determine whether the other phenotypes associated with *luxS* mutagenesis in *H. pylori* are dependent on the metabolic role of LuxS, or its role as the AI-2 synthase. Related work is reported and discussed in following chapters.

CHAPTER 5.
H.PYLORI LuxS IS REQUIRED FOR MOTILITY, AND THIS IS DEPENDENT ON AUTO-INDUCER-2 PRODUCTION RATHER THAN METABOLIC EFFECTS

The work presented this chapter has been published in BMC Microbiology.

5 *H.pylori* LuxS is required for motility, and this is dependent on auto-inducer-2 production rather than metabolic effects

5.1 Introduction

Many bacteria are known to release extra-cellular signalling molecules (auto-inducers) to communicate cell to cell. It is generally assumed that auto-inducers (including auto-inducer-2) are employed to regulate aspects of bacterial behaviour, including modulation of cell population density, response to changes of cellular phenotypes, interaction with hosts and expression of genes crucial for bacterial survival or virulence - a process termed 'quorum sensing (QS)' (Sperandio *et al*., 2003, Camilli & Bassler, 2006, Vendeville *et al*., 2005).

As stated in previous chapters, LuxS catalyses one of the steps in the activated methyl cycle (AMC), a pathway that generates methyl groups with a higher transfer potential (Winzer *et al*., 2003). The precursor of auto-inducer-2 (AI-2), 4, 5-dihydroxy-2, 3-pentanedione (DPD) is generated as a by-product in the conversion of *S*RH to homocysteine. 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 (Winzer *et al*., 2003, Vendeville *et al*., 2005). In some organisms, the AMC is different. For example, in *P. aeruginosa*, LuxS and Pfs are replaced by a single enzyme (*S*AH hydrolase) which converts *S*AH to homocysteine in a one step reaction without the concomitant

production of DPD (or AI-2) (Duerre & Walker, 1977). As described in Chapter 1. Introduction, AI-2 has been extensively studied in *V. harveyi,* in which it regulates bioluminescence and other traits (Camilli & Bassler, 2006, Lerat & Moran, 2004, Bassler *et al*., 1997). Use of a *V. harveyi* reporter strain that expresses bioluminescence in response to addition of AI-2 has become the standard method for detecting AI-2 production by other bacterial species (Bassler *et al*., 1997).

H. pylori, a Gram-negative bacterium which causes peptic ulceration, gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma, contains a *luxS* homologue and possesses an integral homocysteine and DPD (or AI-2) biosynthesis pathway (Atherton, 2006, Forsyth & Cover, 2000, Joyce *et al*., 2000). $luxS_{Hp}$ ($HP0105_{26695}$; $JHP0097_{J99}$) is next to housekeeping genes $mccA_{Hp}$ ($HP0107_{26695}$; $JHP0099_{J99}$) and $mccB_{Hp}$ ($HP0106_{26695}$; $JHP0098_{J99}$) (Tomb *et al*., 1997, Doig *et al*., 1999, Doherty *et al*., 2010). Data from a related study (Doherty *et al*., 2010) 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). This consequently makes the true role of $LuxS_{Hp}$ in bacterial signalling unclear.

To date, information on phenotypic changes exhibited as a result of $luxS_{Hp}$ inactivation remains limited. TwoΔ $luxS_{Hp}$ mutants were reported to form biofilms more efficiently than the parent strain, indicating a possible but counterintuitive role of $luxS_{Hp}$ in biofilm reduction (Cole *et al*., 2004). Studies of two independent groups looked at fitness of Δ $luxS_{Hp}$ mutants *in vivo* using mouse and gerbil models, respectively (Lee *et al*., 2006, Osaki *et al*., 2006). They showed that mutated $luxS_{Hp}$ strains SS1 and TK1402 exhibited diminished motility and reduced fitness in co-

infection experiments. In both cases, restoration of *luxS*$_{Hp}$ by genetic complementation partially restored these phenotypes (Lee *et al*., 2006, Osaki *et al*., 2006). Lee and coworkers argued that the decreased fitness in the Δ*luxS*$_{Hp}$ mutant of strain SS1 was most likely due to the disruption of the cycle of *S*RH consumption and homocysteine synthesis and that AI-2 seemed unlikely to be a QS signal molecule (Lee *et al*., 2006).

In this study, the role of LuxS$_{Hp}$ in AI-2 production was confirmed by a *V. harveyi* bioluminescence assay. Experiments were set up to distinguish between the mechanisms underlying the alteration in motility of Δ*luxS*$_{Hp}$ mutants, and to clarify whether this originated from a disruption of cysteine metabolism or production of the signalling molecule AI-2. The lack of motility in mutants disrupted in components of the reverse transsulphuration pathway other than LuxS, coupled to the inability of cysteine to complement the motility defect of the Δ*luxS*$_{Hp}$ mutant, shows that disruption of cysteine biosynthesis is not able to reduce motility in *H. pylori*.

5.2 Materials and Methods

5.2.1 Construction of the complemented Δ*luxS*$^{+}$ strain

H. pylori J99 wild-type was transformed with the plasmid pGEMTluxSXN396 containing *sacB-km* construct encoding kanamycin resistance (Km^{r}) and (5%) sucrose sensitivity (Suc^{s}) (Loh *et al*., 2004). Disruption of the chromosomal *luxS*$_{Hp}$ gene was accomplished by natural transformation, allelic exchange, and screening for kanamycin-resistance as previously described (Doherty *et al*., 2010), resulting in the J99 Δ*luxS* mutant strain. The presence of the *sacB-km* cassette was verified by amplifying fragments on *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 Δ*luxS* was then transformed with plasmid pGEMTluxS (encoding wild-type *luxS*), and transformants in which *sacB-km* had been replaced with the introduced original *luxS* locus were selected on (5%) sucrose resistance (Suc^{r}) and kanamycin sensitivity (Km^{s}). 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.

5.2.2 Motility bioassays

5.2.2.1 Plate 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 indicator 40μg/ml Tetrazolium Chloride (Sigma). Tetrazolium chloride is a redox dye which may be reduced to a pink compound, a fluorescent formazan derivative (Kaprelyants & Kell, 1993). Plates were seeded with 5μl *H. pylori* liquid culture (forming a circle with 3mm diameter) standardised to an OD_{600nm} of 1.0 and were incubated at 37°C for up to 7 days under the condition

described before. The motility halos were recorded using a digital camera and the area of each halo was measured using GS-800 Calibrated Densitometer (Biorad). When measuring, the entire colour of each halo (increase in diameter) was quantified, including faint colour at the edges of the colonies.

5.2.2.2 Plate motility assay using chemically defined media

The liquid chemically defined media were prepared as previously described (Reynolds & Penn, 1994, Doherty *et al*., 2010). 60ml of sterile chemically defined media were added to 40ml 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. The final concentration of cysteine was 1.0mM, 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).

5.2.2.3 Motility assay with AI-2 complementation

AI-2 was synthesised enzymatically as described previously (Winzer *et al*., 2002a) using purified proteins $LuxS_{E.\ coli}$ and $Pfs_{E.\ coli}$. For complementation of the Δ*luxS*$_{Hp}$ 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 the cell from supernatant (CFS) from *H. pylori* wild-type broth culture standardised to an OD_{600nm} of 1.0 at the time point in the growth curve that maximal AI-2 activity was measured. The AI-2 supplemented plate (ASP) was prepared using the 0.4% soft agar containing *in vitro* synthesised AI-2 (0.25% v/v). The buffer control plate (BCP) was 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 (only buffer containing no AI-2). 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 GS-800 Calibrated Densitometer (Biorad).

5.3 Results

5.3.1 The construction of the complemented Δ*luxS*$_{Hp}^{+}$ strain

To facilitate construction of the complemented Δ*luxS*$^{+}$ mutant, a two-step mutagenesis strategy was used, allowing gene replacement without introducing new selectable markers into the final strain. The method uses a two-gene cassette containing both a selectable marker *km* and a counterselectable marker *sacB*. The *km-sacB* cassette was cloned into the gene *luxS* (yielded the plasmid pGEMTluxSXN396) (Loh *et al*., 2004) and used to replace the J99 wild-type *luxS* gene on the chromosome by allelic exchange. The transformant obtained from the first mutagenesis step was the J99Δ*luxS*-F, a strain with kanamycin resistance (km^{r}) and 5% sucrose sensitivity (Suc^{s}). A second transformation replaces the mutated *luxS* containing the *km-sacB* cassette with an engineered plasmid, pGEMTluxS, containing the original *luxS* gene. The resulting transformant was the complemented Δ *luxS*$^{+}$ mutant, with the phenotype of (5%) sucrose resistance (Suc^{r}) and 5% kanamycin sensitivity (km^{s}) (Figure 5.1).

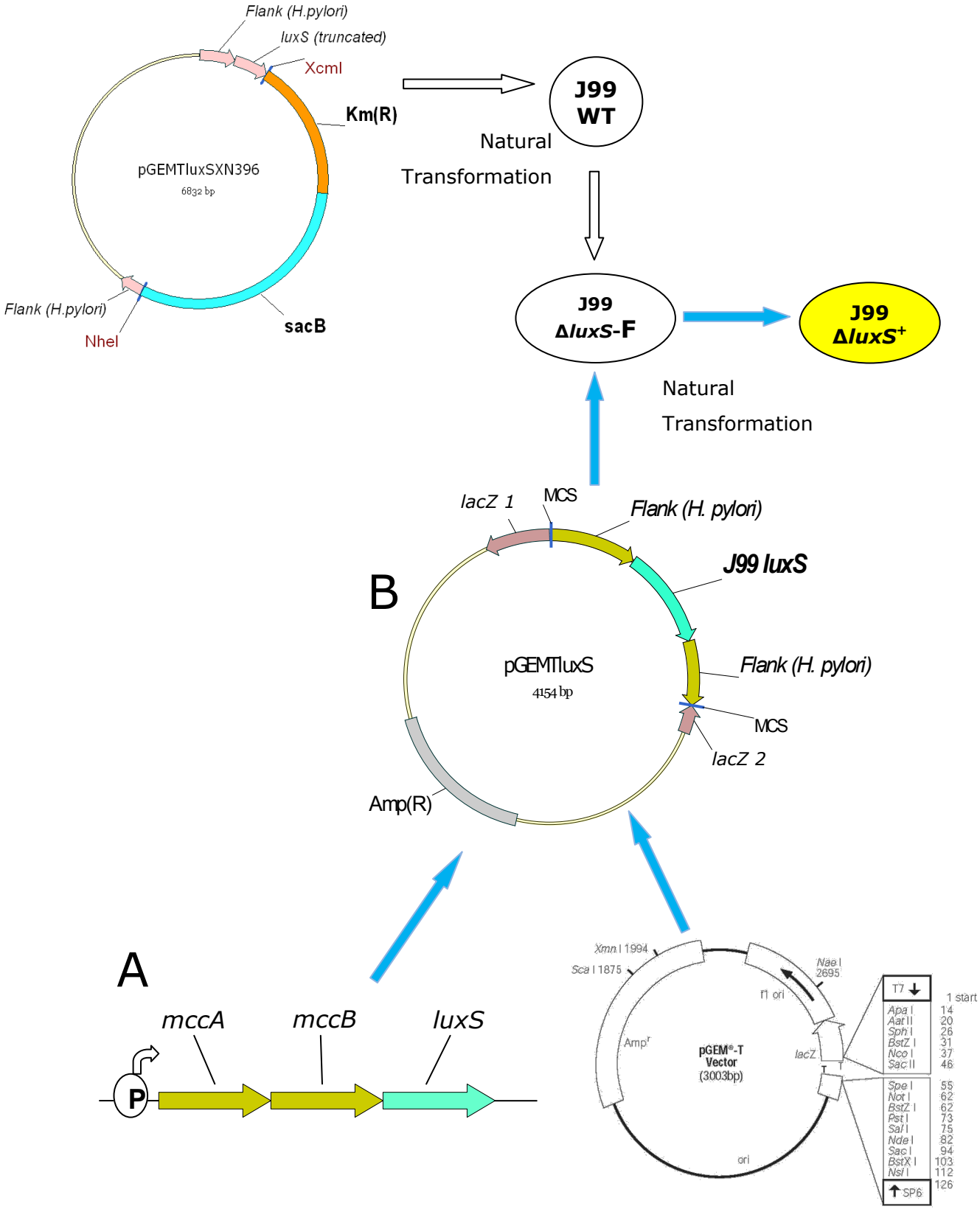


Figure 5.1 Flow diagram of construction of the complemented Δ*luxS*$_{Hp}^{+}$ mutant

In the process of constructing this mutant, steps labelled by white arrows went first, and were then followed by the steps labelled with blue arrows. For details in text see Materials and Method. MCS=multiple cloning site

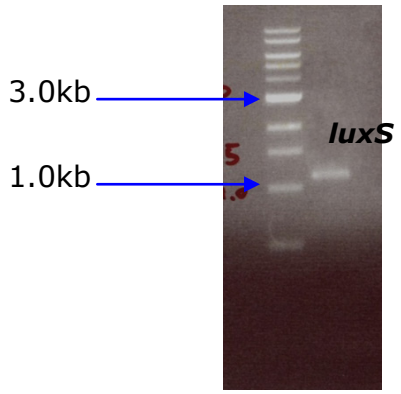


Figure 5.2 PCR product of *luxS*$_{Hp}$

The gel shows the product generated in figure 5.1 Step A. The PCR product of *luxS*$_{Hp}$ was amplified from J99 wild-type genomic DNA. The size of the original *luxS*$_{Hp}$ was 456bp. Around 350bp of additional DNA sequence was included at both ends of the original *luxS*$_{Hp}$ sequence to make a final fragment containing the entire *luxS*$_{Hp}$ plus flanking sequences. Primers *luxS*$_{Hp}$-F/*luxS*$_{Hp}$-R were used in the amplification. The size of the whole *luxS*$_{Hp}$ PCR product was 1.15kb. Marker: Promega 1kb DNA ladder.

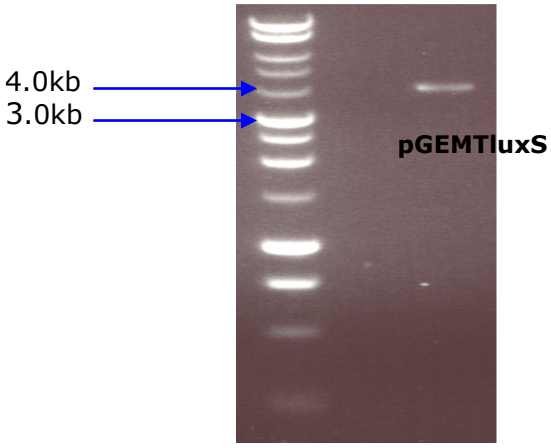


Figure 5.3 Digested plasmid pGEMTluxS

The gel confirms the product generated in figure 5.1 Step B.The plasmid pGEMTluxS (4.15kb) was produced by insertion of the *luxS*$_{Hp}$ PCR product into the vector pGEM-T. This was confirmed by restriction digestion with the enzyme NotI and the size of the digested product was checked on an electrophoresis gel. Marker: Promega 1kb DNA ladder.

5.3.2 The *H. pylori* Δ*luxS* mutant lost the ability to produce AI-2 while the wild-type, Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ mutants did not

The ability of each mutant to produce AI-2 was assayed using the *V. harveyi* AI-2 detection system described by Surette and Bassler (Bassler *et al*., 1997, Winzer *et al*., 2002a). *H. pylori* wild-type J99 and all mutant strains (Δ*luxS*, Δ*mccA* and Δ*mccB*) were grown in Brucella broth containing serum (10% v/v). AI-2 activity in the CFS was detected from the log phase. The wild-type produced AI-2 in a growth dependent manner, in which AI-2 accumulated during the late log phase and onward, reaching maximum levels in the stationary phase. During stationary phase, AI-2 levels decreased and were almost undetectable by 72 h during the death phase. Similar data were obtained with Δ$mccB_{Hp}$ and Δ$mccA_{Hp}$ mutants. After the peak, bioluminescence activity decreased to an undetectable level by 72 h. On the other hand, the Δ$luxS_{Hp}$ mutant, unlike the wild-type and the other two mutant strains, yielded significantly (p<0.001) reduced levels of bioluminescence at each time point, indicating that the production of AI-2 is $luxS_{Hp}$-dependent and that insertion of kanamycin cassette (*aphA3*) into $mccA_{Hp}$ and $mccB_{Hp}$ does not affect expression of the downstream gene $luxS_{Hp}$ (Figure 5.4).

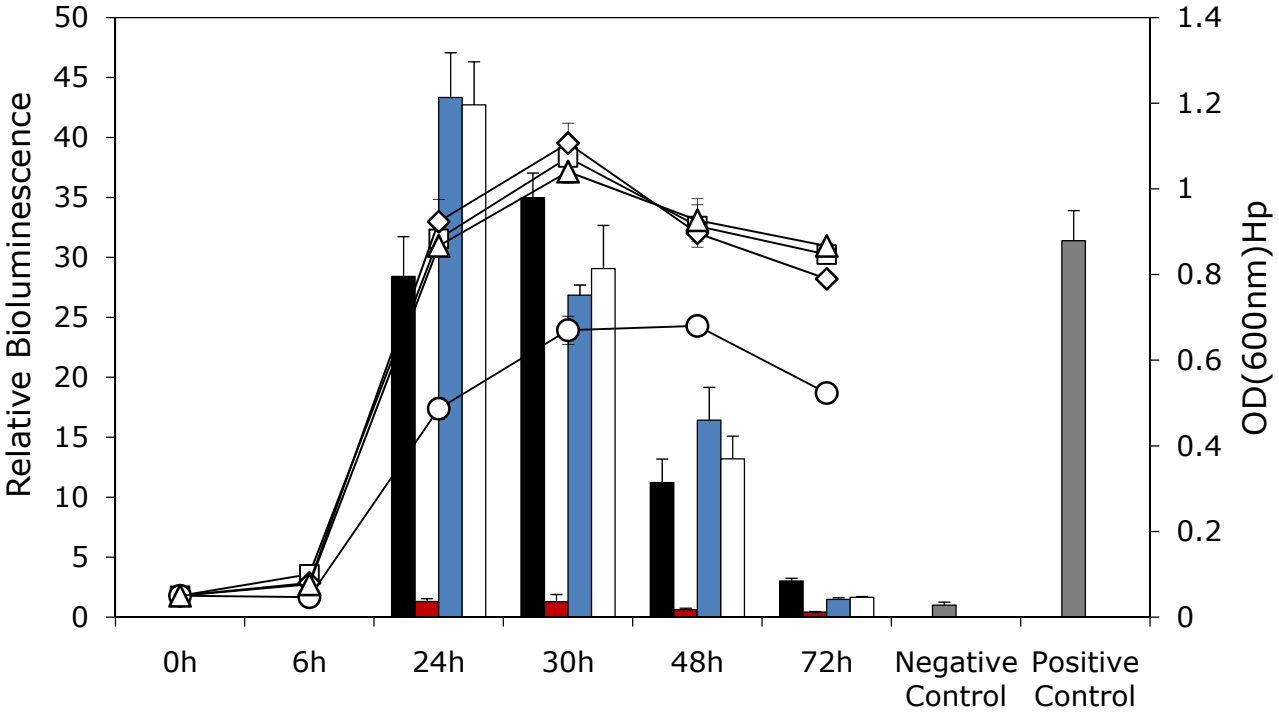


Figure 5.4 AI-2 production in *H. pylori* is *luxS* dependent

AI-2 production in J99 wild-type (black column), Δ*luxS*$_{Hp}$ (red column), Δ*mccB*$_{Hp}$ (blue column) and Δ*mccA*$_{Hp}$ (white column) mutants was measured. Strains were grown in Brucella broth, and aliquots were removed at 24h, 30h, 48h and 72h to assess the optical density (wild-type, square; Δ*luxS*$_{Hp}$, diamond; Δ*mccB*$_{Hp}$, circle; Δ*mccA*$_{Hp}$, 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 OD_{600nm} of *H. pylori*) in the presence of 1/10 diluted *H. pylori* culture supernatants over the negative control (NC, Brucella broth alone). A diluted *in vitro* synthesised AI-2 sample was utilised as a qualitative positive control (PC) (Winzer *et al.*, 2002a). Bioluminescence induced by wild-type, Δ*mccB*$_{Hp}$, and Δ*mccA*$_{Hp}$ strains was significantly greater than that induced by the Δ*luxS*$_{Hp}$ 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). Error bars indicate the standard deviation of triplicate samples for the bioluminescence and OD_{600nm} measurements, respectively.

5.3.3 Deletion of *luxS*$_{Hp}$ abolishes motility while the Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ mutants remained motile

To investigate whether motility of *H. pylori* was affected by cysteine biosynthesis, firstly, motility was compared between wild-type, Δ*luxS*$_{Hp}$, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ mutants. To do this, the 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 swarming halo areas were quantified after 3, 5 and 7 days of incubation. As stated in Materials and Methods, tetrazolium chloride was utilised as an indicator. The mechanism of the tetrazolium staining is that when the colourless chemical tetrazolium chloride diffuses into actively respiring tissues it accepts electrons from the mitochondrial electron transport chain, reducing it to a pink compound, formazan. The accumulation of formazan stains the tissues red, and the intensity of the red colour is proportional to the rate of respiration in those tissues (Kaprelyants & Kell, 1993).

Halo areas that surrounded the wild-type, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ strains kept increasing during continuous incubation, although the latter was slightly delayed in comparison to the others. After 7 days of culture, the Δ*luxS*$_{Hp}$ mutant remained almost non-motile and produced a significantly (p<0.001) reduced swarm halo compared to wild-type, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ strains in 3 independent repeat experiments (Figure 5.5A). After 7 days, the wild-type, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ 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 (Figure 5.5B). 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.

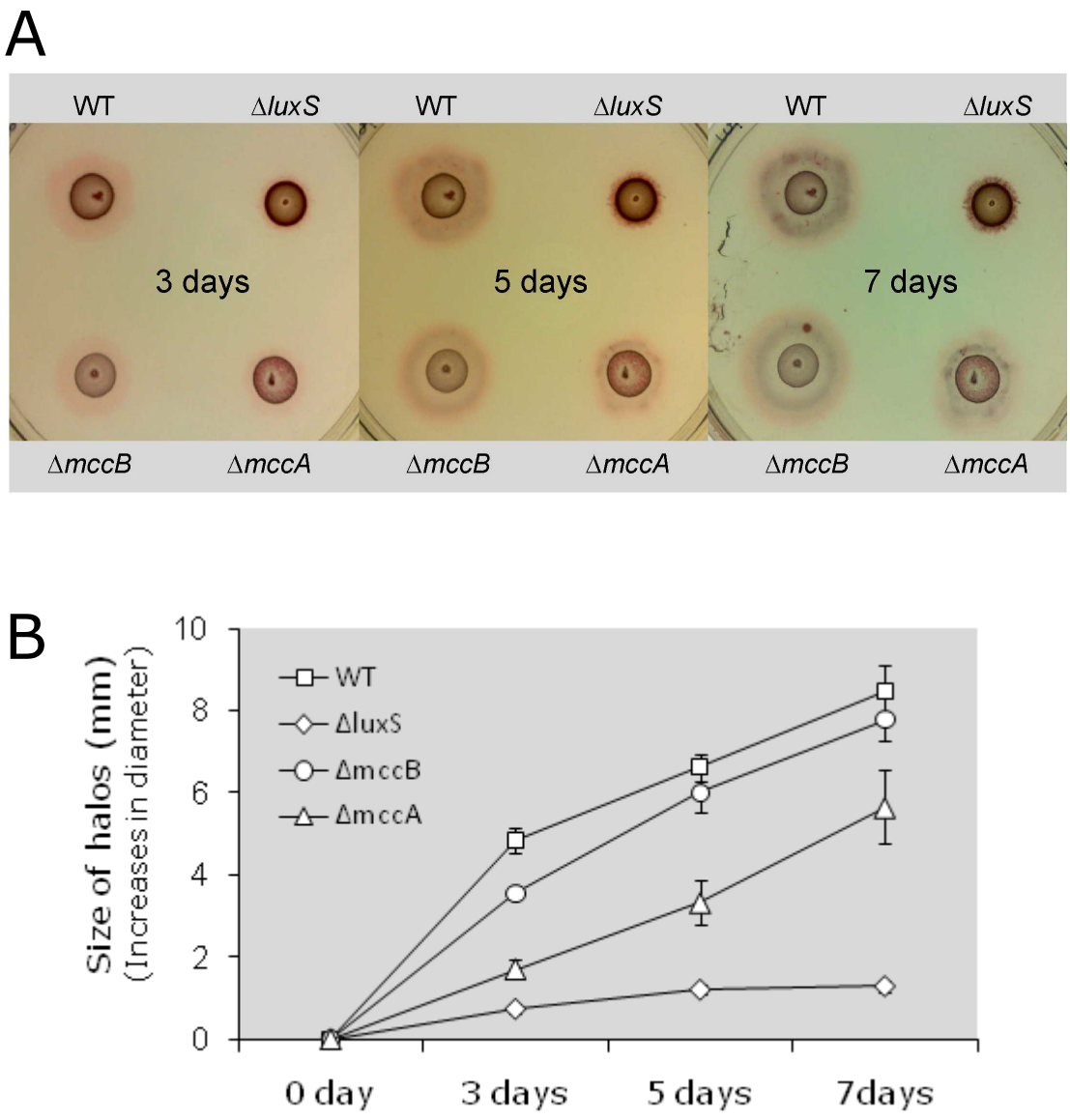


Figure 5.5 Motility bioassay of *H. pylori* strains on the normal soft plate

5μl of liquid growth (24h) of wild-type, Δ*luxS*$_{Hp}$, Δ*mccB*$_{Hp}$ and Δ*mccA*$_{Hp}$ mutants were seeded on each quarter of a soft agar plate. After 3, 5 and 7 days of incubation, the swarming halo of each strain was (A) recorded using a digital camera and (B) the area of each halo (wild-type, square; Δ*luxS*$_{Hp}$, diamond; Δ*mccB*$_{Hp}$, circle; Δ*mccA*$_{Hp}$, triangle) was measured using a GS-800 Calibrated Densitometer (Biorad). Error bars indicate the standard deviation of halo sizes of quadruple measurements in one experiment.

5.3.4 Exogenous AI-2 or genetic complementation can restore the motility defect of the Δ*luxS*$_{Hp}$ mutant, but exogenous cysteine addition can not

To rule out the possibility that second site mutations in the Δ*luxS*$_{Hp}$ mutant were inhibiting motility, genetic complementation was performed to create the Δ*luxS*$_{Hp}^{+}$ strain (see Chapter 5.2 Materials and Methods). The non-motile Δ*flhB* mutant was used as a negative control (Wand *et al*., 2006). 24 h culture of wild-type, Δ*luxS*$_{Hp}$, Δ*luxS*$_{Hp}^{+}$ and Δ*flhB*$_{Hp}$ strains grown in Brucella broth were individually spotted onto normal motility plates. After 5 days of incubation, the mean halo diameter of the Δ*luxS*$_{Hp}^{+}$ strain was (6.9 ± 0.2 mm, *n*=4), which was slightly larger than that of wild-type (4.7 ± 0.7 mm, *n*=4). The Δ*luxS*$_{Hp}$ and Δ*flhB*$_{Hp}$ mutants showed very similar non-motile phenotypes (Figure 5.6A).

To examine whether AI-2 can influence motility of *H. pylori*, motility of the wild-type, Δ*luxS*$_{Hp}$ and Δ*flhB* mutants was assessed using 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 (only 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 plate (compare Figure 5.6A or the right panel of Figure 5.6B with the left panel of Figure 5.6B). Whilst the Δ*luxS*$_{Hp}$ mutant was non motile on the BCP, the halo increased by 4.6 ± 0.4 mm, *n*=4 on ASP (Figure 5.6B). The control Δ*flhB*$_{Hp}$ mutant remained non-motile on the ASP (Figure 5.6B).

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) could behave similarly. Data shown in previous chapters have revealed that exogenous cysteine rescues growth defects of mutants ($\Delta luxS_{Hp}$, $\Delta mccA_{Hp}$ and $\Delta mccB_{Hp}$) unable to complete cysteine biosynthesis via the RTSP of *H. pylori* in chemically defined broth (see Chapter 4.2.1). Chemical complementation of motility was thus performed using chemically defined plates supplemented with 1.0mM cysteine. Methionine was added to the plates as the sulphur source since all *H. pylori* strains are methionine auxotrophs. After 5 days of incubation, wild-type *H. pylori* and $\Delta mccA_{Hp}$ and $\Delta mccB_{Hp}$ mutants formed swarming 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. Th e $\Delta luxS_{Hp}$ mutant remained non-motile (Figure 5.6C).

Taken together, data presented here indicate that the motility defect of the $\Delta luxS_{Hp}$ mutant was restored either genetically or chemically with AI-2, but not with exogenous cysteine. This suggests that *luxS*/AI-2 play a role in enhancing bacterial motility, rather than an intact cysteine biosynthesis pathway, implying a likely role of $luxS_{Hp}$ in signalling.

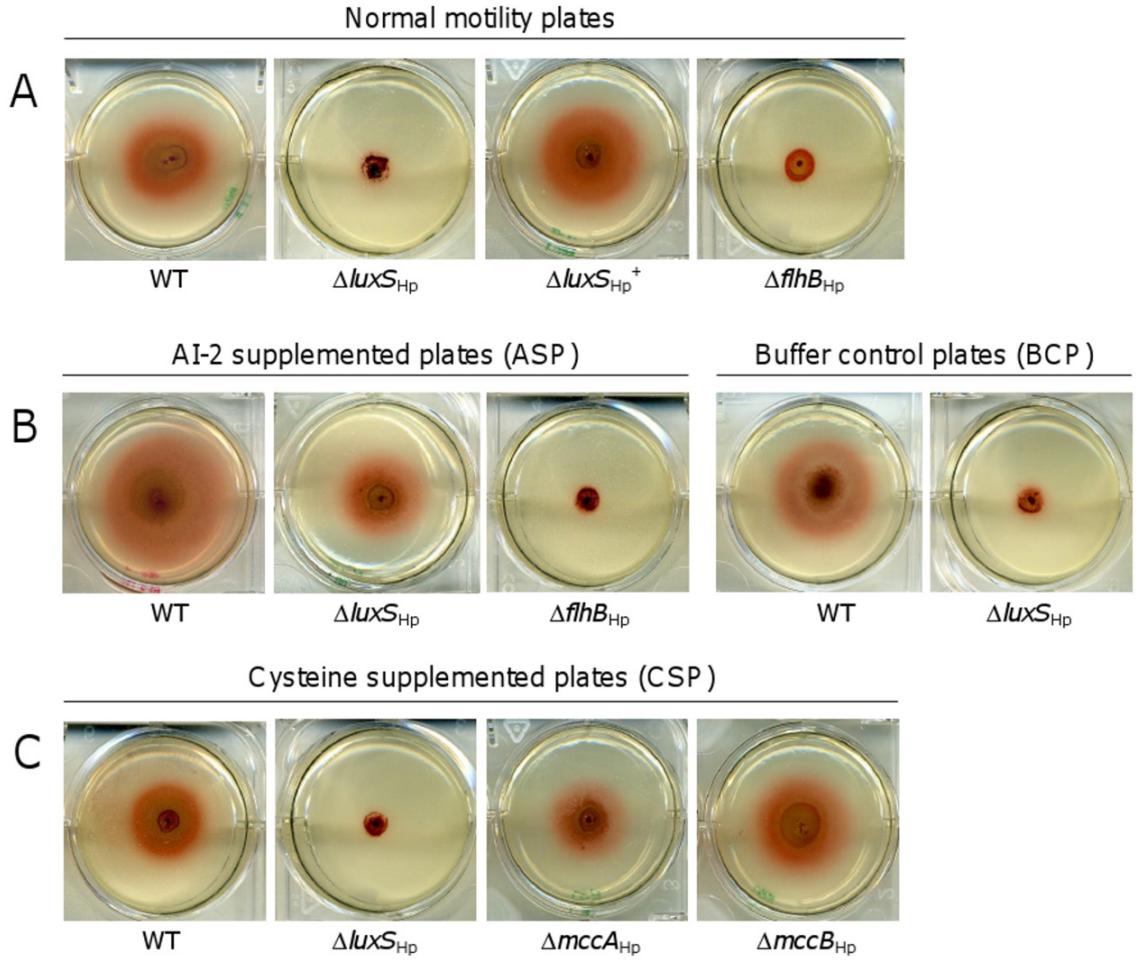


Figure 5.6 AI-2, but not cysteine rescues the motility defect of the LuxS$_{Hp}$ mutant.

(A) Wild-type, Δ*luxS*$_{Hp}$, and Δ*luxS*$_{Hp}^{+}$ bacteria were seeded onto soft plates composed of normal medium. The non-motile Δ*flhB* mutant served as the the negative control. (B) Wild-type, Δ*luxS*$_{Hp}$ and Δ*flhB* bacteria were seeded onto motility plates supplemented with *in vitro* synthsised AI-2. Wild-type and Δ*luxS*$_{Hp}$ were also seeded on motility plates containing buffer control solution used for *in vitro* AI-2 synthesis. (C) Wild-type, Δ*luxS*$_{Hp}$ Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ strains were seeded onto chemically defined motility plates supplemeneted with cysteine. After 5 days of incubation, the swarming halo of each strain on each plate was recorded using a digital camera and the area of each halo was measured using GS-800 Calibrated Densitometer (Biorad).

5.4 Discussion

The function of *luxS*$_{Hp}$ is controversial due to putative roles both in signalling and metabolism. 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 (Winzer *et al*., 2003). In support of this, in some bacteria, production of AI-2 does appear to be associated with metabolic rather than regulatory phenomena (Doherty *et al*., 2006). In *H. pylori,* it has been shown that, rather than functioning in recycling methionine as in most bacteria, LuxS (along with newly-characterised MccA and MccB), synthesises cysteine via a process of reverse transsulphuration. This makes the mechanism underlying phenotypic changes upon *luxS* inactivation unclear, in particular, whether the phenotypic alterations are due to the disrupted cysteine provision pathway, or due to loss of the signalling molecule AI-2. This work shows that AI-2 signalling but not cysteine provision is necessary for normal levels of motility in *H. pylori* strain J99, suggesting a likely role of *H. pylori* AI-2 in signalling.

First of all, the role of LuxS$_{Hp}$ in AI-2 formation was confirmed by doing the *V. harveyi* bioluminescence assay. Experimental data showed that AI-2 activity peaked in the stationary phase and then significantly fell. Previously, Forsyth *et al*. and Loh *et al*. individually reported that maximal AI-2 activity appears in early log phase (5 h ~ 10 h) rather than stationary phase (Loh *et al*., 2004, Forsyth & Cover, 2000). However, similar to our study, Rader *et al*. (2007) found maximum AI-2 activity in stationary phase. Interestingly, they found a very strong luminescence peak in late stationary phase (at 40 h) (Rader *et al*., 2007).

Also, Δ*mccB*$_{Hp}$ and Δ*mccA*$_{Hp}$ strains were shown producing AI-2 in a growth-dependent-manner. AI-2 activity of these two mutants consistently peaked at an earlier stage compared to the wild-type. This suggests two possibilities: firstly, disruption of upstream *mccB* or *mccA* in *H. pylori* could be affecting the transcription of *luxS*, making LuxS accumulate at an earlier time point. Alternatively, inhibition of cysteine biosynthesis by disrupting *mccB*$_{Hp}$ or *mccA*$_{Hp}$ somehow could be stimulating the conversion of *S*RH to AI-2/homocysteine at an earlier growth phase, consequently causing earlier appearance of higher quantities of AI-2. Moreover, it appeared that colour changes of the mutants on motility plates were slightly different from the wild-type (Figure 5.5A and 5.6). This may be due to the fact that respiratory activity of the bacteria is involved in the colour change of the tetrazolium staining, and hence the changes in altering capability of mutants might affect the degree of colour change compared with wild-type. As tetrazolium chloride is a redox dye, the fact that cysteine is a redox active molecule might thus be an additional factor to cause the issue.

Previous studies have shown that mutations of *luxS*$_{Hp}$ 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 (Rader *et al*., 2007, Osaki *et al*., 2006, Lee *et al*., 2006). 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 (Osaki *et al*., 2006, Rader *et al*., 2007). Like these previous reports, our study shows that abolished motility of J99 Δ*luxS*$_{Hp}$ 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.

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 (Doherty *et al*., 2010), it was reasoned that inactivation of any of these three enzymes would have a similar influence upon cysteine biosynthesis, whilst only the Δ *luxS*$_{Hp}$ mutant would be devoid of AI-2. Thus, if the reduced motility of the Δ *luxS*$_{Hp}$ mutant derived from disrupted cysteine biosynthesis, mutants in *mccA*$_{Hp}$ and *mccB*$_{Hp}$ would have a similar motility defect. Therefore, an experiment was performed 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, Δ*luxS*$_{Hp}$, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ 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, 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 Δ*luxS*$_{Hp}$ 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. This, for the first time suggests that motility of *H. pylori* can be blocked by the loss of *luxS*$_{Hp}$ itself, but cannot be affected by disrupting the cysteine provision pathway.

Furthermore, on both normal motility plates and the cysteine supplemented plates, the Δ*mccA*$_{Hp}$ mutant appeared to be less motile than the wild-type and the Δ*mccB*$_{Hp}$ mutant (however, its swarming halo was still significantly larger than the Δ*luxS*$_{Hp}$ mutant). This may be because inactivation of *mccA*$_{Hp}$ directly disrupted the conversion of homocysteine to cystathionine, allowing homocysteine to accumulate in cells. We speculate that this could potentially signal the cells to stop the production of homocysteine; consequently, as a by-product in the production of homocysteine, the synthesis of AI-2 could also be affected.

Motility is an absolute requirement for *H. pylori* colonization in several animal models (Ottemann & Lowenthal, 2002, Eaton *et al.*, 1992, Lee *et al*., 2006, Osaki *et al*., 2006) and is thus considered an important virulence trait of this pathogen. The LuxS associated motility control exists in some other bacteria, for example, in *E. coli* O157: H7, the expression of more than 30 genes related to motility and flagella assembly was down-regulated in the Δ*luxS* mutant, and the mutant had less motility than the wild-type (Sperandio *et al*., 2001). In Gram-negative *C. jejuni* and *V. alginolyticus*, removal of the gene *luxS* altered flallelar gene transcription and bacterial motility (He *et al*., 2008, Tian *et al*., 2008). In *Proteus mirabilis*, however, deletion of *luxS* did not change its motility phenotype, probably because this bacterium has an intrinsic hierarchical system to control its motility (Schneider *et al.*, 2002). All of these altherations led to an assumption that motility of *H. pylori* is likely to be regulated by LuxS via modulating transcription of genes correlated to motility or flagella synthesis.

H. pylori may use AI-2, which has been shown to be produced in a cell density dependent manner, in conjunction with other environmental cues, to regulate motility

as a survival strategy within the human stomach. Patterns of *H. pylori* dispersal within the gastric environment are correlated with disease outcome, with ulcers associated with higher bacterial density in the distal stomach and gastric cancer arising from infections in which the bacteria are distributed throughout the stomach (Blaser & Kirschner, 1999). Consequently the prediction was that, at reasonably high cell densities and when AI-2 concentration researches up to a critical level, it may be beneficial for *H. pylori* to increase flagella production or enhance some flagellar function as a strategy to promote motility and dispersal throughout the stomach. To test this hypothesis, more experimental data are exhibited following this chapter to give compelling evidence on the mechanism of LuxS/AI-2 signalling on regulation of motility in *H. pylori*.

CHAPTER 6.
LuxS-SYNTHESISED AUTO-INDUCER-2 REGULATES MOTILITY BY MODULATING GENE TRANSCRIPTION AND FLAGELLAR SYNTHESIS

The work presented in this chapter has been published in BMC Microbiology.

6 *H. pylori* LuxS-synthesised auto-inducer-2 regulates motility by modulating gene transcription and flagellar synthesis

6.1 Introduction

Auto-inducer-2 (AI-2) production is widespread among bacterial species; its formation is catalysed by the enzyme LuxS (Vendeville *et al*., 2005). It is widely utilised by both Gram-positive and Gram-negative bacteria. So far, known AI-2 dependent processes in bacteria include bioluminescence, biofilm formation, motility, and regulation of virulence factors (Vendeville *et al*., 2005). Good examples include *Vibrio harveyi* and *Vibrio cholerae*, where AI-2 regulates density-dependent bioluminescence and virulence production, respectively (Bassler *et al*., 1997, Camara *et al*., 2002). Although AI-2 production is common among bacterial species, it remains controversial as to how many of these bacteria utilise AI-2 as a signalling molecule, as opposed to producing it as a metabolic byproduct of the *S*AM pathway.

One such controversial case is *Helicobacter pylori*. Previous chapters have clarified the role of $LuxS_{Hp}$ in cysteine metabolism and the role of AI-2 in bacterial motility. It is now important to elucidate the mechanism underlying the motility defect of *H. pylori* induced by LuxS inactivation. There are several possible ways 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 Δ*luxS* mutant but a lack of a functional motor may prevent rotation. Thirdly, both motor and flagellum may be functional, but unable to respond to incoming signals.

In terms of gene transcription related to $LuxS_{Hp}$/AI-2, a previous study has demonstrated that a Δ*luxS*$_{Hp}$ mutant lost growth-phase-dependent regulation of expression of the major flagellin, FlaA, and that cell culture supernatants containing AI-2 could increase *flaA* transcription (Loh *et al*., 2004). More recently, Rader *et al*. have shown that *luxS*$_{Hp}$ mutations affect flagellar morphology in strains that also lack one of the transcriptional regulators (σ^{28}, *flgS* or *flgM*), and that this can be complemented upon the addition of DPD. They reported that loss of *luxS*$_{Hp}$ caused decreased transcription of the flagellar regulator *flhA*, and that expression of *flhA* was induced by DPD (Rader *et al*., 2007). This complementation through the addition of exogenous DPD re-established the possibility that in *H. pylori* there could be a LuxS-dependent signalling network.

In order to clarify the mechanism underlying alterations of motility in the Δ*luxS*$_{Hp}$ mutant, electron microscopy (EM) was employed to examine flagella assembly and the levels of individual components of flagella were assessed at a transcriptional and translational level. By examining motility, flagellar morphogenesis, flagellar gene transcription and protein expression in *H. pylori* wild-type and mutant cells, the demonstration here that exogenously added AI-2 (or DPD) influences motility via regulating flagellar gene transcription (and thus the number and length of flagella) supports the existence of an additional role for LuxS in *H. pylori* as a signalling molecule synthase. Moreover, the inability of cysteine to complement the motility and flagellar gene transcription in the Δ*luxS*$_{Hp}$ mutant, confirms that disruption of cysteine biosynthesis is not the mechanism underlying the reduction in motility.

6.2 Materials and Methods

6.2.1 Motility assay by microscopy

Motility analysis was also carried out by direct observation under phase-contrast microscopy using a Nikon Eclipse E600 after cells were grown in co-culture conditions. Briefly, co-cultures of *H. pylori*-AGS cells were prepared (described below). After 24 h, 10µl culture was placed onto a microscope slide and covered with a coverslip and free-swimming *H. pylori* cells were examined under the microscope.

6.2.2 Tissue culture and bacterial co-culture

6.2.2.1 AGS cells culture

All chemicals were Gibco, UK products. Human gastric adenocasinoma (AGS) cells were grown in culture medium (Ham's F-12 supplemented with L-glutamine (200mM) and FBS [10% v/v]) in a 37°C incubator containing 5% CO_2. After cells have grown to confluency, the flask was washed twice by the sterile Phosphate Buffered Saline (PBS) and filtered trypsin was then added. Cells with trypsin were incubated for 2-3 min. After all cells were suspended in trypsin, they were poured into a 20ml universal tube. The universal tube containing cells was topped up with the culture medium and centrifuged for 3 min at 1500 rpm. The supernatant was poured off into a waste bottle. The universal tube was flicked to resuspend cell pellet and refilled with 20ml medium. The wash step was then repeated once more. Cell pellets were resuspened in 5ml or 8ml of culture medium. The 1 in 5 or 1 in 8 dilution was then added to a 75cm^2 flask containing fresh media mix and incubated at the same condition as before to allow cells to re-grow to confluency.

6.2.2.2 AGS cells-bacteria coculture

AGS cells were counted using the trypan (0.35% v/v) blue dye method. Cells were seeded at a density of 1×10^5 cells/ml into 6 well plates and grown to 80%

confluency. The cells media mix was removed and replaced with 2ml fresh F-12 media. Plates were inoculated with 24 h *H. pylori* liquid cultures standardised to an OD_{600nm} of 0.1 and incubated for one day in a microaerophilic environment. Bacterial cells were then analysed using a phase-contrast Nikon Eclipse E600 microscope and electron microscopy.

6.2.3 Electron microscopy (EM)

H. pylori cells were pre-grown as described for motility analysis. 15µl of culture was allowed to settle on a carbon formvar grid (Agar Scientific) for 1min. The suspension was removed and the grid washed by addition of 15ul of PBS for an additional minute. This was removed and the cells stained with 0.5% Phospho-tungstic acid (PTA) pH7.0 for one minute. Grids were examined and pictures taken using a JEOL JEM1010 Transmission Electron Microscope. Three groups of *H. pylori* cell samples prepared on different dates were examined. Each group of samples contained wild-type, Δ*luxS* and Δ*luxS*$^{+}$ cells treated and not treated with DPD. For each group, 100 *H. pylori* cells from each culture sample were examined.

6.2.4 Western blotting

24 h liquid (Brucella broth) culture of each *H. pylori* strain was adjusted to OD_{600nm} of 1.0. A 500µl 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 1ml sterile PBS. 100µl protein sample was collected. The same volume of 2 × sample buffer was added and boiled for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described in General Materials and Methods (Chapter 2.2.3). For immunoblotting, proteins were transferred from SDS-PAGE gels to nitrocellulose paper by the methanol Tris-glycine system described by Towbin *et al.* (Towbin *et al*., 1992). Briefly, it was run by the electrophoretic method in

Western buffer (14.4g Glycine and 3.03g Tris in 1L dH_2O) at 150mA for 1 h. Transfer of proteins was verified by staining the membrane with Ponceau S for 1 min. The stain was then removed with PBS. The membrane was immersed in blocking buffer (3% skimmed milk in PBS), shaking at room temperature for 20 min. The blots were incubated with rabbit polyclonal antibodies against *H. pylori* flagellin and hook protein[§§] (diluted 1/1,000--1/10,000) (Ryan *et al.*, 2005) in blocking buffer overnight at 4°C (or 1-2 h at room temperature) on a shaker. The blot was then rinsed 3 times using PBS-Tween (0.2%). Bound antibodies were detected using secondary anti-rabbit IgG alkaline phosphatase conjugate antibody (diluted 1/5,000 in 3% skimmed milk) (Sigma, UK). The blot was rinsed three times in PBS-Tween (0.2%) and 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). The primary and secondary antibodies used and rinse conditions are shown Table 6.1.

Table 6.1 Primary and secondary antibodies and rinse conditions

Name	Mono/ Polyclonal	PBS-tween	Secondary antibody
Anti-flagellin and hook protein	Polyclonal	0.2% 3×5 mins	GAR-AP

GAR: goat anti rabbit; AP: alkaline phosphatase conjugate antibody

[§§] The antibody is a generous gift from Paul O'Toole.

6.2.5 Detection and quantitative analysis of RNA expression

6.2.5.1 RNA isolation

RNA for transcriptional test was isolated from *H. pylori* cells after 24 h of incubation in Brucella broth. Culture collects were treated with RNA protection reagent (QIAGEN, UK) and RNA was extracted using RNeasy mini kit (QIAGEN, UK). Contaminating genomic DNA was removed using DNA free kit (Ambion).

6.2.5.2 cDNA synthesis

Synthesis of cDNA was performed using Ominiscript RT kit (QIAGEN, UK) and random hexamers (Roche, Germany). Firstly, template RNA was stored on ice. RNase inhibitor (Ambion) was diluted to a final concentration of 10U/µl in ice-cold 1 × buffer RT (0.1µl 10 × buffer RT, 0.65µl dH_2O and 0.25µl 40U/µl RNase inhibitor) and kept on the ice. 20µl reaction mixtures of cDNA positive samples contained 2µl 10 × buffer RT, 2µl dNTP mix, 2µl random hexamers, 1µl RNase inhibitor (10U/µl), 6µl H_2O, 1µl omniscript and 6µl RNA. cDNA negative samples contained 1µl 10 × buffer RT, 1µl dNTP mix, 1µl random hexamers, 0.5µl RNase inhibitor (10U/µl), 3.5µl H_2O and 3µl RNA. All reaction mixtures were incubated at 37°C for 0.5 h-1 h.

6.2.5.3 Quantitative analysis of transcription by RT-PCR

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 6.2. All RT-PCR reaction mixtures contained 12.5µl of SYBR Green Mix (QIAGEN, UK), 5µl of gene specific primers, 2µl cDNA template (cDNA was diluted 10-fold prior to adding into the RT-PCR reactions) and RNase free water to a final volume of 25µl. 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* and no-template), were run in triplicate. Transcript level of each gene was normalised to the *16S* rRNA in each sample.

Table 6.2 Primers used for RT-PCR and efficiencies

Primer	Sequence (5'-3')	Efficiency
16S_F	CGA TGA AGC TTC TAG CTT GC	1.81
16S_R	ATA GGA CAT AGG CTG ATC TC	
σ^{80}F	GTC AAA ACG ATG GAG ACC ACT TT	2.07
σ^{80}R	GCA CTT GAG CGA TAA TGT CTT CT	
σ^{54}F	CGA AGT TTA TGA GAA AGT ACG CAA	1.90
σ^{54}R	AAA ATC TTT AGA AAA TTC ATG GTG TT	
σ^{28}F	GTC AAT GGG GCA ATG TTA GAT	2.05
σ^{28}R	AAT GCT CAT CGC TAG GCT CT	
flgRF	ACG GAC GCT AAT GTC ATG CTA	1.96
flgRR	ATG TTG ATC GCT ATA AAA GGG TG	
flgSF	AAC AGC ATT CAG GGC AAG C	1.95
flgSR	AAG AGA GAT AGA GCC TAC GG	
flgMF	CGT GTGGAA AAG AAT GAA A	1.87
flgMR	AGA AGT CTC ATG CAA GTT GAT	
flaAF	CAG GTT CGT ATC GCT ACA GGC	1.98
flaAR	ATC ACT TCT GCT AAC ACG CCG	
flaBF	ACT GGG ATT GGG GCG TTA	2.03
flaBR	TCA ACC TCC CGTCAG CGT C	
flgEF	GCT CAG GCA CGA TCA CTC TAA	2.01
flgER	AAC GCC ATG AAA GGT CTT AAT AC	
flhAF	TCA TTG GAG GGT TTT TAG TGG	2.06
flhAR	GGT GCG AGT GGC GAC AAT	
motAF	TGA GTT TAG AGG GGC GAG TG	1.93
motAR	CCA GTA ATG AGC GGC ACC	
motBF	TTC AGG GAA AGA AGA AGA GCA A	1.92
motBR	TCA AAC AGC AAA CTA GAG AAA A	
fliIF	ACG AGC GAT GAT AGC CCT TTA	2.04
fliIR	ACC GAT TTC TCT TTG AGC CAT	
ureAF	GAT GAT GTG ATG GAT GGT GTG G	1.95
ureAR	TAA GAA CAA CTC ACC AGG AAC C	

6.2.5.4 Efficiency Calculation

To calculate relative transcriptional levels of the genes of interest using the Pfaffl method (Pfaffl, 2001), the reaction efficiency of each PCR was calculated (Figure 6.1). The PCR efficiencies were calculated by diluting the positive control cDNA (J99 wild-type in experiments presented here) and calculating the gradient from plots of dilution against CT value (cycle number at which the threshold was crossed). Examples of plots calculation are shown in Figure 6.2.

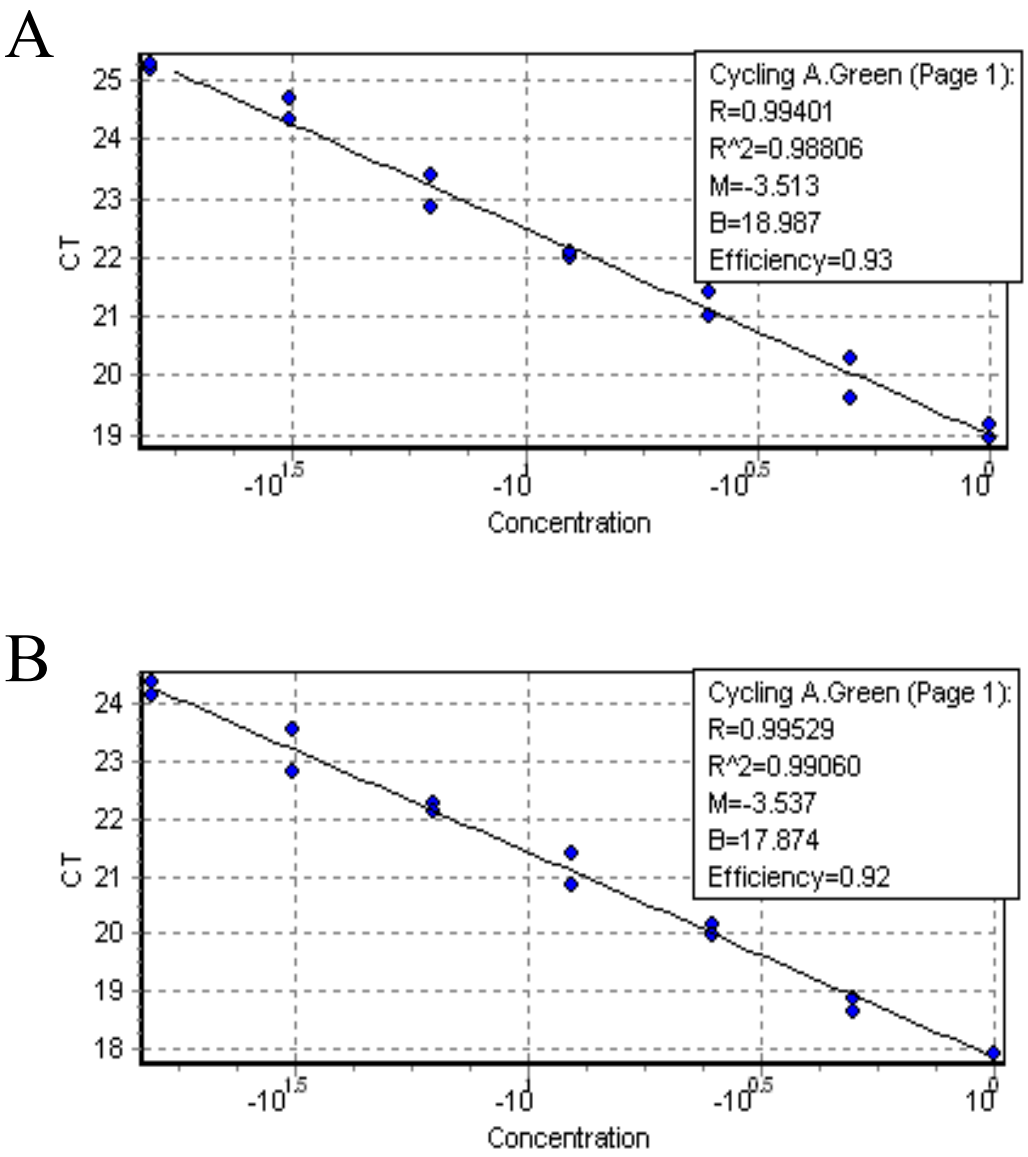


Figure 6.1 Efficiency runs for *motA* (A) and *motB* (B) RT-PCRs.

The PCR efficiencies were calculated by diluting the positive control cDNA (J99 wild-type) and calculating the gradient from plots of dilution against CT value. The efficiency of *motA* reaction is 1.93 and the efficiency of *motB* reaction is 1.92.

A

$$Ratio = \frac{E_{target}^{\Delta CP_{target}(Control-Sample)}}{E_{ref}^{\Delta CP_{ref}(Control-Sample)}}$$

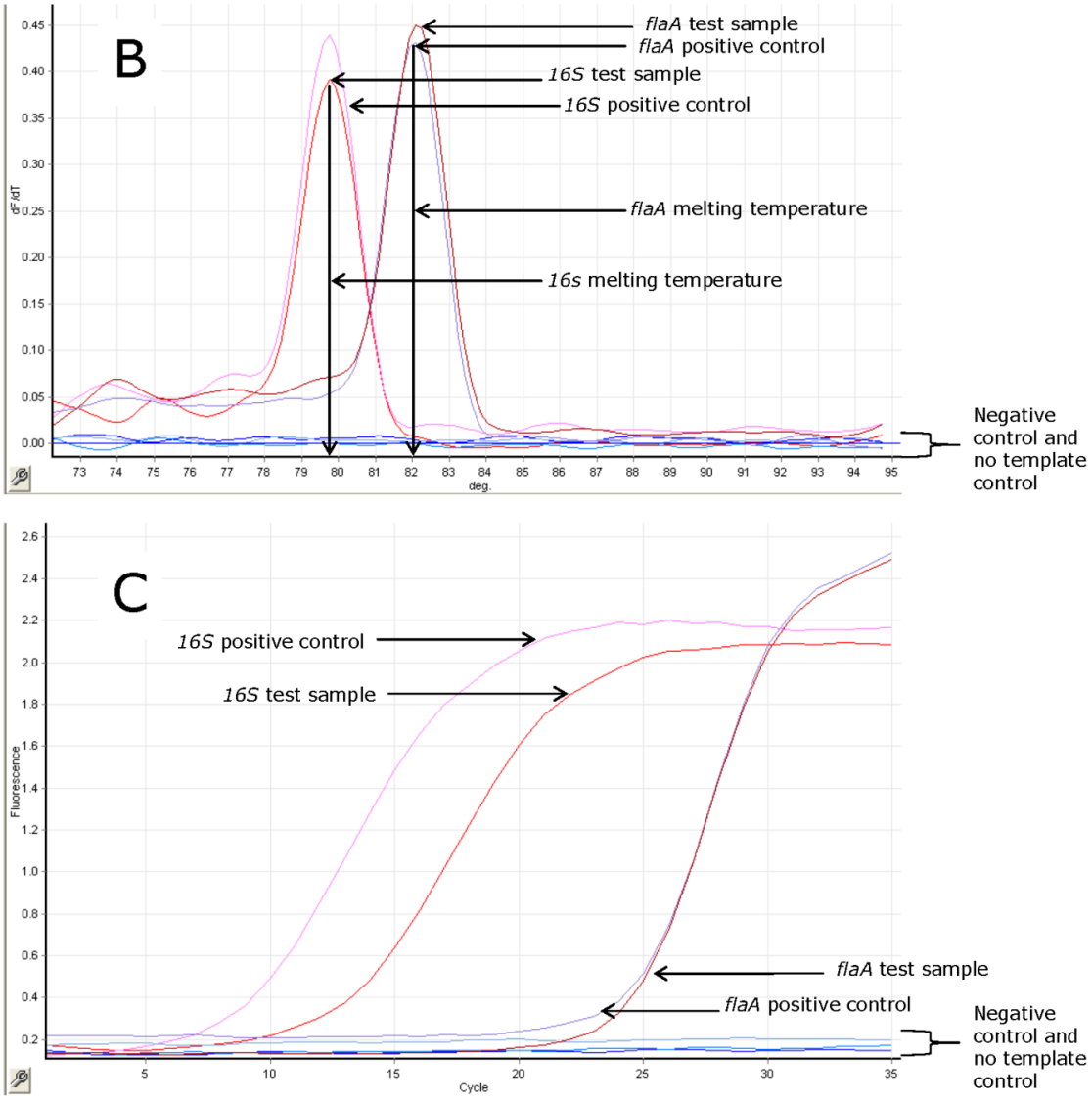


Figure 6.2 Pfaffl's equation and examples of melt and amplification curves produced by *flaA* RT-PCT rotor.

(A) Pffafl's equation; (B) melting temperature for *flaA*; (C) amplification for *flaA*.

Degree: °C

6.3 Results

6.3.1 ΔLuxS$_{Hp}$ mutants have altered flagella morphology and swimming patterns

(Microscopic analyses were collaboration with Laura Hobley and Liz Sockett, Institute of Genetics, University of Nottingham)

Motility plates effectively indicate motility phenotypes of the population, but do not give any indication of the structure of the motility organelle (flagellum), or the swimming pattern of individual cells. To characterise the phenotypes underlying the decreased ability of the Δ*luxS*$_{Hp}$ mutant to swarm in soft agar, we observed motility of individual bacterial cells using phase-contrast microscopy and also examined the flagellar morphology of the cells using electron microscopy (Figure 6.3). Cells tested included wild-type, Δ*luxS*$_{Hp}$, Δ*luxS*$_{Hp}^{+}$ 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.

For microscopic motility assay, bacteria on quite a few different areas of the slides were examined (>200 cells of each strain sample were examined). Phase-contrast microscopy revealed that 40-50% of wild-type cells and 75-80% Δ*luxS*$_{Hp}^{+}$ cells were swimming; whereas fewer than 2% of Δ*luxS*$_{Hp}$ cells could swim. When grown with exogenous DPD, swimming cells were 50% in wild-type, 40% in Δ*luxS*$_{Hp}$ and 70% in Δ*luxS*$_{Hp}^{+}$. Cultures of Δ*luxS*$_{Hp}$ grown with exogenous cysteine consistently contained less than 2% motile cells. To exclude the possibility that the restoration of motility of Δ*luxS*$_{Hp}$ cells was due to an effect of DPD on AGS cells rather than *H. pylori*, a control sample was set up in which the wild-type and Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ cells in these control cultures showed very similar motility phenotypes to those co-cultured with normal AGS cells, indicating that DPD is a functional signaling molecule to *H. pylori* cells rather than eukaryotic cells. Moreover, the approximate swimming speed of the Δ*luxS*$_{Hp}$ cells was visibly lower compared to the wild-type, Δ*luxS*$_{Hp}^{+}$ and all cell samples plus DPD. Notably, the amount of Δ*luxS*$_{Hp}$ cells in co-cultures was visibly less for the other strains; whereas, in rich media (e.g. Brucella broth), the Δ*luxS*$_{Hp}$ mutant grew as fast as wild-type.

For electron microscopy, three groups of *H. pylori* cell samples prepared on different dates were examined. Each group of samples contained wild-type, Δ*luxS* and Δ*luxS*$^{+}$ cells treated and not treated with DPD. For each group, 100 *H. pylori* cells from each culture sample were examined. Electron microscopic images showed that all samples tested (wild-type, Δ*luxS*$_{Hp}$ and Δ*luxS*$_{Hp}^{+}$, 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 Δ*luxS*$_{Hp}$ strain were consistently short and usually fewer in number. Changes were quantified, rounding to the nearest 5% and quoting means ± SD. In the experiments, nearly all of the wild-type cells (95% ± 3%, n=3) (n=3 means 3 independent microscopic experiments) tested had flagella and most of these had multiple flagella, which were usually at one pole and typically 3-4 in number (90% ± 3%, n=3) (Figure 6.3A). In contrast, fewer Δ*luxS*$_{Hp}$ 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 6.3B). The complemented Δ*luxS*$_{Hp}^{+}$ cells (Figure 6.3C) were similar to wild-type, with nearly all cells (95% ± 3%, n=3) possessing 3-4 normal long flagella at least one pole. Addition of DPD to

Δ*luxS*$_{Hp}$ cells (Figure 6.3E) also converted them to a wild-type morphology, with the vast majority (95% ± 3%, n=3) producing 3-4 wild-type length flagella usually present at a single pole. Addition of DPD to wild-type cells (Figure 6.3D) 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. Addition of DPD to the Δ*luxS*$_{Hp}^{+}$ cells (Figure 6.3F) had a similar effect, with more cells with flagella at both poles.

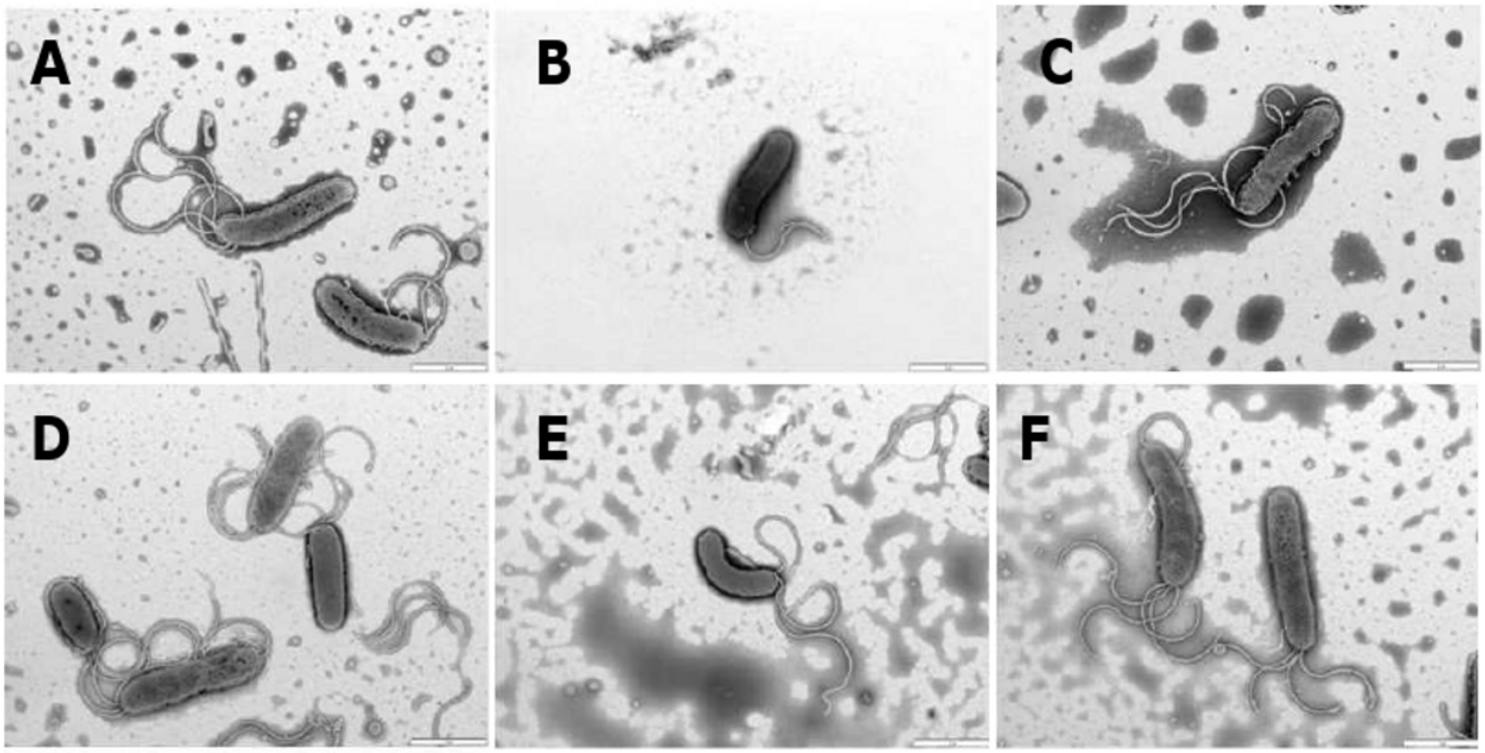


Figure 6.3 *luxS*/DPD modulates flagellar morphogenesis

H. pylori cells were co-cultured with AGS cells. Cells were stained with 0.5% PTA. Scale bars represent 2um. (A) wild-type, (B) Δ*luxS*$_{Hp}$, (C) Δ*luxS*$_{Hp}^{+}$, (D) wild-type with DPD, (E) Δ*luxS*$_{Hp}$ with DPD and (F) Δ*luxS*$_{Hp}^{+}$ with DPD. DPD was added after 10 h of incubation and once again after 18 h of incubation during the co-culture.

6.3.2 Mutation of *luxS*$_{Hp}$ resulted in decreased production of flagellar proteins FlaA and FlgE

The reduced number and length of flagella in Δ*luxS*$_{Hp}$ cells observed by electron microscopy could result from a number of different changes in the proteome. As previous work had suggested possible involvement of major flagella proteins, these were investigated by immunoblotting whole cell lysates with anti-flagellin (FlaA and FlaB) and anti-FlgE (hook protein) antiserum (Figure 6.4). To exclude global differences in protein production between strains, the loading was corrected for numbers of bacteria rather than for total protein levels. However, in practice, FlaB levels were very similar between all wild-type and mutant strains and were not shown to vary in the subsequent transcription analysis, so these acted as a reasonable loading control. Because we were interested in the ratio of flagella proteins within each wild-type or mutant strain, results were expressed relative to FlaB levels within each strain. In these experiments, *H. pylori* wild-type 17874, and derived mutants (Δ*flaA* and Δ*flgE*) were used as positive and negative controls, respectively. *H. pylori* wild-type, its Δ*luxS*$_{Hp}$ mutant, the complemented Δ*luxS*$_{Hp}^{+}$ mutant and controls were grown in Brucella Broth at 37°C for up to 24 h, at which point high levels of AI-2 activity were detected. In the experiments, four repetitions were included, when the reflective density (RD) of each protein band was measured using Quantity One 4.6.5 software (Biorad).

All strains tested were found to produce FlaB at approximately the same level (Figure 6.4). The reflective density of the FlaB bands of the wild-type, Δ*luxS*$_{Hp}$ mutant and the complemented Δ*luxS*$_{Hp}^{+}$ 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. All

other results (FlaA and FlgE) were expressed relative to FlaB in each strain. Mutagenesis of $LuxS_{Hp}$ reduced the expression of FlaA relative to FlaB (from mean 1.60 in the wild-type to 1.23 in the Δ*luxS*$_{Hp}$ mutant, $p<0.01$), and complementation increased the ratio back to wild-type levels (mean 1.70 in the Δ*luxS*$_{Hp}^{+}$ mutant, $p<0.01$ compared with the Δ*luxS*$_{Hp}$ mutant). Next, FlgE expression was examined, and a similar trend was found (wild-type FlgE:FlaB ratio mean 0.74; Δ*luxS*$_{Hp}$ mutant 0.51; complemented Δ*luxS*$_{Hp}^{+}$ mutant 0.77; $p<0.01$ for differences between Δ*luxS*$_{Hp}$ mutant and wild-type and complemented stains). These data show that FlaA and FlgE syntheses were reduced relative to FlaB in the *luxS*$_{Hp}$ mutant and these changes were restored by genetical complementation.

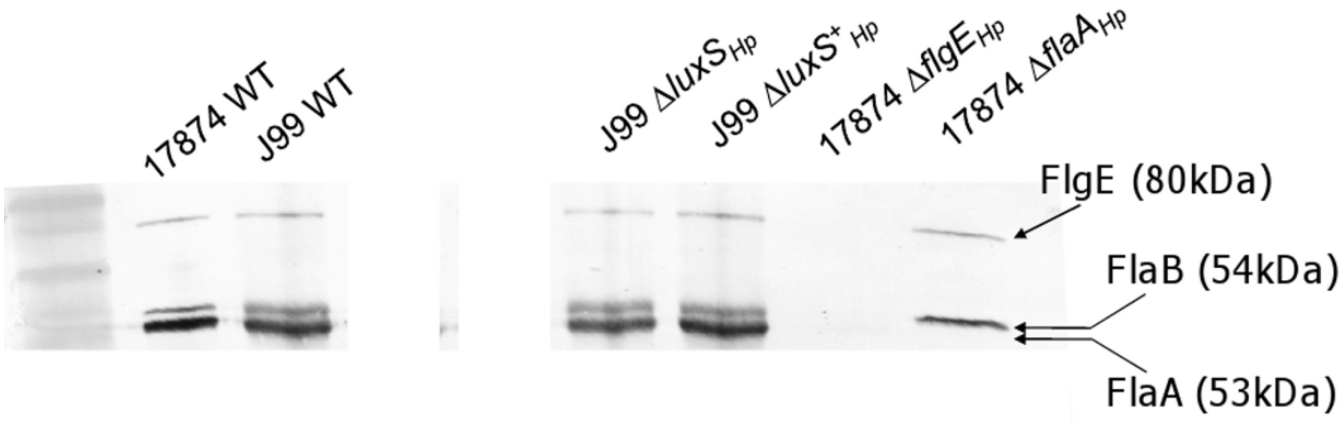


Figure 6.4 Mutation of *luxS* causes altered flagellin and hook protein production

Cell lysates of the strains indicated were subjected to immunoblotting with anti-flagellin (FlaA and FlaB) and anti-hook protein (FlgE) together. The proteins were measured in wild-type, Δ*luxS*$_{Hp}$, Δ*luxS*$_{Hp}^{+}$ cells grown in Brucella broth at 37°C for 24h. *H. pylori* strain 17874 wild-type served as the positive control. Mutants in *flaA* (a kind gift from Paul O'Toole) and *flgE* (O'Toole *et al*., 1994) derived from this strain (17874Δ*flaA* and 17874Δ*flgE*) served as negative controls for identifying FlaA and FlgE, respectively.

6.3.3 AI-2 regulates the transcription of flagellar genes

Previous reports have provided evidence that *luxS*$_{Hp}$-dependent QS may modulate motility via transcriptional regulation of *flaA* or *flhA* (Rader *et al*., 2007). Quantitative RT-PCR (qRT-PCR) was utilised to screen for alterations in transcription of these and other genes involved in flagellar assembly. This may help to extend our understanding of the regulatory mechanisms that might be involved. The same cultures were analysed as in the previous section so that direct comparisons between transcriptional and translational effects could be made in the presence and absence of DPD. To exclude an effect of cysteine biosynthesis, exogenous addition of cysteine was also undertaken. RNA samples were obtained from the same liquid cultures utilised in protein production analysis (see previous section). 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 6.5A).

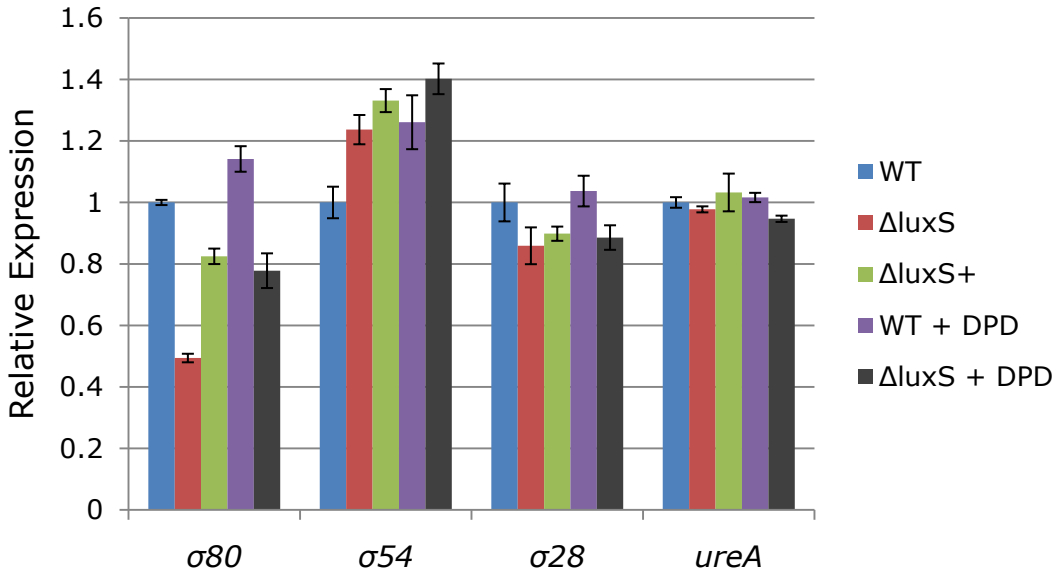
H. pylori wild-type, its Δ*luxS* mutant and the complemented Δ*luxS*$^{+}$ mutant were grown in Brucella broth at 37°C for up to 24 h, a condition in which a high level of AI-2 activity was detected (Figure 5.4). Cultures of wt + DPD and Δ*luxS* + DPD (DPD was added after 10 h of incubation and once again after 18 h of incubation) were also involved in this experiment. Interestingly, we did not find DPD of different concentrations (50μM, 150μM and 500μM) resulted in significant variation in bioluminescence (Figure 6.5E). *16S* RNA transcription was used for normalization. The gene *ureA* was used as a transcriptional gene control.

The flagellar genes tested included: three main sigma factors σ^{28} (intermediate class gene), σ^{54} (class 1 gene) and σ^{80}; class 1 flagellar genes *flhA* (encodes flagellar regulator component), *flgR* (encodes response regulator protein), *flgS* (encodes histidine kinase), *motA* and *motB* (encode flagellar motor proteins); class 2 genes *flaB* (encodes minor flagellin) and *flgE* (enodes flagellar hook protein); and a class 3 gene *flaA* (encodes major flagellin). The flagellar basal body gene *fliI* (encodes membrane-associated export ATPase), *flhF* (functionally equivalent to master regulators), and the anti-sigma factor *flgM* were also examined.

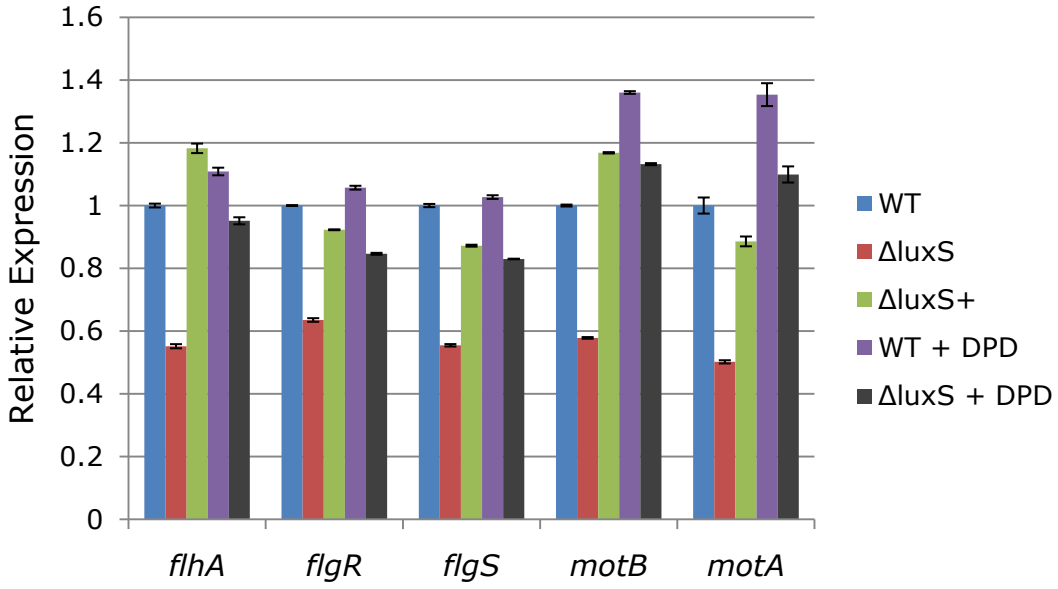
σ^{80} showed a consistent pattern of 1.8-2 fold (p<0.001) reduced transcription in the Δ*luxS*$_{Hp}$ mutant compared to the wild-type; while σ^{54} and σ^{28} in the Δ*luxS*$_{Hp}$ mutant were transcribed at close to wild-type levels (Figure 6.5A). For class 1 genes tested, *flhA*, *flgR* and *flgS* were 1.75 fold, 1.5 fold and 1.7 fold (p<0.001) respectively down-regulated in the Δ*luxS*$_{Hp}$ mutant. Both *motA* and *motB* were over 1.5 fold (p<0.001) down-regulated in the Δ*luxS* mutant compared to the wild-type (Figure 6.5B). For the class 2 genes tested, *flgE* was approximately 1.4 fold (p<0.001) down-regulated in the mutant; while *flaB* did not exhibit any significant change. *flaA* was the only class 3 gene tested in this study, and it was around 3.5-4 fold (p<0.001) down-regulated in the Δ*luxS*$_{Hp}$ mutant (Figure 6.5C). The intermediate class genes *flhF* and *flgM* were transcribed almost at the same level in both wild-type and the Δ*luxS*$_{Hp}$ mutant. The transcription of *fliI* was also significantly (p<0.001) reduced in the Δ*luxS*$_{Hp}$ mutant (Figure 6.5D).

The reduced transcription of σ^{80}, *flhA*, *flgR*, *flgS motA*, *motB*, *flgE*, *flaA* and *fliI* was restored genetically by the complementation of the mutant with the wild type *luxS*$_{Hp}$ gene. Also, 150μM DPD was sufficient to restore the transcription of these genes in

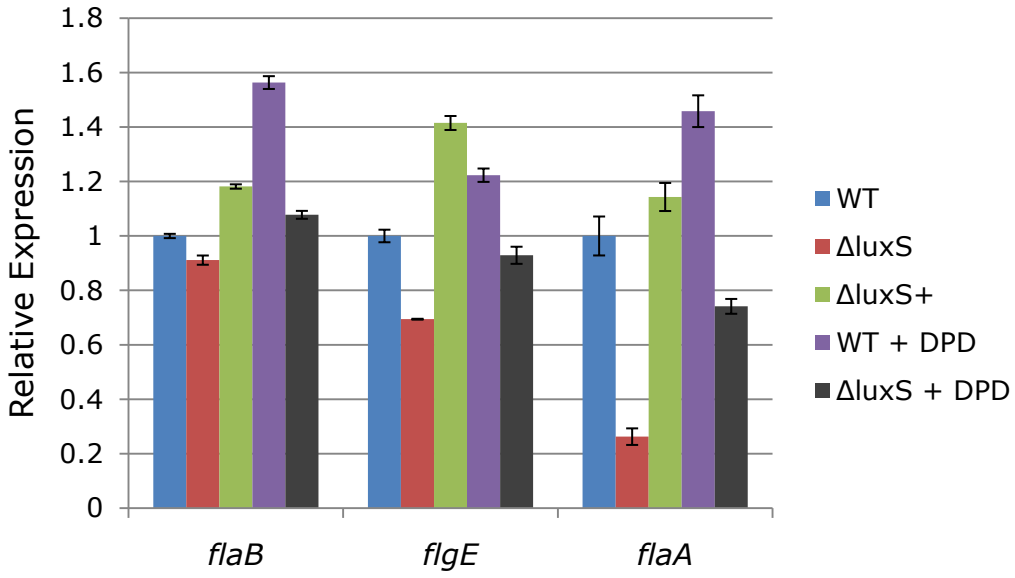
Δ*luxS*$_{Hp}$ cells to at or near the level of the wild-type. In wild-type cells, addition of DPD markedly increased transcription of *flhA*, *motA*, *motB* and *flaA*, whilst σ^{80}, *flgR*, *flgS*, *flgE* and *fliI* only showed a marginal increase. Exogenous addition of cysteine to the Δ*luxS*$_{Hp}$ 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.



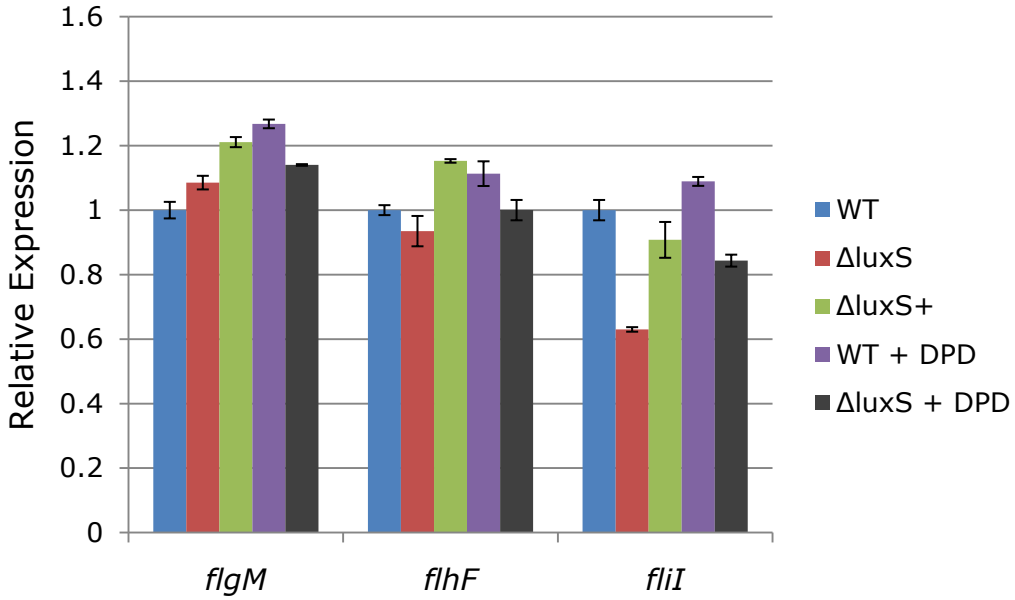
(A) The main flagellar regulator factors and *ureA* (non-flagellar gene control)



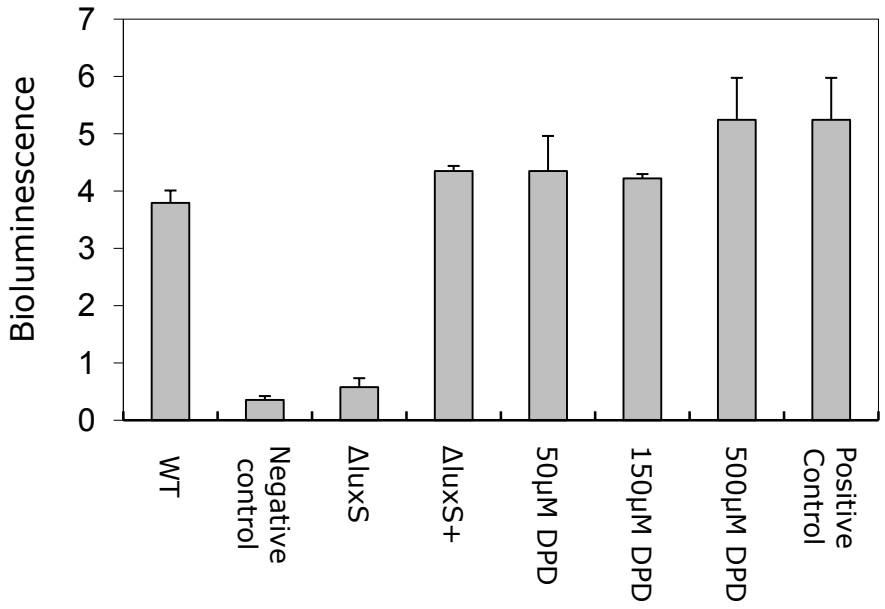
(B) Class 1 flagellar genes tested



(C) Class 2 and 3 flagellar genes tested



(D) The intermediate class genes tested and the basal body gene *fliI*



(E) AI-2 activity bioassay

Figure 6.5 *luxS*/DPD modulates *H. pylori* flagellar gene transcription

Transcript levels of (A) σ^{80}, σ^{54}, σ^{28}, *ureA*; (B) *flhA*, *flgR*, *flgS*, *motA*, *motB*; (C) *flaB*, *flgE*, *flaA*; (D) *flgM*, *flhF*, *fliI* were determined by qRT-PCR normalised to the levels of the *16S* rRNA gene. Relative expression of *ureA* was utilised as a gene control. The Y axis shows the relative transcriptional level of each gene in each strain normalised to the level of the

same gene in the strain control (which is J99 wild-type in every case). Values are mean activities of triplicate RNA samples of each strain. Transcript levels were measured in wild-type and Δ*luxS*$_{Hp}$ cultures grown with or without DPD and in Δ *luxS*$_{Hp}^{+}$ cultures grown without DPD (150μM). (E) AI-2 activity in DPD solution (at concentrations of 50, 150 or 500 μM) and in the cell free culture supernatants (24h) of *H. pylori* wild-type, Δ*luxS*$_{Hp}$ and Δ*luxS*$_{Hp}^{+}$ strains grown in the Brucella broth (corrected by OD_{600nm}). Negative control is Brucella broth alone. A diluted *in vitro* synthesised AI-2 sample was utilised as a qualitative positive control (Winzer *et al*., 2002a). Error bars indicate standard deviation.

6.4 Discussion

In this chapter, the processes underlying the loss of motility of the Δ*luxS*$_{Hp}$ 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.

Firstly, microscopic assay showed significantly reduced motility in the Δ*luxS*$_{Hp}$ mutant and this was completely restored by genetaically complementation and significantly restored by addition of DPD. EM pictures showed that deletion of *luxS*$_{Hp}$ reduced flagellar number and length and that this could be complemented by the *luxS*$_{Hp}$ gene and at least partially by DPD, but not by the provision of cysteine. Also it was noted that addition of DPD to the Δ *luxS*$_{Hp}^{+}$ complemented strains or wild-type promoted flagella formation at the second pole. In the experiments presented here, it was clear that the majority of Δ*luxS*$_{Hp}$ mutant cells had flagella. Although not involved in this study, image analysis can be used, for example the Hobson BacTracker (Morehouse *et al*, 2005) or video imaging and analysis with the ImageJ package (Murray & Kazmierczak, 2006, Collins, 2007). These methods would help to get a more quantitative measure of motility, including not only rotary movement of cells but also translational movement by directed swimming.

The smaller flagella observed in the mutant could result from the observed alteration of FlaA : FlaB ratio as previously described (Suerbaum *et al.*, 1993, Josenhans *et al.*, 1995). However, proving this would require extensive immuno-EM analysis with anti-FlaA and anti-FlaB antisera, which was mot provided due to time constraint. As *flaA* has been confirmed to be essential for motility in *H. pylori* while *flaB* is a structural subunit of filament which increases motility (Josenhans *et al.*, 1995,

Suerbaum *et al*., 1993), the change of the ratio between flagellins FlaA and FlaB may be one factor resulting in the abolished motility of the Δ*luxS* 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.

Loh *et al*. also found that $luxS_{Hp}$ regulates the growth-phase-dependent expression of *flaA*, but they did not find an obvious effect of AI-2 on *flaA* transcription (Loh *et al*., 2004). They explained that this might be since *H. pylori* lacks specific regulatory proteins needed for AI-2 signalling (e.g. LuxP, LuxU, LuxO) (Loh *et al*., 2004). To find out the proposed effects of $LuxS_{Hp}$/AI-2 on signalling, the transcription of some flagellar genes in cells of wild-type, Δ*luxS*, Δ$luxS^{+}$, wt + DPD and Δ*luxS* + DPD was compared using RT-PCR.

The transcriptional data of *flaA*, *flaB* and *flgE* are consistent with phenotypes of bacteria found by EM and immuno-blotting. 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 (σ^{28}, *flgS* or *flgM*) and where wild-type length flagella were reported for the Δ$luxS_{Hp}$ mutant alone (Rader *et al*., 2007). However, surprisingly in that report, the addition of DPD to the double mutants lengthened the flagellar filaments. This was reminiscent of what was found in the single Δ$luxS_{Hp}$ mutant of J99 where DPD addition or $luxS_{Hp}$ complementation restored flagellar length to the wild-type level.

The model of the flagellar regulon suggests that flagellar gene transcription of *H. pylori* is tightly regulated by three RNA polymerase sigma factors σ^{80}, σ^{54} and σ^{28} (Niehus 2004). σ^{80} controls the transcription of class 1 genes (early flagellar genes),

including FlhA (flagellar basal body components), FlgR (response regulator protein), FlgS (histidine kinase), MotA and MotB (flagellar motor proteins) etc.; σ^{54} controls class 2 genes (middle flagellar structural genes), assisted by the histidine kinase FlgS and the response regulator FlgR. The minor flagellin FlaB and hook protein FlgE are involved in this class. σ^{28} and FlgM regulate the transcription of class 3 genes (late flagellar structural genes) containing the major flagellin FlaA. Intermediate class genes (structural and regulatory genes) are generally controlled by all of σ^{80}, σ^{54} and σ^{28}. This class includes FlgM and FlhF (predicted to be flagellar basal body components). Both FlhA and FlhF (functionally equivalent to master regulators) are necessary for full transcription of classes 2, 3 and the intermediate class genes (Figure 6.6).

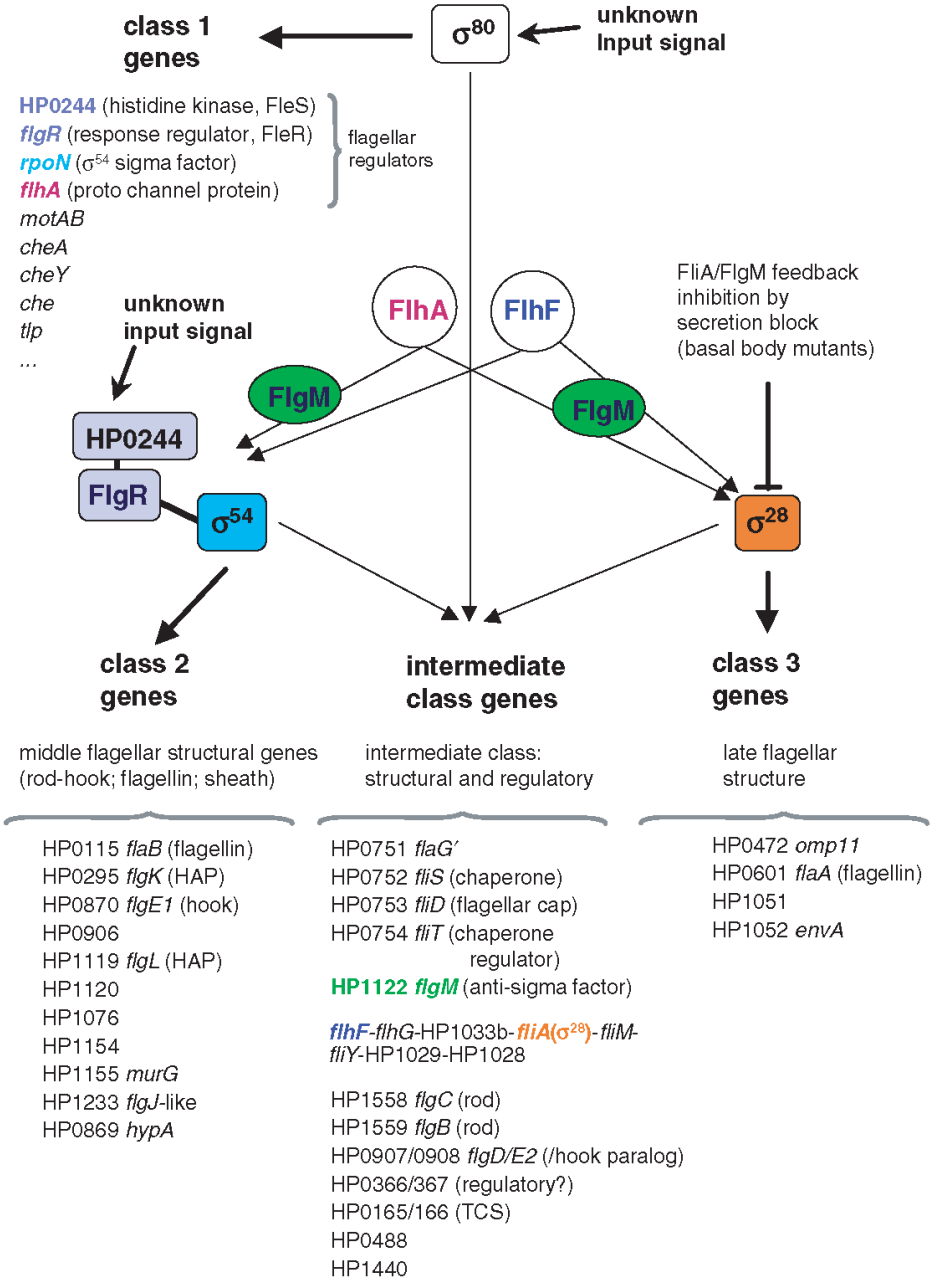


Figure 6.6 Current model of regulation in flagellar biosynthesis of *H. pylori*[***]

The different classes of flagellar genes are regulated by the housekeeping sigma factor σ^{80} (class 1) and the alternative σ^{54} (class 2) and σ^{28} (class 3). The intermediate genes controlled by more than one promoter are grouped in a separate category. The list of putative class 1 genes is not complete, because regulons have not been fully investigated. Transcription of class 2 genes is governed by σ^{54}, assisted by FlgS (or FleS) and FlgR. FlhA and FlhF are both necessary for full transcription of flagellar classes 2 and 3 and the intermediate class. FlgM plays a role in the transcriptional blockade of class 2 and 3 genes

[***] Figure 6.6 is from the paper Niehus *et al.*, 2004.

in *flhA* mutants, but only in the feedback block of class 3 genes in *flhF* mutants (Niehus *et al.*, 2004).

As an upper tier regulator gene in the model of the *H. pylori* flagellar regulon, reduced transcription of σ^{80} in the Δ*luxS*$_{Hp}$ mutant may be the factor leading to the lower expression of some of the class 1 genes including *flhA*, *flgR*, *flgS*, *motA* and *motB*. The reduced expression of flagellar motor genes (*motA* and *motB*) which control flagellar rotation could be one factor contributing to slower motility of the Δ*luxS*$_{Hp}$ mutant although it could also be caused by the lower flagellar number requiring fewer motor units to encircle each flagellar base.

Mutants defective in *flhA* were previously described as being defective in flagellar apparatus assembly and in motility. This is despite FlhA being a membrane bound protein, shown to be present at the base of the flagellum in bacteria such as *Salmonella* (Minamino & Macnab, 1999). Recently Rust and coworkers (2009) reported that the anti-sigma factor for σ^{28}, FlgM, interacts with FlhA at the base of the *Helicobacter* flagellum and this interaction modulates the expression of flagellar genes by σ^{28} (Rust *et al*., 2009). The decrease in *flhA* expression, seen in our Δ*luxS*$_{Hp}$ mutant could explain the change in flagellar length but not via an FlgM-dependent pathway as seen by Rader, as Rust and coworkers report that FlgM levels were wild-type in a Δ*flhA* mutant in *Helicobacter* strains N6 and 88-3887 (Motile variant of 26695) (Rust *et al*., 2009). Also, the data here showed that reduced transcription of *flhA* did not result in transcriptional reduction in σ^{28} and σ^{54}. This could be because σ^{28} and σ^{54} are not only regulated by *flhA* but also by another regulator gene such as *flhF* which was not affected by loss of *luxS*$_{Hp}$ in our experiments.

A previous study suggests that *flhA*, σ^{54}, *flgR* and *flhF* were required for the presence of flagella, motility and the expression of the flagellar proteins (Niehus *et al*., 2004). The data in this chapter showed that only *flhA* and *flgR* of these four genes, were down-regulated when *luxS*$_{Hp}$ was absent, and that although σ^{28} and σ^{54} were not affected by reduced expression of *flhA*, the class 2 gene *flgE* and the class 3 gene *flaA* were still significantly down-regulated in the Δ*luxS*$_{Hp}$ mutant. This might be due to the effect of FlgR/FlgS (reduced in the Δ*luxS*$_{Hp}$ mutant) in co-regulation of σ^{54}-dependent class 2 and intermediate class genes; and also could be due to the role of FlgM in the transcriptional blockade of class 2 and 3 genes when *flhA* is expressed at lower level or mutated (Figure 6.6).

Both Rust and coworkers (Rust *et al*., 2009) and Neihus and co-workers (Niehus *et al*., 2004) show that FlaB is not regulated by the same regulatory pathway as FlaA, and as FlaB levels in ourΔ *luxS*$_{Hp}$ mutant concur with this (Niehus *et al*., 2004), the short flagella observed in the Δ*luxS*$_{Hp}$ 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 *luxS*$_{Hp}$-complemented Δ*luxS*$_{Hp}^{+}$ bacteria or Δ *luxS*$_{Hp}$ bacteria+DPD which have longer flagella.

FlaB and FlgE are part of the same regulon that is controlled by the FlgS/FlgR two component system and the sigma factor σ^{54} (RpoN) (Niehus *et al*., 2004). Interestingly, though no significant change in FlaB was found, FlgE production as well as its gene expression was affected by loss of LuxS/AI-2. This suggests that *luxS* inactivation might differentially affect transcription of flagella genes with the same class. One possibility is that the FlgR/FlgS-σ^{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 *flhF*.

Flagellar assembly needs a type III secretion system which is dependent upon export chaperones that protect and transport structural subunits using the membrane-associated export ATPase, FliI (Jenks *et al.*, 1997, Lane *et al.*, 2006). Therefore, the decreased transcription of *fliI* might be another factor in blocking motility via shortened filament length in the Δ*luxS*$_{Hp}$ mutant, as *Helicobacter* Δ*fliI* mutants are non-motile and synthesise reduced amounts of flagellin (FlaA, FlaB) and hook protein (FlgE) subunits (Jenks *et al*., 1997).

In conclusion, the significantly reduced transcription of *flhA*, *flgR*, *flgS*, *motA*, *motB*, *flaA*, *flgE* and *fliI*, together with decreased FlaA and FlgE synthesis are likely to explain the motility defect in the Δ*luxS*$_{Hp}$ mutant which seems to be due to both fewer and shorter flagella. In the experiments here, the motility defect, down-regulated flagellar gene expression and reduced synthesis of flagellar proteins in the Δ*luxS*$_{Hp}$ mutant were due to loss of AI-2 only, and not to the metabolic effect of *luxS*$_{Hp}$ on biosynthesis of cysteine. These results suggest that LuxS/AI-2 is likely to be a functional signalling system contributing to control motility in *H. pylori*. However, it is still uncertain whether AI-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 AI-2 sensory and regulatory apparatus in *H. pylori* (Tomb *et al*., 1997, Rezzonico & Duffy, 2008). Also, we cannot exclude the possibility that AI-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.

Previously, Lee *et al*. and Osaki *et al*. looked at fitness of Δ*luxS*$_{Hp}$ mutants *in vivo* using mouse and gerbil models, respectively (Lee *et al*., 2006, Osaki *et al*., 2006). The authors did not favour a signalling explanation for the reduced fitness mechanisms but both speculated that it might be caused by metabolic disturbances upon loss of *luxS*$_{Hp}$ (Lee *et al*., 2006, Osaki *et al*., 2006). 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 QS system increasing motility. For example, it is speculated 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.

CHAPTER 7.
GENERAL DISCUSSION

7 General discussion

7.1 Conclusion

The evidence presented in this thesis suggests that the three-gene cluster *cysK*$_{Hp}$-*metB*$_{Hp}$-*luxS*$_{Hp}$ encodes the capacity to generate cysteine from products of the incomplete activated methyl cycle (AMC) of *H. pylori* in a process of reverse transsulphuration. In this pathway, *S*-ribosyl homocysteine (*S*RH) is converted by LuxS$_{Hp}$ to homocysteine (as in the classic AMC), and thence by CysK$_{Hp}$ to cystathionine and MetB$_{Hp}$ to cysteine. The misnamed genes *cysK*$_{Hp}$ and *metB*$_{Hp}$ are recommended to be renamed as *mccA* (methionine-to-cysteine-conversion gene A) and *mccB*, respectively.

As well as being a metabolic enzyme in the reverse transsulphuration pathway (RTSP), this work shows that the third gene of this gene cluster, LuxS$_{Hp}$, has an alternative role in regulation of motility by modulating flagellar transcripts and flagellar biosynthesis. This is through production of the signalling molecule AI-2, rather than the metabolic effect of LuxS in cysteine biosynthesis. These results provide preliminary evidence that AI-2 is also likely to be a functional signalling molecule in *H. pylori*. However, at this stage, data in this thesis have still not shown whether AI-2 is a true quorum sensing (QS) signal or whether LuxS/AI-2 is a real QS system employed by this bacterium.

7.2 Discussion

7.2.1 Comparison of LuxS in cysteine synthesis in *H. pylori* with other bacteria

Like *H. pylori*, several other bacterial species including *P. aeruginosa*, *M. tuberculosis* and *B. subtilis* are capable of cysteine biosynthesis through the reverse transsulphuration pathway (RTSP). However, in these bacteria, in contrast to *H. pylori*, the sulphate assimilatory cysteine biosynthesis pathway (SACBP) is strongly favoured (Vermeij & Kertesz, 1999, Wheeler *et al*., 2005, Hullo *et al*., 2007). Usually, the enzymes *S*-ribosylhomocysteinase (which is LuxS in *H. pylori)*, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL) are responsible for the generation of the intermediates homocysteine and cystathionine, and the final product cysteine, respectively, through the process of the reverse transsulpuration (Hullo *et al*., 2007, Doherty *et al*., 2010). *P. aeruginosa* is slightly different because a single enzyme *S*AH hydrolase is present instead of the Pfs-LuxS combination, converting *S*AH to homocysteine in a one step reaction without the concomitant production of DPD (or AI-2) (Duerre & Walker, 1977). In other bacteria also, in the absence of LuxS and Pfs, the alternative enzyme, *S*AH hydrolase, is often present. Sometimes, both LuxS/Pfs and *S*AH hydrolase can be absent, but there is only one example (*Bifidobacterium*) in which all three proteins (LuxS, Pfs and *S*AH hydrolase) are present (Vendeville *et al*., 2005). However, the analysis of the numerous *Bifidobacterium* genomes available to date (e.g. KEGG http://www.genome.jp/kegg/) suggests that *Bifidobacterium* does not appear to possess a RTSP. In *M. tuberculosis*, MetB is a special case due to its dual function acting as CBS and CGL in both directions of the reaction between cystathionine and cysteine, resulting in its dual role in both directions of the TSP and the RTSP (Wheeler *et al*., 2005). Like the *H. pylori* RTSP, the pathway in *B. subtilis* is completed by its enzymes LuxS, MccA

and MccB. The difference is that *B. sublitis* MccB, is also able to convert homocysteine to sulphide directly. Sulphide can be incorporated into cysteine through the SACBP, a process which is not present in *H. pylori* (Hullo *et al*., 2007).

7.2.2 Role of LuxS in metabolism and signalling between bacteria

Whether generated as a by-product in the activated methyl cycle (AMC) or the RTSP pathway, the function of AI-2 and the physiological role of LuxS remain matters of debate. In *S. aureus*, AI-2 has been thought to be a pure metabolic product without a QS effect (Doherty *et al*., 2006). In the EHEC *E. coli* O157:H7 Δ*luxS* mutant, the type III secretion and motility defects were restored genetically by *luxS* but failed to be restored by addition of synthetic AI-2 (Sperandio *et al*., 2003). In *S. Typhimurium*, *luxS* affects biofilm formation (De Keersmaecker *et al*., 2005) and flagellar phase variation (Karavolos *et al*., 2008), again implying a metabolic but not a signaling role. However, similar to EHEC *E. coli*, the two phenotypic changes seen in *S. Typhimurium* upon loss of *luxS* can be restored genetically but not by addition of synthetic AI-2 (Choi *et al*., 2007, De Keersmaecker *et al*., 2005). These findings indicate that the contributions of *luxS* to type III secretion and motility in EHEC *E. coli* and to biofilm formation and flagellar phase variation in *S. Typhimurium* might not be signal- or QS- dependent, further implying that phenotypes dependent on the gene *luxS* and AI-2 in some bacteria may be secondary to metabolic changes rather than QS (Karavolos *et al*., 2008).

Data presented in this thesis have suggested a likely function of AI-2 in a QS regulatory network in *H. pylori*. Nevertheless, no AI-2 sensory apparatus, for example, the LuxP/Q and the Lsr system has been identified in *H. pylori* (Rezzonico & Duffy, 2008). Therefore, like most species, $luxS_{Hp}$/AI-2 has a proven metabolic

role (Rezzonico & Duffy, 2008, Winzer *et al*., 2002a, Winzer *et al*., 2002b, Winzer *et al*., 2003). Although our work suggests it is likely to have a secondary role in QS signalling, this needs futher analyses as there is no definitive explanation for the mechanism to sense or respond to exogenous AI-2 in *H. pylori* cells.

In contrast, in some bacteria LuxS/AI-2 has an undoubted signalling role. For example, in the marine bacterium *V. harveyi*, in addition to LuxCDABE, AI-1 and AI-2 jointly regulate genes encoding a variety of putative functions including a secreted metalloprotease, three putative type III secretion system components and a conserved hypothetical protein (Surette *et al*., 1999). In *B. subtilis*, LuxS activity is required for biofilm formation, swarming motility and social and pluricellular behavior (Lombardia *et al*., 2006). In EHEC *E. coli* O157:H7, 404 genes were regulated by *luxS* at least 5 fold, which comprises approximately 10% of the array genes, mainly including genes involved in the type III secretion system, cell division, Stx expression, assembly of flagella, chemotaxis, motility and transport (Sperandio *et al*., 2001). In *S. Typhimurium*, the *luxS* gene is necessary for expression of genes in pathogenicity island 1 (Choi *et al*., 2007). To date, alterations in bacterial motility and biofilm formation seem to be the most commonly seen phenotypic changes upon inactivation of *luxS* or loss of AI-2. *H. pylori* appears similar. In addition, it is interesting and important to know if *H. pylori* AI-2 affects pathogenicity associated genes, for example, *vacA*, *dupA* and genes invoved in the *cag*-pathogenicity island. However, due to time constraints, work on putative AI-2 related effects on *H. pylori* pathogenicity was not pursued in this study.

Although the non-cysteine metabolism associated phenotypic changes observed on deletion of $luxS_{Hp}$ could be complemented genetically and also by addition of AI-2,

the exact mechanistic link between *luxS* mutation and altered phenotypes is still unclear. There are several possible explanations other than a signalling effect. One is the increased metabolic burden, which is caused by the inability of the strain to salvage *S*AH to homocysteine, allowing accumulation of the toxic intermediate *S*AH. The other possibility is that AI-2 may be used as a precursor for biosynthetic purposes not connected with signalling. In other systems, at least some of the observed changes are related to the metabolism of sulphur amino acids (Sperandio *et al*., 2005) and others as a response of the cell to the disruption of the AMC.

To date, there is still no explanation for why a signalling molecule AI-2 should be generated in the AMC, or the RTSP. Could this be because QS is actually controlled by the metabolic AMC or RTSP? The provision of AI-2 would be guaranteed through the central AMC (or the incomplete AMC in *H. pylori*) or RTSP as the pathway is significantly meaningful for bacterial survival and normal growth. The linkage of *luxS* to other genes (like *mccA* and *mccB* in *H. pylori*) involved in the AMC and cysteine synthesis suggests that LuxS has an original role in central metabolism, and further clues that relate to the function of AI-2 will probably be obtained from establishing the signal transduction/uptake pathways present in different bacteria.

7.2.3 Role of LuxS in Biology of *H. pylori* and other bacteria

QS systems mediated by AHL (AI-1) signalling molecules are typically species specific (Williams, 2007). In contrast, the LuxS/AI-2 systems of EHEC *E. coli*, EHPC *E. coli*, *S. Typhimurium*, and *H. pylori* do not appear to be species specific. For instance, AI-2 of these species is able to induce bioluminescence in *V. harveyi* (Surette *et al*., 1999, Loh *et al*., 2004). One possible role for such a nonspecific

bacterial QS system could be to detect total viable bacterial biomass as an indication of competition for nutritional resources. Alternatively, a bacterial signalling molecule may not regulate the gene expression of the same bacterium, but may act on other bacterial species. For example, some *S. aureus* strains produce signalling molecules that interfere with QS networks in other *S. aureus* strains (Ji *et al.*, 1997). *S. aureus* may also produce a signalling molecule identical to that produced by *Enterococcus faecalis* (Firth *et al.*, 1994, Muscholl-Silberhorn *et al.*, 1997). For *H. pylori*, up to now there is no evidence for the regulatory function of *H. pylori* AI-2 on other bacterial species (except for *V. harveyi*). However, cells of the gastric system itself may be an alternative target for signalling molecules of *H. pylori*. If gastric epithelial cells were responsive to *H. pylori* AI-2, this might represent a bacterial strategy for altering the gastric environment to allow persistent *H. pylori* colonization (Blaser & Kirschner, 1999, Williams *et al.*, 2000, Williams, 2007).

H. pylori is special in terms of its colonizing place within the gastrointestinal ecosystem. Almost the entire length of the gastrointestinal tract has plentiful and diverse microbial flora, while *H. pylori* is often the only species colonizing the gastric mucosa (Cover & Blaser, 2009). A number of bacteria are able to pass through the stomach, and many of them can colonize other regions of the alimentary channel. However, it is remarkable that *H. pylori* is almost the only species that successfully colonizes the gastric niche, although many other bacteria have robust acid tolerance mechanisms. This might help *H. pylori* to enter the gastric mucus layer and escape from the very acidic pH of the gastric environment (McGowan *et al.*, 1996). In addition, *H. pylori* produces chemicals that may prevent other bacterial species from colonizing (Freeman, 1999). It is thus tempting to speculate that the

capacity of *H. pylori* to produce and detect AI-2 may be an important factor for preventing competing bacterial growth in the gastric mucosa.

7.2.4 LuxS/AI-2 as a therapeutic target

A major goal of bacterial QS studies is the development of potential anti-QS agents, allowing the development of novel anti-microbial strategies. *H. pylori* treatment is complex and antibiotic resistance is necessary, so effective new strategies for *H. pylori* treatment are badly needed. Potential strategies include the use of molecules with a similar structure to auto-inducers which interfere with signalling (QS molecular antagonists or QS blockers), and development of auto-inducer degradation pathways. Due to its widespread distribution, AI-2 potentially offers the possibility to develop effective wide-ranging antimicrobials. Amazingly, both synthetic and natural molecules, as well as mechanisms to take up and degrade AI-2 have been reported to interfere with AI-2 signalling (Manefield *et al*., 1999, Manefield *et al*., 2001, Rasmussen *et al*., 2000, Givskov *et al*., 1996, Novak & Fratamico, 2004, Kim *et al*., 2008).

7.3 Future work

7.3.1 *In vitro* study on the *H. pylori* cysteine biosynthetic pathway

Experiments presented here have identified an *H. pylori* cysteine biosynthesis pathway through a process of reverse transsulphuration. The metabolic functions of the enzymes $LuxS_{Hp}$, $MccA_{Hp}$ and $MccB_{Hp}$ were identified based on a group of bioassays. To further analyse the activities of the key enzymes of this pathway would need replication of the *H. pylori* cysteine biosynthetic pathway *in vitro*. To do this, the enzymes $LuxS_{Hp}$, $MccA_{Hp}$ and $MccB_{Hp}$ should be cloned and expressed in an *E. coli* system. Similar to the experiment of *in vitro* AI-2 synthesis, the *S*AH product

could be used as the substrate in the putative *in vitro* RTSP. The appropriate quantity of $MccA_{Hp}$ and $MccB_{Hp}$ would be added in each step respectively to complete the cysteine *in vitro* synthesis.

7.3.2 Look carefully at the ratio of FlaA: FlaB

Osaki and co-workers have reported that low level infection by Δ*luxS* mutants is due to poorer colonization. In their study, virulence factors and adhesion appeared normal but flagella were inactive (Osaki *et al*., 2006). This could be due to the change of the ratio FlaA: FlaB. The levels of FlaA and FlgE in our experiments are reduced in the Δ*luxS* mutant based on the Western blot (Figure 6.4). With a quantitative scan of the bands, it is difficult to tell accurately if there is a difference in the ratio of FlaA to FlaB in the Δ*luxS* mutant compared to wild-type. More convincing experimental data could be obtained by purifying and analysing flagella that have been sheared from the cells. This would also distinguish between flagellar proteins that are exported and assembled into the flagellum versus subunits that are within the cytoplasm.

7.3.3 Can exogenous AI-2 addition induce transcriptional changes?

It seems that addition of AI-2 to the Δ*luxS* mutant gives a slightly different zone of motility than the wild-type has. The reason for this could be that the cells are simply growing differently; they might be stressed by the AI-2 molecule in some way (e.g. through an oxidative stress response). It is clear that the motility defect is not merely a straightforward lack of flagella. Whether it is in part due to motor dysfunction, different subunit assembly, or some sort of 'start-rotating' signal loss is unclear. Microarray, may give clues to homologues involved in other bacteria, but precise experiment using a candidate approach will likely be needed. Also, this project could be extended to check protein levels of all up-regulated and down-regulated genes.

7.3.4 If AI-2 is available during initial adhesion, can the Δ *luxS* mutant then cause a normal infection?

H. pylori possesses numerous adhesion molecules, which are important for colonization in animal models. Osaki *et al* (2006) in their study using Mongolian gerbils indicate that the decreased infectivity of the Δ*luxS* mutant strain was not due to decreased adhesion activity, but was due to lower motility (Osaki *et al*., 2006). Also, in gerbil stomachs, some of numerous other types of bacteria may possess the *luxS*/AI-2 system. Osaki and co-workers speculated that AI-2 produced by other bacteria affected the *H. pylori* Δ*luxS* mutant strain, thus allowing the mutant to survive in the gerbil stomach (Osaki *et al*., 2006). The question is, if AI-2 is available during initial adhesion, would the Δ$luxS_{Hp}$ mutant lead to a normal infection? This could be investigated by *in vivo* co-infection of wild-type and the Δ $luxS_{Hp}$ mutant. This work could be done by co-innoculating the Δ$luxS_{Hp}$ mutant with wild-type, killing wild-type with antibiotics (as the $luxS_{Hp}$ mutant contains an antibiotic resistance cassette) and seeing if the mutant can maintain infection at the same density.

7.4 Problems with experimental approaches in this thesis

Most of the key problems with experiments have been discussed in each chapter. However, there are still some specific problems as well as general problems with experimental approaches that have not been considered fully. These are now discussed below.

In Chapter 5, the flagella morphology and swimming patterns were examined using electron and phase-contrast microscopy. Previous studies have reported that the

number and length of flagella in the Δ*luxS*$_{Hp}$ mutant was no different compared to the wild-type (Rader *et al*., 2007, Osaki *et al*., 2006); whereas data presented here suggested that both the length of flagella filaments was shortened and the number of flagella was less in the Δ*luxS*$_{Hp}$ mutant. The contradictory results between this study and other studies may be due to the different strain used, so it would have been better if additional strains had been examined, as *H. pylori* is very genetically variable.

The regulatory role of LuxS on flagellar genes has been demonstrated in Chapter 6, and some flagellar genes have been analysed at the RNA level (Figure 6.5). Now more data are needed at the protein level, but this would be difficult or impossible to achieve at the moment as levels of proteins concerned would be too low to visualise without specific antisera, and these antisera are not currently available to us. Also in Chapter 6, data suggest the possible signalling function of AI-2, but it is difficult to entirely exclude the possibility that AI-2 is acting through a different mechanism affecting flagella synthesis (although the low concentrations used make it unlikely that this would be through acting as an energy source). As stated in Chapter 6, the machinery (if any) for sensing or importing AI-2 into *H. pylori* is unknown and it is hard to predict the abundance of protein families with low homology to LuxP/Q or Lsr of *Vibrio* and *Enterococcus* (Taga *et al*., 2001, Xavier & Bassler, 2005, Xavier *et al*., 2007). Therefore, currently it is not possible to clarify the mechanism whereby AI-2 affects flagella synthesis, or to tell if AI-2 acts on a central regulator of flagella synthesis.

Throughout this study, experiments of AI-2 (DPD) complementation were performed in several assays including plate motility bioassays, microscopic analyses and transcriptional tests. In these experiments, 150μM DPD was used because this

concentration was found to induce the same level of bioluminescence as the 24 h wild-type cell free supernatants (CFS) in the *V. harveyi* bioluminescence bioassay. However, although the concentration of DPD (150μM) has been assumed to be biological because it is appropriate to the *Vibrio* system, in truth we do not (and cannot) know whether it is truly relevant to the *H. pylori* system. Data presented suggest that it is the physiologically relevant concentration at least during *in vitro* growth of *H. pylori*. Determining *in vivo* AI-2 concentrations would be complicated by the many resident microbes that produce it.

Also in this study, a mutagenesis approach rather than the approach of looking at actual enzyme activity was used to investigate the physiological function of the three-gene cluster *mccA*$_{Hp}$-*mccB*$_{Hp}$-*luxS*$_{Hp}$. It may be worth further confirming the function of each of the enzymes MccA$_{Hp}$, MccB$_{Hp}$ and LuxS$_{Hp}$ by constructing the RTSP *in vitro* (also see Chapter 7.3.1).

Finally, due to time constraints, no *in vivo* study using animal models was performed in this work. Although the primary metabolic function of the three-gene cluster *mccA*$_{Hp}$-*mccB*$_{Hp}$-*luxS*$_{Hp}$ and the possible secondary role of LuxS$_{Hp}$ in contributing to control motility by signalling have been investigated, it is still not clear how the two effects (metabolic and signalling) of LuxS$_{Hp}$ would affect each other, and how the three-gene cluster of *H. pylori* would function in the host. This could be investigated using Mongolian gerbils by *in vivo* infection of each strain individually, or by *in vivo* co-infection of wild-type and each of the mutants (the wild-type could then be eliminated using antibiotics). Once the initial colonization of each strain was established, the role of related *H. pylori* genes in the host could be studied.

CHAPTER 8. REFERENCES

8 References

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APPENDIX

APPENDIX-I

Neil C. Doherty*, Feifei Shen*, Nigel M. Halliday, David A. Barrett, Kim R. Hardie, Klaus Winzer, and John C. Atherton. **In *Helicobacter pylori,* LuxS is a key enzyme in cysteine provision through a reverse transsulphuration pathway.** *Joint first author. *Journal of Bacteriology* 192: 1184-1192. (2010).

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In *Helicobacter pylori*, LuxS Is a Key Enzyme in Cysteine Provision through a Reverse Transsulfuration Pathway▽

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In many bacteria, LuxS functions as a quorum-sensing molecule synthase. However, it also has a second, more central metabolic function in the activated methyl cycle (AMC), which generates the *S*-adenosylmethionine required by methyltransferases and recycles the product via methionine. *Helicobacter pylori* lacks an enzyme catalyzing homocysteine-to-methionine conversion, rendering the AMC incomplete and thus making any metabolic role of *H. pylori* LuxS ($LuxS_{Hp}$) unclear. Interestingly, $luxS_{Hp}$ is located next to genes annotated as $cysK_{Hp}$ and $metB_{Hp}$, involved in other bacteria in cysteine and methionine metabolism. We showed that isogenic strains carrying mutations in $luxS_{Hp}$, $cysK_{Hp}$, and $metB_{Hp}$ could not grow without added cysteine (whereas the wild type could), suggesting roles in cysteine synthesis. Growth of the Δ$luxS_{Hp}$ mutant was restored by homocysteine or cystathionine and growth of the Δ$cysK_{Hp}$ mutant by cystathionine only. The Δ$metB_{Hp}$ mutant had an absolute requirement for cysteine. Metabolite analyses showed that *S*-ribosylhomocysteine accumulated in the Δ$luxS_{Hp}$ mutant, homocysteine in the Δ$cysK_{Hp}$ mutant, and cystathionine in the Δ$metB_{Hp}$ mutant. This suggests that *S*-ribosylhomocysteine is converted by $LuxS_{Hp}$ to homocysteine (as in the classic AMC) and thence by $CysK_{Hp}$ to cystathionine and by $MetB_{Hp}$ to cysteine. *In silico* analysis suggested that *cysK-metB-luxS* were acquired by *H. pylori* from a Gram-positive source. We conclude that *cysK-metB-luxS* encode the capacity to generate cysteine from products of the incomplete AMC of *H. pylori* in a process of reverse transsulfuration. We recommend that the misnamed genes $cysK_{Hp}$ and $metB_{Hp}$ be renamed *mccA* (methionine-to-cysteine-conversion gene A) and *mccB*, respectively.

Helicobacter pylori is a Gram-negative bacterium which causes peptic ulceration, gastric adenocarcinoma, and gastric lymphoma (2). All *H. pylori* strains possess a homologue of *luxS*, best known as a quorum-sensing molecule synthase. *luxS* homologues have been found in around half of all bacterial genomes sequenced to date (38, 42, 44). Some of the molecules formed in the reaction catalyzed by LuxS are collectively termed autoinducer 2 (AI-2) and have been shown to act as signaling molecules. They have been described as mediating a variety of effects in different bacteria, including on virulence. In *H. pylori*, disruption of *luxS* has been shown to increase biofilm formation (6) and to reduce *in vivo* fitness and infectivity (20, 29). Several motility-associated $luxS_{Hp}$ phenotypes have also been reported, including loss of growth phase-dependent *flaA* regulation (24), reduced motility on soft agar plates, and reduced transcription of the flagellar regulator *flhA* (29, 30).

In most bacteria, the enzyme encoded by *luxS* has another (possibly primary and sometimes sole) function: it is an integral metabolic component of the activated methyl cycle (AMC). The AMC is a key metabolic pathway that generates *S*-adenosylmethionine (*S*AM) as an intermediate product (the full cycle is shown in Fig. 1). *S*AM bears a methyl group with a relatively high transfer potential and is used by numerous methyltransferases to carry out cellular processes, including nucleic acid and protein methylation and detoxification of reactive metabolites (11). The product of the methyltransferase reaction is *S*-adenosylhomocysteine (*S*AH), and in the complete AMC, *S*AM is regenerated from *S*AH via homocysteine and methionine, ready for another round of methylation/transmethylation (35, 44, 47, 49). The role of LuxS in the AMC is to catalyze cleavage of *S*-ribosylhomocysteine (*S*RH) to yield homocysteine and a by-product, 4,5-dihydroxy-2,3-pentanedione (DPD) (Fig. 1) (35, 44, 47, 49). DPD is the precursor of the family of related, interconverting molecules collectively termed "AI-2" (5, 44).

H. pylori is unusual among bacteria in not having a complete AMC. The final step in the classical AMC, the conversion of homocysteine to methionine can be carried out by either of two N^5-methyltetrahydrofolate-dependent methyltransferases, MetE or MetH. However, all *H. pylori* genomes studied to date lack *metE* and *metH* homologues (4). This raises the question of

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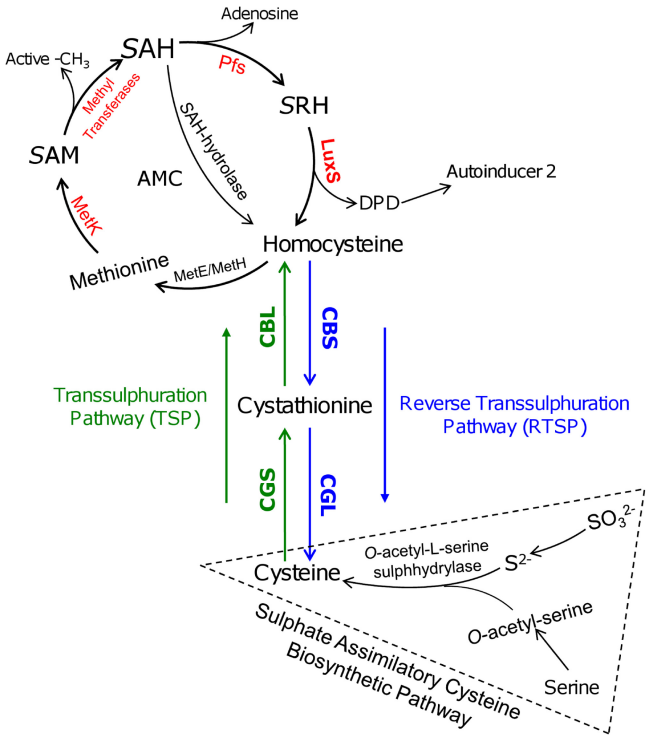

FIG. 1. Bacterial methionine and cysteine interconversion pathways. The activated methyl cycle (AMC) regenerates the active methyl donor, *S*AM, from the toxic methyl transferase product, *S*AH. *S*AH is metabolized in one of two ways. Many bacteria, including *H. pylori*, generate homocysteine from *S*AH in a two-step manner: a nucleosidase, Pfs, converts *S*AH to *S*RH, and then LuxS converts this to homocysteine and DPD. The alternative route produces homocysteine directly from *S*AH in a one-step reaction requiring *S*AH hydrolase (11, 47). Many bacteria synthesize cysteine through the sulfate assimilatory cysteine biosynthetic pathway, which uses inorganic sulfur as a substrate (pathway bounded by a dashed triangle). Enzymes with previously described homologues in *H. pylori* are shown in red. Enzymes that do not have homologues in *H. pylori* are shown in black. The reactions of the transsulfuration and reverse transsulfuration pathways, which are a major focus of this paper, are shown in green and blue, respectively. We show in this paper that $CysK_{Hp}$ is the candidate cystathionine β-synthase and $MetB_{Hp}$ the candidate cystathionine γ-lyase in the reverse transsulfuration pathway. We suggest that these genes be renamed *mccA* (methionine-to-cysteine-conversion gene A) and *mccB*, respectively. CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; CGS, cystathionine γ-synthase; DPD, 4,5-dihydroxy-2,3-pentanedione; LuxS, *S*-ribosylhomocysteinase/autoinducer 2 synthase; MetE/MetH, methionine synthase; MetK, *S*-adenosylmethionine synthase; Pfs, 5′-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase; *S*AM, *S*-adenosylmethionine; *S*AH, *S*-adenosylhomocysteine; *S*RH, *S*-ribosylhomocysteine.

whether the only function of LuxS in *H. pylori* is as a quorum-sensing molecule synthase or whether LuxS in *H. pylori* has another, as yet undescribed, metabolic role.

In searching for a possible metabolic role for $LuxS_{Hp}$, we formed the hypothesis that it is part of a *de novo* cysteine biosynthesis pathway that uses methionine as a reduced sulfur source. Our reasoning for this was based on three observations. First, *H. pylori* has an absolute requirement for methionine (26, 27, 31), in agreement with its lack of *metE* and *metH*. Second, *H. pylori* can synthesize cysteine without apparently making use of oxidized sulfur compounds, such as sulfate: genomic studies show that homologues to the genes which encode the components required for uptake of sulfate and conversion to sulfide are absent (10). Third, all complete *H. pylori* genomes available to date contain homologues of two genes, annotated as *cysK* and *metB*, located immediately upstream of *luxS*. In other bacteria, these are involved in the generation of cysteine and interconversion of cysteine and methionine, respectively. Recently the *metB* gene from *H. pylori* strain SS1 was purified and characterized, but its physiological role was not established (18).

When we pondered the fate of homocysteine, which is not converted to methionine in *H. pylori* due to a lack of MetE and MetH, coupled with the observation that the gene responsible for its production, *luxS*, is linked with two other genes predicted to be involved in methionine and cysteine metabolism, we hypothesized that *H. pylori* is able to utilize homocysteine by converting it to cysteine via the reverse transsulfuration pathway (RTSP), involving the associated genes *metB* and *cysK*. We thus set out to address this hypothesis and more specifically to define the roles of the *cysK*, *metB*, and *luxS* homologues in the putative *H. pylori* RTSP.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains DH5α and DS941 (37, 51) were used in cloning/subcloning experiments. *E. coli* was routinely propagated in Luria-Bertani (LB) broth or on LB agar plates at 37°C under normal atmospheric conditions. *Vibrio harveyi* was grown in either LB or AB medium (12) at 30°C, also under normal atmospheric conditions. *H. pylori* strains were routinely passaged on horse blood agar (Oxoid) every 2 to 3 days and were grown in a MACS VA500 microaerobic workstation (Don Whitley Scientific) using a humidified atmosphere consisting of 6% O_2, 3% H_2, 5% CO_2, and 86% N_2. Antibiotic selection was carried out at 100 μg ml^{-1} with ampicillin (or carbenicillin) and 50 μg ml^{-1} for kanamycin.

For *H. pylori* metabolic supplementation experiments, the complete chemically defined medium (cCDM) of Reynolds and Penn was formulated as previously described (31), with variations as indicated in the text. The concentrations of methionine and cysteine used were 505 μM and 1.0 mM, respectively. For the variations of CDM lacking cysteine (see Results for description of use), different chemicals were added: homocysteine (at 1.0 mM), cystathionine (at 1.0 mM), or potassium sulfide (a range of concentrations up to 1.0 mM were utilized). All chemicals were of high purity (cell culture tested) and were obtained from Sigma-Aldrich Co. CDM batch culture comparative growth studies were carried out as described previously (9).

Measurement of AI-2 activity. AI-2 measurements were performed as described previously (3, 47). Twenty-four-hour culture supernatant samples, corresponding to mid-late log phase, were tested. AI-2 activity was expressed as fold change in induced bioluminescence from the reporter strain compared with that for the negative control. Negative control samples consisted of media which had not been inoculated but otherwise were treated as test cultures. Positive controls were carried out using *in vitro*-synthesized AI-2, prepared as described previously (8).

DNA manipulation. Recombinant DNA techniques were carried out using standard methods (34). PCR amplifications were performed using either standard DNA polymerase (GoTaq; Promega, Madison, WI) or proofreading DNA polymerase (Novagen KOD; Merck Chemicals Ltd., United Kingdom). Restriction digests were performed in accordance with the manufacturer's recommendations (Promega, Madison, WI). *E. coli* genomic and plasmid DNA was isolated using the Qiagen range of preparatory kits (Qiagen, United Kingdom). *H. pylori* genomic DNA was purified as described previously (22). DNA sequencing was conducted using standard fluorescent dye terminator chemistries, and analysis was performed using the Applied Biosystems 3730 DNA analyzer system (Geneservice, Cambridge, United Kingdom, and Applied Biosystems Inc., Foster City, CA.). Results were analyzed using the BioEdit software suite (13).

Mutagenesis of $luxS_{Hp}$, $metB_{Hp}$, and $cysK_{Hp}$. All mutants were made following an insertion-deletion strategy. The *H. pylori* Δ*luxS* mutant strains were made by

TABLE 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/notes	Source/reference
Strains		
Escherichia coli		
DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *relA1 supE44* Δ(*lacZYA-argF*) *U169 F*$^-$ ϕ80d*lacZ*ΔM15 *deoA phoA* λ$^-$	51
DS941	*thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44 rec*$^+$ *recF lacI*^{q} *lacZ* ΔM15	37
Vibrio harveyi		
BB170	*luxN*::Tn*5*; AI-1 sensor negative; AI-2 sensor positive	39
Helicobacter pylori		
J99 (ATCC 700824)	Wild-type strain	1
J99Δ*luxS*	J99; Δ*luxS*::Km^r	This study
J99Δ*metB*	J99; Δ*metB*::Km^r	This study
J99Δ*cysK*	J99; Δ*cysK*::Km^r	This study
NCTC 11637 (ATCC 43504)	Wild-type strain	25
11637Δ*luxS*	11637; Δ*luxS*::Km^r	This study
11637Δ*metB*	11637; Δ*metB*::Km^r	This study
11637Δ*cysK*	11637; Δ*cysK*::Km^r	This study
Plasmids		
pGEM-T	Commercial TA cloning vector; Ap^r	Promega, Madison, WI
pMWA2	Source of apolar *aphA3* cassette; Ap^r Km^r	46
pLUXS	pGEM-T::*luxS*::*aphA*3; Km^r	Theo Verboom
pMETB	pGEM-T bearing the $metB_{Hp}$ gene; Ap^r	This study
pCYSK	pGEM-T bearing the $cysK_{Hp}$ gene; Ap^r	This study
pMF1	pMETB deletion-insertion derivative; Ap^r Km^r	This study
pCF2	pCYSK deletion-insertion derivative; Ap^r Km^r	This study
pProEx-$luxS_{EC}$	pProEX HT containing *luxS* gene of *E. coli* MG1655	47
pProEx HT mtan	pProEX HT containing *pfs* gene of *E. coli*	47

transforming J99 and NCTC11637 with pGEMT::*luxS*::*aphA*3 (a generous gift from Leo Smeets and Theo Verboom).

The *cysK* and *metB* loci were mutagenized as follows. Fragments containing the open reading frames (ORFs) and approximately 400 to 500 bp on either side were amplified from J99 genomic DNA using the following primer pairs: *cysK* (forward, 5′-CACCATTGACAAATCCTTCC-3′; reverse, 5′-TTTGGTGTTGG GCTTGATAG-3′) and *metB* (forward, 5′-CCTGATAATCCCGCAGCCTACT A-3′; reverse, 5′-ACCCCCACTTCAGACCACTCAG-3′). These products were then cloned into pGEM-T Easy (Promega, Madison, WI). Inverse PCR (iPCR) products were generated from these using the following primer pairs, which were designed so that resulting clones would contain a deletion of a large part of the open reading frame of each target gene, preventing gain of function recombination/excision events: *metB* (5′ iPCR, 5′-CGTGAATTCCGGCTAAACCAG-3′ [EcoRI site underlined]; 3′ iPCR, 5′-TGAACAGGATCCGTTAGAAGATT-3′ [BamHI site underlined]) and *cysK* (5′iPCR, 5′-GCTGTTTTTCTGTGCTGAA TTCTT-3′ [EcoRI site underlined]; 3′iPCR, 5′-GTGGATCCGAGGGTTCTA TTTTGA-3′ [BamHI site underlined]). These were prepared for cloning by restriction digestion and purified. The apolar *aphA3* cassette, conferring kanamycin resistance, was isolated from pMWA2 (46) by restriction digestion with EcoRI/BamHI and ligated into the prepared iPCR products. Positive clones were selected on the basis of kanamycin resistance and confirmed by PCR and DNA sequencing. The resultant vector constructs are nonreplicative in *H. pylori* and were used as suicide vectors to transform J99 and NCTC11637.

Bacterial transformations. *E. coli* was transformed with plasmid DNA by electroporation using a Bio-Rad Gene Pulser with pulse controller at a voltage of 1.8 kV, with a resistance of 200 Ω at 25-μF capacitance. *H. pylori* was transformed by natural transformation using established protocols (22). Recombinant *H. pylori* strains were recovered by selection on Columbia agar base containing kanamycin, egg yolk emulsion (5% [vol/vol]), and triphenyl tetrazolium chloride at a working concentration of 40 μg ml^{-1}. Inclusion of this indicator made it easier to see the small recombinant colonies.

Sample preparation and relative quantification of candidate metabolites. The following protocol was developed for sampling *H. pylori* cultures prior to metabolite analysis. Strains were grown in complete CDM for 24 h. All subsequent manipulations were performed on ice or in chilled equipment, and all plastic ware was prechilled prior to use. Three ml of culture at an optical density (OD) at 600 nm (OD_{600}) of 1.0 was taken as a standard sampling volume (variances from this OD were corrected for by using larger or smaller volumes). This was quenched in 5 ml ice-cold phosphate-buffered saline (PBS). Cells were recovered by centrifugation and washed with a further 5 ml ice-cold PBS. The cells were again recovered by centrifugation, and cell pellets were stored at −80°C. Metabolite analysis was performed as described previously (15, 36).

Phylogenetic analyses. Primary amino acid sequences were aligned using the ClustalW algorithm using BioEdit (13). Accession numbers of individual protein sequences used are as follows: *H. pylori*, LuxS, NP222818; MetB, NP222819; CysK, NP222820; RecA, NP222862; *Helicobacter acinonychis*, LuxS, YP665186; MetB, YP665187; CysK, YP665188; RecA, YP664174; *Helicobacter hepaticus*, LuxS, NP859707; MetB, NP859593; CysK, NP860380; RecA, NP860164; *Helicobacter bilis*, LuxS, ZP04580164; MetB, ZP04581342; CysK, ZP04581369; RecA, ZP04580683; *Helicobacter canadensis*, LuxS, ZP03656033; MetB, ZP03656080; CysK, ZP03656337; RecA, ZP03655680; *Helicobacter pullorum*, LuxS, ZP04808703; MetB, ZP03661228; CysK, ZP03660206; RecA, ZP04808545; *Helicobacter winghamensis*, LuxS, ZP04583537; MetB, ZP04583291; CysK, ZP04582871; RecA, ZP04583642; *Helicobacter cinadei*, LuxS, ZP03657497; MetB, ZP03658434; CysK, ZP03658433;RecA, ZP03659220; *Wolinella succinogenes*, LuxS, NP908224; MetB, NP907217; CysK, NP907372; RecA, NP907645; *Campylobacter jejuni*, LuxS, YP002344589; MetB, ZP01699839; CysK, YP002344310; RecA, YP179815; *Enterococcus faecium*, LuxS, ZP00604876; MetB, ZP00604875; CysK, ZP00604874; RecA, ZP00603401; *Staphylococcus aureus*, LuxS, BAB43220; MetB, NP370984; CysK, BAB56621; RecA, NP371809; *E. coli*, LuxS, NP417172; MetB, NP418374; CysK, NP416909; RecA, YP853918; and *Bacillus subtilis*, LuxS, NP390945; MccB, NP390603; MccA, NP390604; and RecA, ZP03591416.

These alignments were used to derive phylogenies based on neighbor-joining methods using the Mega4 suite of programs (40). Bootstrap values were generated based on 1.000 replicates of each calculation (consensus bootstrap trees had identical topologies).

RESULTS

Nonpolar mutation of $metB_{Hp}$, $cysK_{Hp}$, and $luxS_{Hp}$. To determine the functions of the putative homologues of MetB, CysK, and LuxS in *H. pylori* ($MetB_{Hp}$, $CysK_{Hp}$, and $LuxS_{Hp}$), we first constructed a panel of mutant strains deficient in each

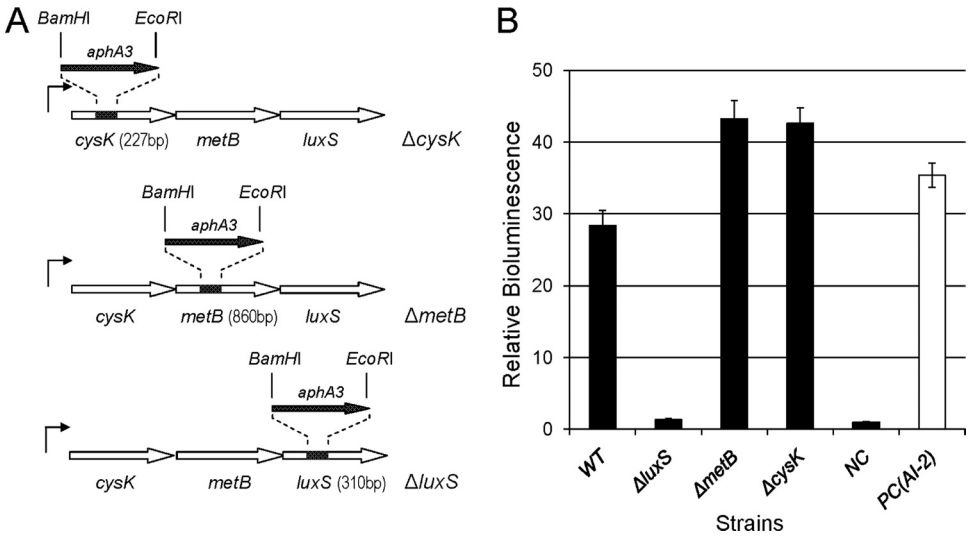

FIG. 2. Mutagenesis strategy and effect of $cysK_{Hp}$ and $metB_{Hp}$ disruption on autoinducer 2 (AI-2) levels. (A) Schematic representation of the $cysK_{Hp}$-$metB_{Hp}$-$luxS_{Hp}$ gene cluster and approximate locations of individual *aphA3* (kanamycin resistance) cassette insertions, indicated in black. The sequence lengths indicated in brackets are the deletion sizes in each gene. A putative σ^{70} promoter lying upstream of $cysK_{Hp}$ was identified using promoter prediction algorithms found at http://www.softberry.ru/berry.phtml and http://www.fruitfly.org/seq_tools/promoter.html and is shown as an arrow. Analysis of the predicted stem-loops found in the 3′ untranslated regions of each of the three genes indicates the presence of a number of putative rho-independent terminator sequences downstream of the $luxS_{Hp}$ ORF but not in those of either $cysK_{Hp}$ or $metB_{Hp}$. (B) Cell supernatants of wild-type *H. pylori* strain 11637 (WT) and Δ*cysK*, Δ*metB*, and Δ*luxS* mutants (grown for 24 h in Brucella broth) were examined for the ability to induce bioluminescence in the *V. harveyi* reporter strain BB170. The AI-2 activity shown is the ratio of relative bioluminescence (corrected by the OD_{600} of *H. pylori* growth) induced by the 1/10-diluted *H. pylori* cell culture supernatants over the negative control (NC), which consisted of broth alone. A diluted *in vitro*-synthesized AI-2 sample was utilized as a qualitative positive control (PC) (8, 47). Bioluminescence induced by WT, Δ$metB_{Hp}$, and Δ$cysK_{Hp}$ strains was significantly greater than that induced by the Δ$luxS_{Hp}$ mutant, as determined by paired Student's *t* test ($P < 0.001$). Error bars indicate the standard deviations within triplicate samples.

one of these gene products in two different *H. pylori* strain backgrounds (J99 and 11637). Each mutant was created using a deletion/insertion approach, using the *aphA3* cassette (kanamycin resistance) as a selectable marker (19). The general approach to this mutagenesis strategy is shown in Fig. 2A, and details are described in Materials and Methods. Mutants were confirmed on the basis of PCR and DNA sequencing.

To avoid polar effects of disrupting either $cysK_{Hp}$ or $metB_{Hp}$, we used a variant of *aphA3* which lacks both the promoter and terminator elements and which we have used successfully in previous studies (46). Since *luxS* comprises the last of the genes in this operon, we were able to use the *V. harveyi* AI-2 assay (3, 47) to demonstrate that disruption of either $cysK_{Hp}$ or $metB_{Hp}$ did not block expression of downstream genes (Fig. 2B). As predicted, insertion of *aphA3* into the *luxS* gene abrogated AI-2 production completely (Fig. 2B).

$LuxS_{Hp}$, $CysK_{Hp}$, and $MetB_{Hp}$ are required for *de novo* cysteine biosynthesis in *H. pylori*. In order to determine the effects of mutagenesis of $cysK_{Hp}$, $metB_{Hp}$, and $luxS_{Hp}$, we composed various chemically defined media (CDM) based on the complete CDM (cCDM) of Reynolds and Penn (31). This is a versatile system which allows precise control of the composition of the medium. cCDM contained both of the sulfur-containing amino acids, methionine and cysteine. We also made 4 variants of this medium, all of which lacked cysteine. One had no additional source of sulfur (we refer to this hereinafter as CDM), while the others contained either homocysteine (CDM+HC), cystathionine (CDM+CTT), or potassium sulfide (CDM+S) (see Materials and Methods for concentrations). Using these media with the wild type and the Δ$cysK_{Hp}$, Δ$metB_{Hp}$, or Δ$luxS_{Hp}$ mutants of both *H. pylori* strain 11637 and J99, we found that all strains grew well in cCDM (Fig. 3A to D for 11637l; J99 not shown). Upon omission of cysteine (CDM), the wild-type strain still grew, although less well than in the complete medium (Fig. 3A). In contrast, all three mutants were auxotrophic for cysteine, with no growth detected (Fig. 3B to D). These observations confirmed that wild-type *H. pylori* is able to grow in the absence of cysteine and so possesses a cysteine *de novo* biosynthesis pathway. They also revealed that $cysK_{Hp}$, $metB_{Hp}$, and $luxS_{Hp}$ are all part of this pathway.

Metabolic complementation of growth defects demonstrates steps in the cysteine biosynthesis pathway catalyzed by $LuxS_{Hp}$, $CysK_{Hp}$, and $MetB_{Hp}$. We next examined which steps in our proposed *H. pylori* reverse transsulfuration pathway (RTSP) (Fig. 1) were catalyzed by $LuxS_{Hp}$, $CysK_{Hp}$, and $MetB_{Hp}$. These experiments gave the same results for mutants constructed in the 11637 and J99 backgrounds; the 11637 results are presented in Fig. 3B to D.

For the Δ$luxS_{Hp}$ mutant, adding homocysteine to the CDM lacking cysteine improved growth of the Δ$luxS_{Hp}$ strain, and CTT restored growth to levels seen with cCDM (CDM with cysteine) (Fig. 3B). This implies that, as expected, $LuxS_{Hp}$ is required for the conversion of *S*RH to homocysteine.

The Δ$cysK_{Hp}$ mutant grew in cCDM (containing cysteine) and in CDM lacking cysteine but containing CTT (CDM+CTT) but not in CDM lacking cysteine (CDM) or CDM lacking

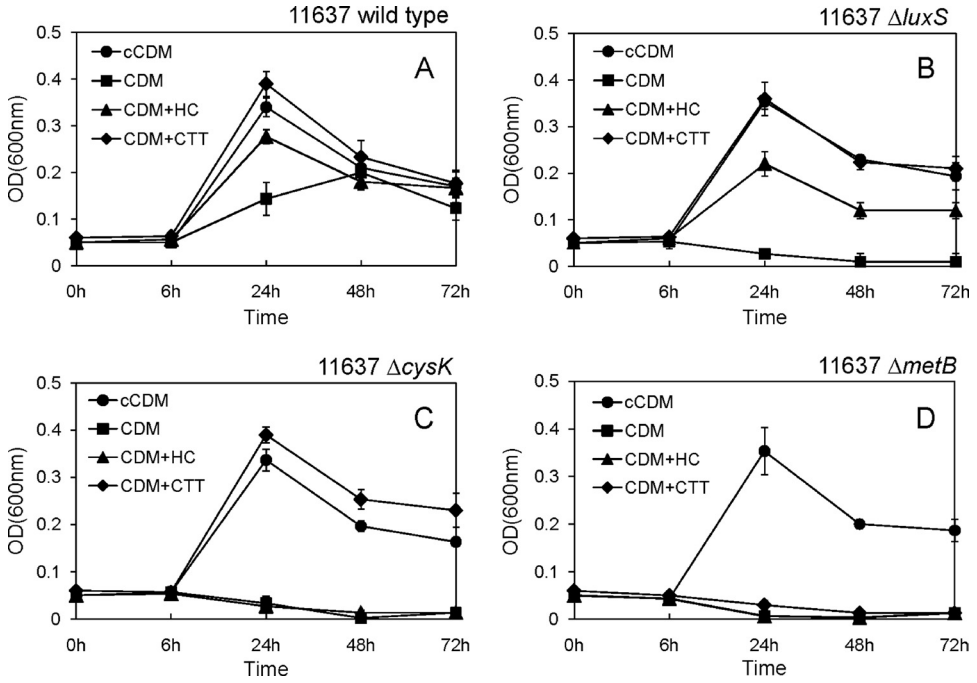

FIG. 3. *H. pylori* Δ*luxS*, Δ*metB*, and Δ*cysK* strains show chemically complementable growth phenotypes. Growth of *H. pylori* strains 11637 (wild type) (A), the Δ*luxS* mutant (B), the Δ*metB* mutant (C), and the Δ*cysK* mutant (D) in complete CDM (cCDM) (circles), CDM lacking cysteine (CDM) (squares), CDM lacking cysteine but containing homocysteine (CDM+HC) (triangles) and CDM lacking cysteine but containing cystathionine (CDM+CTT) (diamonds). The mean OD_{600} values of triplicate culture samples are shown. Error bars indicate standard deviations from the means.

cysteine but containing HC (CDM+HC) (Fig. 3C), suggesting that $CysK_{Hp}$ catalyzes the conversion of HC to CTT.

Finally, the Δ*metB*$_{Hp}$ mutant could grow only in cCDM, indicating an absolute requirement for cysteine (Fig. 3D). This would be consistent with $MetB_{Hp}$ catalyzing the final step from cystathionine to cysteine, although further experimentation is needed to confirm this (see below).

In contrast to results for the wild type, none of the mutants could grow in CDM lacking cysteine but containing potassium sulfide (CDM+S) (data not shown). Thus, it seems that $CysK_{Hp}$ is not acting like CysK homologues in other bacteria (which use sulfide to generate cysteine), in agreement with the reported absence of the assimilatory sulfate reduction pathway in *H. pylori* (10).

Taking these observations together, we propose that *H. pylori* generates cysteine from methionine via components of the AMC, followed by reverse transsulfuration, with $LuxS_{Hp}$ converting *S*RH to homocysteine and $CysK_{Hp}$ converting homocysteine to cystathionine, which is finally converted to cysteine by $MetB_{Hp}$.

Analysis of key metabolite pools confirms the proposed pathway of cysteine biosynthesis and specifically identifies reaction substrates. To investigate the individual steps in the proposed cysteine biosynthetic pathway and specifically to establish the likely substrates for $LuxS_{Hp}$, $CysK_{Hp}$, and $MetB_{Hp}$, we performed metabolite analyses of the wild-type and mutant strains grown in cCDM (Fig. 4). Specifically, we measured the relative levels of the AMC metabolites *S*AM, *S*AH, *S*RH, and HC, plus CTT. Although methionine and cysteine were also

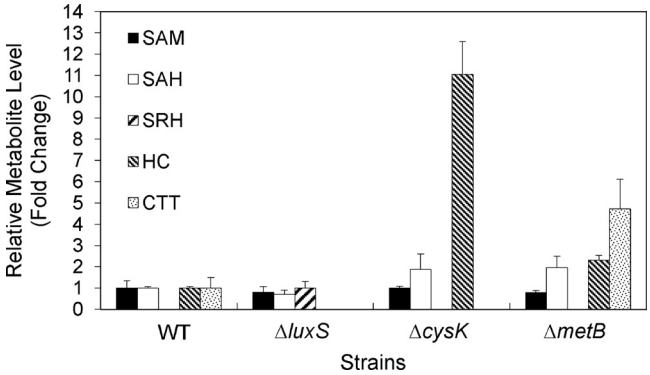

FIG. 4. Analysis of key metabolites of the proposed cysteine biosynthesis pathway. The *H. pylori* wild-type and Δ*cysK*, Δ*metB*, and Δ*luxS* mutant strains were grown in complete CDM (cCDM) in triplicate and harvested after 24 h of incubation. Samples were corrected for OD, and cell extracts were produced. Candidate metabolites involved in the conversion of methionine to cysteine through the RTSP were quantified individually. The fold change of each metabolite was normalized to the level of the wild type unless otherwise indicated (see below). Levels of metabolites that were below the detection threshold were expressed as 0. *S*RH was detected only in the Δ*luxS*$_{Hp}$ strain and was below the detection threshold in the wild-type, Δ*cysK*$_{Hp}$, and Δ*metB*$_{Hp}$ strains. Therefore, the relative *S*RH level was expressed as 1 in the Δ*luxS*$_{Hp}$ strain and as 0 in all other strains. Values given indicate activities derived from triplicate cell extract samples of each *H. pylori* strain. Error bars indicate standard deviations from the means.

measured during this analysis, both amino acids were present in excess in the growth medium (cCDM). As such, their relative levels were not seen to vary significantly between the strains (data not shown).

For the Δ*luxS*$_{Hp}$ mutant, the first important finding was that *S*RH was detectable in the cell extract whereas for the wild type it was undetectable (Fig. 4). The other main differences between the metabolite profiles of the Δ*luxS*$_{Hp}$ mutant and the wild type were that HC and CTT were either undetectable or virtually undetectable in the mutant (0% and 0.4% of wild-type levels, respectively). This confirms that HC and CTT cannot be synthesized in the absence of LuxS$_{Hp}$. Furthermore, the accumulation of *S*RH confirms this to be the *in vivo* substrate of LuxS$_{Hp}$.

For the Δ*cysK*$_{Hp}$ mutant, most strikingly, the levels of HC were 11- to 12-fold greater than those in the wild type, suggesting that this metabolite was accumulating in this strain. Like the Δ*luxS*$_{Hp}$ mutant, the Δ*cysK*$_{Hp}$ mutant produced minimal levels of CTT. The latter observation confirms that CysK$_{Hp}$ is needed for the formation of CTT. The accumulation of HC suggests that this is the substrate for the CysK$_{Hp}$ reaction. As an interesting aside, we found approximately 2-fold more *S*AH in cell extracts from the Δ*cysK*$_{Hp}$ mutant than in those from the wild type. This is discussed below.

Finally, in the Δ*metB*$_{Hp}$ mutant (unlike the case for all other mutants), CTT was present and was at around 5-fold excess relative to the level in the wild type, indicating that this metabolite was accumulating in this strain. This suggests that CTT is the substrate for the MetB$_{Hp}$ reaction. Interestingly, HC was also found to accumulate, although at a lesser level (2- to 3-fold), and, as for the Δ*cysK*$_{Hp}$ mutant, we saw around 2-fold more *S*AH than in the wild-type extract. These findings may indicate a weak feedback inhibition effect of CTT on CysK$_{Hp}$ and of homocysteine on Pfs$_{Hp}$, the enzyme catalyzing the reaction immediately prior to the LuxS$_{Hp}$ reaction in the AMC. Allosteric inhibition of metabolic enzymes by a downstream metabolite(s) is recognized as a common means of regulation of enzyme activity in prokaryotes; thus, these observations are unsurprising.

***Helicobacter pylori luxS* is part of a three-gene cluster which is of Gram-positive origin.** Having demonstrated the metabolic role of the *cysK-metB-luxS* gene cluster in *H. pylori*, we finally turned our attention to its presence in other bacteria and to its likely origin.

Analysis of the available *H. pylori* genomes showed that in all cases *luxS* is located downstream of the two other genes we have examined, annotated as *cysK* and *metB*. *cysK*$_{Hp}$, *metB*$_{Hp}$, and *luxS*$_{Hp}$ lie in the same coding orientation. We found this same arrangement of genes in *Helicobacter acinonychis* (regarded as the most closely related *Helicobacter* species to *H. pylori* (7). However, in all other helicobacters and the related *Campylobacter jejuni* and *Wolinella succinogenes*, this cluster was absent, and homologues of *cysK*, *metB*, and *luxS* were found in diverse genomic locations and were not adjacent to one another.

Protein BLAST searches of CysK$_{Hp}$, MetB$_{Hp}$, and LuxS$_{Hp}$ revealed that their closest known homologues are found in *Enterococcus faecium*, a Gram-positive organism (68%, 74%, and 80% amino acid identities, respectively). Importantly, the *E. faecium* genes exist as a cluster, with the genes lying in the same order as in *H. pylori*/*H. acinonychis.* We conducted phylogenetic analyses using multiple amino acid sequence alignments of LuxS, CysK, and MetB from *H. pylori*, *Helicobacter hepaticus*, *Helicobacter cinaedi*, *Helicobacter bilis*, *Helicobacter pullorum*, *Helicobacter canadensis*, *Helicobacter winghamensis*, *Helicobacter acinonychis*, *W. succinogenes*, *C. jejuni*, and *E. faecium*. We also included candidate Gram-negative sequences (*E. coli*) and two Gram-positive sequences, LuxS, MetB, and CysK from *Staphylococcus aureus* and LuxS, MccA/YrhA, and MccB/YrhB from *Bacillus subtilis*. We reconstructed phylogenies using sequences derived from the housekeeping gene product, RecA, from all 14 organisms. Phylogenetic trees were constructed using neighbor joining methods and are shown in Fig. 5.

We found that with the housekeeping gene product RecA, as expected, the helicobacters all occupy the same cluster, which also contains the *W. succinogenes* sequence. Also as expected, the *C. jejuni* sequence associates with this cluster but is found to be isolated. The Gram-positive representatives group together, and the *E. coli* sequence forms an independent outgroup. We repeated this analysis with a further 7 housekeeping gene products (encoded by *aroE*, *atpA*, *efp*, *engA*, *gapA*, *gyrA*, and *mutY*), which all gave qualitatively similar results, with all of the helicobacter sequences clustering together and the Gram-positive sequences forming separate groups (data not shown). Conversely, with CysK, MetB, and LuxS, the *H. pylori* and *H. acinonychis* sequences grouped more closely with the Gram-positive sequences than with those of the other *Helicobacter* species or species shown to be more closely related to *Helicobacter* species by other phylogenetic methods (7, 14).

These data suggest that in *H. pylori*, the three-gene cluster *cysK-metB-luxS* exists as a conserved syntenic locus that was acquired by horizontal gene transfer from a Gram-positive, enterococcal bacterium after the division of *H. pylori*/*H. acinonychis* from the other *Helicobacter* species but before their division from each other.

DISCUSSION

LuxS is now generally understood to carry out at least two functions, one in metabolism and one in cell-cell signaling (3, 8, 32, 44, 48). Bacterial signaling systems based on LuxS-generated AI-2 are known to regulate gene expression in several members of the *Enterobacteriales* (23, 50), notably *Vibrio* species. Many other bacteria also possess *luxS* homologues and produce AI-2 but appear to lack an AI-2 sensory apparatus (i.e., LuxP/Q and the Lsr system, respectively) (32), in agreement with suggestions that in most species *luxS* fulfils primarily a metabolic role in the AMC (32, 47–49).

In this context, *H. pylori* presents an interesting case: although lacking homologues for the identified AI-2 receptors, the bacterium is known to respond to exogenously added, synthetic AI-2 (30), pointing toward a role of LuxS in cell-cell signaling. On the other hand, Lee and coworkers (2006) proposed that the reduced *in vivo* fitness observed for an *H. pylori* SS1Δ*luxS* mutant was caused by metabolic disturbances, possibly by a disruption of the metabolic flux through the AMC or other LuxS-dependent pathways (20). Interestingly, previous physiological work (26, 27, 31) and genome analyses have re-

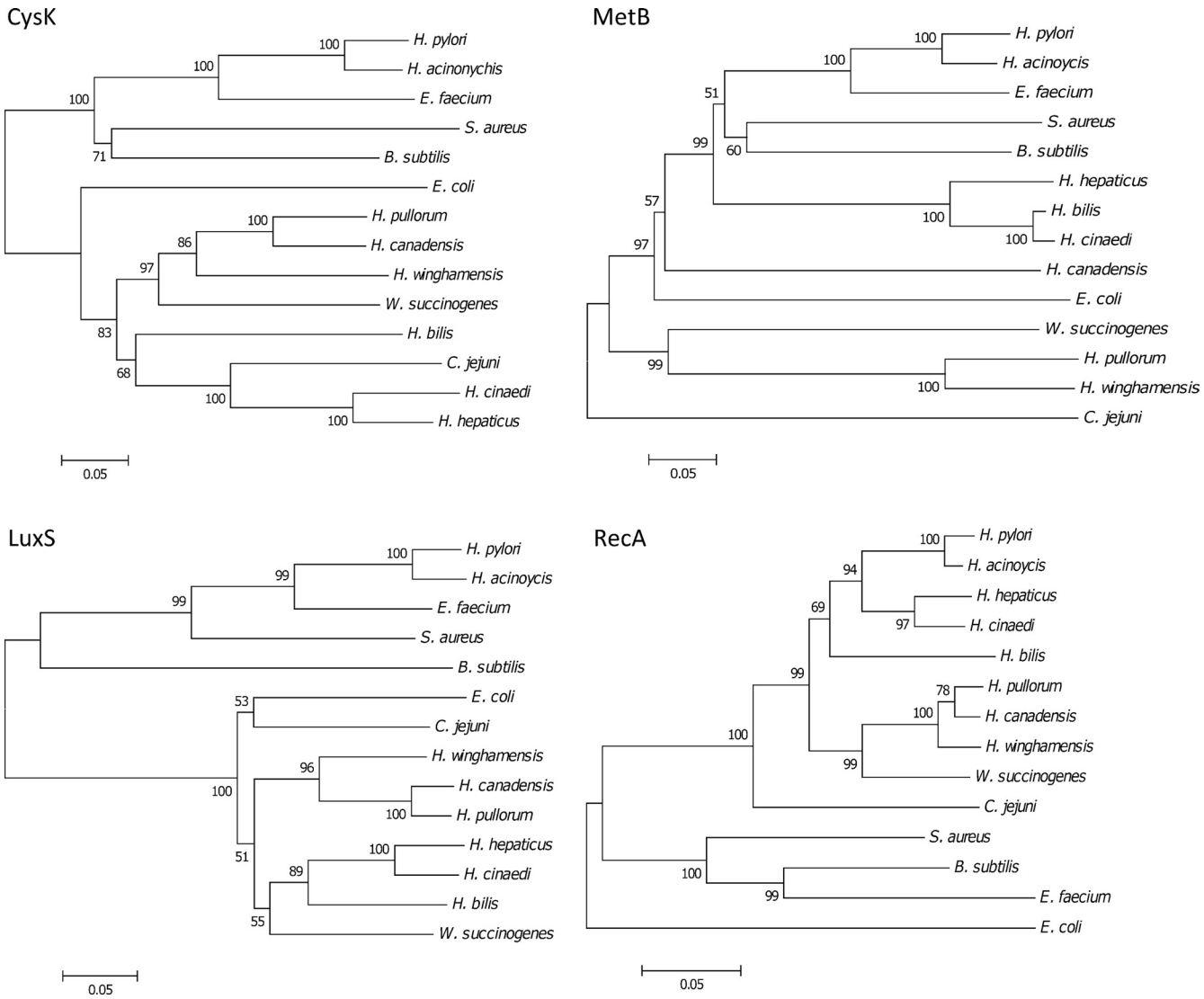

FIG. 5. Neighbor-joining phylogenetic trees based on comparison of LuxS, MetB, CysK, and the housekeeping gene product RecA from *Helicobacter pylori* and other species. Primary amino acid sequences of proteins from *H. pylori* and their closest homologues from selected other species were aligned using the ClustalW algorithm and Bioedit (13). These alignments were used to derive phylogenies based on neighbor-joining methods using the Mega4 suite of programs (40). Bootstrap values were generated based on 1,000 replicates of each calculation (consensus bootstrap trees had identical topologies). Dendrograms were produced indicating evolutionary relationships between the amino acid sequences of CysK, MetB, LuxS, and RecA.

vealed that the AMC in *H. pylori* is incomplete, since the organism is a methionine auxotroph that lacks the genes required for methylation of homocysteine (*metE* or *metH*) (10, 28, 41). This made us question whether LuxS in *H. pylori* had any metabolic roles apart from generating AI-2 and if so, what these roles could be.

Intriguingly, in all currently sequenced *H. pylori* strains, *luxS* is part of a three-gene cluster that in addition to *luxS* contains two other genes with putative functions in methionine and cysteine metabolism (Fig. 2A). These genes, currently annotated as *metB* and *cysK*, until now have been proposed to encode cystathionine γ-synthase and cysteine synthase, respectively (10). The gene cluster (*cysK*, *metB*, and *luxS*) is not only conserved among *H. pylori* strains and *H. acinonychis* but also is present in several Gram-positive bacteria (49), suggesting that $metB_{Hp}$ and $cysK_{Hp}$ are not evolutionary relics of now-inactive metabolic pathways but still fulfil a metabolic function linked to that of $luxS_{Hp}$.

Our hypothesis that the *cysK-metB-luxS* cluster is responsible for cysteine formation was based on several considerations. First, all *H. pylori* strains studied by Reynolds and Penn (1994) and the vast majority of strains characterized by Nedenskov (1994) were able to grow without cysteine (27, 31). Since the organism is not capable of using sulfate as a sulfur source (10), a hitherto-unidentified pathway must exist that makes use of the reduced sulfur present in methionine to generate cysteine. Second, the metabolic fate of the homocysteine generated in the LuxS reaction is unclear. High concentrations of homocysteine are toxic in some organisms (33, 43). Third, only two enzymatic steps are required to convert homocysteine to cys-

teine in the so-called reverse transsulfuration pathway (RTSP) present in some bacteria (for example, in *B. subtilis* and *Pseudomonas putida*), where part of the AMC, including LuxS, is known to contribute to methionine-to-cysteine conversion by generating the required homocysteine (16, 45). In addition, the genes in the *B. subtilis* cluster that show similarity to $cysK_{Hp}$ and $metB_{Hp}$ have recently been shown to encode functional cystathionine β-synthase and cystathionine γ-lyase, respectively, and have therefore been renamed MccA and MccB (*m*ethionine-to-*c*ysteine-*c*onversion genes) (16).

The results of our study are straightforward and in full agreement with the above hypothesis. First, we were able to demonstrate that all genes of the *cysK-metB-luxS* cluster in *H. pylori* (in both 11637 and J99) are required for cysteine prototrophy: mutation of any one of these genes rendered the respective strain auxotrophic for cysteine. Chemical complementation restored the growth of all mutants and also indicated a sequential order for the involved enzymatic activities, which is LuxS-CysK-MetB. Our results suggested that the true physiological function of the $cysK_{Hp}$ gene product lies in the conversion of homocysteine to cystathionine; the latter is then used by $MetB_{Hp}$ to generate cysteine. This interpretation was further corroborated and extended by metabolite profiling. In full agreement with the proposed metabolic functions, the metabolite pools of $\Delta luxS_{Hp}$ mutants showed accumulation of *S*RH and depletion of both homocysteine and cystathionine, whereas $\Delta cysK_{Hp}$ mutants accumulated homocysteine and were depleted for cystathionine. $\Delta metB_{Hp}$ mutants were the only strains that accumulated cystathionine. Thus, it appears that an RTSP is operational in *H. pylori*.

To unequivocally establish the nature of the reactions that link homocysteine with cysteine in *H. pylori* will require purification and detailed characterization of $CysK_{Hp}$ and $MetB_{Hp}$. The latter enzyme has already been studied, but not with respect to its physiological function. Kong et al. (18) cloned the respective gene from *H. pylori* SS1 and purified the recombinant protein. However, the enzymatic activity of $MetB_{Hp}$ was measured only using an unphysiological side reaction that is observed for some enzymes *in vitro* in the presence of *O*-acetylserine and in the absence of other substrates (18). Thus, the true physiological role of $MetB_{Hp}$ remained unclear. With regard to $CysK_{Hp}$, a similar enzyme from *Lactobacillus casei*, also annotated CysK, has recently been proposed to act as a cystathionine β-synthase, converting homocysteine to cystathionine (17). $CysK_{Hp}$ has up to now been thought to act as a cysteine synthase that uses sulfide to generate cysteine (10). However, our finding that sulfide cannot support growth of *H. pylori* in the absence of cysteine, at least under our conditions, suggests that $CysK_{Hp}$ is solely dedicated to homocysteine conversion. Thus, $CysK_{Hp}$ appears to be a cystathionine β-synthase, and $MetB_{Hp}$ appears to be a cystathionine γ-lyase. We therefore suggest that the genes encoding these proteins be renamed. Specifically, we suggest that $cysK_{Hp}$ be renamed *mccA* (methionine-to-cysteine-conversion gene A) and that $metB_{Hp}$ be renamed *mccB*.

The relevance of the established cysteine biosynthesis pathway for the lifestyle, fitness, and virulence of *H. pylori* is not clear. The reduced *in vivo* fitness observed for the *H. pylori* SS1 *luxS* mutant (20) may indeed be linked to its inability to convert methionine to cysteine, although a role of AI-2 cannot be excluded. *H. pylori* and other members of this genus have lost the ability to reduce sulfate (10; our unpublished data). However, it can be assumed that they are well adapted to their specific habitats, which therefore must provide sufficient reduced sulfur sources for these organisms to thrive. Presumably, the conversion of homocysteine to cysteine removes a potentially toxic metabolite and at the same time reduces the (energetically costly) need for cysteine uptake. On the other hand, a similar effect would have been achieved had the organism maintained a functional AMC.

Whatever the selective advantage gained, our genome comparisons revealed that the entire *cysK-metB-luxS* cluster was obtained by horizontal gene transfer from a Gram-positive bacterium closely related to *E. faecium*. Several previous analyses already established the Gram-positive origin of $luxS_{Hp}$ but did not consider the remaining genes of the cluster (21). Interestingly, similar clusters exist in a number of Gram-positive species (49), for instance, *Oceanobacillus iheyensis*, *Clostridium perfringens*, and *Clostridium botulinum*. Intriguingly, in some *Bacillus* species, notably *B. subtilis*, *Bacillus cereus*, and *Bacillus anthracis*, similar clusters contain the *pfs* gene instead of *luxS*. It thus appears that during the course of evolution, the common ancestor of *H. pylori* and *H. acinonychis* has lost its native *luxS* gene and instead acquired a Gram-positive homologue together with the ability to generate cysteine.

Finally, we would point out that in the absence of exogenous cysteine, growth of *H. pylori* will be limited by its capacity to generate this compound from homocysteine. Homocysteine availability, in turn, is determined by the number of methylation reactions carried out by the cells (i.e., the number of *S*AM molecules that can be converted to *S*AH and then further to homocysteine via the action of Pfs and LuxS; see Fig. 1). Under these conditions, generation of the quorum-sensing products comprising AI-2, at least in theory, is directly proportional to growth of the population, and the resulting AI-2 concentration should thus provide a very accurate measure of population density. It is now important to determine whether the other phenotypes associated with *luxS* mutagenesis in *H. pylori* are dependent on the homocysteine biosynthesis role of LuxS or its role as the AI-2 synthase.

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APPENDIX-II

Feifei Shen, Laura Hobley, Neil Doherty, John T. Loh, Timothy L. Cover, R. Elizabeth Sockett, Kim R. Hardie, and John C. Atherton. **In *Helicobacter pylori* auto-inducer-2, but not LuxS/MccAB catalysed reverse transsulphuration, regulates motility through modulation of flagellar gene transcription.** *BMC Microbiology* 10: 210. (2010).

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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 $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ mutant. Immunoblotting identified decreased levels of FlaA and FlgE but not FlaB in the $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ 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

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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 (*S*AM) 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 (*S*AH), 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 *S*AH to *S*-ribosylhomocysteine (*S*RH) 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 (*S*AH hydrolase) which converts *S*AH 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]. $luxS_{Hp}$ ($HP0105_{26695}$; $JHP0097_{J99}$) is positioned next to housekeeping genes $mccA_{Hp}$ ($HP0107_{26695}$; $JHP0099_{J99}$) and $mccB_{Hp}$ ($HP0106_{26695}$; $JHP0098_{J99}$) 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 $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ mutants *in vivo* using mouse and gerbil models, respectively [18,19]. The motility of $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ mutant was most likely due to the disruption of the cycle of *S*RH 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 (σ^{28}, *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 *flhA*, and that expression of *flhA* 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 $\Delta luxS$ 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 $\Delta luxS_{Hp}$ 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 $\Delta luxS_{Hp}$ 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*(r_k^- m_k^+) *relA1 supE44Δ(lacZYA-argF) U169 F*$^-$ *Φ80dlacZΔM15 deoA phoA* λ^-	[21]
DH5α LuxS	DH5α containing the plasmid pProEx-$luxS_{EC}$	[8]
DH5α Pfs	DH5α containing the plasmid pProEx HT mtan	[8]
Helicobacter pylori		
J99 (ATCC700824)	Wild-type motile strain	[44]
J99 Δ*luxS*	J99 derivative; Δ*luxS* :: *km*; Km^r	[15]
J99Δ*luxS*-F	J99 derivative; Δ*luxS* :: *km-sacB*; Km^r Suc^s	This study
J99 $\Delta luxS^+$	J99Δ*luxS*-F derivative; Δ*luxS* :: *km-sacB* replaced with original *luxS* locus; Suc^r Km^s	This study
J99 Δ*mccA*	J99 derivative; Δ*mccA* :: *km*; Km^r	[15]
J99 Δ*mccB*	J99 derivative; Δ*mccB* :: *km*; Km^r	[15]
J99 Δ*flhB*	J99 derivative; ΔHP0770 Lys^{13} to Glu^{347}; Km^r; non-motile	[24]
CCUG 17874*	Wild-type strain	[29]
17874 Δ*flaA*	17874 derivative; Δ*flaA* :: *cat*; Cm^r	Paul O'Toole
17874 Δ*flgE*	17874 derivative; Δ*flgE* :: *km*; Km^r	[30]
Plasmids		
pGEMT	Commercial TA cloning vector; Amp^r	Promega
pGEMTluxSXN396	pGEM-T with inserted 26695 *luxS*; Δ*luxS* :: *km-sacB*; Suc^s Km^r	[17]
pGEMTluxS	pGEM-T with inserted full-length *luxS* fragment	This study
pProEx-$luxS_{EC}$	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 (BB) (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_2, 86% N_2, and 6% O_2 (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 instruction. *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 Δ*luxS*$^+$ strain

H. pylori J99 wild-type was transformed with the plasmid pGEMTluxSXN396 containing a *km-sacB* construct encoding kanamycin resistance (Km^r) and (5%) sucrose sensitivity (Suc^s) [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 Δ*luxS* 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 Δ*luxS* 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 $OD_{600\ nm}$ of 0.05 in fresh BB medium, and grown at 37°C in a VAIN-cabinet with shaking. $OD_{600\ nm}$ 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 $OD_{600\ nm}$ 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).

Motility analysis was also carried out by direct observation under phase-contrast microscopy using a Nikon Eclipse E600 after cells were grown in co-culture conditions as used by Wand *et al.* [24]. Briefly, co-cultures of *H. pylori*-human gastric adenocarcinoma (AGS) cells were prepared (described below). After 24 h, 10 μl culture was placed onto a microscope slide and covered with a coverslip and freely-motile *H. pylori* cells were analysed under the microscope.

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 $\Delta luxS_{Hp}$ 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 $OD_{600\ nm}$ 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% CO_2. After cells had grown to confluency, a 1 in 5 or 1 in 8 dilution was added to a 75 cm^2 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 (0.35% v/v) blue dye method. Cells were seeded at a density of 1×10^5 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, Δ*luxS* and Δ*luxS*$^+$ 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 Δ$luxS_{Hp}$ mutant, the complemented Δ$luxS_{Hp}^+$ mutant and controls (*H. pylori* wild-type 17874 [29], and derived mutants Δ*flaA* (a kind gift from Paul O'Toole) and Δ*flgE* [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 × 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

Table 2 Primers utilised in quantitative RT-PCR

Primers	Sequence (5'-3')
16S_F	CGA TGA AGC TTC TAG CTT GC
16S_R	ATA GGA CAT AGG CTG ATC TC
flaAF	CAG GTT CGT ATC GCT ACA GGC
flaAR	ATC ACT TCT GCT AAC ACG CCG
flaBF	ACT GGG ATT GGG GCG TTA
flaBR	TCA ACC TCC CGTCAG CGT C
flgEF	GCT CAG GCA CGA TCA CTC TAA
flgER	AAC GCC ATG AAA GGT CTT AAT AC
flhAF	TCA TTG GAG GGT TTT TAG TGG
flhAR	GGT GCG AGT GGC GAC AAT
motAF	TGA GTT TAG AGG GGC GAG TG
motAR	CCA GTA ATG AGC GGC ACC
motBF	TTC AGG GAA AGA AGA AGA GCA A
motBR	TCA AAC AGC AAA CTA GAG AAA A
fliIF	ACG AGC GAT GAT AGC CCT TTA
fliIR	ACC GAT TTC TCT TTG AGC CAT
ureAF	GAT GAT GTG ATG GAT GGT GTG G
ureAR	TAA GAA CAA CTC ACC AGG AAC C

controls (*16 S* rRNA and no-template), were run in triplicate. Transcript levels of each gene were normalised to the *16 S* 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* Δ*luxS* mutant lost the ability to produce AI-2 while the wild-type, Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ mutants did not

Our previous study has demonstrated that $luxS_{Hp}$, $mccA_{Hp}$ and $mccB_{Hp}$ genes comprise a reverse transulphuration 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 Δ$mccA_{Hp}$, Δ$mccB_{Hp}$, and Δ$luxS_{Hp}$ 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 Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ mutants, despite the fact that the Δ$mccB_{Hp}$ mutant grew slightly less well than the other mutants and the wild-type. The Δ$luxS_{Hp}$ 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 $mccA_{Hp}$ and $mccB_{Hp}$ did not affect expression of the downstream gene $luxS_{Hp}$ (Figure. 1A).

Deletion of $luxS_{Hp}$ abolishes motility while the Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ 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 Δ$luxS_{Hp}$, Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ 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, Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ strains kept increasing during continuous incubation, although the Δ$mccA_{Hp}$ strain was slightly delayed in comparison to the others. After 7 days of culture, the Δ$luxS_{Hp}$ mutant remained almost non-motile and produced a significantly ($p < 0.001$) reduced motility halo compared to wild-type, Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ strains in 3 independent repeat experiments (Figure. 1B). After 7 days, the wild-type, Δ$mccA_{Hp}$ and Δ$mccB_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ mutant, but exogenous cysteine addition cannot

To rule out the possibility that second site mutations in the Δ$luxS_{Hp}$ mutant were inhibiting motility, genetic complementation was performed to create the Δ$luxS_{Hp}^{+}$ strain (see Materials and Methods). The non-motile Δ*flhB* mutant was used as a negative control [24]. 24 h cultures of wild-type, Δ$luxS_{Hp}$, Δ$luxS_{Hp}^{+}$ and Δ$flhB_{Hp}$ strains grown in Brucella broth were individually spotted onto normal motility plates. After 5 days of incubation, the mean halo diameter of the Δ$luxS_{Hp}^{+}$ 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 Δ$luxS_{Hp}$

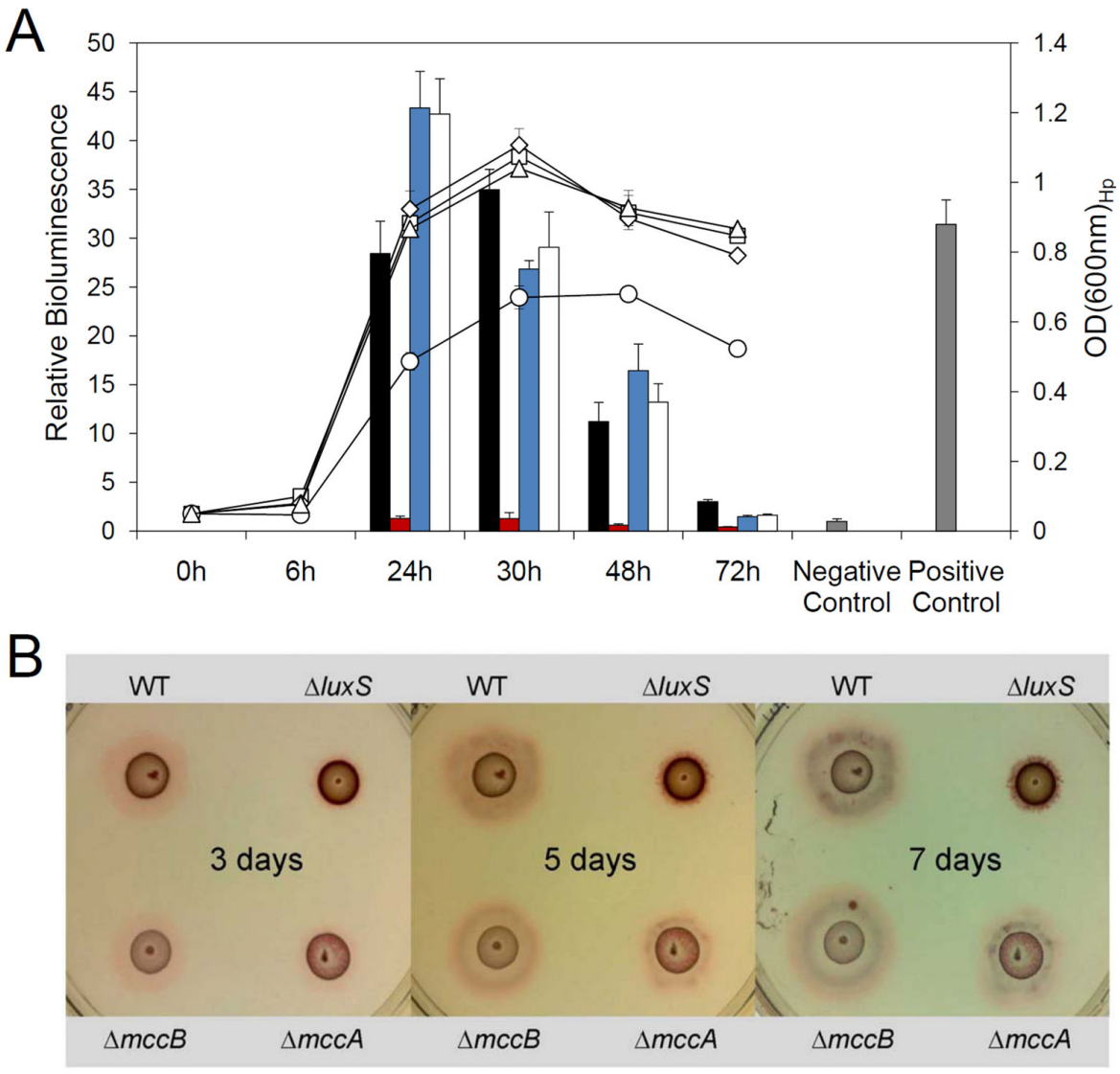

Figure 1 The Δ*luxS*$_{Hp}$ mutant of *H. pylori* strain J99 lacks AI-2 and is non-motile unlike other mutants deficient in cysteine biosynthesis. (A) AI-2 production in J99 wild-type (black column), Δ*luxS*$_{Hp}$ (red column), Δ*mccB*$_{Hp}$ (blue column) and Δ*mccA*$_{Hp}$ (white column) mutants was 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; Δ*luxS*$_{Hp}$, diamond; Δ*mccB*$_{Hp}$, circle; Δ*mccA*$_{Hp}$, 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 OD_{600nm} 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, Δ*mccB*$_{Hp}$, and Δ*mccA*$_{Hp}$ strains was significantly greater than that induced by the Δ*luxS*$_{Hp}$ 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, Δ*luxS*$_{Hp}$, Δ*mccB*$_{Hp}$ and Δ*mccA*$_{Hp}$ 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.

and Δ*flhB*$_{Hp}$ 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, Δ*luxS*$_{Hp}$ and Δ*flhB* 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

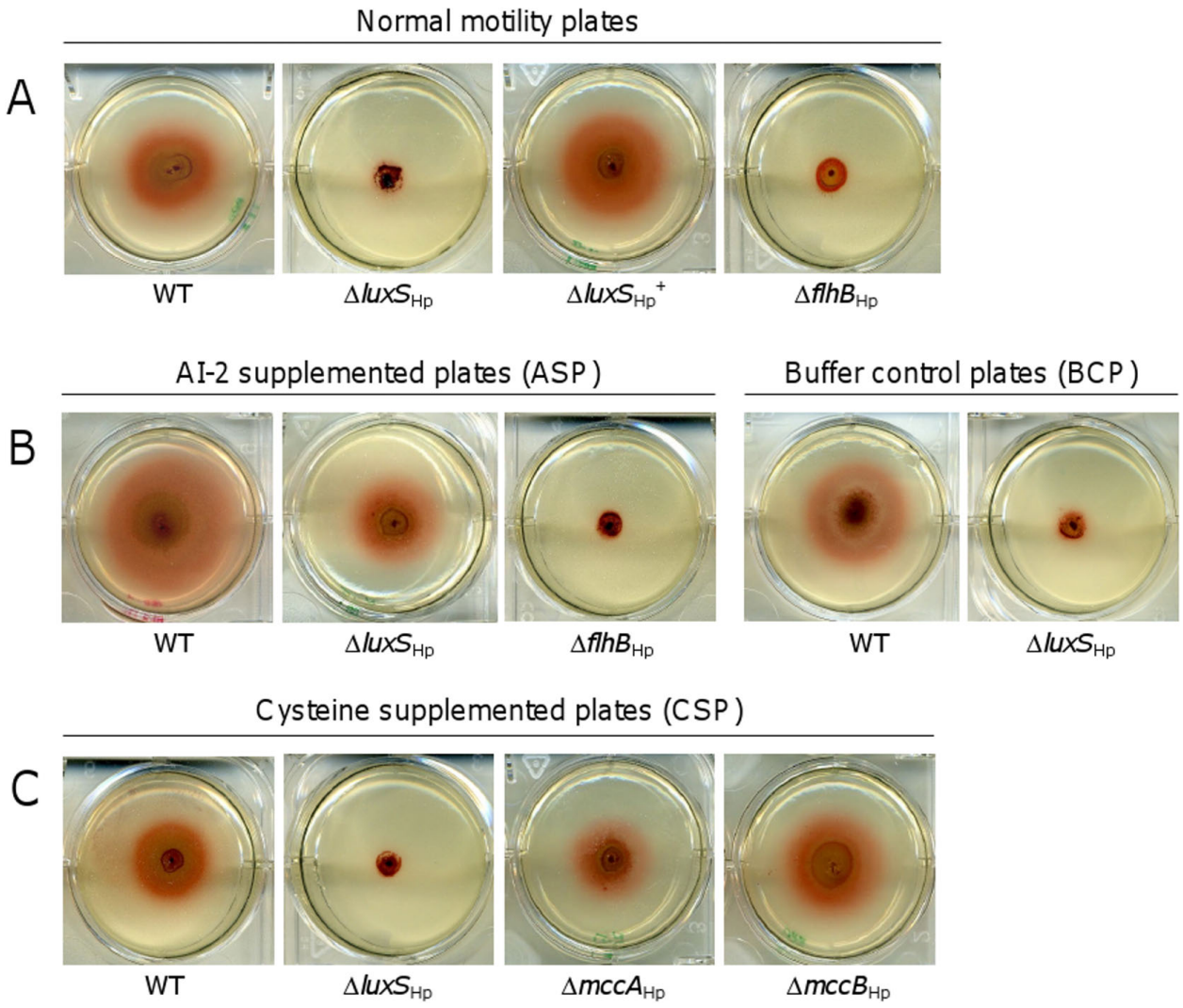

Figure 2 AI-2, but not cysteine rescues the motility defect of the ΔLuxS$_{Hp}$ mutant. (A) Wild-type, Δ*luxS*$_{Hp}$, and Δ*luxS*$_{Hp}^{+}$ bacteria were seeded onto soft plates composed of normal medium. The non-motile Δ*flhB* mutant served as the negative control. (B) Wild-type, Δ*luxS*$_{Hp}$ and Δ*flhB*$_{Hp}$ bacteria were seeded onto motility plates supplemented with *in vitro* synthesised AI-2. Wild-type and Δ*luxS*$_{Hp}$ were also seeded on motility plates containing buffer control solution used for *in vitro* AI-2 synthesis. (C) Wild-type, Δ*luxS*$_{Hp}$ Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ strains were seeded onto chemically defined motility plates supplemented with cysteine. After 5 days of incubation, the motility halo of each strain on each plate was recorded using a digital camera and the area of each halo was measured using a GS-800 Calibrated Densitometer (Biorad).

plate (compare Figure. 2A or the right panel of Figure. 2B with the left panel of Figure. 2B). Whilst the Δ*luxS*$_{Hp}$ 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 Δ*flhB*$_{Hp}$ 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* (Δ*luxS*$_{Hp}$, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ 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 Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ mutant remained non-motile (Figure. 2C).

Taken together, these data indicate that the motility defect of the Δ*luxS*$_{Hp}$ mutant was restored either

genetically or chemically with AI-2, but not with exogenous cysteine. This suggests that *luxS* and AI-2 play a role in enhancing bacterial motility, rather than an intact cysteine biosynthesis pathway, implying a likely role of $luxS_{Hp}$ in signalling.

ΔLuxS$_{Hp}$ 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 Δ$luxS_{Hp}$ 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, Δ$luxS_{Hp}$ and Δ$luxS_{Hp}^{+}$, 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 Δ$luxS_{Hp}^{+}$ cells were motile; whereas less than 2% of Δ$luxS_{Hp}$ cells were motile. When grown with exogenous DPD, motile cells again made up > 40% of the population for wild-type and Δ$luxS_{Hp}^{+}$ cells, but now also made up > 40% of the population for Δ$luxS_{Hp}$ cells. Cultures of the Δ$luxS_{Hp}$ grown with exogenous cysteine consistently contained less than 2% motile cells. To exclude the possibility that the restoration of motility of Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ cells was visibly lower compared to the wild-type, Δ$luxS^{+}$ and all cell samples plus DPD.

Electron microscopic images (Figure. 3) showed that all samples tested (wild-type, Δ$luxS_{Hp}$ and Δ$luxS_{Hp}^{+}$, 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}^{+}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}^{+}$ cells had a similar effect, with more cells with flagella at both poles (Figure. 3F).

Mutation of $luxS_{Hp}$ resulted in the decreased production of flagellar proteins FlaA and FlgE

The reduced number and length of flagella in Δ$luxS_{Hp}$ 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 (Δ*flaA* and Δ*flgE*) 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, Δ$luxS_{Hp}$ mutant and the complemented Δ$luxS_{Hp}^{+}$ 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 $LuxS_{Hp}$ reduced the expression of FlaA relative to FlaB (from

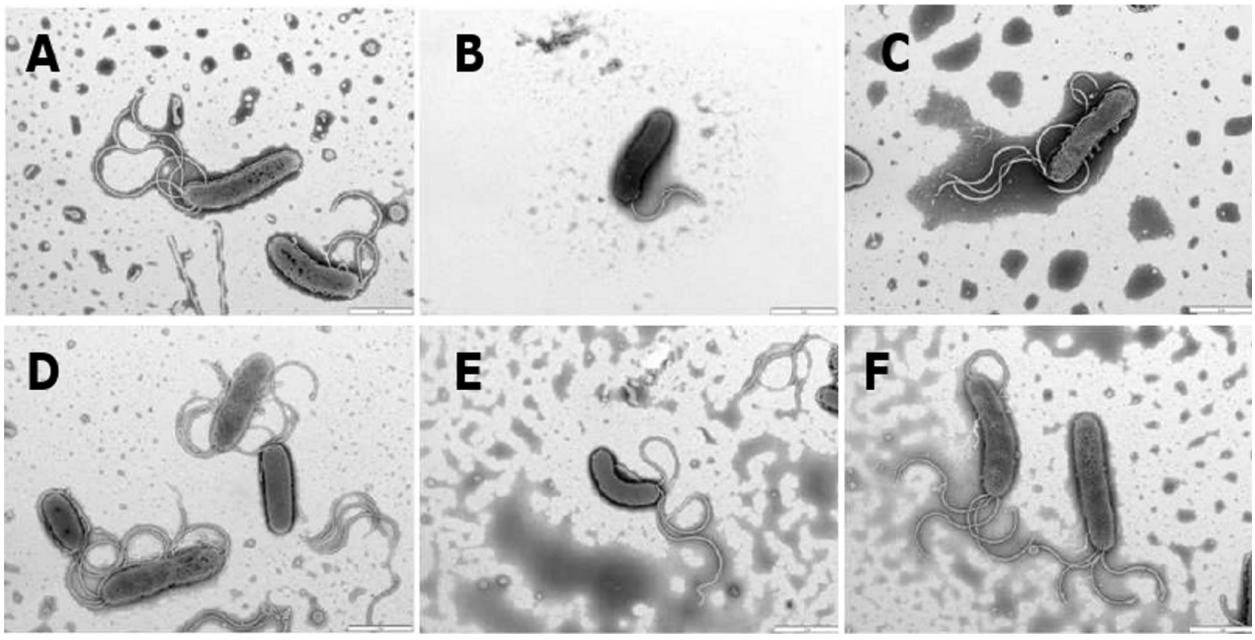

Figure 3 $luxS_{Hp}$/DPD modulates flagellar morphogenesis. *H. pylori* cells were co-cultured with AGS cells. Cells were stained with 0.5% photungstate (PTA). Scale bars represent 2 μm. (A) wild-type, (B) Δ$luxS_{Hp}$, (C) Δ$luxS_{Hp}^{+}$, (D) wild-type with DPD, (E) Δ$luxS_{Hp}$ with DPD and (F) Δ$luxS_{Hp}^{+}$ with DPD. DPD was added after 10 h of incubation and once again after 18 h of incubation during co-cultures.

mean 1.60 in the wild-type to 1.23 in the Δ$luxS_{Hp}$ mutant, $p < 0.01$), and complementation increased the ratio back to wild-type levels (mean 1.70 in the Δ$luxS_{Hp}^{+}$ mutant, $p < 0.01$ compared with the Δ$luxS_{Hp}$ mutant). Next, we examined FlgE expression, and a similar trend was found (wild-type FlgE:FlaB ratio mean 0.74; Δ$luxS_{Hp}$ mutant 0.51; complemented Δ$luxS_{Hp}^{+}$ mutant 0.77; $p < 0.01$ for differences between Δ$luxS_{Hp}$ mutant and wild-type and complemented stains). These data show that FlaA and FlgE synthesis was reduced relative to FlaB in the Δ$luxS_{Hp}$ mutant and these changes were restored by genetic complementation.

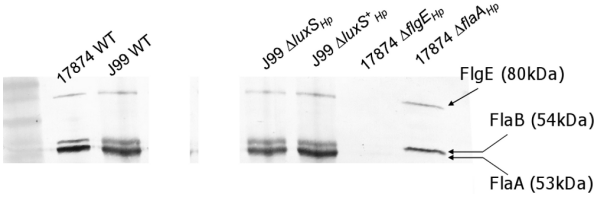

Figure 4 Mutation of $luxS_{Hp}$ causes altered flagellin and hook protein production. Cell lysates of the strains indicated were subjected to immunoblotting with anti-flagellin (FlaA and FlaB) and anti-hook protein (FlgE) together [32]. The proteins were measured in wild-type, Δ$luxS_{Hp}$, Δ$luxS_{Hp}^{+}$ cultures grown in Brucella broth at 37°C for 24 h. *H. pylori* strain 17874 wild-type [29] served as the positive control. Mutants in *flaA* (a kind gift from Paul O'Toole) and *flgE* [30] derived from this strain (17874Δ*flaA* and 17874Δ*flgE*) served as negative controls for identifying FlaA and FlgE, respectively.

AI-2 regulates the transcription of flagellar genes

Previous reports have provided evidence that $luxS_{Hp}$-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. *16 S* 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* (enodes flagellar hook protein); and class 3 gene *flaA* (encodes major flagellin). *fliI* (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 Δ$luxS_{Hp}$ mutant compared to the wild-type (Figure. 5A). For class 2 genes tested, *flgE* was 1.5 fold ($p < 0.001$)

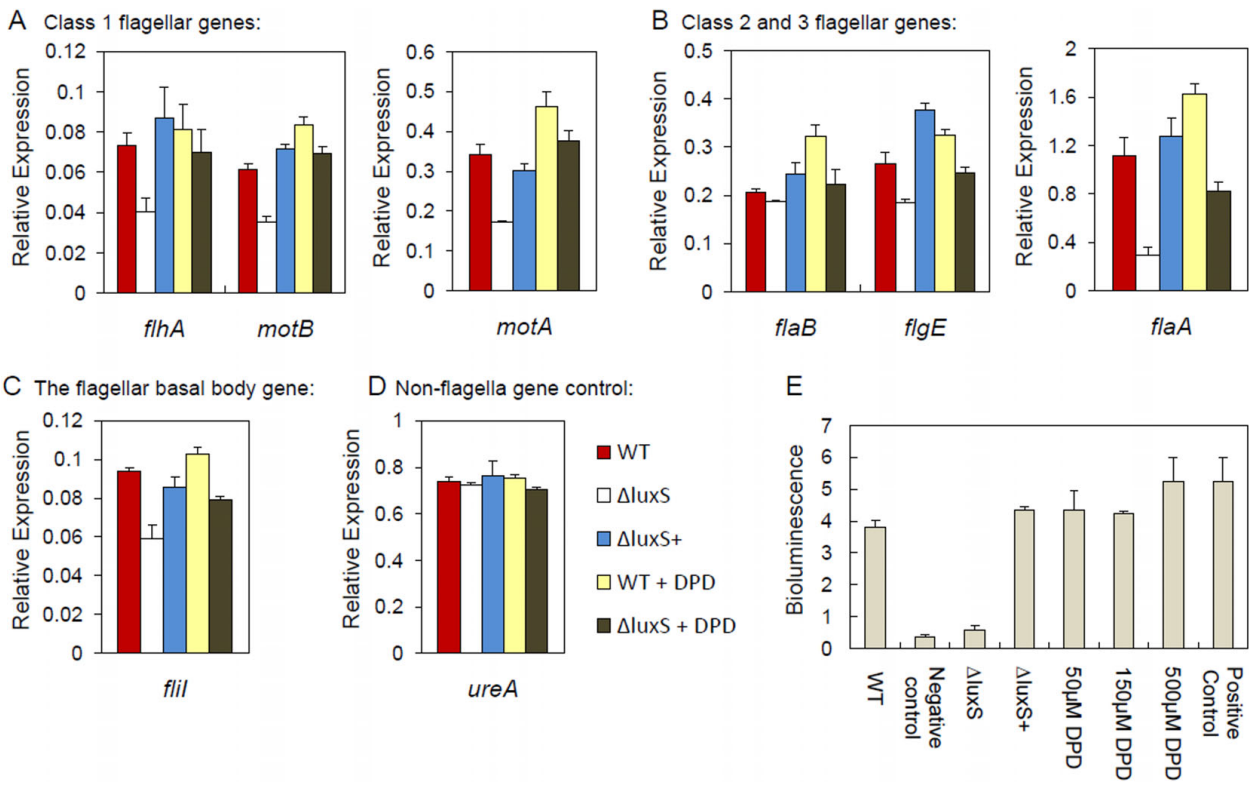

Figure 5 $luxS_{Hp}$/DPD modulates *H. pylori* flagellar gene transcription. Transcript levels of (A) *flhA*, *motA*, *motB*; (B) *flaB*, *flgE*, *flaA*; (C) *fliI* were determined by qRT-PCR normalised to the levels of the *16 S* rRNA gene. (D) Relative expression of *ureA* was utilised as a non-flagella gene control. The Y axis shows the relative transcriptional level of each gene in each strain normalised to the level of the same gene in the strain control (which is J99 wild-type in every case). Values are mean activities of triplicate RNA samples of each strain. Transcript levels were measured in wild-type and Δ$luxS_{Hp}$ cultures grown with or without DPD (150 μM) and in Δ$luxS_{Hp}^{+}$ cultures grown without DPD. (E) AI-2 activity (using the previously-described *V. harveyi* BB170 bioluminescence assay [4]) in DPD solution (at concentrations of 50 μM, 150 μM or 500 μM) and in cell free culture supernatant (24 h) of *H. pylori* wild-type, Δ$luxS_{Hp}$ and Δ$luxS_{Hp}^{+}$ strains grown in the Brucella broth (starting $OD_{600\ nm}$ of 0.05). Negative control is Brucella broth alone. A diluted *in vitro* synthesised AI-2 sample was utilised as a qualitative positive control [8]. Error bars indicate standard deviation.

down-regulated in the Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ$luxS_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 *luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ mutant would be devoid of AI-2. Thus, if the reduced motility of the Δ*luxS*$_{Hp}$ 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, Δ*luxS*$_{Hp}$, Δ*mccA*$_{Hp}$ and Δ*mccB*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ 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 (σ^{28}, *flgS* or *flgM*) and where wild-type length flagella were reported for the Δ*luxS*$_{Hp}$ 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 σ^{28}, FlgM, interacts with FlhA at the base of the *Helicobacter* flagellum and this interaction modulates the expression of flagellar genes by σ^{28} [37]. The decrease in *flhA* expression, seen in our Δ*luxS*$_{Hp}$ 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 Δ*flhA* mutant in *Helicobacter* strains N6 and 88-3887 [37].

Both Rust and co-workers [37] and Neihus and coworkers [33] show that FlaB is not regulated by the same regulatory pathway as FlaA, and as FlaB levels in

our Δ*luxS*$_{Hp}$ mutant concur with this, the short flagella we observe in the Δ*luxS*$_{Hp}$ 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 *luxS*$_{Hp}$-complemented Δ*luxS*$_{Hp}^{+}$ bacteria or Δ*luxS*$_{Hp}$ 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 σ^{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/AI-2. This suggests that *luxS* inactivation might affect transcription of the same class of flagellar genes differently. One possibility is that the FlgR/FlgS-σ^{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 *flhF*.

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 Δ*luxS*$_{Hp}$ 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 Δ*luxS*$_{Hp}$ mutant were due to loss of AI-2 only, and not to the metabolic effect of *luxS*$_{Hp}$ on biosynthesis of cysteine. These results suggest that LuxS/AI-2 is likely to be a functional signalling system contributing to control motility in *H. pylori*. However, it is still uncertain whether AI-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 AI-2 sensory and regulatory apparatus in *H. pylori* [13,40]. Also, we cannot exclude the possibility that AI-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 AI-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, AI-2 does not function as a QS molecule in *C. jejuni* (NCTC 11168) during exponential growth *in vitro* when a high level of AI-2 is produced [42]. Thus, so far there is no good evidence to ascertain whether AI-2 functions as a true QS signal in this species. In *H. pylori*, Lee *et al.* and Osaki *et al.* looked at fitness of Δ*luxS*$_{Hp}$ 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 *luxS*$_{Hp}$ [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 transsulphuration 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 AI-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 transsulphuration 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 Δ*luxS* mutant. JTL and TLC designed and generated the plasmids needed for the construction of the complemented Δ*luxS*$^{+}$ 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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