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Possible involvement of an FKBP family member protein from a psychrotrophic bacterium *Shewanella* sp. SIB1 in cold-adaptation

Yutaka Suzuki, Mitsuru Haruki*, Kazufumi Takano, Masaaki Morikawa and Shigenori Kanaya

Department of Material and Life Science, Graduate School of Engineering, Osaka University, Japan

A psychrotrophic bacterium *Shewanella* sp. strain SIB1 was grown at 4 and 20 °C, and total soluble proteins extracted from the cells were analyzed by two-dimensional polyacrylamide gel electrophoresis. Comparison of these patterns showed that the cellular content of a protein with a molecular mass of 28 kDa and an isoelectric point of four greatly increased at 4 °C compared to that at 20 °C. Determination of the N-terminal amino acid sequence, followed by the cloning and sequencing of the gene encoding this protein, revealed that this protein is a member of the FKBP family of proteins with an amino acid sequence identity of 56% to *Escherichia coli* FKBP22. This protein was overproduced in *E. coli* in a His-tagged form, purified, and analyzed for peptidyl-prolyl *cis-trans* isomerase activity. When this activity was determined by the protease coupling assay using *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as a substrate at various temperatures, the protein exhibited the highest activity at 10 °C with a k_{cat}/K_m value of 0.87 $\mu M^{-1} \cdot s^{-1}$. When the peptidyl-prolyl *cis-trans* isomerase activity was determined by the RNase T_1 refolding assay at 10 and 20 °C, the protein exhibited higher activity at 10 °C with a k_{cat}/K_m value of 0.50 $\mu M^{-1} \cdot s^{-1}$. These k_{cat}/K_m values are lower but comparable to those of *E. coli* FKBP22. We propose that a FKBP family protein is involved in cold-adaptation of psychrotrophic bacteria.

Keywords: psychrotrophic bacterium; 2D-PAGE; FKBP family protein; PPIase; cold-adaptation.

Nascent polypeptides must be folded into their precise 3D structures to become functional proteins. As folding intermediates have a tendency to interact with one another, such that proper folding cannot be completed, protein folding processes must be achieved rapidly and effectively to avoid such aggregation [1]. The protein folding processes are thought to be mediated by two classes of proteins. The first class of the proteins includes molecular chaperones, which typically bind to exposed hydrophobic parts of unfolded polypeptide chains and release their substrates in a controlled manner, thereby preventing aggregation and assisting in proper folding [2]. The other class of proteins includes enzymes that catalyze specific steps of protein folding. This group of proteins includes disulfide isomerases, which catalyze formation and isomerization of disulfide bonds, and peptidyl-prolyl *cis-trans* isomerases (PPIases), which catalyze the *cis-trans* isomerization of peptide bonds N-terminal of the proline residues [3]. For many proteins, the *cis-trans* isomerization of peptide bonds N-terminal of the proline residues is the rate-limiting step in their folding [4–7].

PPIases (EC 5.2.1.8) are divided into three structurally unrelated families, cyclophilin, FK506-binding protein (FKBP), and parvulin families [8]. These PPIases are present in all kingdoms of life, and all species contain multiple PPIases within a single cell. For example, *Escherichia coli* contains two members of the cyclophilin family, five members of the FKBP family, and three members of the parvulin family. *Saccharomyces cerevisiae* contains eight members of the cyclophilin family, four members of the FKBP family, and one member of the parvulin family [9]. PPIases are usually composed of several domains. In each PPIase, one domain is common to the members of each PPIase family and therefore specifies the family to which that PPIase belongs. The others are unique to the particular PPIase and therefore are thought to be related to the protein's distinct function. In many cases, however, disruption of the genes encoding the members of the FKBP and cyclophilin families does not cause any significant phenotypic change [8]. For example, a yeast mutant lacking ESS1, which is the only member of the parvulin family found in yeast, is lethal. A yeast mutant lacking all 12 members of the FKBP and cyclophilin families, however, is viable [9,10]. Similarly, *E. coli* mutants lacking PpiA or FkpA, which are members of the cyclophilin and FKBP families, respectively, exhibit no obvious changes in phenotype [11,12]. Although the enzymatic activities have been demonstrated for all PPIases from *E. coli* [11,13–19], their natural substrates are yet to be identified and their exact biological functions remain unknown.

Correspondence to: S. Kanaya, Department of Material and Life Science, Graduate School of Engineering, Osaka University, 2–1, Yamadaoka, Suita, Osaka 565–0871, Japan.
Fax/Tel.: + 81 6 6879 7938; E-mail: kanaya@mls.eng.osaka-u.ac.jp

Abbreviations: FKBP, FK506-binding protein; PPIase, peptidyl-prolyl cis-trans isomerase.

Enzymes: peptidyl-prolyl cis-trans isomerase (PPIases, EC 5.2.1.8).

Note: The nucleotide sequence reported in this paper has been deposited in DDBJ with accession number AB116100.

**Present address*: Department of Materials Chemistry and Engineering, College of Engineering, Nihon University, Tamura-machi, Koriyama, Fukushima 963–8642, Japan.

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Psychrophiles and psychrotrophs are bacteria that can grow at low temperatures. In these bacteria, a variety of the systems that facilitate protein folding processes must be developed because protein folding reactions are generally slow at low temperatures. Acceleration of the peptidyl-prolyl isomerization reaction by PPIases may be the function of one such system. This reaction is normally slow, especially at low temperatures, if it is not assisted by PPIases. However, only the PPIases from mesophilic bacteria [8] and (hyper)thermophilic archaea [20–22] have so far been isolated and characterized. Neither the involvement of PPIases nor other proteins in protein folding process in psychrophiles or psychrotrophs has been reported, although several proteins have been reported to be induced for synthesis at low temperatures in these bacteria [23–30].

Shewanella sp. strain SIB1 is a psychrotrophic bacterium that grows most rapidly at 20 °C [31]. This strain can grow at temperatures as low as 0 °C but cannot grow at temperatures exceeding 30 °C. Ribonuclease HI from this strain has been shown to exhibit enzymatic properties characteristic of cold-adapted enzymes [32]. In this work, we show that the cellular content of an FKBP family member protein (FKBP22) with PPIase activity increased at 4 °C compared to that at 20 °C in this strain. This protein may facilitate protein folding processes when the SIB1 cells are grown at low temperatures.

Experimental procedures

Cells and plasmids

The psychrotrophic bacterium *Shewanella* sp. strain SIB1 was isolated in our laboratory from water deposits in a Japanese oil reservoir [31]. *E. coli* JM109 [*recA*1, *supE*44, *endA*1, *hsdR*17, *gyrA*96, *relA*1, *thi*, Δ(*lac-ProAB*)/F′, *traD*36, *ProAB*$^+$, *lacI*^{q} *lacZ*ΔM15] was obtained from Toyobo, Kyoto, Japan. *E. coli* BL21(DE3) [*F*$^-$, *ompT*, *hsd*S_B(r_B^-, m_B^-), gal(λcI857, *ind*1, Sam7, *nin*5, *lac*UV5-T7*gene*1), *dcm*(DE3)] and plasmid pET-28a were purchased from Novagen. Plasmid pUC18 was purchased from Takara Shuzo, Kyoto, Japan. The *E. coli* transformants were grown in Luria–Bertani medium containing 50 mg·L^{-1} ampicillin or 35 mg·L^{-1} kanamycin.

Extraction of soluble proteins from SIB1

Cultures of *Shewanella* sp. strain SIB1 were grown at 4 or 20 °C in 200 mL of medium (pH 7.2) containing 1.5% (w/v) Bacto tryptone, 0.1% (w/v) yeast extract, 0.1% (v/v) glycerol, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4{\cdot}7H_2O$, and 3% (w/v) NaCl to the mid-exponential phase (D_{660} = 1.0). Cells were harvested by centrifugation (8000 ***g*** for 10 min) at each cultivation temperature. Cells were then suspended in 50 mM Tris/HCl (pH 7.0), disrupted by sonication, and centrifuged at 15 000 ***g*** for 30 min at 4 °C. The supernatant, which contained total cellular soluble proteins, was pooled and used for 2D gel electrophoresis analysis.

Two-dimensional gel electrophoresis

2D-PAGE was performed with slight modifications according to the protocol of Oh-Ishi *et al.* [33]. The soluble proteins extracted from the SIB1 cells were dissolved in 50 mM Tris/HCl (pH 7.0) containing 5 M urea and 3 M thiourea at a concentration of 3 mg·mL^{-1}, and subjected to isoelectric focusing for the 1D-separation. Isoelectric focusing was conducted at 600 V for 20 h at 4 °C. Then, 12% SDS/PAGE was performed for the 2D separation. The proteins were detected by staining the gel with Coomassie Brilliant Blue. The N-terminal amino acid sequence of the protein was determined with a Procise 491 protein sequencer (Applied Biosystems).

General DNA manipulations

Genomic DNA was prepared from a Sarkosyl lysate of the *Shewanella* sp. SIB1 cells as described previously [34]. This genomic DNA was completely digested with *Kpn*I and *Sac*I, and the resultant DNA fragments were ligated into the *Kpn*I–*Sac*I sites of pUC18. The resultant plasmids were used to transform *E. coli* JM109 to generate a genomic library of SIB1. Southern blot analysis and colony hybridization were carried out by using AlkPhos Direct system (Amersham Pharmacia Biotech) according to the procedures recommended by the supplier. PCR was performed with GeneAmp PCR system 2400 (Perkin-Elmer) using KOD polymerase (Toyobo) according to the procedures recommended by the supplier. The DNA sequence was determined with a Prism 310 DNA sequencer (Applied Biosystems).

Overproduction and Purification of SIB1 FKBP22

Plasmid pSIB1 for overproduction of a His-tagged form of SIB1 FKBP22 (SIB1 FKBP22*) was constructed by ligating the DNA fragment containing the *Sh-fklB* gene into the *Nde*I–*Bam*HI sites of pET-28a. This DNA fragment was amplified by PCR. The sequences of the PCR primers were 5′-AGAGAGAATTCATATGTCAGATTTGTTCAG-3′ for the 5′-primer and 5′-GGCCACTGGATCCAACT ACAGCAATTCTCA-3′ for the 3′-primer, where underlined bases show the positions of the *Nde*I and *Bam*HI sites for the 5′- and 3′-primers, respectively.

For overproduction of SIB1 FKBP22*, *E. coli* BL21(DE3) was transformed with plasmid pSIB1 and grown at 30 °C. When D_{660} reached 0.6, 1 mM of isopropyl thio-β-D-galactoside (IPTG) was added to the culture medium and cultivation was continued at 30 °C for 1 h. The temperature of the growth medium was then shifted to 10 °C and cultivation was continued at 10 °C for an additional 40 h. Cells were harvested by centrifugation at 6000 ***g*** for 10 min at 4 °C, suspended in 20 mM sodium phosphate (pH 8.0) containing 0.5 M NaCl, disrupted by sonication, and centrifuged at 15 000 ***g*** for 30 min at 4 °C. The supernatant was applied to a HiTrap Chelating HP column (5 mL) (Amersham Pharmacia Biotech) charged with Ni^{2+} ions. The protein was eluted from the column with a linear gradient of imidazole from 10 to 500 mM at a flow rate of 2 mL·min^{-1}. The protein fractions at an imidazole concentration of ≈ 330 mM were pooled, dialyzed against 50 mM Tris/HCl (pH 8.0) containing 50 mM NaCl, and applied to a Superdex 200 16/60 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris/HCl (pH 8.0) containing 50 mM NaCl. Elution was

performed at a flow rate of 0.5 mL·min^{-1}. The protein fractions were pooled and used for biochemical characterizations. All purification procedures were performed at 4 °C. The purity of the protein was analyzed by SDS/PAGE [35] on a 12% (w/v) polyacrylamide gel, followed by staining with Coomassie Brilliant Blue.

Overproduction and purification of *E. coli* FKBP22

Plasmid pECOLI for overproduction of a His-tagged form of *E. coli* FKBP22 (*E. coli* FKBP22*), in which the *fklB* gene from *E. coli* (*Ec-fklB*) was introduced into the *Nde*I-*Sal*I sites of pET-28a, was constructed in the following manner. As the *Ec-fklB* gene contains a single *Nde*I site, the plasmid pECOLI could not be constructed by simply amplifying the entire gene by PCR and ligating it into the *Nde*I-*Sal*I sites of pET28a. First, the *Ec-fklB* gene was amplified by PCR by using the 5′- and 3′-primers with the sequences of 5′-TAAGAAAGGAAATCATATGACCA CCCCAAC-3′ and 5′-ATTGCTGAATGCCGGATCCC CTCTCGTTCG-3′, respectively, where underlined bases show the position of the *Nde*I site. The PCR product was ligated into the *Sma*I site of pUC18 to generate plasmid pUCECOLI. In this plasmid, two *Nde*I sites are located within the *Ec-fklB* gene (one at the 5′-terminus), a unique *Eco*RI site is located between these *Nde*I sites, and a unique *Sal*I site is located downstream of the *Ec-fklB* gene. This plasmid was then digested by *Nde*I and *Eco*RI to produce the 450 bp *Nde*I-*Eco*RI fragment containing the 5′-terminal region of the *Ec-fklB* gene, or by *Eco*RI and *Sal*I to produce the 250 bp *Eco*RI-*Sal*I fragment containing the 3′-terminal region of the *Ec-fklB* gene. Ligation of these DNA fragments into the *Nde*I-*Sal*I sites of pET28a produced plasmid pECOLI.

For overproduction of *E. coli* FKBP22*, *E. coli* BL21(DE3) was transformed with pECOLI and grown at 30 °C. When D_{660} reached 0.6, 1 mM IPTG was added to the culture medium and cultivation was continued at 30 °C for 3 h. Disruption of the cells and the purification of the protein by metal chelating affinity chromatography and gel filtration were performed as described above for SIB1 FKBP22*.

Molecular mass

The molecular mass of SIB1 FKBP22* was determined by a LCQ electrospray ionization mass spectrometer (Finnigan Mat). The scan range was 300–4000 *m*/*z*. The ESI-MS spectra were acquired using LCQ NAVIGATOR software, and the scans were deconvoluted using FINNIGAN BIOWORKS software. The molecular mass of SIB1 FKBP22* in solution was determined by sedimentation equilibrium analytical ultracentrifugation. Sedimentation equilibrium experiments were performed at 10 °C with a Beckman Optima XL-A Analytical Ultracentrifuge using an An-60 Ti rotor at a speed of 19 000 r.p.m. Before measurements, the protein solutions were dialyzed overnight against 20 mM sodium phosphate (pH 8.0) at 4 °C. The protein concentration distribution within the cell was monitored by the absorbance at 280 nm. Analysis of the sedimentation equilibria was performed using the program XLAVEL (Beckman, version 2). The molecular masses of SIB1 FKBP22* and *E. coli* FKBP22* in a multimeric form were also estimated by gel filtration column chromatography, which was performed as described above for purification of SIB1 FKBP22*. Thyroglobulin (670 kDa), γ-globulin (158 kDa), and ovalbumin (44 kDa) were used as standard proteins.

Protein concentration

Protein concentrations were determined from the UV absorption on the basis that the absorbance at 280 nm of a 0.1% solution is 0.68 for SIB1 FKBP22* and 0.69 for *E. coli* FKBP22*. These values were calculated by using ε of 1576 M^{-1}·cm^{-1} for Tyr and 5225 M^{-1}·cm^{-1} for Trp at 280 nm [36].

Enzymatic activity

The PPIase activity was determined by protease-coupling assay [37,38] and RNase T_1 refolding assay [39]. For the protease-coupling assay, chymotrypsin was used as the protease and two oligopeptides *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (Wako Chemicals) were used as the substrates. The reaction mixture (2.1 mL) contained 35 mM Hepes buffer (pH 7.8), 25 μM oligopeptide substrate, and the appropriate amount of the enzyme. The reaction mixture was incubated at reaction temperature for 3 min prior to the addition of chymotrypsin. The reaction was initiated by the addition of 30 μL of 0.76 mM chymotrypsin. In the presence of such a high concentration of protease, *p*-nitroaniline is released from the substrate within a few seconds when the peptide bond N-terminal of the proline residue in the substrate assumes the *trans* conformation. However, *p*-nitroaniline is not released from the substrate when this peptide bond is in the *cis* conformation. Therefore, the isomerization reaction of this peptide bond catalyzed by PPIases was measured by monitoring the change in the concentration of *p*-nitroaniline. The increase in the rate of isomerization is implicit in the increased rate of *p*-nitroaniline release, because catalysis of isomerization produces *trans* substrate with increased frequency. The concentration of *p*-nitroaniline was determined from the absorption at 390 nm with the molar absorption coefficient value of 8900 M^{-1}·cm^{-1} using a Hitachi U-2010 UV/VIS spectrophotometer (Hitachi Instruments). The catalytic efficiency (k_{cat}/K_m) was calculated from the relationship $k_{cat}/K_m = (k_p - k_n)/E$, where E represents the concentration of the enzyme, and k_p and k_n represent the first-order rate constants for the release of *p*-nitroaniline from the substrate in the presence and absence of the enzyme, respectively [40]. For accurate calculations of the k_{cat}/K_m values, we used the data of k_n smaller than 7.0×10^{-2}·s^{-1}. When the k_n value exceeded 7.0×10^{-2}·s^{-1}, the linear relationship between the PPIase concentration and the k_{cat}/K_m value was lost. The k_n values were 3.2×10^{-3} at 4 °C, 7.2×10^{-3} at 10 °C, 1.2×10^{-2} at 15 °C, 2.1×10^{-2} at 20 °C, 3.9×10^{-2} at 25 °C and 7.5×10^{-2}·s^{-1} at 30 °C.

For the RNase T_1 refolding assay, RNase T_1 was first unfolded by incubating the solution containing 50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 5.6 M guanidine hydrochloride, and 16 μM RNase T_1 (Funakoshi) at 10 °C overnight. Refolding was then initiated by diluting this solution 80-fold

with 50 mM Tris/HCl (pH 8.0) containing SIB1 FKBP22* or *E. coli* FKBP22*. The final concentrations of RNase T_1, SIB1 FKBP22*, and *E. coli* FKBP22* were 0.2 μM, 8.9 nM and 1.0 nM, respectively. The refolding reaction was monitored by measuring the increase in tryptophan fluorescence with a F-2000 spectrofluorometer (Hitachi Instruments). The excitation and emission wavelengths were 295 and 323 nm, respectively, and the band width was 10 nm. The refolding curves were analyzed with a double exponential fit [41]. The k_{cat}/K_m values were calculated from the relationship described above, where k_p and k_n represent the first-order rate constants for the faster refolding phase of RNase T_1 in the presence and absence of the enzyme, respectively.

Results and discussion

Detection of a protein with increased cellular content at a low temperature

Cellular contents of the proteins in the bacterial cells are often affected by the culture condition of these cells. When the cells are grown at unusual conditions, the cellular contents of the proteins that are associated with adaptation at these conditions usually increase. More specifically, the cellular content of a protein that is involved in cold-adaptation, may increase when the cells are grown at the temperatures much lower than the optimum one. It is uncertain, however, whether such an increase is a cause or effect of adaptation. To examine whether such a cold-adaptation mechanism is present in *Shewanella* sp. strain SIB1, cells were grown at 4 and 20 °C until the D_{660} was 1.0. The total soluble proteins were subsequently extracted from these cells, and then subjected to 2D-PAGE. Comparison of the 2D-PAGE patterns showed that the cellular contents of several proteins increased greatly at 4 °C as compared to those at 20 °C (Fig. 1). They include a protein (P28) with a molecular mass of 28 kDa and an isoelectric point of 4. When the soluble proteins extracted from the SIB1 cells grown at 0, 10, and 15 °C were also analyzed by 2D-PAGE, the cellular content of P28 greatly increased at 0 and 10 °C, but did not significantly increase at 15 °C, when compared to that at 20 °C (data not shown). These results indicate that the cellular content of P28 greatly increases when the SIB1 cells are grown at the temperatures below 10 °C. As increase of the cellular content of P28 at low temperatures is marked and reproducible, we decided to clone the gene encoding P28.

Gene cloning

The N-terminal amino acid sequence of P28 was determined to be SDLFSTMEQHASYGVG. The gene encoding P28 was cloned by Southern blot analysis and colony hybridization using DNA oligomers that are designed from this amino acid sequence information as a probe. Digestion of the SIB1 genome with *Kpn*I and *Sac*I, followed by Southern blot analysis strongly suggested that a 1.3 kb *Kpn*I-*Sac*I fragment contains the gene encoding P28. Construction of the genomic library of SIB1, followed by colony hybridization, allowed us to clone this 1.3 kb *Kpn*I-*Sac*I fragment. Determination of the nucleotide sequence of this DNA

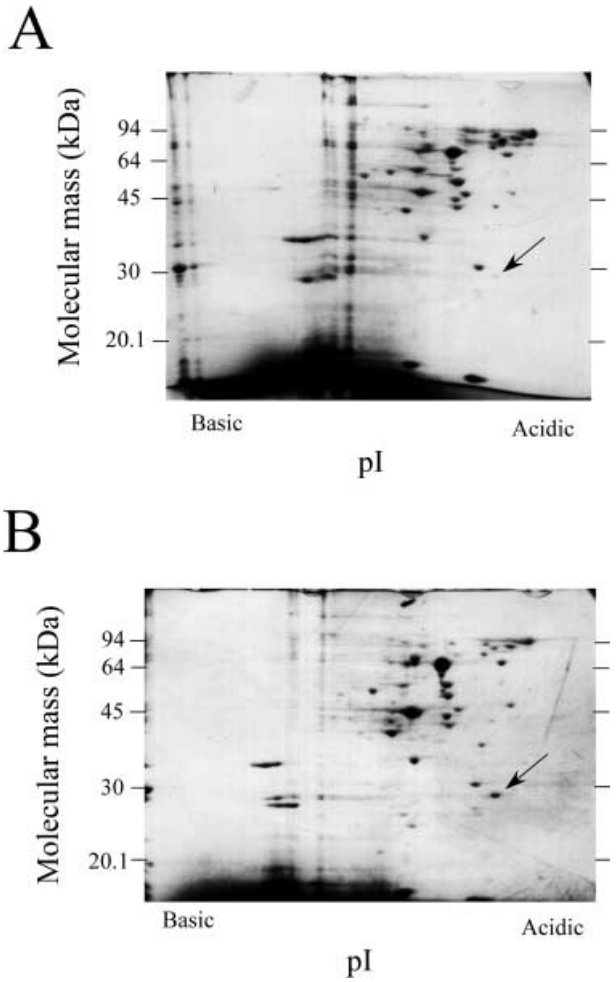

Fig. 1. 2D-PAGE analysis of the proteins extracted from the SIB1 cells. Soluble proteins extracted from the SIB1 cells grown at 20 (A) and 4 °C (B) were applied to 2D-PAGE. Slab gels were stained with Coomassie Brilliant Blue. Arrows indicate the position of P28.

fragment revealed that it contains the entire gene encoding P28. P28 consists of 205 amino acid residues with a calculated molecular mass of 21 783 Da and isoelectric point of 4.3. The deduced N-terminal amino acid sequence of this protein is identical with the determined mass. As P28 shows the highest sequence identity of 85% to FKBP22 from *Shewanella oneidensis* MR-1 (accession number AE015558), which also consists of 205 amino acid residues, P28 and the gene encoding it will be designated as SIB1 FKBP22 and *Sh-fklB*, hereafter.

In addition to the *Sh-fklB* gene, the 1.3 kb *Kpn*I-*Sac*I fragment contains partial *htpX*-like and *dap*B-like genes that are located 79 bp upstream and 84 bp downstream of the *Sh-fklB* gene (Fig. 2). The *htpX*-like and *dap*B-like genes encode a homologue of a zinc protease and a dihydrodipicolinate reductase from *S. oneidensis* MR-1, respectively. The directions of the transcriptions of these genes are the same as that of the *Sh-fklB* gene. The 79 bp noncoding sequence between the *htpX*-like and *Sh-fklB* genes contains a putative σ^{70}-type promoter sequence [42] and a putative Shine-Dalgarno sequence [43], which may function as

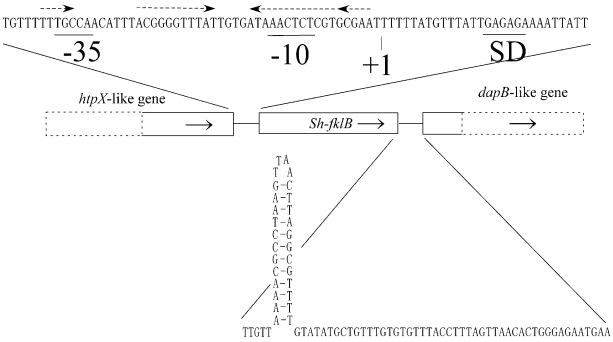

Fig. 2. Localization of the *Sh-fklB* gene. Localization of the *htpX*-like, *Sh-fklB*, and *dapB*-like genes, as well as the nucleotide sequences of the noncoding regions, are shown. The 1.3 kb *Kpn*I-*Sac*I fragment of the SIB1 genome does not contain the entire *htpX*-like and *dapB*-like genes. The truncated regions of these genes are shown by boxes with broken lines. The direction of the transcription for each gene is shown by an arrow. A putative σ^{70}-type promoter site (−10 and −35 regions) and a putative Shine–Dalgarno (SD) sequence are shown. A putative initiation site for transcription is marked by '+1'. A putative stem-loop structure, which may function as a transcription termination signal for the *Sh-fklB* gene, is also shown. Broken arrows represent an inverted repeat of the sequence, which may form a stem-loop structure.

transcriptional and translational signals for the *Sh-fklB* gene, respectively. This noncoding sequence also contains a putative stem-loop structure (from T6 to A50), which is followed by six T residues. This putative stem-loop structure, which is overlapped with a putative promoter sequence for the *Sh-fklB* gene, may function as a transcription termination signal for the *htpX*-like gene. Likewise, the 84 bp noncoding sequence between the *Sh-fklB* and *dap*B-like genes contains a potential stem-loop structure. As this sequence is located 6 nucleotides downstream of the translational termination codon, TAG, it may function as a transcription termination signal for the *Sh-fklB* gene. This noncoding sequence also contains a putative Shine-Dalgarno sequence that may function as a translational signal for the *dap*B-like gene.

Amino acid sequence

In Fig. 3, the amino acid sequence of SIB1 FKBP22 is compared with those of the proteins that show relatively high sequence similarities, as well as human FKBP12, which is one of the most extensively studied FKBP family proteins. In the regions where the amino acid sequences can be aligned, SIB1 FKBP22 shows sequence identities of 56% to *E. coli* FKBP22, 43% to *E. coli* FkpA, 41% to *Legionella pneumophila* MIP, and 43% to human FKBP12. The macrophage infectivity potentiator (MIP) protein was originally detected as an essential virulence factor of *L. pneumophila* associated with macrophage infectivity [44], and was found later to be a FKBP family protein that exhibits PPIase activity [45]. Its crystal structure has been determined [46]. As *E. coli* FKBP22 and *E. coli* FkpA

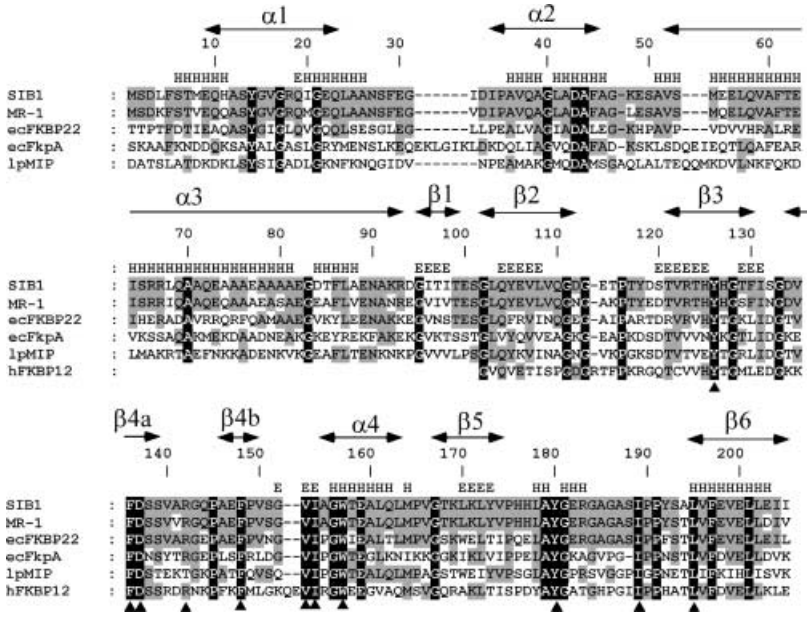

Fig. 3. Alignment of the amino acid sequences of the members of the MIP-like FKBP subfamily and human FKBP12. Fully conserved amino acid residues are shown in white letters on a dark background. Amino acid residues, which are not fully conserved but conserved in SIB1 FKBP22 and at least one of other proteins, are shaded. Numbers above the sequences indicate the positions of the residues relative to the initiator methionine of SIB1 FKBP22. The ranges of the α-helices and β-strands of *L. pneumophila* MIP are shown above the sequences according to Riboldi-Tunnicliffe *et al.* [46]. The amino acid residues forming the hydrophobic active-site pocket of this protein are also denoted by the solid triangles below the sequences. Secondary structures of SIB1 FKBP22 predicted by Chou–Fasman algorism are shown above the sequences (H: helix, E; strand). SIB1, entire sequence of SIB1 FKBP22; MR-1, entire sequence of FKBP22 from *S. oneidensis* MR-1; ecFKBP22, entire sequence of *E. coli* FKBP22 without Met1; ecFkpA, Ser37-Lys249 of *E. coli* FkpA; lpMIP, Asp3-Lys230 of *L. pneumophila* MIP; hFKBP12, entire sequence of human FKBP12. Accession numbers are AE015558 for MR-1 FKBP22, AAC77164 for ecFKBP22, AAC76372 for ecFkpA, S42595 for lpMIP and M34539 for hFKBP12.

have been classified as MIP-like FKBP subfamily proteins [17], SIB1 FKBP22 should also be classified into the MIP-like FKBP subfamily.

According to the crystal structure, *L. pneumophila* MIP is composed of a N-terminal domain, that is involved in dimerization of the protein, and a C-terminal catalytic domain [46]. Three helices (α1–3) comprise the N-terminal domain, and six β-strands (β1–6) and one helix (α4) comprise the C-terminal domain. Tyr125, Phe135, Asp136, Thr141, Phe147, Val152, Ile153, Trp156, Tyr179, Ile188 and Leu194 form the hydrophobic active-site pocket. All of these residues, except for Thr141, are conserved in other members of the MIP-like FKBP subfamily. These results suggest that MIP-like FKBP subfamily proteins, except human FKBP12 which is composed of only a C-terminal catalytic domain, have similar 3D structures and are distinguished from other FKBP family proteins in their unique domain structures. Obviously, the amino acid sequences of these proteins in the C-terminal domain (Gly95–Ile205 for SIB1 FKBP22) are more strongly conserved than those in the N-terminal domain (Met1–Arg93 for SIB1 FKBP22). Indeed, the amino acid sequence identity between SIB1 FKBP22 and *E. coli* FKBP22 in the C-terminal domain is 67%, while it is only 40% in the N-terminal domain.

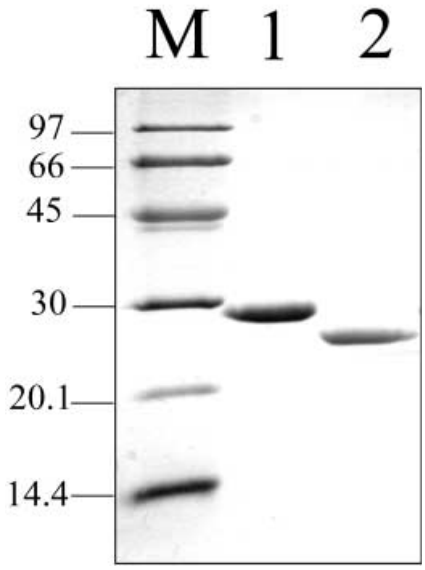

Fig. 4. SDS/PAGE of purified recombinant proteins. Purified SIB1 FKBP22* (lane 1) and *E. coli* FKBP22* (lane 2) were applied to 12% (w/v) SDS/PAGE and stained with Coomassie Brilliant Blue. M, A low molecular mass marker kit (Amersham Pharmacia Biotech).

Overproduction and purification

SIB1 FKBP22 and *E. coli* FKBP22 were overproduced in a His-tagged form at 10 and 37 °C, respectively. These His-tagged forms of the proteins are designated as SIB1 FKBP22* and *E. coli* FKBP22*. SIB1 FKBP22* was overproduced at such a low temperature because it exhibited the maximal PPIase activity at 10 °C (see below). Both proteins accumulated in the *E. coli* cells in a soluble form and were purified to give a single band on SDS/PAGE (Fig. 4). The amount of the protein purified from 1 L culture was typically 4.4 mg for SIB1 FKBP22* and 6.6 mg for *E. coli* FKBP22*. It is noted that the gene expression was induced initially at 30 °C for 1 h for overproduction of SIB1 FKBP22*. However, SIB1 FKBP22* did not accumulate appreciably in the *E. coli* cells when the cells were harvested before the temperature of the growth medium was shifted to 10 °C (data not shown).

The molecular mass of SIB1 FKBP22* was determined by ESI-MS mass spectroscopy to be 23 947.3 ± 3.3 Da, which is identical to that calculated from the amino acid sequence (23 947 Da). However, the molecular mass of SIB1 FKBP22* estimated by SDS/PAGE (29 kDa) is much larger than this value (Fig. 4). The molecular mass of the natural protein estimated by 2D-PAGE (28 kDa) is also much larger than that calculated from the amino acid sequence. The molecular mass of *E. coli* FKBP22* is estimated to be 26 kDa by SDS/PAGE (Fig. 4), which is comparable to that calculated from the amino acid sequence (24 379 Da).

To determine the molecular mass of SIB1 FKBP22* in solution, sedimentation equilibrium analytical ultracentrifugation was performed at three different initial loading concentrations of the protein. The data fitted well to a single-species model with no evidence of aggregation, and apparent molecular masses were determined to be 46 156, 42 999 and 41 150 Da at 0.6, 1.2 and 1.8 $mg \cdot mL^{-1}$ of initial loading concentrations, respectively. Extrapolation to zero concentration gave the molecular mass of 48 441 Da, which was two times larger than the calculated one, indicating that SIB1 FKBP22* exists as a dimer like *L. pneumophila* MIP. According to the crystal structure of *L. pneumophila* MIP, the α1 helix of one monomer makes hydrophobic interactions with the α2 helix of the other. The amino acid sequences in these regions are relatively well conserved in SIB1 FKBP22* and *E. coli* FKBP22* (Fig. 3). Furthermore, at the core of the dimerization domain, two methionine residues (Met38 and Met42) located in the α2 helix of one monomer make hydrophobic interactions with those of the other monomer. In SIB1 FKBP22* and *E. coli* FKBP22*, these residues are replaced by Val, Leu, or Ile, suggesting that the hydrophobic interactions at the core of the dimerization domain are conserved in these proteins. Therefore, SIB1 FKBP22* and *E. coli* FKBP22* probably assumes a similar dimer structure as *L. pneumophila* MIP. By gel filtration column chromatography, however, the molecular masses of SIB1 FKBP22* and *E. coli* FKBP22* were estimated to be 74 000 and 66 000 Da, respectively. These values are 3.1 and 2.7 times larger than those calculated from the corresponding amino acid sequences. This is probably because their molecular shapes are cylindrical rather than globular, as is in the case of *L. pneumophila* MIP [46]. In fact, the molecular mass of *L. pneumophila* MIP estimated from gel filtration column chromatography has been reported to be larger than that calculated from its deduced amino acid sequence by 2.7 times [47].

The molecular mass of SIB1 FKBP22* determined by sedimentation analysis was identical to the calculated value for dimer form because it was not affected by the shape of the protein molecule. In contrast, the molecular mass of

SIB1 FKBP22* estimated from gel filtration analysis was larger than the calculated value for dimer form by 1.6-fold, probably because it was affected by the shape of the protein molecule. Cylindrical proteins usually migrate through the gel filtration column faster than globular proteins, which are used for calibration of molecular mass.

PPIase activity

The peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of SIB1 FKBP22* was determined by protease coupling assay. Its catalytic efficiency (k_{cat}/K_m) was estimated to be 0.87 $\mu M^{-1}\cdot s^{-1}$ for *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide and 0.03 $\mu M^{-1}s^{-1}$ for *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide at 10 °C. This substrate specificity is similar to those of *E. coli* FKBP22 [17] and *L. pneumophila* MIP [48]. Using *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as the substrate, the temperature dependence of the PPIase activity of SIB1 FKBP22* was compared with that of *E. coli* FKBP22* (Fig. 5). SIB1 FKBP22* exhibited the highest catalytic efficiency at 10 °C. This value did not change at 15 °C (0.79 $\mu M^{-1}\cdot s^{-1}$), but decreased significantly at temperatures higher than 20 °C (0.44 $\mu M^{-1}\cdot s^{-1}$ at 20 °C and 0.23 $\mu M^{-1}\cdot s^{-1}$ at 25 °C). In contrast, the k_{cat}/K_m value of *E. coli* FKBP22* increased as the reaction temperature increased from 4 to 25 °C. The PPIase activities of these proteins were not measured at temperatures higher than 30 °C, because the rate for spontaneous prolyl isomerization reaction was too high to accurately determine those catalyzed by PPIases.

The PPIase activity of SIB1 FKBP22* was also measured by RNase T_1 refolding assay. The refolding of RNase T_1 is dominated by the slow isomerization reactions of two peptidyl-prolyl bonds, and is therefore generally used as a model system for investigating PPIase activity [39]. Acceleration of the faster of the two slow prolyl isomerization-limited folding rates was observed in the presence SIB1 FKBP22* (Fig. 6), suggesting that SIB1 FKBP22* catalyzes prolyl isomerization of proteins in a nonspecific manner. Acceleration of this refolding reaction was also observed in the presence of *E. coli* FKBP22* (Fig. 6). The rate constants for the reactions catalyzed by 8.9 nM SIB1 FKBP22* and 1.0 nM *E. coli* FKBP22* at 10 and 20 °C, as well as those for spontaneous reactions, are summarized in Table 1. The results indicate that the catalytic efficiency (k_{cat}/K_m) of SIB1 FKBP22* was greatly reduced at 20 °C (0.13 $\mu M^{-1}\cdot s^{-1}$) as compared to that at 10 °C (0.50 $\mu M^{-1}\cdot s^{-1}$), whereas the catalytic efficiency of *E. coli* FKBP22* was increased at 20 °C (3.2 $\mu M^{-1}\cdot s^{-1}$) as compared to that at 10 °C (1.2 $\mu M^{-1}\cdot s^{-1}$). These results support those obtained by the protease coupling assay that optimum temperature of SIB1 FKBP22 is 10 °C.

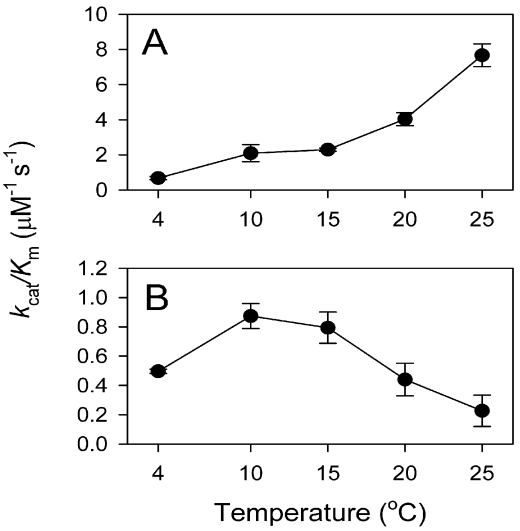

Fig. 5. Temperature dependence of PPIase activity. The PPIase activities of *E. coli* FKBP22* (A) and SIB1 FKBP22* (B) were determined by protease coupling assay at the temperatures indicated using *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as a substrate, as described under Experimental procedures. The catalytic efficiency, k_{cat}/K_m, was calculated according to Harrison and Stein [32]. The experiment was carried out in duplicate. Each plot represents the average value and errors from the average values are shown.

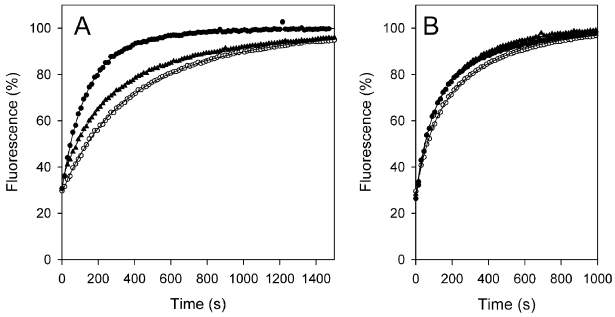

Fig. 6. Catalysis of the slow refolding reactions of RNase T_1 by SIB1 FKBP22* and *E. coli* FKBP22*. The increase in tryptophan fluorescence at 323 nm during refolding of RNase T_1 (0.2 μM) is shown as a function of the refolding time. Refolding reactions were carried out at 10 (A) and 20 °C (B) in the absence (○), or presence of 8.9 nM of SIB1 FKBP22* (●) or 1.0 nM *E. coli* FKBP22* (▲).

Table 1. Rate constants of RNase T_1 refolding assisted by SIB1 FKBP22* and *E. coli* FKBP22*. RNase T_1 (0.2 μM), which had been unfolded in 50 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and 5.6 M guanidine hydrochloride, was refolded by diluting 80-fold with 50 mM Tris/HCl (pH 8.0) in the absence or presence of 8.9 nM SIB1 FKBP22* or 1.0 nM *E. coli* FKBP22*. The refolding curves were analyzed with double exponential fit [41]. The k_{cat}/K_m values were calculated from the relationship $k_{cat}/K_m = (k_p - k_n)/E$, where E represents the concentration of the enzyme, and k_p and k_n represent the first-order rate constants for the faster refolding phase of RNase T_1 in the presence and absence of the enzyme, respectively [40]. Errors are within 6% of the values reported.

Enzyme	Temperature (°C)	k_p or k_n (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
None	10	4.3×10^{-3}	–
	20	1.2×10^{-2}	–
SIB1 FKBP22*	10	8.8×10^{-3}	0.5
	20	1.3×10^{-2}	0.13
E. coli FKBP22*	10	5.4×10^{-3}	1.2
	20	1.5×10^{-2}	3.2

Temperature dependence of the PPIase activity has been analyzed for bovine cyclophilin 18 [40,49], human FKBP12 [49], and a FKBP from a thermophilic archaeon [50], and the optimum temperature has been reported to be ≈ 20 °C for bovine cyclophilin 18 [49]. As the prolyl isomerization is a spontaneous reaction and the rate for this reaction increases as the reaction temperature increases, it is difficult to determine accurately the PPIase activity at temperatures higher than 30 °C. SIB1 FKBP22*, with an optimum temperature of 10 °C, may therefore prove to be an excellent model protein to study structure–function–stability relationships of PPIases.

Possible physiological role of FKBP22

Members of the MIP-like FKBP subfamily seem to be present ubiquitously in both pathogenic and nonpathogenic Gram-negative bacteria. The biological functions of MIP from pathogens have been relatively well understood [51–54], while those from nonpathogens have not yet been understood. These proteins exhibit PPIase activities, but the levels are very low. For example, the PPIase activities of *E. coli* FKBP22 and FkpA are lower than those of *E. coli* Cyps by 20- to 50-fold [13]. Furthermore, disruption of the gene encoding *E. coli* FkpA does not cause any significant phenotypic change [11]. The functional significance, if any, of the MIP-like FKBP subfamily proteins from nonpathogens is therefore difficult to discern.

Several prokaryotic PPIases, such as PpiB from *Bacillus subtilis* [55], Trigger Factor from *E. coli* [56], and a FKBP family protein from hyperthermophilic archaeon *Thermococcus* sp. KS-1 [57], have been reported to be induced by cold-shock. It has also been reported that the PPIase activity of Trigger Factor is responsible for the growth of the *E. coli* cells at low temperatures [58]. These previous results together with ours suggest that the prokaryotic cells require PPIases for growth at low temperatures, regardless of whether they are hyperthermophiles or psychrophiles. It has been reported that parvulin family PPIases, such as human Pin1 and yeast ESS1, specifically isomerize phosphorylated Ser/Thr-Pro bonds and thereby mediate many cellular processes through proline-driven conformational change of a protein [59]. This system has been proposed to be present in interleukin-2 tyrosine kinase SH2 domain [60] and *trans*-membrane channels [61] as well. Existence of a proline-driven signaling system mediated by a specific PPIase may be able to explain why so many different kinds of PPIases are present in a single cell. However, no species of bacteria has been reported to have such a system. Therefore, it is more likely that FKBP22 nonspecifically mediates the protein folding process. At low temperatures, protein folding reactions proceed slowly as do other chemical reactions. It is certainly plausible that organisms living in cold environments develop some systems to enable efficient protein folding. Adaptation to cold conditions can be achieved by the modification of amino acid sequences of proteins so that their folding processes are accelerated [62,63]. Amino acid sequence modification by itself, however, may not be always effective in intramolecularly catalyzing the *cis-trans* isomerization. PPIase activity may facilitate efficient folding of proteins containing proline residues with a *cis* conformation at low temperatures.

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References

1. Netzer, W.J. & Hartl, F.U. (1998) Protein folding in the cytosol: chaperonin-dependent and -independent mechanisms. *Trends Biochem. Sci.* **23**, 68–73.
2. Hartl, F.U. & Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852–1858.
3. Schiene, C. & Fischer, G. (2000) Enzymes that catalyse the restructuring of proteins. *Curr. Opin. Struct. Biol.* **10**, 40–45.
4. Brandts, J.F., Halvorson, H.R. & Brennan, M. (1975) Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis-trans* isomerism of proline residues. *Biochemistry* **14**, 4953–4963.
5. Cook, K.H., Schmid, F.X. & Baldwin, R.L. (1979) Role of proline isomerization in folding of ribonuclease A at low temperatures. *Proc. Natl Acad. Sci. USA* **76**, 6157–6161.
6. Schmid, F.X. & Blaschek, H. (1981) A native-like intermediate on the ribonuclease A folding pathway. 2. Comparison of its properties to native ribonuclease A. *Eur. J. Biochem.* **114**, 111–117.
7. Schmid, F.X., Mayr, L.M., Mucke, M. & Schonbrunner, E.R. (1993) Prolyl isomerases: role in protein folding. *Adv. Protein Chem.* **44**, 25–66.
8. Gothel, S.F. & Marahiel, M.A. (1999) Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell. Mol. Life. Sci.* **55**, 423–436.
9. Dolinski, K., Muir, S., Cardenas, M. & Heitman, J. (1997) All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* **94**, 13093–13098.
10. Hanes, S.D., Shank, P.R. & Bostian, K.A. (1989) Sequence and mutational analysis of ESS1, a gene essential for growth in *Saccharomyces cerevisiae*. *Yeast* **5**, 55–72.
11. Horne, S.M. & Young, K.D. (1995) *Escherichia coli* and other species of the Enterobacteriaceae encode a protein similar to the family of Mip-like FK506-binding proteins. *Arch. Microbiol.* **163**, 357–365.
12. Kleerebezem, M., Heutink, M. & Tommassen, J. (1995) Characterization of an *Escherichia coli rotA* mutant, affected in periplasmic peptidyl-prolyl *cis*/*trans* isomerase. *Mol. Microbiol.* **18**, 313–320.
13. Compton, L.A., Davis, J.M., Macdonald, J.R. & Bachinger, H.P. (1992) Structural and functional characterization of *Escherichia coli* peptidyl-prolyl *cis-trans* isomerases. *Eur. J. Biochem.* **206**, 927–934.
14. Dartigalongue, C. & Raina, S. (1998) A new heat-shock gene, *ppiD*, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. *EMBO J.* **17**, 3968–3980.
15. Hottenrott, S., Schumann, T., Pluckthun, A., Fischer, G. & Rahfeld, J.U. (1997) The *Escherichia coli* SlyD is a metal

ion-regulated peptidyl-prolyl *cis*/*trans*-isomerase. *J. Biol. Chem.* **272**, 15697–15701.
16. Rahfeld, J.U., Schierhorn, A., Mann, K. & Fischer, G. (1994) A novel peptidyl-prolyl *cis*/*trans* isomerase from *Escherichia coli*. *FEBS Lett.* **343**, 65–69.
17. Rahfeld, J.U., Rucknagel, K.P., Stoller, G., Horne, S.M., Schierhorn, A., Young, K.D. & Fischer, G. (1996) Isolation and amino acid sequence of a new 22-kDa FKBP-like peptidyl-prolyl *cis*/*trans*-isomerase of *Escherichia coli*. Similarity to Mip-like proteins of pathogenic bacteria. *J. Biol. Chem.* **271**, 22130–22138.
18. Roof, W.D., Fang, H.Q., Young, K.D., Sun, J. & Young, R. (1997) Mutational analysis of *slyD*, an *Escherichia coli* gene encoding a protein of the FKBP immunophilin family. *Mol. Microbiol.* **25**, 1031–1046.
19. Stoller, G., Rucknagel, K.P., Nierhaus, K.H., Schmid, F.X., Fischer, G. & Rahfeld, J.U. (1995) A ribosome-associated peptidyl-prolyl *cis*/*trans* isomerase identified as the trigger factor. *EMBO J.* **14**, 4939–4948.
20. Furutani, M., Ideno, A., Iida, T. & Maruyama, T. (2000) FK506 binding protein from a thermophilic archaeon, *Methanococcus thermolithotrophicus*, has chaperone-like activity in vitro. *Biochemistry* **39**, 453–462.
21. Ideno, A., Furutani, M., Iba, Y., Kurosawa, Y. & Maruyama, T. (2002) FK506 binding protein from the hyperthermophilic archaeon *Pyrococcus horikoshii* suppresses the aggregation of proteins in *Escherichia coli*. *Appl. Environ. Microbiol.* **68**, 464–469.
22. Iida, T., Furutani, M., Nishida, F. & Maruyama, T. (1998) FKBP-type peptidyl-prolyl *cis-trans* isomerase from a sulfur-dependent hyperthermophilic archaeon, *Thermococcus sp.* KS-1. *Gene* **222**, 249–255.
23. Whyte, L.G. & Inniss, W.E. (1992) Cold shock protein and cold acclimation protein in a psychrotrophic bacterium. *Can. J. Microbiol.* **38**, 1281–1285.
24. Roberts, M.E. & Inniss, W.E. (1992) The synthesis of cold shock and cold accliation proteins in the psychrophilic bacterium *Aquqspirillum arcticum*. *Curr. Microbiol.* **25**, 275–278.
25. Araki, T. (1992) An analysis of the effect of changes in growth temperature on proteolysis in vivo in the psychrophilic bacterium *Viblio* sp. strain ANT-300. *J. Gen. Microbiol.* **138**, 2075–2082.
26. Potier, P., Drevet, P., Gounot, A.M. & Hipkiss, A.R. (1990) Temperaure-dependenct change in proteolytic activies and protein composition in the psychrotrophic bacterium *Arthrobacter globiformis* $S_1$55. *J. Gen. Microbiol.* **136**, 283–291.
27. Hebraud, M., Dubois, E., Potier, P. & Labadie, J. (1994) Effect of growth temperature on the protein levels in a psychrotrophic bacterium, *Pseudomonas fragi*. *J. Bacteriol.* **176**, 4017–4024.
28. Berger, F., Morellet, N., Menu, F. & Potier, P. (1996) Cold shock an cold acclimation proteins in the psychrotophic bacterium *Arthrobacter globiformis* SI55. *J. Bactriol.* **178**, 2999–3007.
29. Michel, V., Lehoux, I., Depret, G., Anglade, P., Labadie, J. & Hebraud, M. (1997) The cold shock response of the psychrotrophic bacterium *Pseudomonas fragi* involves four low-molecular-mass nucleic acid-binding proteins. *J. Bacteriol.* **179**, 7331–7342.
30. Hebraud, M. & Potier, P. (1999) Cold shock response and low temperature adaptation in psychrotrohic bacteria. *J. Mol. Microbiol. Biotechnol.* **1**, 211–219.
31. Kato, T., Haruki, M., Imanaka, T., Morikawa, M. & Kanaya, S. (2001) Isolation and characterization of psychotrophic bacteria from oil-reservoir water and oil sands. *Appl. Microbiol. Biotechnol.* **55**, 794–800.
32. Ohtani, N., Haruki, M., Morikawa, M. & Kanaya, S. (2001) Heat labile ribonuclease HI from a psychrotrophic bacterium: gene cloning, characterization, and site-directed mutagenesis. *Protein Eng.* **14**, 975–982.
33. Oh-Ishi, M. & Hirabayashi, T. (1989) Comparison of protein constituents between atria and ventricles from various vertebrates by two-dimensional gel electrophoresis. *Comp. Biochem. Physiol. B* **92**, 609–617.
34. Imanaka, T., Tanaka, T., Tsunekawa, H. & Aiba, S. (1981) Cloning of the genes for penicillinase, *penP* and *penI*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus licheniformis*. *J. Bacteriol.* **147**, 776–786.
35. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
36. Goodwin, T.W. & Morton, R.A. (1946) The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.* **40**, 628–632.
37. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. & Schmid, F.X. (1989) Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**, 476–478.
38. Takahashi, N., Hayano, T. & Suzuki, M. (1989) Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**, 473–475.
39. Schonbrunner, E.R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N. & Schmid, F.X. (1991) Catalysis of protein folding by cyclophilins from different species. *J. Biol. Chem.* **266**, 3630–3635.
40. Harrison, R.K. & Stein, R.L. (1990) Mechanistic studies of peptidyl prolyl cis-trans isomerase: evidence for catalysis by distortion. *Biochemistry* **29**, 1684–1689.
41. Ramm, K. & Pluckthun, A. (2000) The periplasmic *Escherichia coli* peptidylprolyl *cis,trans*-isomerase FkpA. II. Isomerase-independent chaperone activity *in vitro*. *J. Biol. Chem.* **275**, 17106–17113.
42. Rosenberg, M. & Court, D. (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu. Rev. Genet.* **13**, 319–353.
43. Shine, J. & Dalgarno, L. (1974) The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl Acad. Sci. USA* **71**, 1342–1346.
44. Engleberg, N.C., Carter, C., Weber, D.R., Cianciotto, N.P. & Eisenstein, B.I. (1989) DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. *Infect. Immun.* **57**, 1263–1270.
45. Fischer, G., Bang, H., Ludwig, B., Mann, K. & Hacker, J. (1992) Mip protein of *Legionella pneumophila* exhibits peptidyl-prolyl-*cis*/*trans* isomerase (PPlase) activity. *Mol. Microbiol.* **6**, 1375–1383.
46. Riboldi-Tunnicliffe, A., Konig, B., Jessen, S., Weiss, M.S., Rahfeld, J., Hacker, J., Fischer, G. & Hilgenfeld, R. (2001) Crystal structure of Mip, a prolylisomerase from *Legionella pneumophila*. *Nat. Struct. Biol.* **8**, 779–783.
47. Schmidt, B., Rahfeld, J., Schierhorn, A., Ludwig, B., Hacker, J. & Fischer, G. (1994) A homodimer represents an active species of the peptidyl-prolyl *cis*/*trans* isomerase FKBP25mem from *Legionella pneumophila*. *FEBS Lett.* **352**, 185–190.
48. Ludwig, B., Rahfeld, J., Schmidt, B., Mann, K., Wintermeyer, E., Fischer, G. & Hacker, J. (1994) Characterization of Mip proteins of *Legionella pneumophila*. *FEMS Microbiol. Lett.* **118**, 23–30.
49. Harrison, R.K. & Stein, R.L. (1992) Mechanistic studies of enzymatic and nonenzymatic prolyl cis-trans isomerization. *J. Am. Chem. Soc.* **114**, 3464–3471.
50. Furutani, M., Iida, T., Yamano, S., Kamino, K. & Maruyama, T. (1998) Biochemical and genetic characterization of an FK506-sensitive peptidyl prolyl *cis-trans* isomerase from a thermophilic archaeon, *Methanococcus thermolithotrophicus*. *J. Bacteriol.* **180**, 388–394.
51. Cianciotto, N.P., Eisenstein, B.I., Mondy, C.H. & Engleberg, N.C. (1990) A mutation in *mip* gene results in an attenuation

of *Legionella pneumophila* virulrence. *J. Infect. Dis.* **162**, 121–126.
52. Cianciotto, N.P. & Fields, B.S. (1992) *Legionella pneumophila mip* gene potentiates intercellular infection of protozoa and human macrophages. *Proc. Natl Acad. Sci. USA* **89**, 5188–8192.
53. Wintermeyer, E., Ludwig, B., Steinert, M., Schmidt, B., Fischer, G. & Hacker, J. (1995) Influience of site specifically altered Mip protein on intracellular survival of *Legionella pneumophila* in eukaryotic cells. *Infect. Immune.* **63**, 4576–4583.
54. Swanson, M.S. & Hammer, B.K. (2000) *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annu. Rev. Microbiol.* **54**, 567–613.
55. Graumann, P., Schroder, K., Schmid, R. & Marahiel, M.A. (1996) Cold shock stress-induced proteins in *Bacillus subtilis*. *J. Bacteriol.* **178**, 4611–4619.
56. Kandror, O. & Goldberg, A.L. (1997) Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. *Proc. Natl Acad. Sci. USA* **94**, 4978–4981.
57. Ideno, A., Yoshida, T., Iida, T., Furutani, M. & Maruyama, T. (2001) FK506-binding protein of the hyperthermophilic archaeum, *Thermococcus sp.* KS-1, a cold-shock-inducible peptidyl-prolyl *cis-trans* isomerase with activities to trap and refold denatured proteins. *Biochem. J.* **357**, 465–471.
58. Schiene-Fischer, C., Habazettl, J., Tradler, T. & Fischer, G. (2002) Evaluation of similarities in the cis/trans isomerase function of trigger factor and DnaK. *Biol. Chem.* **383**, 1865–1873.
59. Lu, K.P., Liou, Y.C. & Zhou, X.Z. (2002) Pinning down proline-directed phosphorylation signaling. *Trends Cell Biol.* **12**, 164–172.
60. Mallis, R.J., Brazin, K.N., Fulton, D.B. & Andreotti, A.H. (2002) Structural characterization of a proline-driven conformational switch within the Itk SH2 domain. *Nat. Struct. Biol.* **9**, 900–905.
61. Sansom, M.S. & Weinstein, H. (2000) Hinges, swivels and switches: the role of prolines in signalling via transmembrane α-helices. *Trends Pharmacol. Sci.* **21**, 445–451.
62. Reimer, U., Mokdad, N.E., Schutkowski, M. & Fischer, G. (1997) Intramolecular assistance of *cis*/*trans* isomerization of histidine-proline moiety. *Biochemistry* **36**, 13802–13808.
63. Texter, F.L., Spencer, D.B., Rosenstein, R. & Matthew, C.R. (1992) Intramolecular catalysis of proline isomerization reaction in the folding of dihydrofolate reductase. *Biochemistry* **31**, 5687–5691.