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ORIGINAL PAPER

Proteomic analysis of *Psychrobacter cryohalolentis* K5 during growth at subzero temperatures

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Abstract It is crucial to examine the physiological processes of psychrophiles at temperatures below 4°C, particularly to facilitate extrapolation of laboratory results to in situ activity. Using two dimensional electrophoresis, we examined patterns of protein abundance during growth at 16, 4, and –4°C of the eurypsychrophile *Psychrobacter cryohalolentis* K5 and report the first identification of cold inducible proteins (CIPs) present during growth at subzero temperatures. Growth temperature substantially reprogrammed the proteome; the relative abundance of 303 of the 618 protein spots detected (~31% of the proteins at each growth temperature) varied significantly with temperature. Five CIPs were detected specifically at –4°C; their identities (AtpF, EF-Ts, TolC, Pcryo_1988, and FecA) suggested specific stress on energy production, protein synthesis, and transport during growth at subzero temperatures. The need for continual relief of low-temperature stress on these cellular processes was confirmed via identification of 22 additional CIPs whose abundance increased during growth at –4°C (relative to higher temperatures). Our data suggested that iron may be limiting during growth at subzero temperatures and that a cold-adapted allele was employed at –4°C for transport of iron. In summary, these data suggest that low-temperature stresses continue to intensify as growth temperatures decrease to –4°C.

Keywords Psychrophiles · Proteome · Low-temperature · Psychrobacter · Cold acclimation

Abbreviations
CIP Cold inducible protein
CAP Cold acclimation protein
CSP Cold shock protein

Introduction

A large portion of the Earth's surface exists at temperatures below 4°C and includes the low-temperature environments of sea ice, glacial ice, the deep sea, and permafrost (Graumann and Marahiel 1996; Russell and Hamamoto 1998). These low-temperature environments are inhabited by a variety of cold-adapted organisms such as bacteria, archaea, yeasts, algae, insects, marine and terrestrial invertebrates, fish, and plants—but are dominated by microorganisms in terms of species diversity and biomass (Feller and Gerday 2003). Microorganisms that thrive at temperatures near 0°C are termed psychrophiles and can be classified as steno- or eury-psychrophiles, depending on their ability to withstand a narrow or wide range of temperatures, respectively (Feller and Gerday 2003).

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Microorganisms that live at subzero temperatures must evolve mechanisms to deal with the accompanying thermodynamic constraints. These constraints include lower rates of catalysis and transport, decreased membrane fluidity, stabilization of the secondary structure of nucleic acids (leading to the inhibition of replication, transcription, and translation), and formation of ice crystals (Cavicchioli et al. 2000). Most of these thermodynamic constraints apply to all microorganisms at the lower limits of their growth temperature ranges; while the most severe constraints affect those microorganisms (psychrophiles) that grow at the lowest temperatures. Much has been learned from the study of the growth of mesophiles at low temperatures (20 to 4°C); however, limited data exists about growth at low temperatures by low-temperature adapted organisms.

Both microarray and proteome studies can identify cellular processes required for the growth of psychrophiles at low temperatures. Post-transcriptional and post-translational regulation can, however, confound correlations between transcript and protein levels. For example, a study of 106 *Saccharomyces cervisiae* proteins demonstrated that no correlation could be found between mRNA levels and protein amounts (Gygi et al. 1999). In addition, examination of the proteome and transcriptome of low temperature (15°C) growth of *Bacillus subtilis* revealed that about half of the cold inducible proteins (CIPs) did not have corresponding increases in transcript levels; while another 10% of the CIPs had changes in expression opposite of transcript trends (Budde et al. 2006). Hence, microarray and proteomic approaches to cold acclimation will yield distinct, complementary data about growth at low temperatures. In this study, we employed proteomics to identify CIPs during growth of a eurypsychrophile at subzero temperatures.

CIPs are defined as proteins that are preferentially or uniquely present at low temperatures and are thought to contribute specifically to the ability of organisms to function at low temperatures (Scherer and Neuhaus 2002). CIPs can be further classified into cold shock proteins (CSPs) or cold acclimation proteins (CAPs) depending on the kinetics of their expression. CSPs are induced immediately upon cold shock with peak expression occurring shortly after temperature downshift, while CAPs are present at higher levels as growth resumes at the lower temperature. CSPs assist in overcoming the immediate effects of temperature downshifts (primarily restoring gene expression and translation, Graumann and Marahiel 1996; Hebraud and Potier 1999). Among the known CSPs is the RNA chaperone CspA and the RNA helicase CsdA (Yamanka et al. 1998). Because the same thermal constraints affect both cold shock and cold acclimation, CSPs and CAPs may share functionality at both the molecular and cellular level (Whyte and Inniss 1992; Bayles et al. 1996; Berger et al. 1996; Panoff et al. 1997). The responses of microorganisms to cold shock have been extensively studied by proteomic (and microarray) approaches (for example, Panoff et al. 1994; Graumann et al. 1996; Kaan et al. 2002; Phadtare and Inouye 2004; Weinberg et al. 2005); however, there have been very few studies of growth at or below 4°C (Homma et al. 2003).

Cold acclimation proteins have been identified in the mesophiles *Enteroccoccus faecalis* at 8°C and *Listeria monocytogenes* at 10°C (Panoff et al. 1997; Liu et al. 2002). CAPs have been detected during growth at 0 to 10°C in steno- and eury-psychrophiles, such as *Arthrobacter globiformis* SI55, *Rhizobium leguminosarum, Bacillus psychrophilus, Pseudomonas fragi, Pseudomonas flourescens, Aquaspirillum arcticum*, and *Methanococcoides burtonii* (Roberts and Inniss 1992; Whyte and Inniss 1992; Hebraud et al. 1994; Berger et al. 1996; Colucci and Inniss 1996; Drouin et al. 2000; Goodchild et al. 2004). A homolog of CspA was found to be upregulated during growth at 4°C in *A. globiformus* SI55 (Berger et al. 1997). Proteomic studies of *M. burtonii* during growth at 4°C (relative to its T_{opt} of 23°C) have provided valuable information about cold adaptation in Archaea revealing a need for efficient carbon utilization and relief of stress on transcription, protein folding, and the generation of the proton motive force (Goodchild et al. 2004, 2005). Despite these advances, little is known about the proteome of cold-adapted Bacteria or the identities of most CIPs in Bacteria, particularly during growth at subzero temperatures. Subzero temperatures combine the stresses of low temperature and low water activity as high concentrations of solutes are required to maintain liquid water at subzero temperatures.

In this study, we employed a proteomics approach to examine the effect of subzero temperature on the physiology of the eurypsychrophile *Psychrobacter cryohalolentis* K5 (Bakermans et al. 2006). We report patterns of protein abundance at 16, 4, and –4°C (salinity remained constant at 5%) and the first detection and identification of CIPs present during growth at subzero temperatures in *P. cryohalolentis* K5. Low-temperature adaptations are expected within *P. cryohalolentis* K5 because it survived a 43,000-year burial within Siberian permafrost at approximately –10°C and is capable of reproducing at –10°C (Bakermans et al. 2003).

Materials and methods

Growth and preparation of cells

Broth cultures of *P. cryohalolentis* K5 were grown in 100 ml of defined medium with acetate as the carbon source [20 mM sodium acetate, 7% sea salts (Sigma #S9883), 50 mM MOPS, 1 mM K_2HPO_4, 5 mM NH_4Cl, 1 × Trace Metals Solution, 1 × Wolfe's Vitamin Solution] in Sarstedt 175 cm^2 polystyrene tissue culture flasks without shaking. Three biological replicates were grown at each temperature (16, 4, –4°C); the high salt concentration of the medium prevented freezing at the lowest temperature. Cultures were incubated in a VWR Signature* Low-Temperature Incubator Model 2005 with a temperature uniformity of ±0.2°C as verified by a temperature logger. Cultures were inoculated with 1 ml of cells grown at the respective incubation temperature in the defined medium. Each replicate originated from a different colony on one plate of *P. cryohalolentis* K5. Cells were harvested at an average OD_{600} of 0.22 (±0.04) by centrifugation at 10,000×*g* at 16 or 4°C for 10 min. Supernatants were removed and cell pellets were extracted with an equal volume of urea solubilization buffer [9 M urea, 2% 2-mercaptoethanol, 2% ampholytes (pH 8–10, BioRad), and 4% Nonidet P40]. The soluble, denatured proteins were recovered in the supernatants after centrifugation of the samples at 435,000×*g* for 10 min using a Beckman TL100 tabletop ultracentrifuge. Protein concentrations were determined using a modification of the Bradford protein assay (Ramagli and Rodriguez 1985). After assaying for protein, samples were stored at –80°C until analysis by two-dimensional gel electrophoresis (2DE).

Two-dimensional gel electrophoresis

Aliquots of samples containing 40 μg of protein were separated in the first dimension by isoelectric focusing using polyacrylamide gels containing 50% pH 5–7 with 50% pH 3–10 carrier ampholytes (Anderson and Anderson 1978a). After 14,000 Vh, the first-dimension gels were equilibrated with sodium dodecyl sulfate (SDS) and the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by O'Farrell using a linear gradient of 10–17% acrylamide (O'Farrell 1975; Anderson and Anderson 1978b). Proteins were then detected by staining with silver nitrate (Giometti et al. 1991a). Two gels were examined for each of the three biological replicates at each temperature for a total of six gels examined for each temperature (except for 16°C for which there were five gels from three biological replicates).

Image acquisition and analysis

Images of 2DE protein patterns were digitized by using an Eikonix 1412 charge-coupled device scanner interfaced with a Vax 4000-90 workstation. The images were then transferred to a PC and converted to Tiff format. Gel images at a resolution of 300 dpi were analyzed using the Image Master 4 (trial version) software available from the Swiss Institute of Bioinformatics. Spot detection parameters (smooth, saliency, and minimum area) were varied from gel to gel, while striving to detect the same range of spots on all gels. After manually matching three to four landmark spots to guide the program, spots were automatically matched between gels and matches were verified manually. Relative abundance of spots was compared between gels based on percent volume. Percent volume was calculated as the volume of the spot (calculated from the area and peak intensity of the spot) divided by the volume of all spots detected. Gel images from the same growth temperature were compared with each other in order to produce a master ''composite'' gel. Presence or absence of protein spots was examined over all six gels, and protein spots were only included on a final ''composite'' gel if present on four or more gels (only required on three or more gels for 16°C). The majority (76–84%) of protein spots detected at each temperature were found on four or more of the gels and their abundance at each temperature did not vary considerably from gel to gel (72% of spots examined had coefficients of variation ≤25; while the mean and median coefficient of variation was 21 and 17, respectively).

The composite gels for each growth temperature were then compared with each other to identify cold inducible proteins (CIPs). We defined CIPs as those proteins that were detected (1) at all growth temperatures with increased abundance at –4°C, (2) at all growth temperatures with abundance increasing as temperature decreased (at least 1.5-fold more at –4 than at 4 or 16°C), (3) at 4 and –4°C, with abundance increased at –4 relative to 4°C, and (4) only at –4°C. Changes in abundance were considered significant if $P < 0.05$ using the *t* test. Spots were not included if the volume at –4°C was less than 0.15%, or if examination of the gel showed an irregular spot.

Protein identification

CIPs were identified at the MSU Proteomics Facility. Gel spots were excised and subjected to in-gel diges-

tion with porcine trypsin. The resulting tryptic peptides, for each separate gel spot, were extracted online using a Waters CapLC and desalted on a 1 mm × 0.2 mm Magic C18 Captrap cartridge. The bound peptides were then flushed onto a 15 cm × 75 μm New Objectives Picofrit column packed with Michrom Magic C18 AQ packing material terminating in an 8 μm spray tip. Peptides were separated using the Waters CapLC over 60 min with a gradient of 5% B to 50% B in 45 min with a flow rate of 250 nl/min (Buffer A = 0.1% formic acid, Buffer B = 95% Acetonitrile and 0.1% formic acid) into a Waters Q-Tof Ultima API mass spectrometer. The top four ions in each survey scan were subjected to automatic low energy CID and the resulting uninterpreted MS/MS product ion spectra were searched against the predicted proteins for *P. cryohalolentis* K5 obtained from the Joint Genome Institute Microbial Sequencing program at Oak Ridge National Laboratory and available at http://www.genome.ornl.gov/microbial/pcry/. The proteins were searched using the Mascot database searching software. Scores were considered real if two peptides from each protein had a Mascot score above a significance level of $P = 0.05$. Protein scores were additionally screened for relevance using a cutoff of 46 and requiring that at least one peptide from each protein have a score greater than 30. Proteins were annotated by comparing the predicted *P. cryohalolentis* K5 proteins with the proteins in the NCBI non-redundant protein database using the program BLASTP (Altschul et al. 1997).

Results and discussion

We used 2DE to identify changes that occur in the proteome of *P. cryohalolentis* K5 in response to growth at different temperatures (Fig. 1, 2). A total of 618 unique protein spots were detected during growth at the three temperatures and could represent up to 25% of the 2,485 ORFs predicted from the genome sequence. A total of 311 (51%) of these spots did not vary significantly with growth temperature and accounted for 73% (v/v) of the amount of protein detected at each temperature (Fig. 3). These ''common'' proteins most likely represent housekeeping proteins required for basic cell functions.

Differential production of proteins

Protein patterns demonstrated that growth temperature substantially reprogrammed the proteome. Of 618 proteins examined, 303 exhibited variation with

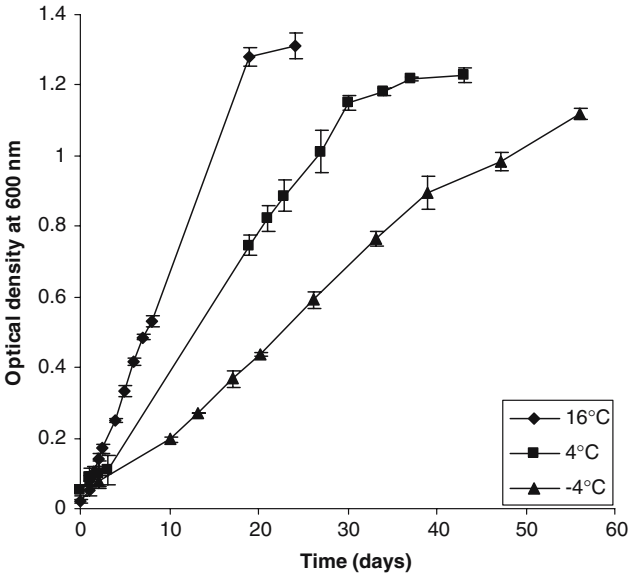

Fig. 1 Growth of *P. cryohalolentis* cultures as monitored by optical density at 600 nm; cultures were harvested at an OD_{600} of ~0.2. Lag phases were omitted from the plot

temperature—that is, the abundance (percent volume) of 303 proteins varied with growth temperature (fold difference was greater than 2 or less than 0.5, $P < 0.05$, Fig. 4). These differentially-produced proteins accounted for about 31% of the number of proteins and about 27% (v/v) of the amount of protein within the cell at each growth temperature. The variation of protein abundance with temperature has not been extensively examined in psychrophiles or mesophiles. Previous studies demonstrated that the abundance of only 10–15% of the proteins in *P. fragi*, *Vibrio* sp. ANT-300, *Escherichia coli*, and *M. burtonii* varied with temperature (Herendeen et al. 1979; Araki 1991; Hebraud et al. 1994; Goodchild et al. 2004). These results contrast with our data that suggests that each temperature regime requires a large set of specialized proteins. However, a recent study of *B. subtilis* found that 45% of protein spots detected changed with growth temperature, 37 versus 16°C (Budde et al. 2006).

Some of these differentially-produced proteins displayed temperature trends; that is, some proteins accumulated to high levels at low temperatures, while other proteins accumulated to high levels at high temperatures. Protein spots could thus be grouped based on trends with temperature (Fig. 5). For example, the relative abundance of 26 spots was higher at –4 and 16°C, indicating that the temperature ''extremes'' induced a common response. The relative abundance of a substantial number of spots (62, 63, and 36) was highest at specific growth temperatures (–4, 4 and

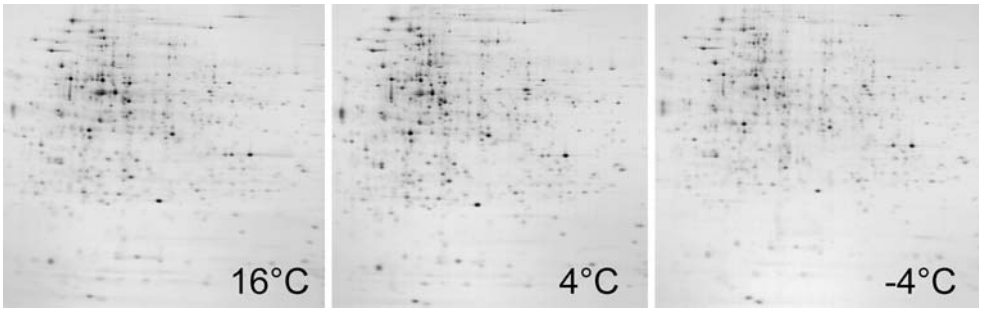

Fig. 2 Representative images of 2DE gels at each growth temperature

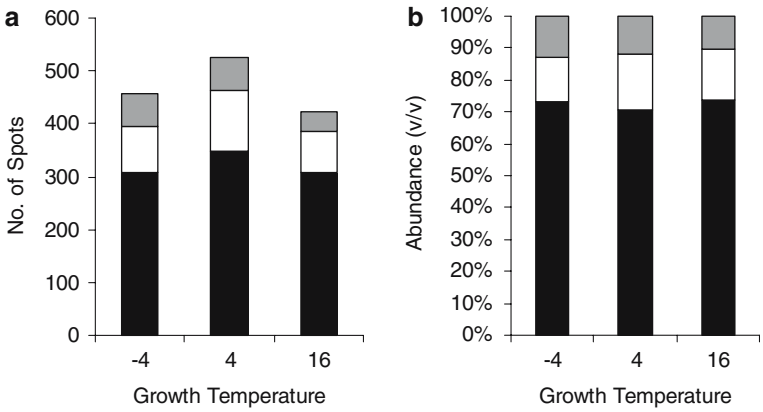

Fig. 3 Overall distribution of protein numbers (**a**) and abundance (**b**) at each growth temperature. *Black bars* represent proteins whose abundance did not vary with temperature; *white bars* represent proteins whose abundance increased significantly at two of the three growth temperatures; and *gray bars* represent proteins whose abundance increased significantly at one growth temperature

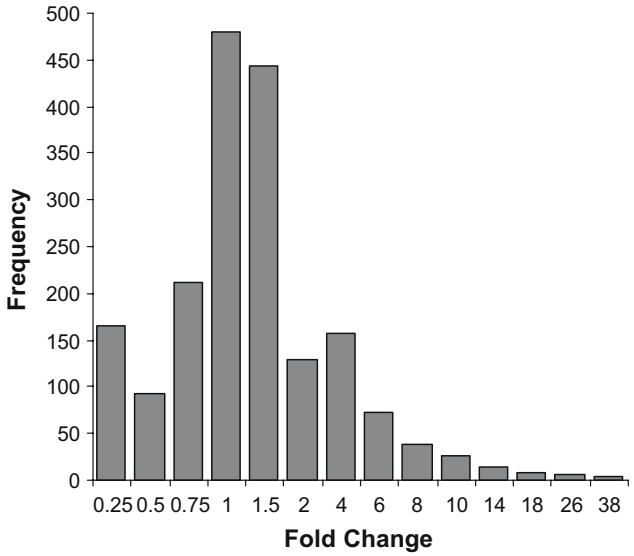

Fig. 4 Histogram of fold changes in relative abundance between growth temperatures. Fold changes are shown for all spots and between all temperatures (–4/4, –4/16, and 4/16; a total of 1,416 comparisons). When no analogous spot was present, fold change was calculated using the detection limit of 0.02%

16°C, respectively) indicating that specific temperatures induced specific responses. For identification purposes, we focused on proteins displaying thermal trends most relevant to growth at subzero temperatures—those proteins that increased in abundance as temperature decreased.

Cold inducible proteins

In this study, we defined cold inducible proteins as those proteins whose relative abundance increased at subzero temperatures and subsequently identified 25 CIPs relevant to growth of *P. cryohalolentis* K5 at –4°C (Table 1). Two additional spots (62 and 119) were included in our analysis because they had also been identified in a preliminary study using a rich growth medium. We suspect that many of these CIPs are cold acclimation proteins; however, without examining the kinetics of CIP abundance with respect to cold shock, we could not differentiate between CSPs and CAPs in this study.

Twenty-six of the CIPs were successfully identified by LC/MS/MS; one CIP could not be identified, likely because insufficient amount of protein was extracted from the gel spot. Only one spot contained two different proteins—spot 348 was identified as both isocitrate lyase and the ATPase of an ABC transporter.

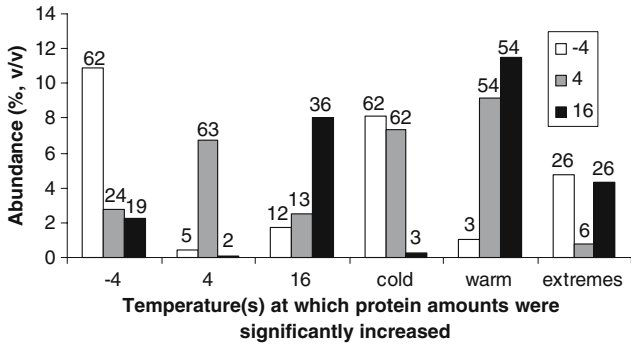

Fig. 5 Abundance of differentially-expressed protein sets at each growth temperature. Proteins with similar trends in relative abundance relative to temperature were grouped into categories that are presented on the *x*-axis. *Column labels* indicate the number of proteins contributing to total protein abundance in each category at each growth temperature

Following identification, spot migration in the 2D gels was reexamined and the following discrepancies were noted. Spots 411 and 415 were identified as the same protein (TypA), but focused at different pI in the 2DE gels. Four CIPs had molecular weights inconsistent with the predicted molecular weight of the ORF. Spot 62 and 82 migrated more slowly (were larger) than expected for RplY and RpsB, respectively; while spots 408 and 409 migrated faster (were smaller) than expected for FecA and AtpF, respectively. These differences in mass and pI may be due to posttranslational modifications, truncated or degraded forms, aberrant migration, and/or error in determining masses from the gels. In addition, errors in genome annotation can result in mismatches between predicted and observed molecular weight; for example, the predicted protein mass could include a leader sequence that was cleaved off in the cell before the samples were prepped.

CIPs detected only at subzero temperatures

Five of the CIPs examined were only detected during growth at –4°C and included: the b subunit of F1/F0 ATP synthase, AtpF; the outer membrane efflux system protein TolC; the elongation factor Ts, EF-Ts; a hypothetical protein with a bacterial Ig-like domain, Pcryo_1988; and the outer membrane receptor for ferric citrate transport, FecA. While only one copy of AtpF, TolC, EF-Ts, and Pcryo_1988 are present in the genome, synthesis of these proteins at higher temperatures is not precluded by our analysis, merely below the detection limit of the 2DE gels. The drastic increase in relative abundance of these proteins at –4°C, relative to 4 and 16°C, suggest that the increases in these proteins counteract increased stress at –4°C.

Based on the identity and known functions of these proteins, we can make some preliminary suggestions about their roles during growth at subzero temperatures. An increase in AtpF at low temperature suggests an increased need for ATP synthase rather than any specific need for the b subunit or its particular function, because other subunits of ATP synthase are notoriously difficult to detect (Kolker et al. 2003). The efflux transporter TolC (as AcrAB-TolC) has a broad substrate range and transports antibiotics, detergents, etc. suggesting an increased need to export potentially harmful molecules at –4°C. EF-Ts regenerates EF-Tu by promoting the dissociation of GDP; thus, the increase in EF-Ts at low temperatures suggests that more EF-Tu must be regenerated. Little can be proposed about the function of Pcryo_1988; the presence of a bacterial Ig-like domain suggests that it is a surface protein. Interestingly, FecA (Pcryo_1280) is a duplication of Pcryo_1281 (or vice versa) that would be transcribed in the opposite direction, is 32 amino acids longer than Pcryo_1281 (which may increase flexibility at low temperatures, Georlette et al. 2004), and was only detected at –4°C suggesting that Pcryo_1280 may be a cold-adapted allele of FecA. While animals commonly use cold-adapted alleles as an adaptation to temperature changes, few examples have been documented in bacteria (Ishii et al. 1987; He et al. 2001).

These data indicate that a variety of processes from ATP synthesis to translation to transport are specifically impacted by subzero temperatures and that adaptation to subzero temperature includes the use of cold-adapted alleles. Functional information about all the CIPs that were upregulated at subzero temperatures in *P. cryohalolentis* K5 was examined to elucidate a more complete understanding of the stress of subzero temperatures on growth.

Putative roles of CIPs in low-temperature growth

The majority of CIPs identified in *P. cryohalolentis* K5 could be classified into three functional groups: gene expression, transport, or energy production.

Gene expression

One of the major effects of cold stress on microbial growth is the inhibition of translation initiation and elongation (Broeze et al. 1978). Some adaptations of bacteria that decrease low-temperature stresses on translation include the induction of CspA, an RNA chaperone that binds to mRNA and destabilizes secondary structure (Jiang et al. 1997), and specialized ribosome-associated proteins or RNAs that alter the

Table 1 Cold inducible proteins

Cellular function	Spot no.	Spot amount (%, v/v)			Relative abundance[a]			ORF	Score[b]	Peptides	Description	Gene	Mass[c] (kDa)
		16°	4°	–4°	–4°/4°	–4°/16°	4°/16°						
Gene expression	62	0.476 ± 0.061	0.525 ± 0.103	0.681 ± 0.162	(1.34)	1.33	(0.99)	184	179	4	Ctc form of ribosomal protein L25	rplY	24.5
	82	0.480 ± 0.204	0.259 ± 0.048	0.593 ± 0.083	2.18	(1.41)	0.65	389	134	4	Ribosomal protein S2	rpsB	28.9
	2		0.347 ± 0.068	0.511 ± 0.056	1.47	[25]	[17]	526	52	2	Cold shock protein	cspA	7.45
	411	0.213 ± 0.031	0.375 ± 0.025	0.453 ± 0.027	1.18	2.00	1.69	373	506	9	GTP-binding protein or elongation factor Tu	typA	68
	415		0.132 ± 0.043	0.212 ± 0.024	1.61	[6.6]	[10]	373	228	4	GTP-binding protein or elongation factor Tu	typA	68
	104			0.179 ± 0.030	[8.9]	[8.9]	(1)	390	90	2	Elongation factor Ts	tsf	31.7
	328	0.066 ± 0.006	0.060 ± 0.010	0.210 ± 0.021	3.37	2.95	(0.88)	73			Antitermination factor	nusA	56.1
Transport	348	0.310 ± 0.183	0.485 ± 0.054	0.630 ± 0.198	(1.63)	2.78	1.70	144	266	6	ATPase of ABC transporters with duplicated ATPase domains	uup	62.4
	189	0.165 ± 0.046	0.164 ± 0.032	0.375 ± 0.040	2.38	2.26	(0.95)	1,728	173	3	ABC-type Fe3+ transporter, periplasmic component	afuA	40.5
	119	0.253 ± 0.074	0.223 ± 0.063	0.317 ± 0.043	1.41	(1.34)	(0.95)	741	318	5	TRAP-T family transporter, substrate binding subunit	dctP	36.6
	343			0.235 ± 0.094	[12]	[12]	(1)	1,759	318	5	Outer membrane efflux system	TolC	65.1
	31		0.106 ± 0.005	0.178 ± 0.009	1.68	[8.9]	[5.3]	788	77	1	ABC lipoprotein exporter, ATPase	lolD	24.6
	408			0.151 ± 0.042	[7.5]	[7.5]	(1)	1,280	84	1	Outer membrane receptor for Fe3+ dicitrate, TonB dependent	fecA	81.4
Energy Production	131	0.377 ± 0.089	0.208 ± 0.037	0.730 ± 0.150	3.34	2.10	0.63	2,044	153	4	Malate/lactate dehydrogenases	mdh	35.3
	348	0.310 ± 0.183	0.485 ± 0.054	0.630 ± 0.198	(1.63)	2.78	1.70	1,860	170	4	Isocitrate lyase	aceA	59.2
	226	0.295 ± 0.110	0.306 ± 0.073	0.526 ± 0.032	1.73	2.11	(1.22)	217	47	1	Acetate kinase	ackA	44.6
	9			0.342 ± 0.030	[17]	[17]	(1)	2,331	242	5	F1/F0 ATP synthase b subunit, H+/Na+ translocating	atpF	17.1
	360	0.034 ± 0.004	0.089 ± 0.011	0.098 ± 0.002	(1.08)	2.70	2.50	296	128	1	NH3-dependent (glutamine-hydrolyzing) NAD(+) synthetase	nadE	62.7
A.A. Metabolism	444	0.058 ± 0.008	0.143 ± 0.041	0.142 ± 0.034	(1.08)	2.37	2.20	803	70	2	Glycine dehydrogenase	gcvP	106.3
	95		0.111 ± 0.021	0.204 ± 0.043	1.84	[10]	[5.5]	150			Shikimate 5-dehydrogenase	aroE	30.5
	225		0.078 ± 0.014	0.142 ± 0.064	1.82	[7.1]	[3.9]	1,434			Amino transferase (also orfs 12, 819)		~45
Other	423	0.169 ± 0.022	0.211 ± 0.023	0.365 ± 0.033	1.82	2.04	1.12	385	307	5	Chemotaxis protein histidine kinase	cheA	81.6
	12		0.248 ± 0.029	0.279 ± 0.021	(1.13)	[14]	[12]	420	202	4	Hydroperoxide detoxification protein	osmC	14.5
	414			0.170 ± 0.053	[8.5]	[8.5]	(1)	1,988	73	1	Hypothetical with bacterial Ig-like domain		71.5
	355		0.070 ± 0.023	0.169 ± 0.037	2.42	[8.4]	[8.4]	1,140			Xanthine dehydrogenase	xdhA	60.2
	71	0.097 ± 0.011	0.135 ± 0.035	0.229 ± 0.027	1.63	2.47	1.52	605			Hypothetical		27.4
	397		0.105 ± 0.032	0.202 ± 0.055	2.01	[10]	[5.2]						
	338	0.035 ± 0.002	0.055 ± 0.010	0.074 ± 0.004	1.34	2.00	1.49	1,036			Pyridine nucleotide transhydrogenase		61.0

[a] Abundance ratios in parenthesis were not statistically significant ($P > 0.05$); ratios in brackets were calculated using a detection limit of 0.02% (the lowest percent volume measured)

[b] Scores are the total protein scores reported by the Mascot searching algorithm 1.9.2. CAPS without scores were identified by comparison to a virtual gel

[c] Mass was calculated from genome sequence information

structure and hence the temperature-dependent abilities of the ribosome itself (Jones and Inouye 1996; von Stetten et al. 1998). For example, genotypic adaptation of 16S ribosomal RNA to low temperatures has been demonstrated in cold tolerant strains of *Bacillus cereus* that were shown to substitute A and T for G and C when compared to mesophilic strains (Pruss et al. 1999).

In *P. cryohalolentis* K5, five CIPs were involved with translation and included two ribosomal proteins (S2 and the Ctc form of L25), two elongation factors (EF-Ts and the EF-Tu related TypA), and the cold shock protein CspA. Both ribosomal proteins were present at all temperatures suggesting that they are essential for cell function. Higher amounts of S2 and Ctc may be required at low temperatures to counteract increased stress, or to compensate for decreased activity, suggesting that these ribosomal proteins may specifically contribute to ribosomal function at low temperatures. The exact role of S2 and Ctc in ribosome function is unknown; however, Ctc has been localized to the ribosome in *B. subtilis* and its N-terminal domain binds the E-loop of 5S ribosomal RNA (Fedorov et al. 2001; Schmalisch et al. 2002). Interestingly, both S2 and Ctc are among the eight ribosomal proteins (of 53 total) that are transcribed individually in *P. cryohalolentis* K5, suggesting that they are regulated independently of the ''core'' of ribosomal proteins. A similar observation was made for ribosomal proteins that were upregulated in response to cold shock in *B. subtilis* (Kaan et al. 2002).

Two elongation factors (the EF-Tu related TypA and EF-Ts) were induced during growth of *P. cryohalolentis* K5 at low temperatures. The exact function of TypA is unknown; however, it is involved in stress responses, binds to ribosomes, has GTPase activity, and has many similarities to EF-Tu—all compelling evidence suggesting that TypA is itself an elongation factor (Farris et al. 1998; Grant et al. 2003). In translation, EF-Tu-GTP binds aminoacyl-tRNA delivering it to the A-site of the ribosome with subsequent hydrolysis of GTP and is regenerated by EF-Ts, which promotes the dissociation of GDP. TypA was present at all temperatures; however, a pI-variant form of TypA (with a lower pI) was detected only at 4 and –4°C suggesting that a different form may provide additional function at low temperatures. Specialized elongation factors may be required during low-temperature growth to facilitate interactions during translation that may be hindered by more stable structures at low temperatures.

Not surprisingly, CspA was upregulated at low temperature suggesting an increased need for RNA chaperone activity as temperature decreased. In addition, the antitermination factor NusA was also present in relatively high abundance at –4°C suggesting that premature termination of transcripts may be a problem at low temperatures. Together these data demonstrated that maintaining gene expression is important to continued growth at subzero temperatures.

Transport

At low temperatures, transport systems are required to counteract lower rates of diffusion and transport across the membrane and for the transport of compatible solutes (Nedwell 1999; Welsh 2000). Six transport-related proteins were up-regulated at low temperatures and included two separate systems for the transport of ferric iron (AfuA and FecA), the lipoprotein transporter LolD, a hydrophobe/amphiphile efflux protein (TolC), a TRAP-T dicarboxylate transporter (DctP), and an ABC transporter with unknown substrate range (Uup). The detection of the lipoprotein transporter LolD suggested an increased need for lipoproteins perhaps for maintaining fluidity of the membrane or activity of membrane proteins at low temperatures. AfuA, DctP, and Uup were present at all temperatures suggesting that they are essential for cell function. Increased amount of these transporters (and FecA) at low temperatures suggests an increased need for the substrates they transport: iron and possibly acetate. Preliminary mutagenesis studies have indicated that the DctP homolog in *P. cryohalolentis* K5 may transport acetate (unpublished data). Because acetate was the only carbon and energy source provided in these experiments, the increase in DctP may indicate an increased need for carbon and energy at low temperatures.

Two of the four available iron transporters were upregulated at low temperatures. The four iron transport systems identified in the genome sequence of *P. cryohalolentis* K5 included: orf1907–1910, the ferric enterochelin ABC transporter CeuABCD; orf1726–1728, a ferric ABC transporter; orf1280–1285, the ferric citrate transporter FecABCDE; and orf1912, a TonB-dependent hemoglobin/transferrin/lactoferrin uptake protein. At low temperatures, more iron may be required to relieve or counter oxidative stress, as in iron superoxide dismutase (Smirnova et al. 2001). In addition, many proteins require iron to carry out enzymatic reactions; therefore, if more enzymes are required at low temperatures to compensate for decreased activity, then more iron will be needed for reaction centers.

Energy production

Little is known about how low temperatures affect energy production in bacteria. Some bacteria use different pathways to generate energy at different growth temperatures; for example, some *Rhizobium* strains switch from respiration to lactate glycolysis at low temperatures (Sardesai and Babu 2000). Temperature-specific carbon source utilization has also been observed (Ponder et al. 2005). While at low temperatures *M. burtonii* upregulated proteins involved in the energy producing processes of methanogenesis (Goodchild et al. 2004).

In *P. cryohalolentis* K5, two proteins from the glyoxylate cycle (malate dehydrogenase and isocitrate lyase) were upregulated at –4°C. The glyoxylate cycle is used for the production of oxaloacetate for the cell's carbohydrate needs and allows the cell to conserve carbon when growing on two-carbon compounds like acetate. Acetate kinase, which feeds acetate into the glyoxylate cycle, was also upregulated at low temperatures in *P. cryohalolentis* K5. In addition, NAD synthetase was upregulated at low temperatures, likely to regenerate the NAD^+ used in the glyoxylate cycle. Glyoxylate cycle enzymes may be upregulated at low temperatures to provide more carbon and energy at low temperatures. The concomitant increase in a possible acetate transporter also supports this hypothesis. Because many proteins involved with the glyoxylate cycle were upregulated at low temperatures, we believe that the glyoxylate cycle itself is important for counteracting increased stress at low temperatures and not that the amount of proteins increased to counteract decreased activity of those proteins. Growth at low temperatures has been shown to require more energy and be less efficient (Bakermans et al. 2003; Bakermans and Nealson 2004).

Alternatively, increased use of the glyoxylate cycle at low temperatures could indicate a need to produce intermediates. For example, during cold stress in *Rhizobium* the amount of malate dehydrogenase increased indicating that the glyoxylate cycle was being utilized to produce oxaloacetate for use in the pentose phosphate pathway (Sardesai and Babu 2001). Another study demonstrated that the glyoxylate cycle was employed (as measured via an increase in isocitrate lyase) for the production of citrate for aluminum detoxification when *Pseudomonas fluorescens* was subjected to aluminum stress (Hamel et al. 2004). Intermediates could possibly be used as compatible solutes; however, in this study we did not identify other proteins that could be involved in compatible solute synthesis.

Miscellaneous

Of the remaining CIPs, only OsmC had a clear role in growth at low temperatures. OsmC detoxifies organic hydroperoxides that are produced during aerobic respiration (Lesniak et al. 2003). Oxidative stress increases at low temperatures because oxygen radicals accumulate to higher steady state concentrations given that oxygen is more soluble and reduced respiration rates consume oxygen more slowly. The remaining CIPs had diverse functions which included chemotaxis, nucleotide metabolism, and a surface protein. In addition, several CIPs (gcvP, aroE, and aminotransferases) were identified which were involved in some aspect of amino acid metabolism; however, no obvious connection to low-temperature stress could be inferred.

Similarities to stress responses in other organisms

Of the 27 CIPs identified in *P. cryohalolentis* K5, only TypA, Mdh, NusA, CheA, NadE, and CspA (proteins or transcripts) had previously been identified as being upregulated in response to cold stress (Jones et al. 1987; Graumann et al. 1996; Jiang et al. 1997; Sardesai and Babu 2001; Kiss et al. 2004; Budde et al. 2006). This is the first report of cold regulation for the remaining 21 CIPs which included 2 ribosomal proteins, EF-Ts, 6 transport proteins, isocitrate lyase, acetate kinase, AtpF, and OsmC. As discussed above, there are plausible roles for these CIPs during growth at low temperatures. These CIPs may not have been identified as cold-regulated in other studies because they may be needed more at subzero temperatures and previous studies have not examined such low temperatures. Indeed, if we had only compared 4 with 16°C, most of these proteins would not have been identified (based on significant differences in relative abundance). Alternatively, the identification of these CIPs in *P. cryohalolentis* K5 may be due to differences in low temperature adaptations of psychrophiles (continued growth at subzero temperatures) versus mesophiles (limited or no growth during exposure to low temperatures).

A substantial number of the CIPs identified in *P. cryohalolentis* K5 have been identified in other stress responses in other organisms. As expected, several CIPs have been identified in response to changes in temperature (see above for cold stress). The deletion of RpsB results in cold sensitivity of *E. coli* (Strocchi et al. 2006). Heat stress induced the expression of Ctc and NadE in *B. subtilis*, (Antelmann et al. 1997; Hecker and Volker 1998). Osmotic stress, which often

shows similarities to cold stress, induced the expression of the TRAP transporter *TeaABC* in *Halomonas elongata* for the transport of the compatible solute ectoine (Grammann et al. 2002); Ctc in *L. monocytogenes* in the absence of osmoprotectants (Gardan et al. 2003) and in *B. subtilis* (Hecker and Volker 1998); OsmC in *E. coli* (Lesniak et al. 2003); and NadE in *B. subtilis* (Antelmann et al. 1997). Ef-Tu, Ef-Ts, and RpsB were upregulated in response to heat shock and pressure in *Lactobacillus sanfranciscensis* (Pavlovic et al. 2005). Oxidative stress, often a component of other stresses, induced the expression of OsmC in *E. coli* (Lesniak et al. 2003) and Ctc in *B. subtilis* (Hecker and Volker 1998). In addition, AckK was induced by phosphate limitation in *S. meliloti* (Summers et al. 1999); TypA was induced during low pH and SDS stress adaptation in *S. meliloti* (Kiss et al. 2004); isocitrate lyase was overexpressed in *P. fluorescens* subjected to aluminum stress (Hamel et al. 2004); and NadE was induced in response to ethanol stress in *B. subtilis* (Antelmann et al. 1997). The involvement of these CIPs in other stress responses in *Psychrobacter* has yet to be determined; however, overlap of proteins between microbial stress responses is common (Hecker and Volker 1998).

Conclusions

We have identified CIPs present during growth of *P. cryohalolentis* K5 at –4°C. To date, CIPs have not been examined during growth at subzero temperatures (CSPs have been detected, but not identified, at subzero temperatures in *Rhizobia*, and no evidence was presented for growth of these bacteria at the subzero temperatures examined; Cloutier et al. 1992). We used 2DE to examine patterns of protein abundance and identify CIPs, hence it is likely that membrane proteins, proteins with unusual physiochemical properties (such as the alkaline pI typical of some ribosomal proteins), and low-abundance proteins were not detected. Slow growth rates can also affect protein levels. However, slow growth rates are an inherent consequence of low temperature; hence any effect of slow growth rate on protein levels is still a response to low temperatures. Despite these limitations and caveats, the following general conclusions remain.

Patterns of protein abundance in *P. cryohalolentis* K5 demonstrated that growth temperature substantially reprogrammed the proteome; ~31% of the proteins at each growth temperature responded to temperature. The identities of CIPs suggested that continual relief of low-temperature stress on translation via specialized ribosomal proteins and elongation factors, on transport via increased amounts of transporters, and on energy production via the glyoxylate cycle may be required for successful growth of *P. cryohalolentis* K5 at subzero temperatures. In addition, our data suggested that iron may be limiting during growth at subzero temperatures and that to ensure adequate transport of iron into the cell a cold-adapted allele was employed. This is the first report of cold regulation for most of the CIPs identified. While the putative functions of these CIPS suggest that similar cellular processes are affected, it appears that different proteins are utilized to combat low-temperature stress in *P. cryohalolentis* K5 than in other psychrophiles and mesophiles. Future studies seek to verify the role of these proteins in combating low-temperature and other stresses facilitating growth at subzero temperatures and identifying the complete subzero proteome of *P. cryohalolentis* K5.

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