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ORIGINAL RESEARCH PAPER

Cold adaptation of a psychrophilic chaperonin from *Psychrobacter* sp. and its application for heterologous protein expression

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Abstract
Objectives A chaperonin, *Psy*GroELS, from the Antarctic psychrophilic bacterium *Psychrobacter* sp. PAMC21119, was examined for its role in cold adaptation when expressed in a mesophilic *Escherichia coli* strain.
Results Growth of *E. coli* harboring *Psy*GroELS at 10 °C was increased compared to the control strain. A co-expression system using *Psy*GroELS was developed to increase productivity of the psychrophilic enzyme *Psy*Est9. *PsyEst9* was cloned and expressed using three *E. coli* variants that co-expressed GroELS from PAMC21119, *E. coli*, or *Oleispira antarctica* RB8^T. Co-expression with *Psy*GroELS was more effective for the production of PsyEst9 compared tothe other chaperonins.
Conclusion *Psy*GroELS confers cold tolerance to *E. coli,* and shows potential as an effective co-expression system for the stable production of psychrophilic proteins.

Keywords Chaperonin · Cold adaptation · Esterase · GroEL · *Psychrobacter* sp. · Psychrophilic bacterium

Introduction

The chaperonin GroELS system is formed by the chaperone GroEL and its co-chaperone GroES. It plays a major role in cell growth and viability. *Escherichia coli* GroELS has been extensively studied and its functional structure and physiological role have been revealed (Dahiya and Chaudhuri 2014; Georgescauld et al. 2014; Koike-Takeshita et al. 2014; Tyagi et al. 2009; Warnecke and Hurst 2010; Weber and Pande 2013). Its chaperone activity is conferred via the double-ring-shaped oligomeric 14-mer chaperonin GroEL, which encapsulates a substrate polypeptide, and the heptameric GroES caps the central cavity to facilitate correct folding of the target protein. This molecular chaperone activity for protein folding is the primary role of the GroELS system in a cell; however, GroEL also has additional functions, including cell-to-cell adherence (Hennequin et al. 2001; Kupper et al. 2014).

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Psychrophilic microorganisms with physiological adaptation to low temperatures colonize all permanently cold environments from the deep sea to mountain and polar regions (D'Amico et al. 2006). The cold-adapted enzymes from psychrophiles have been targeted for their biotechnological potentials due to high activity at low temperature and heat lability enabling heat heat inactivation (Cavicchioli et al. 2011). In bacteria, the overproduction of GroELS confers physiological tolerance to unusual environmental conditions such as thermal and chemical stresses. When transformed in *E. coli,* the GroELS system from the psychrophilic bacterium *Oleispira antarctica* RB8^T promoted cell growth at its low-temperature growth limit (Ferrer et al. 2003). This cold adaptation of *E. coli* is useful for the heterologous expression of cold-adapted enzymes because of its improved cultivation at low temperature and the heat-labile property of the target protein (Kolaj et al. 2009; Yan et al. 2012; Yoshimune et al. 2004; Zhang et al. 2013). To date, co-expression systems using GroELS have focused on those of *E. coli* and *O. antarctica* RB8^T. In this study, we investigated the cold-adaptation abilities and in vivo chaperone activity of a psychrophilic GroELS from *Psychrobacter* sp. PAMC21119 that was isolated from Antarctica.

Materials and methods

Construction of the plasmid vector with *Psy*GroELS and bacterial strains

Psychrobacter sp. strain PAMC21119 isolated from Antarctica (62°13′S, 58°47′W) was used as a donor of the psychrophilic *GroELS* gene (Kim et al. 2012). The plasmid pACYCDuet-1 (Novagen) was used for the *Psy*GroELS co-expression system. *PsyGroEL*(GenBank accession no. KP250986) and *PsyGroES*(GenBank accession no. KP250987) were amplified from genomic DNA by PCR using the specific primer pairs EL/f (5′-ATAAGAAGGAGATATACATATGGCAAAAGACGTAAAATTCGGCA-3′) and EL/r (5′-CCAATTGAGATCTGCCATATGTTACATCATGCCGCCCATTCCACCCA-3′); and ES/f (5′-CTTTAATAAGGAGATATACCATGAATATTCGTCCTTTACATGATCG-3′) and ES/r (5′-TGGTGATGGCTGCTGCCCATGGCTAGCCTTCTAGCACACCCAATACATC-3′), and cloned into the *Nde*I and *Nco*I sites (underlined) of the pACYCDuet-1 plasmid, respectively. Cloning was performed by the one-step sequence- and ligation-independent cloning method (Jeong et al. 2012). The resulting plasmid, designated p*Psy*GroELS, was used for cold-adaptation of *E. coli* and co-expression of the target protein, the esterase *Psy*Est. The plasmid pGro7 (Takara, Japan) with a mesophilic *GroELS* gene from *E. coli* and ArticExpress (DE3) (*E. coli* B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r*gal* λ (DE3) *endA* Hte [*cpn10 cpn60* Gent^r]) competent cells (Agilent Technologies) expressing a cold-adapted chaperonin were used in this study (Table 1). *E. coli* DH5α and BL21(DE3) (F$^-$ *ompT-galdcmlonhsd*S_B(r_B^- m_B^-) λ (DE3 [*lac*I *lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5])) strains were used as the hosts for cloning and co-expression of GroELS and the psychrophilic target protein, respectively.

Cloning of the *PsyEst9* gene in the expression vector

The *PsyEst9* gene was annotated with the help of the RAST annotation server using the draft genome sequence of PAMC21119 (Kim et al. 2012). *PsyEst9* (GenBank accession no. P143767) was amplified from genomic DNA, and a pair of primers was designed for PCR: Est/f 5′-GGTGCCGCGCGGCAGCCATATGGCTAGCGAAGTCAGCTTATCAGAAAC-3′ (with *Nhe*I restriction site underlined), Est/r5′-GGTGGTGCTCGAGTGCGGCCGCAAGCTTTTACGAACAGTCTTTATAAT-3′ (with the *Hin*dIII restriction site underlined). The expression vector pET28a was linearized by *Nde*I and *Hin*dIII restriction sites, and then amplified *PsyEst9* was cloned into the plasmid vector, resulting in pPsyEst9.

Heterologous co-expression and protein purification

The plasmid pPsyEst9 was transformed into the hosts *E. coli* BL21(DE3)or ArcticExpress (DE3) with the chaperonin plasmid p*Psy*GroELS or pGro7, respectively, for protein expression. The resulting recombinant strains were grown in 2 lLB broth (1 % Bactotryptone, 0.5 % yeast extract, 1 % NaCl) containing chloramphenicol (50 mg/l) and kanamycin (25 mg/l) at 37 °C. Induction of the recombinant proteins was as follows. When the OD_{600} reached 0.6, IPTG was added to give 0.1 mM, and the temperature was

Table 1 List of the *E. coli* variants with chaperonin GroELS

Name	Host/chaperone plasmid	Replication origin of plasmids	Promoter for chaperone production	Source
*Psy*GroELS	BL21(DE3)/p*Psy*GroELS	pACYC	T7	This study
EscGroELS	BL21(DE3)/pGro7	pACYC	araB	Takara
OleGroELS	ArticExpress(DE3)	pACYC	Constitutive	Agilent technologies

reduced to 10–30 °C. After 1–2.5 days of cultivation, cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C, and frozen at −80 °C until the next experiment. Cell pellets were suspended in 20 mM Tris/HCl buffer (pH8.0) and disrupted by sonication. Cell debris was separated by centrifugation at 16,000*g* for 20 min at 4 °C. To purify the recombinant PsyEst9 s, the soluble proteins were recovered from the cell extract of the IPTG-induced recombinant cells. All of the recombinant proteins were fused with His-tag peptide at the *N*-terminal region. Purification of the recombinant proteins was carried out by Ni-affinity chromatography on a His Trap HP column connected to an FPLC ÄKTA system. The soluble proteins were loaded on a Ni-affinity column, which had already been equilibrated with buffer A (20 mM Tris/HCl, pH8.0, 500 mM NaCl, and 10 mM imidazole). The target proteins were eluted with buffer B (20 mM Tris/HCl, pH8.0, 500 mM NaCl, and 400 mM imidazole). Fractions showing lipase activity (see below) were combined and concentrated by Vivaspin (Sartorius Stedim Biotech; cutoff, 10,000 kDa). The concentrated sample was applied on a gel filtration column packed with Superdex 75 prep grade equilibrated and eluted with 0.15 M NaCl in 20 mM Tris/HCl buffer (pH8.0). The active fractions from the gel filtration column were pooled, concentrated and equilibrated with 20 mM Tris/HCl buffer (pH 8.0). Protein purity was assessed by SDS-PAGE on a 12 % separating gel. The gels were stained with 0.1 % Coomassie Blue R-250 (in methanol/acetic acid/water, 40:10:50, by vol) followed by destaining with methanol/acetic acid/water.

Enzyme assay

General lipase activity was measured spectrophotometrically using *p*-nitrophenyl caprylate (PNP)-C8. The reaction mixture consisted of 100 μM PNP-substrate, 4 % (v/v) ethanol, 20 mM Tris/HCl buffer (pH8.0), and an appropriate amount of enzyme solution. The enzymatic reaction was performed at 20 °C, and one unit of activity was defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol per min under the assay conditions.

Results and discussion

Molecular analysis and in vivo expression of *Psy*GroELS

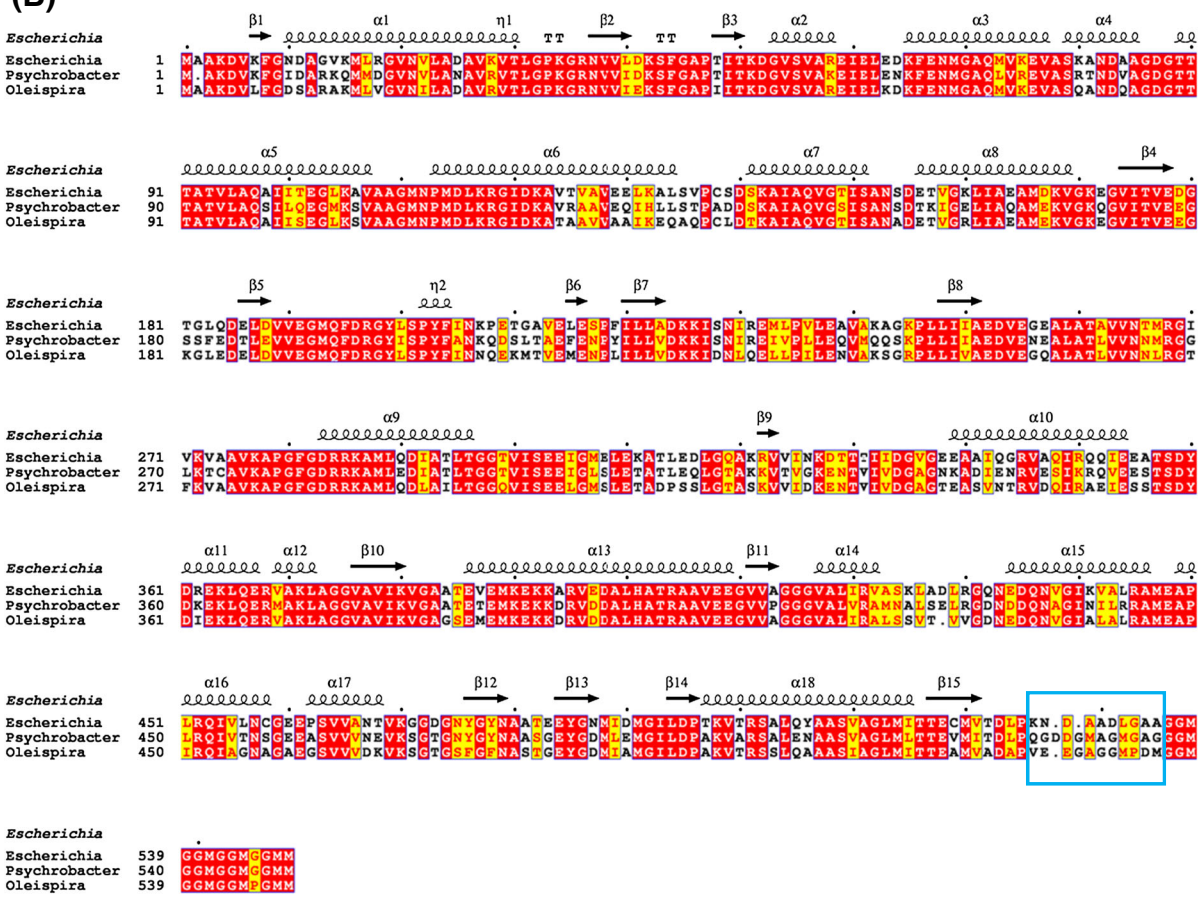
Psychrobacter species are capable of growth at −10 °C to 42 °C and have frequently been isolated from various cold environments, including Antarctic sea ice, sediments, deep seawater, and permafrost. *Psychrobacter* sp. PAMC21119 was isolated from permafrost soil of Antarctica. Its genome has been sequenced (Kim et al. 2012). The chaperonin *Psy*GroEL showed 73 and 70 % sequence homology with *Esc*GroEL from the mesophilic *E. coli* and *Ole*GroEL from the psychrophilic *O. antarctica* RB8^T, respectively (Fig. 1a). There was relatively high primary amino acid sequence identity throughout the sequence, with the exception of the variable *C*-terminal region (residues 526–535 of *Esc*GroEL). This variable region is located inside of the central cavity, which has not been clearly defined in X-ray crystallographic studies because of its flexibility (Clare et al. 2012; Xu et al. 1997). In a functional experiment, Machida et al. (2008) showed that hydrophilicity in the flexible *C*-terminal region is important for proper protein folding within the GroEL cavity; *Psy*GroEL also contains a hydrophilic sequence in the region (Fig. 1).

We evaluated the cold adaptation of recombinant *E. coli* host cells expressing *Psy*GroELS by examining growth at 10 °C. In a 1 l jar fermentor, *E. coli* BL21 (DE3) harboring the plasmid p*Psy*GroELS showed a growth rate approximately twice that of the control strain harboring the plasmid without the inserted gene (Fig. 2).This result suggests that *Psy*GroELS has a

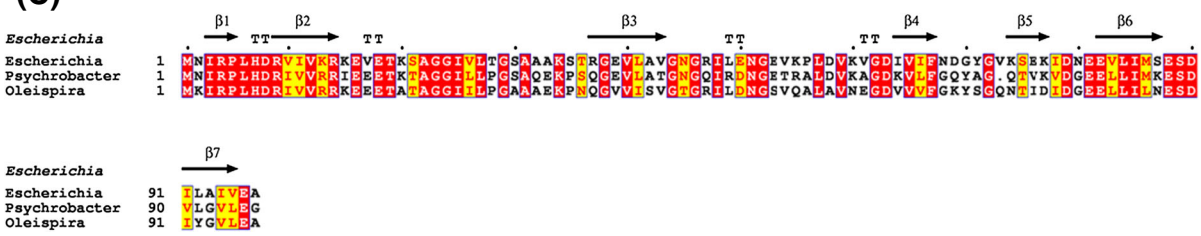
(A)

Identity (%)	PsyGroEL/ES	EcoGroEL/ES	OleGroEL/ES
PsyGroEL/ES	100/100		
EcoGroEL/ES	73/56	100/100	
OleGroEL/ES	70/64	74/55	100/100

(B)



(C)



◀**Fig. 1** The sequence identity matrix based on alignment (**a**). Multi-alignment of the amino acid sequences of three types of GroELs (**b**) and GroESs (**c**). The sequences were aligned with CLUSTALW and displayed in espript along with secondary structure assignments for EscGroELS (PDB ID code: 1GRL, 1AON). Aligned sequences include EcoGroEL (GenBank ID: AAS75782.1), PsyGroEL (GenBank ID: KP250987), OleGroEL (GenBank ID: CCK77137.1), EcoGroES (GenBank ID: ADN49087.1), PsyGroES (GenBank ID: KP250987), and OleGroES (GenBank ID: CAD43723.1). The variable *C*-terminal region of GroEL is highlighted by a *blue box*

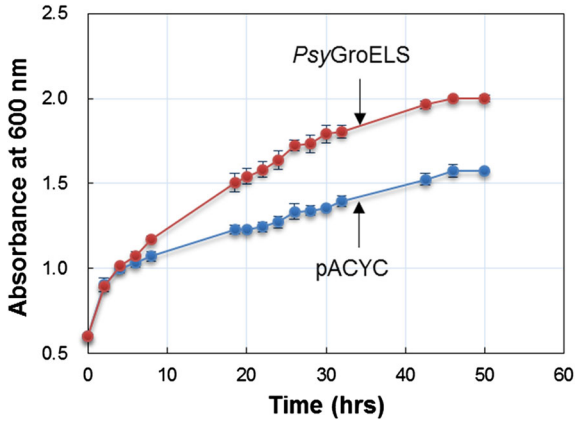


Fig. 2 Comparison of cell growth at 10 °C. Growth of *E. coli* BL21(DE3)/pACYC (*blue circle*) and PsyGroELS (*red circle*) was determined in a 1 l jar fermentor (Biostat Q, Sartorius Stedim Biotech) using LB broth supplemented with the corresponding antibiotic and IPTG. Inocula for the fermenter culture were grown for 1 day on 100 ml of the same media without the inducer. The DO set-point was 30 % air saturation under atmospheric pressure. The pH value was not controlled in the fermentation

physiological role in the cold adaptation of mesophilic *E. coli* cells, as shown in previous studies. In particular, the heterologous expression of psychrophilic GroELS originating from *O. antarctica* RB8^T in *E. coli* was able to enhance growth at low temperature (Ferrer et al. 2003).Furthermore, the flexible *C*-terminal region of GroEL was found to be important in the activity at reaction temperature (Nakamura et al. 2004). Together, these results suggest that the nonconserved *C*-terminal region of *Psy*GroEL may play a role in the cold adaptation of the host.

Co-expression of chaperone and psychrophilic esterase from PAMC21119

The GroELS co-expression system is useful for producing a soluble and active protein in *E. coli* due to the chaperone's activity in assisting protein folding (Kolaj et al. 2009). However, it is generally difficult to express a psychrophilic enzyme in a mesophilic host such as *E. coli* because of its low stability and highly flexible protein structure. Given that *Psy*GroELS had a psychrotolerant role in the mesophilic host, we further evaluated whether this cold-adapted chaperone may be useful for the production of a psychrophilic protein under low-temperature conditions in the *E. coli* expression system. We focused on the esterase gene *PsyEst9* originating from the PAMC21119 strain. A BLAST search showed the PsyEst9 had 82 % amino acid identity with a psychrophilic lipase from *Moraxella* sp. TA114 (GenBank accession no. P19833) (Feller et al. 1990), and 46 % identity with a lipase from the thermophile *Thermobifida fusca* (GenBank accession no. EOR72035.1). When PsyEst9 was expressed in *E. coli* BL21(DE3), the recombinant protein was mainly produced as an inclusion body with protein aggregates, with an insignificant portion in active soluble form. Therefore, this enzyme was a suitable model for evaluating the production efficiency of an active psychrophilic enzyme using a chaperone co-expression system.

Three *E. coli* variants with different GroELSs (*Psy*GroELS, *Esc*GroELS, and *Ole*GroELS) were prepared. Only the *Psy*GroELS co-expression system was more effective for production of active *Psy*Est9 at low temperature (10 °C) compared to the strain without the chaperonin, whereas the *Psy*Est9 produced from the other *E. coli* variants showed reduced activities in the cell lysate compared to the control. The enzyme activity was similar to that of the control strain at 30 °C (Fig. 3). This result demonstrates that *Psy*Est9 shows different solubility and productivity when expressed in *E. coli* variants depending on the kind of GroELS in the co-expression system, and that *Psy*GroELS is most useful for the production of this psychrophilic enzyme.

Biochemical properties of the *Psy*Est9 produced from various chaperonins

When the *Psy*Est9 was purified, the enzymes from all variants were found together with GroEL, even after the gel filtration step, suggesting that *Psy*Est9 was entrapped in GroEL (Fig. 4a). We tried to separate the *Psy*Est9 and GroEL using a high salt, mild detergent, and ATP treatment but were not successful. The

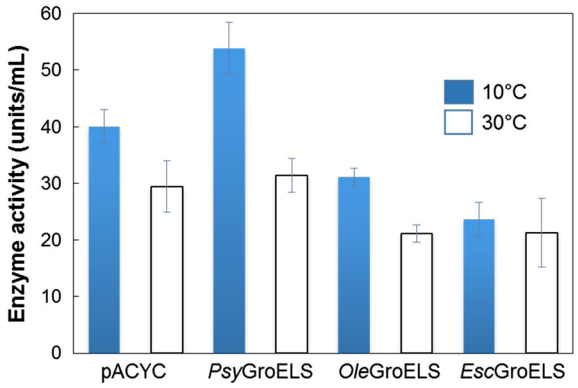


Fig. 3 Influence of the GroELSs co-expression system on the production of PsyEst9 at different temperatures (10 and 30 °C). Enzyme activity was determined with reference tolysate using an equal cell density. The enzyme reaction was performed for 15 min at 20 °C and the increase in p-nitrophenol was determined at 405 nm

PsyEst9 amino acid sequence was annotated as lipase with a BLAST search. However, PsyEst9 showed the highest activity against PNP with mid chain-length fatty acids (PNP-C8) compared to the other PNP substrates tested (PNP-C4, PNP-C10, and PNP-C12), suggesting that this enzyme is in fact an esterase rather than a lipase (data not shown). The properties of the enzymes produced from the three *E. coli* variants (*Psy*Est9/pACYC, *Psy*Est9/*Psy*GroELS, and *Psy*Est9/*Ole*GroELS) were compared. All enzymes had an optimal temperature of 10–20 °C, optimal pH of ~9, and showed about 60 % activity, even at 5 °C, suggesting that *Psy*Est9 is a psychrophilic enzyme. Despite these similarities, the temperature- and pH-dependent activity profiles (Fig. 4b, c) revealed different biochemical properties of the purified enzymes depending on the type of GroELS used for chaperonin co-expression. The enzyme from the *Psy*Est9/*Psy*GroELS system had relatively higher activity at > 20 °C than the others. This suggests that *Psy*GroELS chaperonin-mediated folding of *Psy*Est9 may be more correct and efficient than in the other GroELS co-expression systems.

*Psy*GroELS and *Psy*Est9 both originated from the same strain, PAMC21119. Active *Psy*Est9 production was enhanced by the chaperonin *Psy*GroELS, suggesting that protein folding was mediated by the chaperone activity of GroELS. However, the recombinant active *Psy*Est9 expressed by GroELS existed as an entrapped form in GroEL; the reason for this

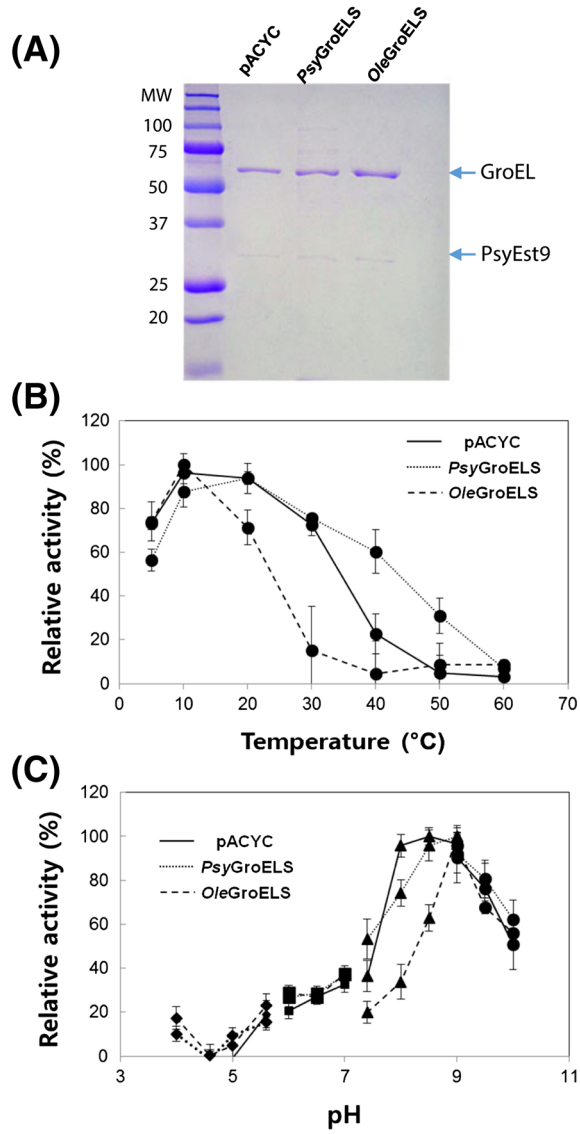


Fig. 4 Enzymatic properties of PsyEst9 s produced by the E. coli variants with pACYC (*solid line*), PsyGroELS (*dotted line*), or OleGroELS (*dashed line*). The purified PsyEst9 s were analyzed by SDS-PAGE (**a**). Effects of temperature (**b**) and pH (**c**) on enzyme activity. In the pH-dependent profile, the following buffers were used: sodium acetate, pH 4–5.6 (*diamonds*); phosphate buffered saline, pH 5.2–7 (*rectangles*); Tris/HCl, pH 7.2–9 (*triangles*); glycine/NaOH, pH 9–10 (*circles*)

phenomenon is not well understood and requires further study. Overall, we demonstrated that *Psy*GroELS could favor the production and correct protein folding of the psychrophilic enzyme *Psy*Est9. To further verify the applicability of a co-expression

system using *Psy*GroELS, the chaperone mechanism of *Psy*GroELS at low temperature should be characterized.

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References

Cavicchioli R, Charlton T, Ertan H, Mohd Omar S, Siddiqui KS, Williams TJ (2011) Biotechnological uses of enzymes from psychrophiles. Microb Biotechnol 4:449–460

Clare DK, Vasishtan S, Stagg S, Quispe J, Farr GW, Topf M, Horwich AL, Saibil HR (2012) ATP-triggered conformational changes delineate substrate-binding and -folding mechanics of the GroEL chaperonin. Cell 149:113–123

Dahiya V, Chaudhuri TK (2014) Chaperones GroEL/GroES accelerate the refolding of a multidomain protein through modulating on-pathway intermediates. J Biol Chem 289:286–298

D'Amico S, Collins T, Marx JC, Feller G, Gerday C (2006) Psychrophilic microorganisms: challenges for life. EMBO Rep 7:385–389

Feller G, Thiry M, Gerday C (1990) Sequence of a lipase gene from the antarctic psychrotroph *Moraxella* TA144. Nucleic Acids Res 18:6431

Ferrer M, Chernikova TN, Yakimov MM, Golyshin PN, Timmis KN (2003) Chaperonins govern growth of *Escherichia coli* at low temperatures. Nat Biotech 21:1266–1267

Georgescauld F, Popova K, Gupta AJ, Bracher A, Engen JR, Hayer-Hartl M, Hartl FU (2014) GroEL/ES chaperonin modulates the mechanism and accelerates the rate of TIM-barrel domain folding. Cell 157:922–934

Hennequin C, Porcheray F, Waligora-Dupriet A, Collignon A, Barc M, Bourlioux P, Karjalainen T (2001) GroEL (Hsp60) of *Clostridium difficile* is involved in cell adherence. Microbiology 147:87–96

Jeong JY, Yim HS, Ryu JY, Lee HS, Lee JH, Seen DS, Kang SG (2012) One-step sequence- and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies. Appl Environ Microbiol 78:5440–5443

Kim SJ, Shin SC, Hong SG, Lee YM, Choi IG, Park H (2012) Genome sequence of a novel member of the genus *Psychrobacter* isolated from Antarctic soil. J Bacteriol 194:2403

Koike-Takeshita A, Arakawa T, Taguchi H, Shimamura T (2014) Crystal structure of a symmetric football-shaped GroEL:-GroES-ATP complex determined at 3.8A reveals rearrangement between two GroEL rings. J Mol Biol 426:3634–3641

Kolaj O, Spada S, Robin S, Wall JG (2009) Use of folding modulators to improve heterologous protein production in *Escherichia coli*. Microb Cell Fact 8:9

Kupper M, Gupta SK, Feldhaar H, Gross R (2014) Versatile roles of the chaperonin GroEL in microorganism-insect interactions. FEMS Microbiol Lett 353:1–10

Machida K, Kono-Okada A, Hongo K, Mizobata T, Kawata Y (2008) Hydrophilic residues 526 KNDAAD 531 in the flexible C-terminal region of the chaperonin GroEL are critical for substrate protein folding within the central cavity. J Biol Chem 283:6886–6896

Nakamura T, Tanaka M, Maruyama A, Higashi Y, Kurusu Y (2004) A nonconserved carboxy-terminal segment of GroEL contributes to reaction temperature. Biosci Biotechnol Biochem 68:2498–2504

Tyagi NK, Fenton WA, Horwich AL (2009) GroEL/GroES cycling: ATP binds to an open ring before substrate protein favoring protein binding and production of the native state. Proc Natl Acad Sci USA 106:20264–20269

Warnecke T, Hurst LD (2010) GroEL dependency affects codon usage–support for a critical role of misfolding in gene evolution. Mol Syst Biol 6:340

Weber JK, Pande VS (2013) Functional understanding of solvent structure in GroEL cavity through dipole field analysis. J Chem Phys 138:165101

Xu Z, Horwich AL, Sigler PB (1997) The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex. Nature 388:741–750

Yan X, Hu S, Guan YX, Yao SJ (2012) Coexpression of chaperonin GroEL/GroES markedly enhanced soluble and functional expression of recombinant human interferon-gamma in *Escherichia coli*. Appl Microbiol Biotechnol 93:1065–1074

Yoshimune K, Ninomiya Y, Wakayama M, Moriguchi M (2004) Molecular chaperones facilitate the soluble expression of *N*-acyl-D-amino acid amidohydrolases in *Escherichia coli*. J Ind Microbiol Biotechnol 31:421–426

Zhang W, Chen S, Liao Y, Wang D, Ding J, Wang Y, Ran X, Lu D, Zhu H (2013) Expression, purification, and characterization of formaldehyde dehydrogenase from *Pseudomonas aeruginosa*. Protein Expr Purif 92:208–213