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ORIGINAL RESEARCH PAPER

Secretion of biologically-active human interferon-*β* by *Bacillus subtilis*

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Abstract Human interferon-*β* (hIFN-*β*) was used as a heterologous model protein to investigate the effects of the *Bacillus subtilis* AmyE propeptide and co-expression of PrsA in enhancing the secretion of heterologous proteins in *B. subtilis*. Secretion and activity of hIFN-*β* with AmyE propeptide increased by more than four-fold compared to that without AmyE propeptide. Moreover, under conditions of co-expressed PrsA, the secretion production and activity of hIFN-*β* with AmyE propeptide increased by more than 1.5-fold. AmyE propeptide and co-expression of PrsA thus have an additive effect on enhancing the production of the hIFN-*β* in *B. subtilis*.

Keywords *Bacillus subtilis* · Human interferon-*β* · Propeptide · PrsA · Protein secretion

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Introduction

Bacillus subtilis and its related species have a high capacity for secreting proteins directly into the culture medium. They are widely used industrially as hosts for the production of various secretion enzymes, such as amylase, protease, xylanase, and lipase (Braun et al. 1999; Tjalsma et al. 2000; Westers et al. 2004). In addition, many are generally regarded as safe (GRAS) microorganisms and do not produce endotoxins. Therefore, the secretion system of these species presents many advantages in terms of production capacity, structural authenticity, product purification, and safety. Nevertheless, the secretion of heterologous proteins from eukaryotes by these species is frequently inefficient (Westers et al. 2004). Considerable efforts have been targeted at developing *B. subtilis* as a host for producing heterologous proteins (Wu and Wong 2002; Li et al. 2004; Westers et al. 2004). Co-expression of an extra-cytoplasmic molecular chaperone, PrsA, enhanced the secretion of several model proteins including α-amylase, single-chain antibody (SCA), and recombinant protective antigen (rPA) (Kontinen and Sarvas 1993; Vitikainen et al. 2001; Wu et al. 1998; Williams et al. 2003). PrsA is essential for viability and protein secretion. In protein secretion, PrsA mediates protein folding at the late stage of secretion (Kontinen and Sarvas 1993, Vitikainen et al. 2001). In addition, we previously demonstrated that the *B. subtilis* AmyE propeptide enhances the secretion of recombinant human

interferon α2b (Kakeshita et al. 2011). The *B. subtilis* AmyE propeptide is a short propeptide (eight amino acids residues) located between the AmyE signal peptide and its mature protein.

In this study, we evaluated whether the fusion of the *B. subtilis* AmyE propeptide could improve the secretion of human interferon-β (hIFN-β) in *B. subtilis*. hIFN-β belongs to the IFN type I family, and is a cytokine produced by macrophages with antiviral, antibacterial, and anticancer activities. In addition, hIFN-β is used for effective treatments in multiple sclerosis (Goodbourn et al. 2000; Randall and Goodbourn 2008). Furthermore, in an attempt to increase secretion of hIFN-β, we examined the effect of co-expression of an extra-cytoplasmic molecular chaperone, PrsA. We demonstrated that the AmyE propeptide and co-expression of PrsA can enhance the secretion production of hIFN-β.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Supplementary Table 1. *E. coli* strains were used for the construction of plasmids, and were grown in L medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) at 30 or 37°C. For the protein expression experiments, *B. subtilis* strains were grown at 30°C and 110 rpm in 500 ml shaking (Sakaguchi) flasks containing 100 ml of 2× L medium (2% tryptone, 1% yeast extract, and 1% NaCl). Antibiotics were as follows: ampicillin, 50 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}; and tetracycline, 15 μg ml^{-1}. Xylose was added to induce protein expression in *B. subtilis* with the expression plasmids. Cell growth was monitored from the OD_{600} value.

Construction of the co-expressing PrsA strain

To construct strain D8C as a control strain, two DNA fragments containing 5′-terminus or 3′-terminus flanking regions of *amyE* were amplified using *B. subtilis* genome as the template, and primers amyE5-F2 and amyE/Cm-R or amyE/Cm-F and amyE3-R2 (See Supplementary Table 2), respectively. A DNA fragment containing a chloramphenicol-resistance gene was also amplified using primers Cm-F and Cm-R (See Supplementary Table 2). To fuse the three DNA fragments, PCR was carried out using primers amyE5-F1 and amyE3-R1 (See Supplementary Table 2). The resulting DNA fragments were introduced into *B. subtilis* Dpr8 (Kodama et al. 2007) by competence transformation and chloramphenicol-resistant clones were selected.

To construct strain D8PA, an additional expressing PrsA with chromosomally encoding PrsA, two DNA fragments containing *spoVG* promoter with Shine–Dalgarno (SD) sequence or-PrsA coding region were amplified using *B. subtilis* genome as the template and primers pVG-F and pVG-R or prsA/PVG-F and prsA/Cm-R (see Supplementary Table 2), respectively. PCR was then performed on the two DNA fragments using primers pVG-F and prsA/Cm-R. Next, another two DNA fragments containing 5′-terminus flanking regions of *amyE* or 3′-terminus flanking regions of *amyE* with *cat* gene were amplified using *B. subtilis* D8C genome as the template, and primers amyE5-F2 and amyE/PVG-R (see Supplementary Table 2) or Cm-F and amyE3-R2, respectively. Finally, to fuse the three DNA fragments, PCR was carried out using primers amyE5-F1 and amyE3-R1. The resulting DNA fragment was introduced into Dpr8 by competence transformation, and chloramphenicol-resistant clones were selected. Correct integration of resistance markers or *prsA* into the *amyE* locus of *B. subtilis* chromosome was verified by PCR.

Construction of the expression plasmids for production of hIFN-β

To express hIFN-β, we used two plasmids, pHKK3100 and pHKK3200 (Kakeshita et al. 2010, 2011). pHKK3100 contained the *B. subtilis amyE* ribosome binding site and the signal peptide (33 amino acids) of *B. subtilis amyE*, while pHKK3200 contained the *B. subtilis amyE* ribosome binding site and the signal peptide with propeptide (41 amino acids) of *B. subtilis amyE*. To construct the expression plasmids, the 524 bp DNA fragment containing the mature region of the human *ifn-β* ORF was amplified using pORF-hIFNB (InvivoGen) as the template, and primers ifnb-F and ifnb-R (see Supplementary Table 2). The PCR product was digested with *Kpn*I and *Bam*HI (sites underlined in the above primer sequences) and cloned into pHKK3100 or pHKK3200, cut with the same enzymes downstream

of the *B. subtilis amyE* signal peptide or the *B. subtilis amyE* signal peptide and propeptide. The resulting plasmids were designated as pHKK3111 and pHK3211, respectively. In pHKK3111, the AmyE SP-hIFN-β fusion contains a junction built from the amino acids alanine, glycine and ghreonine. Similarly, in pHKK3211, the AmyE SP-Pro-hIFN-β fusion contains a junction built from the amino acids glycine and threonine. The two constructed expression plasmids were verified by DNA sequencing, and were introduced into *B. subtilis* by protoplast transformation.

Preparation of anti-PrsA antisera

Peptides corresponding to the region from Gly-216 to Asp-230 and of PrsA (Kontinen et al. 1991) were synthesized and used to raise antisera and prepared by Operon Biotechnologies (Tokyo, Japan).

Protein extraction and immunoblotting

The recombinant *B. subtilis* strains were grown to OD_{600} 0.75 and induced with 0.6% xylose for 20 h. The supernatant fraction from *B. subtilis* cultures were prepared as described previously (Kakeshita et al. 2011). Protein samples were resolved by SDS-PAGE (15% acrylamide) and electrotransferred to a PVDF membrane in a semi-dry system (Bio-Rad). Proteins were visualized using a rabbit polyclonal antibody to hIFN-β (PeproTech EC LTD) or a rabbit polyclonal antibody to PrsA, horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin IgG (GE Healthcare) and an ImmunoStar Kit (Wako), according to the manufacturer's instructions. The intensities of the bands obtained by western blot analysis were converted into numerical values using Image J (National Institutes of Health, USA), for measuring the amount of protein. Commercially purified hIFN-β (PeproTech EC LTD) was used as a control.

Assay of hIFN-β biological activity

Assay of the biological activity of the hIFN-β was performed in the Department of Bio Research, Kamakura Techno-science, Inc. (Kanagawa, Japan). The biological activity of the interferon was determined as described previously (Kakeshita et al. 2011). A standard cytopathic effect inhibition assay, using Sindbis virus challenge of FL cells was performed to quantify the antiviral activity of the hIFN-β in the sample preparation. Specific activity was determined with reference to standard hIFN-β (Japanese Reference-03 of 4×10^2 IU ml^{-1}, from the National Institute of Infectious Diseases, Japan). One unit of activity is defined as the amount of hIFN-β required to produce antiviral activity equivalent to that expressed by one international unit (IU) of the standard.

Results and discussion

Production and confirmation of hIFN-β from *B. subtilis*

To analyze the effect of the AmyE propeptide on secretion, the *ifn-β* gene was cloned into the *B. subtilis* expression vectors pHKK3100 and pHKK3200. The resulting plasmids were designated as pHKK3111, which contained the AmyE SP-hIFN-β fusion protein and pHK3211, which contained the AmyE SP-Pro-hIFN-β fusion protein (Fig. 1).

To identify the expression of hIFN-β, we cultured Dpr8 with pHKK3111 and pHKK3211, respectively. As shown in Fig. 2, no hIFN-β band was detected by western blot analysis in both recombinants without xylose (Fig. 2, lanes 2 and 4). By detection with western blot analysis, striking protein bands at roughly 17 and 18 kDa for pHKK3111 and pKHH3211, respectively, were observed after 20 h induction by xylose (Fig. 2, lanes 1 and 3). The secreted hIFN-β from pHKK3211 exhibited a higher molecular weight than the secreted hIFN-β from pHKK3111 and the commercially purified hIFN-β, due to the presence of the AmyE propeptide. The AmyE propeptide was not cleaved after secretion of hIFN-β with propeptide, the same as reported for hIFN-α2b (Kakeshita et al. 2011).

For further investigation of the recombinant protein secretion level, western blotting analysis was used to detect and quantify the secreted hIFN-β in the supernatant fractions of both strains. The amount of secreted hIFN-β was 3.7 ± 0.6 μg ml^{-1} for Dpr8 with pHKK3211 and 0.9 ± 0.1 μg ml^{-1} for Dpr8 with pHKK3111 (Table 1). We demonstrated that the secretion production of hIFN-β with propeptide increased by four-fold, compared to that without propeptide. This result indicated that the AmyE propeptide enhanced the secretion of the hIFN-β protein from *B. subtilis*.

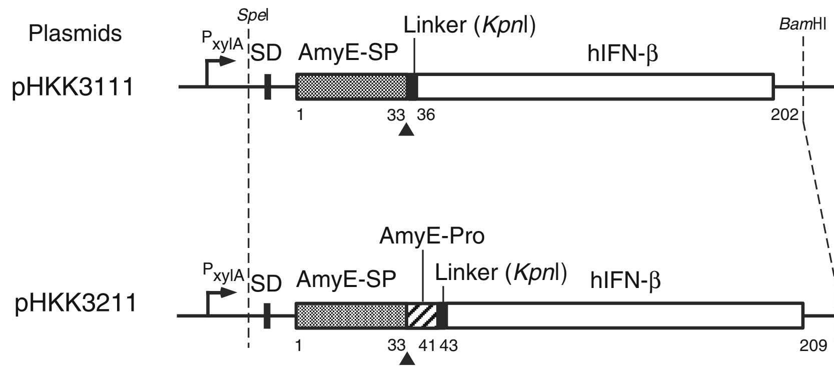

Fig. 1 Schematic representation of the regulatory system for controlled expression of hIFN-*β* by *B. subtilis*. Construction of plasmids pHKK3111 and pHKK3211. The restriction sites used for each construction are indicated. P_{xylA}, xylose-inducible promoter; RBS, ribosome binding site; AmyE SP, *B. subtilis* AmyE signal peptide; AmyE-Pro, *B. subtilis* AmyE propeptide. *Arrowheads* indicate the predicted signal peptide cleavage sites. Numbers denote the amino acid position relative to the *N*-terminus of fusion proteins. See text for detailed descriptions of the construction of the expression plasmids

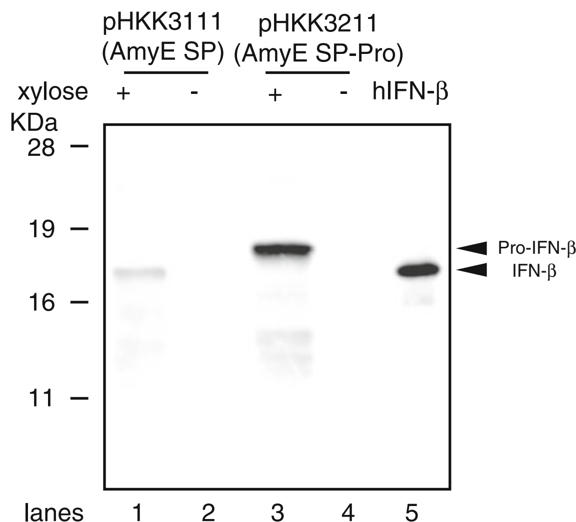

Fig. 2 Western blotting analysis of secreted hIFN-*β* proteins in *B. subtilis*. Samples were collected at 20 h after xylose induction, separated by 15% SDS-PAGE, and western blotting using anti hIFN-*β* polyclonal antibodies. Dpr8 with pHKK3111 (*lanes 1* and 2); Dpr8 with pHKK3211 (*lanes 3* and *4*); 0.6% xylose induced (*lanes 1* and *3*), none induced (*lanes 2* and *4*), commercially purified hIFN-*β* 50 ng (*lane 5*). *Arrowheads* indicate the positions of the Pro-IFN-*β* and hIFN-*β*. Each lane contained 40 µl supernatant mixed with an equal volume of 2× sample buffer

Co-expression of PrsA enhanced the secretion of Pro-hIFN-*β*

To investigate the effect of co-expression of PrsA in enhancing the secretion of hIFN-*β*, we constructed strain D8PA. Insertion of PrsA under the control of the strong expressed *B. subtilis spoVG* promoter (Zuber and Losick 1983) into the chromosome of Dpr8 at the *amyE* locus generated the strain D8PA (Fig. 3a). As a control, strain D8C was constructed in which a chrolamphenicol resistant gene was similarly inserted into the *amyE* locus (Fig. 3a). In D8PA, the expression level of PrsA was increased by 3.2-fold, compared to that of D8C (Fig. 3b). We introduced pHKK3111 or pHKK3211 into D8PA and D8C, and examined amount of secreted hIFN-*β*. As shown Fig. 3c, d, under the conditions of co-expressed PrsA, the amount of secreted hIFN-*β* with AmyE propeptide were increased by 75%, compared to that under the normal expression levels of PrsA. On the other hand, amount of secreted hIFN-*β* without AmyE propeptide was increased by 25%, the increasing rates of hIFN-*β* without propeptide is only one-thirds of that with propeptide (Fig. 3c, d). These results indicated that co-expression of PrsA can enhance the secreted hIFN-*β*, especially, the AmyE propeptide and co-expression of PrsA have an additive effect on enhancing the secretion of the hIFN-*β* protein in *B. subtilis*.

Biological activity of recombinant hIFN-*β*

The biological activities of the hIFN-*β* were measured from the cultures of the induced Dpr8 or D8PA with pHKK3111 and pHKK3211 strains. As shown in Table 1, when Dpr8 was used as host, the total activity increased by 4.9-fold by the insertion of the propeptide. In addition, the specific activity of pro-hIFN-*β*

Table 1 Production of hIFN-β from supernatant by recombinant *B. subtilis* with expression vector

Strains	Plasmid	Sample of supernatant	Total protein (mg/l)	Total activity (IU/ml)	Specific activity (U/mg)
Dpr8	pHKK3111	hIFN-β	0.9 ± 0.1	0.8 ± 0.1 × 10^3	0.9 × 10^6
Dpr8	pHKK3211	Pro-hIFN-β	3.7 ± 0.6	3.9 ± 0.7 × 10^3	1.1 × 10^6
D8PA	pHKK3111	hIFN-β	1.0 ± 0.3	1.1 ± 0.1 × 10^3	1.1 × 10^6
D8PA	pHKK3211	Pro-hIFN-β	5.5 ± 1.3	10.1 ± 2 × 10^3	1.8 × 10^6

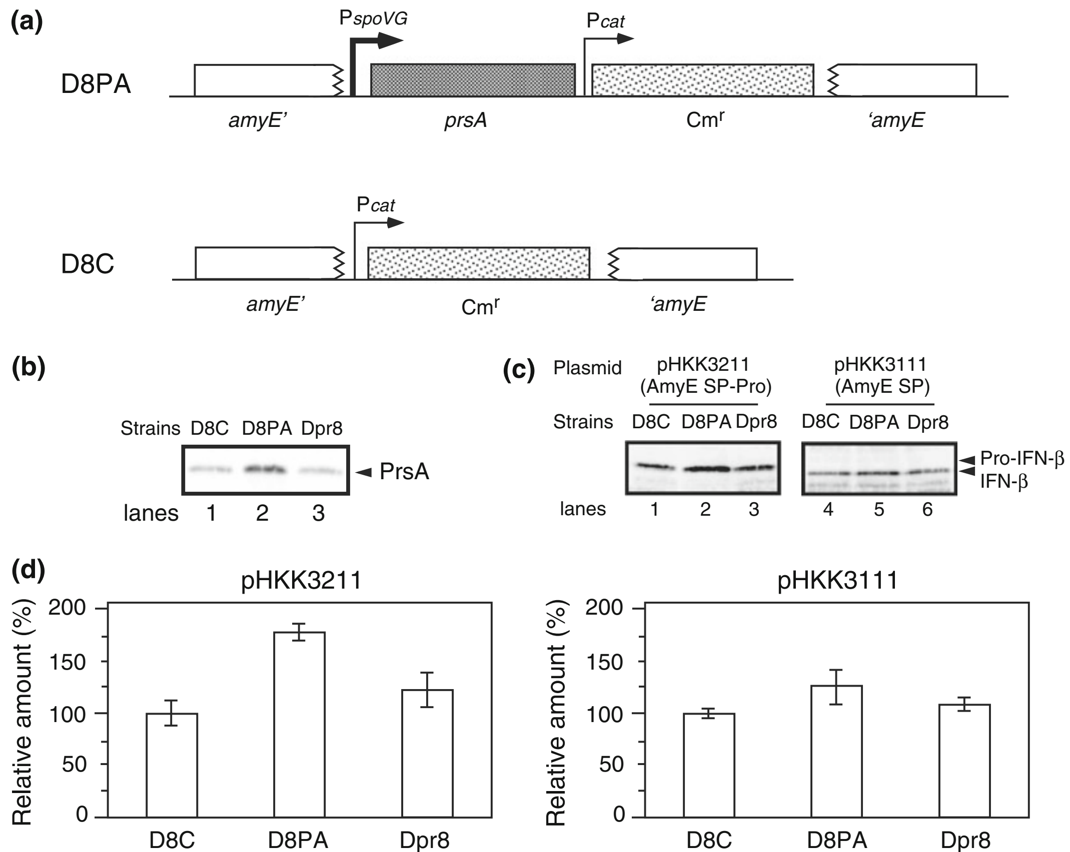

Fig. 3 Comparison of the amounts of secreted hIFN-β from *B. subtilis* D8C and D8PA, PrsA co-expressing strains. **a** Schematic representation of the gene structure around *amyE* locus in the *B. subtilis* mutant strains D8PA and D8C genome. See text for a description of the construction of D8PA and D8C. P_{spoVG} and PrsA represent the *B. subtilis spoVG* promoter and *B. subtilis* PrsA, respectively. P_{cat} and Cm^r represent chloramphenicol-resistant gene promoter and coding region, respectively. **b** Western blot analysis of PrsA protein from *B. subtilis* D8C, D8PA, and Dpr8. At 20 h of culture after xylose induction, cells were harvested and lysed, and the cellular lysates were analyzed by western blotting using anti-PrsA antibodies. **c** Western blot analysis of hIFN-β production by *B. subtilis* D8C, D8PA, and Dpr8. D8C with pHKK3211 (*Lane 1*); D8C with pHKK3111 (*lane 4*); D8PA with pHKK3211 (*lane 2*); D8PA with pHKK3111 (*lane 5*); Dpr8 with pHKK3211 (*lane 3*); Dpr8 with pHKK3111 (*lane 6*). *Arrowheads* indicate the positions of the Pro-IFN-β and hIFN-β. *Lanes 1–3* contained 20 µl supernatant or *lanes 4–6* contained 40 µl supernatant mixed with an equal volume of 2× sample buffer. **d** Quantification of secreted hIFN-β protein production in the culture medium. The hIFN-β production corresponding to the *B. subtilis* D8C strain was set as 100%. Data represent the mean of three experiments, and error *bars* represent standard error

reached 1.1 × 10^6 U mg^{-1}, which is roughly the same as that of the non-propeptide hIFN-β (Table 1). This result indicated that the presence of the propeptide did not inhibit biological activity. In addition, by using D8PA as the host, the specific activity of hIFN-β without propeptide reached 1.1 × 10^6 U mg^{-1}, which

is the same as that of the Dpr8 (Table 1). On the other hand, when D8PA was used as host, the anti-viral activity was increased by 2.5-fold by the co-expression of the PrsA. In addition, the specific activity of pro-hIFN-β reached 1.8×10^6 U mg^{-1} and, this increased by more than 1.6-fold, compared to other specific activity (Table 1). This result raises the possibility that co-expression of PrsA may mediate protein folding in protein secretion.

Conclusion

We found that the fusion propeptide enhanced the secretion of a heterologous protein, hIFN-β. In addition, co-expression of PrsA was enhanced secretion of hIFN-β. Further studies are required to examine the effect of the AmyE propeptide in enhancing the secretion of other heterologous proteins. The work presented herein illustrates a useful method to enhance the extracellular production of a heterologous protein in *B. subtilis*.

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