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An Overlap between the Control of Programmed Cell Death in *Bacillus anthracis* and Sporulation[▽]†

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The *Staphylococcus aureus cid* and *lrg* operons have been shown to control cell death and lysis in a manner thought to be analogous to programmed cell death (apoptosis) in eukaryotic organisms. Although orthologous operons are present in a wide variety of bacterial species, members of the *Bacillus cereus* group are unique in that they have a total of four *cid-/lrg*-like operons. Two of these operons are similar to the *S. aureus cid* and *lrg* operons, while the other two (designated *clhAB*$_1$ and *clhAB*$_2$) are unique to this group. In the present study, the functions and regulation of these loci were examined. Interestingly, the *Bacillus anthracis lrgAB* mutant displayed decreased stationary-phase survival, whereas the *clhAB*$_2$ mutant exhibited increased stationary-phase survival compared to the parental and complementation strains. However, neither mutation had a dramatic effect on murein hydrolase activity or autolysis. Furthermore, a quantitative analysis of the sporulation efficiency revealed that both mutants formed fewer spores than did the parental strain. Similar to *S. aureus*, *B. anthracis lrgAB* transcription was shown to be induced by gramicidin and CCCP, agents known to dissipate the proton motive force, in a *lytSR*-dependent manner. Northern blot analyses also demonstrated a positive role for *lytSR* in the *clhAB*$_2$ transcription. Taken together, the results of the present study demonstrate that *B. anthracis lrgAB* and *clhAB*$_2$ play important roles in the control of cell death and lysis and reveal a previously unrecognized role of this system in sporulation.

Studies have shown that the *Staphylococcus aureus cidABC* and *lrgAB* operons play an important role in the control of cell death and lysis (22). The *cidA* gene exerts its effect by increasing murein hydrolase activity to increase cell lysis (21, 24, 25), while the *lrgAB* operon inhibits murein hydrolase activity and lysis (11). Based on the predicted structural features of the CidA and LrgA proteins, along with the antagonistic effects of *cid* and *lrg* mutations on murein hydrolase activity, CidA and LrgA have been proposed to regulate cell death and lysis in a manner analogous to bacteriophage-encoded holins and antiholins, respectively (4). One biological function of the *cidA* gene is in the control of cell lysis during biofilm development (26). The resulting genomic DNA released (designated eDNA) was found to provide an important structural role in the biofilm matrix. These studies have led to the hypothesis that the *cid* and *lrg* gene products comprise the molecular machinery mediating bacterial programmed cell death (4, 22).

Analysis of *S. aureus cidABC* and *lrgAB* transcription have revealed two overlapping regulatory pathways, one mediated by the LysR-type transcriptional regulator, CidR, and responding to glucose metabolism (33), and the other induced by changes in membrane potential and involving the two-component regulatory system, LytSR (20). The *cidABC* operon lies downstream from the *cidR* gene (25, 33), and analysis of a *cidR* mutant indicated that CidR enhances *cidABC* and *lrgAB* expression in the presence of acetic acid generated by the metabolism of high levels of glucose (33). By comparison, expression of *lrgAB* is positively regulated by the *lytSR* operon (6) located immediately upstream of *lrgAB*. Recent studies in *S. aureus* have shown that this regulatory system is required for the induction of *lrgAB* expression in response to various agents affecting membrane potential (20). Typical of two-component regulatory systems, LytS is predicted to be a membrane protein that upon stimulation interacts with its cognate response regulator, LytR, which then increases the expression of the *lrgAB* operon. Initial studies of the *S. aureus lytSR* operon revealed that this novel regulatory system affects murein hydrolase activity and autolysis, presumably due to its regulation of *lrgAB* expression (6). Furthermore, recent studies demonstrate that *lytSR* is also involved in regulating biofilm development in *S. aureus* (unpublished data).

A sequence analysis of other microbial genomes has revealed the presence of *cidAB* and *lrgAB* homologues, as well as the *cidR* and *lytSR* regulatory genes, in a wide variety of gram-positive and gram-negative bacteria and several archaeal species (4). One such organism is *Bacillus anthracis*, which contains operons with open reading frames similar to the previously characterized *cid* and *lrg* operons of *S. aureus*. As in *S. aureus*, *B. anthracis cid* and *lrg* operons are located downstream of genes encoding a LysR-type transcriptional regulator and a two-component regulatory system, respectively (1). Unlike in *S. aureus*, the *cidR* gene is divergently transcribed in *B. anthracis*, and there is no *cidC* gene, encoding a pyruvate oxidase, in the *B. anthracis* genome (1). The *B. anthracis* CidR homologue was shown to play an important role in the regulation of *cid* and *lrg* expression, as well as cell death in the stationary phase (1). That study also showed that the genes encoding S-layer proteins are regulated by CidR and their gene products, Sap and EA1, possess murein hydrolase activity.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
B. anthracis		
Sterne	*B. anthracis* ($pXO1^+$ $pXO2^-$)	31
KB5000	Sterne *lytSR*::Km; Km^r	This study
KB5050	KB5000 with pLC17	This study
KB5400	Sterne *lrgAB*::Km; Km^r	This study
KB5450	KB5400 with pLC20	This study
KB6000	Sterne *clhAB*$_2$::Km; Km^r	This study
KB6050	KB6000 with pJA22	This study
E. coli		
DH5α	Host strain for construction of recombinant plasmids	Invitrogen
JM110	Dam^- Dcm^- strain	34
Plasmid		
pDG780	Source of Km^r cassette; Km^r Amp^r	12
pKS1	Temperature-sensitive plasmid for *B. anthracis*; Km^r	28
pJA71	Derivative of pKS1 containing the kanamycin cassette flanked by *lytSR* fragments; Km^r	This study
pCN51	Shuttle vector; Em^r Amp^r	4
pLC17	*lytSR* complementation plasmid	This study
pBKJ236	Integration plasmid for *B. anthracis*	14
pJA22	*clhAB*$_2$ complementation plasmid	This study
pLC20	*lrgAB* complementation plasmid	This study

[a] Em^r, erythromycin resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
LytSRF1	GGCGCTCGAGGTTGCTTTTGTTCCCATATTTTCGG
LytSRR1	CCGAAGCTTAGATCTATAAGTCCGACGCGTTCAATC
LytSRF2	CAACTGCAGTCAGCCATGGTTCAACTCTA
LytSRR2	CCGCACTAGTCGATAGCTGATAAAATTTGCCACC
Clh2F1	ATAGGGCCCCCAAGTGACAATCCAACGACA
Clh2R1	TGTCTCGAGATGCCAGTTGCGATGAGTGTT
Clh2F2	GATCTGCAGTGTACCCACGTTCCAACCATA
Clh2R2	GCGTCTAGAATAGAGTTATCAAGTAAAGCG
LrgABKOF3a	GGACTCGAGGTGTAGAGGACGCATTAGAAG
LrgABKOR3	CGGAGATCTAATGACTAACCCGATTACCGATG
LrgABKOF4	TAACTGCAGAACAGCGGGTCACGCATTAGGAG
LrgABKOR4	GCGACTAGTTTATTTTGTTCTAACGTCCTTCTCCAT
5LytSRCOMP	CCCGGATCCGTAAAAGCTCAATACCTCACCTCG
3LytSRCOMP	CCCGAATTCGGAAACGCTCTCTAAATTTCAC
Clh2F3	ACTCTGCAGTCCGTTAGTCACATTCCC
Clh2R3	TGCTCTAGACTTGGCGTACCTCCTACA
5FwdlrgABCOMP	CGCGGTACCAAAGAGGTGGCCAAAATGAG
3RevlrgABCOMP	CCCGGATCCGTTTTCCTATCCAATAAACGGCATA
lrgAF	ATTTACCAATTCCAATGCCCTCA
lrgAR	CGTGCTTGCGTCCTTTATTTACT
lrgBF	AATCGCATACGGAATCGGAACA
lrgBR	TCCAATAAACGGCATAAACATCG
Clh2AB-1F	CTGGATTGCAAAGCTGCTC
Clh2AB-1R	TTCATCGCTCTCATCACACC
Clh2AB-2F	TCATCGCGACTCTCTTTCCT
Clh2AB-2R	TCTTGTCCAAATTGCTGCTC

In the present study, we examined the *B. anthracis* LytSR two-component regulatory system and have begun the characterization of two additional *cid/lrg* homologues (designated *clhAB*$_1$ and *clhAB*$_2$ [for *cid/lrg* homologues AB-1 and AB-2]) within the *B. anthracis* genome. The results presented here confirm that the *lytSR* operon of *B. anthracis*, like that of the *S. aureus lytSR*, positively regulates *lrgAB* expression and that *lrgAB* transcription is induced by dissipation of the proton motive force in a *lytSR*-dependent fashion. In addition, the *lrgAB* and *clhAB*$_2$ mutations were shown to affect stationary-phase survival and lysis. Finally, the results presented reveal for the first time that this system affects sporulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the present study are listed in Table 1. *B. anthracis* Sterne was grown in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth or filter-sterilized NZY broth (3% [wt/vol] N-Z Amine A and 1% [wt/vol] yeast extract - adjusted to pH 7.5). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium. Agar was added (15 g per L) when needed. All liquid cultures were grown at 37°C with constant shaking (250 rpm) with a culture volume to flask ratio of no greater than 1:10. Chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Sporulation assays were carried out in Schaeffer's medium (12). When necessary, antibiotics were used at the following concentrations: ampicillin (50 μg ml^{-1}), erythromycin (5 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and chloramphenicol (10 μg ml^{-1}).

DNA manipulations. *B. anthracis* genomic DNA was isolated by using a Qiagen (Valencia, CA) genomic DNA purification kit according to the manufacturer's instructions. Plasmid DNA was purified by using the Wizard Plus SV DNA purification kit (Promega, Inc., Madison, WI). Restriction endonucleases and T4 DNA ligase were purchased from either New England Biolabs (Beverly, MA) or Invitrogen Life Technologies (Carlsbad, CA). *E. coli* competent cells were prepared as performed by Dagert and Ehrlich (9) and electroporation of *B. anthracis* cells were carried out as described by Koehler et al. (15).

Allele replacement of *lytSR*. A *lytSR* mutation was generated in *B. anthracis* Sterne using an allele replacement strategy as follows. A 1,025-bp DNA fragment spanning a region 5′ to the *lytSR* genes was PCR-amplified using the forward primer, LytSRF1, and the reverse primer, LytSRR1 (Table 2), incorporating XhoI and HindIII-BglII restriction sites, respectively. This DNA fragment was then ligated into the XhoI and BglII sites of pKS1 (28). Next, a 1,000-bp 3′ *lytSR* fragment was amplified by using the forward primer, LytSRF2, and the reverse primer, LytSRR2 (Table 2), incorporating PstI and SpeI restriction sites, respectively. This fragment was subsequently ligated into the PstI and SpeI sites of pKS1 containing the 5′ *lytSR* fragment. This plasmid (designated pJA52) was transformed into *E. coli* JM110, reisolated, and then used for electroporation into *B. anthracis* (17). Single colonies of the transformed *B. anthracis* cells were grown overnight at 37°C in BHI broth containing erythromycin and then subcultured with 1:1000 dilutions in antibiotic-free BHI broth at 30°C each day for 3 days. After the third day, dilutions of the culture were spread on BHI agar plates containing erythromycin and isolated colonies were subsequently screened for an erythromycin-sensitive, kanamycin-resistant phenotype. Replacement of the *lytSR* operon with the kanamycin resistance gene was confirmed by PCR analysis, and the mutant strain was designated KB5000 (Table 1). An *lrgAB* mutation was made by using a similar strategy using the primer sets LrgABKOF3a and LrgABKOR3 and LrgABKOF4 and LrgABKOR4 (Table 2). This strain was designated KB5400 (Table 1). A similar allele replacement strategy was also used to make

a *clhAB*$_2$ mutation using the primer sets Clh2F1/Clh2R1 and Clh2F2/Clh2R2 (Table 2). This strain was designated KB6000 (Table 1).

Complementation plasmid construction. Complementation of the *lytSR* mutation in KB5000 was achieved by PCR amplification of the *lytSR* open reading frames, along with 540 bp upstream region using Platinum *Pfx* High Fidelity DNA polymerase (Invitrogen) and the primers 5LytSRCOMP and 3LytSRCOMP (Table 2), incorporating SphI and BamHI sites, respectively. The resulting DNA fragment was then ligated into the SphI and BamHI sites of pBK123, a derivative of pCN51 (7), in which the erythromycin cassette has been replaced with chloramphenicol cassette from pC194, generating the plasmid pLC17. This plasmid was introduced into KB5000 by electroporation, generating the *lytSR* complementation strain designated KB5050 (Table 1). Complementation of *clhAB*$_2$ in KB6000 was achieved by PCR amplification of the *clhAB*$_2$ open reading frames, along with a 575-bp upstream region, using the primers Clh2F3 and Clh2R3 (Table 2) incorporating PstI and XbaI sites, respectively. The resulting DNA fragment was then ligated into the PstI and XbaI sites of pHT304 (3), generating pJA22. This plasmid was introduced into KB6000 by electroporation, generating the *clhAB*$_2$ complementation strain KB6050 (Table 1). Complementation of the *lrgAB* mutation in KB5400 was attempted by PCR amplification of the *lrgAB* open reading frames, along with a 500-bp upstream region, using the primers 5FwdlrgABCOMP and 3RevlrgABCOMP (Table 2), incorporating SphI and BamHI sites, respectively. The resulting DNA fragment was then ligated into the SphI and BamHI sites of pCN51 (7), generating the plasmid pLC20. This plasmid was introduced into KB5000 by electroporation, generating strain KB5450 (Table 1).

Isolation of RNA. Overnight cultures of *B. anthracis* strains were inoculated into 100 ml of prewarmed NZY broth (with or without the addition of 35 mM glucose) in a 1-liter flask to an optical density at 600 nm (OD_{600}) of 0.1 and incubated with shaking at 250 rpm for 12 h at 37°C. Cells were harvested at 2, 6, and 12 h postinoculation. Harvested cells were transferred to lysing matrix B tubes (Qbiogene, La Jolla, CA) containing 0.1-mm beads and lysed by shaking them in a Fastprep FP120 instrument (Qbiogene) for 23 s at a setting of 6 (19). The lysates were centrifuged at 13,000 × *g* for 10 min at 4°C, and the supernatants were collected for RNA purification by using an RNeasy kit (Qiagen) according to the protocols provided by the manufacturer.

Northern blot analysis. RNA samples (5.0 μg) were separated by electrophoresis in a 1% agarose gel containing 0.66 M formaldehyde and morpholinepropanesulfonic acid running buffer (20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2.0 mM EDTA [pH 7.0]). The RNA samples were subsequently transferred to a nylon membrane (Micron Separations, Inc., Westboro, MA) by capillary transfer in 20× SSC buffer (0.3 M sodium citrate, 3.0 M NaCl [pH 7.0]) and fixed to the membrane by UV cross-linking using a UV Stratalinker 1800 (Stratagene, Cedar Creek, TX). Hybridization with gene-specific probes was performed using the digoxigenin (DIG) system (Roche Applied Science, Indianapolis, ID) according to the manufacturer's recommendations. DIG-labeled DNA probes were PCR amplified using the gene-specific primers listed in Table 2. Northern hybridization experiments were performed on RNA extracted from the *lytSR*, *lrgAB*, and *clhAB*$_2$ mutants and the expected transcripts were absent in each case, confirming the specificity of the probes used (unpublished results). The sizes of each transcript were determined by comparison to an RNA ladder (Invitrogen) run in the same gel.

Murein hydrolase assays. Cell wall-associated proteins were collected essentially as described previously for *B. subtilis* (10). Fresh overnight cultures of *B. anthracis* strains were used to inoculate 50 ml of NZY broth to an OD_{600} of 0.1, followed by incubation for 6 h at 37°C and 250 rpm. The cells were harvested by centrifugation at 12,000 × *g* for 10 min and resuspended in 1/100 volumes of sample buffer as described previously (10). The cell pellet was boiled for 10 min and centrifuged at 13,000 × *g*, and the supernatants were collected. The extracted proteins were analyzed by zymography as described previously (16).

Determination of stationary-phase survival. Overnight cultures of *B. anthracis* were diluted to an OD_{600} of 0.1 in 50 ml of NZY broth (with or without the addition of 35 mM glucose) in 500-ml flasks, followed by incubation for several days at 37°C with shaking at 250 rpm. At intervals, an aliquot of the cultures were collected to assess viable cell counts and the OD_{600}.

Sporulation assay. Cultures were grown in Schaeffer's medium (13) and samples were taken 48 h after inoculation, diluted in phosphate-buffered saline, and plated on BHI agar before and after heat treatment at 65°C for 30 min. The sporulation efficiency was calculated as the total number of cells surviving heat treatment divided by the total number of cells present prior to heat treatment and then multiplying that value by 100 (32).

Fluorescence microscopy. A Live/Dead BacLight bacterial viability kit (Molecular Probes, Inc.) was used to assess differences in cell morphology and to distinguish between live and dead cells. The method utilizes a mixture of Syto-9 and propidium iodide, which results in the staining of live cells green and dead cells red. Cells were stained according to the manufacturer's instructions and then visualized under ×1,000 magnification with a Nikon Eclipse TS 100 microscope (Nikon Instruments, Inc., Melville, NY). Representative images of each strain were captured by using a Nikon Digital Sight DS-L1 camera.

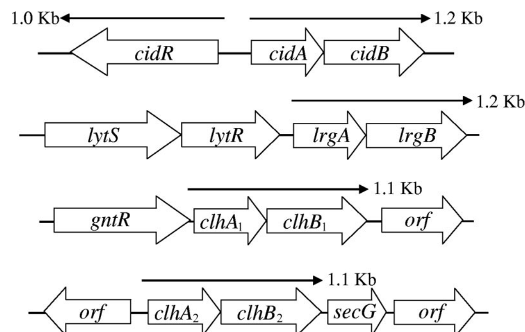

FIG. 1. Schematic diagram of *cid* and *lrg* orthologues of *B. anthracis*. The *cid* and *lrg* operons are located adjacent to *cidR* and *lytSR* loci, respectively, as in *S. aureus*. Two additional loci with similarities to the *S. aureus cid*/*lrg* operons are also present in the *B. anthracis* genome. These genes have been designated *clhA*$_1$ (BAS3599), *clhB*$_1$ (BAS3598), *clhA*$_2$ (BAS4960), and *clhB*$_2$ (BAS4959). The *gntR* (BAS3600) and *secG* (BAS4958) genes encode a potential transcriptional regulator and a subunit of the SecG preprotein translocase, respectively. Open reading frames whose putative products do not match known proteins are labeled "*orf*." Arrows above the genes represent the direction and sizes of transcripts identified by Northern blot analyses presented here and in previous studies (1).

RESULTS

Identification of *cidAB*/*lrgAB* orthologues in *B. anthracis*. A recent study established the existence of the Cid/Lrg regulatory network in *B. anthracis* and has demonstrated its role in the control of cell death and lysis in this pathogen (1). That study also established the importance of the CidR regulatory protein in the control of *cid* and *lrg* expression and demonstrated its impact on cell death in the stationary phase. The goal of the present study was to focus on LytSR-mediated control of this system and to begin to define the transcriptional organization and the functions of the *cid* and *lrg* homologues. Interestingly, a BLAST search of the *B. anthracis* Sterne genome (http://www.ncbi.nlm.nih.gov) revealed the presence of two additional *cidAB*/*lrgAB* orthologues (designated *clhAB*$_1$ and *clhAB*$_2$) besides those associated with the *B. anthracis cidR* and *lytSR* operons (Fig. 1). Sequence analysis of the available genomes revealed that *clhAB*$_1$ and *clhAB*$_2$ are unique to the *B. cereus* group of bacteria, including *B. anthracis* and *Bacillus thuringiensis*. The *B. anthracis clhA*$_1$ and *clhB*$_1$ genes (accession numbers BAS3599 and BAS3598) are predicted to encode 13.2- and 23.3-kDa proteins, respectively, sharing 35 and 30% amino acid sequence identity with the *cidA*- and *cidB*-encoded proteins of *B. anthracis*. The *B. anthracis clhA*$_2$ and *clhB*$_2$ genes (accession numbers BAS4960 and BAS4959) encode proteins with 32 and 37% amino acid sequence identity compared to *B. anthracis cidA* and *cidB*. Based on the sequence similarities of the *clhAB*$_1$ and *clhAB*$_2$ genes to *cidAB* and *lrgAB*, these genes are proposed to participate in the control of cell death and lysis in *B. anthracis*.

Considering the similarities of the *clh*$_1$ and *clh*$_2$ loci to the *cidAB* and *lrgAB* operons, we performed transcription analyses

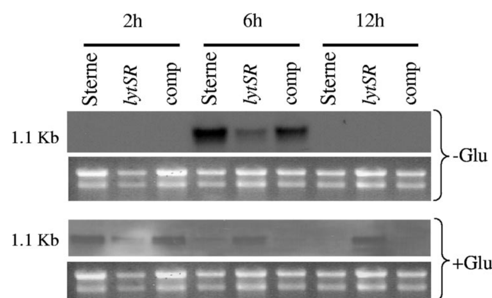

FIG. 2. Northern blot analysis of $clhAB_2$ transcription. *B. anthracis* Sterne, its *lytSR* mutant derivative (*lytSR*), and the *lytSR* complementation strain (comp) were grown in the presence (+Glu) or absence (−Glu) of 35 mM glucose, and RNA was collected at 2, 6, and 12 h. Portions (5 μg) of samples were separated in a 1.0% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to DIG-labeled probes derived from the $clhB_2$ gene. An ethidium bromide-stained gel of the RNA used in these experiments is also shown as a loading control.

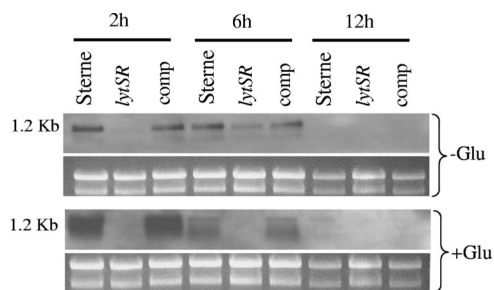

FIG. 3. Northern blot analysis of *lrgAB* transcription. *B. anthracis* Sterne, its *lytSR* mutant derivative (*lytSR*), and the *lytSR* complementation strain (comp) were grown in the presence (+Glu) or absence (−Glu) of 35 mM glucose, and RNA was collected at 2, 6 and 12 h. Portions (5 μg) of samples were separated in a 1.0% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to DIG-labeled probes derived from the *lrgB* gene. An ethidium bromide-stained gel of the RNA used in these experiments is also shown.

to determine whether these genes also form dicistronic operons. Although transcription of the $clhA_1$ and $clhB_1$ genes could not be detected under a variety of growth conditions tested using either Northern blot or reverse transcriptase PCR analyses, transcription of the $clhA_2$ and $clhB_2$ genes was readily detected by Northern blotting (Fig. 2). Furthermore, probes specific for $clhA_2$ and $clhB_2$ genes both hybridized to 1.1-kb transcripts, suggesting that these genes form a dicistronic operon similar to the *cidAB* and *lrgAB* operons (1). We also examined the regulation of $clhAB_2$ in response to glucose, a factor known to affect expression of the *cid* and *lrg* operons. Previously, it has been demonstrated that the *B. anthracis cidAB* transcription is most abundant in the early exponential phase in the presence or absence of glucose (1). Furthermore, *lrgAB* transcription was found to be most abundant in lag to early exponential phase in the presence or absence of glucose. In the absence of glucose, the $clhAB_2$ operon was found to be expressed in the parental strain during late-exponential (6-h) phase, while growth in the presence of glucose resulted in $clhAB_2$ transcription primarily during early exponential growth (Fig. 2).

LytSR-mediated transcriptional control. It was shown previously that the transcription of the *B. anthracis cidAB* and *lrgAB* operons is dependent on the *cidR* gene encoding a putative LysR-type transcription regulator (1). To investigate the impact of *lytSR* on the transcription of *B. anthracis cidAB* and *lrgAB*, we generated a *lytSR* mutant and performed Northern blot analyses on RNA isolated from these cells grown in the presence or absence of glucose. As shown in Fig. 3, dramatically reduced levels of the 1.2-kb *lrgAB* transcripts, both in the presence and in the absence of glucose, were observed in the *lytSR* mutant compared to the parental and complemented strains. In contrast, *cidAB* expression was found to be unaffected by the *lytSR* mutation under the conditions tested (data not shown), which is similar to what was observed in *S. aureus*.

To determine whether transcription of $clhAB_1$ and $clhAB_2$ is affected by *B. anthracis lytSR*, we performed Northern blot analysis as described above using probes specific for these operons. As shown in Fig. 2, transcription of $clhAB_2$ in the *lytSR* mutant was lower than in the parental and complemented strains during the late exponential phase in the absence of glucose. Interestingly, $clhAB_2$ transcription was higher in the *lytSR* mutant in the presence of glucose during late-exponential and stationary phases of growth compared to the parental and complementation strains (Fig. 2), indicating that LytSR can have both positive and negative effects on $clhAB_2$ expression depending on the growth conditions. Finally, to determine the role of *cidR* in the regulation of $clhAB_2$ transcription, RNA was isolated from the previously characterized *B. anthracis cidR* mutant (1) and analyzed by Northern blot analysis. These experiments, however, demonstrated no effect of the *cidR* mutant on $clhAB_2$ expression, indicating that CidR is not involved in the control of this operon under these conditions (data not shown). Again, transcription of the $clhA_1$ and $clhB_1$ genes was not detected in either of the *lytSR* or *cidR* mutants.

Studies in *S. aureus* have also demonstrated that *lrgAB* transcription is induced in a *lytSR*-dependent fashion in response to agents that dissipate membrane potential ($\Delta\psi$) (20). To examine whether *B. anthracis lrgAB* and $clhAB_2$ transcription is similarly regulated, the parental, *lytSR* mutant, and complementation strains were grown to exponential phase and treated with 25 μg of gramicidin/ml or 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to dissipate the $\Delta\psi$, and RNA was collected for Northern blot analyses. As shown in Fig. 4a, *lrgAB* transcription was induced by both of these agents in the parental strain but was not induced in the *lytSR* mutant. Furthermore, $\Delta\psi$-mediated induction of *lrgAB* expression was restored to wild-type levels in the complementation strain. Again, in contrast, $clhAB_2$ expression was found to be repressed by treatment with agents that dissipate the $\Delta\psi$ and was also absent in the *lytSR* mutant and complemented strains (Fig. 4b). Combined, these studies demonstrate that the *B. anthracis* LytSR two-component regulatory system is functionally similar to that of *S. aureus* and that it mediates both $\Delta\psi$-dependent (*lrgAB*) and -independent ($clhAB_2$) transcriptional control in *B. anthracis*.

Assessing the role of *lytSR* in cell death and lysis. Preliminary studies revealed that the *lytSR* mutant grew at the same rate as the Sterne and complemented strains as determined by measurements of optical density and viability in a time course assay (unpublished results). To assess the impact of the *lytSR* mutation on cell death and lysis during the stationary phase, these strains were grown for 9 days, and measurements of the

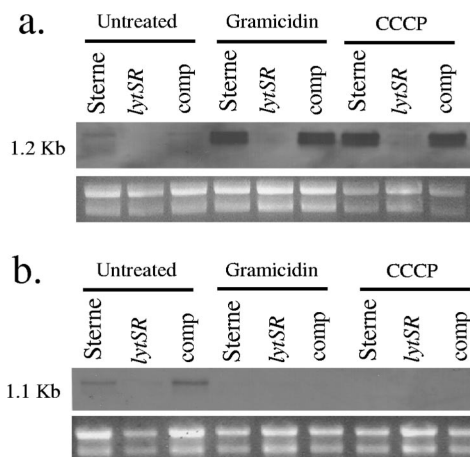

FIG. 4. Δψ-mediated control of *lrgAB* and *chlAB*$_2$ transcription. *B. anthracis* Sterne, its *lytSR* mutant derivative (*lytSR*), and the *lytSR* complementation strain (comp) were grown without glucose for 6 h and treated with gramicidin or CCCP for 15 min prior to RNA isolation. Portions (5 μg) of samples were separated in a 1.0% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to DIG-labeled probes derived from the *lrgB* gene (a) or the *clhB*$_2$ gene (b). An ethidium bromide-stained gel of the RNA used in these experiments is also shown.

cell viability and the optical density were obtained. Unlike the *cidR* mutant, which exhibited a rapid cell death (RCD) phenotype in the stationary phase (1), the *B. anthracis lytSR* mutation did not have a discernible effect on stationary-phase survival and lysis (data not shown). However, the *lytSR* mutant had a significant effect on expression of the 85-kDa Sap S-layer protein (see Fig. S1a in the supplemental material) previously found to contain murein hydrolase activity (1). Similar to results described previously (1), the absence of Sap in the *lytSR* mutant strain corresponded with the loss of an 85-kDa murein hydrolase (see Fig. S1b in the supplemental material). These results are also similar to the finding that the *cidR* mutation in *B. anthracis* caused reduced Sap production (1) and that, as in *S. aureus*, the *B. anthracis* LytSR two-component regulatory system plays a role in regulating murein hydrolase activity. However, this effect on murein hydrolase activity had no discernible impact on cell lysis under the conditions tested.

Analysis of *lrgAB* and *clhAB*$_2$ mutants. Since the *lytSR* mutation in *B. anthracis* only had observable effects on transcription of the *lrgAB* and *clhAB*$_2$ operons, we elected to focus on the functions of *lrgAB* and *clhAB*$_2$. Thus, we generated mutations in the *lrgAB* and *clhAB*$_2$ operons by allele replacement and, as described above, subjected these mutants to assays of cell death and lysis in the stationary phase. Interestingly, similar to the *B. anthracis cidR* mutant (1), the *lrgAB* mutant exhibited a RCD phenotype in the stationary phase, displaying a complete loss of viability after 12 and 48 h of incubation in the presence or absence of glucose, respectively (Fig. 5b). Unlike in *S. aureus*, measurements of the optical density revealed that the *B. anthracis lrgAB* mutant displayed a lysis pattern similar to that of the wild-type strain (Fig. 5a), indicating that while the *lrgAB* mutation had a dramatic effect on cell viability in sthe tationary phase it had little impact on cell lysis. Consistent with this is the observation that the *lrgAB* mutation had little effect on murein hydrolase activity in a zymographic assay (unpublished results). It should also be noted that expression of the *lrgAB* operon from a plasmid failed to restore the wild-type phenotype in the *lrgAB* mutant (Fig. 5a and b), despite the fact that *lrgAB* transcription was restored in this strain (data not shown). Although the reason for the inability to complement this mutation is unknown, these results are similar to studies of the *S. aureus cidA* mutation, which is also not complementable by expression of *cidA in trans* (24, 26). In contrast to the *lrgAB* mutant, the *clhAB*$_2$ mutant exhibited decreased cell death in the stationary phase in the absence of glucose, maintaining a viability that was ~10-fold higher than the parental and complemented strains (Fig. 5d and see Fig. S2 in the supplemental material). In the presence of glucose, the viabilities of the parental, *clhAB*$_2$ mutant, and complementation strains were indistinguishable and remained lower at approximately 8×10^3 CFU per ml. In addition, the optical density measurements of the *clhAB*$_2$ mutant were similar to the parental and complementation strains, both in the presence and in the absence of glucose (Fig. 5c).

To more closely examine the effects of the *lrgAB* and *clhAB*$_2$ mutations on *B. anthracis*, we examined cell viability using a fluorescent Live/Dead BacLight bacterial viability kit (Molecular Probes). In cultures grown overnight in the absence of glucose, both the parental and the *lrgAB* mutant strains showed normal rod-shaped cells (unpublished results). At the 48-h time point, cultures of the parental strain contained a mixture of live and dead cells exhibiting green and red fluorescence, respectively (Fig. 6). However, at 48 h, the time in which the *lrgAB* mutant viability drops to undetectable levels, the majority of the *lrgAB* mutant cells stained red as expected but appeared unevenly stained within the cells. Furthermore, the cells failed to separate normally and thus formed long chains. Strikingly, the staining pattern and the appearance of the *clhAB*$_2$ mutant were drastically different compared to the parental and *lrgAB* mutant strains. As shown in Fig. 6, the *clhAB*$_2$ mutant appeared shorter and formed more rounded cells. Similar to the *lrgAB* mutant, the *clhAB*$_2$ mutant also failed to separate normally. Furthermore, the *clhAB*$_2$ mutant cells were only weakly stained with Syto-9, suggesting that the cells may be less robust and/or contained a compromised cell membrane. Importantly, the wild-type appearance of cells was restored when *clhAB*$_2$ was expressed in *trans*.

Effect of *lrgAB* and *clhAB*$_2$ mutations on sporulation. Given the dramatic effect of the *lrgAB* and *clhAB*$_2$ mutations on the cells in the stationary phase, we predicted that these mutations would also affect the ability of the cells to undergo sporulation. Thus, the abilities of the *lrgAB* and *clhAB*$_2$ mutants to sporulate were assessed. As shown in Fig. 7, the parental strain had a sporulation efficiency of ~88% after 48 h of growth in the Schaeffer's medium. In contrast, the *lrgAB* and *clhAB*$_2$ mutants showed dramatically reduced sporulation efficiencies (ca. 5 and 11%, respectively) after 48 h. As a negative control, the *spo0A* mutant was also tested and found not to form spores in this assay as expected. As described above, the sporulation defect of the *clhAB*$_2$ mutant was restored to nearly wild-type levels in the complementation strain. These results demonstrate that the *lrgAB* and *clhAB*$_2$ gene products have a dramatic impact on sporulation in *B. anthracis*.

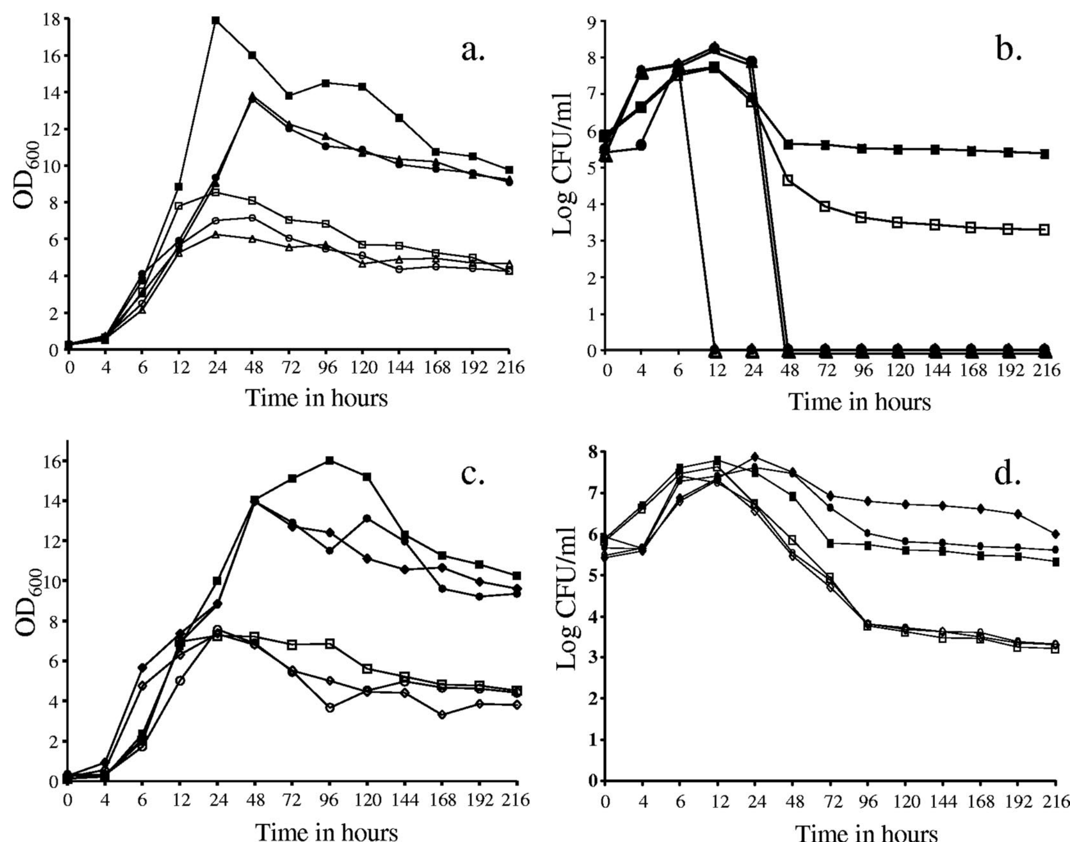

FIG. 5. Stationary-phase survival assays. *B. anthracis* Sterne (squares), its *lrgAB* mutant derivative (a and b, triangles), the *lrgAB* complementation strain (a and b, circles), its $clhAB_2$ mutant derivative (c and d, diamonds), and the $clhAB_2$ complementation strain (c and d, circles) were grown in the presence (open symbols) or absence (solid symbols) of 35 mM glucose, and samples were removed and assayed to determine the optical density (OD_{600} [a and c]) and cell viability (log CFU/ml [b and d]). The data presented are representative of three independent experiments. Additional replicates are presented in Fig. S2 in the supplemental material.

DISCUSSION

Previously, our laboratory has demonstrated that expression of the *B. anthracis cidAB* and *lrgAB* operons is affected by the *cidR* gene encoding an LysR-type transcriptional regulator (1). Disruption of the *cidR* gene resulted in altered murein hydrolase activity and RCD in the stationary phase. In the present study we focused on the *B. anthracis* LytSR two-component regulatory system and show that, as in *S. aureus*, this system plays a positive role in *lrgAB* expression but had no apparent effect on *cidAB* expression under the growth conditions tested. The data presented here also demonstrate that, just as in *S. aureus*, $\Delta\psi$-mediated induction of *lrgAB* expression in *B. anthracis* is dependent on the LytSR two-component regulatory system, indicating that this regulator is involved in sensing changes associated with $\Delta\psi$. Thus, these data indicate that that the LytSR-mediated control of *lrgAB* expression is well conserved between *S. aureus* and *B. anthracis*.

We also examined two additional *cidAB*/*lrgAB* homologues in *B. anthracis*, designated $clhAB_1$ and $clhAB_2$, in addition to those associated with *lytSR* and *cidR*. These homologues are unique to the *B. cereus* group and, based on their sequence similarity to *cid* and *lrg*, were hypothesized to be involved in cell death and lysis. Similar to the *B. anthracis cidAB* and *lrgAB* operons, $clhA_2$ and $clhB_2$ genes form a dicistronic operon and were induced by growth in the presence of glucose. However, in contrast to the *B. anthracis lrgAB* expression, the glucose-dependent induction of $clhAB_2$ was independent of *cidR*. Furthermore, our studies demonstrated that $clhAB_2$ expression is affected by *lytSR*, but whether expression was increased or decreased by this mutation was dependent on the presence of glucose in the culture medium. Our results also demonstrate that $clhAB_2$ expression was repressed by agents that dissipate $\Delta\psi$, unlike *lrgAB* expression in both *B. anthracis* and *S. aureus*, which was induced by these compounds. These data clearly indicate that the regulatory strategies utilized to control *lrgAB* and $clhAB_2$ expression in response to carbohydrate metabolism and $\Delta\psi$ are similar but that subtle species-specific variations exist. As in *S. aureus*, the *B. anthracis* LytSR two-component system may be involved in sensing key metabolic signals including but not limited to dissipation of $\Delta\psi$ to affect *lrgAB* and $clhAB_2$ expression. Although the precise signal and molecular mechanism by which this signal transduction pathway functions is unknown, we speculate that this system provides a means by which the bacteria can assess its overall metabolic state. However, it is likely that the differences in regulation observed reflects the unique metabolic requirements of *B. anthracis* and *S. aureus*. Unfortunately, the expression of $clhA_1$ and $clhB_1$ could not be detected under any of the conditions

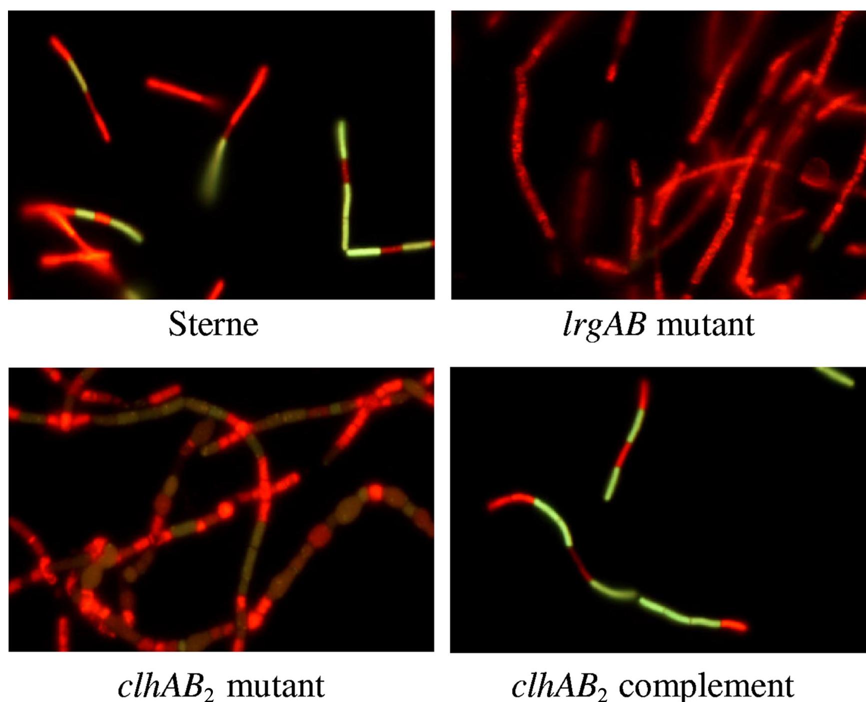

FIG. 6. Microscopic examination of cell viability. Fluorescence microscopy using Live/Dead BacLight staining of the *B. anthracis* Sterne, its *lrgAB* mutant, its *clhAB*$_2$ mutant derivative, and the *clhAB*$_2$ complementation strain after incubation in NZY broth for 48 h. Red staining is indicative of dead or damaged cells, while green staining indicates live cells.

tested, so no information regarding its regulatory control could be assessed.

Based on previous studies in *S. aureus*, we proposed that *cidA* and *lrgA* gene products regulate murein hydrolase activity in a manner similar to holins and antiholins, respectively (5, 11, 22, 23). In contrast to previous findings in *S. aureus*, disruption of *B. anthracis lrgAB* and *clhAB*$_2$ had a minimal effect on murein hydrolase activity, possibly as a result of the redundancy of these genes. However, the *lrgAB* and *clhAB*$_2$ mutants displayed altered stationary-phase viability compared to the parental strain (Fig. 5). Similar to the *B. anthracis cidR* mutant,

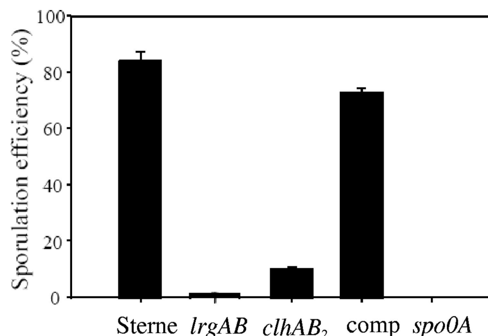

FIG. 7. Impact of *lrgAB* and *clhAB*$_2$ mutations on sporulation. *B. anthracis* Sterne, *lrgAB* mutant, *clhAB*$_2$ mutant, *clhAB*$_2$ complementation strain KB6050 (comp), and *spo0A* mutant were grown in Schaeffer's medium. After 48 h of incubation at 37°C, dilutions were spread on BHI agar before and after heat treatment at 65°C for 30 min. The sporulation efficiency was calculated as the ratio of the number of spores to the number of viable cells for each strain.

the *lrgAB* mutant exhibited an RCD phenotype in stationary phase that was enhanced by growth in the presence of excess glucose. Consistent with this, Live/Dead staining of stationary-phase cells revealed that the *lrgAB* mutant population was comprised of mostly dead cells in comparison to the parental strain. Although the *lrgAB* mutant displayed a dramatic affect on cell viability, it did not have any effect on stationary-phase lysis. In contrast to the *lrgAB* mutant, the *clhAB*$_2$ mutant enhanced stationary phase survival when grown in the absence of glucose (Fig. 5c and d). Interestingly, Live/Dead staining revealed that although the *clhAB*$_2$ mutant strain exhibited enhanced stationary-phase survival after 48 h of incubation, the cells of the *clhAB*$_2$ mutant were visibly shorter and stained weakly with Syto-9, indicating that the cells may be less robust and/or contained a compromised cell membrane. Collectively, these results suggest that, in *B. anthracis*, *lrgAB* and *clhAB*$_2$ operons regulate cell death in the stationary phase in an opposing manner without having a pronounced effect on stationary-phase lysis.

The control of bacterial cell death and lysis is proposed to be important in biofilm adherence and maturation (4). In *S. aureus*, CidA-dependent lysis during biofilm development results in the release of DNA, which becomes a structural component of the biofilm matrix (2, 26). Studies in different organisms have shown that the regulation of cell death and lysis is important for other bacterial developmental processes such as natural competence, fruiting body formation, and sporulation (18, 27). In the present study we demonstrate that mutations in the *B. anthracis lrgAB* and *clhAB*$_2$ operons dramatically reduced sporulation efficiencies compared to the parental strain.

Studies in sporulating bacteria such as *B. subtilis* have shown that the differentiation processes of sporulation require a number of rearrangements and modifications of cell wall peptidoglycan that include at least four events in which autolysins are likely to be involved (29, 30). These four events include (i) asymmetric cell division, (ii) engulfment of the prespore by the mother cell, (iii) lying down of peptidoglycan around the forespore, and (iv) lysis of the mother cell to release the mature endospore (29). It is possible that *lrgAB* and *clhAB*$_2$ mutations may be involved in any of these four events of differentiation, thereby affecting the sporulation potential of the strain. Alternatively, the control of cell death and lysis of a subpopulation of cells may affect sporulation in a manner related to cannibalism as previously described (8, 22). Establishing the molecular mechanism by which the Cid/Lrg system affects sporulation is a current focus of our laboratory.

In conclusion, the results of the present study reveal that the *B. anthracis lrgAB* and *clhAB*$_2$ play important roles in the control of cell death in the stationary phase and reveal a previously unrecognized role of this system in sporulation. Comparison of these systems in *B. anthracis* and *S. aureus* will be essential for elucidating the regulatory strategies underlying the control of cell death and lysis. Furthermore, continued investigation of this system in *B. anthracis* and other bacterial species will likely reveal additional insight into the biological roles of programmed cell death in bacteria.

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