Poly-β-hydroxybutyrate Metabolism Is Unrelated to the Sporulation and Parasporal Crystal Protein Formation in Bacillus thuringiensis

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INTRODUCTION

Bacillus thuringiensis is a ubiquitous Gram-positive, rod-shaped, and spore-forming bacterium that produces poly-3-hydroxybutyrate (PHB) and insecticidal crystal proteins (ICPs). PHB is a linear biopolymer consisting of (R)-3-hydroxybutyrate (3HB) monomers. It can be accumulated as insoluble cytoplasmic granules under over-nutrition state and/or in the absence of one or more essential nutritional elements in B. thuringiensis (Jendrossek, 2009). When energy supplies are exhausted, it can then be served as an alternate energy source (Anderson and Dawes, 1990; Jendrossek and Pfeiffer, 2014; Prieto et al., 2016).
The biochemical pathways for PHB synthesis and degradation have been studied in great details in bacteria such as Ralstonia eutropha H16, B. megaterium, Rhodospirillum rubrum, and so on (Jendrossek and Handrick, 2002). The PHB synthesis requires three enzymatic steps, starting from the condensation of two molecules of acetyl-coenzyme A to form one molecule of acetoacetyl-CoA (condensation reaction, catalyzed by the PhaA thiolase), followed by the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA (3HB-CoA) (reduction reaction, catalyzed by the PhaB reductase), the monomeric precursor for PHB. Finally, the 3HB-CoA monomers are polymerized to form PHB by the PHB synthase (PhaC) (Anderson and Dawes, 1990; Steinbuchel et al., 1992; Madison and Huisman, 1999; Chen, 2009). Likewise, three enzymes of PhaZ, BdhA and AacS are involved for its degradation, in which the most crucial step is the depolymerization of PHB into 3HB-CoA catalyzed by the PHB depolymerase (PhaZ). This enzyme is currently the only known PHB depolymerase observed to date (Tseng et al., 2006; Jendrossek and Pfeiffer, 2014).

The process of sporulation and ICP accumulation requires considerable input of carbon and energy sources (Wang et al., 2013a,b). As early paper (Slepecky and Law, 1961) reported that PHB fueled the sporulation process in B. megaterium. It was also reported that PHB was accumulated maximally prior to the spore formation and was degraded during the process of sporulation in B. cereus (Kominek and Halvorson, 1965; Valappil et al., 2007). However, how PHB affects sporulation and parasporal crystal formation is still controversial to date. It is generally believed that PHB degradation can provide energy and carbon sources required for the sporulation and parasporal crystal formation. For example, Navarro et al. (2006) found a linear relationship between the PHB accumulation and the parasporal crystal formation in B. thuringiensis. Recently, a phaC deletion mutant from B. thuringiensis BMB171 was constructed, and found to result in a growth delay and sporulation-deficient phenotype (Chen et al., 2012). However, another group reported that spore formation is not impaired in a phaC deletion mutant in B. thuringiensis (Chen et al., 2010). In many cases, utilization of PHB does not seem to be imperative for sporulation, because many spore-forming genus Bacillus that cannot synthesize PHB still sporulate normally. Most Bacillus species such as B. subtilis exhibits natural PHB-negative phenotype, and bioinformatic analysis reveals no known pha genes or sequences in their genomes (Singh et al., 2009), indicating that PHB has no direct correlation with the bacterial sporulation. McCool and Cannon (2001) constructed a phaPQRBC operon deletion strain of B. megaterium. However, other than the PHB-negative phenotype, it showed no apparent phenotypic difference and exhibited similar growth rate as its progenitor. Based on these conflicting results, the relationship between PHB accumulation and sporulation and ICP formation still remains obscure. In this study, we have used the markerless gene deletion method, which is believed to cause the least perturbation to genome, to knock-out the phaC and phaZ genes respectively in order to carefully examine the influence of PHB on sporulation and ICP formation in B. thuringiensis BMB171.

MATERIALS AND METHODS

Strains, Plasmids, Primers and Growth Conditions

The strains and plasmids, as well as the primers used in this study, were listed in Table 1 and Table S1, respectively. Escherichia coli were cultured at 37°C in lysogeny broth (LB) medium (g/L: tryptone, 10; yeast extract, 5; NaCl, 10). The medium was adjusted to pH 7.0 before autoclaving at 121°C for 15 min. Unless otherwise specified, B. thuringiensis strains were cultured at 28°C in the GYS medium (g/L: glucose, 1.00; yeast extract, 2.00; K2HPO4·3H2O, 0.66; (NH4)2SO4, 2.00; MgSO4·7H2O, 0.04; MnSO4·H2O, 0.04; CaCl2, 0.08). The medium was autoclaved at 115°C for 30 min after adjusting the pH to 7.8.

Construction of the Integrating Plasmids for Gene Deletion

To construct the plasmids for phaC (BMB171_RS06655, old locus lag BMB171_C1161) and phaZ (BMB171_RS16270, old locus lag BMB171_C2975) deletions, upstream homologous arms of UphaC and UphaD and downstream homologous arms of DphaC and DphaZ (homologous to the 5’ and 3’ uncoding regions of the target genes) of approximately 750 bp were amplified from the BMB171 genomic DNA by PCR, using primer pairs of DphaCU/F/DphaCU R, DphaCD F/DphaCD R, DphaZU/F/DphaZU R and DphaZD F/DphaZD R, respectively (Table S1). Then, UphaC (or UphaZ) was inserted into plasmid pRP1028 between the Mlu I and BamH I sites to construct the plasmid pRP1028-UphaC (or pRP1028-UphaZ). Next, DphaC (or DphaZ) was inserted into plasmid pRP1028-UphaC (or pRP1028-UphaZ) between the BamH I and Kpn I sites to construct the integrating plasmid pRP1028-UDphaC (pRP1161) [or pRP1028-UDphaZ (pRP2975)] (Figure S1). pRP1028 was a shuttle plasmid with a temperature-sensitive suicide B. thuringiensis replicon. The resulting integrating plasmids were further verified by sequencing.

Construction of phaC and phaZ Deletion Strains

The markerless gene deletion system was successfully developed for BMB171 based on an I-SceI mediated replacement method as established in B. anthracis by Janes and Stibitz (2006). The detailed procedures have been well described in a previous publication (Zheng et al., 2015), and will only be briefly accounted here taking deletion of phaC as the example. Firstly, BMB171 (recipient strain), E. coli DH5α containing the integrating plasmid pRP1028-UDphaC (donor strain) and E. coli DH5α containing the helper plasmid pSS1827 (helper strain) used for conjugal transfer (triparental mating) were grown in LB medium. The plasmid pRP1028-UDphaC was integrated into the BMB171 chromosome to form the plasmid-integrated
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strains and plasmids | Characteristics | Source |
|----------------------|-----------------|--------|
| **Strains**          |                 |        |
| E. coli DH5α         | RecA1 endA1 gyrA96 thi hsdR17 (rK- mK +) relA1 supE44 | Invitrogen |
| BMB171               | *B. thuringiensis* strain BMB171; an acrystalliferous mutant strain; high transformation frequency | Li et al., 2000; He et al., 2010 |
| ΔphaC                | Markerless phaC gene deletion mutant of BMB171 | This study |
| ΔphaZ                | Markerless phaZ gene deletion mutant of BMB171 | This study |
| BMB171-pHT           | BMB171 strain harboring empty vector pHT304 | This study |
| BMB171-phaC          | BMB171 strain harboring pBMB43-304 | This study |
| ΔphaC-phaZ           | ΔphaC strain harboring pBMB43-304 | This study |
| DH5α-pRP1028         | DH5α harboring pRP1028 | Janes and Stibitz, 2006 |
| DH5α-pSS4332         | DH5α harboring pSS4332 | Janes and Stibitz, 2006 |
| DH5α-pSS1827         | DH5α harboring pSS1827 | Janes and Stibitz, 2006 |
| **Plasmids**         |                 |        |
| pSS1827              | Helper plasmid for conjugative transfer; Amp<sup>R</sup> | Janes and Stibitz, 2006 |
| pSS4332              | *B. thuringiensis*-E. coli shuttle plasmid; Km<sup>R</sup>; containing gfp and i-SceI restriction enzyme encoding gene | Janes and Stibitz, 2006 |
| pRP1028              | *B. thuringiensis*-E. coli shuttle plasmid; Amp<sup>R</sup>Em<sup>R</sup>; containing temperature-sensitive suicide *B. thuringiensis* replocron, turbo-rfp gene and an-i-SceI recognition site, etc. | Janes and Stibitz, 2006 |
| pRP1161              | pRP1028 with upstream homologous arm of phaC (UpphaC) and downstream homologous arm of phaC (DphaC) | This study |
| pRP2975              | pRP1028 with upstream homologous arm of phaZ (UpphaZ) and downstream homologous arm of phaZ (DphaZ) | This study |
| pHT304               | *B. thuringiensis*-E. coli shuttle plasmid; Amp<sup>R</sup>Em<sup>R</sup> | Arantes and Lereclus, 1991 |
| pBMB43-304           | *B. thuringiensis*-E. coli shuttle plasmid containing ORF of cry1Ac10; Amp<sup>R</sup>Em<sup>R</sup> | Qi et al., 2015 |

strain by homologous single cross-over recombination. Plasmid-integrated strains were verified by PCR using primer pairs IphaC F/UniversalI R (Table S1). Secondly, the expression plasmid pSS4332 (in DH5α-pSS4332), which produced the restriction endonuclease I-SceI, was conjugationally transferred into the plasmid-integrated strain by a triparental mating system with the help of *E. coli* DH5α-pSS1827 as well. The I-SceI endonuclease can recognize and cleave the highly specific 18 bp DNA target site within the integrated plasmid, resulting in chromosomal double-stranded break and thus stimulated the host genetic repair by homologous recombination between the flanking repeat sequences. As a result, there is approximately 50% possibility to delete the target gene from chromosome. Positive deletion strains of *phaC* were verified by PCR and sequenced (Figures S2 and S3). Finally, the plasmid pSS4332 in the *phaC* deletion strain was eliminated by continuous passage for ten times in the LB medium at 28°C. Those strains in which the pSS4332 plasmid was removed were sensitive against kanamycin. Deletion of *phaZ* was performed using the same method (Figures S2 and S4).

**RNA Extraction, cDNA Synthesis and RT-qPCR**

Twenty milliliter of a cultured sample at 48 h in LB medium was collected, and pelleted cells were grounded in liquid nitrogen. The procedures for RNA extraction and cDNA synthesis were carried out as previously described (Wang et al., 2013b; Zheng et al., 2015). Each experiment was repeated in triple.

**Extraction and Determination of ICP Concentrations**

Strains of BMB171-*cry*, ΔphaC-*cry* and ΔphaZ-*cry* were obtained by transformation of the cry1Ac10 promoter and *cry* gene-containing plasmid (pBMB43-304) (Qi et al., 2015) into the BMB171, ΔphaC and ΔphaZ strains, respectively. pBMB43-304 is a derivative of the low copy number shuttle plasmid pHT304 (Arantes and Lereclus, 1991), into which a *cry* gene was inserted between the two *Hind*III sites. The plasmid pBMB43-304 was maintained by growing the bacteria in the presence of 25 μg/mL erythromycin. For the extraction of the ICP (Cry1Ac10), strains were grown at 28°C and 200 rpm for 20 h in GYS medium supplemented with 25 μg/mL erythromycin. After optical density measurement, cells were diluted to a final OD<sub>600</sub> of 1.0, and 20 mL of each culture was separately collected by centrifuge at 6000 × g for 15 min (AG Eppendorf, Hamburg, Germany). Procedure for the extraction of the ICP was carried out according to a previous study (Wang et al., 2013a). Finally, the ICP was visualized by SDS-PAGE and the ICP concentrations were measured by the Bradford method.

**PHB Assays**

The PHB contents of *B. thuringiensis* cells were determined by UV absorption spectroscopy at 245 nm (Law and...
Slepecky, 1961) using PHB extracted from BMB171 as standard.

Transmission Electron Microscopy
Sample preparation for transmission electron microscopy was carried out according to the method described in the literature (Tian et al., 2005) with slight modification. Strains were collected at 9 and 19 h, centrifuged at $6000 \times g$ for 3 min, and washed three times with PBS buffer (pH 7.2). Pellets were then resuspended in 2.5% glutaraldehyde phosphate buffer, fixed at 4°C for 24 h. After washing three times with PBS buffer (pH 7.2), cells were dehydrated gradually using different concentrations of ethanol (20%, 50%, 70%, 80%, 90%, and 100%). Each process was carried out twice for 15 min each. Samples were stored in vacuum freeze drier overnight. Thin sections were examined on a HITACHI H-7000FA transmission electron microscope (Hitachi, Ibaraki, Japan).

Virulence Assays
The larvae of Heliothis armigera were reared at 28°C with a light/dark cycle of 12:12 h. Artificial diet comprised 4 g yeast extraction, 7 g bean flour, 0.5 g vitamin C, 1.5 g agar, 36% acetic acid and 1 g penicillin per 100 mL of water. The medium was transferred into 24-hole cell culture plates (Corning, USA), 1 mL per well. 200 mL of cells grown in GYS medium for 20 h were harvested by centrifugation ($8,500 \times g$, 5 min, 4°C) and suspended in 20 mL distilled water. Cells were then diluted 10,000-fold, with 100 µl of diluted cells applied to each well, allowing it to dry automatically. One first-instar larvae was used for feeding assay per well, with mortality recorded at indicated dates. Meanwhile, body length and weight were measured at the 6th day. Three repeats per bioassay were performed using 24 larvae for each strain (Fiuza et al., 2013).

Spore Count
Besides BMB171-cry, ΔphaC-cry and ΔphaZ-cry strains, BMB171 and BMB171-pHT (BMB171 harboring empty plasmid pH7304) strains were used as controls. OD$_{600}$ of each strain grown in the LB medium for 20 h were harvested by centrifugation ($8,500 \times g$, 5 min, 4°C) and suspended in 20 mL distilled water. Cells were then diluted 10,000-fold, with 100 µl of diluted cells applied to each well, allowing it to dry automatically. One first-instar larvae was used for feeding assay per well, with mortality recorded at indicated dates. Meanwhile, body length and weight were measured at the 6th day. Three repeats per bioassay were performed using 24 larvae for each strain (Fiuza et al., 2013).

RESULTS

PHB Contents Changed upon phaC or phaZ Deletion
In previous reports, phaC and phaZ were identified as the genes encoding PHB synthetase and degradation enzyme in B. thuringiensis respectively (Tseng et al., 2006; Chen et al., 2010). To disrupt the PHB metabolism pathway, phaC and phaZ were deleted from the parent strain BMB171 by the markerless gene deletion method, with the corresponding strains named as ΔphaC and ΔphaZ, respectively. The growth curves of BMB171, ΔphaC and ΔphaZ strains in GYS medium at 28°C were determined. No obvious difference was observed for all three strains, and they all entered the stationary phase at approximate 11 h (Figure 1).

The amount of PHB produced was measured during the whole growth period. For the parent strain BMB171, it was found that its intracellular PHB level started to accumulate from 3 h and reached to a maximum level at 11 h, after which it descended rapidly to half amount at about 16 h and approximately to zero at 21 h (Figure 2). No PHB could be detected in the ΔphaC mutant over the whole growth cycle, indicating that PHB accumulation in the ΔphaC mutant was totally abolished. This data indicated that PhaC was absolutely required for the PHB synthesis (Figure 2). As for the ΔphaZ mutant, the accumulation stage was similar, but
there was a delay in the degradation stage. It began to degrade at 16 h, with about half amount left at 21 h. Since PHB degradation wasn’t completely abolished in ΔphaZ, we speculated that there were some other PHB degradation enzymes or pathways existing in B. thuringiensis.

**Cell Morphology Didn’t Change in the phaC and phaZ Deletion Strains**

BMB171 is an acrystalliferous strain that doesn’t produce ICPs (Li et al., 2000; He et al., 2010). To investigate its morphological changes in the absence of phaC and phaZ, the ICP coding gene cry1Ac10-containing plasmid pBMB43-304 (Qi et al., 2015) needs to be introduced into this strain, and the ΔphaC and ΔphaZ mutants. The corresponding strains were named as BMB171-cry, ΔphaC-cry and ΔphaZ-cry, respectively. Cell morphologies during the exponential phase and sporulation phase were observed using transmission electron microscope. The cell size and shape of the mutants were found to be similar as the parent strain (Figure 3). The spore and parasporal crystal morphologies of ΔphaC-cry and ΔphaZ-cry also exhibited no major difference compared to those of BMB171-cry under the similar growth conditions.

**ICP Formation and Toxicity Were Not Changed in the ΔphaC-cry and ΔphaZ-cry Strains**

To be more quantitative, we have further measured the ICP amounts to investigate the influence of phaC or phaZ deletion on ICP formation. As shown in Figure 4, all the three strains produce the ICP without conspicuous difference in their amounts (“ns” indicates not significant, $P > 0.05$). It is well known that ICPs are virulent to the Helicoverpa armigera larvae. To test whether the toxicities differ in these strains, virulence assays were also performed and the survival rates of the H. armigera larvae fed with these bacteria were measured ($\text{H}_2\text{O}$ and BMB171 were set as control groups). Table 2 shows that the survival rates of BMB171-cry, ΔphaC-cry and ΔphaZ-cry were almost similar even after six days ($ns, P > 0.05$). The body length and weight also didn’t differ much among these three strains ($ns, P > 0.05$). Together, these results indicate that the insecticidal activities of the ICP produced by the three strains were comparable.

**PhaC or PhaZ Is Not Required for Sporulation**

As observed by the transmission electron microscopy in the Figure 3, both the ΔphaC-cry and ΔphaZ-cry strains are able to sporulate similar to the BMB171-cry strain, which is different to the previous result that sporulation was abolished when phaC was deleted from BMB171 (Chen et al., 2012). It is thus important to learn what caused the difference. To make the comparison more objective, three B. thuringiensis strains were cultivated in the same condition as Chen’s experiment, and their spore numbers were counted. No significant difference for the BMB171-cry, ΔphaC-cry and ΔphaZ-cry strains (Figure 5) seems to be present ($ns, P > 0.05$). In addition, several sporulation-related genes were selected to measure their relative expression levels by RT-qPCR experiments using 16S rRNA gene as an internal control. The sporulation-related genes include those expressed in the pre-divisional cells (spoIAB and spoIGA), early mother cells (spoIID) and late mother cells (cotH and gerE) (de Hoon et al., 2010). If the sporulation pathway was impaired in those strains, gene expression level will change. Our data showed that the transcription levels of most sporulation-related genes such as spoIAB, spoIGA, spoIID, cotH and gerE didn’t change significantly (Figure 6, ns, $P > 0.05$). Taken together, these results indicate that the spore-forming ability
and transcription of sporulation-related genes were not much impaired in the three strains demonstrating that there is no relationship between the PHB accumulation and sporulation in *B. thuringiensis*.

**Bioinformatics Analysis Revealed That There Is No Relationship between the PHB Metabolism and the Sporulation in the Genus Bacillus**

To further explore the relationship between PHB metabolism and sporulation, we expanded our investigation further through bioinformatics analysis. Since almost all species in the genus *Bacillus* except *B. infantis* can sporulate, we wondered whether those species can also produce PHB. Distribution of *phaC* and *phaZ* genes in the 79 strains of the genus *Bacillus* with complete genomes from NCBI were investigated (Table S2). Screening of metabolic (KEGG) and genomic (NCBI) database for *phaC* and *phaZ* genes revealed that they were absent in the *B. subtilis* group and some other strains in genus *Bacillus* (Table S3). Since PhaC is required for PHB synthesis, we speculate that there is no PHB production in those strains lacking the *phaC* gene. Therefore,
no relationship between the PHB accumulation and sporulation could be revealed from this bioinformatics study.

**DISCUSSION**

In this study, cell morphologies of BMB171-cry, ΔphaC-cry and ΔphaZ-cry strains during the exponential and sporulation phases were observed using transmission electron microscope and phase contrast microscope. Interestingly, not much difference in their morphologies were observed. Other features, such as spore production rate and ICP concentration, were also carefully checked. Again, no significant change about these two characteristics was observed among the three strains. These observations were consistent with the data reported by McCool and Cannon (2001) and Chen et al. (2010), in which depletion of PHB metabolism pathway did not affect the sporulation and ICP formation. However, Chen et al. (2012) reported a conflict result even in the same *B. thuringiensis* strain. Comparison of methodologies used in these two experiments revealed that the only difference was the gene knockout method. In our study, we employed a markless gene deletion method to construct the ΔphaC and ΔphaZ mutant strains, while in Chen’s publication, a traditional knockout method was used, which required a higher screening temperature (42°C) that is close to the lethal temperature (around 45°C) of *B. thuringiensis* (Wu et al., 2011). This temperature difference could lead to a devastating effect on the physiological and genetic properties of this bacterium. Moreover, a resistance gene that was introduced into the chromosome to replace the target gene also increased the possibility of phenotypic change. Thus, we speculated that mutations in other genes other than *phaC* may lead to the lack of sporulation and ICP formation. As a matter of fact, we occasionally observed strains with abnormal phenotype when traditional knockout method was applied. If these unsure knockout strains were chosen for genetic study, inconsistent conclusions could be drawn. Based on our practice in the deletion of hundreds of genes in BMB171, we concluded that this system is easy to operate and allows for clean deletions of one or more genes within an operon (Zheng et al., 2015; Zhang et al., 2015, 2016). Because there is no need for a higher screening temperature, undesirable mutations could decrease, resulting with much more consistent results. Therefore, we strongly suggest using the 1-secl endonuclease markless gene knockout method for gene knockout studies in *B. thuringiensis* and even in *B. cereus* group strains.

PHB was generally believed to serve as a sink of carbon and reducing equivalents. However, this doesn’t rule out the possibility that bacteria may use other metabolic pathway to provide the energy required for sporulation and ICP formation. PHB oxidation involves a specific NADH dependent dehydrogenase (PhaB) (Madison and Huisman, 1999), which competes for tricarboxylic acid (TCA) cycle intermediates (for example, NADH, NADPH, ATP, and acetyl-CoA) in the electron transport system. When PHB accumulation is disrupted, more resources are accessible to TCA cycle. Transcriptome analyses of the wild type and mutated *Pseudomonas putida* showed that lack of PHA accumulation in the mutant altered transcription of many genes coding for enzymes involved in the central metabolic pathways, including carbohydrate, fatty acid, amino acid, nucleotide, cofactor and prosthetic group synthesis pathways (Escapa et al., 2012). Meanwhile, when the PHB-negative mutant *R. eutrophus* PHB-4 and its wild type were subjected to proteome analyses, higher amounts of acetyl-CoA and pyruvate in the PHB-negative mutant were observed (Raberg et al., 2014). From these results we speculate that when PHB biosynthetic pathway is disrupted, other metabolic pathway could take place to provide the energy required for sporulation and ICP formation (Senior and Dawes, 1973; Jackson and Dawes, 1976; Page and Knosp, 1989; Aldor and Keasling, 2003).

Due to the absence of PHB depolymerase, ΔphaZ-cry may lost the ability to degrade PHB during stationary phase. However, when *phaZ* was deleted, most bacterial cells can still degrade PHB through an unknown mechanism. Thus deletion of *phaZ* didn’t seem to completely abolish PHB degradation. We speculate that some other pathways may be present to degrade PHB. In fact, many have tried to identify and isolate the PHB depolymerase. PHB is present in two different physical forms, an intracellular native PHB granule form (nPHB) and an extracellular denatured PHB granule form (ePHB). The nPHB exists in an amorphous state and is always coated with various proteins and phospholipase, while the ePHB is a partially crystalline polymer (jendrossek and Handrick, 2002). The degradation enzymes for nPHB and ePHB displayed a low sequence similarity with rather different biochemical properties (Saegusa et al., 2001; York et al., 2003). Although the ePHB degradation enzyme has been extensively investigated in *P. lemoignei* (Kumar et al., 2000; Schober et al., 2000), not much is known about the nPHB degradation yet. Most methods to identify new nPHB depolymerase is based on sequence comparison with already known depolymerase, either by comparing their *phaZ* genes in the other species, or *phaC* genes (Tseng et al., 2006; Chen et al., 2009; Sznaider and Jendrossek, 2014), or through the blast search of the consensus motif. To date, only one PHB degradation enzyme (PhaZ) was well characterized in *B. thuringiensis* (Tseng et al., 2006; Wang et al., 2014). Obtained results indicated that these methods all suffer from some limitations. Further investigations are required to identify the novel potential nPHB depolymerase. From the results demonstrated in our work, however, we strongly believe that there must have other PHB depolymerases existent in *B. thuringiensis*, and characterization of these potential PHB depolymerases is currently undergoing.

Taken together, we have verified the relationship between PHB accumulation and sporulation and ICP formation in *B. thuringiensis*. This investigation provides a new perspective about the relationship between the important metabolic processes in *B. thuringiensis*, and supply information for designing novel metabolic engineering strategies for maximizing ICPs production or controlling sporulation process.
AUTHOR CONTRIBUTIONS

XW performed molecular biology work, participated in study design and drafted the manuscript. ZL performed the phenotype assays and study design. XL (third author) performed the transmission electron microscopy observations, extraction and determination of ICP concentrations, and drafted the manuscript. HQ carried out gene deletions and the virulence assays. XC performed PHB assay and spore counting. XL (sixth author) carried out data analysis. JH conceived the study, participated in the study design, coordinated the research, reviewed and edited the manuscript. All authors read and approved the final 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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