Disruption of Two-component System LytSR Affects Forespore Engulfment in *Bacillus thuringiensis*

Qi Peng 1†, Jianbo Wu 1,2†, Xiaomin Chen 1, Lili Qiu 1, Jie Zhang 1, Hongtao Tian 2* and Fuping Song 1*

1 State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China, 2 Institute of Food Science and Technology, Hebei Agricultural University, Baoding, China

Two-component regulatory systems (TCSs) play pivotal roles in bacteria sensing many different stimuli from environment. Here, we investigated the role of the LytSR TCS in spore formation in *Bacillus thuringiensis* (Bt) subsp. *kurstaki* HD73. *lacZ* gene fusions revealed that the transcription of the downstream genes, *lrgAB*, encoding two putative membrane-associated proteins, is regulated by LytSR. The sporulation efficiency of a *lytSR* mutant was significantly lower than that of wild-type HD73. A confocal microscopic analysis demonstrated that LytSR modulates the process of forespore engulfment. Moreover, the transcription of the *lytSR* operon is regulated by the mother-cell transcription factor SigE, whereas the transcription of the sporulation gene *spoIIP* was reduced in the *lytSR* mutant, as demonstrated with a β-galactosidase activity assay. These results suggest that LytSR modulates forespore engulfment by affecting the transcription of the *spoIIP* gene in Bt.

Keywords: two-component system, LytSR, sporulation, *spoIIP*, *Bacillus thuringiensis*

INTRODUCTION

As a type of specialized differentiated cell, spores are used by *Bacillus* to survive starvation and harsh conditions. *Bacillus subtilis* is the best-studied spore-forming bacterium. Its endospore is formed by an unusual mechanism involving asymmetric cell division, followed by the engulfment of the cells and the spore morphogenesis (Errington, 2003). The formation of the asymmetric septum is a key event in spore development. Two sigma factors, σ^E^ and σ^K^, are instrumental in setting the cell-specific programs of gene expression in motion. Some σ^E^- and σ^K^-dependent genes are also involved in the prespore engulfment process (Errington, 2003). σ^E^ is initially produced as an inactive pro-σ^E^-precursor and is specifically activated only in the mother cell. The σ^K^- regulator includes genes necessary for engulfment (Tan and Ramamurthi, 2014). During engulfment, peptidoglycan degradation machinery composed of SpoIID, SpoIM, and SpoIP is initially required for septal-wall thinning and subsequently for the movement of the engulfing membranes (Ohara et al., 2015). The completion of engulfment is a key event governing the later stages of spore development. In the prespore, a third sporulation-specific sigma factor, σ^K^, becomes active at this time, and this sigma factor controls the final stages of development inside the spore. The final mother–cell-specific sigma factor, σ^K^, is regulated at multiple levels and is involved in the formation of the spore coat and in spore maturation (Errington, 2003; Hilbert and Piggot, 2004).

The two-component regulatory system (TCS), which typically consists of a membrane-spanning histidine kinase (HK) sensor and a cytoplasmic response regulator (RR), also plays a critical role in bacterial adaptation, survival, and virulence by sensing changes in the external environment.
and modulating gene expression in response to a variety of stimuli (Skerker et al., 2005). Studies have found that the transition of \textit{B. subtilis} from vegetative growth to sporulation is governed by the master transcription factor Spo0A, which is regulated by a complex phosphorelay involving five autophosphorylating histidine kinases (KinA–E), which respond to different types of environmental stress. Spo0A is not a simple negative regulator of the initiation of sporulation (Burbuly et al., 1991; Jiang et al., 2000; Fujita and Losick, 2003). However, it is not known whether other TCSs are involved in the subsequent spore formation stage, which consists of asymmetric cell division and engulfment in \textit{Bacillus}.

The \textit{Bacillus cereus} group of closely related Gram-positive, spore-forming bacteria includes \textit{B. cereus}, a common cause of human food poisoning, \textit{B. thuringiensis} (Bt), an insect pathogen, and \textit{B. anthracis}, the etiological agent of anthrax in mammals (Schnef et al., 1998; Stenfors Arnesen et al., 2008). The general functions of some TCSs in \textit{B. cereus} strains have been studied. For example, PP2C-type phosphatase RsbY receives its input from the multi sensor hybrid kinase RskK, and RskKY has been shown to regulate the activity of the alternative sigma factor B (van Schaik et al., 2005; de Been et al., 2010). SpSRK is active in response to glucose-6 phosphate and regulates the activity of the \textit{spslABC} operon, which is involved in sugar phosphate transport (Song et al., 2012). In \textit{B. anthracis}, LytSR regulates murein hydrolase activity, whereas the \textit{lrgAB} genes, which are regulated by LytSR, affect stationary-phase survival and sporulation efficiency (Chandramohan et al., 2009). The parental strain has a sporulation efficiency of 88%, whereas the sporulation efficiency of the \textit{lrgAB} mutant is only 5%, suggesting that the \textit{lrgAB} gene products have a dramatic impact on sporulation in \textit{B. anthracis} (Chandramohan et al., 2009). However, how LytSR affects sporulation remains unclear.

The functions of the LytSR TCS in \textit{Bt} were investigated in this study using \textit{Bt} subsp. \textit{kurstaki} HD73. Our results show that the downstream genes, \textit{lrgAB}, are regulated by LytSR, which is under the control of the mother cell transcription sigma factor SigE. LytSR modulates the subsequent forespore engulfment process and regulates the expression of the sporulation gene \textit{spoIID}.

**MATERIALS AND METHODS**

**Bacterial Strains, Media, and DNA Manipulation**

The bacterial strains and plasmids used in this study are listed in Table 1. \textit{Bt} strain HD73 (accession no. CP004069) was used in this study (Liu et al., 2013). The \textit{Bt} strains were transformed by electroporation, as previously described (Lereclus et al., 1989). \textit{Escherichia coli} and the \textit{Bt} strains were cultured in Luria-Bertani (LB) medium or Schaeffer's sporulation medium (SSM, 8 g of nutrition broth, 0.12% MgSO\textsubscript{4} \textit{[m/v]}, 0.1% KCl \textit{[m/v]}, 0.01 M NaOH, 0.1 M MnCl\textsubscript{2}, 0.01 M Ca(NO\textsubscript{3})\textsubscript{2}, and 0.01 M FeSO\textsubscript{4} in 1 L of H\textsubscript{2}O; Schaeffer et al., 1965) with shaking (220 rpm) at 37 and 30°C, respectively. The antibiotic concentrations used for bacterial selection were 100 µg/ml kanamycin and 10 µg/ml erythromycin for \textit{Bt} and 100 µg/ml ampicillin for \textit{E. coli}. DNA manipulation as previously described (Peng et al., 2014). Oligonucleotide primers were listed in Table 2.

**Construction of lytSR and lrgAB Mutants**

DNA fragments corresponding to the downstream and upstream regions of the \textit{lrgAB} genes (HD73_5854 and HD73_5855) were amplified by PCR using chromosomal DNA from \textit{Bt} HD73 as the template and the \textit{lytSR-1F/lytSR-1R} and \textit{lytSR-2F/lytSR-2R} primer pairs, respectively. The corresponding DNA fragments were fused with overlapping PCR using primers \textit{lytSR-1F} and \textit{lytSR-2R}, and the PCR product was digested with BamHI and EcoRI. The fragments were purified and ligated with the temperature-sensitive suicide plasmid pMAD (Arnaud et al., 2004) digested with the same enzymes, to yield the recombinant plasmid pMAD-\textit{ΔlytSR}, which was used to transformed into host strains with electroporation. The transformed transformants were incubated at 39–41°C. Colonies lacking erythromycin resistance were selected and one mutant strain, HD\textit{ΔlytSR}, was verified with PCR.

The upstream (562-bp) and downstream (561-bp) fragments of \textit{lrgAB} (HD73_5853) were PCR amplified using the primer pairs \textit{lrgAB-1F/lrgAB-1R} and \textit{lrgAB-2F/lrgAB-2R}, respectively, and using \textit{Bt} HD73 genomic DNA as the template. The kanamycin (Kan)-resistance gene (1,473 bp) was amplified using primers Kan-R and Kan-F. The deletion-insertion mutant cassette was amplified with overlapping PCR using the upstream and downstream fragments and the Kan-resistance gene as the templates, with primers \textit{lrgAB-1F} and \textit{lrgAB-2R}. The \textit{lrgAB} deletion-insertion mutant cassette was inserted into the \textit{BamHI} and \textit{EcoRI} restriction sites of the pMAD plasmid to generate the recombinant plasmid pMAD-\textit{ΔlrgAB}, which was then used to transform \textit{Bt} HD73 cells with electroporation. Transformants were grown at 30°C in LB plate containing erythromycin and kanamycin, and then transferred to liquid LB containing kanamycin at 39°C. The cells were then plated on LB agar plates. Colonies with kanamycin resistance but lacking erythromycin resistance were selected, and one mutant strain, HD\textit{ΔlrgAB}, was verified with PCR.

**Genetic Complementation of the lrgAB and lytSR Deletion Mutants**

The oligonucleotide primer pairs \textit{lrgABhf-F/lrgABhf-R} and \textit{lytSRhf-F/lytSRhf-R} were used to amplify the \textit{lrgAB} gene with its own promoter \textit{PlrgAB}, and the \textit{lytSR} gene with its promoter \textit{PlytSR}. The resultant fragments were digested with \textit{PstI/BamHI} and \textit{SalI/EcoRI}, respectively, and then integrated into the shuttle vector pHT315 (Arantes and Lereclus, 1991) to generate pHT\textit{lrgAB} and pH\textit{lytSR}, respectively. The genetically complemented mutant strains \textit{ΔlrgAB}(\textit{lrgAB}) and \textit{ΔlytSR}(\textit{lytSR}) were generated by introducing pHT\textit{lrgAB} and pH\textit{lytSR} into HD\textit{ΔlrgAB} and HD\textit{ΔlytSR}, respectively.

**Construction of spoIID, spoIM, and spoIIP Mutants**

\textit{spoIID} (HD73_5692), \textit{spoIM} (HD73_4392), and \textit{spoIIP} (HD73_2232) mutants were constructed similar to \textit{lrgAB} as described above, but using the primer pairs \textit{spoIID-1F/spoIID-1R},
**TABLE 1 | Strains and plasmids used in this study.**

| Strains/plasmids | Relevant genotype and characteristics | Resource |
|------------------|---------------------------------------|----------|
| **STRAINS**      |                                       |          |
| E. coli TG1      | Δ(lac-proAB) supE thi hsd-5 (F’ traD36  
|                  | proA* - 5 proB6 Δ lacI2 ΔM15), general  
|                  | purpose cloning host                  |          |
| E. coli ET 12567 | F− dam-13-Tn9 dcm-6 hasM hsdR recF143  
|                  | z2-202::Tn10 galK2 galT22 ara14  
|                  | pacY1 xylE-5 leuB3 thi-1, for generation  
|                  | of unmethylated DNA                    |          |
| HD73             | B. thuringiensis strain carrying the cry1Ac  
|                  | gene                                   |          |
| HDΔsigE         | HD73 mutant type, ΔsigE                | Du et al., 2012 |
| HDΔlytSR        | HD73 mutant type, ΔlytSR               | This study |
| HDΔlytSR        | HD73 mutant type, ΔlytSR               | This study |
| HDΔspolID       | HD73 mutant type, ΔspolID              | This study |
| HDΔspolIM       | HD73 mutant type, ΔspolIM              | This study |
| HDΔspolIP       | HD73 mutant type, ΔspolIP              | This study |
| ΔsigE(PlySR)    | HDΔsigE carrying pht304PplySR          | This study |
| HD(plySR)       | HD73 carrying pht304PplySR             | This study |
| ΔlytSR(pigAB)   | HDΔlytSR carrying pht304PpigAB        | This study |
| HD(pigAB)       | HD73 carrying pht304PpigAB             | This study |
| ΔlytSR(papsulation) | HDΔlytSR carrying pht304PspolpID     | This study |
| HD(papsulation) | HD73 carrying pht304PspolpID           | This study |
| ΔspolID(pispolp) | HDΔspolID carrying pht304PspolpID      | This study |
| HD(pispolp)     | HD73 carrying pht304PspolpID           | This study |
| ΔspolIM(pispolp) | HDΔspolIM carrying pht304PspolpIM      | This study |
| HD(pispolp)     | HD73 carrying pht304PspolpIM           | This study |
| ΔlrgAB(lrgAB)   | HDΔlrgAB genetic complementation strain  
|                  | carrying pht304A(∆lrgAB) plasmid; Emrift | This study |
| ΔlytSR(lytSR)   | HDΔlytSR genetic complementation strain  
|                  | carrying pht304A(∆lytSR) plasmid; Emrift | This study |

| **PLASMIDS**    |                                       |          |
| pMAD            | AmpR, EryR, temperature-sensitive Bt-E.  
|                  | coli shuttle vector                    | Arnaud et al., 2004 |
| pH7304-18Z      | Promoterless lacI2 Vector, Emrift, AmpR  
|                  |                                      | Agisess and Lereclus, 1994 |
| pH7315          | B. thuringiensis-E. coli shuttle vector | Arantes and Lereclus, 1991 |
| pH7315(lytSR)   | pH7315 with lytSR genetic complementation  
|                  | fragment                               | This study |
| pH7315(pigAB)   | pH7315 with pigAB genetic complementation  
|                  | fragment                               | This study |
| pMAD-ΔlytSR     | pMAD with ΔlytSR deletion fragment     | This study |
| pMAD-ΔlytSR     | pMAD with ΔlytSR deletion fragment     | This study |
| pMAD-ΔspolID    | pMAD with ΔspolID deletion fragment    | This study |
| pMAD-ΔspolID    | pMAD with ΔspolID deletion fragment    | This study |
| pMAD-ΔspolP     | pMAD with ΔspolP deletion fragment     | This study |
| pH7304-18Z(pispolID) | AmpR, EryR, pH7304-18Z carrying  
|                  | promoter upstream from spolIP          | This study |
| pH7304-18Z(pispolIM) | AmpR, EryR, pH7304-18Z carrying  
|                  | promoter upstream from spolIM          | This study |
| pH7304-18Z(pispolP) | AmpR, EryR, pH7304-18Z carrying  
|                  | promoter upstream from spolP           | This study |

**TABLE 2 | Sequences of oligonucleotide primers used in this study.**

| Primer name | Sequence (5' → 3') |
|-------------|-------------------|
| lytSR-1F    | CGCGATCCGACTCCCATTCAACTAA |
| lytSR-1R    | CTCCAGGTGTTTGCGTGGTGAATGGTGGAC |
| lytSR-2F    | GGAAATACGTTATAGTTGAGTACAATTGGAC |
| lytSR-2R    | CGAGATCCGCTGTTGAGTGGTGAATGGTGGAC |
| lytSR-3F    | GGCGATGAGTGGTGAATGGTGGAC |
| lytSR-3R    | CGAGATCCGCTGTTGAGTGGTGAATGGTGGAC |
| lytSR-4F    | GGCGATGAGTGGTGAATGGTGGAC |
| lytSR-4R    | CGAGATCCGCTGTTGAGTGGTGAATGGTGGAC |
| lytSR-5F    | GGCGATGAGTGGTGAATGGTGGAC |
| lytSR-5R    | CGAGATCCGCTGTTGAGTGGTGAATGGTGGAC |
| lytSR-6F    | GGCGATGAGTGGTGAATGGTGGAC |
| lytSR-6R    | CGAGATCCGCTGTTGAGTGGTGAATGGTGGAC |

aRestriction sites are underlined and in bold font.

**spoIID-2F/spoIID-2R, kanD-F/kanD-R, spoIM-1F/spoIM-1R, spoIM-2F/spoIM-2R, kanM-F/kanM-R, spoIIP-1F/spoIIP-1R, spoIIP-2F/spoIIP-2R, and kanP-F/kanP-R, respectively. The recombinant plasmids pMAD-ΔspoIID, pMAD-ΔspoIM, and pMAD-ΔspoIIP were electropropated into Bt HD73 cells. Colonies with kanamycin resistance but lacking erythromycin resistance were selected, and mutant strains, HDΔspoIID, HDΔspoIM, and HDΔspoIIP, were verified by PCR.**

**Growth Curve Assays**

Overnight cultures of each strain grown in LB medium were used as starters for growth curve analyses. The exponential growth phase cells were washed in phosphate-buffered saline and then inoculated into SSM or M9 medium supplemented with tryptophan (50 μg/ml) and pyruvate (6 g/l) to an optical
density at 600 nm (OD\textsubscript{600}) of 0.1. The cultures were incubated at 30°C with shaking at 220 rpm, and growth was monitored by measuring the absorbance at 600 nm at different timepoints. Values represent the means of at least three independent replicates. Error bars represent standard deviations.

**Determination of Sporulation Efficiency**
The HD73, HDΔIrgAB, ΔIrgAB(ΔrgAB), HDΔlytSR, and ΔlytSR(lytSR) strains were grown in SSM to T\textsubscript{28} (T\textsubscript{0} is the end of the exponential phase, and T\textsubscript{n} is n hours after T\textsubscript{0}) at 30°C with vigorous shaking. The number of viable cells was counted as the total colony-forming units (CFU) on the LB plates. The number of spores was determined as the number of heat-resistant (65°C for 30 min) CFU on the LB plates. Sporulation efficiency was defined as the ratio of the number of spores to the number of viable cells, multiplied by 100. Values represent the means of at least three independent replicates. The data were analyzed with SPSS (version 19.0) using a t-test. Error bars represent standard deviations. P-values are indicated in the figure legend.

**Laser Scanning Confocal Microscopy**
The vital membrane dye FM4-64 (Molecular Probes, Inc., Eugene, OR, USA) was dissolved in dimethyl sulfoxide to a final concentration of 100 µM. The cells were stained with FM4-64 (100 µM) for 1 min on ice (Yang J. et al., 2013). To assess engulfment, 0.5 ml of cells cultured to T\textsubscript{12} were pelleted and resuspended in 0.1 ml of H\textsubscript{2}O. An aliquot (2 µl) of this cell suspension was placed on a slide and stained with FM4-64 (100 µM) and MitoTracker Green FM (MTG, 100 nM; from Molecular Probes) for 1 min, and then scanned (476–490 nm excitation and 510–667 nm emission) with a confocal laser scanning microscope (Leica TCS SL; Leica Microsystems, Wetzlar, Germany). Each strain was scanned independently at least three times and each scan was then viewed in at least five fields. The rate of incomplete engulfment was defined as the ratio of the number of incompletely engulfed cells (stained with FM4-64 in the mother cell) to the total number of cells. The values given are the means of at least three independent replicates.

**Construction of Promoter Fusions with lacZ**
To assess the transcriptional activity of PlrgAB and PtytSR promoters, putative promoter fragments (633 and 845 bp, respectively) were cloned from Bt HD73 genomic DNA using the primer pairs PlrgAB-F/PlrgAB-R and PtytSR-F/PtytSR-R, respectively. The PslI/BamHI fragments of PlrgAB and PtytSR were separately integrated into vector pHT304-18Z, which is the Bt-E. coli shuttle harboring a promoterless lacZ gene (Agaisse and Lereclus, 1994) to generate plasmids pHT304PlrgAB and pHHT304PtytSR, respectively. The former was introduced into Bt strain HD73 and the HDΔlytSR mutant, whereas the latter was introduced into Bt strain HD73 and the HDΔsigE mutant (Du et al., 2012). Resultant HD(PlrgAB), ΔlytSR(PlrgAB), HD(PtytSR), and ΔsigE(PtytSR) strains were selected with erythromycin and verified with PCR. The constructions of PspoIID, PspoIM, and PspoIIIP (650, 437, and 668 bp, respectively) with lacZ fusions are similar to PlrgAB as described above, but using the primer pairs PspoIID-F/PspoIID-R, PspoIM-F/PspoIM-R, and PspoIIIP-F/PspoIIIP-R, respectively. The recombinant plasmids pHHT304PspoIID, pHHT304PspoIM, and pHHT304PspoIIIP were introduced into Bt strain HD73 and the HDΔlytSR mutant. The resultant strains ΔlytSR(PspoIID), ΔlytSR(PspoIM), and ΔlytSR(PspoIIIP) were selected with erythromycin and verified with PCR.

**β-Galactosidase Activity Assay**
Bt strains carrying lacZ transcriptional fusions were cultured in liquid SSM and 2-ml samples were collected at 1-h intervals. The cells were pelleted and resuspended in 0.5 ml of Z buffer (Peng et al., 2014) at 4°C, then lysed with a Mini-Beadbeater cell disrupter (BioSpec, Bartlesville, OK, USA) and centrifuged at 10,000 × g for 7 min at 4°C. β-Galactosidase activity was determined as previously described (Perchat et al., 2011). The reported values are the means of at least three independent assays. The data were analyzed with SPSS (version 19.0) using a t-test. Error bars represent standard deviations.

**RESULTS**

**PlrgAB Promoter Transcription Is Regulated by LytSR**
IrgAB is located downstream from the lytSR genes in Bt HD73 (Figure 1A). The Bt lytS (HD73_5856, sensor histidine kinase), lytR (HD73_5855, response regulator), IrgA (HD73_5854, holin-like protein), and IrgB (HD73_5853, holin-like protein) genes encode proteins that share 50, 44, 44, and 54% amino acid sequence identity, respectively, with homologs in Staphylococcus aureus (Patel and Golemi-Kotra, 2015), and 66, 65, 62, and 78% amino acid sequence identity with homologs in B. subtilis (van den Esker et al., 2017). Alignments of these proteins from Bt, S. aureus, and B. subtilis are shown in Supplementary Figure 1. The two-component system LytSR/LytST contained the conserved His\_kinase domain and the response regulator receiver domain in Bt, S. aureus, and B. subtilis (Supplementary Figure 1).

To investigate the transcription from and regulation of the PlrgAB promoter in Bt, Bt strain HD73, and the lytSR mutant HDΔlytSR were transformed with a PlrgAB-lacZ fusion construct. The results of the β-galactosidase assay showed that the transcriptional activity of PlrgAB increased from T\textsubscript{4} to T\textsubscript{8} in the HD73 strain in SSM, whereas it did not increase dramatically in the HDΔlytSR mutant (Figure 1B), suggesting that the transcription of the IrgAB genes is positively regulated by LytSR during the late sporulation process.

**LytSR Modulates Bt Forespore Engulfment**
Previous studies have shown that LytSR/LytST is involved in pyruvate utilization (Zhu et al., 2010; van den Esker et al., 2017). We also compared the growth of the lytSR mutant with that of wild-type strain HD73 in the presence of pyruvate. Results showed that ΔlytSR was unable to grow in M9 medium supplemented with pyruvate, whereas the wild-type reached an OD\textsubscript{600} of 0.9 after 20 h of incubation (Figure 2A), suggesting that LytSR is involved in pyruvate utilization in Bt. However, no
FIGURE 1 | PlrgAB transcription in wild-type Bt HD73 and the lytSR mutant. (A) Gene organization at the lytSR–lrgAB locus in Bt HD73, S. aureus and B. subtilis.

White arrows represent open reading frames (ORFs); small arrows denote the lengths of promoters upstream from the lytS and lrgA genes in Bt. (B) β-galactosidase activity from the lrgAB promoter (PlrgAB) in HD73 (*) and lytSR mutant (+) grown in SSM. T₀ is the end of the exponential phase; Tn is n hours after T₀. Values represent the means of at least three independent replicates; error bars represent standard deviations.

differences in the growth curves of ΔlytSR and the wild-type were observed in SSM (Figure 2B). Thus, in order to eliminate the effects of growth medium, we selected SSM for further analyses of the sporulation efficiency.

Because the lrgAB genes have a dramatic impact on sporulation in B. anthracis (Chandramohan et al., 2009), we predicted that the lytSR or lrgAB mutation would affect the ability of the Bt cells to undergo sporulation. Therefore, the abilities of the lrgAB and lytSR mutants to sporulate were assessed. The wild-type strain HD73 had a sporulation efficiency of 85 ± 4% after growth to T₂₈ in SSM (Figure 3). The sporulation efficiency was not significantly different between HD73 and either HDΔlrgAB (72 ± 11%) or ΔlrgAB(lrgAB) (76 ± 9%), whereas it was significantly reduced in both HDΔlytSR (47 ± 3%, P ≤ 0.001) and, the genetically complemented strain ΔlytSR(lytSR) (54 ±4%, P ≤ 0.01). Based on the P-values (P ≤ 0.05, Figure 3) between ΔlytSR and ΔlytSR(lytSR), ΔlytSR(lytSR) showed a partly restored sporulation function. These results indicate that lytSR affects spore formation and the regulation of the genes involved in sporulation.

To determine the effect of LytSR on sporulation in Bt HD73, the cell membranes of Bt HD73 and its mutants were stained with the vital dye FM4-64, which labels the plasma membranes of living cells, and the process of spore formation was visualized with confocal microscopy. In cells grown to T₃ in SSM, the polar septum was curved in the wild-type and mutant cells, whereas some cells of HDΔlytSR had an incomplete septum at the distal pole (Figure 4). At T₁₂, the process of engulfment was
FIGURE 2 | Growth curves assay. Wild-type HD73 (♦) and lytSR mutant cells (◦) were grown in M9 supplemented with pyruvate (A), and SSM (B). Values represent the means of at least three independent replicates; Error bars represent standard deviations.

FIGURE 3 | Analysis of sporulation efficiency. Sporulation efficiencies of wild-type HD73, ΔlytSR, ΔrgAB, ΔrgAB(rgAB), and ΔlytSR(lytSR) were compared. Sporulation efficiency was defined as the ratio of the number of spores to the number of viable cells, multiplied by 100. Values represent the means of at least three independent replicates. The data were analyzed with SPSS (version 19.0) using a t-test. Error bars represent standard deviations. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

completed in the forespores of the wild-type (Figure 5, arrow 1) and HDΔrgAB cells. In these cases, the spores were not labeled with FM4-64, but were stained with MTG, and only the outer membranous outline of the living cells could be observed. In the mutant HDΔlytSR, a proportion of the cells had completed the process of engulfment, but 52 ± 3% cells were arrested in forespore engulfment (Figure 5, arrow 2), and a bipolar septum phenotype was observed.

**lytSR Transcription Is Controlled by SigE**
HDΔlytSR cells were unable to initiate engulfment or form bipolar septa. The mother–cell-specific sigma factor SigE plays a critical role in the formation of an asymmetric septum and in forespore engulfment (Errington, 2003). Therefore, we predicted that SigE would also affect the transcription of lytSR. The results of the β-galactosidase assay indicate that the transcriptional activity of PlytSR increased rapidly from T4 to T10 in wild-type HD73, whereas it increased much more slowly in the HDΔsigE mutant grown in SSM (Figure 6), suggesting that the transcription of lytSR is controlled by the mother-cell-specific sigma factor SigE.

**LytSR Affects spoIIP Expression**
In *B. subtilis*, the sporulation genes spoIID, spoIIM, and spoIIP are controlled by SigE (Eichenberger et al., 2001) and may also be involved in suppressing septum formation at the distal pole of the sporangium (Chastanet and Losick, 2007). To determine whether LytSR affects the process of spore engulfment by regulating the expression of spoIID, spoIIM, and spoIIP, the promoters of these genes were fused to lacZ and the β-galactosidase activity was assessed in wild-type HD73 cells, lytSR and sigE mutants. The results showed that the transcriptional activities of spoIID, spoIIM, and spoIIP were sharply reduced or abolished in the sigE mutant grown in SSM (Figure 7), suggesting that the transcription of spoIID, spoIIM, and spoIIP is directly controlled by SigE in *Bt*. The transcription of PspoIID and PspoIIM transcription did not differ between the wild-type and mutants grown in SSM (Figures 7A,B). However, PspoIIP activity was dramatically reduced in the lytSR mutant grown in SSM (Figure 7C). These results suggest that the transcription of spoIIP is affected by LytSR.

**LytSR Mainly Modulates Bt Forespore Engulfment by Regulating spoIIP Expression**
To determine whether LytSR modulates *Bt* forespore engulfment by regulating spoIIP expression, we observed the phenotypes of the spoIID, spoIIM, and spoIIP mutants in SSM. In cells...
FIGURE 4 | Sporulation process in Bt HD73 in SSM. Laser scanning confocal micrographs of Bt wild-type HD73 cells and ΔirgAB, ΔlytSR, ΔlytSRΔlytSR, ΔspolII, ΔspolII, ΔspolII, ΔspolII, and ΔspolII cells grown in SSM to T3 and T12 (30°C). Cell membrane is visible as red fluorescence. Yellow arrow indicates a bipolar septum. Bar, 7.5 µm.
FIGURE 5 | Schematic representation of the membrane fusion assay. Laser scanning confocal micrographs of Bt wild-type HD73, ΔlytSR, and ΔspoIIP cells grown in SSM to T12 (30°C). Red lines represent membranes stained with FM4-64 and MitoTracker Green FM (MTG), and green lines indicate membranes stained with MTG only. Arrow 1 points to cells that have completed the process of engulfment; only the mother–cell membranes are stained with FM4-64, but MTG stained both the forespore and mother–cell membranes. Arrow 2 points to cells that have undergone incomplete engulfment, and the membrane fusion is stained with FM4-64 and MTG. Arrow 3 points to the crystal protein stained with MTG only.

grown to T3 or T12 in SSM, the polar septum was curved or had completed the process of engulfment in the wild-type, whereas some HDΔspoIM, and HDΔspoIID cells displayed an incomplete septum at the distal pole (Figure 4). At T12, the phenotype of HDΔspoIP was similar to that of HDΔlytSR, and the only difference was that more HDΔspoIP cells (68 ± 5%) than HDΔlytSR cells (48 ± 3%) had completed the process of engulfment (Figure 5). In contrast, almost all the HDΔspoIID and HDΔspoIM cells arrested in forespore engulfment, and bipolar septa were also observed (Figure 4), so this phenotype is similar to that of the spoIID and spoIM mutants of B. subtilis (Pogliano et al., 1999). The β-galactosidase activity assay also revealed that PspoIIIP was dramatically reduced in the lytSR mutant grown in SSM (Figure 7). All these results indicate that LytSR modulates Bt forespore engulfment, mainly by affecting spoIIP expression.

DISCUSSION

The sporulation efficiency assay and a confocal microscopic analysis showed that spore formation was unaffected in the Bt lrgAB mutant. This differs from the dramatic impact of this mutation on sporulation efficiency observed in B. anthracis (Chandramohan et al., 2009), although orthologues of the lrgAB locus of Bt HD73 are conserved in the genomes of the B. cereus group (Supplementary Figure 2). These genes share high sequence similarity and a similar organization with those of the lrgAB locus. However, in the Bt lytSR mutant, sporulation efficiency was markedly reduced and spore engulfment was lower than wild-type, and a bipolar septum was observed in some cells grown in SSM. These results indicate that LytSR does not modulate the process of spore formation by regulating of lrgAB, but probably by controlling the expression of other genes.

LytSR and LrgAB are widely conserved in both the B. cereus group and amongst other bacterial species (Supplementary Figure 2). In S. aureus, the LytSR are involved in the regulation of bacterial programmed cell death, biofilm formation, and adaptation to cationic antimicrobial peptides (Brunskill and Bayles, 1996; Rice et al., 2005; Sharma-Kuinkel et al., 2009; Yang S. J. et al., 2013; Lehman et al., 2015), while in Staphylococcus epidermidis, they play a role in regulating extracellular murein
hydrolase activity, bacterial cell death, and pyruvate utilization (Zhu et al., 2010). In B. subtilis, the lytSR and ysbA homologs\textit{lytST} and\textit{ysbA} are not involved in programmed cell death, but are essential for pyruvate transport or utilization (van den Esker et al., 2017). We also found that mutation of\textit{lytSR} has an effect on pyruvate utilization in M9 medium in\textit{Bt} (Figure 2A). However, no differences in the growth curves of\textit{ΔlytSR} and the wild-type were observed in SSM (Figure 2B). We further demonstrated that the LytSR are involved in the process of spore engulfment in\textit{Bt} in SSM. These results indicate that LytSR does not modulate the process of spore formation by affecting the pyruvate utilization.

A high proportion (61%) of\textit{B. subtilis}\textit{sigE} mutant cells had complete septa near both the poles and failed to undergo engulfment. SigE directs controls the transcription of the\textit{spolIID},\textit{spolIM}, and\textit{spolIP} (Eichenberger et al., 2001). Single mutants of these genes prevent engulfment as they are defective in the dissolution of the peptidoglycan layer between the two membranes of the polar septum. Instead, the septal membrane bulges through the incompletely degraded cell wall layer. In double mutants, the bulge is less prominent, and only in the absence of all three proteins does septum formation occur at both poles at a frequency similar to that observed in the\textit{sigE} mutant (Eichenberger et al., 2001; Meyer et al., 2010; Tan and Ramamurthi, 2014). The transcription of\textit{spolID},\textit{spolIM}, and\textit{spolIP} is controlled by SigE in\textit{Bt} (Figure 7) as in\textit{B. subtilis}, and the transcriptional activity of\textit{spolIP} was sharply reduced in the\textit{lytSR} mutant compared with that in the wild-type strain grown in SSM. However, the transcriptional activities of\textit{spolIM} and\textit{spolID} in\textit{lytSR} mutant did not differ from those in the wild-type strain. This observation suggests that the effect of\textit{lytSR} on\textit{spolIP} expression does not result from the direct activity of\textit{LytR} on the transcription of\textit{spolIP}. The effect of the\textit{lytSR} mutation on\textit{spolIP} expression might be attributable to the low availability of active SigE in the mother–cell compartment of the mutant strain. The transcription of\textit{spolIP} requires SigE. However, the amount of SigE required for the full expression of\textit{spolIM},\textit{spolID}, and\textit{spolIP} might differ, as has been demonstrated for the genes of the Spo0A regulon, which are distributed in two classes: those that are regulated at a low dose of Spo0A-P and those that require a high dose to be activated or repressed (Fujita et al., 2005). In a similar way,\textit{spolIP} transcription might require larger amounts of SigE than the transcription of\textit{spolIM} and\textit{spolID}. Therefore, the SigE defect in the\textit{lytSR} mutant would have a more dramatic effect on\textit{spolIP} expression than on\textit{spolIM} or\textit{spolID} expression.

The transcriptional analysis of\textit{lytSR} in the\textit{sigE} mutant and of\textit{spolID},\textit{spolIM}, and\textit{spolIP} in the\textit{lytSR} mutant revealed that the\textit{lytSR} operon is controlled by SigE and that the efficacy of\textit{spolIP} transcription depends, directly or indirectly, on\textit{lytSR}. We have demonstrated that LytSR affects spore formation by preventing the correct engulfment of the forespore. However, we did not determine whether this effect is responsible for the defect in\textit{spolIP} expression or, reciprocally, if it is caused by weak\textit{spolIP} expression. In\textit{B. subtilis},\textit{SpolIP} is targeted to the septal membrane by\textit{SpolIM}, where it interacts with\textit{SpoIID}, which also localizes to the membrane via its interaction with\textit{SpolIP}.\textit{SpolIP} and\textit{SpoIID} have complementary enzymatic activities, which are similar to those of\textit{LytB} and\textit{LytC} (CwlB), respectively, the major vegetative autolysins.

![Figure 6](image6.png)

**FIGURE 6** | Transcription of\textit{PlytSR} promoter in\textit{Bt}. Wild-type\textit{HD73} (▲) and\textit{sigE} mutant cells (*) were grown in SSM.\textit{T0} is the end of the exponential phase, and\textit{Tn} is n hours after\textit{T0}. Values represent the means of at least three independent replicates; error bars represent standard deviations.

![Figure 7](image7.png)

**FIGURE 7** | Transcription of\textit{PspolID},\textit{PspolIM}, and\textit{PspolIP} promoters in\textit{Bt}. Transcription of\textit{PspolID} (A),\textit{PspolIM} (B), and\textit{PspolIP} (C) in wild-type\textit{HD73} (▲),\textit{lytSR} mutant (■), and\textit{sigE} mutant (*) cells grown in SSM.\textit{T0} is the end of the exponential phase, and\textit{Tn} is n hours after\textit{T0}. Values represent the means of at least three independent replicates; error bars represent standard deviations.
involved in peptidoglycan degradation (Shida et al., 2001; Chastanet and Losick, 2007; Gutierrez et al., 2010). Therefore, we infer that LytSR modulates sporulation by directly or indirectly inducing the transcription of the sporulation gene spolIP. However, many other genes are also involved in engulfment and must be examined in future studies because they may be more directly responsible for the sporulation phenotype.

**AUTHOR CONTRIBUTIONS**

FS designed the research. JP and JW performed the experimental work. QP drafted the manuscript. JW, XC, and LQ constructed the mutants, analyzed the sporulation efficiency and perform the laser scanning confocal microscopy. FS, JZ, and HT critically revised the manuscript for intellectual content. All authors read and approved the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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