Quorum sensing is the regulation of bacterial gene expression in response to changes in cell density (13). Bacteria that utilize quorum-sensing signaling pathways synthesize signaling molecules (autoinducers [AI]) which have been found in nature as N-homoserine lactones (8) or small peptides (13). AI molecules (autoinducers [AI]) which have been found in nature and have been used to detect AI-2 or AI-2-like molecules in its milieu may be used to detect AI-2 molecules and that a luxS mutant has slowed growth compared to that of a wild-type strain. These observations suggest modalities for both prevention and treatment of anthrax.

**Materials and Methods**

**Bacterial strains and culture conditions.** *B. anthracis* vaccine strain 34F2 (Colorado Serum Company, Denver, Colo.), a derivative of the Sterne strain (21) (Table 1), was routinely grown in brain heart infusion broth (BHI) at 37°C. *E. coli* strain DH5α was routinely grown in Luria-Bertani broth at 37°C. Ampicillin (50 μg/ml) was added for cultivation of DH5α strains harboring recombinant plasmids. *V. harveyi* strain BB170, kindly provided by Bonnie Bassler (Princeton University, Princeton, N.J.), was routinely grown in Auto-Inducer Bioassay medium (AB) (22) at 30°C.

**Generation of cell-free culture medium and *V. harveyi* bioluminescence assays.** *B. anthracis* and *E. coli* strains were grown overnight with aeration at 37°C. Cell-free conditioned culture medium (CFM) was prepared by centrifuging culture supernates at 8,000 × g and passing the medium through a 0.2-μm-pore-size Acrodisc syringe filter (Gelman Laboratory). CFM preparations were stored at −20°C until studied. CFM from *V. harveyi* strain BB170 was prepared in the same manner, except that cultures were grown at 30°C. *V. harveyi* bioluminescence assays were performed essentially as described by Surette and Bassler (22).

**Construction of *B. anthracis* luxS strain.** To construct a luxS mutant, 1.18-kb and 989-bp fragments flanking BA5047 were amplified by using oligonucleotides BAluxSKOF1 (5'-GACTCTTGTAACAGACGTCG-3'), BAluxSKOR1 (5'-GCAATCTCTTACATAAGGTG-3'), and BAluxSKOR2 (5'-GCCACATCATATCTGGTT-3').
The PCR-amplified products were purified by using a Qiagen PCR purification kit and subsequently were digested with HindIII. Digests were cloned into pGEM-T Easy and were screened by PCR with primers BAluxSKOF1 and BAluxSKOR2; the plasmid with the correct insert is designated pMJ301. pMJ301 was digested with HindIII and aphA, conferring kanamycin resistance, and was introduced into pMJ301 to create pMJ301K (9). pMJ301K was digested with EcoRI, releasing the insert region, which was cloned into a pUC19 derivative (pUS19) with spectinomycin resistance to create pMJ301KS. Since methylation inhibits transformation into B. anthracis, pMJ301KS was cloned into dam-defective E. coli strain SCS110. Purified pMJ301KS from SCS110 was electroporated into B. anthracis strain 34F2, and colonies were selected for Kanr and Spe+. Transformants were picked on medium containing 50 μg of kanamycin/ml and 100 μg of spectinomycin/ml and then were subcultured daily in the absence of antibiotics at 37°C with aeration for 15 days. Individual colonies were subsequently screened to identify clones that were both Kanr and Spe+. Clones with the correct antibiotic phenotype were confirmed by PCR to have allelic exchange of aphA in the luxS locus by using oligonucleotides SterneF (5'-GACAATTGAAACAGCCTCAG-3') and SterneR (5'-GTATGCTTATAACATTCCGTCG-3'), with HindIII digestion of the PCR products.

**Construction and screening for pMJ301.** Chromosomal DNA of B. anthracis strain 34F2 was purified by using the Wizard Genomic DNA Purification kit (Promega, Madison, Wis.) and was used as template for PCR amplification of open reading frame (ORF) BA5047. The oligonucleotides used were designated BAluxSKOF1 (5'-ATGCCATCAGTAGAAGACTTG-3') and BAluxSKOR2 (5'-CCAAATTTCTCAAGTTACCTC-3'). In the PCR, DNA was denatured for 1 min at 94°C, with annealing for 1 min at 51°C and extension for 1 min at 72°C. The amplified product was cloned into pGEM-T Easy, yielding pMJ301, which then was transformed into E. coli strain DH5α with selection for ampicillin resistance. The insert from pMJ301 was subjected to sequence analysis by using ClustalW (23). Phylograms based on amino acid alignments were constructed using Paup 4.0b8 (Sinauer Associates, Sunderland, Mass.) with genera-

| Plasmid or strain | Relevant characteristic(s) | Source or reference |
|-------------------|-----------------------------|---------------------|
| pGem-T Easy | Amp' | Promega |
| pMJ301KS | 1.18-kb and 989-bp B. anthracis PCR, products and aphA (Kan') cloned into Spec' pUC19 derivative, pUS19 | This work |
| pMJ501 | 474-bp PCR product of BA5047 cloned into pGEM-T Easy | This work |

**Strain**

| E. coli | DH5α | F' (8080lacZDMD15 D[ lacZ Y-argF]U169 deoR recA1 endA1 hsdR17( rK - mK +) phoA supE44 1-thi-1 gvrA96 relA1) rpsL150 ZDM5 | Promega |
|---------|------|-------------------------------------------------|-----|
| SCS110  | rp4L. (Str') thr leu thi-1 lacY galK gatT ara tonA tdx dam cem sup44 Dlac-proAB | Stratagene |
| B. anthracis | 34F2 | pXO1'/pXO2' | 21 |
| 34F2luxS | pXO1'/pXO2' ΔluxS Kan' | This work |
| V. harveyi | BBB170 | Sensor AI-1'/sensor AI-2' | 1 |

**RESULTS**

Identification and organization of the B. anthracis luxS locus. The unfinished genomic sequence of the Ames strain of B. anthracis has been made publicly available by The Institute for Genomic Research (www.TIGR.org). By using the nucleotide sequence of the 471-bp Bacillus subtilis luxS gene as a template, the partially annotated B. anthracis genome was subjected to BLASTN search, which revealed a 474-bp ORF, BA5047, with 72% similarity to luxS (also known as ytjB) from B. subtilis. To further characterize the putative B. anthracis luxS locus, flanking nucleotide sequences were submitted for BLASTN analysis. Sequence analysis of the region upstream of the B. anthracis luxS ortholog revealed a high level of conservation in which B. subtilis genes yjF4 and yjF5 had homologs with nucleotide similarities of 70 and 67%, respectively. However, the region downstream of the B. anthracis luxS ortholog showed substantial variation compared to B. subtilis. Only one proximate downstream B. subtilis gene, ytkD, had an ortholog in B. anthracis. Immediately downstream of BA5047 are two ORFs (BA5045 and BA5046) of 201 and 231 bp, respectively, with no significant homologs in GenBank. The orientations of the flanking ORFs indicate that BA5047 is in a monocistronic operon (Fig. 1) (18).

Characterization of the B. anthracis luxS ortholog. Further data suggesting that BA5047 may be a functional luxS ortholog are provided by an alignment of the translated sequence with 17 other LuxS protein sequences. Although size variations exist in the luxS products, conserved regions essential for function across prokaryotic genera have been defined (13). Alignment of protein sequences from 17 known luxS orthologs with BA5047 reveal a number of conserved amino acids, including those hypothesized to be essential for LuxS enzymatic activity (10). These data provide evidence that B. anthracis ORF BA5047 encodes a LuxS protein with function. Phylogenetic analysis was done to further characterize the B. anthracis luxS
ortholog (Fig. 2). As expected, the phylogram revealed that the
*B. anthracis* *luxS* translated product is most closely related to
the *luxS* products of *B. subtilis* (10) and *B. halodurans*; the high
bootstrap values indicate that the analysis is robust. Interest-
ingly, the *Helicobacter pylori* *luxS* product (6) was found to be
more closely related to *Staphylococcus aureus* (24) than to *Campylobacter jejuni* (3), suggesting horizontal gene transfer.

**Synthesis of a functional AI-2 molecule by *B. anthracis* cells.**
Utilizing the *V. harveyi* AI-2 reporter assay, liquid cultures of
*B. anthracis* vaccine (Sterne) strain 34F2 were examined to
determine whether *B. anthracis* cells synthesize an AI-2 or
AI-2-like molecule. The AI-2 assay utilizes a deficiency in the
AI-1 sensor in *V. harveyi* strain BB170 (22). Without the *luxN*
AI-1-encoded sensor, strain BB170 can only exhibit biolumi-
nescence in response to AI-2 or an AI-2-like molecule. Grow-
ning a culture of strain BB170 overnight and then diluting it
1:10,000 (to yield low cell density) reduces the level of endog-
enous AI-2 below the threshold required for luminescence. In
this experimental system the addition of exogenous AI-2 from
bacteria possessing *luxS* function can restore the biolumines-
cence phenotype of the BB170 cells (22). As a negative control
the *V. harveyi* reporter strain BB170 was incubated with sterile
CFM alone; as a positive control CFM from a high-density
culture of strain BB170 also was used (Fig. 3). Addition of
sterile CFM to cells of BB170 served as the standard in lumines-
cence, whereas as expected, addition of CFM from the high-density BB170 culture induced greater than 100-
fold increases in luminescence. In multiple experiments, CFM
from *B. anthracis* strain 34F2 had activity similar to that of the
positive control, with substantial increases in luminescence
compared to that of the negative control (Fig. 3). The results of
these experiments indicate that *B. anthracis* synthesizes AI-2 or
an AI-2-like molecule that is involved in the *lux* quorum-sens-
ning system.

**Evidence that BA5047 encodes a functional *luxS*.** To deter-
mine whether BA5047 is the *B. anthracis* ORF responsible for
synthesis of AI-2 or an AI-2-like molecule, we took advantage
of the inability of *E. coli* strain DH5a to synthesize a functional
AI-2 molecule (6). The *B. anthracis luxS* ortholog (BA5047) was
amplified by PCR and was cloned into the *E. coli* shuttle
vector pGEM-T Easy to create pMJ501. Only the ORF was
cloned into pGEM-T Easy, into a site downstream of the
vector’s isopropyl-β-D-thiogalactopyranoside (IPTG)-induc-
able promoter. CFM from high-density cultures of DH5a con-
taining vector pMJ501 were induced with IPTG and then were
screened for the synthesis of AI-2, as measured in the *V.
harveyi* bioluminescence assay (Fig. 4). As negative controls,
the reporter strain BB170 was incubated with sterile CFM

![Fig. 1. Schematic of *B. anthracis* chromosomal organization in the region of the putative *luxS* homolog. The *B. subtilis* nucleotide sequence was used to identify a putative *luxS* homolog in the *B. anthracis* genome. BLASTN analysis revealed an ORF (BA5047) in the partially annotated *B. anthracis* genome that was 72% identical to *luxS* in *B. subtilis*. Arrows indicate the direction of transcription. Black arrows indicate the locations of primers for construction of 34F2ΔluxS.](http://iai.asm.org/)

![Fig. 2. Phylogenetic analysis of translated products of *luxS* orthologs from 17 bacterial species and *B. anthracis* ORF BA5047. Sequences were aligned by using the GCG Pileup program and were subjected to phylogenetic analysis by using PAUP 4.0b4a. Bootstrap values of more than 50% (based on 1,000 replicates) are represented below the phylogram. The genus and species for each sequence are located at the termination of each branch.](http://iai.asm.org/)
FIG. 3. Induction of bioluminescence in *V. harveyi* reporter strain by CFM from *B. anthracis* cells. *V. harveyi* strain BB170 is deficient in the AI-1 sensor encoded by *luxN*, and thus upregulates only the expression of the *lux* operon (measured as RLU) when AI-2 or AI-2-like molecules are present in its milieu (1). CFM obtained from AI-2-synthesizing bacteria grown to high density (including BB170, in which the AI-2-regulated system is intact) can induce expression of the bioluminescence-generating *luxCDABE* operon in BB170. In the experiments shown, sterile CFM alone and CFM from high-cell-density cultures of *V. harveyi* strain BB170 were negative and positive controls, respectively, and CFM from high-density 6-h cultures of *B. anthracis* strain 34F2 and 34F2*luxS* were the unknowns. Cells of BB170 were grown for 2 h (black boxes) or 4 h (white boxes) in the presence of sterile CFM. The baseline is the value for use of uninoculated (sterile) CFM alone at 2 h. By 4 h the endogenous AI-2 activity was substantially higher than that at 2 h. Each bar represents the means (± standard deviations) of triplicate experiments. Compared to the negative control, wild-type 34F2 but not 34F2*luxS* showed substantial AI-2 activity.

FIG. 4. Induction of bioluminescence in the *V. harveyi* reporter strain by cloned BA5047 in *E. coli*. *V. harveyi* strain BB170 upregulates only the expression of bioluminescence when AI-2 or AI-2-like molecules are present in its milieu, as described in the legend to Fig. 3. In the experiments shown, negative controls were BB170 cells incubated for 2 h with sterile CFM alone and CFM from high-cell-density cultures of *E. coli* strain DH5α alone or containing pGEM-T Easy without insert. Positive controls used were CFM from high-density cultures of *V. harveyi* strain BB170 and *B. anthracis* strain 34F2 (6-h culture). The unknown specimen was CFM from DH5α containing pMJ501; all assays were run in triplicate. The dashed line indicates the endogenous RLU for the BB170 cells grown for 2 h in the presence of sterile CFM alone.

FIG. 5. PCR confirmation of the creation of *B. anthracis* 34F2*luxS*. Primers flanking the regions of recombination were used to confirm the construction of 34F2*luxS*. The PCR products were from chromosomal DNA from wild-type strain 34F2 (lane 1) and from a putative 34F2*luxS* clone (lane 2). The size change is indicative of the insertion of *aphA*, which encodes kanamycin resistance (9). The purified PCR products shown in lanes 1 and 2 were digested with *Hind*III and are shown in lanes 3 (wild-type strain) and 4 (putative 34F2*luxS* clone). The new 1.4-kb band in lane 4 confirmed the insertion of *aphA* in the *luxS* locus. The numbers to the right of the gel indicate molecular size in kilobase pairs.

substantial bioluminescence (Fig. 4). As expected, no bioluminescence was induced by CFM from cultures of DH5α or from DH5α containing pGEM-T Easy without insert. In contrast, CFM from DH5α containing pMJ501 induced a high level of bioluminescence, greater than that induced by CFM from the positive controls. Compared to the control *E. coli* CFMs, there was nearly a 1,000-fold mean increase in induction of bioluminescence by pMJ501 (Fig. 4).

*B. anthracis* 34F2*luxS* has a defect in AI-2 activity. To analyze the effect of AI-2 signaling in *B. anthracis*, we created a *luxS* mutant in strain 34F2 by replacement of the *luxS* homolog with a kanamycin resistance cassette (see Materials and Methods). After electroporation of the wild-type strain with the mutated locus on pMJ301KS, kanamycin-resistant transformants were serially passed for 15 days in vitro to select for a double-crossover event. Screening of one such KanR* SpeI* transformant by PCR showed the expected products (Fig. 5), indicating the proper construction. Additionally, in lane 3 of Fig. 5, the ~0.4-kb band corresponds to a fragment of *luxS* amplified from genomic DNA from the wild-type strain. To determine the effect of the mutation on AI-2 synthesis, we utilized the *V. harveyi* bioassay as described above (Fig. 3). Compared to the baseline level of AI-2 activity in reporter strain BB170, CFM from a high-density culture of 34F2*luxS* had no additional AI-2 activity. The data collected provided evidence that *luxS* is necessary for AI-2 synthesis in *B. anthracis* strain 34F2 (Fig. 3).

Growth defect in *B. anthracis* 34F2*luxS*. When cultured in liquid medium, *B. anthracis* 34F2*luxS* exhibits noticeable
Growing overnight in BHI medium and were inoculated with fresh BHI fl
adjusted to reflect an OD 600 of 0.6 to standardize the cell numbers.

and CFM from 34F2 were incubated with strain BB170 (Fig. 6B). Sterile CFM alone
luted in sterile medium to refl
density cultures of 34F2 in the
B. anthracis
growth defects compared to wild-type B. anthracis 34F2 (Fig. 6A). As determined by cell density, 34F2luxS has a brief delay (approximately 30 to 60 min) in the transition between lag and early exponential phase compared to those for wild-type 34F2.

Subsequently, exponential growths for the wild-type and mu-
tant strains are parallel, but the mutant enters into stationary
phase at a much lower cell density. Thus, under the conditions
tested AI-2 function appears necessary for full B. anthracis
growth in vitro.

Growth phase dependence of AI-2 synthesis in B. anthracis.

To determine whether B. anthracis synthesis of AI-2 was growth phase dependent, CFMs were collected from the 34F2 cells at various time points in the growth cycle and were used in the V. harveyi bioluminescence assay. The CFMs were di-
luted in sterile medium to reflect equal numbers of cells and were incubated with strain BB170 (Fig. 6B). Sterile CFM alone and CFM from 34F2luxS were used as negative controls, and CFM from a high-density culture of V. harveyi strain BB170 was used as a positive control. Analysis of CFM collected from B. anthracis showed that AI-2 is maximally synthesized during the mid-exponential phase of growth and diminishes during stationary phase. CFM collected from a 6-h culture of the wild-type strain did not enhance the growth of 34F2 (data not shown).

DISCUSSION

In this study we confirmed that an ORF (BA5047) in the partially annotated B. anthracis genome possesses extensive homology to the luxS ortholog (vigB) in B. subtilis (10). Although the B. anthracis locus for BA5047 is not highly conserved compared to that of B. subtilis, the orientations of the ORFs indicate that BA5047 is in a monocistronic operon, which facilitates examination of its function and regulation. In other organisms, luxS appears essential for the synthesis of a quorum-sensing molecule (AI-2) (12), first identified in the marine bacterium V. harveyi (1). Both multiple protein align-
ments and phylogenetic analyses (Fig. 2) of the B. anthracis luxS ortholog (BA5047) revealed strong evolutionary relationships with those of B. subtilis and B. halodurans, indicating strong conservation of luxS within the genus Bacillus. Phyloge-
netic analysis of luxS orthologs reveals two major groupings, generally clustering gram-positive and gram-negative species separately (Fig. 2); one exception is H. pylori, providing evi-
dence consistent with horizontal transfer of luxS across genera (Fig. 2). Taken together, the phylogenetic studies and the protein alignments indicating the presence of conserved amino acids confirm that BA5047 encodes a luxS ortholog.

That CFM from strain 34F2 was able to stimulate lumines-
cence in V. harveyi strain BB170 (Fig. 3) indicates that B. anthracis produces AI-2 or an AI-2-like molecule, likely similar in structure to AI-2 from V. harveyi (2). With this evidence we next focused on BA5047, the luxS ortholog. Expression of B. anthracis BA5047 in E. coli strain DH15a demonstrates its central role in synthesis of AI-2 or an AI-2-like molecule (Fig. 4) and suggests the capability of B. anthracis to conduct density-
dependent gene expression. Isogenic deletion of luxS (Fig. 5) resulted in an inability of the B. anthracis mutant (34F2luxS) to produce AI-2 or an AI-2-like molecule that could be detected in the V. harveyi bioassay (Fig. 3). Similarly, compared to the wild-type strain the mutant showed delay in the transi-
tion from lag to exponential growth phase and entered stationary
phase early (Fig. 6A). In total, the luxS culture grew more slowly and produced fewer cells compared to the wild type.

B. anthracis synthesis of AI-2 or an AI-2-like molecule med-
ated by luxS thus plays an important role in the regulation of
growth. As such, targets of the hypothesized density-depend-
ent gene expression must include genes regulating vegetative
growth and cell cycle. If B. anthracis regulates gene expression by means of an AI molecule, as do other pathogens (14), cells might have the ability to suppress virulence gene expression until the total population reaches a threshold density. Suppression of virulence gene expression by quorum sensing could allow B. anthracis to evade immune detection until its popu-
lation is at a density sufficiently high to overwhelm the host’s innate and adaptive defenses. That AI-2 synthesis is maximal during exponential phase growth is consistent with this hypoth-
thesis.

This hypothesis suggests that a possible means of treating anthrax could be via inhibitors of AI-2 to downregulate den-
sity-dependent gene expression. Recent data has shown that a
synthetic furanone, (SZ)-4-bromo-5-(bromomethylene)-3-butyl-2(SH)-furanone, has the ability to inhibit AI-2-mediated quorum sensing in *E. coli* and *V. harveyi* (19) as well as swarming and biofilm formation by *B. subtilis* (20). Examination of this or similar molecules could permit ascertainment of the role of AI-2-mediated mechanisms in virulence gene expression of *B. anthracis*.

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