Functional Annotation Analytics of Bacillus Genomes Reveals Stress Responsive Acetate Utilization and Sulfate Uptake in the Biotechnologically Relevant Bacillus megaterium

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Abstract: Bacillus species form an heterogeneous group of Gram-positive bacteria that include members that are disease-causing, biotechnologically-relevant, and can serve as biological research tools. A common feature of Bacillus species is their ability to survive in harsh environmental conditions by formation of resistant endospores. Genes encoding the universal stress protein (USP) domain confer cellular and organismal survival during unfavorable conditions such as nutrient depletion. As of February 2012, the genome sequences and a variety of functional annotations for at least 123 Bacillus isolates including 45 Bacillus cereus isolates were available in public domain bioinformatics resources. Additionally, the genome sequencing status of 10 of the B. cereus isolates were annotated as finished with each genome encoded 3 USP genes. The conservation of gene neighborhood of the 140 aa universal stress protein in the B. cereus genomes led to the identification of a predicted plasmid-encoded transcriptional unit that includes a USP gene and a sulfate uptake gene in the soil-inhabiting Bacillus megaterium. Gene neighborhood analysis combined with visual analytics of chemical ligand binding sites data provided knowledge-building biological insights on possible cellular functions of B. megaterium universal stress proteins. These functions include sulfate and potassium uptake, acid extrusion, cellular energy-level sensing, survival in high oxygen conditions and acetate utilization. Of particular interest was a two-gene transcriptional unit that consisted of genes for a universal stress protein and a sirtuin Sir2 (deacetylase enzyme for NAD+-dependent acetate utilization). The predicted transcriptional units for stress responsive inorganic sulfate uptake and acetate utilization could explain biological mechanisms for survival of soil-inhabiting Bacillus species in sulfate and acetate limiting conditions. Considering the key role of sirtuins in mammalian physiology additional research on the USP-Sir2 transcriptional unit of B. megaterium could help explain mammalian acetate metabolism in glucose-limiting conditions such as caloric restriction. Finally, the deep-rooted position of B. megaterium in the phylogeny of Bacillus species makes the investigation of the functional coupling acetate utilization and stress response compelling.

Keywords: ATP-binding, acetate utilization, Bacillus, Bacillus cereus, Bacillus megaterium, Sir2, sirtuins, sulfate uptake, universal stress proteins
Introduction

*Bacillus* species form an heterogeneous group of Gram-positive bacteria that include members that are disease-causing, biotechnologically-relevant, and can serve as biological research tools.1–5 A common feature of *Bacillus* species is their ability to survive in harsh environmental conditions via the formation of resistant endospores. Regulation of gene activity in response to environmental conditions is a well-defined mechanism in *Bacillus*.2 The formation and germination of highly resistant endospores are known responses to unfavorable environmental conditions.6 These dormant cells allow *Bacillus* species to survive extreme conditions such as elevated temperatures, extreme pH and salinity changes. The physiological feature of spore formation is evident in food processing, where exposure to mild preservation elicits an adaptive stress response, resulting in *Bacillus* spores found in food.7 Because spores can also attach to numerous substrates8 and persist in the soil for years9, detection of genes associated with their survival could be critical in understanding pathogenesis of endospore-forming *Bacillus* species.

Genes encoding the universal stress protein (USP) domain have been detected in diverse organisms, including plants, bacteria and fungi.10,11 The universal stress proteins (defined by the Protein Family (Pfam) accession PF00582) confer survival during extreme conditions such as drought,12 osmolarity,13 oxidative stress due to hydrogen peroxide14 and acid response.15 The universal stress proteins have been classified broadly based on their ability to bind or not bind ATP.16,17 The soil-inhabiting *Bacillus subtilis* must overcome unfavorable conditions in the soil, including nutritional stresses.18 Before the completion of the genome sequence of *B. subtilis* 168, genomic regions containing genes that encode the USP domain were investigated. The *yxiE* gene, which encodes a 148 aa protein, was shown to be part of a three-gene operon for aryl-beta-glucoside utilization and the operon induced only when cells were grown in salicin, a plant-derived carbohydrate.19 Additionally, *yxiE* was revealed by DNA sequencing to be in the genomic region between the histidine utilization operon (*hut*) and wall-associated protein A (*wapA*) loci in the *B. subtilis* 168 genome.20 Another USP domain encoding gene, *nhaX* (also termed *yheK*) was found between a serine protein kinase (*prkA*) and ATP-dependent nuclease (*addAB*) loci in the *B. subtilis* 168 genome.21 Both *yxiE* and *yheK* are induced in phosphate starvation.22 The public availability of the genome sequences of more than 100 *Bacillus* isolates present new opportunities to extensively analyze the functional annotation of the universal stress proteins encoded in *Bacillus* genomes.

As of February 2012, the genome sequences and a variety of functional annotations for at least 123 *Bacillus* isolates, including 45 *Bacillus cereus* isolates, were available in the Integrated Microbial Genomes (IMG) database and comparative analysis system.23 The number of USP gene per *Bacillus* genome ranged from 1 (strains ATCC 7061 and SAFR-032 of *Bacillus pumilus*) to 6 (*Bacillus megaterium* QM B1551 and *B. selenitireducens* MLS10). The draft genome of marine *Bacillus* species NRRL B-1491124 encodes 5 USP genes. Additionally, the genome sequencing status of 10 of the *B. cereus* isolates were annotated as finished with each genome encoded with 3 USP genes. Clustering the USPs from the 10 genomes by amino acid length revealed two USPs (140 aa and 152 aa) that encoded only the universal stress protein domain. The third gene encoded protein where a kinase domain is present with the USP domain.

The reported research focused on the two *B. cereus* genes that encode only the USP domain. The annotated function and direction of transcription of adjacent genes of the two *Bacillus cereus* USP genes were determined in the 10 finished genomes. Further, the identity of the amino acid for binding to ATP and other ligands in orthologous universal stress proteins of *Bacillus cereus* were compared and the transcription of two USP genes in growing cultures of a strain of *B. cereus* were determined. The conservation of the gene neighborhood of the 140 aa USP in the *B. cereus* genomes led to the identification of a predicted plasmid-encoded transcriptional unit that includes a USP gene and a sulfate uptake gene in the soil-inhabiting *B. megaterium*. Additional gene neighborhood analysis of genes for universal stress proteins in *B. megaterium* identified a predicted two-gene transcriptional unit that consists of a USP gene and a sirtuin (deacetylase enzyme for NAD++-dependent acetate utilization). The predicted transcriptional units for stress responsive inorganic sulfate uptake and acetate utilization could explain biological mechanisms for survival of soil-inhabiting *Bacillus* species in sulfate and acetate limiting conditions.
Materials and Methods
Function and transcription direction of adjacent genes for genes encoding universal stress proteins
The function and transcription direction of adjacent genes for two genes encoding universal stress proteins were compared for the following 10 finished Bacillus cereus genomes: 03BB102; AH187 (F4810/72); AH820; B4264; ATCC 10987; ATCC 14579; cytotoxis NVH 391-98; G9842; Q1 and E33L (ZK). The Gene Neighborhoods function in the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov) was used to construct a chromosomal map of the regions with the two genes encoding the 140 aa and 152 aa universal stress proteins. The transcription direction and function (as defined by Cluster of Orthologous Groups [COG] of Proteins; http://www.ncbi.nlm.nih.gov/COG/) were determined for the adjacent genes. To facilitate the comparison of the adjacent genes, we used a 3-digit binary accession to encode the direction of the transcription, 1 in the same transcription direction as the USP gene and 0 in the opposite transcription direction of the USP gene. The transcription direction for the USP gene was assigned a 1. The binary accession allows us to predict stress response equipped biological functions. Therefore, a binary accession of 111, 110, 011 are potential chromosomal encoded pathways. The potential pathways are further verified using transcriptional units provided by the BioCyc Collection of Pathway and Genome Databases.

Prediction of ligand binding sites for Bacillus cereus universal stress proteins
The amino acid residues for ATP binding and other ligands for the 30 Bacillus cereus universal stress proteins were predicted using the web interface to the Batch Conserved Domain (CD)-Search available at http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi. The GenBank protein accession for the protein sequences were obtained from the Integrated Microbial Gemone (IMG) system and submitted to Batch CD-Search. The ligand binding sites predicted for the USPs were processed using a computer script into a data set suitable for comparison of the ligand binding sites. Each record of the dataset consisted of (i) amino acid residue; (ii) position of the amino acid in the sequence; (iii) GenBank Accession; (iv) Ligand Site position; (v) ligand type; (vi) Protein domain type; and (vii) protein domain in Conserved Domain Database. The data set was processed using a Visual Analytics software (http://www.tableausoftware.com/public/) to compare the identity of the amino acid residues involved in ligand binding. A visual analytics approach can enable knowledge building biological insights to be accomplished from analyzing multiple types of datasets.

Bacterial strains and cultures
Strain ATCC 10876 (also referred to as strain 569) was selected as the Bacillus cereus strain for detection of genes for universal stress proteins. Strain ATCC 10876 has been investigated for diverse biological processes, including response to iron limitation and production of metal-dependent beta lactamases. B. cereus ATCC 10876, obtained from the American Type Culture Collection (Manassas VA, USA) was cultured overnight in Brain Heart Infusion (BHI) broth overnight at 37 °C and 200 rpm. The resulting bacterial cultures were diluted to OD600 0.01 in BHI and incubated for four hours at 37 °C and 200 rpm.

Total RNA isolation
Total RNA was isolated according to the instructions in the Fisher BioReagents SurePrep TrueTotal RNA Purification Kit. Bacillus cells were pelleted by centrifuging at 14,000 rpm for 10 minutes. The supernatant was decanted and the cell pellet was mixed thoroughly with lysozyme (3 mg/mL). After a 10 minute incubation at room temperature, Fisher lysis solution was added to the mixture and the sample vortexed vigorously. The sample was centrifuged to remove any intact cells before the samples were applied to the Fisher SurePrep column. The samples were washed with ethanol several times and the cDNA isolated by elution buffer. The RNA concentration was determined by the Nanodrop Spectrophotometers.
End point reverse transcription (RT) polymerase chain reaction (PCR)
The expression of USP genes was examined by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed according to the manufacturer’s directions (Invitrogen, Super Script One Step RT-PCR System). The Invitrogen Oligo Perfect system was used to design primers to genes encoding two *B. cereus* USPs ZP04319786 and ZP04315896 as well as *tuf* gene, encoding elongation factor Tu. RT-PCR was performed as follows: 1 cycle for 15 minutes at 45 °C and 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 57 °C for 30 seconds, and extension for 1 minute at 68 °C.

DNA gel electrophoresis
Gel Electrophoresis was used to visualize RT-PCR products. Agarose (0.32 grams) was dissolved in 1X Tris Borate EDTA (TBE) and heated. After the mixture was allowed to cool, ethidium bromide was added to the solution (4 microliters), mixed, and the warm solution was poured in to the horizontal casting trays. Samples were diluted with running buffer (10 microliters of sample and 2 microliters of loading dye) and separated for 45 minutes at 100 volts. Bands were visualized and photographed using the Versa-Doc (BioRad).

Results
Function and transcription direction of adjacent genes for genes encoding universal stress proteins
The strain designation, GenBank Project ID, Integrated Microbial Genomes (IMG) Taxon ID and gene count for 10 finished (completely sequenced) *Bacillus cereus* genomes are presented in Table 1. Of the strains analyzed, strain ATCC 10987 had the highest gene count of 6,126 genes while strain cytotoxis NVH 391-98 had the lowest gene count of 4,250 genes. The accession numbers of the 2 universal stress proteins in each selected *B. cereus* genome are presented in Table 2. The predicted function of the adjacent genes and the encoding for the transcription direction is presented in Tables 3 and 4 respectively.

| *Bacillus cereus* genome | GenBank Project ID | IMG Taxon ID* | Gene count |
|-------------------------|-------------------|---------------|------------|
| 03BB102                 | 31307             | 643692007     | 5,767      |
| AH187 (F4810/72)        | 17715             | 643348510     | 5,903      |
| AH820                   | 17711             | 643348511     | 5,941      |
| ATCC 10987              | 74                | 637000016     | 6,126      |
| ATCC 14579              | 384               | 637000017     | 5,513      |
| B4264                   | 17731             | 643348512     | 5,557      |
| cytotoxis NVH 391-98    | 13624             | 640753006     | 4,250      |
| E33L (ZK)               | 12468             | 637000018     | 5,886      |
| G9842                   | 17733             | 643348513     | 5,994      |
| Q1                      | 16220             | 643348514     | 5,621      |

Note: *IMG: Integrated Microbial Genomes system.

In the case of the gene for the 140 aa USP, the function of the gene before the USP gene in the 10 genes was COG0659 [Sulfate permease and related transporters (MFS superfamily)] (Table 2). For two strains (cytotoxis NVH 391-98 and E33L [ZK]) the function of the gene was predicted as COG0747 (ABC-type dipeptide transport system, periplasmic component). The gene after the USP in strain ATCC 14579 was annotated as COG2271 (Sugar phosphate permease). For transcription direction, only strains cytotoxis NVH 39-98, E33L (ZK) and ATCC 14579 had the adjacent gene after the USP gene in the opposite direction of the USP gene. The other genomes had the adjacent genes to the USP gene in same direction hence the encoding of 111.

In summary, the *B. cereus* gene for the 140 aa USP is adjacent to the gene for inorganic sulfate uptake in the 10 genomes investigated. Additional inspection of the neighborhood of 92 USP genes from 36 finished genomes of other *Bacillus* species identified in the non-pathogenic, soil-inhabiting *Bacillus megaterium* QM B1551 a plasmid-encoded universal stress protein with locus tag BMQ_pBM70089. The gene BMQ_pBM70089 and the adjacent sulfate transporter (BMQ_pBM70090) form a transcriptional unit according to predictions in the BioCyc database (Fig. 1).
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Table 2. Accession numbers and locus tags of universal stress proteins in selected Bacillus cereus genomes.

| Bacillus cereus genome | GenBank Accession (140 aa USP) | Locus tag (140 aa USP) | GenBank Accession (152 aa USP) | Locus tag (152 aa USP) |
|------------------------|-------------------------------|------------------------|-------------------------------|------------------------|
| 03BB102                | YP_002748010                  | BCA_0692               | YP_002751985                  | BCA_4741               |
| AH187 (F4810/72)       | YP_002336786                  | BCAH187_A0783          | YP_002340677                  | BCAH187_A4754          |
| AH820                  | YP_002449684                  | BCAB820_0711           | YP_002453689                  | BCAB820_4743           |
| ATCC 10987             | NP_977047                     | BCE_0722               | NP_981053                     | BCE_4760               |
| ATCC 14579             | NP_830469                     | BC0655                 | NP_834331                     | BC4625                 |
| B4264                  | YP_002365450                  | BCBB4264_0690          | YP_002369419                  | BCBB4264_4733          |
| Cytotoxin NVH 391-98   | YP_001373894                  | Bcer98_0550            | YP_001376514                  | Bcer98_3301            |
| E33L (ZK)              | YP_082171                     | BCZK0565               | YP_085948                     | BCZK4370               |
| G9842                  | YP_002444119                  | BCG9842_B4648          | YP_002448190                  | BCG9842_B0502          |
| Q1                     | YP_00252867                   | BCQ_0722               | YP_002532148                  | BCQ_4433               |

Table 3. Function of adjacent genes of universal stress protein (140 aa) in selected Bacillus cereus genomes.

| Strain                  | Function before | Function after | Binary accession |
|-------------------------|-----------------|----------------|------------------|
| Cytotoxin NVH 391-98    | COG0659         | COG0747        | 110              |
| E33L (ZK)               | COG0659         | COG0747        | 110              |
| 03BB102                 | COG0659         | COG0000        | 111              |
| AH187 (F4810/72)       | COG0659         | COG0000        | 111              |
| AH820                   | COG0659         | COG0000        | 111              |
| B4264                   | COG0659         | COG0000        | 111              |
| ATCC 10987              | COG0659         | COG0000        | 111              |
| ATCC 14579              | COG0659         | COG2271        | 110              |
| G9842                   | COG0659         | COG0000        | 111              |
| Q1                      | COG0659         | COG0000        | 111              |

Table 4. Function of adjacent genes of universal stress protein (152 aa) in selected Bacillus cereus genomes.

| Strain                  | Before | After | Binary accession |
|-------------------------|--------|-------|------------------|
| AH187 (F4810/72)       | COG1028| COG0000| 010              |
| AH820                  | COG1028| COG0000| 010              |
| B4264                  | COG1028| COG0000| 010              |
| ATCC 10987             | COG1028| COG0000| 010              |
| ATCC 14579             | COG1028| COG0000| 010              |
| Cytotoxin NVH 391-98   | COG1028| COG0000| 010              |
| G9842                  | COG1028| COG0000| 010              |
| E33L (ZK)              | COG1028| COG0000| 010              |
| 03BB102                | COG1028| COG0000| 010              |
| Q1                     | COG1028| COG0000| 011              |

Notes: *The before and after gene is based on numerical order of the locus tag. COG: Cluster of Orthologous Groups of Proteins. COG0659: Sulfate permease and related transporters (MFS superfamily); COG0747: ABC-type dipeptide transport system, periplasmic component; COG227: Sugar phosphate permease.
strains encode a two-gene transcriptional unit encoding a silent information regulator 2 (Sir2) family transcriptional regulator and a universal stress protein. The USPs are BMQ_3990 and BMD_3975 from QM B1551 and DSM 319 respectively.

Gene neighborhood and chromosomal cassette analyses performed on the IMG resource revealed that the USP-Sir2 transcriptional unit is unique to *B. megaterium*. The chromosomal cassette showing the unique presence of the Sir2 and USP as adjacent genes in strains of *B. megaterium* can be viewed at [http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=GeneCassette&page=geneCassette&gene_oid=646729802&type=pfam](http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=GeneCassette&page=geneCassette&gene_oid=646729802&type=pfam).

**Figure 1.** Gene neighborhood and schematic diagram of inorganic sulfate transport in *Bacillus megaterium*. Chromosomal location and (A) Gene local context and transcription unit in *Bacillus megaterium* plasmid pBM700 with gene for universal stress protein (BMQ_pBM70089) and gene for inorganic sulfate transport (BMQ_pBM70090). A dashed baseline indicates that there is no high-quality evidence to confirm the extent of this transcription unit. (B) Enzymatic reaction of inorganic anion transporter, sulfate permease (SulP) family. Source of images: [http://biocyc.org/](http://biocyc.org/).

Prediction of ligand binding sites for *Bacillus cereus* universal stress proteins
The ligand binding site position, the amino acid position and the amino acid for the ligand binding sites for the 140 aa and 152 aa universal stress proteins of *B. cereus* genomes are presented in Tables 5 and 6 respectively. In the 140 aa USP, nine of the strains had 12 conserved ligand binding sites while strain cytotoxins NVH 391-98 had only 8 of the 12 ligand binding sites. The first ligand binding positions in NVH 391-98 and the other nine 140 aa USPs are 108 and 8 respectively. Further, there was no agreement of the amino acid types in the two USP groups despite having identical sizes (Table 5).

Comparative analysis of chemical ligand binding sites and gene neighborhood of universal stress proteins in *Bacillus* genomes
The amino acids sites for chemical ligand binding for the sequences of the universal stress proteins from *B. cereus* 03BB102 (3 USP genes) were compared to *B. megaterium* QM B1551 and *Bacillus* sp. NRRL B-14911. The latter two genomes were selected because of they have 6 and 5 USP genes respectively and form a clade when their 16S rRNA genes were analyzed. A total of 12 ligands binding sites is expected for a complete USP domain (PF00582) following that of *Methanococcus jannaschii* MJ0577 protein. Clustering the features of ligand binding sites (position, amino acid and amino
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Figure 2. Multi-genome alignment of gene neighborhood of genes for universal stress protein representative *Bacillus cereus* genomes. **Notes:** Universal stress protein (USP) gene in blue with diagonal lines. *Bacillus cereus* Q1 is only genome with transcriptional regulator of the ArsR family. Source of image: [http://biocyc.org/](http://biocyc.org/).

Table 5. Ligand binding sites for 140 aa universal stress proteins of selected *Bacillus cereus* strains.

| Ligand binding position | Amino acid position | Amino acid |
|-------------------------|---------------------|------------|
| 1                       | 8                   | A          |
| 2                       | 9                   | C          |
| 3                       | 13                  | D          |
| 5                       | 108                 | A          |
| 6                       | 109                 | G          |
| 7                       | 111                 | R          |
| 8                       | 112                 | G          |
| 9                       | 112                 | G          |
| 10                      | 123                 | S          |
| 11                      | 124                 | V          |
| 12                      | 125                 | S          |

Notes: Conserved in all finished *B. cereus* strains except for *B. cereus* cytotoxin NVH 391-98. The 8 binding sites and associated amino acids for strain NVH 391-98 were 108 (V); 109 (C); 111 (R); 112 (G); 123 (V) and 125 (S). The protein domain architecture for Bcerc98_0550 of strain NVH 391-98 can be retrieved from: [http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?INPUT_TYPE=live&SEQUENCE=YP_001373894.1](http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?INPUT_TYPE=live&SEQUENCE=YP_001373894.1).

Table 6. Ligand binding sites for 152 aa universal stress proteins of selected *Bacillus cereus* strains.

| Ligand binding position | Amino acid position | Amino acid |
|-------------------------|---------------------|------------|
| 1                       | 11                  | A          |
| 2                       | 12                  | V          |
| 3                       | 13                  | D          |
| 4                       | 41                  | I          |
| 5                       | 112                 | C          |
| 6                       | 113                 | G          |
| 7                       | 115                 | T          |
| 8                       | 116                 | G          |
| 9                       | 126                 | G          |
| 10                      | 127                 | S          |
| 11                      | 128                 | V          |
| 12                      | 129                 | S          |
channel histidine kinase sensor). BMQ_3025 is in the same transcriptional unit with BMQ_3024 which encodes a protein with cystathionine-beta-synthase (CBS) domain known for binding to adenosine for cellular energy-sensing.

Multi-genome alignment of several Bacillus genomes revealed the gene for CBS domain in several Bacillus genomes. However, transcriptional unit encoding possible stress responsive potassium uptake and energy sensing was found in Bacillus megaterium QM B1551. The 165 aa BMQ1871 is not part of a transcriptional unit but is adjacent to a high oxygen, responsive, 4-member transcriptional unit encoding subunits of cytochrome o quinol oxidase (cyoABCD), homolog of one of the terminal oxidases identified in Escherichia coli.

Detection of genes encoding universal stress proteins in B. cereus ATCC 10876
Vegetative B. cereus cells were isolated during exponential growth in BHI at 37 °C. Total RNA

Figure 3. Visualization of clustering of ligand binding sites from universal stress protein sequences of selected Bacillus species.
Notes: An interpretation of the visualization is that of the 14 sequences, 8 sequences have Alanine (A) in ligand position 1 of the universal stress protein ligand binding signature. It could be inferred that proteins B14911_13742 and BMQ_1747 have unique ligand binding sites of Glycine (G) and Threonine respectively.
was extracted from cells for end point reverse transcription PCR. Both putative genes for two USPs (ZP04319786 and ZP04315896) and a control gene, tuf, were detected to be transcribed during the latter stages of exponential growth in BHI (Fig. 4).

Discussion

The public availability of at least 100 genome sequences of Bacillus species presents new opportunities to use bioinformatic approaches to predict biological processes. These predictions could lead to diverse societally beneficial applications, including disease treatment and prevention, improved agricultural productivity and industrial biotechnological applications. At least 45 genomes of Bacillus cereus are in the Integrated Microbial Genomes (IMG), making it the species with the highest number of genomes sequenced within the Bacillus genera. Each of these genomes have 3 USP genes. One of the USP genes encode protein domains for the enzyme kinase function. The B. cereus USP genes of interest in the reported research were those that encodes only the USP domain (Pfam Identifier: PF00582). Functional annotation analytics were performed on 10 finished B. cereus genomes: 03BB102; AH187 (F4810/72); AH820; B4264; ATCC 10987; ATCC 14579; cyto-toxins NVH 391-98; G9842; Q1 and E33L (ZK). All the 10 B. cereus genomes have genes encoding the 140 aa and 152 aa USPs. Comparing the predicted function and direction of transcription of adjacent genes of USP genes could identify similarities and differences between the chromosomal regions containing the USP genes in multiple genomes from the same species. Furthermore, the comparisons could provide information on biological pathways in which universal stress proteins function. The findings could better define differential physiological responses to environmental stresses of B. cereus strains.

The soil-inhabiting B. megaterium QM B1551 has 6 USP genes, including one on a plasmid. Unlike B. subtilis (another soil-inhabiting Bacillus species), where the two USP genes have been characterized, the functions of the B. megaterium USPs have not been investigated. Gene neighborhood analysis (Figs. 1 and 2) combined with visual analytics of chemical ligand binding sites data (Fig. 3) provided knowledge-building insights on possible cellular functions of B. megaterium universal stress proteins. These functions include sulfate and potassium uptake, acid extrusion, cellular energy-level sensing, survival in high oxygen conditions and acetate utilization. Of particular interest was a transcriptional unit found in only Bacillus megaterium that consisted of a USP gene and a sirtuin (Sir2) gene. The Sir2 is a highly conserved deacetylase enzyme from bacteria to higher eukaryotes that depends on a nicotinamide adenine dinucleotide ion (NAD+) to remove acetyl groups from lysine amino acids in proteins. In general, Sir2 proteins have roles in cell aging, chromosomal stability, energy metabolism in response to nutrient signals, gene silencing and stress response. In mammals, members of the sirtuin gene family have been extensively investigated, including as therapeutic targets for age-related disorders, cancer, cardiovascular diseases and obesity. In B. subtilis, the NAD+ dependent sirtuin (SrtN) and another non-NAD+-dependent deacetylases AcuC are required to maintain the acetyl coenzyme A (Ac-CoA) synthetase (AcsA) enzyme as active (i.e. deacetylated) so that the cell can grow with low concentrations of acetate. The conversion of acetate into Ac-CoA is an inducible pathway catalyzed by AcsA and expressed in low extracellular concentrations of acetate. In Escherichia coli K12, in the absence of other substrates acetate can serve as sole source of carbon and energy.

The function of the unique USP-Sir2 transcriptional unit in the physiology of B. megaterium has not been

![Figure 4](image-url)

**Figure 4.** Confirmation of expression of genes for universal stress proteins in Bacillus cereus ATCC 10876. (A) Bands in the DNA gel demonstrate that both genes are expressed during the latter stages of exponential growth in Brain Heart Infusion broth. (B) Expression of tuf (elongation factor Tu) was used as a control to measure RNA isolation.
reported. The transcriptional unit is present in the all three strains (DSM 319, QM B1551 and WSH-002) with a published genome sequence. One possibility is the efficient utilization of acetate as a soil-inhabiting bacteria or in conditions of low acetate. Considering the key role of sirtuins in mamalian physiology, additional research on the USP-Sir2 transcriptional unit of \textit{B. megaterium} could help explain mammalian acetate metabolism in glucose-limiting conditions such as caloric restriction. Additional gene transcriptional and protein structure investigations are needed to determine the genomic regulation and protein–protein interaction of the USP-Sir2 transcriptional unit. Finally, the deep-rooted position of \textit{B. megaterium} in the phylogeny of the \textit{Bacillus} species makes the investigation of the functional coupling acetate utilization and stress response compelling.

The comparison of the chromosomal region of the gene for the 140 aa USP revealed unique gene function annotation and transcriptional direction for two strains: cytotoxis NVH 391-98 and E33L (ZK). Strain NVH 391-98 was isolated during a severe food (vegetable puree) borne disease outbreak in France in 1998. Recently, strain NVH 391-98 has been proposed as a new \textit{Bacillus} species called \textit{cytotoxicus}. Strain E33L (ZK) was isolated from a zebra carcass in Namibia and the nearest relative to \textit{B. anthracis}. The prediction of amino acids in ligand binding sites helped to distinguish the 140 aa USP of NVH 391-98 from the other \textit{B. cereus} strains. In the case of the 152 aa USP, the ligand binding sites were conserved. We previously used ligand binding to cluster the 152 aa USP, the ligand binding sites were conserved.

\textbf{Conclusion}

\textit{Bacillus} species form an heterogeneous group of Gram-positive bacteria that include members that are disease-causing, biotechnologically-relevant, and can serve as biological research tools. A common feature is their ability to survive in harsh environmental conditions by formation of resistant endospores. Universal stress proteins have been demonstrated to be important during survival in unfavorable conditions in numerous pathogenic microbes. The bioinformatics strategy of mining functional annotations from multiple databases and multiple \textit{Bacillus} genomes led to the identification of sulfate uptake and acetate utilization pathways that are predicted to be coupled with universal stress proteins in \textit{Bacillus megaterium}.

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Author Contributions
Conceived and designed the experiments: BSW, RDI, BLG. Analyzed the data: BSW, BLG, SSS, WKA, ALH, ANM, COB, RDI. Wrote the first draft of the manuscript: BLS, BSW, RDI, WKA. Contributed to the writing of the manuscript: RDI, BLG, BSW. Agree with manuscript results and conclusions: BSW, BLG, SSS, WKA, ALH, ANM, COB, RDI. Jointly developed the structure and arguments for the paper: BSW, BLG, SSS, WKA, ANM, RDI. Made critical revisions and approved final version: BSW, RDI. All authors reviewed and approved of the final manuscript.

Competing Interests
Author(s) disclose no potential conflicts of interest.

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As a requirement of publication author(s) have provided to the publisher signed certification of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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