# Proteomics Viewed on Stress Response of Lipolytic Thermophilic Bacterium *Geobacillus* sp. NTU 03

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## Abstract:

**Background**: Microbial lipolytic enzymes have attracted considerable attention owing to their biotechnological potential. Thermophiles usually produce many enzymes that are very useful for industrial applications, and have already some reports about their thermostable lipolytic enzyme sources. However, the functional properties and mechanisms of thermophiles are rarely reported and still need more understanding on their physiology to expand the application. The objective of the present study is to isolate lipolytic thermophiles and employ differential proteomic approach to investigate their stress responses. **Methods:** Lipolytic thermophiles were isolated by tributyrin agar plate from local area at 65$^{o}$C and identified by 16S rRNA analysis. Acid (pH 5), salt (NaCl 2% w/v) and acid-salt (pH 5 and NaCl 2% w/v) were used as stress condition. 2-DE was employed to investigate the bacterial stress responses and proteins were identified by LC-MS/MS. **Results:** Twenty two isolates displayed lipolytic activity, of these NTU 03 showed high lipolytic enzyme activity and was chosen as the target of the study. The partial sequence of 16S rRNA showed 99% similarity to *Geobacillus* sp. After different stress condition treated, dramatic changes of the intracellular protein expression patterns were found in 2-DE gel. Many of proteins were down-regulated by each stress. About 17%, 23% and 18% of proteins were up-regulation under acid, salt and acid-salt treatment, respectively. More than three proteins were up-regulation under acid treatment but down-regulation under salt treatment, ex the F0F1 ATP synthase subunit beta, but some spots showed opposite situation. One spot, identified as pyridoxine biosynthesis protein, showed dramatic down-regulation under salt stress but slightly decreased under acid and acid-salt condition. **Conclusion:** The 2-DE provided an overview of cell adaptation to stress responses of *Geobacillus* sp. NTU 03 by the dramatic changes in the protein expression patterns. These changes will contribute to connect regulation pathway of different stress responses.

Keyword: thermophiles, stress response and proteome

## Introduction

The need for alternative energy sources that combine environmental friendliness with biodegradability, low toxicity, renewable, and less dependence on petroleum products increased rapidly. One such energy source is referred to as biofuel, including biodiesel and bioethanol. Biodiesel can be produced from natural oils, fats, waste products of vegetable oil refinery or animal rendering, and used frying oils through enzymatic reactions involving lipase or others chemical reactions. (Ragauskas, *et al.*, 2006; Akoh, *et al*., 2007)

Lipases are found in all living organisms and are classified on the basis of the sources from which they are obtained, such as microorganism, animal, and plant. In practice, microbial lipases are commonly used by the industry, because of high yield and separation easily. Lipases constitute a group of enzymes having the ability to catalyze both the hydrolysis and synthesis of long-chain acylglycerols. These reactions usually proceed with high regioselectivity and enantioselectivity, and, therefore, lipases have become very important stereoselective biocatalysts used in organic chemistry or additives in laundry detergents (Jaeger, *et al*., 1994, Nthangeni, *et al*., 2001).

The thermostability of biocatalysts is an important criterion when dealing with industrial bioprocesses at high temperature for sustainable operation. Values of elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates, for example: vegetable oils and animal fats, higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reaction.

Enzyme stability is dictated by its three-dimensional configuration, which in turn is determined by genetic and environmental factors. Therefore thermophilic bacteria are promising sources of heat-stable enzymes, ex. thermostable lipases (Abd Rahman, *et al*., 2007). Lipases from thermophiles have the advantage of thermal stability at 65-70$^{o}$C, which makes them good candidates for lipid modifications at high temperatures (Abdel-Fattah, *et al*., 2002). In addition to higher thermostability, proteins from thermophiles often showed higher stability toward organic solvents and higher activity at elevated temperature (Abd Rahman, *et al*., 2007). Recently,

thermophilic bacilli, *Geobacillus* spp. such as *Geobacillus* sp. TW1 (Li and Zhang, 2005), *Geobacillus* sp.T1 (Leow, *et al*., 2007), *Geobacillus zalihae* sp. nov. (Abd Rahman, *et al*., 2007) were reported as thermostable lipase producers, and *Geobacillus thermodenitrificans* NG80-2 was reported the presence of a long-chain alkane degradation pathway. (Feng, *et al*., 2007).

'Stress response' refers to the reaction of a living cell to potentially harmful physical conditions, chemical agents, or nutritional starvation condition. It is well-known that living cell are able overcome some stresses by qualitative and quantitative adjustments in protein synthesis. The synthesis or the overexpression of a subset of proteins thus allows living cell to face harsh conditions and to maintain essential cellular functions. Because of the relatively low complexity of bacterial systems, many of the proteins involved in basic physiological reactions such as stress/starvation responses or metabolic pathways can be visualized through the gel-based proteomics, and so many bacterial systems developed into valuable model sytem for physological research (Vasseur, *et al*., 1999; Hecker and Völker, 2004; Höper, *et al*., 2006). According to the nature of the stress, the bacterial response at the level of protein synthesis can be completely different and produce variations in the expression of different sets of proteins on a few two-dimensional electrophoresis gels rapidly. A comprehensive understanding of the physiology of industrially relevant microorganisms during bioprocesses, thus leading to a more successfully directed strain and process optimization, was hindered in the past by the lack of reliable information on the physiological status of the cells at the molecular level under cultural conditions. (Schweder and Hecker, 2004; Schuster, 2000)

The objective of the present study is to isolate lipolytic thermophiles and employ differential proteomic approach to analyze their protein expression of stress responses. Through compared the two dimensional electrophoresis (2-DE) patterns of proteins synthesized after the different stress treatments, we try to point out the main proteins involved in each mechanism of adaptation and the regulation of thermophiles under stress may be understood. Stress-induced proteins will be the molecular markers for detection the fitness of cultures and used as positive indicators for a culture that is

fully adapted to resisting an upcoming stress condition. As more information about the mechanisms involved in the adaptation of thermophiles to stress conditions are understood, a better knowledge of stress physiology may be useful to optimize the culture conditions affecting thermophiles physiology to enhance yield of correlated bioproducts.

## Material and Methods

### Enrichment and isolation of lipolytic thermophiles

Soil, compost, wasted or addled-oils/fats samples were collected from different local areas. The enrichment culture of samples were performed at 65°C with rotary-shaking water bath (150 rpm) in nutrient broth (NB) containing 1% olive oil for 12 hours at an initial pH 7.0. The enriched cultures were further screened by using tributyrin agar plate (Sigma, St. Louis, MO, USA). Lipolytic activities rely on the formation of a clear zone on tributyrin agar plate assay as a result of lipase production (Lawerence, *et al*., 1967).

### DNA extraction, PCR sequencing of 16S rDNA region and lipase gene

DNA was isolated from the strain using the Promega Wizard genomic DNA kit (Promega, Madison, WI, USA), following the manufacturer's instructions for gram-positive bacteria. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using primers designed to amplify almost 1500 base pair fragment of the 16S rRNA gene region. The forward primer 27F was 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 1522R was 5'-AAGGAGGTGATCCAACCGCA-3'. The PCR mixture consisted of 30 picomoles of each primer, 10 ng of chromosomal DNA, 200 μM dNTPs and 2.5 uits of *Taq* DNA polymerase in 50 μL of polymerase buffer. The PCR was carried out for 94$^{o}$C for 10 min, 30 cycles at 94$^{o}$C for 1 min, 55$^{o}$C for 1 min, 72$^{o}$C for 2 min and 72$^{o}$C for 30 min.

The lipase gene was amplified by PCR using degenerate primers CJH-F1 (5'-AGSRTGATGAAAKGCTGYGGGCTKATGK-3') and CJH-R1 (5'-KYWTTAAGGCYGCAARCTCGCCA-3') with following PCR condition: 94$^{o}$C for 4 min, 35 cycles at 94$^{o}$C for 1 min, 60$^{o}$C for 2 min, 72$^{o}$C for 1 min and 72$^{o}$C for 7 min (Leow, *et al*., 2004).

After completion, a fraction of the PCR mixture was examined using agarose gel

electrophoresis (Sambrook and Russell, 2001). The remnant mixture purified using QIAquick PCR purification reagents (Qiagen, Hamburg, Germeny). DNA sequences were obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM BigDye Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA, USA).

Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using Vector NTI 10.3 software (Invitrogen, Taipei, Taiwan).

**Application of stressing conditions**

Pre-cultures were grown in previously autoclaved Hinton flasks filled with 100 mL NB. They were incubated for overnight on rotary shaking water bath at 150 rpm and 60$^{o}$C.

For the three stress challenges [acid (pH 5), salt (2% NaCl) and combined treatments (acid-salt, pH 5 + 2% NaCl)], 5 mL pre-culture broth were transferred to 100 mL fresh NB in Hinton flasks. The bacterium was cultured on rotary shaking water bath at 150 rpm and 65$^{o}$C until mid-log phase ($OD_{600}$ ~ 0.7-0.5) and subsequently treated by three stress challenges. After 10 minutes of stress treating, the bacterial cell were observed by spectrophotometry ($OD_{600}$) and harvested by centrifugation at 4$^{o}$C, 8,000 x g for 10 minutes. The bacterial cells were washed three times with preparation buffer pH 8.0 containing 10 mM Tris-HCl, 1 mM EDTA and 0.1mM PMSF.

**Protein extraction**

The protein extraction procedure will follow the protocol of Topanurak published at 2005 (Topanurak, *et al*., 2005). Washed Cell pellets were resuspended in the same buffer disrupted on ice by sonication (five cycles of 50 s). Cell debris was removed by centrifugation at 12,000 x g at 4$^{o}$C for 30 min. The sample solutions are precipitated

by addition of pre-chilled 10% trichloroacetic acid (TCA) and 0.1% dithiothreitol (DTT) in acetone and stored overnight at -20$^{o}$C to precipitate the proteins and to remove the salt and nucleic acid. The suspensions were centrifuged at 20,000 x g for 30 min. Protein pellets are resuspended in ice-cold acetone containing 0.1% DTT and stored at -20$^{o}$C for at least 30 min. The protein suspensions were centrifuged again at 20,000 x g for 30 min and the pellets are resuspended in ice-cold acetone without DTT. The protein suspensions are stored at -20$^{o}$C for 30 min and then centrifuge at 20,000 x g for 30 min. The obtained pellets are immediately dissolved in lysis buffer [7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS), 1% **IPG** buffer pH 4–7 and 65 mM DTT]. Each sample is sonicated and centrifuged, and the protein concentration is determined using a PlusOne 2-D Quant Kit (Amersham Biosciences, Buckinghamshire, UK) with bovine serum albumin (BSA) as a standard (Topanurak, *et al*., 2005).

**Two-dimensional electrophoresis (2-DE)**

Each sample is applied onto immobilized pH gradient (IPG) strips (13 cm, pH 4-7; Amersham Biosciences) with a final concentration of 350 µg protein in 250 µL. Ettan IPGphorII isoelectric focusing system (IEF system, Amersham Biosciences) is performed under following condition: IPG strips are rehydrated passively and/or actively for 12 hrs at 30 V followed by ramping to 250 V for 1 hr, 500 V for 1 hr, 1000 V for 1 hr, 1500 V for 1 hr, 2000 V for 1 hr, 3000 V for 1 hr, 6000 V for 1 hr and focusing at 8000 V for 40000 Vhr. After IEF, the IPG strips are equilibrated in equilibration buffer I (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% DTT and a trace of bromophenol blue) for 15 min, and then subsequently alkylated in equilibration buffer II (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% w/v iodoacetamide and a trace of bromophenol blue) for 15 min. Each equilibrated IPG strip is placed on top of the 12.5% polyacrylamide gel and covered with 0.5% agarose. The second-dimensional separation is performed using the SE 600 Ruby standard dual cooled gel exectrophoresis unit (Amersham Biosciences) and

carried out at 40 mA per gel at 15$^{o}$C until the bromophenol blue dye front reached the bottom of the gel. At the end of each run, the 2-D gels are staining by colloidal CBB stains method (Candiano, *et al*., 2004). In addition, the 2-D gel images were exported to the image analysis software program, using ImageMaster$^{TM}$ 2D platinum software (Amersham Biosciences).

**In gel digestion**

Protein spots are manually excised from the polyacrylamide gels and transferred to 500-μL siliconized eppendorf tubes. The gel pieces were washed twice with 200 μL 50% acetonitrile (ACN)/25 mM ammonium bicarbonate (ABC) buffer pH 8.0 for 15 min each. The gel pieces were then washed once with 200 μL 100% ACN and dried using a SpeedVac concentrator (Savant, Ramsey, MN, USA). Dried gel pieces were swollen in 10 μL 25 mM ABC containing 0.1 mg trypsin (sequencing grade; Promega, Madison, WI, USA). Gel pieces were then crushed with a siliconized blue stick and incubated at 37$^{o}$C for at least 16 h. Peptides were subsequently extracted twice with 50 μL 50% ACN/5% trifuloro acetic acid (TFA), and the extracted solutions were combined and dried using SpeedVac concentrator. The peptides or pellets were then resuspended in 20 μL 0.1% TFA and the suspended solutions were purified using ZipTip C18 (Millipore, Bedford, MA, USA). Briefly, 10 μL samples were drawn up and down in the ZipTip ten times, and the ZipTip was washed with 10 μL 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were eluted with 5 μL 75% ACN/0.1% formic acid.

**Protein identification by LC-MS/MS**

LC-MS/MS analysis was performed on an integrated nano-LC-MS/MS system (QSTAR XL) comprising a LC Packings Nano-LC system with an autosampler, and a QSTAR XL Q-TOF mass spectrometer (Applied Biosystems) fitted with nano-LC

sprayer. Injected samples were first trapped and desalted on a LC-Packings PepMap™ C18 μ-Precolumn™ Cartidge (5 μm, 30 μm I.D. x 5 mm; Dionex, Sunnyvale, CA, USA) after that the peptides were eluted off from the precolumn and separated on an analytical LC-Packings PepMap C18 column (3 μm , 15 cm x 75 μm i.d.,) connected inline to the mass spectrometer, at 200 μL/min using a 40 min gradient of 5% to 60% ACN in 0.1% formic acid. Online nanoESI-MS survey scan and data dependent acquisition of CID MS/MS were fully automated and synchronized with the nanoLC runs under the full software control of AnalystQS. Prior to online analysis, the nanoLC sprayer source parameters were tuned and optimized. Argon was used as the collision gas for CID MS/MS. Calibration was performed using the product ions generated from fragmentation of the doubly charged molecular ion of renin. For routine protein identification analysis, the 1 s survey scans were acquired over the mass range m/z 400 – 1600 and a maximum of 2 concurrent MS/MS acquisitions could be triggered for 2+, 3+ and 4+ charged precursors detected at an intensity above the predefined threshold. Each MS/MS acquisition will be completed and switch back to survey scan when the precursor intensity fell below a predefined threshold or after a maximum of 6 s acquisition. After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were combined and output as a single MASCOT-searchable peak list file. The peak list files were used to query the NCBI database using the MASCOT program with the following parameters : peptide mass tolerance, 150 ppm; MS/MS ion mass tolerance, 0.15 Da; allow up to one missed cleavage. Only significant hits as defined by MASCOT probability analysis will be considered initially. In addition, a minimum total score of 20 comprising at least a peptide match of ion score more than 20 was arbitrarily set as the threshold for acceptance.

# Result

## Isolation and selection of lipolytic thermophiles

Out of 27 thermophilic aerobic bacterial isolates, 22 colonies representing different morphotypes were able to hydrolyze tributyrin as determined by plate assay. The biotype NTU 03 strain was selected as the most potent organism based on zone of hydrolysis formed and the PCR product of lipase gene (Figure 1), NTU 03 was named G*eobacillus* sp. NTU 03 and therefore used for further experiments

## Characterization, molecular identification and phylogenetic analysis of G*eobacillus* sp. NTU 03

The cellular morphology of G*eobacillus* sp. NTU 03 cells were gram positive, endospore forming rods commonly observed in unbranched chains of 4-8 cells. Spores were spherical and formed terminally. The growth condition for G*eobacillus* sp. NTU 03 was 45-75$^{o}$C and between pH 4.5 and 10 with the optimum growth temperature at 65$^{o}$C and pH 6.5-7.0 in nutrient broth, respectively. The partial sequencing of the 16S rRNA gene shows 93-99.5% to validly described *Geobacillus* sp. and showed the highest similarity with *G. vulcani* BGSC 97A1, *G. zalihae* T1 and *G. lituanicus* BGSC W9A89. Construction of phylogenetic trees using the neighbour-joining method in determining the evolutionary relationship among a group of validly described closely related species is indicated in Figure 2.

## Proteomic analysis of intracellular proteins of G*eobacillus* sp. NTU 03

To investigate transient metabolic adaptation for bacterial survival and its physiological activity, the proteome of G*eobacillus* sp. NTU 03 under acid, salt or combined stress was studied immediately after stress treated. The growth profile of this bacterium is shown in Figure 3. When challenged with stress conditions during

exponential growth, G*eobacillus* sp. NTU 03 immediately arrested growing. After an adaptation period of about 30-60 min growth resumed at a reduced rate.

After stress conditions treated within a short period time of 10 min, 2-DE analysis of the intracellular proteins of G*eobacillus* sp. NTU 03 demonstrated some changes in the protein patterns (Figures 4A-D). Many of proteins were down-regulated by each stress. About 17%, 23% and 18% of proteins were up-regulated under acid, salt and acid-salt treatment, respectively. In this work, a total of 22 proteins determined with different expressions in protein content were cut and analyzed using LC-MS/MS. By a search of the protein database against all bacterial species through NCBI on MASCOT, 22 proteins were identified (Table 1). Of the 22 protein spots identified, 14 were up-regulated under acid stress condition; 16 were up-regulated under salt stress condition; and 11 were up-regulated under acid-salt stress condition; 1 was down-regulated under all of three stress conditions. Among the 22 identified proteins, 2 were up-regulated under acid and acid-salt stress condition but down-regulated under salt stress condition, 3 were up-regulated under salt and acid-salt stress condition but down-regulated under acid stress condition.

## Discussion

Since lipases of thermophiles are usually more thermostable, they have attracted considerable attention owing to their biotechnological potential. Thermophilic bacilli, including *Geobacillus*, are widely distributed and have been successfully isolated from all continents where geothermal or artificial thermal areas occur. (McMullan *et al*., 2004). But there were some strains of *Geobacillus* readily isolated from temperate soil environments. Initial studies surprisingly showed that thermophilic aerobic bacilli could be readily isolated in large number from a range of temperate Irish soils. Subsequently the similar findings had been repeated in Europe (McMullan *et al*., 2004, Marchant, *et al*., 2002a and b). In this work, different samples were collected from local thermal or temperate areas to screen lipolytic thermophiles. The isolated strains had checked by PCR of lipase gene and characterized by 16S rRNA sequences. *Geobacillus* sp. was a phenotypically and phylogenetically coherent group of thermophilic bacilli displaying very high similarity among their 16S rRNA sequences (98.5-99.2%) (Nazina, *et al*., 2001). Identification these highly similarity species for lower order taxa, the resolving power of 16S rRNA sequence analysis may be poorly, and need more traditional biochemical evidences or others molecular biotechniques, for example, *recN* sequence similarity analysis or amplification profiles of the 16S-23S internal spacer region (Zeigler, 2005; Flint, *et al*., 2001)

Under the low pH condition, by the identified acid induced proteins, we suggested that may show three different responses to overcome the acid stress. F0F1 ATP synthase, phosphoyruvate hydratase, dehydroenase E1 component and inorganic pyrophosphatase involved into the intracellular proton balance, carbohydrate metabolism and energy supply. The second response was lysine metabolism pathway (Figure 5), including tetrahydrodipicolinate succinylase, and L-alanine dehydrogenase, and the other down-regulated protein dihydrodipicolinate synthase. Overexpression of the first two proteins, made the cell metabolism tendency to lysine biosynthesis. In *E. coli*, when acidic or lysine rich conditions happened, the *cadBA* operon inducted by cadC to increase the level of cadB and cadA. CadB was lysine/cadaverine antiporter,

transport lysine inside, and cadaverine outside the cell. CadA was lysine decarboxylase, used lysine as substrate to produce cadaverine and cost a proton, and so intracellular proton gradient would be adjusted. (Neely and Olson, 1996; Huang, *et al*., 2007). The other acid induced proteins included oligopeptide ABC transporter, ABC transporter (lipoprotein), flagellin protein, elongation factor Tu, perodiredoxin, thioreduction like protein and co-chaperonin GroES. After stress treated, many of normal cellular metabolisms were down-regulated, due to the metabolic proteins misfold or translated abnormally. Many cellular substrates for survival became shortage, so the transporter protein families may be the substitute way for satisfied cellular requirements. Furthermore, for assisting new translated proteins with folding correctly, besides the co-chaperonin GroES, elongation factor Tu (EF-Tu) was overexpression, too. EF-Tu had been shown the function as a molecular chaperone and to play a role in the protection of other proteins from thermal denaturation (Caldas *et al*., 1998).

Many proteins induced by salt stress were same as acid condition, for example: phosphopyruvate hydratase, L-alanine dehydrogenase, ABC transporter (lipoprotein), flagellin protein, tetrahydrodipicolinate succinylase, peroxiredoxin, inorganic pyrophosphatase, and co-chaperonin GroES. Among them, L-alanine dehydrogenease and tetrahydrodipicolinate succinylase may defense salt stress by different mechanism, due to the dihydrodipicolinate synthase, another lysine biosynthesis pathway member was induced but not in acid stress condition. N-Succinyl-LL-2,6-diaminoheptanedioate, the downstream product of tetrahydrodipicolinate succinylase, could be the substrate of N-Succinyl-L-2,6-diaminoheptanedioate:2-ocoglytarate amino-transferase reacted with 2-oxoglutarate to form L-glutamate. Glutamate, as the precursor for the preferred intracellularly synthesize compatible solute proline, is probably required in higher concentrations after salt stress when extracellular compatiable solutes were not available in the growth medium (Kuhlmann and Bremer, 2002; Whatmore, *et al*., 1990; Höper, *et al*., 2006).

Thioredoxin, thioredoxin reductase and NADPH, the thioredoxin system, is ubiquitous from Archea to man. Thiol-disulfide bond balance is generally maintained in bacteria by thioredoxin reductase-thioredoxin and/or glutathione-glutaredoxin systems. (Arnér and Holmgren, 2000; Vido, *et al*., 2005). Therefore, they also served as electron donors for enzymes such as ribonucleotide reductase, thioredoxin peroxidase (peroxiredoxin) and methionine sulfoxide reductase. Since, peroxiredoxin helped cell to overcome the intracellular oxidative imbalance caused by stress condition, and the thioredoxin system did the same as peroxiredoxin and promoted protein refolding correctly by the thiol-disulfide bond balance.

From the fold change of identified proteins, co-chaperonin, GroES, translation initiation inhibitor and the thioredoxin system were more expression under salt condition than low pH condition, and the translation initiation inhibitor was induced by salt but not by acid. We suggested problem about proteins misfolding under salt stress condition was more serious.

Protein spot 135, 182, 197, 249, 262, 320, 357, 364, 373 and 427 were overexpression under three stress conditions, these proteins may belong with the general stress response. Protein spot 77 and 199 were induced only by acid and 267, 408, 419 and 423 were induced only by salt may belong with specific responses, respectively. One of the earliest responses of the cell to growth arrest caused by stress or starvation is the increased production of a common group of very prominent proteins. This particular induction pattern led to the assumption that these proteins may have a rather non-specific, but nevertheless essential, protective function under stress, regardless of the specific growth-restricting factor. Therefore, these proteins were referred to as general stress proteins (Hecker and Völker, 2001). Besides these, each stress or starvation condition induced the synthesis of a specific set of proteins call stress/starvation-specific proteins. These groups of proteins might have a specific protective function against a single stimulus that might allow the cell to neutralize the stress factor, adapt to its presence or repair damage caused by it. Starvation-specific

proteins may allow uptake of limiting substrates with higher affinity, searching for alternate substrates and replacing the limiting substrates by others or moving to new nutrient sources by chemotaxis (Hecker and Völker, 2001).

## Concluding remarks

The use of 2-DE to study bacterial stress responses is an interesting approach and bring more than more attractive challenges, because it provides the essential basic information on the global regulation of gene expression under different stress conditions. In our work, we identified 22 proteins induced by stress responses, and connect to stress responses by their natural function or mechanism. An extension of this work could be the study of different stress level, treatment period, and others different stress or starvation conditions. The network between the stress responses and their regulation could be construction more completely.

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Table 1. Protein identification of intracellular proteins in *Geobacillus* sp. NTU 03, after different stress conditions treated, by LC-MS/MS and MASCOT software, based on NCBI database (http://www.ncbi.nlm.nih.gov/)

| Spot no. | Protein names | Sources | Mr [a] (kDa) | pI [a] | Mr [b] (kDa) | pI [b] | Sequence coverage (%) | Score | Accession number | Fold change under stress condition | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Acid | Salt | Acid-Salt |
| 77 | oligopeptide ABC transporter | *Geobacillus kaustophilus* HTA426 | 60351 | 8.42 | 60 | 5.4 | 57 | 1627 | gi\|56419346 | 2.13 | 0.00 | 1.65 |
| 118 | F0F1 ATP synthase subunit beta | | 51821 | 5.03 | 50 | 5.2 | 80 | 1381 | gi\|56421893 | 1.69 | 0.22 | 0.74 |
| 135 | phosphopyruvate hydratase | | 46575 | 4.8 | 50 | 5 | 70 | 759 | gi\|56421589 | 3.24 | 2.18 | 1.94 |
| 182 | L-alanine dehydrogenase | | 39696 | 5.37 | 43 | 5.3 | 52 | 664 | gi\|56421983 | 1.69 | 2.02 | 1.51 |
| 197 | ABC transporter (lipoprotein) | | 39113 | 6.38 | 40 | 5.5 | 61 | 1183 | gi\|56419818 | 2.24 | 1.68 | 1.69 |
| 199 | dehydrogenase E1 component, beta subunit | | 35418 | 4.96 | 40 | 5.1 | 70 | 889 | gi\|56419594 | 2.73 | 0.67 | 1.22 |
| 247 | pyridoxine biosynthesis protein | | 31576 | 5.62 | 35 | 5.7 | 44 | 743 | gi\|138893690 | 0.77 | 0.18 | 0.89 |
| 249 | flagellin protein | | 31902 | 5.24 | 35 | 4.8 | 49 | 494 | gi\|56421666 | 2.07 | 1.04 | 1.57 |
| 262 | flagellin protein | | 31902 | 5.24 | 36 | 5.1 | 15 | 167 | gi\|56421666 | 2.44 | 1.97 | 1.74 |
| 267 | dihydrodipicolinate synthase | | 30100 | 5.33 | 33 | 5.4 | 32 | 375 | gi\|56419812 | 0.89 | 2.18 | 1.34 |
| 306 | elongation factor Tu | | 43279 | 4.87 | 30 | 5 | 15 | 295 | gi\|56418639 | 2.28 | 1.05 | 2.91 |
| 320 | tetrahydrodipicolinate succinylase | | 24820 | 4.89 | 29 | 5.1 | 31 | 310 | gi\|56419584 | 1.17 | 2.24 | 1.45 |
| 357 | peroxiredoxin | | 20952 | 4.99 | 25 | 5.2 | 62 | 255 | gi\|56421110 | 1.27 | 1.66 | 1.24 |
| 364 | inorganic pyrophosphatase | | 19158 | 4.69 | 25 | 4.9 | 37 | 165 | gi\|56420781 | 2.66 | 1.96 | 1.77 |
| 373 | thioreduction like protein | | 19710 | 6.43 | 26 | 6 | 31 | 205 | gi\|138896609 | 1.27 | 1.66 | 1.24 |
| 408 | translation initiation inhibitor | | 13598 | 5.62 | 17 | 5.7 | 24 | 123 | gi\|56418576 | 0.59 | 1.24 | 1.05 |
| 419 | thioredoxin (TRX) | | 11542 | 4.88 | 14.4 | 5 | 41 | 187 | gi\|56421220 | 0.00 | 1.97 | 0.64 |
| 423 | thioredoxin (TRX) | | 11542 | 4.88 | 14.4 | 5.1 | 41 | 177 | gi\|56421220 | 0.00 | 2.26 | 2.32 |
| 427 | co-chaperonin GroES | | 10204 | 5.01 | 14 | 5.2 | 55 | 255 | gi\|56418783 | 1.45 | 1.66 | 1.65 |
| 429 | co-chaperonin GroES | | 10204 | 5.01 | 13 | 5.1 | 45 | 198 | gi\|56418783 | 0.00 | 1.90 | 0.91 |

[a] Theoretical Mr of the matched protein and theoretical pI of the matched protein in the database. [b] Estimated molecular mass (Mr) and pI values estimated from 2-DE PAGE. The pI values correspond to the middle of the spots.

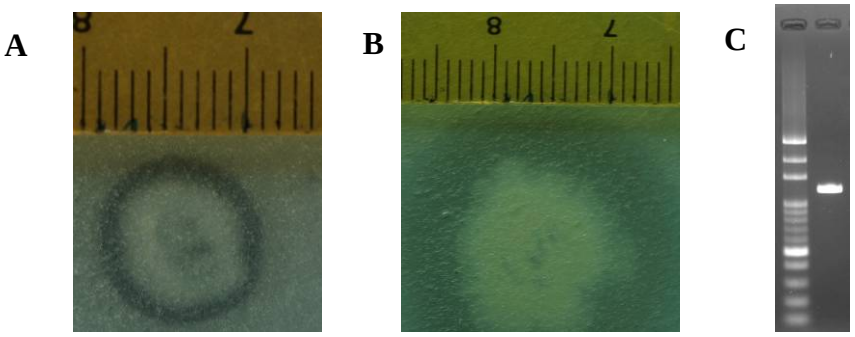



Figure 1. Lipolytic activity assay of NTU 03 by tributyrin agar plate and PCR of lipase gene. A and B: clear zone of tributyrin hydrolysis formed by NTU 03 on tributyrin agar plate at 65$^{o}$C through 24 h and 36 h cultural period. C: amplicon of thermostable lipase gene. M: 100 bp marker; amplicon size: 1260 bp.

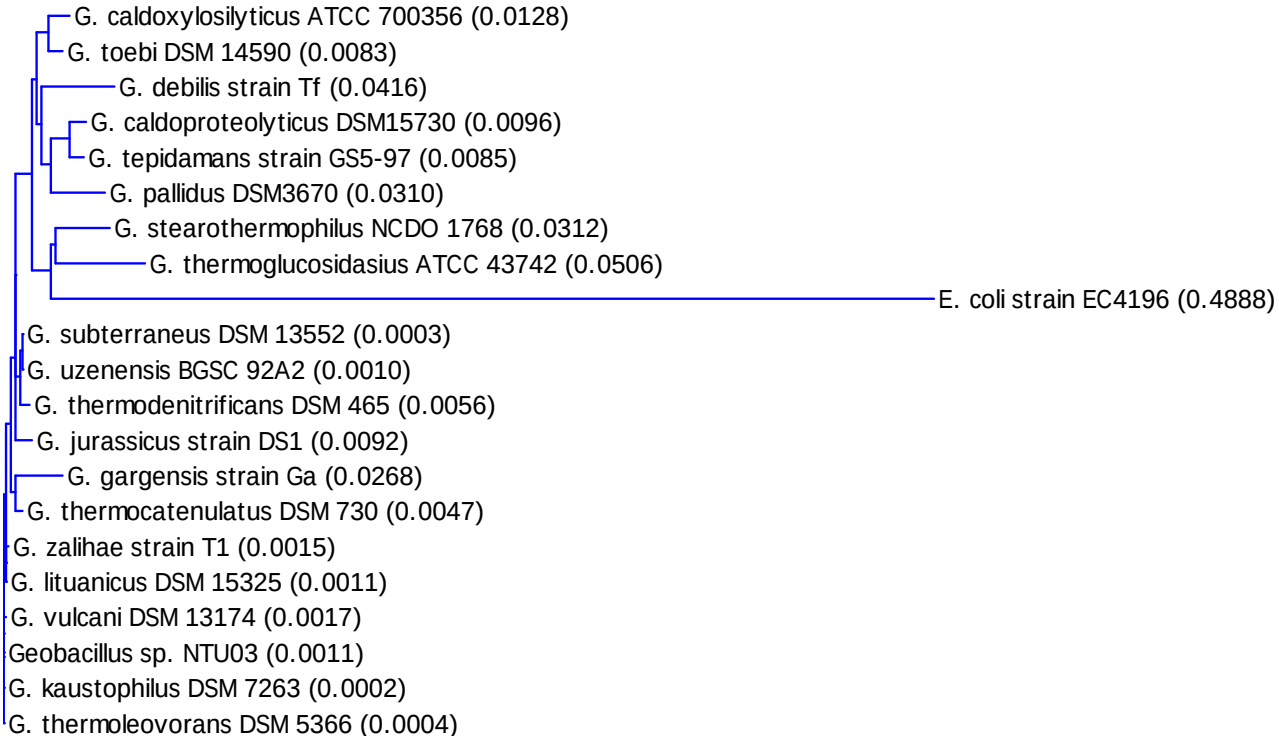



Figure 2. Phylogenetic position of *Geobacillus* sp. NTU 03 with other validly described species of the genus *Geobacillus*. The members of genus *Geobacillus* used include *G. thermoleovorans* (DSM 5366$^T$, Z26923); *G. kaustophilus* (DSM 7263$^T$, AY608934); *G. vulcani* (DSM 13174$^T$, AY608940); *G. lituanicus* (DSM 15325$^T$, AY608945); *G. thermocatenulatus* (DSM 730$^T$, AY608935); *G. gargensis* (strain Ga$^T$, AY193888); *G. stearothermophilus* (NCDO 1768$^T$, X60640); *G. uzenensis* (BGSC 92A2, AY608959); *G. jurassicus* (strain DS1$^T$, AY312404); *G. subterraneus* (DSM 13552$^T$, AY608956); *G. thermodenitrificans* (DSM 465$^T$, AY608960); *G. caldoxylosilyticus* (ATCC 700356$^T$, AF067651); *G. toebi* (DSM 14590$^T$, AY608982); *G. thermoglucosidasius* (ATCC 43742$^T$, X60641), *G. tepidamans* (strain GS5-97$^T$, AY563003); *G. caldoproteolyticus* (DSM15730$^T$, AY327448); *G. pallidus* (DSM3670$^T$, Z26930); *G. debilis* (strain Tf$^T$, AJ564616) and *G. zalihae* (strain T1, AY166603). *Escherichia coli* (strain EC4196, NZ_ABHO01000053) were used as an out-group. Phylogenetic tree was inferred by using the neighbour-joining methods. The software package Vector NTI 10.3 was used for analysis

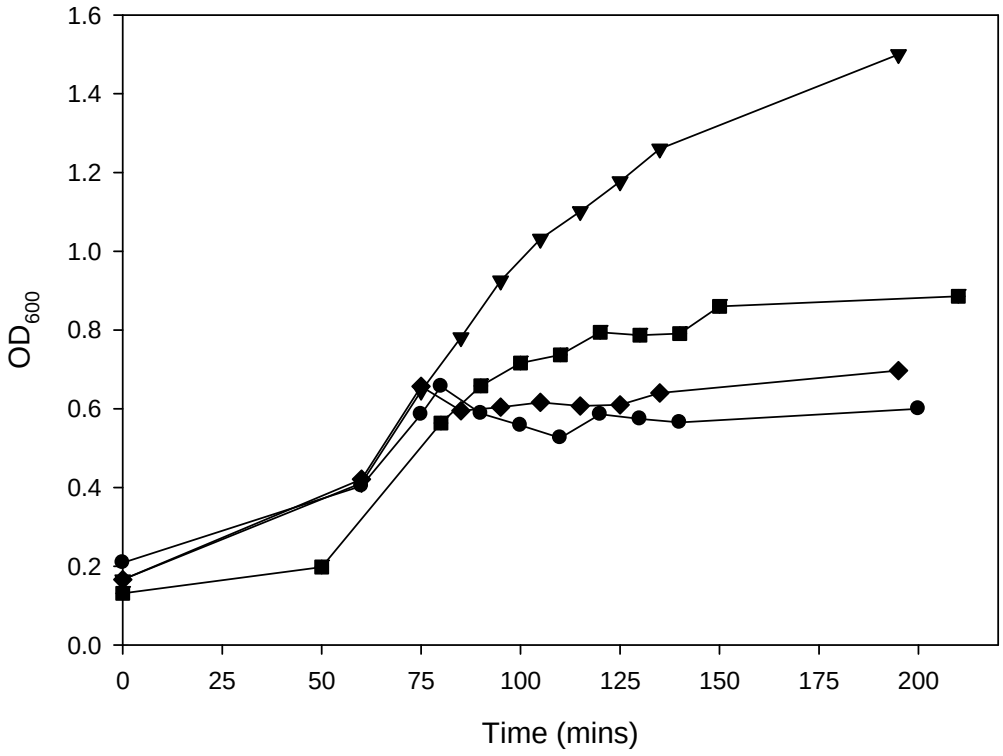
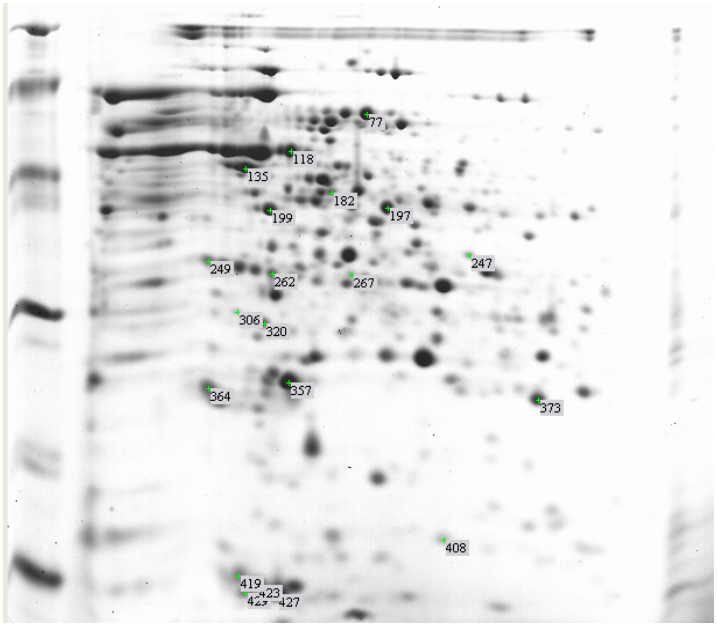



Figure 3. Growth profile of *Geobacillus* sp. NTU 03 in NB before and after challenge with different stress conditions. When cultures reached an optical density of 0.5-0.7 different stress condition were treated. –▼- control, -■- salt stress (2% NaCl), –●- acid stress (pH 5), -◆- combined stress (acid-salt, pH 5+2% NaCl)

A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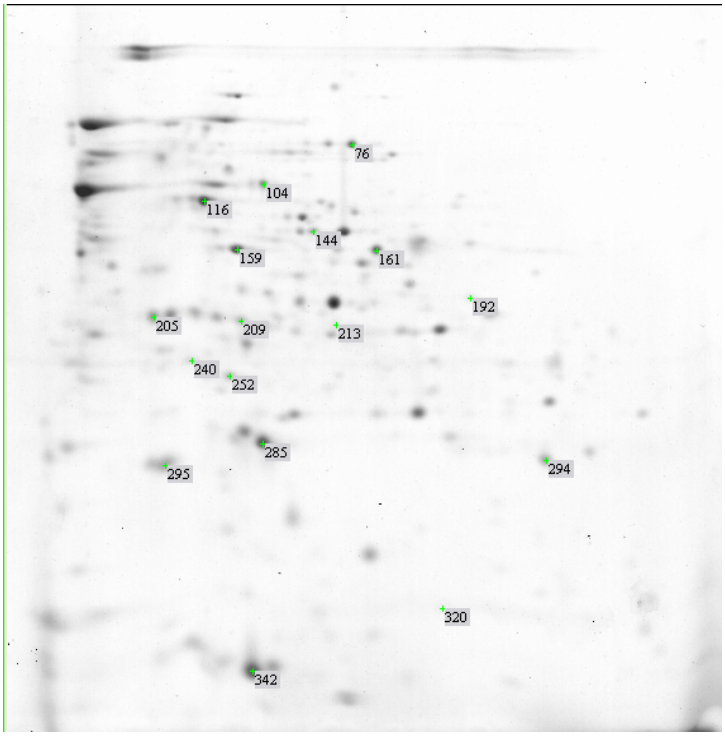
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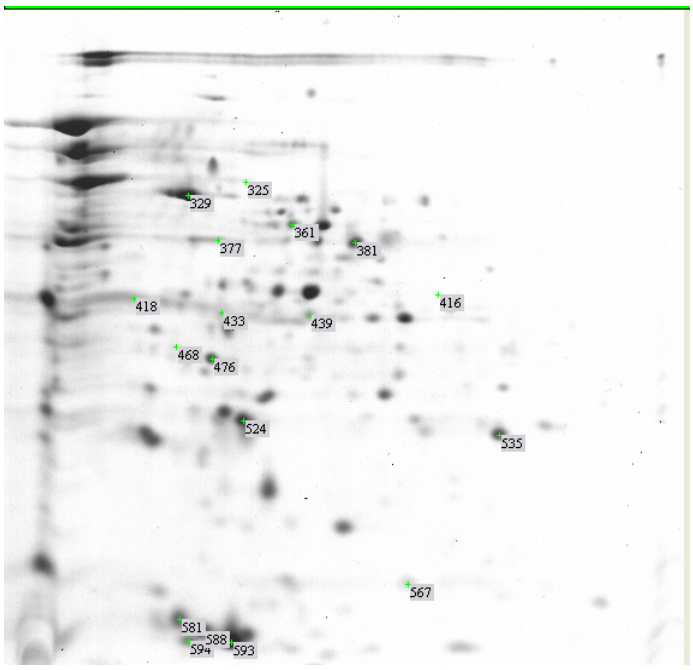
C




D

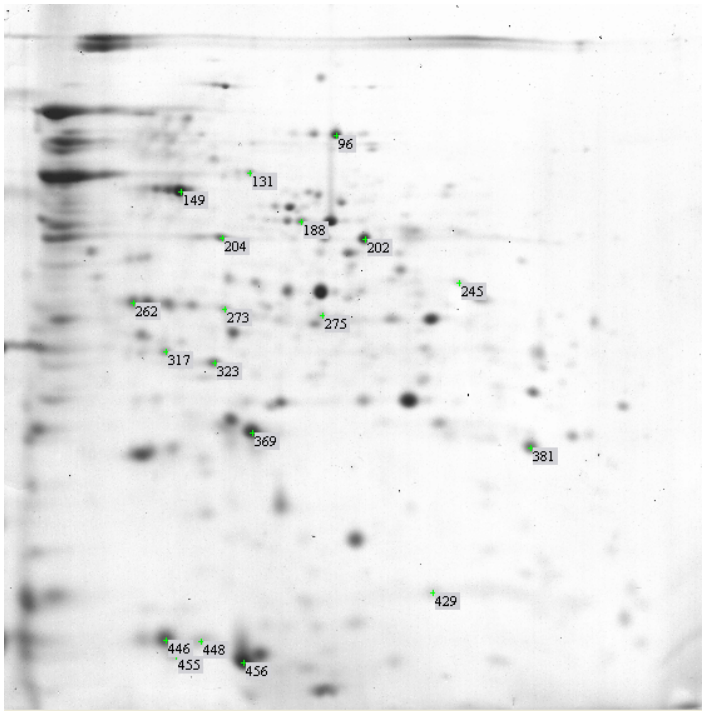



Figure 4. 2-D gel images of cell extracts from *Geobacillus* sp. NTU 03 at three different stress conditions; A: normal condition, B: acid stress condition (pH 5), C: salt stress condition (2% NaCl), D: acid-salt stress condition (pH 5 + 2% NaCl). The number labeled show the spots of proteins, which were identified by LC-MS/MS.

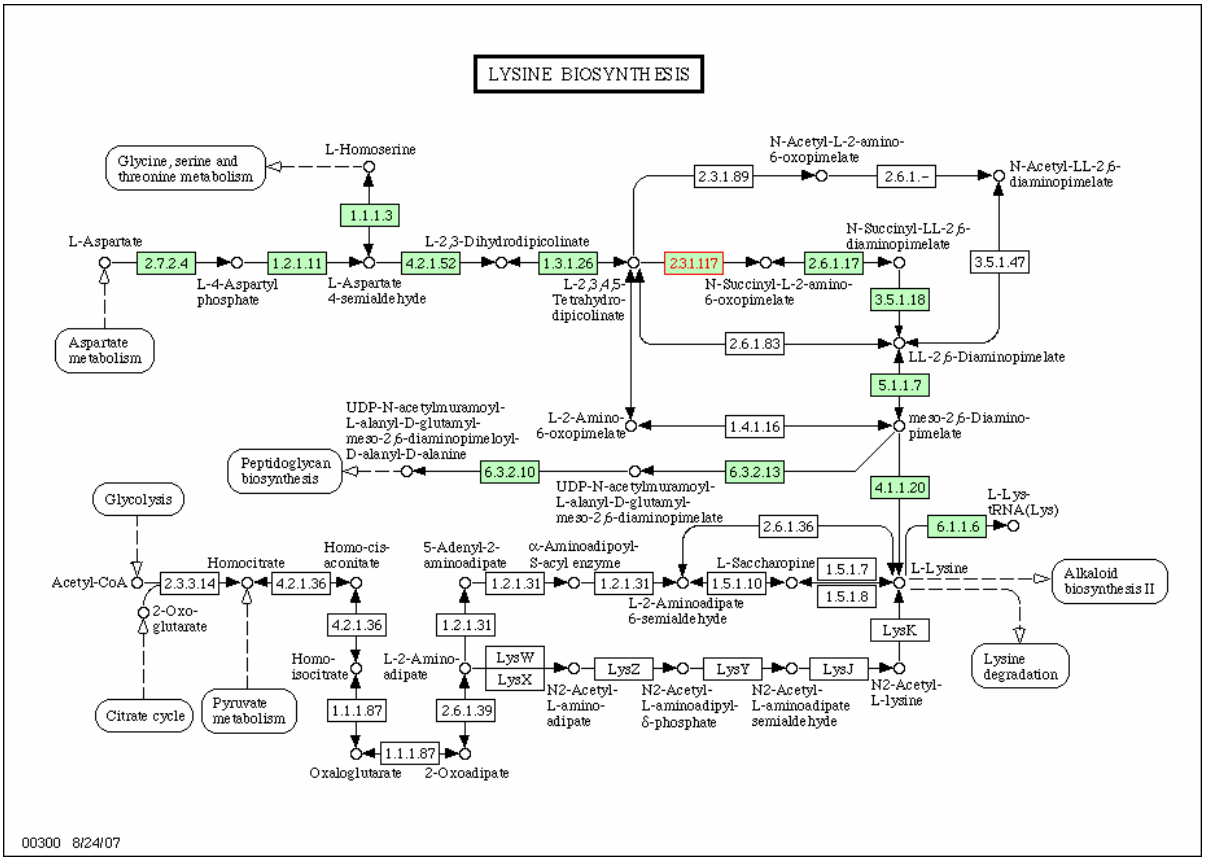



Figure 5. Lysine biosynthesis pathway. There were two identified proteins were including in this pathway, that may be involing into acid or salt stress responses from different mechanism. Tetrahydrodipicolinate succinylase: E.C. 2.3.1.117; dihydrodipicolinate synthase: E.C. 4.2.1.52. (http://www.genome.ad.jp/kegg/)