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Improvement of Heterologous Protein Secretion by *Bacillus subtilis*

Hiroshi Kakeshita[1,2], Yasushi Kageyama[1], Katsuya Ozaki[1], Kouji Nakamura[2] and Katsutoshi Ara[1]

[1]*Biological Science Laboratories, Kao Corporation,*

[2]*Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan*

1. Introduction

The Gram-positive bacterium, *Bacillus subtilis* and related species are widely used as hosts for the extracellular production of industrially worthy enzymes, such as amylases, proteases, xylanase, and lipases (Braun et al., 1999; Tjalsma et al., 2000; Westers et al., 2004). These species possess a very high capacity for secreting a variety of exoenzymes into the growth medium, thereby reducing downstream purification processes. In addition, many of these 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 (Table1). Hence, these species are never selected as the best cell factory for pharmaceutical proteins (Westers et al., 2004).

In pharmaceutical industry, the production of recombinant proteins in *Escherichia coli* is well established. In many cases, proteins are produced in cytoplasm of *E. coli*, and therefore, the production of recombinant proteins involves refolding and purification from inclusion bodies. However, the production of soluble recombinant proteins is relatively more cost-effective and less time-consuming. In fact, many studies have been performed regarding methods to overcome the problem of inclusion bodies and to improve protein solubility for the expression of heterologous proteins (Kapust & Waugh, 1999; Baneyx & Mujacic, 2004; Sørensen & Mortensen, 2005; Rabhi-Essafi et al., 2007). Therefore, developing human protein producing hosts is a major challenge in the field of biotechnology and protein production in *Bacilli*.

In *B. subtilis*, one long-standing major problem is the presence of high levels of extracellular protease for the production of heterologous proteins. In recent years, many proteases have been identified via the completed genome sequence of *B. subtilis* (Kunst et al., 1997; Westers et al., 2004), thereby allowing the construction of many protease-depleted strains for the production of heterologous proteins.

In addition, considerable efforts have been targeted at developing *B. subtilis* as a host for the production of heterologous proteins (Wu & Wong, 2002; Li et al., 2004; Westers et al., 2004; Kodama et al., 2007a, 2007b). However, many problems still remain for the secretion of human proteins, and these should be analyzed from the complementary perspectives of both the target protein and the secretion pathway, in order to improve human protein secretion.

We have used human interferon-α and interferon-β as heterologous model proteins to investigate the effects of *B. subtilis* secretion.

In this report, the knowledge which has become available in recent years aimed at improving heterologous protein secretion is discussed, and co-production of a Tat system is shown to provide a useful tool to enhance the secretion of heterologous proteins.

Product	Yield	Reference
α-amylase (AmyQ)	1 – 3 g/L	Palva, 1982
Avid-stable α-amylase	3.1 g/L	Heng et al., 2005
Cutinase	20 mg/L	Brockmeier et al., 2006
Proinsulin (PI)	1 g/L	Olmos-Soto and Contreras-Flores, 2003
LipaseA	600 mg/L	Lesuisse et al., 1993
Streptavidin	35 -50 mg/L	Wu et al., 2002a
scFv	10 -15 mg/L	Wu et al., 2002b
Interleukin (IL) -3	100 mg/L	Westers at al., 2006
hEGF	7.0 mg/L	Lam et al., 1998
human Interferon (IFN)-α2b	0.5-1.0 mg/L	Palva et al., 1983
human Interferon (IFN)-γ	20 mg/L	Rojas Contreras et al., 2010

Table 1. Protein products from *B. subtilis*

2. Signal peptide and propeptide

The major of Bacterial secreted proteins are translocated across the cytoplasmic membrane via the Sec pathway (Antelmann et. al. 2004). Secretory proteins are identified by a signal peptide at the protein's N-terminus. A signal peptide consists of a positively charged N-domain, a hydrophobic H domain, and a C domain containing a specific cleavage site. Most signal peptides are Sec dependent signal peptides, which are cleaved by a type I signal peptidase at the AXA cleavage site (Tjalsma et al., 2000), as an example, *B. subtilis* α-amlyase (AmyE) (Fig. 1).

2.1 Signal peptide

For the production of a heterologous protein in the culture medium of *B. subtilis*, it is necessary to use a signal peptide that directs the protein very efficiently to the translocase. However, heterologous protein secretion often results in inefficient and unsatisfyingly low

yields. The relationship between signal peptides and target proteins remains unknown. Accordingly, previous studies have indicated the need for individually optimal signal peptides for every heterologous secretion target.

Recently, Brockmeier et al. (2006) established a new strategy for the optimization of heterologous protein secretion in *B. subtilis*, by screening a library of all natural signal peptides of the strain. Accordingly, the best signal peptide for the secretion of one target protein is not automatically the best, or even sufficient, for the secretion of a different target protein (Brockmeier et al., 2006).

In our study, human interferon-α (hIFN-α) was used as a heterologous model protein, to investigate the secretion of the *B. subtilis* several major signal peptides. (Fig. 2). We found that for the secretion production of hIFN-α, the AmyE signal peptide is one of the best signal peptides (unpublished data).

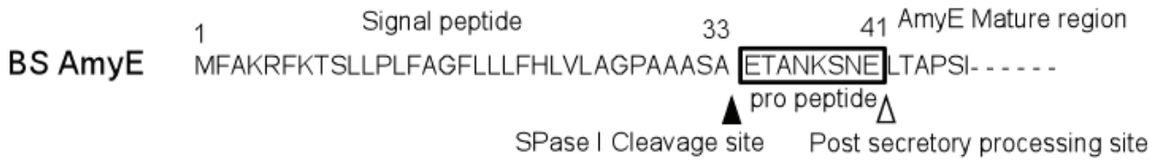


Fig. 1. The amino acid sequence of N-terminus-pre-pro AmyE. The putative SPase cleavage site is indicated by a closed arrowhead, and the post-secretory processing site is indicated by an open arrowhead, as described in the references (Takase et al., 1988; Sasamoto et al., 1989). Numbers above the AmyE amino acid sequence refer to the locations of the encoded amino acid residues of AmyE (adapted from Kakeshita et al., 2011a).

2.2 Propeptide

Some secreted bacterial proteins have cleavage propeptides located between their signal peptide and the mature protein. The propeptide is processed after translocation. Long propeptides (60 to 200 residues) are present for most bacterial extracellular proteases, which are auto-catalytically cleaved and possess intramolecular chaperon activities, for example, *B. subtilis* AprE (Braun et al., 1996; Ikemura & Inouye, 1998; Wang et al., 1998; Yabuta et al., 2001; Yabuta et al., 2002; Zhu et al., 1989). On the other hand, short propeptides (with fewer than 60 residues) are present for a few secreted proteins, including *B. subtilis* α-amylase (AmyE) (Davis et al., 1977; Mezes et al., 1983; Takase et al., 1998) (Fig. 1). In *B. subtilis*, the AmyE propeptide is cleaved by unknown proteins, and is dispensable for secretion, folding, and stability (Takase et al., 1998; Sasamoto et al., 1989).

However, the secretion efficiency of the *Staphylococcus aureus* nuclease (Nuc) was found to be enhanced by a propeptide in *E. coli* (Suciu & Inouye 1996) and *Lactococcus lactis* (Le Loir et al., 1998). In addition, in *L. lactis*, the nine-residue synthetic propeptide, LEISSTCDA, which is fused immediately after the signal peptide cleavage site, is known to enhance heterologous protein secretion (Le Loir et al., 1998; Le Loir et al., 2005; Zhuang et al., 2008; Zhang et al., 2010). Therefore, we evaluated whether the fusion of the AmyE signal peptide and the propeptide could improve the secretion of hIFNα-2b, compared to that with only AmyE signal peptide.

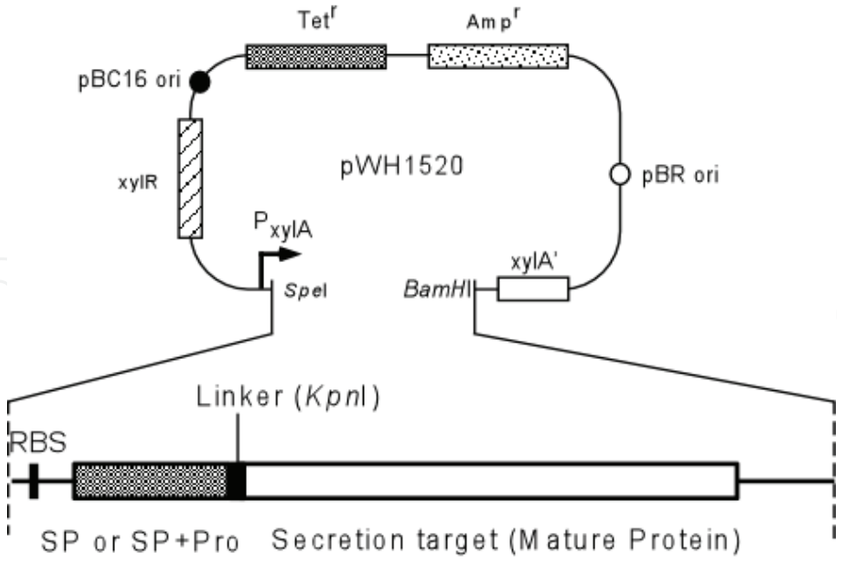


Fig. 2. Construction of plasmids for production and secretion of heterologous proteins. The restriction sites used for each construction are indicated. P_{xylA}, promoter of *xylA*; RBS, ribosome binding site; SP, signal peptide; Pro, propeptide.

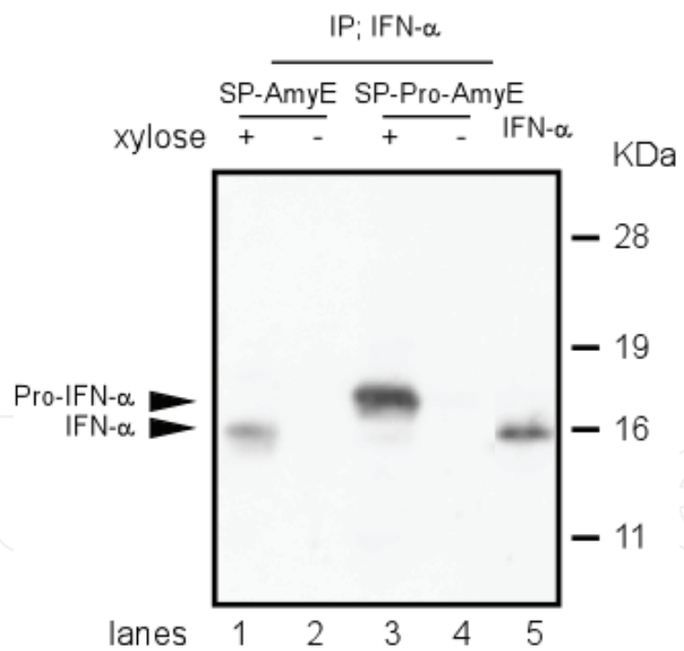


Fig. 3. Western blot analysis of hIFN-α production by *B. subtilis* Dpr8 with pHKK3101 (AmyE SP-hIFN-α) or pHKK3201 (AmyE SP-Pro-hIFN-α). Samples were collected at 20 h after xylose induction, separated by 15% SDS-PAGE, and stained with Western blotting using anti hIFN-α2b polyclonal antibodies. Dpr8 with pHKK3101 (lanes 1 and 2); Dpr8 with pHKK3201 (lanes 3 and 4); 0.6% xylose induced (lanes 1 and 3), none induced (lanes 2 and 4), and commercially purified hIFN-α 10 ng (lane 5). Arrowheads indicate the positions of the Pro-hIFN-α2b and hIFN-α2b. (adapted from Kakeshita et al., 2011a)

We showed that the secretion production and activity of hIFN-α2b with propeptide increased by more than 3-fold, compared to that without propeptide. The amount of secreted hIFN-α2b with propeptide was 15mg /L. This result indicated that the propeptide of AmyE enhanced the secretion of hIFNα-2b (Fig. 3, Kakeshita et al., 2011a).

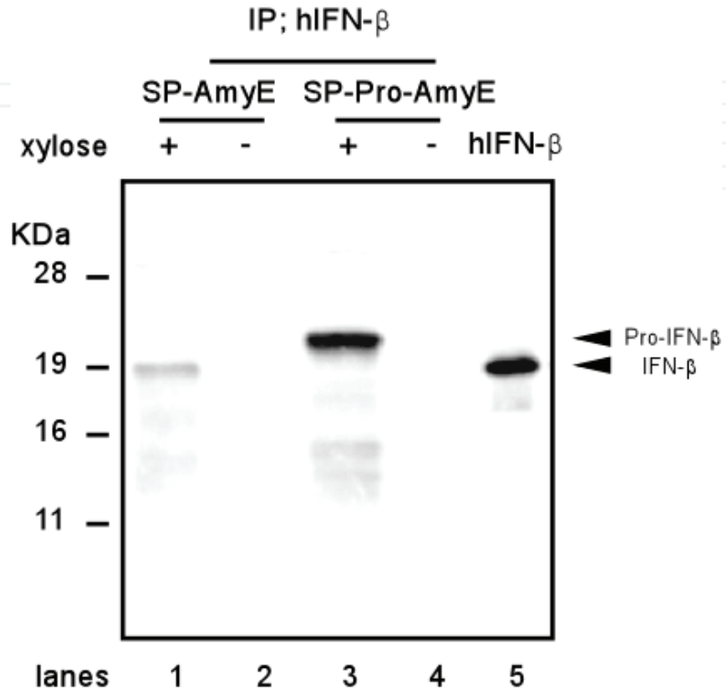


Fig. 4. Western blot analysis of hIFN-β production by *B. subtilis* Dpr8 with pHKK3111 (AmyE SP-hIFN-β) or pHKK3211 (AmyE SP-Pro hIFN-β). Samples were collected at 20 h after xylose induction, separated by 15% SDS-PAGE, and stained with Western blotting using anti hIFN-β polyclonal antibodies. Dpr8 with 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), and commercially purified hIFN-β 50 ng (lane 5). Arrowheads indicate the positions of the Pro-hIFN-β and hIFN-β. (adapted from Kakeshita et al., 2011b)

In *L. lactis*, directed mutagenesis experiments demonstrated that the positive effect of LEISSTCDA on protein secretion was due to the insertion of negatively charged residues in the N-terminus of the mature moiety (Le Loir et al., 2001). In hIFN-α2b with AmyE propeptide, the first 10 amino acid residues of this mature protein have a net charge of -1. On the other hand, hIFN-α2b without propeptide has a net charge of 0. In addition, we demonstrated that propeptide mutants of neutral or positive charge resulted in a reduction in the amount of secreted hIFN-α2b, compared with propeptides of negative charge. This result suggested that negative charges in the mature protein can enhance the secretion of hIFN-α2b (Kakeshita et al., 2011a).

We then indicated that the AmyE propeptide enhanced the secretion of the hIFN-β protein from *B. subtilis*, as well. The secretion production and activity of hIFN-β with propeptide increased by more than 4-fold (Fig. 4, Kakeshita et al., 2011b). The amount of secreted hIFN-

β with propeptide was 3.7mg /L. These results indicated that the propeptide of AmyE enhanced the secretion and extracellular production of a heterologous protein in *B. subtilis*.

2.3 Deletion of the C-terminus of SecA

In *B. subtilis*, most secreted proteins are translocated across the cytoplasmic membrane via the Sec system (Tjalsma et al., 2000; Tjalsma et al., 2004; Yamane et al., 2004). Nearly all of the components of the Sec system identified in *E. coli* have also been identified in *B. subtilis* and are biochemically well-characterized (van Wely et al., 2001; Harwood et al., 2008). Among these components, the peripheral membrane protein, SecA is considered to play a pivotal role in secretion. The SecYEG complex acts as a receptor for SecA, and functions as a preprotein conducting channel (Hartl et al., 1990; Fekkes et al., 1997). In *E. coli*, SecB is a molecular chaperone that functions in the post-translational protein translocation pathway, and binds to the C-terminal SecB binding site of *E. coli* SecA. In *B. subtilis*, this region of SecA is highly conserved. However, genome sequencing revealed that SecB is absent in *B. subtilis* (Kunst et al., 1997). *B. subtilis* Ffh interacts directly with SecA, and promotes the formation of soluble SecA-preprotein complexes (Bunai et al., 1999). These results suggest that the signal recognition particle (SRP) of *B. subtilis* not only acts as a targeting factor in co-translational translocation, but also stimulates the process of post-translocation across the membrane (Harwood & Cranenburgh, 2008; Ling et al., 2007; Tjalsma et al., 2000; Yamane et al., 2004). In additon, it has been shown that SecB binding site of *B. subtilis* SecA is not essential for viability and protein secretion (van Wely et al., 2000). The SecB binding site is connected by a C-terminal Linker (CTL) with the α-helical scaffold domain (HSD) in SecA. A cross-species comparison of the amino acid sequence of SecA revealed that the CTL is not well-conserved between *B. subtilis* and other species, including *E. coli*. We examined the effects of modifying the C-terminal region of SecA on growth and the extracellular production of heterologous proteins in *B. subtilis*, and demonstrated that the C-terminal domain (CTD) of SecA is not essential for viability or protein secretion. Furthermore, we showed that the productivity of hINF-α2b increased by 2.2-fold, compared to wild type SecA (Kakeshita et al., 2010). The crystal structure of *B. subtils* SecA indicated that CTL binds to the surface of NBF-I. The CTL-binding grove is a highly conserved and hydrophobic surface, and this grove is predicted to be one of the mature preprotein binding sites in SecA (Hunt et al., 2002). Therefore, deletion of the CTL of SecA is likely to affect SecA - preprotein interaction, and likely caused an increase in the secretion of heterologous proteins.

2.4 Co-expression of PrsA

PrsA is essential for viability and protein secretion. In protein secretion, PrsA is suggested to mediate protein folding at the late stage of secretion (Konitinen et al., 1991; Kontinen & Sarvas, 1993; Vitikainen et al., 2001). We examined the effect of co-expression of an extra-cytoplasmic molecular chaperone, PrsA. It is known that co-expression of an extra-cytoplasmic molecular chaperone, PrsA enhances the secretion of several model proteins: α - amylase, Single-chain antibody (SCA), and recombinant Protective antigen (rPA) (Kontinen & Sarvas, 1993; Vitikainen et al., 2001; Wu et al., 1998; Williams et al., 2003).

We demonstrated that co-expression of PrsA can act in concert with the AmyE propeptide to enhance the secretion production of hIFN-β. The amount of secreted hIFN-β with propeptide was 5.5mg /L. (Fig. 5, Kakeshita et al., 2011b).

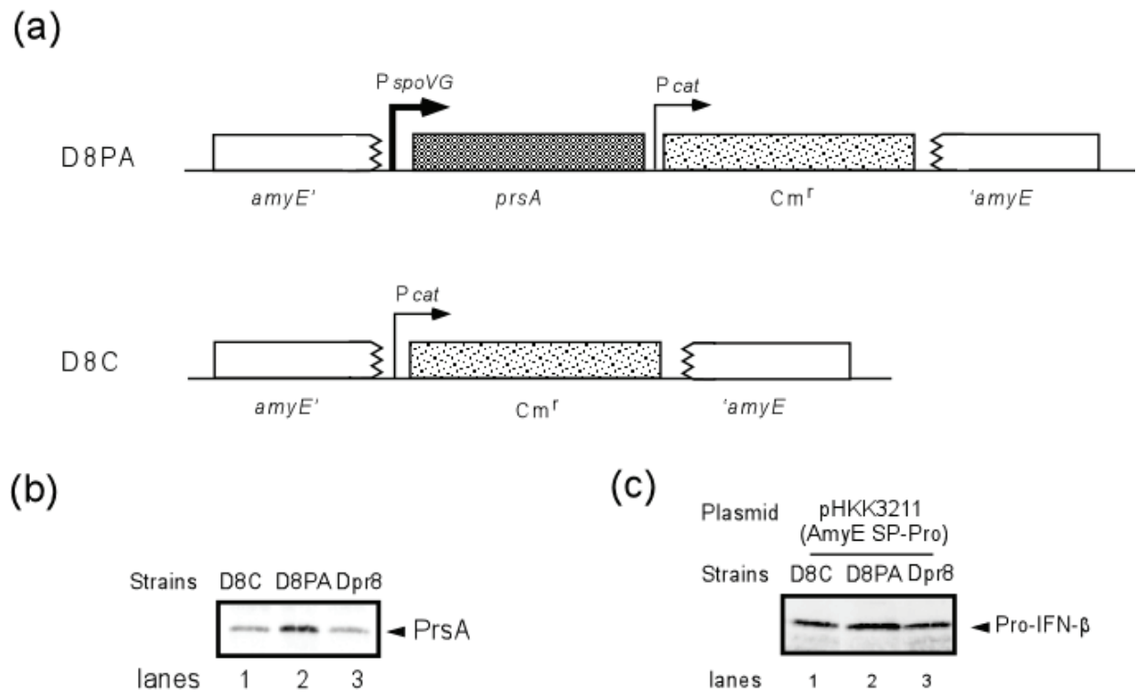


Fig. 5. Comparison of the amounts of secreted hIFN-β from *B. subtilis* D8C and D8PA, PrsA co-expressing strains. (a) Schematic representation of the gene structure around the amyE locus in the *B. subtilis* mutant strains D8PA and D8C. P_{spoVG} and *prsA* represent the *B. subtilis spoVG* promoter and *B. subtilis PrsA*, respectively. P_{cat} and Cmr represent the chloramphenicol-resistant gene promoter and coding region, respectively. (b) Western blot analysis of PrsA protein from *B. subtilis* D8C, D8PA, and Dpr8. (c) Western blot analysis of hIFN-β production by *B. subtilis* D8C, D8PA, and Dpr8. D8C with pHKK3211 (lane 1); D8PA with pHKK3211 (lane 2); Dpr8 with pHKK3211 (lane 3). Arrowheads indicate the positions of Pro-IFN-β. (Adapted from Kakeshita et al., 2011b).

3. Tat pathway

The majority of bacterial secreted proteins are translocated across the cytoplasmic membrane via the Sec pathway, which acts on unfolded proteins using the energy provided by ATP hydrolysis (Tajalsma et al., 2000; Antelman et al., 2000). Recently, a novel and different secretion protein translocation pathway, the twin-arginine translocation (Tat) pathway was discovered (Santini et al., 1998; Berks et al., 2000; van Dijl et al., 2002). The bacterial twin-arginine translocation (Tat) machinery is able to transport folded proteins across the cytoplasmic membrane (Robinson et al., 2001). The Tat pathway might have advantages over the Sec pathway for the production of heterologous proteins, because many proteins fold tightly before they reach the Sec machinery, and thus cannot engage with it for translocation across the cytoplasmic membrane.

B. subtilis contains two substrate specific Tat systems, TatAyCy and TatAdCd. The TatAyCy translocase is required for translocation of YwbN. On the other hand, a TatAdCd translocase translocates the phosphodiesterase PhoD (Jongbloed JD et al., 2002; Pop et al., 2002).

3.1 Twin-arginine signal peptide

Proteins are targeted to the Tat pathway by tripartite N-terminal signal peptides, the amino-terminal portion (n region) of which contain a conserved twin-arginine (RR) motif (R-R-X-#-#, where # is a hydrophobic residue).

In a previous study by Jongbloed et al., a database search for the presence of this motif in amino-terminal protein sequences identified a total number of 27 putative RR-signal peptides.

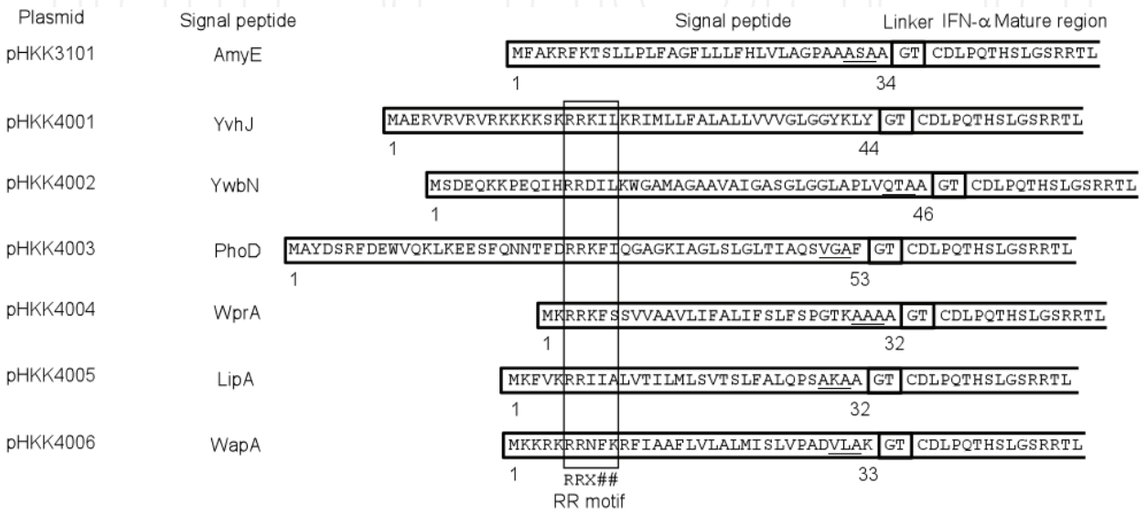


Fig. 6. Schematic representation of the signal sequences used for secretion of human Interferon-α in *B. subtilis*. Schematic structure of the proteins encoded by each indicated plasmid. The twin-arginine motif is boxed, and the residues at positions -3 to -1 relative to the predicted SPase I cleavage site are underlined. The six base pairs of the KpnI site add the amino acids Gly–Thr to the end of each signal peptide coding sequence; therefore, in the table, each sequence ends with GT. Numbers under the signal peptides refer to the respective locations of the encoded amino acid residues.

We therefore selected six candidate Tat signal peptides, shown in Fig. 6, from the list generated by Jongbloed et al. for testing in the hIFN-α secreted assay. To determine the secretion ability for hINF-α2b, the six signal peptide genes considered to belong to the Tat pathway of *B. subtilis* were PCR-amplified. The PCR-amplified signal peptide genes were inserted upstream of the hIFN-α mature peptide gene in pHKK3101, yielding six secretion expression vectors. pHKK3101 expressing hIFN-α with the AmyE signal peptide, as the Sec-type signal peptide, was used as the control plasmid. The resultant recombinants were transformed into *B. subtilis* Dpr8, respectively, and the secretion expression of hIFN-α mediated by these signal peptides was detected by immunoblotting analysis. The hIFN-α was expressed in these strains and hIFN-α production was induced with the addition of 0.6% of xylose to the exponentially growing cultures (OD660 = 0.3), and both culture supernatants and intracellular lysates were analyzed as described in Kakeshita et al. (2010). As shown in Fig. 7a, in the extracellular fraction, only one band corresponding to mature protein (16 kDa) was detected for the samples of *B. subtilis* Dpr8 cells harboring

pHKK3101 (AmyE signal), pHKK4004 (WprA), pHKK4005 (LipA), and pHKK4006 (WapA) by Western blot and immunoblot. This result suggested that the obtained three signal peptides (WprA, LipA, WapA) directed efficient secretion expression.

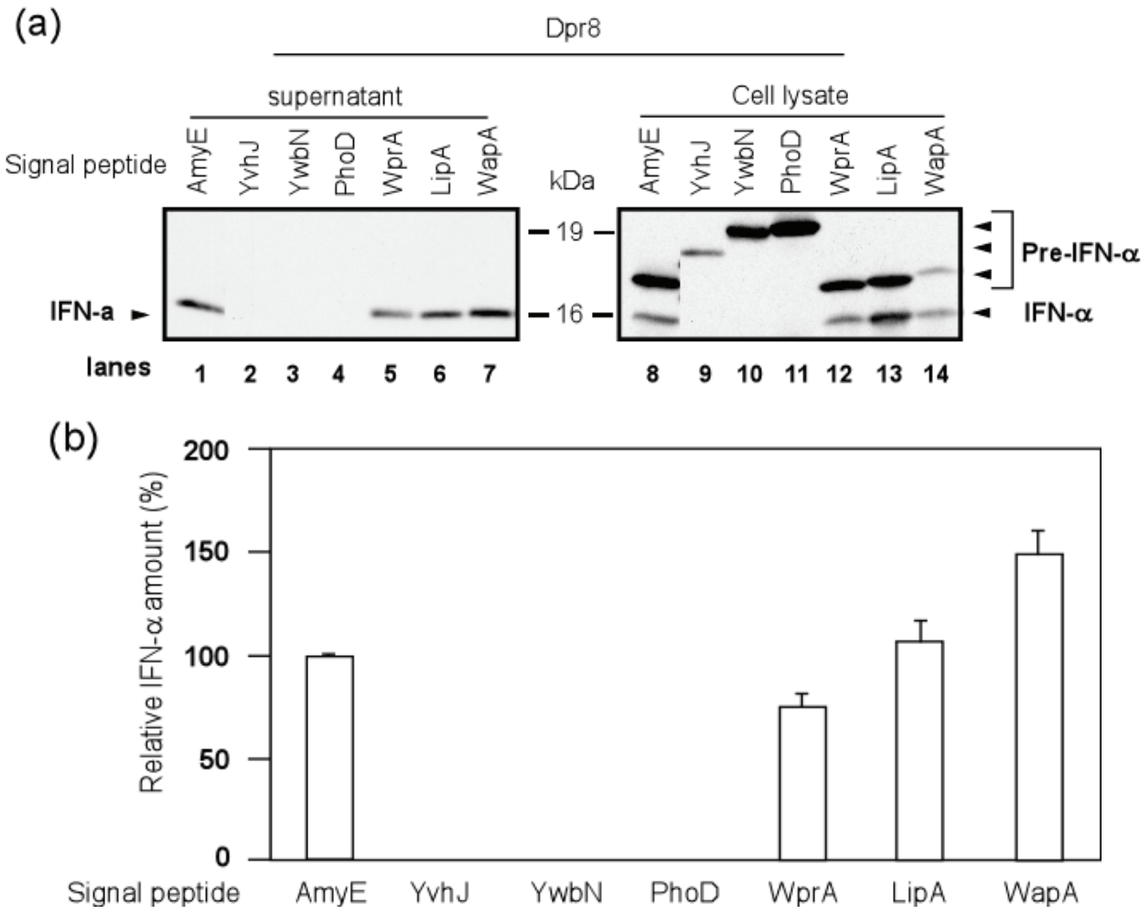


Fig. 7. Comparison of the amounts of secreted hIFN-α using the Twin arginine signal peptides from *B. subtilis* Dpr8. (a) Western blot analysis of hIFN-α production in *B. subtilis* Dpr8 harboring seven recombinants. Cells were grown at 30 °C in 2xL medium. Samples were collected at 20 h after xylose induction, separated by 15% SDS-PAGE, and subjected to Western blotting using anti hIFN-β polyclonal antibodies. Protein samples present in the supernatant (lanes 1, 2, 3, 4, 5, and 6) and cell fractions (lanes 7, 8, 9, 10, 11, and 12) of stationary-phase cultures were prepared by centrifugation, analyzed by SDS-PAGE, and immunodetected with anti-hIFN-α antibodies. Dpr8/pHKK3101 (lanes 1 and 8); Dpr8/pHKK4001 (lanes 2 and 9); Dpr8/pHKK4002 (lanes 3 and 10); Dpr8/pHKK4003 (lanes 4 and 11); Dpr8/pHKK4004 (lanes 5 and 12); Dpr8/pHKK4005 (lanes 6 and 13); Dpr8/pHKK4006 (lanes 7 and 14); precursor, pre hIFN-α; mature, hIFN-α. S, supernatant; C, cell fractions. (b) Quantification of secreted hIFN-α mature form in the culture medium and cell fraction. The hIFN-α production corresponding to the supernatant of *B. subtilis* Dpr8 carrying pHKK3101 (AmyE signal peptide) was set as 100%. Data represent the mean of three experiments, and error bars represent standard error.

Especially, WapA demonstrated the highest efficiency of hIFN-α secretion expression, which was 1.5-fold as high as the Sec dependent signal peptide, AmyE (Fig. 7b).

However, No hIFN-α was detected in the supernatants of Dpr8/pHKK4001 (YvhJ), Dpr8/pHKK4002 (YwbN), or Dpr8/pHKK4003 (PhoD). In the intracellular lysates of Dpr8/pHKK3101, Dpr8/pHKK4004, Dpr8/pHKK4005, and Dpr8/pHKK4006, two bands were detected. As deduced from the molecular mass of each band, these bands ware assigned to the unprocessed precursor (17 kDa) and the mature protein (16 kDa), respectively. On the other hand, only one band corresponding to the unprocessed protein was detected for the samples of Dpr8/pHKK4001 (YvhJ), Dpr8/pHKK4002 (YwbN), and Dpr8/pHKK4003 (PhoD).

These results suggested that the three obtained signal peptides, YvhJ, YwbN, and PhoD cannot be secreted hIFN-α2b into the supernatant.

3.2 Co-expression of the tat system

We examined the effect of co-expression of the Tat-machinary, TatAd/Cd or TatAy/Cy. To examine the effects of the co-expression of *B. subtilis* tat genes on hIFN-α secretion, we constructed TatAd/TatCd and TatAy/TatCy under the control of the *spoVG* promoter in plasmids. It is known that the *spoVG* promoter is a powerful promoter (Zuber & Losick 1983). The resulting constructs were subsequently integrated into the chromosome of *B. subtilis* strain Dpr8 via a double crossover event at the *amyE* locus, leaving the native tat genes intact (Fig. 8a).

The resultant strains, D8tatD and D8tatY were transformed with pHKK3101, pHKK4001, pHKK4002, pHKK4003, pHKK4004, pHKK4005, and pHKK4006 for expression of hIFN-α.

As shown in Fig. 8b and c, when the LipA signal peptide was fused to hIFN-α, a densitometric analysis of the western blotting demonstrated that the amounts of hIFN-α secreted by D8tatD and D8tatY were increased by roughly 2-fold, compared with that in strain Dpr8 (Fig. 8c). When the WprA signal peptide was fused to hIFN-α, in D8tatD, the amount of secreted hIFN-α was increased by 71% compared with that in the parental strain, Dpr8, whereas the enhanced production of hIFN-α increased by 29%. On the other hand, When the WapA signal peptide was fused to hIFN-α, the amounts of hIFN-α secreted by D8tatD and D8tatY were increased by only 10-20%, compared with that in strain Dpr8 (Fig. 8c). Then, when the AmyE signal peptide was fused to hIFN-α, the amounts of hIFN-α secreted by D8tatD and D8tatY were increased by 37% and 25%, respectively compared with that in strain Dpr8 (Fig. 8c). Therefore, WapA signal peptide and AmyE signal peptide are not able to enhance of secretion by co–expression of Tat system. In addition, when the YvhJ, YwbN, and PhoD signal peptides, respectively were fused to hIFN-α, the bands of hIFN-α secreted by D8tatD and D8tatY could not be detected in the resulting supernatants (data not shown).

We demonstrated that co-expression of TatAd/Cd or TatAy/Cy with LipA signal peptide can act in concert to enhance the secretion production of hIFN-α. In addition, WprA signal peptide was enhanced the secretion production of hIFNα by co-expression of TatAd/Cd, not TatAy/Cy. On the other hands, AmyE signal peptide and WapA peptide are Tat pathway independent.

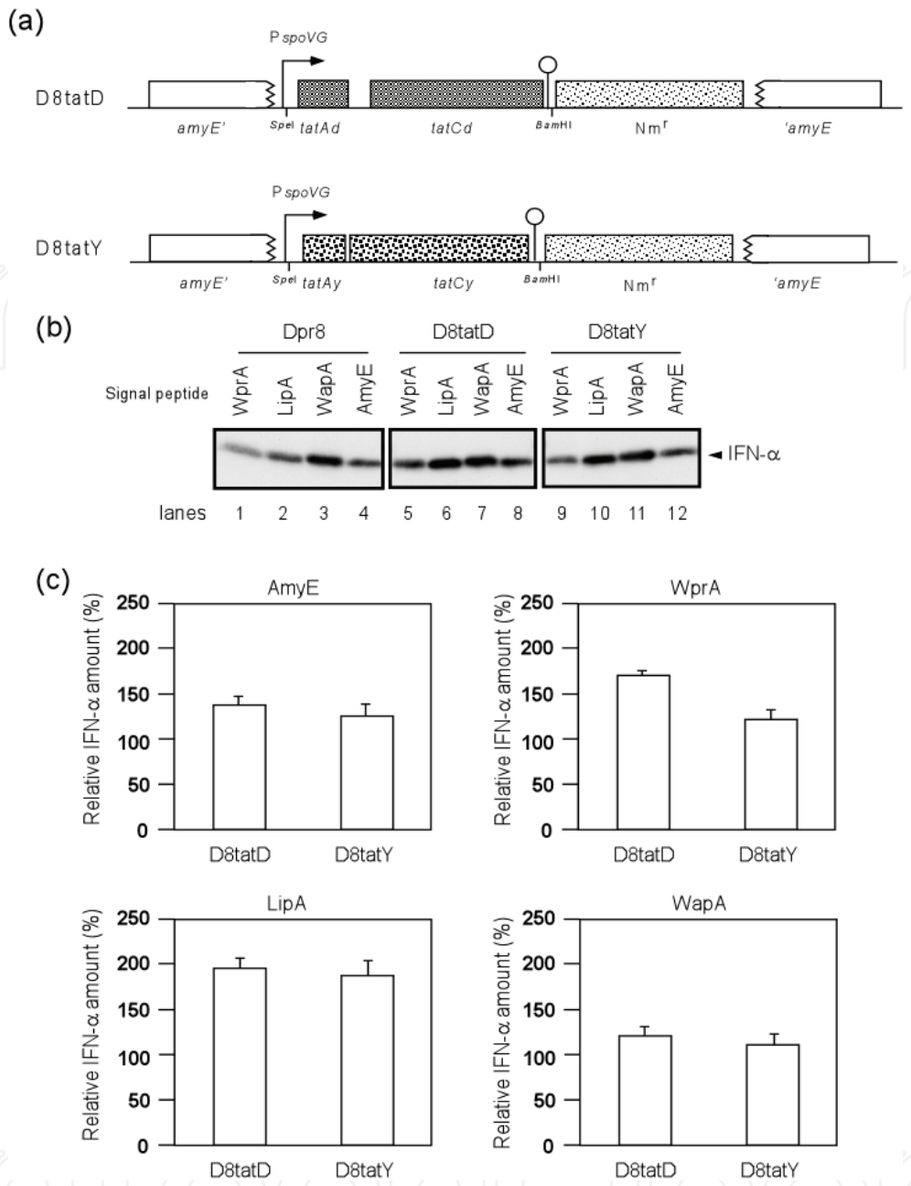


Fig. 8. Comparison of the amounts of secreted hIFN-α from *B. subtilis* Dpr8 and Tat overexpressing strains. (a) Schematic representation of the gene structure around the *amyE* locus in the *B. subtilis* D8tatD and D8tatY mutant strain genomes. Construction of strains D8tatD and D8tatY was by double crossover integration of plasmids pHKK2001 (*tatAd-Cd*) and pHKK2002 (*tatAy-Cy*) into the *amyE* locus of *B. subtilis* Dpr8. The resulting strain contains the native *phoD-tatAd-tatCd* locus, as well as one copy of *tatAd-Cd* and *tatAy-Cy* under the control of the P_{spoVG} promoter. The stem-loop structures and the bent arrows indicate the putative Rho-independent terminators and promoters, respectively. (b) Western blot analysis of hIFN-α production by *B. subtilis* Dpr8, D8tatD, and D8tatY (carrying pHKK3101, pHKK4004, pHKK4005, or pHKK4006) was performed in the same manner as for hIFN-α. (c) Quantification of secreted hIFN-α in mature form in the culture medium. The hIFN-α production corresponding to the *B. subtilis* Dpr8 strain was set as 100%. Data represent the mean of three experiments, and error bars represent standard error.

4. Conclusions

In recent years, considerable efforts have been targeted at developing *B. subtilis* as a host for the production of heterologous proteins. However, the secretion of heterologous proteins from eukaryotes by these species produces small yields and is frequently inefficient. Initially, we considered the major problem to be the presence of high levels of extracellular protease in *B. subtilis*. Nevertheless, even after obtaining many depleted protease strains, the problem of inefficient secretion was not resolved. Currently, it is considered that the largest problem is the detection of the pre-mature form of human protein in cell lysate, when human proteins with signal peptide are over expressed in *B. subtilis* (Fig. 7a). Normally, the pre-mature forms of target secretion proteins are not detected in cell lysates. If the pre-mature form of target a secretion protein is detected, it indicates a problem in the secretion pathway, for example, non-functional or depleted SecA, SecY, Ffh, or FtsY (Sadaie et al. 1991; Takamatsu et al., 1992; Honda et al., 1993; Oguro et al., 1995; Tjalsma et al., 2000; Tjalsma et al., 2004; Yamane et al., 2004). Therefore, we must solve this primary problem, which is the accumulation of the precursor of human proteins in *B. subtilis* cells.

We indicated that the propeptide of AmyE enhanced the secretion of the extracellular production of a heterologous protein in *B. subtilis*. In *L. lactis*, the nine-residue synthetic propeptide, LEISSTCDA, which is fused immediately after the signal peptide cleavage site, is known to enhance heterologous protein secretion (Le Loir et al., 1998). In addition, LEISSTCDA enhances secretion efficiency (Le Loir et al., 2001). Therefore, it is considered that a short type propeptide may be one answer to improve the accumulation of precursor.

On the other hand, we indicated that the deletion of the C-terminal domain of SecA enhanced the secretion of heterologous proteins. *secA* is an essential gene, and SecA is considered to play a pivotal role in secretion (Sadaie et al. 1991; Takamatsu et al., 1992; Tjalsma et al., 2000; Tjalsma et al., 2004; Yamane et al., 2004). In addition, we exhibited that the co-expression of PrsA or the Tat system can be able to enhance the secretion production. In the future, it may be necessary to modify the components of the secretion machinery for higher secretion efficiency.

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Biotechnology is the scientific field of studying and applying the most efficient methods and techniques to get useful end-products for the human society by using viable micro-organisms, cells, and tissues of plants or animals, or even certain functional components of their organisms, that are grown in fully controlled conditions to maximize their specific metabolism inside fully automatic bioreactors. It is very important to make the specific difference between biotechnology as a distinct science of getting valuable products from molecules, cells or tissues of viable organisms, and any other applications of bioprocesses that are based on using the whole living plants or animals in different fields of human activities such as bioremediation, environmental protection, organic agriculture, or industrial exploitation of natural resources. The volume Advances in Applied Biotechnology is a scientific book containing recent advances of selected research works that are ongoing in certain biotechnological applications. Fourteen chapters divided in four sections related to the newest biotechnological achievements in environmental protection, medicine and health care, biopharmaceutical producing, molecular genetics, and tissue engineering are presented.

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Phone: +86-21-62489820
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