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Divergent Effects of Chaperone Overexpression and Ethanol Supplementation on Inclusion Body Formation in Recombinant *Escherichia coli*

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The proper folding of aggregation-prone recombinant proteins in *Escherichia coli* can be facilitated by co-overexpressing specific molecular chaperones or by culturing the cells in the presence of ethanol or other agents that upregulate the synthesis of all heat-shock proteins (hsps). We have investigated the effect of combining direct chaperone overproduction with ethanol supplementation on the cytoplasmic folding of two aggregation-prone model proteins, preS2-S′-β-galactosidase and human SPARC. In 25-ml shake flask cultures grown at 30°C, addition of 3% (v/v) ethanol to the growth medium prior to inoculation improved the chaperone-mediated increase in the yields of active preS2-S′-β-galactosidase 1.5- to 2-fold. When cultures overexpressing the *dnaKJ* operon were grown in the presence of ethanol, the levels of enzymatic activity were 5-fold higher relative to control cells and preS2-S′-β-galactosidase aggregation was almost entirely abolished. Combining DnaK–DnaJ overexpression and growth of the cells at temperatures lower than 30°C did not result in a comparable increase in activity. Although the individual effects of ethanol supplementation and *dnaKJ* overproduction were more limited when the culture volume was raised, a synergistic improvement in preS2-S′-β-galactosidase activity was observed when the two approaches were used in concert. In contrast, ethanol supplementation promoted the aggregation of human SPARC, a protein exhibiting a chaperone dependency similar to that of preS2-S′-β-galactosidase. Our results show that ethanol can exert complex and divergent effects on inclusion body formation and that the beneficial effect of the solvent on recombinant protein folding cannot simply be explained by an increase in the intracellular concentration of molecular chaperones.

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The high-level expression of recombinant proteins in *Escherichia coli* often leads to their misfolding and the formation of insoluble aggregates known as inclusion bodies. Since inclusion bodies can be easily isolated by centrifugation and consist primarily of the protein of interest, protein aggregation has been widely exploited to simplify purification schemes (1). Unfortunately, many polypeptides are difficult to refold following chemical denaturation, and *in vitro* refolding may become prohibitively expensive for high-throughput operations. Thus, it is often desirable to produce recombinant proteins in a soluble conformation *in vivo* and to purify the biologically active polypeptides by traditional chromatography techniques. Several parameters have been shown to influence inclusion body formation in *E. coli.* They include the transcription rate of the gene of interest, the growth temperature, the composition and pH of the culture medium, and the cellular localization of the overexpressed protein (2). More recently, it has become clear that alterations in the intracellular concentration of folding modulators can have a significant impact on the folding of many recombinant gene products (reviewed in 3–5).

When *E. coli* is subjected to a variety of stresses, including temperature upshift, exposure to organic solvents and the accumulation of misfolded proteins, the synthesis of 20–30 heat-shock proteins (hsps) is transiently upregulated in order to repair cellular damage (6). The increased transcription of hsps results from higher concentrations of the heat-shock transcription factor σ^{32}, which directs the RNA polymerase core enzyme to heat-shock gene promoters (reviewed in 7,8). Although the cellular function of many hsps remains unclear, several are known to be ATP-dependent proteases and molecular chaperones (7). The latter proteins are of particular interest for biotechnology applications since their role is to help nascent and partially folded polypeptides reach a proper conformation or cellular

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location. The two major chaperone systems present in the cytoplasm of *E. coli* are the DnaK–DnaJ–GrpE and GroEL–GroES folding machines (reviewed in 9). Co-overexpression of components of either set of chaperones can significantly improve the solubility and/or secretion of many structurally and functionally unrelated recombinant polypeptides (3–5). In addition, indirect methods leading to an increase in the concentration of chromosomal hsps, including growth of the cells at high temperatures (10,11), mutations in negative regulators of the heat-shock response (12), and co-overexpression of plasmid-encoded σ^{32} (13,14), have also proven beneficial to the folding and secretion of certain overexpressed proteins.

Ethanol is one of the most powerful elicitors of the heat-shock response in *E. coli* (6). Its addition to the growth medium has been shown to increase the recovery yields of active RNA polymerase β-subunit (11), soybean seed lipoxygenase L-1 (15), and preS2-S′-β-galactosidase (14). In this report we have investigated the effect of combining direct chaperone overproduction and ethanol supplementation on the folding of two aggregation-prone recombinant proteins synthesized in the cytoplasm of *E. coli:* preS2-S′-β-galactosidase, which consists of the preS2 and S′ domains of the hepatitis B surface antigen fused to the N-terminus of *E. coli* β-galactosidase, and human SPARC, a 35-kDa monomeric glycoprotein containing 14 cysteine residues. We show that co-overexpression of the *dnaKJ* operon in cultures supplemented with 3% ethanol increases the recovery yields of preS2-S′-β-galactosidase fivefold relative to control cells by almost entirely suppressing aggregation. In contrast, the presence of the solvent is deleterious to the proper folding of SPARC. Optimization and scale-up issues are addressed.

MATERIALS AND METHODS

Strains, plasmids, and media. The *E. coli* strains JM105 (*endA1 thi rpsL sbcB15 hsdR4* Δ(*lac-pro*) [F′ *traD36 proAB lacI*^{q} *lacZ* Δ*M15*]) (16) and BL21(DE3) (F^- *ompT* [*lon*] *hsdS*$_B$ (r_B- m_B-) λ DE3 lysogen) (17) have been described. Plasmid pTBG(H+) encodes the preS2-S′-β-galactosidase fusion protein under *tac* promoter control (18), while pSPARCwt places human SPARC under control of the bacteriophage T7 promoter (19). Plasmid pTG10 is a pACYC184-derived cloning vector (10). Plasmids pGroESL (10), pDnaK/J (A. A. Gatenby), and pσ^{32} (14) are pTG10 derivatives carrying the *groE* operon, the *dnaKJ* operon, and the *rpoH* gene, respectively. Transformants were obtained by the RbCl method and selected at 30°C. LB medium was supplemented with 0.2% glucose, 34 μg/ml chloramphenicol, and 50 μg/ml ampicillin. Carbenicillin was substituted for ampicillin in experiments involving pSPARCwt, in 500-ml shake flask cultures and in fermentations. M9 minimal salts (pH 7.0) were supplemented with 0.1 mM $CaCl_2$, 2.5 mM $MgSO_4$, 0.2% casamino acids (Difco), and the above concentrations of glucose and antibiotics.

Shake flask cultures and cell fractionation. Overnight cultures grown at 30°C in LB medium were used to inoculate 25 ml of supplemented LB medium in 125-ml Erlenmeyer shake flasks at a 1:50 dilution. Absolute ethanol was added to the medium at various concentrations (v/v) prior to inoculation, except in the experiments of Fig. 2B where ethanol was added at the times indicated in the text. The cells were grown at 30°C to midexponential phase ($A_{600} \approx 0.4$), induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and held at 30°C or shifted to water baths maintained at the indicated temperatures. Immediately before addition of IPTG and at the specified times postinduction, 3 ml samples were withdrawn and the absorbance at 600 nm (A_{600}) was recorded. The cells were centrifuged at 6500*g* for 8 min, resuspended in 3 ml of 50 mM potassium phosphate monobasic, pH 6.5, and disrupted with a French press at 10,000 psi. Soluble fractions were immediately clarified by centrifugation at 13,000*g* for 10 min; 500-ml LB cultures were grown and induced in 2-L Erlenmeyer flasks as above. Fermentations were conducted in 1.3 L of supplemented LB medium at pH 7.0 and 30°C using a BioFlo C32 fermentor (New Brunswick) with automated pH and temperature control. Translation arrest experiments were performed as described (14). Briefly, the cells were grown and induced as above and 200 μg/ml neomycin was added 1 h postinduction to halt further protein synthesis. Culture samples were collected every 20 min for 2 h following antibiotic addition.

β-Galactosidase enzymatic assays. β-Galactosidase assays were performed in triplicate using the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described (14). β-Galactosidase activities are reported in Miller units ($1000 \times \Delta A_{420}/A_{600}$ of culture/ml of culture/min of reaction). To verify that the concentrations of ethanol used in this study did not affect enzymatic assays, purified β-galactosidase (Sigma) was assayed as above in the presence of 3 and 6% ethanol. In both cases, activity levels were similar to those obtained in the absence of the solvent (data not shown).

Electrophoresis techniques. Samples used for electrophoresis were prepared from 25-ml LB cultures grown and induced as above. At specified time points, 1-ml aliquots of whole cells were resuspended into 1× SDS/DTT loading buffer, and 3 ml samples were collected and fractionated into soluble and insoluble fractions as described above. The soluble cell extracts were concentrated by methanol–chloroform extraction (20) before resuspension, while the insoluble fractions were

resuspended directly in 1× SDS/DTT loading buffer. Aliquots corresponding to identical A_{600} units of cells from whole-cell, soluble, and insoluble fractions were resolved on 8% (preS2-S′-β-galactosidase) or 15% (SPARC) SDS–PAGE minigels and visualized by Coomassie blue staining or immunoblotting.

Immunoblotting. For SPARC immunoblots, soluble and insoluble fractions corresponding to identical amounts of culture were fractionated on 15% SDS minigels before transfer to nitrocellulose. The membranes were probed with murine monoclonal antibodies to human platelet SPARC (Haematologic Technologies) followed by incubation with alkaline-phosphatase-conjugated goat anti-mouse IgG (Sigma). The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). Identical gels were stained with Coomassie blue to verify that the cellular fractions were loaded evenly.

RESULTS

Hsp Overexpression and Ethanol Supplementation Improve the Production of Soluble and Active PreS2-S′-β-galactosidase in an Additive Manner

Plasmid pTBG(H+) encodes the preS2-S′-β-galactosidase fusion protein under *tac* promoter control (18). Enzymatically active preS2-S′-β-galactosidase is a homotetramer of ~125 kDa subunits, each consisting of the preS2 and hydrophobic S′ domains of the hepatitis B surface antigen fused to the N-terminus of *E. coli* β-galactosidase (18). This fusion protein aggregates extensively in the cytoplasm of *E. coli* at temperatures as low as 30°C and becomes increasingly less soluble as the growth temperature is increased (18). We have previously reported that co-overexpression of DnaK–DnaJ can improve the yields of soluble and active preS2-S′-β-galactosidase 3- to 6-fold over a temperature range from 30 to 42°C, whereas higher levels of the GroEL–GroES chaperonins lead to a 1.5-fold increase in activity at 30°C only (14). When the intracellular concentration of all chromosomal hsps was increased by direct overproduction of σ^{32}, or addition of 3% ethanol (v/v) to the growth medium, a 2- to 3-fold higher recovery of active enzyme was observed at 30 and 42°C, but not at 37°C (14).

In order to determine whether ethanol addition would prove beneficial when used in concert with direct methods leading to an increase in the intracellular concentration of molecular chaperones, JM105 cells cotransformed with pTBG(H+) and either pTG10, pDnaK/J, pGroESL, or pσ^{32} were grown at 30°C in LB medium supplemented or not with 3% ethanol. Synthesis of preS2-S′-β-galactosidase was induced by addition of 1 mM IPTG to midexponential phase cultures, and clarified extracts prepared 2 h after induction were assayed for β-galactosidase activity. Figure 1A shows that the presence of ethanol in the growth medium increased the recovery of active preS2-S′-β-galactosidase 1.5- to 2-fold relative to identical cells grown without the solvent in all cases. In particular, the activity levels in ethanol-treated cells overproducing the *dnaKJ* operon were almost 5-fold higher compared to pTG10 transformants grown in the absence of the solvent. SDS–PAGE analysis of the soluble and insoluble cellular fractions of the former cells showed that preS2-S′-β-galactosidase aggregation was almost completely suppressed under these conditions (Fig. 1B). The beneficial effect of ethanol supplementation on the accumulation of active fusion protein was independent of the growth medium since a similar increase in activity was

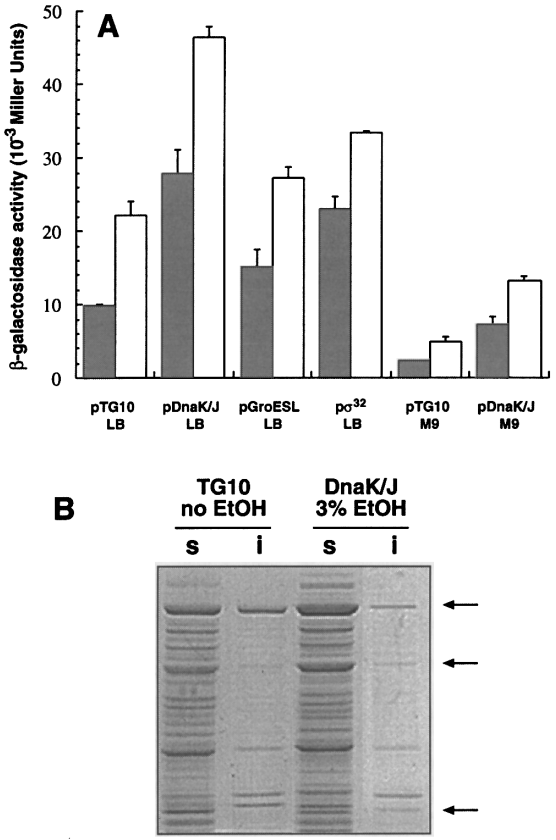

FIG. 1. Additive effects of ethanol addition and molecular chaperone overexpression on the recovery of enzymatically active preS2-S′-β-galactosidase. (A) β-Galactosidase activity in JM105 cells harboring pTBG(H+) and the indicated plasmids grown 2 h postinduction at 30°C in the absence (shaded bars) or presence (open bars) of 3% ethanol. The growth medium is indicated. (B) SDS–PAGE fractionation of cells harboring pTG10 or pDnaK/J grown 1 h postinduction in the absence or presence of 3% ethanol as indicated. Soluble (s) and insoluble (i) fractions are shown. The positions of preS2-S′-β-galactosidase and DnaK are indicated by the upper and middle arrows, respectively. A decrease in the intensity of the band corresponding to OmpC/F (lower arrow) was observed in all experiments when the cells were grown in the presence of ethanol.

TABLE 1

Effect of the Ethanol Concentration on the Growth Rate of Induced Cultures and the Yields of Active PreS2-S′-β-galactosidase

	pTG10		pDnaK/J	
% Ethanol	μ (h^{-1})[a]	10^{-3} Miller units[b]	μ (h^{-1})	10^{-3} Miller units
0	0.95 (100)	9.88 (100)	1.01 (100)	24.5 (100)
1	0.87 (91)	13.1 (133)	0.95 (95)	28.7 (117)
2	0.88 (93)	17.6 (178)	0.86 (86)	30.7 (125)
3	0.81 (86)	21.3 (216)	0.76 (76)	39.3 (160)
4	0.72 (76)	25.8 (261)	0.64 (63)	25.1 (98)
5	0.58 (61)	ND[c]	0.46 (46)	ND
6	0.41 (42)	ND	0.29 (29)	ND

[a] JM105 cells harboring pTBG(H+) and the indicated plasmids were grown in the presence of increasing concentrations of ethanol at 30°C and the growth rates (μ) were determined from turbidity measurements. The percentage decrease in growth rates relative to untreated cultures is shown in parentheses.

[b] β-Galactosidase activities were determined 2 h postinduction. The percentage change in activity relative to untreated cultures is shown in parentheses.

[c] ND, not determined.

observed when pTG10 or pDnaK/J transformants were grown in M9 medium containing 3% ethanol (Fig. 1A). The lower activity levels obtained in minimal medium are likely to result from a reduction in recombinant protein expression under suboptimal nutritional conditions.

Optimizing the Conditions for Ethanol Supplementation

To determine the optimal conditions for the use of ethanol in cells overexpressing the *dnaKJ* operon, JM105 cultures harboring pTBG(H+) and pDnaK/J were grown at 30°C in LB medium supplemented with 0 to 6% (v/v) ethanol. Whereas ethanol concentrations of 2% or less had little beneficial effect on the recovery of enzymatic activity (Fig. 2A), the use of more than 3% ethanol led to a significant inhibition of cell growth (Table 1) and declining levels of enzymatic activity at late time points (Fig. 2A). We therefore conclude that an ethanol concentration of 3% is optimum to maximize the recovery of active preS2-S′-β-galactosidase. The influence of the time of ethanol addition was next investigated. Figure 2B shows that addition of 3% ethanol at the time of IPTG induction (Fig. 2B, h) had little beneficial effect on the recovery of enzymatic activity when measured 1 h postinduction and was 50% less efficient than preinoculation treatment (Fig. 2B, ◇) when the activity was measured 2 h postinduction. Since the presence of ethanol in the growth medium may induce the misfolding of host proteins, the above results may be explained by a sudden excessive demand on molecular chaperone activity. Because the high-level transcription of hsps is a transient process that stops 20–30 min after ethanol addition (21), this time period may be sufficient to repair any major cellular damage caused by the solvent. To test this possibility, the cultures were treated with 3% ethanol 30 min prior to induction of preS2-S′-β-galactosidase synthesis. Under these conditions, the 1-h postinduction levels of activity were 35% higher relative to cultures supplemented with ethanol before inoculation (Fig. 2B, s and ◇). While this moderate improvement in yields may be related to a higher concentration of molecular chaperones at the time of preS2-S′-β-galactosidase induction, it was not sustained at later time points for unclear reasons. Similar results were obtained with JM105 cells cotransformed with pTBG(H+) and pTG10 (data not shown). Taken together, the above data indicate that the greatest improvements in the recovery of active preS2-S′-β-galactosidase take place when 3% ethanol is added to the growth medium prior to inoculation.

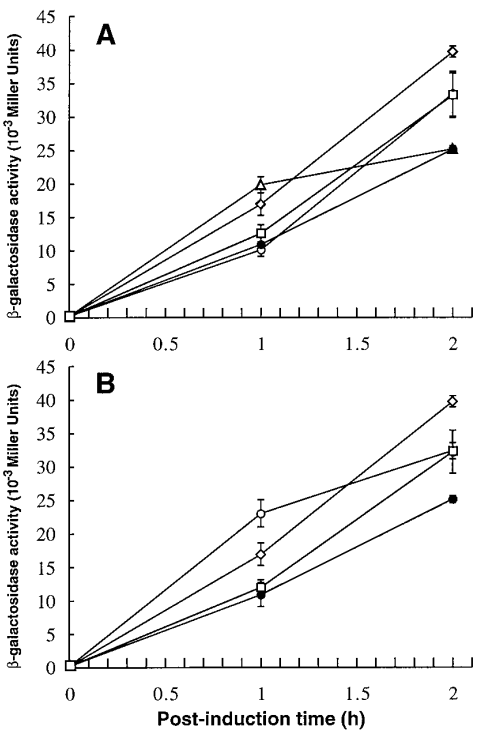

FIG. 2. Optimal conditions for the use of ethanol. (A) β-Galactosidase activity in JM105 cells cotransformed with pTBG(H+) and pDnaK/J grown at 30°C in LB medium supplemented with the following concentrations of ethanol: 0% (●), 1% (s), 2% (h), 3% (◇), 4% (n). (B) JM105 cells harboring pTBG(H+) and pDnaK/J were grown at 30°C in LB medium in the absence (●) or presence of 3% ethanol added prior to inoculation (◇), at the time of IPTG induction (h), or 30 min prior to IPTG induction (s).

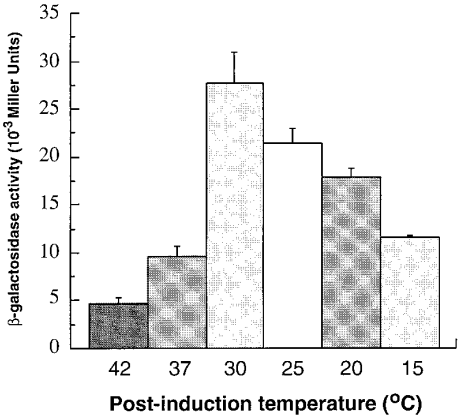

FIG. 3. Combined effects of DnaK–DnaJ cooverexpression and growth temperature. β-Galactosidase activities were measured 2 h postinduction in JM105 cells cotransformed with pTBG(H+) and pDnaK/J grown in LB medium. The postinduction growth temperature is indicated.

Low-Temperature Cultivation Does Not Further Improve the Yields of Active PreS2-S′-β-galactosidase in DnaK–DnaJ Overexpressing Cells

It is well documented that the proper *in vivo* folding of many recombinant proteins can be improved by reducing the culture growth temperature (2,22). To investigate the influence of this parameter on the recovery of active preS2-S′-β-galactosidase from cultures overexpressing DnaK–DnaJ, JM105 cells harboring pTBG(H+) and pDnaK/J were grown to midexponential phase at 30°C and transferred to water baths held at various temperatures immediately after IPTG induction. In contrast to the results obtained by adding ethanol to 30°C cultures, the combined use of growth temperatures lower than 30°C and *dnaKJ* overexpression did not further improve the yields of active preS2-S′-β-galactosidase (Fig. 3). SDS–PAGE analysis of cellular fractions showed that the decline in activity with temperature was related to a decrease in the synthesis rate of the fusion protein (data not shown). In agreement with these results, the highest yields of active preS2-S′-β-galactosidase in pTBG(H+) single transformants cultured over a temperature range of 10 to 37°C were obtained at 30°C (22). Thus, ethanol addition is superior to the use of lower growth temperature in enhancing the recovery yields of preS2-S′-β-galactosidase in either control cells or in cultures overexpressing the DnaK–DnaJ molecular chaperones.

DnaK–DnaJ Overexpression and Ethanol Supplementation Synergistically Improve the Recovery of Active PreS2-S′-β-galactosidase in Large-Scale Cultures

In an effort to determine if the additive effects of *dnaKJ* overexpression and ethanol supplementation would scale-up to larger culture volumes, the experiments of Fig. 1 were repeated using 500 ml of growth medium in 2-L Erlenmeyer flasks. Figure 4 shows that, under all conditions examined, the recovery yields of active preS2-S′-β-galactosidase were reduced two- to threefold when the culture volume was increased from 25 to 500 ml (compare to Fig. 1A). Furthermore, the beneficial effects of DnaK–DnaJ overexpression were much more modest than those observed in small shake flasks, and a sharp reduction in activity was observed 3 h postinduction in ethanol-treated pTG10 control cells (Fig. 4). SDS–PAGE analysis of culture samples revealed that the synthesis level preS2-S′-β-galactosidase remained high for the duration of these experiments, and that the decrease in yields relative to the 25-ml cultures resulted from increased aggregation of the fusion protein (data not shown). We also found that overexpression of the *dnaKJ* operon had less positive influence on the recovery of active preS2-S′-β-galactosidase in 1.3-L batch fermentations, whereas ethanol had essentially no effect under these conditions (unpublished data). Ethanol addition may be particularly ineffective in a fermentation set-up since sparged air may rapidly scrub the solvent from the medium. Despite these limitations, the combined use of ethanol supplementation and *dnaKJ* overexpression had a synergistic effect on the production of active preS2-S′-β-galactosidase in 500-ml shake flask cultures, leading to a more than twofold increase in the levels of enzymatic activity relative to control cells for at least 3 h (Fig. 4, m).

Ethanol Supplementation Promotes the Aggregation of Human SPARC

Secreted protein acidic and rich in cysteine (SPARC) is a 35-kDa monomeric glycoprotein that is believed to

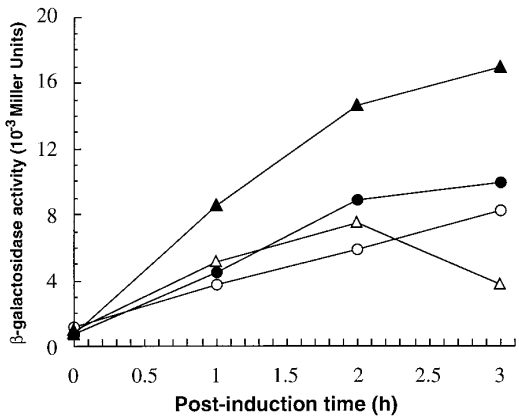

FIG. 4. Chaperone overexpression and ethanol supplementation are less effective in large shake flask cultures but synergistically improve folding when used in concert. β-Galactosidase activity in JM105 cell harboring pTBG(H+) and either pTG10 (open symbols) or pDnaK/J (filled symbols) grown at 30°C in 2-L shake flasks containing 500 ml of LB medium supplemented (m, n) or not (●, s) with 3% ethanol.

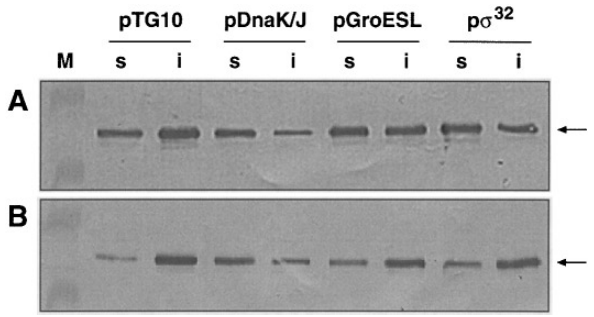

FIG. 5. Ethanol is detrimental to the solubility of human SPARC. BL21(DE3) cotransformed with pSPARCwt and the indicated plasmids were grown in LB medium at 37°C in the absence (A) or presence (B) of 3% ethanol. Soluble (s) and insoluble (i) fractions corresponding to identical amounts of cell culture collected 2 h postinduction were resolved by SDS–PAGE and transferred to nitrocellulose. The membranes were probed with anti-SPARC antibodies and developed by colorimetric reaction. The position of SPARC is indicated by the arrows.

play an important role in tissue remodeling and wound repair. When produced under full induction conditions from plasmid pSPARCwt, which places the cDNA for human SPARC under control of the bacteriophage T7 promoter (19), about half of the newly synthesized protein accumulates in an inclusion body form (23). Because overexpression of DnaK–DnaJ significantly increases the solubility of both preS2-S′-β-galactosidase and SPARC, whereas GroES–GroEL or σ^{32} overproduction have little effect on the aggregation of either polypeptide (Figs. 1A and 5A; Refs. 14,23), both proteins appear to exhibit similar chaperone requirements for reaching a properly folded conformation *in vivo*. We therefore investigated the influence of ethanol supplementation on the folding and aggregation of SPARC. In contrast to the results obtained with preS2-S′-β-galactosidase, ethanol treatment was found to promote, rather than reduce, the aggregation of SPARC at 30, 37, and 42°C (Fig. 5; data not shown). Co-overexpression of GroEL-GroES or σ^{32} failed to suppress the ethanol-induced decrease in SPARC solubility, while higher levels of DnaK–DnaJ were still effective in protecting this protein from misfolding in the presence of ethanol (Fig. 5B). The possibility that heat-shock proteases induced by ethanol were degrading soluble SPARC—thus leading to the apparent increase in protein aggregation—was ruled out since the stability of SPARC in cells treated or not with ethanol was comparable following translational arrest with 200 μg/ml neomycin (data not shown). Furthermore, aggregation of soluble SPARC species was not observed after translational arrest, irrespective of the fact that the cells had been grown or not in ethanol-supplemented medium. Thus, ethanol treatment is likely to affect the solubility of nascent or newly synthesized SPARC chains through complex mechanisms. The above results highlight the fact that the use of ethanol can facilitate the folding of certain, but not all, proteins in the cytoplasm of *E. coli*.

DISCUSSION

While the direct co-overexpression of components of the DnaK–DnaJ–GrpE and GroEL–GroEL molecular chaperone machines can improve the proper folding or secretion of a number of recombinant proteins in *E. coli*, chaperone overproduction remains ineffective in other cases (3–5). Possible explanations for this phenomenon include (i) an incorrect choice of the overproduced chaperone(s), (ii) a need for additional cofactors which may not have been identified to date, or (iii) a requirement for the cooperative or network action of several chaperone systems. Since most known molecular chaperones are also hsps, their synthesis can be induced in a stoichiometric fashion by triggering the heat-shock response with ethanol or other agents that stress the cell. In this report, we have examined the effect of combining ethanol treatment with the direct overexpression of specific chaperone operons, or of the σ^{32} heat shock transcription factor, on the production of two model recombinant proteins that misfold when expressed at high levels in *E. coli*.

In the case of preS2-S′-β-galactosidase cultures grown in 25-ml shake flasks, ethanol treatment and chaperone overexpression exerted additive effects on the recovery of active protein (Fig. 1A). More importantly, when optimal conditions were used (i.e., co-overexpression of the *dnaKJ* operon and supplementation of the medium with 3% ethanol prior to inoculation) preS2-S′-β-galactosidase aggregation was almost entirely suppressed (Fig. 1B). In contrast, the use of suboptimal growth temperatures (i.e., below 30°C) did not lead to a comparable increase in activity when combined with DnaK–DnaJ overexpression (Fig. 3). In larger 500-ml shake-flask cultures, the individual improvements brought about by *dnaKJ* overproduction or ethanol supplementation were significantly reduced (Fig. 4). Nevertheless, combining the two approaches led to a synergistic improvement in the yields of active fusion protein (Fig. 4, m).

In addition to ethanol, a number of compounds are known to induce the synthesis of heat-shock proteins in *E. coli* (6,21). These include certain antibiotics (e.g., puromycin and aminoglycosides), nalidixic acid, heavy metals, peroxides, and oxidants. Although some of these compounds may improve the folding of recombinant proteins in a manner similar to ethanol treatment, the majority of these agents are not likely to be useful for the production of recombinant proteins since they are generally much less effective than ethanol in inducing the heat-shock response (21), lead to translational errors, and can severely inhibit cell growth.

Many such compounds are also toxic or present environmental hazards (e.g., benzene).

Kashlev and coworkers have reported that experimental conditions which induce a stress response in *E. coli* improve the activity of plasmid-encoded RNA polymerase β-subunit in wild-type strains, but not in an *rpoH* mutant deficient in the induction of the heat-shock response (11). Since we have previously shown that supplementing the growth medium with 3% ethanol results in almost identical improvements in the yields of active preS2-S′-β-galactosidase over the 30–42°C temperature range compared to direct co-overexpression of σ^{32} (12), it was reasonable to assume that ethanol exerts its beneficial effect on the folding of heterologous proteins by increasing the intracellular concentration of chromosomally encoded molecular chaperones. Nevertheless, only minor increases in the relative synthesis rates of DnaK and GroEL (10–20%) were detected by pulse-labeling ethanol-treated JM105 cells cotransformed with pTBG(H+) and pTG10 after IPTG induction (unpublished data). For comparison, co-overexpression of plasmid-encoded σ^{32} leads to a twofold increase in the synthesis rates of these chaperones (14). In addition, ethanol supplementation was deleterious to the folding of human SPARC, a protein exhibiting a chaperone dependency similar to that of preS2-S′-β-galactosidase (Figs. 1A and 5A; Refs. 14,23). Taken together, the above results suggest that the positive influence of ethanol on recombinant protein folding cannot be solely explained by higher intracellular concentrations of molecular chaperones. An alternate possibility would be that the lower synthesis rates of preS2-S′-β-galactosidase in ethanol-treated cultures (see Table 1) facilitate the correct folding of the fusion protein. We were, however, unable to increase the specific activity of preS2-S′-β-galactosidase in control cells by inducing its synthesis with lower concentrations of IPTG (data not shown) or by reducing the cell growth temperature (22). Since the beneficial effect of ethanol on the folding of preS2-S′-β-galactosidase cannot be fully explained by either higher levels of hsps or a decrease in protein synthesis rates, a complex and synergistic combination of these mechanisms is likely to be responsible for the observed increase in solubility.

Although our results demonstrate that ethanol can be a powerful tool to improve the solubility of certain recombinant proteins when used together with chaperone overexpression, the solvent may also promote the aggregation of the target polypeptide. Such divergent effects suggest that the beneficial influence of ethanol depends on the folding pathway of the overproduced protein and must be evaluated on a case-by-case basis.

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