Overexpression, Purification, and Analysis of Complementation Behavior of *E. coli* SuhB Protein: Comparison with Bacterial and Archaeal Inositol Monophosphatases†

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ABSTRACT: The *E. coli suhB* gene product, which has been suggested to participate in posttranscriptional control of gene expression, also possesses inositol-1-phosphatase (I-1-Pase) activity. To test if SuhB I-1-Pase activity is sufficient for its function in cells, we have cloned the genes for three other I-1-Pases (from the archaea *Methanococcus jannaschii* and *Archaeoglobus fulgidus*, and from the bacterium *Thermotoga maritima*) into the *E. coli* expression vector pET23a(+) and examined if these extragenic I-1-Pases could complement the *suhB* mutation in *E. coli* strain CG1307 (which also has a mutation in *dnaB* and a cold-sensitive phenotype). None of these I-1-Pase genes restored growth at 30 °C although they generated active I-1-Pase enzymes (as measured by I-1-Pase specific activities of crude protein extracts from the transformed CG1307 cells). In contrast, the pET23a(+) recombinant plasmid with the wild-type *E. coli suhB* gene complemented the cold sensitivity of the chromosomal mutant *suhB* and restored the temperature-sensitive growth of the *dnaB* mutation in the double mutant strain CG1307. Further evidence that this relief of the suppressor behavior of the *suhB* mutation is not related to the I-1-Pase activity of the SuhB protein was provided by construction of the *E. coli* SuhB mutant D87N. This mutant protein is inactive as an I-1-Pase but fully functional in changing the temperature sensitivity of the *E. coli* double mutant strain. Therefore, I-1-P phosphatase activity is neither sufficient nor required for complementation of *suhB* mutant suppressor effects. The wild-type *E. coli* SuhB protein was also overexpressed to very high levels and purified to homogeneity in high yield (1 mg/10 mL of culture). The major differences of the *E. coli* I-1-Pase from all the other characterized I-1-Pases are that it exists as a monomer (rather than a dimer or tetramer) in solution and is more hydrophobic. These physical differences, rather than the I-1-Pase activity, may be involved in the biological role of wild-type SuhB in *E. coli*.

Mutations localized to the *Escherichia coli suhB* gene product have appeared under a variety of conditions as suppressors of various temperature-sensitive mutations that do not share any characteristics in gene function, including DNA replication mutation *dnaB121* (*1*), protein secretion mutation *secY24* (*2, 3*), and heat shock response mutation *rpoH15* (*4, 5*). A summary of these different mutant phenotypes in presented in Table 1. *E. coli* mutant strain B178ts121 (*1*) contains an insertion in the *dnaB* gene that affects DNA replication at higher temperature (e.g., 42 °C). This strain, which has a wild-type chromosomal *suhB* gene, forms normal colonies at 30 °C but cannot form colonies at 42 °C. A double mutant *E. coli* strain (CG1307) derived from it contains mutations in both *dnaB* and *suhB* genes (*1*), and was found to exhibit a cold-sensitive phenotype (inability to form colonies at 30 °C). The introduction of a plasmid containing the wild-type *suhB* gene into the double mutant allows the cells to now grow at 30 °C and not 42 °C. A *secY24* mutant with a single base change is defective in secretion of envelope proteins across the cytoplasmic membrane at higher temperature (42 °C) and accumulates precursors of envelope proteins. The *ssyA3* mutation, identified as a suppressor for the *secY24* mutant, turned out to be a mutated *suhB* gene (*3, 4*). In the *secY24*−*ssyA3* double mutant, growth at 30 °C was rescued by a plasmid containing wild-type *suhB*. The *rpoH15* mutant produces altered σ^{32} protein that results in a deficiency in the transcription of heat shock genes; these cells can only grow at 34 °C or lower. A mutation of the *suhB* gene enhances σ^{32} synthesis and alters the growth temperature of the *rpoH15* mutant to high temperature (40 °C) (*4, 5*). Thus, all three known *suhB* suppressor mutations cause a cold-sensitive growth pheno-

Table 1: Effect of Wild Type and Mutant *suhB* on the Growth Temperature of Different *E. coli* Strains Containing Diverse Mutations

		growth at		effect of extragenic WT *suhB* on growth	
strain	mutation	30 °C	42 °C	30 °C	42 °C
dnaB121	DNA replication (*dnaB*)	+	−		
CG1307	DNA replication and *suhB*	−	+	+	−
secY24	protein secretion	+	−		
secY24/ ssyA3	protein secretion and *suhB*	−	+	+	−
rpoH15	heat shock response	+	−		
rpoH15/ suhB2	heat shock response and *suhB*	−	+	+	−

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type. The mechanism by which *suhB* mutations suppress these other rather diverse mutations is unclear, although it has been suggested that wild-type SuhB may participate in posttranscriptional control of gene expression (*6, 7*).

An interesting characteristic of the SuhB protein that is potentially relevant to how its mutants suppress diverse temperature-sensitive mutations is its significant sequence homology with mammalian inositol monophosphatase, a key enzyme involved in phosphoinositide-dependent signal transduction pathways (*8*−*10*). I-1-Pases are ubiquitous in a wide range of organisms. When the *E. coli* SuhB protein sequence is used to search the protein database for organisms with sequenced genomes, one can find proteins ranging from 245 to 292 residues that are homologous to SuhB. Several of these organisms (e.g., *S. cerevisiae*) utilize inositol signaling pathways; others synthesize inositol-containing lipids as components of their cell membrane. In other organisms, inositol is part of small solutes synthesized and accumulated in response to osmotic or thermal stress (*11*). However, in many cells including *E. coli*, inositol compounds (small soluble molecules or lipids) are undetected or represent very minor components. For example, phosphatidylinositol in *E. coli* strains represents less than 0.01% of total phospholipid in the cells (*12*).

The question arises as to what is the true function of these homologous proteins, and how can one distinguish between a cellular role as an I-1-Pase, whose primary function is hydrolysis of I-1-P and related compounds, and a protein likely to be involved in the regulation of RNA processing (*6, 7*), whose mutants can suppress temperature-sensitive growth. The putative identifications of hypothetical proteins as either SuhB homologues or inositol monophosphatases appear sometimes arbitrary, especially when the sequence homologies drop to the lower range of the threshold (25−30% identity) for most sequence recognition software. For example, the MJ0109 gene product from *Methanococcus jannaschii* was initially identified as an inositol monophosphatase and later revised to be a SuhB homologue (*13*) based on its 56.5% sequence similarity with a hypothetical protein of *Haemophilus influenzae* (HI0937) (*14*) which has 81.1% sequence similarity with the *E. coli* SuhB protein. If the protein encoded by MJ0109 is compared with both human I-1-Pase and the *E. coli suhB* gene product directly, it has a slightly higher score with human I-1-Pase than with SuhB, but both scores have ∼30% identity. As the recombinant *E. coli* SuhB protein (*15*) and MJ0109 gene product (*16*) clearly have I-1-Pase activity with kinetic parameters (K_m, V_{max}) similar to other known I-1-Pase enzymes, in vitro I-1-Pase activity is not valid for distinguishing between cellular function as an I-1-Pase or SuhB homologue.

The objectives of the present work include the following: (i) in vivo testing of genes from three thermophiles that have been putatively identified as coding for SuhB or I-1-Pase to see if they are able to complement the *suhB* mutation and allow cell growth at 30 °C in an *E. coli* double mutant strain (CG1307); (ii) overexpression and purification of pure SuhB protein for kinetic and physical characterization; and (iii) determination of other significant similarities and differences between SuhB, thermophiles, and other I-1-Pase proteins. Stringent testing of the effect of I-1-Pase/SuhB proteins in their natural host requires the development of mutant cell lines from those thermophilic organisms. However, the mutant *E. coli* cell lines are an excellent tool to probe if the effect of the *suhB* gene is absolutely related to I-1-Pase activity. The in vivo complementation test shows that *M. jannaschii*, *Archaeoglobus fulgidus*, and *Thermotoga maritima* I-1-Pases cannot complement the *suhB* mutation in *E. coli* CG1307 even though at the *E. coli* growth temperature they have levels of expression and I-1-Pase activity comparable to the extragenic *E. coli* SuhB I-1-Pase control. For the second objective, the *suhB* gene was cloned into *E. coli* expression vector pET23a(+), overexpressed to very high levels, purified to homogeneity in high yield (1 mg of pure enzyme per 10 mL of cultured medium), and characterized kinetically. A mutation in the *suhB* gene that converts aspartate-87 to asparagine (D87N) was constructed based on sequence alignment with human I-1-Pase for which a crystal structure exists (*17*−*19*). D87 is aligned with D93 of human I-1-Pase and is predicted to be a residue involved in both substrate and metal ion binding. This mutant SuhB protein was inactive as an I-1-Pase. However, this mutant *suhB* gene introduced on a plasmid could still complement the *suhB* mutation in CG1307 and allow the cells to grow at 30 °C. These studies indicate that I-1-Pase activity is neither required nor sufficient for complementing the suppressor behavior of *suhB* mutations.

MATERIALS AND METHODS

Chemicals. D,L-Inositol-1-phosphate, D-inositol-1-phosphate, inositol-2-phosphate, 2-AMP, 5-AMP, *p*-nitrophenolphosphate (pNPP), β-glycerophosphate (βGP), D-glucose-1-phosphate (G-1-P), D-glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), NAD^+, SDS−PAGE molecular weight markers, gel filtration molecular weight markers, and Coomassie brilliant blue 250 were obtained from Sigma. The Q-Sepharose fast flow and Phenyl-Sepharose resins were obtained from Pharmacia; BioGel HTP was obtained from BioRad. Restriction enzymes were obtained from New England BioLab. The ligation kit, BL21(DE3)pLysS, and Novablue *E. coli* strains were purchased from Novagen. The PCR kit was obtained from Perkin-Elmer. The *pfu* DNA polymerase was purchased from Stratagene. The *E. coli* double mutant strain CG1307 and the plasmid pLB170 were provided by Prof. Costa Georgopoulos (Centre Medical Universitaire, Geneva, Switzerland). The *A. fulgidus* genomic DNA was provided by Prof. Harold Schreier from the University of Maryland. L-Inositol-1-phosphate was synthesized enzymatically using *A. fulgidus* I-1-P synthase (*20*). The construction of plasmid pBC/MJ, which has the *M. jannaschii* I-1-Pase gene inserted into pET23a(+), and pBC/TM, which has the *T. maritima* I-1-Pase gene in pET23a(+), was described previously (*16, 21*). The recombinant plasmid, HGBDI64, which contains human I-1-Pase in pBluescript SK-phagemid vector, was obtained from ATCC (ATCC #103219). The oligonucleotide primers were purchased from Operon Technology.

Media and Plates. LB broth has 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter. L medium contains 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. The pH was adjusted to 7.4. The L-plates contained 50 μg/mL ampicillin and 50 μg/mL kanamycin; LB plates had 100 μg/mL ampicillin and 34 μg/mL chloroamphenicol when used for recombinant plasmid transformed BL21(DE3)-

pLysS strain and 100 μg/mL ampicillin for transformed Novablue cells.

Subcloning the E. coli suhB Gene into pET23a(+) and in Vivo Complementation Tests. Two oligonucleotides, 5′-ccagtgagagagaCATATGcatccgatgctg-3′ (with *Nde*I site) and 5′-gggcgggtgagtGAATTCacccgcctgag-3′ (with *Eco*RI site), were used to reconstruct and amplify the *E. coli suhB* gene from plasmid pLB170 which contains a 5 kilobase *Kpn*I DNA fragment of *E. coli* in vector pSP6/T719 (*7*). The 25 cycle *pfu* DNA polymerase-amplified PCR products were digested with *Nde*I and *Eco*RI, ligated to the *Nde*I−*Eco*RI-cut pET23a(+) vector to form plasmid pBC/EC-wt, and transformed to Novablue competent cells to maintain the plasmid. The positive clones were identified by restriction mapping. The plasmid from a positive recombinant clone was transformed into the CG1307 strain, a double mutant having an insertion in the *dnaB* gene and a mutation in the *suhB* gene. The *dnaB* mutant (ts121) alone does not form colonies on L-plates at 42 °C, but can form colonies at 30 °C. Strain CG1307, with a mutation in *suhB* as well, is a revertant of ts121 that now forms colonies at 42 °C but not at 30 °C. When this double mutant takes up plasmid containing the wild-type *suhB* gene, it forms colonies on L-plates (with 50 μg/mL ampicillin and 50 μg/mL kanamycin) only at 30 °C. The plasmids pBC/MJ [*M. jannaschii* I-1-Pase gene in pET23a(+)], pBC/TM [*T. maritima* I-1-Pase gene in pET23a(+)], pBC/AF [*A. fulgidus* I-1-Pase gene in pET23a(+)], pLB170, HGBDI64, pET23a(+), and pBC/EC-D87N (see mutagenesis of *E. coli* I-1-Pase for its construction) were also introduced into the *E. coli* CG1307 strain by standard $CaCl_2$ transformation methods. The plates were incubated at 30 and 42 °C for 20−30 h, and colonies counted.

Cloning of A. fulgidus I-1-Pase Gene. Two oligonucleotide primers were designed based on the DNA sequence of flanking regions of the AF2373 fragment that was putatively identified as the *A. fulgidus suhB* gene. 5′-ccctacgc-ccCATATGgatgaaagg-3′ (containing an *Nde*I site) and 5′-gcttcctcaatcGAATTCacgtgacc-3′ (containing a *Eco*RI site) were used to amplify the AF2372 gene from *A. fulgidus* genomic DNA with high-fidelity *pfu* DNA polymerase. The 25-cycle PCR products were cut with *Nde*I−*Eco*RI and ligated to the *Nde*I- and *Eco*RI-digested pET23a(+). Positive clones were identified by restriction mapping, and DNA sequence was confirmed by automatic double strand DNA sequencing.

Expression Levels of I-1-Pase Genes in CG1307. The percent of total protein in CG1307 that is represented by I-1-Pase was measured by comparing the observed specific activity (measured with a colorimetric phosphate assay) in the crude extract (nmol min^{-1} mg^{-1}) toward D-I-1-P at a given temperature to the specific activity for each of the pure I-1-Pases toward D-I-1-P at that temperature (30 or 40 °C): [specific activity of crude extracts/specific activity of pure I-1-Pase] × 100 = % expression of I-1-Pase of the total protein (weight by weight basis). Specific activity values for the *M. jannaschii* and *T. maritima* I-1-Pases toward D-I-1-P have been published previously (*20, 21*).

Overexpression of Recombinant SuhB Protein. The recombinant plasmid pBC/EC was transformed into BL21(DE3)pLysS-competent cells for expression. A single colony of BL21(DE3)pLysS containing the recombinant *suhB* gene−pET23a(+) plasmid was grown in 5 mL of LB medium with 50 μg/mL ampicillin and 34 μg/mL chloroamphenicol until the optical density at 560 nm reached 0.6. Cell pellets from 5 mL of culture were used to inoculate 2 L of fresh LB medium containing 100 μg/mL ampicillin and 34 μg/mL chloroamphenicol. These cultures were grown to OD_{560} ∼0.7 by rapid shaking (200 rpm) at 37 °C. Overexpression of recombinant protein in the cultures was induced by the addition of 4 mL of 200 mM IPTG (to a final concentration of 0.4 mM). Over the course of 3−4 h, the cultures grew to OD_{560} ∼1.5. Aliquots of cultured cells (300 mL) were harvested by centrifugation and stored at −20 °C until needed. The time course for expression of protein was monitored by SDS−PAGE and detection of a band corresponding to 32 kDa. The crude cell extract had high I-1-Pase activity, whereas the BL21(DE3)/pET23a(+) cell extract (cells containing the plasmid without the *suhB* gene insert) did not have the corresponding 32 kDa band on SDS−PAGE and no detectable I-1-Pase activity.

Purification of SuhB Protein from Cell Pellets. The initial purification of this protein turned out to be problematic if the columns and buffers used for the purification of *M. jannaschii* and *T. maritima* I-1-Pase were used (*16, 21*). When the cells were harvested and broken immediately, little cell debris was detected after centrifugation at 12400*g* for 20 min. However, after dialysis and storage, significant amounts of the overexpressed protein precipitated. When cell pellets were frozen and stored for several weeks, the protein was much less soluble when the cells were opened. This low solubility at low ionic strength is likely to account for the very low yield (25 μg/500 mL of cultured cells) of purified protein reported previously (*15*). The inclusion of 50 mM KCl in the breakage buffer increased enzyme solubility in the crude homogenate from both fresh and frozen cell pellets. For a typical preparation, frozen cells (∼0.8 g from 300 mL of culture) were thawed and resuspended in 60 mL of buffer A (50 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) with 50 mM KCl. The suspensions were sonicated for 10 × 30 s on ice, and the supernatant was separated from cell debris by centrifugation (12400*g* for 20 min). The solubilized protein was applied to a Q-Sepharose Fast Flow column (2.5 × 12 cm) and eluted with a gradient of 0.05−0.50 M KCl in buffer A (total 400 mL). The I-1-Pase fractions, eluting at 0.1 M KCl as detected by I-1-Pase activity and SDS−PAGE, were pooled (∼100 mL) and then loaded onto a BioGel HTP column (1.6 × 25 cm) preequilibrated with buffer A. Protein was eluted with a linear gradient of 0−1.2 M $(NH_4)_2SO_4$ in Buffer A (total volume ∼200 mL). The fractions of pure of SuhB protein as judged on SDS−PAGE were combined and dialyzed against buffer A with 200 mM KCl and concentrated to 6 mL with a protein concentration of 5 mg/mL or concentrated to ∼30 mg/mL without dialysis.

Mutagenesis of E. coli SuhB I-1-Pase Activity. Residue D87 of *E. coli* I-1-Pase is aligned with D93 in the well-conserved active site region of human enzyme (*17*−*19*). Mutation of human I-1-Pase D93 to N93 resulted in complete loss of I-1-Pase activity (*22*). D87 in the *E. coli* enzyme is an excellent target for mutagenesis because a similar mutation (D87N) should also eliminate its I-1-Pase activity completely if the *E. coli* and human enzymes have similar active site structures. The two-step overlap extension PCR mutagenesis method (*23, 24*) was used to introduce the specific mutation

(D87N) in the *suhB* gene. In the first-step PCR, two pairs of primers, primer **a**, 5′-gatcccgcgaaattaatacgactcac-3′, and primer **b**, 5′-aaagttggtagtgccATTcagtggatcgataacc-3′; primer **c**, 5′-ggttatcgatccactgAAT-ggcactaccaacttt-3′, and primer **d**, 5′-ggggttatgctagttattgct-3′, were used to amplify the *suhB* gene fragment from the wild-type recombinant plasmid. Primers **a** and **b** resulted in a 713 bp fragment that had overlapping ends with the 369 bp fragment amplified by primers **c** and **d**. One GAT codon was modified to AAT in the overlapping region of both fragments. In the second-step PCR reaction, the equal molar gene-cleaned 713 and 369 bp fragments from the first-step amplification were used as templates and amplified with primers **a** and **d**, which gave a 1050 bp product. The gene-cleaned 1050 bp fragments were cut with *Nde*I and *Eco*RI and ligated to the *Nde*I−*Eco*RI-digested pET23a(+) vectors to form the mutant plasmid pBC/EC-D87N.

Inositol Monophosphatase Assays. I-1-Pase activity was measured by colorimetric determination of released inorganic phosphate (P_i) (*25*). For use in monitoring I-1-Pase activity of column fractions, the reaction mixture contained 2 μL of D,L-I-1-P (10 mM stock in 50 mM Tris, pH 8.0), 2 μL of $MgCl_2$ (200 mM stock in 50 mM Tris, pH 8.0), and 44 μL of enzyme solution. The amount of enzyme added was adjusted to give an A_{660} of 0.3−0.4 within 1−2 min of incubation time. The final concentrations of substrate and Mg^{2+} were 0.4 and 8 mM in most assay mixtures. For the V_{max} and K_m determinations, the sensitivity range of the colorimetric P_i assay and the lower substrate concentrations needed in the assay required increasing the total assay volume to 0.5 mL. After incubation at 37 °C (the assay temperature used unless specified) for 1 min, 1 mL of ammonium molybdate Malachite Green reagent was immediately added to the mixtures (*25*). The color reagent contains ∼1.2 M HCl and is sufficient to stop the reaction. A comparison of observed A_{660} changes to standard P_i samples was used to calculate the I-1-Pase rate. All the substrates were quite stable for at least 1 h in the dye solution as evidenced by the lack of P_i in controls without the addition of the enzyme. Specific activity values were calculated by using the protein concentration determined by the Bradford method (*26*) with bovine serum albumin (obtained from BioRad) as the standard. A calculated extinction coefficient $\epsilon_{280} = 23\,000$ mol·cm based on the predicted amino acid composition (*27*) was used to determine the concentration of homogeneous recombinant protein.

Gel Filtration. A column (1.6 × 72 cm) of Biogel A 0.5m equilibrated with buffer A or buffer A with 0.25 mM KCl was used to determine the native molecular weight of *E. coli* SuhB protein in low and moderate ionic strength media. The void volume was determined with Blue Dextran, and the column was standardized with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), *M. jannaschii* I-1-Pase (56 kDa) (*16*), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Samples (0.5 mL of A_{280} 2∼3) were applied to the column and eluted at a flow rate of 0.4 mL/min. Fractions of 2 mL each were collected and assayed for I-1-Pase activity and absorbance at 280 nm.

RESULTS

In Vivo Complementation of suhB Mutation. To test if other microbial I-1-Pase gene products can function in *E. coli* in complementing the chromosomal *suhB* mutation in CG1307, we transformed three recombinant plasmids, pET23a-(+) containing known functional I-1-Pase genes (pBC/MJ, pBC/TM, and pBC/AF), into the double mutant strain CG1307. pBC/MJ contains the cloned I-1-Pase gene from *M. jannaschii*, pBC/TM has the *T. maritima* I-1-Pase gene, and pBC/AF contains the *A. fulgidus* I-1-Pase gene. The effects of these plasmids on the growth temperature of CG1307 are shown in Table 2. Clearly, those I-1-Pase genes did not function to complement the *E. coli* mutant, because the transformed host cells still formed colonies on L-plates at 42 °C only; no visible colonies appeared when the plates were incubated at 30 °C. The pET23a(+) and all the pET vector series (*28*) were specifically designed to be used with *E. coli* strains, such as BL21DE3 and its derivatives, that have an inducible chromosome copy of the T7 RNA polymerase gene under the control of lacUV promoter (*29*). To get expression of the cloned genes with any other strains, the T7 RNA polymerase gene required must be introduced by helper phage infection. The genes cloned downstream of the T7 promoter, which are not used by *E. coli* host RNA polymerase, have a low chance of being transcribed, and the base line transcription is expected to be extremely low. One might argue that those cloned genes might not be functional in *E. coli* because the CG1307 strain does not have any source of T7 RNA polymerase, and pET 23a(+) does not have an *E. coli* promoter before its polycloning site. The promotor for the β-lactamase gene in that plasmid is also far away from the cloned I-1-Pase genes. With this potential complication in mind, we subcloned the wild-type *E. coli suhB* gene into the same plasmid. The CG1307 strain transformed with the plasmid containing wild-type *E. coli suhB* (designated pBC/EC-wt) exhibited growth at 30 °C (Table 2) and not at 42 °C. As a positive control, the plasmid pLB170 was introduced into CG1307; as expected, cell growth now occurred at 30 °C and not 42 °C. When CG1307 was transformed with plasmid pET23a(+) that did not contain the wild-type *suhB* gene, the cells could only grow at 42 °C, implying the *suhB* gene introduced with pBC/EC-wt was responsible for complementing the chromosomal *suhB* mutation. The cells transformed with pET23a(+) did,

Table 2: Results of in Vivo Complementation of the *suhB* Mutation in *E. coli* CG1307 upon Transformation with Plasmids Containing Various I-1-Pase Genes[a]

designation	host plasmid	I-1-Pase gene	colonies (30 °C)	colonies (42 °C)
pLB170	PLB170	*E. coli suhB*	500	0
pET23a(+)	pET23a(+)	−	0	1000
pBC/EC-wt	pET23a(+)	wild type *suhB*	2000−3000	10
pBC/EC-D87N	pET23a(+)	D87N mutant *suhB*	2000	0
pBC/MJ	pET23a(+)	*M. jannaschii* I-1-Pase	0	1000
pBC/TM	pET23a(+)	*T. maritima* I-1-Pase	0	1000
pBC/AF	pET23a(+)	*A. fulgidus* I-1-Pase	0	1000
HGBDI64	pBluescript SK-phagemid vector	human I-1-Pase	0	600

[a] Plasmids (1 μL) were transformed into CG1307 competent cells using the standard $CaCl_2$ transformation method. After the preincubation at 37 °C for 1 h, the cell pellets were resuspended in 200 μL of fresh L media, and 100 μL of each was plated on L-plates. The plates were then incubated at 30 or 42 °C for 20−30 h.

Table 3: I-1-Pase Activity in Protein Extracts of *E. coli* CG1307 Transformed with Plasmids [pET23a(+)] Containing Various I-1-Pase Genes[a]

I-1-Pase gene source	sp act. (nmol min^{-1} mg^{-1}) 30 °C	42 °C	expression level (%)[b]
E. coli	1.86 ± 0.04	4.21 ± 0.06	0.096[c]
M. jannaschii	0.65 ± 0.06	1.47 ± 0.06	0.25[d]
T. maritima	11.9 ± 0.08	27.2 ± 0.2	0.13[d]

[a] Cultures were grown in 10 mL of media at the permissive temperature from a single colony; I-1-Pase activity was assayed with D-I-1-P as the substrate. [b] Expression level was estimated by comparing the specific activity of the I-1-Pase in protein extracts from *E. coli* CG1307 containing the plasmid to the specific activity of the purified enzyme (see Materials and Methods). [c] Cells of *E. coli* CG107 with the *E. coli suhB* gene introduced on the pET23a(+) plasmid were grown at 30 °C. [d] *E. coli* strain G1307 with the *M. jannaschii* I-1-Pase or *T. maritima* I-1-Pase gene introduced on the pET23a(+) plasmid were grown at 42 °C.

however, acquire ampicillin resistance from the plasmid. Interestingly, the commercial plasmid HGBDI64 that contains the human I-1-Pase gene was also not functional in altering the growth temperature of the double mutant CG1307.

Since the complementation assays relied on basal levels of expression from a T7 promoter in the absence of the T7 polymerase, expression levels of the different I-1-Pase proteins could differ significantly. Thus, differences in protein expression could be the reason that the plasmids with the I-1-Pase genes from the two thermophiles could not complement the *suhB* mutation in CG1307. To test this, expression levels of the different I-1-Pase proteins (at the permissive temperature for growth) introduced into the CG1307 cells were quantified by measuring I-1-Pase activity (at both 30 and 42 °C) in protein extracts and comparing this to specific activity for the pure enzymes at the relevant temperature. As shown in Table 3, expression of the three I-1-Pases for which data are available for the specific activity of the pure I-1-Pase (*16*, *21*) was comparable (0.1−0.25%). Furthermore, enzyme activity was detected for each enzyme at both 30 and 42 °C. These results strongly suggest that I-1-Pase activity alone cannot complement the *suhB* mutation in CG1307.

Overexpression, Purification, and Mutation of Recombinant Protein. The apparently unique ability of extragenic *suhB* to complement the *E. coli* CG1307 mutation and alter the growth temperature of the cells suggested that some other facet aside from I-1-Pase activity was linked to its behavior in *E. coli*. To study the SuhB protein and to compare it to the other better studied I-1-Pases, the SuhB protein was overexpressed in *E. coli* to very high levels (60−70% of total cellular protein). About 30 mg of pure enzyme (Figure 1A) could be obtained from 300 mL of cultured cells; a summary of specific activities and yields after each step is shown in Table 4. The purified SuhB protein was characterized by a single band on SDS−PAGE with an apparent subunit size of ∼32 kDa; that value is considerably larger than the 29.2 kDa predicted from the DNA sequence. The abnormal electrophoretic mobility for this protein was first observed by Matsuhisa et al. (*15*); the reason for this behavior is unknown although it may reflect enhanced hydrophobicity of the protein compared to other I-1-Pases. Gel filtration showed the native *E. coli* SuhB to have a molecular mass of

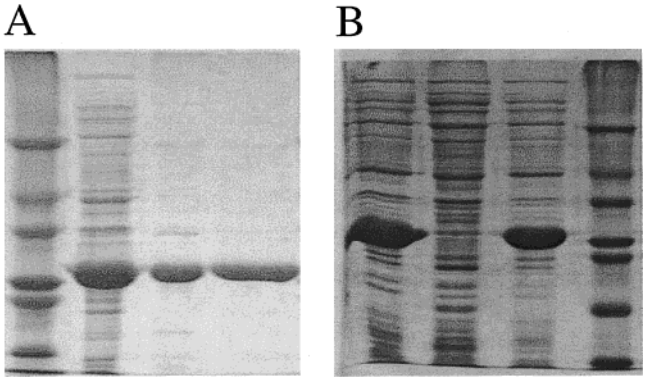

FIGURE 1: (A) *E. coli* SuhB protein expression and purification analyzed by 12% acrylamide SDS−PAGE stained with Coomassie brilliant blue: lane a, molecular mass standards (kDa): 66, 45, 36, 29, 24, 20.1, 14.2; lane b, crude extract; lane c, combined fractions from QFF column; lane d, purified *E. coli* SuhB. (B) SDS−PAGE analysis of D87N SuhB mutant overexpression: solubilized cells in lanes a and c indicate high levels of soluble proteins at 32 kDa which are inactive as I-1-Pase enzymes because of the mutation of active site residue D87. Lane b represents protein from a mutant plasmid that did not overexpress the recombinant protein. In lane d are shown molecular mass standards (66, 45, 36, 29, 24, 20.1, and 14.2 kDa).

Table 4: Purification of *E. coli* SuhB Protein

	total protein (mg)	specific activity[a]	total units[b]	yield (%)	purification (*x*-fold)
crude extract	92.8	2.05	190	100	1
Q-Sepharose fast flow	40	3.1	125	66	1.52
Phenyl-Sepharose	31	3.3	101	53	1.59

[a] Specific activity units are μmol min^{-1} mg^{-1} (determined with D,L-I-1-P at 1 mM concentration); assays were carried out at 37 °C. [b] One unit is defined as 1 μmol of P_i produced per milligram of protein per minute at 37 °C.

30.5 ± 1.5 kDa in low ionic strength buffer (buffer A) and 39 kDa in buffer A supplemented with 250 mM KCl. Since the SuhB protein has I-1-Pase activity, the gel filtration results strongly suggest this I-1-Pase is active as a monomer, a striking difference from all other known I-1-Pase proteins which are oligomeric (usually dimers). Interestingly, the activity of mammalian I-1-Pase has been detected in 8 M urea (*30*). This observation suggests that partially folded conformations of mammalian I-1-Pase monomers still have the catalytic site preserved and are functional.

Aligning the sequence of *E. coli* SuhB with the human I-1-Pase (Figure 2), for which there is a crystal structure (*17*−*19*), suggests that the *E. coli* protein has most of the key active site residues of the human enzyme. Specifically, *E. coli* I-1-Pase has D87, A188, E205, P158, and D212 aligned with D93, A196, E213, S165, and D220 of the human enzyme (these residues form the inositol binding site). In particular, D93 of the human enzyme has been shown to be critical for activity (*22*). The similarity in inositol binding motifs between human and *E. coli* proteins suggested to us that the *E. coli* D87N mutant would be inactive. If the mutant is indeed inactive [evaluated by overexpressing the altered gene in BL21(DE3)pLysS and assaying for mutant protein specific activity], it can be used to transform the double mutant strain CG1307. If colonies still only form at 42 °C, then I-1-Pase activity is necessary for the *suhB* gene product to complement the chromosomal *suhB* mutation in CG1307.

```
                10        20        30        40        50
Human  MADPWQECMDYAVTLARQAGEVVCEAIK--NEMNVMLKSSPVDLVTATDQKVEKMLISSI
                .. ::  ::.::... .  .  . ...  :.:  :.:: .:. .: ..:..:
E.coli     MHPMLNIAVRAARKAGNLIAKNYETPDAVEASQKGSN-DFVTNVDKAAEAVIIDTI
                  10        20        30        40        50

       60        70        80        90       100       110
Human  KEKYPSHSFIGEESVAAGEKSILTDNPTWIIDPIDGTTNFVHRFPFVAVSIGFAVNKKIE
       ...::.:..: :::   ::     ..  :.::::.:::::..:.:  ::::.  .. . :
E.coli RKSYPQHTIITEES---GELEGTDQDVQWVIDPLDGTTNFIKRLPHFAVSIAVRIKGRTE
          60        70        80        90       100       110

      120       130       140       150       160       170
Human  FGVVYSCVEGKMYTARKGKGAFCNGQKLQVSQQEDITKSLLVTELGSSRTPETVRMVLSN
        .:::. ......:: .:.::  :: .:. :  .:.  ..:.: .  .. . .  ..
E.coli VAVVYDPMRNELFTATRGQGAQLNGYRLRGSTARDLDGTILATGF-PFKAKQYATTYINI
            120       130       140       150       160       170

      180       190       200       210       220       230
Human  MEKLF--CIPVHGIRSVGTAAVNMCLVATGGADAYYEMGIHCWDVAGAGIIVTEAGGVLM
       . :::  :     .: .:.::...  ::.: .:...:.:.. :: :.. ..: :::::.
E.coli VGKLFNECAD---FRRTGSAALDLAYVAAGRVDGFFEIGLRPWDFAAGELLVREAGGIVS
            180       190       200       210       220

       240       250       260       270
Human  DVTGGPFDLMSRRVIAANNRILAERIAKEIQVIPLQRDDED
       : :::   ...  ..:.: :..   .:.
E.coli DFTGGHNYMLTGNIVAGNPRVVKAMLANMRDELSDALKR
      230       240       250       260
```

FIGURE 2: Sequence alignment of human I-1-Pase and *E. coli* SuhB; the likely active site residues, based on the human enzyme crystal structure, are labeled in boldface.

If, however, the transformed cells now grow at 30 °C, then the ability of the protein to complement the mutation is independent of I-1-Pase activity. A plasmid, designated pBC/EC-D87N, was constructed with the mutant *suhB* gene. Overexpression of the mutant in BL21(DE3)pLysS yielded large amounts of soluble D87N protein (Figure 1B), comparable to what can be generated with the wild-type *suhB* gene in that expression strain. Similar to the human D93N mutant enzyme, *E. coli* SuhB D87N had no detectable I-1-Pase activity (the upper limit on I-1-Pase activity was 0.0003 μmol min^{-1} mg^{-1}, or less than ~0.01% of the wild-type activity). Transformation of CG1307 with pBC/EC-D87N caused colonies to grow at 30 °C instead of 42 °C (Table 2). Thus, the loss of I-1-Pase activity had virtually no effect on the ability of the extragenic mutant *suhB* to complement the CG1307 mutation. Whatever the SuhB protein is doing in the *E. coli* cells, it does not require I-1-Pase activity.

Kinetic Characterization of the E. coli SuhB I-1-Pase Activity. Like all the other I-1-Pase enzymes, the *E. coli* SuhB protein required Mg^{2+} for I-1-Pase activity (Figure 3A). The optimal Mg^{2+} concentration was around 6 mM, which is close to the previously reported value of 10 mM (*15*). This Mg^{2+} requirement is slightly higher than that for most mammalian and plant I-1-Pase enzymes (1−3 mM) (*31*−*34*) but much less than the optimum required by I-1-Pase enzymes from thermophilic organisms (50−100 mM) (*16*, *21*). Other divalent metal ions that led to partial activity included Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+} (Figure 3B). When these metal ions were included in an assay containing Mg^{2+}, they all inhibited the enzyme activity, indicating that they could bind tightly to the enzyme but were not as effective as Mg^{2+}. While high concentrations of divalent metal ions inhibited I-1-Pase, small monovalent Li^+ was the most potent inhibitor (Figure 4). The IC_{50} for Li^+ with 0.4 mM substrate was 2 mM, a value higher than the K_i for mammalian I-1-Pases (0.2−0.3) (*22*, *32*) and the value of

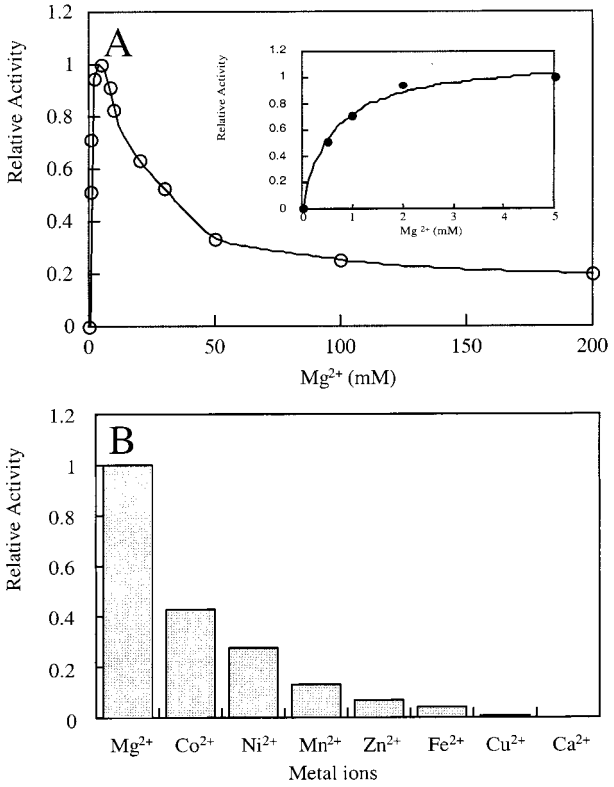

FIGURE 3: (A) Dependence of *E. coli* SuhB I-1-Pase activity (toward 0.4 mM I-1-P in 50 mM Tris-HCl, pH 8.0, at 37 °C) on Mg^{2+} concentration. Activities are normalized to the value obtained with 6 mM $MgCl_2$. (B) Ability of other metal ions (at 6 mM) to activate *E. coli* extragenic suppressor I-1-Pase activity in the absence of Mg^{2+}. Activities are normalized to the value obtained with 6 mM $MgCl_2$.

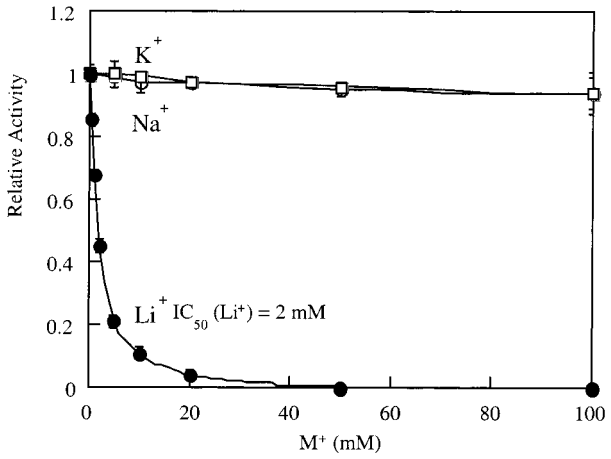

FIGURE 4: Inhibition of *E. coli* SuhB I-1-Pase activity by Li^+ (●), Na^+ (○), and K^+ (□). Assay mixtures containing 0.4 mM D,L-I-1-P in 50 mM Tris-HCl, pH 8.0, with 6 mM $MgCl_2$ and 1.4 μg of I-1-Pase were incubated at 37 °C for 2 min. Relative activities are normalized to the value for the assay without the monovalent cation salt added.

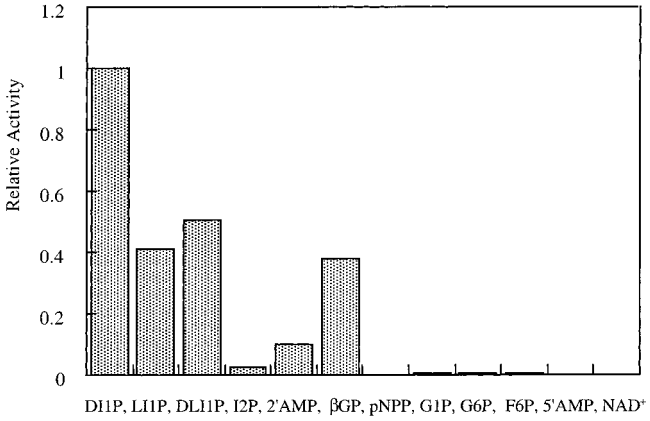

FIGURE 5: Substrate specificity of *E. coli* SuhB phosphatase activity. The assay mixtures, containing 1.0 mM substrate, 6 mM $MgCl_2$, in 50 mM Tris-HCl, pH 8.0, and enzyme (1.4 μg) in a total volume of 20 μL, were incubated at 37 °C for 2 min. Activities are normalized to the value for D-I-1-P. Errors in enzyme activity were 3−5%.

0.3 mM that had previously been estimated for Li^+ inhibition of SuhB protein (*15*). Neither Na^+ nor K^+ had any significant effects on activity in the concentration range of 0−100 mM and only inhibited the enzyme at very high concentrations.

The substrate specificity of *E. coli* SuhB I-1-Pase activity is shown in Figure 5. In contrast to previous reports (*15*), we found this enzyme can discriminate between D- and L-isomers of I-1-P under the assay conditions used. The *E. coli* I-1-Pase was about 2.5-fold more active toward D-I-1-P than toward L-I-1-P. This difference in activity remains over a substrate concentration range of 0.03−2.0 mM and for assay temperatures as high as 85 °C. The only other known I-1-Pase that has a large preference for the D-isomer is the *T. maritima* I-1-Pase, which is about 20 times more active toward D-I-1-P than L-I-1-P (*21*). The K_m and V_{max} values for the *E. coli* I-1-Pase toward I-1-P isomers are listed in Table 5. At 37 °C, the V_{max} was 2−3-fold lower than the previously reported value [12.2 μmol min^{-1} mg^{-1} (*15*)], but the K_m was almost identical with that work. The V_{max} increased dramatically when the temperature was increased to 70 °C while the K_m remained almost the same. The I-1-Pase activity of SuhB was also less efficient in hydrolyzing I-2-P, with only ∼2% of the activity toward I-1-P [this should be compared with 77% in the earlier report (*15*)]. Similar to other I-1-Pases, the *E. coli* enzyme can hydrolyze other phosphate monoesters including β-glycerophosphate and 2′-AMP (both with $CH-O-PO_3^{2-}$ groups). However, pNPP, 5′-AMP, NAD^+, G-6-P, and G-1-P were not substrates (at 0.4 mM) for the *E. coli* enzyme. For comparison, I-1-Pases from thermophilic organisms possess broader substrate specificity and exhibit high activity toward pNPP and G-1-P (*16, 21*). The kinetic differences between the SuhB characterized here and that reported earlier cannot be explained by experimental errors based on the assay methods used, and might suggest that there is a slight difference in SuhB enzymes between different cell lines. The *suhB* gene that was expressed, purified, and characterized in this work was identified by its ability to complement the chromosomal *suhB* mutation in the double mutant CG1307. The previously characterized SuhB protein (*15*) was isolated as the protein whose mutation acted as a suppressor of the protein secretion mutation *secY24*. Clearly, there could be slight differences in the two enzymes.

Table 5: Kinetic Parameters for *E. coli* SuhB I-1-Pase Activity

substrate	temp (°C)	K_m (mM)	V_{max} [μmol of P_i min^{-1} (mg of protein)$^{-1}$]
D-I-1-P	37	0.064 ± 0.003	6.9 ± 0.1
L-I-1-P	37	0.079 ± 0.011	2.66 ± 0.09
D,L-I-1-P	37	0.069 ± 0.010	3.38 ± 0.06
D,L-I-1-P	70	0.092 ± 0.018	27.6 ± 1.4

Temperature Dependence of Activity and Heat Stability of E. coli I-1-Pase. The specific activity of *E. coli* I-1-Pase increased significantly with increasing temperature (Figure 6A) for assays with short incubation time (2 min). An Arrhenius plot of these data was linear to 75 °C, and the estimated activation energy of this enzyme was 46 kJ/mol. The enzyme itself was also moderately heat stable for longer periods of time. Most plant and mammalian I-1-Pase enzymes, although they inhabit an environment with a temperature of 37 °C or lower, are very heat stable and can survive long incubation periods at 60−70 °C (*31, 34*) and 15 min at 80 °C (*34*). The recombinant I-1-Pases from the hyperthermophilic microorganisms have even more pronounced heat stability (*16, 21*). Since the *E. coli suhB* gene product has significant sequence homology with those I-1-Pases, it was expected to be at least moderately heat stable. As seen in Figure 6B, about 90% activity survived after heating for 5 min at 70 °C.

DISCUSSION

Mutations in the *E. coli suhB* gene have the ability to suppress diverse mutations [e.g., defects in DNA replication (*dnaB121*), protein secretion (*secY24*), and heat shock response (*rpoH15*)] that affect the growth temperature of the organism. The mechanism by which this occurs is unclear. Rescue of the original mutant phenotype occurs with the introduction of wild-type *suhB* on a plasmid. The complementation test described here confirms that the *suhB* gene only, without the need of any flanking region, is fully functional in complementing the suppressor mutation in the CG1307 double mutant strain. More critically, mutation of

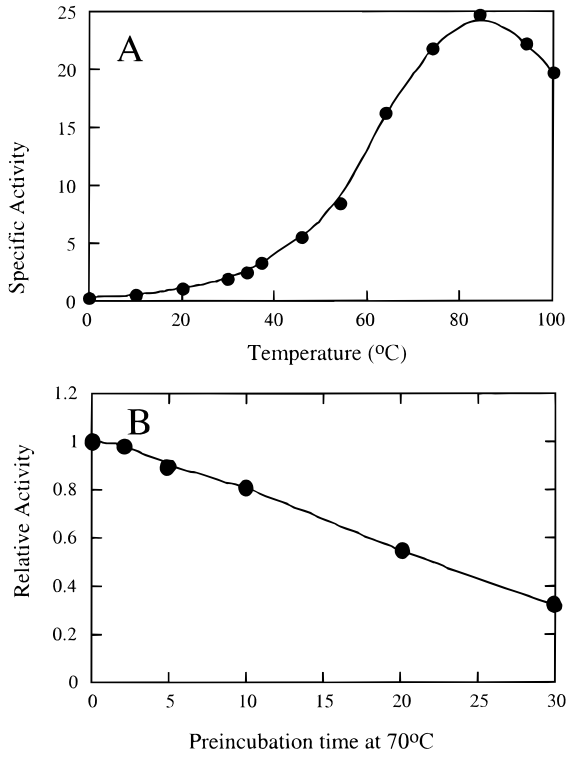

FIGURE 6: (A) Temperature dependence of *E. coli* SuhB I-1-Pase activity. (B) Thermal stability of *E. coli* I-1-Pase activity after preincubation at 70 °C for various times. The enzyme activity was measured after the preincubation by adding protein to the standard assay mix and incubating at 37 °C for 1 min.

the *E. coli* protein at D87 generated an inactive I-1-Pase that still complemented the mutation. This result clearly indicates that I-1-Pase activity is not required for complementation of mutant *suhB* in *E. coli* CG1307. On the other hand, neither the archaeal *M. jannaschii* and *A. fulgidus* I-1-Pase nor the eubacterial *T. maritima* I-1-Pase protein was functional in complementing the suppressor mutation in that *E. coli* strain. Considering that *T. maritima* I-1-Pase has comparable I-1-Pase activity with the *E. coli* enzyme at 30 °C [and a similar activation energy (*21*)], and that even though they are expressed at low levels in *E. coli* CG1307, all I-1-Pase specific activities are comparable, we can conclude that I-1-Pase activity is neither sufficient nor required for the complementation behavior of this protein.

A possible scenario for how SuhB complements these temperature mutants has been outlined by Inada and Nakamura (*6*, *7*), who propose that SuhB possesses an anti-RNAse III activity that affects mRNA stability. The double strand RNA (dsRNA) cleavage ability of RNase III is thought to be lethal at nonpermissive temperatures. SuhB could act as an anti-RNAse III factor by (i) direct interactions with RNase III to modulate its activity or (ii) interactions with target RNAs to protect them from RNase III degradation. If there is a direct interaction of SuhB with RNase III, it does not require SuhB phosphatase activity in the *dnaB121* mutant. Could there be an alternative activity of SuhB that regulates RNase III? The protein encoded by *suhB* has been noted to have high homology to that coded for by the *cysQ* or *amtA* gene (needed for ammonium transport and aerobic cysteine biosynthesis) (*35*). However, exactly what type of 'activity' the proteins share will require more detailed physical studies of SuhB. An alternative explanation has SuhB protein binding to dsRNA and protecting it from RNase III degradation (in essence acting as a 'dsRNA chaperone'). This could explain why *suhB* mutations always cause cold-sensitive growth. RNase III most effectively interacts with dsRNA at lower temperatures; hence, wild-type SuhB is required to protect the RNA and allow cell growth. At higher temperatures, dsRNA secondary structure is destabilized and effective complex formation with RNAse III blocked.

One unique characteristic of the SuhB I-1-Pase (compared to all other known I-1-Pase proteins) is that it can exist in solution as a monomer as well as form large insoluble aggregates that can be solubilized by increased salt. All other characterized I-1-Pases are dimers (or a tetramer in the case of the enzyme from *T. maritima*) and are extremely soluble in low ionic strength buffers and show no tendency to precipitate from solution. The *E. coli* I-1-Pase is also a more hydrophobic protein. SuhB as overexpressed in *E. coli* does not appear to be a membrane-associated protein. However, when the cells are lysed by sonication, SuhB is sufficiently amphipathic to solubilize all the membrane debris. That the intact cells containing the SuhB are viable suggests that the SuhB must initially be soluble in the cytoplasm or possibly aggregated but in either case not associated with the membrane. It is tempting to suggest that the more hydrophobic monomeric structure of this protein is linked to its biological role in *E. coli* and that mutations in this protein that lead to altered SuhB (not mutations that reduce expression) might alter this characteristic and lead to suppressor behavior. However, a definitive understanding of the differences between I-1-Pases, wild-type SuhB, and SuhB mutations that function as suppressors is likely to be answered only with high-resolution structural data. In this paper, we have developed a protocol for very efficient overexpression and purification of *E. coli* SuhB protein that can provide enough pure protein for crystallization purposes.

These results also provide the possibility of using pET23a-(+) vector in a cell-based high-throughput screening assay for proteins that complement the suppressor mutations. Although this vector normally needs T7 RNA polymerase for high-level gene expression, the very efficient *E. coli* ribosome binding site located before the polycloning site must compensate partially for the low transcription efficiency and thus can still provide enough expressed protein for cellular function. Two major advantages to using pET23a-(+) as a vector in the high-throughput screening assay are that (i) it can be directed to produce only native protein by just cloning the gene of interest in its *Nde*I site while other vectors (e.g., pUC) often need to produce N-terminal fusion proteins, and (ii) the plasmid isolated from positive clones (those that grow at 30 rather than 42 °C) can be transformed directly into the expression host to overexpress the protein of interest without successive subcloning. The proteins overexpressed from the plasmid selected from positive clones are functional in vivo in complementing the suppressor behavior of the *suhB* mutation in CG1307. Any plasmids coding for mutant inactive proteins would be eliminated by the functional screening.

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