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Proteomics of life at low temperatures: trigger factor is the primary chaperone in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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Summary

The proteomes expressed at 4°C and 18°C by the psychrophilic Antarctic bacterium *Pseudoalteromonas haloplanktis* have been compared using two-dimensional differential in-gel electrophoresis, showing that translation, protein folding, membrane integrity and anti-oxidant activities are upregulated at 4°C. This proteomic analysis revealed that the trigger factor is the main upregulated protein at low temperature. The trigger factor is the first molecular chaperone interacting with virtually all newly synthesized polypeptides on the ribosome and also possesses a peptidyl-prolyl *cis-trans* isomerase activity. This suggests that protein folding at low temperatures is a rate-limiting step for bacterial growth in cold environments. It is proposed that the psychrophilic trigger factor rescues the chaperone function as both DnaK and GroEL (the major bacterial chaperones but also heat-shock proteins) are downregulated at 4°C. The recombinant psychrophilic trigger factor is a monomer that displays unusually low conformational stability with a *Tm* value of 33°C, suggesting that the essential chaperone function requires considerable flexibility and dynamics to compensate for the reduction of molecular motions at freezing temperatures. Its chaperone activity is strongly temperature-dependent and requires near-zero temperature to stably bind a model-unfolded polypeptide.

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Introduction

Cold biotopes on Earth are more abundant than generally appreciated. Indeed, ~70% of our planet surface is covered by oceans that, irrespective of the latitude, have a constant temperature of 4°C below a depth of 1000 m. Permafrost represents more than 20% of terrestrial soils and has revealed a high biodiversity in cryopegs, e.g. salty water pockets that have remained liquid for about 100 000 years at –10°C (Gilichinsky *et al.*, 2005; Steven *et al.*, 2006). Polar regions account for another 15% of the Earth's surface, possessing unusual microbiotopes such as porous rocks in Antarctic dry valleys hosting microbial communities surviving at –60°C (Friedmann, 1982; de los Rios *et al.*, 2007) or the liquid brine veins between sea ice crystals harbouring metabolically active microorganisms at –20°C (Deming, 2002). Cryoconite holes on glacier surfaces represent another permanently cold biotope hosting complex microbial communities (Stibal *et al.*, 2006). These examples illustrate the unsuspected ability of microorganisms to adapt to low temperatures but also imply that psychrophiles (cold-loving microorganisms) are the most abundant extremophiles in terms of biomass, diversity and distribution (D'Amico *et al.*, 2006).

Previous studies of psychrophiles at the molecular level were mainly focused on cold-active enzymes and on maintenance of membrane fluidity. It was shown that the high specific activity at low temperatures of cold-adapted enzymes is a key adaptation to compensate for the exponential decrease of chemical reaction rates as temperature is reduced. Such high biocatalytic activity arises from the disappearance of various non-covalent stabilizing interactions, resulting in an improved flexibility of the enzyme conformation (Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006). Whereas membrane structures

are rigidified in cold conditions, an adequate fluidity is required to preserve the integrity of their physiological functions. This homeoviscosity is achieved by steric hindrances introduced in the lipid bilayer via incorporation of *cis*-unsaturated and branched-chain lipids, for instance (Russell, 2007). More recently, the genome sequences of five Proteobacteria (Rabus *et al.*, 2004; Methe *et al.*, 2005; Duchaud *et al.*, 2007; Riley *et al.*, 2008), including the strain used in the present study (Medigue *et al.*, 2005), and draft genome sequences of two Archaea (Saunders *et al.*, 2003) have been analysed in the context of cold adaptation (Danchin, 2007; Bowman, 2008). Several genome features, frequently revealed as multiple gene copies, have been related to these adaptations. The solubility of gases, especially oxygen, is increased at low temperatures while radicals are stabilized. As a consequence, psychrophiles are exposed to higher concentrations of reactive oxygen species (ROS) but various mechanisms to cope with these toxic species have been proposed. This includes a slightly lower frequency of oxidizable amino acids in proteins, the occurrence of specific reductases to repair oxidized residues, the presence of dioxygenases incorporating dioxygen into oxidized macromolecules and even the deletion of ROS-producing metabolic pathways. Interestingly, acyl desaturases (that introduce a double bond into fatty acyl chains with dioxygen as substrate) couple the elimination of toxic oxygen to the improvement of membrane fluidity. Secondary structures in nucleic acids are strengthened by low temperatures leading to reduced efficiency in transcription and translation processes. The importance of RNA-folding processes adapted to cold is underlined by the large number of RNA helicases in some genomes and by genes for cold-shock (nucleic acid-binding) proteins that might participate in the adaptive response. Biases in the amino acid composition of the proteomes have been noted, with a major trend for a high content in polar residues and a low content in charged residues. This is in line with a similar trend reported by comparison of hyperthermophilic proteomes with thermophilic and mesophilic proteomes (Suhre and Claverie, 2003). The ability to survive in seasonally frozen environments is also indicated by pathways for cryoprotectant synthesis and compatible solutes accumulation, by the capacity to secrete copious amounts of exopolysaccharides and by synthesis of polymers acting as carbon and nitrogen energy reserves during starvation. However, the lack of common features shared by all these psychrophilic genomes has suggested that cold adaptation superimposes on pre-existing cellular organization and, accordingly, the adaptive strategies may differ between the various microorganisms (Bowman, 2008).

Earlier proteomic studies of cold-loving bacteria have revealed a distinct cold-shock response when compared with mesophilic microorganisms (Hebraud and Potier, 1999). In the former, synthesis of housekeeping genes is not repressed upon temperature downshift while continued growth at low temperature is characterized by induction of the so-called cold-acclimation proteins (CAPs). A central role in cold adaptation has been attributed to this set of proteins because the sustained overexpression of CAPs distinguishes cold-loving bacteria from mesophilic microorganisms. In this context, we have performed a proteomic study of these upregulated proteins in the psychrophilic Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 using two-dimensional differential in-gel electrophoresis (2D-DIGE) and we have identified the trigger factor as the main CAP upregulated at low temperature in this Antarctic bacterium.

Results and discussion

Bacterial growth at low temperatures

The strain *P. haloplanktis* TAC125 has been isolated from sea water sampled along the Antarctic ice-shell. The ability of this psychrophilic bacterium to grow at low temperatures is illustrated in Fig. 1. While the mesophile *Escherichia coli* fails to grow exponentially below ~10°C (Strocchi *et al.*, 2006), the Antarctic strain displays a doubling time of ~4 h at 4°C when cultivated in a marine broth. This fast growth rate is primarily achieved by a low temperature dependence of the generation times when compared with the mesophilic bacterium, i.e. the generation time of *P. haloplanktis* is moderately increased when the culture temperature is decreased.

It should be stressed that enzymes from cold-adapted organisms are characterized by both a high specific

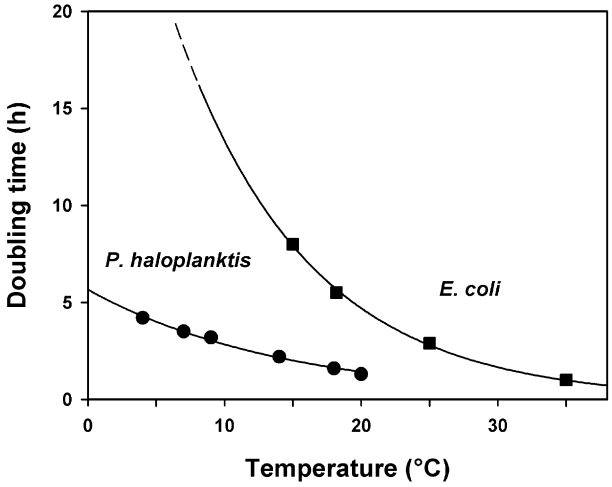

Fig. 1. Temperature–dependence of growth for the Antarctic psychrophile *P. haloplanktis* and for the mesophile *E. coli*. *P. haloplanktis* was grown in a marine broth and *E. coli* was grown in LB broth. Solid lines are best-fit of the data to a single-exponential equation. Below ~10°C, *E. coli* fails to grow exponentially (dashed).

activity at low temperatures and a low temperature dependence of their activity (formally, a weak activation enthalpy), i.e. reaction rates of psychrophilic enzymes are less reduced by a decrease in temperature as compared with mesophilic enzymes (D'Amico *et al*., 2003; Feller and Gerday, 2003). Accordingly, the growth characteristics of the Antarctic bacterium (Fig. 1) appear to be governed by the properties of its enzymatic machinery: high enzyme-catalysed reaction rates maintain metabolic fluxes and cellular functions at low temperatures, whereas the weak temperature dependence of enzyme activity counteracts the effect of cold temperatures on biochemical reaction rates.

Proteomic analysis of cold-acclimation proteins

The proteomes expressed by the Antarctic bacterium at 4°C and 18°C have been compared by 2D-DIGE. Figure 2 illustrates an example of 2D-DIGE pattern after differential CyDye labelling and co-migration of cell extracts from cultures at 4°C and 18°C. As protein extracts have been prepared from cells growing exponentially at these temperatures, all upregulated proteins at 4°C are regarded as CAPs. Five similar experiments, each involving triplicate gels, were performed in the present study. On the typical gel shown in Fig. 2, 142 protein spots were more intense for cell extracts obtained at 4°C when compared with extracts from 18°C. Therefore, the sensitivity of the method reveals a more complex pattern of CAPs than previously reported (Hebraud and Potier, 1999). Among these 142 CAPs, only 40 proteins were retained, satisfying both statistical biological variation analysis and mass spectrometry identification scores (Table 1). Accordingly, the function of the identified proteins should be analysed as markers of a pathway, rather than for their specific function as they represent 28% of the detected upregulated proteins at 4°C.

Thirty per cent of the identified proteins are directly related to protein synthesis (Table 1) and cover all essential steps, from transcription (including RNA polymerase RpoB) to translation and folding (Tig, PpiD). Genes *pnp* and *rpsA* also encode components of the degradosome that regulates transcript lifetimes. The Rho termination factor is a RNA/DNA helicase that can contribute to relieve nucleic acid secondary structures strengthened in cold conditions. Interestingly, mutations in the ribosomal protein L6 (RplF) have been reported to cause loss of *E. coli* cells viability at 0°C (Bosl and Bock, 1981). Methionyl-tRNA synthetase MetG displays one of the highest upregulation ratio: this can be tentatively related to the requirement of an increased pool of initiation tRNA to promote protein synthesis. In the last step of protein synthesis, the folding catalyst Tig acts on proteins synthesized by the ribosome while PpiD is involved in the folding of outer membrane proteins. Peptidyl-prolyl *cis-trans* isomerization appears therefore as a limiting factor for a wide range of proteins. Two putative proteases (PSHAa2492, PSHAa2260) were also identified as CAPs and can potentially participate to proteolysis of misfolded proteins. These observations strongly suggest that low

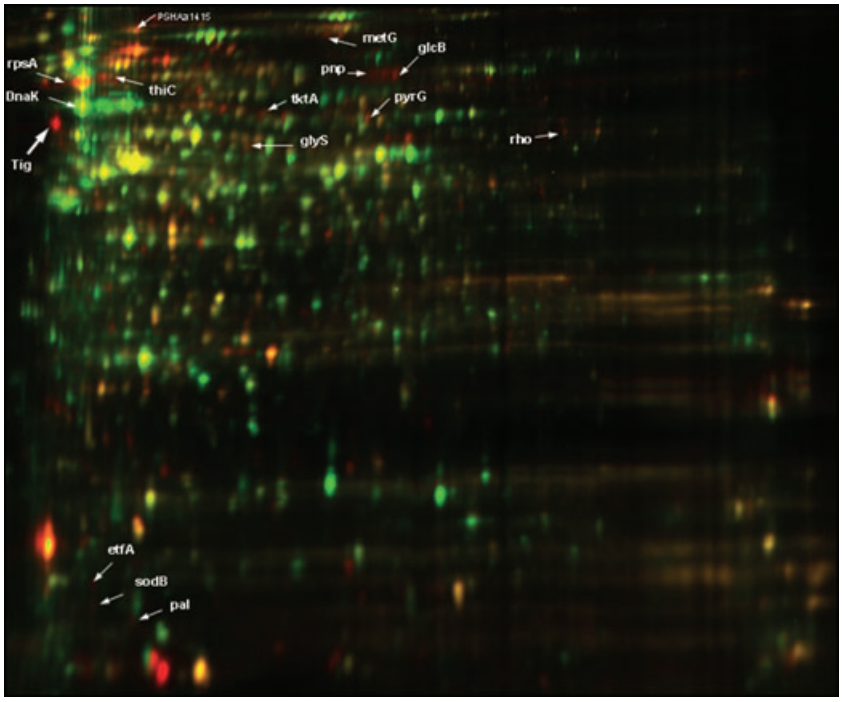

Fig. 2. Comparison of intracellular soluble proteins from *Pseudoalteromonas haloplanktis* TAC125 grown at 4°C (red) and 18°C (green) on 2D-DIGE gels analysed by fluorescence. From left to right, non-linear gradient from pH 3 to pH 10. From top to bottom, mass scale from ~150 to ~15 kDa. The large white arrow indicates the trigger factor spot. The intense red fluorescence correlates with its upregulation at 4°C. Some proteins identified in this experiment are also labelled.

Table 1. Proteins identified by MALDI-TOF or LC MS-MS (*) in upregulated spots selected at 4°C from 2D-DIGE of *Pseudoalteromonas haloplanktis* TAC125.

Protein	Accession number	Gene number	Gene	Upregulation ratio	*t*-test	Nominal Mr	Calculated pI	Mowse score	% sequence coverage	Peptides matched
Protein synthesis and folding										
Transcription, translation										
DNA-directed RNA polymerase subunit beta	gil77359193	PSHAa0222	*rpoB*	1.71	0.0050	150049	5.17	301	28	43
Putative transcriptional regulator, LysR familly	gil77361231	PSHAa2308		1.97	0.0001	31502	6.60	144	14	3
Transcription termination factor Rho	gil77359089	PSHAa0112	*rho*	2.47	0.0500	47060	7.19	157	44	16
Polynucleotide phosphorylase (also CSP)	gil77359947	PSHAa1001	*pnp*	4.08	0.0040	75552	5.00	247	12	5
30S ribosomal subunit protein S1; site specific RNA helicase	gil77360368	PSHAa1425	*rpsA*	2.16	0.0022	60988	4.79	226	17	6
50S ribosomal protein L6	gil77361719	PSHAa2815	*rplF*	1.62	0.0009	19190	9.72	61	20	2
Putative ribosomal modification enzyme (present in *P. putida*)	gil77360320	PSHAa1377		2.15	0.0140	52650	6.24	232	23	6
Glycyl-tRNA synthetase subunit beta	gil77358988	PSHAa0006	*glyS*	3.50	0.0033	75842	5.14	50	5	2
Methionyl-tRNA synthetase	gil77360260	PSHAa1317	*metG*	7.59	0.0057	75764	5.24	72	4	2
Translation-associated GTPase	gil77359994	PSHAa1051	*engD*	2.49	0.0006	39426	4.69	96	36	14
Folding										
Trigger factor* (also CSP)	gil77360987	PSHAa2063	*tig*	37.62	0.0120	47534	4.58	413	18	8
Peptidyl-prolyl cis-trans isomerase D	gil77360982	PSHAa2058	*ppiD*	1.59	0.0200	69864	4.49	123	27	21
Oxydative stress										
Glutathione synthetase	gil77361514	PSHAa2599	*gshB*	13.18	0.0090	35230	4.98	111	38	12
Superoxide dismutase [Fe]	gil73621931	PSHAa1215	*sodB*	1.61	0.0011	21237	4.97	256	26	5
Membrane proteins and receptors										
Outer membrane lipoprotein	gil77360808	PSHAa1870	*pal*	3.01	0.0028	19845	5.17	138	23	3
GTP-binding protein, LPS core synthesis* (also CSP)	gil77359139	PSHAa0167	*typA*	5.46	0.0110	66893	5.07	144	4	2
Putative TonB-dependent receptor	gil77361103	PSHAa2180		2.67	0.0460	90529	4.60	74	4	2
Putative TonB-dependent receptor protein	gil77361393	PSHAa2478		2.28	0.0480	79084	4.72	61	4	2
Putative outer membrane protein	gil77360358	PSHAa1415		2.30	0.0370	86922	4.86	492	19	8
Putative outer membrane associated transporter component	gil77360913	PSHAa1989		3.54	0.0160	94338	4.49	69	3	2
General Metabolism										
Degradation, assimilation										
Phosphoglucomutase	gil77360575	PSHAa1634	*pgm*	1.65	0.0049	59385	5.04	153	11	4
Glycine dehydrogenase	gil77361388	PSHAa2473	*gcvP*	1.63	0.0052	104625	5.65	91	5	3
Propionyl-CoA:succinate-CoA transferase	gil77360926	PSHAa2002	*ygfH*	6.17	0.0022	54901	5.65	124	28	20
Putative aminopeptidase	gil77361407	PSHAa2492		2.86	0.0420	95140	5.42	129	9	5
Generation of precursor metabolites and energy										
Citrate synthase	gil77360594	PSHAa1653	*gltA*	1.80	0.0089	47748	5.54	56	10	3
Bifunctional aconitate hydratase 2/ 2-methylisocitrate dehydratase	gil77359155	PSHAa0184	*acnB*	3.84	0.0270	95442	5.10	127	8	4
Fumarate hydratase, class I	gil77360109	PSHAa1166	*fumB*	2.72	0.0033	55223	5.49	250	45	27
Malate synthase G	gil77361983	PSHAb0061	*glcB*	4.42	0.0120	80322	5.73	382	12	6
Electron transfer flavoprotein alpha-subunit	gil77360558	PSHAa1617	*etfA*	1.62	0.0009	32074	4.75	77	5	1
Biosynthesis										
DAHP synthetase (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase)	gil77362467	PSHAb0559	*aroH*	4.64	0.0049	50831	5.53	161	41	16
Acetylornithine transaminase	gil77362337	PSHAb0428	*dapC*	1.77	0.0020	43302	5.61	128	44	16
AcetylCoA carboxylase, carboxytransferase subunit alpha	gil77360938	PSHAa2014	*accA*	2.82	0.0053	35675	5.58	124	36	17
Thiamine biosynthesis protein thiC	gil77359444	PSHAa0480	*thiC*	3.50	0.0033	73268	5.82	81	9	3
Transketolase 1	gil77359623	PSHAa0671	*tktA*	1.81	0.0210	72250	5.12	128	6	3
Spermidine synthase	gil77361750	PSHAa2847	*speE*	2.49	0.0095	32114	4.95	80	40	12
CTP synthetase	gil77359692	PSHAa0741	*pyrG*	1.70	0.0250	60288	5.68	88	27	13
Unknown function										
Conserved protein of unknown function with carboxypeptidase C domain	gil77361183	PSHAa2260		1.89	0.0004	57707	7.72	106	9	4
Putative secreted hydrolase	gil77362422	PSHAb0513		1.67	0.0002	45690	8.48	286	18	5
Hypothetical protein ; coiled-coil protein	gil77360302	PSHAa1359		2.81	0.0050	55330	6.10	180	11	4
Hypothetical protein	gil77360130	PSHAa1187		4.58	0.0089	28866	5.63	84	46	10

temperatures impair protein synthesis and folding, resulting in upregulation of the associated cellular processes.

Cold-shock proteins (CSPs) have been well documented in mesophiles. Interestingly, three proteins classified as CSPs in mesophiles have been identified as CAPs in *P. haloplanktis*. These proteins (Pnp, TypA and Tig) display high upregulation ratios and are involved in distinct functions (degradosome, membrane integrity and protein folding respectively). Sustained synthesis of various CSP-homologues has been also reported in other cold-adapted bacteria (Bakermans *et al.*, 2007; Kawamoto *et al.*, 2007; Bergholz *et al.*, 2009). There are therefore striking similarities between the cold-shock response in mesophiles and cold adaptation in psychrophiles. From an evolutionary point of view, it can be proposed that one of the adaptive mechanisms to growth in the cold was to regulate the cold-shock response, shifting from a transient expression of CSPs to a continuous synthesis of at least some of them.

Glutathione synthetase is the second main upregulated protein at 4°C and superoxide dismutase was also detected as a CAP. This is a clear indication of a cellular response to an oxidative stress arising from increased oxygen solubility and ROS stability. Considering the upregulation ratio of glutathione synthetase, the primary adaptive strategy in *P. haloplanktis* appears to be an improvement of the redox buffering capacity of the cytoplasm.

Six CAPs are either components or regulator of the outer membrane architecture. The occurrence of TonB-dependent receptors is indicative of sensing and exchanges with the external medium while TypA (involved in LPS core synthesis) and Pal (a peptidoglycan-associated protein) are required for outer membrane stability and integrity (Abergel *et al.*, 2001).

The main body of the identified CAPs consists of enzymes belonging to the general metabolism of the bacterium. Although these CAPs are involved in various metabolic pathways, the emerging picture is that of an oxidative energy production. This is suggested by catabolic activities (Pgm, GcvP), various components of the Krebs cycle (citrate synthase, aconitase, fumarase) and of related pathways (malate synthase) and by an electron transfer protein (EtfA). It is worth mentioning that several enzymes in this group complement the above-mentioned functions stimulated by cold growth conditions. For instance, AroH and DapC are involved in aromatic and basic amino acid biosynthesis respectively. This is in line with improved protein synthesis. Acetyl-CoA carboxylase provides the malonyl-CoA substrate for biosynthesis of fatty acids and can contribute to the maintenance of membrane integrity. Activities of ThiC and TktA are coupled in the pentose phosphate pathway that generates reducing equivalent in the form of NADPH. The latter is used to reduce glutathione therefore complementing the oxidative stress response. Polyamines can be implicated in cold adaptation as evidenced by detection of spermidine synthase. Polyamines are effectively involved in various functions such as nucleic acid protection, regulation of RNA and protein synthesis or membrane stability. Finally, four proteins of unknown function have been identified as CAPs, suggesting that additional adaptive mechanisms remain to be elucidated.

The peptidome of cell extracts from 4°C and 18°C cultures was also investigated by SELDI-TOF-MS allowing peptide profiling for masses from 1 to 18 kDa. However, no significant differences in peptide peak intensities were noted as SELDI profiles of biological replicas were dominated by variable degradation peptides.

The trigger factor is the major cold acclimation protein and the primary chaperone

In the case of the Antarctic bacterium, the major CAP overexpressed at 4°C by a factor 37x has been unambiguously identified as the trigger factor (TF) Tig (Table 1 and Fig. 3). The latter is the first molecular chaperone interacting with virtually all newly synthesized polypeptides on the ribosome. It delays premature chain compaction and maintains the elongating polypeptide in a non-aggregated state until sufficient structural information for productive folding is available and subsequently promotes protein folding (Merz *et al.*, 2008; Hartl and Hayer-Hartl, 2009; Martinez-Hackert and Hendrickson, 2009). Furthermore, TF also contains a domain catalysing the *cis-trans* isomerization of

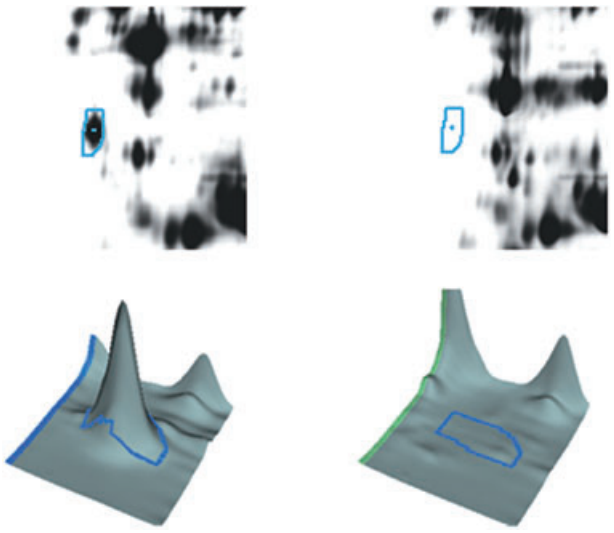

Fig. 3. Comparative analysis of spot containing the trigger factor from *Pseudoalteromonas haloplanktis* TAC125 grown at 4°C (left panels) and 18°C (right panels). Spot view on 2D-gel seen in fluorescence (upper panels) and three-dimensional images (lower panels) obtained with DeCyder software.

peptide bonds involving a proline residue (Kramer *et al*., 2004). This *cis-trans* isomerization is a well-known rate-limiting step in protein folding (Baldwin, 2008). Considering the high level of upregulation and the essential functions of TF, it can be concluded that protein folding at low temperatures is a rate-limiting step for bacterial growth in cold environments. It should be mentioned that another peptidyl-prolyl *cis-trans* isomerase (PPiase) is also upregulated at 4°C in *P. haloplanktis* (Table 1). Furthermore, some previous studies on cold-adapted microorganisms have reported either PPiases (Goodchild *et al*., 2004; Suzuki *et al*., 2004) or the trigger factor (Qiu *et al*., 2006; Kawamoto *et al*., 2007) as potential CAPs. It seems therefore that the constraint imposed by protein folding in the cold and the cellular response are common traits in psychrophilic microorganisms. It is also worth mentioning that TF is induced upon cold shock in *E. coli* (Kandror and Goldberg, 1997), indicating that this transient CSP in the mesophile is continuously overexpressed in the psychrophile to adapt to cold temperatures.

However, the unusually high upregulation level of the psychrophilic TF in *P. haloplanktis* is appealing and could be part of a more general adaptive mechanism. We propose the following analysis. In bacteria, the three main chaperones are TF that stabilize nascent polypeptides on ribosomes and initiate ATP-independent folding, DnaK that mediates co- or post-transcriptional folding and the GroEL/ES chaperonin that acts downstream in folding assistance (Hartl and Hayer-Hartl, 2009). Both latter chaperones are also well-known heat shock proteins. In *E. coli* it has been shown that synthesis of heat shock proteins is repressed during growth at low temperatures, but also that DnaK and GroEL are harmful to cells at 4°C as their induced expression reduces cell viability at this temperature (Kandror and Goldberg, 1997). Searching for downregulated proteins in the proteome of *P. haloplanktis* TAC125 grown at 4°C, we indeed found that the synthesis of DnaK and GroEL is reduced by factors of 13 and 3 respectively (Table S1). Very significantly also, it has been demonstrated that GroEL from *P. haloplanktis* TAC125 is not cold-adapted, it is inefficient at low temperatures as its activity is reduced to the same extent than that of its *E. coli* homologue (Tosco *et al*., 2003). Considering the downregulation of DnaK and GroEL in *P. haloplanktis* at 4°C, the harmful effect of these chaperones on *E. coli* at 4°C and the inefficiency of GroEL from *P. haloplanktis* TAC125, we propose that TF rescues the chaperone function at low temperatures, therefore explaining its unusual overexpression. Accordingly, TF becomes the primary chaperone of the Antarctic bacterium for growth in the cold. Furthermore, its GroEL chaperonin appears to be well suited to function during sudden temperature increases of the environment and also demonstrates that cold-adapted and non cold-adapted proteins can coexist within the same microorganism.

There is only one gene copy of the trigger factor in both *P. haloplanktis* and *E. coli* genomes. In the mesophilic bacterium, the trigger factor gene is not essential for growth at any temperature (Deuerling *et al*., 1999; Kramer *et al*., 2004). Attempts to inactivate this gene in *P. haloplanktis* TAC125 were carried out using recently developed methods involving a suicide vector derived from a psychrophilic cryptic plasmid and successfully used to knockout non-essential genes (Parrilli *et al*., 2008; 2010). It was, however, not possible to obtain TF deletion mutants of the Antarctic bacterium after recovery at either 4°C or 18°C. Although a technical artefact cannot be ruled out, this suggests that the trigger factor gene is essential for this psychrophilic microorganism. This is in line with our hypothesis that the transient expression of some CSPs in mesophiles has evolved towards an essential and continuous expression in psychrophiles.

Structure analysis of the psychrophilic trigger factor

The amino acid sequence of the *P. haloplanktis* TF (47 534 Da) displays 61% identity (85% similarity) on 434 residues with its homologue from *E. coli* (Fig. S1). When compared with other TF of known crystal structure, it displays 65% identity with *Vibrio cholerae* TF but only 22% with *Thermotoga maritima* TF. Its sequence is also close to that of some TF from psychrophilic bacteria: *Colwellia psychrerythraea* (65% identity), *Psychromonas arctica* (65%), *Psychromonas ingrahamii* (58%) or *Psychrobacter frigidicola* (43%). The pronounced sequence similarity and predicted secondary structure conservation with *E. coli* TF suggest that the psychrophilic chaperone should also fold into an extended 'crouching dragon' conformation (Ferbitz *et al*., 2004) comprising three domains (Fig. S2). The N-terminal domain (aa 1-144) mediates ribosome attachment *via* an exposed loop containing the signature motif ^{43}GFRxGKVP, the PPIase activity domain (aa 145-247) located at the opposite end of the molecule (Kramer *et al*., 2004) and the C-terminal domain (aa 248-434) forming the body of the protein and bearing the central module of chaperone activity (Merz *et al*., 2006). Within the PPiase domain, all residues that have been implicated in this activity are also strictly conserved (F168, F177, E178, F185, M194, I195, F198, V215, F217, P218, Y221 and F233). Among the nine residues that have been used to probe the path followed by a nascent polypeptide into the chaperone cavity in *E. coli* TF (Merz *et al*., 2008), six are identical in *P. haloplanktis* TF (Q9, F233, F256, F322, A377, E404) whereas the three different residues (S61A, I76Y, H205K) are located within groups of conserved amino acids (Fig. S1).

Pseudoalteromonas haloplanktis TF isoelectric point (pI = 4.58) is slightly more acidic than that of the *E. coli* homologue (pI = 4.83). Such differences in pI values (more acidic for low pI or more basic for high pI) have been reported for numerous psychrophilic proteins (Feller, 2003) but their significance has remained elusive. It can now be proposed that these differences in pI values are related to the necessity of improving protein solubility that decreases at low temperatures. Indeed, it has been recently demonstrated that hypercharged proteins display a drastic increase of solubility (Lawrence *et al.*, 2007; Vendruscolo and Dobson, 2007). This physico-chemical constraint is expected to exert a selective pressure on amino acid composition of cold-adapted proteins, especially in the crowded cytoplasm. The low content in Arg residues in *P. haloplanktis* TF (16 versus 26 residues in *E. coli*) contributes to its lower pI but also suggests a lower propensity to form stabilizing electrostatic interactions (Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006). Finally, the higher content in Asn and Gln (44 versus 32 in *E. coli*), both residues harbouring a heat-labile side-chain prone to thermal deamination, can be related to the lack of selective pressure for heat-stable side-chain in cold environments, as also noted at the genome level of *P. haloplanktis* TAC125 (Medigue *et al.*, 2005).

The psychrophilic trigger factor is a marginally stable monomeric protein

In order to analyse the psychrophilic TF, its gene has been cloned and overexpressed in *E. coli* without purification tag. The recombinant protein was purified to homogeneity and its identity was confirmed by automated Edman N-terminal sequencing (indicating a non-processed fMet) and by ESI-Q-TOF mass spectrometry (47 540 Da measured). Its thermal stability was further investigated by differential scanning calorimetry. Figure 4A shows that TF from the Antarctic bacterium is a marginally stable protein, exhibiting a melting point T_m at 33°C. It follows that at a typical mesophilic temperature of 37°C, almost all the protein population is already in the unfolded state. In addition, the calorimetric enthalpy is also very weak (ΔH_{cal} = 82.5 kcal mol^{-1}, the sum of all enthalpic contributions to protein stability disrupted during unfolding and calculated from the area under the transition). By comparison, a T_m of 54°C and a calorimetric enthalpy of 178 kcal mol^{-1} have been reported for the *E. coli* trigger factor analysed by DSC (Fan *et al.*, 2008). To the best of our knowledge, *P. haloplanktis* TF is the least stable protein reported so far. This strongly suggests that the essential chaperone function requires considerable flexibility and dynamics to compensate for the reduction of molecular motions at freezing temperatures.

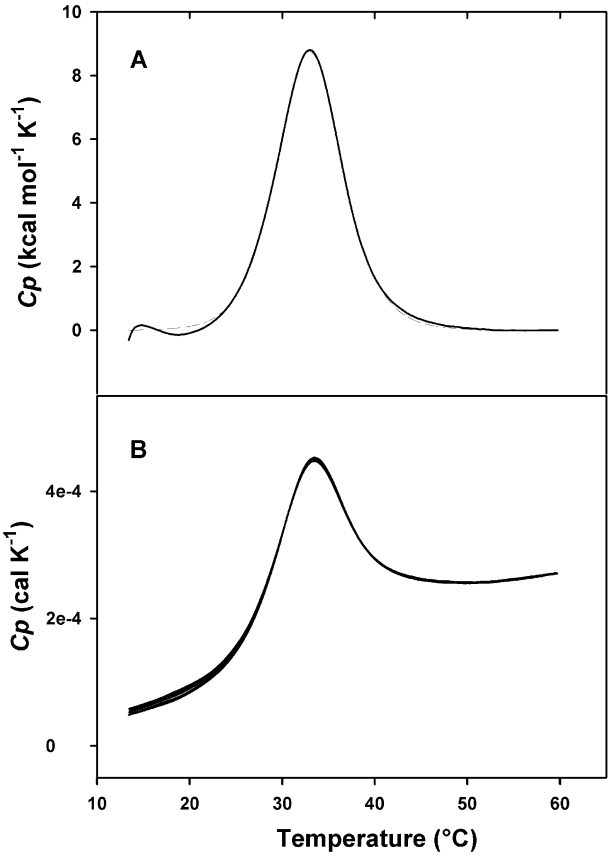

Fig. 4. Microcalorimetric analysis of the trigger factor from *P. haloplanktis*.
A. Normalized microcalorimetric record in 30 mM MOPS, 250 mM NaCl, pH 7.6. Baseline-subtracted data have been normalized for protein concentration (2.6 mg ml^{-1}). The dashed line corresponds to the fit of the DSC data to a two-state unfolding transition.
B. Reversibility of the unfolding transition. Raw thermograms of five consecutive unfolding transitions without refilling. Superimposition of the five thermograms demonstrates full reversibility of unfolding-refolding cycles.

Trigger factors are large, multidomain proteins (Ferbitz *et al.*, 2004). Despite this modular structure (Fig. S1), *P. haloplanktis* TF unfolds according to a perfect 2-state transition (Fig. 4A), i.e. without significantly populated intermediates between the native and the unfolded states, as indicated by the ratio $\Delta H_{cal}/\Delta H_{vH}$ = 1.04 at pH 7.6 (ΔH_{vH} = 79.0 kcal mol^{-1}, van't Hoff enthalpy calculated from the slope of the normalized transition). This indicates that the psychrophilic TF is uniformly unstable and unfolds cooperatively. Furthermore, its unfolding is fully reversible. Figure 4B displays five consecutive unfolding-cooling cycles: the perfect superimposition of thermograms shows that, after unfolding, the protein regains the native state upon cooling. More surprisingly, this psychrophilic protein can be heated up to 90°C and fully refolded upon cooling (not shown). Such unique unfolding reversibility can be explained in part by the low hydrophobicity of

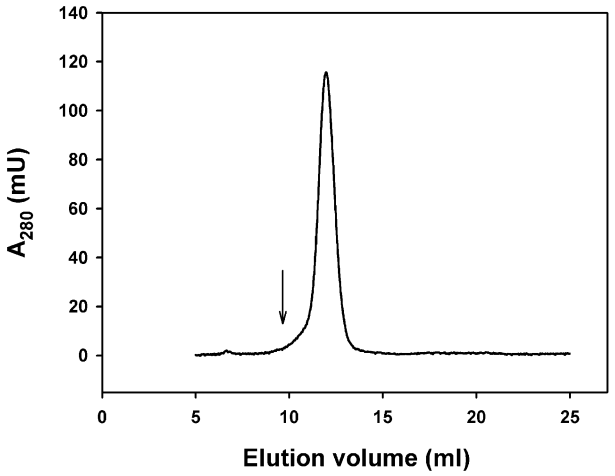

Fig. 5. Analytical size exclusion FPLC on a Superdex 200H/R 10/30 column. The trigger factor from *P. haloplanktis* was loaded at 61 μM concentration (1 ml). The arrow indicates the theoretical elution volume of a dimer.

the protein core clusters, as observed in cold-adapted polypeptides (Feller and Gerday, 2003), making them less prone to aggregation. However, the intrinsic chaperone function should also be taken into account: as a fraction of the TF refolds in the native state, it may be able to protect the remaining population from misrefolding.

A $\Delta H_{cal}/\Delta H_{vH}$ ratio close to unity also indicates that this protein unfolds as a monomer. Indeed, a dimer would have a ratio of 0.5 as the calorimetric enthalpy is expressed here in kcal per mole of monomer (Privalov, 1979). This was further confirmed by analytical size exclusion chromatography (Fig. 5). The psychrophilic TF (47.5 kDa) is eluted with a slightly higher apparent mass (55.4 kDa), indicating that its hydrodynamic dimensions are higher than for a compact globular protein of equivalent mass. Such deviation is expected considering the extended domain organization (122 × 59 × 63 Å) adopted in the crystal structure (Ferbitz *et al.*, 2004). However, the chromatogram displayed no evidence for dimeric species (Fig. 5) as the protein is eluted into a single symmetrical peak. This is in contrast with the *E. coli* trigger factor that has been reported to undergo *in vitro* a concentration-dependent dynamic equilibrium between the monomeric and the dimeric forms with dissociation constant in the range of 1–18 μM (Patzelt *et al.*, 2002). In Fig. 5, the mean peak concentration (loaded concentration divided by peak volume) was 15.2 μM and the maximal concentration (from absorbance) was 40 μM, i.e. well above the expected *Kd* value. Furthermore, static light scattering experiments performed in batch mode (~50 μM TF solutions) provided a mean particle mass of 51 kDa and 106 kDa for *P. haloplanktis* and *E. coli* TF respectively. This is in agreement with a monomeric psychrophilic TF and a dimeric *E. coli* TF. However, in dynamic light scattering (batch mode) the particle polydispersity (size distribution) of *P. haloplanktis* TF was twice that of *E. coli* TF, suggesting that the psychrophilic TF may possibly perform transient intermolecular interactions. Our observations are in line with the recent suggestion that the trigger factor from *Psychrobacter frigidicola* is a monomeric chaperone (Robin *et al.*, 2009). The interpretation of these differences in oligomerization state remains to be properly explained but suggest noticeable differences between psychrophilic and mesophilic bacteria for the TF function in the cytoplasmic fraction, when not bound to the ribosome.

Chaperone and antichaperone activities of the psychrophilic trigger factor

In a typical GAPDH refolding assay, the GdmCl-unfolded protein is diluted in non-denaturing buffer, giving rise to aggregation of misfolded species, as recorded by light scattering. Stoichiometric excess of *E. coli* TF has been reported to inhibit aggregation by binding to the unfolded protein (Huang *et al.*, 2000), as also shown in Fig. 6. However, under these experimental conditions the psychrophilic TF displays an antichaperone behaviour as it increases both the amount of aggregated material (Fig. 6A) and the rate of aggregation (Fig. 6B), revealing its inability to support its chaperone function under mesophilic conditions. As shown in Fig. 7A, the chaperone activity of *P. haloplanktis* TF was recovered at 15°C under lower residual GdmCl concentration (30 mM). However, higher stoichiometric ratios were required to inhibit aggregation, as compared with *E. coli* TF, and furthermore full aggregation arrest was not obtained. This can be attributed to a still sub-optimal temperature (GAPDH fails to aggregate reproducibly below 15°C). Surprisingly, under the same conditions but at 20°C, the chaperone activity of the psychrophilic TF is lost, whereas the antichaperone activity prevails. This is a clear indication of a strong temperature dependence of its chaperone function. This has been addressed by a temperature shift experiment. The refolding mixtures were first incubated 30 min in melting ice, and then transferred to the spectrophotometer cell thermostated at 15°C: temperature equilibration was reached in 4 min (Fig. 7B). GAPDH alone does not aggregate significantly during cold incubation but initiates aggregation when the temperature is raised. By contrast, in the presence of the psychrophilic TF, aggregation is totally inhibited for 10–15 min at 15°C, before it starts at a slow rate. When pre-incubation was performed at 10°C, inhibition but not aggregation suppression was observed. It can be concluded that (i) *P. haloplanktis* TF requires near-zero temperatures to efficiently bind unfolded GAPDH, (ii) it can maintain this association for a limited period of time at 15°C, (iii) then it slowly dissociates,

allowing the unbound unfolded GAPDH to aggregate. This illustrates a remarkable cold adaptation of the chaperone function in the psychrophilic TF.

In an attempt to delineate the structural domain bearing the chaperone activity, a mutant of the psychrophilic TF deleted of the C-terminal 55 amino acid residues was constructed. In *E. coli* TF, deletion of the corresponding residues caused the complete loss of the chaperone activity *in vitro* (Merz *et al.*, 2006). However, when produced in *E. coli* this *P. haloplanktis* TF mutant is not stably folded and is quickly proteolysed. This behaviour can be related to the fragile and unstable psychrophilic TF structure that cannot tolerate large deletion in its main structural domain.

Conclusions

The CAPs identified in this study and those previously reported for an Antarctic bacterium (Kawamoto *et al.*, 2007), three Siberian permafrost bacteria (Qiu *et al.*, 2006; Bakermans *et al.*, 2007; Zheng *et al.*, 2007; Bergholz *et al.*, 2009) and an Antarctic archaeon (Goodchild *et al.*, 2004; Goodchild *et al.*, 2005) do not constitute a conserved set of proteins in terms of identification and expression level. Such heterogeneous upregulation of CAPs supports the view that cold-adaptation mechanisms are constrained by the species-specific cellular structure and organization, resulting in distinct adaptive strategies. Nevertheless, a survey of these data shows that the main upregulated functions for growth at low temperatures are protein synthesis (transcription, translation), RNA and protein folding, membrane integrity, anti-oxidant activities and regulation of specific metabolic pathways. In the case of *P. haloplanktis* TAC125, the strong overexpression at 4°C of the trigger factor can be interpreted with respect to the strain lifestyle. This bacterium can reach high cell densities when cultivated in rich media. It has been proposed that the split of its genome into two chromosomes

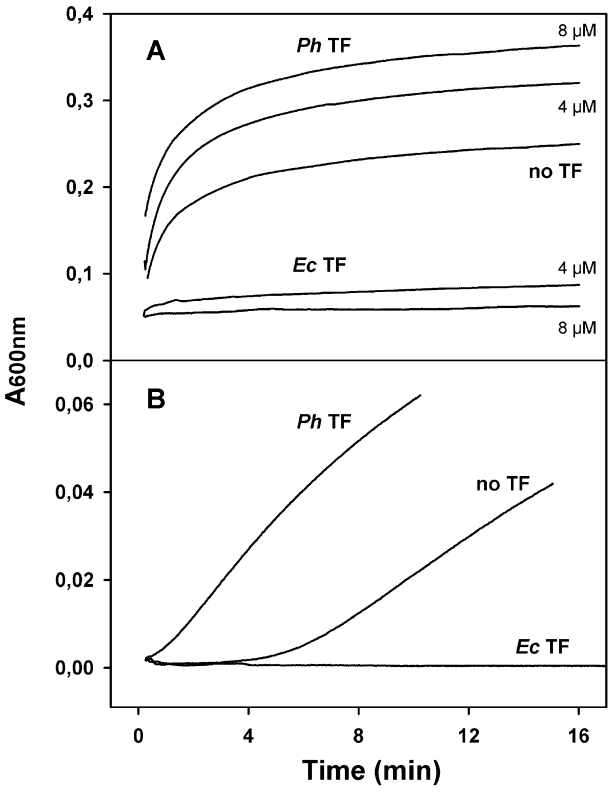

Fig. 6. Antichaperone activity of the psychrophilic TF at 20°C.
A. Aggregation of GAPDH is recorded by a turbidity increase after 50-fold dilution of the denatured enzyme (2.5 µM final concentration). Addition of *E. coli* TF (*Ec* TF) prevents aggregation whereas *P. haloplanktis* TF (*Ph* TF) promotes aggregation.
B. As in (A) with 0.6 µM GAPDH final concentration in order to record initial aggregation rates. A sixfold molar excess of *Ec* TF (4 µM) suppresses aggregation whereas *Ph* TF (4 µM) significantly increases the aggregation rate. Residual GdmCl concentration was 60 mM.

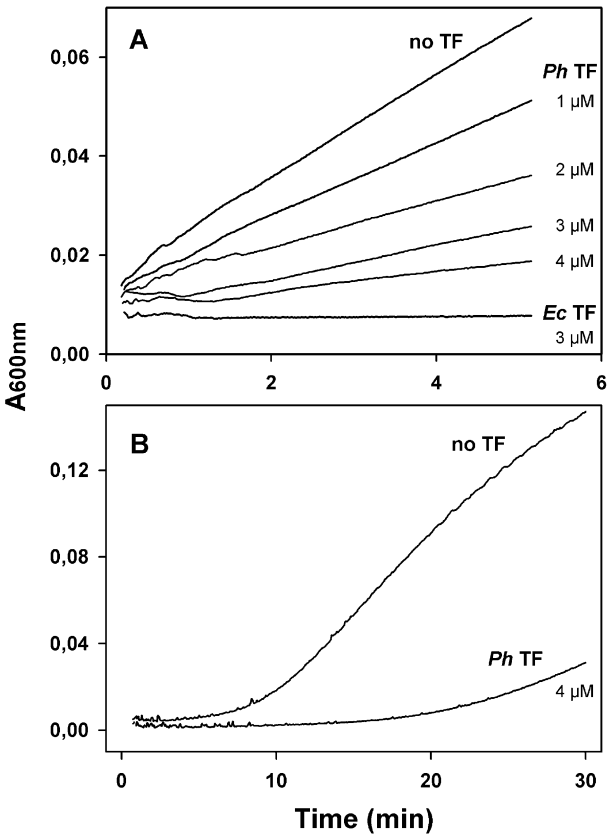

Fig. 7. Chaperone activity of the psychrophilic TF at 15°C. Abbreviations as in Fig. 6 and 1.25 µM final GAPDH concentration. Residual GdmCl concentration was 30 mM.
A. Addition of *Ec* TF suppresses aggregation whereas *Ph* TF inhibits aggregation. Only transient aggregation arrest is observed at 3 and 4 µM *Ph* TF.
B. As in A with 30 min incubation of the refolding mixtures in melting ice. After cold incubation, *Ph* TF suppresses aggregation for 10–15 min.

and the very high number of tRNA genes might increase the speed of replication and growth when abundant nutrients become available (Medigue *et al.*, 2005; Danchin, 2007). In this context, fast and efficient folding assistance of newly synthesized polypeptides by the trigger factor at low temperatures would be also required, especially under conditions that repress synthesis and activity of DnaK and GroEL.

Experimental procedures

Bacterial strain and growth conditions

The Gram-negative bacterium *P. haloplanktis* TAC125 is available from the Collection de l'Institut Pasteur (Paris, France) under accession number CIP 108707. This strain was grown at 4°C and 18°C in broth containing 5 g l^{-1} Peptone N-Z-Soy BL 7 (Sigma), 1 g l^{-1} NH_4NO_3, 0.14 g l^{-1} KH_2PO_4, 10 g l^{-1} sea salts at pH 7.0 in 500 ml of flasks containing 50 ml of culture medium under 250 r.p.m. orbital agitation. These cultures were inoculated from a preculture grown at the same temperature and in the same conditions.

Cell preparations for 2D-DIGE

Four analytical cultures were performed at either 4°C or 18°C. At the mid-exponential phase (A_{550} between 1 and 2), the four cultures grown at the same temperature were pooled and cells were harvested by centrifugation at 12 000 *g* during 20 min. The pellets were washed with a solution of 10 g l^{-1} NaCl and then cells were lysed by sonication in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). After centrifugation at 48 000 *g* during 20 min, soluble proteins were collected in the supernatant and precipitated with the 2D Clean-Up Kit from GE Healthcare in order to prepare suitable samples with low conductivity for isoelectric focusing. Precipitated proteins were solubilized in DIGE labelling buffer (7 M urea, 2 M thiourea, 2% ASB14 zwitterionic detergent, 30 mM Tris-HCl at pH 8.5); the insoluble material was removed by centrifugation and the pH was adjusted to 8.5 with NaOH.

CyDye labelling and 2D-DIGE

Protein concentration was determined with the RC/DC Protein Assay (Bio-Rad). CyDye labelling was performed according to the manufacturer instructions (GE Healthcare). Briefly, 25 µg of each protein sample (from cultures at 4°C and 18°C) was labelled separately with 200 pmol of CyDye (Cy3 and Cy5) and a pooled sample, composed of an equal amount of all samples from cultures at 4 and 18°C, was labelled with Cy2. The latter was used as the internal standard for matching and normalization between gels. All samples were incubated 30 min in the dark and the labelling reaction was stopped with 10 mM lysine. Pairs of Cy3 and Cy5 labelled samples together with 25 µg of Cy2 labelled internal standard were pooled and were brought to 1% DTT and 2% 3–10 NL IPG buffer (GE Healthcare) and the total volume was adjusted to 450 µl with standard rehydration buffer (7 M urea, 2 M thiourea, 2% w/v ASB 14, 1% DTT, 2% pH 3–10 NL IPG buffer). This protein solution was used to rehydrate 24 cm IPG strip, pH 3–10, NL (GE Healthcare) during 8 h. Proteins were separated on IPGphor III isoelectric focusing unit (GE Healthcare). This isoelectric focalization (IEF) was successively conducted at 500 V (constant) during 1 h, 1000 V (gradient) during 3 h, 8000 V (gradient) during 3 h, 8000 V (constant) during 8h45 with a maximum current setting of 50 µA per strip. Three independent IEF separations were performed on the pooled sample. Prior to second-dimension separation, the IPG strips were equilibrated according to Gorg *et al.* (1995). They were then sealed with 0.5% agarose in SDS running buffer on top of 12.5% w/v acrylamide gels. These gels were poured between silane-treated low fluorescence glass plates (GE Healthcare) to minimize background fluorescence during scanning. The second-dimension electrophoresis was performed at 20°C in an Ettan Dalt 6 system (GE Healthcare) at 2.5 W per gel during 30 min and then 25 W per gel during 4 h. Each gel was then scanned with the Typhoon 9400 scanner (GE Healthcare) at the wavelengths corresponding to each CyDye. One additional gel was run in the same conditions but was loaded with 25 µg of internal standard and 100 µg of non-labelled extract from 4°C culture. This preparative gel was used for spot excision and mass spectrometry identification.

Image analysis

Images were analysed with the DeCyder software (GE Healthcare) according to the manufacturer instructions. In brief, co-detection of the three CyDye labelled forms of each spot was performed using DIA (Differential In-gel Analysis) software module and allowed to calculate ratios between samples and internal standard abundances. Inter-gel variability was corrected by matching and normalization of the Cy2 internal standard spot maps by the BVA (Biological Variance Analysis) module of the DeCyder software. Protein spots that showed a statistically significant ($P < 0.05$) Student's *t*-test for an increased intensity with minimum threshold of 1.5 were accepted as being differentially expressed in the extracts under comparison.

Mass spectrometry and protein identification

Proteins were identified by LC MS-MS or MALDI-TOF. Protein spots that significantly changed were excised from the preparative gel by the SpotPicker (GE Healthcare). In-gel trypsin digestion of the proteins and extraction of peptides were made on the Ettan Spot Handling Workstation (GE Healthcare). Samples were analysed either with an UltraFlex II MALDI-TOF-TOF (Bruker Daltonics) or with an LC ion trap system (Ultimate Dionex, Esquire HCT Ultra, Bruker Daltonics). Peptide mass fingerprints and MS-MS spectra were analysed using Biotools software (Bruker) and the integrated Mascot (Matrix Science) search engine in NCBI database restricted to proteobacteria. All the proteins discussed were significantly identified with a probability for random identification $P < 0.05$. Peptide profiling was performed on a Bio-Rad SELDI-TOF-MS PCS4000 using NP20 chips.

Gene cloning, production and purification of the trigger factor

Genomic DNA was extracted from a batch culture of *P. haloplanktis* TAC 125 using the Wizard Genomic DNA purification kit (Promega). The trigger factor gene was PCR-amplified using Vent DNA polymerase (New England Biolabs) by standard molecular biology techniques. Primers were designed in order to introduce a NdeI site overlapping the initiation codon and a BamHI site after the stop codon (forward: 5′-ttgaggtaacatatgcaagtttctgttgag-3′, reverse: 5′-taa gtcaatggatccttaagcaccttgctgtggatt-3′, restriction sites underlined). The PCR product was digested by these enzymes and cloned into the pET22b expression vector (Novagen). The resulting recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells. The trigger factor gene from *E. coli* RR1 was cloned using the same strategy (forward: 5′-tgaggtaaccatatgcaagtttcagttgag-3′, reverse: 5′-gttatgctgggatccttacgcctgctggttcat-3′).

Colonies of the *E. coli* BL21 (DE3) cells transformed with this vector were used to inoculate a 25 ml preculture of Terrific broth [12 g l^{-1} Bactotryptone (Difco), 24 g l^{-1} yeast extract (Difco), 4 ml l^{-1} glycerol, 12.54 g l^{-1} K_2HPO_4, 2.31 g l^{-1} KH_2PO_4, pH 7.2] containing 100 mg l^{-1} ampicillin and run at 18°C for 1 h. 2 ml of aliquots were used to inoculate 250 ml of TB in 2-liter flasks and the cultures were incubated overnight at 18°C and 250 r.p.m. Protein expression was induced by 1 mM isopropyl-β-D-1-thiogalactopyranoside and following further 24 h incubation at 18°C, the cells were harvested by centrifugation at 10 000 *g* for 30 min at 4°C. The cell pellet was resuspended in 35 mM HEPES, pH 7.6, then disrupted in a pre-chilled high-pressure cell disrupter (Constant Systems) at 28 kpsi, and centrifuged at 30 000 *g* for 30 min. The supernatant was incubated for 1 h in the presence of Benzonase and a protease inhibitor cocktail (Complete, Roche) and was then brought to 90% saturation in $(NH_4)_2SO_4$. The precipitated proteins collected by centrifugation were suspended in 35 mM HEPES, pH 7.6, 30% saturation in $(NH_4)_2SO_4$ and the soluble fraction was recovered after centrifugation at 15 000 *g*. This fraction was loaded on a Phenyl-Sepharose CL-4B column (2.5 × 20 cm) equilibrated in 35 mM HEPES, 30% saturation in $(NH_4)_2SO_4$, 1 mM PMSF, pH 7.6. Proteins were eluted with a decreasing gradient (450 ml) from 30% to 0% saturation in $(NH_4)_2SO_4$. Fractions containing the trigger factor were then loaded on a DEAE-agarose column (2.5 × 40 cm) equilibrated in 35 mM HEPES, 1 mM PMSF, pH 7.6 and eluted with a linear gradient (1 l) from 0 to 0.5 M NaCl. Purified proteins samples (~5 mg ml^{-1}) were stored at −70°C. The trigger factor from *E. coli* RR1 was purified by the same procedure.

Analytical procedures

The N-terminal amino acid sequence of the recombinant protein was determined by automated Edman degradation using a pulsed-liquid-phase protein sequencer Procise 494 (Applied Biosystems) fitted with an on-line phenylthiohydantoin analyser. Mass determination was performed by ESI-Q-TOF mass spectrometry (Waters, Micromass) in 25% acetonitrile, 0.5% formic acid. Static and dynamic light scattering experiments were performed in 35 mM HEPES, 250 mM NaCl, pH = 7.6 on a DynaPro NanoStar (Wyatt Technology). Prevention of GAPDH aggregation was recorded as described (Kramer *et al*., 2004) using an Uvikon XS spectrophotometer (Bio-Tek) at 620 nm.

Differential scanning calorimetry measurements were performed using a MicroCal VP-DSC instrument at a scan rate of 60 K h^{-1} and under ~25 psi positive cell pressure. Samples were dialysed overnight against 30 mM MOPS, 250 mM NaCl, pH 7.6. Reference baselines were recorded using the dialysis buffer. Protein concentration was determined by the bicinchoninic acid protein assay reagent (Pierce) after dialysis and was ~55 μM. Calorimetric enthalpies ($\Delta Hcal$) were determined as the area of the transitions, normalized for protein concentration and limited by a progress baseline, using the MicroCal Origin v.7 software. The non-2-state fitting model was applied to calculate van't Hoff enthalpies, ΔH_{vH}.

Analytical size exclusion chromatography was performed on a Superdex 200H/R 10/30 prepacked fast protein liquid chromatography column connected to an Äkta Explorer. The column was eluted by 35 mM HEPES, 250 mM NaCl, pH 7.6 at 0.5 ml min^{-1} and calibrated with hen egg lysozyme (14.3 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31.0 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and rabbit muscle phosphorylase b (97.4 kDa). 1 ml of trigger factor at 61 μM was loaded on the analytical column.

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