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Extracellular production of an intact and biologically active human growth hormone by the *Bacillus brevis* system

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The characteristic features of the *Bacillus brevis* system are very high productivity of heterologous proteins and very low extracellular protease activity. However, degradation of some heterologous proteins, especially mammalian proteins, can be observed and resulted in a lowering of protein productivity. By using a mutant expressing low levels of proteases and the addition of EDTA to the medium, intact human growth hormone (hGH) was successfully produced with the *B. brevis* system. Signal peptide modification with higher basicity in the amino terminal region and higher hydrophobicity in the middle region brought about a twelve-fold increase in hGH production. The hGH yield was further elevated to 240 mg L^{-1} by optimization of culture conditions. Thus, biologically active and mature hGH can be efficiently produced directly in the medium with the *B. brevis* system.

Keywords: human growth hormone; *Bacillus brevis*; secretion; protease inhibitor

Introduction

A host-vector system for efficient extracellular production of heterologous proteins has been developed by using *Bacillus brevis* as the host [14]. Many heterologous proteins such as bacterial and human α-amylase [5,10], human epidermal growth factor [16] were successfully produced by this system. The system has two prominent features: proteins are secreted directly into the culture medium in soluble and biologically active forms, and the secreted proteins are usually stable because of a very low level of proteolytic activity in the extracellular milieu [16]. Although proteolytic activity was not detected in the culture supernatant of *B. brevis* HPD31, which has been used as a potential host for heterologous protein production, with casein or bovine serum albumin as a substrate, several lines of evidence suggested that proteases were secreted and active. For example, the amount of some animal protein in the culture supernatant decreased considerably after its amount reached maximum. The degradation of secreted protein by extracellular protease (exoprotease) may impair the production level of mature protein, and proteolytic modification of heterologous proteins would be a serious problem especially in the production of medically useful proteins. To circumvent these problems, we isolated a mutant deficient in one of the major proteases of *B. brevis* HPD31 (unpublished data).

In this paper, we report that we succeeded in protecting the degradation of heterologous protein caused by exoprotease of *B. brevis* and achieved very efficient production of mature and biologically active human growth hormone (hGH).

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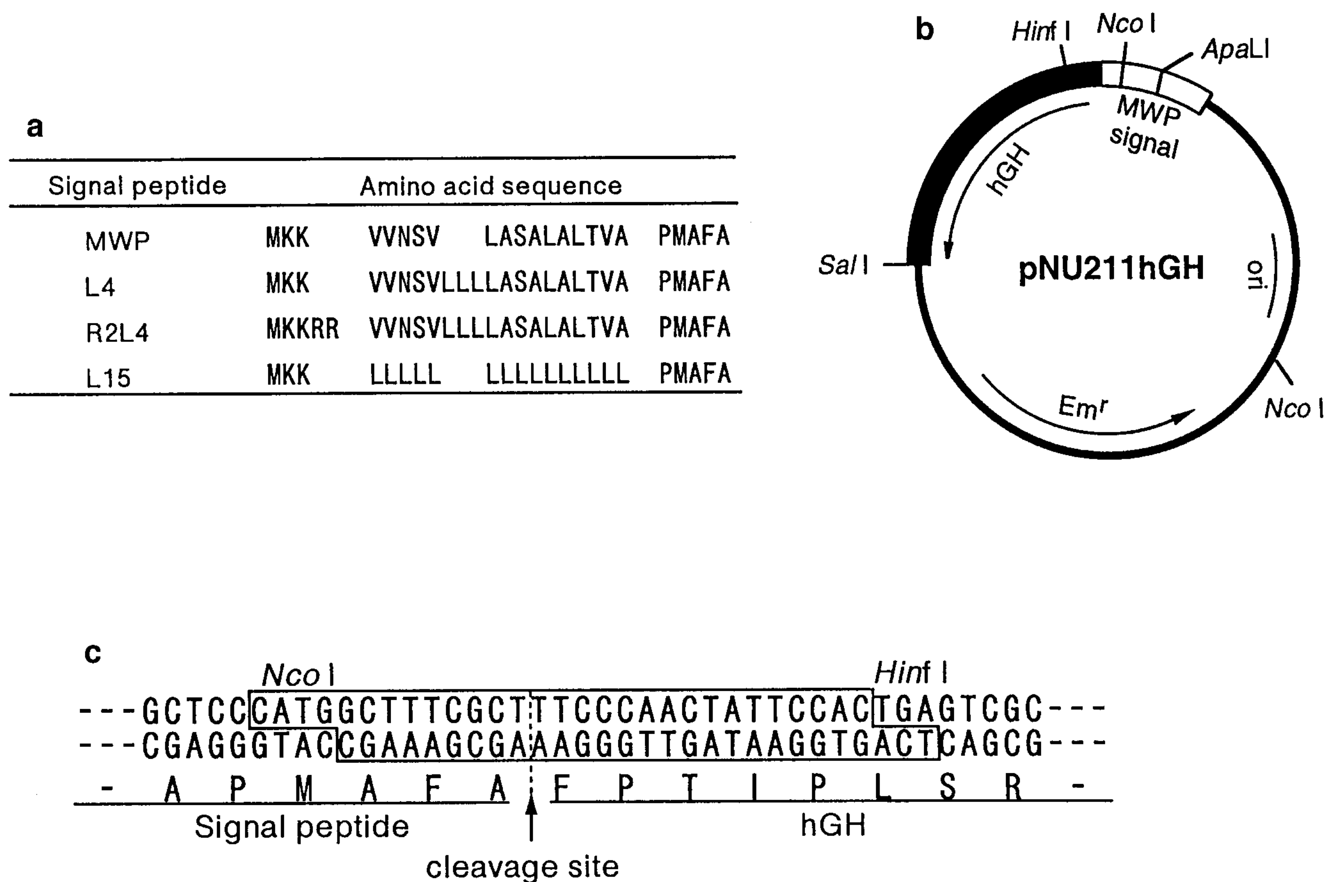
Materials and methods

Strains, plasmids and media

B. brevis HPD31 [11] and *B. brevis* 31-OK, an exoprotease-deficient mutant of the HPD31 [4], were used as a host of protein production. The general methodology for manipulation of *B. brevis* was described by Udaka and Yamagata [14]. pNU210 and pNU211 are *B. brevis* expression-secretion vectors containing a multiple promoter region, translation initiation sites, and the signal peptide-encoding region, which are derived from the middle wall protein (MWP) gene of *B. brevis* 47 [17]. In pNU211L4, pNU211R2L4 and pNU211L15, the MWP signal peptide-encoding sequence was replaced by L4, R2L4 and L15 signal peptide-encoding sequence (Figure 1a), respectively. The hGH expression vectors were constructed as follows. The 561-bp *hinf*I-*Sal*I fragment encoding hGH was isolated from pGH-L9 [2], which was kindly supplied by Professor Morio Ikehara. The isolated fragment was inserted between *Sal*I and *Nco*I sites of pBR-AN3 [14] using appropriate synthetic linker DNA. The *Apa*LI-*Sal*I fragment of the resulting plasmid was inserted between *Apa*LI and *Sal*I sites of corresponding plasmids. On the plasmids thus constructed, namely, pNU200GH, pNU211L4GH, pNU211R2L4GH and pNU211L15GH (Figure 1b), the sequence encoding signal peptide are directly followed by the mature hGH sequence (Figure 1c).

Plasmid was introduced into *B. brevis* by the Tris-PEG method [12]. *B. brevis* cells carrying the expression-secretion vector were grown with shaking at 30°C. YC medium containing 30 g of polypeptone P1 (Nihon Pharmaceuticals, Tokyo, Japan), 2 g of yeast extract, 30 g of glucose, 0.1 g of $CaCl_2 \cdot 2H_2O$, 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; pH 7.2. Erythromycin (10 mg L^{-1}) was added for cultivation of the plasmid-carrying strain.



a

Signal peptide	Amino acid sequence			
MWP	MKK	VVNSV	LASALALTVA	PMAFA
L4	MKK	VVNSVLLLLASALALTVA		PMAFA
R2L4	MKKRR	VVNSVLLLLASALALTVA		PMAFA
L15	MKK	LLLLL	LLLLLLLLLL	PMAFA

Figure 1 *Bacillus brevis* expression vectors. (a) The amino acid sequence of MWP, L4, R2L4 and L15 signal peptides. (b) Schematic diagram of plasmid pNU211hGH. pNU211hGH contains multiple promoters, the MWP signal peptide sequence and the DNA encoding the mature portion of hGH. The erythromycin resistance gene (Em^r) and the replication origin (Ori) were derived from pE194 and pUB110, respectively. In pNU211L4hGH, pNU211R2L4hGH or pNU211L15hGH, the L4, R2L4 or L15 signal sequence was used instead of the MWP signal sequence, respectively. (c) The nucleotide and deduced amino acid sequences around the signal peptide cleavage site of the fused gene. The synthetic linker DNA is boxed.

Purification of hGH from culture supernatant of B. brevis

B. brevis carrying pNU211R2L4GH was grown in YC medium for 2 days. The culture supernatant was concentrated by ultrafiltration (10 kDa cut off; Fuji filter, Tokyo, Japan) and dialyzed against buffer A (20 mM Tris-HCl, pH 7.5) at 4°C. The dialyzed solution was applied to a Q-Sepharose Fast Flow (Pharmacia) column equilibrated with buffer A, and then eluted with a linear gradient of 0–0.5 M NaCl in buffer A. The active fraction containing 0.1% trifluoroacetic acid (TFA) was applied to RPC 5/10 column (Pharmacia) equilibrated with 0.1% TFA, and eluted with a linear gradient of 0–70% acetonitrile in 0.1% TFA. The active fraction was then applied to a C4P-100 column (Waters), and eluted with a linear gradient of 30–70% acetonitrile in 0.1% TFA. hGH was purified to a single band on SDS-PAGE in a final yield of 16%.

Analysis of hGH

The N-terminal amino acid sequence was determined by a protein sequencer ABI model 473A. The C-terminal amino acid was determined by hydrazinolysis [8]. The amount of hGH was routinely measured by ELISA using anti-hGH monoclonal antibody (ZYMED) and anti-hGH rabbit serum (UCB) as primary antibodies, horseradish peroxidase conjugated goat anti-rabbit IgG (VECTOR) as a secondary antibody and *o*-phenylenediamine as a substrate for peroxidase. The hGH purchased from UCB-bioproducts was used as an authentic sample. Adipogenic activity of hGH was measured by the method of Morikawa *et al* [6] with the following modifications. The 3T3-L1 cell was used as a preadipose cell, and the serum supplement for differentiation was changed to 2.5% cow serum with insulin (5 mg L^{-1}), biotin (1 μM), transferrin (5 mg L^{-1}), triiodothyronine (2 nM), mercaptoethanol (40 μM), EGF (30 μg L^{-1}), and vitamin C (0.2 mM). 3T3-L1 cells were plated at 1×10^4 cells per 35-mm dish and grown at 37°C for 4 days in 5% CO_2 in air. hGH was added to the culture at the indicated concentrations, and incubation was continued for 14 days. Assay of glycerophosphate dehydrogenase (GPDH) activity was carried out by the measurement of oxidation of NADH [15]. One unit of enzyme activity was defined as the oxidation of 1 nmol NADH per min. Protein concentration was determined by protein Assay kit (Bio-Rad).

Results

Host-vector system for hGH production

The expression vector, pNU200GH, was introduced into *B. brevis* HPD31 or *B. brevis* 31-OK. Each transformant was

cultivated in YC medium at 30°C with shaking. Except for a faint band showing smaller molecular mass than that of authentic hGH, no band could be detected on Western blot analysis of the culture supernatant of *B. brevis* HPD31 (pNU200GH) (Figure 2a). On the other hand, a band showing the same molecular mass as that of authentic hGH was detected in the 2-day-old culture supernatant of *B. brevis* 31-OK (pNU200GH), but a degradation product, with a molecular mass slightly smaller appeared in the 3- to 6-day-old culture supernatant (Figure 2b). N-terminal amino acid sequence analysis of the degradation product revealed that the first N-terminal amino acid, phenylalanine, was deleted.

Prevention of the hGH degradation by protease inhibitor

Although exoprotease activity of *B. brevis* 31-OK is one fourth that of the parent, *B. brevis* HPD31, the activity of the 31-OK is not negligible. The degradative activity in the culture medium of *B. brevis* HPD31 and 31-OK was inhibited by chelating agents such as EDTA, though neither by serine protease inhibitors, nor by thiol protease inhibitors (unpublished data). Chelating agent was added to YC medium at an appropriate concentration where cell growth was not inhibited, and *B. brevis* carrying the expression vector was cultivated in such a medium at 30°C for 6 days. EDTA was most effective among the inhibitors effective to exoprotease of *B. brevis*. In the case of the HPD31 as a host, the addition of EDTA partially prevented the degradation of hGH, ie the hGH showing native size was accumulated, but the degradation product was also detected in the culture supernatant (Figure 2c). For the 31-OK, the addition of EDTA prevented hGH degradation even after 6 days of cultivation. The amount of hGH in the 6-day-old culture supernatant of the 31-OK was about 13 mg L^{-1}. Other chelating agents were also examined for their ability to prevent hGH degradation. By the addition of EGTA (5 mM), almost all hGH produced by *B. brevis* 31-OK showed native size, but *o*-phenanthrolin showed no effect at a concentration which did not inhibit cell growth (Figure 2d).

Effect of signal peptide modification on hGH production

To examine the effect of signal peptide modification on hGH production, the expression vectors, pNU211L4GH, pNU211R2L4GH and pNU211L15GH were constructed and introduced into *B. brevis* 31-OK. Figure 3 shows the distribution of hGH produced by this system. The mature-sized hGH was detected in both cellular and extracellular fractions of all transformants tested. The R2L4 signal sequence was most effective for hGH secretion into culture supernatant, and the hGH production yield was increased 12-fold (148 mg L^{-1}) on replacement of the MWP signal

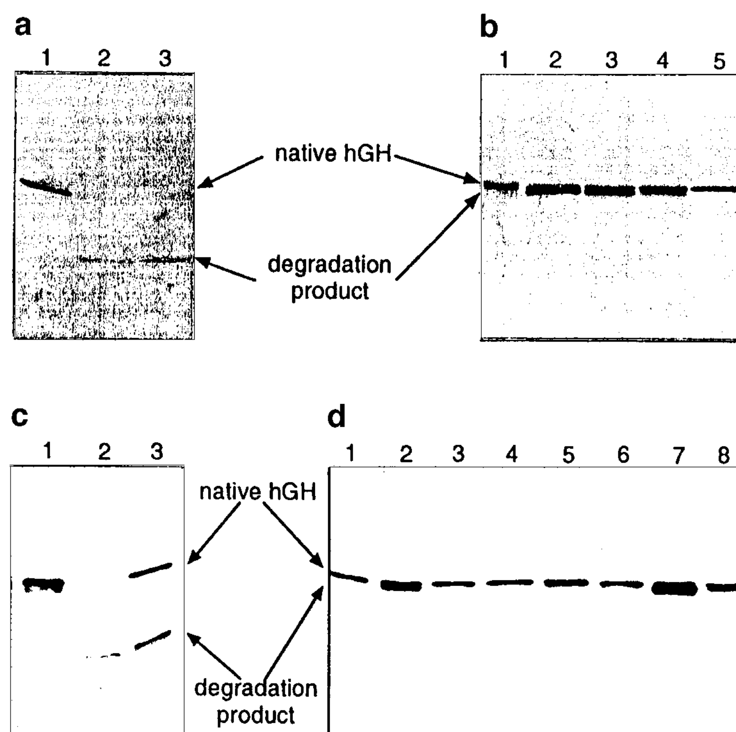


Figure 2 Immunoblot analysis of hGH secreted by *B. brevis*. *B. brevis* carrying pNU211hGH was cultivated at 30°C in YC medium. The culture supernatants were subjected to SDS-PAGE followed by immunoblot analysis with anti-hGH serum. (a) Lane 1: authentic hGH (150 ng); lanes 2, 3: 10 and 20 μl, respectively, of culture supernatant *B. brevis* HPD31 (pNU211hGH) grown in YC medium for 6 days. (b) Lane 1: authentic hGH (150 ng); lanes 2–5: 10 μl of 6-, 4-, 3- and 2-day-culture, respectively, of supernatant *B. brevis* 31-OK (pNU211hGH). (c) *B. brevis* HPD31 (pNU211hGH) was grown for 6 days in YC medium with supplement described below. Ten microlitres of culture supernatant were subjected to the analysis. Lane 1: authentic hGH (100 ng); lane 2: no EDTA; lane 3: 1 mM EDTA. (d) *B. brevis* 31-OK (pNU211hGH) was cultivated for 6 days in YC medium with supplement described below. Ten microlitres of culture supernatant were subjected to the analysis. Lane 1: authentic hGH (100 ng); lane 2: no EDTA; lanes, 3, 4: 0.25 and 0.5 mM EDTA, respectively; lane 5, 6: 1 and 5 mM EGTA respectively; lane 7, 8: 0.075 and 0.125 mM *o*-phenanthrolin, respectively.

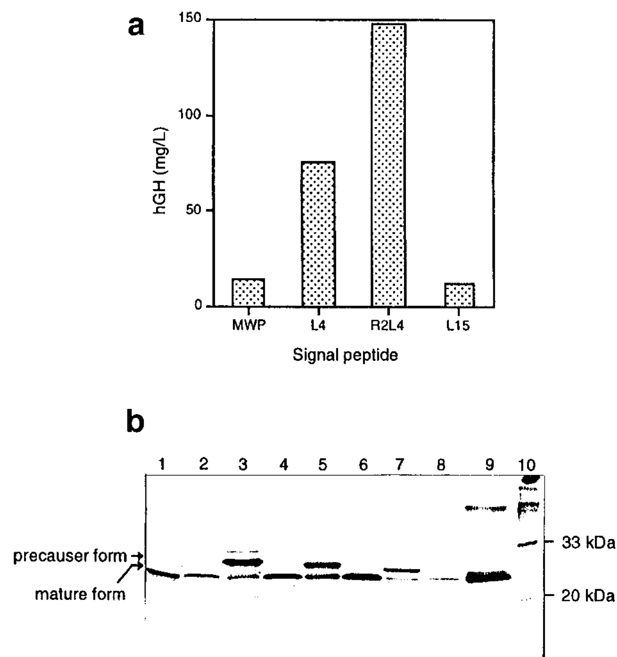


Figure 3 Effect of signal peptide modification on hGH production. *B. brevis* 31-OK carrying expression vectors was grown for 6 days at 30°C in YC medium. The culture broth was centrifuged and the supernatant was used as the extracellular fraction. The cell pellet was suspended in the same volume as the culture broth of 50 mM Tris-HCl (pH 7.5) and then sonicated. The suspension was centrifuged and the supernatant was used as the cellular fraction. (a) Production of hGH. The amount of hGH in the culture supernatant was measured by ELISA. (b) Localization of hGH. One microlitre of both fractions was analyzed by SDS-PAGE and Western analysis was done with anti-hGH serum. Lane 1: authentic hGH (100 ng); lanes 2, 4, 6 and 8: extracellular fraction of *B. brevis* 31-OK carrying pNU211hGH, pNU211L4hGH, pNU211R2L4hGH and pNU211L15hGH, respectively; lanes 3, 5, 7 and 9: cellular fraction of *B. brevis* 31-OK carrying pNU211hGH, pNU211L4hGH, pNU211R2L4hGH and pNU211L15hGH, respectively; lane 10: prestained marker proteins.

peptide with a more basic and more hydrophobic signal peptide. The precursor protein which has the correct N-terminal sequence was detected in the cellular fraction, and the amount of precursor protein was reduced as the amount of mature hGH increased in the culture supernatant, except for *B. brevis* carrying pNU211L15 (Figure 3b). In the cellular fraction of *B. brevis* carrying pNU211L15, a large amount of precursor lacking N-terminal 19 amino acid residues was detected.

Improvement of culture condition

To improve the hGH productivity, culture conditions such as cultivation temperature and concentration of medium components, and the addition of some chemicals in the medium were examined. The 30°C used generally for heterologous protein production in the *B. brevis* system was the best temperature for hGH production as far as tested (data not shown). The production level was increased to 240 mg L^{-1} by changing the concentration of polypeptone P1 in YC medium to 2% instead of 3%. The addition of amphipathic chemicals such as Tween 40 and polyethylene glycol to the medium showed almost no effect (data not shown).

Characterization of the hGH produced by B. brevis

We purified hGH from the culture supernatant of *B. brevis* 31-OK (pNU11R2L4GH) by an efficient method described under Materials and Methods. The N-terminal 12 residues and a C-terminal residue of the purified sample were identical with those of authentic hGH. The hGH fraction was shown to be completely biologically active, as determined by adipogenic activity on 3T3-L1 cells, compared to the authentic hGH (Figure 4).

Discussion

Recombinant hGH is industrially produced and widely used as a potential therapeutic agent against dwarfism, bone fractures, skin burns etc. Although several production procedures are known [1], direct high-level secretion of hGH into the culture medium is not reported, except for the *B. subtilis* system [7]. However, exoprotease of *B. subtilis* is too active to allow industrial production.

Protection against extracellular protease of *B. brevis* was essential for efficient production of mature intact hGH, which is known to have a short half-life (about 30 min in humans). *B. brevis* HPD31, which was useful for the production of many heterologous proteins, has at least two weak extracellular proteases, a 40-kDa and a 48-kDa protease (unpublished data). The 48-kDa protease is a metalloprotease which is inhibited by metal chelators such as EDTA, and it was indicated that the 48-kDa protease is involved in the degradation of secreted hGH. The use of *B. brevis* 31-OK, a 48-kDa protease-deficient mutant of HPD31, led to the accumulation of intact hGH in the culture supernatant. However, a degraded hGH lacking one N-terminal amino acid, phenylalanine, appeared in the culture supernatant of *B. brevis* 31-OK after 3 days of incubation. The extracellular protease activity of *B. brevis* 31-OK was

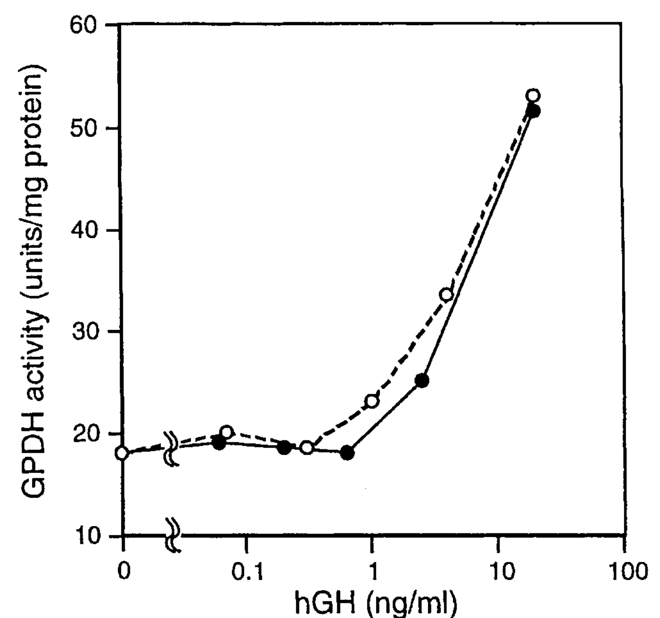


Figure 4 Adipogenic activity of hGH produced by *B. brevis*. The adipogenic activity was determined by the GPDH activity in the cells treated with hGH. Purified hGH fraction from *B. brevis* (●) or authentic hGH (○) was added into the differentiation medium.

inhibited by metal chelators, whereas the divalent cations, especially Mn^{2+} and Fe^{2+}, in the medium were essential for efficient hGH production. For EDTA, we found a proper concentration which satisfied requirements to prevent hGH degradation and to achieve efficient cell growth and hGH production.

Studies concerning the structure and functional relationship of signal peptides have mostly been performed in *E. coli* systems. We report profound effects of signal peptide modification on the efficiency of heterologous protein production in the *B. brevis* system for the first time [9], and report that modified signal peptide, R2L4 signal, achieves the efficient secretion of hGH in this study. But this signal peptide is not so effective for the secretion of a fungal protein disulfide isomerase (unpublished data). It should be noted that signal peptide modifications for improvement of the protein production efficiency appeared to depend on the kind of protein to be secreted. We do not know the mechanism underlying the increased secretion with modified signal peptides, but speculate that higher basicity and hydrophobicity allow the signal peptide to interact with the cellular secretory machinery, so that the modified signal peptide can allow heterologous proteins to be secreted more efficiently.

The intact hGH has two disulfide bonds in the molecule, and the disulfide bond between Cys 53 and Cys 165 is known to be necessary to express full activity [13]. Since all the hGH produced in the *B. brevis* system was fully biologically active, it suggested that the hGH produced, folded correctly and its disulfide bond was formed properly. Disulfide-bond forming enzyme, Bdb, was found on the cell surface of *B. brevis*, and was functionally identical to *E. coli* DsbA [3]. Whether Bdb is essential for disulfide bond formation of proteins in *B. brevis* is not known, but we presume that Bdb plays an important role in the disulfide bond formation of heterologous proteins. Here, we show that the *B. brevis* system has the potential for producing biologically active human proteins directly in the culture supernatant.

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