Extremophiles (2006) 10:97–104
DOI 10.1007/s00792-005-0476-x

ORIGINAL PAPER

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Identification of genes regulated by changing salinity in the deep-sea bacterium *Shewanella* sp. WP3 using RNA arbitrarily primed PCR

Received: 24 April 2005 / Accepted: 25 July 2005 / Published online: 24 August 2005

Abstract The differential gene transcription of a deep-sea bacterium *Shewanella* sp. WP3 in response to changing salinity was analyzed by RNA fingerprinting using arbitrarily primed PCR (RAP-PCR). Ninety primer sets were used to scan two different RNA pools derived from cultures of 1% and 7% NaCl concentrations. Forty-three putative differential-expressed fragments were identified, cloned, and sequenced. Six out of the 43 fragments were confirmed to be truly differentially transcribed in terms of changing salinity. The deduced amino acid sequences of the six gene fragments showed highest identities (66–96%) with ribosomal protein L24, ATP binding protein, and chaperon protein HscA of *Shewanella oneidensis* MR-1 (Y6, Y9, and Y29); isocitrate lyase of *Pseudomonas aeruginosa* (Y15); peptidylprolyl *cis–trans* isomerase of *Shewanella* sp. SIB1 (Y21), glutamine synthetase of *Shewanella violacea* (Y25), respectively. Four genes (Y6, Y15, Y21, and Y25) were up regulated in 7% NaCl, while the other two (Y9 and Y29) contained more abundant transcripts in 1% NaCl. The data suggested that strategies involved in controlling protein synthesis, protein folding and/or trafficking, glutamate concentration, fatty acid metabolism, and substance transporting were used for salt adaptation in *Shewanella* sp. WP3. The expression patterns of the six genes in response to transient stress shocks including salt shock (3% NaCl shift to 12%), cold shock (15°C shift to 0°C), and high-hydrostatic pressure shock (0.1 MPa shift to 50 MPa) were further examined. Y29 encoding the putative HscA chaperon protein was indicated to be involved in adaptation of all the stresses tested.

Keywords *Shewanella* sp. WP3 · RAP-PCR · Changing salinity · Differential-expressed fragments · Stress shock

Communicated by K. Horikoshi

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Introduction

Most of the deep sea consists of dark, high pressure, cold, and nutrient limited environments. Plenty of bacteria have been isolated from the cold deep sea, *Shewanella* sp. being among the most frequently isolated deep-sea bacteria collected at varying depths (Kato 1999). The diversity of piezophilic *Shewanella* sp. has been proposed to be related to ocean circulation (Kato and Nogi 2001). Antarctic bottom water has a major influence on ocean circulation and potentially on the biota of deep oceans (Bowman et al. 2003). It brings various environmental changes to the microbes within the deep sea, including changes in temperature, pressure, and salinity. Deep-sea *Shewanella* sp. may have evolved specific adaptation mechanisms for dealing with these environmental changes. The adaptation mechanisms of *Shewanella* sp. towards temperature, high-hydrostatic pressure have been partly characterized (Gao et al. 2004; Ishii et al. 2002; Nakasone et al. 1999, 2002). However, the osmoregulatory mechanisms of *Shewanella* sp., and their possible cross regulation with temperature or pressure, are poorly understood.

Osmoregulatory mechanisms have been studied in different groups of organisms, particularly in *Escherichia coli* (Culham et al. 2001; Ramirez et al. 1989), and halophilic prokaryotes (Mojica et al. 1997). During osmoregulation, bacteria have evolved to accumulate a high level of a certain class of solutes (termed compatible

solutes) that does not interfere extensively with the functioning of cytoplasmic enzymes. Compatible solutes can be accumulated by de novo biosynthesis or by uptake from the environment (Gouffi et al. 1999). In *E. coli*, it has been observed that osmotic stress dramatically inhibits active transport of carbohydrates (Roth et al. 1985) and DNA replication (Meury 1988). Modification of lipid composition depending on salinity has also been reported in halophilic organisms, including *Halomonas* (Vreeland et al. 1984) and *Haloarchaea* (Konrad and Eichler 2002).

Recently, we isolated two novel deep-sea *Shewanella* strains from deep-sea sediment within the west Pacific, and they were supposed to be novel deep-sea species (Wang et al. 2004). *Shewanella* sp. WP3 (hereafter referred as WP3) had a broad temperature between 4 and 28°C, with optimum at 15–20°C. Meanwhile, preliminary tests showed that it is pressure tolerant and could grow over a relatively broad range of salt concentration (unpublished data). WP3 is an ideal microbe to study the osmoadaptation mechanisms of deep-sea *Shewanella* sp. and their possible cross regulating pathways with temperature and/or pressure.

In this study, RNA arbitrarily primed PCR (RAP-PCR) approach was chosen to screen for the differentially expressed genes responding to changing salinities. RAP-PCR has been proven to be an effective method to detect new genes under different environmental stresses in prokaryotes (Benson et al. 2000; Bidle 2003; Fleming et al.1998; Du and Kolenbrander 2000). This technique uses random oligonucleotide primers to create a unique cDNA fingerprint for a given microorganism in a particularly physiological state, thus providing a powerful tool for assessing differential gene expression in prokaryotes. Through RAP-PCR screening and RT-PCR confirmation approaches, six genes were identified to be involved in the osmotic adaptation of WP3. Some genes were indicated for the first time to be regulated by salinity, and subsequent analyses also indicated that several of these genes are also regulated by other stresses.

Materials and methods

Bacterial strains and culture conditions

WP3 was cultured in modified marine medium 2216E (5 g/l tryptone, 1 g/l yeast extract, 0.1 g/l $FePO_4$, 34 g/l NaCl). The strain was cultivated with vigorous shaking (200 rpm) at 15°C. Culture medium 2216E modified with a gradient salt concentration from 0.5 (w/v) to 12% (w/v) NaCl was used to check the growth salinity range for WP3. The growth of WP3 was monitored by measuring the optical absorption value at OD_{600}. For osmotic shock tests, mid-exponential-phase cultures from modified 2216E of 3.4% NaCl were divided into two aliquots, one was harvested and treated with 12% (w/v) NaCl for 30 min, the other one was remained untreated as control. For cold shock tests, mid-exponential-phase cultures at 15°C were transferred and incubated at 0°C for 30 min. The high-hydrostatic pressure shocks were carried out by transferring the cells from 0.1 to 50 MPa for 1 h. Overnight log phase cultures of WP3 cells were diluted tenfold by the same medium, transferred to polyethylene bulbs, sealed with no air space and placed inside pressure vessels. The pin-closure pressure vessels (Yayanos and Dietz 1982) were used in this study (constructed by Wuxi Jinliu Petrochemical Instrument Equipment Inc., China). Pressure was applied using a hand operated pump and a quick fit connector to the pressure vessel. Cultures were then incubated in a cold house with the temperature of about 15°C at either atmospheric pressure or at 50 MPa.

E. coli strain XL-1 blue used for cloning was grown in liquid LB medium at 37°C. When necessary, ampicillin was added in the concentration of 100 μg/ml.

RNA preparation and treatments

WP3 was cultivated in triplicate using modified 2216E containing 1% (low) or 7% (high) NaCl. Mid-exponential-phase cultures ($OD_{600} = \sim 0.5$) from the three independent cultures of low or high-salt concentrations were mixed and used for RNA isolation. Meanwhile, the transient osmotic shock/cold shock/high-pressure shock aliquots (three independent cultures by each stress condition) and their controls were also used for RNA isolation. Cells were harvested by centrifugation at 5000 *g*, RNA was extracted using TRI reagent-RNA/DNA/protein isolation kit (Molecular Research Center Inc., OH, USA) according to the manufacturer's instructions. RNA was quantified using a UV spectrophotometer (Ultrospec2100, Amersham Pharmacia Biotech., Chalfont, UK) and its integrity was assessed by agarose gel electrophoresis prior to experiments. When necessary, RNA was treated with DNase I (MBI, VA, USA) and purified using RNeasy spin columns (Qiagen, Crawley, UK) according to the manufacturer's recommendations. Purified RNA was eluted in RNase-free water and adjusted to a final concentration of 100 ng/μl and stored at −80°C.

RAP-PCR

RAP-PCR was performed essentially as described by Fleming et al. (1998). Arbitrary 11mer RT primers and 10mer PCR primers from published sequences (Fislage et al. 1997) were synthesized by Sangon Inc. (Shanghai, China). First-strand cDNA synthesis was performed using 200 ng of heat-denatured (5 min, 70°C) purified RNA in 20-μl reaction mixtures containing 200 mM of each deoxynucleoside triphosphate; 5 mM dithiothreitol, 50 U of AMV-reverse transcriptase (RT) (MBI), one time RT reaction buffer, and 0.4 mM arbitrary primers. The first-strand reaction was performed in a thermocy-

cler (Biometra, Germany) as follows: touchdown from 50°C to 30°C in 45 s increments followed by 1 h at 42°C. Second strand synthesis was performed in 30-μl reactions containing the following: 3 μl of first-strand reaction, 0.5 U of *Taq* polymerase (Takara, Tokyo, Japan), 20 mM of each dNTP, 2 mM concentrations of each primer (primer 1 is from first-strand synthesis; primer 2 is a different randomly chosen primer), one time PCR buffer, and 1.5 mM $MgCl_2$. Program for RAP-PCR were 94°C for 30 s, 40°C for 2 min, and 72°C for 1 min for 40 cycles, with a 10-min extension at 72°C on the final cycle. RAP-PCR samples were heated at 92°C for 2 min after the addition of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were then loaded onto a 6% denaturing acrylamide gel (20×42 cm) containing 7 M urea with the CBS adjustable sequencer apparatus (CBS Scientific Co. Ltd., San Francisco, CA, USA) and were electrophoresed at 1700 V for about 2 h until the xylene cyanol band reached the bottom of the gel. The gels were subsequently silver-stained for visualization as described by White et al. (2004).

Isolation, cloning, and sequencing of RAP-PCR fragments

Following identification of differential-expressed fragments, bands of interest were excised from denatured PAGE gels and placed into a micro-centrifuge tube containing 20 μl of sterile water for 95°C 20 min. A portion of the eluted fragment was used in a subsequent PCR containing the original primers used in RAP-PCR amplification. The secondary PCR fragments were gel purified and cloned into the T-vector (Takara) according to the manufacturer's instructions. Clones of correctly sized insert were sequenced (Sangon, Shanghai, China). Sequence similarity searches were performed with the BLASTx network service in NCBI.

Confirmation of regulated fragments

To verify the regulated fragments identified in RAP-PCR, the RT-PCR approach described by Cabanes et al. (2000) was used. The gene-specific RT-PCRs were performed using SuperScript One-Step RT-PCR kit (Invitrogen, UK) according to the manufacturer's instructions. All RT-PCR experiments were performed in triplicate independently to ensure reproducibility. During the PCR amplification of the genes, different numbers of cycles were used to avoid PCR saturation.

Accession numbers

The sequences of the partial gene fragments identified in this work have been deposited in databank under accession numbers from AJ890358 to AJ890363.

Results

Salinity range for growth of WP3

The growth salinity range of *Shewanella* sp. WP3 was tested with a gradient salt concentration from 0.5% to 12 % as described above. It was found that WP3 could grow at a NaCl concentration range between 1% and 7%, with optimal growth occurring at 3–4% NaCl (data not shown). Compared to the optimal salt growth condition, bacterial cultures in low and high-NaCl concentrations (1% and 7% each) showed an increased lag phase. The cultures had a similar growth rate in the media of high and low-NaCl concentrations.

RNA integrity determination

To screen differentially expressed genes under changing salinities, RNAs of WP3 grown at two extreme salt concentrations (1% and 7%) were extracted as described in the materials and methods. The integrity and size distribution of total RNA was checked by 1% denaturing agarose gel electrophoresis and ethidium bromide staining. 23S rRNA bands appeared with an intensity approximately twice that of the 16S rRNA band (data not shown), indicating no obvious RNA degradation during preparation. The RNA samples were further treated by RNAase-free DNase I and purified to remove any contamination of DNA during the RNA isolation. The purified RNA was tested using as PCR templates directly, no PCR product was got (photo not shown), indicating that there was no DNA contamination in RNA samples. The DNA-free RNA was crucial for further RAP-PCR experiments.

RAP-PCR to identify differentially expressed genes

One hundred primer combinations (Fislage et al. 1997) were first tested using for differential gene screening in WP3. Ten primer combinations were found not able to amplify adequate bands, and therefore, only the remaining 90 primer combinations were used later (photo not shown). On average, each primer combination yielded more than 70 visible bands on the sequencing gel. In total, more than 6000 bands were generated, they were supposed being able to cover nearly all the genome transcripts. Forty-three candidates for differentially expressed genes were identified in gels as shown in Fig. 1. The differentially expressed RAP-PCR products were recovered and re-amplified by standard PCR program as described in the materials and methods with the same arbitrary primers used in RAP-PCR. After re-amplification, the products were gel purified, cloned, and sequenced.

Out of 43 putative differential-expressed fragments sequenced, 16 of them were rejected, as they corre-

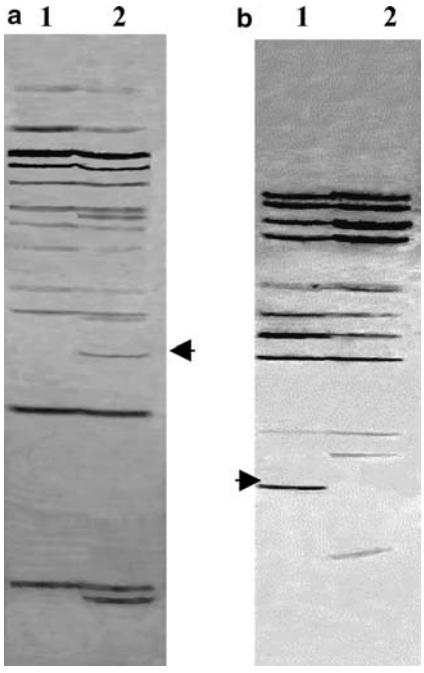

Fig. 1 Standard RAP gels from changing salinity with primer combination A4S7 (**a**) and A4S8 (**b**). RAP-PCR reactions were performed as described in the *materials and methods* section using RNA templates isolated from cultures of 1% (low) and 7% (high) NaCl concentrations. The *arrows* indicate the putative differentially expressed fragments. *1* 1% Nacl concentration, *2* 7% NaCl concentration

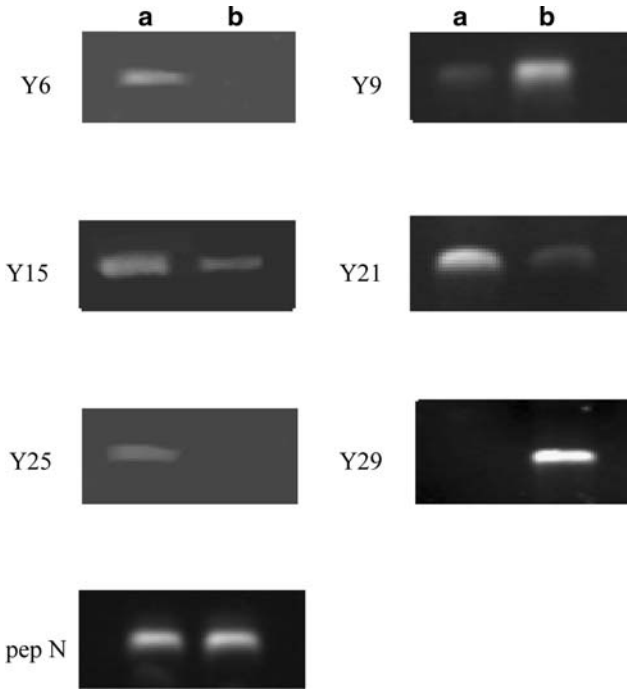

Fig. 2 Confirmation of regulated RAP-PCR products. Confirmation of the selected RAP-PCR products was conducted by RT-PCR as described in the *materials and methods* section. All reactions were performed under stringent conditions using gene-specific primers. RT-PCR was performed with WP3 cultures of both 7% (**a**) and 1% (**b**) NaCl concentrations. The constitutive expression of *pepN* under different salinity conditions was used as control

sponded to bacterial 16S or 23S rRNAs. Of the 27 remaining clones, five of them didn't show any sequence similarity to known sequences in database, and that they were not subjected to further analysis. In the remaining 22 RAP cDNAs, three (Y15, Y17, and Y18) corresponded to the different parts of the same gene encoding putative isocitrate lyase from *Pseudomonas aeruginosa* PAO1 (accession no. AAG06022, Stover et al. 2000); Y4, Y5, and Y30, Y31 were the other two category of the same gene encoding putative ammonia permease and hypothetical protein, respectively. Thus, these 22 transcripts represent 18 distinct genes.

Confirmation of differential gene expression

Confirmation of the differential gene expression was performed by RT-PCR using different total RNA preparations. The transcription of a gene *pepN* encoding a putative aminopeptidase N, which was found constitutively transcribed in different culturing conditions (our unpublished data). In addition to 16S (or 23S) rRNA normalization prior to reverse transcription, the *pepN* gene transcription pattern was used as a control in RT-PCR (Fig. 2). Of the 18 distinct RAP-PCR products selected for further confirmation, 12 were identified as false positive (data not shown). Six RAP-PCR products were confirmed to be differentially expressed as seen in the original RAP-PCR (Fig. 2). Among these six fragments, four (Y6, Y15, Y21, and Y25) were up regulated in 7% NaCl; while the other two (Y9 and Y29) were over-expressed in 1% NaCl.

Characterization of the differentially expressed genes

The differentially expressed gene fragments and their closest relatives are presented in Table 1. The deduced amino acid sequence of the four up-regulated fragments (Y6, Y15, Y21, and Y25) at high-salt concentration had 68–96% identity with 50S ribosomal protein L24; isocitrate lyase; peptidyprolyl *cis–trans* isomerase; glutamine synthetase, respectively. Y9 and Y29 corresponded to genes whose expressions were up regulated in low-NaCl concentration, they encoded putative ABC transporter ATP binding protein and chaperone protein HscA, respectively.

Gene expression patterns under different stress shock conditions

To test whether the differentially expressed genes identified above were regulated by other stress conditions, RT-PCRs were performed in triplicate using RNAs isolated from salt shock, cold shock, and high-pressure shock conditions. The results of RT-PCRs are presented

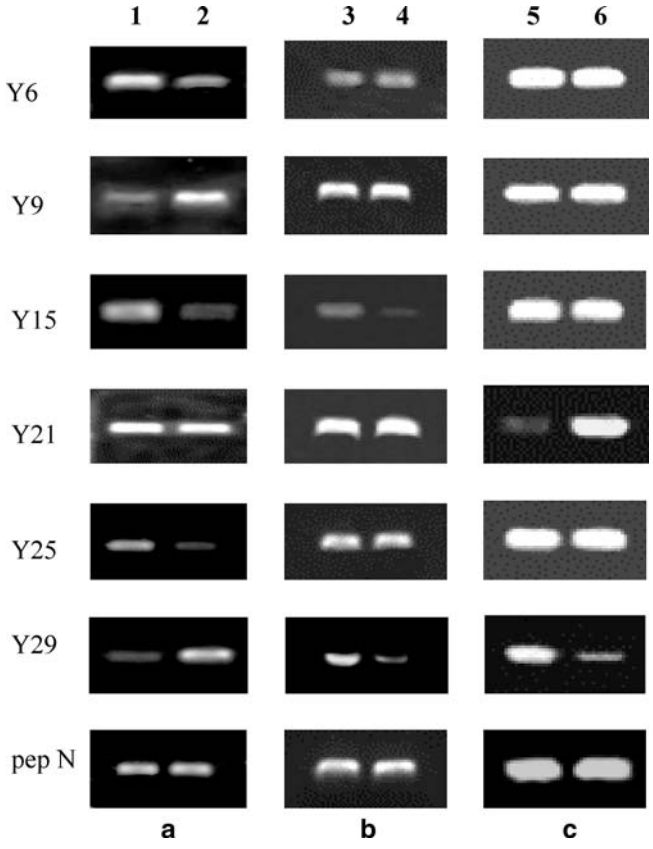

Fig. 3 Examination of gene expression under different stress shock conditions. The differentially expressed gene fragments identified under changing salinity conditions were further checked under salt shock (**a**), cold shock (**b**), and pressure shock (**c**) conditions as described in the *materials and methods* section. RNAs isolated from shocked cells and their untreated controls were used for RT-PCR, as described above. All RT-PCR experiments were repeated three times independently to ensure reproducibility. The constitutive expression of *pepN* under stress shock conditions and normal conditions was used as control. *1* Salt shock, *2* untreated control, *3* cold shock, *4* untreated control, *5* high-pressure shock, *6* untreated control

in Fig. 3. It is shown that under high-salt shock condition (Fig. 3a), Y6, Y15, and Y25 were up regulated and Y9, Y29 were down regulated, consistent with the results obtained by changing salinity (Figs. 2 and 3a). However, the transcription of Y21 under the optimal and salt shock condition was unchanged (Fig. 3a). As for the cold shock condition, only Y15 and Y29 were differentially expressed, with the abundance of their transcripts increasing under cold shock condition (Fig. 3b). Under high-pressure shock condition, Y29 was induced whereas Y21 was partially inhibited (Fig. 3c).

Discussion

The RAP-PCR approach has been known having high proportion of false positive results among clones obtained from differentially expressed bands. A major concern is the fact that one band may actually be composed of cDNAs derived from multiple genes. Some of these genes may be differentially expressed, but others may be constitutively expressed (Frias-Lopez et al. 2004). Another concern is that some of the random primers used for synthesizing cDNAs are likely to anneal to rRNAs, which constitute the largest fraction of the total RNA in the samples, thus preventing efficient conversion of the small portion of mRNA into cDNA. It has been reported that when prokaryotic RNA was used, up to 40% of differentially expressed bands identified using differential display analysis were of ribosomal origin (Nagel et al. 1999). Also, in this study, among 43 candidate genes identified by sequencing gels, there had a large portion (37%, 16/43) of 16S or 23S rRNA genes. Nevertheless, RAP-PCR has been shown to be a powerful tool to screen for differentially expressed genes in prokaryotes (Frias-Lopez et al. 2004). In this study, 90 primer combinations were used for global screening, around 6000 bands were generated, 43 putative differential-expressed fragments were screened out, and six fragments were confirmed to be truly of differential transcript abundance in terms of changing salinity.

The deep-sea bacterium *Shewanella* sp. WP3 is a psychrotolerant, pressure tolerant bacterium, and it grows over a salinity range of between 1% and 7% NaCl. We suspect that there could exist some general stress factors facilitating WP3 cells to acclimate to different environmental stresses such as low temperature, high pressure, and salinity changing. This study is the start of our project to understand how WP3 cells cope with different stresses. By using a RAP-PCR approach, six genes were found to be salinity regulated in WP3, encoding a putative ribosomal protein L24, an ATP binding protein, chaperon protein HscA, isocitrate lyase, peptidylprolyl *cis–trans* isomerase, and glutamine synthetase.

The amino acid sequence of Y6 had 92% identity with *rplX* encoding the ribosomal protein L24 from *Shewanella oneidensis* MR-1 (Table 1). L24 is known essential for early steps of the assembly of the 50S ribosomal subunit but it is not involved in both the late assembly and the ribosomal functions (Charollais et al. 2003). The phenotypic features of the mutant lacking L24 of *E. coli*, are temperature sensitivity and a very low-growth rate already at permissive temperatures (Herold et al. 1986). Genome wide transcription analysis of the cold shock response in *Bacillus subtilis* found that *rplX* was twofold to threefold cold induced. It was shown that not all ribosomal components are synthesized de novo upon cold shock, only a selected subset of ribosomal components is required, whose individual functions might have special importance after cold shock (Beckering et al. 2002). In this study, *rplX* gene was found induced under high-salinity concentration and salt shock conditions but remained unchanged under cold shock and pressure shock conditions (Fig. 3), this implied that L24 may have function in helping the assembly of ribosomes in WP3 under high-salt conditions, but not under cold or high-pressure conditions.

Table 1 Homology analyses of nucleotide sequences of DNA fragments derived from RAP-PCR products of changing salinity

Clone	Length (bp)	Closest relative	Accession No.	P	Identity: similarity (%)	Positive (%)	Changing salinity	
							1%	7%
Y6	163	50S ribosomal protein L24 [*S. oneidensis* MR-1]	NP_715882	9e-05	46/50 (92%)	47/50 (94%)	+[a]	
Y9	324	ABC transporter, ATP-binding protein [*S. oneidensis* MR-1]	NP_719490	1e-46	94/108 (87%)	101/108 (93%)		+
Y15	212	Probable isocitrate lyase [*P. aeruginosa* PAO1]	AAG06022	2e-31	64/70 (91%)	69/70 (98%)	+	
Y21	283	Peptidylprolyl *cis–trans* isomerase [*Shewanella* sp. SIB1]	BAD12460	4e-43	84/122 (68%)	98/122 (80%)	+	
Y25	184	Glutamine synthetase [*S. violacea*]	BAA85980	2e-26	58/60 (96%)	59/60 (98%)	+	
Y29	420	Chaperone protein HscA [*S. oneidensis* MR-1]	NP_717864	7e-43	88/133 (66%)	109/133 (81%)		+

[a] "+" represents higher gene transcription

The deduced amino acid of Y9 showed highest identity (87%) with an ATP binding-ABC transporter from *S. oneidensis*. However, it is still unknown what substrates the ABC transporter transports. It has been observed in *E. coli*, and perhaps in many other bacteria that the transport of carbohydrates, potassium, and other solutes are influenced by salt stress (Roth et al. 1985), thus it is possible that Y9 may be involved in transporting one or some of these substances.

Clone Y15 was up regulated during high salinity and presented 91% homology with the *icl* gene for isocitrate lyase (an enzyme involved in the glyoxylate pathway). In *Mycobacterium tuberculosis*, using microarray analysis, *icl* was found induced by a factor of 2–2.5 after an acid shock of 15 or 30 min, but was induced to significantly higher levels after acid treatment for 18 h. This suggested that low pH may be sensed by the cell as a signal to regulate fatty acid metabolism for survival (Fisher et al. 2002). The *icl* gene from *Colwellia maris* was induced by low temperature under the condition of growing on succinate as the carbon source (Watanabe et al 2002). It was suggested that the *icl* gene of *C. maris*, encoding thermolabile isocitrate lyase, was important for cold adaptation of the cells. Our study indicated that gene for isocitrate lyase was induced at high-NaCl concentration and by salt shock, and this was the first clear evidence showing that isocitrate lyase could be involved in salt adaptation. Furthermore, it was also shown that Y15 was induced under cold shock conditions in WP3. These data together suggest that the isocitrate lyase is important for the survival of bacteria under different stresses including pH, temperature, and salinity.

Y21 exhibits high identity to the *fklB* gene for peptidylprolyl-*cis*/*trans*-isomerase (PPIase) of *Shewanella* sp. S1B1. PPIases, which catalyze the *cis*/*trans* isomerization of peptide bonds N-terminal of prolines, have been suggested to play an important role in protein folding or trafficking. PPIases are present in all life forms; however, their exact biological functions remain to be found. Several PPIases, such as PpiB from *B. subtilis* (Graumann et al. 1996), Trigger Factor from *E. coli* (Kandror and Goldberg 1997), a FKBP family protein from hyperthermophilic archaeon *Thermococcus* sp. KS-1 (Ideno et al. 2001), a PPIase protein (FKBP22) belonging to FKBP family from psychrotolerant bacterium *Shewanella* sp. SIB1 have been reported to be cold induced. This suggests a possible involvement of PPIases in the cold adaptation of cells through the enhancement of protein folding at low temperatures. In the present study, Y21, whose deduced amino acids has 68% identity with FKBP22 of *Shewanella* sp. S1B1, was up regulated under high-salinity conditions. This indicates that the WP3-FKBP22 may involve in the salt adaptation of the bacterium, the first report indicating that PPIase may be involved in the salt adaptation of bacteria. However, Y21 was not induced both by salt shock or cold shock for 30 min (Fig. 3a, b). This would argue that the PPIase in WP3 was not involved in the early stage of salt or cold adaptation. Interestingly, the transcription of Y21 was found to be repressed under high-hydrostatic pressure shock (Fig. 3c). Further studies are needed to elucidate the underlying regulatory mechanisms.

In *E. coli* (Schiller et al. 2000), *Salmonella typhimurium* (Botsford et al. 1994), and perhaps in a number of other bacteria, glutamate has been found accumulated in response to osmotic stress, and it was found that glutamate plays its role in osmoregulation by retaining an optimal intracellular level of K^+ (Yan et al. 1996). In a number of bacteria, the synthesis of glutamate has two pathways: the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle and biosynthetic glutamate dehydrogenase (GDH). The GS/GOGAT cycle is controlled by modulation of the synthesis and catalytic activity of GS. Clone Y25 encoding a putative GS, was induced at high-salt medium (7%) and by salt shock (Figs. 2 and 3a). The data suggest that in WP3, the *glnA* gene is osmo-regulated to control the glutamate pool in the cell. In deep-sea bacterium *Shewanella violacea*, the transcription of *glnA* was found pressure regulated (Nakasone et al. 2002), while in *Photobacterium profundum*

SS9, which grows optimally at 20–30 MPa, the glutamate concentration in the cells was found to remain relatively constant at 0.1 MPa and at its optimal pressure (Martin et al. 2002). Our data showed that in WP3, the transcription of the putative *glnA* gene was not regulated under cold shock and high-hydrostatic pressure shock conditions (Fig. 3b, c), implying that *glnA* may not be involved in the cold or hydrostatic pressure adaptation of WP3.

Y29 was identified as a putative chaperone involved in the maturation of iron–sulfur cluster-containing proteins. Genetic studies in *E. coli* and *Saccharomyces cerevisiae* have demonstrated crucial roles for IscS, IscU, IscA, HscB, HscA, and Fdx in Fe–S cluster biosynthesis, since mutations in the respective genes decrease the activity of many Fe–S proteins (Tokumoto et al. 2002). Meanwhile, it was found that Hsc66 encoded by *hscA* in *E.coli* is induced by cold shock but not by heat shock (Lelivelt and Kawula 1995). Surprisingly, Y29 was found differentially expressed under all stress conditions we examined so far, which was up regulated in cold shock/hydrostatic pressure shock conditions and down regulated in high salinity and salt shock conditions. We speculate that the putative HscA protein encoded by Y29 may serve as a molecular chaperon helping the correct folding of proteins during different stresses. It has been reported that elevated hydrostatic pressure had opposing effects on enzyme activity and stability versus elevated osmotic pressure (Robinson and Sliger 1995). The differential regulation of WP3 Y29 may be caused by the opposing effects of high-hydrostatic pressure and high-osmotic pressure on proteins. Low temperature and high pressure had similar effects on protein synthesis and membrane structure (Bartlett 2002). The biochemical function of HscA in WP3 cells in response to different stresses need to be further investigated.

In conclusion, six genes, possibly involved in protein synthesis (Y6), protein folding or trafficking (Y 21 and Y29), glutamate biosynthesis (Y25), fatty acid metabolism (Y15), and substance transport (Y9) were indicated to facilitate *Shewanella* sp. WP3 survival under changing salinity conditions. Some of these genes were found to be co-regulated under several other stress conditions including osmotic shock, cold shock, or hydrostatic pressure shock.

Acknowledgment We thank the anonymous reviewers for help in editing the manuscript. This work is partly supported by the Chinese high Tech (863) program (2004AA621010), COMRA Fund (DY105-04-02-7).

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