Research Article

Proteomic Analysis of Bacillus thuringiensis Strain 4.0718 at Different Growth Phases

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The growth process of Bacillus thuringiensis Bt4.0718 strain was studied using proteomic technologies. The proteins of Bt whole cells at three phases—middle vegetative, early sporulation, and late sporulation—were extracted with lysis buffer, followed with separation by 2-DE and identified by MALDI-TOF/TOF MS. Bioactive factors such as insecticidal crystal proteins (ICPs) including Cry1Ac(3), Cry2Aa, and BTRX28, immune inhibitor (InhA), and InhA precursor were identified. InhA started to express at the middle vegetative phase, suggesting its contribution to the survival of Bt in the host body. At the early sporulation phase, ICPs started their expression. CotJC, OppA, ORF1, and SpoIVA related to the formation of crystals and spores were identified, the expression characteristics of which ensured the stable formation of crystals and spores. This study provides an important foundation for further exploration of the stable expression of ICPs, the smooth formation of crystals, and the construction of recombinant strains.

1. Introduction

Bacillus thuringiensis (Bt) is a ubiquitous Gram-positive, spore-forming bacterium that produces parasporal crystalline inclusions named insecticidal crystal proteins (ICPs) during its sporulation phase [1, 2]. Its insecticidal activity is largely attributed to its insecticidal crystal proteins, which were used in the development of important microbial insecticidal pesticides [3] widely used in agriculture and forestry [4].

Genome sequencing and annotation of Bt. spp. kurstaki 97-27 was achieved [5]. Compared to genome studies, proteome investigation can provide an insight into factors such as protein presence, abundance, and posttranslational modification. With such information, we can gain an understanding of its physiology, pathogenesis, and mechanism of avoiding host immune systems. Two-dimensional gel electrophoresis (2-DE) combining with mass spectrometry (MS) has been used to study many bacteria, such as E. coli [6–8], Bacillus anthracis [9, 10], and Bacillus cereus [11, 12].

There have been several studies on Bacillus thuringiensis at the protein level [13, 14]. However, most of these efforts have been centered on the identification of ICPs [15] and bacteriocin [16, 17] using SDS-PAGE, Western blot, and MS. Meanwhile, 2-DE was used to identify Bt crystal proteins [18].

To date, characterization of the process of spore and crystal formation of Bacillus thuringiensis has not been reported. The aim of our study was to identify the proteins expressed during the growth of Bt cells and to find more bioactive components including insecticidal crystal proteins (ICPs) in the Bt4.0718 strain. In this study, several kinds of bioactive factors such as Cry1Ac(3), Cry2Aa, InhA, InhA precursor, and bacillolysin were identified in Bt4.0718 strain. The expression differences of the bioactive factors between the three phases and their effect on virulence of bacteria cell were analyzed. Threonine synthase, CodY protein, ORF1, CotJC, OppA, and SpoIVA which may take part in or regulate the expression of crystal proteins or spore formation were identified, some of these proteins have not been reported by previous studies on Bt, and their sequences and functions in Bacillus thuringiensis need to be studied in depth. This research provides an important foundation for the steady...
expression of crystal proteins in Bt and the construction of recombinant Bt strains with high toxicity.

2. Materials and Methods

2.1. Culture and Observation of Bt Cells. Bacillus thuringiensis 4.0718 strain was used in this study. It produces two major kinds of parasporal crystals, Cry1 (130 kDa) and Cry2 (65 kDa) [19]. Cells were grown in fermentation medium at 30°C with rotary shaking at 200 rpm. Bt cells were collected every two hours for 28 hours after cells had been transferred into the fermentation medium. The cell growth density was determined at OD600 nm by using blank culture medium as a control. The changes in the Bt cells during their life cycle were monitored by observation of samples under contrast-phase microscopy.

2.2. Preparation of Protein Samples. Bt Cells were harvested at different growth phases (middle vegetative phases (T1), early sporulation phase (T2), and late sporulation phase (T3)) and centrifuged for 9 min at 9,600 g at 4°C. The pellets were washed four times for 7 min at 9,600 g with low-salt washing buffer. The pellets were vacuum-dried and stored at −20°C. Dried pellets weighing 0.036 g were resuspended in 1 mL Milli-Q water containing 1 mM PMSF and ruptured by sonication for 10 min at 0°C. After adding 15 U RNase A and 15 μL 10 mM RNase A to 540 μL of the lysed cell suspension, which was then kept in ice-cold water for 1 h, solid thiourea, CHAPS, urea, Tris and DTT were added to final concentration of 2 M, 4%, 8 M, 40 mM, and 60 mM, respectively. The solution was kept at 4°C for 30 min and then at room temperature for 30 min to solubilize proteins efficiently. It was then centrifuged for 15 min at 16,000 g to precipitate the insoluble components. The supernatant was collected, and its protein concentration was determined by the Bradford method [20] and the 2D Quant Kit (GE Biosciences) [21] and then stored in aliquots at −70°C till used for 2-DE.

2.3. 2-DE. 2-DE was performed according to the methodology described previously [22]. Isoelectric focusing was performed in Immobiline IPG strips (18 cm) on an IPGphor system (GE, formerly Amersham Pharmacia Biotech). Protein samples of 1 mg were mixed with a rehydration solution containing 8 M urea, 2 M Thiourea, 4% CHAPS, 40 mM Tris, IPG buffer (0.5% V/V, pH 4–7), 18 mM DTT, and traces of bromophenol blue, to a total volume of 350 μL. The mixture was pipetted into the strip holder. Isoelectric focusing (IEF) was carried out at 20°C on the IPGphor unit under the following steps: (1) hydration for 12 h, (2) 200 V for 1 h, (3) 500 V for 1 h, (4) 1000 V for 1 h, (5) Grad 8000 V for 0.5 h, and (6) 8000 V for 7 h. After focusing, the strips were soaked for 15 min in reduction solution (2% SDS, 6 M urea, 30% glycerol, and 125 mM DTT) followed by 15 min in alkylation solution (2% SDS, 6 M urea, 30% glycerol, and 125 mM iodoacetamide). The SDS-PAGE step was performed in 10% polyacrylamide gel run on a Protean II system (Bio-Rad, Hercules, CA, USA) at 10°C. After 2-DE, gels were stained with a Coomassie Brilliant Blue (CBB) solution. Spot detection, quantification, and matchset were performed using Melanie 6.0 software.

2.4. In-Gel Protein Digestion. The CBB-stained protein spots were cut out from the gels and washed twice with 50 μL water and then destained three times with 50 μL 25 mM ammonium bicarbonate and 50% ACN for 30 min at 37°C. The destained gel pieces were washed with 25 mM ammonium bicarbonate, 50% ACN, and 100% ACN in turn. These dehydrated pieces were lyophilized in a vacuum centrifuge. Vacuum-dried Gel pieces were reswolled with 5 μg 25 mM ammonium bicarbonate containing trypsin at low temperature for 45 min followed by added buffer. After incubation at 37°C for 16 hours, the supernatant was transferred into another tube and 50 μL 67% acetonitrile (ACN) was added to extract peptides in the gel pieces. After 3 h incubation at 37°C, extracted supernatant was combined with the first one. The combined supernatant was lyophilized for MS.

2.5. MALDI-TOF/TOF MS and Protein Identification. The digested peptides were solubilized in deionized water containing 0.1% TFA. A 1 μL sample was mixed with 0.5 μL matrix solution (saturated solution of CCA in 50% ACN and 0.1% TFA). The mixture was applied to a target well and introduced into the mass spectrometer after air-drying. Bovine serum albumin products were used for external calibration.

For proteomic identification, MS/MS spectra were searched by MASCOT (Matrix Science, http://www.matrix-science.com/) using the no-redundant NCBI Gram-positive bacterial sequence database. Mass tolerance was set at 50 ppm for the mass of peptide precursors and at 0.6 Da for the mass of fragment ions. Carbamidomethylation of cysteines and methionine oxidation were set as the fixed and variable modifications, respectively.

3. Results

3.1. Morphology of Bt Cells, Spores, and Crystals at Different Growth Phases. The growth curve of Bt4.0718 according to the optical density determinations at OD600 nm is presented in Figure 1(a). The optical density increased exponentially from 2 to 12 h (T1), indicating that bacteria during this period are in the logarithmic phase. From 12 to 26 h (T2), the optical density changes little, showing that bacteria during this period are in the stationary phase. After 26 h (T3), the optical density value declined sharply, which means that bacteria reached the death phase.

These observations (Figure 1(b)) showed that, at T1 phase, no spore or crystal could be observed. At T2 phase, spores of atactic shape existed in Bt cells while the crystals were absent in the majority of cells. At T3 phase, cell walls became thin; the mature spores and crystals with orderly shape were present in the cells, making up most of the cell volume.
detected on the 2-DE gels of the three phases of T1, T2, and T3, respectively. All the protein spots with different expression were excised but only 56 of them presenting 49 proteins were identified using MALDI-TOF/TOF MS and Gram-positive bacteria database search. Meanwhile, an important protein without significant regulation between the three different phases was identified. There are same proteins (Table 1) identified in the three phases. According to the functions of these identified proteins (Table 2), all the protein spots were categorized into five major groups: bioactive factors, metabolism, crystal and spore formation, amino acid and protein synthesis, and other functions.

4. Discussion

4.1. Physiology of Bt4.0718. One goal of proteomic studies consists in providing a global comprehensive view of cellular physiology and cellular adaptation reactions [23]. In the proteomic studies of Bacillus, the proteins of cells at mid-exponential phase had been separated and identified to resolve the rapid growth of the entire spectrum of proteins [9, 24]. Also, proteins extracted from cells growing in cultures with high salt concentration [25] or facing other high-pressure environments [26] were compared with proteins from cells in normal cultures using the proteomic approach, to find proteins closely related to resistance to environmental stress. The function of these proteins was analyzed to understand the internal regulation reaction. In our study, proteins extracted from cells at different growth phases were separated and identified. The proteins identified from cells at T1 were mainly enzymes involved in metabolism, similar to the protein profiling of Bacillus subtilis [24]. To study the proteome of whole cells in Bt was to study the changes of protein expressions of Bt cells during their lives. And the formation and maturation of spores and crystals are the direct results of protein expression changes. Both of them are important parameters according to appropriate phases we chose as research targets.

The regulation reactions were also studied by analyzing proteins with different expression during the lifetime of Bt4.0718. The results showed that, at T1, the enzymes involved in metabolism sustained rapid cell growth, and crystal and spore formation did not commence. At T2, some proteins participating in the citric acid cycle enzymes were downregulated; spores were formed while ICPs started to express in the phase, which were regulated by the expression of threonine synthase upregulated significantly, and CodY inhibited the expression of proteins at the early stability phase, providing a large number of raw materials for ICPs. Therefore, although at T2 the nutrition for growth of Bt cells has already begun to be restricted, ICPs can have a fast and efficient expression. As the components of nutrients in the medium changed, organic tolerance proteins started to express, helping cells to adapt to the environment, and nutrition is also the important prerequisite of spore formation. At T3, the mature spores and crystals were formed, providing a lot of pressure on space in the cells. The study found that PspA protein was induced to express, which played an important role in the survival of adversity [27], and organic tolerance protein has a certain expression in Bt cells. The expression could help Bt cells adapt to the
Figure 2: (a) Representation of 2-DE gel separation of the total proteins of whole cell of *Bacillus thuringiensis* according to predicted pI and molecular masses. (b) 2-DE maps of the proteins of whole cells at T1, T2, and T3 from Bt4.0718 strain. The protein spots marked with arrows were identified by MALDI-TOF/TOF MS.
Table 1: The same proteins identified in the three phases.

| Accession no. | Protein description |
|---------------|---------------------|
| gi | 30020418 | 2-methylcitrate dehydratase (Bacillus cereus ATCC 14579) |
| gi | 30018379 | 3OS ribosomal protein S10 (Bacillus cereus ATCC 14579) |
| gi | 30018375 | 3OS ribosomal protein S12 (Bacillus cereus ATCC 14579) |
| gi | 30018386 | 3OS ribosomal protein S3 (Bacillus cereus ATCC 14579) |
| gi | 30022730 | 3OS ribosomal protein S4 (Bacillus cereus ATCC 14579) |
| gi | 30018397 | 3OS ribosomal protein S5 (Bacillus cereus ATCC 14579) |
| gi | 30023498 | 3OS ribosomal protein S6 (Bacillus cereus ATCC 14579) |
| gi | 30018412 | 50S ribosomal protein L13 (Bacillus cereus ATCC 14579) |
| gi | 30018407 | 50S ribosomal protein L17 (Bacillus cereus ATCC 14579) |
| gi | 30018383 | 50S ribosomal protein L2 (Bacillus cereus ATCC 14579) |
| gi | 30022519 | 50S ribosomal protein L21 (Bacillus cereus ATCC 14579) |
| gi | 30018385 | 50S ribosomal protein L22 (Bacillus cereus ATCC 14579) |
| gi | 30260309 | 5OS ribosomal protein L29 (Bacillus anthracis str. Ames) |
| gi | 30018392 | 5OS ribosomal protein L5 (Bacillus cereus ATCC 14579) |
| gi | 30021657 | Aldehyde dehydrogenase (Bacillus cereus ATCC 14579) |
| gi | 30021917 | ATP-dependent protease ATP-binding subunit (Bacillus cereus ATCC 14579) |
| gi | 167936927 | Cell envelope-bound metalloprotease (camelysin) (Bacillus cereus AH1134) |
| gi | 161511213 | co-chaperonin GroES (Bacillus cereus ATCC 14579) |
| gi | 30022058 | dihydrolipoamide dehydrogenase (Bacillus cereus ATCC 14579) |
| gi | 30022244 | dihydrolipoamide dehydrogenase (Bacillus cereus ATCC 14579) |
| gi | 30260298 | elongation factor G (Bacillus anthracis str. Ames) |
| gi | 30018377 | elongation factor G (Bacillus cereus ATCC 14579) |
| gi | 30018378 | elongation factor Tu (Bacillus cereus ATCC 14579) |
| gi | 30023337 | F0F1 ATP synthase subunit beta (Bacillus cereus ATCC 14579) |
| gi | 67866498 | InhA (Bacillus thuringiensis serovar kurstaki) |
| gi | 30022668 | isocitrate dehydrogenase (Bacillus cereus ATCC 14579) |
| gi | 30022246 | Leucine dehydrogenase (Bacillus cereus ATCC 14579) |
| gi | 30019663 | nucleoside diphosphate kinase (Bacillus cereus ATCC 14579) |
| gi | 30021685 | Oligopeptide-binding protein oppA (Bacillus cereus ATCC 14579) |
| gi | 30019304 | ornithine-oxo-acid transaminase (Bacillus cereus ATCC 14579) |
| gi | 30022061 | Pyruvate dehydrogenase E1 component alpha subunit (Bacillus cereus ATCC 14579) |
| gi | 168144706 | response regulator aspartate phosphatase (Bacillus cereus G9842) |
| gi | 157690926 | ribosomal protein S11 (Bacillus pumilus SAFR-032) |
| gi | 30018384 | SSU ribosomal protein S19P (Bacillus cereus ATCC 14579) |
| gi | 42779595 | tellurium resistance protein (Bacillus cereus ATCC 10987) |
| gi | 30022339 | Transcriptional regulator, ArsR family (Bacillus cereus ATCC 14579) |
| gi | 30022700 | Universal stress protein family (Bacillus cereus ATCC 14579) |

environment. This is the first preliminary study of the Bt cell physiology by use of proteomic technologies, and the intracellular physiological changes during the lifetime of Bt were analyzed, laying an important foundation for the in-depth study of Bt physiology.

4.2. Bioactive Factors and Bt Virulence. Besides the analysis of Bt cell physiological change during its lifetime, many bioactive factors such as insecticidal crystal proteins (ICPs), immune inhibitor A (InhA), and InhA precursor separated by 2-DE in the cells of Bt4.0718 were analyzed, which will help to understand the relationship between their expression and Bt virulence.

In Bt4.0718, ICPs included Cry1Ac(3), BTRX28, and Cry2Aa which were separated and identified by SDS-PAGE (figure not shown). The clustalX analyses showed that BTRX28 shared 98% homology with Cry1Ac. At T2, ICPs started to express, and the protein concentration was the highest or maximal. Therefore, ICPs were the most important part of Bt virulence at the sporulation phase. The proteins classified into Cry1 can specifically kill insects of Coleoptera, while Cry2 can specifically kill insects of
### Table 2: Proteins identified by MALDI-TOF/TOF MS.

| Spot no. | Protein description                                      | Accession no. | Score | Sequence coverage (%) | Peptide                        | Ion score |
|----------|----------------------------------------------------------|---------------|-------|-----------------------|--------------------------------|-----------|
| A. Bioactive factors |                                                                 |               |       |                       |                                |           |
| 1        | Cry1A(c)3 (Bacillus cereus G9241) proteinase VCA0223     | gi | 142742 | 342  25    | IFTAFSLYDAR                    | 94        |
| 3        | immune inhibitor A (Bacillus thuringiensis)             | gi | 47568584 | 166 21   | R.NYAGSDTALQYAR.G              | 54        |
| 6        | Immune inhibitor A precursor camelysin (Bacillus thuringiensis serovar konkukian str. 97-27) | gi | 9858110 | 263  19  | K.FEVGQADDNSAGAVR.L              | 85        |
| 7        | Immune inhibitor A precursor camelysin (Bacillus thuringiensis serovar konkukian str. 97-27) | gi | 124464 | 70   4     | K.TYINQQIPDAGR.I                 | 58        |
| 14       | Bacillolysin (Bacillus cereus ATCC 14579)                | gi | 30023075 | 208  24  | K.AAYLVSEGGDHYGVK.V              | 79        |
| 40       | Cell envelope-bound metalloprotease (camelysin) (Bacillus cereus ATCC 14579) | gi | 30019432 | 102  30  | K.DIFAPEWGEK.G                   | 62        |
| 41       | insecticidal crystal protein BTRX28 (Bacillus thuringiensis serovar kunthalaRX28) | gi | 13173240 | 104  15  | K.NGIVLFHDEVR.S                  | 72        |
| 46       | Camelysin (Bacillus cereus)                             | gi | 24474855 | 130  19  | K.EFLQNQSLTIK.D                 | 81        |
| 54, 53   | Camelysin (Bacillus cereus)                             | gi | 47678765 | 131  22  | R.GNSNYFPDYFIR.N                 | 19        |
| *        | insecticidal crystal protein Cry2Aa (Bacillus thuringiensis serovar kurstaki) | gi | 47678765 | 131  22  | R.GNSNYFPDYFIR.N                 | 19        |
| B. Metabolism |                                                                 |               |       |                       |                                |           |
| 2        | succinate dehydrogenase flavoprotein subunit (Bacillus thuringiensis str. Al Hakam) | gi | 11847969 | 245  32  |                                |           |
| 5, 20    | transketolase (Bacillus thuringiensis serovar konkukian str. 97-27) | gi | 49478325 | 215  22  | R.LVVLHDSNDLSDLGDLNR.S            | 93        |
| 17       | methylmalonic acid semialdehyde dehydrogenase (Bacillus anthracis str. Ames) | gi | 30262359 | 146  27  | R.TVQGVIQSAFSGER.C                | 59        |
| 18       | adenylsuccinate synthetase (Bacillus cereus G9241)      | gi | 47568666 | 88   11    | R.VGDGPFFPTLHDIEQHZRE           | 27        |
| 19       | UDP-N-acetyl-D-mannosamine 6-dehydrogenase (Bacillus thuringiensis serovar israelensis ATCC 35646) | gi | 75760242 | 128  8    | K.VGEDFYIGYSER.I                | 54        |
| 21, 22   | isocitrate dehydrogenase (Bacillus cereus ATCC 14579)   | gi | 30022668 | 309  27  | K.DSIADIFQQLTRPR.E               | 80        |
| 36       | thiamin biosynthesis ThiG (Bacillus cereus G9241)       | gi | 47564664 | 226  27  | K.AIDVSEAELTFAVR.R               | 88        |
| 42       | fructose-bisphosphate aldolase (Bacillus cereus G9241) | gi | 47568789 | 298  25  | K.LDSIEDALNTYNALQEGAVGDGR.N     | 161       |
| 52       | 3-ketoacyl-(acyl-carrier-protein) reductase (Bacillus cereus ATCC 14579) | gi | 30020097 | 86   19    | K.FGVLGLTESLAMEVR.K             | 55        |
| 34       | malate dehydrogenase (Bacillus anthracis str. Ames)     | gi | 30264663 | 145  12  | R.YSYAGGIPLETLPK.E               | 72        |
| C. Crystal and spores formation |                                                                 |               |       |                       |                                |           |
| 12       | oligopeptide ABC transporter, substrate-binding protein (Bacillus thuringiensis serovar konkukian str. 97-27) | gi | 49481243 | 101  13  | K.GIANHTFFGDFSYK.W               | 50        |
| 13       | Oligopeptide-binding protein oppA (Bacillus cereus ATCC 14579) | gi | 30021687 | 195  30  | K.TLLEDVAVPILYQR.G               | 70        |
| 24, 33   | stage IV sporulation protein A (Bacillus cereus G9241) | gi | 47565971 | 226  21  | K.IEYDQVADALRM                  | 77        |
| Spot no. | Protein description | Accession no. | Score | Sequence coverage (%) | Peptide | Ion score |
|---------|---------------------|--------------|-------|-----------------------|---------|-----------|
| 30      | Threonine synthase  | gi | 135811 | 307 | 40 | K.GHVIEEPETIATAIR.I | 94 |
| 43, 47  | CotJC protein (Bacillus thuringiensis serovar israelensis ATCC 35646) | gi | 75760539 | 373 | 52 | K.LLIEQYGGADGELAAALR.Y | 92 |
| 30, 47, 56, 57 | Uncharacterized 20 kDa protein in cryB1 5′ region (ORF1) | gi | 140467 | 143 | 26 | K.YSVQQLPHYVIDGDIHQV.R | 82 |

(D) Amino acid and protein synthesis

| Spot no. | Protein description | Accession no. | Score | Sequence coverage (%) | Peptide | Ion score |
|---------|---------------------|--------------|-------|-----------------------|---------|-----------|
| 4       | 5-methyltetrahydropteroylglutamate-homocysteine methyltransferase (Bacillus cereus ATCC 14579) | gi | 30022091 | 298 | 29 | R.YQEEIGLDVLVHGEFER.T | 102 |
| 10      | bifunctional GMP synthase/glutamine amidotransferase protein (Bacillus anthracis str. Ames) | gi | 30260444 | 93 | 26 | K.AREDHFVGNLYFN.R | 33 |
| 11      | 1-pyrrline-5-carboxylate dehydrogenase (Bacillus anthracis str. Ames) | gi | 30260481 | 99 | 14 | K.YSVQQLPHYVIDGDIHQV.R | 81 |
| 26      | elongation factor Ts (Bacillus cereus ATCC 14579) | gi | 30021914 | 184 | 52 | K.TDADAFGAYLMGGRQ.I + Oxidation (M) | 22 |
| 27      | translation elongation factor Ts (Bacillus cereus G9241) | gi | 47569104 | 78 | 5 | K.TDADAFGAYLMGGRQ.I | 73 |
| 35      | Cysteine synthase (Bacillus cereus ATCC 14579) | gi | 30018339 | 92 | 21 | K.EHGFIPQFKN.E | 38 |
| 37      | branched-chain amino acid aminotransferase (Bacillus anthracis str. Ames) | gi | 30261496 | 59 | 14 | K.YSVQQLPHYVIDGDIHQV.R | 81 |
| 45      | transcriptional repressor CodY (Bacillus cereus ATCC 14579) | gi | 30021916 | 216 | 40 | R.EFQGQLTIVIPVGUI.G | 76 |
| 51      | 30S ribosomal protein S4 (Bacillus cereus ATCC 14579) | gi | 30022730 | 167 | 39 | K.YSVQQLPHYVIDGDIHQV.R | 44 |

(E) Other functions

| Spot no. | Protein description | Accession no. | Score | Sequence coverage (%) | Peptide | Ion score |
|---------|---------------------|--------------|-------|-----------------------|---------|-----------|
| 8, 9    | 5′-nucleotidase (Bacillus thuringiensis serovar israelensis ATCC 35646) | gi | 75761007 | 395 | 47 | K.GANFPYVAANFYNK.S | 85 |
| 15      | ATP synthase subunit B (Bacillus cereus ATCC 14579) | gi | 30023337 | 257 | 64 | R.ALSPEIVGEEHYEVAR.Q | 75 |
| 23      | electron transfer flavoprotein, beta subunit (beta-ETF) (Bacillus thuringiensis serovar konkukian) | gi | 49481481 | 527 | 66 | R.DAQGGEVTVTVGDESEKLR.T | 148 |
| 25      | NAD(+) synthetase (Bacillus anthracis str. Ames) | gi | 30262026 | 254 | 38 | K.GFVLGSGGQDDLTRQ.R | 104 |
| 28      | aminomethyltransferase (Bacillus cereus ATCC 14579) | gi | 30022309 | 326 | 41 | K.ZAAYDETEIEIR.Q | 76 |
| 29      | acyl-CoA dehydrogenase (Bacillus cereus ATCC 10987) | gi | 42781609 | 213 | 51 | K.TAEFPYETFQK.M | 51 |
| 31      | ketol-acid reductoisomerase (Bacillus anthracis str. Ames) | gi | 30261892 | 413 | 58 | R.HSISDTAEGDYTVGSR.I | 86 |
| 32      | 3-ketoacyl-CoA thiolase (Bacillus thuringiensis serovar israelensis ATCC 35646) | gi | 75760332 | 156 | 17 | R.YCSSGLQSIAGI.GER.I | 64 |
| 38      | organic solvent tolerance protein (Shewanella oneidensis MR-1) | gi | 24375135 | 94 | 56 | K.INSEEWGEI.NAK.L | 56 |
Diptera. The kinds of ICPs in Bt4.0718 strain determined its insecticidal spectrum, consistent to the results of bioactivity tests [19]. InhA is a kind of secret neutral metalloproteinase containing zinc metal [28], which can destroy antibacterial peptides secreted by insect hosts, such as cecropins and attacins from the immune hemolymph of Hyalophora cecropia [29]. Consequently, it can impress the humoral immune system of insect hosts. Because of this characteristic, InhA can protect Bt cells by prohibiting the lysis action of antibacterial proteins to Bt cells [30]. In our study, at T1, InhA and InhA precursor protein started to express when the ICPs did not express, at T2, the expression amount of InhA and InhA precursor protein was the greatest, but at T3, they could not be identified from 2-DE gel. The investigation suggested that, after invading the insect host, Bt expressed InhA that inhibited the cell lysis action of antibacterial peptides produced by the host, in favor of the subsistence of Bt in the host body. It laid a basis for the formation of crystals and spores and the exertion of Bt virulence at the sporulation phase. Camelysin and bacillolysin are also important bioactive factors. Camelysin first discovered in Bacillus cereus (Bs) was about the harm of Bacillus cereus to humans [31]. In Bacillus thuringiensis subsp. israelensis, the sequence of a putative protease shared high homology with camelysin of the closely related Bs species. Recently it is reported [32] that the camelysin has a positive effect on regulating the expression of InhA. The protease can make cleavage between amino acid residues 34 and 35 of Cyt2Ba, to obtain rather pure and active toxin species quickly and simply [33]. In this study, three camelysins were identified in Bt4.0718; the amount of one camelysin remains unchanged while the other two only synthesized at T3. It demonstrated to some degree that camelysin in Bt is not only related to spore formation but also to the virulence activity of crystals. Bacillolysin can catalyze the hydrolysis of peptide bonds of amino ground terminals of leucine or phenylalanine. Some studies have shown that thermolysin-like metalloproteinases such as aurelysin, pseudelysin, and bacillolysin presented virulence factors of diverse bacterial pathogens. These particular microbial metalloproteinases mediate sensing of invading microbes and elicit innate immune responses in insects. They played a predominant role as virulence factors and promoted the development of bacteria or fungi within the infected hosts [34]. The functions of bacillolysin in Bt should be studied in depth. It was found that at any phase, the expression of ICPs was accompanied with other bioactive factors’ expression, though Bt virulence was sustained mainly by ICPs. It was demonstrated that these bioactive factors with weak biological activity played an important role in Bt virulence.

The diversity of bioactive factors of Bt4.0718 provided a scientific basis and practical ideas to study its insecticide mechanisms to broaden its insecticide spectrum and to enhance its killing effect.

4.3. Regulation Reaction in the Formation of Crystals and Spores. The important role of regulator was usually studied with mutation methods. For example, the important role of SpoIVF involved in the crystal formation process of Bt was researched by the observation of crystal and spore formation in a SpoIVF deficient mutant [35]. Also, proteomic technologies were used to detect the protein expression differences between mutant and wild strains [14]. In our study, parts of regulators associated with the formation of crystals and spores, such as OppA, CotJC protein, SpoIV, and ORF1, were separated and identified by proteomic technologies. The expression and function of the regulators were analyzed to gain a relatively clear understanding about their roles in the formation of crystals and spores during Bt’s lifetime. As crystals and spores began to form at T2, certain proteins associated with crystal and spore formation were identified at T2 first. CotJC protein and OppA were identified at T2. In Bacillus subtilis (Bs), CotJC protein manipulation is necessary for the normal formation of spore coat, and some of them are even the composition of spores [36, 37]. In our study, CotJC protein in Bt was only expressed in the

| Spot no. | Protein description | Accession no. | Score | Sequence coverage (%) | Peptide | Ion score |
|----------|---------------------|---------------|-------|-----------------------|---------|-----------|
| 39       | inosine hydrolase (Bacillus thuringiensis serovar kurstaki) | gi | 156081515 | 217 23 | K.LIEPCPIDIIIIVATGR.L | 80       |
| 44       | purine nucleoside phosphorylase (Bacillus cereus ATCC 14579) | gi | 30019611 | 144 30 | K.YIAETFLEDVTCYNNVR.G | 80       |
| 48       | hypothetical protein BC1708 (Bacillus cereus ATCC 14579) | gi | 30019852 | 225 45 | K.NGIVLFHDIEVR.S | 65       |
| 49       | Phage shock protein A (Bacillus thuringiensis serovar israelensis ATCC 35646) | gi | 75758863 | 286 57 | K.ILFEEQEAIVK.K | 42       |
| 50       | hypothetical protein BC2244 (Bacillus cereus ATCC 14579) | gi | 30020376 | 116 25 | K.YGQEVTTQEQLAR.Y | 88       |
| 55       | Alkyl hydroperoxide reductase C22 (Bacillus cereus ATCC 14579) | gi | 30018585 | 123 37 | R.TITTNFNVLMEEEGLAAR.G | 70       |

*The protein was separated by SDS-PAGE.*
process of spore formation, which noted that the role of CotJC protein in Bt should be similar to that in Bs, but it needed further confirmation. Oligopeptide transport system (Opp system) in Bs is important at the early sporulation phase [38]. It was found that in Bacillus thuringiensis with opp mutation, the rate of sprouting was reduced, and the phosphorylation of Spo0A can be controlled by Opp protein in both Bt and Bs [39]. The oligopeptide ABC transporter protein and oppA identified in this study both belong to the oligopeptide transporter system. The two proteins were specifically expressed in the sporulation phase, so that they may play an important role in the formation of spores in Bt4.0718. At T3, uncharacterized 20 kDa protein in CryB1 5′ region (ORF1) and phase IV sporulation protein A (SpoIVA) started to express specifically. The gene of ORF1 (orf1) is one of open reading frames at upstream of cry2A operon; orf1 is 33% homology with the p19 gene of cry11Aa operon, but the expression of orf1 could not help the expression of Cry2Aa. However, Ge proved that the participation of ORF2, the gene of which (orf2) is also another open reading frame next to orf1 at upstream of cry2A operon, cannot only enhance the expression of Cry2Aa but also help the formation of crystals [40]. ORF2 duplication unit for Cry2Aa may provide attachment (matrix) or scaffold (scaffold) to the formation of crystals and can also help misfolded protein crystals refold into the right structure to immunize it to the risk of degradation, so as to enhance the protein crystal structure and stability. So it demonstrated that ORF2 ought to specifically express at T3 as ORF1. The results show that ICPs had started to express when spores began their formation, but the contrast-phase microscopic observation showed that when it was hard to observe the existence of crystals, the structure of spores had been clear at T2, but at T3, the structures of crystals and spores were both clearly observed. Therefore, it was suggested that the specific expression of ORF1 and ORF2 at T3 played an important role in the crystal formation. SpoIVA in Bacillus subtilis (Bs) is a conservative cytoplasmic protein which has a specialized role in the morphogenesis of spore cortex and the attachment of spore coat to the spores surface [41]. SpoIVA is expressed soon after the formation of the asymmetric septum during sporulation and acts in the mother cell compartment. Up till now, the function of SpoIVA in Bt had not been described in detail, but according to the relationship between Bt and Bs, the specific expression of SpoIVA in Bt suggested its importance during spore formation.

Through comparative analysis of the expression differences of proteins in whole Bt cells at different growth phases, the impact on the regulation and control to the strains in the course of the formation of crystals and spores could be understood. It lays an important foundation for further exploration of the stable expression of ICPs, the smooth formation of crystal, and the application in the production in Bt recombinant strains.

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