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Protein Expression Analysis of *Halobacillus dabanensis* D-8^T Subjected to Salt Shock

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To investigate the mechanism of salt tolerance of gram-positive moderately halophilic bacteria, two-dimensional gel electrophoresis (2-D PAGE) was employed to achieve high resolution maps of proteins of *Halobacillus dabanensis* D-8^T. Approximately 700 spots of proteins were identified from these 2-D PAGE maps. The majority of these proteins had molecular weights between 17.5 and 66 kDa, and most of them were distributed between the isoelectric points (pI) 4.0 and 5.9. Some protein spots were distributed in the more acidic region of the 2-D gel (pI <4.0). This pattern indicated that a number of proteins in the strain D-8^T are acidic. To understand the adaptation mechanisms of moderately halophilic bacteria in response to sudden environmental changes, differential protein profiles of this strain were investigated by 2-D PAGE and Imagemaster^{TM} 2D Platinum software after the cells were subjected to salt shock of 1 to 25% salinity for 5 and 50 min. Analysis showed 59 proteins with an altered level of expression as the result of the exposure to salt shock. Eighteen proteins had increased expression, 8 proteins were induced, and the expression of 33 proteins was down-regulated. Eight of the up-regulated proteins were identified using MALDI-TOF/MS and MASCOT, and were similar to proteins involved in signal transduction, proteins participating in energy metabolism pathways and proteins involved in stress.

Keywords: *Halobacillus dabanensis*, two-dimensional gel electrophoresis, salt shock, protein expression

Moderately halophilic bacteria form a versatile group adapted to life at a wide range of salinity (0.1 - 32.5 % salt) and with the possibility of rapid adjustment to changes in the external salt concentration (Ventosa *et al.*, 1998). They inhabit a wide range of habitats that are much less restricted than the habitats in which the halophilic archaea thrive. The importance of research into these organisms' natural habitats and mechanisms of adaptability to a wide range of salinities has only recently been recognized (Ventosa *et al.*, 1998). This interest has resulted in many recent reports on moderate halophilic bacteria isolated from halophilic ecosystems.

It is difficult to screen for salt sensitive mutants of moderately halophilic bacteria by transposon induced mutagenesis because of their intrinsic resistance to many antimicrobial agents. Up to now, only a few mutants have been obtained in gram-negative moderately halophilic bacteria such as *Halomonas elongata* (Canovas *et al.*, 1997), but no similar reports have been found in literature with regard to gram-positive moderately halophilic bacteria. For many years we have been trying to explore the salt-tolerance mechanisms of bacteria at the genetic level, and to produce salt sensitive mutants of gram-positive moderately halophilic bacteria by transposon induced mutagenesis. However, we failed to obtain any mutants in spite of using Tn5, Tn1732 and Tn916. Nevertheless, we recently cloned the gene encoding the transport system for the compatible solute glycine betaine of the gram-positive moderately halophilic bacterium *Halobacillus trueperi* (Lu *et al.*, 2004). However, by genomic manipulation, it is difficult to discern general osmoadaptation mechanisms of moderately halophilic bacteria in response to environmental challenges. Two-dimensional gel electrophoresis (2-D PAGE) is the most efficient way to find proteins involved in adaptability to high salt conditions.

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2-D PAGE allows the analysis of complex protein mixtures, the discovery of novel proteins and can provide clues about the biological function of proteins (Graves *et al.*, 2002). Up to now, 2-D PAGE has been used to investigate bacterial global gene expression in a number of specific research areas including bacterial stress responses in *Escherichia coli, Bacillus subtilis,* and *Listeria monocytogenes* (Cash, 1998). Unfortunately, 2-D PAGE has not been widely used to separate proteins from moderately halophilic bacteria due to specific technical problems. For instance, salt, which is essential for the growth of halophiles, interferes with isoelectric focusing of the samples. To our knowledge, no reports have been published concerning the expression profiles of global proteins after moderately halophilic bacteria have been subjected to salt shock by 2-D PAGE. Mojica *et al.* (1997), however, studied the expression of proteins of *Halomonas elongata* in response to salt stress using 1-D PAGE.

In this work, 2-D PAGE was employed to investigate the differential protein expression profiles of *Halobacillus dabanensis* D-8^T (Liu *et al.*, 2005) cells subjected to salt shock from 1% to 25% salinity for 5 min or 50 min. We expected to find new proteins related to salt stress and to collect information about quantities and categories of differentially displayed proteins after salt shock by identifying some up-regulated proteins by MALDI-TOF. In fact the results reveal a unique salt tolerance mechanism of this strain and provide a general picture of the osmoregulation networks of moderately halophilic bacteria in response to high salt conditions.

Materials and Methods

Bacterial growth conditions

Halobacillus dabanensis D-8^T was isolated from the saline deposits of Daban lake in Xinjiang, China (Liu *et al.*, 2005), and is able to grow in salt concentrations ranging from 0.5% to 23% in SW medium. In our study, this strain was incubated in 10% SW medium. This medium contains 0.5% yeast extract and 10% salts (8.1% NaCl, 0.7% $MgCl_2$, 1% $MgSO_4 \cdot 7H_2O$, 0.2% KCl, all w/v) at a pH of 8.0. Cells were incubated in 100 ml cultures in 250 ml flasks with rotary shaking at 200 rpm at 37°C.

Salt shock from 1 to 25% salinity

For the up-shift experiments, 600 ml cells growing for 18 h (exponential phase) in 1% SW medium were collected by centrifugation at 5,800 rpm for 10 min at 37°C. To induce the salt shock, the pellets were resuspended in 25% SW medium, pre-warmed to 37°C to the original volume and incubated for 5 min or 50 min at 37°C. Cells were harvested at 5,800 rpm for 10 min at 4°C and cell pellets of all samples are collected from 600 ml cultures. The pellets were suspended and washed for four times in 50 mmol/L Tris-HCl (pH 7.4). As control, bacterial cells were suspended and incubated for 5 or 50 min in 1% SW medium and washed as described above.

Protein sample preparation

Bacterial pellets were resuspended in 1 ml lysis buffer and incubated for 30 min on ice. The lysis buffer contained 9.5 M urea, 1% Triton X-100, 4% CHAPS, 2 M thiourea, 0.5% ampholines (4-6:3-9.5=4:1), 100 mM DTT, 10 μg/ml DNase I and 200 μg/ml lysozyme. After sonication, suspensions were centrifuged at 80,000 × g for 15 min at 15°C to pellet unbroken bacteria and cell fragments.

Two-dimensional gel electrophoresis

Proteins were separated using the protocol of O'Farrell (1975) with the following modifications: Ampholines (4-6:3-9.5=4:1) Triton X-100 was used instead of NP-40, and CHAPS was added to the gel mixture before casting the gels. The size of isoelectric focusing (IEF) tubes was 3.5 mm (inner diameter) × 13 cm, and the length of IEF gel was 11 cm. 2-D SDS-PAGE standards (containing 7 proteins of known isoelectric points and molecular weights) were purchased from Bio-Rad (USA). The IEF procedure was as follows: 200 V 30 min, 400 V 16 h and 800 V 1 h. The IEF gels were fixed for 30 min before equilibrium. SDS-PAGE was performed in 3% stacking gels and 16% separating gels. Three parallel gels of every sample were dried between cellophane.

Image collection and analysis

Dried gels were scanned with an Imagemaster scanner at 600 dpi. Analysis of gel images was carried out by ImageMaster^{TM} 2D Platinum (version 5.0, Amersham Bioscience, UK).

In-gel tryptic digestion of protein spots

Protein spots of interest were excised from the gels and put into 1.5 ml eppendorf tubes. The gel pieces were destained in a solution containing 25% ethanol and 7% acetic acid overnight or until they looked colorless. In-gel digestion was carried out using trypsin, and peptides were extracted from gels in 0.1% trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile. Peptides that came into the solution were transferred into new eppendorf tubes. Gel pieces were subsequently extracted with 0.1% TFA in H_2O (HPLC grade) and these extracts were added to the corresponding eppendorf tubes. Extracts were dried using a Speed Vac Concentrator. Dried peptides were analyzed with a protein mass spectrometer. (www.promega.com)

Identification of proteins by MALDI-TOF/MS and database matching

Dried peptides were dissolved again in 10 μl 0.5% TFA, desalted and spotted onto the MALDI target. Peptides were analyzed with an Ettan mass spectrometer (Amersham Bioscience, UK), and the peptide fingerprint spectrum of protein spots was identified in the protein database of bacteria using the MASCOT search engine, http://www.matrixscience.com

Results and Discussion

Differentially displayed expression of proteins after salt shock

For this study, we compared the protein patterns of cells of the strain D-8^T grown under 1% salinity (used as control), to that of cells subjected to salt shock (25% salinity) treatments for 5 and 50 min by 2-D PAGE. We analyzed three parallel gels of each treatment using Imagemaster^{TM} 2D Platinum software. Approximately 700 protein spots were detected on 2-D gels, and most of these proteins had molecular weights of between 17.5 and 66 kDa, and isoelectric points ranging from 4.0 to 5.9 (Fig. 1). After salt shock treatments for 5 min and 50 min at 25% salinity, 59 proteins of the strain D-8^T had altered expression level. The expression of 26 proteins was up-regulated by exposure to high salinity including eight new proteins induced. Compared to 1% salinity, 33 proteins were down-regulated, some of which even disappeared after the shock of 25% salinity.

Eight proteins were up-regulated by just 5 min exposure to high salinity and still had a high expression level after 50 min (Fig. 2, u1-8); this included 5 new proteins (u1-5). Also, we observed a set of 18 proteins that were specifically up-regulated after a 50 min salt shock (Fig. 3, s1-18) including 3 new proteins (s1-3). Compared to the expression of proteins in strain D-8^T under 1% salinity, the expression of two proteins was reduced by 5 min salt shock as well

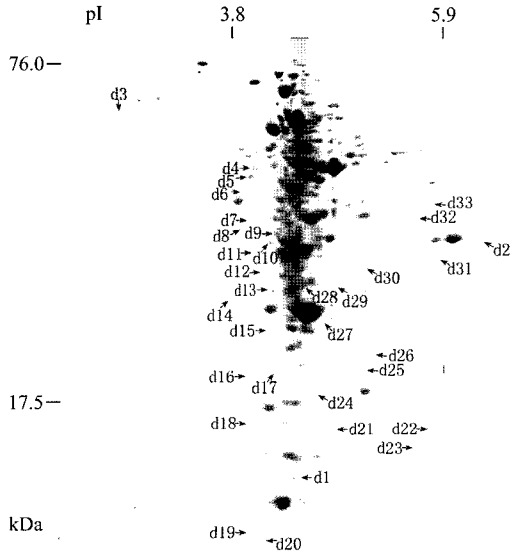

Fig. 1. 2-D PAGE map of proteins of *H. dabanensis* D-8^T grown in 1% salinity medium. "d" represents down-regulated protein spots after salt shock.

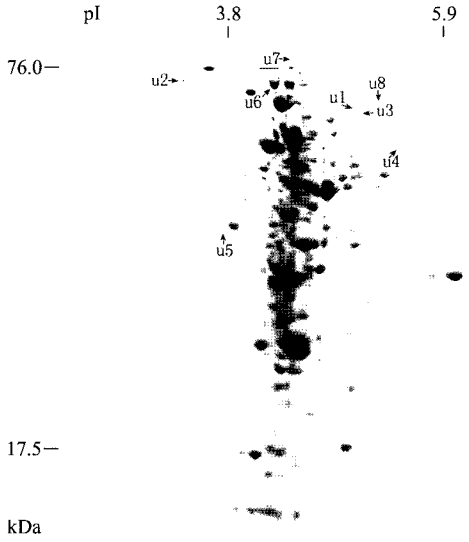

Fig. 2. 2-D PAGE map of proteins after *H. dabanensis* D-8^T grown in 1% salinity medium was subjected to salt shock of 25% salinity for 5 min. "u" represents up-regulated protein spots overexpressed after salt shock of both 5 min and 50 min.

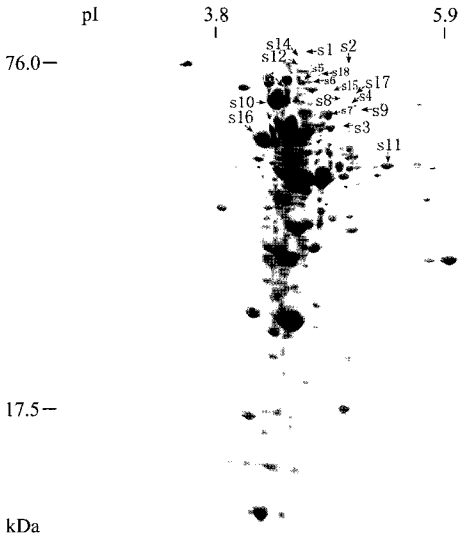

Fig. 3. 2-D PAGE map of proteins after *H. dabanensis* D-8^T grown in 1% salinity medium was subjected to salt shock of 25% salinity for 50 min. "s" represents up-regulated protein spots overexpressed after salt shock of 50 min.

as by 50 min of salt shock (Fig. 1, d1-2). In addition, 31 proteins had reduced expression after 50 min of salt shock (Fig. 1, d3-33).

This work provides clear evidence that the majority of the proteins in gram-positive moderately halophilic bacteria *H. dabanensis* D-8^T is acidic and is similar to the proteins of the extremely halophilic archaea (Cho *et al.*, 2003) though they are more weakly acidic. This suggests that these two distinct microorganisms possess certain similar salt tolerance mechanisms. Therefore, our finding shows that acidic proteins contribute to high salt toleration of moderately halophilic bacteria to a certain extent. In addition, studies suggest that the acidic nature of halophilic proteins somehow causes electroendosmotic flow and high conductivity during isoelectric focusing, which can make separation difficult to achieve (Cho *et al.*, 2003). In the present study, we also observed that horizontal streaking often appeared in the 2-D PAGE gels. This seemed to be an effect of the acidic nature of the proteins during the separation of the global proteins of the strain D-8^T (data not shown).

Differential expression analysis of proteins shows that this strain responds to salt shock with changes in the expression levels of many genes involved in the adaptation to a new osmotic environment. Synthesis of some proteins alters immediately after salt shock of 25% salinity, and this results in overexpression, induction or repression of proteins within 5 min of exposure. Synthesis of some proteins, however, is only affected after longer salt shock (seen after 50 min). Moreover, salt shock induced some new proteins, supporting Mojica's study of *Halomonas elongata* using 1D SDS-PAGE (1997), which found immediate synthesis of a set of high-salt-related proteins after the stress of an up-shift from 0.3% to 23% NaCl. At the same time, synthesis of many proteins decreased markedly or even ceased completely. This indicates that cells have to lower or to stop synthesis of some proteins for survival under these adverse conditions. A more detailed analysis of the differential synthesis of proteins in this strain in response to stress conditions would be of great value for the understanding of the molecular mechanisms involved in osmoregulation.

Characterization of proteins identified as related to salt shock by MALDI-TOF/MS

After analyzing the differential expression of proteins of the strain D-8^T in response to salt shock from 1% to 25% salinity, the interesting protein spots were excised and digested with trypsin. Eight overexpressed proteins resulting from salt shock at 25% salinity were identified by MALDI-TOF/MS and MASCOT as

Table 1. Proteins identified after exposure to 25% salinity from 2-D PAGE patterns of *Halobacillus dabanensis* D-8^T by MALDI-TOF/MS using the MASCOT search engine

Spot No.	Homologous protein	Number of mass values matched	Putative M_r (kDa)/p*I*	Theoretical M_r (kDa)/p*I*	Accession No.
u2	CheY-like receiver [*Desulfovibrio desulfuricans* G20]	10	72/3.2	72/4.9	ZP_00130545
s12	Hypothetical protein plu3513 [*Photorhabdus luminescens* subsp. laumondii TTO1]	9	57/5.2	115/6.3	NP_930730
s1	ATPases with chaperone activity, ATP-binding subunit [*Clostridium thermocellum* ATCC 27405]	10	90/4.7	92/4.8	ZP_00313438
s2	ClpC ATPase [*Listeria monocytogenes* str. 4b F2365]	8	85/5.4	91/7.0	YP_012854
s10	Class I heat shock protein [*Oceanobacillus iheyensis* HTE831]	8	63/4.5	58/4.6	NP_691577
u8	Oxaloacetate decarboxylase, putative [*Porphyromonas gingivalis* W83]	8	61/5.6	70/5.6	AAQ65474
s16	Enolase [*Oceanobacillus ihey)ensis* HTE831]	6	50/4.4	46/4.5	NP_693355
s11	Ornithine transcarbamylase [*Bacillus cereus* ATCC 14579]	5	42/5.6	38/5.4	NP_830246

u: up-regulated proteins after salt shock of both 5 min and 50 min; s: up-regulated protein spots after salt shock of 50 min. Differential expression of every protein spot is the statistical result of four parallel gels of every sample by ImageMaster^{TM} 2D Platinum. The value of overproduction is at least 2 fold, and the proteins analyzed by MALDI-TOF were expressed over 4-fold more than the control. Protein scores greater than 72 are significant ($p<0.05$) when every protein was matched by MASCOT.

a CheY-like receiver; a probable outer membrane receptor; enolase; ATPases with chaperone activity; a class I heat shock protein; ClpC ATPase; putative oxaloacetate decarboxylase and ornithine carbamoyltransferase (Table 1). These eight proteins were characterized as follows. Protein spot u2 was a CheY-like receiver. CheY has been extensively studied as a response regulator. Previous studies suggest that adaptive responses of microorganisms are governed by two-component signal transduction systems, and these regulatory systems are highly homologous throughout the bacterial kingdom (Hubbard *et al.*, 2003). In addition, Graumann *et al.* (1996) found that CheY is a cold shock stress-induced protein in *Bacillus subtilis*. Protein spot s12 was the hypothetical protein plu3513, a probable outer membrane receptor. Protein spot s16 was an enolase. This is a glycolytic enzyme which provides cells with energy during the time that they are subjected to osmotic stress (Prasad *et al.*, 2003). It can be up-regulated under a variety of stress conditions. For instance, enolase was up-regulated in *Lactobacillus rhamnosus* HN001 by heat and osmotic stress (Prasad *et al.*, 2003). Protein spot s1 was an ATPase with chaperone activity, and a ATP-binding subunit. ATPases can unfold proteins and disaggregate preformed protein aggregates to target them for degradation in order to restore their functions. Protein spot s2 was a ClpC ATPase. This is a stress protein belonging to the Hsp100/Clp family, which is a class of highly conserved proteins implicated in stress tolerance mechanisms of many prokaryotic organisms (Schirmer *et al.*, 1996). Rouquette *et al.* (1996) found that under stress conditions *L. monocytogenes* produces a ClpC ATPase, and Tn917 insertions that inactivated *clpC* only resulted in mutants that were highly susceptible to high osmolarity stress. Protein spot s10 was a class I heat shock protein. Studies have demonstrated that heat shock proteins are induced when a cell undergoes environmental stresses such as heat or salt shock. An example of this was described by Hecker *et al.* (1998), who demonstrated that salt stress in *Bacillus subtilis* was very effective in the induction of general stress proteins including heat shock protein. In addition, expression of the protein of spot u8 was increased after salt shock, both at 5 min and at 50 min, and the protein of spot s11 had an increased expression after salt shock of 50 min. These two proteins were identified as a putative oxaloacetate decarboxylase and an ornithine carbamoyltransferase, respectively.

From the identification of these proteins we can conclude that proteins induced in response to salt shock are mainly those involved in signal transduction, energy-production and heat shock. In the present study, the CheY-like receiver was induced very rapidly upon exposure to 25% salinity, and its concentration presumably continuously increased up to that observed after 50 min in high salt. Overexpression of CheY during exposure of this strain to salt shock is normal for a stress protein (Graumann *et al.*, 1996). Moreover, a probable outer membrane receptor was also overexpressed in response to salt shock for 50 min. Receptor overexpression indicates that signal receivers are essential for cells to be able to give a swift response to adverse life conditions like a salt shock.

Proteins which are involved in energy metabolism were induced very soon after exposure of strain D-8^T to high salinity. For instance, oxaloacetate decarboxylase was found to be up-regulated within 5 min of exposure to a salt shock, and continued to be up-regulated during the 50 min that this strain was subjected to shock. Enolase was also increased but only after 50 min of exposure to 25% salt. It seems that the TCA and glycolytic pathways supply more energy to the cells in salt stress. Energy metabolism should be very active when this strain is coping with high salt challenges. Our result is consistent with the conclusion of Duche *et al.* (2002), who suggested that some of the up-regulated proteins of *L. monocytogenes* are probably related to metabolic pathways that provide osmoregulation mechanisms with energy under salt stress conditions.

In addition, three of the proteins overexpressed after salt shock were identified as heat shock proteins. Among them, ATPase with chaperone activity and a ClpC ATPase were induced, and a class I heat shock protein was overexpressed after salt shock for 50 min. These general stress proteins assist proper folding for other proteins and/or ensure synthesis of new induced proteins as part of the cells' adaptation to salt stress.

Sporulation is the one of the strategies used by endospore forming organisms under extremely severe challenges. In the present study, salt shock may have induced a rigorous response, but no spore was observed. This is probably because the salt shock might not have been long enough to induce the forming of spores. Accordingly, expression of proteins related to sporulation was not observed. In addition, in spite of the fact that *H. dabanensis* D-8^T is an endospore forming organism, it is difficult for cells cultured in a complex medium to form spores even if they are incubated to the stationary phase. We observed the cells from different culture experiments many times by microscope (data no shown) and failed to find endospores unless the cells were cultivated in a sea water medium for a long time.

In conclusion, we found differential expression profiles of global proteins in strain D-8^T subjected to salt shock to 25% salinity using 2-D PAGE, and

suggest that the acidic nature of the proteins confer moderately halophilic bacteria with the capability of salt tolerance to a certain extent. In addition, proteins involving in signal transduction, energy pathways and general stress proteins were found to be induced and/or increased during severe salt stress. These proteins for the most part play primary roles in protecting cells enabling them to survive in adverse environments.

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