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Secretion of *Escherichia coli* DsbA and DsbC proteins from *Brevibacillus choshinensis*: stimulation of human epidermal growth factor production

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Abstract

DsbA and DsbC, members of the thioredoxin super-family of redox proteins, which are expressed in the periplasmic space of *Escherichia coli*, were cloned into and successfully secreted from *Brevibacillus choshinensis* at $\sim$100 μg ml^{-1}. Both proteins were active in exchanging disulfide bonds of bovine insulin *in vitro*. Furthermore, DsbA secreted by *B. choshinensis* promoted the conversion of non-native human epidermal growth factor to the native form.

Introduction

Brevibacillus choshinensis (formerly *Bacillus brevis*) is a useful host-vector system with which to produce intact and biologically active foreign proteins. Udaka (1976) was the first to isolate *Bacillus brevis* reporting the secretion of endogenous proteins at up to 20 g l^{-1} into culture medium. On development of an efficient host-vector system, several recombinant mammalian and bacterial proteins (1–3.5 g per liter) were successfully produced extracellularly (Udaka *et al*. 1989, Takagi *et al.* 1989, Udaka & Yamagata 1993). Since the efficient secretion of active proteins requires many factors, including intracellular molecular chaperones which allow secretory precursor proteins to maintain secretion-competent conformations for export, we have characterized the major molecular chaperones DnaK and GroEL, and their co-chaperones, and cloned their genes (Tokunaga *et al*. 1998, 2001). The exported proteins fold to assume the native conformation with the assistance of foldases, such as thiol-disulfide oxidoreductase. The mechanisms of disulfide bond formation in the periplasmic space of *E. coli* have been studied extensively (Rietsch & Beckwith 1998) and it was reported that the overproduction of *E. coli* periplasmic redox protein, Dsbs, facilitated production of recombinant heterologous proteins which contain several disulfide bonds (Kurokawa *et al.* 2000, 2001). In *B. choshinensis*, although a thiol-disulfide oxidoreductase, Bbd, was identified at the periphery of the cells (Ishihara *et al.* 1995), secretion of a large amount of heterologous protein containing multi-disulfide bonds, such as human epidermal growth factor (hEGF), resulted in the formation of incorrect disulfide bonds (Miyauchi *et al*. 1999). The oxidoreductase system for disulfide bond formation in *B. choshinensis* is not well understood.

In the present study, we attempted to construct *B. choshinensis* cells which secrete *E. coli* DsbA or DsbC protein in order to enhance the extracellular catalytic activities of thiol-disulfide exchange reactions for correct disulfide-bond formation in heterologous proteins secreted by *B. choshinensis*.

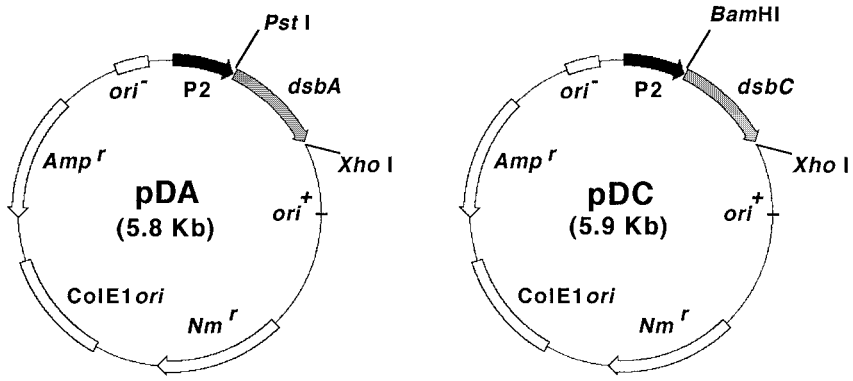

Fig. 1. Schematic illustration of expression-secretion plasmids, pDA and pDC. Plasmids pDA and pDC were constructed from the *E. coli-B. choshinensis* shuttle vector pNCMO2 as described in the text. *Amp*^{r}, ampicillin resistance; *Nm*^{r}, neomycin resistance; *ori*, replication origin; P2, promoter of the cell wall protein gene.

Materials and methods

Strains, plasmids and medium

Bacillus choshinensis HPD-31 transformants were grown in T2M medium (10 g glucose, 10 g polypeptone, 5 g meat extract, 2 g yeast extract, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot 4H_2O$, and 1 mg $ZnSO_4 \cdot 7H_2O$ per liter) containing neomycin at 60 μg ml^{-1} at 30 °C. *E. coli* JM109 was used for DNA manipulations.

Construction of B. choshinensis *secretion vectors for* E. coli DsbA *or* DsbC

DNA manipulations proceeded as described by Maniatis *et al.* (1988). The *E. coli dsbA* gene was amplified from *E. coli* chromosomal DNA by PCR with a forward primer (5′-CCGGATCCGCGCAGTATGAAGATGGT-3′) which encodes a *Bam*HI site followed by the coding sequence starting at Ala_1 of mature DsbA, and a backwards primer (5′-CCCTCGAGTTATTTTTTCTCGGACAG-3′) which encodes the sequence up to the stop codon and *Xho*I site. The *dsbC* gene was also amplified by PCR with a forward primer (5′-GGCTGCAGATGACGCGGCAATTCAACAAAC-3′) which encodes a *Pst*I site followed by the coding sequence starting at Asp_1 of mature DsbC, and a backward primer (5′-GGCTCGAGTTATTTACCGCTGGTCATTTTT-3′) which contains the coding sequence up to the stop codon and *Xho*I site. The *B. choshinensis* secretion vector pNCMO2, containing the P2 promoter and an improved secretion signal of surface layer protein, was digested by *Bam*HI/*Xho*I for subcloning of *dsbA* or *Pst*I/*Xho*I for *dsbC*, and ligated with restriction enzyme-digested PCR fragments to construct pDA for the *dsbA* gene, and pDC for the *dsbC* gene, respectively (Figure 1).

Assay for oxidoreductase activity of DsbA and DsbC

The reaction mixture (500 μl) containing 0.13 mM bovine insulin (Sigma) in 100 mM phosphate buffer, pH 7, and 2 mM EDTA was mixed with 100 μl culture supernatant adjusted to pH 7, and incubated at 25 °C. The reaction was started by adding 5 μl 100 mM dithiothreitol (DTT), and the absorbancy increase at 650 nm was monitored (Holmgren 1979).

Assay of the conversion from non-native to native hEGF

B. choshinensis, expressing or not expressing Dsb proteins, was incubated at 30 °C with non-native hEGF, prepared as described (Miyauchi 1999). An aliquot of the mixture was withdrawn, centrifuged to remove cells, and subjected to Tricine SDS-PAGE to measure the formation of native hEGF.

Other methods

SDS-PAGE was carried out according to Laemmli (1970), and Schägger & Jagow (1987). The amount of protein was measured by the Lowry method. The amino terminal amino acid sequence was determined with a Protein sequencer Procise 492 (Applied Biosystems) after blotting of the protein band of SDS-PAGE to Problot membranes.

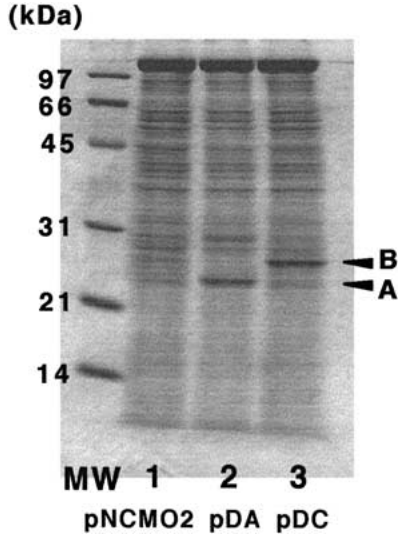

Fig. 2. SDS-electrophoretogram for the culture supernatants of *B. choshinensis* harboring pDA or pDC. Extra protein bands, bands A (lane 2) and B (lane 3), were detected. Lane 1, HPD31(pNCMO2); lane 2, HPD31(pDA); lane 3, HPD31(pDC). MW, molecular weight standard.

Results and discussion

Expression, secretion, and identification of E. coli *DsbA and DsbC proteins from* B. choshinensis

B. choshinensis HPD31(pDA) or *B. choshinensis* HPD31(pDC) were grown, and 10 μl culture supernatant obtained by centrifugation at 10 000 $\times$ *g* for 10 min was subjected to SDS-PAGE (Figure 2). HPD31(pDA) and HPD31(pDC) cells secreted an extra protein with an apparent molecular mass of around 23 kDa (lane 2, band A) and 25 kDa (lane 3, band B), respectively, compared with the control (lane 1). For identification, these proteins were subjected to SDS-PAGE in 10% (w/v) polyacrylamide, blotted on to Problot membranes, and their amino terminal sequences were analyzed. As shown in Figure 3, the amino terminal sequences of bands A and B were identical to the expected sequences, those of *E. coli* DsbA and DsbC preceded by the amino acids Ala-Gly-Ser for DsbA and Ala for DsbC, both derived from nucleotides of cloning sites. These results indicated that signal sequence of *B. choshinensis* surface layer protein-*E. coli* Dsb fusion proteins were correctly recognized, processed and secreted by the secretory machinery and signal peptidase of *B. choshinensis*.

Assay for redox activity of DsbA and DsbC proteins

To examine whether DsbA and DsbC proteins secreted into the *B. choshinensis* culture medium were active, the oxidoreductase activity was assayed as the enzymatic conversion of insulin in the presence of DTT. As shown in Figure 4, the culture supernatants containing DsbA (curve c) or DsbC (curve b) clearly showed a reduction of native insulin. As a positive control, 0.25 μM bovine liver protein disulfide isomerase (Sigma) was also assayed (curve a). After enzymatic assay, the aggregated insulin was precipitated by centrifugation, washed briefly with phosphate buffer, pH 7, and subjected to SDS-PAGE analysis without β-mercaptoethanol: an insulin ladder representing the formation of inter-molecular disulfide bonds was observed (data not shown). These results clearly indicate that both of the *E. coli* Dsb proteins secreted into the *B. choshinensis* culture medium were active in the disulfide exchange reaction with insulin.

Effects of DsbA and DsbC proteins on the conversion of non-native to native form of hEGF

High-level expression and secretion of a large amount of heterologous protein containing multi-disulfide bonds by *B. choshinensis* resulted in the formation of incorrect disulfide bonds (Miyauchi *et al.* 1999). Miyauchi *et al*. (1999) found that incubation of a non-native multimer of hEGF with fresh cells of *B. choshinensis* catalyzed the conversion of non-native to native hEGF, indicating that *B. choshinensis* cells possess a novel thiol-disulfide exchange system that has not been identified. They also described that the sonicated cells lost this redox activity, suggesting that the existence of intact cells was essential for the reaction. Here, we have further examined the effects of these active DsbA and DsbC proteins which have been secreted by *B. choshinensis* on the correct formation of disulfide bonds in hEGF by the same procedures reported by Miyauchi *et al*. (1999). As shown in Figure 5, *B. choshinensis* (pDA) cells (lanes III-1 to 5) formed about 1.8-fold more native hEGF than *B. choshinensis* (pNCMO2) cells (lanes II-1 to 5). DsbC protein showed slightly (maximum 1.06-fold) promotive effects (lanes IV-1 to 5).

These findings indicated that DsbA functioned more effective than DsbC to convert non-native hEGF to native one. It was suggested that thiol-oxidizing activity of DsbA was more effective than disulfide-reducing activity of DsbC for the formation of native hEGF under these assay conditions. Alterna-

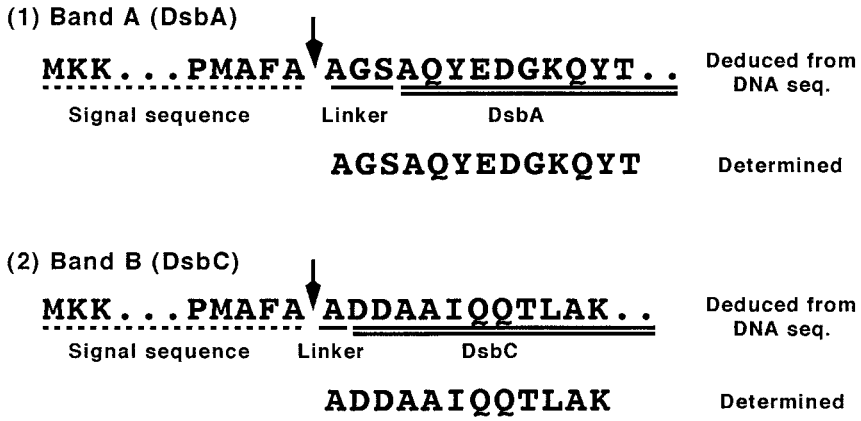

Fig. 3. Amino terminal amino acid sequence analysis of *E. coli* DsbA and DsbC proteins secreted from *B. choshinensis*. A broken line represents the signal sequence of the cell wall protein of *B. choshinensis*. Underline represents the linker sequences derived from the cloning sites. A double underline shows the mature sequence of DsbA and DsbC proteins. An arrow shows the signal cleavage site.

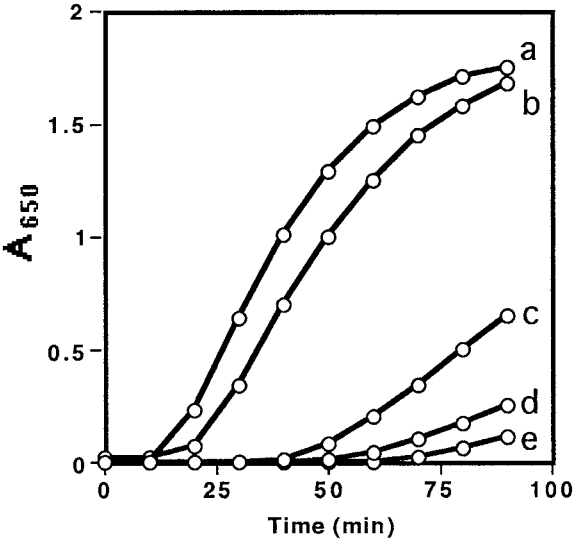

Fig. 4. Enzyme assay for oxidoreductase activities of secreted DsbA and DsbC proteins. (a) Protein disulfide isomerase (Sigma P3818, 2.5 μM) as a positive control. (b) Culture supernatant of *B. choshinensis* HPD31(pDC). (c) Culture supernatant of HPD31(pDA). (d) Culture supernatant of HPD31(pNCMO2). (e) Culture medium (before inoculation of cells) was used to measure non-enzymatic reactions.

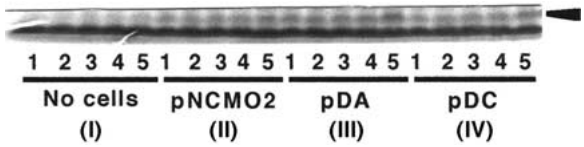

Fig. 5. Effects of DsbA and DsbC proteins on the production of native hEGF. *B. choshinensis* cells (cell culture of $A_{660} = 8$) with or without secretion of DsbA or DsbC were incubated with non-native hEGF (0.2 mg) in an incubation mixture (200 μl) containing 20 μg of erythromycin and chloramphenicol, 1 mM reduced glutathione and 30 mM 2-[*N*-morpholino]ethanesulfonic acid buffer, pH 6, for 0 (lane 1), 10 (lane 2), 30 (lane 3), 60 (lane 4) and 180 (lane 5) min at 25 °C. An aliquot was analyzed by Tricine SDS-PAGE. An arrow head shows the position of native hEGF.

tively, secreted DsbA, but not DsbC, might cooperate with putative thiol-disulfide oxidoreductase systems in *B. choshinensis* reported by Miyauchi *et al.* (1999). Apparent ineffectiveness of DsbC might be due to the absence of DsbD-like factor (Rietsch & Beckwith 1998) in *B. choshinensis*.

We have succeeded in the expression and secretion of active *E. coli* DsbA and DsbC proteins using a protein-hyperproducing bacterium, *Brevibacillus choshinensis*, with a production level of ~100 μg ml^{-1} culture broth. Secreted DsbA protein improved the efficiency of the converting reaction of hEGF from non-native to native form by *B. choshinensis*. Our results open a prospect that we can obtain a correctly folded native protein which has multiple disulfide bonds by coexpressing these heterologous oxidoreductases using *B. choshinensis* expression-secretion system.

References

Holmgren A (1979) Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J. Biol. Chem.* **254**: 9627–9632.

Ishihara T, Tomita H, Hasegawa Y, Tsukagoshi N, Yamagata H, Udaka S (1995) Cloning and characterization of the gene for a protein thiol-disulfide oxidoreductase in *Bacillus brevis. J. Bacteriol.* **177**: 745–749.

Kurokawa Y, Yanagi H, Yura T (2000) Overexpression of protein disulfide isomerase DsbC stabilizes multi-disulfide-bonded re-

combinant protein produced and transported to the periplasm in *Escherichia coli*. *Appl. Environ. Microbiol.* **66**: 3960–3965.

Kurokawa Y, Yanagi H, Yura T (2001) Overexpression of bacterial protein disulfide isomerase (DsbC) and its modulator (DsbD) markedly enhances periplasmic production of human nerve growth factor in *Escherichia coli*. *J. Biol. Chem.* **276**: 14393–14399.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Maniatis, Fritsch EF, Sambrook J (1988) *Molecular Cloning*. New York: Cold Spring Harbor Laboratory Press.

Miyauchi A, Ozawa M, Mizukami M, Yashiro K, Ebisu S, Tojo T, Fujii T, Takagi H (1999) Structural conversion from non-native to native form of recombinant human epidermal growth factor by *Brevibacillus choshinensis*. *Biosci. Biotechnol. Biochem.* **63**: 1965–1969.

Rietsch A, Beckwith J (1998) The genetics of disulfide bond metabolism. *Annu. Rev. Genet.* **32**: 163–184.

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**: 368–379.

Takagi H, Kadowaki K, Udaka S (1989) Screening and characterization of protein-hyperproducing bacteria without detectable exoprotease activity. *Agric. Biol. Chem.* **53**: 691–699.

Tokunaga H, Yamakawa M, Mizukami M, Takagi H, Tokunaga M (1998) Molecular cloning of the *dnaK* locus, and purification and characterization of a DnaK protein from *Bacillus brevis* HPD31. *Biochim. Biophys. Acta* **1387**: 65–79.

Tokunaga M, Shiraishi Y, Odachi M, Mizukami M, Tokunaga H, Philo JS, Arakawa T, Ishibashi M, Tanaka R, Takagi H (2001) Molecular cloning of *groESL* locus, and purification and characterization of chaperonins, GroEL and GroES, from *Bacillus brevis*. *Biosci. Biotechnol. Biochem.* **65**: 1379–1387

Udaka S (1976) Screening for protein-producing bacteria. *Agric. Biol. Chem.* **40**: 523–528.

Udaka S, Yamagata H (1993) Protein secretion in *Bacillus brevis*. *Antonie van Leeuwenhoek* **64**: 137–143.

Udaka S, Tsukagoshi N, Yamagata H (1989) *Bacillus brevis*, a host bacterium for efficient production of useful proteins. *Biotechnol. Gent. Eng. Rev.* **7**: 113–146.