Role of *luxS* in *Bacillus anthracis* growth and virulence factor expression

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Quorum-sensing (QS) activity in bacterial gene expression in response to changes in cell density, involves pathways that synthesize signaling molecules (auto-inducers). The *luxS/AI-2*-mediated QS system has been identified in both Gram-positive and Gram-negative bacteria. *Bacillus anthracis*, the etiological agent of anthrax, possesses genes involved in *luxS/AI-2*-mediated QS, and deletion of *luxS* in *B. anthracis* Sterne strain 34F2 results in inhibition of AI-2 synthesis and a growth defect. In the present study, we created a *ΔluxS* *B. anthracis* strain complemented in trans by insertion of a cassette, including *luxS* and a gene encoding erythromycin resistance, into the truncated *plcR* regulator locus. The complemented *luxS* strain has restored AI-2 synthesis and wild-type growth. A *B. anthracis* microarray study revealed consistent differential gene expression between the wild-type and *ΔluxS* strain, including downregulation of the *luxS* allele, *AI-1* encoded sensor, strain BB170 only exhibits bioluminescence in the *V. harveyi* bioassay. Furthermore, analysis of the *B. anthracis* genome indicated the presence of a gene, *luxS*, which is involved in QS. Disruption of *luxS* resulted in the inability of *B. anthracis* to synthesize a functional AI-2 or AI-2-like molecule recognizable in the *V. harveyi* bioassay, and in a defect in growth in vitro for the *B. anthracis luxS* mutant. These data suggest that *B. anthracis* may utilize the *luxS/AI-2* QS system to regulate growth as well as density-dependent gene expression.

In the present report, we characterize the differential gene expression of *Bacillus anthracis* strains 34F2, 34F2:*ΔluxS* and 34F2:*luxS:comp*. To further characterize the role of QS in *B. anthracis*, by microarray analysis we analyzed *B. anthracis* gene expression in wild-type cells grown in the presence or absence of halogenated furanones. Finally we utilize a custom tiled genome Affymetrix array to identify possible small RNAs differentially expressed in the *luxS* mutant compared to the wildtype.

Results

Complementation of AI-2 deficiency. Cell-free medium (CFM) was collected from *B. anthracis* strains 34F2, 34F2:*ΔluxS* and 34F2:*luxS:comp* for assessment in the *Vibrio harveyi* BB170 bioluminescence assay. The AI-2 bioassay utilizes a deficiency in the AI-1 sensor in *V. harveyi* strain BB170. Without the *luxN* AI-1 encoded sensor, strain BB170 only exhibits bioluminescence in response to AI-2 or an AI-2-like molecule. Growth of strain
tion can restore the bioluminescence phenotype of the BB170 as a positive control, CFM from a high-density culture of strain was incubated with sterile cell-free medium (CFM) alone, and controls included were CFM from (negative control) and strain 34F2, strain 34F 2 luxS whether the growth defect was directly related to the deletion of 34F2, strain 34F 2 luxS:comp exhibits AI-2 activity compared to wild-type 34F 2. (B) In the experiment shown, CFM from 5-h cultures of wild-type 34F 2 and 34F2 (positive control). CFM B. anthracis luxS:comp shows restored AI-2 activity compared to wild-type 34F 2. (C) In the experiment shown, CFM from 5-h cultures of wild-type 34F 2, and 34F,luxS:comp were serially diluted 1:1, 1:10 and 1:100. Each bar represents the mean (±SD) of triplicate experiments. Additional gene expression of a B. anthracis 34FΔluxS strain grown aerobically. To identify genes regulated by the luxS/AI-2 QS system, B. anthracis microarrays were utilized. Total RNA was isolated from B. anthracis strains 34FΔ and 34FΔluxS grown in BHI in the absence of sodium bicarbonate. Isolated RNA samples were hybridized to spotted B. anthracis array slides and analyzed using TM4 software (www.tm4.org). Significance of microarray (SAM) analysis of array data revealed that 576 genes were differentially expressed in the 34FΔluxS strain, compared to the wild-type 34FΔ strain based on a false discovery cutoff of 7%. Select upregulated genes ≥2-fold (Table 2) and downregulated genes ≥2-fold (Table 3) are listed. Genes that were not downregulated ≥2-fold, but are in the middle of a presumed operon in which other genes have a ≥2-fold change are included. Among the genes downregulated in the 34FΔluxS strain are the luxS gene (as expected), the S-layer virulence gene EA1, encoding putative S-layer proteins, and genes involved in phosphotransferase systems (PTS) (Table 3). Genes that were upregulated ≥2-fold included those encoding a phosphoprotein C, a bacillus protease and resistance protein (Table 2). These data suggest that luxS/AI-2 is involved in regulating peptide transport and S-layer expression. To further elucidate impact of luxS/AI-2 on B. anthracis gene expression we performed an analysis of down and upregulated genes to determine cellular and functional roles. Analysis of downregulated genes revealed that a significant portion are involved in energy metabolism (23%) and transport and binding proteins (12.7%), and analysis of upregulated genes revealed a significant portion are involved with cellular processes (18.8%). Closer examination of genes downregulated in B. anthracis 34FΔluxS strain revealed several operons involved in nitrate/nitrite metabolism (Table 3) when grown either aerobically or in the presence of sodium bicarbonate. Nitrate reduction has three fundamental roles: (1) utilization as a nitrogen source (nitrate assimilation); (2) maintenance of oxidation-reduction balance (nitrate dissimilation), and (3) utilization as a terminal electron acceptor (nitrate respiration). In-depth analysis of nitrate/nitrite respiration regulation in B. subtilis revealed the ability of the bacterium to convert nitrate or nitrite to ammonium when grown aerobically. The conversion of these nitrogen sources to ammonium is catalyzed by assimilatory nitrate and nitrate reductases. Mutation in nasD or nasE or nasF (assimilatory nitrite reductases) led to growth defects in B. subtilis when glucose was depleted. These observations coupled with the transcriptional profile of the 34FΔluxS strain, suggests that luxS/AI-2 may play a role in regulating nitrate/nitrite metabolism in B. anthracis, and that the observed growth defect in the luxS mutant is due to the inability to utilize nitrate/nitrite. It is interesting to note that complementation of luxS in B. anthracis strain 34FΔluxS did not fully restore the transcriptional expression of the nitrate/nitrite regulon to wildtype ΔluxS strain (Fig.2). These data suggest that luxS/AI-2-mediated quorum sensing is involved in regulating metabolic functions in B. anthracis under aerobic conditions. Based on these findings, we sought to characterize the growth defect in 34FΔluxS by examining the transcriptional profile of the 34FΔluxS strain compared to the wild type parental strain.

Figure 1. Induction of bioluminescence in V. harveyi reporter strain by CFM from B. anthracis cells. V. harveyi strain BB170 only upregulates the expression of the lux operon [measured as relative light units (RLU)], when AI-2 or AI-2-like molecules are present in its milieu. Cell free medium (CFM) obtained from AI-2-synthesizing bacteria can induce expression of the bioluminescence-generating luxCDABE operon in BB170. (A) In the experiments shown, CFM from 5-h cultures of B. anthracis strains 34FΔ and 34FΔluxS and sterile CFM alone were used as positive and negative controls. The baseline is the value when uninoculated (sterile) CFM alone at 5 h were used. Each bar represents the mean (±SD) of triplicate experiments. Compared to the negative and positive controls, 34FΔluxS:comp showed restored AI-2 activity compared to wild-type 34FΔ. (B) In the experiment shown, CFM from 5-h cultures of wild-type 34FΔ and 34FΔluxS:comp were serially diluted 1:1, 1:10 and 1:100. Each bar represents the mean (±SD) of triplicate experiments.

BB170 overnight, followed by dilution 1:10,000 (to yield low cell density), reduces the level of endogenous 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 bioluminescence phenotype of the BB170 cells. As a negative control, the V. harveyi reporter strain BB170 was incubated with sterile cell-free medium (CFM) alone, and as a positive control, CFM from a high-density culture of strain BB170 was used (Fig. 1). Addition of sterile CFM to cells of BB170 served as the standard for baseline luminescence, whereas, as expected, addition of CFM from the high-density BB170 culture induced a >100-fold increase in luminescence. Additional controls included were CFM from B. anthracis strain 34FΔluxS (negative control) and B. anthracis strain 34FΔ (positive control). CFM from B. anthracis strain 34FΔluxS:comp exhibits AI-2 activity comparable to that of the wild-type strain (Fig. 1). These data demonstrate that the luxS chromosomal complementation fully restored AI-2 production that was deficient in B. anthracis strain 34FΔluxS (Fig. 1).

Complementation of the growth defect in B. anthracis strain 34FΔluxS. When cultured in liquid medium, B. anthracis 34FΔluxS exhibited a moderate, but reproducible, growth defect compared to wild-type B. anthracis 34FΔ. To determine whether the growth defect was directly related to the deletion of luxS, we compared the growth of B. anthracis wild-type strain 34FΔluxS, strain 34FΔluxS, and the 34FΔluxS:comp strain. As determined by triplicate cell densities and based on OD 500, the 34FΔluxS:comp strain showed restored growth compared to the
slightly downregulated in the 34F₂ΔluxS strain compared to the wild type, suggesting that luxS/AI-2 influence on expression of the toxin components is regulated by factors in addition to atxA.

Analysis of the transcriptional profile of the 34F₂ΔluxS complement strain revealed near wildtype levels of toxin components pagA, lef and cya compared to the luxS mutant strain (Table 5). Based on these data, we hypothesize that luxS/AI-2 may play a role in influencing additional regulators that modulate toxin expression.

Effect of halogenated furanones on B. anthracis gene expression.

To better understand the mechanism by which B. anthracis may utilize luxS/AI-2-mediated QS to regulate gene expression, we studied the effect of halogenated furanones in microarray experiments. Previous studies showed that halogenated furanones can inhibit AI-2-regulated genes in E. coli without inhibiting bacterial growth. However, our recent data showed that halogenated furanones inhibit B. anthracis growth in a dose-dependent manner. To further elucidate the potential role of luxS/AI-2 in nitrate/nitrite metabolism we will make use of inhibitors of AI-2-mediated quorum-sensing.

Differential gene expression of B. anthracis 34F₂ΔluxS grown with 0.8% sodium bicarbonate. QS has been implicated in the regulation of virulence gene expression for a number of pathogenic bacteria. To identify genes regulated by the luxS/AI-2 QS system and CO₂/bicarbonate, total RNA was isolated from B. anthracis strains 34F₂ and 34F₂ΔluxS grown in BHI in the presence of 0.8% sodium bicarbonate. CO₂/bicarbonate is critical for activation of toxin gene expression in B. anthracis. Isolated RNA samples were hybridized to spotted B. anthracis array slides and analyzed using Spotfinder and TMeV software. Analysis of the microarray data revealed substantial differential expression of virulence genes on plasmid pXO1 in the 34F₂ΔluxS strain compared to the parental strain 34F₂. Genes downregulated on pXO1 include the protective antigen (pagA), pagR, lethal factor (lef) and the calmodulin-sensitive adenylate cyclase (cya) (Table 4, Fig. 3). Figure 3 demonstrates temporal changes in gene expression within the pathogenicity island on pXO1 in strain 34F₂ΔluxS with regard to the downregulation of the toxin components. The virulence regulator atxA was only slightly downregulated in the 34F₂ΔluxS strain compared to the wild type, suggesting that luxS/AI-2 influence on expression of the toxin components is regulated by factors in addition to atxA. Analysis of the transcriptional profile of the 34F₂ΔluxS complement strain revealed near wildtype levels of toxin components pagA, lef and cya compared to the luxS mutant strain (Table 5). Based on these data, we hypothesize that luxS/AI-2 may play a role in influencing additional regulators that modulate toxin expression.
post-exposure to fur-2 (Table 6). These data are consistent with the observation that fur-2 significantly inhibits expression of lacZ fusions to the promoters of the toxin components. In addition to the toxin components, furanone treatment of B. anthracis cells

(Fig. 4). Inhibition and stimulation by fur-2 was not a global event, with only ~5% of the genome showing ≥3-fold differential expression. Expression of the B. anthracis toxin components (lef and pagA) on pXO1 were significantly downregulated two hours post-exposure to fur-2 (Table 6). These data are consistent with the observation that fur-2 significantly inhibits expression of lacZ fusions to the promoters of the toxin components. In addition to the toxin components, furanone treatment of B. anthracis cells
inhibited respiratory nitrate systems as mentioned above.

To determine whether fur-2 was inhibiting genes regulated by the luxS/AI-2 QS system, the differentially expressed genes in cells treated with fur-2 were compared to those differentially expressed in the B. anthracis ΔluxS strain (Tables 2 and 3). Analysis of microarray data revealed that B. anthracis toxin components and respiratory nitrate reductase systems were downregulated under both experimental conditions (Fig. 4). However, fur-2 did not inhibit the expression of luxS directly. These data indicate that fur-2 does not directly inhibit the transcriptional activity of luxS, but may interact with the luxS product at the cellular level. Furthermore, fur-2 inhibits genes that may be regulated by luxS/AI-2 mediated QS in B. anthracis. These data support the hypothesis that luxS/AI-2-mediated QS may play a role in the regulation of the B. anthracis toxin components (pagA, lef and cya) and nitrate/nitrite metabolism.

Analysis of small RNAs in B. anthracis 34F2ΔluxS strain. Small RNAs have been implicated in regulating pathways involved in QS.49 To investigate the effect of a luxS mutation on small RNAs in B. anthracis, we utilized an Affymetrix custom genome tiled array. This platform permitted us to investigate the transcriptional activity of small and antisense RNAs. Analysis of pXO1 revealed several regions on the virulence plasmid demonstrating differential transcriptional activity in intergenic regions (Fig. 5). The intergenic region between locus BXA0122 and BXA0124 had substantial transcriptional activity. Furthermore, a comparison of the 34F2ΔluxS strain with the wildtype demonstrated a lower transcriptional profile, suggesting that luxS/AI-2 may play a role in regulating the transcriptional activity of small RNAs in B. anthracis. However, to better understand the regulatory network of luxS/AI-2-mediated QS, additional experiments will be necessary.

Discussion

In this study, we were able to complement the luxS/AI-2 deficiency in the 34F2ΔluxS mutant. First, we assessed whether insertion of the luxS ORF plus its putative promoter into the plcR locus, creating 34F2ΔluxS:comp strain, would restore luxS/AI-2 activity. Cell-free medium collected from the 34F2ΔluxS:comp strain was able to fully stimulate luminescence in the V. harveyi reporter strain (Fig. 1), indicating that the B. anthracis 34F2ΔluxS:comp strain produces AI-2 or an AI-2-like molecule, likely similar in structure to AI-2 from V. harveyi. Serial dilutions of CFM collected from the 34F2ΔluxS:comp strain were able to stimulate luminescence in the V. harveyi strain to a similar extent as the wild-type 34F2 strain, providing strong evidence that the strain is fully complemented for AI-2 activity. The growth defect observed in the B. anthracis ΔluxS strain suggests that luxS is involved in regulating B. anthracis growth. That the growth of B. anthracis strain 34F2ΔluxS:comp was restored to wildtype levels (Fig. 2) provides direct evidence that luxS is in fact involved in regulating B. anthracis growth. Regulation of growth by luxS
toxins in concert with small RNAs (sRNA). Furthermore, recent data has demonstrated that small RNAs play a critical role in regulating virulence and sporulation in \textit{V. cholerae} and \textit{B. subtilis}, respectively. One potential mechanism for \textit{luxS}/AI-2-mediated quorum-sensing modulation of \textit{B. anthracis} toxin expression is through the regulation of sRNAs. Detection of AI-2 by bacterial cells could potentially up or downmodulate regulatory small RNAs, leading to differential expression of the \textit{B. anthracis} toxins.

To further understand the mechanisms by which \textit{B. anthracis} utilizes the \textit{luxS}/AI-2-mediated QS system to regulate growth-phase-dependent gene expression, we studied halogenated furanones, known inhibitors of QS. Furanone-1 [(5Z)-4-bromo-5-\{bromomethylene\}-3-butyl-2(5H)-furanone] has been shown to inhibit >70% of the AI-2 regulated genes in \textit{E. coli}, has been observed in \textit{Streptococcus pyogenes}. In total, these data demonstrate that the altered phenotypes in the ΔluxS strain are due to \textit{luxS} disruption and not to other undefined coincident mutations.

To better understand the role of \textit{luxS}/AI-2-mediated QS in regulating \textit{B. anthracis} gene expression and growth regulation, we utilized microarray analysis. Our use of microarrays permitted us to identify a number of genes potentially regulated by the \textit{luxS}/AI-2 QS system, but which in total represent <1% of those in the \textit{B. anthracis} genome. Downregulation in the ΔluxS strain of key virulence genes \textit{pagA}, \textit{pagR}, \textit{lef} and \textit{cya}, is notable. Inhibition of nitrate metabolism might explain the growth defect present in the 34F₄ luxS strain. The observed inhibition of the plasmid-encoded toxin gene expression may or may not be direct; AI-2 can modulate the expression of \textit{Vibrio cholerae} toxins in concert with small RNAs (sRNA). Furthermore, recent data has demonstrated that small RNAs play a critical role in regulating virulence and sporulation in \textit{V. cholerae} and \textit{B. subtilis}, respectively. One potential mechanism for \textit{luxS}/AI-2-mediated quorum-sensing modulation of \textit{B. anthracis} toxin expression is through the regulation of sRNAs. Detection of AI-2 by bacterial cells could potentially up or downmodulate regulatory small RNAs, leading to differential expression of the \textit{B. anthracis} toxins.

![Figure 5. Analysis of small non-coding RNA of strain 34F₄ΔluxS compared to strain 34F₄ using an Affymetrix tiled array. A select region (between BXA0122 and BXA0124) based on pXO1 from \textit{B. anthracis} strain A2012, demonstrating transcriptional activity in intergenic regions. Red indicates the transcriptional activity of the parental strain and blue represents strain 34F₄ΔluxS.](image-url)
without affecting bacterial growth rate.\textsuperscript{31} We recently showed that halogenated furanones inhibit \textit{B. anthracis} growth in a dose-dependent manner, and can inhibit the expression of \textit{lacZ} when fused to the promoters of the toxin components.\textsuperscript{30} The microarray analyses now reveal that treatment of cells with furanone (fur-2) results in inhibition of the transcription of virulence genes \textit{lef} and \textit{cya} on pXO1, and respiratory nitrate systems (Open reading frames BA2125-28, BA2133-38 and BA2142-46). Upstream sequence analysis of each potential operon revealed a conserved promoter motif, suggesting coordinate regulation of these operons (Suppl. Table 1). However this motif was not found upstream of \textit{atxA} or other virulence genes. These data indicate that fur-2 inhibits \textit{B. anthracis} virulence gene expression and may lead to the development of a novel therapeutic agent against anthrax infections. In \textit{Pseudomonas aeruginosa}, respiratory nitrate regulation has been implicated to be QS-dependent.\textsuperscript{30}

We conclude that \textit{B. anthracis} utilizes \textit{luxS} to regulate AI-2-dependent QS and bacterial growth; with specific regulation of expression of the S-layer protein EA1, respiratory nitrate-dependent QS and bacterial growth; with specific regulation of expression of \textit{fur-2} suggesting coordinate regulation of these operons. Further interrogating these small RNAs may illuminate greater understanding on how the toxins are expressed, since no \textit{atxA} binding motif has ever been identified.

\section*{Materials and Methods}

\textbf{Bacterial strains and culture conditions.} \textit{B. anthracis} strains 34F\textsubscript{2}, (Colorado Serum Company, Denver, CO), 34F,\textit{ΔluxS} and 34F,\textit{ΔluxS::comp} were routinely grown in Brain Heart Infusion broth (BHI) or BHI with 0.8\% sodium bicarbonate at 37\textdegree C. \textit{Escherichia coli} strain DH5\textalpha was routinely grown in Luria-Bertani broth (LB) at 37\textdegree C. Ampicillin (50 \textmu g/ml) was added for cultivation of DH5\textalpha strains harboring recombinant plasmids. \textit{V. harveyi} strain BB170, kindly provided by Bonnie Bassler (Princeton University, Princeton, NJ), was routinely grown in Auto-inducer Bioassay medium (AB) at 30\textdegree C.\textsuperscript{37,58}

\textbf{Generation of cell-free culture medium and \textit{V. harveyi} bioassays.} \textit{B. anthracis} strains were grown overnight with aeration at 37\textdegree C. Cell-free conditioned culture medium (CFM) was prepared by centrifugation of cultures at 4,000 rpm (Eppendorf Centrifuge 5810R) and passing the medium through a 0.2 \textmu m pore-size MILLIPORE syringe filters (Carrigwohill Company, Cork, Ireland). CFM preparations were stored at -20\textdegree C until used. CFM from \textit{V. harveyi} strain BB170 was prepared in the same manner, except that cultures were grown at 30\textdegree C. \textit{V. harveyi} bioluminescence assays were performed essentially as previously described.\textsuperscript{37} Briefly, \textit{V. harveyi} strain BB170 was grown at 30\textdegree C with aeration for 16 h, cultures were diluted 1:10,000 in fresh AB broth, and then 10\% CFM or dilutions thereof of the bacterial cultures to be tested was added. Aliquots of 1.0-ml were taken 5 h after CFM was added, and bioluminescence measured, expressed as relative light units (RLU), using a luminometer.

\textbf{Construction of a \textit{B. anthracis} \textit{ΔluxS}: complement strain.} To complement the \textit{luxS} deletion in \textit{B. anthracis} strain 34F,\textit{ΔluxS}, the \textit{plecR} operon, which encodes a pleiotropic regulator of hemolysins and phospholipases in \textit{Bacillus cereus}, was selected as the site for integration of the \textit{luxS} complementation cassette. In wild-type \textit{B. anthracis} strains, \textit{plecR} contains a nonsense mutation making it naturally inactive and thus a neutral spot for an in trans complementation locus.\textsuperscript{59} The \textit{luxS} ORF including its putative promoter were cloned into the \textit{plecR} ORF. PCR was used to amplify a 3 kb fragment from the \textit{plecR} locus, including 1.05 kb upstream and 1.1 kb downstream of the \textit{plecR} ORF using primers P1 and P2 (Table 1). The PCR-amplified product was purified using a PCR purification kit (Qiagen, Valencia, CA), and subsequently cloned into pGEM-T easy. A plasmid with the correct insert and orientation was designated pMJ601. pMJ601 was modified by inverse PCR to add \textit{BamHI} sites (primers P3 and P4; Table 1) in the middle of the cloned insert for insertion of the \textit{luxS} complementation cassette. The \textit{luxS} complementation cassette was created by using purified chromosomal DNA of \textit{B. anthracis} strain 34F\textsubscript{2}. \textit{B. anthracis} \textit{luxS} was cloned using primers P9 and P10 to generate a 715-bp fragment including the \textit{putative luxS} promoter, \textit{ErmR} and, subsequently cloned into pGEM-T easy. A plasmid with the correct insert was designated pMJ602ET. Purified pMJ602ET from SCS110 was electrotransferred into \textit{B. anthracis} strain 34F\textsubscript{2}:\textit{ΔluxS} and colonies selected

\begin{table}[h]
\centering
\caption{Oligonucleotide primers used in this study} \label{table1}
\begin{tabular}{|c|c|}
\hline
\textbf{Primer designation} & \textbf{Nucleotide sequence (5' \to 3')} \tabularnewline \hline
P1F & TTTGACACGTGCGATTAGAAAGAGCAGGCTC \\
P2R & GTTTGAGAACTGATGTAACAGGCTC \\
P3F & GCGGATCCATTGAAGAAACTGCTGAAGCTC \\
P4R & GCGGATCCATTGAAGAAACTGCTGAAGCTC \\
P5F & GTTTGAGAACTGATGTAACAGGCTC \\
P6R & CGTTAAACCTTGATGTAACAGGCTC \\
P9F & GCGGATCCATTGAAGAAACTGCTGAAGCTC \\
P10R & GCTTCGAAATATCAGTGCGAAGGCTC \\
P11F & GCGGGTACCTTTGAACGTGCGATGAAAG \\
P12R & GCGGGTACCTTTGAACGTGCGATGAAAG \\
BAluxSF & ATGCGCATGCGAAGGCTC \\
BAluxSR & TATTCGTTATATCAGGTTTCTCAAGGCTC \\
\hline
\end{tabular}
\footnote{Restriction sites underlined; \textit{BamHI} (GGATCC) and \textit{KpnI} (GGTACC).}
\end{table}
Generation of probes for microarray experiments. DNA probes for microarray experiments were generated by adding 2 µg of total RNA in a mixture containing 6 µg of random hexamers (Invitrogen), 0.01 M dithiothreitol, an aminoallyl-deoxynucleoside triphosphate mixture containing 25 mM each dATP, dCTP and dGTP, 15 mM dTTP, and 10 mM amino-allyl-dUTP (aa-dUTP) (Sigma), reaction buffer, and 400 units of SuperScript III reverse transcriptase (Invitrogen) at 42°C overnight. The RNA template then was hydrolyzed by adding NaOH and EDTA to a final concentration of 0.2 and 0.1 M, respectively, and incubating at 65°C for 15 min. Unincorporated aa-dUTP was removed with a Minelute column (Qiagen). The probe was eluted with a phosphate elution buffer (4 mM KPO4, pH 8.5, in ultrapure water), dried and resuspended in 0.1 M sodium carbonate buffer (pH 9.0). To couple the amino-allyl cDNA with fluorescent labels, normal human serum-Cy3 or normal human serum-Cy5 (Amersham) was added at room temperature for 1 h. Uncoupled label was removed using the Qiagen Minelute column (Valencia, CA) (intranet.jtc.jcvsf.org/sops/M007.pdf).

Microarray hybridization, scanning, image analysis, normalization and analysis. Aminosilane-coated slides printed with a set of 15,552 B. anthracis open reading frame sequences (www.jcvi.org) were prehybridized in 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (Invitrogen), 0.1% sodium dodecyl for erythromycin resistance. Transformants were initially picked on medium containing 5 µg/ml of erythromycin, and then subcultured twice 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 Erm<sup>+</sup> and Tet<sup>+</sup>. This would suggest loss of pMJ602ET and incorporation of the modified plc<sup>R</sup> allele into the chromosome. For clones with the appropriate antibiotic phenotype, the correct insertion was confirmed by PCR using primers luxSF/P6 and luxSR/P5 (Table 1).

RNA isolation for microarray analysis. B. anthracis RNA was isolated from bacterial cultures grown to mid-log in BHI media with or without sodium bicarbonate, RNAprotect (Qiagen) was directly added to the growth media at a concentration of 2:1 (volume of RNAprotect to bacterial culture). RNA was isolated from bacterial cultures grown in the presence of or the absence of furanone-2 (fur-2; 3-butyl-5-(dibromomethylene)-2-(5H)-furanone), as stated above. Cells treated with RNAprotect were pelleted and stored at -80°C until RNA extraction, using the Ambion mirVana RNA kit (Austin, TX) in combination with the Barocycler (Pressure BioSciences, South Easton, MA). RNA quantity and quality was assessed by measuring total RNA using a nanodrop 1000 spectrophotometer (Thermo Scientific) and visualizing RNA on an agarose gel. Purified RNA was stored at 80°C.

Table 2. B. anthracis chromosomal genes upregulated in the 34F,ΔluxS strain as determined by microarray analysis, sorted by locus

| Locus<sup>a</sup> | Gene Name<sup>b</sup> | 30 min<sup>c</sup> | 60 min<sup>c</sup> | 90 min<sup>c</sup> | 120 min<sup>c</sup> |
|-------------------|------------------------|------------------|------------------|------------------|------------------|
| BA0194            | oligopeptide ABC transporter, oligopeptide-binding protein | 1.42             | 5.03             | 4.44             | 7.67             |
| BA0369            | methyl-accepting chemotaxis protein | 6.15             | 11.47            | 6.68             | 6.28             |
| BA0558            | methyl-accepting chemotaxis protein | 12.47            | 11.47            | 11.00            | 9.85             |
| BA0575            | methyl-accepting chemotaxis protein | 4.86             | 7.16             | 6.45             | 8.51             |
| BA0677            | phospholipase C (plc) | 4.14             | 7.06             | 5.28             | 6.54             |
| BA0682            | hypothetical protein | 3.46             | 5.70             | 7.06             | 6.36             |
| BA0796            | conserved hypothetical protein | 2.20             | 5.39             | 6.82             | 16.56            |
| BA2025            | hypothetical protein | 9.32             | 14.22            | 10.85            | 12.73            |
| BA2114            | RNA polymerase sigma-70 factor, ECF subfamily | 1.17             | 3.61             | 6.82             | 12.21            |
| BA2115            | hypothetical protein | 1.68             | 3.48             | 7.84             | 9.99             |
| BA2363            | transcriptional regulator, ArsR family | 3.78             | 11.63            | 12.38            | 7.67             |
| BA2606            | hypothetical protein | 7.52             | 11.63            | 7.94             | 6.41             |
| BA2732            | RNA polymerase sigma factor SigX, putative | 1.35             | 2.77             | 15.67            | 64.00            |
| BA3029            | succinylornithine transaminase, putative | 1.23             | 4.89             | 7.41             | 7.78             |
| BA3034            | hypothetical protein | 1.55             | 5.06             | 4.23             | 8.22             |
| BA3114            | membrane protein, putative | 13.45            | 10.78            | 7.36             | 11.24            |
| BA3305            | transcriptional regulator, ArsR family | 3.76             | 7.67             | 5.06             | 10.34            |
| BA3405            | ABC transporter, permease protein | 1.28             | 1.83             | 8.57             | 11.63            |
| BA3406            | ABC transporter, ATP-binding protein | 0.71             | 2.46             | 6.77             | 18.13            |
| BA3407            | hypothetical protein | 1.08             | 2.22             | 7.11             | 8.28             |
| BA4264            | nitroreductase family protein | 1.52             | 2.81             | 3.97             | 7.16             |
| BA5481            | conserved domain protein | 7.31             | 14.03            | 4.89             | 7.26             |
| BA5628            | iron compound ABC transporter | 1.85             | 4.11             | 3.76             | 7.01             |

<sup>a</sup>Numbers represent fold change over the 120 min time course of the experiments. <sup>b</sup>Functional annotations were obtained from the B. anthracis complete genome sequence (www.jcvi.org); <sup>c</sup>Locus based on Ames strain of B. anthracis.
A selected set of genes that appeared to be co-regulated in the linear expression map were analyzed to discover motifs in upstream areas that may identify sulfate and 1% bovine serum albumin at 42°C for 60 min. The slides then were washed at room temperature with distilled water, dipped in isopropanol and allowed to dry. Equal volumes of the appropriate Cy3- and Cy5-labeled probes were combined, dried and then resuspended in a solution of 40% formamide, 5x SSC and 0.1% sodium dodecyl sulfate. Resuspended probes were heated to 95°C prior to hybridization. The probe mixture then was added to the microarray slide and allowed to hybridize overnight at 42°C. Hybridized slides were washed sequentially in solutions of 1x SSC-0.2% SDS, 0.1x SSC-0.2% SDS and 0.1x SSC at room temperature, then dried in air and scanned with an Axon GenePix 4000 scanner (intranet.itc.jcvi.org/sops/M008.pdf). All wash buffers were supplemented with 1-ml of 0.1 M DTT per liter of wash buffer. Individual TIFF images from each channel were analyzed with TIGR Spotfinder (available at pfgrc.jcvi.org/index.php/bioinformatics.html). Microarray data were normalized by LOWESS normalization and with in-slide replicate analysis using TM4 software MIDAS (available at pfgrc.jcvi.org/index.php/bioinformatics.html).38,60 We selected genes in which comparison of wild-type cells exposed to diluent alone and cells exposed to 20 µg/ml of fur-2 yielded log₂ value \(|\geq 1.5|\) in all samples. We also selected genes that had a log₂ value \(|\geq 1.5|\) in all samples, comparing wild-type cells to ΔluxS cells. Genes with a log₂ value \(|\leq 1.5|\) but a log₂ value \(|\geq 1|\) were selected if the gene appeared in the middle of an operon.

**Analysis of upstream promoter motifs.** A selected set of genes that appeared to be co-regulated in the linear expression map were analyzed to discover motifs in upstream areas that may identify

### Table 3. *B. anthracis* chromosomal genes downregulated in the 34F,ΔluxS strain as determined by microarray analysis, sorted by locus

| Locus | Gene name | 30 min | 60 min | 90 min | 120 min |
|-------|-----------|--------|--------|--------|---------|
| BA0238 | hypothetical protein | -3.41 | -29.65 | -5.78 | -15.78 |
| BA0239 | hypothetical protein | -3.86 | -22.16 | -5.86 | -19.29 |
| BA0240 | 4-hydroxyphenylpyruvate dioxygenase (hppD) | NaN | -11.63 | -9.51 | -6.41 |
| BA0509 | formate acetyltransferase (pf) | -33.13 | -125.37 | -56.89 | -35.51 |
| BA0510 | pyruvate formate-lyase-activating enzyme (pflA) | -1.26 | -23.26 | -36.00 | -21.71 |
| BA0668 | ribose ABC transporter, permease protein (rbsC) | -1.42 | -14.42 | -12.91 | -7.94 |
| BA0669 | ribose ABC transporter, ribose-binding protein (rbsB) | -1.58 | -15.89 | -11.88 | -6.96 |
| BA0887 | S-layer protein EAI (eog) | -7.26 | -25.63 | -46.85 | -3.97 |
| BA1086 | sugar-binding transcriptional regulator, Lacl family | -3.14 | -19.29 | -11.08 | -4.20 |
| BA2125 | respiratory nitrate reductase, alpha subunit (narG) | -6.68 | -4.20 | -52.71 | -42.22 |
| BA2126 | respiratory nitrate reductase, beta subunit (narH) | -1.13 | -3.18 | -29.24 | -17.39 |
| BA2128 | respiratory nitrate reductase, gamma subunit (narL) | -1.25 | -3.12 | -38.59 | -18.13 |
| BA2133 | molybdenum cofactor biosynthesis protein A (narA-1) | -1.84 | -6.36 | -45.25 | -14.83 |
| BA2134 | molybdopterin biosynthesis protein MoeB, putative | -1.07 | -11.91 | -11.01 | -7.96 |
| BA2135 | molybdopterin biosynthesis protein MoeA (moeA-1) | -1.12 | -2.19 | -35.26 | -13.64 |
| BA2136 | molybdopterin converting factor, subunit 2 (moeE-1) | -1.13 | -2.95 | -48.84 | -20.53 |
| BA2137 | molybdopterin converting factor, subunit 1 (moeD-1) | -1.16 | -1.91 | -22.78 | -29.86 |
| BA2138 | nitrate transporter (narK) | -1.24 | -1.92 | -37.53 | -21.11 |
| BA2146 | nitrite reductase [NAD(P)H], large subunit (narB) | -1.33 | -76.11 | -60.97 | -210.84 |
| BA2267 | alcohol dehydrogenase, zinc-containing | -3.61 | -12.91 | -6.45 | -9.19 |
| BA2295 | acetate CoA-transferase, subunit A (atoD) | -1.01 | NaN | -8.11 | -22.16 |
| BA2350 | carboxyvinyl-carboxyphosphonate phosphorylmutase (yqiQ) | -1.52 | -5.24 | -12.13 | -19.16 |
| BA2547 | acyl-CoA dehydrogenase | 2.01 | -13.36 | -19.70 | -10.48 |
| BA2548 | acetyl-CoA carboxylase, biotin carboxylase, putative | -1.66 | -7.06 | -17.27 | -9.99 |
| BA2552 | carboxyl transferase domain protein | -1.24 | -16.45 | -11.39 | -10.27 |
| BA3479 | transcriptional regulator, ArsR family | -36.00 | -44.94 | -37.27 | -136.24 |
| BA3481 | hypothetical protein | -38.85 | -315.17 | -137.19 | -69.55 |
| BA3482 | conserved hypothetical protein | -32.67 | -36.00 | -64.45 | -140.07 |
| BA3483 | conserved hypothetical protein | -30.48 | -67.18 | -53.82 | -219.79 |
| BA3663 | anaerobic ribonucleoside-triphosphate reductase | NaN | -14.03 | -4.66 | -9.92 |
| BA5047 | autoinducer-2 production protein LuxS (luxS) | -14.72 | -28.84 | -29.86 | -494.56 |
| BA5696 | superoxide dismutase, Mn (sodA-2) | -1.77 | -7.62 | -14.62 | -7.11 |

*Numbers represent fold change over the 120 min time course of the experiments; Functional annotations were obtained from the *B. anthracis* complete genome sequence (www.jcvi.org); Locus based on Ames strain of *B. anthracis*; NaN indicates a miss data point.*
the discriminative motif finding tool, DEME. DEME requires two input sequence lists; one input corresponds to upstream areas believed to possibly contain a shared motif and the other set consists of upstream sequences of genes that are thought to not contain the motif within the positive group. The algorithm attempts to find a motif that best separates the positive group of upstream sequences corresponding to the co-regulated common transcription factor binding sites. For motif discovery analysis, 200 base sequences upstream of each gene in the genome were collected. These upstream sequences were truncated when necessary to exclude sequences that crossed a neighboring gene boundary. Upstream sequences less than 30 bases were excluded. If the gene was transcribed on the minus strand, the reverse complement of the upstream region was analyzed. Motif finding was performed using the discriminative motif finding tool, DEME. DEME requires two input sequence lists; one input corresponds to upstream areas believed to possibly contain a shared motif and the other set consists of upstream sequences of genes that are thought to not contain the motif within the positive group. The algorithm attempts to find a motif that best separates the positive group of upstream sequences corresponding to the co-regulated gene

**Table 4.** Select genes downregulated on pXO1 in *B. anthracis* 34FΔluxS as determined by microarray analysis, sorted by locus

| Locus  | Gene name                        | 30 min | 60 min | 90 min | 120 min |
|--------|----------------------------------|--------|--------|--------|---------|
| BXA0142| calmodulin-sensitive adenylate cyclase (cya) | 1.03   | -2.00  | -3.46  | -4.69   |
| BXA0146| transcriptional activator AtxA (atxA)      | -1.34  | -1.16  | -1.25  | -1.83   |
| BXA0164| protective antigen (pagA)              | -1.02  | -1.53  | -7.21  | -26.17  |
| BXA0165| hypothetical protein                 | 1.21   | -1.74  | -7.52  | -20.97  |
| BXA0166| transcriptional repressor PagR (pagR)     | -1.13  | -2.13  | -6.11  | -13.36  |
| BXA0167| hypothetical protein                | 1.36   | -1.39  | -5.70  | -10.27  |
| BXA0168| hypothetical protein                | -1.20  | -1.13  | -2.46  | -2.62   |
| BXA0169| hypothetical protein                | -1.23  | -1.20  | -2.10  | -2.89   |
| BXA0170| hypothetical protein                | -1.24  | 1.16   | -1.71  | -4.23   |
| BXA0171| ribonuclease domain protein         | -1.59  | -1.49  | -2.99  | -7.62   |
| BXA0172| lethal factor (lef)                 | -1.02  | 1.05   | -2.71  | -4.92   |

*aNumbers represent fold change over the 120 min time course of the experiments; Bolded genes indicate genes involved in *B. anthracis* toxin production or its regulation; *Functional annotations were obtained from the *B. anthracis* complete genome sequence (www.jcvi.org); *Locus based on Ames strain of *B. anthracis*.

**Table 5.** Select virulence genes downregulated on pXO1 in 34FΔluxS compared to 34FΔluxS:comp

| Locus  | Gene name                        | 120 min |
|--------|----------------------------------|---------|
| BXA0124| S-layer protein                 | -5.16   |
| BXA0142| calmodulin-sensitive adenylate cyclase (cya) | -3.97   |
| BXA0146| transcriptional activator AtxA (atxA)      | -2.9    |
| BXA0164| protective antigen (pagA)              | -13.78  |
| BXA0165| hypothetical protein                | -16.83  |
| BXA0166| transcriptional repressor PagR (pagR)     | -14.66  |
| BXA0172| lethal factor (lef)                 | -4.32   |

*aNumbers represent fold change; Bolded genes indicate genes involved in *B. anthracis* toxin production or its regulation; *Functional annotations were obtained from the *B. anthracis* complete genome sequence (www.jcvi.org); *Locus based on *B. anthracis* strain A2012.

**Table 6.** Select *B. anthracis* genes on pXO1 downregulated by fur-2

| Locus  | Gene name                        | Symbol | 120 min |
|--------|----------------------------------|--------|---------|
| BXA0124| S-layer protein                 | cyaA    | -16.63  |
| BXA0142| calmodulin-sensitive adenylate cyclase (cya) | -1.41   |
| BXA0146| transcriptional activator AtxA (atxA)      | -1.34  |
| BXA0164| protective antigen (pagA)              | -13.48 |
| BXA0166| transcriptional repressor PagR (pagR)     | -5.81  |
| BXA0172| lethal factor (lef)                 | lef     | -2.03   |

*aNumbers represent fold change; *Functional annotations were obtained from the *B. anthracis* complete genome sequence (www.jcvi.org); *Locus based on *B. anthracis* strain A2012.
set from the negative set, representing all other upstream sequences in the genome. A 15 base window was used and the reverse complement of each upstream sequence also was analyzed. DEGEM reports a consensus motif and upstream sequence matches for each positive and negative sequence. NCBI refseq was the source for the whole genome sequence file and the coordinate file (ptf file) used to extract upstream sequences.

**Analysis of small RNAs in *B. anthracis* 34F AluxS strain.**

Cells were grown to early log phase and pellets collected at 30, 60, 90 and 120 minutes. Total RNA was extracted from cell pellets previously treated with RNAProtec (Qiagen), and the extracted RNAs were treated with Turbo DNase-free DNase (Ambion), then converted to cDNA and labeled according to the Affymetrix protocol. Labeled probes were hybridized onto the Affymetrix platform and incubated overnight in an Affymetrix oven. Chips were washed, scanned and .CEL files were imported into genomeMTV (www.genomeMTV.org) for analysis. Data were normalized by the genomeMTV software, based on quantum normalization. Analysis of pXO1 using the sliding window of genomeMTV permitted us to visually scan the virulence plasmid and inspect for intergenic transcriptional activity.

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**Note**

Supplementary materials can be found at:

- www.landesbioscience.com/supplement/JonesVIRU1-2-Sup1.xls
- www.landesbioscience.com/supplement/JonesVIRU1-2-Sup2.pdf

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