

# Overproduction of Bacterial Protein Disulfide Isomerase (DsbC) and Its Modulator (DsbD) Markedly Enhances Periplasmic Production of Human Nerve Growth Factor in *Escherichia coli*\*

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**Production of eukaryotic proteins with multiple disulfide bonds in the *Escherichia coli* periplasm often encounters difficulty in obtaining soluble products with native structure. Human nerve growth factor  $\beta$  (NGF) contains three disulfide bonds between nonconsecutive cysteine residues and forms insoluble aggregates when expressed in *E. coli*. We now report that overexpression of Dsb proteins known to catalyze formation and isomerization of disulfide bonds can substantially enhance periplasmic production of NGF. A set of pACYC184-based plasmids that permit *dsb* expression under the *araB* promoter were introduced into cells carrying a compatible plasmid that expresses NGF. The efficiency of periplasmic production of NGF fused to the OmpT signal peptide was strikingly improved by coexpression of DsbCD or DsbABCD proteins (up to 80% of total NGF produced). Coexpression of DsbAB was hardly effective, whereas that of DsbAC increased the total yield but not the periplasmic expression. These results suggest synergistic roles of DsbC and DsbD in disulfide isomerization that appear to become limiting upon NGF production. Furthermore, recombinant NGF produced with excess DsbCD (or DsbABCD) was biologically active judged by the neurite outgrowth assay using rat PC12 cells.**

secretion proteins often exhibit multiple and complex patterns of disulfide bonds (7), and such structural features have been thought to create difficulties in obtaining active proteins, because incorrect disulfide bond formation is likely to yield inactive and insoluble products (13).

Recent studies revealed several novel enzymes and factors involved in disulfide bond formation in the *E. coli* periplasm, which include at least four proteins, DsbA, DsbB, DsbC, and DsbD (4, 5, 14–17). These Dsb proteins contain one or more highly conserved thioredoxin-like Cys-Xaa-Xaa-Cys motifs that are crucial for the activity of disulfide oxidoreductases (5, 18). DsbA is a periplasmic enzyme that can act on nascent polypeptide chains in the formation of disulfide bonds during their folding (16, 19, 20). DsbC is another periplasmic enzyme known as a disulfide isomerase and can convert aberrant disulfide bonds to correct ones (16, 21–24). DsbB and DsbD are associated with the inner membrane and modulate activities of DsbA and DsbC, respectively (14, 15, 24, 25). Thus, an efficient chain of reactions for disulfide bond formation and isomerization seems to be operated in the periplasm during normal growth.

Based on the above findings, overexpression of Dsb proteins has been employed to increase efficiency of periplasmic expression of a number of heterologous proteins with multiple disulfide bonds in *E. coli* (4, 9, 10). So far, only limited success was reported; periplasmic expression of some proteins was improved by coexpression of DsbA, together with addition of reduced glutathione or *N*-acetylcysteine to the medium (26). DsbC can become overloaded upon production of heterologous proteins with multiple disulfide bonds, leading to insufficient conversion of aberrant disulfide bonds to the correct forms (4, 7, 27). A *dsbC* deletion reduced the production of urokinase (15) or insulin-like growth hormone I (28) that has disulfide bonds formed between nonconsecutive cysteine residues but hardly affected production of alkaline phosphatase or OmpA (15) or human growth hormone that has disulfide bonds between consecutive cysteine residues (28). These results indicated potentially important roles of DsbC in folding of proteins carrying multiple disulfide bonds, especially those involving nonconsecutive cysteine pairs. It should be noted that DsbC exhibits a disulfide reductase activity (the active site cysteine residues must be kept reduced to attack incorrectly formed disulfide bonds and catalyze isomerization (14, 15, 24)) in the highly oxidative periplasmic environment as compared with the endoplasmic reticulum of eukaryotes (2, 5, 13); the difference in the redox environment between the two compartments may be responsible for inefficient expression of some eukaryotic proteins in *E. coli* (13). The redox potential of the active site of DsbC is controlled by DsbD (14, 15, 24) in a fashion quite distinct from that of protein disulfide isomerase regulated by reduced glutathione in eukaryotes (5, 13, 29).

In this study, we systematically examined the effects of

The periplasm of *Escherichia coli* contains enzymes that can assist protein folding such as Dsb (disulfide bond formation) proteins (1–6), like endoplasmic reticulum of eukaryotes, and can provide an oxidative environment potentially useful for production of heterologous secretory proteins (*e.g.* eukaryotic cytokines and peptide hormones) that have a number of disulfide bonds (1, 7). Such proteins can be secreted to the *E. coli* periplasm as in frame fusion proteins with a bacterial signal peptide (8–10). It is often difficult, however, to obtain good yields of proteins by periplasmic production for a number of reasons (4, 9, 10). By promoting protein translocation (through the inner membrane) with altered translocation machinery (11, 12) or with coexpression of secretion-promoting factors such as molecular chaperones (*e.g.* SecB) (4, 9, 10), appreciable improvements were made for some proteins that contain relatively simple patterns of disulfide bonds. However, eukaryotic

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icontrolled coexpression of sets of Dsb proteins on periplasmic production of human nerve growth factor  $\beta$  (NGF).<sup>1</sup> NGF carries three disulfide bonds with nonconsecutive cysteine pairs and was previously shown to aggregate upon periplasmic expression in *E. coli* (30). The set of newly constructed plasmids provided convenient means of assessing the effects of controlled coexpression of *dsb* genes on specific target proteins expressed from compatible plasmids. The results revealed a striking enhancement of periplasmic production of NGF, particularly when both DsbC catalyzing the isomerization and its membrane-associated modulator DsbD are simultaneously overexpressed.

#### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Plasmids**—*E. coli* K12 strain JM109 (*F*<sup>-</sup>, *recA1*, *endA1*, *gyrA96*, *thi-1*, *supE44*, *relA1*,  $\Delta$ (*lac*  $\Delta$  *proAB*), (*F'* *traD36*, *proAB*, *lacIq lacZ* $\Delta$ M15), *mcrA*) (Takara, Kyoto, Japan) was used as the expression host throughout the experiments. DM391 (*dsbB* null mutant of CA8000) (31) and SR2612 (*dsbD* null mutant of MC4100) (14) were kindly provided by D. Missiakas. Plasmid pT7Blue<sup>®</sup> was purchased from Novagen. Expression vector pARK2 for Dsb proteins was constructed by inserting a fragment of pTrc99A containing a *rrnBT1T2* terminator region into a derivative of pAR3, pACYC184-based arabinose-inducible expression plasmid compatible with ColE1-derived plasmids (32). Expression vectors for the target protein NGF are derivatives of pTrc99A (Amersham Pharmacia Biotech), which allowed expression of NGF fused in frame to downstream of *OmpA*, *OmpT*, or *MalE* signal peptide, respectively, under controls of the *trc* promoter and the *lacIq* repressor and designated pTrc-*OmpA*, pTrc-*OmpT*, and pTrc-*MalE*, respectively. Vectors carrying each of the signal sequences (synthetic oligonucleotides) were constructed, and the coding region of the NGF gene (R & D Systems) was inserted downstream of pTrc-*OmpA*, pTrc-*OmpT*, and pTrc-*MalE*, and the resulting plasmids were designated pTrc-*OmpA*-NGF, pTrc-*OmpT*-NGF, and pTrc-*MalE*-NGF, respectively. Some codons rarely used in *E. coli* were replaced by those used more frequently to improve translational efficiency of the mature form of the NGF gene; TCA (Ser<sup>1</sup> and Ser<sup>2</sup>), CCC (Pro<sup>5</sup>), and AGG (Arg<sup>9</sup>) were replaced by AGC, CCG, and CGC, respectively.

**Construction of Dsb Expression Plasmids**—Each *dsb* gene (plus the respective or slightly modified Shine-Dalgarno sequence) was cloned by polymerase chain reaction (PCR) as follows: *dsbA*, *dsbB*, and *dsbC* genes were amplified with KOD DNA polymerase (Toyobo, Osaka, Japan), and *dsbD* was amplified with LA *Taq* DNA polymerase (Takara, Kyoto, Japan) and cloned into pT7Blue<sup>®</sup>. *dsbA* was amplified using 5' CGGGAGCTCATCGGAGAGAGTAGA 3' and 5' GGCCCGGG-AATTATTATTTTTTCTCGGA 3' as primers and pSK220 (33) as template, and the PCR product was digested with *SacI* and *AvaI*, purified, and cloned into the *SacI*-*AvaI* site of pT7Blue<sup>®</sup>. Similarly, *dsbB* was amplified using 5' GGCCCGGGCTGCGCACTATGCATATTGCAGGG 3' and 5' GGCATATGGATTATTAGCGACCGAACAGATCACGG 3' as primers and pSS51 (34) as template, and the PCR product was digested with *AvaI* and *NdeI*, purified, and cloned into the *AvaI*-*NdeI* site of pT7Blue<sup>®</sup>. *dsbC* was amplified using 5' GGATATGAGGAGGAAGATTATGAAGAAAGG3' and 5' CCGTCGACGATTATTATT-TACCGCTGGTCATTTTTTGGTGTTCG 3' as primers and Kohara  $\lambda$  clone number 468 as template, and the PCR product was digested with *NdeI* and *SalI*, purified, and cloned into the *NdeI*-*SalI* site of pT7Blue<sup>®</sup>. *dsbD* was amplified using 5' CCGTCGACGAGGCCGACATGCAGCTGCCGAAGGCGTCTGGC 3' and 5' CCGCATGCTTATCACGGTTGGC-GATCGCGC 3' as primers and Kohara  $\lambda$  clone number 648 as template, and the PCR product was digested with *SalI* and *SphI*, purified, and cloned into the *SalI*-*SphI* site of pT7Blue<sup>®</sup>. The structure of the resulting plasmids were confirmed by sequencing and designated pT7*dsbA*, pT7*dsbB*, pT7*dsbC*, and pT7*dsbD*, respectively. Plasmids carrying an artificial subset of the *dsb* genes in four combinations were then constructed. The *SacI*-*AvaI* fragment (~0.6 kbp) of pT7*dsbA* was inserted into the *SacI*-*AvaI* site of pT7*dsbB* or pT7*dsbC* to yield pT7*dsbAB* or pT7*dsbAC*, respectively. The *SalI*-*SphI* fragment (~1.5 kbp) of pT7*dsbD* was inserted into the *SalI*-*SphI* site of pT7*dsbB* or pT7*dsbC* to yield pT7*dsbBD* or pT7*dsbCD*, respectively. For construction of the entire set of *dsbABCD* genes, the *SacI*-*NdeI* fragment (~1.1 kbp) of

pT7*dsbAB* was inserted into the *SacI*-*NdeI* site of pT7*dsbCD* to yield pT7*dsbABCD*.

Each subset of the genes obtained above was placed under the control of the *araB* promoter and the *araC* regulatory gene on a pACYC184-based vector (pARK2); the *SacI*-*HindIII* fragment of pT7*dsbAB*, pT7*dsbAC*, pT7*dsbBD*, pT7*dsbCD*, or pT7*dsbABCD* was inserted into the *SacI*-*HindIII* site of pARK2, yielding pDbAB1, pDbAC1, pDbBD1, pDbCD1, or pDbABCD1, respectively. pDbABCD2 is a kanamycin-resistant version of pDbABCD1 that was constructed by inserting the *BstBI*-*BstBI* fragment of pDbABCD1 (~4.8 kbp) into pAR3*kan* carrying kanamycin (instead of chloramphenicol) resistance gene.

**Culture Conditions and Protein Expression**—*E. coli* JM109 cells carrying an NGF expression plasmid such as pTrc-*OmpT*-NGF and a pDb plasmid were grown in L broth (35) supplemented with ampicillin (50  $\mu$ g/ml) and chloramphenicol (34  $\mu$ g/ml) at 37 °C. When a culture reached 20 Klett units (number 66 filter), Dsb proteins were induced by adding L-arabinose to the medium, and 30 min later, NGF was induced by adding 50  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). All chemicals were of analytical grade supplied by Wako Pure Chemical (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

**Fractionation of Proteins**—Samples of cells (200  $\mu$ l) were harvested by centrifugation, and periplasmic proteins were obtained by osmotic disruption of spheroplasts; cells were first resuspended into 100  $\mu$ l of 30 mM Tris-HCl (pH 8.0), 20% sucrose, lysozyme was added to 0.1 mg/ml, and cells were incubated at 4 °C for 30 min. After adding MgCl<sub>2</sub> to 50 mM, the sample was centrifuged at 20,000  $\times g$  for 10 min, and the supernatant was taken as periplasmic fraction. The spheroplast pellet was resuspended in 100  $\mu$ l of 5 mM MgCl<sub>2</sub>, sonicated for 10 min, centrifuged at 100,000  $\times g$  at 4 °C for 1 h on a Beckman TLA 120.2 rotor, and the supernatant was withdrawn as the cytoplasmic fraction. The resulting pellet was resuspended in 100  $\mu$ l of 5 mM MgCl<sub>2</sub>, 1% octylglucoside, incubated at 4 °C for 10 min, and centrifuged again at 100,000  $\times g$  at 4 °C for 1 h. The supernatant was used as the membrane fraction, and the pellet was used as the insoluble fraction. Whole-cell proteins were prepared separately by precipitating a portion (200  $\mu$ l) of the culture directly with trichloroacetic acid (36).

**Analysis of Proteins**—Each of the above fractions was resuspended in SDS sample buffer, either directly or after trichloroacetic acid precipitation and washing with acetone, and heat-treated essentially as described (37). Proteins (corresponding to equal optical density) were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) followed by visualization with Coomassie Brilliant Blue or by immunoblotting with specific polyclonal antibody against NGF (Santa Cruz Biotechnology, Inc.), DsBA (kindly donated by Y. Akiyama),  $\beta$ -lactamase (5 Prime  $\rightarrow$  3 Prime, Inc., Boulder, CO), alkaline phosphatase (Nordic Immunological Laboratories), or DnaJ (StressGen, Inc.). The detection system used was either horseradish peroxidase-conjugated anti-rabbit or -mouse antibody and an ECL kit (Amersham Pharmacia Biotech) or alkaline phosphatase-conjugated anti-rabbit or -mouse antibody, nitroblue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad, Inc.). Quantification was carried out by an Intelligent Quantifier apparatus (BioImage Systems Co., Tokyo, Japan).

**Bioassay for NGF Activity**—*E. coli* JM109 cells carrying pTrc-*OmpT*-NGF and pDbCD1 (or pDbABCD1 or vector alone) was grown in L broth (400 ml  $\times$  2) and induced for expression of Dsb proteins followed by that of *OmpT*-NGF for 8 h. Cells were harvested and washed with 0.85% NaCl, and 4 g of wet cells were resuspended in 40 ml of buffer containing 0.1% (v/v) protease inhibitor mixture (Sigma). Periplasmic fraction was prepared and concentrated by a Centiprep 10 concentrator (Amicon), and the buffer was adjusted to 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and adsorbed to Affi-prep Polymixin Matrix (Bio-Rad) by shaking overnight on a rotary shaker at 4 °C. After centrifugation at 10,000  $\times g$  for 10 min, the supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and applied to a DEAE Toyopearl column (Tosoh, Tokyo, Japan). After washing the column with the same buffer containing 0.2 M KCl, NGF was eluted with the buffer containing 0.5 M KCl. The eluate was concentrated on a Centricon 10 concentrator (Amicon) to ~2 ml and dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA. This procedure removed >80% of proteins without apparent loss of activity.

The partially purified NGF thus obtained was assayed for activity by adding serial dilutions of sample to rat pheochromocytoma (PC12) cells (30) grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 10% horse serum using 24-well collagen-coated plates. Mouse NGF (Biomedical Technologies Inc.) served as a standard for comparison. Neurite outgrowth was observed under a microscope after incubation of plates at 37 °C in 5% humidified CO<sub>2</sub> for 7 days.

<sup>1</sup> The abbreviations used are: NGF, nerve growth factor; PCR, polymerase chain reaction; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair.

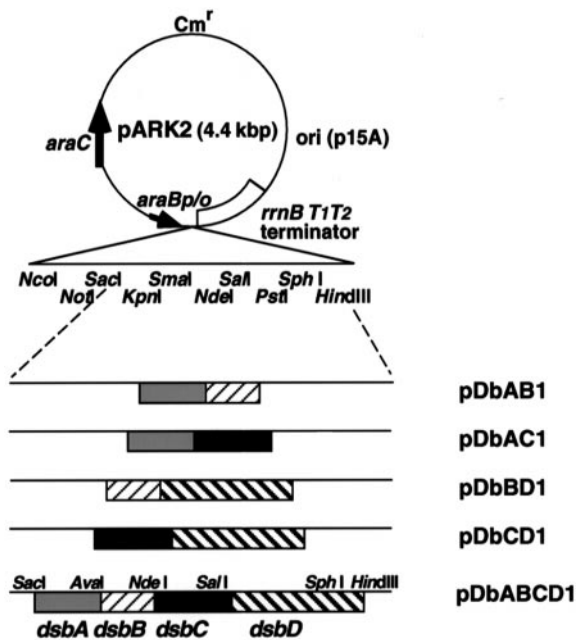


FIG. 1. Construction of Dsb expression plasmids. Organizations of clustered *dsb* genes are schematically shown. The *dsb* genes were cloned and tandemly joined on the pT7Blue® vector and placed under the control of the *araB* promoter on a pARK2 expression vector as described under "Experimental Procedures."

## RESULTS

**Construction of the *dsb* Expression Plasmids**—The *dsbA*, *dsbB*, *dsbC*, and *dsbD* genes were cloned and tandemly joined in various combinations to obtain artificial operons under the *araB* promoter on a pACYC184-based plasmid, pARK2. Each of the *dsb* genes contained the entire coding region with short flanking regions on both sides including a putative Shine-Dalgarno sequence but not a transcription terminator. Several pairwise combined genes (*dsbAB*, *dsbAC*, *dsbCD*, and *dsbBD*) and the complete set of genes (*dsbABCD*) were initially cloned on a high copy plasmid as described under "Experimental Procedures." Each set of the genes was then placed under the *araB* promoter controlled by the *araC* regulatory gene on pARK2 vector, and the resulting plasmids were designated pDbAB1, pDbAC1, pDbBD1, pDbCD1, and pDbABCD1 (Fig. 1). These plasmids are compatible with ColE1-type plasmids generally used for expression of recombinant proteins.

When L-arabinose was added to an L broth culture of *E. coli* carrying each of the expression plasmids, the respective Dsb proteins were induced to various levels depending on the arabinose concentration used (Fig. 2B). DsbA protein (21 kDa) was detected by Western blotting with DsbA-specific antibody, whereas DsbC protein (24 kDa) was detected by staining with Coomassie Brilliant Blue on SDS-PAGE (Fig. 2A), although the mobility observed was appreciably slower than that predicted from the primary structure as observed previously by Missiakas *et al.* (21). On the other hand, expression of DsbB and DsbD from the plasmids was confirmed by their abilities to complement the defective phenotypes (higher sensitivity to dithiothreitol or to CuSO<sub>4</sub>) of the respective deletion mutants (data not shown). The growth of host bacteria carrying pDbABCD1 that can express all the Dsb proteins was not affected significantly by addition of L-arabinose up to 200 µg/ml, which was adopted as the standard condition.

**Inhibition of Cell Growth by NGF and Its Relief by Dsb Coexpression**—The human NGF gene was fused with a signal peptide of *ompA*, *ompT*, or *malE* to facilitate membrane transport and was expressed in JM109 cells. When these NGF fusion

proteins were induced by adding 0.1 mM IPTG at 37 °C, their production was detected within 30 min, gradually increased for about 90 min, and cell growth was retarded depending on the kind of signal peptide used. When the *ompA* or *malE* signal was used, a marked growth inhibition was observed, whereas the *ompT* signal caused only slight inhibition (Fig. 3A). Such differential effects of the various signal peptides suggested that certain anomaly in membrane transport of NGF fusion protein caused inhibition of cell growth. Indeed, the amount of periplasmic enzyme β-lactamase was clearly reduced upon induction of OmpA-NGF or MalE-NGF (data not shown).

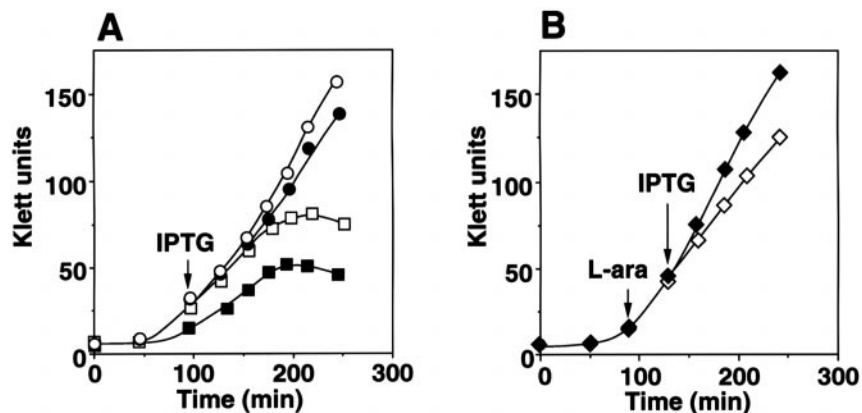
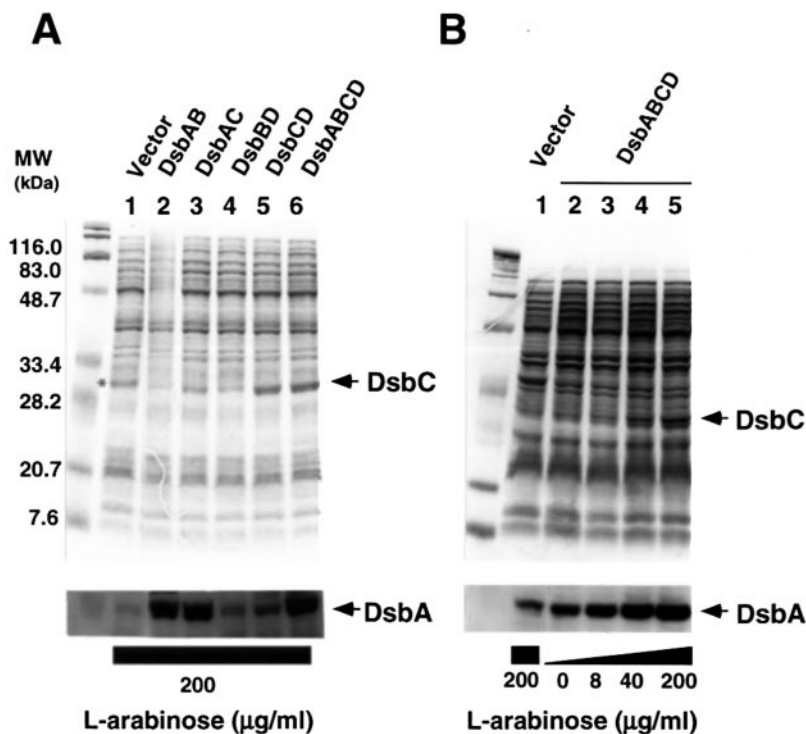
We then introduced the Dsb expression plasmid (pDbABCD1) into the above strains and examined the effects of Dsb coexpression on cell growth. The growth inhibition observed upon OmpA-NGF or MalE-NGF expression was found to be exacerbated when Dsb proteins were coexpressed; no clones carrying both the OmpA-NGF and pDbABCD1 (or pDbAB1) expression plasmids were obtained even after prolonged incubation. Although a clone carrying both OmpA-NGF and pDbCD1 expression plasmids was obtained, no accumulation of NGF or β-lactamase was observed (data not shown). In contrast, the slight but significant growth inhibition observed upon OmpT-NGF expression was completely relieved when the Dsb proteins were coexpressed by addition of L-arabinose (Fig. 3B). These results suggested that OmpT-NGF fusion protein can be successfully transported to the periplasm at least to some extent and that membrane transport of this, as well as other periplasmic proteins, is enhanced by overproduction of Dsb proteins in the periplasm.

**Effects of Dsb Coexpression on the Production and Localization of NGF**—To determine whether overexpression of the Dsb proteins enhances production of NGF possibly through assisting the folding of OmpT-NGF, various sets of Dsb proteins were coexpressed from the expression plasmids, and their effects on the amount of OmpT-NGF produced and on periplasmic localization were compared. Coexpression of DsbAB or DsbCD should enhance the efficiencies of either disulfide bond formation or isomerization, respectively, whereas that of DsbAC increases both the disulfide bond formation and isomerization activities. Under these conditions, coexpression of OmpT-NGF did not affect the levels of DsbA and DsbC significantly (data not shown).

As shown in Fig. 4, the control cells carrying the pACYC184 vector produced NGF of which about 30% was found in the periplasmic fraction after induction for 3.5 h (*lanes 1 and 6*). Coexpression of DsbAB hardly affected the total or periplasmic expression of NGF (*lanes 2 and 7*), whereas that of DsbAC increased the total yield nearly 2-fold but hardly increased the periplasmic production (*lanes 3 and 8*). Essentially the same result was obtained when the level of DsbAC coexpression was further enhanced by using a higher concentration of arabinose (data not shown). In contrast, coexpression of DsbCD enhanced the total NGF production by about 2-fold and the periplasmic expression by about 3-fold over the vector control; namely, about 60% of total NGF was recovered in the periplasm (*lanes 4 and 9*). Even higher periplasmic production of NGF (~80%) was observed when all the Dsb proteins (DsbABCD) were overexpressed (*lanes 5 and 10*). In the presence of excess DsbCD or DsbABCD (but not DsbAB), NGF was significantly stabilized, which probably explains the increase in total NGF production (data not shown).

**Effects of Varying Levels of DsbABCD Coexpression on Localization of NGF**—To further assess the effects of DsbABCD coexpression on OmpT-NGF production, we varied the level of Dsb coexpression and analyzed its effect on distribution of NGF into several distinct subcellular fractions. The extent of Dsb

**FIG. 2. Expression of Dsb proteins from the expression plasmids.** Strain JM109 carrying each of the Dsb expression plasmids or the vector (pARK2) was grown in L broth containing chloramphenicol (34  $\mu\text{g/ml}$ ) at 37  $^{\circ}\text{C}$ , and Dsb proteins were induced with L-arabinose for 1 h. Expression of Dsb proteins was analyzed by SDS-PAGE using a 12.5% acrylamide gel followed by staining with Coomassie Brilliant Blue (*upper panels*) or by immunoblotting of the same gel using DsbA-specific antibody (*lower panels*). Numbers to the left indicate molecular mass (kDa) of protein markers (Bio-Rad). A, expression of DsbA and DsbC proteins with 200  $\mu\text{g/ml}$  of L-arabinose. The asterisk (\*) represents a nonspecific band and not DsbC. B, dependence of Dsb expression on L-arabinose concentration.



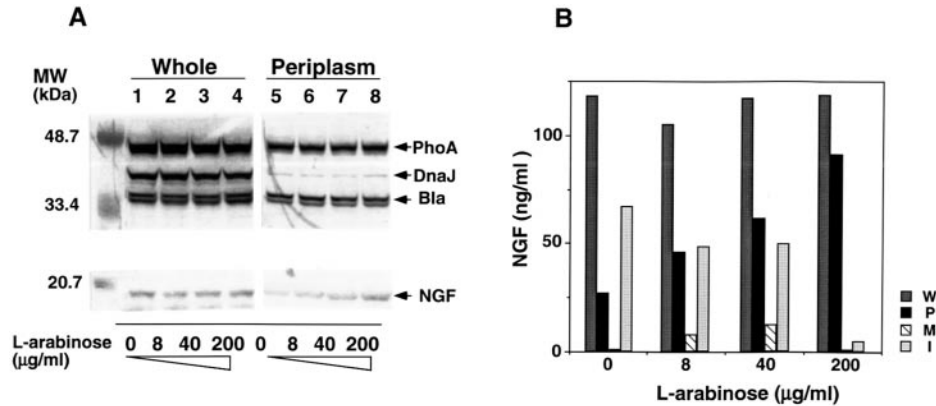
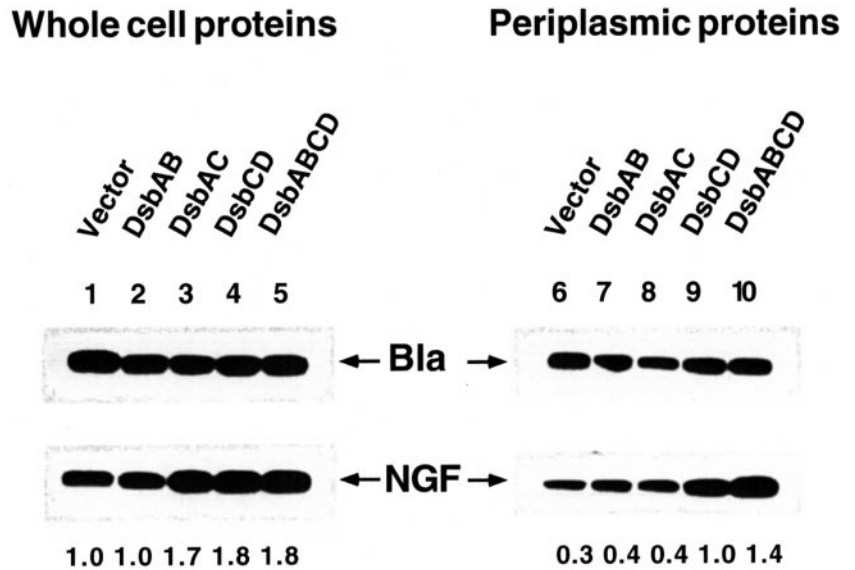
**FIG. 3. Effects of NGF production and overexpression of Dsb proteins on cell growth.** A, effects of production of NGF fused with diverse signal peptides. Derivatives of strain JM109 carrying pTrc-OmpA-NGF ( $\square$ ), pTrc-OmpT-NGF ( $\bullet$ ), pTrc-MalE-NGF ( $\blacksquare$ ), or pTrc-OmpT vector ( $\circ$ ) were grown in L broth containing ampicillin at 37  $^{\circ}\text{C}$ . When the culture reached 20 Klett units, expression of NGF was induced by adding 50  $\mu\text{M}$  IPTG. B, effects of coexpressing OmpT-NGF and DsbABCD. JM109 cells carrying both pTrc-OmpT-NGF and pDbABCD1 ( $\blacklozenge$ ) or pTrc-OmpT-NGF and pACYC184 vector ( $\diamond$ ) were grown in L broth containing ampicillin and chloramphenicol at 37  $^{\circ}\text{C}$ , and when the culture reached 20 Klett units, expression of Dsb proteins was induced by L-arabinose (L-ara; 200  $\mu\text{g/ml}$ ), followed by induction of OmpT-NGF with 50  $\mu\text{M}$  IPTG after 30 min.

coexpression, within the range tested, hardly affected the total amount of NGF produced, whereas NGF obtained in the periplasmic fraction increased markedly with increasing level of Dsb coexpression (Fig. 5A), concomitant with the decrease in the insoluble fraction (Fig. 5B). Again, about 80% of the total NGF produced was found in the periplasm at the maximum Dsb coexpression. Only the band with mobility characteristic of the mature NGF was detected, suggesting that the signal peptide was effectively removed from the precursor OmpT-NGF by processing. The periplasmic fraction analyzed contained most of  $\beta$ -lactamase and alkaline phosphatase as expected but very little cytoplasmic protein, DnaJ (Fig. 5A). Thus, NGF found in the periplasmic fraction most probably represents soluble forms of protein that had been successfully processed and correctly folded in the periplasm.

**Periplasmic Production of Active NGF by Dsb Coexpression**—To further substantiate the above possibility, we deter-

mined the biological activity of soluble NGF found in the periplasm by using neurite outgrowth assay with rat PC12 cells. To remove proteins toxic to the cells while minimizing possible denaturation or refolding of NGF produced, the periplasmic fraction was concentrated without salt precipitation followed by treatments with affinity and ion-exchange columns. Bioassays with serial dilutions of product revealed that NGF found in the periplasm upon DsbCD (or DsbABCD) overexpression exhibit activity comparable with that of authentic mouse NGF (Fig. 6, B and C). On the other hand, the NGF recovered from similar periplasmic fraction obtained without overexpression of Dsb proteins, even after 4-fold concentration, failed to show any detectable activity (Fig. 6A), indicating that NGF produced under these conditions is hardly active. These results strongly suggest that NGF produced and transported to the periplasm in *E. coli* become biologically active only when assisted by extensive overexpression of DsbCD (or DsbABCD).

**FIG. 4. Effects of coexpression of different subsets of Dsb proteins on OmpT-NGF production.** Derivatives of strain JM109 carrying both pTrc-OmpT-NGF and a Dsb expression plasmid (or pACYC184 vector) were grown in L broth, and expression of Dsb proteins and OmpT-NGF was induced as described in the legend to Fig. 3. After induction of OmpT-NGF for 3.5 h, whole-cell proteins (lanes 1–5) and periplasmic proteins (lanes 6–10) were prepared separately from equal volumes of each culture. Proteins were analyzed by SDS-PAGE (15% gel) followed by immunoblotting for NGF and  $\beta$ -lactamase (*Bla*) and quantified as described under “Experimental Procedures.” Values shown below the blots indicate amounts of NGF obtained relative to that found in whole-cell proteins from the vector control (lane 1).



**FIG. 5. Effects of varying levels of DsbABCD coexpression on intracellular distribution of OmpT-NGF.** Strain JM109 carrying both pTrc-OmpT-NGF and pDbABCD1 was grown, and Dsb proteins and OmpT-NGF were induced as described in the legend to Fig. 3. After a 1-h induction of OmpT-NGF, cells were collected, fractionated, and analyzed as described under “Experimental Procedures.” *A*, effects of Dsb coexpression level on the periplasmic expression of OmpT-NGF. Whole-cell proteins (lanes 1–4) and periplasmic proteins (lanes 5–8) were analyzed by SDS-PAGE (15% gel) followed by immunoblotting with a mixture of antibodies against NGF, DnaJ,  $\beta$ -lactamase (*Bla*), alkaline phosphatase (PhoA) and PhoA-conjugated secondary antibody. *B*, effects of Dsb coexpression level on the intracellular localization of OmpT-NGF. Whole-cell proteins (*W*), periplasm (*P*), cytoplasm (*C*), membrane (*M*), and insoluble fractions (*I*) were prepared from equal volume of cultures, analyzed by SDS-PAGE, immunoblotted as in *A*, except that the secondary antibody used was horseradish peroxidase-conjugated antibody, and quantified as described under “Experimental Procedures.” Cytoplasmic fraction is not shown, because the amount of NGF detected was negligible for all cultures examined.

DISCUSSION

We have constructed a set of versatile plasmids for controlled expression of Dsb proteins to assess the effects of coexpression on periplasmic production of recombinant proteins in *E. coli*. These plasmids permit coordinate induction of different sets of Dsb proteins by manipulating their expression levels and timing independently from that of the target recombinant protein. The maximum extents of overproduction for DsbA and DsbC proteins were severalfold higher than the normal levels but hardly affected cell growth under the conditions employed.

Among the three signal peptides tested, the OmpT signal turned out to be most effective for producing soluble NGF with apparently little effects on translocation of periplasmic proteins and host cell growth, and the slight inhibitory effect on growth was overcome by overexpressing the whole set of Dsb proteins (Fig. 3). In contrast, when the OmpA or MalE signal was used, marked growth inhibition was observed upon induction of NGF presumably because of defective membrane transport of secretory proteins; however, this defect was not rescued by overproduction of Dsb proteins. Although the mechanism of secretion defects remains obscure, the N terminus of mature

NGF containing three consecutive serine residues tends to form a  $\beta$ -turn structure and could be involved in translocation inhibition of NGF precursor. The present results revealed that such inhibition can be alleviated by using an appropriate signal peptide.

Among the subsets of Dsb proteins tested, coexpression of DsbCD but not DsbAB was effective and that of DsbABCD was most effective for obtaining soluble NGF in the periplasm (Fig. 4). Thus, although coexpression of DsbAB alone has little effect, that of both DsbAB and DsbCD are highly effective perhaps through synergistic function between two pairs of proteins in assisting folding of NGF. Although DsbCD could assist transport of the target protein directly or indirectly by pulling the precursor into the periplasm (17), it seems more likely that overexpressed DsbCD proteins efficiently promoted isomerization of aberrant disulfide bonds formed on nascent NGF, thus yielding correctly folded products. Sone *et al.* (16) demonstrated that overexpressed DsbC acts as disulfide isomerase on the mutant alkaline phosphatase, which contains aberrant disulfide bonds formed between consecutive cysteine residues. However, only a limited success was reported on disulfide isomer-

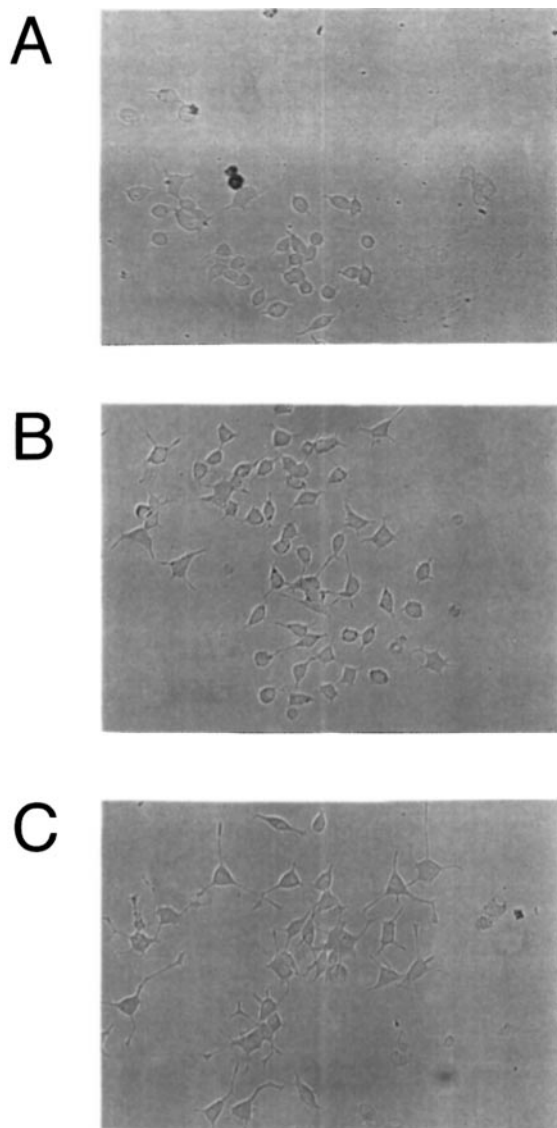


FIG. 6. **Bioassay for NGF produced under Dsb overexpression.** PC12 cells were treated with partially purified recombinant NGF (100 ng/ml) produced in the absence (A) or presence of excess DsbCD (B) or authentic mouse NGF (100 ng/ml) (C). Microscopic observation was made after a 7-day incubation.

ase-assisted periplasmic production of heterologous proteins that contain multiple disulfide bonds between nonconsecutive cysteine residues (nonconsecutive-type) (17, 29). The success probably depends not only on overexpression of the DsbC isomerase but also on maintaining the disulfide-reductase activity of DsbC in the oxidized environment of periplasm. Coexpression of rat protein disulfide isomerase in *E. coli* significantly increased the total yield of bovine pancreatic trypsin inhibitor, which has three disulfide bonds of nonconsecutive type but failed to reduce the amount of incorrectly folded products (29). When DsbC was overexpressed with insulin-like growth hormone I, which also has three nonconsecutive disulfide bonds, the total or insoluble products increased but not the periplasmic products, despite the fact that the active site cysteine residues of DsbC were kept partially reduced (17).

In the course of the present study, Qiu *et al.* (38) reported production of a soluble and active human tissue plasminogen activator that has 527 amino acid residues and 17 nonconsecutive disulfide bonds in the *E. coli* periplasm by overexpressing DsbC (38). However, in this case, overexpressed DsbC might act stoichiometrically rather than catalytically, and the inacti-

ated DsbC could inhibit the membrane transport of precursor DsbC and other proteins essential for cell growth. The present results based on the differences between DsbAC and DsbCD coexpression suggested that overexpression of DsbC is necessary but not sufficient for obtaining maximum periplasmic production of NGF; coexpression of DsbC with DsbD, which is presumably required for regenerating DsbC, appeared to be crucial for efficient isomerization of disulfide bonds in the *E. coli* periplasm. Consistent with this proposal, expression of a bovine pancreatic trypsin inhibitor containing three disulfide bonds (nonconsecutive-type) yielded intermediates mostly with aberrant disulfide bonds in a *dsbD* deletion mutant (14). Similarly, the yield of active human placental alkaline phosphatase was low, whereas bacterial alkaline phosphatase was normally produced in the *dipZ* (*dsbD*) mutant presumably because of formation of aberrant disulfide bonds (39). It thus seems clear that DsbD, as well as DsbC, can become overloaded upon overexpression of heterologous proteins having multiple disulfide bonds.

Because the increasing levels of DsbABCD coexpression enhance the amount of soluble NGF found in the periplasm with concomitant decrease in insoluble products, the overproduced Dsb proteins are most likely to enhance the periplasmic folding of NGF by preventing aggregation. Coexpression of DsbAB was hardly effective in this respect, suggesting that enhanced disulfide-forming activity alone is not sufficient for facilitating the protein folding. In contrast, coexpression of DsbAC, DsbCD, or DsbABCD markedly enhanced the total amount of NGF produced (Fig. 4). Overexpression of DsbC therefore appears to protect the product in some way from proteolysis; in fact, the NGF product was stabilized in the presence of excess DsbCD or DsbABCD proteins. On the other hand, increased periplasmic production of NGF was observed only when DsbC and DsbD were simultaneously coexpressed (DsbCD or DsbABCD). This suggests that DsbC can assist conversion of aberrant disulfides of NGF to the native form but cannot release native, soluble products in the absence of sufficient amounts of DsbD. The results of bioassay revealed that recombinant NGF produced with DsbCD overexpression has activity similar to that of authentic NGF, suggesting that correct folding of NGF was efficiently catalyzed by overexpressed DsbCD proteins. Detailed mechanisms of disulfide isomerization including the mode of modulation of DsbC activity by DsbD or other factors should have important bearings on further understanding and improving of periplasmic production of recombinant proteins that require complex disulfide bond formation.

After completion of this work, two laboratories reported correction of the primary structure of DsbD (40, 41), which indicated that translation of the *dsbD* gene begins at a start codon 76 codons upstream of that previously thought. Because the present expression plasmids were constructed on the basis of previously reported gene structure, the excess DsbD proteins obtained here lack the N-terminal 76 amino acid residues (including 26 possible signal sequences). However, the *dsbD* gene used did complement the defective phenotype of *dsbD* null mutant, and overexpression of DsbCD (but not DsbAC) exerted distinct effects on the periplasmic expression of NGF (Fig. 4), suggesting active participation of the excess DsbD produced; the truncated DsbD should retain all the cysteine residues that may be required for the disulfide oxidoreductase activity. We therefore believe that the essential finding and conclusion of this work remains unaffected, although the effect of DsbD overexpression observed here may have been underestimated.

In conclusion, our results strongly suggest that disulfide bond isomerization of NGF can be efficiently and synergistically catalyzed by overexpression of DsbC and its modulator

DsbD. Excess DsbC protein appears to be successfully transported to the periplasm, and its activity can be effectively maintained by simultaneous supply of excess DsbD. We also found recently that periplasmic production of horseradish peroxidase containing multiple disulfide bonds and unstable in *E. coli* is much improved by overexpressing Dsb proteins (42). The Dsb coexpression plasmids such as those reported here should prove useful for studying production of heterologous proteins with multiple disulfide bonds and prone to aggregation or degradation upon secretion to the periplasm.

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