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Life in the Cold: a Proteomic Study of Cold-Repressed Proteins in the Antarctic Bacterium *Pseudoalteromonas haloplanktis* TAC125▽†

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The proteomes expressed at 4°C and 18°C by the psychrophilic Antarctic bacterium *Pseudoalteromonas haloplanktis* were compared using two-dimensional differential in-gel electrophoresis with special reference to proteins repressed by low temperatures. Remarkably, the major cold-repressed proteins, almost undetectable at 4°C, were heat shock proteins involved in folding assistance.

The Gram-negative bacterium *Pseudoalteromonas haloplanktis* is a typical representative of the *Gammaproteobacteria* found in cold marine environments, and strain TAC125 has been isolated from seawater sampled along the Antarctic ice shelf. Such strains thrive permanently in seawater at about −2°C to +4°C but are also anticipated to endure long-term frozen conditions when entrapped in the winter ice pack. The genome of *P. haloplanktis* TAC125 has been fully sequenced (11). This work has further allowed a proteomic study of its cold acclimation proteins (CAPs), i.e., proteins that are continuously overexpressed at a high level during growth at low temperatures (14). This has demonstrated that protein synthesis and protein folding are the main upregulated functions, suggesting that both cellular processes are limiting factors for bacterial development in cold environments. Here we report a proteomic survey of cold-repressed proteins at 4°C in order to complete the metabolic pattern of the bacterium's growth at low temperature.

Temperature dependence of growth. The Antarctic bacterium maintains a doubling time of ~4 h at 4°C in a marine broth, with an extrapolated generation time of 5 h 15 min at 0°C (Fig. 1a). When the culture temperature is raised to 20°C, the generation time decreases moderately (e.g., 1 h 40 min at 18°C) with a concomitant increase in the biomass produced at the stationary phase (Fig. 1b). At temperatures higher than 20°C, a drastic reduction in cell density at the stationary phase is recorded (Fig. 1b), indicating heat-induced stress on the cell. *P. haloplanktis* fails to grow above 29°C. According to this growth behavior, the temperatures of 4°C and 18°C were selected here for the differential comparison of the proteomes, as 18°C does not induce excessive stress as far as growth rate and biomass are concerned.

Cold-induced versus cold-repressed proteins. The proteomes expressed by the Antarctic bacterium at 4°C and 18°C during the logarithmic phase of growth were compared by two-dimensional (2D) differential in-gel electrophoresis as described previously (14). In a typical single 2D gel (see Fig. S1 in the supplemental material), 142 protein spots were more abundant at 4°C (CAPs), whereas 309 protein spots were less intense at 4°C than at 18°C. This unexpectedly large number of cold-repressed proteins already indicates that numerous cellular functions are downregulated during growth at low temperature. The repression factors (or induction factors for CAPs), given by relative spot abundance between 4°C and 18°C, are illustrated in Fig. 2. This distribution shows that 21% of cold-repressed proteins display a downregulation factor of between 5 and 28, revealing that some key cellular functions are severely affected. Of the 309 cold-repressed proteins, 83 were retained, which satisfied both statistical biological variation analysis and mass spectrometry identification scores. These cold-repressed proteins are listed in Table S1 in the supplemental material along with their repression factors, and their distribution in the main cellular functions is given in Table S2 in the supplemental material. It is worth mentioning that cold-repressed proteins could formally correspond to proteins that are overexpressed at 18°C. This feature should be taken into account when analyzing the proteomic results presented below.

HSPs and protein folding. The major heat shock proteins (HSPs) such as the chaperone DnaK, the chaperonin GroEL, and the chaperone Hsp90, as well as the so-called small HSPs (IbpA and -B), were identified here as strongly cold-repressed proteins in the proteome of *P. haloplanktis*. The overexpression of bacterial HSPs at elevated temperatures is well recognized as being indicative of a heat-induced cellular stress (5, 16), and this is obviously relevant for the Antarctic bacterium grown at 18°C. In *Escherichia coli*, it has been shown that synthesis of HSPs is repressed during growth at low temperatures but also that these HSPs are harmful to cells at 4°C, as their induced expression reduces cell viability at this temperature (9). Analysis of the *P. haloplanktis* HSP spots (Fig. 3; see Fig. S2 in the supplemental material) shows that these HSPs are present in only trace amounts at 4°C and are therefore true cold-repressed proteins in the Antarctic strain. However, downregulation of this protein group in the Antarctic bacterium severely impairs an essential

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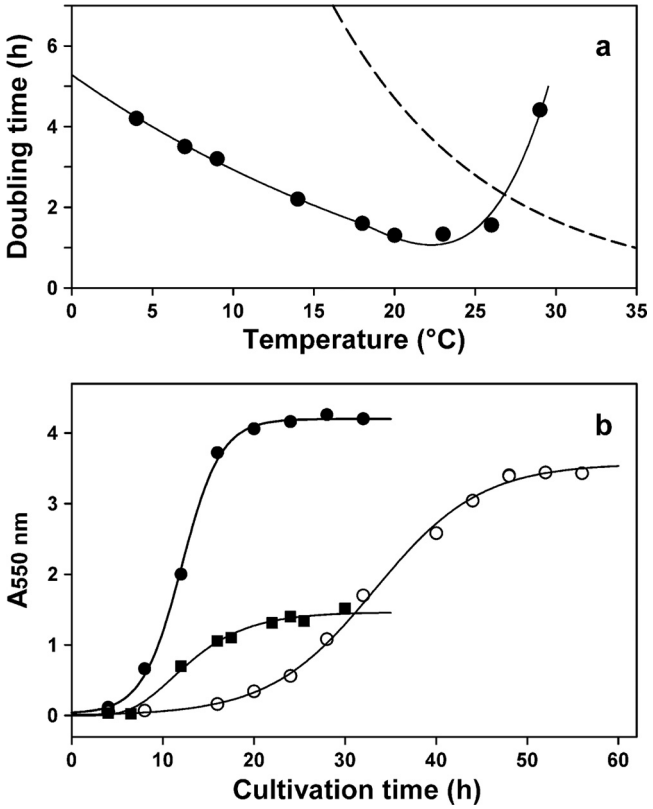

FIG. 1. (a) Temperature dependence of the generation time of *Pseudoalteromonas haloplanktis* TAC125 grown in marine broth (solid line and circles). A typical curve for *E. coli* RR1 in LB broth is shown for comparison (dashed line). (b) Growth curves of *P. haloplanktis* at 4°C (○), 18°C (●), and 26°C (■).

cellular function, as these HSPs are chaperones assisting co- or posttranslational protein folding (7). Such a detrimental situation reinforces our previous suggestion that overexpression of the trigger factor (37-times upregulation; see Fig. S2 in the supplemental

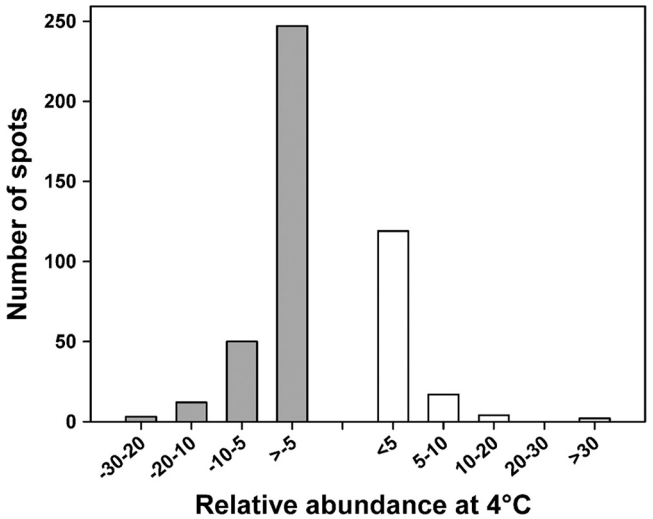

FIG. 2. Distribution of the relative abundance of cold-repressed proteins (dashed, negative values, 18°C/4°C spot volume ratio) and of cold acclimation proteins (positive values, 4°C/18°C spot volume ratio) in the proteome of *P. haloplanktis* grown at 4°C.

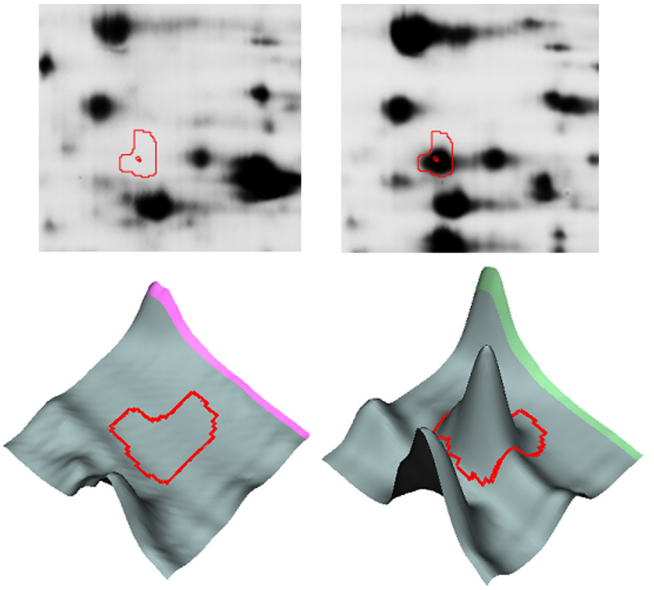

FIG. 3. Comparative analysis of spots containing the chaperone DnaK from *Pseudoalteromonas haloplanktis* grown at 4°C (left panels) and 18°C (right panels). Spot view on a 2D gel seen in fluorescence (upper panels) and three-dimensional images (lower panels) obtained with DeCyder software.

material) by *P. haloplanktis* TAC125 at 4°C may rescue the chaperone function at low temperatures (14). Indeed, the trigger factor is the first chaperone interacting with virtually all newly synthesized polypeptides on the ribosome (12) but it is also a cold shock protein in *E. coli* (9). Low temperature slows down the folding reaction and is well known to reduce misfolding and aggregation (10), possibly contributing to a limiting of the detrimental effects of HSP repression. Nevertheless, it is now clear that regulation of the expression of these proteins involved in thermal stress is a primary adaptation to bacterial growth at low temperatures.

Metabolism depression at low temperatures. Nearly half of the proteins downregulated at 4°C are related to functions involved in general bacterial metabolism (see Table S2 in the supplemental material). This includes the degradation or biosynthesis of compounds and the production of energy. Most of these proteins are involved in oxidative metabolism, in particular to glycolysis, the pentose phosphate pathway, the Krebs cycle, and electron chain transporters. Accordingly, the Antarctic bacterium depresses its general metabolism when grown at low temperature. This is in agreement with the reduced biomass produced at 4°C (Fig. 1b). It is worth mentioning that ancient bacterial survival has been reported in frozen samples up to half a million years old and such viability has been correlated with minimal cellular metabolic activity and the capacity to slowly repair DNA (8). We have previously shown that protein synthesis and folding are limiting factors in the growth of *P. haloplanktis* at cold temperatures (14). The present proteomic data indicate that when these limitations are alleviated at 18°C, the bacterium proliferates by the activation of its general metabolism and therefore divides actively and produces more biomass. This can also be regarded as an adaptive strategy to increase the viable population during short

warmer periods. From an ecological point of view, and in the context of possible global warming, a rise in environmental temperature would result mainly in the proliferation of bacteria such as *P. haloplanktis*.

Downregulation of iron metabolism at low temperatures. Iron uptake and iron-related proteins are clearly downregulated at 4°C. Two transport systems of this essential element were found to be downregulated at 4°C: the ABC transporter FbpA and a TonB-dependent receptor. The first is involved in the uptake of weakly soluble ferric ion (Fe^{3+}) directly from the environment, and the second is required for the transport of heme complexes and ferric siderophores through the cell membrane (3). The reduced need of *P. haloplanktis* for iron at 4°C can be partly explained by the downregulation of the Krebs cycle and the respiratory chain (and their iron-containing complexes such as SdhB), by the repression of HmgA, which requires Fe^{2+} to degrade cyclic amino acids, or by the strong downregulation of catalase (which is made up of four heme groups). Hemes are tetrapyrroles that have porphobilinogen as a precursor: this is in agreement with the downregulation of both GltX (glutamyl-tRNA synthetase) and HemB (5-aminolevulinate dehydratase), which are responsible for porphobilinogen synthesis.

Various metallic ions are essential for cell metabolism, and therefore, the fact that proteomic data only point to cold repression of iron-related proteins is puzzling. Iron in a redox-active form (Fe^{2+}) is potentially deleterious, as it is able to induce oxidative cell damage by the Fenton reaction, for instance (17). It can be tentatively proposed that, as a result of the improved stability of ROS (reactive oxygen species) at low temperatures, the downregulation of iron-related proteins could contribute to an avoidance of such detrimental iron-based reactions. In this respect, it should be mentioned that the genome of *P. haloplanktis* entirely lacks the ubiquitous ROS-producing molybdopterin metabolism (11). This suggests that the Antarctic bacterium tends to avoid ROS production involving metallic ions.

Oxidative stress-related proteins. The second group of proteins that displays the highest repression factors at 4°C is represented by the oxidative stress-related proteins catalase, glutathione reductase, and peroxiredoxin (see Table S1 in the supplemental material). At first sight, this may be regarded as a conflicting result because conclusive findings have indicated that psychrophiles, including *P. haloplanktis*, are exposed to permanent oxidative stress at low temperatures, which originates from improved oxygen solubility and increased ROS stability (1, 2, 4, 11, 13–15). However, it should be recalled that the general aerobic metabolism of the Antarctic bacterium is stimulated at 18°C, also resulting in ROS production. Accordingly, the identified oxidative stress-related proteins would be better regarded as being induced at 18°C rather than repressed at 4°C.

The upregulation of catalase and peroxiredoxin at 18°C shows that the bacterium needs to be protected against ROS like H_2O_2 as both enzymes catalyze its decomposition into O_2 and H_2O. Under oxidative stress, the NADPH supply for reduced glutathione regeneration is also dependent on glucose-6-phosphate dehydrogenase (Zwf) in the first step of the pentose phosphate pathway, which is upregulated at 18°C. Glutathione reductase (Gor) plays a central role in the reoxidation of NADPH from the pentose phosphate pathway, allowing the formation of reduced glutathione, an important cellular antioxidant. The upregulated DNA-binding DPS protein DpsB plays a major role in the protection of bacterial DNA from damage by ROS and is induced under stress conditions (6). There is obviously a finely tuned balance between the cellular mechanisms protecting against oxidative stresses generated by low temperatures (resulting from ROS stability and oxygen solubility) and those generated by high temperatures (resulting from stimulated metabolic activity) which deserves further investigation.

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Supplementary Material to:

Life in the cold: a proteomic study of cold-repressed proteins in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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Content

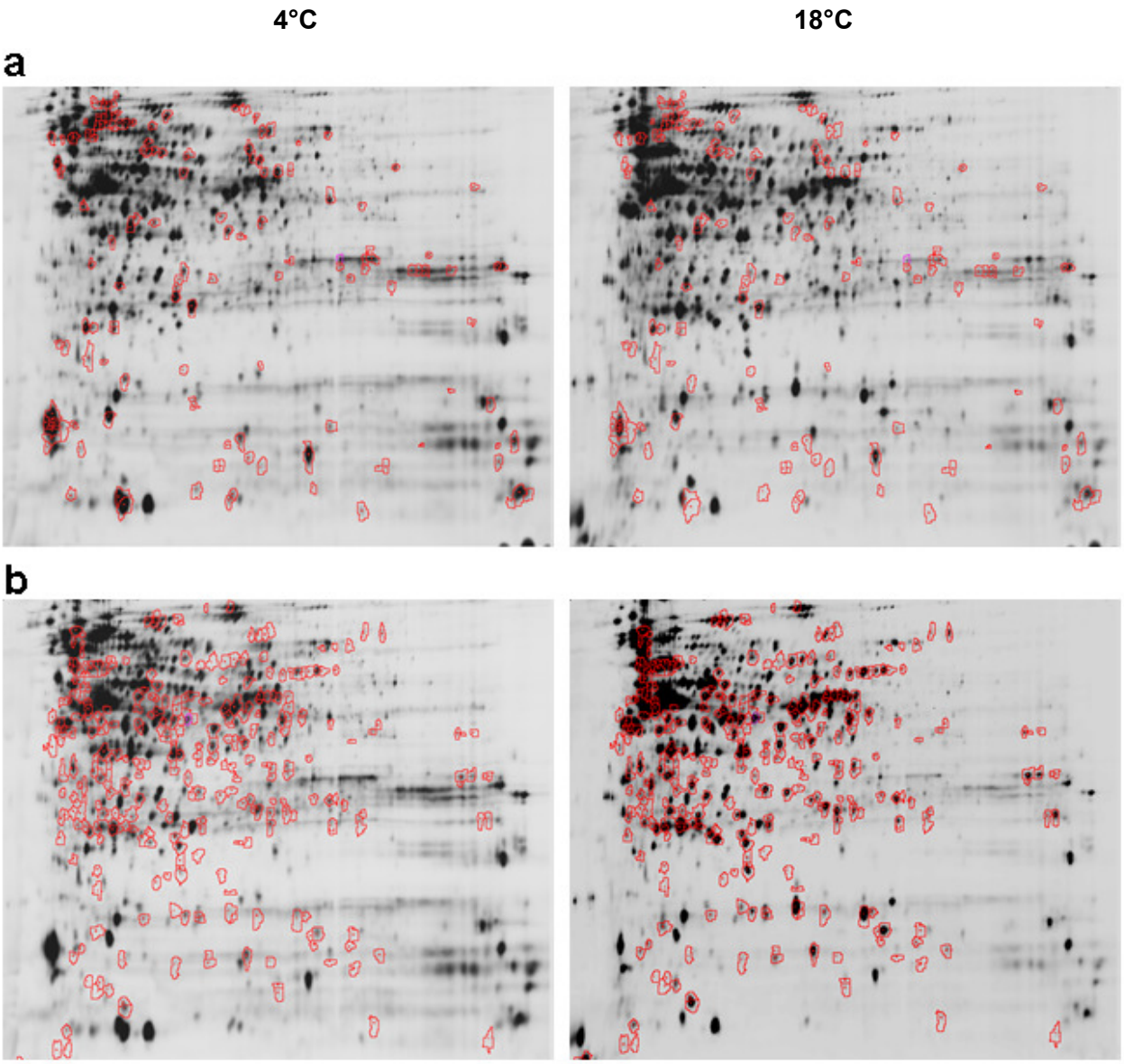
Supplementary Figure 1

Supplementary Figure 2

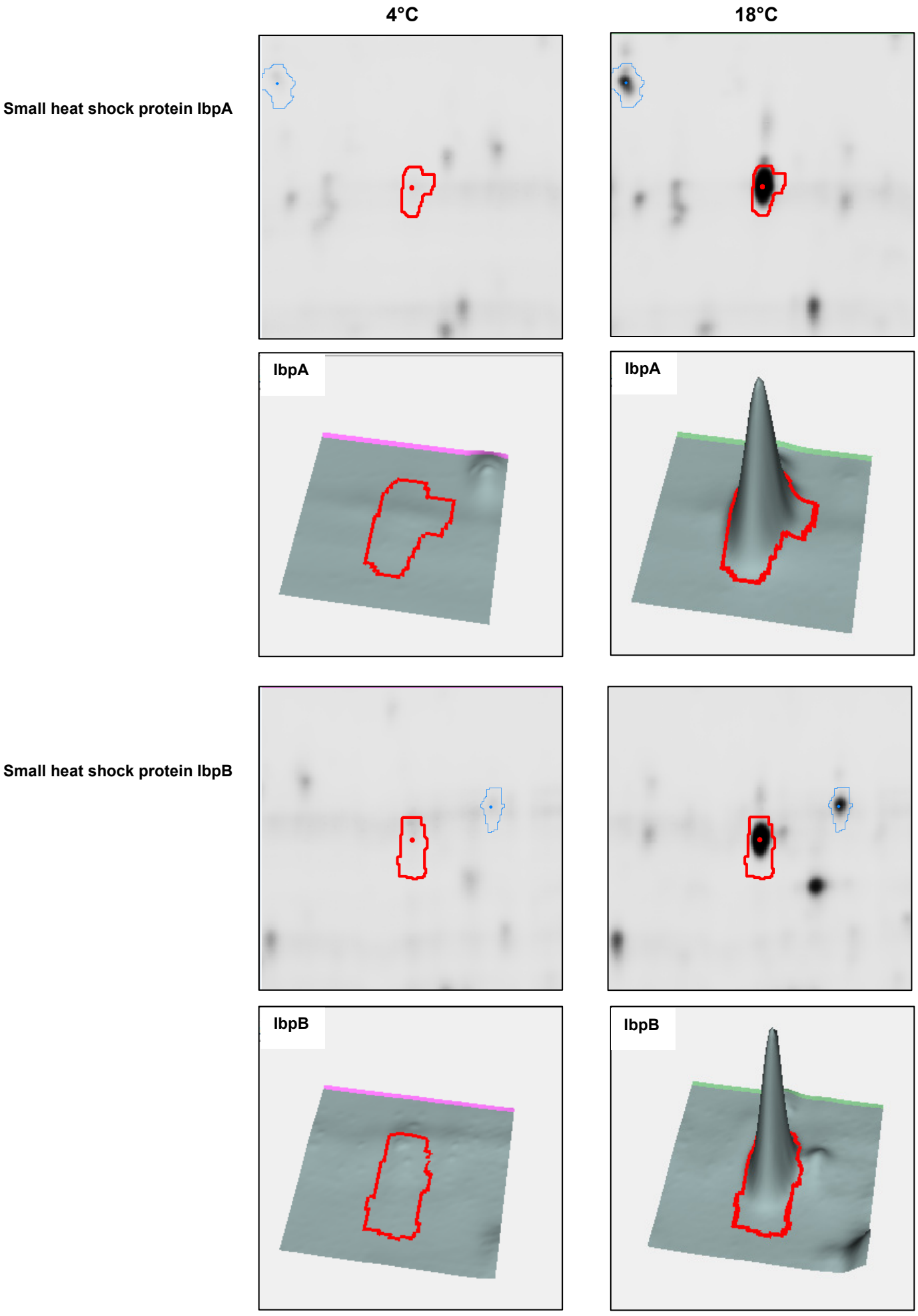
Supplementary Table 1

Supplementary Table 2

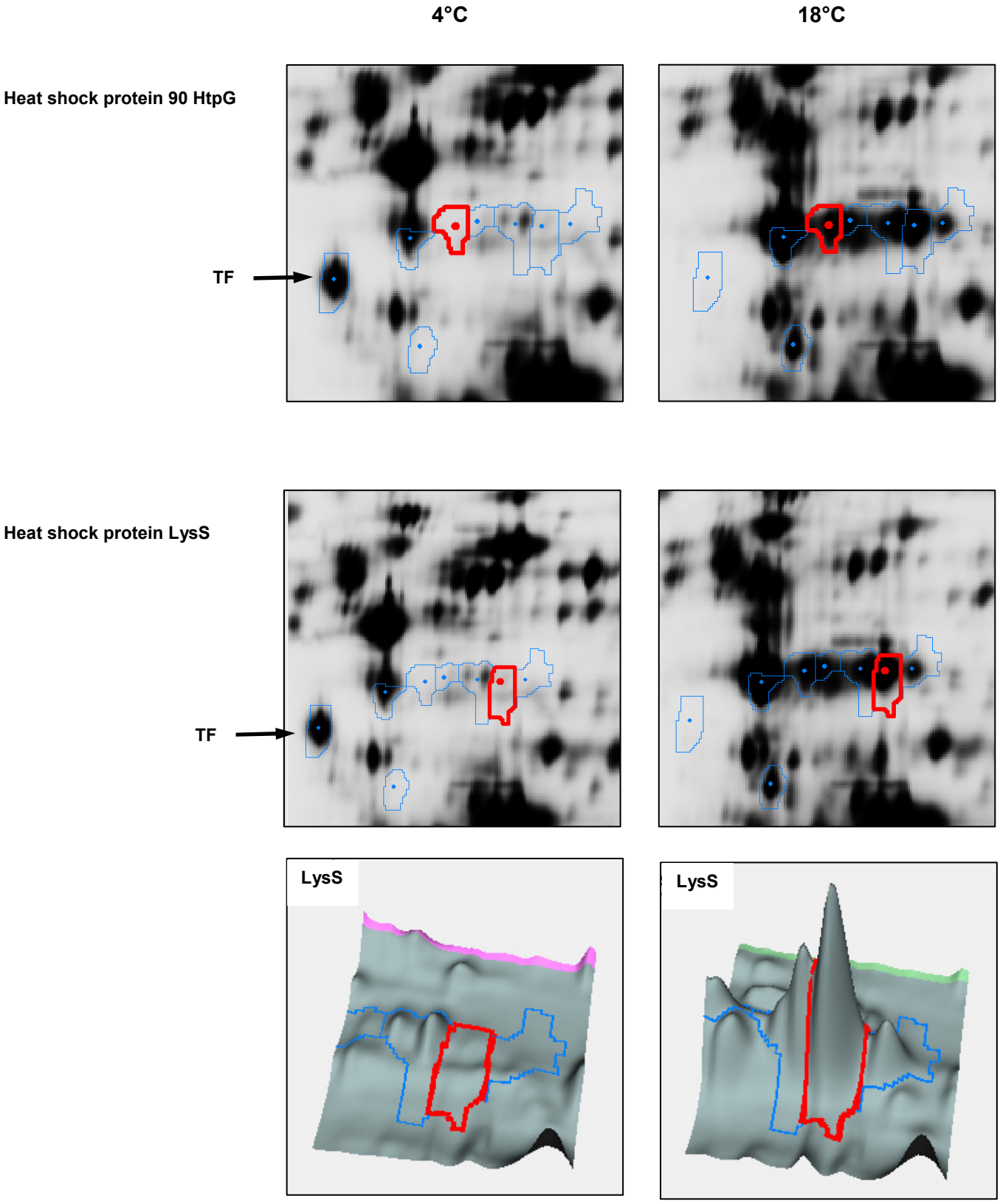
Supplementary Figure 1 Differential analyses of soluble cellular proteins from *Pseudoalteromonas haloplanktis* TAC125 grown at 4°C (left panels) and 18°C (right panels) on 2D-DIGE gels analyzed by fluorescence. **(a)** 142 protein spots that are more intense at 4°C are indicated. **(b)** 309 protein spots that are less intense at 4°C are indicated. On each gel, from left to right, non-linear gradient from pH 3 to pH 10, from top to bottom, mass scale from ~150 to ~15 kDa.



Supplementary Figure 2 Comparative analysis of spots containing heat shock proteins (in red) from *Pseudoalteromonas haloplanktis* grown at 4°C (left panels) and 18°C (right panels). Spot view on 2D-gel seen in fluorescence and three-dimensional images obtained with DeCyder software.



Supplementary Figure 2 (continued) Note the overexpression of the cold shock chaperone trigger factor (TF) at 4°C in the following gels.



Supplementary Table 1 Cold-repressed proteins identified by MALDI-TOF or LC MS-MS (*) in down-regulated spots at 4°C from 2D-DIGE of *Pseudoalteromonas haloplanktis* TAC125.

Protein	Gene number	Gene	Down-regulation ratio	*t*-test	Nominal Mr	Calculated pI	Score	% sequence coverage	Queries matched
Protein synthesis and folding									
Transcription, translation									
30S ribosomal subunit protein S2	PSHAa2036	*rpsB*	1,51	0,0079	26827	6,61	102	22	2
30S ribosomal protein S4	PSHAa2807	*rpsD*	2,03	0,00043	23469	9,90	76	14	2
30S ribosomal subunit protein S7*	PSHAa0225	*rpsG*	23,82	0,00059	17582	10,37	156	15	2
50S ribosomal protein L4	PSHAa0145	*rplD*	3,02	0,00066	21816	9,52	143	63	13
50S ribosomal subunit protein L13	PSHAa2532	*rplM*	2,33	0,0062	15934	9,78	245	36	4
50S ribosomal protein L25	PSHAa1053	*rplY*	3,28	0,036	22329	5,39	345	33	5
Lysyl-tRNA synthetase *	PSHAa0518	*lysS*	16,85	0,00011	58601	4,95	393	13	6
Glutamyl-tRNA synthetase	PSHAa0635	*gltX*	3,65	0,0036	56377	5,41	124	7	2
DNA-directed RNA polymerase alpha chain	PSHAa2806	*rpoA*	2,45	0,002	36208	4,67	410	25	6
Elongation factor EF-2	PSHAa2940		2,08	0,023	76038	4,93	228	12	5
Elongation factor Ts	PSHAa2035	*tsf*	1,60	0,004	30388	5,16	90	14	3
Elongation factor Tu	PSHAa0227	*tufA*	13,14	0,0014	43450	4,98	250	65	26
Elongation factor Tu	PSHAa2911	*tufB*	13,14	0,0014	43425	4,95	227	60	24
Folding and Heat Shock Proteins									
Chaperonin GroEL	PSHAa0259	*groL*	3,41	0,0001	57164	4,76	162	51	18
Chaperone protein DnaK	PSHAb0357	*dnaK*	13,01	0,047	68966	4,69	78	27	13
Heat shock protein 90*	PSHAa1207	*htpG*	27,78	0,0069	72457	4,88	147	3	2
Peptidyl-dipeptidase Dcp	PSHAa2184	*dcp*	9,93	0,0014	79373	6,51	139	28	17
Disulfide bond formation protein	PSHAa0248	*dsbA*	2,56	0,017	22977	6,20	234	30	3
Disulfide bond formation protein	PSHAa0249	*porA*	2,30	0,00014	23695	9,16	69	8	2

Small heat shock protein*	PSHAa0992	*ibpB*	18,15	0,02	16549	6,08	232	14	5
Small heat shock protein*	PSHAa0993	*ibpA*	23,82	0,00059	16325	5,66	272	20	8
Oxydative stress									
Glutathione reductase*	PSHAa0360	*gor*	8,15	0,0073	48719	5,84	195	10	3
Catalase*	PSHAa1737		6,50	0,00097	57385	6,05	322	11	5
Peroxiredoxin 2	PSHAa0839	*ahpCB*	15,66	0,013	22272	5,07	175	63	15
DNA-binding DPS protein	PSHAa2954	*dpsB*	2,55	0,0016	17622	4,97	87	13	1
Membrane proteins and receptors									
TonB-dependent receptor	PSHAa0108		2,51	0,00015	77108	4,59	124	22	13
TonB-dependent receptor protein	PSHAa1987		2,78	0,033	107300	4,44	249	37	28
Iron-regulated outer membrane virulence protein homolog	PSHAb0251	*irgA*	1,76	0,0094	72335	4,85	95	4	2
Iron(III) ABC transporter periplasmic iron(III)-binding protein	PSHAa2663	*fbpA*	1,74	0,0022	37132	8,77	243	20	4
Putative Hemin receptor protein HmuR	PSHAb0072	*hmuR*	1,72	0,00077	76706	4,84	280	16	6
Putative outer membrane protein with a TonB box	PSHAb0341		7,26	0,0014	118270	4,56	170	25	21
General Metabolism									
Degradation, assimilation									
Pyruvate dehydrogenase decarboxylase subunit*	PSHAa0391	*aceE*	5,45	0,0016	99462	5,29	617	12	14
Dihydrolipoamide dehydrogenase	PSHAa0393	*lpd*	1,73	0,021	50262	5,22	207	57	24
Leucine dehydrogenase	PSHAa1167	*bcd*	4,20	0,014	44509	5,36	154	41	15
Alanine dehydrogenase	PSHAa1718		4,84	0,0037	39623	5,87	226	69	25
Enoyl-CoA hydratase/isomerase	PSHAa1450		1,61	0,0002	27698	5,88	218	30	5
Beta subunit of a coenzyme A carboxylase protein	PSHAa1451		1,89	0,000045	57883	5,85	495	21	7
Isovaleryl-CoA dehydrogenase	PSHAa1452		2,02	0,0036	42497	5,26	487	35	8
Lactoylglutathione lyase	PSHAa1601	*gloA*	2,25	0,039	19825	4,69	49	32	3
Succinyl-CoA synthetase, alpha subunit, NAD(P)-binding	PSHAa1644	*sucD*	1,59	0,03	30031	5,32	173	17	3
Succinyl-CoA synthetase subunit beta	PSHAa1645	*sucC*	1,71	0,012	41676	4,89	248	62	24
4-hydroxyphenylpyruvate dioxygenase	PSHAa2168	*melA*	1,83	0,00019	39518	4,59	305	22	5
Fumarylacetoacetase	PSHAa2169	*faH*	1,72	0,0035	48486	5,22	123	42	14

Homogentisate 1,2-dioxygenase	PSHAb0338	*hmgA*	1,98	0,0023	48715	5,30	117	39	15
2-amino-3-ketobutyrate coenzyme A ligase	PSHAa2316	*kbl*	2,43	0,00016	43263	5,10	82	33	11
Generation of precursor metabolites and energy									
Electron transfer flavoprotein beta-subunit	PSHAa1618	*etfB*	4,76	0,000075	26957	6,23	476	51	8
Fumarate hydratase	PSHAa0048	*fumC*	3,65	0,0036	48707	5,44	299	22	5
Fructose-bisphosphate aldolase	PSHAa0596	*fbaA*	3,87	0,0034	38290	5.44	154	21	6
Glucose-6-phosphate 1-dehydrogenase	PSHAa1140	*zwf*	1,84	0,0015	55604	5,49	2,95	20	6
Succinyl-CoA transferase, subunit A	PSHAa1447		2,36	0,028	25046	5,17	226	32	4
Acyl-CoA dehydrogenase protein	PSHAa1456		2,23	0,0067	42271	5,18	171	57	22
Glyceraldehyde 3-phosphate dehydrogenase*	PSHAa1900	*gapA*	6,50	0,00097	52872	6,06	94	3	1
Na(+)-translocating NADH-quinone reductase subunit A	PSHAa2241	*nqrA*	1,71	0,0028	48116	5,88	116	9	3
Transaldolase B	PSHAa2559	*talB*	1,60	0,004	35012	5,23	167	20	5
Malate dehydrogenase	PSHAa2658	*mdh*	1,58	0,0018	31900	4,97	330	29	4
F0F1 ATP synthase subunit alpha	PSHAa3010	*atpA*	1,72	0,0086	55524	5,09	129	43	15
Isocitrate lyase	PSHAb0062	*aceA*	16,85	0,00011	59267	5,03	267	10	5
Phosphoenolpyruvate synthase	PSHAb0557	*ppsA*	3,77	0,00005	85805	4,82	169	30	27
Succinate dehydrogenase iron-sulfur subunit	PSHAa1648	*sdhB*	2,76	0,000015	27162	5,60	115	36	8
Biosynthesis									
GTP cyclohydrolase I	PSHAa0073	*folE*	2,03	0,015	20780	6,31	141	17	3
Phosphoribosylglycinamide synthetase	PSHAa0343	*purD*	1,61	0,0026	45795	4,70	194	15	4
Hypoxanthine-guanine phosphoribosyltransferase	PSHAa0609	*hpt*	2,00	0,02	21373	5,36	104	23	2
Carbamoyl phosphate synthetase, glutamine amidotransferase small subunit	PSHAa1228	*carA*	1,98	0,016	39787	5,43	119	9	2
Branched-chain amino acid aminotransferase	PSHAa1270	*ilvE*	1,99	0,022	35612	5,68	52	6	2
NAD-linked malate dehydrogenase	PSHAa1565	*sfcA*	4,19	0,001	62590	5,36	164	15	5
Adenylosuccinate lyase	PSHAa1692	*purB*	2,14	0,043	51465	5,47	260	15	5
Uridylate kinase	PSHAa2034	*pyrH*	2,68	0,0014	27272	5,45	152	68	19
Aspartate-semialdehyde dehydrogenase	PSHAa2078	*asd*	1,55	0,043	36897	4,62	129	19	4
3,4-dihydroxy-2-butanone 4-phosphate synthase	PSHAa2372	*ribB*	1,58	0,00088	40364	5,26	156	46	16

Serine hydroxymethyltransferase	PSHAa2376	*glyA*	2,03	0,001	45082	5.76	220	16	4
2-hydroxyacid dehydrogenase protein	PSHAa2619		1,72	0,024	34129	5.89	98	35	10
Bifunctional GMP synthase/glutamine amidotransferase protein	PSHAa0649	*guaA*	1,69	0,00005	59019	5,4	185	32	21
Delta-aminolevulinic acid dehydratase	PSHAa2935	*hemB*	3,72	0,018	36991	4.97	89	35	11
Unknown function									
Hypothetical protein PSHAa1142	PSHAa1142		1,70	0,0011	26391	5.24	130	47	15
Hypothetical protein PSHAa1751*	PSHAa1751		12,06	0,00013	22712	5.22	159	14	2
Hypothetical protein PSHAa1921	PSHAa1921		1,69	0,013	29728	4,38	73	29	5
Hypothetical protein PSHAa2755	PSHAa2755		1,98	0,0024	20956	5,4	59	7	1
Putative acyltransferase	PSHAa0174		2,09	0,032	33895	5.76	76	13	3
Putative hydrolase	PSHAa1854		2,25	0,012	25962	8.76	160	19	3
Putative cystathionine gamma-synthase or beta-lyase, PLP-dependent	PSHAb0477		2,06	0,00051	45313	5.96	198	11	3
Periplasmic protein*	PSHAa1298		6,35	0,042	20896	5.70	50	5	2
Secreted protein	PSHAa2972		2,07	0,003	30771	9	108	22	4
Conserved protein of unknown function ; putative signal peptide	PSHAb0473		2,98	0,000000054	46283	7,08	228	13	4

Supplementary Table 2 Distribution of the identified cold-repressed proteins in the main cellular functions of *P. haloplanktis* TAC125 (*n*, number of identified proteins; %, per cent of total)

Function	*n*	%
Degradation	14	17
Biosynthesis	14	17
Energy production	14	17
Protein synthesis	13	16
Folding and HSPs	8	10
Receptors	6	7
Detoxification	4	5
Unknown function	10	12