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Disrupting the *luxS* quorum sensing gene does not significantly affect *Bacillus anthracis* virulence in mice or guinea pigs

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Many bacterial species use secreted quorum-sensing autoinducer molecules to regulate cell density- and growth phase-dependent gene expression, including virulence factor production, as sufficient environmental autoinducer concentrations are achieved. *Bacillus anthracis*, the causative agent of anthrax, contains a functional autoinducer (AI-2) system, which appears to regulate virulence gene expression. To determine if the AI-2 system is necessary for disease, we constructed a LuxS AI-2 synthase-deficient mutant in the virulent Ames strain of *B. anthracis*. We found that growth of the LuxS-deficient mutant was inhibited and sporulation was delayed when compared with the parental strain. However, spores of the Ames *luxS* mutant remained fully virulent in both mice and guinea pigs.

Introduction

Bacillus anthracis is a rod-shaped, Gram-positive bacteria and the etiological agent of anthrax.[1] In response to nutrient depletion, *B. anthracis* forms highly resistant spores and can remain dormant and viable in the soil for decades.[2] When these spores are deposited in the lungs, gastrointestinal tract, or skin lesions of a susceptible animal, they germinate[3] to form toxin-producing, vegetative bacilli, which can rapidly proliferate and overwhelm the host.[4] Improved and novel anthrax treatments are needed to address the risks posed by possible antibiotic- and/or vaccine-resistant *B. anthracis* strains.[5]

Many Gram-negative and -positive bacteria use methods of intracellular communications, referred to as quorum sensing (QS), to coordinate gene expression in response to local cell density. One QS pathway that is common to both Gram-negative and -positive bacteria is the autoinducer-2 (AI-2) pathway. This system has been well described in *Vibrio harveyi* where the LuxS synthase produces the AI-2 signal molecule from S-ribosylhomocysteine, a byproduct of S-adenoxylmethionine metabolism.[6] The membrane-permeable AI-2 signal molecule binds to LuxP in neighboring cells to initiate a phosphate transfer cascade, which leads to the deactivation of the negative response regulator LuxO.[7] AI-2 signal molecules have been shown to regulate genes encoding a variety of functions, including toxin expression and other bacterial virulence determinants.[8-12]

A functional AI-2 molecule was identified in the Sterne strain of *B. anthracis*.[8] Researchers found subsequently that AI-2 QS inhibitors limit growth and toxin gene expression in the bacterium.[9] Recently, a *luxS*-deficient mutant of the *B. anthracis* Sterne strain was shown to exhibit similar phenotypic defects.[13] These findings suggest that opportunities might exist to treat *B. anthracis* infections using QS inhibitors. Therefore, we challenged mice and guinea pigs with a *luxS*-deficient mutant of the fully virulent Ames strain to determine whether disrupting the *B. anthracis* AI-2 QS pathway limited the severity of *B. anthracis* infections in small animal models. As reported here, our study failed to reveal statistically-significant differences in survival rates between animals challenged with *B. anthracis* Ames wild-type or *luxS* mutants.

Results

Disrupting the *luxS* gene eliminates AI-2 production in *B. anthracis* Ames. The *luxS* gene (BA5047) encodes an AI-2 synthase, which produces the AI-2 QS reporter molecule. Because the *luxS* gene is monocistronic and downstream open reading frames (BA5045 and BA5046) are encoded in the opposite direction,[14,15] disruption of the *luxS* gene would not have a downstream polar effect. Therefore, using a technique previously applied by ourselves and others to study *B. anthracis* pathogenesis,[16-20] we disrupted the *B. anthracis* Ames *luxS* gene by inserting

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the erythromycin-resistant pEO3 plasmid into the middle of the coding region by homologous recombination. We then confirmed proper formation of the merodiploid mutant by PCR-amplifying a gene fragment that spanned the junction of the pEO3 plasmid and the *luxS* open reading frame. Similarly assaying multiple colony isolates from media- and animal passaged-mutants confirmed stable retention of the insert, even in the absence of the selective antibiotic (data not shown).

We created the *B. anthracis* Ames *luxS* mutant to eliminate endogenous production of the AI-2 reporter molecule and evaluate the effect of AI-2-deficient bacteria on mouse and guinea pig survival. Therefore, to show that we successfully eliminated AI-2 signal molecule production in the *luxS* mutant, we used filtered conditioned medium from wild-type and mutant *B. anthracis* cultures to induce bioluminescence in the *V. harveyi* BB170 reporter strain.[21] Filtered medium collected from stationary phase cultures of both the wild-type and *luxS* bacteria were added to the reporter strain and luminescence measured. The conditioned medium from the wild-type strain was able to induce bioluminescence, thus demonstrating the production of a functional AI-2 molecule in the Ames strain. However, when conditioned medium from the Ames *luxS* mutant was added to the reporter strain, the bioluminescence detected was no greater than what was observed with buffer alone (**Fig. 1**). These data confirm that the AI-2 signal molecule is not produced by the Ames *luxS* mutant.

The *luxS* mutant is deficient in growth and delayed in sporulation. A previous report indicated that a *luxS* mutation in the attenuated Sterne strain of *B. anthracis* led to decreased growth in liquid culture.[13] Given this relationship between LuxS activity and bacterial growth, we compared the growth kinetics of the Ames wild-type and *luxS* mutant strains in brain heart infusion (BHI) broth. Consistent with the findings for *B. anthracis* Sterne, the Ames *luxS* mutant grew slower than wild-type Ames in liquid culture (**Fig. 2**).

We also found that the *luxS* mutant was impaired for growth in Difco Sporulation Medium (DSM) when compared with the parental strain (data not shown) and measured about a log reduction in the total number of colony forming units (CFU)/ml at the 24 h time point (**Fig. 3**). While the drop in measured CFU/ml is likely due in part to slower growth, it may also be attributable to the more extensive aggregation of vegetative bacilli in *luxS* cultures than in wild-type cultures, which exhibited more free-floating spores under a light microscope (data not shown). Although *luxS* mutant was impaired for growth in DSM, the time at which the culture left exponential growth and began to sporulate was similar to that of the wild-type bacteria.

To confirm that the *luxS* mutant was producing fewer spores than wild-type bacteria, we analyzed sporulation by measuring the number of heat-resistant spores at 24 and 48 h after the onset of sporulation. Heat-resistance appears at an intermediate point in sporulation and is a reliable measure of progression through all but the final stages of spore formation.[22] At 24 h, only 15% of the viable *luxS* mutant cells were heat-resistant compared with 92% of wild-type bacteria. However, by 48 h, 100% of the *luxS* cells were resistant to high temperature (**Fig. 3**). These data suggest that while the *luxS* mutant is not significantly defective in spore production, it is defective in the progression through at least the early stages of the process. As a result, *luxS* cells progress through sporulation less efficiently or less rapidly than wild type.

Finally, to assess the stability of *luxS* cointegrate mutants, 200 colonies from the sporulation assay were picked and patched onto Luria-Bertani (LB) agar plates with and without erythromycin. No erythromycin-sensitive colonies were detected, indicating that the cointegrate mutant was genetically stable during the in vitro culture conditions. We further validated mutant stability by PCR analysis of DNA derived from several of the antibiotic-resistant colonies. PCR analysis indicated that the *luxS* gene in each of the sampled colonies were disrupted by a pEO3 integrant (data not shown).

LuxS-deficient mutants remain as virulent as wild-type bacteria in mouse and guinea pig models. Because QS has been linked to virulence gene expression in many pathogens, including toxin gene expression in *B. anthracis*,[9,13] we evaluated the Ames *luxS* mutant in multiple small animal models of anthrax infection. First, we compared survival of BALB/c mice challenged with either wild-type or *luxS* spores via intranasal (~2.65×10^6 spores in 50 µl of water) or intraperitoneal (~2,700 spores in 100 µl of water) delivery. For these challenge models, previous studies calculated the Ames strain LD_{50} for mice to be 3.7×10^4 and 500 spores, respectively.[23,24] As shown in **Figure 4**, there were no statistically significant differences in survival or time to death between mice challenged with wild-type or *luxS* mutant bacteria, regardless of challenge route.

In addition to the mouse models of infection, guinea pigs (n = 10 for *luxS* and n = 5 for Ames wild-type) were challenged intramuscularly (~350 spores in 200 µl of water). A previous study calculated the Ames strain LD_{50} for a guinea pig to be ~100 spores.[25] Our study used a relatively low challenge dose so as to avoid overwhelming the animals and to ensure that a low level of attenuation in the virulence of spores could be observed. However, all animals similarly succumbed to infection with either strain by day 3, and no differences in survival or time to death were observed (data not shown).

Finally, to exclude the possibility that reversion of the *luxS* mutant to the wild-type form could be occurring during infection, guinea pig spleens were removed and bacteria recovered. A representative sample of bacteria (100 CFU from each mutant-challenged animal) was screened on LB agar plates with and without erythromycin. All plated CFU remained antibiotic resistant, indicating that the guinea pigs had succumbed to infection by *luxS* mutants and not wild-type revertants.

Discussion

Many bacterial species possess density- and growth phase-dependent genes, which respond to extracellular signaling molecules that accumulate in the environment. These QS signals regulate various bacterial functions and have been shown to affect the expression of numerous virulence factors.[8-12] *B. anthracis* contains a functional AI-2 system that regulates the gene encoding the S-layer protein as well as other virulence genes, such as *pagA*,

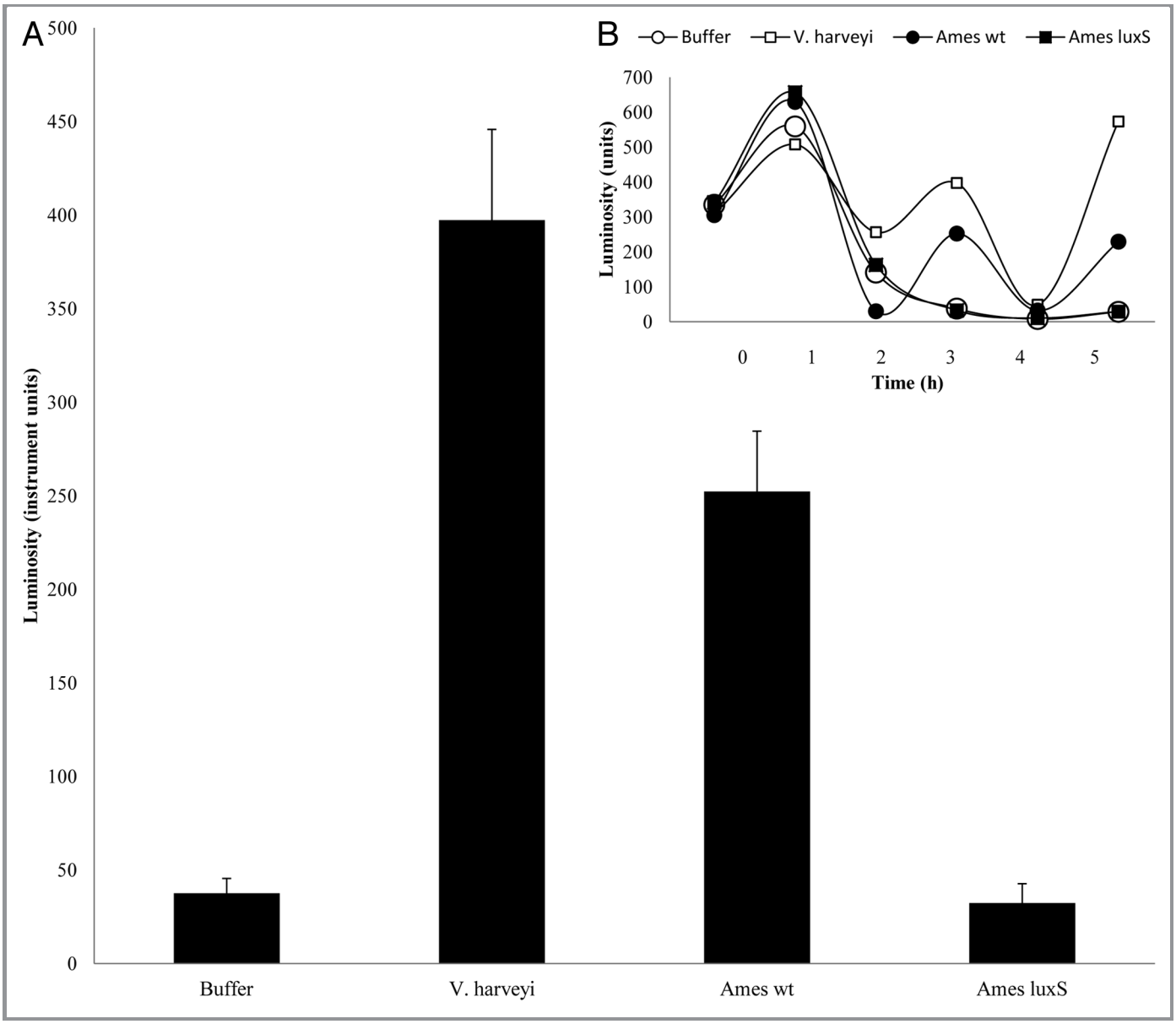

Figure 1. Conditioned medium from the *B. anthracis luxS* mutant does not induce a *V. harveyi* QS response. AI-2 activity is shown as luminosity expressed from *V. harveyi* strain BB170 during growth in response to the addition of autoinducer buffer or filtered, conditioned medium from *V. harveyi* (BB170), the wild-type *B. anthracis* strain (Ames wt), or the *B. anthracis luxS* mutant (Ames *luxS*). (A) Luminosity values at 3 h of growth. The error bars represent the standard deviation from the readings of six replicates. The difference between the wild-type Ames and *luxS* mutant was statistically significant ($p = 3.15 \times 10^{-6}$). (B) Luminosity values at various growth times of the assay. These data are representative of the results of two independent experiments.

pagR, *lef* and *cya*.[8,9,13] These findings suggest that the AI-2 QS system might provide an effective therapeutic target against *B. anthracis* infection. To further evaluate this possibility, we constructed a LuxS-deficient mutant of the fully virulent *B. anthracis* Ames strain to determine if the LuxS AI-2 synthase is necessary for virulence in mouse and guinea pig models of infection.

The *B. anthracis* Ames *luxS* mutant exhibited an in vitro growth defect (**Fig. 2**) similar to that previously observed in a *luxS*-deficient Sterne strain.[8] Additionally, a temporary delay was observed for completion of sporulation with the *luxS* mutant when compared with the parental strain (**Fig. 3**). To date, a role for the LuxS AI-2 synthase protein in *B. anthracis* sporulation has not been demonstrated. Furthermore, a recent microarray study, which compared the parental and *luxS* mutant Sterne strains,[13] did not reveal expression differences of known sporulation genes. However, a *luxS* mutant in the related *Bacillus subtilis* natto strain exhibited delayed formation of fruiting bodies during spore development.[26] *B. subtilis* sporulation is regulated by both the ComX peptide autoinducer[27] and small RNAs (sRNA).[28] Because

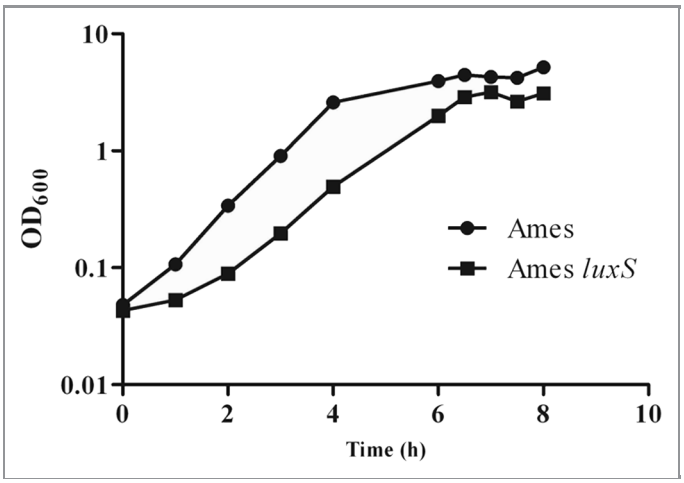

Figure 2. The *B. anthracis luxS* mutant exhibited impaired growth in BHI broth. Comparison is made of growth between the Ames wild-type (circles) and the *luxS* (squares) bacteria in BHI broth. These data are representative of the results of at least three independent experiments.

AI-2 and sRNA are synergistic in regulating *Vibrio cholera*[29] and possibly *B. anthracis*[13] toxin expression, perhaps such combined regulation may explain the delayed sporulation results observed with the *luxS* mutant of *B. anthracis*.

The main goal of this study was to determine if the AI-2 QS pathway is significant for *B. anthracis* virulence. Therefore, we created and then evaluated virulence of an Ames *luxS* mutant in several small animal models for *B. anthracis* infection. BALB/c mice were tested by both intranasal and intraperitoneal routes of infection. In addition, guinea pigs were challenged intramuscularly. Despite previous studies linking AI-2 signal molecules to toxin and virulence factor regulation and the fact that our *B. anthracis luxS* mutant was deficient in growth, we saw no statistically significant differences in either the time to death or survival rates for animals challenged with mutant and wild-type isolates (**Fig. 4**). These results demonstrate that the intact AI-2 QS pathway does not play a significant role in *B. anthracis* infection in these animals and suggest that the AI-2 synthase enzyme would not provide an effective therapeutic target. Although LuxS activity is known to affect virulence in other bacterial species,[30-32] our study using these rodent models of anthrax infection demonstrates that *B. anthracis* may be among those bacteria for which the AI-2 synthase does not play a significant role in infection.[33-36]

Methods

Bacterial growth and sporulation. *Escherichia coli* were cultured in LB medium supplemented with 50 μg/ml of ampicillin. The Ames strain of *B. anthracis*[37] was cultured in either LB medium or

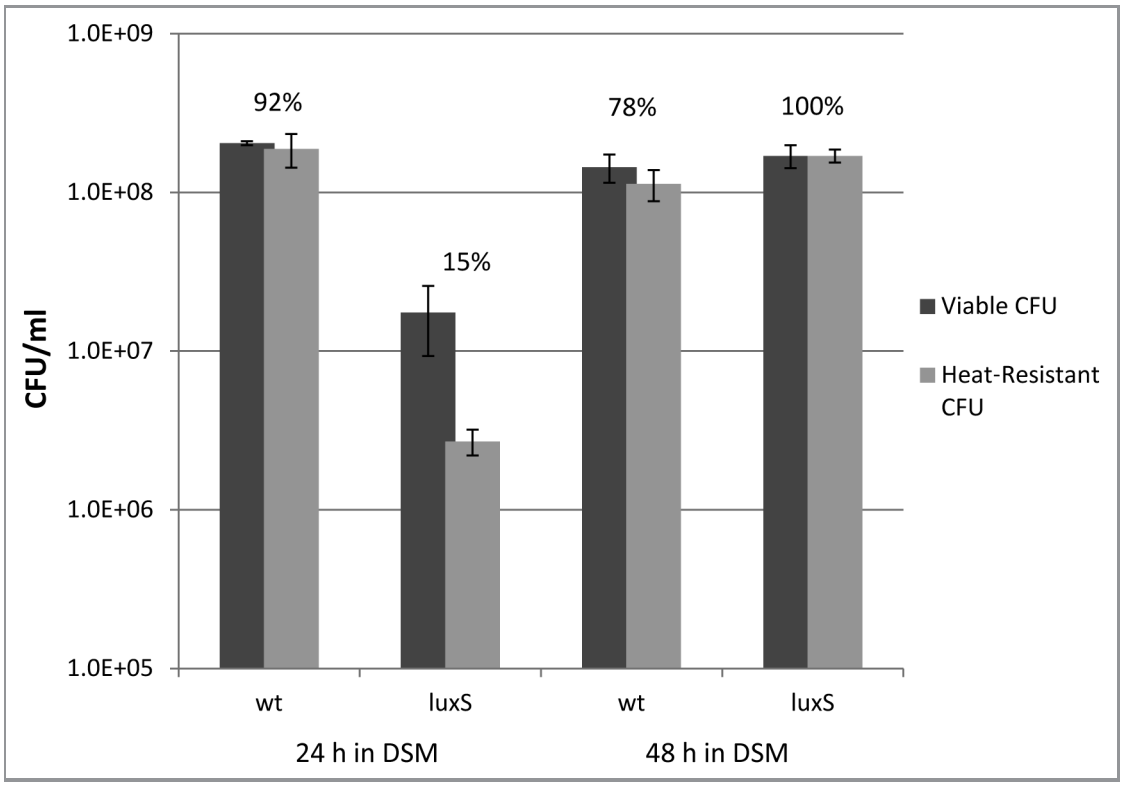

Figure 3. *LuxS*-deficient bacteria show signs of delayed sporulation. Ames wild-type and *luxS* bacteria were inoculated at equal concentrations (by OD_{600}) in DSM. CFU counts were obtained at 24 h and 48 h by plating culture samples before and after heating for 30 min at 65°C. The percentages of heat-resistant spores at each time point are indicated above the colony counts and are based on the fraction of heat-resistant cells in the sampled media. These data are representative of three independent experiments.

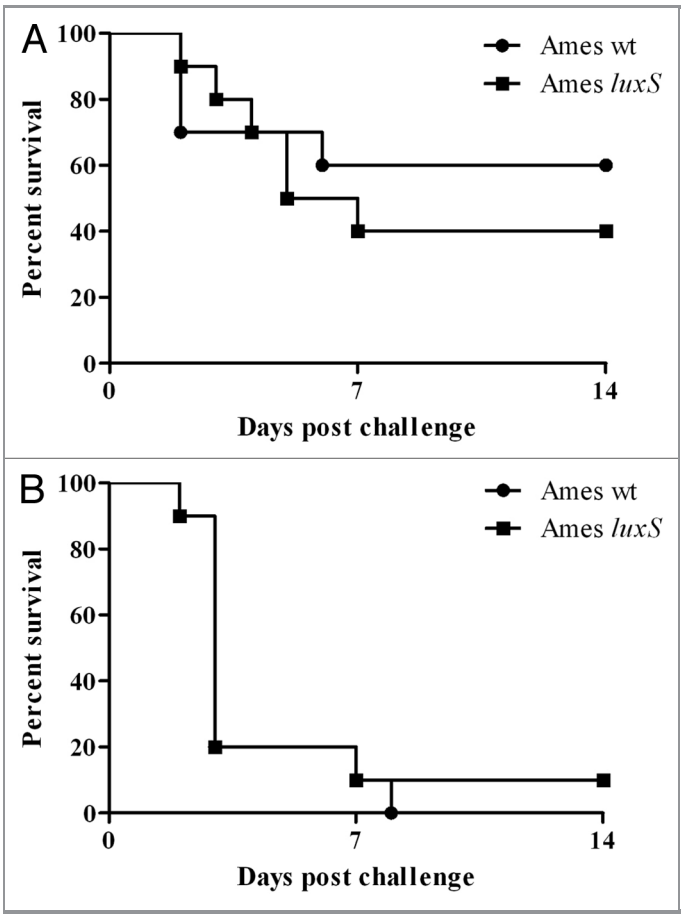

Figure 4. Loss of LuxS AI-2 synthase activity does not affect *B. anthracis* virulence in mice. There was no statistical difference between the survival curves for BALB/c mice challenged with *B. anthracis* Ames *luxS* spores (squares; n = 10) or wild-type spores (circles; n = 10) regardless of whether the spores were administered intranasally (A) (~2.65 × 10^6 spores; p = 0.72) or intraperitonally (B) (~2,700 spores; p = 0.49).

BHI broth. For construction and selection of the *luxS* mutant, medium was supplemented with 5 μg/ml of erythromycin, otherwise the mutant was grown under the same conditions as the wild-type strain. To induce sporulation, *B. anthracis* was grown in DSM[38] and spores were purified as previously described.[16] Because *B. anthracis* spores are resistant to heat, spore formation was measured by counting CFUs before and after incubating the bacterial cultures at 65°C for 30 min.

Mutant construction. The *luxS* mutant was constructed by PCR-amplifying an internal fragment of the *B. anthracis* Ames *luxS* gene (nucleotides 120–224, where ATG = 1–3) using two internal primers (5'-TTGCCAACCGAATAAAC-3' and 5'-TCAAAATGTGGATAACGAT-3') and cloning the PCR product into the pEO3 plasmid.[19] The resulting construct was integrated into the *B. anthracis* Ames chromosome to form a merodiploid mutant as previously described.[16] PCR analysis was used to confirm stabile disruption of the *luxS* gene in both culture- and animal-passaged bacteria.

AI-2 production. A previously published bioluminescence assay was used, with minor modifications, to detect endogenously produced AI-2 signal molecules in wild-type and *luxS B. anthracis* cultures.[21] Briefly, an overnight culture of *V. harveyi* strain BB170 (ATCC) was grown in LB at 30°C, diluted 1:5,000 into autoinducer buffer, and 990 μl aliquots were distributed into optical-grade micro-titer plates preloaded with 10 μl of conditioned medium. The filtered, conditioned medium was prepared from liquid bacterial cultures of *B. anthracis* Ames wild-type or *luxS* strains grown to stationary phase in BHI at 37°C. Similarly prepared filtered medium from *V. harveyi* was used as a control. The assay plate was incubated at 30°C with orbital shaking, and luminescence was measured at 490 nm every hour using a Victor^2 Multilabel Counter (Perkin-Elmer). Each test subject was averaged from six replicates across a single microtiter plate.

Animal challenges. To assess potential changes in virulence associated with disrupting the *B. anthracis* Ames AI-2 QS pathway, spores from both wild-type and mutant strains were used in mouse intraperitoneal and intranasal models,[39] as well as the guinea pig intramuscular model.[25] Research was conducted under an IACUC-approved protocol in compliance with the Animal Welfare Act, PHS Policy and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Statistics. Survival rates were compared between groups by Fisher exact tests with permutation adjustment for multiple comparisons using SAS Version 8.2 (SAS Institute Inc., SAS OnlineDoc, Version 8). For comparing data from bioluminescence experiments and time to death studies of mouse challenges, statistical significance ($p < 0.05$) was determined by the two-tailed Student's t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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