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GroEL from the psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC 125: molecular characterization and gene cloning

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Abstract The heat shock response of the psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC 125 (*Ph*TAC 125) gives rise to the production of several inducible proteins. Among these, the protein corresponding to a 55-kDa band on SDS-PAGE was purified to homogeneity and identified as a GroEL-like protein. The gene coding for this protein (*Ph*GroEL) was cloned and sequenced; the deduced amino acid sequence shows 82% sequence identity to GroEL from *Escherichia coli* (*Ec*GroEL). The ORF found in the 5' upstream region codes for a homologue of the GroES from *E. coli* (*Ph*GroES, 71% sequence identity to *Ec*GroES). *Ph*GroEL shows a chaperone activity and can use GroES from *E. coli* as a co-chaperone. *Ph*GroEL melting temperature, 6 °C lower than that of *Ec*GroEL, and equilibrium unfolding experiments in urea showed a less stable protein architecture for the psychrophilic GroEL. The data herein reported demonstrate that *Ph*GroEL cold adaptation consists in a shift of the protein properties toward lower temperatures without increasing catalytic efficiency at low temperatures. Primary extension analysis depicted a complex organization of regulative elements for the operon containing the genes coding for *Ph*groES and *Ph*groEL (*PhgroE*), suggesting that a fine-tuning of transcription can also be involved in thermal adaptation of *Ph*TAC 125.

Keywords Adaptation to cold · Antarctic bacteria · GroEL · GroES · Heat shock · *Pseudoalteromonas haloplanktis*

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Introduction

Induction of a specific subset of highly conserved proteins, i.e., heat-shock proteins (hsps), is a universal response among prokaryotic and eukaryotic organisms to the exposure to a mild, non lethal heat shock (Lindquist 1986). An understanding of the mechanisms whereby microorganisms from extreme environmental conditions adapt to temperature changes should further contribute to the knowledge of hsp functioning. In this respect, hsps from different thermophilic organisms have already been isolated and characterized (Osipiuk and Joachimiak 1997; Motohashi et al. 1994; Trent et al. 1991, 1994; Cross et al. 1996; Roy et al. 1999), whereas few data have been reported so far on the heat shock response in organisms growing at low temperatures.

Specific heat shock-inducible protein patterns have been reported for different cold-adapted bacteria (McCallum et al. 1986; McCallum and Innis 1990) and yeasts (Berg et al. 1987; Julseth and Inniss 1990; Deegenaars and Watson 1997, 1998), and some attempts have been made to demonstrate relationships to known mesophilic hsps. McCallum and coworkers (McCallum et al. 1986; McCallum and Innis 1990) have shown that two psychrophilic bacteria possess genomic DNA sequences homologous to the *dnaK* gene of *Escherichia coli*. Different strains of Antarctic yeasts showed the presence of some proteins crossreacting with antibodies raised against the major hsps from *Saccharomyces cerevisiae* (Deegenaars and Watson 1997, 1998). Nevertheless, no attempts have been made so far to purify homogeneous "cold" hsps in order to perform structural-functional comparison with their mesophilic counterparts.

During a study aimed at analyzing the heat shock response of the gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC 125 (*Ph*TAC 125) isolated from Antarctic seawater, we purified a hsp that resulted in a GroEL homologue. An intriguing characteristic of *Ph*TAC 125 is its ability to grow "optimally" in quite a

broad range of temperatures, thus adapting the whole cellular machinery to work efficiently in "deep" cold as well as at moderate temperatures (Tutino et al. 1999a, where *Ph*TAC 125 is still named *Moraxella* TAC 125). This feature makes *Ph*TAC 125 a truly cold-adapted bacterium, making it an interesting example of a psychrophilic organism that is suitable to investigation of the molecular basis of adaptation to life at low temperatures and to biotechnological exploitation.

The GroEL proteins, belonging to the class of hsp60 chaperones, are essential for cell growth and for survival in stress conditions in many bacteria, and their synthesis is highly sensitive to growth temperature and temperature changes. GroEL, together with its co-chaperone GroES, appears to be critical for proper protein folding in vivo (Goloubinoff et al. 1989), and they have been shown to promote the refolding of several denatured proteins in vitro (Mendoza et al. 1992). The corresponding genes *groEL* and *groES* are typically arranged as an operon (*groE*), and their translation products are assembled into single or double heptameric rings, respectively. In the presence of nucleotides, GroEL forms a 1:1 complex with GroES and binds the protein substrate in its central cavity. Substrate release is contingent upon ATP hydrolysis, and multiple cycles of binding and release may be necessary for a protein to reach its native conformation (for recent reviews on structure and functioning of GroEL, see Sigler et al. 1998; Fenton and Horwich 1997; Grantcharova et al. 2001).

In this paper we report a molecular and functional characterization of GroEL from *P. haloplanktis* TAC 125 (*Ph*GroEL). Moreover, the genes coding for *Ph*GroEL and its co-chaperone *Ph*GroES were cloned and sequenced, and primary extension analysis was performed at several temperature conditions in order to gain information on the transcription of the corresponding operon (*PhgroE)*.

Materials and methods

Bacterial sources and proteins

Pseudoalteromonas haloplanktis TAC 125 (*Ph*TAC 125) (from the C. Gerday collection, University of Liege), was collected in 1992 from seawater near the French Antarctic Station Dumont d'Urville (60°40';40°01'E). *Escherichia coli* DH5α (*sup*E44, Δ*lac*U169 [ϕ80 *lac*Z Δ M 15], *hsd*R17, *rec*A1, *end*A1, *gyr* A96, *thi*-1 *rel*A1) was the host strain for DNA manipulation. GroEL and GroES from *E.coli* were a kind gift from Prof. J. Buchner (Institute of Organic and Biological Chemistry, Technical University of Munich). Synthetic oligonucleotides were from Ceinge Biotecnologie Avanzate s.c.r.l.

Heat shock and cell labeling

P. haloplanktis TAC 125 cells were grown at 15 °C to mid-log phase, (optical density at 600 nm = 0.3), in the minimal medium Shatz (KH_2PO_4 1 g/l, NH_4NO_3 1 g/l, NaCl 10 g/l, $MgSO_4$ $7H_2O$ 0.2 g/l, $FeSO_4$ $7H_2O$ 10 mg/l, $CaCl_2$ $2H_2O$ 10 mg/l, pH 7.0) with 0.2% of galactose and then rapidly shifted to 37 °C. As the desired temperature was reached, 19 μCi of [^{35}S] methionine and [^{35}S] cysteine (Pro-Mix, Amersham) were added to 1 ml of cell culture that was further incubated at 37 °C. Radiolabeled cells were harvested by centrifugation at 4,000 *g* for 15 min at 4 °C, and pellets were resuspended in Laemmli sample buffer (Laemmli 1970) and loaded on a 12.5% (w/v) SDS-PAGE. Radiolabeled proteins were detected by exposing dried gels to Fuji Medical X-ray Films.

Purification of GroEL from *P. haloplanktis* TAC 125 (*Ph*GroEL)

The pellet of 3 ml of cell culture submitted to heat shock and radiolabeling as described above was combined with the pellet of 250 ml of cell culture that had been heat shocked for 30 min at 37 °C but not radiolabeled. After a freeze and thawing cycle at –80 °C, cells were resuspended in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication with a Misonix, Ultrasonic Processor. Cell debris was removed by centrifugation at 11,000 *g* for 20 min at 4 °C. The cell-free crude extract was fractionated with ammonium sulfate at 30% of saturation, and the soluble supernatant, separated by centrifugation (11,000 *g*, 20 min, 4 °C), was concentrated by ammonium sulfate precipitation at 80% saturation. Protein pellet was resuspended, extensively dialyzed against 50 mM Tris-HCl, pH 7.5, and loaded on a Mono-Q column (PC 1.6/5 Pharmacia) equilibrated in the same buffer. Proteins were eluted with a linear NaCl gradient (0–0.5 M in equilibration buffer), and fractions were analyzed for radioactivity in a Beckman Ready Safe liquid scintillation cocktail with a scintillation counter (Beckmann LS 6000 SC). The most radioactive fractions were loaded on a SDS-PAGE, and those showing a major band at about 55 kDa (eluting between 74% and 82% of the gradient) were pooled, concentrated (Amicon 30 kDa membrane filter), dialyzed against 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, and loaded on a Superose 6 (PC 3.2/30) gel-filtration column equilibrated in the same buffer. The column was calibrated in the same buffer with the following protein of known molecular mass: bovine thyroglobulin (669 kDa), equine apoferritin (420 kDa), aspartate aminotransferase from *E. coli* (90 kDa), and bovine serum albumin (66 kDa). Fractions corresponding to a peak of radioactivity and containing a major band of 55 kDa when analyzed by SDS-PAGE were pooled, concentrated, and loaded again on Superose 6 in the same conditions as above as a further purification step.

For preparative protein purification, cells were grown at 15 °C in TYP medium (16 g/l yeast extract, 16 g/l tryptone, 10 g/l marine salts, pH 7.5) to stationary phase and were not heat-shocked or radiolabeled. The cells were harvested and processed as above, this time using a Poros 50 HQ (PerSeptive Biosystems) in the anion exchange step and a 125-ml Superose 6 as the first gel-filtration column. Protein concentration was determined with the Bio-Rad Protein Assay (Bradford 1976) with BSA as standard.

Reduction and alkylation of cysteine residues

Protein samples were reduced in 0.25 M Tris-HCl pH 8.5 and 1.25 mM EDTA, containing 6 M guanidinium chloride, by incubation at 37 °C for 2 h, under nitrogen atmosphere with a 10:1 molar excess of dithiothreitol (25 mM). The free cysteine residues were alkylated by using a 10:1 molar excess of iodoacetamide over the total -SH groups at room temperature for 1 h, in the dark, under nitrogen atmosphere. Protein samples were freed from salt and reagent excess by passing the reaction mixture through a PD10 prepacked column, equilibrated, and eluted in 0.4% ammonium bicarbonate, pH 8.5.

Enzymatic hydrolysis and peptide purification

Endoprotease LysC digestion was performed on a carboxamidomethylated protein sample (100 μg) in 0.4% ammonium bicarbonate, pH 8.5 at 37 °C, overnight, using an enzyme/substrate ratio of 1:50. The peptide mixture was separated on a narrow bore Vydac C_{18} column (The Separation Group), using a linear gradient

from 5% to 60% of acetonitrile in 0.1% trifluoroacetic acid, over a period of 80 min, at a flow rate of 0.2 ml/min.

Protein sequence analysis

Automated N-terminal degradation of the electroblotted protein or purified peptides was performed using a Perkin Elmer-Applied Biosystems 477A pulsed-liquid protein sequencer equipped with a model 120A analyzer for the online identification and quantification of phenylthiohydantoin amino acids.

Mass spectrometric analysis

ESI-MS spectra were recorded by using an API-100 single quadrupole mass spectrometer (Applied Biosystems) equipped with an atmospheric pressure ionization source or a BIO-Q triple quadrupole mass spectrometer (Micromass) equipped with an electrospray ion source. Protein molecular weight was determined by injecting a protein solution (10 pmol/µl) directly into the ion source at a flow of 5 µl/min. Data were elaborated using the BioMultiView program version 1.3 (Applied Biosystems). All masses were reported as average values.

MALDI spectra were recorded by using a Voyager DE MALDI-TOF spectrometer (Perkin Elmer-Perseptive Biosystem); a mixture of analyte solution, α-ciano-4-hydroxy-cinnamic acid or 2,5-dihydroxy-benzoic acid, bovine insulin, and horse heart myoglobin was applied to the sample plate and dried in vacuo. Mass calibration was performed using the molecular ions from the horse myoglobin (16,951.50 *m*/*z*), the bovine insulin (5,734.59 *m*/*z*), and the α-ciano-4-hydroxy-cinnamic acid (379.06 *m*/*z*) as internal standards. Raw data were analyzed by using software provided by the manufacturer and reported as average masses.

Cloning of *P. haloplanktis* TAC *groESL* operon

A DNA fragment of 1,130 bp of the gene coding for *Ph*groEL was amplified by polymerase chain reaction (PCR) on genomic DNA (extracted as in Birolo et al. 2000). Two degenerated oligonucleotides were designed on the basis of amino acid sequence information [5'PCR, 5'-GARGAYAARTTYGARAAYATGGG; 3'PCR, 5'-CCRTGRTTYTGRTCYTCRTTRTC (where Y = T, C; R = A, G)]. A mixture containing 1 µg genomic DNA, 2.5 pmol/µl oligo 5'PCR and 5 pmol/µl oligo 3'PCR, 1.75 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, and 200 µM dNTP in a final volume of 50 µl was incubated at 95 °C for 10 min, after which 2.5 units *Taq* DNA polymerase were added. Thirty-five cycles of amplification consisting of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C were carried out and followed by a cycle in which the extension reaction at 72 °C was prolonged for 15 min in order to complete DNA synthesis. The amplified fragment was subsequently purified by agarose gel electrophoresis, ligated into a pCAP^s plasmid (Boheringer), and sequenced. The nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger et al. 1977) with T7 Sequenase (Amersham). The regions upstream and downstream the amplified fragment were obtained by inverse PCR. Ten micrograms of genomic DNA was digested with *Pst*I in 1-ml final volume, phenol/chloroform/isoamylic alcohol extracted, and ethanol precipitated. Five micrograms of *Pst*I-digested DNA was ligated in 66 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM ATP in 1-ml final volume with 60 units of T4 DNA ligase (Boehringer). Ligated DNA was precipitated with ethanol and resuspended in 100 µl 50 mM Tris-HCl pH 7.5. A DNA fragment of about 2,000 bp was amplified on ligated DNA using two primers that had been designed on the sequence of the 1,130-bp fragment (5'invPCR, 5'-GTAGTACCTGGCGGCGGCG; 3'invPCR, 5'-CCATCGCCGGCTGCATCGT). A mixture containing 230 ng ligated DNA, 0.7 pmol/µl each primer, 14 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 50 mM Tris-HCl pH 9.0, and 360 µM dNTP in a final volume of 50 µl was incubated for 3 min at 93 °C, for 1 min at 60 °C, and for 10 min at 68 °C, during which 3.5 units of a mixture containing *Taq* and *Pwo* DNA polymerases (Boehringer) were added. Thirty-nine cycles of amplification consisting of 10 s at 93 °C, 1 min at 60 °C, and 7 min at 68 °C were carried out. The amplified fragment was purified by agarose gel-electrophoresis, ligated into a pCAP^s plasmid, and sequenced, giving the entire *groEL* gene sequence and a fragment *groES* gene. The remainder of the operon was cloned in two steps by single specific primer PCR.

In the first step, a PCR carried out on a *Hind*III-genomic library of *Ph* TAC125 in pGEM4Z (Promega) allowed us to amplify the remaining portion of the *PhgroES* gene. A DNA fragment of 763 bp was amplified on the genomic library using the synthetic oligonucleotide 3'invPCR and the M13/pUC universal sequencing forward oligonucleotide (5'-GTTTTCCCAGTCACGAC) as primers. The PCR was carried out in 50 µl of a mixture containing 1.5 µg plasmid DNA, 1 pmol/µl oligo 3' invPCR, 2 pmol/µl pUC forward oligonucleotide, 1.75 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% mM $MgCl_2$, and 200 µM dNTP, following the same procedure as described above for the amplification on the genomic DNA, except that the annealing temperature was set at 62 °C. The amplified fragment was purified by agarose gel electrophoresis and sequenced, allowing us to complete the *PhgroES* gene sequence.

In the second step, a PCR carried out on a *Pst*I-genomic library of *Ph* TAC125 in pGEM4Z allowed us to amplify a region upstream of *PhgroES*. A DNA fragment of 274 bp was amplified on the genomic library using the synthetic oligonucleotide PromRevGroE (5' CGCGATCATGTAAAGGACG 3') and the M13/pUC universal sequencing reverse oligonucleotide (5' AGCGGATAACAATTTCACACAGGA 3') as primers. The PCR was carried out following the same procedure as described above for the amplification on *Pst*I-genomic library, except that the annealing temperature was set at 58 °C. The amplified fragment was gel purified and sequenced, allowing us to complete the *PhgroE* sequence. A fragment containing the whole *PhgroE* operon was then amplified on genomic DNA and ligated to the vector pUC18 (Stratagene), giving rise to plasmid pGTO. The EMBL Databank accession number for GroEL and GroES is AJ243594.

Primer extension mapping

Duplicate cultures of *Ph*TAC125 were grown at the desired temperature conditions in TYP medium to an optical density at 600 nm (OD_{600}) of 2.5, chilled briefly on ice, collected by centrifugation at 6,000 *g*, and stored at −80 °C. Total RNA was isolated from 1.5-ml cultures. A 1-ml volume of TRIreagent (Sigma) was added to each cell pellet. Total RNA was separated from genomic DNA and proteins by centrifugation following the addition of 0.2 ml of chloroform. After isopropyl alcohol precipitation, RNA samples were resuspended in 30 µl of RNase-free water and recovered as specified by the manufacturer.

Nine picomoles of the oligonucleotide PromRevGroE (5' CGCGATCATGTAAAGGACG 3') was labeled with 30 µCi of (γ-^{32} P)ATP (Amersham), 10 U of polynucleotide kinase (Roche), and 1 µl of 10X polynucleotide kinase buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM DTT, 1 mM spermidine, pH 8.2) in a final volume of 10 µl for 1 h at 37 °C. Unincorporated nucleotides were removed using the Removal Nucleotides KIT (Quiagen).

For the primer extension reactions, 50 µg of each total RNA was incubated at 65 °C for 7 min with 1.6 µl of 5X avian myeloblastosis virus reverse transcriptase (AMV) buffer (250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine) and 2.5×10^5 cpm of oligonucleotide in a final volume of 8 µl. Annealing was accomplished by freezing the total RNA-oligonucleotide solutions in a dry ice-ethanol bath and by slowly defrosting in ice. The primer extension reactions were carried out for 60 min at 42 °C following the addition of 3.0 µl of a master mix containing 1.4 mM dCTP, 1.4 mM dATP, 1.4 mM dTTP, 1.4 mM dGTP, 7 U of AMV (Promega), and 1X AMV buffer. Each reaction was stopped by addition of 8 µl of sequencing stop solution.

The length of the primer extension products was calculated by comparing the electrophoretical mobility in a 6.0% polyacrylamide gel with that of the product of a sequencing reaction generated with plasmid pGTO template and the same primer as used for the reverse transcription reactions.

CD spectroscopy

A JASCO J-715 spectropolarimeter equipped with a Peltier thermostatic cell holder (Jasco model PTC-348) was used. Temperature was measured directly in the 0.1-cm path length quartz cell, the solutions were filtered just before use, and data were corrected by subtracting a control from which the protein was omitted.

Spectra were recorded at 20 °C from 250 to 184 nm at 0.2 nm resolution, 8 s response, and protein concentration was 10 μM in 50 mM Tris-HCl pH 7.0. All data are averages of two measures. Thermal denaturation of the protein was followed by recording the ellipticity at 220 nm as temperature was varied from 5 °C to 95 °C at a rate of 1 °C min^{-1}. Linear baselines were fitted above and below the transition zone, and the apparent fraction of molecules in the unfolded state (F_u) was derived from the experimental mean residue ellipticity according to the equation:

$$Fu = \frac{(y_n - y_{obs})}{(y_n - y_u)}$$

where y_n and y_u are the pre- and post-transitional baselines that are assumed to depend linearly on temperature:

- $y_n = y_n + m_n T$
- $y_u = y_u + m_u T$

The midpoint of thermal transition, T_m, was defined as the temperature at which the apparent fraction F_u is = 0.5.

Fluorescence spectroscopy

Fluorescence measurements were carried out on a Perkin Elmer LB50S fluorimeter in 10-mm cells with thermostatically controlled cell holder, and temperature was measured directly in the cuvette. The solutions were filtered just before use, and data were corrected by subtracting a control from which the enzyme was omitted. Native protein (0.45 μM as monomer concentration, in 50 mM Tris-HCl, NaCl 0.15 M, pH 7.5) was incubated at 25 °C in the presence of various concentrations of urea for 30 min before adding 1-anilinonapthalene-8-sulfonate (ANS) to a final concentration of 5 μM. Fluorescence emission spectra were collected, between 430 and 500 nm (emission slit 10 nm) with an excitation wavelength set at 395 nm (excitation slit 5 nm), and recorded as the average of two scans. Fluorescence at 460 nm was reported as a function of urea concentration.

Chaperone activity of *Ph*GroEL

Chaperone-mediated protein folding activity was performed in buffer A (0.2 M KH_2PO_4, 1.8% glycerol, 7.7 mM NaCl, 5.4 mM $MgCl_2$, 1.5 mM DTT, 10 mM EDTA). Porcine mitochondrial malate dehydrogenase [mMDH (Sigma)], was denatured at room temperature in 4.0 M GdmCl, 38 mM Tris-HCl pH 7.5, and 10 mM dithiothreitol (DTT) for 1 h. Refolding at 20 °C was initiated by 50-fold rapid dilution (to a subunit concentration of 87 nM) in buffer A containing 0.30 μM *Ph*GroEL or *Ec*GroEL. After 120 min, 5 mM ATP and 0.86 μM *Ec*GroES were added. Aliquots were taken at different time intervals from each refolding mixture and assayed for mMDH activity by diluting 8-fold into cuvettes containing 0.2 mM NADH and 0.6 mM oxaloacetate and measuring the rate of conversion of NADH to NAD^+ at 340 nm with a DU7500 Beckmann spectrophotometer.

ATPase assay

The malachite green phosphate assay developed to determine nanomolar amounts of inorganic phosphate (Lanzetta et al. 1979) was used to measure ATPase activity. Phosphate standard solutions were prepared to give a linear standard curve from 0 to 4.0 μM. One microgram of chaperone was incubated in 150 μl buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, and 1 mM ATP at the desired temperature. Aliquots of 50 μl were withdrawn at 20, 40, and 60 min, and phosphate content was determined by adding 850 μl of assay solution (prepared as in Lanzetta et al. 1979) and stopping the reaction after 1 min with 100 μl of 1.15 M sodium citrate. Absorbance was then recorded at 660 nm.

Polyacrylamide gel electrophoresis

Denaturing 12.5% PAGE was carried out in the presence of SDS according to Laemmli (1970). Nondenaturing PAGE was done at room temperature as above but without SDS.

Computer data fitting

Micromath Scientist for Windows was used to analyze the data.

Results and discussion

Setting up of temperature up-shift conditions to purify hsp from *Pseudoalteromonas haloplanktis* TAC 125

P. haloplanktis TAC 125 is a psychrotrophic bacterium able to grow below 4 °C, with an optimal growth temperature at about 20 °C (Tutino et al. 1999a).

Patterns of protein synthesis in "control" and heat-shocked cells were examined using [^{35}S]methionine and [^{35}S]cysteine labeling as described in the Materials and methods section. Changes in protein pattern were most pronounced in cell cultures subjected to heat shock at 37 °C for 30 min, probably also because of concomitant denaturation of several proteins. Moreover, this high temperature is in agreement with previous data on cold-adapted microorganisms that require a large up-shift of temperature to induce massive synthesis of hsps (Deegenaars and Watson 1997). Autoradiography of SDS-PAGE of the whole cell extract revealed that the organism responds to the heat shock by inducing the synthesis of a set of proteins. Several bands increased in intensity during shock treatment; the induction of the synthesis of five of them, of approximately 65, 55, 50, 15, and 14 kDa, is most evident (Fig. 1).

We focused our attention on the protein of about 55 kDa on the assumption that it was related to the class of hsp60. It was purified by taking advantage of the radioactivity incorporated during heat shock as described in the Materials and methods section; about 200 μg of pure protein was obtained from 15 g of wet cells of *Ph*TAC 125.

The homogeneity of protein was assessed by SDS-PAGE. Electrospray mass spectrometry revealed a single component having a molecular mass of 57,011.9 ± 4.3 kDa. N-terminal sequence of the 57-kDa electroblotted protein (double underlined sequence in Fig.2) was

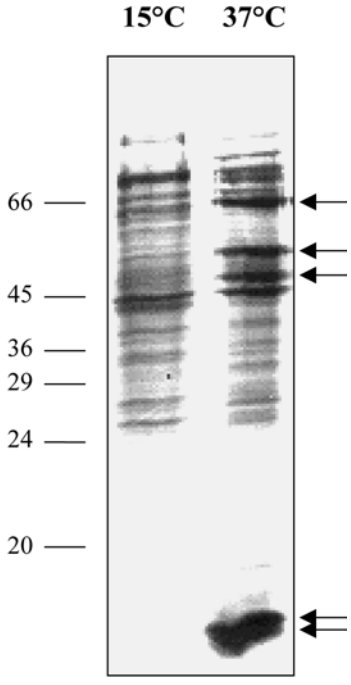

Fig. 1 Autoradiography of L-[^{35}S]methionine pulse-labeled total proteins of *Ph*TAC 125 at normal growth temperature (15 °C) and after 30 min of heat-shock exposure (30 °C). *Arrows* indicate some of the proteins induced by heat shock. Molecular weight standard positions (kDa) are indicated on the *left*

compared with the protein entries of Swiss Prot Data Bank and turned out to be 73.3% identical to the N-terminal sequence of GroEL from *E. coli*.

Some internal peptides of *Ph*GroEL, obtained by endoprotease LysC digestion, were sequenced (Fig. 2). The fragments to be sequenced were selected on the basis of a MALDI mapping experiment among those peptides with a mass that had no correspondence in the expected mapping of *Ec*GroEL.

Cloning of *PhgroEL* and *PhgroES*

Taking advantage of primary structure information, the sequence of the entire *PhgroE* operon was obtained by means of PCR and inverse PCR techniques. The nucleotide sequence obtained is shown in Fig. 2 with its translation. Analysis of the sequence revealed two ORFs separated by 48 nt. A complete ORF of 1,641 nt encodes a GroEL protein of 547 residues that exhibits 82% sequence identity to GroEL from *E. coli* (Hemmingsen et al. 1988). The expected molecular mass (57,012.4 kDa), calculated on the basis of the sequence reported in the figure, was in agreement with the value experimentally determined. A second ORF of 288 nt encodes a protein of 95 amino acids that appears to be a GroES homologue since it exhibits 71% sequence identity to GroES from *E.coli* (Hemmingsen et al. 1988).

The *PhgroES* and *PhgroEL* translational start codons (ATG) are preceded (10 nt) by putative ribosome-binding sites (AGGAG and AGAGG, respectively), while 30 nt after the *PhgroEL* stop codon there is a putative hairpin or stem-loop structure that can be involved in transcription termination. No potential promoter sequence was identified between the two genes, suggesting their organization in a bicistronic operon as found in most bacterial species (Segal and Ron 1996).

Codon usage within the two ORFs shows a marked bias for A or T in the wobble position, as already reported for the gene coding for the aspartate aminotransferase from the same organism (Tutino et al. 1999b) and in agreement with the frequencies observed for all the other genes from *Ph*TAC 125 so far identified, namely, the elongation factor Tu (EMBL accession number AJ249258), the thioredoxin (data to be published), and several gene fragments (Tutino and Duilio, personal communications).

The deduced sequence of *Ph*GroEL, confirmed by MALDI mapping experiments, shows considerable similarity to previously reported sequences from other organisms, as shown from the alignment of *Ph*GroEL to a consensus sequence (Fig.3) derived by Brocchieri and Karlin from a multiple alignment of 43 hsp60 sequences (Brocchieri and Karlin 2000). This hsp60 multiple alignment consists of sequences from α-, β-, γ-, and ϵ-proteobacteria; Rickettsiales; high and low G + C gram-positive bacteria; other bacterial sequences, including Cyanobacteria; and eukaryotic sequences, such as *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. In particular, there are 21 gram-negative sequences in the multiple alignment; thus, they represent 49% of the sequences aligned. This sequences analysis represents a valid tool for the comparison of *Ph*GroEL to known hsp60 sequences and for the integration and analysis of sequence, structure, and function information.

All the amino acids that make contact with ATP/ADP in *consensus* sequence are conserved in *Ph*GroEL; those amino acids interacting with GroES or the bound substrate that do not match the *consensus* sequence are nevertheless the same as those found in *Ec*GroEL (Brocchieri and Karlin 2000). A few substitutions with respect to both the *consensus* and *Ec*GroEL sequences can be observed among the residues at the inter-ring and within-ring contacts: in position 438 there is a histidine instead of a valine, but this position has quite a low conservation index (CI = 0.16, Brocchieri and Karlin 2000); a methionine can be found instead of a valine in position 387 (CI = 0.46, Brocchieri and Karlin 2000); an isoleucine substitutes the highly conserved valine 521 (CI = 0.85, Brocchieri and Karlin 2000) that is at the interface between the E domains of contiguous monomers within the same ring.

Transcriptional analysis of the *PhgroE* operon

The sequence of the entire regulatory region of the *PhgroE* operon was analyzed preliminarily for the

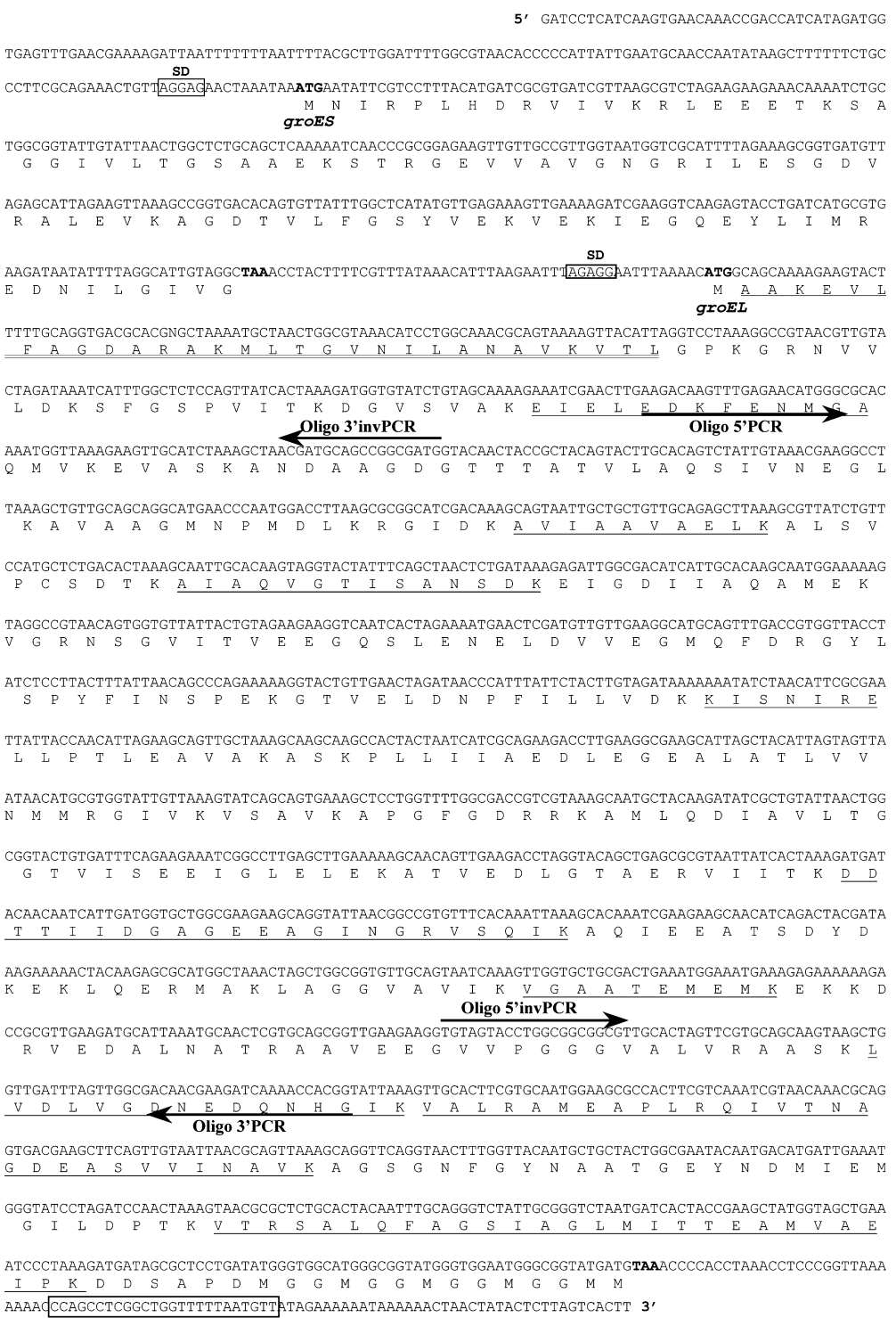

Fig. 2 Nucleotide and amino acid sequences of *Ph*GroEL and *Ph*GroES. Peptides sequenced are *underlined* (the N-terminal sequence is *double underlined*). Sequence of peptides used to design primers for the PCR amplification are shown with *arrows* below the amino acidic sequence (oligo 5'PCR and oligo 3'PCR), while oligonucleotides designed as primers for inverse PCR are indicated with *arrows* above the nucleotide sequence (oligo 5'invPCR and oligo 3'invPCR). Initiation and stop codons are in *bold*. Putative regions of ribosome binding site and palindromic sequences of transcriptional termination are *boxed*

presence of promoters (Neural Network Promoter: http://www.fruitfly.org/seq_tools/promoter.html), revealing the presence of four putative –10 and –35 regions (Fig. 4 C). When the same analysis was carried out on the regulatory region of the *E. coli groE* operon, only two putative promoter regions could be predicted, corresponding to the two regulative elements experimentally identified: a σ^{70}-dependent promoter and a σ^{32}-dependent promoter (Zhou et al. 1988). The latter plays a key role in *E. coli* heat-shock response, since it is recognized by the alternative factor σ^{32}, whose level dramatically increases during the first few minutes following heat shock (Yura et al. 1993).

A primer extension analysis was carried out in order to verify the functionality of identified promoters. Reaction was carried out on RNA samples isolated from cells permanently grown at different temperatures (4–15–20–25 °C) and from cells grown at 15 °C and shifted

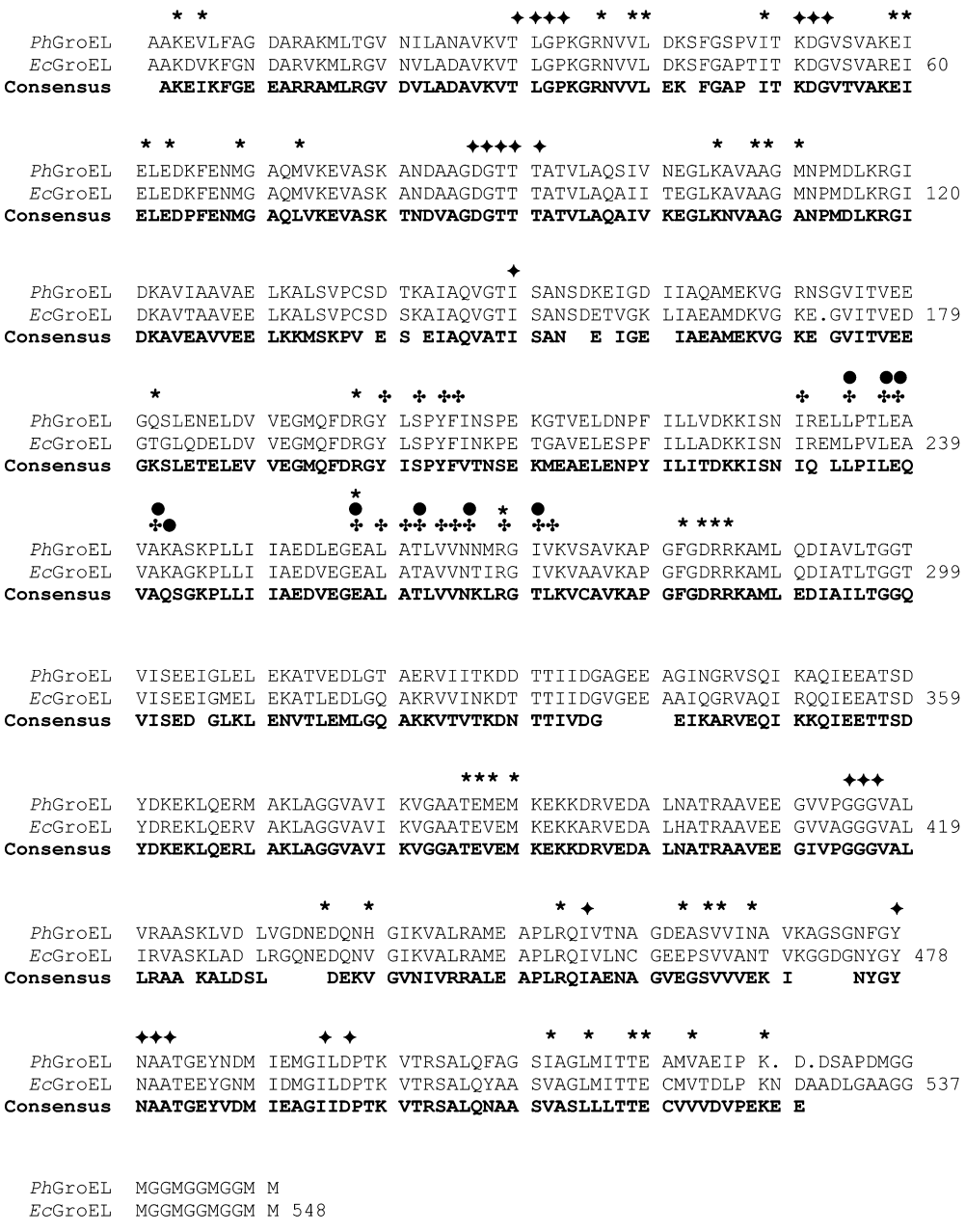

Fig. 3 Amino acid alignment between GroEL from *Pseudoalteromonas haloplanktis* TAC 125 (*Ph*GroEL), GroEL from *Escherichia coli* (*Ec*GroEL), and a consensus sequence of hsp60 proteins. *Diamond* Residues involved in ATP/ADP binding, *star* residues proposed to be involved in substrate binding, *filled circle* residues interacting with GroES, asterisk residues involved in intersubunits interactions. *Ec*GroEL numbering is indicated on the side

to 27 °C for 15 min (Fig. 4A, B), using an oligonucleotide complementary to the 5' end of *PhgroES*. At all of the temperature conditions tested, the same four transcriptional start sites are detected, at the –106, –67, –54, and –23 positions from the *PhgroES* start codon, corresponding to the promoters named P_1, P_2, P_3, and P_4, respectively (Fig. 4C). Promoter P_3 displays the characteristic sequence motifs of a heat-inducible, σ^{32}-dependent promoter; the –10 and –35 regions are, in fact, 87.5% and 69% similar to the corresponding regions in *E. coli* σ^{32}-dependent promoters, whereas promoter P_4 resembles the corresponding regions of the σ^{70}-dependent promoter in the *E. coli groE* operon (Hawley and McClure 1993) (Table1). Therefore, because of their higher efficiencies, P_3 and P_4 could represent the canonical promoters of the *PhgroE* operon. P_1 and P_2 promoters, corresponding to the two additional transcription start sites located at the –106 and –67 position, respectively, are evident only at exposure times longer than requested for visualizing the other signals (Fig. 4B) and show a low degree of similarity with the consensus σ^{70} promoters from *E. coli*. These two additional promoters might possibly represent typical transcriptional elements of cold-adapted bacteria. Further investigations are needed for the functional analysis of these promoter regions in *Ph*TAC125. As far as the other signals in the primer extension analysis are concerned, those between the two start points at the –23 and –54 positions (indicated in Fig. 4A by asterisks) do not correspond to any transcription start signal, but they can be attributed to the result of interferences of the reverse transcriptase with secondary structures located

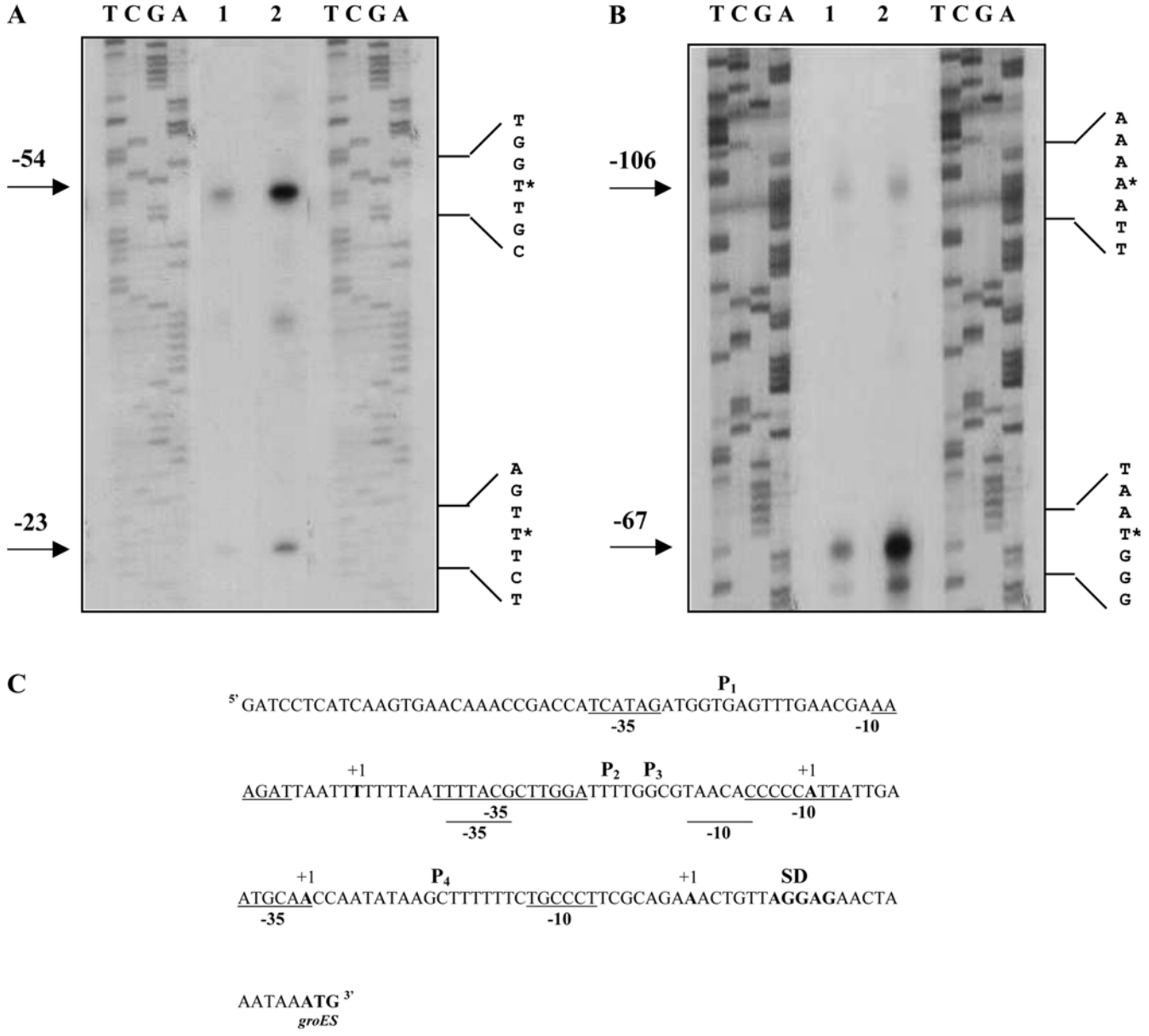

Fig. 4A–C Determination of *PhgroE* transcription start sites by primer extension. The PromRevGroE primer (5' CGCGATCA TGTAAAGGACG 3') was 5'end-labeled with (γ^{32}-P)-ATP and hybridized to 50 μg of total RNA from *Ph*TAC 125 cells grown at 15 °C (*lane 1*) and exposed at 27 °C for 3 min (*lane 2*), 6 min (*lane 3*), 10 min (*lane 4*), 15 min (*lane 5*), 25 min (*lane 6*), 40 min (*lane 7*), and 60 min (*lane 8*). The hybrids were then extended using reverse transcriptase and the lengths of the extension products were estimated by comparing mobility in a 6.0% polyacrylamide gel. *Lanes T*, *C*, *G*, and *A* indicate the dideoxy termination lanes. The signals corresponding to the transcription start sites were resolved by autoradiography after 4 h (**A**) and 40 h (**B**) of exposition. The transcription start sites are numbered according to the sequence of the 5' region of the *groE* genes shown in **C**. The –10 and –35 regions of the P_1, P_2, P_3, and P_4 are *underlined*. The Shine-Dalgarno sequence is indicated in *bold*

downstream ($^{5'}$TTTCTGCCCTTCGCAGAAA$^{3'}$, ^{5}TATTGAATGCAACCAATA$^{3'}$) or to specific processing of primary transcripts. The same pattern of signals is also obtained from primer extension experiments carried out after heat shock, thus indicating that stress conditions do not switch any of the signals on or off.

Moreover, transcripts relative to the promoter P_3 are always more abundant than transcripts relative to P_4, both during isothermal growths and after heat-shock treatments, suggesting a physiological function of P_3 and, therefore, of σ^{32} factor in all conditions, not only in response to heat shock. Nevertheless, this behavior is in agreement with data reported from transcriptional and genetic analysis of the *E. coli groE* operon, indicating that the σ^{32} factor is able to transcribe the *groE* heat-shock operon at all temperatures (Zhou et al 1988).

*Ph*GroEL characterization

The hsp60-like protein from *Ph*TAC 125 is structurally and functionally related to *Ec*GroEL. Gel-filtration

Table 1 Comparison of *Ph*TAC 125 and *Escherichia coli* putative promoter sequences located upstream of the *groE* operon

	-35 region		-10 region		Start site
*Ph*TAC125 σ^{32}-dep **P_3**	TTTTACGCTTGGA-	(14 bp)	-CCCCATTA-	(9 bp)	-A (-54)
E. coli σ^{32}-dep	TTTCCCCCTTGAA-	(15 bp)	-CCCCATTT-	(9 bp)	-C
E. coli σ^{32} consensus [a]	T-C-C-CTTGAA-	(13–15 bp)	-CCCCAT-T-		
*Ph*TAC125 σ^{70}-dep **P_4**	ATGCAA-	(18 bp)	-TGCCCT-	(7 bp)	-A (-23)
*Ph*TAC125 **P_2**	TTTACG-	(14 bp)	-TAACAC-	(4 bp)	-A (-67)
*Ph*TAC125 **P_1**	TCATAG-	(17 bp)	-AAAGAT-	(5 bp)	-T (-106)
E. coli σ^{70}-dep	TGGTCA-	(17 bp)	-TAAGCT-	(7 bp)	-C
E. coli σ^{70} consensus [a]	TTGACA-	(16–19 bp)	-TATAAT-		

[a] *E. coli* consensus sequences for σ^{32} and σ^{70} promoter region as reported in Hawley and McClure (1993)

chromatography analysis and far UV-CD spectra of *Ph*GroEL, in comparison to *Ec*GroEL, suggest similar quaternary and secondary structure organization for these two proteins (data not shown). *Ph*GroEL possesses a chaperone activity similar to that of *Ec*GroEL. In fact, it is able to bind unfolded mMDH (an often-used in vitro substrate of chaperones [Miller et al 1993]), inhibiting its spontaneous refolding when it is present alone and releasing it in a conformation committed to recover up to 93% of initial activity when Mg-ATP and *Ec*GroES are added (data not shown). This experiment also demonstrates that *Ec*GroES can replace *Ph*GroES as a co-chaperone of *Ph*GroEL. Moreover, the same kinetics and yield of reactivation of mMDH at 20 °C in the presence of *Ph*GroEL or *Ec*GroEL (with Mg-ATP and/or *Ec*GroES added) were observed. This indicates that there is no difference between the psychrophilic and mesophilic chaperones in efficiency as a folding helper.

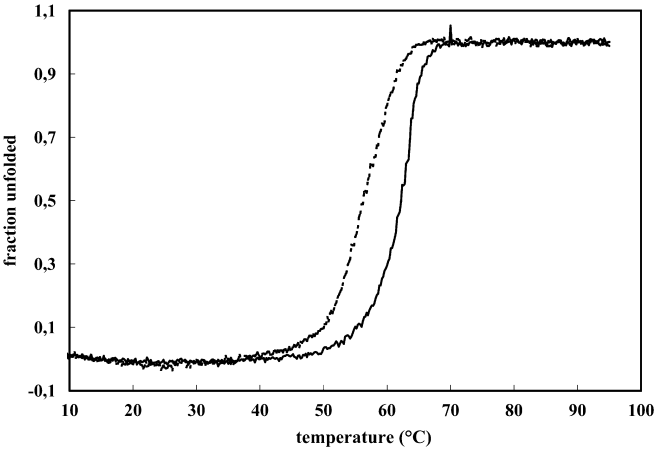

Fig. 5 Thermal unfolding of *Ph*GroEL (- - -) and *Ec*GroEL (- -). Enzyme concentration was 10 μM in 50 mM Tris-HCl pH 7.0. Thermal transitions were followed at 220 nm as temperature was varied at a rate of 1 °C min^{-1}. The fraction of unfolded protein was calculated from experimental data as described in the Materials and methods section

*Ph*GroEL stability

In order to compare the stability of the two chaperones, we evaluated structural changes induced by temperature variations and by the addition of a chaotropic agent such as urea. The temperature-induced unfolding was monitored following the loss of secondary structure by means of peptide ellipticity at 220 nm (Fig. 5). The midpoint of thermal transition (T_m) was 56 °C, as compared to 62 °C for *Ec*GroEL in the same conditions. A psychrophilic protein with such a relatively high unfolding temperature may not be suprising if the physiological role of this chaperone as a hsp is taken into account.

The urea-induced unfolding transition of both *Ph*GroEL and *Ec*GroEL proteins was followed by monitoring of the exposure of hydrophobic surfaces as a function of urea concentration, using the extrinsic fluorescence probe ANS. Similar experiments, carried out with bis-ANS for *Ec*GroEL, showed that increasing urea concentration induces a two-phase unfolding process: the dissociation of the 14-mer followed by the unfolding of the resulted monomers are indicated by an increase followed by a decrease in the bis-ANS fluorescence intensity, thus suggesting that organized hydrophobic surfaces are first exposed and then disrupted (Mendoza et al 1994). The same behavior can be observed for *Ph*GroEL, but at a lower concentration of urea in comparison to *Ec*GroEL, as shown in Fig. 6 A. The peak of ANS fluorescence occurs at 2.25 M urea for the psychrotrophic protein and at 3 M urea for the mesophilic one.

Native gel electrophoresis analysis (Fig. 6B) confirms the lower stability of the homotetradecameric structure of *Ph*GroEL, showing the appearance of the monomeric species of *Ph*GroEL at 2.0 M urea while the monomeric *Ec*GroEL is detected only at 2.75 M urea. No species of intermediate oligomeric state can be detected in this experiment, as already reported for *Ec*GroEL (Mendoza et al 1994). This suggests a highly cooperative transition under these conditions and the likely instability of native-like monomers, which unfold during the gel electrophoresis run.

The shift of urea transitions to lower concentrations for *Ph*GroEL could indicate a more-flexible structure for the psychrotrophic protein that has to overcome the low temperature constraints.

The most significant amino acid substitutions in *Ph*GroEL that could account for the difference in stability between the two proteins seem to be in hydrophobic inter-monomer contacts, namely, in positions 387, 438, and 521 (Fig. 3). Moreover, it seems to be

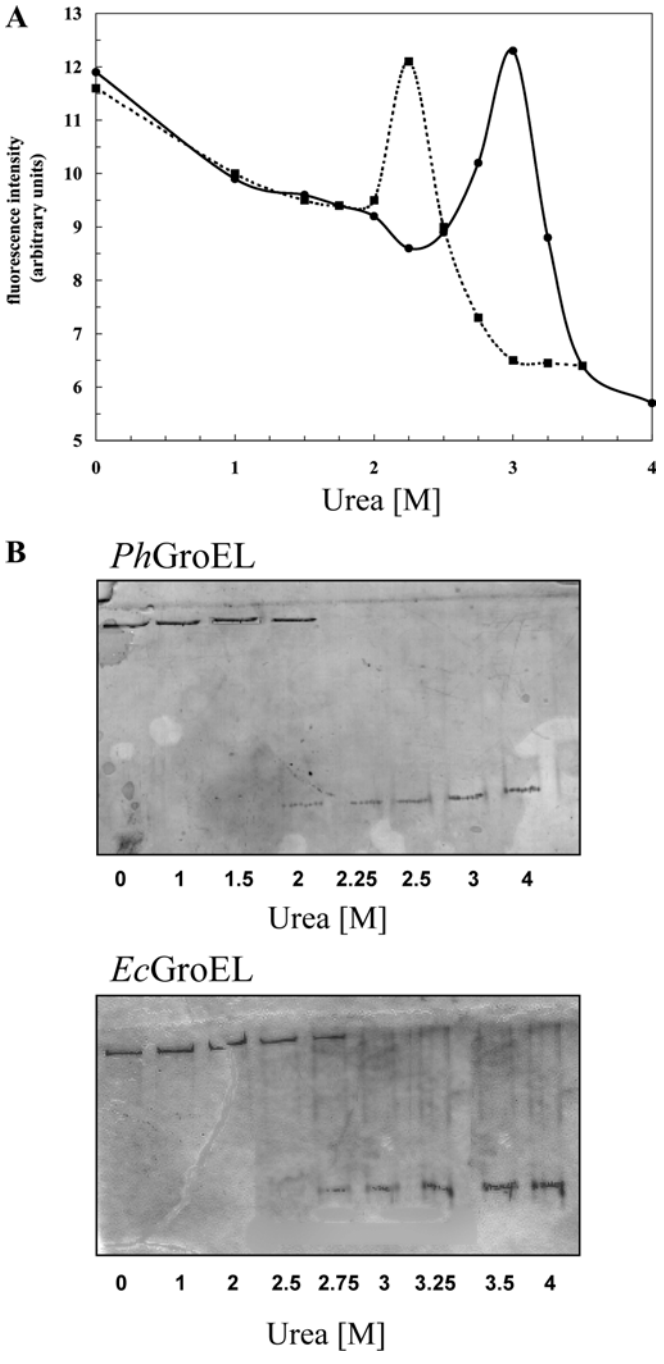

Fig. 6A, B Urea-induced unfolding transition of *Ph*GroEL and *Ec*GroEL. Protein samples, 0.45 μM monomer concentration in 50 mM Tris-HCl, NaCl 0.15 M, pH 7.5, were incubated at 25 °C for 30 min in the presence of various concentrations of urea and analyzed (**A**) by extrinsic fluorescence emission at 460 nm (exc. at 395 nm) in the presence of ANS 5 μM and (**B**) by nondenaturing gel electrophoresis

worth mentioning the substitution in position 518, where an alanine replaces the cysteine of the *E. coli* sequence, since mutagenesis of Cys 518 in *Ec*GroEL has been shown to affect the stability of quaternary structure without influencing its function (Luo and Horowitz 1994).

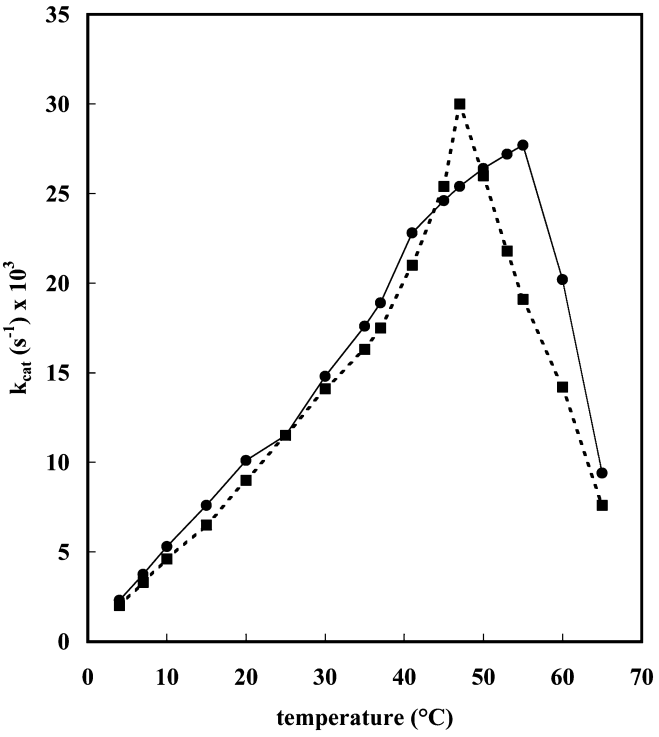

Fig. 7 Temperature dependence of ATPase activity. k_{cat} for the ATPase activity of GroEL from *Pseudoalteromonas haloplanktis* TAC 125 (*filled square*) and from *Escherichia coli* (*filled circle*) were determined at increasing temperature as described in the Materials and methods section

ATPase activity

ATPase activity for both *Ph*GroEL and *Ec*GroEL was measured in the range of 5–65 °C (Fig. 7). The k_{cat} values determined for *Ph*GroEL are comparable to those of *Ec*GroEL, and the only difference observed is that the maximum of activity of *Ph*GroEL occurs at 47 °C, some 8 °C lower than for the mesophilic chaperone. Therefore, in seeking adaptation to cold, *Ph*GroEL very slightly shifts the curve of activity as a function of temperature toward low temperatures, in agreement with the behavior usually observed for proteins isolated from cold-adapted microorganisms with respect to mesophilic ones. Nevertheless, it is worth noting that at low temperatures catalytic efficiency is not higher than for *Ec*GroEL, as would have been expected for an axiom of psychrophilic enzymes (Feller et al. 1996; Gerday et al. 2000). However, the activity of aspartate aminotransferase from *Ph*TAC125 is always lower than that of the mesophilic enzyme from *E. coli* in the whole temperature range 0–30 °C (Birolo et al. 2000), and similar results have been also reported for the elongation factor Tu from the same organism (Masullo et al. 2000). This suggests that, at least in this organism, the adaptation to life in the cold does not necessary imply a higher catalytic efficiency at low temperatures for all the enzymatic mechanisms.

Conclusions

We have characterized a heat shock protein from the psychrotrophic bacterium *Pseudoalteromonas haloplanktis* TAC 125, which turned out to be a GroEL-like protein. The primary structure of the protein from the cold-adapted bacterium was shown to share a high degree of identity with the mesophilic GroEL from *E. coli* (82%). This result is not unexpected, since chaperones belong to a very highly conserved class of proteins; furthermore, the proteins of *Ph*TAC125 so far characterized show a high percentage of residues identical to the corresponding gene products of *E. coli*.

The melting temperature of the psychrotrophic GroEL is 6 °C lower than that of the mesophilic one, and the maximum of ATPase activity of *Ph*GroEL is also 8 °C lower than that of *Ec*GroEL. These data are in agreement with the general tendency of psychrophilic proteins to optimally shift their function to lower temperatures. However, the not-so-impressive difference suggests that *Ph*GroEL is not one of those classically "cold-adapted" proteins for which an improvement in physiological efficiency through optimization of the catalytic parameters is claimed to compensate for the reduction of reaction rates induced by low temperatures. Moreover, the rather marginal lower stability of the psychrophilic GroEL could well arise from a lack of selective pressure toward thermostability rather than from specific adaptation needs. Similarly, the aspartate aminotransferase form *Ph*TAC 125 does not exhibit increased catalytic efficiency at low temperature and reduced thermostability (Birolo et al. 2000), nor do the elongation factor Tu (Masullo et al. 2000) or the thioredoxin from the same organism (data to be published). Should we conclude that the proteins in *Ph*TAC 125 are not cold-adapted? If our answer is positive, then we should ask ourselves how *Ph*TAC 125 managed to be among the best cold-adapted bacteria in terms of duplication times at low temperatures and range of growth temperature (Tutino et al. 1999a, where *Ph*TAC 125 is named *Moraxella* TAC 125). It seems to be a paradox; however, it is interesting to observe that the usually claimed rules of protein cold adaptation are derived from the characterization of enzymes that in several cases were purified from organisms specifically selected to fulfill biotechnological requests, namely, high catalytic performances. This is, for instance, the case of β-galactosidase from *P. haloplanktis* TAE 79, a strain that was selected after a screening of about 300 bacterial isolates collected in Antarctica for the highest intracellular β-galactosidase activity (Hoyoux et al. 2000). This is also the case of cold-adapted lipases (Feller et al. 1989) and of the alkaline metalloprotease from a psychrophilic *Pseudomonas* species (Chessa et al. 2000). We wonder whether a selective pressure seeking for biotechnological performances could have forced the interpretation of general rules of protein cold adaptation. Another working hypothesis is that the adaptation of extracellular proteins (such as lipases, α-amylase, proteases) can differ from that of intracellular proteins (such as GroEL, aspartate aminotransferase, thioredoxin, and the elongation factor Tu from *Ph*TAC 125), showing less pronounced molecular adaptations that nevertheless allow appropriate metabolic fluxes at the environmental cell working temperatures. The set of available data is not, of course, large enough to formulate a consistent answer. However, one can also guess that many of the characteristics of an organism are determined more by the regulative features of gene expression than by only the protein's structural aspects. In these respects, the complexity of the promoter region of *PhGroESL* operon would suggest such a hypothesis.

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