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Comparative proteomic analysis of *Bacillus thuringiensis* wild-type and two mutant strains disturbed in polyphosphate homeostasis

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Abstract: Polyphosphate polymer (polyP) plays a very important role in every living cell. Synthesis of this linear polymer of phosphate (P_i) residues is catalyzed by the polyphosphate kinase (PPK) enzyme. It was shown that high levels of intracellular polyphosphate stimulated endotoxin production by *Bacillus thuringiensis* subsp. *israelensis* (*Bti*). In this study, proteomic analysis of the wild-type and two mutant strains, overexpressing the *ppk* gene (*Bti* pHT*ppk*) and without the *ppk* gene (*Bti* Δ*ppk*), were used to clarify the relation between polyP and endotoxin production. Intracellular proteins were separated by two-dimensional gel electrophoresis; 41 spots of interest (proteins differentially expressed) were obtained and 35 of them were identified by mass spectrometry. Analysis of the protein profiles showed that there is a general decrease in the expression levels of proteins related with energy metabolism, amino acid metabolism, and purine biosynthesis in both *Bti* pHT*ppk* and *Bti Δppk*. Gluconeogenesis and fatty acid metabolism were also slowed down in both strains, whereas expression of stress response proteins increased compared to the wild-type. These results suggested that changes in polyP concentration cause a general stress condition inside the cell, which in turn stimulates secondary metabolite synthesis.

Key words: Polyphosphate polymer, polyphosphate kinase, two-dimensional gel electrophoresis, endotoxin, bioinsecticide, *Bacillus thuringiensis israelensis*, secondary metabolite, regulation, proteomics

1. Introduction

Polyphosphate (polyP), which is a linear polymer of phosphate (P_i) residues linked by high-energy phosphoanhydride bonds, is present in all organisms from bacteria to humans (Rao et al., 2009). PolyP has been shown to have important roles in a variety of cellular processes including regulation of enzyme activities, storage of P_i and energy, adaptive responses to physical and chemical stresses, bacterial survival during the stationary phase, chelation of cations, maintenance of optimal translation efficiency, gene transcription control, and formation and function of cell membrane, channels, and pumps (Kornberg et al., 1999). PolyP has also been shown to be necessary for motility, biofilm formation, and other virulence properties of different bacteria such as *Salmonella* spp. (Kim et al., 2002), *Shigella flexneri* (Kim et al., 2002), *Pseudomonas aeruginosa* (Rashid et al., 2000), and *Mycobacterium tuberculosis* (Singh et al., 2013). The effect of polyP on the biosynthesis of secondary metabolites by *Streptomyces* (Chouayekh and Virolle, 2002; Yalım Camcı et al., 2012) and *Bacillus thuringiensis israelensis* (*Bti*) (Doruk et al., 2013) has also been demonstrated. There are a few studies about the function of polyP in eukaryotes: it has a role in blood coagulation (Smith et al., 2006), inflammation (Muller et al., 2009), innate immunity, and cancer metastasis (Wang et al., 2003; Tammenkoski et al., 2008).

The main polyP synthetic enzyme in many bacteria is polyP kinase 1 (PPK1), which catalyzes the reversible transfer of P_i from ATP to polyP and from polyP to ADP (Brown and Kornberg, 2008).

Although polyP has been shown to have important functions in different organisms, to our knowledge, there have been only two studies on the role of this polymer in *Bacillus*. Shi et al. (2004) showed that polyP is important for motility, biofilm formation, and sporulation in *Bacillus cereus*. In another study, the *Bti* strain, which overexpress the *ppk* gene, was found to be about 7.7 times more toxic against late 2nd instar *Culex quinquefasciatus* than the wild-type (Doruk et al., 2013). To clarify the relation between polyP metabolism and endotoxin production, the proteomes of the wild-type and two mutant strains, one overexpressing the *ppk* gene (*Bti* pHT*ppk*) (Doruk et al., 2013) and the other without the *ppk* gene (*Bti* Δ*ppk*) (Doruk and Gedik, 2013), were analyzed in this study.

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2. Materials and methods

2.1. Media and growth conditions

Bti strains were grown in Difco sporulation medium (DSM) (4 g/L nutrient broth, 25 mM K_2HPO_4, 25 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 µM $FeSO_4$, 10 µM $MnCl_2$, 5 g/L glucose) (Donovan et al., 1988) for endotoxin production and were grown in both DSM and Luria broth for proteomic analysis at 30 °C. Experiments were started from overnight cultures, which were diluted to equalize inoculum size by using a spectrophotometer (OD_{600}). Where appropriate, 25 µg/mL erythromycin was added to growth media. Liquid cultures were aerated on a rotary shaker at 220 rpm.

2.2. Strains and plasmids

Bti ATCC 35646 and pHT315 were kindly provided by Gwo-Chyuan Shaw (National Yang-Ming University, Taiwan). The full list of strains and plasmids is given in Table 1.

2.3. Protein isolation

Protein extraction for toxin isolation was performed by the procedure of Donovan et al. (1988). Proteins of 10 mL of cells (spore and toxins) from cultures grown for 72 h were extracted and equal volumes of protein solution from each sample were serially diluted (from 50 µg/mL to 1.56 µg/mL) and used in the bioassay experiments.

For the total protein isolation for two-dimensional electrophoresis (2-DE), cultures grown for 10 h (in both DSM and LB media) were harvested and washed with a previously chilled TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA). The pellets were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, cOmplete protease inhibitor cocktail (Roche, Switzerland)) and ruptured by sonication for 10 min at 0 °C. After adding 1 mg/mL DNase and 0.25 mg/mL RNase, the lysed cell suspension was centrifuged at 13,800 rcf for 10 min to precipitate the insoluble components. The supernatant was collected, its protein concentration was determined using the Bradford method (Bradford 1976), and it was then stored at –80 °C until used for 2-DE.

2.4. Mosquito larvicidal activity

The method for larvicidal activity was adapted from Promdonkoy et al. (2005). Basically, 10 late 2nd instar *Culex quinquefasciatus* larvae (supplied by Öner Koçak, Hacettepe University, Turkey) were exposed to serially diluted toxins in each well of 24-well plates (well diameter: 1.5 cm) containing 1 mL of sterile tap water. LC_{50} values were determined by using probit analysis (Finney and Stevens, 1948) at the end of 24 h by taking the average results of three independent experiments.

2.5. Two-dimensional electrophoresis

Protein samples of 400 µg were mixed with a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, immobilized pH gradient (IPG) buffer (2% v/v, pH 3–10), and 65 mM dithiothreitol (DTT) to a total volume of 400 µL. The mixture was loaded on IPG strips (17 cm, pH 3–10 nonlinear gradient, Bio-Rad, USA) and rehydrated without current for 2 h by passive rehydration and with current of 50 V for 16 h by active rehydration. Isoelectric focusing was carried out at 20 °C on the IPGphor unit under the following steps: 1) 200 V for 300 Vh, 2) 500 V for 500 Vh, 3) 1000 V for 1000 Vh, 4) 4000 V for 4000 Vh, 5) 8000 V for 24,000 Vh, and 6) 8000 V for 30,000 Vh. After focusing, the strips were subsequently equilibrated for 15 min in reduction solution (2% SDS, 6 M urea, 0.375 M Tris (pH 8.8), 20% glycerol, and 2% DTT) followed by 15 min in alkylation solution (2% SDS, 6 M urea, 0.375 M Tris (pH 8.8), 20% glycerol, and 125 mM iodoacetamide). After isoelectric focusing, the second dimension was performed in 12% polyacrylamide gels. After 2-DE (Bio-Rad), gels were stained with colloidal Coomassie brilliant blue (CBB) solution (Candiano et al., 2004) and the gel image was transferred to a computer using a digital imaging system (VersaDoc MP 4000, Bio-Rad).

2.6. Image analysis of 2-DE gels

PDQuest 8.0.1 2-DE gel analysis software (Bio-Rad) was used for spot quantification. Spot detection parameters were optimized in order to minimize false positive detection and maximize real spot detection. Analysis was performed to identify spots with qualitative (presence/absence) and quantitative ≥1.5-fold increase/decrease.

2.7. Protein identification by matrix-assisted laser desorption/ionization tandem time of flight (MALDI-TOF-TOF)

CBB-stained spots were excised from the gel, cut into pieces, and washed twice with 50% (v/v) methanol and

Table 1. Strains and plasmids used in this study.

	Strain/plasmid	Relevant genotype/comments	Source/reference
Plasmid	pHT*ppk*	Contains *ppk* gene cloned into pHT315	(Doruk et al., 2013)
B. thuringiensis strains	*Bti*	Wild-type (ATCC 35646)	ATCC
	Bti pHT*ppk*	*Bti* carrying pHT*ppk* plasmid	(Doruk et al., 2013)
	Bti Δ*ppk*	*Bti* without *ppk* gene	(Doruk and Gedik, 2013)

5% acetic acid until they became colorless. Destained gel pieces were dehydrated with acetonitrile (ACN), treated with 10 mM DTT in 100 mM NH_4HCO_3 for 30 min at room temperature, and finally alkylated with 100 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min in the dark. After being dehydrated with ACN and rehydrated with NH_4HCO_3, gel pieces were digested with 30 µL of trypsin solution (20 ng/µL prepared in 100 mM NH_4HCO_3) and incubated at 37 °C overnight. The peptides were extracted twice from gel slices with 5% formic acid in 50% ACN. Desalting of peptide solution was performed by using a ZipTip.

Mass spectrometry (MS) analysis was performed on a MALDI-TOF-TOF instrument (Bruker Autoflex III Smartbeam, USA) and spectra were processed and analyzed using the BioTools software (Bruker Daltonics, USA). Database searching was carried out individually using an in-house MASCOT server (Matrix Science, London, UK).

3. Results

Previously, Doruk et al. (2013) showed that the toxicity of the *ppk*-overexpressing *Bti* pHT*ppk* strain against late 2nd instar *C. quinquefasciatus* was about 7.7 times higher than that of *Bti*, as determined by a larvicidal activity test. Toxicity of *Bti* without the *ppk* gene (*Bti* Δ*ppk*) was found to be 2.4 times higher than that of *Bti* against late 2nd instar *C. quinquefasciatus* (Table 2) in the present study, suggesting that changes in polyP concentration (increases or decreases) stimulate secondary metabolite synthesis.

To clarify the relationship between polyP and endotoxin production, the proteomes of the wild-type, the *ppk*-overexpressing strain (*Bti* pHT*ppk*) (Doruk et al., 2013), and the strain without the *ppk* gene (*Bti* Δ*ppk*) (Doruk and Gedik, 2013) were compared by 2-DE (Figure 1). *Bti* strains were grown in both LB and DSM media and samples were collected at the 6th and 10th hours of fermentation where the *ppk* activity of *Bti* is high (data not shown). All samples were analyzed using biological and experimental duplicates. The protein profiles of 6-h and 10-h samples grown in both LB and DSM were similar, although the spots were more clear in 10-h samples grown in LB. Figure 1 shows the gels of the proteins extracted from each strain grown in LB for 10 h. Forty-one proteins were found to be differentially expressed and 35 of them were identified by MS. Compared to the wild-type, expression of 9 proteins increased, that of 17 decreased, and 4 of them ceased in the *Bti Δppk* mutant strain (Figures 2 and 3; Table 3). The expression of 11 proteins increased, that of 12 decreased, and 1 of them ceased in the *Bti* pHT*ppk* strain compared to the wild-type strain (Figure 2 and 4; Table 3). Proteins identified by MALDI-TOF-TOF were grouped according to their functions (Table 3; Figures 3 and 4).

Table 2. Mosquito larvicidal activity of *Bti* and *Bti* Δ*ppk* strains against late 2nd instar *Culex quinquefasciatus* larvae. LC_{50} values were determined by using probit analysis.

Strain	Mosquito larvicidal activity (24 h) (LC_{50} ng/mL)*
Bti (wild-type)	44.8 ± 2
Bti Δ*ppk*	18.4 ± 4.7

*: LC_{50} is the concentration of inclusion that causes 50% mortality. n = 10.

3.1. Energy metabolism

Three proteins, dihydrolipoamide dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and inosine-5'-monophosphate dehydrogenase, were found to be less abundant in the *Bti* pHT*ppk* strain (overexpressing the *ppk* gene) than in the wild-type and enolase was found overrepresented in the same strain.

Dihydrolipoamide dehydrogenase (DLD) (spot 5) is a vital enzyme of energy metabolism catalyzing NAD^+-dependent reoxidation of dihydrolipoamide in a number of multienzyme complexes, which are primarily involved in important steps of aerobic and anaerobic metabolism and also in the conversion of 2-oxo acids to their corresponding acyl-CoA derivative (Perham et al., 1987; de Kok et al., 1998). It is also known that DLD functions in the glycine cleavage multienzyme complex and in the acetoin dehydrogenase complex in some bacteria such as *Bacillus subtilis* and *Clostridium magnum* (Kruger et al., 1994; Huang et al., 1999). Moreover, mutations in the cell are known to stimulate the ability of DLD to produce superoxide radical and hydrogen peroxide in vitro (Ambrus et al., 2011) and this enzyme is an important source of reactive oxygen species (ROS) also in living cells, particularly under conditions that increase the NADH/NAD ratio (Starkov et al., 2004; Tretter and Adam-Vizi, 2004).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 12) catalyzes the sixth step of glycolysis and also plays a role in gluconeogenesis. It is known that stress conditions cause the inactivation of GAPDH. This inactivation results in generation of more antioxidant cofactor NADPH, which is needed by some antioxidant systems (Ralser et al., 2007).

Inosine-5'-monophosphate dehydrogenase (IMPDH) (spot 16) is an important enzyme to regulate the intracellular guanine nucleotide pool, which is essential for maintaining normal cell function and growth. As a purine biosynthetic enzyme IMPDH is essential for DNA and RNA synthesis, signal transduction, energy transfer, glycoprotein synthesis, and other cellular proliferation processes (Shah and Kharkar, 2015).

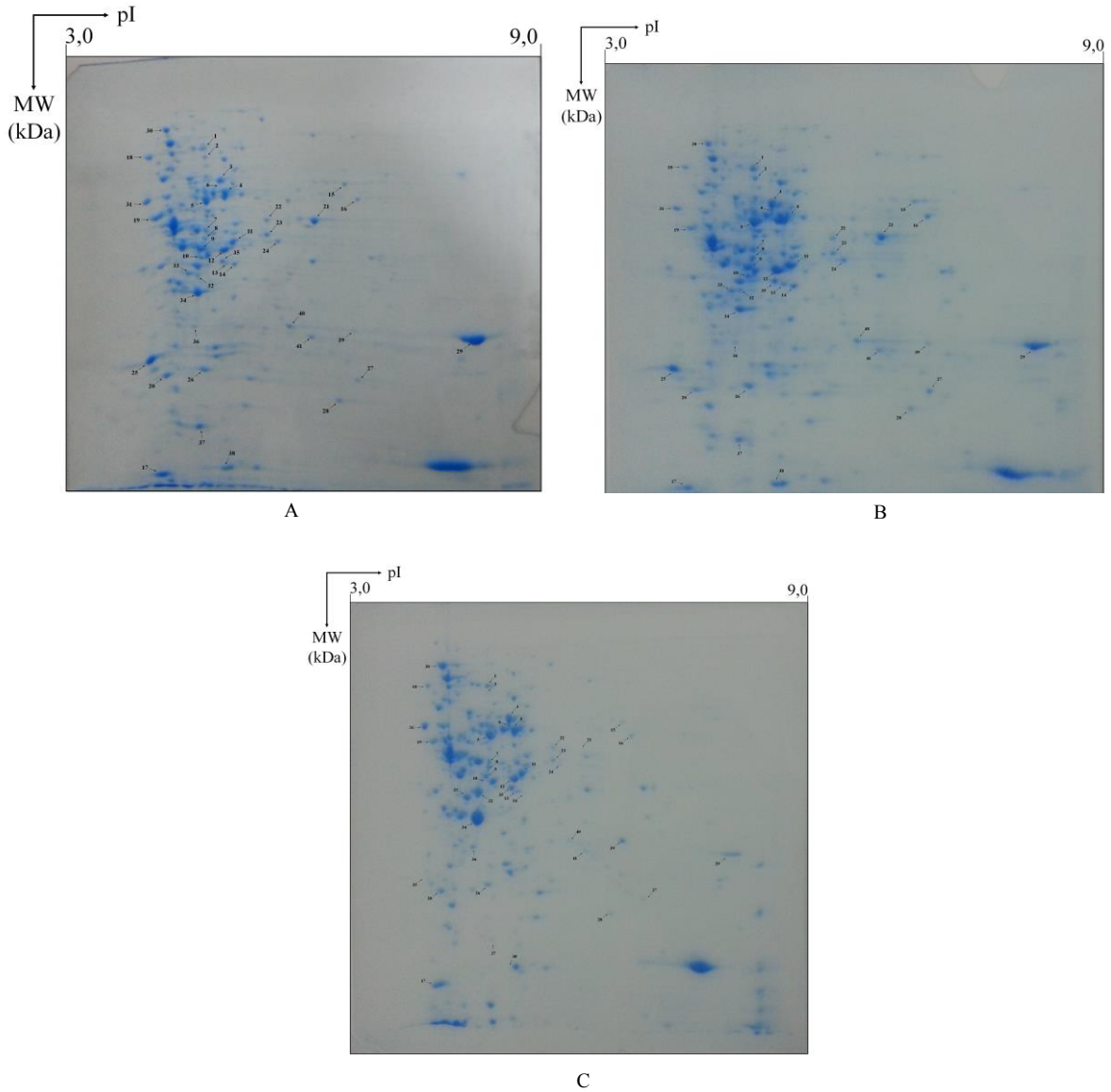

Figure 1. Representative 2-DE gels of proteins extracted from *Bti* (A), *Bti* pHT*ppk* (B), and *Bti* Δ*ppk* (C) strains grown for 10 h in LB medium. The spots differentially represented are numbered and correspond to the proteins listed in Table 3. All samples were analyzed by using biological and experimental duplicates.

Enolase (phosphopyruvate hydratase) (spot 19), which is overrepresented in *Bti* pHT*ppk*, is a glycolytic enzyme involved in carbon metabolism. This metalloenzyme catalyzes the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). It is also involved in RNA processing and gene regulation. Recently it was shown that enolase influences tolerance to oxidative stress and virulence in *Pseudomonas aeruginosa* (Weng et al., 2016). Enolase is also known to be important for sporulation of *Bacillus subtilis* (Leyva-Vazquez and Setlow, 1994).

DLD, GAPDH, IMPDH, and citrate synthase proteins were found to be less abundant in the *Bti* Δ*ppk* strain. Nucleoside diphosphate kinase, on the other hand, was not expressed in this strain.

Citrate synthase (spot 24) catalyzes the first step of the Krebs cycle. Oxaloacetate and acetyl-CoA are the substrates of the reaction. High ratios of ATP:ADP, acetyl-CoA:CoA, and NADH:NAD are known to inhibit the enzyme (Wiegand and Remington, 1986).

Nucleoside diphosphate kinases (NDPKs) (spot 38) are enzymes required for the synthesis of nucleoside triphosphates (NTPs) other than ATP. They have important roles in bacterial growth, virulence, protein elongation, lipid synthesis, cell signaling, and polysaccharide synthesis

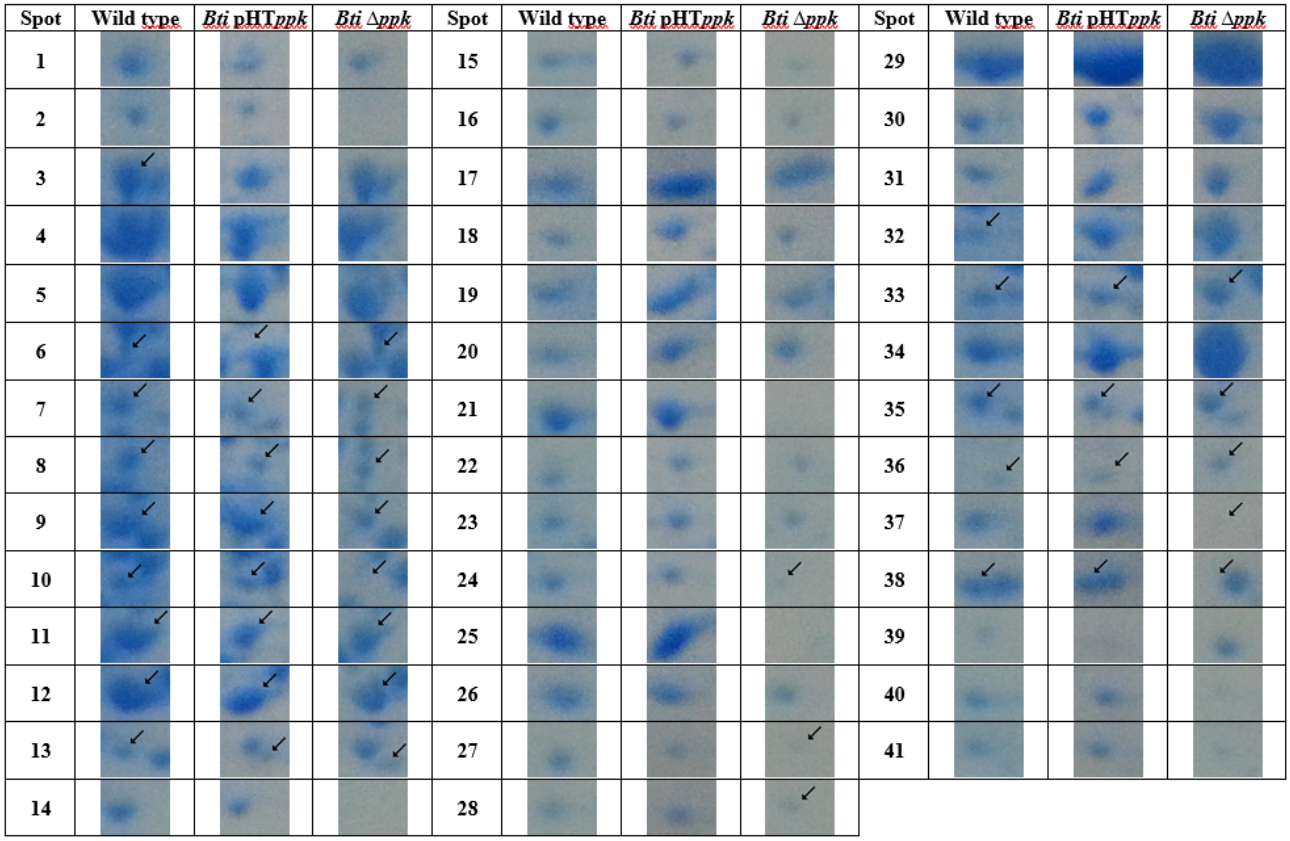

Figure 2. Close-up view of the spots differentially represented in the gels of Figure 1. The spots differentially represented are numbered and correspond to the proteins listed in Table 3.

(Chakrabarty, 1998). Attwood and Wieland (2015) discovered that NDPKs also act as a protein histidine kinase, which involves reversible histidine phosphorylation. This enzyme also serves an important role in the synthesis of (p)ppGpp, an alarmone of the stringent response (Kim et al., 1998).

3.2. Protein folding and stress response

Three proteins (chaperone protein dnaK, alkyl hydroperoxide reductase, elongation factor Ts) that function in stress response and two others (aconitate hydratase, trigger factor) that function in protein folding were found to be more abundant in the *Bti* pHT*ppk* strain.

Chaperone protein dnaK (spot 18) is responsible for correct folding of proteins by inhibiting unsuitable molecular interactions (Deuerling and Bukau, 2004) and is a source of mutational robustness (Aguilar-Rodríguez et al., 2016).

Alkyl hydroperoxide reductase (peroxiredoxin) (spot 20) protects the cell against ROS, which are related to the TCA cycle and respiration chain, by reducing peroxides to water or alcohol. Moreover, this enzyme renews the NAD pool and protects the oxidation/reduction balance (Nishiyama et al., 2001; Seib et al., 2006).

Elongation factor proteins (spot 32) were shown to fold proteins like stress chaperones in *E. coli* (Caldas et al., 1998).

Aconitate hydratase (spot 30) functions in the TCA cycle and is also responsible for posttranslational modifications necessary for correct protein folding (Gupta et al., 2009).

Trigger factor (spot 31) is a ribosome-related bacterial chaperone that folds proteins without ATP (Merz et al., 2008).

In the *Bti* Δ*ppk* strain, elongation factor Ts, phage shock protein, aconitate hydratase, and trigger factor were found to be more abundant compared to the wild-type.

Phage shock protein (spot 39) plays important roles in the stress response in the cell, especially when shortages of nutrient and energy are present (Darwin, 2005).

3.3. Metabolic pathways

In both the *Bti* pHT*ppk* and *Bti* Δ*ppk* strains, phosphoenol pyruvate carboxykinase, acetate/propionate kinase, acyl-CoA dehydrogenase, and fructose 1,6-bisphosphatase proteins were found to be less abundant compared to the wild-type.

Other than those proteins, propionyl-CoA carboxylase beta chain, 3-ketoacyl-(acyl-carrier protein) reductase,

Table 3. MALDI-TOF-TOF results of differentially expressed proteins. "I" denotes proteins whose expression increased, "D" denotes proteins whose expression decreased, "N" denotes proteins that are not expressed, and "E" denotes proteins whose expressions are equal.

Energy metabolism									
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession	Mass	Score	Protein name	Title	Biological process
Spot 5	D	D	1848.8	gi/30022058	49409	109	Dihydrolipoamide dehydrogenase	A flavoprotein enzyme that oxidizes dihydrolipoamide to lipoamide.	Cell redox homeostasis
Spot 12	D	D	1703.7	gi/30023174	35176	112	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis and gluconeogenesis	
Spot 16	D	D	1544.8	gi/6561887	55358	93	IMP dehydrogenase	Converts inosine monophosphate to xanthosine monophosphate, the first committed and rate-limiting step in de novo synthesis of guanine nucleotides, therefore playing an important role in regulation of cell growth	GMP biosynthesis, purine biosynthesis
Spot 19	I	E	1911.8	gi/30023172	46343	140	Enolase	Catalysis of the conversion of 2-PG to PEP, the ninth and penultimate step of glycolysis	Glycolysis, sporulation resulting in formation of a cellular spore
Spot 24	E	D	2081.9	gi/30020417	42358	60	Citrate synthase	Exists in nearly all living cells and stands as a pace-making enzyme in the first step of the citric acid cycle (or Krebs cycle)	Cellular carbohydrate metabolic process
Spot 38	E	N	975.6	gi/30019663	16631	54	Nucleoside diphosphate kinase		CTP biosynthetic process, GTP biosynthetic process, UTP biosynthetic process
Protein folding and stress response									
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 18	I	E	1254.7	gi/118721	65211	53	Chaperone protein dnaK (heat shock protein 70)	Acts as a chaperone	Protein folding, response to stress
Spot 20	I	E	2008.9	gi/30018585	20693	52	Alkyl hydroperoxide reductase (peroxiredoxin) C22	Cell redox homeostasis, response to reactive oxygen species	Response to oxidative stress
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 32	I	I	1758.9	gi/16078 713	32334	112	Elongation factor Ts	A set of proteins that are used in protein synthesis in the cell	Protein biosynthesis

Table 3. (Continued).

Spot 30	I	I	1286.7	gi/30263563	98977	61	Aconitate hydratase		Cell cycle, cell division, protein folding, protein transport
Spot 31	I	I	903.5	gi/16800374	47869	59	Trigger factor	Involved in protein export; acts as a chaperone by maintaining the newly synthesized protein in an open conformation (cell division)	
Spot 39	E	I	1627.8	gi/30019585	26508	94	Phage shock protein A	Stress response	
Metabolic pathways									
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 3	D	D	1939.8	gi/30022835	57915	128	Phosphoenol pyruvate carboxykinase	In the lyase family used in the metabolic pathway of gluconeogenesis	Gluconeogenesis
Spot 7	D	D	1005.6	gi/30022712	43178	55	Acetate/propionate kinase		Acetate kinase activity
Spot 11	D	D	1318.7	gi/30262538	41606	75	Acyl-CoA dehydrogenase	Acyl-CoA dehydrogenase activity; involved in the degradation of long-chain fatty acids	Acyl-CoA dehydrogenase activity, fatty acid β-oxidation
Spot 13	D	D	1761.8	gi/30023364	33993	106	Fructose 1,6-bisphosphatase	Converts fructose-1,6 bisphosphate to fructose 6-phosphate in gluconeogenesis and the Calvin cycle, which are both anabolic pathways	Gluconeogenesis
Spot 15	E	D	1407.7	gi/30020616	56527	72	Propionyl-CoA carboxylase beta chain	Catalyzes the carboxylation reaction of propionyl CoA (great importance as a glucose precursor)	Propionyl-CoA carboxylase activity
Spot 28	I	D	973.6	gi/30019378	19801	59	2'-5'-RNA ligase	RNA metabolic process	Metabolic process
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 33	E	I	1255.7	gi/9297081	30799	67	Fructose-bisphosphate aldolase	Catalyzing a reversible reaction that splits the aldol, fructose 1,6-bisphosphate, into the triose phosphates - dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP)	Carbohydrate metabolic process
Spot 40	E	D	1171.7	gi/30019468	26461	100	3-Ketoacyl-(acyl-carrier protein) reductase(3-oxoacyl-[acyl-carrier protein] reductase)	Participates in fatty acid biosynthesis and polyunsaturated fatty acid biosynthesis	Oxidation-reduction process
Spot 41	E	D	1690.8	gi/30022601	27934	71	Enoyl-CoA hydratase	Hydrates the double bond between the second and third carbons on acyl-CoA	Fatty acid metabolic process

Table 3. (Continued).

Nitrogen and amino acid metabolism									
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 4	D	D	1241.7	gi/30020418	53363	61	2-methyl citrate dehydratase	Participates in propanoate metabolism	
Spot 6	N	E	1590.7	gi/6094396	56909	82	Glutamate-tRNA ligase	Participates in 3 metabolic pathways: glutamate metabolism, porphyrin and chlorophyll metabolism, and aminoacyl-tRNA biosynthesis	Glutamyl-tRNA amino acylation
Spot 8	D	D	1507.7	gi/30022309	40200	77	Aminomethyltransferase	The glycine cleavage system catalyzes the degradation of glycine	Glycine catabolic process
Spot 14	D	N	1848.8	gi/30018617	35051	111	Carbamate kinase	Participates in 4 metabolic pathways: purine metabolism, glutamate metabolism, arginine and proline metabolism, and nitrogen metabolism	Arginine metabolic process
Spot 21	E	N	1796.9	gi/30018614	46850	131	Arginine deiminase	Participates in arginine and proline metabolism (expression could be regulated by various environmental factors)	Arginine catabolic process to ornithine, protein citrullination
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 22	E	D	1175.6	gi/30023347	45230	52	Serine hydroxymethyltransferase	Catalyzes the reversible interconversion of serine and glycine with tetrahydrofolate (THF) serving as the one-carbon carrier; this reaction serves as the major source of one-carbon groups required for the biosynthesis of purines, thymidylate, methionine, and other important biomolecules	Glycine biosynthetic process from serine, tetrahydrofolate interconversion
Spot 23	E	D	1812.9	gi/30018778	40209	96	Alanine dehydrogenase	Participates in taurine and hypotaurine metabolism and reductive carboxylate cycle (CO_2 fixation); catalyzes reversible oxidative deamination of L-alanine to pyruvate; this enzyme is a key factor in assimilation of L-alanine as an energy source through tricarboxylic acid cycle during sporulation	Alanine catabolic process, sporulation resulting in formation of a cellular spore
Spot 35	D	E	1577.8	gi/30022243	36760	74	2-Oxoisovalerate dehydrogenase alpha subunit	Oxidoreductase activity	Catabolism of the branched-chain amino acids, isoleucine, leucine, and valine

Table 3. (Continued).

Other proteins									
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 17	I	I	2486.2	gi/30018370	12510	136	50S ribosomal protein L7/L12	Forms part of the ribosomal stalk, which helps the ribosome interact with GTP-bound translation factors, and is thus essential for accurate translation	Translation
Spot 25	I	N	1449.8	gi/24474855	21588	129	Camelysin	Novel surface metalloproteinase camelysin (casein-cleaving metalloproteinase)	Proteolysis
Spot 27	D	D	973.5	gi/30019489	20252	61	Hypothetical protein BC 1339		
Spot 29	I	I	1697.8	gi/75758638	21361	81	Hypothetical protein RBHT_07739		
Name	*Bti* pHT*ppk*	*Bti* Δ*ppk*	Parent ion	Accession #	Mass	Score	Protein name	Title	Biological process
Spot 34	I	I	1573.8	gi/47565426	32684	125	Flagellin		Ciliary or bacterial-type flagellar motility
Spot 36	E	I	1943	gi/30021916	28775	118	Transcriptional repressor CodY	DNA-binding protein that represses the expression of many genes that are induced ascells making the transition from rapid exponential growth to stationary phase and sporulation	Regulation of transcription, DNA-dependent

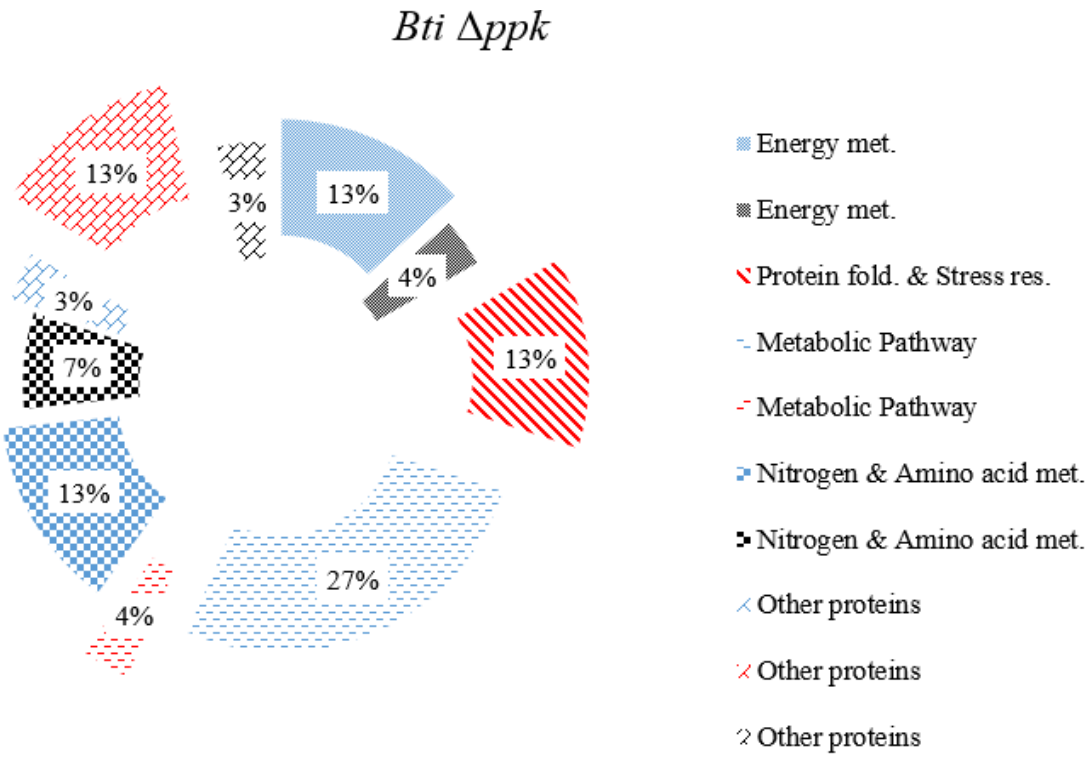

Figure 3. Summary of differentially expressed proteins in *Bti* Δ*ppk*. Red patterns show proteins whose expression increased, blue patterns show proteins whose expression decreased, and black patterns show proteins that are not expressed.

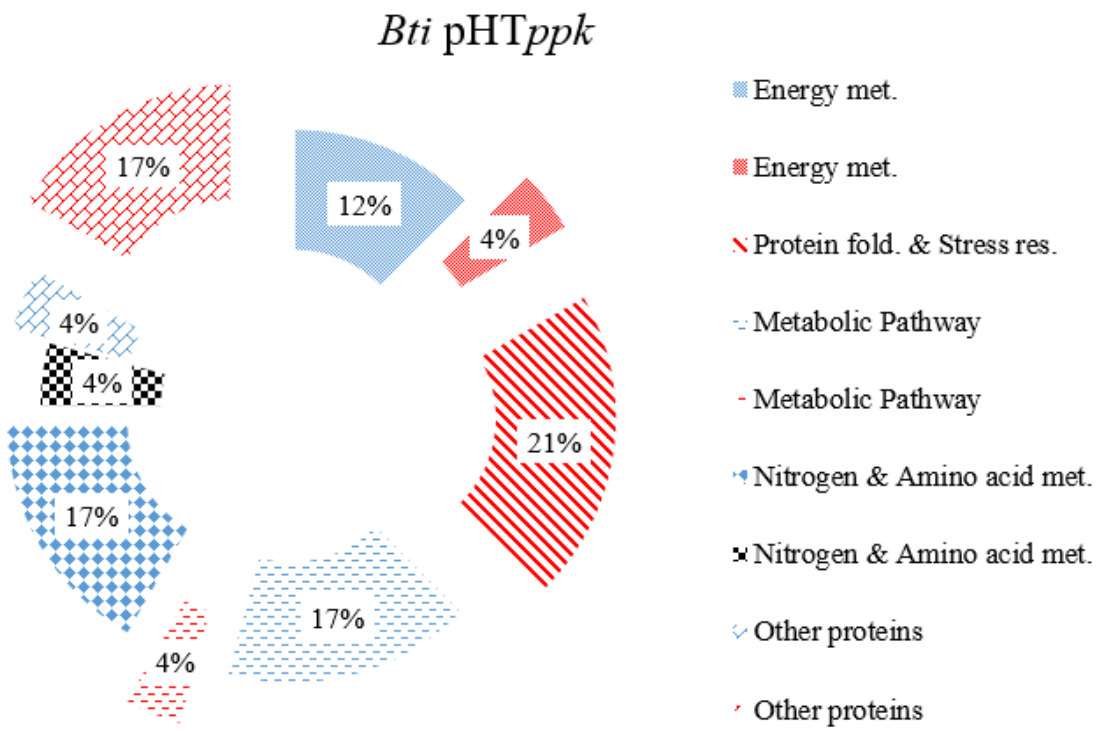

Figure 4. Summary of differentially expressed proteins in *Bti* pHT*ppk*. Red patterns show proteins whose expression increased, blue patterns show proteins whose expression decreased, and black patterns show proteins that are not expressed.

and enoyl-CoA hydratase were also less abundant in the *Bti* Δ*ppk* strain. Fructose-bisphosphate aldolase enzyme, on the other hand, was expressed more in the same strain. The expression level of 2'-5'-RNA ligase in the *Bti* pHT*ppk* strain was more than in the wild-type and vice versa for the *Bti* Δ*ppk* strain.

Phosphoenol pyruvate carboxykinase (spot 3) functions in gluconeogenesis and catalyzes the reversible decarboxylation and mononucleotide-dependent phosphorylation of oxaloacetate to PEP and CO_2 (Matte et al., 1996). It has been shown that this enzyme plays a role not only in gluconeogenesis but also in production of glutamine and lysine through the TCA cycle intermediates in *Corynebacterium glutamicum* (Aich et al., 2003).

Acetate/propionate kinase (spot 7), which is an important enzyme for glycolysis, is responsible for β-oxidation of fatty acids. It is known that the enzyme level increases in the case of high glucose concentrations (Grundy et al., 1993).

Acyl-CoA dehydrogenase (spot 11) functions in fatty acid and amino acid catabolism by catalyzing the α,β-dehydrogenation of acyl-CoA esters. Several members of the acyl-CoA dehydrogenase family are involved in fatty acid β-oxidation; these are short, medium, long, and very long chain acyl-CoA dehydrogenases. Short chain fatty acids are an important carbon and energy source in bacteria (Swigonova et al., 2009).

Fructose 1,6-bisphosphatase (spot 13) is a key enzyme of the gluconeogenic pathway, hydrolyzing fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate (Hines et al., 2007).

Propionyl-CoA carboxylase beta chain (spot 15) catalyzes the carboxylation of propionyl CoA. Propionyl CoA, as an important metabolite in lipid metabolism and in valine, isoleucine, and methionine metabolisms, is crucial as a glucose precursor (Rodriguez and Gramajo, 1999; Rodriguez-Pombo et al., 2002).

3-Ketoacyl-(acyl-carrier protein) reductase (spot 40) functions in the biosynthesis of fatty acids by performing NADPH-dependent reduction of β-ketoacyl–ACP substrates to β-hydroxyacyl–ACP products (Nomura et al., 2005).

Enoyl-CoA hydratase (spot 41) is an important enzyme of fatty acid metabolism that catalyzes the second step of the β-oxidation pathway. It adds a water molecule to the double bond between the 2nd and 3rd carbon of acyl-CoA. This enzyme is responsible for production of acetyl-CoA and energy by metabolizing fatty acids (Agnihotri and Liu, 2003).

Fructose-bisphosphate aldolase (spot 33) is an important enzyme in carbohydrate metabolism. In glycolysis, this enzyme catalyzes the cleavage of fructose-1,6-bisphosphate (FBP) to a ketose, dihydroxyacetone phosphate (DHAP), and an aldose, glyceraldehyde-3-phosphate (G3P). In gluconeogenesis it catalyzes the reverse condensation reaction (Cooper et al., 1996).

2'-5'-RNA ligase (spot 28) is an important enzyme in the RNA metabolic process. It ligates half-tRNA molecules with 2',3'-cyclic phosphate and 5-hydroxyl termini, giving rise to a product containing a 2'-5' phosphodiester linkage. This enzyme has been shown to perform cleavage or ligation in vivo (Arn and Abelson, 1996).

3.4. Nitrogen and amino acid metabolism

In the *Bti* pHT*ppk* strain, 2-methyl citrate dehydratase, aminomethyltransferase, carbamate kinase, and 2-oxoisovalerate dehydrogenase alpha subunit proteins were found to be less abundant. Glutamate-tRNA ligase, on the other hand, was not expressed at all in the same strain.

In the *Bti* Δ*ppk* strain, 2-methyl citrate dehydratase, aminomethyltransferase, serine hydroxymethyltransferase, and alanine dehydrogenase proteins were found to be less abundant. Carbamate kinase and arginine deiminase, on the other hand, were not expressed at all in the same strain.

2-Methyl citrate dehydratase (spot 4) is required for propionate catabolism via the methyl citrate cycle. Propionate is oxidized via this cycle to pyruvate (Blank et al., 2002).

Aminomethyltransferase (spot 8) is one of the four enzymes of the glycine cleavage system, which catalyzes the oxidative decarboxylation of glycine in bacteria. This tetrahydrofolate-dependent enzyme is responsible for the synthesis of methylenetetrahydrofolate (Lee et al., 2004; Okamura-Ikeda et al., 2010).

Carbamate kinase (spot 14) functions in purine, glutamate, arginine, and proline metabolisms. It also has important functions in nitrogen metabolism (Marina et al., 1999).

2-Oxoisovalerate dehydrogenase alpha subunit (spot 35) functions in the catabolism of branched-chain amino acids isoleucine, leucine, and valine (Debarbouille et al., 1999).

Glutamate-tRNA ligase (spot 6) catalyzes the attachment of glutamate to tRNA(Glu) in a two-step reaction: glutamate is first activated by ATP to form Glu-AMP and then transferred to the acceptor end of tRNA(Glu). It functions in glutamate, porphyrin, and chlorophyll metabolisms. At the same time this enzyme also plays a role in aminoacyl-tRNA biosynthesis (Breton et al., 1990; Gagnon et al., 1994).

Serine hydroxymethyltransferase (spot 22), which is an important enzyme that supplies single carbon units to the cell, catalyzes the reversible interconversion of L-serine into glycine and tetrahydrofolate into 5,10-methylenetetrahydrofolate.

5,10-Methylenetetrahydrofolate, being a vital link between amino acid and nucleotide metabolism, is a key

intermediate for the biosynthesis of purines, thymidylate, choline, and methionine (Pai et al., 2009).

Arginine deiminase (spot 21) functions in arginine and proline metabolisms. The expression of this enzyme is known to be regulated by environmental factors (Li et al., 2008).

Alanine dehydrogenase (spot 23) catalyzes the reversible oxidative deamination of L-alanine to pyruvate. This enzyme is important for energy generation during sporulation (Siranosian et al., 1993).

3.5. Other proteins

In the *Bti* pHT*ppk* strain, 50S ribosomal protein L7/L12, flagellin, and camelysin proteins were found to be expressed more.

50S ribosomal protein L7/L12, flagellin, and transcriptional repressor CodY proteins were found to be expressed more in the *Bti* Δ*ppk* strain. Camelysin protein, on the other hand, was not expressed at all in the same strain.

50S ribosomal protein L7/L12 (spot 17), which is among the most important components of prokaryotic ribosomes, interacts with GTP-dependent translation factors during protein biosynthesis in bacteria (Pettersson and Kurland, 1980).

Flagellin (spot 34) is the most abundant (as many as 20,000) structural protein of flagella. Motility, chemotaxis, and biofilm formation require the expression of flagellin (Pratt and Kolter, 1998).

Camelysin (casein-cleaving metalloproteinase) (spot 25) is a zinc-containing metalloprotease that is responsible for proteolysis (Fricke et al., 2001). Camelysin preferentially cleaves peptide bonds in front of aliphatic hydrophobic and hydrophilic amino acids. It has been shown that this protein can activate cytotoxic toxin proteins (Cyt1Aa and Cyt2Ba) produced by *Bti* (Nisnevitch et al., 2010).

Transcriptional repressor CodY (spot 36) is a highly conserved DNA binding protein in gram-positive bacteria with low G+C content. It regulates the expression of many genes involved in the transition of cells from rapid exponential growth to the stationary phase and sporulation (Ratnayake-Lecamwasam et al., 2001).

4. Discussion

In a previous study, polyphosphate metabolism of *Bti* was manipulated by overexpressing the *ppk* gene (encoding polyP synthesizing enzyme) under the control of its native promoter (Doruk et al., 2013). This strain (*Bti* pHT*ppk*) was found to produce more toxin than the wild-type and exhibited significantly higher toxicity against late 2nd instar *Culex quinquefasciatus* larvae (7.7 times) (Doruk et al., 2013) and 3rd instar wild *Culex pipiens* larvae (10 times) (Doruk et al., 2016) than control strains.

The *Bti* strain without the *ppk* gene (*Bti Δppk)* was also found to be more toxic (2.4 times) than the wild-type against late 2nd instar *C. quinquefasciatus* larvae in the present study. These results clearly demonstrate that overexpression or deletion of the *ppk* gene affects toxicity of *Bti* in a positive manner, although *Bti* pHT*ppk* is much more toxic than *Bti Δppk*.

The elicitation mechanism of secondary metabolism is a complex process and the precise mechanism is poorly understood. Changes in the levels of intracellular molecules such as ions (Murphy et al., 2011), alarmones (Kawai et al., 2007), or ROS (Radman et al., 2006) serve as internal signals to direct the regulation of secondary metabolites either directly influencing the transcription of the secondary metabolite gene cluster or inducing a transcriptional activator of the target gene cluster (Kawai et al., 2007; Rigali et al., 2008; Nair et al., 2009; Tanaka et al., 2010).

To explain the phenotypes of *Bti* pHT*ppk* and *Bti Δppk*, proteomes of wild and mutant strains were analyzed. For this, intracellular proteins were separated by 2-DE and 41 spots differentially expressed were determined; 35 of those were identified and analyzed by MS. Compared to the wild-type, expression of 9 proteins increased, that of 17 decreased, and 4 of them ceased in the *Bti Δppk* mutant strain. The expression of 11 proteins increased, that of 12 decreased, and 1 of them ceased in *Bti* pHT*ppk*.

When we look into the whole proteome, the protein profile of the *Bti* pHT*ppk* strain was found to be closer to the wild-type compared to the *Bti Δppk* strain, which has more dramatic changes in its proteome. There is a general decrease in the expression levels of proteins related to energy metabolism, amino acid metabolism, and purine biosynthesis in both *Bti* pHT*ppk* and *Bti Δppk*. Gluconeogenesis and fatty acid metabolism were also slowed down in both strains. Decrease in expression levels of a larger number of proteins functioning in those metabolisms were seen in *Bti Δppk*.

Contrary to our results, Varela et al. (2010) found that polyP deficiency in *Pseudomonas* sp. B4 caused an increase in the energy-generating pathways (TCA, β-oxidation, oxidative phosphorylation). As it is a sporulating bacterium, we know that a great majority of genes involved in the glycolysis and TCA cycle are significantly downregulated during sporulation, implying an overall decrease in the activities of these pathways (Wang et al., 2013). Decrease in energy metabolisms of both *Bti* recombinant strains can be associated with sporulation, although we found that *Bti* Δ*ppk* and *Bti* pHT*ppk* are sporulating as well as the wild-type (data not shown). Shi et al. (2004) suggested that polyP depolymerization may induce or at least promote efficient sporulation in *B. cereus*. Although sporulation efficiency was impaired in

the *ppx* mutant strain, sporulation did not appear to be defective in the *ppk* mutant in their study. Similarly, we also found that *ppk* is not necessary for sporulation in *Bti*. Transcriptional repressor CodY (spot 36), which regulates expression of many genes involved in the transition of cells from rapid exponential growth to the stationary phase and sporulation (Ratnayake-Lecamwasam et al., 2001), was expressed more in *Bti* Δ*ppk*. The reason for efficient sporulation of the *ppk* mutant strain could be the high expression of this protein.

Although a general decrease in the expression of proteins functioning in all metabolic pathways was seen in both recombinant strains, an increase in the expression of proteins that function in protein folding and stress response was observed. For example, elongation factor Ts, which can fold proteins like stress chaperons, and aconitate hydratase and trigger factor, which are protein folding proteins, were found to be expressed more in both *Bti Δppk* and *Bti* pHT*ppk*. Moreover, stress response proteins chaperone protein dnaK and alkyl hydroperoxide reductase were also found to be expressed more in the *Bti* pHT*ppk* strain. In the *Bti Δppk* strain phage shock protein, which is known to have very important tasks in the case of shortages of nutrient and energy, was also expressed more than in the wild-type.

There is other evidence for the occurrence of stress conditions inside both mutant cells. Dihydrolipoamide dehydrogenase (DLD or E3) is an enzyme of energy metabolism, but it is also an important source of ROS in living cells (Starkov et al., 2004; Tretter and Adam-Vizi, 2004). It is possible to hypothesize that decreasing the expression of DLD by *Bti* pHT*ppk* and *Bti* Δ*ppk* strains can be a protection mechanism to avoid ROS accumulation inside the cell, which is already under stress because of the disturbance of polyP homeostasis. It is known that chemical molecules repressing ROS production in microbes could serve as elicitors of microbial secondary metabolites (Abdelmohsen et al., 2015), although we do not know if there is a specific chemical that negatively affects DLD expression and elicits toxin production in this specific case.

Another energy metabolism enzyme, glyceraldehyde-3-phosphate dehydrogenase, is inactivated under stress conditions. This inactivation results in the generation of more antioxidant cofactor NADPH, which is needed by some antioxidant systems (Ralser et al., 2007). This enzyme is also less abundant in both mutant strains.

Moreover, it was shown that enolase, which is a glycolytic enzyme involved in carbon metabolism, influences tolerance to oxidative stress and virulence in *Pseudomonas aeruginosa* (Weng et al., 2016). High expression of this enzyme in *Bti* pHT*ppk* could give us a clue to claim that this strain may be under oxidative stress.

High expression of camelysin protein in *Bti* pHT*ppk* could be another reason for the hypertoxicity of this strain. Nisnevitch et al. (2010) demonstrated that camelysin enzyme can activate the protoxins Cyt1Aa and Cyt2Ba by truncating their polypeptide chains. Therefore, protoxin activation is carried out not only by insect gut proteases (Al-Yahyaee and Ellar, 1995), but also by the camelysin protein in *Bti*. It is interesting that this protein was not expressed at all in the *Bti* Δ*ppk* strain, which can explain the difference of the toxicity levels of both recombinant strains.

Deletion of the *ppk* gene caused more severe effects in *Bti* compared to the strain overexpressing the *ppk* gene. NDPK could be one of the responsible enzymes for these effects since it is not expressed at all in the *Bti* Δ*ppk* strain. This enzyme has important roles in bacterial growth, virulence, protein elongation, lipid and polysaccharide synthesis, and cell signaling by synthesizing NTPs other than ATP (Chakrabarty, 1998). NDPK also plays a critical role in the synthesis of (p)ppGpp during stringent response (Kim et al., 1998).

One of the proteins whose expression increased in both *Bti* pHT*ppk* and *Bti Δppk* was flagellin. This is the unit that constitutes the filament of the bacterial flagella. It is known that prior to sporulation *Bti* cells increase their flagellin levels inside the cell in order to search for nutrients. Increased expression of flagellin protein was particularly seen in *Bti Δppk* (spot 40).

Fructose-bisphosphate aldolase catalyzes the cleavage of fructose-1,6-bisphosphate to DHAP and G3P (Cooper et al., 1996). In *E. coli*, to replenish a low concentration of needed inorganic phosphate, DHAP is converted to methylglyoxal and an inorganic phosphate is given off by this reaction (Kayser et al., 2005). It can be speculated that one of the reasons for high expression of fructose-bisphosphate aldolase in the *Bti* Δ*ppk* strain is to replenish the cell with inorganic phosphate.

In summary, the results of this study suggest that changes in polyP concentration (increases or decreases) cause a general stress condition inside the cell, which in turn stimulates secondary metabolite synthesis. General stress in cells caused by lack of polyP has been shown in previous studies (Varela et al., 2010; Le Marechal et al., 2013). However, creation of a general stress condition by increased concentration of polyP was shown for the first time in this study.

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