

# Proteomic and transcriptomic investigations on cold-responsive properties of the psychrophilic Antarctic bacterium *Psychrobacter* sp. PAMC 21119 at subzero temperatures

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## Summary

***Psychrobacter* sp. PAMC 21119, isolated from Antarctic permafrost soil, grows and proliferates at subzero temperatures. However, its major mechanism of cold adaptation regulation remains poorly understood. We investigated the transcriptomic and proteomic responses of this species to cold temperatures by comparing profiles at  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  to understand how extreme microorganisms survive under subzero conditions. We found a total of 2,906 transcripts and 584 differentially expressed genes ( $\geq$  twofold,  $P < 0.005$ ) by RNA-seq. Genes for translation, ribosomal structure and biogenesis were upregulated, and lipid transport and metabolism was downregulated at low temperatures. A total of 60 protein spots ( $\geq 1.8$  fold,  $P < 0.005$ ) showed differential expression on two-dimensional gel electrophoresis and the proteins were identified by mass spectrometry. The most prominent upregulated proteins in response to cold were involved in metabolite transport, protein folding and membrane fluidity. Proteins involved in energy production and conversion, and heme protein synthesis were downregulated. Moreover, isoform exchange of cold-shock proteins was detected at both temperatures. Interestingly, pathways for acetyl-**

**CoA metabolism, putrescine synthesis and amino acid metabolism were upregulated. This study highlights some of the strategies and different physiological states that *Psychrobacter* sp. PAMC 21119 has developed to adapt to the cold environment in Antarctica.**

## Introduction

Cold environments on Earth are considerable in scale. Nearly 80% of Earth's environments are permanently cold at temperatures below  $5^{\circ}\text{C}$ , particularly in most worldwide oceans and high latitude soils (Russell, 2000). These environments are also stressful in other ways to microorganisms, including low water availability, limited organic and inorganic nutrient availability and long-term exposure to high energy radiation (Bakermans *et al.*, 2012). Permafrost makes up  $> 20\%$  of terrestrial soils, and permafrost exposed to temperatures from  $-10^{\circ}\text{C}$  to  $-60^{\circ}\text{C}$  has low water and carbon contents, depends on soil types in permafrost (Rivkina *et al.*, 2004; Steven *et al.*, 2006). Despite these challenging habitats, a variety of microbial life has been found throughout polar environments (Rivkina *et al.*, 2000; Vishnivetskaya *et al.*, 2000). Their cellular functions should have adapted physiologically and metabolically to cold temperatures and such environmental parameters would drive microorganisms to establish their own physiological adaptations for selective survival in cold regions (Bakermans *et al.*, 2012).

Previous studies have attempted to define the molecular and biochemical aspects of unique cold adaptation mechanisms, using methods such as next-generation sequencing and proteomics technologies. In bacteria, upregulation of gene expression or induction of differential expression of isozymes was observed to recover the activities of diminished biochemical reactions or to recover functionally at various temperature ranges (Somero, 1995; Maki *et al.*, 2006). Reactive oxygen species (ROS) are highly solubilized and their concentrations increase at lower temperatures. Consequently, in general, the gene

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expression of antioxidant enzymes is upregulated, and the pathways of ROS production are suppressed (Medigue *et al.*, 2005; Methe *et al.*, 2005). Trehalose is a versatile disaccharide in cold adaptation mechanisms that acts by preventing protein denaturation and aggregation, stabilizing cellular membranes and scavenging free radicals (Kandror *et al.*, 2002). The genes involved in trehalose synthesis, *otsA* and *ostB*, were upregulated in *Escherichia coli* at cold temperatures (Phadtare and Inouye, 2004). Previous studies have reported accumulation of compatible solutes in *Psychrobacter* during cold stress (Amato and Christner, 2009; Ayala-del-Río *et al.*, 2010; Ewert and Deming, 2014). Accumulation of compatible solutes, such as proline, glutamate and glycine betaine, increases at low temperatures. These solutes neutralize osmotic pressure and control cell turgor pressure in cold environments (Ko *et al.*, 1994). A transcriptomic approach of the *Psychrobacter arcticus* identified genes associated in transportation system and revealed elevated gene expressions at low temperatures (Ponder, 2005). Cold shock proteins (CSPs) are one of the most common proteins, that are expressed under cold environments. It was reported that CspA, the major CSP, destabilizes secondary structures of RNA to a single-stranded state (Barria *et al.*, 2013). CspE was shown to bind poly-A tails and inhibit RNA degradation (Feng *et al.*, 2001). *Psychrobacter arcticus* possess three CSPs, that remove the secondary structures in the mRNA, so that the ribosome stability and translation efficiency can be enhanced at low temperatures (Ayala-del-Río *et al.*, 2010). In other studies, several cold adaptation mechanisms were described involving membrane fluidity, transport systems, cell proliferation, gene expression, protein expression, chaperones, enzyme activities, energy metabolism and inhibition of intracellular ice crystal formation (Cavicchioli *et al.*, 2000; Deming, 2002; D'Amico *et al.*, 2006; Piette *et al.*, 2010; Novototskaya-Vlasova *et al.*, 2012).

*Psychrobacter* sp. PAMC 21119 was isolated from permafrost soil on Barton Peninsula, King George Island, Antarctica (Kim *et al.*, 2012). This strain is closely related to *Psychrobacter arcticus* 273-4, *Psychrobacter cryohalensis* K5 and *Psychrobacter* sp. PRwf-1 (Kim *et al.*, 2012). The genus *Psychrobacter* contains a group of Gram-negative, rod-shaped, heterotrophic bacteria. Most *Psychrobacter* species are able to grow at a wide range of temperatures between  $-10^{\circ}\text{C}$  and  $42^{\circ}\text{C}$ , and they have frequently been isolated from diverse cold environments. Several members of this genus have successfully adapted to cold temperatures and have developed various strategies at the molecular level to survive at low temperatures. In *Psychrobacter arcticus*, expression of genes involved in transcription, translation, energy production and most biosynthetic pathways was downregulated, whereas the genes for biosynthesis of amino acids such as proline,

tryptophan and methionine were upregulated at cold temperatures (Bergholz *et al.*, 2009). Another study reported the proteomic identification of cold-inducible proteins in *Psychrobacter cryohalensis* K5 in subzero temperature culture conditions, and suggested that low temperatures may induce the successful growth of *P. cryohalensis* K5 by influencing translation, transport and energy production (Bakermans *et al.*, 2007).

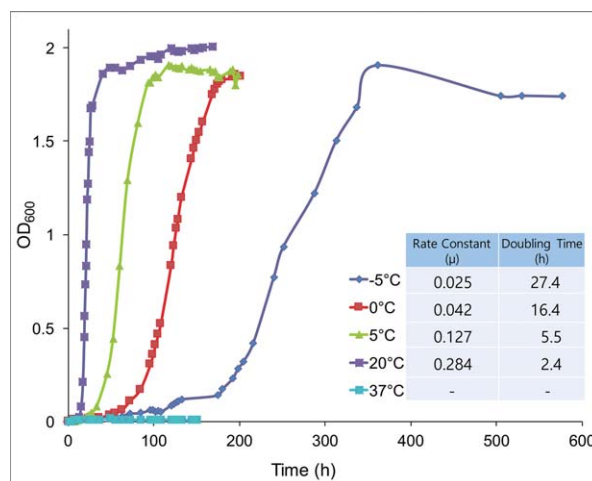
In the present study, we analysed the transcript and protein expression profiles of *Psychrobacter* sp. PAMC 21119 under two temperature conditions:  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$ . This study aims to reveal adaptation strategies employed by psychrophilic microorganisms for survival at subzero temperatures.

## Results

### Bacterial cell culture, next-generation sequencing data and two-dimensional gel electrophoresis (2-DE) analyses

The bacterial cells were cultured at five different temperatures (Fig. 1). The cultures grew successfully from  $-5^{\circ}\text{C}$  to  $20^{\circ}\text{C}$  but not at  $37^{\circ}\text{C}$ . The rate constants ( $\mu$ ) of each temperature group were shown to be 0.025, 0.042, 0.127 and 0.284. The  $\mu$  of the  $-5^{\circ}\text{C}$  was lower than  $20^{\circ}\text{C}$ . The cells grew faster at higher temperatures in the experimental treatments.

The cDNAs purified from cultures at  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  were subjected to next-generation sequencing using an Illumina HiSeq instrument. After low-quality regions, adaptors, and possible contaminants were eliminated, a total of 26,270,292 high-quality reads from the  $-5^{\circ}\text{C}$  group and



**Fig. 1.** Growth curves of *Psychrobacter* sp. PAMC 21119 at different temperatures.

Cells were cultured in marine broth, and the OD<sub>600</sub> values were measured spectrophotometrically. Doubling time and rate constant of each culture temperature are shown. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**Table 1.** Summary of transcript reads from *Psychrobacter* sp. PAMC 21119, obtained using the Illumina HiSeq.

	P21119_−5°C	P21119_20°C
Total reads	26,270,292	27,487,823
rRNA reads	75,779	174,371
mRNA reads	19,550,792	18,582,564

27,487,823 high-quality reads from the 20°C group, were obtained (Table 1). After *de novo* assembly with trimmed reads using the Trinity software, size-selected reads were assembled into 2,906 transcripts comprising 3.36 Mbp (Table 2). The transcripts ranged in size from 200 to 15,042 bp with an average size of 1,367 bp and an N50 of 2,468. The top-hit species distribution of BLAST matches to the National Center for Biotechnology Information (NCBI) 'nr' protein database is shown in Supporting Information Fig. S1. These sequence data have been submitted to NCBI and can be accessed in the Short Read Archive (SRA) under the accession number SRP071712.

The two-dimensional SDS-PAGE gels of *Psychrobacter* sp. PAMC 21119 proteins extracted from the cells grown at −5°C and 20°C revealed a total of 830 protein spots (Fig. 2), of which 60 spots had significant changes in mean intensity ( $\geq 1.8$ -fold) (Table 3). These spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS/MS). Among the 60 differentially expressed proteins, 31 were downregulated and 29 were upregulated at −5°C (Supporting Information Table S1). Each enlarged spot and expression fold-change is shown in Fig. 3.

#### Differential expression and enrichment analyses using protein and gene expression profiles

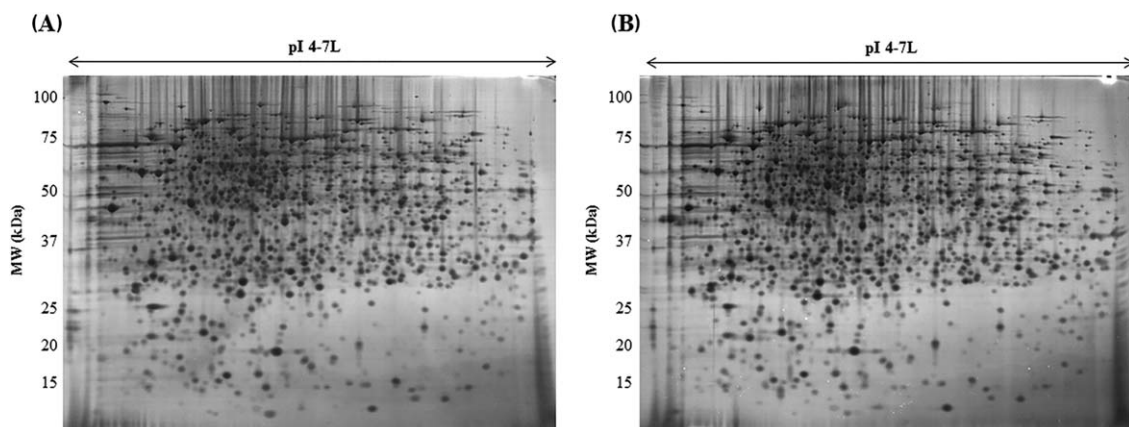
The 60 protein spots exhibiting  $\geq 1.8$ -fold changes in expression were classified into different categories by

**Table 2.** *De novo* transcript assembly statistics of *Psychrobacter* sp. PAMC 21119.

Size	3,362,348
CDS	2,906
G + C Ratio	43.38
N50	2,468
Average length (bp)	1,367
Longest transcript (bp)	15,042

utilizing the clusters of orthologous groups of proteins (COG) databases, implying that these proteins may regulate different cellular functions during cold adaptation (Figs. 4 and 5, Table 3). COG analysis revealed that amino acid transport and metabolism (33%), cell wall/membrane/envelope biogenesis (7%), and post-translation modification, protein turnover and chaperones (13%) were the most prevalent in the −5°C cultures (Fig. 4A). Upregulated and downregulated groups are depicted with the number of proteins per category in Fig. 5. The percentage of expressed proteins in each category indicates the COG classes that were mainly affected by cold stress. The 584 genes showing  $\geq 2.0$ -fold changes were categorized to COG classes (Supporting Information Table S2). The major categories were cluster of translation, ribosomal structure and biogenesis (10%) in upregulated genes that include ribosomal proteins and translation elongation factors. Lipid transport and metabolism (12%) which contains oxidoreductases was predominant in downregulated genes. Components of energy production and conversion were similarly expressed in upregulated (9%) and downregulated (11%) groups. Genes involved in amino acid transport and metabolism showed almost the same expression percentage in both upregulated (11%) and downregulated (12%) groups (Supporting Information Table S2).

The overlap analysis between the transcriptome and proteome data enriched the information on differential

**Fig. 2.** Representative two-dimensional gel electrophoresis (2-DE) gel images (pH 4–7) of whole cell lysates of *Psychrobacter* sp. PAMC 21119, incubated at two different temperatures: −5°C (A) and 20°C (B).

**Table 3.** Functional categorization of differentially expressed proteins of *Psychrobacter* sp. PAMC 21119 at two different temperatures.

SSP	Protein	Accession number <sup>a</sup>	KEGG ID <sup>b</sup>	Gene name	Theoretical Mr	Theoretical pI	Theoretical Mr	Observed Mr	Observed pI	Observed	Sequence coverage (%)	Change (fold) <sup>c</sup>	t-test <sup>d</sup>
	Energy production and conversion (C)												
8730	NADH:ubiquinone oxidoreductase	gi648256035	K00332	nuoC	67469	6.18	64440	64440	5.99	60	2.3	0.00096	
5537	Methylmalonate-semialdehyde dehydrogenase	gi497886961	K00140	mmsA	53180	5.21	53360	53360	5.12	33	2.2	0.0039	
5326	NADPH:quinone reductase	gi497886587	K19267	qorB	35801	5.27	39760	39760	5.16	21	2.0	0.0373	
6639	Succinate dehydrogenase flavoprotein subunit	gi497886639	K00239	sdhA	68083	5.52	67610	67610	5.33	25	-1.9	0.027	
4547	F0F1 ATP synthase subunit alpha	gi497883797	K02111	atpA	56130	5.04	56180	56180	5.02	23	-1.9	0.049	
	Amino acid transport and metabolism (E)												
1843	Aminopeptidase N	gi648256178	K01256	pepN	98974	4.6	90310	90310	4.44	19	2.6	0.0046	
4540	Isopropylmalate isomerase	gi648256092	K01703	leuC	51280	4.95	54960	54960	4.89	37	2.0	0.0052	
2437	Aspartate aminotransferase	gi648256254	K00817	hisC	41508	4.57	41510	41510	4.45	27	2.2	0.0045	
9225	Transglutaminase	gi497883290		tpgA	30053	6.21	33650	33650	6.18	37	1.9	0.0032	
4549	Arginine deiminase	gi497886422	K01478	arcA	21193	5.34	52360	52360	5.06	42	7.7	0.0437	
7246	5,10-Methylenetetrahydrofolate reductase	gi648256251	K00297	metF	27308	5.65	31980	31980	5.6	16	2.3	0.0025	
8637	Gamma-glutamylputrescine oxidoreductase	gi648256018	K09471	puuB	50942	6.06	54520	54520	6.08	37	2.3	0.0055	
7234	Arginine ABC transporter ATP-binding protein	gi497887681	K10004	aatP	29565	5.62	32070	32070	5.68	20	1.9	0.0013	
4538	Glutamine synthetase	gi497884507	K01915	glnA	49623	4.98	56240	56240	4.91	20	4.7	0.0004	
9614	Flavin monoamine oxidase	gi497887013	K00274	aofH	59887	6.76	56270	56270	6.4	37	2.0	0.036	
9611	Flavin monoamine oxidase	gi497887013	K00274	aofH	59887	6.76	57910	57910	6.19	33	-2.6	0.002	
7732	Aminotransferase class-iii	gi497887536	K01845	hemL	95511	5.77	83780	83780	5.62	18	-2.4	0.008	
6538	Aminotransferase	gi648256062	K14260	alaA	60784	5.79	52020	52020	5.38	8	-1.8	0.0004	
6439	3-Phosphoglycerate dehydrogenase	gi497887587	K00058	serA	44850	5.47	44350	44350	5.33	23	-2.3	0.037	
	Nucleotide transport and metabolism (F)												
4139	ADP-ribose pyrophosphatase	gi497887500	K03426	nudC	21749	5.02	29030	29030	4.98	31	-2.2	0.04	
6248	Thymidylate synthase	gi497883759	K00560	thyA	32429	5.62	32800	32800	5.59	25	-1.9	0.001	
	Carbohydrate transport and metabolism (G)												
6636	Glyceraldehyde-3-phosphate dehydrogenase	gi497886004	K00134	gapA	51191	5.57	57070	57070	5.45	15	-2.6	0.001	
6631	Glyceraldehyde-3-phosphate dehydrogenase	gi497886004	K00134	gapA	51191	5.57	57240	57240	5.32	12	-2.3	0.01	
	Coenzyme transport and metabolism (H)												
4433	Delta-aminolevulinic acid dehydratase	gi497885019	K01698	hemB	36977	5.03	42260	42260	4.9	20	-2.2	0.012	
7353	NAD(P)-binding enzyme	gi497887178	K11996	moeB	31515	5.7	36560	36560	5.62	18	-2.1	0.004	
7640	Coproporphyrinogen III oxidase	gi497883084	K02495	hemN	58527	5.73	56590	56590	5.7	44	-1.9	0.004	
6440	Quinolinate synthetase	gi648256043	K03517	nadA	41949	5.43	45310	45310	5.36	19	-1.8	0.008	
6439	3-Phosphoglycerate dehydrogenase	gi497887587	K00058	serA	44850	5.47	44350	44350	5.33	23	-2.3	0.037	
	Lipid transport and metabolism (I)												
1133	Succinyl-CoA:3-ketoacid-CoA transferase	gi497885767	K01029	scoB	22469	4.48	27990	27990	4.39	49	2.1	0.0228	
8739	Hypothetical protein	gi648256041	K02111	esX	105429	6.24	72440	72440	6.02	9	1.8	0.001	
7345	Esterase	gi648256132	K10218	galC	34597	5.77	39690	39690	5.77	20	-2.3	0.001	
7347	4-Diphosphocytidylyl-2C-methyl-Derythritol kinase	gi648255899	K00919	ispE	34697	5.66	35070	35070	5.59	7	-2.2	0.021	
	Translation, ribosomal structure and biogenesis (J)												
9615	Amidase	gi497882332	K02433	gatA	53334	6.41	53260	53260	6.48	17	1.9	0.0049	
8245	Methionine aminopeptidase	gi648255921		map	29557	5.84	34120	34120	5.97	45	3.0	0.008	
4635	Glutaminyl-tRNA synthetase	gi497885096	K01886	glnS	65952	5	67330	67330	4.89	22	-2.6	0.013	

Table 3. cont.

SSP	Protein	Accession number <sup>a</sup>	KEGG ID <sup>b</sup>	Gene name	Theoretical Mr	Theoretical pI	Theoretical Mr	Observed Mr	Observed pI	Sequence coverage (%)	Change (fold) <sup>c</sup>	t-test <sup>d</sup>
Transcription (K)												
9233	LysR family transcriptional regulator	gi 497885697	K17850	lysR	32156	6.49	32200	32200	6.44	46	1.9	0.0001
4025	RNA chaperone/anti-terminator	gi 497887505		csp	7402	5.11	10680	10680	5.05	91	1.9	0.0056
3033	Cold-shock protein	gi 648256010	K03704	csp	8080	4.97	13050	13050	4.74	80	-3.4	0.0002
5023	ArsR family transcriptional regulator	gi 497883864		arsR	12844	5.25	14100	14100	5.12	74	-4.7	0.0107
Replication, recombination and repair (L)												
6340	DNA-binding protein	gi 497887899	K02039	phoU	29088	4.93	39070	39070	5.44	36	-2.3	0.004
Cell wall/membrane/envelope biogenesis (M)												
1227	Membrane protein	gi 497882173	K07274	ompV	29414	4.66	32850	32850	4.42	40	2.1	0.0045
6435	16S rRNA methyltransferase	gi 497886387	K03438	rsmH	42409	5.53	45900	45900	5.44	19	2.1	0.0001
Post-translational modification, protein turnover and chaperones (O)												
6242	Peptidase	gi 497883292	K03432	prcA	27919	5.64	29590	29590	5.54	34	3.1	0.0000
8124	Starvation protein A	gi 497885736	K03599	sspA	24137	5.89	28150	28150	5.88	28	2.6	0.00002
5243	Glutathione S-transferase	gi 497885101	K00799	gst	24874	5.3	33280	33280	5.22	14	2.0	0.0002
5232	Glutathione S-transferase	gi 497887398		gst	27828	5.27	29820	29820	5.23	73	2.0	0.0013
General function prediction only (F)												
4326	N-carbamoylputrescine amidase	gi 497884501	K12251	agdB	32908	5.15	37150	37150	5.07	27	4.0	0.0002
2229	Arylesterase	gi 497883645	K05714	MhpC	30602	4.64	33380	33380	4.48	24	2.6	0.0342
2735	Phosphatase	gi 497887694	K07093	phoX	80020	4.84	75060	75060	4.61	30	2.5	0.0124
5326	NADPH:quinone reductase	gi 497886587	K19267	qorB	35801	5.27	39760	39760	5.16	21	2.0	0.0373
4133	Pyridoxamine 5'-phosphate oxidase	gi 497881841	K00275	pdxH	18371	5.06	20830	20830	4.96	87	-4.0	0.0000
6253	Carbon-nitrogen hydrolase	gi 497881780	K01501	nit	33530	5.53	34570	34570	5.43	19	-2.5	0.0003
4034	Peptidase	gi 497885738	K03101	sspB	16160	5.11	18380	18380	5.08	27	-2.1	0.008
Function unknown (S)												
5145	Hypothetical protein	gi 497887516			16704	5.28	23280	23280	5.15	14	2.0	0.0188
6129	Hypothetical protein	gi 497884057	K01091	gph	19038	5.59	28650	28650	5.5	16	-16.4	0.031
7013	Hypothetical protein	gi 497885819	K05601	Hcp	18987	6.52	18450	18450	5.67	26	-5.9	0.0003
6020	Hypothetical protein	gi 497884556			20869	6.04	20860	20860	5.57	42	-3.2	0.0012
5133	Alkaline phosphatase	gi 497885207	K03975	DcdA	21982	5.3	27760	27760	5.27	33	-2.8	0.00001
8033	Hypothetical protein	gi 497885819	K05601	Hcp	18987	6.52	18790	18790	5.9	47	-2.5	0.016
3023	Endonuclease	gi 648256110	K03470	rnhB	12666	4.81	12780	12780	4.65	46	-2.3	0.034
Not Assigned												
6336	Hypothetical protein	gi 497887892	K00806	uppS	33752	5.51	35920	35920	5.51	40	-6.3	0.046
9026	Hypothetical protein	gi 497884722	K00600	glyA	13313	6.81	14400	14400	6.17	68	-2.7	0.042

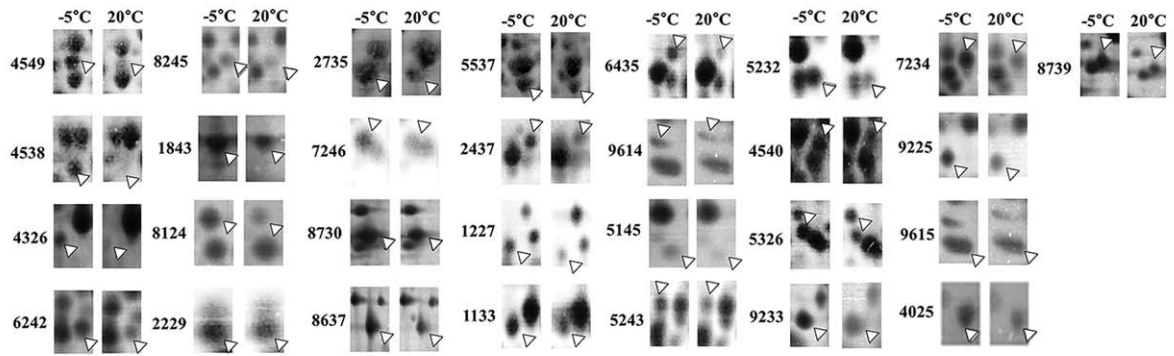
a. Protein accession numbers from *Psychrobacter* sp. PAMC 21119.

b. KEGG numbers from *Psychrobacter* sp. PAMC 21119.

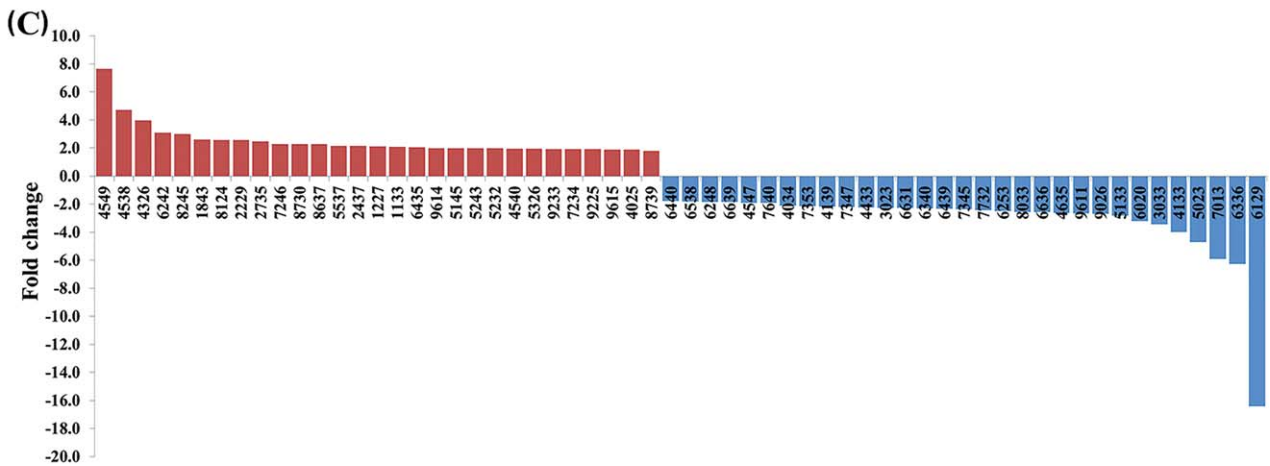
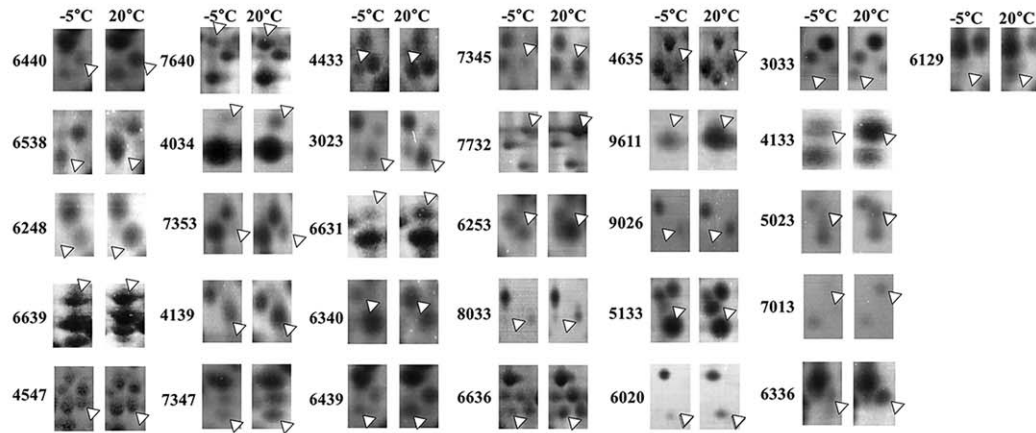
c. Changes in protein levels are reported as the ratio between the normalized protein spot volume from cells grown at  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  ( $V_{-5^{\circ}\text{C}}/V_{20^{\circ}\text{C}}$ ), for proteins present at a higher level. For proteins present at a lower level, it is reported as the negative reciprocal values ( $-V_{20^{\circ}\text{C}}/V_{-5^{\circ}\text{C}}$ ).

d. The statistical significance of the spot intensity difference between  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  was established using Student's *t*-test ( $P < 0.05$ ). Proteins that did not have COG assignments were sorted into the Not Assigned category.

(A) Up-regulated proteins



(B) Down-regulated proteins



**Fig. 3.** Identification of differential abundances of proteins in *Psychrobacter* sp. PAMC 21119 incubated at two different temperatures. Partial enlarged profiles show the differential abundances of the same proteins at  $-5$  and  $20^{\circ}\text{C}$ .

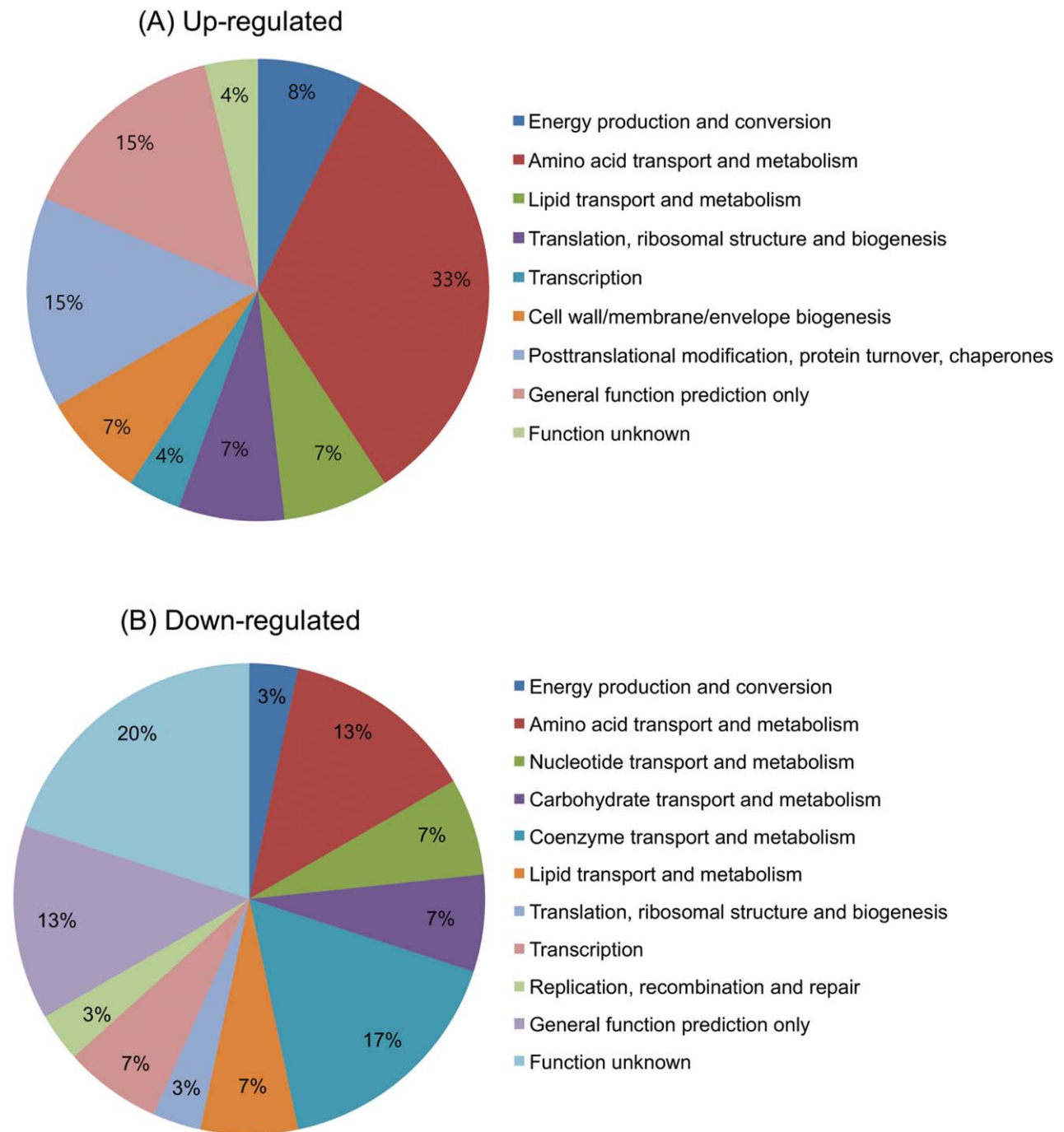
A. Upregulated proteins (B) Downregulated proteins.

C. Histogram displays showing both (A) and (B). Fold-change is reported as the ratio between the normalized protein spot volume from cells grown at  $-5$  and  $20^{\circ}\text{C}$  ( $V_{-5^{\circ}\text{C}}/V_{20^{\circ}\text{C}}$ ), for proteins presenting upregulated levels. For proteins presenting downregulated levels, fold-change is reported as the negative reciprocal value ( $-V_{20^{\circ}\text{C}}/V_{-5^{\circ}\text{C}}$ ). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

expression induced by cold stress in *Psychrobacter* sp. PAMC 21119. A schematic overlap was depicted with a Venn diagram (Fig. 6). The data indicate the numbers of genes showing  $>$  twofold changes in the transcriptome

and  $>$  1.8-fold changes in the proteome at  $-5^{\circ}\text{C}$  compared to the  $20^{\circ}\text{C}$  cultures. Among the upregulation data, 29 proteins and 312 genes responded to cold stress ( $-5^{\circ}\text{C}$ ). Only eight were commonly upregulated in

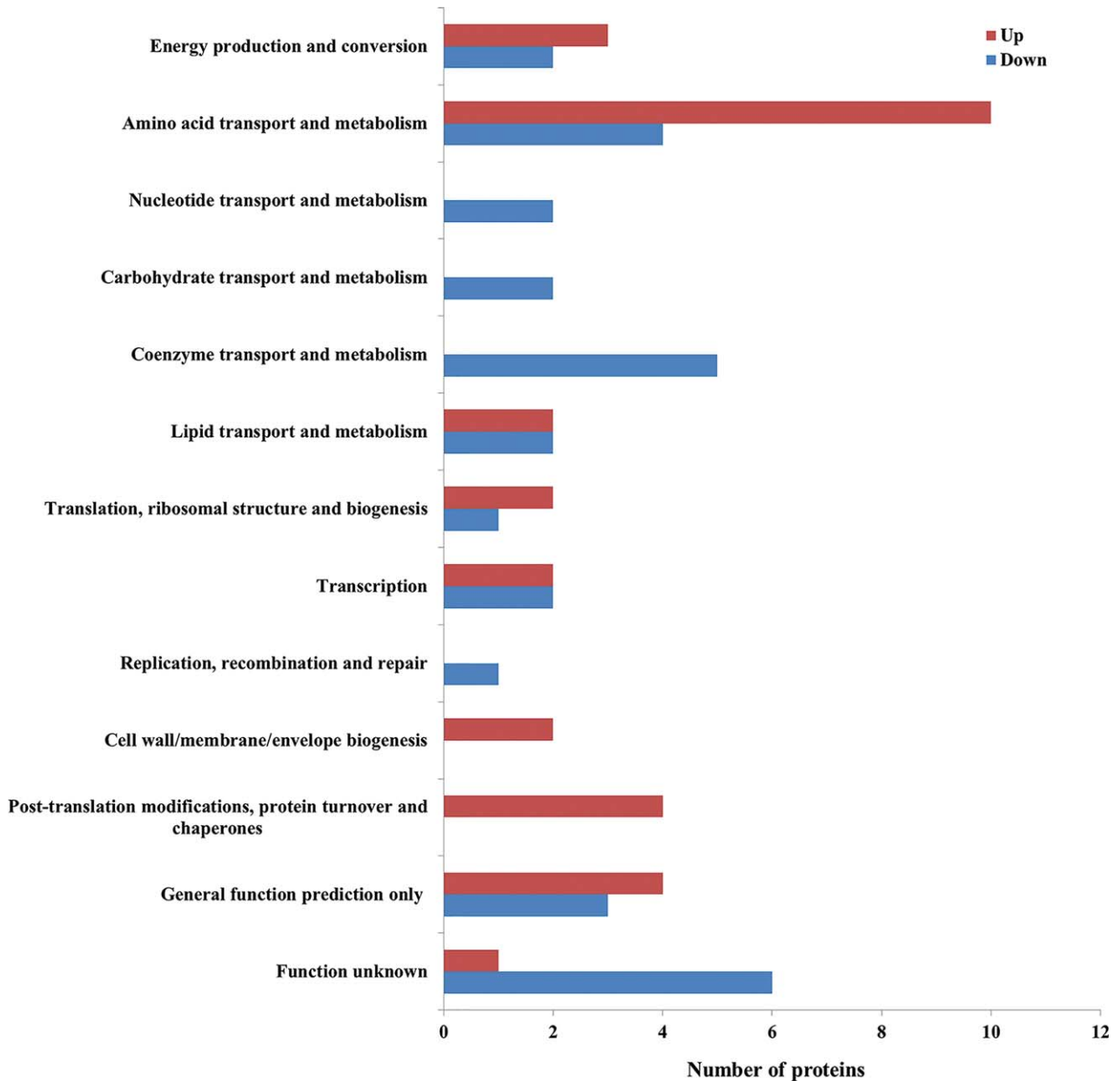




**Fig. 4.** Functional classification of 60 protein spots selected for protein identification by mass spectrometry/mass spectrometry (MS/MS) analysis. Each pie-chart shows functional categories of these varied abundances of upregulated and downregulated proteins according to clusters of orthologous groups of proteins (COG) analysis. Amino acid transport and metabolism, post-translational modification, turnover and chaperones were highly present in upregulated profiles, whereas nucleotide transport and metabolism, carbohydrate transport and metabolism and coenzyme transport and metabolism dominated downregulated profiles. A. Upregulated proteins and (B) downregulated proteins at  $-5^{\circ}\text{C}$ . [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

both the proteome and transcriptome groups. In the downregulation list, 31 proteins and 272 genes showed decreased expression in response to cold stress. Eleven were downregulated in both the proteome and

transcriptome groups. However, six genes were increased in the proteome but decreased in the transcriptome, while one was increased in the transcriptome but decreased in the proteome. The gene IDs in each

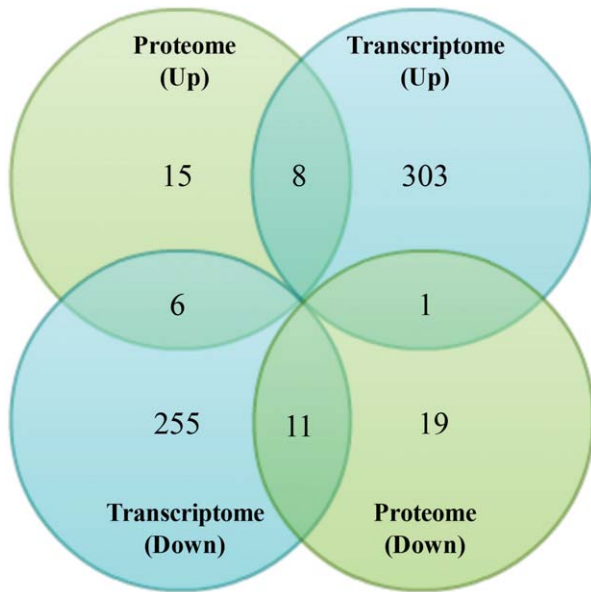


**Fig. 5.** Proteins with significant differential abundances sorted by COG categories. Proteins with upregulated abundances are shown as red bars; proteins with downregulated abundances at  $-5^{\circ}\text{C}$  are indicated as blue bars. Proteins without COG assignments were not included in this analysis. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

group are listed in Supporting Information Table S2, and the numbers of overlaps are shown in Table 4 and Supporting Information Table S3. The differentially expressed transcripts and proteins were subjected to gene ontology (GO) analysis and classified into three major functional categories (biological process, molecular function and cellular component) and 25 subcategories using the complete set of GO terms (Supporting Information Fig. S2). The upregulated transcriptome revealed that cellular processes

(GO:0009987, 68.8%) comprised the largest proportion, followed by translation (GO:0006412, 12%) in the biological process category. Moreover, in the proteome results, metabolic process (GO:0008152, 95.2%) in the biological process category, and catalytic activity (GO:0003824, 95.2%) in the molecular function category were upregulated. The downregulated groups did not reveal any distinct categories. All enriched GO terms of the transcripts and proteins in each group are listed in Supporting Information Table S4.





**Fig. 6.** Venn diagram showing differentially expressed genes and proteins of *Psychrobacter* sp. PAMC 21119. Common numbers among each profile are presented. P-UP, protein upregulated ( $\geq 1.8$  fold); P-Down, protein downregulated ( $\leq -1.8$  fold); T-UP, transcript upregulated ( $\geq 2.0$  fold); T-Down, transcript downregulated ( $\leq -2.0$  fold). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

#### Specific molecular components and pathways involved in cold stress

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) online resource, functional roles of differentially

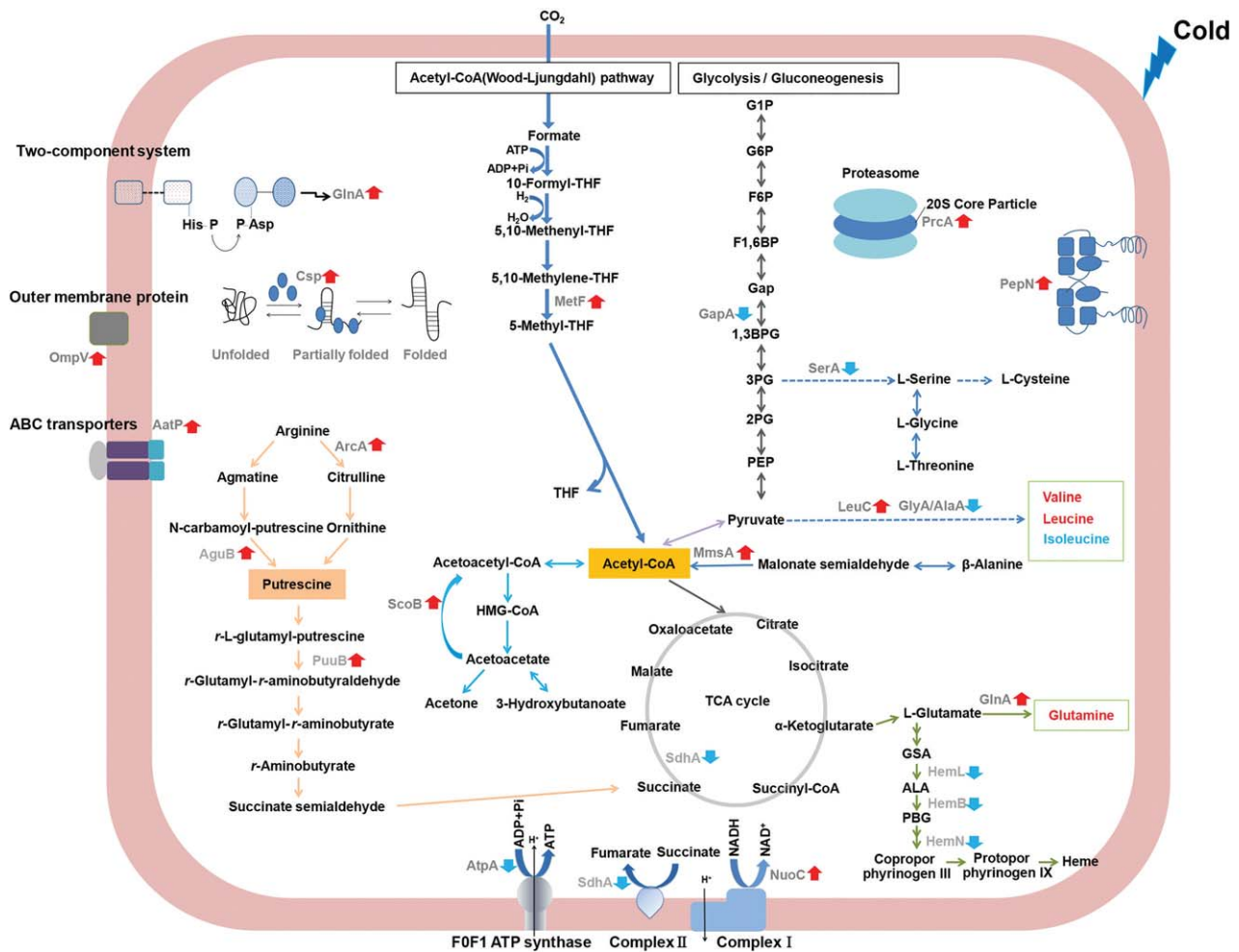
abundant proteins were interpreted with respect to exposure at  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$ . Fifty three protein names were mapped onto the KEGG pathways (Table 3). It was revealed that NuoC, involved in electron transport system complex I, showed a 2.3-fold increase. In addition, expression of the SdhA and AtpA proteins, which are found in electron transport system complex II and ATP synthase, respectively, increased 1.9-fold. Moreover, expression of heme proteins (HemL, 2.4-fold decrease; HemB, 2.2-fold decrease; and HemN, 1.9-fold decrease) was reduced. Heme proteins make up a pathway for electron transfer across membranes. Proteasome subunits (PrcA) increased 3.1-fold, which indicates that abnormal proteins caused by misfolding or denaturation increase at subzero temperatures, thereby inducing protein degradation machinery. Proteins involved in amino acid metabolism showed increased expression at  $-5^{\circ}\text{C}$ , including aminopeptidase N (PepN), isopropylmalate isomerase (LeuC), methylmalate-semialdehyde dehydrogenase (MmsA) and glutamine synthetase (GlnA). The ATP-binding cassette (ABC) transporter, which mediates recruitment of short molecules from outside of the cells, exhibited elevated expression (AatP, 1.9-fold). Outer membrane protein (OmpV), which can be regulated by small RNA, showed increased expression (Fig. 7).

Cold-shock proteins (CSPs) are known as RNA chaperones, and two isozymes were differentially expressed in this study. The amino acid compositions of the two forms were illustrated and compared (Fig. 8). The isozyme that increased at  $-5^{\circ}\text{C}$  contained fewer arginine

**Table 4.** A list of common differentially expressed genes between transcriptome and proteome.

Contig no.	SSP	Accession no.	T-fold change <sup>a</sup>	P-fold change <sup>a</sup>	Gene name	Protein name
I. Upregulated						
P21119_03600	4549	gil497886422	12.3	7.7	arcA	Arginine deiminase
P21119_01390	6242	gil497883292	2.8	3.1	prcA	Peptidase
P21119_24850	1843	gil648256178	2.1	2.6	pepN	Aminopeptidase N
P21119_23670	7246	gil648256251	2.5	2.3	metF	5,10-methylenetetrahydrofolate reductase
P21119_12360	5232	gil497887398	2.0	2.0	gst	Glutathione S-transferase
P21119_07640	4540	gil648256092	6.5	2.0	leuC	isopropylmalate isomerase
P21119_20460	7234	gil497887681	2.4	1.9	aatP	Arginine ABC transporter ATP-binding protein
P21119_12960	4025	gil497887505	2.9	1.9	csp	RNA chaperone/anti-terminator
II. Downregulated						
P21119_08130	7013	gil497885819	-2.4	-5.9	Hcp	Hypothetical protein
P21119_04930	3033	gil648256010	-3.8	-3.4	csp	Cold-shock protein
P21119_06460	6020	gil497884556	-3.1	-3.2		Hypothetical protein
P21119_28730	5133	gil497885207	-2.1	-2.8	DedA	Alkaline phosphatase
P21119_24670	9611	gil497887013	-3.8	-2.6	aofH	Flavin monoamine oxidase
P21119_08130	8033	gil497885819	-2.4	-2.5	Hcp	Hypothetical protein
P21119_19160	6253	gil497881780	-3.5	-2.5	nit	Carbon-nitrogen hydrolase
P21119_13300	7732	gil497887536	-3.6	-2.4	hemL	Aminotransferase class III
P21119_28410	4433	gil497885019	-5.7	-2.2	hemB	Delta-aminolevulinic acid dehydratase
P21119_18670	7347	gil648255899	-2.5	-2.2	ispE	4-diphosphocytidyl-2C-D-erythritol kinase
P21119_15690	7640	gil497883084	-2.5	-1.9	hemN	Coproporphyrinogen III oxidase

a. T, transcript; P, proteome.



**Fig. 7.** Depiction of low temperature-dependent molecular states in *Psychrobacter* sp. PAMC 21119. Periplasm and cytoplasm are shown in pink and white respectively.

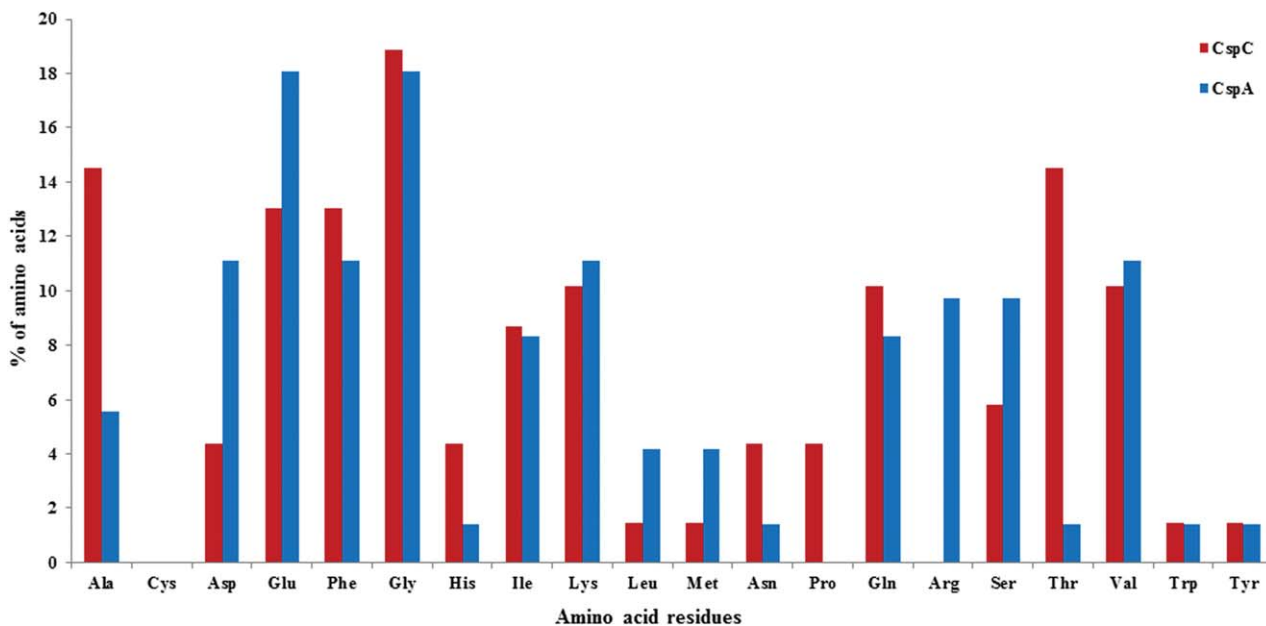
The temperature-responsive differentially expressed proteins involved in each metabolic pathway are shown. Thick arrows indicate the differentially expressed proteins. Upward and downward arrows represent upregulation and downregulation respectively. Solid thin arrows indicate metabolic flows. Dotted arrows indicate the precursor provider pathways. Arrows of the same colour are associated with the same pathway. The direction of reactions has not been rigorously determined and is generally as reported in Kyoto Encyclopedia of Genes and Genomes (KEGG) reactions. G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6BP, fructose 1,6-bisphosphate; Gap, glyceraldehyde 3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; THF, tetrahydrofolate; HMG-CoA,  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA; GSA, glutamate-1-semialdehyde; ALA, 5-amino levulinic acid; PBG, porphobilinogen; MetF, 5,10-methylenetetrahydrofolate reductase; PrcA, peptidase; PepN, aminopeptidase N; GapA, glyceraldehyde-3-phosphate dehydrogenase; SerA, 3-phosphoglycerate dehydrogenase; LeuC, isopropylmalate isomerase; GlyA, hypothetical protein; AlaA, aspartate aminotransferase; MmsA, methylmalonate-semialdehyde dehydrogenase; GlnA, glutamine synthetase; HemL, aminotransferase class-III; HemB, delta-aminoevulvic acid dehydratase; HemN, coproporphyrinogen III oxidase; SdhA, succinate dehydrogenase flavoprotein subunit; NuoC, NADH:ubiquinone oxidoreductase; AtpA, F0F1 ATP synthase subunit alpha; ScoB, succinyl-CoA 3-ketoacid-CoA transferase; AatP, arginine ABC transporter ATP-binding protein; Csp, RNA chaperone/anti-terminator; OmpV, membrane protein; ArcA, arginine deiminase; AguB, N-carbamoylputrescine amidase; PuuB, gamma-glutamylputrescine oxidoreductase. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

and more lysine residues. In addition, the contents of acidic residues such as aspartate and glutamate were relatively lower when compared to isozymes at 20°C, which implies that the flexibility of CSP is enhanced at subzero temperatures.

Acetyl-CoA is an important molecule in metabolism, used in many biochemical reactions. It is a fundamental resource in the biosynthesis of fatty acids, cholesterol and

ketone bodies. In this study, elevated expressions of MetF (2.3-fold) and ScoB (2.1-fold) were detected at -5°C; they are involved in acetyl-CoA synthesis and re-utilization of ketone bodies respectively (Fig. 7).

Putrescine and spermidine, the major two polyamines found in bacteria, are accumulated when cells undergo proliferation or stress conditions. It was found that the expressions of ArcA (7.7-fold) and AguB (4.0-fold)



**Fig. 8.** Amino acid composition of cold-shock protein (Csp) isoforms in *Psychrobacter* sp. PAMC 21119. The number of amino acid residues of each protein is shown as percentage in the entire Csp sequences. Red bars represent upregulated proteins and blue bars indicate downregulated proteins at  $-5^{\circ}\text{C}$ , compared with  $20^{\circ}\text{C}$ . [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

increased at  $-5^{\circ}\text{C}$ , and these proteins are associated with the putrescine synthesis pathway (Fig 7).

#### Effect of temperature on cellular metabolic activity

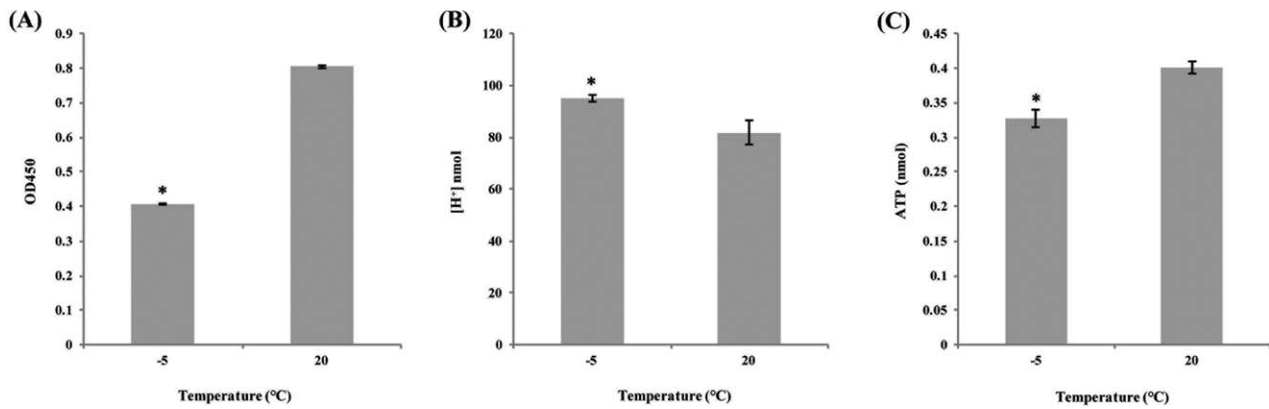
Several enzymes identified by proteome analysis were subjected to measurements of their cellular metabolic activity. Cells were harvested from the logarithmic phase of growth, and the same amount of cells was used for each assay. Expression of SdhA, a main component of the electron transport system complex II, was downregulated at  $-5^{\circ}\text{C}$  (Table 3) and succinate dehydrogenase activity decreased significantly at  $-5^{\circ}\text{C}$  compared to  $20^{\circ}\text{C}$  (Fig. 9A). On the other hand, protein expression of NuoC, which is involved in formation of proton ions in the electron transport system complex I, was upregulated at  $-5^{\circ}\text{C}$  (Table 3, Fig. 3). The amount of cellular proton ions was measured by a pH meter and was higher at  $-5^{\circ}\text{C}$  than at  $20^{\circ}\text{C}$  (Fig. 9B). In addition, the concentration of intracellular ATP was checked, because expression of AtpA associated with ATP synthesis was downregulated at  $-5^{\circ}\text{C}$ . We found that the amount of ATP was lower at  $-5^{\circ}\text{C}$  than at  $20^{\circ}\text{C}$  (Fig. 9C).

#### Discussion

To survive under cold temperature conditions, microorganisms express several cold-induced proteins. It has been reported that CSPs have been found in almost all types of bacteria, including thermophiles, mesophiles and psychrophiles (Mueller *et al.*, 2000; Ermolenko and Makhatazde,

2002; Phadtare *et al.*, 2003). Nine CSP homologues, *cspA-I*, were found in *E. coli* (Yamanaka *et al.*, 1998); three *csp* genes, *cspB*, *cspC* and *cspD*, were identified in *Bacillus subtilis* (Schindler *et al.*, 1999); and two genes, *cspA* and *cspG*, were reported from the deep-sea psychrophilic bacterium *Shewanella violacea* (Fujii *et al.*, 1999). Nine homologs of the *cspA* family in *E. coli* stabilize secondary structures of mRNAs as RNA chaperones; consequently, they can enhance translation efficiency at subnormal temperatures as transcription regulators (Bae *et al.*, 2000). Two isoforms of CSP were differentially expressed at  $-5^{\circ}\text{C}$  in this study (Table 3). In a previous study, the role of temperature-dependent isozyme exchange was described as the ability to catalyse similar reactions at different growth temperatures (Maki *et al.*, 2006). A similar isozyme exchange was observed in the current study by comparing the proteome datasets from  $-5^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  (Fig. 3). Considering the typical functions of CSP, it is postulated that the selective expression of CSP isozymes in *Psychrobacter* sp. PAMC 21119 would also be involved in ribosome assembly, translation initiation and proper folding of proteins and nucleic acids, etc., in cold environments. Further three-dimensional protein structure analysis will be necessary to define the detailed action mechanism of these proteins.

Acetyl-CoA production could be a critical factor in the adaptive response to cold. It was revealed that protein expressions of MetF, ScoB and MmsA were induced at subzero temperatures (Fig. 3). Acetyl-CoA is transformed



**Fig. 9.** Colorimetric assay for assessing cellular metabolic activity in *Psychrobacter* sp. 21119.

A. The activity of succinate dehydrogenase.

B. The H<sup>+</sup> amount of each temperature group.

C. Amount of cellular ATP in lysed whole cells. An asterisk indicates a significant difference, according to *t*-test ( $*P < 0.05$ ). Error bars represent standard deviation based on three biological replicates.

to ketone bodies, and ketone bodies such as acetoacetate, acetone and  $\beta$ -hydroxybutyrate ( $\beta$ -OHB) have signaling functions and can be resources for cellular energy. A number of bacterial species synthesize a polymer of  $\beta$ -OHB for energy storage (Yang and Seto, 2008). Moreover, acetyl-CoA is a precursor of fatty acid and necessary for membrane fluidity, which is related to cell resistance to freezing (Annous *et al.*, 1999). Interestingly, the tricarboxylic acid (TCA) cycle was not highly activated in the  $-5^{\circ}\text{C}$  condition and acetyl-CoA input into the TCA cycle seems to be shunted to glutamine synthesis (Fig. 7). The overall numbers of each glutamine, valine and leucine residue in the 29 upregulated proteins were greater than in the 31 downregulated proteins at  $-5^{\circ}\text{C}$ , whereas the total number of isoleucine residue was less (Table 5 and Supporting Information Table S5). This clearly indicates that the expression of these four amino acid is basically controlled by Acetyl-CoA to make a reservoir for specific amino acid utilization to the cold-induced protein expressions.

The electron transport chain (ETC) and ATP synthase clearly appear to be affected by cold stress. Expression of a complex I component (NuoC) was induced, but a component of complex II (SdhA) was downregulated (Fig. 7). The enzyme activity of succinate dehydrogenase declined to

**Table 5.** The total numbers of four amino acid residues in the proteins regulated under cold stress conditions in *Psychrobacter* sp. PAMC 21119.

	Upregulated	Downregulated
Total protein number	29	31
Glutamine	474	434
Valine	751	689
Leucine	948	908
Isoleucine	592	678

nearly 50% at  $-5^{\circ}\text{C}$  (Fig. 9A), a component of ATP synthase was downregulated, and the amount of cellular ATP decreased significantly (Fig. 9C). Moreover, the amount of cellular proton ion was relatively higher at  $-5^{\circ}\text{C}$  (Fig. 9B). It was reported that the first molecules responding to acidity are two components of the  $F_1F_0$  ATP synthase, downregulating its gene expression (Booth, 1985). Interestingly, a downregulated alpha subunit of ATP synthase (AtpA) in the present study is in accordance with previous results (Wen *et al.*, 2003), which suggests that a strategy for limiting proton uptake is downregulation of ATP synthase. ATP production is essential in cellular energy maintenance and reservation, but in cold stress, even for psychrophiles, ATP synthesis does not seem to be very active, which implies that ATP utilization is not necessary as great as in higher temperature environments. Previous studies reported increased ATP amounts and suggested that this resulted from decreased utilization of ATP in *P. cryohalolentis* at lower temperatures (Feniouk *et al.*, 2007; Amato and Christner, 2009). However, *P. cryohalolentis* was isolated from a cryopeg (Bakermans *et al.*, 2006), and in this different habitat it might have developed a different energy maintenance strategy due to the extreme environment. Considering that *Psychrobacter* sp. PAMC 21119 was isolated from permafrost soil, this study suggests more evidence of species-specific cold adaptive mechanisms.

In general, most bacterial cells synthesize two natural polyamines, putrescine and spermidine (Tabor and Tabor, 1985; Cohen, 1998). It is known that these small cationic amines contribute to enhanced macromolecular function by stabilizing the structure of DNA and RNA (Cohen, 1998) and this function appears to be related to protective roles against toxic effects of ROS (Shah and Swiatlo, 2008). Polyamines are involved in a broad range of cell

growth activities (Tabor and Tabor, 1985; Cohen, 1998) and their concentrations correlate with growth rates (Tweeddale *et al.*, 1998). Putrescine stimulates some transcription factors and affects more than 300 genes (Yoshida *et al.*, 2004; Terui *et al.*, 2009). Interestingly, in a previous study, putrescine increased under stress conditions (Tabor and Tabor, 1984). Putrescine is produced from either ornithine or arginine by ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) respectively. In the current study, two upregulated proteins (ArcA and AguB) at subzero temperatures were associated with these two pathways. Further studies could clarify the specific role of putrescine and its target genes triggered in cold environments.

Transport protein systems may play a significant role in the living strategy of psychrophilic bacteria. For example, short peptides in the culture medium can be imported into bacterial cells by ABC transporters and can be reutilized as building blocks in nutrient-limited environments. In the current study, the expression level of ABC transport protein was upregulated at  $-5^{\circ}\text{C}$  (Table 3). ABC transporters are a major family of membrane transport proteins, which transport a broad range of nutrients or metabolites into and out of the cells (Mauchline *et al.*, 2006; Giuliani *et al.*, 2011). It was shown that the expression of one ABC transporter (aatP) is controlled by the two-component system AauR/S (Singh and Röhm, 2008), but none of these proteins were expressed differentially in this study. The most frequent transcripts of *Psychrobacter* sp. PAMC 21119 were amino acid transporters and enzymes involved in amino acid metabolism (Table 3). The COG category E proteins in *Psychrobacter* sp. PAMC 21119 included nine enzymes involved in the transport, biosynthesis and degradation of amino acids (Table 3): aminopeptidase N (EC 1.4.1.1), isopropylmalate isomerase (EC 4.2.1.33), aspartate aminotransferase (EC 2.6.1.9), transglutaminase (EC 2.3.2.13), 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20), gamma-glutamylputrescine oxidoreductase (EC 1.4.3.-), arginine ABC transporter ATP-binding protein (EC 3.6.1.3), glutamine synthetase (EC 6.3.1.2) and flavin monoamine oxidase (EC 1.4.3.2). These findings suggest that the role of transporter systems in amino acid metabolism might be critical in psychrophilic bacteria. In nutrient-limited subzero temperature environments, proteolytic systems can be associated with protein recycling as carbon and nitrogen sources. Thus, these proteins can contribute to the enrichment of amino acid pools in the cells. Indeed, degradation of intracellular proteins increased in bacterial cells that were exposed to cold-shock (Jozefczuk *et al.*, 2010). Similarly, therefore, differentially expressed enzymes involved in amino acid synthesis may also be an explainable feature (Fig. 7).

Nine protein spots that showed differential expressions in 2-DE analysis were not assigned to any category (Table

3). These functionally unknown proteins may be involved in other unknown mechanisms of microbial cold adaptation. In other studies, many differentially expressed transcripts had unknown functions under subzero temperature growth conditions in the psychrophilic bacteria *Exiguobacterium sibiricum* and *Planococcus halocryophilus* Or1 (Rodrigues *et al.*, 2008; Mykytczuk *et al.*, 2013). Moreover, several hypothetical proteins were upregulated and downregulated (Table 3, groups S) and these types of proteins have also been differentially expressed under subzero conditions in the *P. arcticus* (Ayala-del-Río *et al.*, 2010). Although the exact functions of these unknown proteins have not yet been revealed, it is obvious that cold-driven expression of many proteins with unknown functions has played an important role in the physiological regulation of *Psychrobacter* sp. PAMC 21119 in Antarctic subzero environments for a long time.

Comparison analysis of proteome and transcriptome profiles showed that 34.8% of protein expression overlaps with 2.5% of transcript expression in upregulation profiles, and 36.7% of protein expression overlapped with 3.9% of transcript expression in downregulation profiles (Fig. 6). Moreover, 0.3% of upregulated genes were translated to 5% of downregulated proteins, and 2.2% of downregulated genes were expressed as 28.6% of upregulated proteins (Fig. 6). Considering that transcription occurs in an upstream step and translation to proteins is an end-point event in molecular response, this discrepancy is not surprising. Nevertheless, the differences and expression shifts between the transcriptome and proteome are still greater. Accumulating more comparison data of both proteome and transcriptome expression profiles in subzero conditions would enable a more comprehensive understanding of cold acclimation in psychrophiles.

In conclusion, this study examines the molecular response of *Psychrobacter* sp. PAMC 21119 to cold stress in subzero temperature environments. These psychrophilic bacteria were culturable and viable at  $-5^{\circ}\text{C}$ . The bacterial cells were exposed to  $-5^{\circ}\text{C}$  and the analysis was carried out in comparison with cells cultured at  $20^{\circ}\text{C}$ . The cell growth was not very active, which is similar to several previous studies. The cells experienced cold stress and regulated their response, which was expressed in transcripts and proteins. Isozyme exchange of CSP would suggest proper folding mechanisms of proteins and nucleic acids at subzero temperatures. Production of cellular energy (e.g., ATP) was suppressed, but intermediate accumulations (e.g., acetyl-CoA and amino acids) were distinct. It seems that cell proliferation is not efficient for cells that are undergoing cold stress. Instead, the cells minimize energy consumption and prepare for a better environment later, similar to pre-hibernation in higher animals. Even though the current study has suggested a possible regulatory mechanism of *Psychrobacter* sp.

PAMC 21119 in cold environments, the general response of psychrophiles is still vague, and it is unclear whether 'pre-hibernation' is the best choice for 'psychrophilic' bacteria. Nonetheless, the current study clearly shows a regulation event of bacterial response to subzero temperatures through analysis of transcriptome and proteome changes in *Psychrobacter* sp. PAMC 21119, isolated from Antarctic soil. Moreover, the remaining unknown proteins in this study might provide insight into more novel mechanisms of cold-adaptation in the near future.

## Experimental procedures

### Bacterial strain and culture conditions

*Psychrobacter* sp. PAMC 21119 was isolated from permafrost soil on Barton Peninsula, King George Island, Antarctica (62°13' S, 58°47' W) (Kim *et al.*, 2012). A single colony was propagated in marine broth (MB) medium at 20°C for 24 h and 1:100 dilutions were prepared using fresh MB medium. The cells were cultured at five different temperatures (−5°C, 0°C, 5°C, 20°C and 37°C) with shaking at 150 r.p.m. Cell growth was monitored spectrophotometrically by measuring the OD<sub>600</sub>. At the mid-exponential phase (OD<sub>600</sub> = 0.8), the bacterial cells were collected by centrifugation at 10,000 × *g* for 15 min at 4°C. After removing the supernatants, the pellets were frozen in liquid nitrogen and stored at −80°C. Triplicate cultures were prepared at both −5°C and 20°C.

### RNA and protein extraction

Total RNA was isolated using the Easy Blue Kit (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions, followed by resuspending dried total RNA pellets in 0.1 mL nuclease-free water. To precipitate RNA, 0.1 volume of 3 M sodium acetate (pH 5.0) and 2.5 volumes of 100% ethanol were added sequentially to the eluted RNA, mixed by vortexing and incubated for 1 h at −80°C. After incubation, the tubes were centrifuged at 12,000 × *g* for 20 min at 4°C. Pellets were rinsed twice with 80% ethanol and resuspended in 30 µL of nuclease-free water. RNA quantification was done spectrophotometrically using an ND-1000 UV/visible spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and electrophoresed on a 1.5% w/v agarose gel to verify RNA integrity. The purified RNA was stored at −80°C. Approximately 10 µg of total RNA was subjected to poly-A selection and a cDNA library was constructed using the TruSeq RNA sample prep kit (Illumina, CA, USA). RNA-seq was performed on an Illumina HiSeq 2500 (Illumina, CA, USA) at ChunLab (Seoul, Korea) according to the manufacturer's protocol.

For protein profiling, cell pellets maintained at −80°C were homogenized using a bullet blender homogenizer (Qiagen, Hildenberg, Germany) and resuspended in 2-DE lysis solution composed of 7 M urea, 2 M thiourea, 1% (w/v) dithiothreitol (DTT), 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 2% (v/v) ampholyte and 1 mM benzamide-HCl. The supernatant containing the soluble protein fraction was collected after centrifugation at 14,000 × *g* for 30 min at 14°C to remove cell debris containing insoluble proteins. The protein concentration of each sample was

determined using the Bradford method (Sigma-Aldrich, St. Louis, MO, USA) (Bradford, 1976) with bovine serum albumin as a standard (Thermo, Waltham, MA, USA). An aliquot of each supernatant containing ~100 µg of soluble protein was then stored at −80°C until further analysis.

### Two-dimensional gel electrophoresis (2-DE) and image analysis

Whole cell lysates were subjected to 2-DE as previously described (Mykytczuk *et al.*, 2011). Briefly, 200 µg of protein was applied to the first dimensional separation by isoelectric focusing using Immobiline DryStrip gels (pH 4–7 linear gradient, 24 cm; GE Healthcare, Milwaukee, WI, USA) by the cup-loading method using a Multiphor II system (GE Healthcare), and then subjected to second dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10–15% gradient, 26 × 20 cm). Protein spots were visualized using the VisPRO™ Protein Stain Kit (Visual Protein Biotechnology, Taipei, Taiwan) according to the manufacturer's protocol. The gel images were obtained using a GS-900 densitometer (Bio-Rad, Hercules, CA, USA) and analysed manually with PDQuest software (ver. 8.1; Bio-Rad). The quantity of protein in each spot was normalized to the total valid spot intensity. Significantly changed spots were selected based on a rate increased/decreased ≥ 1.8-fold or complete appearance or disappearance.

### In-gel trypsin digestion and mass spectrometry

Spots were collected from the replicate gels. The gel plugs were destained and fixed in 10% acetic acid, washed three times with distilled water, and incubated with 100 mM ammonium bicarbonate. After dehydration with 100% acetonitrile (ACN), the gel pieces were vacuum dried. In-gel digestion was performed using 25 ng µL<sup>−1</sup> sequencing grade trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate (pH 7.8) and incubated at 37°C for 12–15 h. All samples were desalted and concentrated with a ZipTip<sub>µ-C18</sub> (Millipore, Bedford, MA, USA) (Erdjument-Bromage *et al.*, 1998), according to the instructions provided by the manufacturer. One microliter of the peptide eluate was mixed (1:1) with a matrix solution of α-cyano-4-hydroxycinnamic acid (10 mg mL<sup>−1</sup>) in 50% ACN, 0.1% trifluoroacetic acid (TFA) and 1 µL was then spotted onto an Opti-TOF MALDI stainless steel target plate (AB SCIEX; Framingham, MA, USA).

The MALDI plates were analysed on a 4800 MALDI TOF/TOF instrument (ABSCIEX) with the 4000 series explorer software (ver. 3.7; ABSCIEX). Data were acquired in positive reflector mode over a mass range of 850 to 4,000 m z<sup>−1</sup> using external calibration spots with TOF/TOF calibration mixture (ABSCIEX). Mass spectra were obtained from each spot using a fixed laser intensity, 1,050 shots per spectrum, with a uniformly random spot search pattern. A 1 kV mass spectrometry/mass spectrometry (MS/MS) operating mode was used, the relative precursor mass window was set at 200 (FWHM), and collision induced dissociation (CID) using air was turned on, with metastable suppression enabled. Peaks with a signal/noise (S/N) ratio greater than 70 were selected for MS/MS. Up to 12 MS/MS spectra could be obtained from each spot,



starting with the most intense peak and ending with the least intense peak. MS/MS acquisition of selected precursors was set to total of 1,800 laser shots. ProteinPilot software (ver. 4.0; ABSCIEX) was used to process MS and MS/MS spectra to submit the peak list to the MASCOT search engine (ver. 1.9; [http://www.matrixscience.com:Matrix Science](http://www.matrixscience.com:Matrix%20Science)) for peptide identification against the NCBI databases (*Psychrobacter* sp. PAMC 21119; entry number 2592). The following parameters were selected in the analysis method: iodoacetamide (IAA) as the alkylating agent of cysteine, oxidation of methionine and trypsin as a digesting enzyme. Parent and daughter ion tolerance were set to 50 p.p.m. and 0.1 Da, respectively, and two missed cleavages were allowed. All proteins were identified by at least two unique peptides from the MS/MS search (Supporting Information Table S6).

#### Bioinformatics, functional annotation and classification

Quantitative transcriptome and proteome data were filtered and sorted based on expression ratios  $\geq 2.0$ -fold and  $\geq 1.8$ -fold respectively (Supporting Information Tables S1 and S2). The GO annotations were obtained by BlastP in Blast2GO (Conesa *et al.*, 2005). GO enrichment analysis was carried out using Blast2GO, a GO analysis toolkit and Fisher's exact test with a threshold of 0.05. Prokaryotic COG annotations were obtained by BlastP in the WebMGA server (<http://weihong-lab.ucsd.edu/metagenomics-analysis/>) (Wu *et al.*, 2011). We used *P*-values after Benjamini correction and considered only those where the corrected *P*-value was  $< 10^{-7}$ . Biochemical pathways were determined using the COG and KEGG databases.

#### Succinate dehydrogenase activity assay

The succinate dehydrogenase activity was measured using the CCK-8 kit (Dojindo, Tabaru, Japan). The bacterial cells from two different temperatures ( $-5$  and  $20^{\circ}\text{C}$ ) were adjusted to  $6.4 \times 10^8$  cells per mL, and 90  $\mu\text{L}$  containing  $5.76 \times 10^7$  cells was transferred to a 96-well flat-bottomed microplate. Then, 10  $\mu\text{L}$  CCK-8 solution was added and incubated for 1 h at room temperature in the dark. The absorbance was measured at 450 nm in a Multiskan GO microplate spectrophotometer (Thermo). All measurements were performed in triplicate.

#### Measurement of ATP amounts

ATP amounts in cells were measured using an ATP colorimetric assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly,  $6.4 \times 10^7$  cells were harvested by centrifugation, followed by disruption with a homogenizer in 50  $\mu\text{L}$  ATP assay buffer. The lysate was centrifuged at 13,000 r.p.m. for 5 min at room temperature and 50  $\mu\text{L}$  supernatant was transferred to a 96-well flat-bottomed microplate. Then, 50  $\mu\text{L}$  reaction mixture (44  $\mu\text{L}$  ATP assay buffer, 2  $\mu\text{L}$  probe, 2  $\mu\text{L}$  ATP converter and 2  $\mu\text{L}$  developer) was added and incubated for 30 min at room temperature in the dark. Absorbance was measured at 570 nm in Multiskan GO microplate spectrophotometer (Thermo). The amount of

ATP was calculated using an ATP standard curve and is expressed in nmol.

#### Determination of cellular proton amounts

The proton amounts in cells were determined by measuring the intracellular pH. The culture medium was centrifuged at 13,000 r.p.m. for 5 min at  $4^{\circ}\text{C}$  and the pellets were resuspended in 3 mL MB. The cell suspension was sonicated in the presence of a constant current for 6 min, and a sonicator (SONICS, Danbury, CT, USA) was programmed to run 2-s sonication pulses followed by a pulse-off period of 6 s. After sonication, the cleared lysate was centrifuged at 13,000 r.p.m. for 15 min at  $4^{\circ}\text{C}$ . The supernatant was then collected and used for pH measurement with a pH meter (JENWAY, Felsted Dunmow, UK). All measurements were performed in triplicate. The proton amount was calculated by the following formula below and is expressed in nmol.

$$[\text{H}^+] = 10^{-\text{pH}}$$

#### Growth rate calculations

The rate constants ( $\mu$ ) were obtained using the equation described previously (Neidhardt *et al.*, 1990).

#### Statistics

All data are expressed as means  $\pm$  SD from three replicates. Student's *t*-test was employed to investigate statistical differences using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA);  $P < 0.05$  was considered to be statistically significant.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Top BLASTx hit species distribution obtained from the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database.

**Fig. S2.** Gene ontology (GO) enrichment analysis of the differentially expressed transcriptome and proteome at different temperatures. (A) upregulated transcriptome, (B) downregulated transcriptome, (C) upregulated proteome and (D) downregulated proteome. BP, biological process; MF, molecular function; CC, cellular component.

**Table S1.** Detailed data for the protein identification summary in Table 3.

**Table S2.** Differentially expressed gene profiles.

**Table S3.** A list of differentially expressed genes between the transcriptome and proteome.

**Table S4.** Enrichment of GO terms of differentially expressed genes in *Psychrobacter* sp. PAMC 21119 under two different temperature conditions.

**Table S5.** Detailed data for the summary of four amino acid residues in Table 5.

**Table S6.** Summary of MS/MS analysis.