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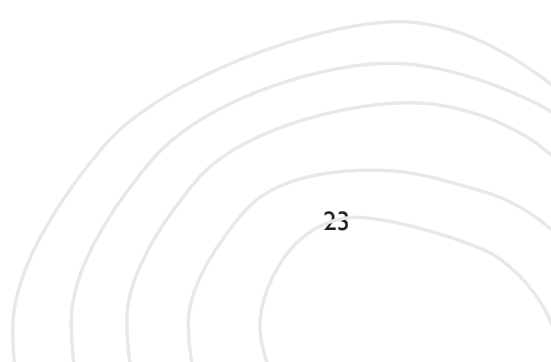
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Chapter 2 - Temperature dependence of the proteome profile of the psychrotolerant pathogenic food spoiler *Bacillus weihenstephanensis* type strain WSBC 10204

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Abstract

Bacillus weihenstephanensis is a subspecies of the *Bacillus cereus sensu lato* group of spore forming bacteria known to cause food spoilage or food poisoning. The key distinguishing phenotype of *B. weihenstephanensis* is its ability to grow below 7°C or, from a food safety perspective, to grow and potentially produce toxins in a refrigerated environment. Comparison of the proteome profile of *B. weihenstephanensis* upon its exposure to different culturing conditions can reveal clues to the mechanistic basis of its psychrotolerant phenotype, as well as elucidate relevant aspects of its toxigenic profile. To this end, the genome of the type strain *Bacillus weihenstephanensis* WSBC 10204 was sequenced and annotated. Subsequently, the proteome profiles of cells grown at either 6°C or 30°C were compared which revealed considerable differences and indicated several hundred (uncharacterized) proteins as being subproteome and/or temperature specific. In this manner several processes were newly indicated to be dependent on growth temperature such as varying carbon flux routes and a different role for the urea cycle. Furthermore, a possible post-translational regulatory function for acetylation was suggested. Toxin production was determined to be largely independent of growth temperature.

Introduction

Bacillus weihenstephanensis is a spore-forming gram-positive bacterium known for its ability to cause food poisoning and food spoilage. The phenotype which distinguishes *B. weihenstephanensis* from other members of the *B. cereus* group is its psychrotolerant growth profile, i.e. its ability to grow at 4-7°C but not at 43°C with an optimum growth temperature of 30°C. From a food safety perspective, characterization of this species is important as the psychrotolerant phenotype introduces an increased potential hazard in an environment reliant on refrigerated storage of products (i.e. 4°C) for microbial inactivation.⁵

The most important vehicle by which *B. weihenstephanensis* manages to contaminate food is through (endo)spores. Spores are a metabolically dormant, highly resistant survival form of the cell capable of withstanding common processing techniques used in the food industry such as heat or acid treatment. Though spores themselves are harmless, once the bacteria return to their vegetative state through a process called germination they can resume growth and produce toxins. Beyond its ability to grow at lower temperatures than its close relatives, *B. weihenstephanensis* has been indicated to be able to produce spores, germinate and produce toxins at lower temperatures as well.⁸⁶ Reports of outbreaks of *B. weihenstephanensis* are rare, as it is not a specifically reportable illness and is not always diagnosed. Furthermore, little effort is made to characterize isolates enough to distinguish it from *B. cereus*.⁶

Lechner et al.¹¹ originally distinguished the species from the rest of the *Bacillus cereus sensu lato* group based on sequence differences in the 16S rDNA, 23S rDNA, the 16S-23S rDNA spacer region and in the gene of the major cold-shock protein homologue *cspA*. Growth characteristics are not enough to classify isolates, as several variants of *B. cereus* are in fact capable of growth at 6°C.⁸⁷ There is still some controversy as to whether or how the *B. cereus*

sensu lato group should be subdivided into different species, as there is no true phylogenetic basis.^{6,88,89} While other members in the *B. cereus* group are studied in great detail with genome sequences available for many strains,⁹⁰ *B. weihenstephanensis* falls behind with only one completed genome⁹¹ and until now no sequence for the type strain.

In order to make *B. weihenstephanensis* more tractable for studies the type strain *Bacillus weihenstephanensis* WSBC10204 (DSM11821) has been sequenced. Annotation of the newly created genome sequence allowed for a complete proteome prediction and subsequent analysis by bottom-up mass spectrometry based proteomics. In order to assess how *B. weihenstephanensis* copes with low growth temperature and determine its toxigenic profile, the proteome expressed at a growth temperature of 6°C and 30°C was analysed. Comparison of the cellular proteome and secretome isolated from late-exponential phase cultures indicated many temperature specific proteins. These differentially expressed proteins indicated several processes which were expected to be growth temperature dependent, as well as processes newly correlated to growth temperature.

Materials & methods

DNA isolation

An overnight culture of *Bacillus weihenstephanensis* WSBC10204 (available at DSMZ under DSM-11821) grown in 25 mL Tryptic Soy Broth medium was harvested by centrifugation, the pellet was resuspended in 2 mL re-suspension buffer (10 mM Tris-HCl pH 7.5, 50 mM EDTA, 100 mM NaCl). Cells were lysed for 30 min at 37°C by addition of 2 mg lysozyme. 200 µL 2% SDS was added and the solution was incubated on ice for 5 min. DNA was extracted by addition of 1 volume of phenol/chloroform. The phases were separated by centrifugation (15 min 13.000 rpm), 1/10th volume of 3 M sodium acetate was added to the aqueous phase. DNA was precipitated with 1 volume of cold 100% ethanol, washed once with 70% ethanol and air dried before resuspension in water.

Genome Sequencing and Annotation

Illumina HiSEQ2500 sequencing and assembly into scaffolds was performed by BaseClear BV, Leiden, The Netherlands. This resulted in a roughly 5.6MB sequence with a coverage of 400x. Scaffolds were aligned to the *B. weihenstephanensis* KBAB4 sequence [GenBank:NC_010184.1] to produce a draft genome using MAUVE.⁹² Residual gaps were closed by PCR amplification and 454 GS FLX+ sequencing with 1 out of 10 PCR products not resulting in a sequence, probably due to a high number of repeats. The sequence is available at [GenBank:CP009746].

The IGS Annotation Engine was used for structural and functional annotation of the sequences.^{93,94} Manatee was used to view annotations.⁹⁵ After machine annotation, each open reading frame was checked manually to verify the annotation. Furthermore, a second annotation was also performed using the RAST annotation server⁹⁶ and was used for comparison. Finally, the annotation was compared with the published genome annotation of *B. weihenstephanensis*

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KBAB4 in order to improve the quality. Annotations were manually verified by comparing against non-redundant databases of NCBI and Uniprot. In order to associate a function with a predicted gene, a minimum cut-off of 30% identity and 80% coverage of the gene length were used, checking at least two best hits in non-redundant protein databases. The BLASTp algorithm was used to search for protein similarities to other deposited sequences, following these criteria: > 50% similarity at the amino acid level and >50% coverage of protein length.

Culturing and Protein sample preparation

Duplicate batch cultures of *Bacillus weihenstephanensis* WSBC10204 were grown in TSB medium at either 6°C or 30°C until late exponential phase as determined by optical density at 600 nm ($OD_{600} = 10$) synchronizing growth phase but not growth rate. Cells were harvested by centrifugation and freeze dried. 50 mg of dried cell pellet was resuspended in 100 mM dithiothreitol, 100 mM Tris-HCl pH 6.8, 4% SDS, disrupted by 3 cycles of sonication consisting of 3 min sonication (50% duty cycle, Output Control 7), 5 min rest on ice in a Branson Sonifier 250 (Branson Ultrasonics, Danbury, USA) and freeze dried.

For secretome preparation the supernatants of duplicate cultures grown as described above were filtered through a 0.2 µm Polyethersulfone filter to remove remaining cells. Secreted proteins were precipitated from the supernatant by addition of up to 10% Trichloroacetic acid and incubation overnight at 4°C. Precipitated proteins were washed three times with 95% acetone and freeze dried.

Preparation for Mass Spectrometry (MS) analysis

5 mg of lyophilized cellular extracts were dissolved in sample buffer, loaded onto a 12% SDS-PAGE gel and run for 2 hrs at 100V until the dye ran off the gel. Gels were stained with Coomassie Brilliant Blue to confirm separation. Gel lanes were excised and cut into approximately 20 slices of equal size. Slices were diced into small pieces and dehydrated by vortexing in 25 mM NH_4HCO_3 , 50% acetonitril twice for 30 min. After drying fully in a centrifugal vacuum concentrator (miVac DNA Concentrator, Genevac Ltd, Ipswich, UK), proteins were reduced by incubation with 10 mM dithiothreitol in 25 mM NH_4HCO_3 for 60 min. at 55°C followed by alkylation with 55mM iodoacetamide for 45 min. in the dark. Gel pieces were washed with 25 mM NH_4HCO_3 , dehydrated and dried fully as above. Proteins were subjected to in-gel tryptic digestion by rehydration with 2.5 µg of trypsin in 25 mM NH_4HCO_3 per fraction and incubation at 37°C overnight. After digestion, the supernatant containing peptides was collected and peptides were further extracted from the gel by vortexing in 50% acetonitril/5% formic acid twice for 30 min. The combined supernatants were partially dried to lower the acetonitrile concentration before desalting as described below. Secreted proteins were resuspended in 8 M urea, 0.5 M ammonium bicarbonate, pH 8.0, 4 mM dithiothreitol and incubated at 55°C for 60 min. Afterwards 25 µl of 20 mM iodoacetamide was added and the samples were incubated in the dark for 45 minutes. The samples were diluted 4 times with water to lower the concentration of urea to avoid inhibition of trypsin after which 2.5 µg of trypsin was added and proteins were digested at 37°C overnight.

Tryptic digests were desalted using Omix µC18 tips (80 µg capacity, Varian, Palo Alto, CA) according to the manufacturer's protocol with final elution of peptides in 50% acetonitril, 0.1% trifluoroacetic acid. Prior to analysis by liquid chromatography mass spectrometry the peptide concentration was estimated on a Nanodrop

ND1000 spectrophotometer (Isogen Life Sciences, De Meern, The Netherlands) at 205 nm.⁹⁷

Liquid Chromatography-Fourier Transform Ion Cyclotron Tandem Mass Spectrometry (LC-FT-ICR MS/MS) analysis
LC-MS/MS data were acquired with an Bruker ApexUltra Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a nano-electrospray Apollo II DualSource™ coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA, USA) High-Performance Liquid Chromatography system. For each digest, samples containing up to 200 ng of tryptic peptides were injected as a 20 µl 0.1% trifluoroacetic acid, 3% acetonitril aqueous solution and loaded onto a PepMap100 C18 (5 µm particle size, 100 Å pore size, 300 µm inner diameter x 5 mm length) precolumn at a flow rate of 50 µL/min. Following injection, the peptides were eluted via an Acclaim PepMap 100 C18 (3 µm particle size, 100 Å pore size, 75 µm inner diameter x 250 mm length.) analytical column (Thermo Scientific, Etten-Leur, The Netherlands) to the nano-electrospray source. For the third biological replicate analyses a similar 500 mm column at 60°C was used. LC Gradient profiles of up to 120 minutes were obtained using 0.1% formic acid / 99.9% H₂O (A) and 0.1% formic acid / 80% acetonitril / 19.9% H₂O (B) (0 min 97%A/3%B, 2 min 94%A/6%B, 110 min 70%A/30%B, 120 min 60%A/40%B, 125 min 100%B) at a flow rate of 300 nL/min. Data dependent Q-selected peptide ions were fragmented in the hexapole collision cell at an Argon pressure of 6x10⁻⁶ mbar (measured at the ion gauge) and the fragment ions were detected in the ICR cell at a resolution of up to 60000. In the MS/MS duty cycle, 3 different precursor peptide ions were selected from each survey MS. The MS/MS duty cycle time for 1 survey MS and 3 MS/MS acquisitions was about 2 s. Instrument mass calibration was better than 5 ppm over an m/z range of 250 to 1500.

Raw FT-MS/MS data were processed with the MASCOT DISTILLER program, version 2.4.3.1 (64bits), MDRO 2.4.3.0 (MATRIX science, London, UK), including the Search toolbox and the Quantification toolbox. Peak-picking for both MS and MS/MS spectra were optimized for the mass resolution of up to 60000. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7, with minimum signal to noise of 2. For each replicate, the combined processed data of gel fraction digests were combined and searched in a MudPIT (Multidimensional Protein Identification Technique) scoring approach with the MASCOT server program 2.3.02 (MATRIX science, London, UK) against the newly created *B. weihenstephanensis* WSBC 10204 predicted proteome database. Processed data from the secretome digests were searched separately. The database was complemented with its corresponding decoy database for statistical analyses of peptide false discovery rate. Trypsin/P was used as enzyme and 1 missed cleavage was allowed. Carbamidomethylation of cysteine was used as a fixed modification. The peptide mass tolerance was set to 40 ppm and the peptide fragment mass tolerance was set to 0.03 Dalton. MASCOT MudPIT peptide identification score was set to a cut-off of 20. At this cut-off and based on the number of assigned decoy peptide sequences, the significance threshold was adjusted to obtain a peptide false discovery rate of 2% for all analyses. The MASCOT protein identification reports were exported as XML and then imported in a custom made VBA software program running in Microsoft Excel. The program facilitates organization and data mining of large sets of proteomics data.

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The identified cellular and secretome fraction associated proteins for the three replicates grown at 6°C and 30°C are listed in Supplementary Table S1, together with their protein MASCOT scores and number of peptide spectrum matches. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium⁹⁸ via the PRIDE partner repository with the dataset identifier PXD002007 and 10.6019/PXD002007.

Results and discussion

Sequencing and annotation

Illumina sequencing resulted in a 5.6 MB sequence with an overall GC% content of 35.3%. The genome carries 5678 coding sequences (CDS), 77 tRNA and 9 rRNA genes. Of the predicted CDSs, 4697 (82.7%) have a predicted biological known function. The rest are either similar to hypothetical proteins in other genomes or have no substantial similarity to other predicted proteins. The CDSs with known biological function were also classified into TIGR (The Institute for Genomic Research) biological roles for further analysis.

The draft sequence contained 44 contigs and 35 scaffolds. Mapping the scaffolds against *Bacillus weihenstephanensis* strain KBAB4 [GenBank:NC_010184.1] using MAUVE allowed for design of PCR primers for genome closing. Using LR-PCR, fragments were obtained for all of the assumed gaps. Further sequencing and annotation of the resulting fragments revealed the presence of tRNA operons. However, one gap between two scaffolds remained resistant to sequencing likely due to a high number of repeats and/or secondary structure of the DNA or a possible insertion of a phage sequence. Alignment with the published sequence of *B. weihenstephanensis* KBAB4 indicated this region of the genome to consist exclusively of tRNA sequences. This allowed for a full proteome prediction of the type strain of *Bacillus weihenstephanensis*. Automatic annotation by IGS Annotation Engine⁹³ predicted 5723 encoded proteins. Manual curation and start codon correction were executed using the Manatee annotation tool.⁹⁵ The high coding density (82.7%) also accounted for the higher number of protein coding sequences in this strain as compared to strain KBAB4 (5155) which indicates that genomic rearrangements have taken place as a result of the acquisition of mobile genetic elements. This insertion might be involved in niche adaptation as well as its contribution to pathogenicity of the current strain.⁹⁹

Proteome profiling and categorization

To assess how *B. weihenstephanensis* responds to growth at low temperatures, the proteome profile was determined at 6°C and 30°C. Proteins were identified in three replicates of cellular isolate and three replicates of secreted protein isolates for each temperature. A total of 2112 individual proteins were detected at least once, across all isolates and replicates. Predicted proteins were grouped to TIGR role categories automatically by the Manatee annotation tool⁹⁵ with proteins not attributed to any category being listed as Uncategorized. The number of detected proteins per category is displayed in Figure 2.1. This indicated that the obtained proteome profiles covered a very large variety of processes. In particular, the majority of proteins ascribed to growth related processes were detected, as is to be expected from late-exponential phase cell cultures. These categories included Nucleotide Synthesis, covering purine and

pyrimidine metabolism; Protein Synthesis, which covers for instance ribosomal proteins and tRNA ligases; as well as Energy Metabolism, which includes glycolysis, the TCA (tricarboxylic acid) cycle, the pentose phosphate pathway, etcetera.

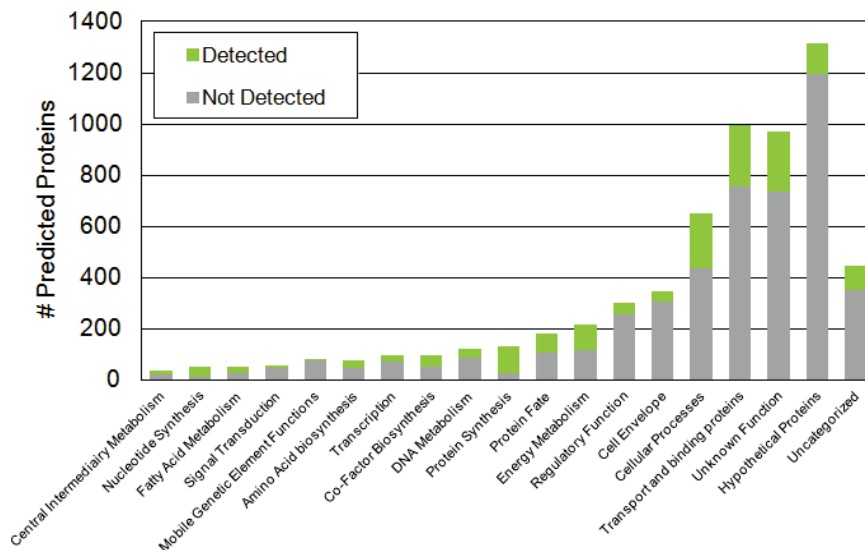


Figure 2.1 – TIGR categorization of predicted proteins.

All predicted proteins were divided into TIGR categories automatically by the Manatee annotation tool⁹⁵. Note that a singular protein may occupy multiple categories. 2112 proteins were detected spanning all TIGR categories. Proteins which were not appointed a category are listed as uncategorized.

A protein was considered positively identified if it had been detected in at least two replicates. Specific localization of identified proteins, either cellular, secreted, or both, is indicated in Figure 2.2. Only 88 proteins were identified exclusively in the secretome, these did however include produced toxins.

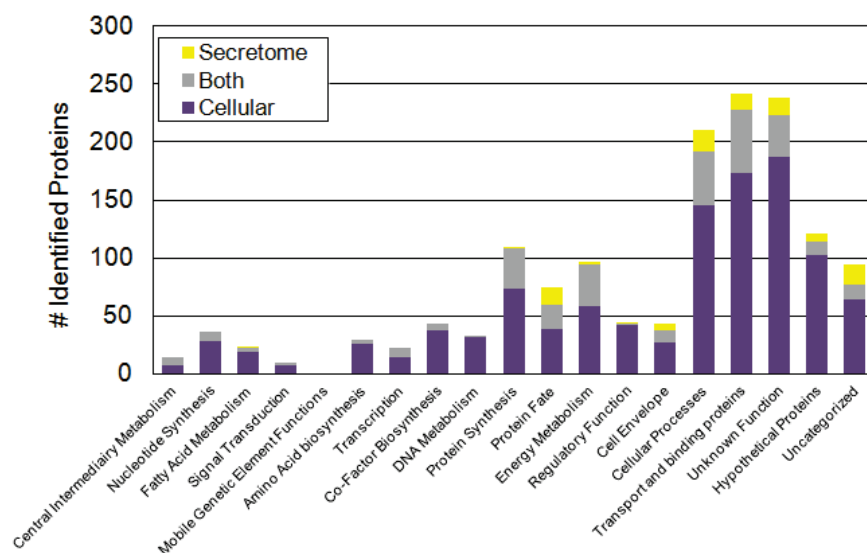


Figure 2.2 – Subproteome localization of identified proteins.

Identified proteins are indicated according to the isolate(s) they were detected in. A total of 976 proteins were specific to the cellular proteome, 88 proteins were secretome specific and 262 proteins were identified in both subproteomes.

Identified proteins divided into the same categories as above are indicated according to their growth temperature predominance and/or specificity in Figure 2.3. A total of 505 proteins showed temperature specific behavior being identified in isolates from either 6°C or 30°C exclusively. Another 129 proteins showed a predominance at 6°C or 30°C determined as follows. In label-free mass spectrometric quantification methods, more abundant peptides are assumed to be selected for fragmentation more often thereby increasing the number of times peptides are identified for abundant proteins in a sample.⁵⁴ It therefore serves as an indication of temperature dependent expression of proteins. Using this spectral counting approach, the number of peptide spectrum matches for a given protein was normalized to the total number of matches in each singular analysis to correct for variation in sample quality. Subsequently, the sum spectral count was averaged for all three replicates and compared between the two experimental conditions. Proteins with a threefold difference in their associated spectral count were considered to be predominant at a certain temperature. Individual proteins with MASCOT scores and number of peptide spectrum matches are listed in Supplementary Table S1. Proteins from across all categories were influenced by growth temperature to a certain extent.

PROTEOME PROFILE OF *B. WEIHENSTEPHANENSIS*

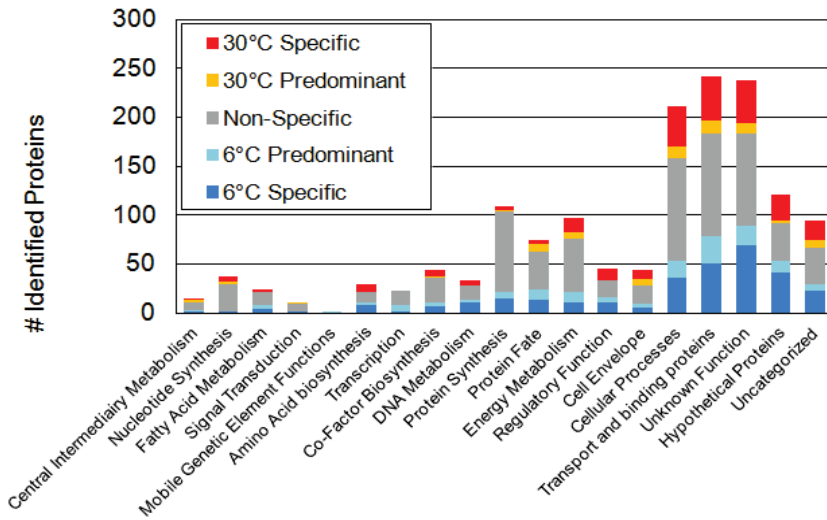


Figure 2.3 – Temperature dependence of identified proteins.

Identified proteins with indicated temperature specificity or predominance showed growth temperature dependent expression of proteins across all categories.

Growth temperature dependent processes

The batch culture setup used for acquiring cell material allowed for a difference in growth rate as a result of the imposed difference in growth temperature. In order to separate a growth temperature effect from a growth rate effect, it is imperative to find some sort of normalization and establish whether cells are in the same growth phase. A good indicator for this would be the ribosomal protein content which can be considered proportional to the growth rate.^{100–103} If the ribosomal proteins are present in similar amounts, the inclination of the cell to grow and divide can be considered the same as well, though actual growth rate may still differ as a result of the temperature dependence of ribosomal activity. By harvesting all cell cultures in the late-exponential phase and determining the ribosomal protein content to be largely temperature independent (Supplementary Table S1) it was concluded that cells were in the same growth phase, i.e. maximum growth rate for that respective temperature. This allowed for an appreciation of growth temperature dependent effects despite the growth rate difference.

Upon encountering a downshift in temperature, bacteria need to adjust in order to maintain viability and/or resume growth. Several hurdles must be overcome including hampered translation, increased superhelicity of DNA, reduced membrane fluidity, and more.¹⁰⁴ Several proteins related to these processes were identified to be expressed in a growth temperature dependent manner. Major Cold Shock proteins CspA (GenelD: *bwei_3875*, GenBank accession number: AIW86483) and CspE (*bwei_2608*, AIW85234) were found to be predominantly or exclusively present at 6°C and are known to aid in preventing premature folding, which can occur during translation at low temperatures.^{105–107}

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DNA gyrase subunit B (*bwei_4278*, AIW86876) was predominant at 6°C as well, allowing for restoration of correct superhelicity of DNA. Furthermore, enzymes responsible for membrane remodelling, such as Fatty Acid Desaturase (*bwei_2028*, AIW84669) a short-chain specific Acyl-CoA Hydrolase (*bwei_1913*, AIW84555) were identified in 6°C isolates exclusively, indicating a tendency towards shorter unsaturated fatty acids which should result in a more fluid membrane. These findings indicated known candidate proteins related to a cold shock or cold adaptation response were indeed detected.

Beyond known candidates, several processes were newly indicated to be correlated to growth temperature. Looking at Energy Metabolism, the proteome profiles showed a few differences. Fermentative pathways, in the form of several Lactate Dehydrogenases (*bwei_0009*, AIW82688; *bwei_3088*, AIW85707; *bwei_4768*, AIW87353) and Pyruvate Formate Lyase (*bwei_5272*, AIW87817), were identified exclusively or predominantly in 30°C isolates, with Pyruvate Formate Lyase showing the clearest semi-quantitative difference among all proteins identified. However, enzymes pertaining to glycolysis and the TCA cycle were present irrespective of temperature. This points to the notion that oxygen limitation is likely not a major factor as aerobic metabolism presumably takes place under the same conditions. Rather than a direct growth temperature effect, induction of fermentative pathways may be a response to alleviate higher levels of pyruvate. This in turn may be caused by a higher flux through glycolysis as a result of higher enzyme activity at 30°C. In essence, the data indicated that a bacterial Crabtree effect occurs at 30°C where glucose consumption cannot be matched by full aerobic conversion of pyruvate, diverting carbon flux to lactate instead.¹⁰⁸⁻¹¹⁰

Looking at cold-induced Energy Metabolism on the other hand, the glyoxylate bypass of the TCA cycle in the form of Isocitrate Lyase (*bwei_3878*, AIW86486) and Malate Synthase (*bwei_3879*, AIW86487) was identified in 6°C isolates only. Isocitrate Lyase can cleave isocitrate into succinate and glyoxylate after which succinate is readily used in the remainder of the TCA cycle. The glyoxylate, however, first needs to be condensed by Malate Synthase using an acetyl group from Acetyl-CoA to form Malate before this can re-enter the TCA cycle. During adjustment of the membrane to low temperatures, fatty acid chains are shortened to increase fluidity as mentioned above. The glyoxylate bypass could be induced to enable the cell to partition the acetyl fragments released during fatty acid chain degradation for either anabolic processes or full oxidation.

PROTEOME PROFILE OF *B. WEIHENSTEPHANENSIS*

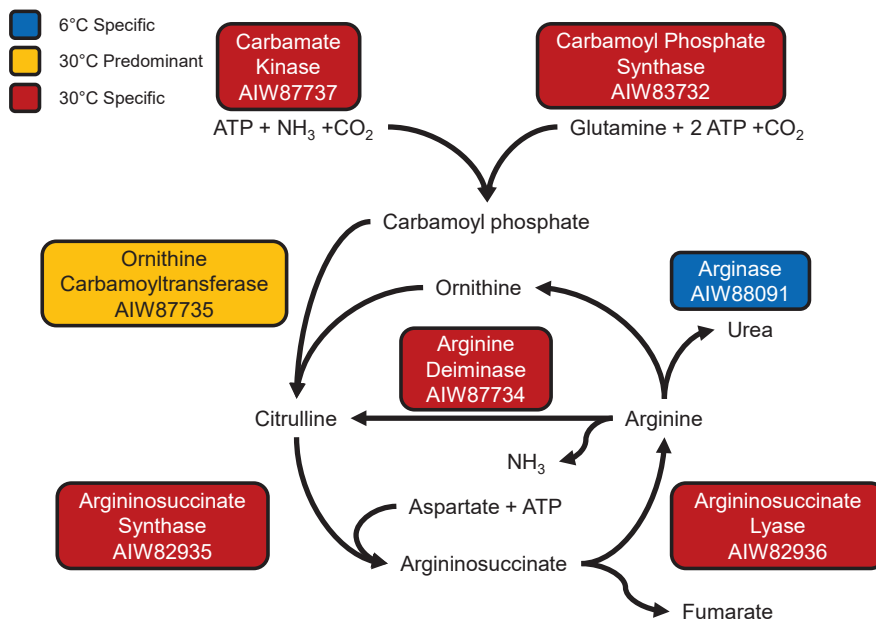


Figure 2.4 – Differential expression of proteins in the urea cycle at different growth temperatures.

Schematic representation of proteins involved in the urea cycle and their corresponding GenBank accession numbers. Most enzymes of the urea cycle were found to be present at 30°C exclusively or predominantly. Only Arginase deviated from this trend, being identified only in isolates of cultures grown at 6°C.

As displayed in Figure 2.4, most urea cycle related proteins were detected exclusively in 30°C isolates. In fact, these proteins were among the most dominant proteins identified at 30°C and, together with Pyruvate Formate Lyase mentioned above, showed the clearest growth temperature dependence (Supplementary Table S1). Of the proteins involved in this cycle, only Arginase (*bwei_5574*, AIW88091) deviated from this behavior being identified exclusively in 6°C isolates. The mechanistic rationale behind this remains unclear, but it appears that the urea cycle is used in a different manner depending on the growth temperature. The net result of the activity of the proteins identified at 30°C would be production of fumarate while at 6°C the suggested end product is urea. Perhaps, in line with the oversaturation of the TCA cycle mentioned above, at 30°C the urea cycle is used to feed fumarate into the TCA cycle to allow it to keep up with a high pyruvate production better. Conversely, urea is known for its chaotropic capabilities which might be beneficial at low temperatures. As a so called compatible solute, it may counteract the tendency of macromolecules to rigidify and/or aggregate at lower temperatures, thereby increasing the bacterium's viable growth temperature range.^{104,111} To put this in perspective, in the related species *Bacillus subtilis*, Budde et al.¹¹² have shown that arginine biosynthetic pathways were in fact chill-induced at the transcriptional level. Here, an opposite effect is observed at the translational level for Argininosuccinate Synthase and Lyase in *Bacillus weihenstephanensis*.

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Apart from the species differences, this may have resulted from a difference in growth medium, as a minimal growth medium was used in the Budde study compared to the rich culture medium used here. Interestingly, conversion of urea to ammonium and CO₂ was shown to be chill-induced in *B. subtilis* as well yet *B. weihenstephanensis* lacks the Urease required for this process. This may signify the alternative strategies available to these organisms which allow one to be psychrotolerant while the other is mesophilic.

A very striking discovery was the fact that a substantial number of acetyltransferases with undetermined substrate specificity were detected (Table 2.1). Acetylation of proteins by acetyltransferases can occur at α - or ϵ -amino groups and is known to be a key post-translational regulatory mechanism in eukaryotes. In prokaryotes, this has only recently gained some traction.¹¹³ In *Escherichia coli*, studies have shown certain stimuli (hypoxic growth) can induce altered acetylation profiles, as monitored by immunoblotting with an acetyl-lysine-specific antibody.¹¹⁴ Furthermore, changes in growth phase were also linked to changes in acetylation. Though the specific role of acetylation remains unclear, there have been indications that carbon source utilization and metabolic flux can be regulated by acetylation.¹¹⁴⁻¹¹⁶ As most of the identified acetyltransferases were temperature specific (Table 2.1), this indicated a possible regulatory function. MASCOT searches of the dataset using Protein N-terminal acetylation and Lysine Acetylation as a variable modification indeed revealed acetylated peptides. However, no clear relation to growth temperature relation was apparent as both modified and unmodified versions of the same peptides were detected at both temperatures (data not shown). Modified peptides are suggested to be of low abundance, in addition to the modification being transient, thus requiring dedicated methods for analysis beyond the profiling approach used in this study. What role acetylation and the temperature specific acetyltransferases play in *B. weihenstephanensis* remains to be determined.

6°C Specific		30°C Specific		30°C Predominant		Non specific	
Gene ID	GenBank	Gene ID	GenBank	Gene ID	GenBank	Gene ID	GenBank
<i>bwei_1911</i>	AIW84553	<i>bwei_0856</i>	AIW83521	<i>bwei_5021</i>	AIW87606	<i>bwei_2238</i>	AIW84871
<i>bwei_2079</i>	AIW84718	<i>bwei_1314</i>	AIW83970			<i>bwei_2813</i>	AIW85435
<i>bwei_2936</i>	AIW85555	<i>bwei_1413</i>	AIW84068				
<i>bwei_2938</i>	AIW85557	<i>bwei_2597</i>	AIW85223				
<i>bwei_3397</i>	AIW86013	<i>bwei_2618</i>	AIW85244				
		<i>bwei_2958</i>	AIW85577				

Table 2.1. Growth temperature dependence of identified Acetyltransferases.

Toxigenic profile largely temperature independent
 From a food safety perspective, it is important to highlight the toxigenic profile. *B. weihenstephanensis* can potentially produce a variety of toxins associated with diarrhoeal disease: haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K.⁶ In this strain, two copies of the genes encoding the homologous three-component pore-forming cytotoxins Hbl and Nhe have been found. As displayed in Table 2.2, component proteins of both copies of Hbl were identified in the secretome regardless of the growth temperature, with copies of Hemolysin BL lytic components L1 and L2 being exclusive or predominant at 30°C. On the other hand, not all components of Nhe were detected, with only one of the copies of NheC (*bwei_3144*, AIW85763) being identified at 6°C.

Toxin	Component Protein	Gene ID	GenBank	30°C	6°C
Hbl1	Hemolysin BL lytic component L1	<i>bwei_1967</i>	AIW84609	+	-
	Hemolysin BL lytic component L2	<i>bwei_1966</i>	AIW84608	++	+
	Hemolysin BL-binding component	<i>bwei_1968</i>	AIW84610	+	+
Hbl2	Hemolysin BL lytic component L1	<i>bwei_2505</i>	AIW85131	++	+
	Hemolysin BL lytic component L2	<i>bwei_2506</i>	AIW85132	++	+
	Hemolysin BL-binding component	<i>bwei_2504</i>	AIW85130	+	+
Nhe1	Hemolytic enterotoxin NheA	<i>bwei_3146</i>	AIW85765	+	+
	Hemolytic enterotoxin NheB	<i>bwei_3145</i>	AIW85764	+	+
	Hemolytic enterotoxin NheC	<i>bwei_3144</i>	AIW85763	-	+
Nhe2	Hemolytic enterotoxin NheA	<i>bwei_5368</i>	AIW87907	+	+
	Hemolytic enterotoxin NheB	<i>bwei_5367</i>	AIW87906	+	+
	Hemolytic enterotoxin NheC	<i>bwei_5366</i>	AIW87905	-	-

Table 2.2. Toxins produced by *B. weihenstephanensis* WSBC 10204.

+ = Identified, ++ = Predominant - = Not Identified

Lindbäck et al.¹¹⁷ have previously shown maximal cytotoxic activity when NheA, NheB and NheC were incubated in a molar ratio of 10 : 10 : 1. This implies NheC is likely to be present at much lower levels than the other components, thereby evading the detection methods used in this study. Gohar et al.¹¹⁸ previously were also not able to detect NheC in the secretome of *Bacillus cereus* using two-dimensional gel electrophoresis combined with MALDI-TOF MS. NheA and NheB were shown to have low toxic activity in absence of NheC^{6,117} implying that regardless of whether NheC is not present or simply not detected due its low abundance, *B. weihenstephanensis* WSBC 10204 is indeed toxigenic at low growth temperatures.

Conclusions

The psychrotolerant food spoiling pathogenic bacterium *Bacillus weihenstephanensis* poses a significant health risk due to its propensity to infect and survive in food processing lines. This, combined with its ability to grow and potentially produce toxins at lower temperatures than related species, begets the question how it manages to maintain growth in a refrigerated environment and if it indeed produces toxins. To open up molecular investigations into the behavior of *B. weihenstephanensis* the type strain WSBC 10204 (DSM 11821) has been sequenced and annotated. Complete proteome prediction followed by analysis of the cellular proteome and secretome profiles at 6°C and 30°C revealed differential expression of proteins related to processes known to be induced at low temperatures, such as the cold shock response, homeoviscous adaptation of the membrane or adjustment of superhelicity of DNA. Furthermore, new processes have been indicated to be dependent on growth temperature, in the form of varying carbon flux, as well as a different role for the urea cycle. Finally, a possible post-translational regulatory function via protein acetylation has been suggested as a result of growth temperature dependent expression of a large variety of acetyltransferases. Toxin production was found to be largely independent of growth temperature with encoded tripartite toxins showing only partial differential expression of component proteins. Regardless, toxins were produced at refrigerated temperatures once again stressing the importance of proper control and further characterization of this species.

Associated content

Supplementary Table S1: all proteins identified in cellular isolates and secretome isolates of *Bacillus weihenstephanensis* type strain WSBC 10204 grown at 6°C and 30°C with the accumulated MASCOT scores and peptide spectrum matches of each protein per isolate. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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