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Quantitative proteomic analysis of the *Bacillus thuringiensis* BGSC-4AW1 strain (serovar *andalousiensis*)

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ABSTRACT

Analysis of the proteome of any *Bacillus thuringiensis* (*Bt*) strain should provide important information about mechanisms of infection, about interactions with the host organisms, and about molecular mechanisms allowing the bacterium to survive detrimental conditions. To address these important issues, we analyzed the proteome of the crystal-forming *Bacillus thuringiensis* strain BGSC-4AW1 (var. a*ndalousiensis*). This Quantitative Proteomics-based analysis revealed the presence of important proteins for cell survival and cell proliferation associated with exosporium, coat, and crystal complexes. Currently, it is not possible to discriminate among the specific sub-proteomes associated with the *Bt*'s developmental stages; however, the information provided by this proteomic analysis is potentially useful for mapping the cellular mechanisms involved in cell survival and adaptation to deleterious environmental conditions. The presence of an insecticidal toxin, as well as a cancer cell-killing Cry protein add to the spectrum of biotechnological applications of *Bacillus thuringiensis.*

Keywords: *Bacillus thuringiensis*, proteomics, systems biology, biotechnology, cancer cell-killing Cry protein

Abbreviations: *Bt*: *Bacillus thuringiensis*; ser: serovar; Cry: crystal; MS: Mass spectrometry; CC-KCP: Cancer cell-killing Cry protein; PI-PLC: phosphatidylinositol-specific phospholipase C.

1. Introduction

The Bacillus cereus group consists of six species: *B. anthracis (Ba), B. cereus (Bc), B. mycoides (Bm), B. pseudomycoides (Bp), B. thuringiensis (Bt)*, and *B. weihenstephanensis (Bw)* [1]. Of these, *Bt* has been used for a long time as a bio-pesticide for insect control [2]. The insecticidal capacity of *Bt* resides in the inclusion bodies (parasporal crystalline inclusions) which are usually formed during the sporulation stage [2, 3]. These inclusion bodies contain proteins of various forms, many of which are toxic to several insect species. Many of these toxins are known as *Bt* δ -endotoxins or Cry toxins; other *Bt* crystal proteins have cytolytic activity and are known as Cyt toxins [2].

Another group of *Bt* toxins has been reported as having specific toxicity against human cancer cells without being hemolytic [4]; these proteins have been named "Parasporins" [5]. Six parasporin types, PS1 to PS6, have being described ([6], see http://parasporin.fitc.pref.fukuoka.jp/, for nomenclature of parasporin proteins). The mode of action of *Bt* Cry toxins has been a matter of intensive research for more than three decades [7-15]. The study of the mode of action of parasporins is a nascent field, in which pore formation [6, 16] and calcium-regulated cell death [17] have been proposed as potential mechanisms for their mode of action.

Here we report the Quantitative Proteomics-based characterization of the *Bt* BGSC-4AW1 (var. *andalousiensis*) strain, including the crystal inclusions. There is a great variety of proteins enclosed in these structures, particularly three toxins in the isolated crystals, i.e., the insecticidal toxin

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Cry8Ca, the Cancer Cell-Killing Cry Protein (CC-KCP), and the phosphatidylinositol-specific phospholipase C (PI-PLC). Many of the proteins identified have specific functions associated with spore formation, coat stability, trans-membrane transport, chaperoning activity, and cell homeostasis.

2. Materials and Methods

2.1. Cell cultures and purification of crystals

Bacillus thuringiensis strain 4AW1 was obtained from the Bacillus Genetic Stock Center (BGSC, Biochemistry Department - The Ohio State University, Columbus, OH). The original code is: *Bacillus thuringiensis* subspecies *andalousiensis* T37001 (=EA10192); genotype: wild type isolate (Note: Serotype 37; isolated in Spain; antisera standard. Shotgun whole genome sequence available from http://www.ebi.ac.uk/ena/data/view/ACNG01000080.1; [18]). The filter disk obtained from the BGSC was placed in a 1.5 mL Eppendorf tube and 1.0 mL of LB medium was added and vortexed vigorously. Four hundred µL of this solution was dispersed on an LB agar plate and left to grow overnight at 28 °C in a dry incubator.

The next day, a single colony from the agar plate was transferred to a flask containing 20 mL LB medium, which, after a 12-hr growth period, was transferred into 500 mL of G-tris medium for growing and sporulation. G-tris medium (10 mM Tris-HCl, pH 7.6; $FeSO_4{\cdot}7H_2O$, 0.00005%; $CuSO_4{\cdot}5H_2O$, 0.0005%; $ZnSO_4{\cdot}7H_2O$, 0.0005%; $MnSO_4{\cdot}H_2O$, 0.005%; $MgSO_4$, 0.02%; $CaCl_2{\cdot}2H_2O$, 0.008%; K_2HPO_4, 0.05%, $(NH_4)_2$ SO4, 0.2%; Glucose, 0.1%) was prepared as previously described [19]. After five days of growth in a shaker/incubator at 28 °C and 250 rpm, the cell/spore/crystal mix was collected as described below.

The bacterial culture was centrifuged at 4,500 rpm for 10 min at 4 °C. The resulting pellet containing spores and crystals was washed once with 1M NaCl containing 1% SDS, followed by centrifugation. The resulting pellet was washed twice in 1M NaCl and then with distilled water until no foam was observed, followed by sonication for 5 min at 4 °C in a Fisher sonic dismembrator model 100 (sonication for 30 s followed by 30 s of cooling). The concentrated solution of the spore-crystal pellet was separated into crystals and spores by sedimentation using a discontinuous sucrose gradient (5 ml of 60%, 3ml of 40%, 5 ml of 30% and 5 ml of 10% in $d_{ii}H_2O$) as described [19]. The sucrose-containing tubes were centrifuged at 4,500 rpm for 30 min at 4 °C. As previously described [19], the 10 – 30% sucrose interphase was collected to obtain the inclusion crystal bodies.

The particulate contents of the 10 – 30% interphase fraction was pelleted by centrifugation and the pellet was washed with $d_{ii}H_2O$, followed by washing with 1M NaCl containing 1% SDS, followed by two washes of 1M NaCl, and with $d_{ii}H_2O$ until no foam was observed. The resulting pellet was divided into two fractions, and one of the fractions was taken for imaging by electron microscopy. The other fraction was sonicated as before, and then washed with a solution of 50:50 acetone:ethanol to remove any remaining lipid material. A fraction of this pellet was also used for imaging by electron microscopy. The final pellet was resuspended in 200 µL $d_{ii}H_2O$ and stored at 4 °C for quantitative proteomic analysis.

2.2. Electron microscopy

Ten µL of the pellet from fraction one and 10 µL of the pellet from fraction two were stained for electron microscopy as previously described [20]. Briefly, carbon-coated copper grids were glow-discharged at 300 mesh in a Harrick plasma cleaner, model PDC 32G for 90 seconds at medium power. The sample was applied to the grid and allowed to sit for 2 min, then washed with 5 drops of 2 % aqueous uranyl acetate and allowed to stain for 1 min. The stain was wicked-off with filter paper and the sample was allowed to dry for several minutes. Images were collected with a Tecnai G2 Transmission Electron Microscope (FEI, Hillsboro, OR). Digital images were taken with a Gatan Multiscan 794 camera using Gatan DM3 Software (Gatan Inc., Pleasanton, CA).

2.3. Protein solubilization for proteomic analysis

The solubilization of the crystal-containing pellets was achieved by mixing 5 µL of pellet (in $d_{ii}H_2O$) with an equal amount of Laemmli loading buffer (100 mM Tris-HCl, pH 7.0, 20 % glycerol, 4 % SDS, 0.02 % bromophenol blue; 0.05 % β-mercaptoethanol), followed by boiling for 5 min in a water bath at 95 °C. The mixture was centrifuged at maximum speed on a table-top centrifuge, and the supernatant was used to determine the presence of protein bands using sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) with pre-cast gels (Bio-Rad, Hercules, CA). The apparent protein molecular weight was determined with "Kaleidoscope" pre-stained protein standards (Bio-Rad).

2.4. Manual trypsin "in-gel" protein digestion for protein identification by mass spectrometry

Manual "in-gel" protein digestion was done in four steps: i) destaining, ii) reduction of disulfide bridges and alkylation of resulting sulfhydryl groups, iii) proteolytic cleavage of the protein (trypsin digestion), and iv) extraction of resulting peptides. The bands of interest were excised from the Coomassie-stained gel and each gel band was cut into 1.0 x 1.0 cm gel pieces and transferred into Axygen 1.7 mL tubes. The gel pieces were washed with 100 µL HPLC-grade water (Sigma, St. Louis, MO) for 5 min at 25 °C. The water was removed and replaced with ~100 µL of 50:50 acetonitrile (ACN):25 mM ammonium bicarbonate (ABC), followed by shaking and centrifugation and removal of the destaining

solution. These steps were repeated twice. The solution was replaced with 75:25 (ACN:25 mM ABC), vortexed, centrifuged, and washed with HPLC-grade water. These two steps were repeated one more time. After washing, the solution was replaced with 100 % 25 mM ABC, shaken, and removed; 100 % ACN was added and shaken for 5 –10 min, and then removed. The last step was repeated until the gel pieces were small and white. Destained gel plugs were lyophilized for 30 min (with a hole punched in each tube cap).

Reduction was performed with DTT as follows: 25 µL of 50 mM ABC was added to each lyophilized gel piece, then 50 µL of 10 mM freshly-prepared DTT was added to each tube (the DTT stock was prepared in 100 mM ABC). The mixture was incubated at 50 °C for 30 min after which the DTT mixture was removed. For alkylation, 25 µL of 50 mM ABC was added to each gel piece, followed by 50 µL of 50 mM iodoacetamide (freshly-prepared in 100 mM ABC, and kept in the dark). The mixture was incubated in the dark for 20 min, after which the iodoacetamide solution was removed.

In-gel digestion was performed by first washing the gel plugs with 100 µL of 50 mM ABC for 5 min. The ABC was removed, and the gel plugs were washed with 100 µL ACN for five minutes. The washing step was repeated twice, the ACN was then removed, and the gel plugs were dried on a speedvac. Then 100 – 200 µL trypsin (from stock 20 µg/µL, Promega, in HCl) was added (enough to cover the gel plugs), and the tubes were kept on ice for ~50 min. Excess trypsin solution was removed, and the plugs were washed with 25 mM ABC, and finally enough 25 mM ABC was added to cover the gel pieces. The digestion was allowed to continue overnight at 37 °C on a shaker.

The peptides were extracted as follows: 50 µl ACN was added to each gel plug and shaken for 10 min at 25 °C. The combined solution was removed and the plug was transferred to a different Axygen tube. Approximately 100 µL of HPLC-grade water was added to each gel plug, and shaken in a thermomixer for 10 min at 25 °C. The solution was removed and combined with the previous extract. Then an extra 30 µL of HPLC-grade water and 50 µL of ACN were added to each gel plug, and shaken in a thermomixer for 10 min at 25 °C. The last solution was removed and combined with the two previous extracts. The last step was repeated with 100% ACN, and all the solutions were combined and dried in a speedVac.

2.5. Identification of proteins by mass spectrometry (MS)

Extracted peptides were desalted using PepClean C18 spin columns (Pierce, Rockford, IL), according to the manufacturer's instructions, and re-suspended in an aqueous solution of 0.1 % formic acid. Identification of proteins was done using reversed-phase LC-MS/MS on a 2D -nanoLC Ultra system (Eksigent Inc, Dublin, CA) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). The Eksigent system was configured to trap and elute peptides via an injection of ~250 fmol of sample. The trapping was performed on a 3 cm-long 100 µm i.d. C18 column while elution was performed on a 15 cm-long 75 µm i.d., 300 Å particle ProteoPep II integraFrit C18 column (New Objective Inc, Woburn, MA). The analytical separation of the tryptic peptides was achieved with a 70-min linear gradient of 2 – 10% buffer B at a 200 nL/min, where buffer A is an aqueous solution of 0.1 % formic acid and buffer B is a solution of 0.1 % formic acid in acetonitrile.

Mass spectrometric data acquisition was performed in a data-dependent manner on a hybrid LTQ-Orbitrap mass spectrometer. A full scan mass analysis on an Orbitrap (externally calibrated to a mass accuracy of < 1 ppm, and a resolution of 60,000 at m/z 400) was followed by intensity-dependent MS/MS of the 10 most abundant peptide ions. Collision induced dissociation (CID)-MS/MS was used to dissociate peptides with normalized collision energy of 35 eV, in the presence of He bath gas atoms at a pressure of 1 mTorr. The MS/MS acquisition of each precursor m/z was repeated for 30 s and subsequently excluded for 60 s. Monoisotopic precursor ion selection (MIPS) and charge state screening were enabled for triggering data-dependent MS/MS scans.

Mass spectra were processed, and peptide identification was performed using Mascot ver. 2.3 (Matrix Science Inc.) implemented on Proteome Discoverer Ver 1.3 software (Thermo-Fisher Scientific). All searches were performed against a curated *Bt* serovar *andalousiensis* data base (GSC-4AW1; downloaded from http://patricbrc.org/portal/portal/patric/Downloads?cType=taxon&cId=, selecting *Bacillus thuringiensis* serovar *andalousiensis* BGSC 4AW1 [21]). Peptide-based protein identification was done using a target-decoy approach with a false discovery rate (FDR) of 1 % [22]. A precursor ion mass tolerance of 200 ppm and a product ion mass tolerance 0.5 Da were used, with a maximum of two missed tryptic cleavages [23]. Methionine oxidation was selected as a variable modification.

2.6. Spectral counting-based quantitative proteomics

Spectral counting was performed on the Mascot DAT files using ProteoIQ: ver 2.3.02 (NuSep Inc., Athens, GA). Proteins, identified as explained above, were subjected to "probability-based" confidence measurements using an independent implementation of the statistical models Peptide and Protein Prophet deployed in Proteo IQ [24, 25]. Protein hits were filtered with a probability of 0.5 and a Mascot identity with a significant score cut-off greater than 26.

3. Results

Separation of the 10 – 30% sucrose gradient interphase produced mostly crystals Fig. 1 (B, C). The proteome of the *Bacillus thuringiensis* serovar *andalousiensis* BGSC-4AW1

strain was obtained by first identifying the proteome of the more complex structures shown in Figure 1A, and then the proteome of the purified crystals shown in Figure 1B. The complex structures were not affected by washing first with 1 M NaCl and then with $d_{ii}H_2O$. As shown in Figure 1A, these washing steps resulted in a combination of crystal-, and spore-containing structures surrounded by a membrane. These complex structures were dissolved in Laemmli loading buffer with β-mercaptoethanol, boiled for five minutes, and the proteins were separated on a 1D gel by electrophoresis (Fig. 2). As indicated in Figure 2A, the gel was cut into 10 equal bands, and each band was submitted to in-gel tryptic digestion, followed by protein identification as described under methods. Three hundred and forty-two unique proteins were identified. These proteins are presented in Table 1 (a detailed and extended table containing all the peptides and protein parameters is included in Supplemental Material S1).

The crystal inclusions were cleaned with NaCl, SDS, diiH2O, acetone, and ethanol, producing the crystals shown in Figure 1B (and inset in Fig. 1). These crystals were dissolved in Laemmli buffer with β-mercaptoethanol, boiled for five minutes and separated on a 1D PAGE gel (Fig. 2B).

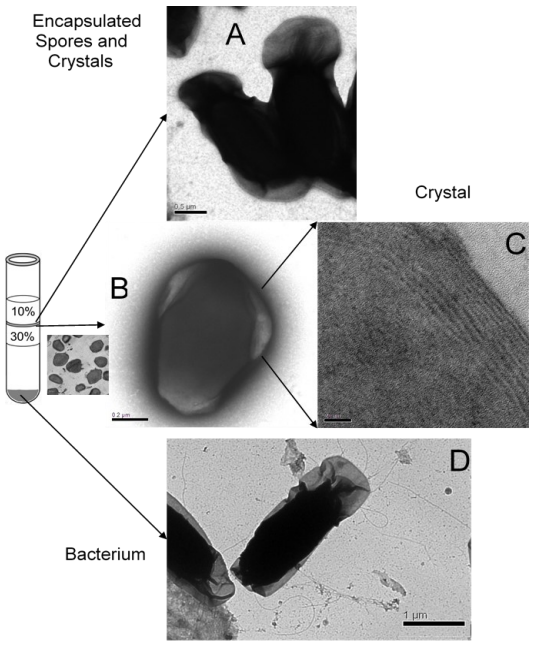

Figure 1. Purification of *Bt* ser. *andalousiesis crystals*. The diagram on the left represents a centrifugation tube with a sucrose gradient from which the 10 – 30 % interphase and the cell pellet were collected as previously described [19]. The interphase contains the structures shown in panels A and B. Panel A shows spores and crystals encapsulated in membranes that were obtained by washing these pellets with 1M NaCl; washing further with 1M NaCl, SDS, and acetone/ethanol produced the clean crystals shown in panel B (several crystals are shown in the inset next to the centrifugation tube); magnification indicates that these crystals are deposited in layers (Panel C). The pellet contained bacterium as indicated in panel D. Notice the sizes of each structure as indicated from the measuring bars at the bottom of each figure.

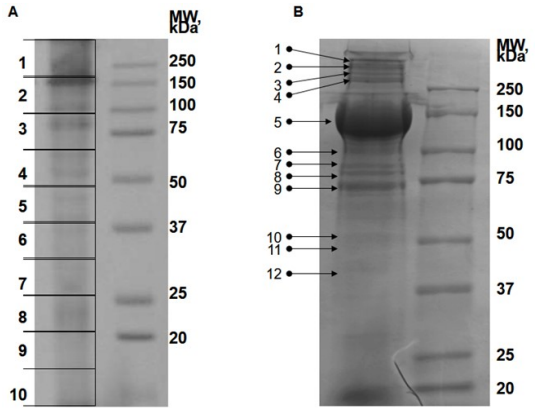

Figure 2. The fraction obtained by washing the 10 – 30 % interphase with 1.0M NaCl, and shown in Figure 1A, was mixed with 10 µL SDS loading buffer, boiled, and the proteins were separated by electrophoresis (A). This gel was cut in 10 equal bands, and each band was subjected to in-gel digestion. The crystals (Figure 1B), obtained by removing the membrane from the structure shown in Figure 1A, were dissolved in 10 µL SDS loading buffer, and boiled for five minutes. The proteins resulting from this separation were removed by cutting the bands as shown (B) and subjected to in-gel protein digestion. Resulting peptides were used to identify proteins.

As indicated in Figure 2B, the separation resulted in 12 major bands, which were excised from the gel and digested with trypsin. The peptides extracted from the gel bands were submitted to MS for protein identification, resulting in twenty-five proteins positively identified in these crystals (Table 2). Seven of these proteins have been reported as structural components of the exosporium on *Bacillus subtilis* preparations [26]. By far the most abundant proteins in these crystalline formations (as calculated from the intensity of the bands in the SDS-PAGE gel (Fig. 2B), and from peptide counting (Table 2), were the pesticidal crystal protein Cry8Ca (26 unique), the phosphatidylinositol-specific phospholipase C (PIPLC, 30 peptides), and the Cancer Cell-Killing Cry Protein (CC-KCP, 22 peptides). The characteristic mass spectra for these three proteins are shown in Figure 3 (a more detailed table with signal intensities and peptides is included in Supplemental Material S2) .

4. Discussion

Knowing the proteome of the crystal inclusions of any of the multiple *Bt* strains is of great importance because this proteome determines the specificity of the toxins, the interactions with the host organism, the virulence of the bacterium, and its capacity to survive deleterious environmental conditions. Many *Bt* strains produce crystal inclusions during their sporulation stage. Presumably, these crystals are responsible of triggering mechanisms that elicit survival responses to detrimental environmental conditions [3, 27-30].

The cellular proteome is dynamic, and there is not a simple way to describe "the proteome" per se; the proteomic

Table 1. *Bacillus thuringiensis* ser. *andalousiensis* (BGSC-4AW1) proteome.

Accession # *	Protein Name †	Coverage	Uniprot Accession Number ‡	Unique peptides
26130616	Heat shock protein 60 family chaperone GroEL	44.67	C3FXI3_BACTU	18
26130562	Alanine racemase	36.76	C3FXG9_BACTU	15
26137067	Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL	37.21	C3G3U6_BACTU	15
26135399	Phage lysin, glycosyl hydrolase, family 25	20.54	*Q637L8_BACCZ	14
26137609	ATP-dependent hsl protease ATP-binding subunit HslU	27.65	*C3I4U8_BACTU	12
26137169	Aldehyde dehydrogenase	35.63	C3G012_BACTU	11
26138644	Chaperone protein DnaK	28.97	C3G8C2_BACTU	11
26130540	4-hydroxyphenylpyruvate dioxygenase	29.30	C3FXF9_BACTU	10
26139154	Acetyl-coenzyme A synthetase	20.24	C3G997_BACTU	9
26135459	Acetoin dehydrogenase E1 component beta-subunit	31.40	*D5TMV5_BACT1	8
26140541	ATP synthase alpha chain	20.12	C3GB10_BACTU	7
26137175	Inosine-uridine preferring nucleoside hydrolase	32.91	C3G468_BACTU	7
26141141	N-acetylmuramoyl-L-alanine amidase	25.90	C3G524_BACTU	7
26130266	Translation elongation factor G	15.03	C3FX39_BACTU	7
26135461	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex	17.00	**Q97QN9_STRPN	6
26138664	forespore-specific protein, putative	34.11	*Q6HD99_BACHK	6
26137107	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	17.08	C3FXF4_BACTU	6
26132353	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1)	28.94	C3FXB7_BACTU	6
26137723	Pyruvate dehydrogenase E1 component alpha subunit	23.99	C3G7D7_BACTU	6
26137925	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	9.95	*C3CND0_BACTU	5
26140537	ATP synthase beta chain	21.96	C3GB10_BACTU	5
26141005	Dehydrogenase	15.37	*C3DYK1_BACTU	5
26134329	Methylmalonate-semialdehyde dehydrogenase	13.79	C3G373_BACTU	5
26140559	Serine hydroxymethyltransferase	16.71	C3GB19_BACTU	5
26130268	Translation elongation factor Tu	16.96	C3FX40_BACTU	5
26137565	3-oxoacyl-[acyl-carrier protein] reductase	28.86	C3G634_BACTU	4
26140715	alternate gene name: ipa-62r	28.28		4
26131779	Long-chain-fatty-acid--CoA ligase	12.55	C3FZH6_BACTU	4
26130278	LSU ribosomal protein L2p (L8e)	21.01	*C3FX36_BACTU	4
26132905	N-acetylmuramoyl-L-alanine amidase, family 2	34.09	C3FYZ3_BACTU	4
26140201	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	17.96	C3FYX5_BACTU	4
26131657	Polypeptide composition of the spore coat protein CotJC	33.86	*D5TQU6_BACT1	4
26136306	Possible response regulator aspartate phosphatase	13.48	A0RGR9_BACAH	4
26137725	Pyruvate dehydrogenase E1 component beta subunit	16.62	C3G7D6_BACTU	4
26132289	response regulator aspartate phosphatase	13.81	C3G382_BACTU	4
26130284	SSU ribosomal protein S3p (S3e)	22.83	*Q3EJF8_BACTI	4
26136897	Transketolase	8.68	C3G6G6_BACTU	4
26134341	2-methylcitrate synthase	12.87	*Q3EM07_BACTI	3
26138258	6,7-dimethyl-8-ribityllumazine synthase	26.14	C3G7S7_BACTU	3
26131175	Alanine dehydrogenase	12.20	C3FY87_BACTU	3
26140539	ATP synthase gamma chain	17.13	C3GB09_BACTU	3
26138646	Chaperone protein DnaJ	10.51	C3G8C1_BACTU	3
26139305	Citrate synthase (si)	9.42	C3G2R2_BACTU	3
26134974	Cysteine dioxygenase	29.69	C3G3I8_BACTU	3
26138156	Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase	8.25		3
26139637	DUF124 domain-containing protein	17.69	C3G9U8_BACTU	3
26132379	Enoyl-[acyl-carrier-protein] reductase [NADH]	19.53	C3FZV0_BACTU	3

26138156	Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase	8.25		3
26139637	DUF124 domain-containing protein	17.69	C3G9U8_BACTU	3
26132379	Enoyl-[acyl-carrier-protein] reductase [NADH]	19.53	C3FZV0_BACTU	3
26138854	Enoyl-CoA hydratase	16.67	C3G2S0_BACTU	3
26137611	GTP-sensing transcriptional pleiotropic repressor codY	13.90	C3G743_BACTU	3
26139551	hydrolase, alpha/beta fold family	14.87	A0RK75_BACAH	3
26139899	Iron-sulfur cluster assembly protein SufD	11.86	*F2H0D8_BACTU	3
26130246	LSU ribosomal protein L11p (L12e)	23.40	**Q3EK30_BACTI	3
26130282	LSU ribosomal protein L22p (L17e)	40.71	*A0R8I8_BACAH	3
26132911	NAD-specific glutamate dehydrogenase	7.71	*D5TUX8_BACT1	3
26133615	Peptidoglycan N-acetylglucosamine deacetylase	26.55	C3G1S2_BACTU	3
26140199	Phosphoglycerate kinase	12.18	C3GAJ4_BACTU	3
26130214	Putative ATP:guanido phosphotransferase YacI	10.45	C3FX13_BACTU	3
26133329	Putative symporter YjcG	7.17		3
26137249	response regulator, putative	10.99	*F0PW98_BACT0	3
26140713	Spore cortex-lytic enzyme CwlJ	32.86	*Q3F0G3_BACTI	3
26137163	Squalene--hopene cyclase	8.27	C3G636_BACTU	3
26130300	SSU ribosomal protein S8p (S15Ae)	30.30	**A0RBV3_BACAH	3
26140947	Tellurium resistance protein TerD	19.59	C3GBK3_BACTU	3
26131185	transcriptional regulator/TPR domain protein	7.11	*F0PUF9_BACT0	3
26133927	Tryptophan 2-monooxygenase	7.35	***	3
26139104	UDP-N-acetylmuramate--alanine ligase	9.40	C3G9C2_BACTU	3
26137481	Zinc protease	9.43	C3G6T9_BACTU	3
26138061	2,3-diketo-5-methylthiopentyl-1-phosphate enolase	6.04	C3G7K1_BACTU	2
26132843	2-isopropylmalate synthase	7.11	C3G0C8_BACTU	2
26137345	2-oxoglutarate oxidoreductase, beta subunit	8.68	*D5TUR1_BACT1	2
26134331	3-hydroxyisobutyrate dehydrogenase	12.16	C3G2R7_BACTU	2
26134325	3-hydroxyisobutyryl-CoA hydrolase	6.84	*Q3EKW7_BACTI	2
26137991	3-ketoacyl-CoA thiolase @ Acetyl-CoA acetyltransferase	9.21	*Q6HBP8_BACHK	2
26135847	5-Enolpyruvylshikimate-3-phosphate synthase	6.06	C3G4C0_BACTU	2
26139295	6-phosphofructokinase	9.09	C3G931_BACTU	2
26132847	Acetolactate synthase small subunit	13.61	C3G0C6_BACTU	2
26139961	acyl-CoA dehydrogenase	5.89	C3G2R6_BACTU	2
26130796	Alkyl hydroperoxide reductase protein C	15.51	C3FXT6_BACTU	2
26140225	ATP-dependent Clp protease proteolytic subunit	7.77	C3GAK6_BACTU	2
26133091	Biosynthetic Aromatic amino acid aminotransferase beta @ Histidinol-phosphate aminotransferase	7.03	*Q3EU27_BACTI	2
26135839	Chorismate mutase I / 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta	11.73	C3G4C4_BACTU	2
26139749	conserved domain protein	11.34	**Q630Y0_BACCZ	2
26136258	D-3-phosphoglycerate dehydrogenase	5.90	C3G515_BACTU	2
26130868	Delta-1-pyrroline-5-carboxylate dehydrogenase	5.44	*Q3EJ13_BACTI	2
26137729	Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex	6.38	*Q3ESR0_BACTI	2
26140193	Enolase	8.82	C3GAJ1_BACTU	2
26136157	FOG: TPR repeat	16.67	C3G8K3_BACTU	2
26135195	Glucose dehydrogenase [pyrroloquinoline-quinone]	7.35	*Q3EMX4_BACTI	2
26130070	Inosine-5'-monophosphate dehydrogenase	7.19	C3FWU7_BACTU	2
26135959	Inosine-uridine preferring nucleoside hydrolase in exosporium	11.92	C3G468_BACTU	2
26139307	Isocitrate dehydrogenase [NADP]	5.81	C3G924_BACTU	2
26139937	lipoprotein, putative	13.71	C3FXA6_BACTU	2
26133679	L-lactate dehydrogenase	6.69	C3GA71_BACTU	2

26130338	LSU ribosomal protein L13p (L13Ae)	15.86	*Q3EK30_BACTI	2
26130248	LSU ribosomal protein L1p (L10Ae)	13.48	**F9ZM33_9GAMM	2
26139347	LSU ribosomal protein L20p	17.80	**F9ZSH6_9GAMM	2
26139002	LSU ribosomal protein L27p	27.08	***F9XYU2_BIFBR	2
26130296	LSU ribosomal protein L5p (L11e)	13.97	**F9ZM55_9GAMM	2
26130302	LSU ribosomal protein L6p (L9e)	13.97	***G0AGC3_9BURK	2
26137959	Maltose/maltodextrin transport ATP-binding protein MalK	6.56	***G0DAV7_ECOLX	2
26138788	MaoC family protein	17.60	*A0RIX7_BACAH	2
26139651	Naphthoate synthase	8.46	C3G9U1_BACTU	2
26140743	O-acetylhomoserine sulfhydrylase / O-succinylhomoserine sulfhydrylase	6.94	C3GBA8_BACTU	2
26132333	Oligoendopeptidase F	4.61	C3FZS6_BACTU	2
26132355	Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)	7.49	C3FXB7_BACTU	2
26137351	Outer spore coat protein E	13.89	**E5W466_9BACI	2
26130462	oxidoreductase of aldo/keto reductase family, subgroup 1	7.94	**F7SFD7_LACJH	2
26139351	Peptidase, M42 family	5.26	**F0PL34_BACT0	2
26136256	Phosphoserine aminotransferase	6.39	C3G516_BACTU	2
26137655	Polyribonucleotide nucleotidyltransferase	3.93	C3G723_BACTU	2
26137767	Predicted ATPase related to phosphate starvation-inducible protein PhoH	5.66	***D6D447_9BACE	2
26139214	Protein ecsC	10.83	*Q3EZS0_BACTI	2
26137743	Protein-glutamine gamma-glutamyltransferase	10.87	C3G7C8_BACTU	2
26134986	putative cytochrome P450 hydroxylase	7.06	***A4FDF8_SACEN	2
26130074	Pyridoxine biosynthesis glutamine amidotransferase, synthase subunit	8.14	***G0V494_9CLOT	2
26137775	Pyruvate carboxyl transferase	2.09	**F5VEX4_9LACO	2
26138658	Quinolinate phosphoribosyltransferase [decarboxylating]	10.11	C3G9C3_BACTU	2
26140875	Single-stranded DNA-binding protein	11.56	C3GBG8_BACTU	2
26130774	Spore coat protein B	18.12	C3FXU6_BACTU	2
26135521	Spore coat protein F	17.50	C3G208_BACTU	2
26130132	Sporulation-specific protease YabG	8.36	C3FWX6_BACTU	2
26130270	SSU ribosomal protein S10p (S20e)	31.37	**F9ZM42_9GAMM	2
26130324	SSU ribosomal protein S11p (S14e)	21.71	**F9ZMC9_9GAMM	2
26138628	SSU ribosomal protein S20p	34.12	*Q3EJD4_BACTI	2
26130306	SSU ribosomal protein S5p (S2e)	14.46	**F9ZM59_9GAMM	2
26132873	Stage IV sporulation protein A	7.85	C3G0N6_BACTU	2
26137595	Succinyl-CoA ligase [ADP-forming] beta chain	7.51	C3G6V7_BACTU	2
26130210	Transcriptional regulator CtsR	13.73	*F0PQ24_BACT0	2
26137473	Unspecified monosaccharide ABC transport system, substrate-	7.04	***F3Y8A0_MELPT	2
26137713	Zn-dependent hydrolase, RNA-metabolising, CPSF 73 kDa analog	5.41	C3G129_BACTU	2
26134339	2-methylcitrate dehydratase	2.51	C3G2R3_BACTU	1
26132617	3-hydroxybutyryl-CoA dehydratase	8.16	C3FZ01_BACTU	1
26140615	3-hydroxybutyryl-CoA dehydrogenase	6.36	C3GB46_BACTU	1
26132841	3-isopropylmalate dehydrogenase	3.67	C3G0C9_BACTU	1
26140617	3-ketoacyl-CoA thiolase [isoleucine degradation]	3.82	*A0RKJ8_BACAH	1
26136135	3-Oxoadipate enol-lactonase, alpha/beta hydrolase fold family	4.33	*F0PJ99_BACT0	1
26138874	4-hydroxybenzoyl-CoA thioesterase family active site	5.41	***C6X3K5_FLAB3	1
26130956	A/G-specific adenine glycosylase	1.64	*D5TNJ4_BACT1	1
26130932	ABC transporter, permease protein, putative	4.21	C3G2K1_BACTU	1
26139208	Acetate kinase	2.77	C3G971_BACTU	1
26134726	Acetoacetyl-CoA synthetase [leucine]	1.86	***D4YLV6_9MICO	1
26136545	acetyltransferase, GNAT family	11.84	C3G3Q3_BACTU	1

26139192	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases, YtcI homolog	3.03	***A4VZ54_STRS2	1
26140863	Adenylosuccinate synthetase	1.40	C3GBG2_BACTU	1
26134287	alcohol dehydrogenase, iron-containing	3.00	C3G8H9_BACTU	1
26138176	Amino acid ABC transporter, amino acid-binding protein	4.63	*D5TZE9_BACT1	1
26134834	Aminoacyl-histidine dipeptidase (Peptidase D)	3.27	C3G359_BACTU	1
26138127	Arginine pathway regulatory protein ArgR, repressor of arg regulon	7.38	***F1W4T9_9BURK	1
26139226	Argininosuccinate lyase	4.33	C3G963_BACTU	1
26130844	Aspartyl-tRNA(Asn) amidotransferase subunit A @ Glutamyl-tRNA(Gln) amidotransferase subunit A	3.30	C3FXR2_BACTU	1
26132833	ATP phosphoribosyltransferase catalytic subunit	9.48	C3G0D3_BACTU	1
26138944	ATP-dependent Clp protease ATP-binding subunit ClpX	2.15	C3G8S7_BACTU	1
26130216	ATP-dependent Clp protease, ATP-binding subunit ClpC / Negative regulator of genetic competence clcC/mecB	1.60	***D3QG46_STALH	1
26134176	ATP-dependent RNA helicase YfmL	3.08	**YFML_BACSU	1
26136015	Bacillus cereus group-specific protein, uncharacterized	10.34	**Q6HQP9_BACAN	1
26130636	Beta-galactosidase	4.17	*C3HT03_BACTU	1
26136410	bifunctional P-450:NADPH-P450 reductase 1	1.03	*F0PJ37_BACT0	1
26138109	Biotin carboxylase of acetyl-CoA carboxylase	3.11	C3G932_BACTU	1
26133669	Branched-chain amino acid transport ATP-binding protein LivF (TC 3.A.1.4.1)	6.87	*Q3EWR4_BACTI	1
26132059	Catalase	3.07	C3FZM4_BACTU	1
26137519	cation-transporting ATPase, E1-E2 family	1.77	C3FXW8_BACTU	1
26130970	Cell division inhibitor	4.98	*D5TNI9_BACT1	1
26137857	Cell division protein FtsZ	3.65	*D5TVH6_BACT1	1
26138942	Cell division trigger factor	5.41	C3G8S8_BACTU	1
26132299	CMP-binding-factor 1	5.10	*C3GT26_BACTU	1
26139006	COG0536: GTP-binding protein Obg	3.50	C3G8P6_BACTU	1
26137185	conserved repeat domain protein	0.36	A0RH48_BACAH	1
26137435	Copper-translocating P-type ATPase	0.99	*A0RH48_BACAH	1
26138770	Cystathionine gamma-lyase	5.31	*A0RIY5_BACAH	1
26130946	Cysteinyl-tRNA synthetase related protein	6.82	*SYC_BACHK	1
26130746	Cystine-binding periplasmic protein precursor	8.02	**D3QWU1_ECOCB	1
26137785	Cytochrome c oxidase polypeptide III	4.35	*D5TVL2_BACT1	1
26138898	Cytosine deaminase	3.22	*D5TJA1_BACT1	1
26132697	D-alanine--poly(phosphoribitol) ligase subunit 1	3.17	*DLTA_BACAH	1
26131761	Deblocking aminopeptidase	3.15	*D5TRT4_BACT1	1
26135759	Delta5 acyl-lipid desaturase	1.75	C3G4G3_BACTU	1
26137491	Dihydrodipicolinate synthase	5.14	C3G6U9_BACTU	1
26137727	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	3.50	Q3ESR0_BACTI	1
26138840	DinB family protein	13.77	**A7GTJ0_BACCN	1
26137665	Dipicolinate synthase subunit B	9.59	C3G6V2_BACTU	1
26130220	DNA integrity scanning protein disA	3.36	C3FX16_BACTU	1
26130056	DNA polymerase III beta subunit	2.90	*RPOB_BACHK	1
26134553	DNA-binding protein HBsu	12.22	**D3QCS2_STALH	1
26130326	DNA-directed RNA polymerase alpha subunit	6.37	*RPOA_BACAH	1
26139763	Dolichol-phosphate mannosyltransferase MtrA	2.09	**C7UHF8_ENTFA	1
26132381	dTDP-4-dehydrorhamnose reductase	4.23	C3FZU9_BACTU	1
26135215	DUF1696 domain-containing protein	12.12	**F7RKW6_9GAMM	1
26139967	Enoyl-CoA hydratase	2.14	*A0RLC8_BACAH	1
26134868	Exosporium protein F	5.99	*Q81TN4_BACAN	1
26140593	Fructose-1,6-bisphosphatase, GlpX type	5.30	*D5TQJ5_BACT2	1
26133385	Fumarate hydratase class II	3.25	C3G187_BACTU	1

26139120	General stress protein	8.24	*D5TKV6_BACT1	1
26138121	Geranyltranstransferase (farnesyldiphosphate synthase)	2.03	C3G7Z3_BACTU	1
26130388	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	2.00	C3FX89_BACTU	1
26139711	Glucose-6-phosphate isomerase	4.44	C3G9W1_BACTU	1
26138966	Glutamate-1-semialdehyde aminotransferase	5.13	C3G8R6_BACTU	1
26131267	Glutamine ABC transporter, periplasmic glutamine-binding protein	5.80	**Q98FF6_RHILO	1
26139088	Glutamyl aminopeptidase; Deblocking aminopeptidase	4.48	C3G9D0_BACTU	1
26138470	Glycine dehydrogenase [decarboxylating] (glycine cleavage system	2.68	*C3CNZ8_BACTU	1
26139745	Glycyl-tRNA synthetase	2.40	C3G9X8_BACTU	1
26132979	GTP cyclohydrolase I type 1	5.29	C3G0N8_BACTU	1
26132885	GTP-binding protein EngA	2.75	C3G0N0_BACTU	1
26138520	GTP-binding protein Era	3.32	C3G8A9_BACTU	1
26138640	Heat-inducible transcription repressor HrcA	6.21	C3G8C4_BACTU	1
26132235	Histidine triad (HIT) nucleotide-binding protein, similarity with	8.33	*HINT_CAEEL	1
26132831	Histidinol dehydrogenase	2.80	*HISX_BACHK	1
26132817	Histidinol-phosphatase	3.25	C3G2F7_BACTU	1
26138708	Histidyl-tRNA synthetase	2.52	*SYH1_BACHK	1
26140739	Homoserine dehydrogenase	3.02	*D5TRH2_BACT1	1
26133603	Homoserine kinase	6.40	C3G1S8_BACTU	1
26139467	Homoserine O-acetyltransferase	4.28	C3G9H6_BACTU	1
26130180	Hypoxanthine-guanine phosphoribosyltransferase	6.11	C3FWZ8_BACTU	1
26140901	Inner membrane protein translocase component YidC, short form OxaI-like	4.31	**C1AJ61_MYCBT	1
26131102	Inner membrane protein YqiK	3.44		1
26134738	Isovaleryl-CoA dehydrogenase	2.89	**C3VMZ0_BACPU	1
26138234	Lipase/Acylhydrolase with GDSL-like motif	5.79	C3GBA3_BACTU	1
26137939	lipoprotein, putative	6.36	C3FXQ7_BACTU	1
26132303	Macrocin O-methyltransferase	4.63	D5TTE7_BACT1	1
26141217	Mannose-6-phosphate isomerase	9.52	C3GAX9_BACTU	1
26133083	Menaquinone-cytochrome C reductase iron-sulfur subunit	7.65	C3G0P9_BACTU	1
26133081	Menaquinone-cytochrome c reductase, cytochrome B subunit	7.59	C3G0Q0_BACTU	1
26130972	Metallo-dependent hydrolases, subgroup C	5.32		1
26134728	Methylcrotonyl-CoA carboxylase carboxyl transferase subunit	2.34		1
26134337	Methylisocitrate lyase	3.31	A0RDV8_BACAH	1
26140235	Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase)	7.24	C3FY36_BACTU	1
26136436	N-acyl-L-amino acid amidohydrolase	3.24	C3G5A7_BACTU	1
26139198	NAD kinase	6.37	Q3EUG8_BACTI	1
26140051	NADH dehydrogenase	4.59	C3FXT5_BACTU	1
26137453	NADH-dependent butanol dehydrogenase A	4.39	A0RH94_BACAH	1
26131183	Nicotinate phosphoribosyltransferase	2.46	C3G9C3_BACTU	1
26134116	Nitrite reductase [NAD(P)H] large subunit	1.37	*D5TY66_BACT1	1
26132567	oligoendopeptidase F, putative	2.66	C3FZS6_BACTU	1
26132359	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	4.21	C3FYF1_BACTU	1
26132357	Oligopeptide transport system permease protein OppC (TC 3.A.1.5.1)	3.55	C3G2Y2_BACTU	1
26138318	outer membrane protein CC2294	7.62		1
26135311	Penicillin-binding protein	5.00	C3G2H1_BACTU	1
26137317	peptidase, M16 family	17.14	Q3ELP4_BACTI	1
26132119	peptidase, M48 family	2.61	C3DCG3_BACTU	1
26132615	phaP protein	5.75	Q3EJ17_BACTI	1
26138798	Phenylalanine-4-hydroxylase - Long	2.57	A0RIX2_BACAH	1

26132825	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	7.11	HIS4_BACAH	1
26134918	Possible caffeoyl-CoA O-methyltransferase	8.25	*A0RE47_BACAH	1
26132773	Protein LiaH, similar to phage shock protein A	6.36	‡C3G9P8_BACTU	1
26130998	PTS system, N-acetylglucosamine-specific IIB component / PTS	2.62	C3FXZ7_BACTU	1
26139297	Pyruvate kinase	2.39	C3G930_BACTU	1
26130148	Ribose-phosphate pyrophosphokinase	4.10	C3FWY4_BACTU	1
26140307	Ribosomal subunit interface protein	6.11	Q6HB98_BACHK	1
26141003	RNA polymerase sigma-70 factor, ECF subfamily	4.15	C3GBN1_BACTU	1
26137861	RNA polymerase sporulation specific sigma factor SigE	6.28	C3G8E8_BACTU	1
26138982	Rod shape-determining protein MreB	5.31	C3G8Q8_BACTU	1
26139481	S-adenosylmethionine synthetase	3.76	C3G9L2_BACTU	1
26139190	Small acid-soluble spore protein, alpha/beta family, SASP_5	21.54	*F0PTE1_BACT0	1
26135122	Spore cortex-lytic enzyme, lytic transglycosylase SleB	7.11	C3G3U6_BACTU	1
26141029	Spore germination protein GerKC	2.81	*F0PIP2_BACT0	1
26130322	SSU ribosomal protein S13p (S18e) [Bacillus thuringiensis serovar andalousiensis BGSC 4AW1] - [locus|VBIBacThu67491_3297|]	9.09	*Q3EJF8_BACTI	1
26132897	SSU ribosomal protein S1p	3.40	*Q3ENL3_BACTI	1
26138508	Stage IV sporulation protein	1.75	C3G8Q3_BACTU	1
26137339	Stage V sporulation protein required for dehydratation of the spore core and assembly of the coat (SpoVS)	19.77	*Q6HJH0_BACHK	1
26138870	Succinate dehydrogenase iron-sulfur protein	6.32	*Q6HD09_BACHK	1
26130832	Succinate-semialdehyde dehydrogenase [NADP+]	2.48	C3G2M0_BACTU	1
26137083	Succinyl-CoA synthetase, alpha subunit-related enzymes	9.49	*D5TTV7_BACT1	1
26132957	Superoxide dismutase [Fe] @ Exosporium SOD	4.61	‡C3G885_BACTU	1
26135303	ThiJ/PfpI family protein	8.42	A0RA98_BACAH	1
26139204	Thiol peroxidase, Tpx-type	7.83	C3G973_BACTU	1
26137055	Thiol:disulfide oxidoreductase related to ResA	5.24	*RESA_BACHK	1
26139793	Thioredoxin reductase	5.78	C3GAL3_BACTU	1
26139341	Threonyl-tRNA synthetase	1.40	C3G907_BACTU	1
26140591	Transcription termination factor Rho	2.60	C3GB35_BACTU	1
26135155	Transcriptional regulator, AraC family	10.69	C3G9S1_BACTU	1
26138402	Transcriptional regulator, ArsR family	11.46	C3G4F2_BACTU	1
26137615	Translation elongation factor Ts	3.73	C3G741_BACTU	1
26141297	Transposase	8.33	C3GBR5_BACTU	1
26140197	Triosephosphate isomerase	5.18	C3GAJ3_BACTU	1
26136541	Ubiquinone/menaquinone biosynthesis methyltransferase UBIE	2.39	C3FYB2_BACTU	1
26138546	VrrA protein	6.80	C3G896_BACTU	1

*: Accession number from http://patricbrc.org
†: Protein name from http://patricbrc.org
‡: Accession number from http://www.uniprot.org

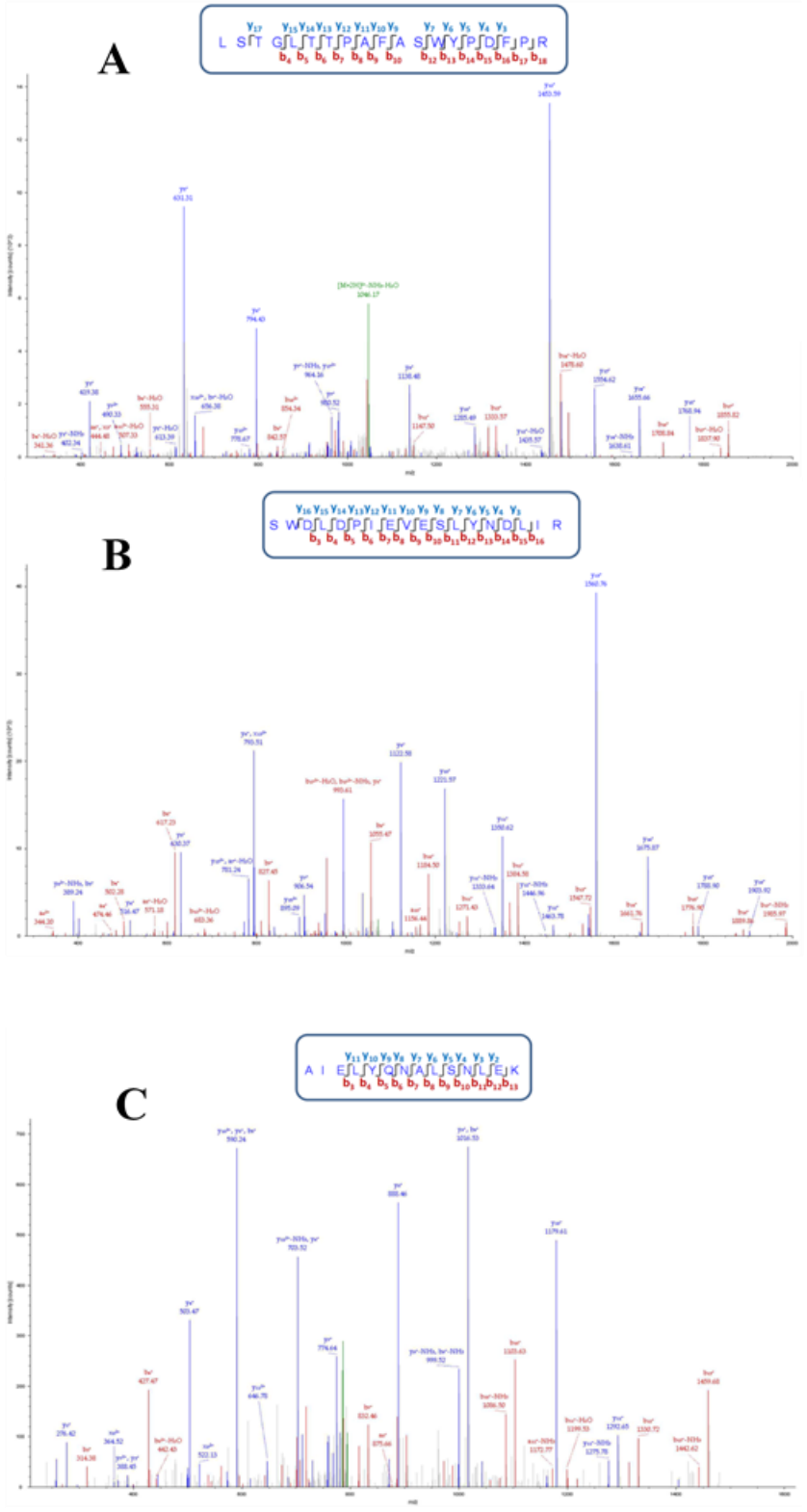

Figure 3. A doubly-charged peptide representing (A) Phosphatidylinositol-specific phospholipase C (PI-PLC); (B) Cry8Ca insecticidal protein; and (C) Cancer cell-killing Cry protein (CC-KCP). b- and y-types fragment ions for each sequence are shown in the sequence map above each MS/MS spectrum. All of the identified peptides along with the protein IDs and the protein parameters are included in Supplemental data S1.

Table 2. The proteome of purified crystal inclusions from *Bt* ser. *andalousiensis* (BGSC-4AW1) .

Accession Number	Protein name	Peptide Counts	Coverage	Number of Unique peptides	MW [kDa]	Calc. pI
26141719	Phosphatidylinositol-specific phospholipase C	334	32.38	30	142.9	5.85
26141725	Pesticidal crystal protein cry8Ca	384	43.80	26	87.8	5.97
26141099	cancer cell-killing Cry protein	254	34.21	22	95.5	5.54
26141097	hypothetical protein	89	27.56	9	68.5	5.20
26138644	Chaperone protein DnaK	30	20.79	8	65.7	4.74
26137169	Aldehyde dehydrogenase	10	19.23	8	53.7	5.45
26137067	Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL	19	19.77	7	48.1	9.20
26130562	Alanine racemase	24	21.59	7	57.4	4.84
26130616	Heat shock protein 60 family chaperone GroEL		9.56	4	43.6	5.73
26138942	Cell division trigger factor		8.47	3	47.2	4.58
26137105	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)		2.28	2	63.9	8.31
26140541	ATP synthase alpha chain		3.39	2	54.6	5.44
26140537	ATP synthase beta chain		5.33	2	51.2	5.05
26141301	hypothetical protein		7.94	2	29.2	4.45
26130268	Translation elongation factor Tu		7.34	2	43.5	6.02
26135461	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex		4.50	2	42.9	5.01
26137435	Copper-translocating P-type ATPase		0.99	1	86.7	5.74
26130266	Translation elongation factor G		1.88	1	76.3	4.98
26130904	hypothetical protein		1.35	1	51.3	9.07
26137611	GTP-sensing transcriptional pleiotropic repressor codY		3.09	1	28.8	5.12
26130296	LSU ribosomal protein L5p (L11e)		4.47	1	20.2	9.70
26135447	hypothetical protein		4.76	1	14.8	9.17
26134587	Exosporium protein D		9.68	1	40.3	8.32
26132369	Exosporium protein F		5.99	1	17.6	5.77
26138646	Chaperone protein DnaJ		3.23	1	17.4	4.49

content of a cell is time- and environment-dependent, therefore we are limited to describing the proteome under specific conditions. In the case of *Bt*, it should be extremely useful knowing the proteome under life-sustaining conditions, and how this proteome changes when the bacterium has to adapt to food-depleted, or extreme environmental conditions, which ultimately result in spore development, and/or formation of crystalline inclusion bodies.

The *Bt* ser. *andalousiensis* proteome presented here points to sets of proteins that may be involved in multiple functions associated with cell growth, including spore formation, spore, coat and exosporium functions, and crystal formation. These protein sets may also be part of the protein mechanisms associated with cellular adaptation to nutrient depletion and adaptation to changes in environmental conditions. The proteomic analysis resulted in the confident identification of 342 distinct proteins (130 of these proteins were identified with two or more peptides, Table 1. See supplemental data for detailed peptide information). Many of these proteins are involved in exosporium, spore, and coat functions, as well as cellular function (Tables 1 and 2; and Fig. 4).

Some proteins presented in Table 1 deserve special attention. Using annotated databases (http://www.uniprot.org/; NCBI, and ExPaSy), we classified 18 proteins as belonging to the spore/coat/exosporium complex (Table 2 and Figure 4A; extended results in Supplemental Material S2). Twenty-nine proteins (Fig. 4 and Supplemental Material S1) are involved in cell division; these proteins must have functions associated with normal cellular growth and development, as well as re-adaptation following a period of deleterious conditions. Two groups of proteins, the Large and Small Ribosomal Subunits (LSU, and SSU, with 14 and 12 proteins, respectively) were found in this study, suggesting that their high numbers should provide the bacterium with the ability to trigger cell growth and cell division when the conditions become favorable.

Identification of all the proteins in this study, and analysis of the protein interaction networks were performed by interrogating NCBI and the "Patric" database (http://patricbrc.org/). Proteins were mapped to the identical proteins or to their closest match in the NCBI database via UNIPROT (http://www.uniprot.org). UNIPROT accession

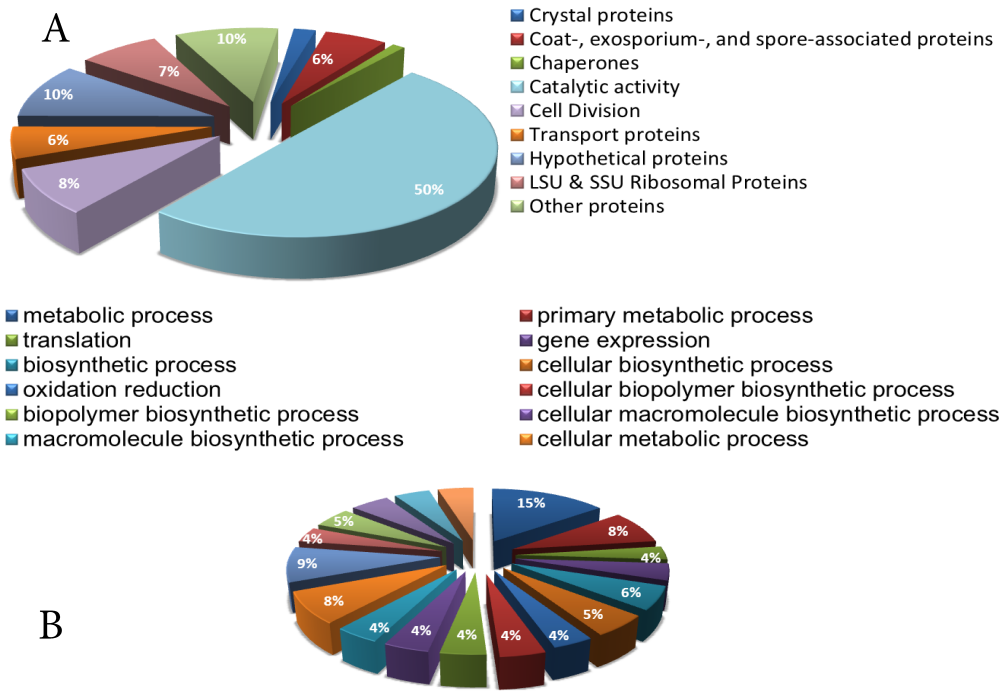

Figure 4. Pie distribution of *Bt* ser. *andalousiensis* proteins.

numbers for most of the proteins found are indicated in Table 1, column 3. Many of these proteins are still not described in this database. In Table 1, one asterisk (*) indicates that the protein was not found in *andalousiensis*; however, it has been identified in another *Bt* strain; two asterisks (**) indicate that the protein was not found in *Bt*, but it was found in other bacilli species, such as *Bacillus cereus*; and three asterisks (***) indicate a protein found in a non-*Bt*-related organism. Two proteins were not found in any database.

The proteins found in the exosporium/crystal complex were classified according to the information available in UNIPROT. The exact number of proteins that are specifically associated with the crystals may be difficult to elucidate because many non-specific proteins become entrapped either during the inclusion-body formation process, or during the crystal purification steps. We therefore counted the peptides identified by MS, and considered only those proteins with 7 or more peptides identified in two independent experiments as being uniquely associated with the crystals. Most of the proteins in the complexes possess catalytic function (50%, Fig. 4A), while 5 (1.5 %) are chaperones. A functional classification of proteins was performed using the online server "Pandora" (http://www.pandora.cs.huji.ac.il/) [31], Fig. 4B.

A large portion of the crystal proteome consists mainly of (See Figure 2B, Table 2; and extended information in Supplemental Material S2 and S3) pesticidal crystal protein Cry8Ca (Accession # from http://patricbrc.org, 261417259; the UNIPROT accession numbers are included in Table 1), phosphatidylinositol-specific phospholipase C (PI-PLC, 26141719), and cancer cell-killing Cry protein (CC-KCP, 26141099). These three proteins represented the majority of the protein content in the crystal and were consistently found in several independent crystal preparations. The remaining significant (>7 peptides in two independent MS runs) proteins in the crystal included hypothetical protein (26141097), chaperone protein DnaK (26138644), aldehyde dehydrogenase (26137169), and spore cortex-lytic enzyme, N-acetylglucosaminidase SleL (26137067). Five of the proteins found in our crystal preparations were previously found as constitutive proteins of the *Bacillus subtilis* exosporium [26] (Supplemental Material S1, and S2).

The presence of three toxins with a wide spectrum of action such as PI-PLC, Cry8Ca, and CC-KCP confers this bacterium the possibility of poisoning a large variety of target organisms. Specifically, Cry8Ca has shown toxicity against cupreous chafer (*Anomala cuprea, coleoptera Scarabaeidae* [32] and *Anomala corpulenta* [33]), *Bt* ser. *andalouisiensis* has shown toxic activity against the coleopteran *Tenebrio molitor* [34], and our preliminary studies on glioblastoma cells (data not included here) shows that purified lysates of BGSC-4AW1 are able to dissolve these cells in culture .

5. Concluding Remarks

The proteome of the *Bt* ser. *andalousiensis* presented here provides data that should be useful for determining the specificity of the bacterium towards target organisms, for understanding the capacity of the spore to survive for long periods of time under non-favorable conditions, as well as

for its ability to "revive" when the conditions turn favorable. Even though *Bt* has been used in biotechnology applications for several decades, the data presented here, and in other publications referenced above, demonstrate further that this is a rich mine of molecules with a wide functional spectrum that could offer endless opportunities for biotechnological applications.

6. Supplementary Material

1. Supplemental Material S1: Mass spectrometry data, protein IDs, identification parameters for complex *Bt* ser. *andalousiensis mixtures*. 2. Supplemental Material S2: Mass spectrometry data, protein IDs, identification parameters for complex Bt. *ser. andalousiensis* crystals. 3. Supplemental Material S3: Complementary mass spectrometry data, protein IDs, identification parameters for *Bt* ser. *andalousiensis*.

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