Production of Human Epidermal Growth Factor by *Bacillus brevis* Increased with Use of a Stable Plasmid from *B. brevis* 481

Shogo Ebisu, Hiroaki Takagi, Kiyoshi Kadowaki, Hideo Yamagata,* and Shigezo Udaka*

Research Laboratory, Hitetsu Shoyu Co., Ltd., Choshi, Chiba 288, Japan
* Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

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A host vector system has been developed for the efficient production of heterologous proteins with *Bacillus brevis* as a host.11 This system has two advantages: proteins are secreted directly into the culture medium in soluble, biologically active form, and the secreted protein is usually stable because of the lack of proteolytic activity in the culture supernatant. Bacterial proteins such as amylases12 can be produced in large amounts by *B. brevis*, but mammalian proteins such as human α-amylase are produced at a low level (one or two orders of magnitude less than for bacterial proteins) unless certain improvements of the host or vector (or both) are made.39 Of the mammalian proteins studied, human epidermal growth factor (hEGF) has been produced in a high yield with such improvements (0.24 g/liter).48 Here, we report that a still higher secretion level of hEGF was obtained with *B. brevis* HPD3139 as the host and plasmid pHY48149 as the vector.

The plasmid pHY700EGF was constructed from plasmid pHY481,71 derived from pHY481,69 as follows. With synthetic linker DNAs, the BamHI and SalI sites in pHY481 were converted to AccI and BamHI sites, respectively, giving pHY481AB. The 640-bp ClaI–HpaI fragment containing the 5′ region of the gene coding for cell wall protein,84 the 230-bp HpaI–BamHI fragment containing the part of the cell wall protein gene encoding the signal peptide,72 and the hEGF gene41 were inserted between the AccI and BamHI sites of pHY481AB. The resulting plasmid, pH700EGF, contained the signal-peptide-encoding region of the cell wall protein gene directly fused to the sequence encoding mature hEGF, and had the promoter of the gene encoding cell wall protein upstream of the fused gene (Fig. 1). This plasmid was introduced into *B. brevis* HPD3139 by electroporation.99 The production of EGF by *B. brevis* HPD31 (pHY700EGF) over time is shown in Fig. 2. The amount of hEGF in the culture medium increased in the early stationary phase of growth, and production was maximum (1.1 g/liter) at 6 days. The hEGF synthesized in *B. brevis* HPD31 was one of its major extracellular proteins, with the same apparent molecular weight as that of standard hEGF as shown by Coomassie brilliant blue staining and immunoblot analysis after SDS–polyacrylamide gel electrophoresis (Fig. 3). The hEGF synthesized in *B. brevis* HPD31 was purified as described by Yamagata et al.41 It gave a single peak on HPLC, the elution time of which was the same as that of authentic hEGF (data not shown). The 40 residues at the NH₂-terminal of the amino acid sequence and the five residues at the COOH-terminal of this sequence of the purified hEGF were identical with those of authentic hEGF.

The amount of hEGF produced by *B. brevis* HPD31 (pHY700EGF) was four times the amount produced by the previously reported *B. brevis* HPD31 (pNU2000EGF). The same host–vector system produced a thermophilic α-amylase from *Bacillus licheniformis* extracellularly at the rate of 3.7 g/liter (data not shown), suggesting the general usefulness of the system. Plasmid pH700EGF differs from pNU2000EGF essentially only in its replication origin (ori). The plasmid pNU2000EGF has the ori of pUB110, from *Staphylococcus aureus*, and has a high copy number, but is an unstable plasmid in *B. brevis*. pH700EGF has the ori of pWT48139 from *B. brevis* 481, which hyperproduces proteins. It has a low copy number, but is stable in *B. brevis*. Thus, for the efficient production of heterologous proteins in *B. brevis*, stability of the vector

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**Fig. 1. Structure of Expression Vector pH700EGF.**
Promoter, the 5′ region of the cell wall protein gene of *B. brevis* HPD31; sp, the signal sequence region of the cell wall protein gene of *B. brevis* 47; EGF, the hEGF gene; Em', erythromycin resistance gene. The main restriction sites are indicated.

**Fig. 2. Course of hEGF Production by *B. brevis* HPD31 (pHY700EGF).**
Cells were grown at 30°C in 5PY medium23 containing 10 μg/ml erythromycin. Extracellular hEGF was measured periodically by enzyme immunoassay.

**Fig. 3. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblot Analysis of hEGF Produced by *B. brevis* HPD31.**
*B. brevis* HPD31 (pHY700EGF) was grown at 30°C in 5PY medium.23 Samples (6 μl) were treated by SDS–polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (A) or immunoblotted with anti-hEGF serum (B). Lane 1, standard hEGF (0.1 g/liter). Lanes 2, 3, and 4, culture supernatant at 2, 4, and 6 days of cultivation, respectively.
seemed to be a more important factor than the copy number of the vector.

References