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Protein secretion in *Bacillus brevis*

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Abstract

Many strains of *Bacillus brevis* were isolated from nature as very efficient producers of extracellular proteins. Strains identified as *B. brevis* including these protein-hyperproducers were reclassified into at least 6 species according to numerical analysis, DNA base composition, and DNA-DNA hybridization.

We developed a host-vector system using appropriate strains of these *Bacillus brevis* as a host, which is excellent for the secretion of heterologous proteins. Utilizing the powerful promoters and signal peptide-coding regions of the cell wall protein gene, various expression-secretion vectors were constructed. The cell wall protein genes of these *B. brevis* are transcribed from multiple and tandemly arranged promoters. Transcription from P2, one of the major promoters among them, was enhanced at the early stationary phase of growth, when divalent cations in the medium was depleted and the cell wall protein layers started to be shed. Translation of the cell wall protein gene transcripts starts at the two sites located tandemly in the same reading frame. The two forms of secretory precursors, translation products from the two sites, are cleaved at the same position giving rise to the same mature proteins. The nucleotide sequence from the promoter to the translation start site is highly conserved in protein-hyperproducing *B. brevis*.

For the efficient secretion of some heterologous proteins, protein-hypersecreting mutants had to be selected. The engineering of the signal peptide was also often necessary to obtain a good secretion of heterologous proteins. Thus, high levels of secretion were achieved only after extensive improvements were made for host, vector and culture conditions. From these experimental results, a great deal of diversity has been observed in various aspects of protein secretion.

Introduction

Protein secretion is a cellular process that comprises a series of reactions with the cellular machinary and is accurately controlled. Since secretion is a process of protein targetting, it intimately related to the components and structural organization of the cells. Therefore, it is natural that a great deal of diversity has been realized in various aspects of protein secretory pathways.

We developed a host-vector system using *Bacillus brevis* as a host, which is excellent for the secretion of heterologous proteins. Utilizing the powerful promoter and signal peptide-coding regions of the cell wall protein gene, we constructed various expression-secretion vectors. Various heterologous proteins were efficiently produced in this system. Bacterial proteins were generally produced in amounts larger than 1 g/liter of culture, as shown in Table 1. Amounts of animal proteins produced were generally one or two order less than those of bacterial proteins with an exception of human epidermal growth factor (EGF) (About 3 g/l), but were much larger than amounts produced in other hosts (Table 1). The *B. brevis* system is prominent in that heterologous proteins are produced in biologically active native form.

In this chapter, we describe the characteristic features of the protein-secretion system with *B. brevis*,

Table 1. Production of heterologous proteins by *Bacillus brevis*.

Protein	Origin	Production (g/l)	Localization
Prokaryotes			
α-Amylase	*Bacillus licheniformis*	3.5	Extracellular
α-Amylase	*B. stearothermophilus*	3	Extracellular
β-Amylase	*Clostridium thermosulfurogenes*	1.6	Extracellular
Cyclodextrin glucosyltransferase	*B. macerans*	1	Extracellular
Sphingomyelinase	*B. cereus*	3	Extracellular
Cholera toxin B subunit	*Vibrio cholerae*	1.4	Extracellular
Xylose (glucose) isomerase	*Thermus thermophilus*	3	Intracellular
Eukaryotes			
Epidermal growth factor	Human	3	Extracellular
Growth hormone	Human	0.2	Extracellular
Growth hormone	Tuna	0.24	Extracellular
Interleukin-2	Human	0.1	Extracellular
α-Amylase	Human, salivary	0.06	Extracellular
Pepsinogen	Human	0.02	Extracellular
Pepsinogen	Swine	0.01	Extracellular
Protein disulfide isomerase	Humicola insolens	0.3	Extracellular
Taka-amylase	*Aspergillus oryzae*	0.02	Extracellular

which has been constructed by us within the last 20 years. Apparently we could observe a good example of the genetic diversity in protein secretion from and related reactions of *B. brevis*.

Properties of the host bacterium

Isolation and classification

As a result of extensive screening, many strains of *Bacillus brevis* were isolated from nature as very efficient producers of extracellular proteins. Since these *B. brevis* strains has the ability to secrete and accumulate proteins in the culture medium to very high levels and shows only a weak extracellular proteinase activity, we developed a host-vector system using this organism for the production of heterologous proteins (Udaka 1976; Takagi et al. 1989).

The heterogeneity of *B. brevis* has been indicated by the wide range of maximum growth temperature, the results of numerical classification, and others. The *B. brevis* strains including protein hyperproducers had guanine-plus-cytosine (G + C) contents ranging from 42 to 53 mol%, although the G + C contents of members of a species should not differ by more than about ± 1 mol%. All these phenotypic heterogeneity of *B. brevis* appeared to be due to variability introduced by the presence of genetically different strains (Takagi et al. 1993). On the other hand, all strains of *B. brevis* tested accumulated one or two major high-molecular-weight proteins in the culture fluid. Extracellular proteins of these strains were shown to cross-react with the antibody against the cell wall protein of *B. brevis* HPD31, except for 2 strains out of 35 strains tested.

Based on the results of numerical analysis, DNA base composition, and DNA-DNA hybridization, the *B. brevis* strains were separated into at least 6 species (Takagi et al. 1993). Figure 1 is the dendrogram showing the phenotypic relationships among the strains studied. Although they were classified to different species, these six species have the specific S-layer (cell wall protein layer) on the cell surface and protein productivity in common. Furthermore, 4 strains from two species were found to have very similar transcription and translation control regions in the 5' upstream of the

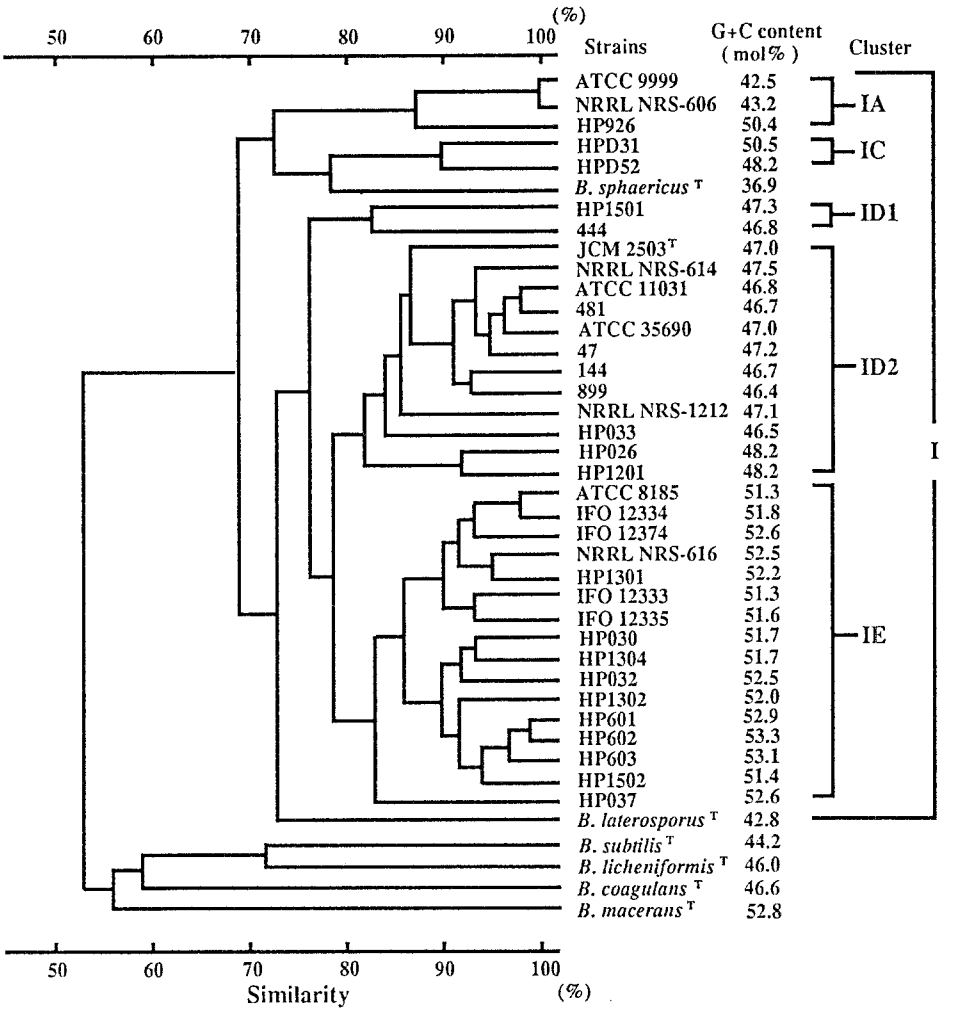

Fig. 1. Dendrogram showing the relationship among the strains of *Bacillus brevis* (only strain numbers are shown) and other *Bacilli* (from Takagi et al. 1993). Similarity among strains was estimated by using the simple matching coefficient of 57 phenotypic characteristics.

cell wall protein gene(s) as described later. Therefore, we proposed to designate them the '*B. brevis* group'.

Secretion of cell wall proteins

B. brevis secretes mainly one or two proteins that were indistinguishable from major protein(s) found in the outer protein layers of the cell wall (Yamada et al. 1981). The major cell wall proteins synthesized at the logarithmic phase form hexagonal arrays on the cell surface (Tsuboi et al. 1982). At the early stationary phase of growth, the protein layers start to be shed concomitantly with a prominent increase in protein secretion. During the stationary phase of growth, cells continue to synthesize and secrete the cell wall proteins, which do not stay on the cell surface but accumulate instead in the medium as extracellular proteins up to 30 g/l. The amount of extracellular proteins reaches more than twice of that of intracellular proteins.

The cell surface structure of the protein producing *B. brevis* is different from those of many Gram-positive bacteria. *B. brevis* has either two or three layered cell wall consisting of one or two protein layers and a thin peptidoglycan layer (Gruber et al. 1988). In the case of two protein layers of *B. brevis* 47, the outer most layer (termed the outer wall) is composed of the outer wall protein (OWP) and the layer between the outer wall layer and the peptidoglycan layer (termed the middle wall) is composed of the middle wall protein (MWP) (Tsuboi et al. 1982). OWP and MWP are serologically different. The antiserum against MWP cross-reacted with the cell wall protein of *B. brevis* HPD31, which has only one protein layer, whereas the antiserum to

OWP did not cross-react with any of the cell wall proteins of strains examined. This indicates that the middle wall layer, the protein layer adjacent to the peptidoglycan layer is well conserved among *B. brevis* strains, but that the OWP may be lost or replaced.

Characteristics of gene expression

Transcription

In *B. brevis* 47, the genes for cell wall proteins, OWP and MWP, constitute a cotranscriptional unit (*cwp* operon) and are transcribed from several tandem promoters (P1, P2, P2*, P3, P4 and P5) located upstream of the *mwp* gene (Adachi et al. 1989, Fig. 2). The nucleotide sequence of this promoter region was conserved in the cell wall protein genes of other three protein-producing *B. brevis*, HPD31, HPD52, and HPO33, with homologies higher than 85%, suggesting that transcription of the cell wall protein genes is regulated through a similar mechanism (Ebisu et al. 1990). The - 10 and - 35 regions of P1, P3, and P4 resemble the consensus sequence recognized by the sigma-A type RNA polymerase of *Bacillus subtilis*. P2 resembles only the consensus sequence in the - 10 region. P5 does not resemble the consensus sequence but the overall sequence around P5 (- 50 to + 8) showed homology to the promoter of the *tycA* gene of *B. brevis* ATCC8185 (Marahiel et al. 1987), the structural gene for tyrocidine synthetase I. Homologous sequences of 8 to 9 bp were found around the transcription start site of P1, P2, and P3 (Fig. 2). Another well characterized *B. brevis* promoter, the promoter of gramicidin S biosynthesis operon (*grs*, Krätzschmar et al. 1989), does not resemble any of the *cwp* promoters.

P1 and P3 were used to the same extents in *B. subtilis* as in *B. brevis*, whereas P2 was used much less frequently in *B. subtilis* than in *B. brevis*. P2 and P3 play major roles in transcription of the *cwp* operon. P3 was used mainly at the exponential phase of growth (Adachi et al. 1989). Transcription from P2 was markedly enhanced at the early stationary phase of growth. $MgCl_2$, when added at 1 to 5 mM to the medium, inhibited this enhancement of transcription. $MgSO_4$ and $CaCl_2$ showed an effect similar to that of $MgCl_2$. Because the addition of Mg^{2+} or Ca^{2+} to the medium also inhibited shedding of the cell wall protein layers at the stationary phase of growth, the coordination in regulation of transcription of the *cwp* operon with the cell wall structure was proposed as follows: when the cell wall protein layers occupy the cell surface, transcription of the *cwp* operon is repressed to a moderate level to supply cell wall proteins of just enough amounts for the growth of cell surface. Loss of the cell wall protein layers from the cell surface at the stationary phase of growth (presumably due to depletion of divalent cations) derepresses the transcription from P2, thus permitting increased synthesis and secretion into the medium of the cell wall proteins (coordinated model; Adachi et al. 1991, Fig. 3). This model is of interest because it involves a pathway that recognizes the situation of the cell surface and transduces a signal to the cytoplasm to repress excess transcription from P2. Such a signal-transducing pathway should play an important role in regulation of the synthesis, transport, and assembly of cell wall proteins in procaryotes.

Translation

Downstream from the multiple and tandem promoters, the *cwp* operon has the two translation initiation sites in the same reading frame. Each of the sites contains an initiation codon, TTG and ATG, and a sequence highly homologous to the 3' end of *B. brevis* 16S rRNA (SD1 and SD2, respectively, Yamagata et al. 1987). The nucleotide sequence of this dual translation initiation sites was perfectly conserved in four protein-producing *B. brevis* strains so far investigated, *B. brevis* 47, HPD31, HPD52, and HPO33, suggesting that the translation as well as transcription of the cell wall protein genes is regulated through the same mechanism in protein-producing *B. brevis* (Ebisu et al. 1990). Use of both translation initiation sites was confirmed by fusing each of the sites to the *B. licheniformis* α-amylase gene (Adachi et al. 1990). The translation frequency from the first site (SD1 and TTG) was estimated to be about one third of that from the second site (SD2 and ATG). Since the TTG codon is used rather often as initiation codon in *Bacillus* but is not in *E. coli*, it might be more suitable to the formation of the translation initiation complex in *B. brevis* than in *E. coli*.

The translation from the second site give rise to a precursor of the cell wall protein with a typical procaryotic signal peptide. The translation product from the first site has an NH_2-terminal extension of 31 amino acid residues preceding the signal peptide. The extension is rich in charged residues.

The two forms of secretary precursors are cleaved at the same position and give rise to mature proteins with the same NH_2-terminus. The cell wall protein gene with the deletion of the first translation initiation

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                          -35                      -10        +1
VEGII       GTCAAAATAATTTTATTGTCAACGTCTTATTAACGTTGATATAATTTGCAAGCTTGCAAA
VEGI/II     TAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTACTGACCTGG
P1          GGAAAAAGGTTCAGTCGTGACAGCCCGCCATATGTCCCCTATAATACGGATTGTGGCGGA
P2          TTTTTTTCGAAGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTCACACGA
P2*         AGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTCACACGAAAAACGGGGA
P3          GAATTGTGTAAAAAAGATTCACGAATTCTAGCAGTTGTGTTACACTAGTGATTGTTGCAT
P4          GATTGTTGCATTTTACACAATACTGAATATACTAGAGATTTTTAACACAAAAAGCGAGGC
P5      ACACAATACTG-AATATACTAGAGATTTTTAACACA---AAAAGCGAGGCTTTCCTGCGAAAGG
          : : ::  :  :::  :::    ::::::  : :    : ::  :::  ::::  :::  ::
tycA    ATCCCTATTTTTTAATCGACTTCCAATTTTT--CTCTGCTATAAT-GAG--TTTCA-GCGTCAGTA
                    -35                      -10        +1
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Fig. 2. Comparison of the sequences of the *cwp* promoters with known promoter sequences. Possible - 35 and - 10 regions are underlined. *veg II* and *veg I/II* are *B. subtilis* promoters (Le Grice & Sonenshein 1982), and *tycA* is a *B. brevis* promoter (Marahiel 1987). Homologous sequences found around transcription initiation sites (+ 1) of P1, P2, and P3 are denoted by asterisks. The P5 and *tycA* sequences are aligned to give maximum matching. Homologous bases are denoted by colons between the sequences (from Adachi et al., 1989)

site tends to be lost from the plasmids, suggesting its deleterious effect on host cells. The first site and the NH_2-terminal extension of the cell wall protein might be important under some specific conditions in nature (Adachi et al. 1990).

Characteristics of protein secretion

Host mutant

Since long time ago, numerous hyperproducing mutants of protein (mostly enzyme) have been isolated, but characterization of their genetic alterations has been done only in a few cases. For example, certain mutation in Deg S and Deg U in *B. subtilis* resulted in an increase of extracellular enzymes such as α-amylase and protease.

In *B. brevis* host-vector system, we use routinely host mutants which show superior productivity of heterologous proteins and higher transformability. These *B. brevis* mutants were selected by a random screening and have been successfully used to produce various proteins from either bacterial or mammalian origins. We do not know the site of the change in these mutants.

In the case of the mutant 47K, which was obtained by screening for hyperproductivity of human α-amylase, the mutation affected the composition of the cell wall and cytoplasmic membrane proteins and the sensitivity toward various antibiotics (Konishi et al.

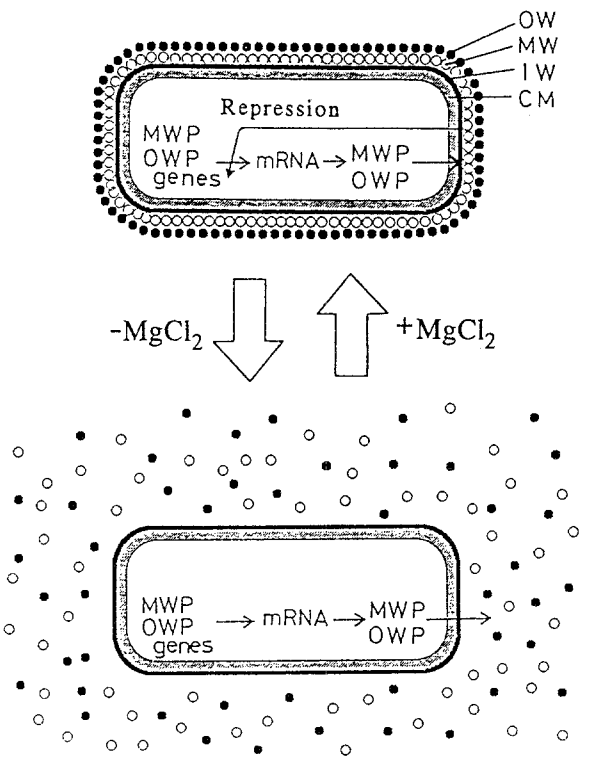

Fig. 3. Coordinated model for the effect of $MgCl_2$ on the cell wall structure and transcription from P2. OW and MW, outer wall and middle wall layers composed of OWP and MWP, respectively. IW, inner wall (peptidoglycan layer); CM, cytoplasmic membrane (from Adachi et al. 1991).

Table 2. Signal peptide sequences from *Bacillus brevis*.

Strain/gene	Sequence[a]	Reference
Strain 47		
Middle wall protein	MKKVVNSVLASALALTVAPMAFA/AEE	Tsuboi et al., 1988
Outer wall protein	MNKKVVLSVLSTTLVASVAASAFA/APK	Tsuboi et al., 1988
Strain HPD31		
Cell wall protein	MKKVVNSVLASALAITVAPMAFA/AED	Ebisu et al. 1990
Protease inhibitor	MKTIRTGMMTLAALAVLGTNVVSA/TSE	Shiga et al. 1992

[a] The cleavage sites are marked by the "/" sign.

1990). Therefore, the mutation probably caused some alterations in the secretory machinery that enhance the protein transport across the membrane.

Vectors

Plasmids pHW1 and pUB110 of *Staphylococcus aureus* origin can be used in *B. brevis*. pHW1 is a low-copy-number plasmid in *B. brevis* and is useful as a cloning vector, especially when products of the cloned gene are deleterious to the host cells. The erythromycin-resistance gene on this plasmid (originally found in pE194) is useful for selection of transformants. pUB110 is a high-copy-number plasmid in *B. brevis*, useful for overproduction of polypeptides from the cloned gene. Expression-secretion vectors such as pNU200 and pNU210 were constructed by using the replication origin of pUB110 and the promoter to the signal peptide regions of the cell wall protein gene of *B. brevis* 47 (Udaka & Yamagata 1993).

Another series of vectors was constructed from cryptic plasmids, pWT481 and pSU926, found in *B. brevis* 481 and 926, respectively. Vectors pHY481 and pHT100 were constructed by using the replication origin of pWT481 and pSU926, respectively. These vectors were very stably maintained in *B. brevis* even in the absence of the selective drug (Yamagata et al. 1985). Human epidermal growth factor was produced in a strikingly large amount (up to 3 g per liter of the culture medium) by using these plasmids as vectors.

Signal peptide

Secretory proteins are characterized to be synthesized as larger precursors with signal peptides at their NH_2-termini. The signal peptides are then cleaved during or immediately after the translocation of the proteins across the cytoplasmic membrane. Amino acid sequences of signal peptides of *B. brevis* proteins are listed in Table 2. The sequences are rather short compared to those of other Gram-positive bacteria (usually 30 amino acid residues), but they have common features of the signal peptide, i.e., the NH_2-terminal basic region, the central hydrophobic region, and the cleavage region. Although no consensus sequence in signal peptides was found, the importance of each region for the function of signal peptide has been well documented. A considerably different signal peptide suitable to each secretory protein and to the membrane of each organism seemed to be evolved. In the case of the secretion of β-amylase from *Clostridium thermosulfurogenes*, MWP (cell wall protein of *B. brevis*) signal peptide directed secretion of the β-amylase in *B. brevis* about 5 fold more efficiently than the *Clostridium* signal peptide of β-amylase (Mizukami et al. 1992).

Folding enzyme

Until very recently, much attention has not been paid to the folding process of the polypeptide after its translocation across the cytoplasmic membrane. Since human proteins such as EGF, which has three disulfide bonds in its molecule, are produced in such large amounts, while only unfolded polypeptide is presumed to pass the membrane, we predicted the presence of some enzymes catalyzing the correct folding of the secreted polypeptide and formation of the disulfide bond in order to form a biologically active native protein. The activity of peptidylprolyl cis-trans isomerase (PPIase), which enhances cis-trans isomerization at the proline residue of the protein, was in fact found in *B. brevis*. A similar amount of the activity was found both in extracellular and intracellular fluid (unpublished results). Furthermore, a disulfide bond forming enzyme (Dsb) activity was detected in the culture supernatant.

The structural gene for Dsb of *B. brevis* was cloned in *Escherichia coli* (unpublished data). The cloned

gene complemented the *dsb A* mutation of *E. coli* on cell motility test. The gene encodes a polypeptide of 118 amino acid residues with a possible signal peptide of 25 amino acid residues at its NH_2-terminus. A sequence characteristic of the disulfide oxidoreductase active site, Cys-Gly-Tyr-Cys, was found in its amino acid sequence.

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