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Cloning and Characterization of the Gene for a Protein Thiol-Disulfide Oxidoreductase in *Bacillus brevis*

TATSUYA ISHIHARA, HIDEAKI TOMITA, YUKO HASEGAWA, NORIHIRO TSUKAGOSHI, HIDEO YAMAGATA, AND SHIGEZO UDAKA*

Department of Applied Biological Sciences, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

Received 29 August 1994/Accepted 21 November 1994

The gene (*bdb*) for protein thiol-disulfide oxidoreductase cloned from *Bacillus brevis* was found to encode a polypeptide consisting of 117 amino acid residues with a signal peptide of 27 residues. Bdb contains a well-conserved motif, Cys-X-X-Cys, which functions as the active center of disulfide oxidoreductases such as DsbA, protein disulfide isomerase, and thioredoxin. The deduced amino acid sequence showed significant homology with those of several bacterial thioredoxins. The *bdb* gene complemented the *Escherichia coli dsbA* mutation, restoring motility by means of flagellar and alkaline phosphatase activity. The Bdb protein overproduced in *B. brevis* was enzymatically active in both reduction and oxidization of disulfide bonds in vitro. Immunoblotting indicated that Bdb could function at the periphery of the cell.

Disulfide bonds play an important role in producing the three-dimensional structures responsible for the specific properties of proteins. Although disulfide bonds in a protein can form spontaneously in vitro, the process is much slower and less effective than that in vivo (20), suggesting the existence of proteins which catalyze native disulfide bond formation in vivo. Disulfide oxidoreductases such as thioredoxin and protein disulfide isomerase (PDI) contain the Cys-X-X-Cys motif as the catalytic active site (16) and facilitate disulfide exchange in vitro (10).

The *dsbA* gene in *Escherichia coli* has been discovered independently by two groups (3, 12). DsbA is a periplasmic protein with the sequence motif Cys-Pro-His-Cys, which resembles the active site of thioredoxin and PDI. The *dsbA* mutation causes a defect in the disulfide bond formation of several periplasmic proteins such as alkaline phosphatase, the OmpA protein, β-lactamase (3), and the P-ring protein of the flagellar basal body (7). DsbA appears to be required for the formation of disulfide bonds. DsbA homologs are also found in bacteria such as *Vibrio cholerae* (19) and *Haemophilus influenzae* (26). They are thought to form a new family of bacterial disulfide oxidoreductase.

All bacterial disulfide oxidoreductases have been isolated only from gram-negative bacteria, while no data have been obtained on those of gram-positive bacteria. In gram-positive bacteria, secretory nascent polypeptides are transported to the external medium and must fold into precise shapes to produce functional proteins outside or on the surface of the cell. It is of interest to examine how extracellular proteins acquire disulfide bonds in the protein secretory pathway. We describe here the cloning and characterization of a gene termed *bdb* (for *Bacillus* disulfide bond formation), which encodes the protein thiol-disulfide oxidoreductase of *Bacillus brevis*, a gram-positive bacterium used as a host for heterologous protein production (31).

MATERIALS AND METHODS

Bacterial strains, media, plasmids, and transformation. The bacterial strains used were *B. brevis* HPD31 (24) and *E. coli* XL1-Blue (Stratagene), JCB502 (λ D69 *lacZ*::Tn*10 tetS* by fusaric acid) (3), JCB572 (JCB502 *dsbA*::*kan-1*) (3). *E. coli* JCB572 and JCB502, which were supplied by J. Bardwell, were used as the cloning host and reference strain, respectively. XL1-blue was used for DNA manipulation. *E. coli* and *B. brevis* were grown in L broth (22) at 37°C and in T2 (30) or 3PY medium at 30°C, respectively. 3PY medium contained 40 g of polypeptone P1 (Nihon Pharmaceutical), 5 g of yeast extract (Difco), 30 g of glucose, 0.1 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4H_2O$, and 1 mg of $ZnSO_4 \cdot 7H_2O$ per liter; the pH was adjusted to 7.0 with NaOH. When required, ampicillin and neomycin were added at concentrations of 50 and 60 mg/liter, respectively. For molecular cloning and sequencing, pUC and M13 vectors were used. Plasmids pKK233-2 (2) and pNH400 were used for gene expression in *E. coli* and *B. brevis*, respectively. Plasmid pNH400 was constructed by inserting the 636-bp *Mfl*I-*Hin*dIII fragment of pNU210 (32), which contains the promoter and signal peptide-encoding region of the cell wall protein gene (1, 33) of *B. brevis* 47 and a multicloning site, into *Bam*HI-*Hin*dIII-digested plasmid pBAM101 (8, 29) and deleting the *Nsp*V-*Eco*RI fragment containing the P2 and P3 promoters of cell wall protein gene. *E. coli* and *B. brevis* were transformed by the methods of Hanahan (9) and Takagi et al. (25), respectively.

Isolation and analysis of DNAs. Chromosomal DNA of *B. brevis* was prepared by the method of Saito and Miura (21). Plasmid DNAs were prepared from *B. brevis* by the method of Birnboim (5). DNA digestion with restriction enzymes and ligation were carried out under the conditions specified by the supplier, and DNA fragments were analyzed by electrophoresis in 0.7% agarose and 5% polyacrylamide gels.

Cloning of the *bdb* gene. Chromosomal DNA from *B. brevis* HPD31 was partially digested with *Sau*3AI and then ligated to pUC119 cleaved with *Bam*HI. The ligated molecules were used to transform *E. coli* JCB572 to ampicillin resistance. Transformants harboring the gene complementing the *E. coli dsbA* mutation were screened for motility on L plates containing 0.22% agar (Difco).

Complementation of the *E. coli dsbA* mutation by the *bdb* gene. The 0.4-kb fragment containing the *bdb* gene was amplified by PCR using p412 as the template. Primer oligonucleotides 5′-GGTCCATGGCACGAGCA AAATG GCT-3′ (primer 1; sense) and 5′-GGAAACAGCTATGACCATG-3′ (primer 2; antisense) were synthesized at the Center for Gene Research of Nagoya University. Primer 1 was designed to introduce an *Nco*I site just in front of the putative ATG translation initiation codon of the *bdb* gene. The amplified fragment was digested with *Sal*I, filled in with the Klenow fragment, and then digested with *Nco*I. The resultant *Nco*I-*Sal*I fragment was inserted between the *Nco*I and *Hin*dIII site filled with the Klenow fragment on pKK233-2 to construct plasmid pKK-Bdb, where the *bdb* gene was placed downstream of the *trc* promoter and the ribosomal binding site of pKK233-2. *E. coli* JCB572 harboring pKK-Bdb was grown with isopropyl-β-D(−)-thiogalactopyranoside (IPTG), and its motility and alkaline phosphatase activity (6) were examined.

To induce alkaline phosphatase, cells were cultured in medium containing 14.4 g of Tris, 2.0 g of glycerol, 4.7 g of NaCl, 1.1 g of NH_4Cl, 1.5 g of Na_2SO_4, 0.4 g of $MgCl_2 \cdot 7H_2O$, 0.3 mg of $FeCl_2$, 0.3 mg of $ZnCl_2$, 1 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of thiamine, and 4 mg of KH_2PO_4 per liter; the pH was adjusted to 7.2 with HCl. Alkaline phosphatase activity was calculated as follows: [(optical density at 420 nm with substrate − optical density at 420 nm without substrate)/min] × 10^3.

Purification and amino-terminal amino acid sequencing of the Bdb protein produced by *B. brevis*. The 0.6-kb *Sau*3AI fragment containing the entire *bdb* gene was inserted into the *Bam*HI site of pNH400 to be placed under the control of the cell wall protein promoter (pNH-Bdb). *B. brevis* HPD31 harboring pNH-Bdb was cultured for 3 days at 30°C in 3PY medium supplemented with neomy-

* Corresponding author. Mailing address: 1-24-3 Uezono-cho, Meito-ku, Nagoya 465, Japan. Phone and Fax: 81-52-782-1537.

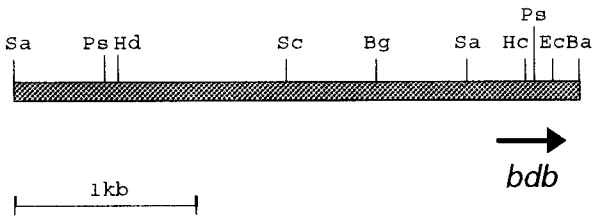

FIG. 1. Restriction map of the *bdb* gene. The arrow indicates the position of the *bdb* gene and the direction of transcription. Restriction sites: Sa, *Sau*3AI; Ps, *Pst*I; Hd, *Hin*dIII; Sc, *Sac*I; Bg, *Bgl*II; Hc, *Hin*cII; Ec, *Eco*RV; Ba, *Bam*HI.

cin at a concentration of 60 mg/liter. After removing cells by centrifugation (3,000 × *g*, 10 min, 4°C), Bdb was precipitated by 55 to 65% saturation with ammonium sulfate, dissolved in a small volume of 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl, and dialyzed extensively against the same buffer. The dialyzed sample was then applied to a Sephadex G-75 column (Pharmacia) previously equilibrated and eluted with the same buffer. The fractions containing Bdb were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0), and applied to a DEAE-cellulose column (Whatman) equilibrated with the same buffer. The Bdb protein was eluted from the column with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Bdb was eluted at about 0.15 M NaCl. The fractions containing the Bdb protein were pooled, concentrated as described above, and dialyzed against 20 mM triethanolamine-HCl (pH 8.0). The dialysate was applied to a HiTrapQ column (Pharmacia) equilibrated with the same buffer and eluted from the column with a linear gradient of 0 to 0.3 M NaCl in the same buffer. Bdb was eluted again at about 0.15 M NaCl and gave a single band on sodium dodecyl sulfate–17.5% polyacrylamide gel electrophoresis (SDS-PAGE). The N-terminal amino acid sequence of purified Bdb was determined by using a gas phase protein sequencer (ABI 470-120A) after treating the Bdb protein as described by Matsudaira (18).

Insulin reduction assay. Insulin (Sigma) at a final concentration of 0.13 mM was incubated at 30°C in 0.1 M sodium phosphate (pH 7.5) containing 2 mM EDTA and 0.33 mM dithiothreitol in the presence or absence of 1.5 μM purified Bdb protein. The reduction of insulin and its resulting precipitation were monitored by measuring the A_{650}.

Assay of the oxidative refolding of reduced RNase A. Reduced RNase A (Sigma) was prepared as previously described (11). RNase A activity was measured as follows. Reduced RNase (final concentration, 20 μg/ml) was incubated at 30°C in 45 mM Tris-acetate (pH 8.0) containing 10 mM $MgCl_2$ and 0.2 mM oxidized glutathione in the presence or absence of 3 μM Bdb. Portions (10 μl) were withdrawn from the reaction mixture at various intervals, mixed with 1 ml of *Saccharomyces cerevisiae* RNA (80 μg/ml; KOHJIN) dissolved in 50 mM Tris-HCl (pH 7.5) containing 25 mM KCl and 5 mM $MgCl_2$ and then incubated at 30°C for 1 min. The amount of RNA hydrolysis was measured by the increase in A_{260} after the precipitation of unhydrolyzed RNA with trichloroacetic acid.

Other methods. Rabbit anti-Bdb protein was prepared with Bdb protein purified as described above by a standard procedure. The products were resolved by SDS-PAGE and then immunoblotted as described by Towbin et al. (27). Protein was determined by the Bio-Rad protein assay with bovine plasma gamma globulin as the standard. Nucleotide sequences were determined by dideoxy-chain termination as described by Sanger et al. (23). Amino acid sequence comparison was performed with the program FASTP (13).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession number D37936.

RESULTS

Cloning of the *bdb* gene from *B. brevis*. *dsbA* mutant *E. coli* JCB572 cannot move by means of flagella because of defective disulfide bond formation in the FlgI protein, a constituent of the P ring of the flagellar motor (17). By using the *E. coli dsbA* mutant strain as a cloning host, clones harboring a gene that complemented the *dsbA* mutation were screened for motile colonies as described in Materials and Methods. Of about 10^4 transformants, one clone was found which moved to the same extent as the *dsbA*$^+$ strain on L plates containing 0.22% agar and contained a 3.2-kb insert derived from *B. brevis*. A restriction map of p412, the plasmid of that clone, is shown in Fig. 1. The 3.2-kb insert was sequenced in both directions, revealing an open reading frame (ORF) between the *Sau*3AI and *Bam*HI sites. To determine whether or not this ORF complements the *E. coli dsbA* mutation, plasmid pKK-Bdb was constructed, in which the ORF was placed under the transcriptional and translational control of the *trc* promoter on pKK233-2 as described in Materials and Methods, and the motility of the transformants was determined. *E. coli* JCB572 carrying pKK-Bdb moved smoothly on soft agar plates, whereas none of the clones carrying pKK233-2 were motile (Fig. 2A). Furthermore, introducing plasmid pKK-Bdb restored the alkaline phosphatase activity of strain JCB572 (Fig. 2B). These data indicated that the protein encoded by the ORF could functionally substitute for *E. coli* DsbA. Thus, the ORF was designated *bdb* (for *Bacillus* disulfide bond formation).

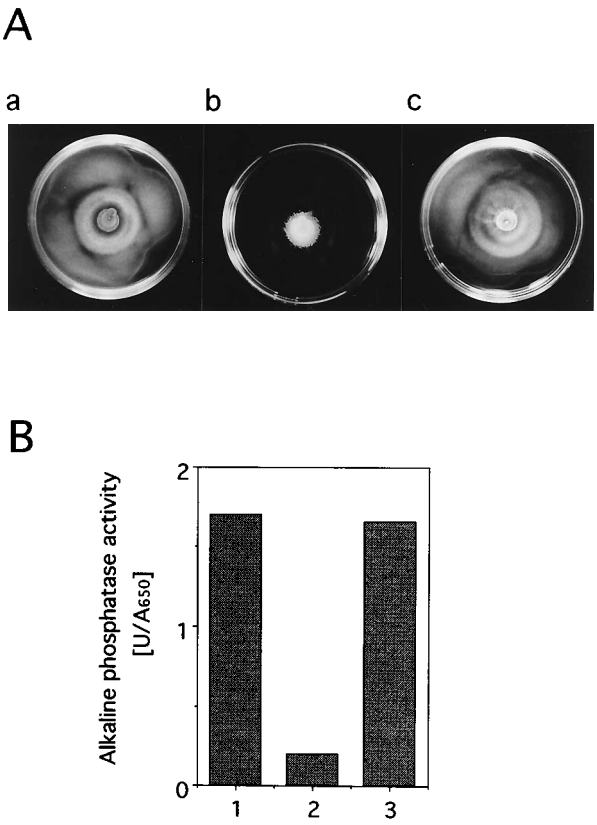

FIG. 2. Complementation of the *E. coli dsbA* mutation by the *bdb* gene. (A) Recovery of the motility of the *E. coli dsbA* mutant. *E. coli* JCB572(pKK-Bdb) (a) was tested for motility on a soft L agar plate. *E. coli* JCB572(pKK233-2) (b) and *E. coli* JCB502(pKK233-2) (c) served as negative and positive controls, respectively. (B) Alkaline phosphatase activity of the *E. coli dsbA* mutant harboring pKK-Bdb. In each cell, alkaline phosphatase activity per unit of A_{650} was measured. Columns: 1, *E. coli* JCB572(pKK-Bdb); 2, *E. coli* JCB572(pKK233-2); 3, *E. coli* JCB502(pKK233-2).

Sequence analysis of the *bdb* gene. The nucleotide and deduced amino acid sequences of the *bdb* gene are shown in Fig. 3. The 354-bp gene encoded 117 amino acids with a molecular weight of 13,326. The N-terminal 27 amino acids had characteristics typical of the signal peptides of secretory precursors, suggesting that the *bdb* gene product is extracellularly localized in *B. brevis*. A comparison of the predicted amino acid sequence of Bdb with that of *E. coli* DsbA revealed no sequence similarity whatsoever. However, Bdb contained, near the amino terminus, the short and characteristic amino acid sequence Cys-X-X-Cys, which is highly conserved among disulfide oxidoreductases such as DsbA, PDI, and thioredoxin (Fig. 4A). The sequence motif Cys-X-X-Cys functions as the active

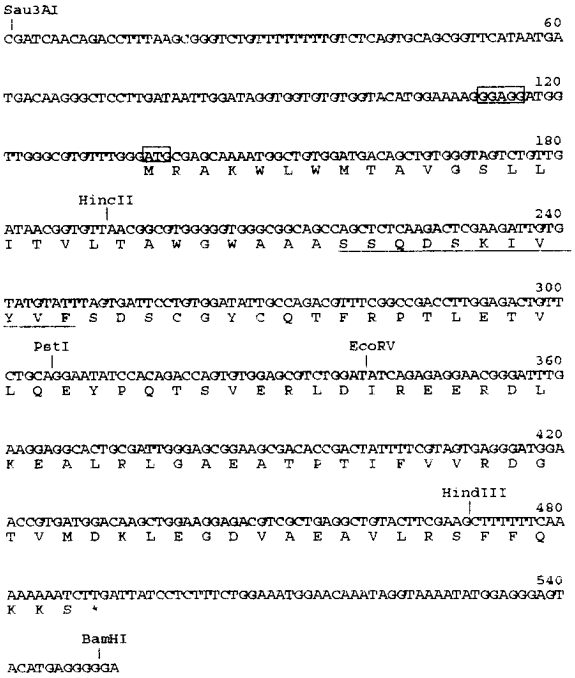

FIG. 3. Nucleotide and deduced amino acid sequences of the *bdb* gene. Only the sequence of the antisense strand is shown. A potential Shine-Dalgarno sequence and a possible initiation codon are boxed. The 11-residue N-terminal amino acid sequence of Bdb isolated from *B. brevis* carrying pNH-Bdb, determined by the Edman method, is underlined.

site of the thiol-disulfide exchange of protein (16). The Cys-Gly-Tyr-Cys sequence of *B. brevis* Bdb could also function as the active center of thiol-disulfide exchange. The deduced amino acid sequence of Bdb showed significant homology to those of several different bacterial thioredoxins bearing the Cys-Gly-Pro-Cys motif, for example, 23% homology with *E. coli* thioredoxin (Fig. 4B).

Overexpression of the *bdb* gene in *B. brevis* and its purification. To characterize the *bdb* gene product biochemically, the Bdb protein was overproduced by constructing and introducing pNH-Bdb into *B. brevis* as described in Materials and Methods. A major protein with an approximate molecular mass of 10 kDa detected in the culture broth was purified to homogeneity as judged by SDS-PAGE (Fig. 5), and its N-terminal amino acid sequence was determined. The 11 N-terminal residues of the Bdb protein determined chemically, SSQDSKIVYVF, were in complete agreement with those deduced starting at Ser. This demonstrated that the first 27 amino acids would function as a signal peptide. The mature Bdb protein is composed of 90 amino acids with a molecular weight of 10,252.

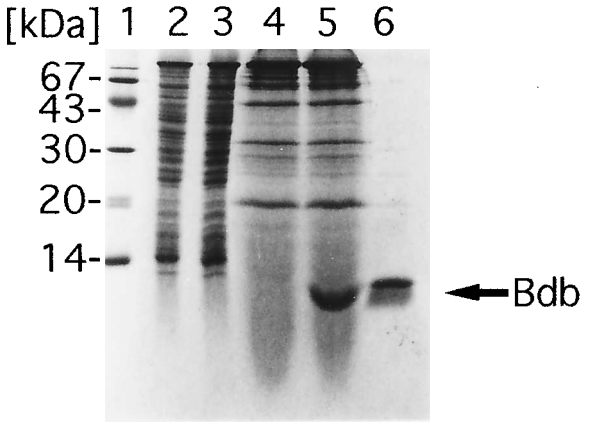

FIG. 5. Overproduction of Bdb in *B. brevis* and purification of the protein. Cell lysates and culture supernatants (10 μl of each) at the stationary phase (72 h) were resolved by SDS-PAGE and then stained with Coomassie brilliant blue. Lanes: 1, molecular size marker proteins; 2 and 3, cell lysates of *B. brevis* carrying pNH400 and pNH-Bdb, respectively; 4 and 5, culture supernatants of *B. brevis* carrying pNH400 and pNH-Bdb, respectively; 6, Bdb purified from *B. brevis* harboring pNH-Bdb (2 μg).

Enzymatic activity of the Bdb protein. The disulfide-reducing and -oxidizing activities of Bdb were examined by using insulin and reduced RNase A, respectively, as the substrates. The Bdb protein stimulated insulin reduction, which resulted in insulin precipitation (Fig. 6A). Furthermore, Bdb facilitated the oxidative refolding of reduced RNase A, which was determined by RNase activity (Fig. 6B). These data indicate that the Bdb protein isolated from *B. brevis* is active in vitro in both reduction and oxidization of disulfide bonds.

Western blotting (immunoblotting) of Bdb. Culture supernatants and cell lysates of *B. brevis* were resolved by SDS-PAGE and then Western blotted. A protein that cross-reacted with the antibody to Bdb was detected mainly in cell lysates, whereas negligible amounts of Bdb were secreted into the culture broth (Fig. 7A). The molecular mass of cellular Bdb was consistent with that of mature Bdb. This suggested that Bdb is localized at the periphery of the cell. In addition to mature Bdb, 15- and 30-kDa proteins in the cell lysates cross-reacted with the anti-Bdb antibody. They might be homologs of Bdb or unrelated proteins that reacted with the Bdb antibody. The Bdb protein overproduced in *B. brevis* accumulated mainly in the culture broth. However, the amount of Bdb detected in the cell lysate of *B. brevis* carrying pNH-Bdb was almost equivalent to that of *B. brevis* carrying pNH400 (Fig. 7B).

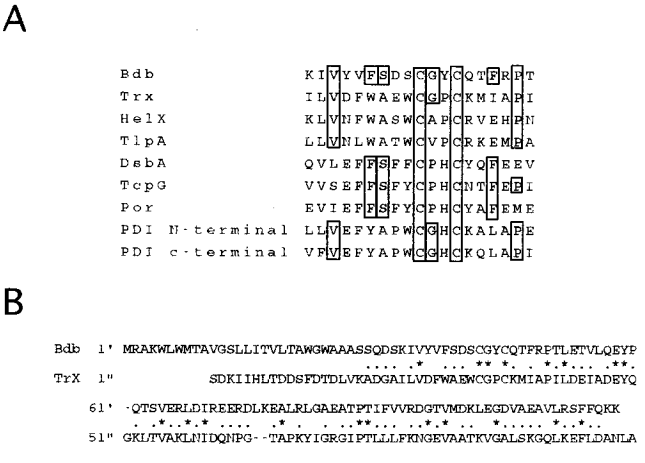

FIG. 4. Comparison of the deduced amino acid sequence of Bdb with those of other disulfide oxidoreductases. (A) Homology at the active site. Bdb residues 33 to 51 are aligned with the region surrounding the catalytic site of *E. coli* thioredoxin (Trx), *R. capsulatus* HelX, *B. japonicum* TlpA, *E. coli* DsbA, *V. cholerae* TcpG, *H. influenzae* Por, and rat hepatic PDI. (B) Overall homology between Bdb and *E. coli* Trx. Amino acid identities are marked with asterisks, and analogous amino acids are marked with dots.

DISCUSSION

The *bdb* gene, encoding a protein with a Cys-Gly-Tyr-Cys sequence which is highly conserved and known to function as

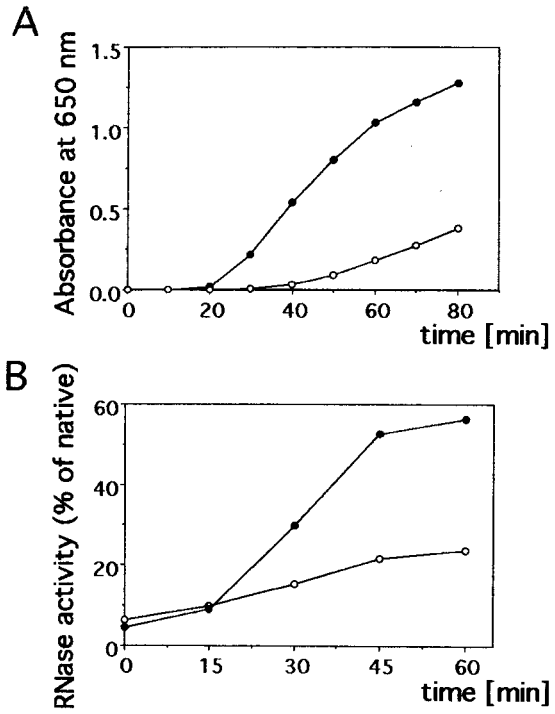

FIG. 6. Enzymatic activity of purified Bdb. (A) Stimulation of insulin reduction by Bdb. The reaction was done with (●) or without (○) 1.5 μM Bdb protein as described in Materials and Methods. (B) Stimulation of oxidative refolding of reduced RNase by Bdb. Reduced RNase was incubated with (●) or without (○) 3 μM Bdb, and the RNase activities of samples were compared with that of an equivalent amount of native RNase.

the active center of disulfide oxidoreductases, was cloned from *B. brevis*. The Bdb protein stimulated the oxidization and reduction of disulfide bonds in vitro. This indicated that Bdb is a new protein thiol-disulfide oxidoreductase.

The amino acid sequence of Bdb resembles neither that of PDI in eukaryotes nor that of DsbA in *E. coli*, except for the sequence around Cys-X-X-Cys. The *dsbA* homologous genes, such as *tcpG* (19) and *por* (26) cloned from *V. cholerae* and *H. influenzae*, respectively, are highly similar (40 to 45% homology) to *E. coli dsbA* and form a new gene family of protein thiol-disulfide oxidoreductases. On the other hand, the amino acid sequence of Bdb is significantly similar to those of several thioredoxins which act in the cytoplasm as a dithiol reductant. Thioredoxin-like proteins encoded by *helX* from *Rhodobacter capsulatus* (4) and *tlpA* from *Bradyrhizobium japonicum* (14) have also been identified. HelX is a periplasmic protein involved in the biogenesis of *c*-type cytochromes. The TlpA protein is involved in cytochrome aa_3 formation and is anchored to the cytoplasmic membrane by an amino-terminal domain, exposing the active site to the periplasm. HelX and TlpA are 23 to 30% identical at the amino acid sequence level to various bacterial thioredoxins and are thought to form another class of disulfide oxidoreductases, the so-called thioredoxin family (15). Therefore, the sequence similarity of Bdb to thioredoxin rather than DsbA suggests that Bdb can be included in the thioredoxin family, although it is functionally similar to DsbA *E. coli*.

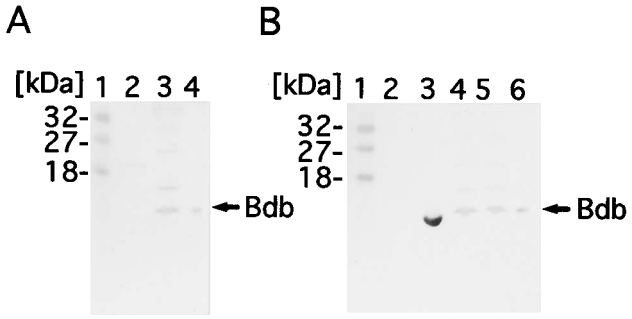

FIG. 7. (A) Immunoblot of cell lysates and culture supernatants of *B. brevis*. Cell lysates and culture supernatants (30 μl of each) at the stationary phase (24 h) were precipitated with trichloroacetic acid and resolved by SDS-PAGE and then immunoblotted against anti-Bdb serum. Lanes: 1, molecular size marker proteins; 2, culture supernatant; 3, cell lysate; 4, purified Bdb (100 ng). (B) Immunoblot of cell lysates and culture supernatants of Bdb-overproducing *B. brevis*. Culture supernatants (10 μl) and cell lysates (30 μl) at the stationary phase (72 h) were resolved by SDS-PAGE and then immunoblotted. Lanes: 1, molecular size marker proteins; 2 and 3, culture supernatants of *B. brevis* carrying pNH400 and pNH-Bdb, respectively; 4 and 5, cell lysates of *B. brevis* carrying pNH400 and pNH-Bdb, respectively; 6, purified Bdb (100 ng).

We do not know whether or not Bdb is essential for disulfide bond formation in proteins in vivo, because an effort to disrupt the *bdb* gene failed. However, the *bdb* gene complemented the *E. coli dsbA* mutation, since *E. coli dsbA* carrying *bdb* recovered motility by flagellar and alkaline phosphatase activity. This suggests that the Bdb protein expressed in *E. coli* can be transported to the periplasmic space, where it catalyzes disulfide bond formation like *E. coli* DsbA. Thus, Bdb seems to be functionally identical to *E. coli* DsbA.

Bdb was detected mostly in the cell lysate by immunoblotting, and its molecular mass was in good agreement with that of mature Bdb. However, most of the Bdb protein, when overproduced, was secreted into the medium, although the cellular Bdb remained constant at a low concentration. On the basis of these results, it is reasonable to assume that Bdb could be translocated across the membrane and localized at the periphery of the cell. Bdb may interact with its substrate proteins and facilitate disulfide bond formation, presumably on the cell surface during or immediately after translocation of the substrate proteins across the cytoplasmic membrane.

Since no proteins, including cell wall proteins (28), secreted by *B. brevis* have any disulfide bonds, identification of true substrate proteins for Bdb is essential to characterize the biological role of Bdb in *B. brevis*. We cannot, however, rule out the possibility that Bdb has unknown functions besides disulfide oxidoreductase activity in *B. brevis*.

A host-vector system for efficient production of heterologous proteins has been developed by using *B. brevis* as the host (31). By using this system, several proteins with multiple disulfide bonds and the same activity as native proteins can be efficiently produced (34). Bdb may play an important role in the disulfide bond formation of these heterologous proteins on the cell surface of *B. brevis*.

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