Title: The impact of the LuxS mutation on phenotypic expression of factors critical for *Campylobacter jejuni* colonization

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Abstract

Studies have collectively shown the wide impact that *luxS* mutation has on the expression and function of various aspects of *Campylobacter jejuni* virulence. Previous work from our group demonstrated that LuxS mutagenesis negatively impacts colonization of the gastrointestinal tract of several host species. To determine what is responsible for the colonization defect, we used a mechanistic approach to understand how the *luxS* mutation affects the expression of key physiologic factors important to the colonization ability of *C. jejuni*. This included expression of genes from the CmeABC efflux system, cell morphology, and motility through mucin substrate between wildtype, *luxS* mutant, and *luxS* complement of the *C. jejuni* strains 11168 and/or IA3902. We also measured and compared the activated methyl cycle (AMC) metabolite levels of the IA3902 *luxS* mutant to wildtype. Results showed that mutagenesis of the *luxS* gene completely disrupted the AMC with altered concentrations of AMC metabolites both upstream and downstream of LuxS. Multidrug efflux pump genes *cmeABC* and *cmeR* showed no significant changes in expression levels within the *luxS* mutant. Though motility through mucin was not completely unaffected by the *luxS* mutation, the lack of differences in cell morphology between wildtype and *luxS* mutant suggest that morphology is not responsible for the slight changes in mucin penetration observed in one of our *luxS* mutants. Though

additional studies are warranted, these findings suggest that the CmeABC multi-drug efflux pump, cell morphology and mucin penetration are not major mechanisms responsible for the *luxS* mutant's colonization defect in its host.

Key Words: *Campylobacter jejuni*, AI-2/LuxS, efflux pump, cell morphology, mucin, motility

Introduction

The gram-negative microaerophilic bacterium *Campylobacter jejuni* is the most common cause of bacterial-associated food borne diarrhea in the United States (CDC, 2013). Of even greater concern to both human and animal health is the emergence of a highly virulent *C. jejuni* clone (clone SA or Sheep Abortion) (Sahin et al., 2008) and other antibiotic resistant *C. jejuni* strains (Alfredson and Korolik, 2007; Wieczorek and Osek, 2013), leading to a dire need for novel approaches to control *C. jejuni* infections and minimize development of additional antibiotic resistance phenotypes.

One area of research that has gained a foothold as a promising alternative drug target for controlling bacterial infections is quorum sensing. This cell density-dependent mechanism has been demonstrated to play a central regulatory role in bacterial communities (Bhardwaj et al., 2013). Most *Campylobacter* species possess the autoinducer-2 (AI-2)/LuxS system, which consists of the LuxS enzyme and its product, the signaling molecule AI-2 (Gözl et al., 2012a). LuxS is part of the activated methyl cycle (AMC) (Figure 1), which functions to recycle *S*-adenosylmethionine (SAM), a primary methyl donor for various biological processes including DNA methylation (Winzer et al., 2003).

In *C. jejuni*, LuxS has been associated with a wide range of virulence traits (Gözl et al., 2012b; Plummer, 2012), including its importance in the fitness and/or colonization ability of *C. jejuni* in the host gut environment (Plummer et al., 2012). However, the exact mechanism underlying the benefit of a functional LuxS system in the critical stage of *C. jejuni* colonization of the intestinal tract has yet to be defined. In an attempt to better delineate the role of LuxS in maintaining normal intracellular levels

of AMC metabolites and host colonization, we set out to characterize the impact of the LuxS system on several colonization factors important to *C. jejuni*, including the expression of the *cmeABC* efflux system operon, expression of the *cmeR* transcriptional repressor of *cmeABC* efflux genes, mucin penetration, and cell shape.

Due to the well-known role of LuxS in AMC synthesis, and the absence of predicted alternative metabolic pathways in *C. jejuni*, we hypothesized that mutagenesis of LuxS would disrupt the AMC cycle resulting in altered intracellular concentrations of other AMC metabolites. Additionally, because previous studies demonstrated a decreased ability of *luxS* mutants to colonize the animal host and induce systemic infection we hypothesized that it would be deficient in its ability to resist bile salts (associated with *cmeABC* efflux pump activity) or in its ability to penetrate the intestinal mucin layer (largely mediated by the cell shape and flagella). Using a mechanistic approach, we characterized the phenotypic effects of the *luxS* mutation on the expression or functions of these colonization factors. These effects included analysis of gene expression of the *cmeABC* system in the presence of a *luxS* mutation, cell shape, motility through mucin, as well as the intracellular effect of the *luxS* mutation on the AMC via measuring AMC metabolite levels.

Materials and Methods

Bacterial strains and culture conditions

Campylobacter jejuni strains were routinely cultured in Mueller-Hinton (MH) broth or agar plates (Difco) at 42°C in gas jars with a microaerobic atmosphere (5% O₂, 10% CO₂, 85% N₂). A list of all *C. jejuni* strains used in this study is described in Table 1. W7 is a highly motile spontaneous variant of the commonly studied *C. jejuni* NCTC 11168 (ATCC 700819) (Plummer et al., 2012). IA3902 is a clinical isolate of *C. jejuni* clone SA (Sheep Abortion) obtained from a sheep abortion

outbreak in Iowa in 2006 (Sahin et al., 2008) and has been sequenced (Wu et al., 2013). The only strain created in this study was the chromosomally encoded *luxS* complement of W7 (W7 Δ luxSc). This strain was created by natural transformation of W7 Δ luxS with genomic DNA from IA3902 Δ luxSc as performed previously (Jeon and Zhang, 2007). Transformants were selected on MH agar plates supplemented with chloramphenicol (4 μ g/ml) and kanamycin (30 μ g/ml) and confirmed by PCR. Strains containing an antimicrobial resistance marker were grown in media containing 4 μ g/ml chloramphenicol or 30 μ g/ml kanamycin.

Expression of cmeB and cmeR genes in Campylobacter jejuni luxS mutant grown under basal conditions

Growth studies

C. jejuni strains W7, W7 Δ luxS, and W7 Δ luxSc were each prepared for growth study inoculum using confluent lawns grown to 24 hours, harvested in a small volume of MH broth media and adjusted to an optical density (OD₆₀₀) of 0.5. Inocula were then further diluted 1:100 by adding 600 μ l of normalized cultures to 60ml MH broth (approximate cell densities of 10⁵-10⁷ colony forming unit (CFU)/ml) in 250ml flasks. Suspensions were grown in gas jars (Mitsubishi Gas Chemical Co., Inc.) under microaerobic conditions at 42°C for 48 hours. Sample aliquots from each strain were harvested at the time of inoculation and at time points 3, 8, 16, 24, 32, and 48 hours for bacterial enumeration. Aliquots from time points 8, 16, 24, and 32 hours were also used for quantitative real-time PCR (qPCR) studies. Three independent growth studies were performed for each strain. Growth of each strain was assessed by using the drop plate method (Chen et al., 2003) to plate serial dilutions (1:10) of bacterial suspensions on MH agar. Log 10 transformation of CFU/ml was performed and assessed for any significant growth differences between strains using two-way analysis of variance (ANOVA) with

repeated measures in GraphPad Prism (La Jolla, CA, USA). A *p*-value of 0.05 or less was considered significant. Results are the average of three independent experiments.

*Quantitative real-time PCR analysis of *cmeB* and *cmeR* transcription under basal conditions*

C. jejuni cultures were collected at time points 8, 16, 24 and 32 hours from the growth studies and mixed with 2 volumes of RNeasy Protect Bacteria Reagent (Qiagen, Valencia, CA). RNeasy Protect cultures were stored in -80°C for no more than a week before total RNA isolation was performed using an RNeasy Protect Bacteria Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was further treated with the TURBO DNA-free kit (Life Technologies, Carlsbad, CA) to remove any residual DNA contamination. RNA concentration was then measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Quality and size distribution of RNA was determined using gel electrophoresis with ethidium bromide staining. RNA samples were stored in -80°C until ready for quantitative real-time PCR (qPCR) analysis. Primers used in the qPCR assay for amplifying expression levels of *cmeB*, *cmeR*, and 16S rRNA are listed in Table 2. The primers made in this study were designed using Primer3 online interface (<http://bioinfo.ut.ee/primer3/>). qPCR assays were run using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) and the MyiQ Single-Color Real-Time PCR detection system (Bio-Rad) as done previously with some modifications described here (Lin et al., 2005). Each dilution of the RNA template was run in triplicate with reaction volumes of 15 µl. Thermal cycling conditions consisted of cDNA synthesis at 50°C for 10 min and 60°C for 5 min followed by reverse transcriptase inactivation at 95°C for 3 min. Amplification then proceeded with 40 cycles of denaturation at 95°C for 10 s followed with annealing for each primer pair (Table 2) at 58°C for 30 s. Relative changes in *cmeB* and *cmeR* expression between W7 wildtype, W7ΔluxS, W7ΔluxSc were calculated using the “ISU Equation” described in previous methods (Gallup and Ackermann, 2006) and modified for this project by the Iowa State University Office of Biotechnology’s DNA Facility ISU qPCR Consultation Service (Ames, IA). Each gene was

normalized using 16S rRNA expression levels as a control. Statistical analyses were performed using two-way ANOVA with repeated measures (GraphPad Prism) to assess significance of changes in gene expression levels. A *p*-value of 0.05 or less was considered significant. Results are the average of three independent experiments.

Cell image capture and morphology analysis using ImageStream

Cultures of bacterial strains were grown at 37°C or 42°C for 24 hours. Cultures were then adjusted to an approximate OD₆₀₀ of 0.1, fixed with 3.6% formalin, washed with DPBS (1X concentration), and stained with BacLight Green stain (Life Technologies, Carlsbad, CA) to a final concentration of 100nM following the manufacturer's protocol. Images of cells for each strain were captured with a 60x objective using the ImageStream^X® multispectral imaging flow cytometer (Amnis Corp., Seattle, WA). Gating was applied on area and intensity to select for images containing single cells positive for fluorescence under channel 2, which contains the blue (488nm) excitation field. Morphological features (area, aspect ratio intensity, aspect ratio, diameter, elongatedness, height, length, major axis intensity, major axis, minor axis intensity, minor axis, perimeter, shape ratio, thickness min/max, width) were calculated for at least 1500 cells of each strain using channel 2 filter images by IDEAS® image analysis software (version 4.0, Amnis Corp., Seattle, WA). Further calculations were made using the Fisher's Discriminant ratio or RD value (difference of means/sum of standard deviation) to compare features between wildtype and *luxS* mutant or wildtype and *luxS* complement in W7 or IA3902 grown at the same temperature. A value of over 1 represented significant difference between the compared samples. At least three independent experiments containing three technical replicates of each strain were performed.

Transmission electron microscopy

Aliquots of bacterial strains and their respective isogenic *luxS* mutants were analyzed by electron microscopy on at least three separate occasions. Strains were grown to log or stationary phase in MH broth under basal conditions. 3µl of each strain were added to Formvar/carbon-coated copper grids and air-dried for 1-3 minutes. Remaining liquid was removed using a filter paper and the grids were washed with deionized distilled water. Grids were air-dried again for the same amount of time and then negatively stained with 1% uranyl acetate for one minute. Excess stain was removed using filter paper and grids were air dried for at least a minute. Samples were then observed in a JEOL JM-2100 scanning/transmission electron microscope at an accelerating voltage of 200kV at the Microscopy and NanoImaging Facility at Iowa State University in Ames, IA.

Mucin penetration assay

Mucin penetration was simulated for bacterial strains using methods adapted from previous studies (Liu et al., 2008; Naresh and Hampson, 2010). One-ml syringes containing 1ml porcine gastric mucin (Sigma-Aldrich) dissolved in PBS buffer to a concentration of 30mg/ml were used as mucin columns. The concentration 30mg/ml was selected as it was the highest concentration of mucin that could be used without the mucin viscosity interfering with the syringe function of the assay. One hundred microliters of log-phase bacterial cultures were added to the top of mucin columns and allowed to settle for 30 min at either 37°C or 42°C. Fractions of 0.1ml (the first 0.1ml collected is fraction 1, with subsequent fractions in chronological order until the last 0.1ml-fraction or fraction 10) were collected from the bottom of mucin columns, plated on MH agar containing Oxoid™ Preston *Campylobacter* selective supplement (Thermo Scientific, Wilmington, DE), incubated for at least 24 hours in microaerobic conditions, and then enumerated for bacterial quantification. At least three independent experiments were performed for each strain at each temperature and statistically analyzed using two-way ANOVA with repeated measures (GraphPad Prism). Data were assessed for significant differences in number of bacteria (CFU or colony forming units) compared to wildtype at each fraction

and total CFU from fractions 1-9. Fraction 10 was not included in any of the analysis as this fraction included the very top of the mucin layer mixed with culture inoculum, thus making it impossible to determine if the bacteria in this sample had truly penetrated the mucin. A *p*-value of 0.05 or less was considered significant.

Analysis of activated methyl cycle metabolites levels in C. jejuni cell pellets using LC-ESI-MS/MS.

The relative levels of AMC metabolites in IA3902, *luxS* mutant (IA3902Δ*luxS*), and *luxS* complement (IA3902Δ*luxS*c) were profiled using a previously described LC-ESI-MS/MS (liquid chromatography-electrospray ionization-mass spectrometry) method (Halliday et al., 2010). To extract metabolites, *C. jejuni* strains were grown from freezer stocks at 42°C with microaerophilic gas and passaged to MH broth. After overnight growth, the optical densities of broth cultures were diluted to an OD₆₀₀ of 0.1-0.15 in MH broth. Cell pellets were collected as described by Halliday et al. (Halliday et al., 2010) and were stored at -80°C until time of submission to the Centre for Analytical Bioscience at the University of Nottingham (Nottingham, England) for metabolite analysis. Chemicals, preparation of stock solutions and standards, metabolite extractions, chemical derivation, instrumentation, and calibration and validation of methods used in the LC-ESI-MS/MS analysis were performed exactly as done by Halliday et al. (Halliday et al., 2010).

Results

Correlation in gene expression of luxS and efflux pump genes cmeB and cmeR

Only one prior study has published findings correlating the functions of LuxS and *C. jejuni* efflux systems. The authors found that under stress-induced conditions, a non-functional LuxS in *C. jejuni* strain 81-176 altered the expression of several key virulence genes, including efflux pump genes (*cmeDEF*) (He et al., 2008). The observations from He et al. and our preliminary work merited further

study into deciphering the mechanisms of the role of LuxS in efflux pump expression. We hypothesized that mutation of the *luxS* gene in *C. jejuni* would decrease the expression of efflux pump genes from the CmeABC efflux system, thus making the organism more sensitive to the bile salts in the intestine and limiting colonization. This hypothesis was tested by evaluating the expression of the *cmeABC* efflux gene in the *luxS* gene mutant. To do this, *C. jejuni* W7 wildtype, W7 mutant containing a non-functioning *luxS* gene (W7 Δ luxS), and a chromosomally encoded genetic complement of the W7 *luxS* mutant (W7 Δ luxSc) were grown at 42°C in broth culture under basal conditions for 48 hours. No growth differences were found between the strains, which were consistent with growth results from previous studies (Plummer et al., 2012).

No statistically significant differences in *cmeB* and *cmeR* transcriptional levels were found between wildtype and W7 Δ luxS over the average of three independent growth experiments (Figure 2). *cmeB* gene expression in W7 Δ luxS fluctuated slightly from 16 to 32 hours with fold-changes (compared to wildtype) of 1.328 (16h) down to 0.8941 (24h) and then returning to 1.044 (32h) (Figure 2a). However, none of these differences were statistically significant. We observed steadily decreasing levels of *cmeR* transcription from 8 to 32 hours with fold changes (compared to wildtype) starting at 1.51 down to 0.529 from an average of three independent experiments (Figure 2b); however, these changes did not reach the level of statistical significance.

These data support the conclusion that the absence of a functional LuxS enzyme (W7 Δ luxS) had no effect on the expression of efflux genes *cmeB* and *cmeR*. Despite the lack of significant differences in efflux gene expression, the consistency of the decreasing expression levels of *cmeR* suggest a potential trend that could have biological significance in different environments to those tested in this study and would warrant additional work in the future.

Effect of luxS mutation on cell morphology

Cell morphology is critical for motility and overall colonization of *C. jejuni* in the host intestinal environment. To date, only one study has looked at cell morphology of a *luxS* mutant in *C. jejuni* 81116 using transmission electron microscopy and found no differences in cell shape or flagellar morphology compared to wildtype (Jeon et al., 2003). We utilized two approaches to measure cell morphology of *luxS* mutants at the population level and individual cell level. To compare morphology within a population, we used the ImageStream^X® multispectral imaging flow cytometer to measure 17 different morphological features for W7 and IA3902 wildtype, *luxS* mutant and *luxS* complement strains. The RD value, which is the difference of means between two strains divided by the sum of standard deviation, was calculated to compare features between W7 or IA3902 strains grown in the same temperature. As shown in Table 3, none of the comparisons for any of the morphological features had an RD value over 1, which suggests that the *luxS* mutation had no impact on the cell morphology features measured in this study.

Transmission electron microscopy was also used for closer inspection of individual cell morphology. In agreement with the ImageStream^X® results, we also found no subjective evidence of changes in cell and flagellar morphology between wildtype, *luxS* mutant, and complement of both strain backgrounds in three separate replicates (Figure 3).

Effect of luxS mutation on motility through mucin

Motility is vital for *C. jejuni* to penetrate the mucins and establish colonization of the intestinal mucosal lining (Alemka et al., 2012). Though numerous studies have found differences in motility between a *C. jejuni* wildtype and *luxS* mutants on motility agar (Elvers and Park, 2002; He et al., 2008; Holmes et al., 2009), none have tested the motility of a *luxS* mutant in mucin *in vitro*. Given the biological importance and relevance of mucin penetration in the physiology of *C. jejuni*, we simulated motility through mucin, comparing the wildtype, *luxS* mutant and *luxS* complement strains of both W7

and IA3902 using a mucin column incubated at 37°C or 42°C. When we compared the bacterial counts at each fraction of the mucin column, we found no significant differences in most of the fractions regardless of the incubation temperature (Figures 4A, 5A and 5C). The only fraction that showed statistically significant differences between wildtype and *luxS* mutant mucin penetration was that of W7Δ*luxS* and the wildtype in fraction 9 when incubated at 42°C (Figure 4C). In this fraction the W7Δ*luxS* strain showed a decreased CFU count compared to the wildtype. Given this result it seemed that there were two plausible possibilities that would explain this finding. Either less of the mutant cells were able to penetrate the mucin at all, or the mutant cells had readily penetrated the mucin and were now located in the deeper fractions of the mucin column. We therefore calculated the total CFU (colony forming units) of W7Δ*luxS* from fractions 1 through 9 (i.e. the sum of the CFU counts in all fractions) and compared to W7 and W7Δ*luxS*Δ*Sc*. While we did not find any statistically significant differences in the mean total CFU of W7Δ*luxS* compared to wildtype (Figure 4D), we did see that the mean CFU of W7Δ*luxS* in fractions 1-9 was noticeably lower than W7 and W7Δ*luxS*Δ*Sc*. Taken together we believe that this may contribute to the significantly lower number of W7Δ*luxS* observed at fraction 9 of the mucin column and suggest that the W7Δ*luxS* mutant does have a mucin penetration defect at 42°C. The mucin penetration data of W7Δ*luxS*Δ*Sc* at 42°C show that complementing the *luxS* gene partially restored the mucin penetration ability of the strain suggesting that the mucin penetration defect observed in W7Δ*luxS* is indeed LuxS-mediated. We also compared the total CFU count from fractions 1-9 for W7Δ*luxS* at 37°C, and IA3902Δ*luxS* incubated at 37°C or 42°C to wildtype to see if there were any differences in total number of *luxS* mutants that were present in these fractions. When we compared with their respective wildtype, we found no significant differences in total CFU counts (Figures 4B, 5B and 5D), consistent with the findings of the individual fraction analysis for each of these samples.

Though the number of W7 Δ luxS cells in fraction 9 (42°C) was statistically lower than W7, the biological significance of this observation regarding the physiology of W7 Δ luxS remains unclear. Regardless, evidence strongly suggests that the motility of the *luxS* mutant through mucin is not affected by morphology based on the lack of morphological differences found between wildtype and *luxS* mutants.

Effect of luxS mutation on the AMC function

Lastly, to assess whether the *luxS* mutation affected the function of the AMC, and to what extent, we utilized the LC-ECI-MS/MS method to analyze intracellular levels of AMC metabolites in IA3902 wildtype, *luxS* mutant (IA3902 Δ luxS), and *luxS* complement (IA3902 Δ luxSc). Results of the metabolite levels, shown as a ratio of metabolite peak areas to internal standards, support our predicted results for our strains (Table 4).

We expected the *luxS* mutation to cause a decrease in homocysteine levels, as this metabolite requires a functional LuxS enzyme for its synthesis. Indeed, this metabolite was almost undetectable in IA3902 Δ luxS while IA3902 and IA3902 Δ luxSc showed normal levels (Table 4). Homocysteine is the product of LuxS catalysis of *S*-ribosylhomocysteine (SRH) to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (Winzer et al., 2002). In addition, because LuxS is non-functional in the mutant, SRH should accumulate at higher levels in the cell. This is clearly evident in IA3902 Δ luxS with SRH levels being three times that of wildtype (Table 4).

Cystathionine levels were at least 5 times lower in IA3902 Δ luxS (0.0030) compared to wildtype (0.0159) and IA3902 Δ luxSc (0.0162), which is expected as homocysteine can be a precursor for the formation of cystathionine (Selhub, 1999). Methionine levels were also reduced in IA3902 Δ luxS (1.7195) compared to wildtype (2.8010), although far from complete depletion. We attribute this finding to the fact that homocysteine levels are already low in IA3902 Δ luxS and any

remaining homocysteine would be converted to methionine, which will also be comparably lower than wildtype.

We found the *S*-adenosylmethionine (SAM) levels reduced by roughly 25% in IA3902 Δ luxS compared to wildtype and IA3902 Δ luxSc (Table 4). SAM is a critical methyl donor for a variety of metabolic and biosynthetic reactions, including methylation of macromolecules such as DNA (Parveen and Cornell, 2011). The slightly lower levels of SAM suggest that the *luxS* mutation could disrupt the primary function of the AMC in recycling SAM.

Slightly higher levels of the toxic metabolite *S*-adenosylhomocysteine (SAH) were observed in IA3902 Δ luxS in contrast to wildtype and IA3902 Δ luxSc (Table 4). This is assumed to be due to the rapid depletion of SAM levels while simultaneously accumulating more SAH. The lack of a functional LuxS enzyme may then slow down or halt the conversion of SAH to SRH. Accumulation of SAH can also inhibit various methylation reactions and polyamine synthesis, thereby altering growth and potentially killing the organism (Cornell et al., 1996). However, because we did not observe any growth differences between our wildtype and mutant, we can only assume that the levels of SAH accumulating in IA3902 Δ luxS are not high enough to prohibit growth. However, the SAH levels may have enough impact to negatively affect the functions of one or more cellular processes including methylation and polyamine synthesis.

The wildtype and IA3902 Δ luxSc showed a complete AMC with comparable levels of almost all major AMC metabolites. The only exception was that no SRH was detected in the complement. This is not a major surprise as the complement strain is believed to have an inherently higher level of LuxS activity than wildtype, due to being expressed in the complement strain by a constitutive promoter instead of its native promoter. This would, in turn, yield a higher rate of conversion of SRH to homocysteine and DPD than wildtype, and thus quickly depleting the supply of available SRH.

Discussion

We set out to investigate the impact of the *luxS* mutation on colonization factors that have not been examined previously and are critical to *C. jejuni* pathogenesis. These factors included multi-drug efflux pump systems, cell morphology, motility, and the metabolite levels of the AMC cycle. The results of this study partially supported our hypothesis that *luxS* mutation has a phenotypic effect on some of the traits we analyzed. For one, the *luxS* mutation does cause a phenotypic change in the AMC metabolite levels observed in the mutant. This was confirmed by analysis of AMC metabolite levels in IA3902 Δ *luxS*, which contained lower levels of homocysteine and higher levels of SRH than wildtype and complement. This is expected as LuxS is responsible for the conversion of SRH to homocysteine and DPD (De Keersmaecker et al., 2006). The absence of a functional LuxS would lead to accumulation of SRH and simultaneous decreased levels of available homocysteine as it is continually used to form methionine and subsequently the SAM methyl donor.

Recently we profiled the methylome of our IA3902 and IA3902 Δ *luxS* strains (Mou et al., 2015). We hypothesized that the *luxS* mutation would severely reduce the level of methylation in the *luxS* mutant compared to wildtype. The logic behind the hypothesis was that the *luxS* mutation would disrupt the AMC cycle and, as a result, reduce the amount of available SAM to donate methyl groups for DNA methylation, which relies on SAM as its primary methyl donor (Parveen and Cornell, 2011). However, we found the *luxS* mutant methylome profile was unchanged compared to wildtype, suggesting the *luxS* mutation had no effect on DNA methylation. Our AMC metabolite results further support those results as there was only a 25% reduction in SAM metabolite levels in our *luxS* mutant compared to other metabolites that had much higher percentages of reduction than wildtype. The smaller reduction level of SAM in the *luxS* mutant suggests that the *luxS* mutant still had a sufficient amount of SAM to allow normal DNA methylation. Although we cannot speculate on the minimum level of SAM required for biological processes like DNA methylation, the fact that there were

measurable levels of methionine in the *luxS* mutant also confirms that there is still some source providing the methionine other than the AMC cycle. Another possibility is precursors such as cystathionine that are being fed into the cycle from an outside source that would allow for the synthesis of methionine (Howlett et al., 2014). This would thus enable processes like DNA methylation to function relatively normally. These findings have additional significance when one considers the dual role of LuxS and phenotypic changes associated with its mutagenesis. If these phenotypes are associated with alterations in metabolic function of the AMC, these data would argue that it is not likely to be associated with significant changes in methionine or SAM.

As for the *cmeB* and *cmeR* efflux gene expression levels in our *luxS* mutant, no significant differences from wildtype expression levels were found. However, there was a general trend of decreased expression of *cmeR* efflux gene in the *luxS* mutant in the conditions tested. While any potential biological significance of this trend is unclear, it does provide a rationale for evaluating these changes in different environmental conditions, especially *in vivo*. One could speculate that the decreased expression of *cmeR* gene in W7 Δ luxS may be triggered by accumulating levels of SAH, as evidenced from the AMC metabolite levels analysis. The lower levels of *cmeR* gene expression would result in less CmeR protein and thus decreased repression of CmeABC. This in turn might lead to increased efflux expression and function that would then assist in extruding the toxic SAH metabolite out of the cell. A similar type of response is evident in *P. aeruginosa* where multi-drug efflux systems were shown to be important in removing toxic metabolites linked to the quorum sensing system function of the organism (Aendekerk et al., 2005). However, the unchanged levels of *cmeB* expression is puzzling as the decreasing *cmeR* expression would also be expected to lead to fewer CmeR proteins to repress the CmeABC operon, and therefore increased opportunity for more *cmeB* expression. This lack of change in *cmeB* expression would argue that the trend of decreased *cmeR* expression is not likely biologically significant in the conditions tested. Preliminary work from our group (data not

shown) found no changes in *cmeB* expression when we exposed broth cultures of W7Δ*luxS* to lethal or sublethal doses of extracellular SAH. This, along with our W7Δ*luxS* *cmeB* expression data grown under basal conditions, suggest that CmeABC expression is not induced by SAH and that the cell may not recruit CmeABC efflux activities in response to high extracellular SAH levels accumulating inside W7Δ*luxS*. Despite the lack of change in *cmeB* expression, there is potential for the *luxS* mutation to trigger expression of other efflux systems associated with the CmeR regulator (Guo et al., 2008). These other efflux transporters could potentially play a role in extrude toxic SAH metabolites out of the cell.

In regards to motility through mucin and cellular morphology, motility through mucin was mostly unaffected by the *luxS* mutation, especially for IA3902. However, motility through mucin was slightly affected in one of our *luxS* mutants (W7Δ*luxS*) when incubated at 42°C, but not at 37°C. Plummer et al. previously found that the IA3902 and W7 *luxS* mutants showed decreased motility compared to wildtype (but with no statistical difference) on a motility agar assay (Plummer et al., 2012). Follow-up studies of those same mutants using real-time imaging flow cytometry and transmission electron microscopy in our experiments found no evidence of changes in morphology. This suggests that morphology is not responsible for the decreased mucin motility. We did observe significantly lower number of W7Δ*luxS* at fraction 9 of the mucin column compared to wildtype, as well as low, but not statistically significant, average total CFU of W7Δ*luxS* in fractions 1 through 9 at 42°C. Complementation of the *luxS* gene partially restored mucin penetration in W7Δ*luxS*, indicating that the mucin motility defect is correlated with the *luxS* mutation. The decreased mucin motility of W7Δ*luxS* may be associated or caused by the motility decrease seen in W7Δ*luxS* (also incubated at 42°C) by Plummer et al. (Plummer et al., 2012). However, it is unknown what mechanism of the *luxS* mutation would lead to the observed mucin penetration results for W7Δ*luxS*. These observations may be specific to the assay as motility is assessed through the use of plates rather than through a homogenous liquid medium.

Other studies have also witnessed similar motility and morphology phenotypes in a *luxS* mutant in *C. jejuni* 81116. Jeon et al. found decreased motility on the motility agar test in a *C. jejuni* 81116 *luxS* mutant, but no changes in cell shape or flagellar morphology under transmission electron microscope analysis (Jeon et al., 2003). Though 81116 is not genetically identical to 11168, the fact that our IA3902 and W7 *luxS* mutant strains exhibited the same motility and cell morphology phenotypes as the 81116 *luxS* mutant indicates that what we observed in our *luxS* mutants is not unique and may share similar LuxS-mediated mechanisms as 81116 *luxS* mutants. However, no further studies looking at the phenotypic effects and mechanisms of the LuxS system on other colonization factors in 81116 have been examined.

The findings from this study reveal how limited our understanding is of the underlying mechanisms of LuxS on the colonization step of *C. jejuni* pathogenesis. The effects of the *luxS* mutation on other aspects of *C. jejuni* pathogenesis steps are also needed to provide a more comprehensive look of the potential regulatory role of LuxS in *C. jejuni*. Potential *C. jejuni* virulence attributes that need to be tested include *in vitro* adhesion and invasion abilities, taxis towards chemoattractants like chicken mucin, L-asparagine, formate, and D-lactate (Vegge et al., 2009); and how stress conditions, like oxidative stress (He et al., 2008), would change the efflux expression in a *luxS* mutant.

Conclusion

In conclusion, we did not find any significant differences in *cmeABC* and *cmeR* expression and cell morphology between our *luxS* mutant and wildtype, which suggest that these phenotypes are not affected by the *luxS* mutation. While largely unaffected in both strains and temperatures, mucin penetration ability was altered in a single condition tested, the W7 Δ *luxS* strain at 42°C. The *luxS* mutation also significantly disrupted the AMC cycle. Notably, it reduced the available levels of

metabolites like homocysteine and cystathionine that require the immediate enzymatic function of LuxS to catalyze the reactions. The results presented here, collectively with previous LuxS studies in *C. jejuni*, call attention to the complexity and our limited understanding of the LuxS system's role in *C. jejuni* colonization and general pathobiology. The questions postulated above will help the field move towards complete characterization of the LuxS mechanisms in *C. jejuni* disease and enable utilization of that knowledge towards development of novel therapeutics against *C. jejuni* diseases.

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Tables

Table 1. List of all bacterial strains used in this study.

Bacterial strains	Description	Source
W7	Highly motile variant of NCTC 11168	(Plummer et al., 2012)
W7ΔluxS	W7 mutant with <i>kan</i> ^R inserted into luxS	(Plummer et al., 2012)
W7ΔluxSc	W7ΔluxS with a chromosomal complement of LuxS, contains <i>cm</i> ^R and <i>kan</i> ^R genes (acquired from IA3902ΔluxSc)	This study
IA3902	<i>C. jejuni</i> isolate of clone SA	(Sahin et al., 2008)
IA3902ΔluxS	IA3902 mutant with <i>kan</i> ^R inserted into luxS	(Plummer et al., 2012)
IA3902ΔluxSc	IA3902ΔluxS with a chromosomal complement of LuxS, contains <i>cm</i> ^R and <i>kan</i> ^R genes	(Plummer et al., 2012)

Table 2. Primers used in this study.

Primer	Sequence (5' – 3')	Reference
cmeB-F	AGAAGCGATTGCTCAAGTAG	Qijing Zhang (Eva Barton)
cmeB-R	TCATATTGGGCAGCTAGAAT	Qijing Zhang (Eva Barton)
cmeR-F	CACAAAAAGTTTTAGCCAGACAAG	This study
cmeR-R	CACTCAAACCTTGTTTCTTGGTATCC	This study
16S-rRNA-F	TACCTGGGCTTGATATCCTA	(Han et al., 2008)
16S-rRNA-R	GGACTTAACCCAACATCTCA	(Han et al., 2008)

Table 3. Normalization scores of 17 morphological characteristics of W7 and IA3902 strains grown at 37°C (A) and 42°C (B). At least 1500 cell images for each strain were captured and calculated for morphology differences using 17 different morphological features for comparison. The resulting normalization scores or RD values of *luxS* mutant and *luxS* complement were normalized to their respective wildtype. RD values of over 1 were considered significant differences, although no significant differences were detected in any of the morphology characteristics compared here.

A.

Strain	Area	Aspect Ratio Intensity	Aspect Ratio	Circularity	Diameter	Elongatedness	Height	Length
W7Δ <i>luxS</i>	0.06998	0.4554	0.1304	0.3051	0.3309	0.2283	0.2651	0.2797
W7Δ <i>luxSc</i>	-0.09066	0.1092	0.1053	-0.01149	-0.08819	-0.09615	-0.1239	-0.1239
IA3902Δ <i>luxS</i>	0.01424	-0.009920	-0.01094	0.0004532	0.01496	0.006580	0.01801	0.01801
IA3902Δ <i>luxSc</i>	-0.1048	-0.08953	-0.09179	-0.1003	-0.09447	0.04537	-0.07641	-0.07665

Strain	Major Axis Intensity	Major Axis	Minor Axis Intensity	Minor Axis	Perimeter	Shape Ratio	Thickness Maximum	Thickness Minimum	Width
W7Δ <i>luxS</i>	0.24602	0.3472	0.2194	0.09199	0.5902	0.2269	0.3115	0.2055	0.1410
W7Δ <i>luxSc</i>	-0.1272	-0.1281	-0.04804	-0.04933	-0.09979	0.07446	-0.05032	-0.04750	-0.05486
IA3902Δ <i>luxS</i>	0.02357	0.02311	0.009048	0.007882	0.01576	-0.02327	0.001237	-0.002118	0.009762
IA3902Δ <i>luxSc</i>	-0.07936	-0.07229	-0.1096	-0.1081	-0.09158	-0.05175	-0.1015	-0.08291	-0.1038

Table 3 (continued).

B.

Strain	Area	Aspect Ratio Intensity	Aspect Ratio	Circularity	Diameter	Elongatedness	Height	Length
W7ΔluxS	0.01058	0.1674	0.1544	0.06220	0.01261	-0.1124	-0.02258	-0.02258
W7ΔluxSc	0.01324	-0.03536	-0.03291	-8.587E-05	0.01336	0.02314	0.01922	0.01922
IA3902ΔluxS	-0.07202	-0.09764	-0.09970	-0.09270	-0.05331	0.07671	-0.01310	-0.01310
IA3902ΔluxSc	0.0004616	-0.04469	-0.04301	-0.01429	0.009081	0.02322	0.02238	0.02238

Strain	Major Axis Intensity	Major Axis	Minor Axis Intensity	Minor Axis	Perimeter	Shape Ratio	Thickness Maximum	Thickness Minimum	Width
W7ΔluxS	-0.03814	-0.03353	0.04360	0.04602	0.004558	0.06395	0.02634	0.02209	0.03860
W7ΔluxSc	0.02197	0.02189	0.004262	0.004865	0.01408	-0.008287	0.004899	0.0086479	0.0069818
IA3902ΔluxS	-0.005244	-0.005037	-0.07815	-0.07883	-0.04493	-0.07250	-0.07911	-0.06668	-0.07372
IA3902ΔluxSc	0.01922	0.02632	-0.01177	-0.006327	0.01199	-0.03115	-0.005518	-0.004038	-0.002413

Table 4. LC-ESI-MS/MS analysis shows expected AMC metabolite levels within *luxS* mutant compared to wildtype and *luxS* complement. Values are presented as ratios of metabolite peak areas to internal standards corrected to the endogenous concentration of the metabolites in a standard cell pellet. Metabolite abbreviations: SAM (*S*-adenosylmethionine), SAH (*S*-adenosylhomocysteine), SRH (*S*-ribosylhomocysteine), Hcyst (Homocysteine), Met (Methionine), Cysta (Cystathionine).

Strain	SAM	SAH	SRH	Hcyst	Met	Cysta
IA3902	0.4090	0.0506	0.0092	0.2842	2.8010	0.0159
IA3902Δ<i>luxS</i>	0.3050	0.0594	0.0332	0.0020	1.7195	0.0030
IA3902Δ<i>luxSc</i>	0.3614	0.0494	0.0000	0.2345	1.3403	0.0162

Figure Captions

Figure 1.

Diagram showing role of LuxS in the activated methyl cycle. In the activated methyl cycle, the LuxS enzyme catalyzes the conversion of *S*-ribosylhomocysteine to homocysteine while also producing the by-product 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor of the autoinducer-2 (AI-2) signaling molecule. Metabolite abbreviations: Hcyst (Homocysteine), Cysta (Cystathionine), Met (Methionine), SAM (*S*-adenosylmethionine), SAH (*S*-adenosylhomocysteine), SRH (*S*-ribosylhomocysteine).

Figure 2.

Expression of *cmeB* (A) and *cmeR* (B) in W7 Δ luxS and W7 Δ luxSc compared to W7 wildtype over a 48-hour growth period. Samples of broth culture grown at 42°C were taken at time points 8, 16, 24, and 32 hours to measure transcript levels of *cmeB* and *cmeR* using qPCR. Bars represent the mean transcript fold-change $\pm$ SD of three independent experiments, relative to W7, with 16s rRNA used as an internal control. No significant differences were found.

Figure 3.

Transmission electron micrographs reveal no morphological changes in cell shape or flagella in strains W7 wildtype (A), *luxS* mutant (Δ luxS) (B), *luxS* complement (Δ luxSc) (C); IA3902 wildtype (D), Δ luxS (E), and Δ luxSc (F).

Figure 4.

Penetration of W7, W7 Δ luxS, and W7 Δ luxSc through mucus while incubating at 37°C (A, B) or 42°C (C, D). Columns of 30mg/ml porcine gastric mucin in PBS were prepared in 1-ml tuberculin syringes, with bacteria inocula applied to the top of the column. The columns were incubated at 37°C (A, B) or 42°C (C, D) for 30 min. After the incubation period, each fraction consisting of 0.1ml mucin was collected from the bottom of the column and plated for bacterial quantification. Bars represent the mean CFU/ml $\pm$ SD of five independent experiments. Fraction 1 represents the bottom of the column, fraction 9 at the top. Graphs for Figures 3A and 3C depict mean CFU/ml $\pm$ SD at each fraction. Figures 3B and 3D depict mean CFU/ml $\pm$ SD of total bacteria from fractions 1 through 9. Asterisks indicate significantly lower number of W7 Δ luxS at the specific fraction of mucin column compared to wildtype.

Figure 5.

Penetration of IA3902, IA3902 Δ luxS, and IA3902 Δ luxSc through mucus while incubating at 37°C (A, B) or 42°C (C, D). Assay was the same as described in Figure 3. Bars represent the mean CFU/ml $\pm$ SD of three independent experiments. Fraction 1 represents the bottom of the column, fraction 9 at the top. Graphs for Figures 4A and 4C depict mean CFU/ml $\pm$ SD at each fraction. Figures 4B and 4D depict mean CFU/ml $\pm$ SD of total bacteria from fractions 1 through 9. No significant differences were found.

A **B**