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Critical Role of LuxS in the Virulence of *Campylobacter jejuni* in a Guinea Pig Model of Abortion

Paul Plummer,[a,b] Orhan Sahin,[b] Eric Burrough,[c]* Rachel Sippy,[b] Kathy Mou,[b] Jessica Rabenold,[b] Mike Yaeger,[c] and Qijing Zhang[b]

Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA[a]; Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA[b]; and Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA[c]

Previous studies on *Campylobacter jejuni* have demonstrated the role of LuxS in motility, cytolethal distending toxin production, agglutination, and intestinal colonization; however, its direct involvement in virulence has not been reported. In this study, we demonstrate a direct role of *luxS* in the virulence of *C. jejuni* in two different animal hosts. The IA3902 strain, a highly virulent sheep abortion strain recently described by our laboratory, along with its isogenic *luxS* mutant and *luxS* complement strains, was inoculated by the oral route into both a pregnant guinea pig virulence model and a chicken colonization model. In both cases, the IA3902 *luxS* mutant demonstrated a complete loss of ability to colonize the intestinal tract. In the pregnant model, the mutant also failed to induce abortion, while the wild-type strain was highly abortifacient. Genetic complementation of the *luxS* gene fully restored the virulent phenotype in both models. Interestingly, when the organism was inoculated into guinea pigs by the intraperitoneal route, no difference in virulence (abortion induction) was observed between the *luxS* mutant and the wild-type strain, suggesting that the defect in virulence following oral inoculation is likely associated with a defect in colonization and/or translocation of the organism out of the intestine. These studies provide the first direct evidence that LuxS plays an important role in the virulence of *C. jejuni* using an *in vivo* model of natural disease.

Campylobacter jejuni is a leading cause of food-borne bacterial enteritis in the United States and worldwide. Generally, it is associated with self-limiting enteritis that is indistinguishable from other bacterial causes of diarrhea, including *Salmonella* and shigellosis; however, it can also cause extraintestinal infections, such as bacteremia and abortion (3). In recent years, the rising incidence of antimicrobial resistance in *C. jejuni* to both macrolides and fluoroquinolones has prompted concerns over the use of these agents in food-producing animals and the future utility of these compounds in disease therapy (48). In a recent registry-based cohort study, it was determined that patients with antimicrobial-resistant strains of *C. jejuni* had a higher risk of a postinfection adverse event within 30 days of sample submission than patients with antimicrobial-sensitive strains (22). These findings highlight the clinical consequences of antimicrobial-resistant *C. jejuni* and demonstrate the need for additional research focused on identification of alternate molecular targets that may be useful in therapeutic interventions of resistant *C. jejuni* strains.

Despite the enhanced research efforts, the pathogenic mechanisms and virulence factors, especially those responsible for systemic dissemination, of *C. jejuni* infections remain poorly understood. Recent studies have demonstrated that the intestinal colonization of *C. jejuni* is a complex event involving multiple factors and pathogen-host interactions (2, 8, 9, 12, 14–18, 23, 24, 28, 29, 32, 33). However, the detailed mechanisms responsible for initial translocation of *C. jejuni* across the gastrointestinal mucosa and the subsequent movement and seeding of distant organs have not been well described. With an increasing incidence of treatment failures resulting from antibiotic-resistant isolates, there is a vital need for an improved understanding of the pathogenic mechanisms responsible for the disease manifestations of this organism.

One target for therapeutic control being explored in a variety of bacterial species is the autoinducer-2 (AI-2)-mediated quorum-sensing pathway (40, 44). AI-2 is synthesized as a by-product of the LuxS enzyme, a key enzyme in the activated methyl cycle of bacteria, where it is responsible for converting *S*-ribosylhomocysteine into homocysteine (36). It is known that *C. jejuni* produces functional AI-2 and that mutagenesis of the *luxS* gene results in decreases in motility, agglutination, cytolethal distending toxin production, and chicken colonization (10, 13, 26, 27, 39).

Certain *C. jejuni* strains have a unique affinity for the uteroplacental unit in pregnant livestock and are known to be an important cause of abortions in ruminants (1, 6, 42). *C. jejuni* has also been reported to be a sporadic cause of abortion in humans (4, 37, 43). Work in our laboratory has demonstrated that a single clone of *C. jejuni*, named SA (for *s*heep *a*bortion), has emerged in recent years to be the predominant cause of sheep abortions and has also been identified to be a cause of bovine, caprine, and canine abortions (42). This clone has also been linked to a significant number of human disease cases, suggesting that it poses an emerging zoonotic threat (O. Sahin et al., unpublished data). IA3902, an isolate of clone SA, is highly virulent in the guinea pig abortion model and consistently results in very high levels of uteroplacental infec-

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Address correspondence to Qijing Zhang, zhang123@iastate.edu.
* Present address: Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA.

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tions and abortion (7). The highly stable virulent phenotype of this strain combined with a reliable animal model of pathogenicity provides us with a unique opportunity to evaluate the role of LuxS in the virulence of *C. jejuni*.

The purpose of this study was to determine the role of LuxS in the *in vivo* pathogenicity of *C. jejuni* IA3902 using two animal models: intestinal colonization in chickens and abortion induction in guinea pigs. Our results clearly demonstrate that the *luxS* mutant of *C. jejuni* was completely attenuated in the guinea pig abortion model when inoculated via oral gavage, suggesting that this gene does play a significant role in the pathogenesis of *Campylobacter*-associated abortions. These findings provide compelling evidence that the *luxS* gene is important for the pathogenicity of *C. jejuni*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Campylobacter jejuni* strains were grown in Mueller-Hinton (MH) broth or agar in a microaerophilic (5% O_2, 10% CO_2, and 85% N_2) environment and incubated at 42°C. Chicken studies were performed using wild-type strain W7 (a highly motile variant of NCTC 11168 [ATCC 700819]), its isogenic LuxS mutant (W7ΔluxS), IA3902 (3902), its LuxS mutant (3902ΔluxS), and a chromosomally encoded LuxS complement of IA3902 (3902ΔluxSc). For the guinea pig abortion model, *C. jejuni* strain IA3902, its LuxS mutant (3902ΔluxS), and a chromosomally encoded complement strain (3902ΔluxSc) were cultured as described above. When necessary for selection, the strains were grown on MH agar containing either kanamycin (30 μg/ml) or chloramphenicol (4 μg/ml). *Escherichia coli* strains used for genetic manipulation were grown on Luria-Bertani (LB) broth or agar containing kanamycin or chloramphenicol as necessary for selection. For isolation of *C. jejuni* from *in vivo* samples, MH agar was supplemented with Preston *Campylobacter* selective supplement (Oxoid, Hampshire, United Kingdom) and *Campylobacter* growth supplement (Oxoid, Hampshire, United Kingdom) as per the manufacturer's recommendations. This selective medium is referred as MH+sel+sup in this paper.

Recombinant DNA techniques. Natural transformation of the W7 and IA3902 strains with genomic DNA from a previous LuxS mutant developed in our laboratory was utilized to construct the W7ΔluxS and 3902ΔluxS strains, respectively, as previously described (38). For complementation of the *luxS* mutants, a copy of the *luxS* gene (including its putative promoter) was inserted into W7ΔluxS by homologous recombination into the intergenic region of the rRNA gene operon as described by Karlyshev and Wren with some modifications (30). Briefly, the ribosomal DNA sequence was amplified and cloned into the pGEM-T Easy plasmid (Promega), which was subsequently digested with MfeI. The chloramphenicol acetyltransferase gene was amplified from pUOA18 (47) and inserted into the digested plasmid to produce pRRC (35). The *luxS* gene, along with 500 bp of its upstream sequence containing the putative promoter region, was cloned into an XbaI site of pRRC located upstream of the chloramphenicol resistance determinant. This plasmid was then utilized as a suicide vector and electroporated into W7ΔluxS. Homologous recombination of this construct with the chromosomal rRNA gene cluster resulted in insertion of a wild-type copy of the *luxS* gene into W7ΔluxS. The transformants were selected using chloramphenicol and confirmed by both PCR and the AI-2 assay using the *Vibrio harveyi* bioluminescence bioassay. Natural transformation with genomic DNA purified from the W7ΔluxS complement was used to obtain a complement of the 3902ΔluxS mutant strain, generating 3902ΔluxSc. PCR and sequence analysis confirmed that the expected mutation and complementation constructs were present (data not shown).

AI-2 bioluminescence assay. The *Vibrio harveyi* bioluminescence assay was performed as previously described (45). Luminescence was measured every 15 min for 6 h, and the values (relative light units [RLUs]) utilized for analysis were those occurring at the time point with the lowest RLU value for the negative-control wells.

Guinea pig abortion model. A previously described model for *Campylobacter*-induced abortion was used in this study (7). Time-bred female Hartley guinea pigs were obtained from a commercial supplier (Elm Hill Labs, Chelmsford, MA) at 20 to 24 days of pregnancy. Animals were allowed a 5-day acclimation period, and fecal swab specimens were collected to confirm *Campylobacter*-negative status prior to inoculation. Three strains were used for inoculation in the guinea pig studies, including IA3902, a highly virulent ovine abortion isolate (42), its *luxS* mutant (3902ΔluxS), and, in the oral challenge model, a chromosomally encoded *luxS* complement of the 3902ΔluxS strain (3902ΔluxSc). The inoculum was prepared by growing strains as a lawn on MH agar plates for 16 h at 42°C. The bacterial cells were harvested and resuspended in MH broth, which was adjusted to an optical density at 600 nm (OD_{600}) of approximately 0.5 to provide an inoculum of approximately 1×10^8 CFU/ml. Each guinea pig was administered 1.0 ml of inoculum by oral gavage. Guinea pigs were observed twice daily for signs of vaginal bleeding, aborted fetuses, or significant weight loss. At the first sign of impending abortion, affected guinea pigs were euthanized using an intraperitoneal injection of pentobarbital sodium and phenytoin sodium (Beuthanasia-D). Animals were subjected to an immediate necropsy with collection of uterus, placenta, fetal liver and lungs, blood, bile, and fecal material for *Campylobacter* culture. At 21 days postinoculation, those guinea pigs that had not previously aborted were euthanized and necropsied as described above. During the study, guinea pigs were housed in individual cages with *ad libitum* access to food and water. Cages were grouped by inoculation strain. For intraperitoneal inoculation, the procedure was essentially identical to that described above, except that 1 ml of 1×10^6-CFU/ml suspension was injected into the abdominal cavity instead of delivered by oral gavage. Guinea pigs were monitored and necropsied as described above, and tissues were processed as follows.

For the oral challenge model, feces, bile, blood, maternal liver and uterus, fetal lung/liver, and placenta were collected and immediately refrigerated until processing, which occurred within 4 h of collection. At necropsy, a rayon swab was used to sample the rectum and distal colon of each guinea pig and then used for direct plating onto MH agar. For culture of bile and blood, 2 to 3 drops of the fluid (approximately 0.1 ml) was directly placed on the MH+sel+sup plates and distributed using a sterile spreader. Maternal liver, placenta, and uterus and fetal lung/liver homogenate were macerated using a sterile scalpel blade and cultured by streaking the tissue with a sterile cotton swab to a MH+sel+sup plate. Plates were incubated in a microaerophilic environment at 42°C for 48 to 72 h. In all cases, *C. jejuni* colonies were identified on the basis of morphological appearance, and a portion of the representative colonies was confirmed using PCR and biochemical testing.

In the intraperitoneal challenge study, samples were processed in a manner identical to that described above, with the exception that for this portion of the study up to three placentas (when available) were pooled and used for quantitative bacterial enumeration. Briefly, the pooled placentas were weighed and diluted 1 to 1 (wt/vol) with MH broth. They were then macerated and mixed using a stomacher. The macerated samples were allowed to sit briefly, and the supernatant was used in a 10-fold serial dilution prior to plating on MH+sel+sup plates for enumeration.

Chicken colonization and competitive fitness. For colonization studies, 1-day-old Ross × Cobb chicks were obtained from a commercial hatchery. All chicks were screened and found to be negative for *Campylobacter* shedding upon arrival using cloacal swabs cultured on MH+sel+sup medium. On day 3 of age, chicks were inoculated by oral gavage with 100 μl of bacterial suspension containing a total of approximately 1×10^7 CFU/ml of either the wild-type or mutant strain. The groups were housed in separate rooms with no contact. Randomly selected birds were sacrificed at predetermined time intervals using carbon dioxide asphyxiation. Necropsy was promptly performed and cecal contents were aseptically collected and placed on ice until further processing.

Samples were subject to 10-fold serial dilution and cultured as described above. For competitive fitness experiments, the strains were grown for 16 h on MH agar plates, harvested into MH broth, and adjusted to an OD_{600} of 0.1, and equal volumes of each strain were mixed in a 1:1 ratio for inoculation (0.2 ml per bird). Monoinoculated birds were given an equal dose (0.1 ml) of the individual cultures. Cecal contents were collected and processed as described above. To confirm that there was not cross-contamination of the bacterial strains between treatment groups, representative isolates from each group were confirmed to be the inoculated strain by antimicrobial selection. In the chicken experiments, the detection limit of the plating methods was 100 CFU/g of feces. A sample from which no *Campylobacter* colonies were detected was arbitrarily assigned a value of 100 for the purposes of calculating means and statistical analysis. All animal experiments conducted as part of the study were approved by and in accordance with the Institutional Animal Care and Use Committee of Iowa State University prior to initiation of the project.

Motility. All strains used in the experiment were assessed for motility using semisolid MH agar medium. Briefly, the strains were grown overnight, and the OD_{600} was adjusted to 0.5. A single stab inoculation was made in the center of the plate, and the plate was incubated at 42°C for 30 h. The distance of swarming motility was measured at the widest portion of the swarm halo.

Statistics. For simultaneous comparison of monoinoculated and coinoculated colonization rates of chickens, an analysis of variance (ANOVA) was performed. If the null hypothesis that the groups were identical was rejected, then Bonferroni's posttest was performed for each of the individual paired groups, with the null hypothesis being that the colonization rates were identical between comparison groups. For simultaneously testing for a difference in the incidence of fetal/placental infection between all three comparison groups (i.e., the wild-type, *luxS* mutant, and complement strain groups), Pearson's chi-square test was utilized. Due to the low number of observations in some of the groups associated with lower than expected pregnancy rates, a simulation was performed with 100,000 iterations using the R statistical package to determine the *P* value (R, R Foundation for Statistical Computing, Austria). Subsequently, Fisher's exact test was used to test for a difference between all potential combinations of group pairs using R. For this component, the null hypothesis tested was that the true odds ratio for the two groups was 1. In all circumstances, a *P* value of less than 0.05 is considered significant and one of less than 0.01 is considered very significant.

RESULTS

Growth, AI-2 activity, and motility. The growth of wild-type strains, *luxS* mutants, and the complemented *luxS* mutants was measured in MH broth over a period of 48 h. According to OD_{600} readings, no statistically significant differences in growth kinetics were observed among the evaluated strains (data not shown). All wild-type and mutant strains were evaluated for the AI-2 phenotype using the *Vibrio harveyi* bioluminescence assay (Fig. 1). The wild-type strains (W7 and 3902) were both found to have luminescent values comparable to the value for the positive-control strain, demonstrating active AI-2 production. In contrast, both *luxS* mutant strains (W7ΔluxS and 3902ΔluxS) had baseline luminescence comparable to that of the negative control (MH broth alone). Interestingly, the complemented strain (3902ΔluxSc) had bioluminescence values significantly higher than those of both the wild-type and positive-control strains.

All isolates were screened for motility prior to use in the animal studies. The isolates were found to be highly motile, and the average motility observed at 30 h postinoculation did not vary between isolates, based on an ANOVA (data not shown). The *luxS* mutants had a trend toward decreased motility compared to the wild-type strains, consistent with previous reports; however, this trend did not result in statistical significance.

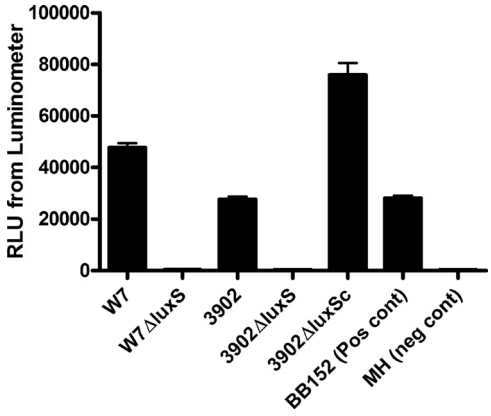

FIG 1 Quantification of AI-2 production by bacterial strains used in this study. AI-2 activity was measured by the bioluminescent *Vibrio harveyi* bioassay. Values are reported as RLUs. *Vibrio harveyi* strain BB152 is the positive control (Pos cont), and uninoculated MH medium serve as a negative control (neg control).

Guinea pig oral challenge. In the first study using pregnant guinea pigs, three groups of 12 time-bred guinea pigs were inoculated orally with 3902, 3902ΔluxS, and 3902ΔluxSc, respectively. Given the stage of gestation when the animals were purchased, the provider was unable to guarantee pregnancy in every guinea pig, and accordingly, some of the animals were determined to have not been pregnant on the basis of a lack of weight gain during the study and necropsy at the conclusion of the study. Three guinea pigs in the 3902 group, 5 guinea pigs in the 3902ΔluxS group, and 2 guinea pigs in the 3902ΔluxSc group were excluded from the data analysis due to a nonpregnant status. Additionally, one animal in the 3902ΔluxS group aborted on day 19 of the study but was negative for *C. jejuni* on all cultures (cecum, bile, blood, uterus, fetus, placenta, and fetal stomach contents) and lacked histopathologic features consistent with *C. jejuni*-induced abortions. In light of these findings, it was deemed that this abortion was unlikely to be associated with the test organism and the data for that animal were not utilized in subsequent analyses. The results showed that 3902 was abortifacient, resulting in a 56% abortion rate, while 3902ΔluxS was completely attenuated as a cause of abortion (Fig. 2). The 3902ΔluxSc complement restored virulence to the level of the wild-type strain, producing a 50% abortion rate (Fig. 2). A significant difference in fetal/placental infection rates was observed between the 3902 and 3902ΔluxS groups ($P = 0.034$), and a very significant difference was observed between the 3902ΔluxS and 3902ΔluxSc complement groups ($P = 0.0023$). No differences between the wild-type and complement groups were observed ($P = 0.34$). These results demonstrate that the mutagenesis of *luxS* attenuates the ability of *C. jejuni* to cause fetoplacental infection and abortion in the guinea pig model and that restoration of *luxS* function by gene complementation fully restores the virulence.

To facilitate understanding the cause of this loss of virulence, we evaluated the fecal shedding patterns of the three groups throughout the study. Fecal carriage results revealed on the basis of fecal shedding that the 3902ΔluxS strain was not able to colonize the inoculated guinea pigs and that the defect in colonization

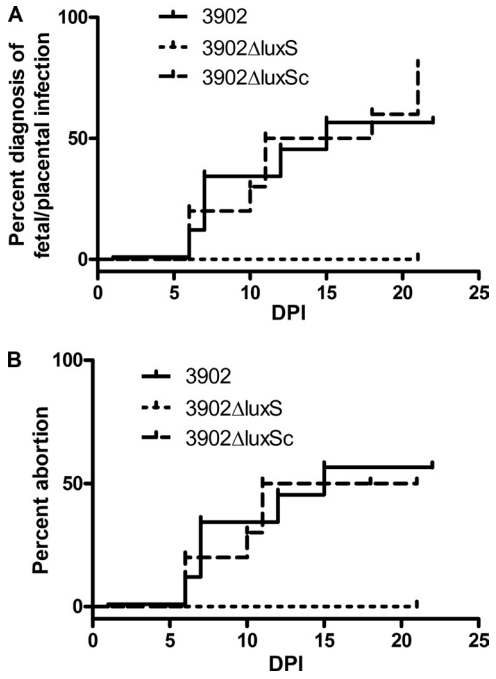

FIG 2 Cumulative fetoplacental infection rate (A) and abortion rate (B) following oral inoculation of *C. jejuni*. Fetoplacental infection was defined as a positive *C. jejuni* culture for one or more of the placental, uterine, or fetal samples collected at necropsy. Abortion was defined as the identification of significant vaginal bleeding or expelled fetuses that were culture positive for *C. jejuni*. For this data analysis, only pregnant guinea pigs are included ($n = 9$ for IA3902, $n = 7$ for 3902ΔluxS, and $n = 10$ for 3902ΔluxSc).

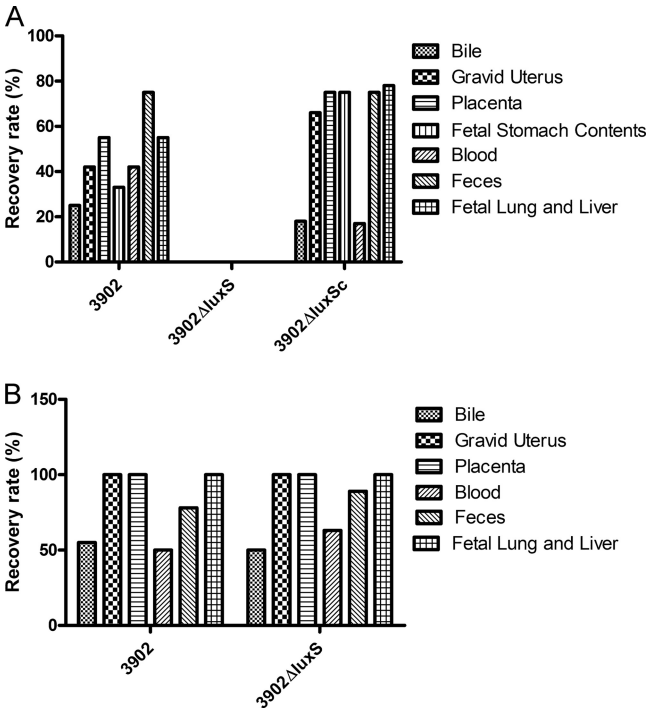

FIG 3 Recovery rate of *C. jejuni* following oral (A) or intraperitoneal (B) inoculation of pregnant guinea pigs. Recovery rates were calculated as the percentage of cultured samples for a given tissue that were culture positive at the time of necropsy. The individual number of samples cultured for each tissue varied on the basis of the ability to aseptically collect the sample at necropsy. The specific numbers of samples cultured and the number positive with semiquantitative counts can be found in Tables 2 and 3.

was fully complemented in the 3902ΔluxSc strain (Table 1). At 3 days postinoculation, 92% of the 3902 group and 75% of the 3902ΔluxSc group were positive for fecal shedding of the inoculation strain, while only 8% of the 3902ΔluxS guinea pigs were shedding *C. jejuni*. At all later time points, there were no 3902ΔluxS isolates found in the feces of the inoculated guinea pigs, while the shedding remained above 75% in the two remaining groups.

The results of the *C. jejuni* culture performed at the time of necropsy are provided in Fig. 3A. For the 3902 group, the recovery rates (positivity with *Campylobacter*) of individual tissue samples ranged from 25% for bile up to 75% for feces. The placenta, feces, and fetal lung/liver homogenate showed the highest recovery rates, with more than 50% of the sample being positive at the time of necropsy. A similar trend was seen for the 3902ΔluxSc strain, which had recovery rates from individual tissues ranging from 16% for bile and blood up to 78% for the fetal liver/lung homogenate. The recovery rates for tissues from the 3902ΔluxSc group approximately mirrored those observed in the 3902 group. In contrast, no tissue samples collected from any of the 3902ΔluxS animals at the time of necropsy yielded a positive culture of *C. jejuni* (Fig. 3A). The semiquantitative culture results for each tissue are also presented in Table 2. These results indicate that blood and bile tend to have lower rates of isolation and tend to have lower bacterial counts on culture. In contrast, the uterus, fetus, and placental samples yielded higher CFU counts in both the 3902 and 3902ΔluxSc strains. This is particularly true for placenta, where all cultured samples yielded greater than 1,000 CFU from a single swab (Table 2).

TABLE 1 Culture-positive fecal samples obtained from pregnant guinea pigs orally inoculated with *C. jejuni*

Strain	Culture-positive rate[a]				
	3 dpi[b]	7 dpi	10 dpi	14 dpi	Necropsy[c]
3902	11/12	7/9	6/9	5/6	8/12
3902ΔluxS	1/12	0/12	0/12	0/12	0/12
3902ΔluxSc	8/12	9/10	8/9	6/7	8/12

[a] Values reported represent the number of samples with positive culture results/number of samples tested.
[b] dpi, days postinoculation.
[c] Necropsy was performed when abortion occurred or at the end of the experiment.

Guinea pig intraperitoneal challenge. Given the complete ablation of the abortion phenotype in the *luxS* mutant strain, we were interested in further defining the general stage of pathogenicity responsible for the defect. In order to test the *luxS* mutant's ability to induce abortions once it reaches the systemic circulation, we repeated the study using an intraperitoneal inoculation route. Following intraperitoneal challenge, both the abortion and fetal-placental infection rates were essentially identical between the 3902 and 3902ΔluxS groups over the period of the study and were not found to be statistically significantly different (Fig. 4), indicating that the *luxS* mutant was capable of causing abortion when given intraperitoneally. This inoculation route resulted in induction of bacteremia, as evidenced by the positive blood cultures obtained from the first guinea pig to abort, at 18 h postinocula-

TABLE 2 Semiquantitative *C. jejuni* recovery data from the orally inoculated guinea pigs

Sample source	No. of culture-positive samples[a]	Recovery[b]		
		Low (<50 CFU)	Moderate (50–1,000 CFU)	High (>1,000 CFU)
Blood[c]				
3902	5/12	3/5	1/5	1/5
3902ΔluxS	0/12	0/0	0/0	0/0
3902ΔluxSc	2/12	2/2	0/2	0/2
Bile[c]				
3902	3/12	3/3	0/3	0/3
3902ΔluxS	0/12	0/0	0/0	0/0
3902ΔluxSc	2/11	2/2	0/2	0/2
Feces[d]				
3902	8/12	3/8	2/8	3/8
3902ΔluxS	0/12	0/0	0/0	0/0
3902ΔluxSc	8/12	4/8	3/8	1/8
Gravid uterus[d]				
3902	5/12	0/5	1/5	4/5
3902ΔluxS	0/12	0/0	0/0	0/0
3902ΔluxSc	8/12	0/8	2/8	6/8
Fetal liver and lung (pooled[e])[d]				
3902	5/9	1/5	1/5	3/5
3902ΔluxS	0/7	0/0	0/0	0/0
3902ΔluxSc	7/9	2/7	3/7	2/7
Placenta[d]				
3902	5/9	0/5	0/5	5/5
3902ΔluxS	0/7	0/0	0/0	0/0
3902ΔluxSc	6/8	0/6	0/6	6/6

[a] Values reported represent the number of samples with positive culture results/number of samples tested.
[b] Values reported represent the number of samples within that category/number of samples with positive culture results.
[c] The detection limit is approximately 10 CFU/ml.
[d] The detection limit is approximately 100 CFU/g.
[e] Pooled, samples from all fetuses of a specific guinea pig were pooled for microbial culture.

tion, and from many of the guinea pigs at the time of abortion (Fig. 3B and Table 3). In comparison to the oral inoculation group, the recovery rates of *C. jejuni* from various tissue samples were higher in the intraperitoneal challenge model (Fig. 3A and B). The recovery rates for the 3902 strain ranged from 44% for blood up to 100% for uterus, fetal lung/liver homogenate, and placenta. Similarly, the recovery rates of the 3902ΔluxS strain ranged from 50% in bile up to 100% for uterus, fetal liver/lung homogenate, and placenta (Fig. 3B). The intraperitoneal challenge resulted in a considerable increase in the recovery rate of the organisms from blood and bile compared with the oral challenge (Table 2), which is not surprising, given that the intraperitoneal route bypasses the necessity of translocation across the intestinal epithelium.

On the basis of the high level of recovery of significant bacterial numbers from placenta, we were interested in further quantifying the bacterial load carried by the placenta in the guinea pigs challenged intraperitoneally. Using quantitative bacterial culture methods, we demonstrated that *C. jejuni* counts ranged from 5×10^6 to 2.2×10^8 CFU/gram of placenta for the 3902 group (Fig. 5). Similarly, the range of counts for the 3902ΔluxS group was from 3×10^6 CFU/gram of placenta to 2.4×10^8 CFU/gram. Interestingly, within 1 day postinoculation, two guinea pigs' placentas contained 5×10^6 and 2.5×10^7 CFU of *C. jejuni* per gram of placental tissue, respectively (Fig. 5), suggesting rapid colonization and replication of this organism in placenta.

Chicken colonization and competitive fitness. To assess the role of *luxS* and AI-2 in the colonization of chickens, two nonconcurrent experiments were performed. In the first chicken study, we compared the ability of the IA3902 strain, its *luxS* mutant, and the genetic complement to colonize the chicken cecum. In this case, like the results for the guinea pig studies described above, the *luxS* mutant strain was unable to colonize, while the wild-type and complement strains colonized the chicken cecum to high levels (Fig. 6), indicating that mutagenesis of *luxS* in IA3902 abolished its ability to colonize the intestinal tract of chickens.

To assess if LuxS confers the same phenotype in another strain, a second experiment was performed, in which the *C. jejuni* W7 strain (a motile clone of NCTC 11168) was used to either monoinoculate or coinoculate chicks. The monoinoculated groups were rapidly colonized by the wild-type or mutant strain and remained steadily colonized for the duration of the project (Fig. 7A). There were no statistically significant differences observed between the two groups at any given time point ($P = 0.47$). In the coinoculation group (Fig. 7B), the mutant strain (W7ΔluxS) was outcompeted by the wild-type strain starting at 8 days postinoculation (dpi) ($P < 0.01$). Pairwise comparisons of the competition pair

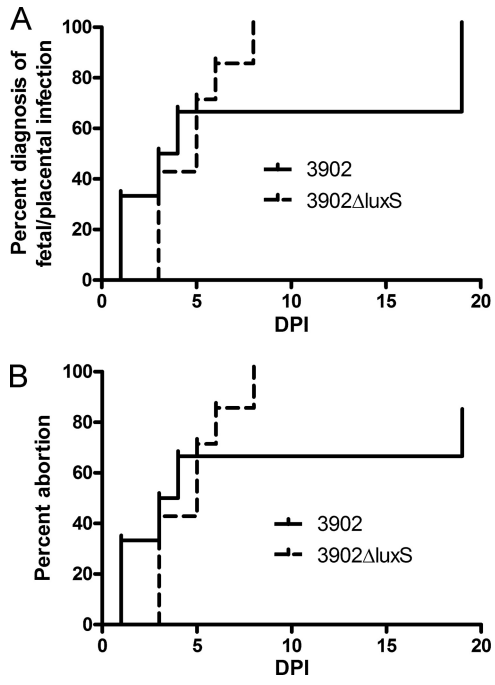

FIG 4 Cumulative fetoplacental infection rate (A) and abortion rate (B) following intraperitoneal inoculation of *C. jejuni*. Fetoplacental infection was defined as a positive *C. jejuni* culture for one or more of the placental, uterine, or fetal samples collected at necropsy. Abortion was defined as the identification of significant vaginal bleeding or expelled fetuses that were culture positive for *C. jejuni* ($n = 6$ for IA3902 and $n = 7$ for 3902ΔluxS).

TABLE 3 Semiquantitative *C. jejuni* recovery data from the intraperitoneally inoculated guinea pigs

Sample source	No. of culture-positive samples[a]	Recovery[b]		
		Low (<50 CFU)	Moderate (50–1,000 CFU)	High (>1,000 CFU)
Blood[c]				
3902	4/9	1/4	2/4	1/4
3902ΔluxS	5/8	4/5	1/5	0/5
Bile[c]				
3902	5/9	3/5	2/5	0/5
3902ΔluxS	4/8	0/4	4/4	0/4
Feces[d]				
3902	7/9	3/7	2/7	2/7
3902ΔluxS	8/9	3/8	4/8	1/8
Gravid uterus[d]				
3902	7/7	1/7	1/7	5/7
3902ΔluxS	7/7	0/7	3/7	3/7
Fetal liver and lung (pooled[e])[d]				
3902	6/6	0/6	0/6	6/6
3902ΔluxS	4/4	0/4	0/4	4/4
Placenta[d]				
3902	7/7	0/7	0/7	7/7
3902ΔluxS	7/7	0/7	0/7	7/7

[a] Values reported represent the number of samples with positive culture results/number of samples tested.
[b] Values reported represent the number of samples within that category/number of samples with positive culture results.
[c] The detection limit is approximately 10 CFU/ml.
[d] The detection limit is approximately 100 CFU/g.
[e] Pooled, samples from all fetuses of a specific guinea pig were pooled for microbial culture.

using Bonferroni's multiple-comparison test demonstrated a statistically significant difference between W7 and W7ΔluxS at all time points, with W7ΔluxS consistently having a lower colonization rate. The mean $\log_{10}$ differences (95% confidence intervals for that difference) at each of the time points are as follows: 4.929 (1.946 to 7.911) for 8 dpi, 4.782 (1.8 to 7.765) for 14 dpi, 7.320 (4.338 to 10.3) for 21 dpi, and 6.970 (4.158 to 9.782) for 28 dpi (Fig. 7B).

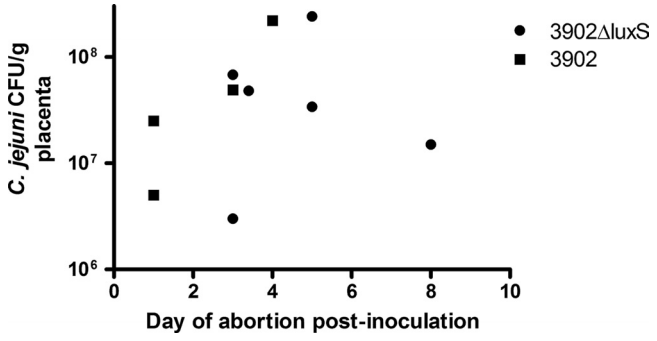

FIG 5 Quantitative *C. jejuni* culture of placental tissue derived from intraperitoneally inoculated pregnant guinea pigs. The numbers of CFU/gram of placenta versus the day of necropsy following inoculation are shown. Not all placentas were available for bacterial quantification.

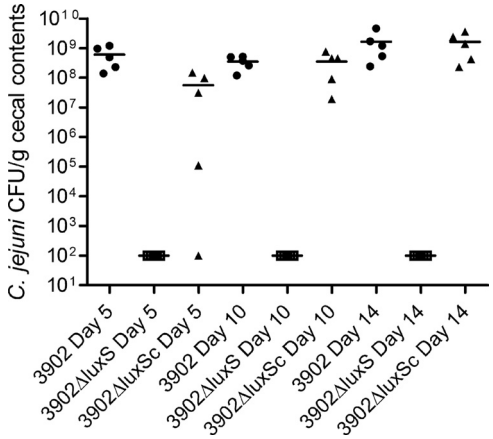

FIG 6 Cecal colonization of chickens by *C. jejuni* IA3902 and its derivatives. Results are reported as the CFU count per gram of cecal contents at 5, 10, and 14 days postinoculation (detection limit, 100 CFU/g of feces). Each symbol represents the data for a single bird, and the horizontal line represents the average of the birds in a given group. The wild type, *luxS* mutant, and complement are 3902, 3902ΔluxS, and 3902ΔluxSc, respectively.

DISCUSSION

This study provides the first evidence for a role of the *luxS* gene in the pathogenesis of *C. jejuni*-induced abortion in a guinea pig model. Previous studies have demonstrated that mutation of *luxS* led to *in vitro* phenotypic changes in virulence factor expression or changes in nondisease colonization (10, 13, 21, 25–27, 39, 41). Inactivation of *luxS* in *C. jejuni* IA3902 completely abolished its ability to induce abortion in pregnant guinea pigs, while in *cis* complementation fully restored this virulence trait (Fig. 2). In addition, we showed that LuxS plays an important role in intestinal colonization or fitness (Fig. 6 and 7). These results clearly demonstrate the key role of LuxS in *Campylobacter*-induced disease and infection.

The chick colonization model does not provide any measure of *Campylobacter*-induced disease since the organism colonizes the chicken host subclinically (11). In contrast, the pregnant guinea pig model provides a consistent and easily measured pathogenic outcome, namely, fetoplacental infection and subsequent abortion (5–7). Such a process is characteristic of *C. jejuni* abortion storms (epidemic abortion outbreaks) in small ruminants (sheep and goats). When performed using oral inoculation, the model also provides an indirect measure of the organism's ability to cross the enteric epithelium and disseminate systemically. Using IA3902, an abortifacient strain of *C. jejuni* (7, 42), it was shown that the *luxS* mutant totally lost the ability to induce abortion in guinea pigs when inoculated by the oral route. This finding provides evidence for a necessary role of the *luxS* gene in the pathogenicity of *C. jejuni* following oral exposure. Furthermore, the experiment utilizing an intraperitoneal inoculation demonstrated that the *luxS* mutant remained as virulent as the wild-type strain. In the group intraperitoneally challenged with the wild-type strain, one guinea pig aborted later than the others and one did not abort but showed a positive fetal-placental tissue culture at necropsy (Fig. 4). This delay in abortion is probably due to animal vari-

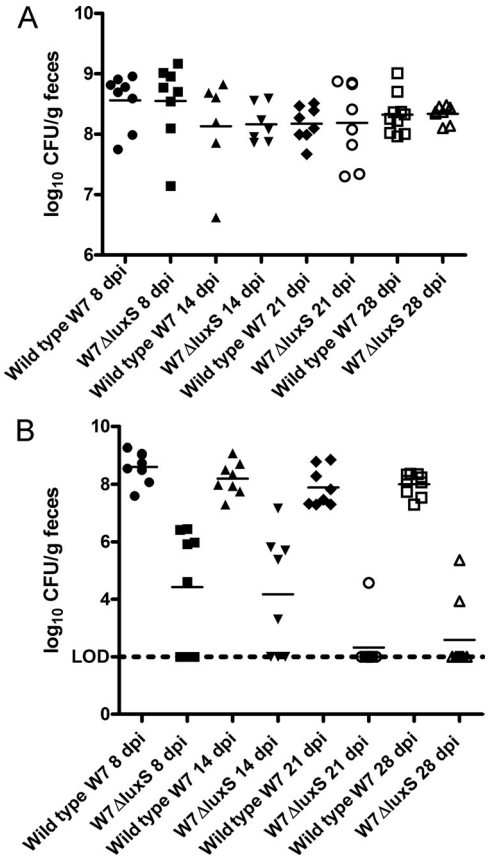

FIG 7 Cecal colonization of chickens by *C. jejuni* W7 and its *luxS* mutant. Results are reported as the $\log_{10}$ CFU count per gram of cecal contents (detection limit, 100 CFU/g of feces). Each symbol represents the data for a single bird, and the horizontal line represents the average of the birds in a given group. (A) Cecal colonization of *C. jejuni* following monoinoculation of pure culture of either W7 or W7ΔluxS at 8, 14, 21, and 28 days postinoculation; (B) cecal colonization following coinoculation with a 1:1 ratio of W7 and W7ΔluxS.

ations or differences in the gestation stages and should not be interpreted to be an indicator for differential virulence between the strains. On the basis of the results of these studies, it is plausible to state that the primary defect of the *luxS* mutant in abortion induction occurs at the level of either colonization or translocation across the intestinal mucosa. Once the *luxS* mutant becomes systemic, it becomes capable of inducing abortions.

Interestingly, the fecal shedding rates following intraperitoneal inoculation were 100% for both IA3902 and its *luxS* mutant. Since no organism was given orally in this challenge, the results provide evidence of *Campylobacter*'s ability to move out of the systemic circulation into the gastrointestinal tract. While the most likely scenario for this movement is colonization of the gallbladder and excretion of the organism through the biliary tract into the intestine, we cannot rule out the possibility that the organism can translocate out of the blood directly across the intestinal mucosa.

The rates of recovery of *C. jejuni* from tissue samples collected at necropsy revealed some interesting findings that may provide additional insight into the pathogenesis of *C. jejuni*-associated abortions. The most obvious of these findings is the complete lack of culture-positive results from any of the tissues collected from guinea pigs inoculated with 3902ΔluxS via the oral route (Fig. 3A and Table 2). When the intraperitoneal route of inoculation was utilized, the strain was readily isolated from tissues at rates and in amounts similar to those for the wild type (Fig. 3B and Table 3). Collectively, these results suggest that the loss of 3902ΔluxS's ability to induce abortions and fetoplacental infections following oral challenge is primarily associated with differences in pathogenesis prior to dissemination out of the intestine. Once the organism becomes systemic, it is capable of rapidly inducing bacteremia, placental infection, and abortion.

The relatively lower rate of recovery of *C. jejuni* from the bile and blood samples (Fig. 3 and Tables 2 and 3) was another interesting finding observed during both guinea pigs studies. We speculate that this is probably due to two reasons. First, these sites are likely less hospitable to bacterial growth than other tissues. Although *C. jejuni* is capable of living in environments that contain bile conjugates, the concentration of these compounds in the gallbladder and concentrated bile is far above that observed in the small intestine and thus may play a role in limiting bacterial replication. Likewise, the bacteria present in the blood are in direct contact with large numbers of innate and acquired immune effector cells and factors, as well as filtered through the hepatic and splenic immune network, potentially limiting their accumulation to large numbers. Second, on the basis of previous studies, this particular strain appears to have a tissue-specific tropism to the fetoplacental unit (7). This tropism, combined with the solid tissue matrix for growth, may allow rapid replication and colonization before immune effector cells can effectively infiltrate the site.

C. jejuni W7 is unable to induce abortion in guinea pigs (7). Thus, this strain and its *luxS* mutant were not evaluated using pregnant guinea pigs and were examined only using the chicken model. The chicken experiments showed clear evidence of strain variability in the *luxS* phenotype as it relates to colonization of the chicken gastrointestinal tract. For IA3902, mutation of *luxS* abolished its ability to colonize chickens (Fig. 6). However, W7 and W7ΔluxS were capable of stably colonizing chicks to similar levels and for similar durations when monoinoculated (Fig. 7A). This result is in contrast to the results obtained for IA3902 and the finding by Quinones et al., who observed a significant difference in rates of colonization of monoinoculated chicks between the wild type and *luxS* mutants of *C. jejuni* 81-176 (39). The reasons for the strain-specific differences are unknown, but it is possible that the metabolism of methionine or *S*-adenosylmethionine (SAM) pathways for these strains differ to certain extents, resulting in differences in the phenotype or their ability to utilize methyltransferases in chemotaxis. Additional work is under way in our laboratories to determine what differences between the strains are responsible for this phenotypic change.

Given that the *luxS* mutant and its wild-type strain, W7, colonized chickens at similar levels when monoinoculated, an additional competitive experiment was performed using coinoculation to assess the fitness of the mutant strain. The results showed a consistent outcompeting of the W7 *luxS* mutant by the wild-type W7 strain (Fig. 7B). We speculate that this fitness disadvantage is possibly associated with multiple factors, including reduced motility, decreased metabolic recycling of

S-adenosylmethionine with its associated alterations in normal metabolism, and the loss of AI-2-mediated quorum sensing. Given that the fitness disadvantage is observed in a model that should contain exogenous enteric levels of AI-2 produced by other bacteria, it is likely that quorum sensing is not the significant mechanism responsible for the loss of colonization.

In conclusion, results from this study demonstrate that *luxS* is important for intestinal adaptation and virulence of *C. jejuni* in abortion induction, identifying a promising target for control of *Campylobacter* infections. The exact role of the dual *luxS* functions (metabolically recycling SAM and producing AI-2) in these adaptation and pathogenicity phenotypes remains unclear. Further experiments focusing on dissecting the role of both functions in conferring the phenotypes should provide additional insights into the pathogenic and adaptive mechanisms of *C. jejuni*.

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