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clpC* operon regulates cell architecture and sporulation in *Bacillus anthracis

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Abstract

The *clpC* operon is known to regulate several processes such as genetic competence, protein degradation and stress survival in bacteria. Here, we describe the role of *clpC* operon in *Bacillus anthracis*. We generated knock-out strains of the *clpC* operon genes to investigate the impact of CtsR, McsA, McsB and ClpC deletion on essential processes of *B. anthracis*. We observed that growth, cell division, sporulation and germination were severely affected in *mcsB* and *clpC* deleted strains, while none of deletions affected toxin secretion. Growth defect in these strains was pronounced at elevated temperature. The growth pattern gets restored on complementation of *mcsB* and *clpC* in respective mutants. Electron microscopic examination revealed that *mcsB* and *clpC* deletion also causes defect in septum formation leading to cell elongation. These vegetative cell deformities were accompanied by inability of mutant strains to generate morphologically intact spores. Higher levels of polyhydroxybutyrate granules accumulation were also observed in these deletion strains, indicating a defect in sporulation process. Our results demonstrate, for the first time, the vital role played by McsB and ClpC in physiology of *B. anthracis* and opens up further interest on this operon, which might be of importance to success of *B. anthracis* as pathogen

INTRODUCTION

Bacillus anthracis, a causative agent of anthrax, has gained importance in the past decade owing to its potential use as a bio warfare agent. The initiation of *B. anthracis* infection

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starts with *B. anthracis* spores inhalation followed by spore germination and toxin secretion in the host (Dixon *et al.*, 1999). Most of the research about this pathogen has involved studying various aspects of sporulation and mechanism of toxin action; however, the underlying networks governing these processes are not fully understood. Clp-ATPases are conserved in all organisms and function as molecular chaperones in association with ClpP proteases to regulate proteolysis (Squires and Squires, 1992; Gottesman, 1996; Gottesman *et al.*, 1997, 1998). The functioning of these proteolytic complexes is well studied in *Bacillus subtilis*, wherein, the chaperone assisted protease complexes (ClpCP, ClpEP and ClpXP) play a key role in several physiological processes by regulating protein quality (Gerth *et al.*, 2004; Kock *et al.*, 2004; Kirstein *et al.*, 2009). However, the role of *clpC* operon in physiology of *B. anthracis* is not understood.

The *clpC* operon encodes four proteins, namely, CtsR, McsA, McsB and ClpC (Figure S1 A). The first encoded protein of *clpC* operon, CtsR possesses a DNA binding helix-turn-helix motif and acts as a negative regulator of *clpC* operon under normal conditions (Kruger and Hecker, 1998; Derre *et al.*, 1999). Temperature elevation leads to change in CtsR conformation followed by its release from the *clpC* operon promoter, hence transcribing all genes in the operon (Elsholz *et al.*, 2010). McsA encoded by the second gene of the operon is an activator of the arginine kinase McsB. McsB phosphorylates its cognate substrates in the operon forming functional proteolytic machinery (Fuhrmann *et al.*, 2009; Elsholz *et al.*, 2012). ClpC has been reported to be required for various processes including stress response, sporulation and competence in *B. subtilis* (Kong and Dubnau, 1994; Kruger *et al.*, 1994; Gerth *et al.*, 1998; Msadek *et al.*, 1998; Nanamiya *et al.*, 1998).

In the present study, we aimed to investigate the role of *clpC* operon in *B. anthracis* Sterne strain. We generated mutants having in-frame deletions in individual genes and the entire *clpC* operon in *B. anthracis*. We demonstrate that McsB and ClpC play essential roles in viable spore formation and germination. The microscopic analysis revealed altered morphology and cell septation pattern in *mcsB* and *clpC* mutants. This study provides the evidence that proteins encoded by *clpC* operon regulate essential processes in the life cycle of *B. anthracis*, which is associated with the pathogenesis.

RESULTS

Generation of knockout strains of *clpC* operon genes in *B. anthracis*

The *clpC* operon in *B. anthracis* encodes four proteins namely, CtsR, McsA, McsB and ClpC (Figure S1 A). This ubiquitous operon is known to regulate stress response and proteolysis in various bacteria. To understand the role of this operon in the physiology of *B. anthracis* Sterne strain, the *Cre-loxP* genetic modification method was used to generate deletion and mutants were confirmed by PCR analysis (Figure S1 B). Individual gene as well as the whole operon deletion mutants (*ctsR*, *mcsA*, *mcsB*, *clpC*, operon) were generated, as described earlier (Pomerantsev *et al.*, 2006). To confirm the in-frame deletions, sequencing was carried out for all the mutants and expression of ClpC (last gene of the operon) was checked by immunoblotting (Figure S1 C).

McsB and ClpC are required for growth during heat stress

ClpC has been reported to be essential for growth of *B. subtilis* during heat stress (Msadek *et al.*, 1994). To evaluate the contribution of *clpC* operon genes during heat stress in *B. anthracis* Sterne strain, we compared the growth of mutant strains with the wild type Sterne strain under physiological and heat stress conditions (Figure 1A, 1B). Comparison of growth kinetics showed similar growth curve pattern of all the mutants except the strains lacking ClpC, at 37 °C (Figure 1A). At elevated temperature of 43 °C, all the mutants except *mcsA* grew slowly in comparison with the wild type Sterne strain. However, growth of *clpC* and operon was severely affected and even after 9 h, the growth was only one-fourth in comparison to the wild type and the mutant lacking McsA (Figure 1B). These results suggest that ClpC is required for normal growth and becomes essential during heat stress whereas, McsA had no apparent role in growth at both physiological and heat stress conditions. Complementation of *mcsB* and *clpC* strains was able to revert the effect of temperature on growth (Figure 1C). Complementation of *mcsB* and *clpC* was confirmed by PCR (Figure S2). These results proved that growth defect was due to lack of McsB and ClpC proteins.

Role of *clpC* operon proteins in sporulation and germination

Sporulation is an adaptive response required for survival of *Bacillus* species in stress conditions. Therefore, *clpC* operon mutants were employed to understand the role of each gene in both sporulation and germination. It is well known that *B. anthracis* Sterne sporulates and germinates with high efficiency (~98 %) (Turnbull, 2008). We also observed approximately 90 % sporulation efficiency in the wild type Sterne strain in 72 h; however, both *mcsB* and *clpC* mutants showed reduced sporulation even after 144 h (Figure 2A, S3). In case of *ctsR* and *mcsA* mutants, the sporulation and germination efficiencies were similar to the wild type (Figure 2A, 2B). The *mcsB* strain showed 60 % sporulation out of which only 40 % spores germinated whereas, *clpC* strain showed only 40 % sporulation and 9 % germination (Figure 2A, 2B). The operon mutant was most severely affected in both sporulation and germination as it showed only 10 % sporulation and 3 % germination. Complementation of *mcsB* and *clpC* in the respective mutant restored the sporulation and germination efficiencies (Table 1).

It has also been reported that mutations get introduced in bacterial genes to compensate for the loss of fitness caused due to the earlier mutations (Levin *et al.*, 2000; De Vos *et al.*, 2013). In order to examine whether the function of McsB and ClpC is taken over by compensatory mutations/other cellular proteins, the process of sporulation and germination was carried out. The spores formed by *mcsB*, *clpC* and operon strains were germinated and subsequently sporulated for three consecutive cycles. We observed that in the third cycle, sporulation efficiency declined to 13 % and 7 % in *clpC* and operon strains. Germination efficiencies were significantly reduced in *mcsB* (26 %), *clpC* (9 %) and operon (1.4 %) strains (Figure 2C, 2D). These results suggest that McsB and ClpC are essential for sporulation and germination. Moreover, *clpC* and operon strains required longer time period (96 h) for sporulation (data not shown). The *clpC* and operon strains also required longer time period for germination (22–30 h), while *mcsB* along with other two mutant strains, *ctsR* and *mcsA*, germinated in 16 h similar to the wild type. Spores

formed by all the mutant strains had retained the pXO1 plasmid (as confirmed by PCR up to three generations, data not shown). Our results showed that in each consecutive cycle, efficiency of sporulation and germination of *mcsB* and *clpC* strains were reduced (Figure 2C, 2D).

***clpC* operon is required for septum formation and cell shape maintenance**

Electron microscopic examination was performed to unravel the possible reason for growth defect. Scanning and transmission electron microscopy revealed that deletion of *mcsB* or *clpC* caused elongation of cells (Figure 3A–F and 3G–L). The *mcsB* and *clpC* mutants showed altered cell division pattern with irregular septum formation across the chain length (Figure 3D–E, 3J–K). The average cell length of wild type, *ctsR* and *mcsA* bacilli is 3–4 µm, whereas, the average cell length of *mcsB* and *clpC* were found to be 6–7 µm and 8–11 µm, respectively (Figure 3). In case of the *operon* strain, the normal morphology of bacilli was altered in the terminal cells of the chain suggesting that absence of the operon affects both the cell shape and cell length (Figure 3).

Deletion of *clpC* operon results in defective spore formation

Bacillus spores are dormant, multilayered, partially hydrated structure formed during stress/starvation conditions. Spores of *B. anthracis* contain multiple protective layers which include exo-sporium (ES), outer spore coat (OSC), inner spore coat (ISC), cortex (C) and core wall (CW) (Giorno *et al.*, 2007; McKenney *et al.*, 2013). The ultrastructure of spores was investigated by transmission electron microscopy (TEM) to observe any visible changes (Figure 4). Although, deletion of *ctsR* and *mcsA* had no apparent effect on growth, sporulation and germination efficiency, the spores formed by these mutants were dissimilar from the wild type spores (Figure 4B, 4C). The spores formed by *ctsR* and *mcsA* strains showed all the layers; however, the thickness of spore coat was reduced than those of wild type Sterne spores. The spores formed by *mcsB* showed absence of core wall, however, spore coat layers were intact. As a result, there was no obvious partition between the core and cortex (Figure 4D). Similarly, spores formed by *clpC* and *operon* strains also failed to form the intact spore coat layers. In addition, the spore compartment was not engulfed by mother compartment (Figure 4E, 4F). In the course of sporulation, engulfment of fore-spore into mother compartment requires protein degradation (Jiang *et al.*, 2005; Broder and Pogliano, 2006). It has been earlier shown in *B. subtilis* that ClpCP dependent proteolysis of sigma factor antagonist, SpoIIAB, plays a regulatory role in fore-spore development (Pan *et al.*, 2001; Pan and Losick, 2003). This probably explains the loss of fore-spore engulfment phenotype in *clpC* and *operon* strains and not in case of *mcsB* strain suggesting that McsB might not be directly involved in this process.

Defect in sporulation leads to polyhydroxybutyric acid accumulation

Sporulation is an energy driven process carried out by members of Firmicutes family to overcome stress/starvation conditions. This regulated process requires a high pool of ATP under conditions of nutritional imbalance. Lipophilic polymers such as polyhydroxybutyrate (PHB) have been reported to serve as an endogenous energy reservoir under such conditions (Wu *et al.*, 2001; Singh *et al.*, 2009). The energy required for sporulation is usually derived

from degradation of PHB and catabolism of other nutrients. Our results also showed biosynthesis of PHB in wild type, *mcsB*, *clpC* and in operon-deleted mutants (Figure 5). Wild type cells formed more than 90 % spores after 72 h and utilized the accumulated PHB during sporulation. The *mcsB* strain showed two different peaks of PHB synthesis at 24 and 96 h (Figure 5A). In *clpC* and operon strains, PHB level increased continuously and accumulated 10–15 folds higher than the wild type strain suggesting that PHB is not utilized during incomplete and inefficient sporulation (Figure 5A, 5B).

Effect of *clpC* operon deletion on lethal toxin secretion

B. anthracis is known to secrete lethal toxin consisting of two proteins, protective antigen and lethal factor (Leppa, 1982). TEM examination revealed discontinuity in the cell wall of *mcsB* and *clpC* strains. To probe whether the defect in membrane integrity and structure of cell adversely affects the secretion of the toxin proteins, we looked for the secretion profile of these proteins. Anthrax lethal toxin was found to be secreted in all the mutants including operon by immunoblotting (Figure 6A and 6B). These results suggest that lack of McsB and ClpC adversely affects the formation of viable spores without influencing toxin secretion in *B. anthracis* (Figure 2, 4 and 6).

DISCUSSION

Prokaryotes contain a variety of ATP-dependent serine protease complexes involving ClpP and Lon proteases that perform regulated protein degradation (Frees *et al.*, 2007). In Gram negative organisms ClpA and ClpX and in Gram-positive organisms ClpC, ClpE and ClpX act as interacting partners of ClpP (Kruger *et al.*, 2000). ClpC operon has been shown to be instrumental in stress physiology, competence and sporulation of *B. subtilis*. However, the role of this conserved operon in the pathogenic *B. anthracis* has not been studied.

In the present work, we aimed to explore the physiological importance of *clpC* operon in *B. anthracis* Sterne strain utilizing in-frame deletion mutants. The growth was not affected by deletion of *ctsR* or *mcsA*, while *clpC* and operon deleted mutants showed impaired growth particularly at 43 °C (Figure 1B). This result is in conformation with previous studies on *B. subtilis* where ClpC was found to be essential for growth at elevated temperature (Msadek *et al.*, 1994). In addition, we also found that the growth was moderately affected by deletion of *mcsB* suggesting that McsB is also required for normal growth during heat stress (Figure 1B). Further experiments with *mcsB* and *clpC* deletion mutants showed cell elongation due to irregular septum formation (Figure 3). The operon mutant lacking both *mcsB* and *clpC* exhibited bulging at cell poles (Figure 3F and S4 B). A possible explanation for the terminal bulging of cells could be aggregation of misfolded proteins due to deletion of *clpC*. It is known that misfolded proteins tend to aggregate at the poles and mid-cell locations during stress conditions and ClpC adaptors are shown to be involved in degradation of misfolded proteins in bacteria (Kruger *et al.*, 2000; Kirstein *et al.*, 2008). Some “mini-cell” formations were also seen in *clpC* and operon deletion mutants (Figure S4 A, S4 B).

Drastic reduction was observed in sporulation and germination efficiencies in *clpC* and operon deletion mutants; however, no difference was observed in *ctsR* and *mcsA* deletion mutants (Figure 2A, 2D). Sporulation is an adaptive response by *Bacillus* species during

stress conditions. Sporulation process comprises of two major morphological changes, which include asymmetrical cell division and engulfment of fore-spore into mother cell. Compensatory mutations have been reported in prokaryotes under selection pressure (Levin *et al.*, 2000; De Vos *et al.*, 2013). However, no increase in sporulation and germination efficiencies of *clpC* and operon mutants was observed even after three cycles indicating lack of any compensatory mutations in these strains (Figure 2C, 2D).

Investigation of spore ultra-structure indicated that *mcsB* and *clpC* failed to form intact spore coat layers (Figure 4D, 4E). Engulfment of fore-spore was hindered in *clpC* and operon deleted mutants (Figure 4E, 4F). It has been reported that the ClpCP complex moves to the fore-spore while ClpXP remains in the mother cell compartment for cellular degradation processes (Kain *et al.*, 2008). Engulfment of fore-spore into mother cell compartment occurs after substrates are preferentially degraded first in the fore-spore compartment (Broder and Pogliano, 2006). Therefore, it appears that absence of ClpC results into engulfment failure as observed in the spores formed by *clpC* deleted mutants. In *mcsB* deleted mutant, core wall was absent and there was no clear partition between the spore core and cortex (Figure 4D). In an earlier study, a different adaptor than McsB, YpbH has been reported to cause defect in sporulation and competence when eliminated or overproduced in *B. subtilis* (Persuh *et al.*, 2002). Being a signaling molecule, an optimum level of McsB is necessary for producing viable spores therefore, lack of McsB in mutant strain caused spores that were devoid of core wall (Figure 4D). We complemented *mcsB* and *clpC* under their native promoter and observed normal growth rate and sporulation pattern after complementation (Figure 1C; Table 1). The role of McsB (an arginine kinase which phosphorylates itself as well as CtsR, McsA and ClpC) (Kirstein *et al.*, 2005; Fuhrmann *et al.*, 2009) appears similar to YpbH, which has been reported earlier to cause defect in sporulation and competence when eliminated or overproduced in *B. subtilis* (Persuh *et al.*, 2002). The defective spore formation in the absence of *mcsB* and *clpC* indicates the fine tuning of the sporulation process by *clpC* operon in *B. anthracis*.

Absence of ClpC is also known to have a pleiotropic effect on *B. subtilis* physiology (Pan *et al.*, 2001; Pan and Losick, 2003). ClpC is known to mediate degradation of SpoIIAB, an antagonist of sporulation-specific sigma factor, sigma factor F. Deletion of *clpC* leads to inhibition of degradation of SpoIIAB in turn leading to impaired activation of sigma F and defect in fore-spore formation. The defective fore-spore eventually results in sporulation arrest (Pan *et al.*, 2001). Apart from affecting the sporulation process, *clpC* deletion is also known to affect the growth of *B. subtilis* during heat stress (Kruger and Hecker, 1998).

During sporulation, the lipophilic polymer PHB has been reported to serve as an energy source in *Bacillus* (Wu *et al.*, 2001; Kumar *et al.*, 2013). Since sporulation efficiency in *mcsB*, *clpC* and operon mutants were compromised; the PHB utilization in these strains was also evaluated. We found PHB content in *clpC* and operon deletion mutants to be 10 to 15 folds higher than the wild type (Figure 5). The accumulation of PHB could be due to lack of energy utilization owing to defective sporulation process.

Cell wall integrity is crucial for maintaining the secretion profile of bacterial cells. TEM examination showed cell wall discontinuity in the *mcsB* and *clpC* deletion mutants. These

results prompted us to speculate whether the morphologically altered vegetative cells of *mcsB* or *clpC* mutant would secrete the anthrax lethal toxin. Interestingly, we found that both *mcsB* and *clpC* mutants secreted anthrax lethal toxin (Figure 6A, 6B) (Leppla, 1982).

It may be advantageous to use *mcsB/clpC* knockout strains for improving the current vaccine. Non-lethal strains displaying defective germination efficiency with production of adequate toxin for protective immunization could serve as vaccine candidates. This study demonstrates that *clpC* operon governs the multifunctional aspects in both vegetative and dormant phases of *B. anthracis* life cycle.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The primers used for plasmids construction are described in the supplementary Table S1. All the plasmids were sequenced to check the fidelity and integrity of the cloned genes mentioned in the Table S2. *B. anthracis* Sterne 34F₂ was obtained from National Institute of Allergy and Infectious Disease, NIH, USA. The in-frame deletion mutants (*ctsR*, *mcsA*, *mcsB*, *clpC*, operon) were made in the background of *B. anthracis* Sterne strain using *Cre-lox* double recombination system and the gene deletions were confirmed by sequencing. The *Escherichia coli* BL21 (DE3) and DH5 α strains were procured from Invitrogen (USA), while *E. coli* SCS110 from Stratagene (Table S3).

The genes *mcsB* and *clpC* were cloned under their native promoter in shuttle vector (pYS5) of *B. anthracis* and *E. coli* to construct the respective complementation strains (Singh *et al.*, 1989). For over-expression of the hexa-histidine tagged proteins, the genes were cloned into pProEx-HTc (Invitrogen, USA) in *E. coli*. All deletion mutants were grown in Luria-Bertani (LB) broth (BD Difco, USA) at 37 °C and 43 °C during heat stress. The sporulation experiments were performed on low carbon and high salt containing sporulation medium at 30 °C. While for the light and electron microscopic studies, vegetative cells were harvested from mid-log phase grown in LB broth.

Generation of *B. anthracis* Sterne *clpC* operon gene mutants and their characterization

To understand the role of *clpC* operon in the physiology of *B. anthracis* Sterne strain, the *Cre-loxP* genetic modification method was used to introduce precise genetic deletions as reported earlier by Pomerantsev *et al.* (2006). In brief, the pSC vector was used to produce a deletion in each gene or whole operon by a two-step recombination using the primer pair sequence shown in supplementary Table S1 (Figure S1 A). Plasmid pCrePAS was used for the elimination of DNA regions containing a spectinomycin resistance cassette located between two similarly oriented *loxP* sites. The deleted region of gene in each mutant strain was confirmed by DNA sequencing (data not shown) and PCR (Figure S1 B). The expression of ClpC protein in each mutant showed that all mutants had in-frame deletions (Figure S1 C).

***B. anthracis* spore preparation**

The wild type and knock-out strains were grown in 10 mL LB broth up to mid-log phase ($OD_{595} = 0.8$). Each culture was inoculated into 100 mL sporulation media containing 0.6 mM $CaCl_2 \cdot 2H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM $MnSO_4 \cdot H_2O$, 85.5 mM NaCl, 0.025 mM $ZnSO_4$, 0.02 mM $CuSO_4$, and 8 g of LB broth/L (pH=6.0) as described earlier (Barua *et al.*, 2009).

Cultures were incubated at 30 °C for 72 h on constant shaking at 200 rpm. The spores were harvested at $12,000 \times g$ for 15 min at 4 °C and transferred to 50 mL autoclaved milliQ water to complete sporulation at 30 °C at 200 rpm. Spores were harvested at 120 h and washed thrice with autoclaved milliQ water and finally re-suspended in 5 mL milliQ water. Spores were heat activated at 65 °C for 30 min and stored at -20 °C. Sporulation and germination efficiencies were calculated as described by Giebel *et al.* (2009) and Burns *et al.* (2010).

Transmission electron microscopy

B. anthracis Sterne strain and its deletion mutants were grown at 37 °C in LB broth and harvested at mid-log phase. The cells were pelleted at $12,000 \times g$ for 10 min and washed three times with 100 mM sodium phosphate buffer (pH=7.4). The samples were immediately transferred for primary fixation to Karnovsky's fixative [2.5 % Glutaraldehyde (TAAB, UK) + 2 % Paraformaldehyde (Sigma-Aldrich, USA) in 100 mM sodium phosphate buffer pH=7.4] for 12 h at 4 °C. The samples were rinsed thoroughly with sodium phosphate buffer to wash off the un-reacted excess fixative. Secondary fixation was performed with 1 % osmium tetroxide (TAAB, UK) for 1 h at 4 °C and then dehydrated with graded acetone (Merck Chemicals, Germany) [30 %, 50 %, 70 %, 80 % ($\times 2$), 90 %, 100 % ($\times 3$); 30 min each at 4 °C]. Absolute xylene (Merck Chemicals, Germany) was used for clearing process and dehydrating agent was replaced from the samples. The bacterial samples were in-filtered and embedded into araldite resin mixture (TAAB, UK). Infiltration was carried out by gradually decreasing the concentration of clearing agent and increasing the concentration of embedding medium proportionately. Curing was processed at 55 °C for 24 h and then 65 °C for 48 h. Sectioning was performed with Leica UC6 ultra-cut and grids were observed in FEI Tecnai G² Spirit at 200 KV (Putnam *et al.*, 2013).

Scanning electron microscopy

The deletion mutants and wild type strains were grown at 37 °C in LB broth and harvested at mid-log phase and processed as earlier described by Dubey *et al.* (2011). The cells were pelleted at $12,000 \times g$ at 4 °C, washed three times with 100 mM sodium phosphate buffer (pH=7.4). The samples were fixed with Karnovsky's fixative (2.5 % Glutaraldehyde + 2 % Paraformaldehyde in 100 mM sodium phosphate buffer pH=7.4) for 6 h at 4 °C and again washed with sodium phosphate buffer thrice. Secondary fixation was performed with 1% osmium tetroxide for 10 min at 4 °C and then dehydrated with graded ethanol [30 %, 50 %, 70 %, 80 % ($\times 2$), 90 %, 100 % ($\times 3$); 30 min each at 4 °C]. Samples were dried with a critical point drying technique and subjected to the gold coating of 10 nm thickness with an Agar sputter coater on aluminium stubs. Samples were observed in Zeiss Scanning Electron Microscope EVO LS15 at 20 KV.

Polyhydroxybutyrate assay during sporulation

Primary inoculum for PHB production was prepared using single bacterial colony from LB agar plate into 10 mL LB broth at 37 °C for 16–20 h at 200 rpm. It was used as inoculum in sporulation media (0.6 mM CaCl₂·2H₂O, 0.8 mM MgSO₄·7H₂O, 0.3 mM MnSO₄·H₂O, 85.5 mM NaCl, 0.025 mM ZnSO₄, 0.02 mM CuSO₄ and 8 g of LB broth/liter, pH–6.0). PHB production was monitored in *mcsB*, *clpC* and operon deleted strains and compared to *B. anthracis* Sterne as control. Cultures were grown in 200 mL sporulation media by incubating at 37 °C and 200 rpm. Aliquots of 100 mL were analyzed for cell dry weight (CDW) and PHB content at various time periods upto 144 h after inoculation. Samples (100 mL) were centrifuged at 8,000 × g at 4 °C for 20 min. The pellet was washed with 10 mL saline solution (0.9 % NaCl). The pellet was dried at 85 °C for 36 h and weighed to estimate CDW. Cell pellet (40 mg) was processed and treated for propanolysis. Propyl-esters of β-hydroxyacids from PHB hydrolysis were analyzed by gas chromatograph (GC) equipped with dimethylpolysiloxane capillary column DB–1 (30 m × 0.25 mm × 0.25 μm) and flame ionization detector by the method described earlier (Patel *et al.*, 2012).

Assay of anthrax lethal toxin in supernatants

Wild type and deletion mutants were inoculated in 10 mL LB broth and incubated overnight at 37 °C. Secondary inoculation (10 %) was performed in 50 mL Brain-Heart Infusion media (BD Difco, USA) containing 1 % sodium bicarbonate. The cultures were incubated at 37 °C for 4 h at 200 rpm shaking. The cultures were pelleted at 12,000 × g for 30 min and supernatants were collected followed by their filter sterilization. Precipitation was performed by adding 20 % trichloroacetic acid (Merck Chemicals, Germany) and incubating at –20 °C for 1 h. Proteins were pelleted at 12,000 × g for 30 min at 4 °C. Protein pellets were washed thrice with 5 mL ice-chilled acetone. The pellets were dried at 95 °C and re-suspended in 1 mL PBS containing 1 × protease-inhibitor cocktail (Roche Applied Sciences, USA). The resuspended protein pellets were loaded along with purified protective antigen/lethal factor on 12 % SDS-PAGE followed by immunoblotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Growth kinetics of *Bacillus anthracis* Sterne and mutants strains

Wild type and mutant strains were grown in LB broth at (A) 37 °C; (B) 43 °C; and (C) *mcsB* and *clpC* complemented strains grown at 43 °C. Absorbance at 595 nm was recorded at various time periods. Each value is average of three independent experiments with three replicates in each experiment.

Figure 2. Sporulation and germination efficiency of *Bacillus anthracis* spores

(A) Sporulation and (B) germination profile of spores of *B. anthracis* Sterne and mutant strains for one cycle. Each bar is average $\pm$ SE of nine replicates representing sporulation or germination efficiency and calculated from three independent replicates of three experiments. (C) Sporulation and (D) germination profile for three cycles. To study sporulation and germination for three cycles, cultures of *B. anthracis* Sterne and mutants were grown and subjected to sporulation (spore of 1st cycle). The spores of 1st cycle were germinated and cultured to obtain spores of 2nd cycle and the obtained spores were again germinated and cultured to produce spores of 3rd cycle. Each value is average of three replicates. Two tailed unpaired t test indicated that means are significantly different. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, non significant.

Figure 3. Scanning and transmission electron micrograph of vegetative cells of *Bacillus anthracis* (Upper Panel) Cells were grown at 37 °C in LB broth and harvested at mid-log phase. Cells were washed with 0.1 M sodium phosphate buffer and fixed primarily with Karnovsky's fixative and then treated with 1 % osmium tetroxide. The samples were dehydrated, subjected to critical point drying and coated with gold. Cells were visualized under Zeiss Evo LS15 and shown as wild type (A); *ctsR* (B); *mcsA* (C); *mcsB* (D); *clpC* (E) and operon (F). White arrows show the septum; Red arrows show bulging at poles; Blue arrows show mini cells. (Scale bar: A, B, C, E = 2 μm; D, F = 10 μm.) **(Lower Panel)** Cells

were grown at 37 °C in LB broth and harvested at mid-log phase. Cells were washed with 0.1 M sodium phosphate buffer and fixed primarily with Karnovsky's fixative and then treated with 1 % osmium tetroxide. The samples were dehydrated and infiltrated with araldite resin mixture and incubated at 65 °C for three days. Ultramicrotomy was performed using Leica Ultracut UC6 and stained with uranyl acetate and lead citrate. Grids were visualized under FEI G² Spirit at 200 KV. Wild type (G); *ctsR* (H); *mcsA* (I); *mcsB* (J); *clpC* (K) and *operon* (L). Black arrows show the septum formed. (Scale bar: G, J, K = 1 µm; H, I, L = 0.5 µm.)

Figure 4. Transmission electron micrographs of spores of *Bacillus anthracis*

The spores were prepared and harvested as described in materials and methods. Wild type (A); *ctsR* (B); *mcsA* (C); *mcsB* (D); *clpC* (E) and operon (F). Various layers of spores are shown by arrow: exo-sporium (ES), outer spore coat (OSC), inner spore coat (ISC), cortex (C), core wall (CW), spore compartment (SPC), mother compartment (MOC). (Scale bar: A, B, C, E = 200 nm; D = 100 nm; F = 0.5 μ m.)

Figure 5. Polyhydroxybutyrate (PHB) formation during sporulation of *Bacillus anthracis*

Each strain was grown in sporulation medium at 37 °C and at each time point 100 mL culture was harvested to isolate PHB as described in materials and methods. The level of PHB in each isolate was measured by Gas Chromatography. Two tailed unpaired t test indicated that means are significantly different. ****P < 0.0001. (A). TEM micrographs of spores/cells at 96 h. Arrows shows PHB granules in the cells (B).

Figure 6. Secretion of anthrax lethal toxin by *Bacillus anthracis* Sterne and mutant strains
Each strain was grown in Brain-Heart Infusion media supplemented with 1 % sodium bicarbonate for 4 h at 37 °C. Culture was centrifuged and proteins of supernatant were precipitated by 20 % cold trichloroacetic acid. Proteins were pelleted at $12,000 \times g$ for 30 min at 4 °C and washed thrice with 5 mL ice-chilled acetone. The pellets were dried at 95 °C and re-suspended in 1 mL PBS containing $1 \times$ protease-inhibitor cocktail. The resuspended protein pellets were loaded along with purified protective antigen/lethal factor on 12 % SDS-PAGE followed by immunoblotting. Blot was developed with PA or LF antibodies and ECL reagents.

Table 1
Sporulation efficiency of complemented strains

Bacillus anthracis Sterne, *mcsB*, *clpC* mutants along with complemented strains were subjected to sporulation in sporulation media and incubated at 30 °C for 120 h. Sporulation efficiency was calculated by Neubauer hemacytometer chamber and Nikon phase contrast microscope. Each value is average $\pm$ SE of three independent experiments.

Strain Name	% Sporulation (SE)
<i>Wild type</i>	96.81 $\pm$ 1.62
<i>mcsB</i>	61.29 $\pm$ 2.62
<i>mcsB complement</i>	90.87 $\pm$ 1.93
<i>clpC</i>	41.02 $\pm$ 1.48
<i>clpC complement</i>	88.07 $\pm$ 2.25