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Bacillus brevis, a Host Bacterium for Efficient Extracellular Production of Useful Proteins

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Introduction

The work described in this review was begun in 1971, when attempts were made to isolate micro-organisms that produce proteins extracellularly in large amounts (Udaka, 1976). The original idea was that among micro-organisms, which have such diverse and extraordinary properties, there must be some that have the ability to secrete large amounts of proteins. Such organisms should be quite useful not only for practical application, such as in the field of food production, but also for basic work to elucidate the mechanism underlying protein secretion. Fortunately, *in vitro* recombinant DNA technology emerged a few years after we had started this work. It was thus quite natural that we tried to utilize this splendid technology to obtain protein-hyperproducers to yield various important proteins. Although it took many years to explore the new technology, we succeeded in constructing an excellent host-vector system for producing foreign proteins, using protein-hyperproducing *Bacillus brevis* as the host.

Needless to say, proteins are the most important constituents of living matter, and many are now manufactured for use as pharmaceuticals and industrial enzymes. Gene technology is already being used to induce synthesis of such proteins by bacteria and yeasts. However, there are many problems with these organisms. Many of the proteins become insoluble and inactive when produced in large amounts in the cytoplasm, and their conversion to active forms requires additional time- and cost-consuming processes, or may not be possible at all (Harris, 1983; Schoemaker, Brasnett and Marston, 1985).

Abbreviations: CM, cytoplasmic membrane; hEGF, human epidermal growth factor; HPI, hexagonally packed intermediate layer; IW, inner wall; MW, middle wall; MWP, middle wall protein; OW, outer wall; OWP, outer wall protein; PEG, polyethylene glycol; PG, peptidoglycan; SL, surface layer; TCA, trichloroacetic acid.

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In contrast, proteins secreted into the culture medium or the periplasmic space tend to be soluble and active. However, the amounts of foreign proteins accumulated in the periplasmic space are usually small, probably because they undergo proteolytic degradation or the space is limited. Thus, secretion into the medium is considered to be the best way of obtaining foreign proteins in both soluble and active forms. Although *Bacillus subtilis* is an efficient extracellular enzyme producer and was expected to be a good protein-secreting host, mammalian proteins produced by this bacterium are rapidly degraded by its own extracellular proteases, even with a *B. subtilis* mutant lacking major proteases (Kawamura and Doi, 1984; Shiroza *et al.*, 1985; Honjo *et al.*, 1986). *Saccharomyces cerevisiae* has also been well studied as to the secretion of human proteins in native forms, but productivity, so far reported, is low (Brake *et al.*, 1984; Smith, Duncan and Moir, 1985).

A protein-producing system involving *B. brevis* overcomes many of these problems, so that the practical application of this system is becoming a reality.

Isolation and characterization of protein-hyperproducers

SCREENING

Initially, five potential protein-producers were discovered by means of a novel screening method (Udaka, 1976). Plates inoculated with many isolates from natural sources were incubated for a few days and then flooded with a protein-denaturing reagent, such as 5% trichloroacetic acid (TCA). Bacteria from colonies which were then surrounded by an opaque area after TCA treatment were selected from replicate plate cultures. The advantage of this method is that a large number of different bacteria can be tested in a simple and rapid manner on agar plates. Many bacteria can be spotted or replicated at one time on several plates using media of various compositions and cultured under different conditions, e.g. at different temperatures. Experiments proved that a more efficient protein-producer gave a denser precipitate beneath the colony on addition of TCA. Autolysis of bacteria usually does not occur under these conditions, so that the formation of an opaque area by acid is a measure of true protein secretion rather than the consequence of cellular lysis. Following primary screening on plates, the bacteria were further selected by assay of extracellular protein in liquid media after culture in shake flasks.

Of 1200 isolates tested, about 15% showed opaque areas with TCA. In general, much more protein secretion was observed when bacteria were cultured in nutrient-rich media than in chemically defined media containing glucose and an inorganic nitrogen source as main components. Among these bacteria, five isolates exhibiting particularly marked responses in this test were found to secrete large amounts of protein (Udaka, 1976).

More recently, a very extensive screening of protein-hyperproducers was carried out by a similar method to that described above (Takagi, Kadowaki and Udaka, 1989). Among 100 000 bacterial isolates from 2000 samples, about 80 isolates showed a productivity of over 1·2 g l^{-1} of protein in liquid culture. Thirty-two of these and the previous five strains produced more than 5 g l^{-1} of

protein when cultured in liquid media of various compositions. Even at this stage of research, we found that some isolates produced 12 g l^{-1} of extracellular proteins.

IDENTIFICATION

Various phenotypic properties of these 37 protein-hyperproducers and the DNA content (mol %) of guanine plus cytosine (G+C content) were examined (unpublished data). Based on these properties, 21 strains were identified as *B. brevis*. Although three out of 21 strains are Gram-negative, almost all other characteristics of the three strains, including spore formation, are the same as those of *B. brevis*. The G+C content in DNA ranges from 46 to 55%, while that of *B. brevis* 8246 (type strain) is 47·4%. Prominent features of these 21 strains of *B. brevis* are that they produce protease extracellularly in insignificant amounts and accumulate one or two high molecular mass proteins (around 100 kDa) in large amounts in the culture medium. These proteins are derived from cell wall proteins which form layers on the bacterial surface (the S-layers) as described in detail later. Strains 47 and HPD31 of *B. brevis* have been used most frequently in the following studies.

Seven isolates were identified as *Bacillus circulans*. This group of bacteria showed a DNA G+C content of 38–43% and had many phenotypic properties in common with *B. brevis*. Another three isolates belonged to *Bacillus* species having about 62% G+C content and properties similar to *B. circulans*. The remaining six isolates were to *Pseudomonas fluorescens* and *P. putida*. Therefore, the majority of the protein-hyperproducing bacteria we isolated belonged to the *Bacillus* species, especially *B. brevis*.

Cultural conditions of *B. brevis*

IN NUTRIENT-RICH MEDIA

B. brevis 47 was used for the detailed studies on cultural conditions for protein production. A large amount of protein can be produced relatively easily in nutrient-rich media. However, the efficiency of protein production varied greatly, depending on the medium composition (Shaku, Koike and Udaka, 1980). *B. brevis* grows well in a medium containing yeast extract or meat extract, but significant amounts of protein are not accumulated. On the other hand, 'Polypepton' (Nihon Pharmaceutical, Tokyo), which is en enzymatic hydrolysate of casein, was found to be a good nutrient, suitable for protein production. 'Polypepton' appeared to contain factors which stimulate protein secretion, since it showed a marked effect on protein production but relatively small effect on growth when added to a medium containing other nutrients. These stimulatory factors seem to consist of polypeptides with molecular masses in the region of a few thousand kDa.

Although optimal cultural conditions depend on the *B. brevis* strains used and the kind of protein (homologous or heterologous) to be secreted, experimental data suggest that media containing 1–5% glucose, 1–3%

'Polypepton', 0–0·2% yeast extract, 0–0·02% $MgCl_2$ are generally satisfactory for the production of bacterial or mammalian protein. In some cases, KH_2PO_4 and/or $CaCl_2$ must be added. Temperature, pH of the medium, and aeration are also important factors.

Glycine and L-isoleucine were found to be prominent among various amino acids tested to enhance protein production by *B. brevis* 47 in nutrient-rich media (Miyashiro *et al.*, 1980a). The simultaneous addition of appropriate amounts of these amino acids approximately doubled the production of proteins, to about 12 g l^{-1} (*Figure 1*). Isoleucine stimulated the synthesis of both extracellular and intracellular proteins, while glycine caused an increase of only extracellular protein production. Under optimal conditions, *B. brevis* HPD31 produced up to 30 g l^{-1} of extracellular proteins.

It may be noted that *B. brevis* cells rarely sporulate in ordinary nutrient medium and spores are not found in the culture during protein production.

IN CHEMICALLY DEFINED MEDIA

A simple chemically defined medium was devised for protein production by *B.*

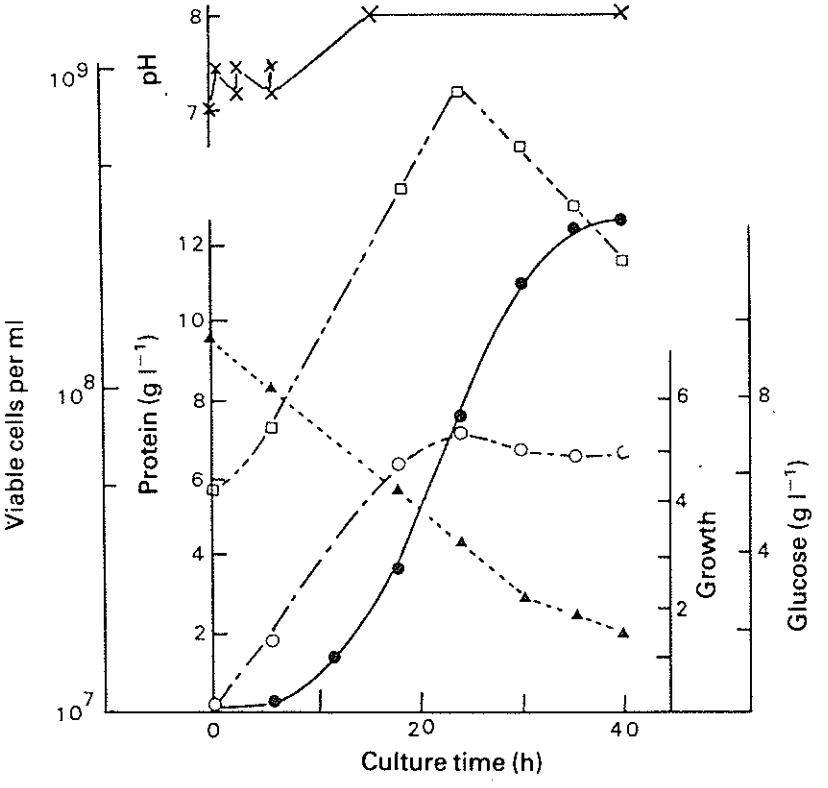

Figure 1. Time course of protein production by *B. brevis* 47 (Miyashiro *et al.*, 1980a). Cultivation was carried out at 34°C on a shaker. Culture medium was a nutrient-rich medium supplemented with 0·25% glycine and 0·5% isoleucine. The pH was controlled by acetic acid. ●, extracellular protein; ○, growth (OD at 660 nm); □, viable cell number; ▲, glucose; ×, pH.

brevis 47 (Tsuchida *et al.*, 1980). About 2 g l^{-1} of proteins were secreted in the synthetic medium, which contained 4% glucose, 1% ammonium sulphate and some inorganic salts including phosphate, Mg^{2+} and Ca^{2+}. An essential component of the medium was Ca^{2+}, which is required by this organism for assimilating glucose.

Protein production was increased still further by addition of certain anti-metabolites to the medium (Miyashiro *et al.*, 1980b). Among various drugs tested, the most effective were those such as bacitracin, D-cycloserine and β-lactam antibiotics, which inhibit synthesis of cell wall components such as peptidoglycans and teichoic acids (murein). When 60 mg l^{-1} of bacitracin was added to the culture medium (50 g glucose/l) at the early logarithmic growth phase, about 9 g l^{-1} of proteins were produced, as compared with 3 g l^{-1} without bacitracin. In this case, total protein production was 12 g l^{-1} (9 g l^{-1} extracellular protein plus 3 g l^{-1} intracellular protein) from 50 g l^{-1} of glucose. Since, generally, the cell yield from glucose (on a dry weight basis and without protein secretion) is known to be at most about 50% and the average protein content of bacterial cells is about 60%, we may expect about 15 g l^{-1} as total protein yield from 50 g l^{-1} glucose. Therefore, the protein yield was calculated to be about 80% on the basis of glucose consumption.

The effect of phosphate on protein production in a synthetic medium was tested (Tsukagoshi *et al.*, 1981). Unexpectedly, the composition of extracellular proteins was altered quantitatively to a considerable extent depending on the concentration of external phosphate. Proteins with smaller molecular masses were accumulated extracellularly in the medium containing less phosphate.

FACTORS TO ENHANCE PROTEIN PRODUCTION

As described above, some drugs, such as inhibitors of murein synthesis, were found to enhance the production of extracellular protein, without having a major action on cell growth. These drugs caused a large decrease in the phosphate content of the murein fraction, suggesting that a decrease in negatively charged phosphate may enhance protein passage through the cell wall (Miyashiro *et al.*, 1980b). Glycine is also known to interfere with peptidoglycan synthesis and may enhance extracellular protein production by mechanisms similar to those of inhibitors of murein synthesis.

Other chemicals found to promote protein production to some extent are surfactants, such as Tween 40 or 60. These drugs may influence the cell membrane, but the mechanism involved is not clear.

Mechanism of protein production

CELL SURFACE STRUCTURE

The cell wall structure of the protein-producing *B. brevis* is quite different from that of most Gram-positive bacteria, in which the cell wall appears as a 15–80

nm thick, fairly homogeneous, electron-dense layer (Sleytr, 1978). Based on the cell wall structure, *B. brevis* strains were classified into two groups (*Figure 2*): Group I with a two-layered cell wall consisting of a middle wall protein layer (MW) and a peptidoglycan layer (PG), as in the case of strains HPD31 and HPD52; Group II with a three-layered cell wall consisting of two protein layers [outer wall (OW) and middle wall (MW)], as well as a peptidoglycan layer, as in strains 47 and HPO33 (Yamada, Tsukagoshi and Udaka, 1981; Gruber *et al.*, 1988). A similar multilayered cell wall structure has been described for non-protein-producing bacteria also, such as *Bacillus* species CIP 76–111, *Bacillus polymyxa* and *Clostridium nigrificans* (Nermut and Murray, 1967; Sleytr, Adam and Klaushofer, 1968; Leduc, Rousseau and Heijenoort, 1977).

B. brevis 47, which has been best characterized among protein-producing *B. brevis* strains, belongs to the second group described above. The outer two protein layers, termed the outer wall (OW) and the middle wall (MW) layers, are composed of proteins with approximate molecular masses of 130 kDa (OWP, outer wall protein) and 150 kDa (MWP, middle wall protein), respectively, as judged by SDS-polyacrylamide gel electrophoresis (Yamada, Tsukagoshi and Udaka, 1981). The OWP and MWP are serologically different (Tsuboi, Tsukagoshi and Udaka, 1982; Ohmizu *et al.*, 1983). The antibody to MWP of strain 47 cross-reacted with cell wall proteins of strains HPD31, HPD52 and HP033, while the antibody to OWP of strain 47 did not cross-react

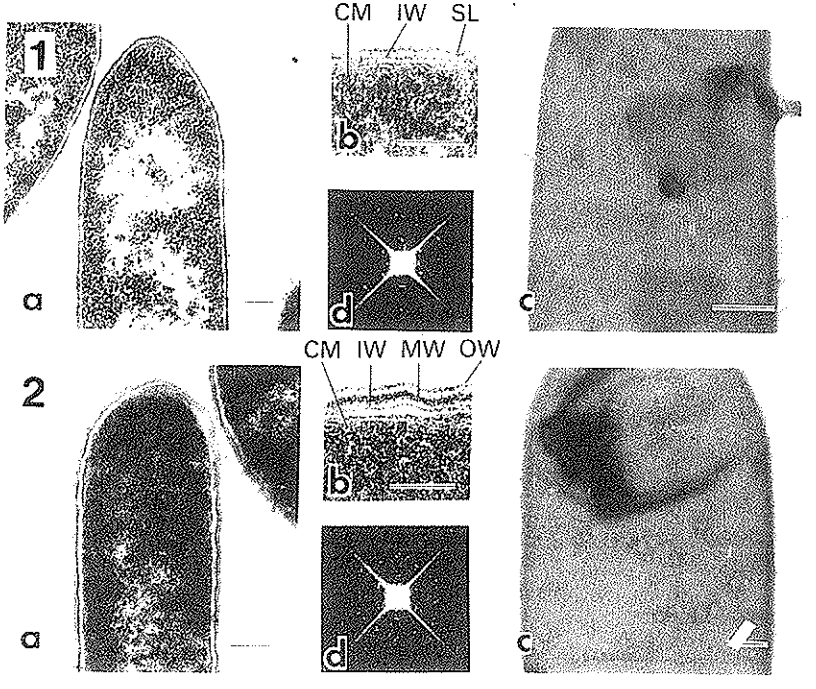

Figure 2. *Bacillus brevis* HPD31 (1) and HP033 (2) in ultrathin sectioned preparations of intact cells (a, b) and negatively stained preparations of cell envelopes (c), with the respective optical diffraction patterns (d) (Gruber *et al.*, 1988). CM, cytoplasmic membrane; PG, peptidoglycan; SL, surface layer; OW, outer wall; MW, middle wall. Bars in (a) and (c) represent 200 nm; bars in (b) represent 50 nm.

with any of the cell wall proteins of strains examined so far. This indicates that the protein of the middle wall layer, the protein layer adjacent to the peptidoglycan layer, appears to be well conserved among protein-producing *B. brevis* strains but that the OWP may be lost or replaced (Gruber *et al.*, 1988).

Protein-producing *B. brevis* displays ordered structures (S-layers) on the cell

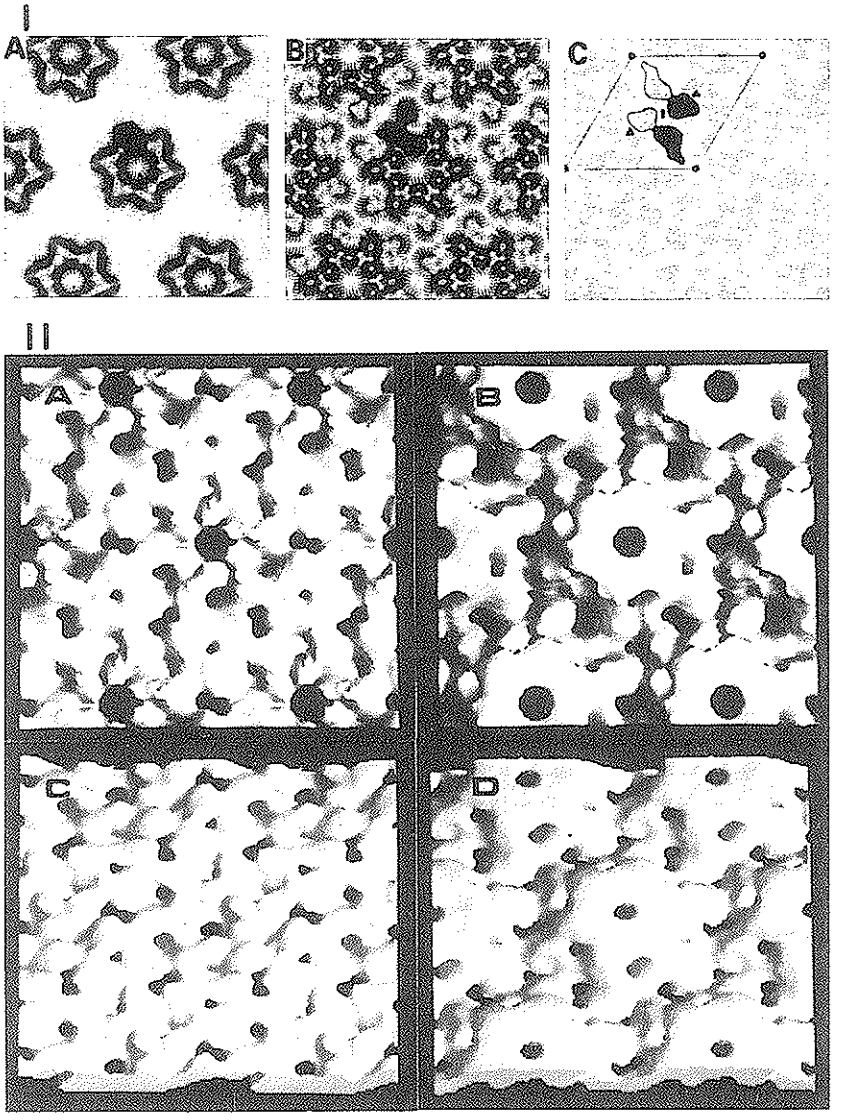

Figure 3. I. Stacked horizontal layers. (A) Inner, (B) central, (C) outer regions of the MW layer. Two putative monomers of neighbouring morphological complexes are marked to illustrate the shapes and the contact regions of the protein domains. The border of the unit cell and the axes of sixfold, threefold and twofold symmetry are marked in (C). II. Computer-generated views of the two surfaces of the three-dimensional reconstruction of the *Bacillus brevis* MW layer. Views of the outer (A, C) and inner (B, D) surfaces are shown. The dimensions of each image = 36 nm × 36 nm.

surface (Yamada, Tsukagoshi and Udaka, 1981; Tsuboi, Tsukagoshi and Udaka, 1982; Tsukagoshi *et al.*, 1982). The S-layers observed in all *B. brevis* strains have a hexagonal array with lattice constants of 14·5–18 nm. Since the discovery of bacterial S-layers in 1953, crystalline surface arrays composed of protein or glycoprotein subunits have been demonstrated in nearly all phylogenetic divisions of the eubacteria and archaebacteria (Sleytr and Messner, 1983). They have been shown to function as protective coats, molecular sieves or promoters for cell adhesion, surface recognition features or frameworks, determining and maintaining cell shape or envelope rigidity. The surface subunits of Gram-positive bacteria predominantly show a tetragonal order, whereas Gram-negative bacteria have mostly hexagonal arrays (Sleytr, 1978). Three-dimensional structures of S-layers have also been demonstrated in a few bacteria (Baumeister and Engelhardt, 1987). In the case of *B. brevis* 47, MWP forms a hexagonal array with a lattice constant of 14·5 nm in the cell wall, though it is at present uncertain whether the OWP forms a regular array. The three-dimensional structure of the MWP (reconstructed from tilted-view electron micrographs after correlation averaging to a resolution of 2 nm) shows a distinct domain structure (*Figure 3*). The heavier domain of six monomers jointly forms a massive core and the lighter domains interconnect adjacent unit cells. Furthermore, the pores of the MWP penetrate through the whole core and appear to determine the porosity of the S-layer. They appear to share some common architectural features with those of other eubacteria (Tsuboi *et al.*, 1989).

In *B. brevis* 47, it was found that the fine cell wall structure is morphologically altered to various extents depending on the growth periods (*Figure 4*). Cells shed the outer two protein layers concomitantly with a prominent increase in protein production (Yamada, Tsukagoshi and Udaka, 1981). Both the 130 kDa and 150 kDa proteins isolated from the culture broth had almost identical amino-acid compositions and, upon limited proteolysis, generated the same peptide fragments as the respective proteins isolated from the cell wall. Furthermore, antisera prepared against the 130 kDa and 150 kDa proteins purified from the culture broth cross-reacted with the respective cell wall proteins. Antisera to the 130 kDa and 150 kDa proteins purified from the cell wall also cross-reacted with the respective extracellular proteins. However, antisera to the 130 kDa protein isolated from both extracellular and cell wall proteins formed no precipitation lines with the 150 kDa protein isolated from the same sources, and vice versa (Tsuboi, Tsukagoshi and Udaka, 1982; Ohmizu *et al.*, 1983). These data proved that the two major extracellular proteins were derived from the respective cell wall proteins.

GENES FOR CELL WALL PROTEINS

Immunological procedures were used to clone the genes coding for the cell wall proteins (MWP and OWP), since no easily measurable biological activities were detected for either protein. Portions of the structural genes for the MWP and OWP were cloned in *E. coli* with pBR322 as a vector (pNT200 designates the plasmid obtained) and were found to direct the synthesis of some

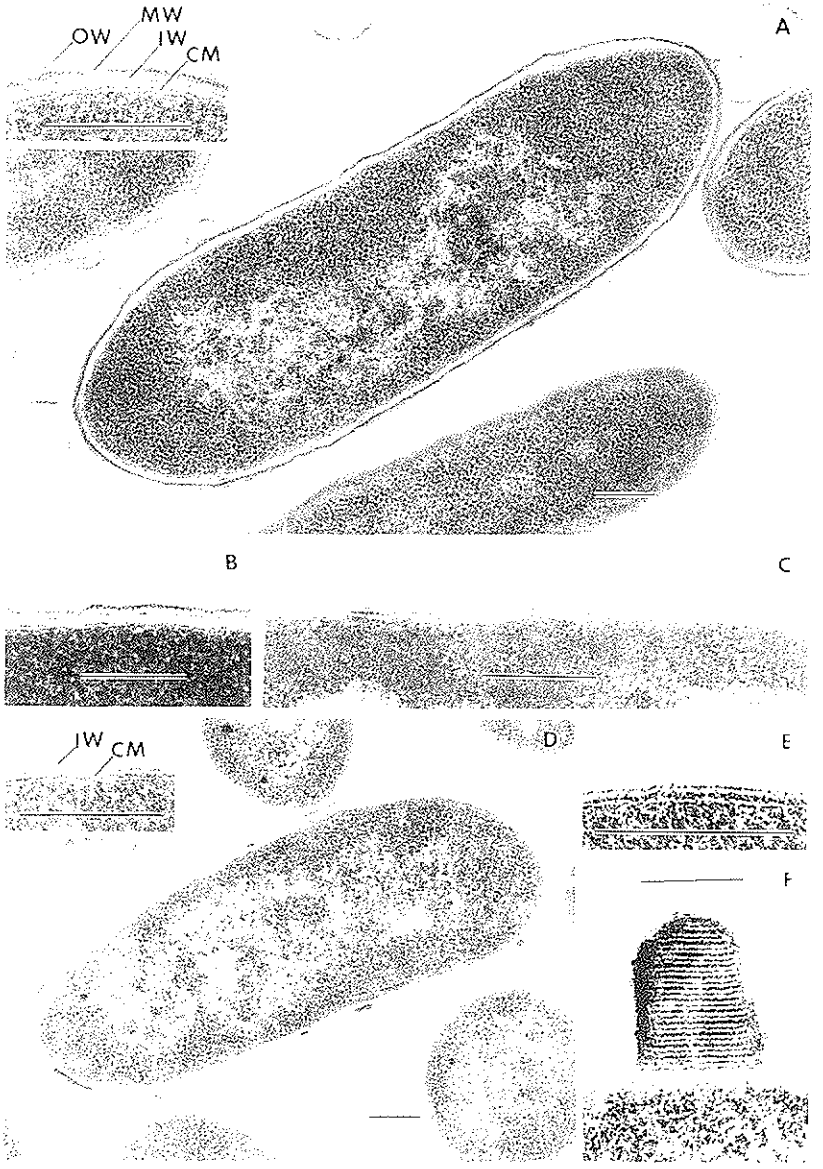

Figure 4. Alterations of fine cell wall structure of *B. brevis* 47–5 during growth (Yamada, Tsukagoshi and Udaka, 1981). At the logarithmic phase, cells were surrounded by a three-layered cell wall (A). A highly magnified picture of the surface (inset) shows clearly three layers above the cytoplasmic membrane (CM). At the early stationary phase, the MW layer changed morphologically to contain spikes (B), and the outer two layers began to be shed (C). At the late stationary phase most of the cells shed the outer two layers and were surrounded only by a thin layer (D). The inset shows only one layer above the CM. After 24 h of growth, shedding of the two layers was complete in most of the cells (E), and cell wall fragments adhering to the surface were seen in some cells (F). Bars represent 200 nm.

polypeptides reactive with anti-MWP serum and others reactive with anti-OWP serum (Tsukagoshi *et al.*, 1984a). pNT200 contained two *Hin*dIII inserts of 1·9 and 0·16 kilobase pairs (kbp). With the cloned DNA fragment as a hybridization probe, the restriction map of the region surrounding the cell wall protein genes on the *B. brevis* 47 genome was constructed (*Figure 5*). This work showed that these genes form a gene cluster, while Northern blot analysis of *B. brevis* 47 RNA indicated that these genes constitute a cotranscriptional unit and that they are transcribed from a promoter(s) located upstream of the MWP gene (Tsuboi *et al.*, 1986). Therefore, the cell wall protein genes of *B. brevis* 47 seem to constitute an operon (*cwp* operon) under co-ordinated control of their expression.

Based on the molecular weights of the cell wall proteins, the 5′ region of the operon, containing transcription and translation initiation sites, was deduced to be in the 3 kbp *Bcl*I–*Bgl*II fragment shown by the solid bar in *Figure 5*. This fragment was cloned into *B. subtilis*, although many efforts to clone the fragment were unsuccessful when *E. coli* was used as the cloning host (Yamagata *et al.*, 1987). Then, the entire nucleotide sequence of both cell wall protein genes was determined (Tsuboi *et al.*, 1986; Yamagata *et al.*, 1987; Tsuboi *et al.*, 1988). MWP and OWP genes were interrupted by an untranslated sequence of 130 bp and consisted of 3090 bp (1030 amino-acid residues with a molecular mass of 114 830 Da) and 2940 bp (980 amino-acid residues with a molecular mass of 103 740 Da), respectively. Both MWP and OWP were synthesized in precursor forms with signal peptides of 23 and 24 amino acids, respectively. When the hydropathy profiles of both proteins were calculated, based on the predicted amino-acid sequences, MWP appears to be surprisingly

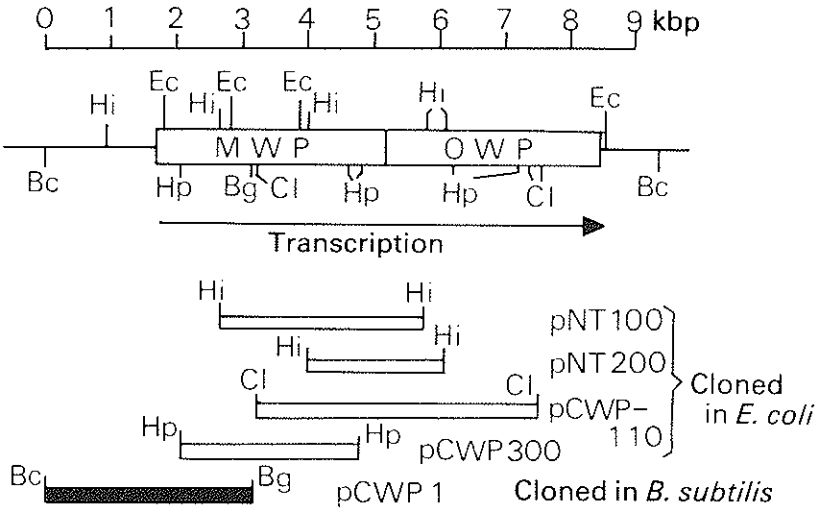

Figure 5. Restriction map of the region around the cell wall protein gene operon of *B. brevis* 47 (Yamagata *et al.*, 1987). Bc, *Bcl*I; Hi, *Hin*dIII; Ec, *Eco*RI; Hp, *Hpa*I; Bg, *Bgl*II; Cl, *Cla*I. The arrow indicates the start point and the direction of transcription, determined by Northern blot analysis of *B. brevis* 47 RNA. In the bottom part, DNA fragments cloned into *E. coli* are denoted by open bars (pNT200 and pCWP110). The DNA fragment containing the 5′ region of the cell wall protein gene operon cloned in *B. subtilis* is denoted by a closed bar.

hydrophilic compared to OWP, except for the NH_2-terminal region. The charged amino-acid residues are distributed rather densely and uniformly in MWP. The predicted amino-acid compositions of MWP and OWP were similar to the chemical amino-acid compositions of other S-layer proteins in the predominance of acidic amino acids compared to basic amino acids and in the very low content of sulphur-containing amino acids.

The nucleotide sequence of the MWP gene, established in strain 47, was found to be well conserved among protein-producing *B. brevis* strains such as HPD31, HPD52 and HP033 (unpublished data). This is consistent with the data that the antibody to MWP of strain 47 cross-reacted with cell wall proteins of strains HPD31, HPD52 and HP033. Although no homologous sequences with the OWP gene were found among cell wall protein genes of protein-producing *B. brevis* examined so far, a statistically significant local homology was observed in the 3′ region between the OWP gene and hexagonally packed intermediate (HPI) layer protein gene of *Deinococcus radiodurans* (Peters *et al.*, 1987).

PROMOTERS

The nucleotide sequence of the 5′ region of the *cwp* operon is shown in *Figure 6*. The positions of the 5′ ends determined by S1 and primer extension analyses are shown. The results of both analyses matched well and suggested the existence of six tandemly arranged promoters within 300 bp upstream from the MWP coding sequence (Yamagata *et al.*, 1987; Adachi *et al.*, 1989).

The putative promoters were designated P1, P2*, P3, P4 and P5 according to the positions of the 5′ ends of the corresponding transcripts. Deletion analysis of the promoter region with the aid of promoter–probe vectors carrying the promoter-less *Bacillus licheniformis* α-amylase (EC 3.2.1.1) gene (Yuuki *et al.*, 1985) showed that P1, P2 and P3 promoters are located within three distinct fragments derived from this region. P2 and P3 promoted efficient synthesis of the α-amylase when they were placed upstream from the α-amylase gene, whereas P1 promoted the synthesis only at a very low level. The use of P2*, P4 and P5 remains uncertain at present (Adachi *et al.*, 1989).

Figure 7 compares the sequences of the *cwp* promoters with known promoter sequences. The P1, P2 and P3 promoters have similar sequences around the transcription initiation site (TACGGATTGT, TAGGATGT and TAGTGATTGT, respectively). The −35 and −10 regions of P1 and P3 resemble the consensus sequence of the *B. subtilis* vegetative promoters recognized by the sigma-43 RNA polymerase (TTGACA in the −35 and TATAAT in the −10 regions, Moran *et al.*, 1982) and the spacer between the −35 and −10 regions corresponds to the preferred internal length (16–18 bp) for *B. subtilis* promoters. Although the P1 promoter (GTGACA in −35 and TATAAT in −10 regions) resembles the consensus sequences most closely of these promoters, it is used much less frequently than P2 and P3 when it is inserted into the promoter–probe vector and analysed for its promoter activity in both *B. brevis* 47 and *B. subtilis*. One possible explanation is that at the first position

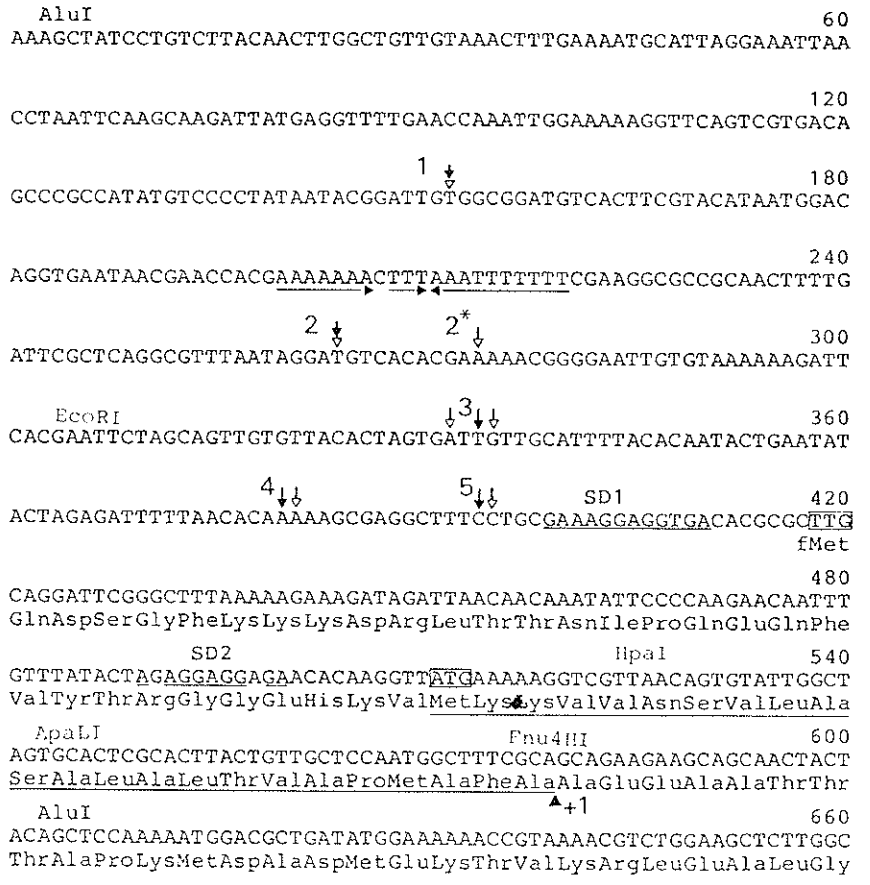

Figure 6. Nucleotide sequence of the 5′ region of the cell wall protein gene operon, and the positions of the 5′ ends of the transcripts (Yamagata *et al.*, 1987; Adachi *et al.*, 1989). Only the sequence of the antisense strand is shown. Vertical arrows numbered 1 through 5, and 2* at the top of the DNA sequence, indicate the positions of the 5′ ends of the transcripts, determined by the S1 nuclease (EC 3.1.30.1) assay (▼) and primer extension assay (▽). Two potential ribosome-binding sites (SD1 and SD2) are underlined below the DNA sequence, and two possible initiation codons are boxed. A 12-nucleotide palindromic sequence is indicated by horizontal arrows below the DNA sequence. The amino-acid sequence deduced from the DNA sequence is numbered from −54, so that the NH_2-terminal Ala of the mature MWP is numbered +1. The cleavage site of the signal sequence (underlined) is indicated by a vertical arrowhead below the amino-acid sequence.

of the −35 sequence, T is important for promoter activity in both bacteria. The lack of PuTPuTG in the −16 region (Henkin and Sonenshein, 1987) or A + T-rich sequence upstream from the −35 region (Moran *et al.*, 1982) might be other reasons for the low activity of P1.

The P3 promoter resembles the *B. subtilis* vegetative promoters not only in the −35 and −10 regions but also in the −16 region and in A-rich sequences located upstream of the −35 region. P3 promoter expressed the α-amylase gene efficiently in *B. subtilis* as well as in *B. brevis*. These results suggests that P3 is recognized in *B. brevis* by an RNA polymerase (EC 2.7.7.6) similar to sigma-43 RNA polymerase of *B. subtilis*.

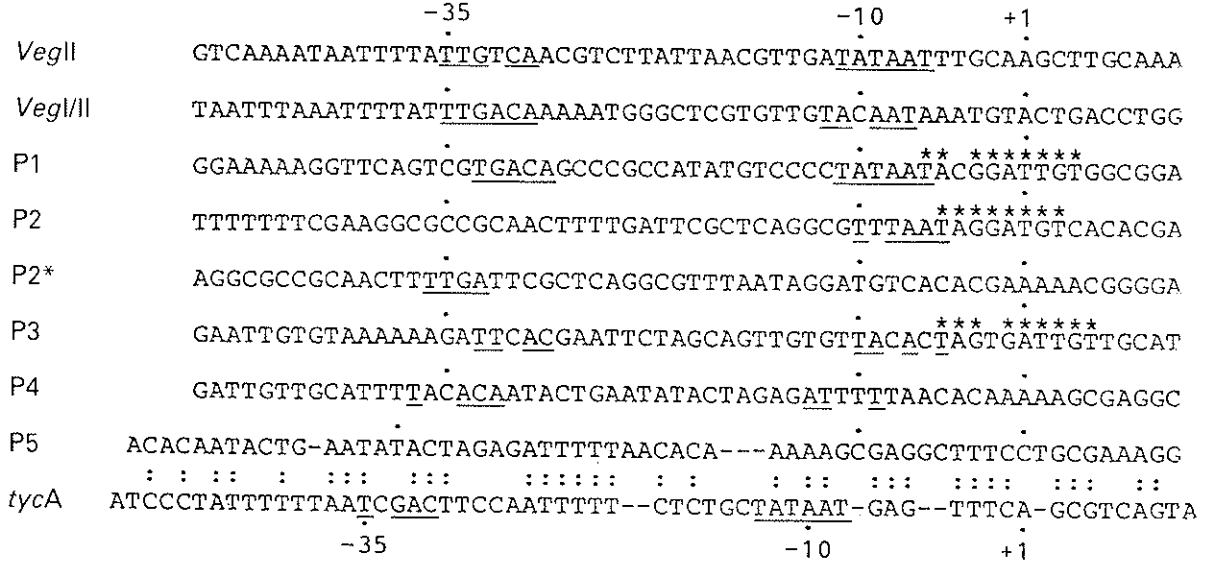

Figure 7. Comparison of the sequences of the *cwp* promoters with known promoter sequences. The sequences between −50 and +10 are presented. Possible −35 and −10 regions are underlined. *vegII* and *vegI/II* are *B. subtilis* promoters (LeGrice and Sonenshein, 1982), and *tycA* is a *B. brevis* promoter (Marahiel *et al.*, 1987). Homologous sequences found around the transcription initiation sites 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.

The P2 promoter led to the very efficient and constitutive expression of the α-amylase gene even in the stationary phase of growth in *B. brevis* (Adachi *et al.*, 1989), suggesting that it plays a major role in the extracellular production of cell wall proteins during the stationary phase of growth (Miyashiro *et al.*, 1980a). In contrast with the frequent use in *B. brevis*, P2 was infrequently used in *B. subtilis* (Adachi *et al.*, 1989). P2 is not homologous with the consensus sequence for sigma-43 type RNA polymerase nor with sequences of any other known promoters in the −35 region, although it shows homology with the consensus sequence in the −10 region. From these features, P2 is considered to be distinct from the *B. subtilis* vegetative promoters and to be a unique promoter used preferentially for the production of extracellular proteins in *B. brevis*.

A homology search between the *cwp* promoters and the *tyc*A promoter of *B. brevis* (Marahiel *et al.*, 1987) showed homologous sequences around the putative P5 promoter (*Figure 7*). From the intensities of the corresponding bands in the S1 nuclease and primer extension assays, the P5 promoter appeared to be a strong promoter that was utilized at all growth phases. So far, attempts to localize this promoter by deletion analysis have been unsuccessful, thus the nature of the P5 is not clear at present.

Tandem or overlapping promoters have been found in several genes of *B. subtilis* (Johnson, Moran and Losick, 1983; Tatti and Moran, 1985; Igo *et al.*, 1987; Wang and Doi, 1987). Several genes expressed over relatively long periods are transcribed by more than one form of RNA polymerase (EC 2.7.7.6) which initiate transcription from either tandem or overlapping promoters (Johnson, Moran and Losick, 1983; Wong, Shnepf and Whiteley, 1983). The sigma-37 RNA polymerase in *B. subtilis* is used under nutritional conditions that inhibit the activity of the tricarboxylic acid cycle (Igo *et al.*, 1987). In the *cwp* operon, one possible sigma-37 type promoter-like sequence was found just downstream of the P2 promoter (AGGATGTCA in the −35 region, nucleotides 260–268; GGAATTGTGT in the −10 region, nucleotides 281–290, *Figure 6*). Analysis of the transcripts under different culture conditions should provide further information about the function of this unique and very complex promoter region. The promoters in cell wall protein genes of *B. brevis* HPD31, HPD52 and HP033 were found to be almost identical to that of strain 47 (unpublished data).

A large fraction (up to 50%) of pulse-labelled RNA in *B. brevis* 47 was found to be polyadenylated RNA (Hussain, Tsukagoshi and Udaka, 1982). An interesting possibility that some of the polyadenylated RNA is involved in the synthesis of extracellular protein in this organism has not yet been examined.

TRANSLATION AND SECRETION SIGNALS

The 5′ region of the *cwp* operon contains two possible translation start sites located tandemly in the same reading frame. The first site (the one located upstream) contains a possible ribosome-binding site designated SD1 (nucleotides 399–410, *Figure 6*) and an initiation codon TTG (nucleotides 418–420). TTG is known to be used as an initiation codon in *Bacillus* species

(Ramakrishna, Dubnau and Smith, 1984; Mezes, Blacher and Lampen, 1985). The second site also contains a ribosomal-binding site, designated SD2 (nucleotides 490–500) and an initiation codon ATG (nucleotides 511–513). SD1 has 12 bases and SD2 has nine bases complementary to the 3′ ends of *B. brevis* and *B. subtilis* 16S rRNA (McLaughlin, Murray and Rabinowitz, 1981; Kop *et al.*, 1984). The amino-acid sequence from Met at −23, the NH_2-terminus of the translational products from the second initiation site, to Ala at −1 shows characteristics of signal peptides of secretory precursors (Inouye and Halegoua, 1980) and is highly homologous with the putative signal peptide of the outer cell wall protein (OWP) of *B. brevis* 47 (Tsuboi *et al.*, 1988). The amino-acid sequence from Ala at +1 to Ala at +9 is in agreement with the NH_2-terminal amino-acid sequence of MWP determined chemically (Tsuboi *et al.*, 1988). A plasmid carrying the MWP-α-amylase fusion gene with a deletion of the first translation start site directed the efficient α-amylase synthesis (Yamagata *et al.*, 1987). A similar deletion analysis showed that the 5′ region of the MWP gene with a deletion of the second translation start site could promote the α-amylase synthesis (unpublished data). Although both of the two possible translation start sites are utilized in *B. brevis*, the translation appears to start at the second site much more frequently than at the first site. The translational products from the first site contain an extra peptide of 31 amino-acid residues preceding a typical MWP signal sequence. The extra peptide is rich in charged amino-acid residues (seven positively and four negatively charged residues). Short open reading frames that could encode oligopeptides preceding the structural gene have been found and were assumed to play a role in the efficient translation of the downstream gene (Green *et al.*, 1985). The first translational start site of the MWP gene may also facilitate efficient translation by increasing the ribosomal initiation rate.

SCHEME OF PROTEIN PRODUCTION

As described on p. 120, the proteins secreted by *B. brevis* 47 are derived from the respective cell wall proteins. At the stationary phase of growth, *B. brevis* 47 cells undergo a remarkable morphological change, in which the OWP and MWP layers are shed from the bacterial surface. The morphological change occurs concomitantly with a prominent increase in protein secretion (Yamada, Tsukagoshi and Udaka, 1981). The synthesis and secretion of the cell wall proteins continue for a long period after the morphological change. As a result, the amount of extracellular proteins reaches 12 g l^{-1} under optimal conditions, which is more than twice in amount of total cellular proteins (Miyashiro *et al.*, 1980a).

Shedding of cell wall protein layers is inhibited by the addition of divalent cations such as Mg^{2+} and Ca^{2+} into the medium. Under such growth conditions *B. brevis* 47 does not accumulate proteins in the medium. A non-protein-producing mutant, *B. brevis* 47-5-25, does not show any morphological change of the cell surface even at the late stationary phase of growth (Yamada, Tsukagoshi and Udaka, 1981). The expression of the *B. stearothermophilus* α-amylase gene cloned in *B. brevis* 47 was inhibited by the addition of large

amounts of Mg^{2+} into the medium (Tsukagoshi *et al.*, 1985). The expression of the *B. licheniformis* α-amylase gene placed downstream of the P2 promoter was also inhibited by the addition of divalent cations (unpublished data). The P2 promoter is one of the major promoters of the *cwp* operon and is considered to be responsible for the constitutive synthesis of the cell wall proteins at the stationary phase of growth (see p. 126).

These results suggest that the regulation of synthesis and secretion of cell wall proteins is somehow linked with structural changes at the cell surface. At the exponential phase of growth, or in the presence of excess divalent cations, cell wall proteins form regular hexagonal arrays on the cell surface. This cell surface structure might affect cellular processes regulating gene expression and protein secretion, and so lead to repression of the *cwp* operon. Loss of cell wall protein layers from the cell surface at the stationary phase of growth and in the absence of divalent cations, may inhibit this regulation, thus permitting continuous synthesis and loss of cell wall proteins into the medium (*Figure 8*). The cause of shedding of cell wall proteins might be the depletion of divalent cations at the end of the logarithmic phase, but the exact mechanism remains to be clarified.

Cell wall proteins are probably secreted by the same process known in other bacteria, as assumed by the presence of signal peptide in the cell wall protein precursor. The composition of extracellular proteins is entirely different from that of intracellular proteins. Only a small portion of pre-labelled cellular proteins and nucleic acids is released into the culture medium during the production of extracellular proteins, suggesting that protein production by *B. brevis* is not due to cellular lysis (unpublished data). Approximately 10% of the total lipid was released into the medium, regardless of the cultural conditions under which various amounts of protein were produced; i.e. protein secretion occurs independently of lipid release (Tsukagoshi *et al.*, 1983).

Gene technology

TRANSFORMATION

It was essential to establish a method of transformation, especially by plasmid DNA, for performing gene manipulation in *B. brevis*. Protoplast transformation has been successful in many bacteria, such as *Bacillus subtilis* (Chang and Cohen, 1979) and *Bacillus stearothermophilus* (Imanaka *et al.*, 1982), in the presence of polyethylene glycol. However, this technique could not be applied to *B. brevis* 47 because of the extreme difficulty in regenerating its protoplasts. Therefore, we developed a new method for transforming *B. brevis* 47 by plasmid DNA, which involves treatment of *B. brevis* cells with Tris-hydrochloride buffer of alkaline pH followed by induction of DNA-uptake with polyethylene glycol. Rationale of the method is as follows: As described earlier, *B. brevis* 47 has two protein layers, an outer wall (OW) and a middle wall (MW), outside a thin peptidoglycan layer on the cell surface. The two protein layers can be removed by incubation with Tris-hydrochloride buffer

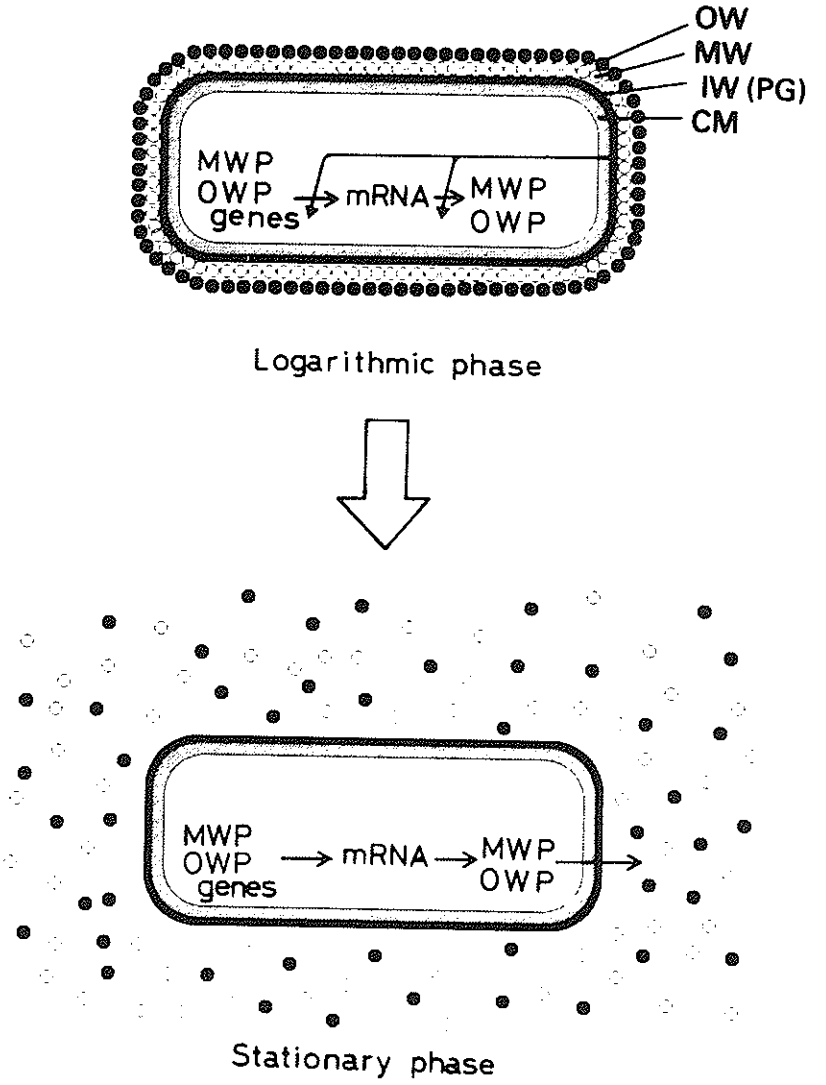

Figure 8. Schema for the coupling of cell wall protein synthesis and secretion with the alteration of the cell wall structure.

(pH 7·5–8·5). The resultant cells, designated 'stripped cells' are surrounded only by a thin peptidoglycan layer and a cytoplasmic membrane. Since the peptidoglycan layer is very thin, DNA-uptake can be induced with polyethylene glycol in stripped cells as in the case of protoplasts. Furthermore, existence of the peptidoglycan layer should facilitate the regeneration of cells after polyethylene glycol treatment (*Figure 9*).

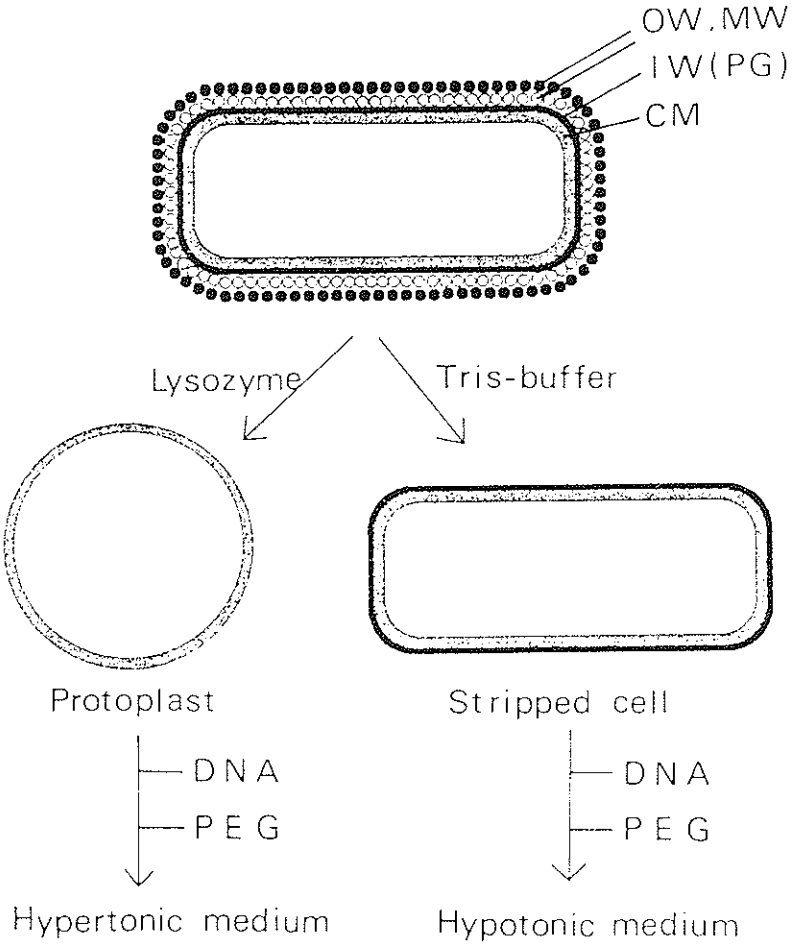

Figure 9. Schema for transformation of *B. brevis* (Tris-PEG method).

To develop the method of plasmid transformation, the following several critical problems must generally be solved:

1. Is the plasmid DNA incorporated into the cells?
2. Can the vector plasmid replicate in the new host cells?
3. Is the gene used for selection of transformants expressed?
4. Can transformants regenerate?

To circumvent the above problems 2–4, we first tried to transfect *B. brevis* 47 with DNA prepared from bacteriophage C7 which was isolated by us from soil using *B. brevis* 47 as a host. Under certain conditions, C7 DNA was incorporated into *B. brevis* stripped cells in the presence of polyethylene glycol and gave rise to infective phage particles. We next tried, under the same conditions as above, to transform *B. brevis* 47 by various plasmid DNAs.

We found that plasmids pHW1 (Horinouchi and Weisblum, 1982) and

pUB110 (Gryczan, Contente and Dubnau, 1978) of *Staphylococcus aureus* origin can be used to transform *B. brevis* 47 to erythromycin and neomycin resistance, respectively (Takahashi *et al.*, 1983). The experimental conditions of this method were optimized and the frequency of transformation was improved to obtain 10^5–10^6 transformants per 1 μg of plasmid DNA. Other *B. brevis* strains, such as HPD31 (Takagi, Kadowaki and Udaka, 1989) could be transformed by essentially the same method with some modification. Stripped cells regenerate very rapidly and form colonies within 1–2 days even on hypotonic media. This method may also be useful to transform other micro-organisms in which the cell surface structure is similar to that of *B. brevis* (Heierson *et al.*, 1987).

The frequency of transformation depends on the strains and plasmids. In some cases, we were unable to obtain transformants. Recently, we found that 'electroporation' can be utilized to transform *B. brevis* having one cell wall protein layer, if the conditions are appropriate (unpublished data).

VECTORS

As described above, plasmids pHW1 and pUB110 can be used for transformation of *B. brevis* 47. 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 host cells. pHW1 has also been used for cloning the MWP and OWP genes of *B. brevis* 47 into *B. subtilis* (Tsuboi *et al.*, 1986, 1988; Yamagata *et al.*, 1987). The erythromycin-resistance gene on this plasmid [originally found in pE194 (Horinouchi and Weisblum, 1982)] is useful for selection of transformants because almost no spontaneous erythromycin-resistant mutants appear under the standard transformation conditions. pHW1B, pHW1E and pRU100 were derived from pHW1. A unique *Hin*dIII site of pHW1, which is convenient as a cloning site, was used to introduce *Bam*HI and *Eco*RI sites in pHW1B and pHW1E, respectively. An *Eco*RI–*Hin*dIII fragment containing a multi-cloning site derived from mp19 was inserted between *Eco*RI and *Pvu*II sites of pHW1E to construct pRU100.

pUB110 is a high-copy-number plasmid in *B. brevis*, useful for overproduction of polypeptides from the cloned gene. The neomycin-resistance gene on this plasmid can be used as a selective marker for transformation. However, *B. brevis* 47 spontaneously gives rise to mutants resistant to this drug at a relatively high frequency, so that care must be taken to distinguish transformants from the spontaneous resistant mutants. On the other hand, neomycin-resistant *B. brevis* HPD31 did not occur spontaneously under the conditions employed. pHT1 was constructed by combining the replication origin of pUB110 and the erythromycin-resistance gene of pHW1 (unpublished data).

Another series of vectors was constructed from a low-copy-number cryptic plasmid, pWT481, found in *B. brevis* 481 (Yamagata *et al.*, 1984). pHY481 was constructed by inserting a fragment containing the erythromycin-resistance gene derived from pHW1 into one of the three *Hin*dIII sites of pWT481. pHY481 is very stably maintained in *B. brevis* 47 even in the absence of the selective drug. Its copy number could be amplified by isolating *B. brevis* 47

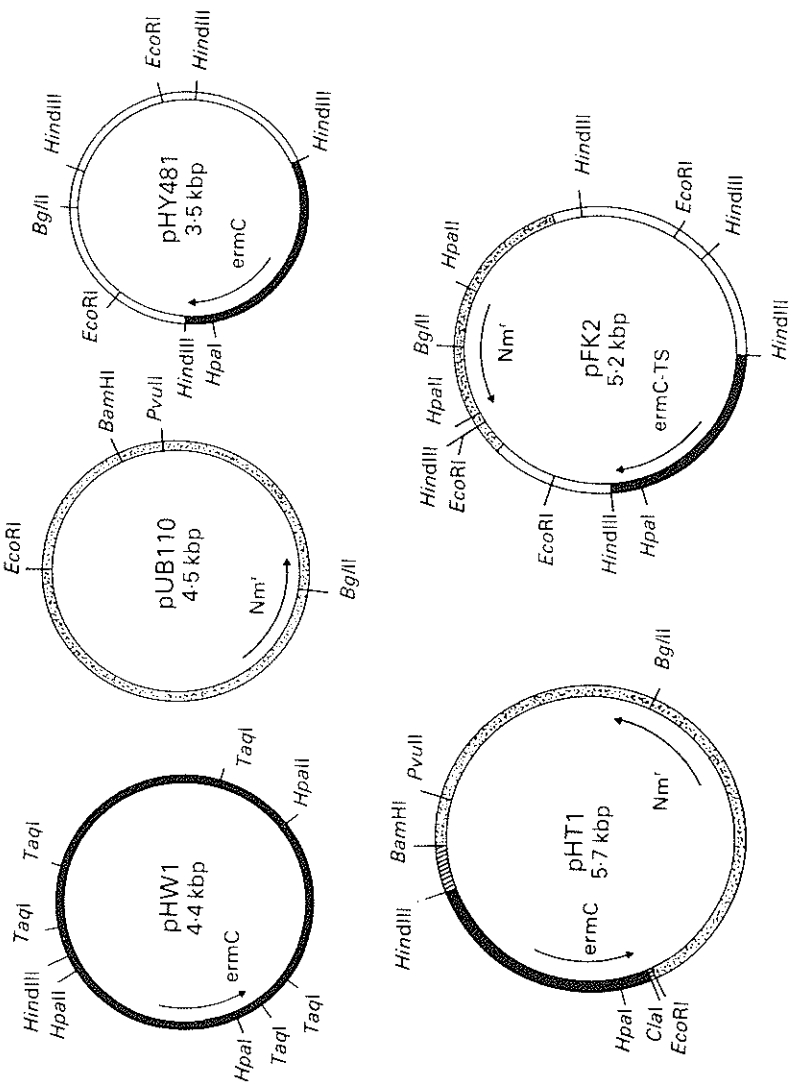

Figure 10. Structures of the several plasmid vectors used in *B. brevis*.

mutants (Yamagata *et al.*, 1985). pFK2 was constructed by inserting a fragment containing a neomycin-resistance gene derived from pUB110 between the *Hpa*II sites of pHY481, and pFK2copT is a mutant plasmid obtained from pFK2. The copy-number of pFK2copT can be amplified severalfold by raising the temperature of the culture medium (unpublished data).

The structures of several of the plasmids described in this section are shown in *Figure 10.*

HOST BACTERIA

Since protein-producing *B. brevis* has the ability to produce extracellular proteins in amounts not found, as yet, for other micro-organisms, we examined the efficiency of the production of a foreign protein, a thermophilic α-amylase, in strain 47. The α-amylase gene of *B. stearothermophilus* was cloned in *E. coli*, using pBR322 (pHI301), and sequenced (Tsukagoshi *et al.*, 1984b; Ihara *et al.*, 1985). The entire α-amylase gene, containing its promoter, SD (ribosome binding site) and the signal peptide sequences on pHI301, was subcloned into pUB110 (pBAM101), which was then introduced to both *B. subtilis* 1A289 (*amy*$^-$) and *B. brevis* 47 (Tsukagoshi *et al.*, 1985).

The α-amylase synthesized in *E. coli* (pHI301), *B. subtilis* (pBAM101) and *B. brevis* 47 (pBAM101) had the same NH_2-terminal amino-acid sequences and thermal stability as that produced by the donor strain. Both *B. subtilis* and *B. brevis* carrying pBAM101 produced significantly large amounts of α-amylase in medium supplemented with glucose as a carbon source. The average enzyme production in *B. subtilis* (pBAM101) and *B. brevis* 47 (pBAM101) was approximately 0·1 and 0·5 g l^{-1}, respectively. Compared with glucose, other carbon sources such as starch, maltose, mannitol, lactose and fructose were less effective for α-amylase production in both hosts. *B. brevis* 47 (pBAM101) always produced three to five times more α-amylase than *B. subtilis* (pBAM101). *E. coli* (pHI301) and *B. stearothermophilus* produced even less, approximately 0·03 and 0·005 g l^{-1}, respectively. Since the activity of the same promoter may differ depending on the bacterial cells used, it may not be valid directly to compare enzyme production in *B. stearothermophilus* with that in other hosts carrying the enzyme gene on plasmids. Yet *B. brevis* 47 (pBAM101) produced the largest amoun: of the enzyme, i.e. 100, 15 and 5 times more than *B. stearothermophilus*, *E. coli* (pHI301) and *B. subtilis* (pBAM101), respectively. Furthermore, the other protein-oriducing strain, HPD31, carrying pBAM101 also produced strikingly large amounts of the enzyme, 3 g l^{-1} (Takagi *et al.*, 1989). These results prove that protein-producing *B. brevis* strains are extremely efficient for foreign protein production. This is further confirmed below.

From many experiments, it has been realized that mutants of *B. brevis* may be selected and used for the efficient production of foreign proteins.

CONSTRUCTION OF EXPRESSION AND SECRETION VECTORS

Since the cell wall proteins are synthesized and secreted into the medium very

efficiently, even during the stationary phase of growth, in *B. brevis* 47, the 5′ region of the *cwp* operon should greatly facilitate the expression of downstream heterologous genes and secretion of the gene products. A 600 bp *Alu*I–*Alu*I fragment (nucleotides 5–604, *Figure 6*) containing all the tandem promoters, dual translation initiation sites and the MWP signal peptide-coding region was isolated and used to construct expression and secretion vectors. The 600 bp fragment was inserted between the *Hpa*II sites of pFK2copT and between *Bam*HI and *Bgl*II sites of pHT1 with the aid of synthetic oligonucleotide linkers, so as to create a unique *Bam*HI site immediately downstream from the insert (pHY500 and pNU100).

One of the *Eco*RI sites, the one originally contained in pHT1, was eliminated from pNU100 to obtain pNU200 by partial cleavage with *Eco*RI, followed by treatment with a Klenow fragment of DNA polymerase I (EC 2.7.7.7) and T4 ligase (EC 6.5.1.1). The structures of the expression–secretion vectors thus constructed, pHY500 and pNU200, are shown in *Figure 11*. The *Bam*HI site on both plasmids is convenient for the insertion of foreign genes to construct transcriptional fusion with the *cwp* operon or translational fusion with the 5′ terminal portion of the MWP gene. In the latter case, the gene product will be a fused protein of the MWP signal peptide, the NH_2-terminal portion (nine amino-acid residues) of mature MWP and the foreign protein. Provided that the fusion protein does not have any structures that interfere with its translocation across the cytoplasmic membrane, the gene products should be efficiently secreted into the medium after cleavage of the MWP signal peptide.

An *Apa*LI site located within the MWP signal peptide-coding region is also unique on both pHY500 and pNU200 and useful to construct transcriptional or translational fusion of the MWP gene with foreign genes. By inserting the appropriate synthetic DNA encoding the COOH-terminal portion of the MWP signal peptide between the *Apa*LI site and the foreign gene, the foreign proteins directly fused with the MWP signal peptide can be synthesized and processed efficiently, resulting in accumulation in the medium of the foreign proteins with no additional amino-acid residues at their NH_2-termini. A *Fnu*4HI site located at the cleavage site of the MWP signal sequence can also be used for the production of foreign proteins with the correct NH_2-terminus, although it is not unique on both plasmids.

Foreign proteins thus secreted into the medium mostly retain their natural conformation and biological activities, in contrast with those synthesized in the microbial cytoplasm, which are often insoluble and denatured (Smith, Duncan and Moir, 1985).

Production of heterologous proteins

BACTERIAL PROTEINS

B. licheniformis α-amylase gene was cloned in *E. coli* and sequenced (Yuuki *et al.*, 1985). The gene comprised the coding sequences of a signal peptide of 29 amino acids and the mature enzyme of 483 amino acids. A plasmid, pHY483,

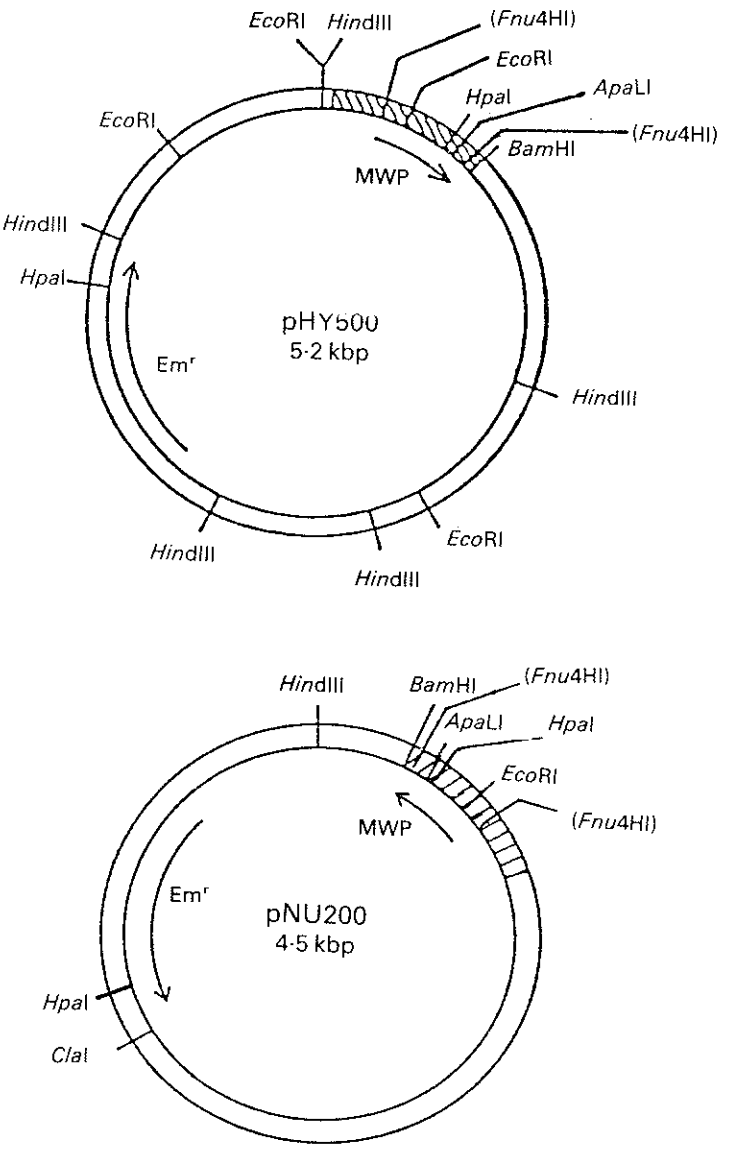

Figure 11. Structures of the expression–secretion vectors, pHY500 and pNU200. Hatched areas indicate the 5′ region of the *cwp* operon, including the multiple promoters and the signal peptide-coding region. As for *Fnu*4HI sites, only those located in the 5′ region are shown.

was constructed by subcloning the entire gene to pHY481 described already. The promoter region of *cwp* operon, 600 bp *Alu*I–*Alu*I fragment shown in *Figure 6*, was inserted to the signal sequence region of the α-amylase gene (pHY4831). pHY4831 carries the multiple promoter region, signal sequence and the coding sequence for the nine NH_2-terminal amino acids of MWP, joined through a linker DNA to the coding sequence for the α-amylase, starting

from amino-acid residue −3 (Ala). *B. brevis* 47 (pHY4831) produced 50 times more extracellular α-amylase (0·25 g l^{-1}) than did *B. brevis* 47 (pHY483) under the same cultivation conditions (Yamagata *et al.*, 1987). Further increase in the enzyme production (0·7 g l^{-1}) was attained by introducing pHY4831 into *B. brevis* 47 cop 11, a mutant where the copy number of the plasmid increases about tenfold (Yamagata *et al.*, 1985).

Similar attempts with an expression–secretion vector such as pNU200 have been made with other enzyme genes, such as cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) of *Bacillus macerans*, thermophilic β-amylase (EC 3.2.1.2) of *Clostridium thermosulfurogenes* (Kitamoto *et al.*, 1988), β-amylase of *Bacillus polymyxa* (Kawazu *et al.*, 1987; Uozumi *et al.*, 1989) and β-lactamase (EC 3.5.2.6) encoded on pBR322. In all cases, a large amount of each enzyme (0·3–1 g l^{-1}) was produced under appropriate culture conditions when *B. brevis* 47 or HPD31 was used as the host. The amount of enzymes produced by *B. brevis* carrying the genes under the control of the *cwp* promoter was at least ten times larger than that produced by *B. brevis* carrying the genes under the control of their own promoters (foreign to *B. brevis*).

MAMMALIAN PROTEINS

Human epidermal growth factor

Human epidermal growth factor (hEGF) is a polypeptide comprised of 53 amino acids with three intramolecular disulphide linkages and is identical with human urogastrone (Gregory, 1975). It is a potent inhibitor of gastric acid secretion and a mitogen for a number of cell types in culture or *in vivo* (Carpenter and Cohen, 1979). Since it is expected to be an important pharmaceutical and its production in other host–vector systems has been low, we investigated its production in our *B. brevis* system (Yamagata *et al.*, 1989).

A synthetic hEGF gene was inserted into the *Fnu*4HI site at the cleavage site of the MWP signal sequence on pNU200, so that the fused gene encodes exactly the same amino-acid sequence as that of mature hEGF directly following the MWP signal peptide (pNU200EGF) (*Figure 12*). *B. brevis* HPD31 (pNU200EGF) in 5YC medium produced as much as 240 mg l^{-1} of hEGF (determined by radioimmunoassay) under optimal conditions. hEGF synthesized in *B. brevis* was one of the major extracellular proteins, with the same apparent molecular weight as that of authentic hEGF, as shown by both Coomassie Brilliant Blue staining and immunoblot analysis after SDS-polyacrylamide gel electrophoresis (*Figure 13*). From the intensity of the bands in *Figure 13*, 1 μl of the culture supernatant was estimated to contain more than 0·2 μg of hEGF, which was in agreement with the result obtained by radioimmunoassay described above. Almost no intracellular hEGF was found, indicating that hEGF synthesized in *B. brevis* was very efficiently secreted into the medium. *Figure 14* shows the time course of hEGF production by *B. brevis* HPD31 (pNU200EGF). The amount of hEGF in the culture medium increased markedly from the early stationary phase of growth. After 3 days, it remained

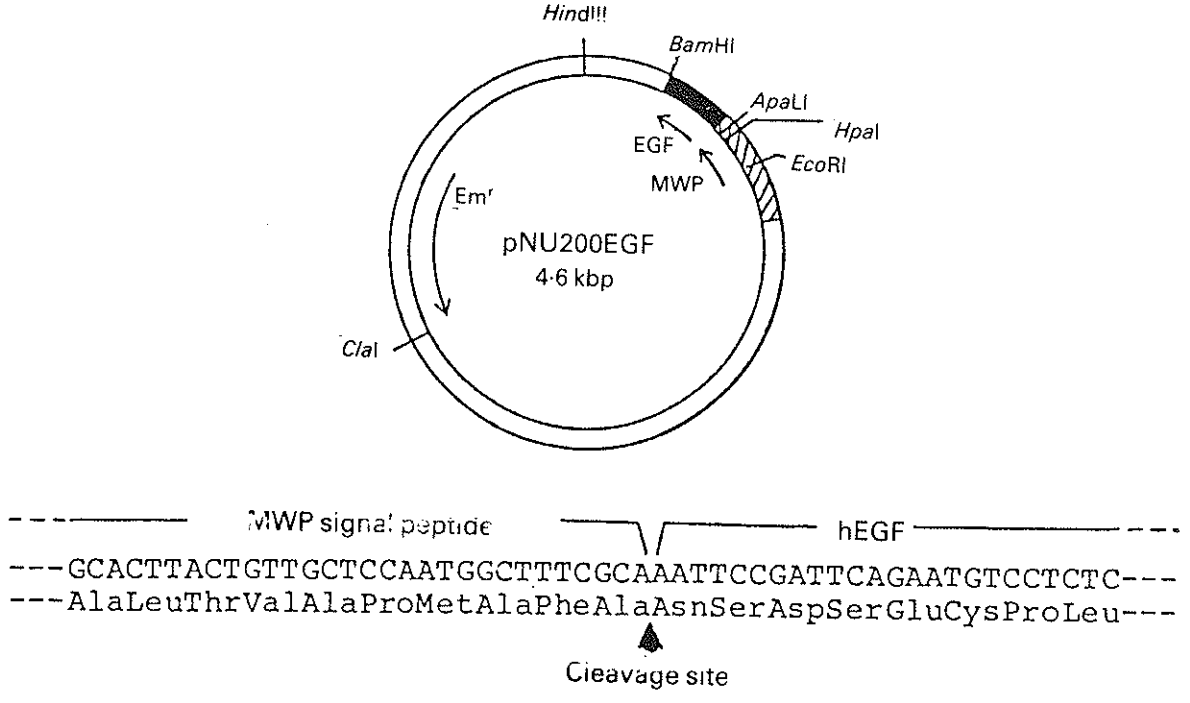

Figure 12. Structure of pNU200EGF. The hatched bar and closed bar indicate the 5′ region of the *cwp* operon and the synthetic hEGF gene, respectively. The nucleotide and amino-acid sequences of the junction region of the fused gene are shown at the bottom. The cleavage site of the MWP signal peptide is shown by a vertical arrow below the amino-acid sequence.

almost constant, suggesting that hEGF accumulated in the medium did not undergo proteolytic degradation.

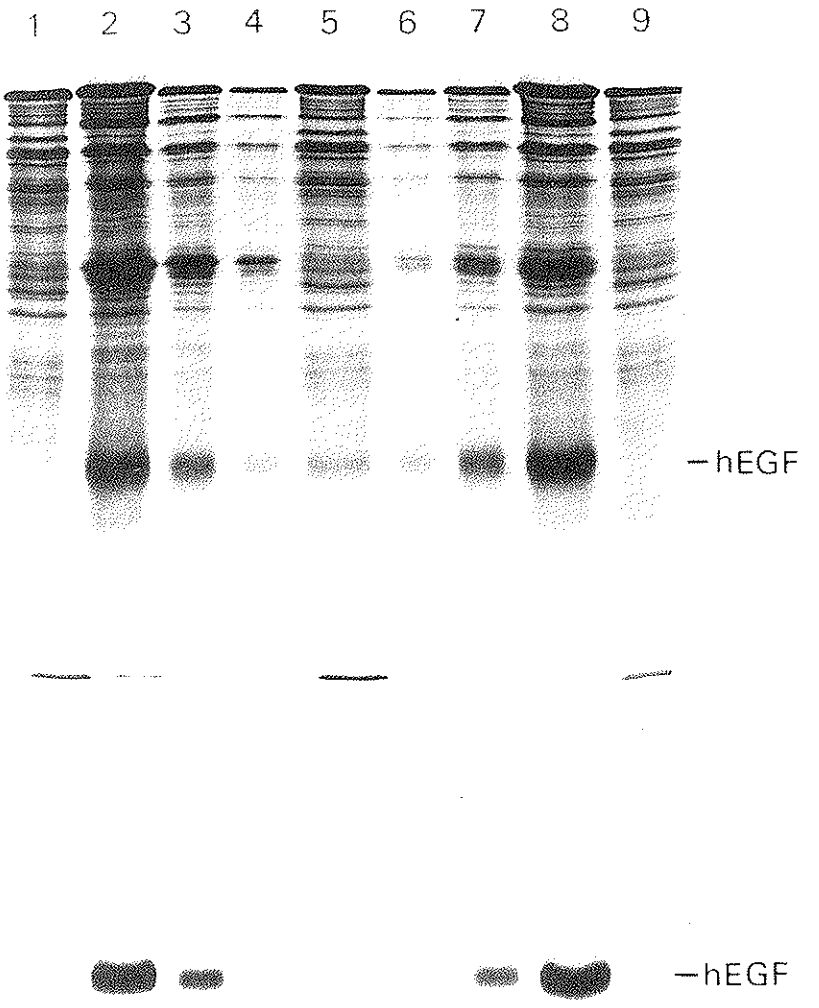

Figure 13. SDS-polyacrylamide gel electrophoresis and immunoblot analyses of hEGF secreted by *B. brevis* (Yamagata *et al.*, 1989). *B. brevis* HPD31 and that carrying pNU200EGF were grown for 4 days at 33°C in 5YC medium (Takagi *et al.*, 1989b) with or without supplements as described below. Culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue (upper part) or immunoblot analysis with anti-hEGF serum (lower part). Lanes 1 and 9, 7·5 μl of the culture supernatants obtained from *B. brevis* HPD31 grown in 5YC medium and that with 0·01% Tween 40, respectively. Lanes 2–4, 7·5 μl, 2·5 μl and 0·8 μl, respectively, of the culture supernatant obtained from *B. brevis* HPD31 (pNU200EGF) grown in 5YC medium plus 0·01% Tween 40. Lane 5, as lane 1 except that 500 ng of authentic hEGF was added. Lanes 6–8, 0·8 μl, 2·5 μl and 7·5 μl, respectively, of the culture supernatant obtained from *B. brevis* HPD31 (pNU200EGF) grown in 5YC medium plus 0·3% glycine.

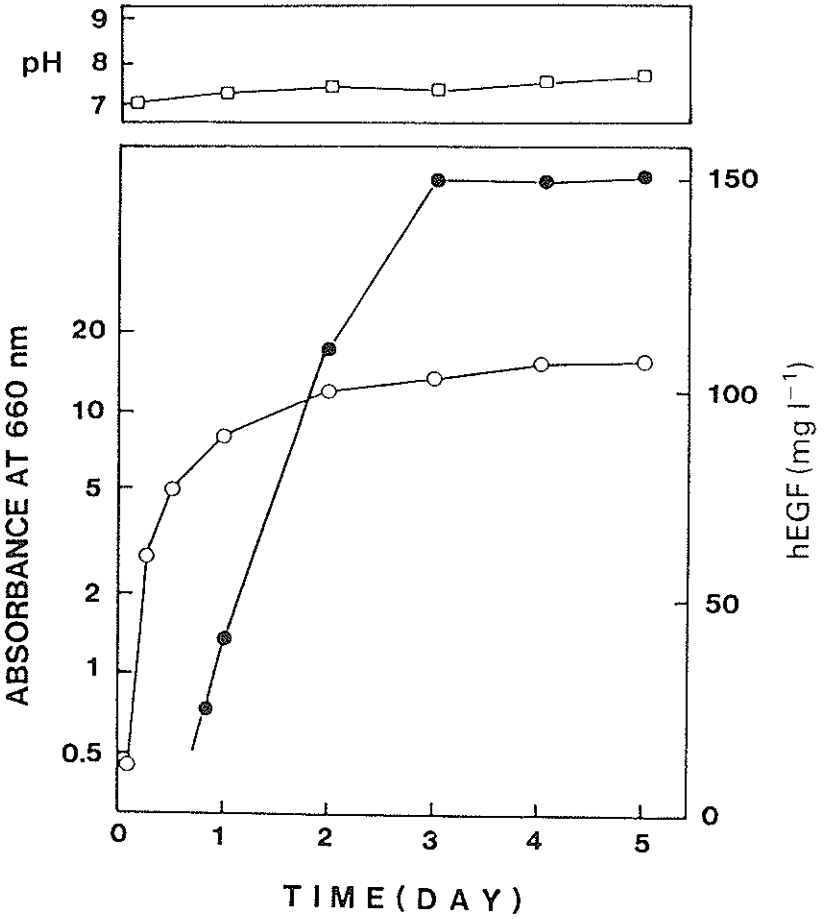

Figure 14. Time course of hEGF production (Yamagata *et al.*, 1989). *B. brevis* HPD31 (pNU200EGF) was grown with shaking in 5YC medium supplemented with 0·3% glycine at 37°C. The amount of extracellular hEGF was determined periodically by radioimmunoassay (●). Cell growth (absorbance at 660 nm, ○) and the pH of the medium were also monitored.

hEGF synthesized in *B. brevis* was purified and analysed for its structure and biological activities. The nine NH_2-terminal amino-acid residues and the amino-acid composition of the purified hEGF (except Cys, which was not determined) matched those of authentic hEGF. Both hEGFs had the same COOH-terminal amino acid, Arg. Even under conditions where disulphide linkages were not reduced, both hEGFs migrated to the same position on SDS-polyacrylamide gel electrophoresis. Furthermore, both hEGFs showed the same activities when their growth-inhibitory effect on human squamous cell carcinomas and stimulatory effect on the initiation of murine fibroblast cell division were examined.

These results indicate that hEGF synthesized in *B. brevis* is correctly processed and secreted efficiently into the medium, forming the correct disulphide linkages and retaining full biological activities. The amount of

secreted hEGF, 240 mg l^{-1}, is about 100 times greater than the amounts observed in other host–vector systems (Brake *et al.*, 1984; Oka *et al.*, 1985).

Human α-amylase

α-Amylase is one of the major secretory products of the pancreas and salivary gland in man, playing a role in digestion of starch and glycogen. cDNA for human salivary α-amylase has been cloned and sequenced (Nishide *et al.*, 1986). The α-amylase consists of 511 amino-acid residues, including 11 Cys residues, with a molecular mass of about 56 000 Da. Since it is a relatively large protein, its synthesis and secretion in microbial hosts may be difficult, compared to small proteins such as EGF. The expression of cDNA for the human salivary α-amylase has been examined in *Saccharomyces cerevisiae* and *B. subtilis*, but the amount of the α-amylase secreted has been low, 0·4 mg l^{-1} and none, respectively (Himeno, Imanaka and Aiba, 1986; Nakamura *et al.*, 1986).

We directly fused the MWP signal peptide-coding sequence and the mature α-amylase-coding sequence, taking advantage of the *Fnu*4HI site and the *Ban*II site located, respectively, at the signal peptide cleavage sites of the MWP gene and the α-amylase gene. The fused gene was inserted into the *Pvu*II site of pHW1 (pHAMY5). pHAMY5 was then introduced into *B. brevis* 47. *B. brevis* 47 (pHAMY5) secreted only a small amount (about 1 mg l^{-1}) of the enzyme, but a host mutant (*B. brevis* 47K) secreting increased amounts of the enzyme was isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. This organism, *B. brevis* 47K (pHAMY5), secreted 6 mg l^{-1} of the enzyme. Further increase in the enzyme production (40 mg l^{-1}) was attained when pHAMY5 was inserted into a multicopy vector, pNU200, and introduced into *B. brevis* 47K (unpublished data). Although this amount is about 6 times less than that of hEGF produced in *B. brevis*, it is 100 times more than that produced in other systems (Nakamura *et al.*, 1986). The elucidation of the mechanism underlying the low efficiency of human α-amylase production as compared to that of hEGF production, and the isolation and analysis of supersecreting mutants of *B. brevis* should facilitate the further improvement of this host–vector system in the production of human proteins.

Swine pepsinogen

The full-length cDNA of swine pepsinogen A, the major component of swine pepsinogens, was isolated from the swine gastric mucosa cDNA library and sequenced (Tsukagoshi *et al.*, 1988). *E. coli* carrying pAS5, which contains the *lac* promoter and the coding sequence for the four NH_2-terminal amino-acids of β-galactosidase (EC 3.2.1.23) joined to the activation peptide segment starting from amino-acid residue +5 (Pro), produced intracellular pepsinogen as so-called inclusion bodies. After appropriate renaturation steps, the pepsinogen was isolated from the *E. coli* strain. The pepsinogen cDNA fragment on pAS5 was subcloned to the expression–secretion vector pNU100 (pSS100). pSS100 carried the coding sequence for the nine NH_2-terminal

amino acids of mature MWP, joined through a linker DNA to pepsinogen cDNA (Takao *et al.*, 1989).

Protein which cross-reacted with the antibody to swine pepsinogen was secreted by this *B. brevis* 47 (pSS100) strain and was found exclusively in the extracellular fraction. Furthermore, the culture broth exhibited pepsin (EC 3.4.23.1) activity, which was completely inhibited by pepstatin, a specific inhibitor of pepsin. We further determined whether or not the pepsinogen produced by *B. brevis* 47 (pSS100) could be converted autocatalytically to pepsin. Under acidic conditions a new band appeared at the same position as that of pepsin produced on activation of authentic swine pepsinogen (*Figure 15*). The rate of the activation process was the same as that in the case of authentic pepsinogen. All these data clearly show that *B. brevis* 47 (pSS100) produced extracellular pepsinogen with the correct conformation. Since the productivity of pepsinogen in *B. brevis* 47 (pSS100) was rather low, approximately 1 mg l^{-1}, pSS100 was introduced into strain HPD31. *B. brevis* HPD31 (pSS100) produced the enzyme efficiently, approximately 11 mg l^{-1}, under the appropriate growth conditions. The enzyme produced by *B. brevis* remained stable on cultivation for a long period, up to 40 h, since *B. brevis* produces no detectable extracellular protease, as described above.

Other proteins

We have been trying to obtain the production by a *B. brevis* system of some mammalian proteins (interleukin-2, etc.) other than those described above. A

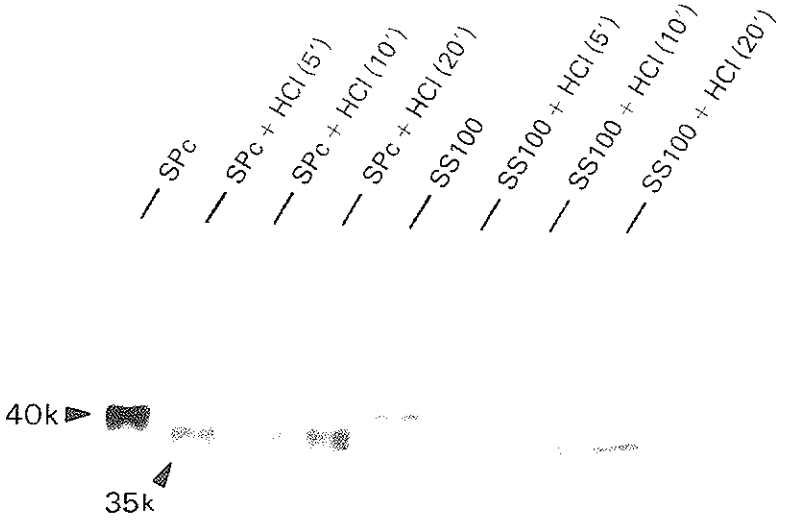

Figure 15. Immunoblot analysis, during the activation process, of pepsinogen (SS100) produced by *B. brevis* 47 (pSS100) (Takao *et al.*, 1989). An enzyme solution was incubated at pH 2 and 12°C for 0 min, 5 min, 10 min and 20 min. Each lane contained protein equivalent to a 1·0 ml culture. Authentic swine pepsinogen, SPc, (0·5 μg protein in each lane) was also incubated under the same conditions. The 40K and 35K protein bands correspond to pepsinogen and pepsin bands, respectively.

level of production of 1 mg l^{-1} is not so difficult to obtain, provided that the target protein is of a secretory nature. As shown in preceding sections, combined improvements in the expression system, plasmid structure, bacterial host and culture conditions were necessary to achieve the maximum production of a particular protein, especially mammalian proteins.

Proteins produced by *B. brevis* remained mostly stable, even on prolonged cultivation. This is quite different from *B. subtilis*, which has also been widely examined as a host to produce foreign proteins. In most cases only small quantities of full-size foreign proteins were detected extracellularly upon prolonged cultivation of *B. subtilis*, since *B. subtilis* produces various proteases.

Conclusion

Using *B. brevis* strains that secrete large amounts of proteins into the medium but hardly any proteases, we have developed a novel host–vector system for the very efficient synthesis and secretion of foreign proteins. The multiple promoters and the signal peptide-coding region of the gene encoding one of the major cell wall proteins of *B. brevis* 47 were used to construct expression–secretion vectors.

With this system, the genes for both bacterial and mammalian secretory proteins are expressed efficiently, and large amounts of structurally correct and biologically active proteins are secreted into the medium. Mammalian proteins can be produced in active forms 10–100 times more efficiently in *B. brevis* than has been reported with other systems.

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