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Biochemistry

Use of *Bacillus brevis* for efficient synthesis and secretion of human epidermal growth factor

(heterologous gene expression system)

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ABSTRACT **Using previously isolated *Bacillus brevis* strains that secrete large amounts of proteins but little protease into the medium, we have developed a host–vector system for very efficient synthesis and secretion of heterologous proteins. The multiple promoters and the signal-peptide-coding region of the MWP gene, a structural gene for one of the major cell wall proteins of *B. brevis* strain 47, were used to construct expression–secretion vectors. With this system, a synthetic gene for human epidermal growth factor (hEGF) was expressed efficiently and a large amount (0.24 g per liter of culture) of mature hEGF was secreted into the medium. hEGF purified from the culture supernatant had the same NH_2-terminal amino acid sequence, COOH-terminal amino acid, and amino acid composition as natural hEGF, and it was fully active in biological assays. These results, in combination with previous results, showed that mammalian proteins can be produced in active form 10–100 times more efficiently in *B. brevis* than has been reported in other systems.**

One of the outstanding achievements of current biotechnology is the production of human proteins such as epidermal growth factor (1–3), lymphokines (4, 5), thrombolytic agents (6, 7), and other plasma proteins (8). However, many of these proteins become insoluble and inactive when produced in large amounts in the cytoplasm of microorganisms (9–12), and their conversion to the active form requires additional time and expense or may be impossible. In contrast, proteins secreted into the medium or the periplasmic space tend to remain soluble and active. However, the amounts of foreign proteins accumulated in the periplasmic space are usually small (13, 14), probably because they suffer proteolytic degradation by periplasmic proteases (15) or their accumulation within the limited periplasmic space is deleterious for the host cells. Accordingly, secretion into the medium is considered to be the favored means to obtain useful proteins in future industrial fermentation processes (16). *Bacillus subtilis* has been well studied as a candidate for use as a protein-secreting host. However, mammalian proteins produced by this organism are rapidly degraded by extracellular proteases (17), even with a mutant lacking major proteases (18). *Saccharomyces cerevisiae* has also been widely studied for secretion of human proteins in native form (2, 16, 19, 20), but productivity has been low.

In previous work, strains of another *Bacillus* species, *B. brevis*, that secrete large amounts of protein but little or no detectable protease were isolated from soil. Among them, *B. brevis* 47 and HPD31, used in this work, accumulate 12–25 g of extracellular proteins per liter of medium under appropriate conditions (refs. 21 and 22; H. Takagi, A. Miyauchi, S. Kagiyama, K. Kadowaki, and S.U., unpublished data). In the case of *B. brevis* 47, the protein secreted consisted mainly of two proteins that were indistinguishable from two major proteins found in the outer two protein layers of the cell wall. The major cell wall proteins synthesized at the logarithmic phase form hexagonal arrays on the cell surface, and the protein layers are shed concomitantly with a prominent increase in protein secretion. At the stationary phase, cells continue to synthesize and secrete the surface proteins, which do not stay on the cell surface but accumulate instead in the medium as extracellular proteins (23, 24). The entire set of genes coding for the cell wall proteins was cloned, and an operon of cell wall protein genes was found (25, 26).

In this paper, we describe the development of a host–vector system in which these protein-hyperproducing *B. brevis* strains are used as host bacteria and the 5′ region of a cell wall protein gene is utilized to construct expression–secretion vectors. We have used this system to produce large amounts of biologically active human epidermal growth factor (hEGF).

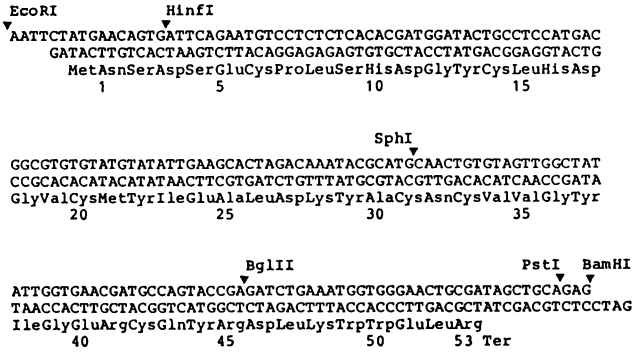

FIG. 1. The nucleotide sequence of the synthetic hEGF gene carried by pTB361 and the amino acid sequence of the polypeptide encoded by the synthetic gene. The NH_2-terminal amino acid of mature hEGF, asparagine, is numbered 1. Ter, termination.

MATERIALS AND METHODS

Bacteria, Plasmids, and Media. *B. brevis* 47 and HPD31 were isolated by Udaka (21) and Takagi *et al.* (27), respectively. Plasmid pTB361 (pBR322 with an inserted synthetic hEGF gene, Fig. 1) was described previously (3); other plasmids are described in *Results*. T2 medium was as described (21). T3 medium contained 5 mM $MgCl_2$, 4 g of yeast extract (Difco), 20 g of Polypeptone (Nihon Pharmaceutical, Tokyo, Japan), 5 g of meat extract (Wako Pure Chemical Industries, Osaka, Japan), 0.1 g of uracil, and 10 g of glucose per liter; pH was adjusted to 7 with NaOH. 5YC medium

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Abbreviation: hEGF, human epidermal growth factor.

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contained 30 g of proteose peptone (Nihon Pharmaceutical), 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 was adjusted to 7.2 with NaOH. When cells were grown for a long period in 5YC medium, 20 g of glucose was added at 48 hr. In some experiments, 0.3% glycine or 0.01% Tween 40 (Katayama Chemical Industries, Osaka, Japan) was added to maximize the production of hEGF. To grow *B. brevis* 47 and HPD31 bearing plasmid pNU200 or pHY500, erythromycin was added at 10 or 30 μg/ml, respectively.

Purification of hEGF Produced by *B. brevis*. HPD31 carrying pNU200EGF was grown for 4 days at 30°C in 5YC medium. The culture supernatant was applied to a Sep-Pak C_{18} cartridge (Waters). The cartridge was washed with water and with 20% acetonitrile containing 0.1% trifluoroacetic acid, and then hEGF was eluted with 50% acetonitrile containing 0.1% trifluoroacetic acid. The eluate was dried under reduced pressure. The residue was dissolved in phosphate-buffered saline and subjected to reverse-phase high-performance liquid chromatography (HPLC) on a μBondasphere C_{18} column (Waters) with a linear gradient of 25–50%

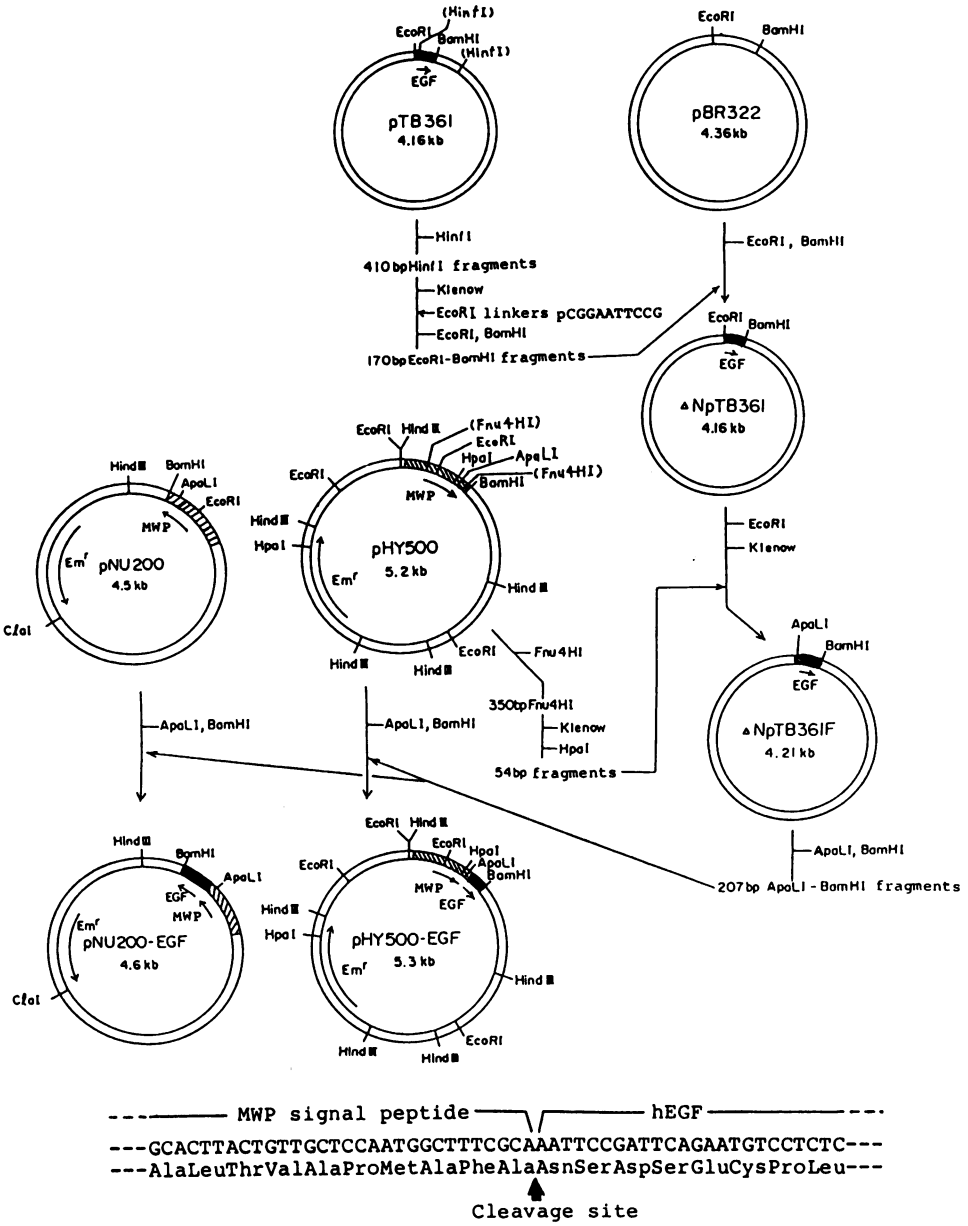

FIG. 2. Construction of hEGF expression–secretion vectors. Shadowed bars denote the 5′ region of the MWP gene, a structural gene for a major cell wall protein of *B. brevis* 47, including its multiple promoters and the signal-peptide-coding region (25). The plasmid pTB361, containing the synthetic hEGF gene, was cleaved with *Hin*fI and the resultant cohesive ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of the four dNTPs. After attachment of *Eco*RI linkers (pCGGAATTCCG), the DNA was cleaved with *Eco*RI and *Bam*HI. A 170-base-pair (bp) *Eco*RI–*Bam*HI fragment containing the hEGF gene was isolated and inserted between the *Eco*RI and *Bam*HI sites of pBR322 (ΔNpTB361). A 54-bp *Fnu*4HI–*Hpa* I fragment containing a portion of the MWP gene coding for the COOH-terminal part of the signal peptide was isolated from pHY500 DNA and inserted into the *Eco*RI site of ΔNpTB361 after the cohesive ends were filled in (ΔNpTB361F). A 207-bp *Apa*LI–*Bam*HI fragment containing the hEGF gene fused with the signal-peptide-coding region of the MWP gene was isolated from ΔNpTB361F DNA and was inserted between the *Apa*LI and *Bam*HI sites of pHY500 and pNU200 (pHY500EGF and pNU200EGF). Arrows indicate the start points and the directions of transcription. Em^r, *ermC* gene (erythromycin-resistance marker); kb, kilobases. As for *Fnu*4HI and *Hin*fI sites, only those used in this study are shown. The nucleotide and amino acid sequences of the junction region of the fused gene finally constructed are shown at the bottom. Cleavage site of the MWP signal peptide is shown by a vertical arrow below the amino acid sequence.

acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing hEGF were dried under reduced pressure. The residue was dissolved in water and subjected to ion-exchange HPLC on a column of TSK DEAE-5PW (Tosoh, Tokyo) with a linear gradient of 0.1–0.3 M ammonium acetate. The peak fraction was subjected to reverse-phase HPLC as described above.

Amino Acid Sequence and Composition Analyses of Purified hEGF. The NH_2-terminal amino acid sequence was determined by a gas-phase protein sequencer (model 470A; Applied Biosystems). The COOH-terminal amino acid was determined by hydrazinolysis (28). The amino acid composition was determined after hydrolysis for 24 hr with 5.7 N HCl at 110°C in the presence of 4% thioglycolic acid. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer.

Colony Immunoassay and Other Analytical Methods. An *in situ* colony immunoassay was performed by the method of Helfman *et al.* (29) except that horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) and the chromophore 4-chloro-1-naphthol were used instead of the ^{125}I-labeled second antibody. Anti-hEGF rabbit serum (Wakunaga Pharmaceutical, Osaka) was used as a first antibody. A hEGF reagent pack (Amersham) was used for radioimmunoassay of hEGF synthesized in *B. brevis*. NaDodSO$_4$/polyacrylamide gel electrophoresis and immunoblot analysis were carried out as described by Laemmli (30) and Burnette (31), respectively. hEGF was detected by the use of the same first and second antibodies as those used in the colony immunoassay.

RESULTS

Construction of Expression–Secretion Vectors in *B. brevis*. Plasmids pHY500 and pNU200 (Fig. 2) were constructed to use for heterologous protein production in *B. brevis*. Both plasmids carry *ermC* (32) as a selective drug-resistance gene. Replication origins of pWT481, a plasmid found in *B. brevis* 481 (33), and pUB110, found in *Staphylococcus aureus* (34), were used in pHY500 and pNU200, respectively. The 600-bp *Alu* I–*Alu* I fragment containing the 5′ region of the *B. brevis* 47 MWP gene, a structural gene for one of the major cell wall proteins (25), was inserted in both plasmids with the aid of *Bam*HI linkers. The 5′ region includes the multiple promoters and ribosomal binding sites, as well as the region encoding the signal peptide and the NH_2-terminal portion of the mature form of MWP (25). The *Apa*LI and *Bam*HI sites, which are unique on both plasmids, are convenient for insertion of a heterologous gene. Since the MWP gene is expressed very efficiently even at the stationary phase of growth (22), its 5′ region should greatly facilitate the expression of heterologous genes placed downstream.

Efficient Production of hEGF in *B. brevis*. The 5′ portion of the synthetic hEGF gene (Fig. 1) was trimmed by *Hin*fI and inserted into the *Fnu*4HI site located at the cleavage site of the MWP signal sequence on pNU200 and pHY500, so that the fused gene encoded exactly the same amino acid sequence as that of mature hEGF directly following the MWP signal peptide (Fig. 2). The resulting plasmids, pNU200EGF and pHY500EGF, were introduced into *B. brevis* 47 and HPD31 by a Tris/PEG method developed by us for transformation of *B. brevis* (35). Clones producing hEGF, identified by colony immunoassay, were grown under various conditions, and the amount of extracellular hEGF was determined by radioimmunoassay (Table 1). *B. brevis* HPD31 carrying pNU200EGF produced as much as 240 mg of extracellular hEGF per liter of culture under optimal conditions. Addition of 0.3% glycine or 0.01% Tween 40 to 5YC medium appreciably stimulated the production of hEGF. Glycine was added because it enhanced the production of cell wall proteins (22). The efficiency of hEGF production by *B. brevis* HPD31 was higher than that by *B. brevis* 47 under the various culture conditions so far examined.

Table 1. Amount of hEGF secreted by *B. brevis* under various growth conditions

Host/plasmid	Medium	Temp., °C	Time, hr	hEGF,* mg/liter
47/pHY500EGF	T3	30	72	7.4
		33	96	37
		37	72	15
47/pNU200EGF	T3	33	96	52
HPD31/pNU200EGF	T3	30	90	61
	5YC	30	90	160
			110	180
			144	180
	5YC + 0.01% Tween 40	30	90	170
			110	210
			144	210
	5YC + 0.3% glycine	30	90	160
			110	240
			144	240
		33	96	220
		37	72	150

*Determined by radioimmunoassay.

Fig. 3 shows the time course of hEGF production by *B. brevis* HPD31 carrying pNU200EGF. The amount of hEGF in the culture medium increased markedly from the early stationary phase of growth. After 3 days, it remained almost constant. 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 NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 4). From the intensity of the bands, 1 μl of the culture supernatant was estimated to contain >0.2 μg of hEGF, which was

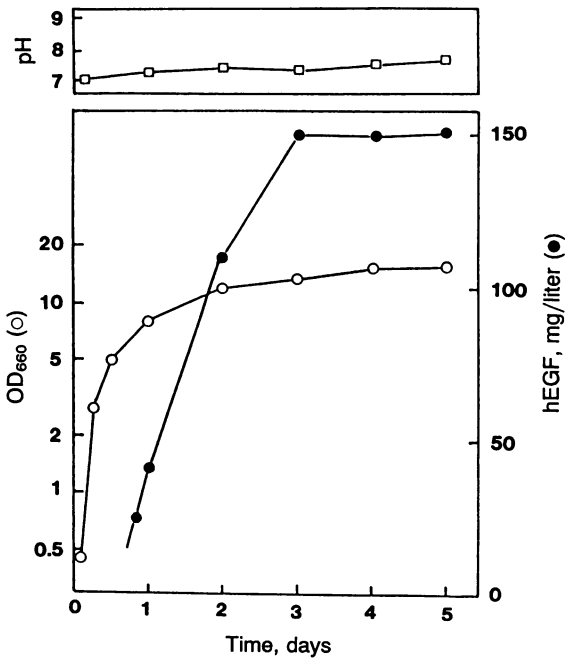

FIG. 3. Time course of hEGF production. *B. brevis* HPD31 carrying pNU200EGF was grown with shaking in 5YC medium supplemented with 0.3% glycine at 37°C. Extracellular hEGF was determined periodically by radioimmunoassay. Cell growth (optical density at 660 nm) and pH of the medium were also monitored.

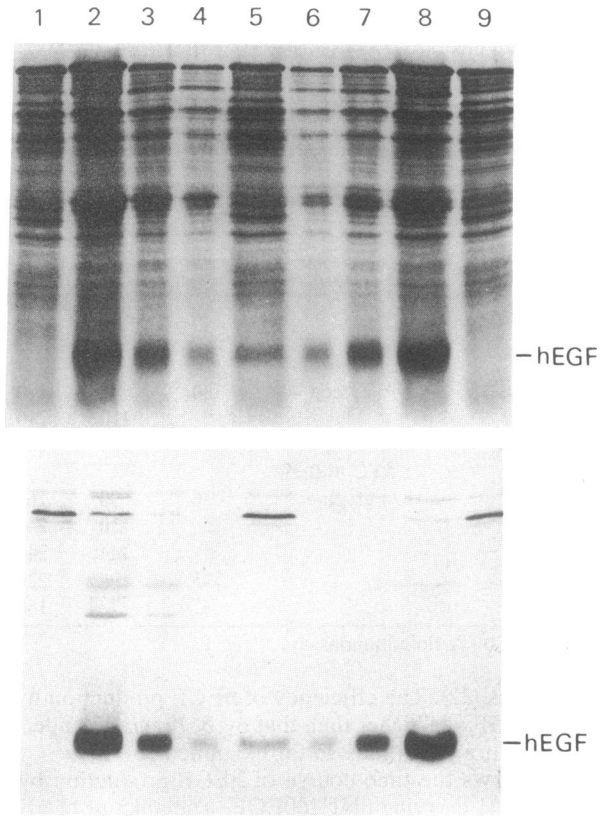

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis and immunoblot analyses of hEGF secreted by *B. brevis*. HPD31 and HPD31 carrying pNU200EGF were grown for 4 days at 33°C in 5 YC medium with or without supplements as described below. Culture supernatants were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue (*Upper*) or immunoblot analysis with anti-hEGF serum (*Lower*). Lanes 1 and 9, 7.5 μl of culture supernatants obtained from *B. brevis* HPD31 grown in 5YC medium without or with 0.01% Tween 40, respectively; lanes 2–4, 7.5 μl, 2.5 μl, and 0.8 μl, respectively, of culture supernatant obtained from *B. brevis* HPD31 carrying pNU200EGF grown in 5YC medium plus 0.01% Tween 40; lane 5, same 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 culture supernatant obtained from *B. brevis* HPD31 carrying pNU200EGF grown in 5YC medium plus 0.3% glycine.

in agreement with the result obtained by radioimmunoassay described above.

Cellular Location of hEGF Synthesized in *B. brevis*. Cells were disrupted by sonic treatment and the amount of hEGF was determined by radioimmunoassay. Almost none was detected in the intracellular soluble fraction (<1% of the amount of extracellular hEGF) indicating that hEGF synthesized in *B. brevis* was secreted efficiently into the medium. Although a small amount of material (<10% of soluble hEGF) that reacted with anti-hEGF serum was found in the insoluble fraction, it has not been characterized yet.

Purification and Structural Analysis of hEGF Synthesized in *B. brevis*. hEGF synthesized in *B. brevis* HPD31 was purified as described in *Materials and Methods*. It gave a single peak on HPLC and its yield was 14% of the total secreted hEGF. The nine NH_2-terminal amino acid residues and the amino acid composition of the purified hEGF (except for cysteine, which was not determined) matched those of authentic hEGF. Furthermore, both had the same COOH-terminal amino acid, arginine. On $NaDodSO_4$/polyacrylamide gel electrophoresis, the purified hEGF migrated to the same position as authentic hEGF, with or without 2-mercaptoethanol pretreatment. These results indicated that the polypeptides synthesized from the MWP–hEGF fusion gene were correctly processed in *B. brevis* and secreted into the medium, probably forming the correct disulfide linkages.

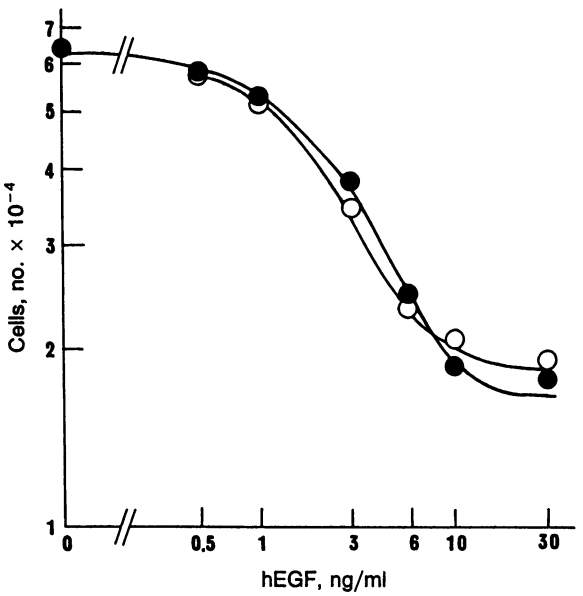

FIG. 5. Inhibition of growth of HSC-1 cells by purified hEGF. HSC-1 cells (36) were plated at 10^4 cells per 35-mm dish and grown at 37°C for 1 day in 5% CO_2 in air. Purified hEGF from *B. brevis* (●) and authentic hEGF (○) were added to the cultures at the indicated concentrations, and incubation was continued for 2 days. Living cells were counted with a Coulter Counter.

Biological Activities of hEGF Synthesized in *B. brevis*. The growth-inhibitory effect of the hEGF on human squamous-cell carcinoma (HSC-1 cells; ref. 36) was examined. hEGF produced by *B. brevis* showed almost the same activity as authentic hEGF (Fig. 5). The stimulatory effect of hEGF on the initiation of murine fibroblast cell division (37) was also similar for the two preparations (data not shown).

DISCUSSION

As mentioned above, *B. brevis* strains used in this work continue to synthesize and secrete a large amount of extracellular proteins even in the stationary phase of growth. The 5′ region of the extracellular protein (cell wall protein) operon of *B. brevis* 47 has multiple and tandemly arranged promoters and two translation initiation sites. Recently, one of the tandem promoters, *P2*, was shown to play a major role in synthesis of the cell wall proteins in the stationary phase of growth (38). Since the time course of hEGF production was similar to that of cell wall protein production, the 5′ region seemed to promote expression of heterologous genes placed downstream by the same mechanism as that for cell wall protein synthesis.

In addition to their excellent protein-producing capability, the *B. brevis* strains used in this study have the advantage of low production of extracellular proteases. The protease activities in the culture supernatants of *B. brevis* 47 and HPD31 were 1.6% and <0.02% (not detectable), respectively, of that of *B. subtilis*, when these bacteria were grown in T2 medium (27). Owing to this unique feature, heterologous proteins secreted by *B. brevis* retained their activity during a long period of cultivation.

It has already been demonstrated that several bacterial proteins, such as α- and β-amylase, can be rather easily produced in large amounts by using our system; e.g., 0.5–3 g/liter (refs. 25 and 39; H. Takagi, A. Miyauchi, S. Kagiyama, K. Kadowaki, and S.U., unpublished data). We used a synthetic hEGF gene to examine whether or not a human

protein could be efficiently produced in our system, since hEGF is expected to be an important pharmaceutical and its production in other host–vector systems has been low. As reported here, hEGF could be produced by *B. brevis* in a large amount (≈0.24 g/liter) even in a laboratory shake-flask culture, whereas active swine pepsinogen and human salivary α-amylase were produced up to 11 and 22 mg/liter, respectively (ref. 40 and unpublished results). These amounts are smaller than those of the bacterial proteins but are considerably (10–100 times) larger than the amounts of the same proteins produced by other hosts: only a few milligrams of hEGF per liter of culture was secreted by *E. coli* and *S. cerevisiae* (1–3), only 0.4 mg of human salivary α-amylase per liter of culture was secreted by *S. cerevisiae* (41) and none by *B. subtilis* (42), and no active swine pepsinogen was synthesized in *E. coli* (43).

The *B. brevis* host–vector system should be useful for large-scale production of human proteins not only for medical uses but also for basic study of these proteins. Also, easy preparation of various altered proteins through site-directed mutagenesis should contribute to the study of structure and function relationship as well as to x-ray crystallographic study. Studies on the mechanism regulating expression of the MWP cell wall protein gene, as well as on factors affecting the efficiency of secretion in *B. brevis*, should facilitate further improvement of the host–vector system. It may be possible to further improve heterologous protein production in *B. brevis* by isolating appropriate mutants, similar to those of *E. coli* lacking an intracellular protease that degrades heterologous proteins specifically (44) or to supersecreting mutants of *S. cerevisiae* (16).

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