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Approaches for Improving Protein Production in Multiple Protease-Deficient *Bacillus subtilis* Host Strains

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1. Introduction

Bacillus subtilis is a Gram-positive, nonpathogenic organism which is widely used as a host for enzyme production, due to its ability to secrete large amounts of proteins into the growth medium (Simonen et al., 1993; Westers et al., 2004). The secretion of a target protein leads to the natural separation of the product from cell components, which simplifies downstream processing of the protein. Accordingly, there has been a great deal of research performed regarding protein production in *B. subtilis* (Simonen et al., 1993; Westers et al., 2004). Nevertheless, the yields of heterologous protein obtained from this organism are often insufficient (Harwood, 1992). Several bottlenecks in the *B. subtilis* secretion pathway have been reported, including poor targeting to the translocase, degradation of the secretory protein, and incorrect folding (Westers et al., 2004). One of the major bottlenecks involves the degradation of the produced protein by extracellular proteases; therefore, inactivation of extracellular proteases is essential for improvement of protein production with *B. subtilis* as the host.

2. Inhibition of proteolysis of heterologous and nature proteins after the translocation process by inactivation of multiple proteases

Eight extracellular proteases have been identified in *B. subtilis* to date, which are encoded by the following genes: *aprE* (Stahl et al., 1984; Wong et al., 1984), *bpr* (Sloma et al., 1990b; Wu et al., 1990), *epr* (Bruckner et al., 1990; Sloma et al., 1988), *mpr* (Rufo et al., 1990; Sloma et al., 1990a), *nprB* (Tran et al., 1991), *nprE* (Yang et al., 1984), *vpr* (Sloma et al., 1991), and *wprA* (Margot et al., 1996). Deletions in the *aprE* (encoding subtilisin, alkaline protease) and *nprE* (encoding neutral protease) genes were the first such mutations, whose mutants show lower activities of extracellular proteases (Sloma et al., 1991). In addition, a deletion mutation in the *epr* gene resulted in low protease activity in the culture supernatant. *wprA* encodes a 96-kDa protein that is processed to the CWBP23 propeptide and CWBP52 mature protease, forming a complex associated with the cell wall (Margot et al., 1996). This complex was also
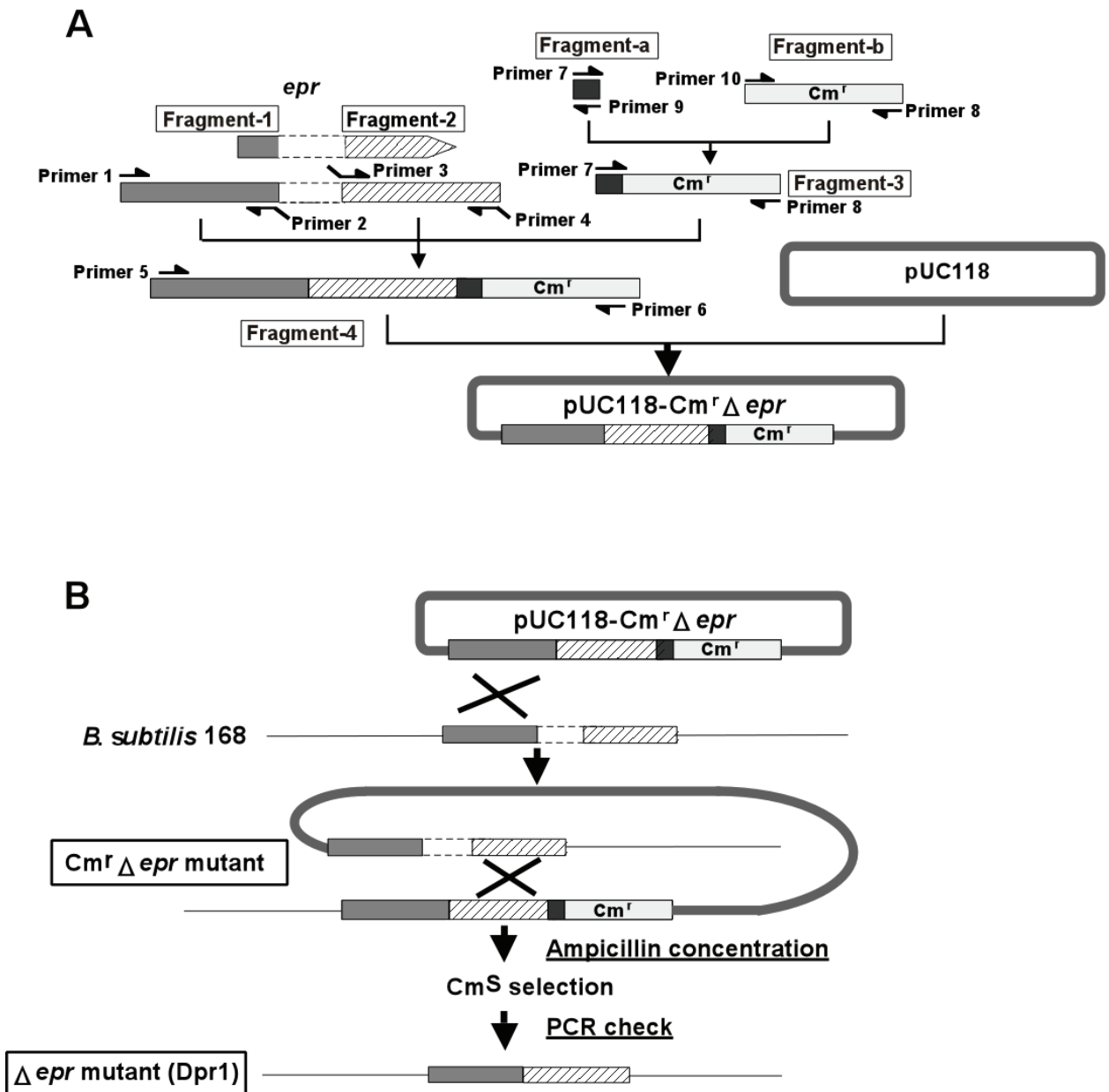
Fig. 1. Strategy for construction of a Δepr mutant. (A) Construction of a chloramphenicol-resistance (Cm^r) plasmid, pUC118-Cm^{r}Δepr. Fragment 3 was amplified with fragment a (containing the repU promoter of pUB110) and fragment b (containing the chloramphenicol resistance gene of pC194), and primers 7 and 8. Fragment 4 was amplified with fragments 1, 2, 3, and primers 5 and 6. The amplified fragment 4 was prepared by blunting and kination, and then cloned into the SmaI site of pUC118 to generate pUC118-Cm^{r}Δepr. (B) Construction of the Δepr mutant. B. subtilis 168 cells were transformed with pUC118-Cm^{r}Δepr, followed by selection for chloramphenicol resistance, obtaining Cm^{r}Δepr. To obtain a Δepr mutant (deleted chloramphenicol-resistance cassette), the ampicillin concentration method was used (Kodama et al., 2007a). The chloramphenicol-sensitive (Cm^s) Δepr mutant was confirmed by PCR using primers 5 and 4.

found in the culture supernatant of *B. subtilis* WB600 (Babe et al., 1998; Wu et al., 1991). Whether it is present in the cell wall or in the culture medium is therefore a critical factor in the degradation of heterologous proteins (Lee et al., 2000). Strains with deletion mutations in multiple extracellular proteases have since been constructed with extracellular protease activities of less than 0.5%, compared to the parental strain (Wu et al., 1991). It was recently reported that an eight protease-deficient strain, WB800, was a useful host for the production of various heterologous proteins (Murashima et al., 2002; L. Westers et al., 2006). However, the use of *B. subtilis* as a host has remained limited to bulk industrial enzyme production. Further optimization is necessary to develop production systems for heterologous proteins. This chapter focuses on the inhibition of proteolysis of secreted proteins after the translocation process by inactivation of multiple proteases which are extracellular (AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr, and WprA), leaked outside from intracellular (AprX), and membrane-bound (HtrA and HtrB).

2.1 The intracellular protease, AprX is involved in degradation of a heterologous protein

In *B. subtilis*, extracellular protease-deficient mutants have been used in attempts to increase the productivity of heterologous proteins. We detected the protease activity of AprX using protease zymography in the culture medium at the late stationary growth phase. Construction of multiple-protease-deficient mutant without antibiotic-resistance markers and the effect of AprX on the heterologous protein production are descrived in detail in the following sections.

2.1.1 Construction of an eight-extracellular-protease-deficient mutant by marker-free deletion in *B. subtilis*

Antibiotic-resistance marker genes were used to create new bacterial strains. However, the number of markers available for use in *B. subtilis* and other bacteria is limited. We used the "ampicillin concentration" method for the creation of eight-extracellular-protease-deficient mutant with marker-free deletion (Fig. 1, Kodama et al., 2007a). Recently, several useful methods were developed to produce unmarked mutations in *B. subtilis* (Liu et al., 2008; Morimoto et al., 2008; Morimoto et al., 2009). These systems are more convenient for the introduction of multiple mutations.

2.1.2 Detection of AprX activity in the culture supernatant with protease zymography

Zymography has been used to detect proteolytic enzymes after electrophoretic separation in gels. Recently, the activities of some proteases, including Vpr have been detected by fibrin zymography of the extracellular proteins of *B. subtilis* (Murashima et al., 2002; L. Westers et al., 2006). The supernatant proteins of *B. subtilis* culture in modified 2xL broth (Kodama et al., 2007b) at 8 h (exponential growth phase), 25 h (early stationary phase), 50 h (mid-stationary phase), and 75 h (late stationary phase) (Fig. 2A) were analyzed by gelatin zymography (Fig. 2B). The resulting zymogram shows the protease activities as clear bands (or zones). In the exponential growth phase (8 h), no protease activity was detected (Fig. 2B, lane 1). However, protease activity increased during the stationary phase, and was highest

at 75 h of the late stationary phase (Fig. 2B, lane 4). We examined the zymogram profile of the supernatant from the eight-extracellular-protease-deficient mutant (Dpr8) at 75 h, and found one clear band in the zymogram (Fig. 2C, lane 2). Protease activity disappeared in the aprX mutant at 75 h (Fig. 2C, lane 3). All of the protease activities completely disappeared in the KA8AX strain, in which nine genes (eight extracellular protease genes and *aprX*) were disrupted (Fig. 2C, lane 4). These results support the idea that the protease is serine protease AprX. To determine the serine and metal protease activities of this protease by zymography, PMSF and EDTA (2 mM each) were added to the supernatant of the 75 h culture of Dpr8 (Fig. 2D). EDTA decreased the activity of the protease slightly, whereas 2 mM PMSF completely inhibited the protease activity (Fig. 2D). These results suggest that the gelatin-degrading protease from the supernatant of Dpr8 culture is AprX. To determine whether AprX is the gelatin-degrading protease in the supernatant of the Dpr8 culture at 75 h, the AprX-FLAG fusion protein was constructed. The fusion gene was expressed with the original promoter and ribosomal binding site. On a zymographic gel, the activity bands corresponding to AprX-FLAG from both 168/AprX-FLAG and Dpr8/AprX-FLAG strains were located at slightly larger positions in size than those of AprX (Fig. 3). The size of the band corresponded to the size of the FLAG peptide. These results indicate that the activity of AprX is detectable as a single band by gelatin zymography of the supernatant of a 75 h culture of *B. subtilis* strains.
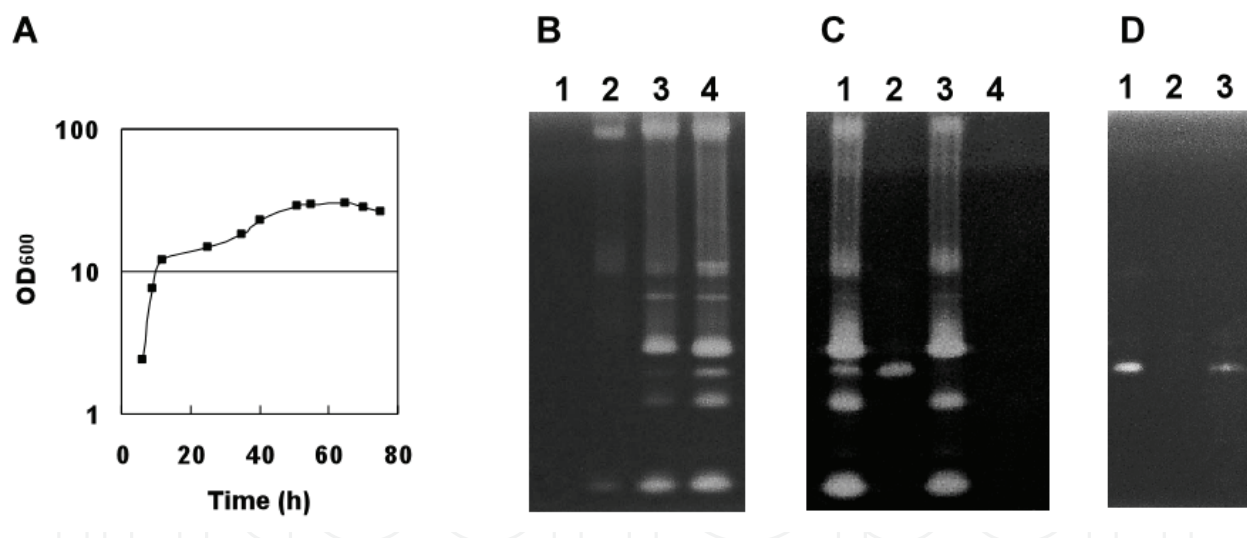

Fig. 2. (A) Cells from the wild-type were cultured in modified 2xL broth at 30°C. (B) Protein samples were prepared from the supernatants of the wild-type, cultured for various incubation times. Lane 1, 8 h culture; lane 2, 25 h; lane 3, 50 h; lane 4, 75 h. (C). Protein samples were prepared from the supernatants of the protease-deficient mutants after a 75 h culture. Lane 1, 168; lane 2, Dpr8; lane 3, AprXdd; lane 4, KA8AX. (D) PMSF or EDTA (2 mM each) was added to the supernatants of Dpr8 after a 75 h culture. Lane 1, Control (no addition); lane 2, addition of 2 mM PMSF; lane 3, addition of 2 mM EDTA. The samples were analyzed on SDS-12% polyacrylamide gels with 0.1% (w/v) gelatin. Proteins from the culture supernatants (equivalent to 0.3 μl) were applied to each lane for panels B, C, and D.
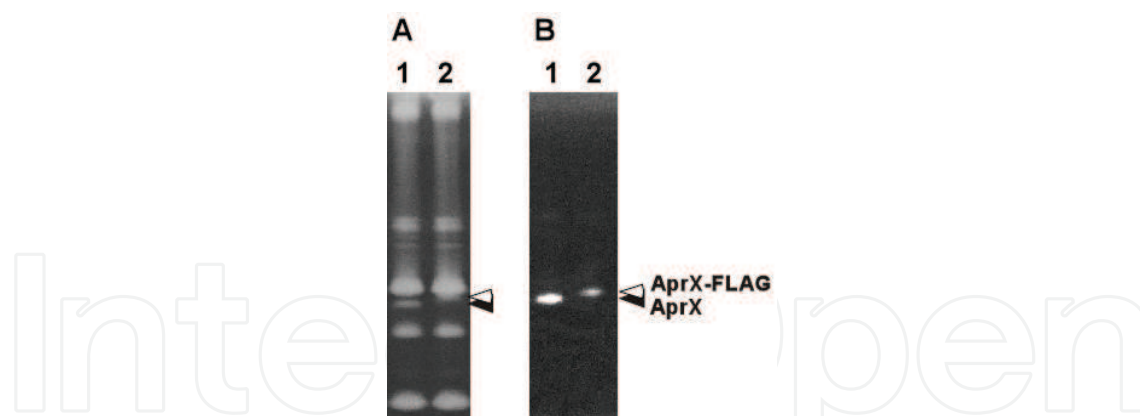
Fig. 3. Zymography of AprX-FLAG proteases. (A) Lane 1, 168; lane 2, 168/AprX-FLAG. (B) Lane 1, Dpr8; lane 2, Dpr8/AprX-FLAG. Proteins from the supernatants of the 75-h cultures (equivalent to 0.3 μl) were applied to the lanes for panels A and B. Arrowheads indicate the positions of AprX (closed symbol) and AprX-FLAG (open symbol).

2.1.3 Intracellular AprX leaked to the culture medium during the late stationary phase

It has been supposed that AprX is a serine protease belonging to the subtilase superfamily, and that it is an intracellular protease, because a canonical signal sequence for secretion has not been found in this protease (Valbuzzi et al.; 1999). However, AprX was detected in the culture medium by gelatin zymography (Fig. 3). *aprX* is transcribed during the stationary phase, and the regulator of SinR exerts negative effect on its transcription directly or indirectly (Valbuzzi et al.; 1999). However, *aprX* is not essential for either growth or sporulation (Valbuzzi et al.; 1999). As a result, the function of AprX has remained poorly understood. The Western blotting of AprX-FLAG from the intracellular fraction showed that the expression of AprX-FLAG began at 25 h, and that the expression level markedly increased after 50 h (Fig. 4). Our results agreed with a previous report that *aprX* is transcribed during the stationary phase. In contrast, a weak AprX-FLAG expression was detected in the supernatant only in the late stationary phase at 75 h (Fig. 4). This result agreed with the zymogram pattern of wild-type AprX (Fig. 2B). A slight decrease in cell density was observed after 50 h for the wild-type (Fig. 2A) and 168/AprX-FLAG strains (data not shown). The bands corresponding to AprX-FLAG from both the intra- and extracellular fractions were located at the same position. This result suggests that there is insufficient secretion of AprX, due to the absence of an obvious signal sequence. These observations also suggest that AprX is localized intracellularly by nature, and is leaked to the culture medium during the late stationary phase due to cell lysis.
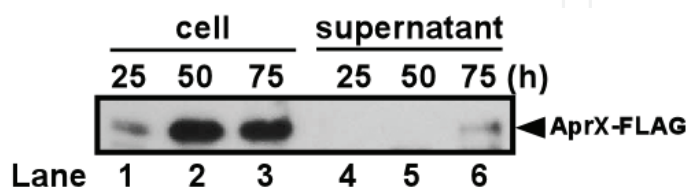

Fig. 4. Western blot analysis of AprX-FLAG. Western blot analysis was carried out to detect AprX-FLAG in the 168/AprX-FLAG strain with the anti-FLAG antibody. Proteins of cells (lanes 1-3) and supernatants (lanes 4-6) from 168/AprX-FLAG (0.02 OD600 units) were prepared as described in Materials and Methods. The arrowhead indicates the position of AprX-FLAG. The times of harvest of cells and supernatants are shown at the top.

2.1.4 AprX involved in degradation of the α-amylase-A522-PreS2 hybrid protein

AprX in the supernatant was able to degrade gelatin. Therefore, we considered that AprX may affect the production of secreted proteins. pTUBE522-preS2 has already been developed for the extracellular production of small peptides of the human hepatitis B virus preS2 antigen (42 amino acids) fused with *B. subtilis* α-amylase (deleting the C-terminal region to construct a peptide carrier) (Honda et al., 1993). To confirm the effect of AprX on the degradation of heterologous proteins, we examined the production of α-amylase-A522-PreS2 as a model of heterologous proteins, in multiple-protease-deficient *B. subtilis* strains. Cells carrying pTUBE522-preS2 were cultured in modified 2xL broth for 25, 50, and 75 h. α-amylase-A522-PreS2 in the supernatants from the cultures of Dpr7, Dpr8, and KA8AX strains was analyzed by Western blotting with the anti-PreS2 antibody (Fig. 5A). The Dpr7 strain lacked seven extracellular proteases (AprE deficiency excluded). No positive band corresponding to α-amylase-A522-PreS2 was detected at any phase for Dpr7 (pTUBE522-preS2) (Fig. 5A, lanes 1-3). Dpr8 (pTUBE522-preS2) produced α-amylase-A522-PreS2 at detectable levels, and production of the hybrid protein attained high levels after 50 h (Fig. 5A, lane 5). However, when AprX was produced in the supernatant of Dpr8 (pTUBE522-preS2) at 75 h, the amount of α-amylase-A522-PreS2 decreased markedly (Fig. 6A, lane 6). As expected, the degradation of α-amylase-A522-PreS2 was markedly inhibited in KA8AX (pTUBE522-preS2) at 75 h, with the relative amount of the hybrid protein produced by this strain being 1.8-times higher than that of Dpr8 at 50 h (Fig. 5A, lanes 5 and 9; Fig. 5B). KA8AX produced α-amylase-A522-PreS2 up to 80 mg/L, which is at least eightfold higher than the amount produced by the improved strain in a previous study (Lee et al., 2000; Fig. 5, lane 9). We also examined the degradation of α-amylase-A522-PreS2 by AprX protease. First, we prepared AprX protease from KA8AX (pDG-AprX) that was grown in a medium containing 1 mM IPTG for 4 h. The overexpression of AprX was confirmed by gelatin zymography (Fig. 6A, lane 2). Afterwards, the α-amylase-A522-PreS2 protein prepared from the supernatant of KA8AX (pTUBE522-preS2) at 75 h was mixed with AprX protease, and the mixture was incubated at 37°C for 60 min. The degradation of α-amylase-A522-PreS2 was analyzed by Western blotting using the anti-PreS2 antibody. H_2O and intracellular proteins extracted from KA8AX (pDG-AprX) cells cultured without IPTG did not decrease the amount of the α-amylase-A522-PreS2 protein (Figs. 6B, C, lanes 2 and 4), but intracellular proteins extracted from KA8AX (pDG-AprX) cells cultured with 1 mM IPTG decreased the amount to 70% (Figs. 6B, C, lanes 5 and 6).

These results indicate that the AprX protease directly degraded the α-amylase-A522-PreS2 protein. One bottleneck of the production of α-amylase-A522-PreS2 was partially solved by the disruption of eight extracellular proteases and AprX, as shown in this chapter. However, the supernatant from the KA8AX culture at 75 h contained not only a small amount of α-amylase-A522-PreS2, but also a large amount of α-amylase protein (determined by Western blotting with the anti-α-amylase antibody; data not shown). On other hand, no PreS2 peptide was detected by Western blotting with the anti-PreS2 antibody (Fig. 5A). These results indicate that the degradation of α-amylase-A522-PreS2 was not inhibited completely in the KA8AX strain, and that there were as yet unidentified protease(s) involved in the proteolysis of the PreS2 region. Therefore, there is still room for improving the inhibition of hybrid protein degradation. It has been reported that IspA (Isp) was identified as a major intracellular serine protease (Koide et al., 1986). We evaluated the inhibition of the

degradation of α-amylase-A522-PreS2 by the inactivation of IspA in the KA8AX strain. However, the productivity of α-amylase-A522-PreS2 in the ten-protease deficient mutant was almost same as that in the KA8AX strain. A search in the GenoList database for *B. subtilis* 168 genome (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList) of proteases and peptidases revealed the presence of 31 known and 11 putative proteases, and 38 known and 12 putative peptidases, respectively. Section 2.2 describes the investigation of membrane-bound proteases involved in protein degradation.
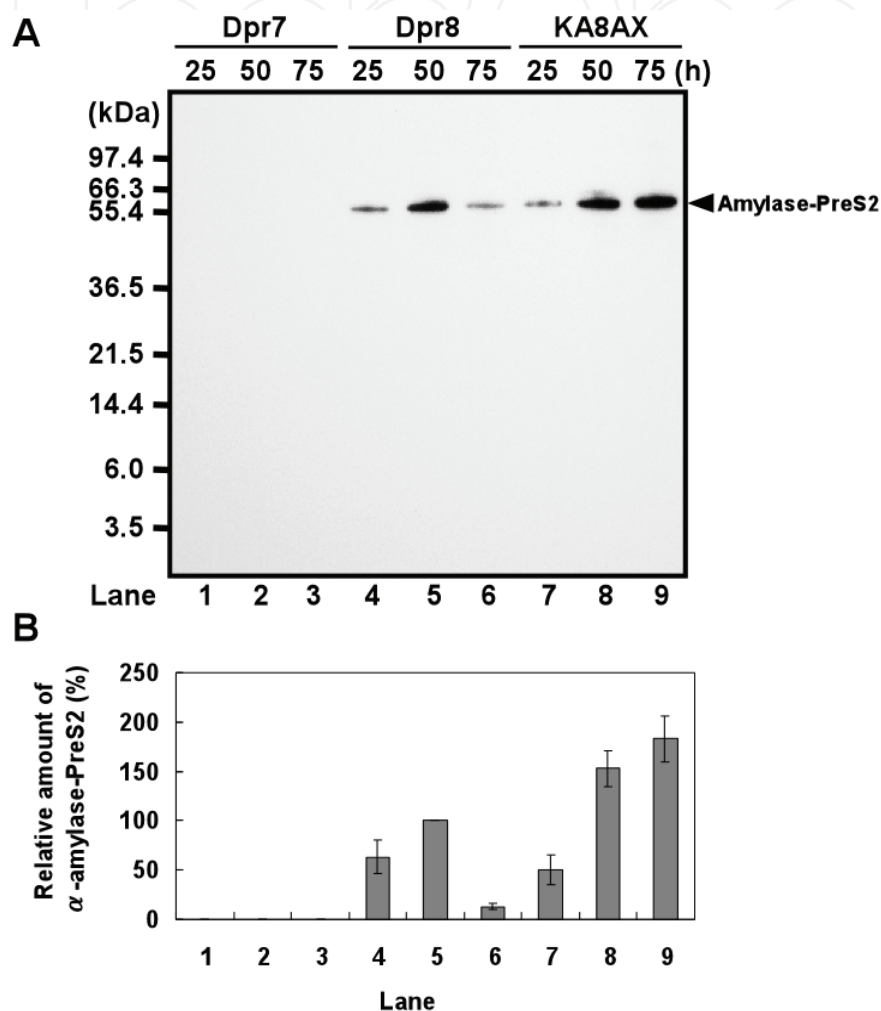
Fig. 5. Western blot analysis of the α-amylase-A522-PreS2 hybrid protein in the extracellular fractions of Dpr7, Dpr8, and KA8AX. (A) Western blot analysis was carried out to detect α-amylase-A522-PreS2 with the anti-PreS2 antibody. Culture supernatants from Dpr7 (lanes 1-3), Dpr8 (lanes 4-6), and KA8AX (lanes 7-9) were collected after 25, 50 h, and 75 h of cultivation, and subjected to Tricine-SDS-PAGE and Western blotting, as described in the Materials and Methods. Proteins from the culture supernatants (equivalent to 1 μl) were applied to each lane. The arrowhead indicates the position of α-amylase-A522-PreS2. The times of harvest of supernatants are shown at the top. (B) The relative α-amylase-A522-PreS2 protein amounts were compared on the basis of band intensities on Western blots (the amount of α-amylase-A522-PreS2 at 50 h in the Dpr8 strain was set to 100%). The presented results are the average of three individual experiments. Error bars correspond to the standard errors of the means (SEM). Lane numbers in panel A correspond to those in panel B.
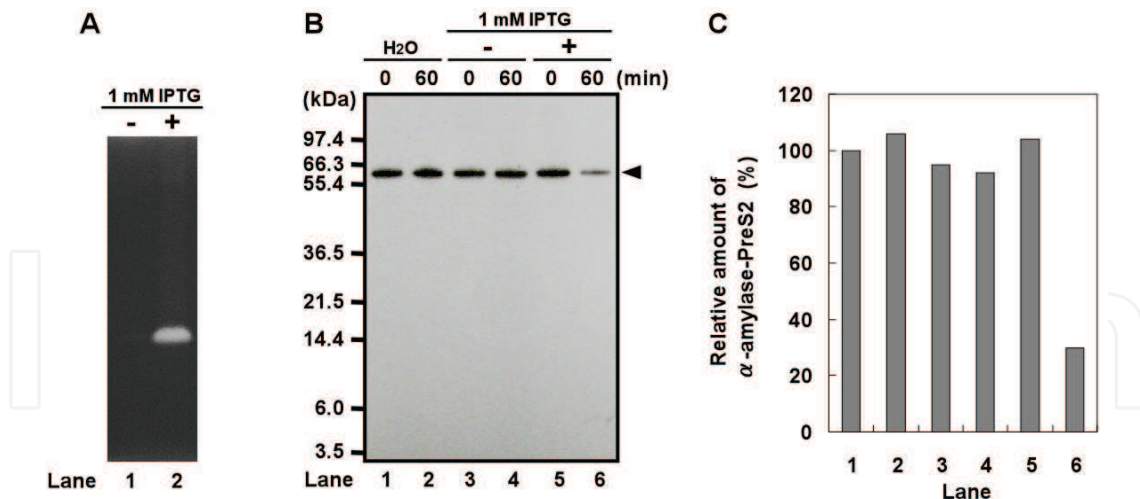
Fig. 6. The α-amylase-A522-PreS2 hybrid protein was degraded by AprX. (A) Zymography of supernatants from the KA8AX(pDG-AprX) strain. Lane 1, without IPTG; lane 2, with IPTG. (B) Western blot analysis of degradation of α-amylase-A522-PreS2 by AprX. AprX from KA8AX (pDG-AprX) mutant cells, cultured for 4 h with or without 1 mM IPTG was prepared as described in the Material and Methods. α-Amylase-A522-PreS2 from 10 µl supernatants of the KA8AX (pTUBE522-preS2) mutant (at 75 h cultivation) was mixed with 10 µl of AprX solution. After incubation at 37°C for 60 min, PMSF (final concentration, 10 mM) was added to the samples to stop the reaction. Western blot analysis was carried out to detect α-amylase-A522-PreS2 with the anti-PreS2 antibody; +, addition of 1 mM IPTG (AprX); -, no addition. The reaction mixture (equivalent to 1 µl) was applied to each lane. The arrowhead indicates the position of α-amylase-A522-PreS2. (C) The relative amounts of α-amylase-A522-PreS2 were obtained by comparing the band intensities on Western blots (the α-amylase-A522-PreS2 amount in lane 1 was set as 100%). Lanes 1 to 6 in panel C correspond to lanes 1 to 6 in panel B.

2.2 The effect of HtrA and HtrB on the degradation of secreted proteins

In this section we describe the effects of membrane-bound proteases and a two-component system on degradation of secreted proteins, and transcriptional regulation of the membrane-bound protease genes.

2.2.1 Cell envelope-associated quality control proteases

In *B. subtilis*, the accumulation of misfolded proteins at the membrane-cell wall interface is sensed by the CssR–CssS two-component system, which consists of the membrane-embedded sensor kinase, CssS and the response regulator, CssR (Hyyryläinen et al., 2001). This system responds to general protein secretion stresses, which can be triggered by either homologous (e.g., overproduction of LipA) or heterologous (e.g., overproduction of AmyQ and hIL-3) proteins, and consequently activates the transcription of the monocistronic *htrA* and *htrB* genes (Darmon et al., 2002; H. Westers et al., 2006; Hyyryläinen et al., 2007). HtrA and HtrB are membrane-bound serine proteases that are responsible for the degradation of misfolded proteins, and can thereby rescue the cell from a lethal accumulation of misfolded proteins in the cell envelope. In addition, HtrA has a dual localization, because it can be detected in the membrane-associated cellular fraction as well as the growth medium. Therefore, HtrA has a chaperone-like activity that might assist misfolded proteins in

recovering their conformation, while also targeting unsuccessful protein for degradation (Antelmann et al., 2003). Induction of *htrA* and *htrB* expressions is responsive to secretion stress in a manner dependent on the CssRS two-component system. In addition, *htrA* and *htrB* expressions are negatively autoregulated and reciprocally cross-regulated (Noone et al., 2000, Noone et al., 2001). Therefore, the absence of HtrA leads to the increased synthesis of HtrB, and vice versa (Noone et al., 2001).

2.2.2 High-level lipase A (LipA) production in eleven proteases mutant

We examined the production of lipase A (LipA) of *B. subtilis* (van Pouderoyen et al., 2001), as a valuable model for industrial enzyme production, in a nine-protease-deficient *B. subtilis* strain. Therefore, we constructed the pHLApm plasmid, in which LipA with the promoter and ribosomal binding site of an alkaline cellulase gene, *egl-237* (Hakamada et al., 2000) was cloned into pHY300PLK (Takara, Japan). LipA was overproduced in *B. subtilis*. Cells carrying pHLApm were cultured in modified 2xL broth for 12, 24, 36, 48, 60, and 75 h. The productivity of LipA in the supernatants from cultures of the 168 and Dpr9 (in which nine genes encoding eight extracellular proteases and AprX were precisely and completely deleted from the chromosome) strains was calculated based on the activity of LipA (Fig. 7). In 24 h cultivation, the production level of the LipA in 168 and Dpr9 could be obtained at 860 mg/L, an excellent yield which is 1.4-times higher than that of previously reported (Lesuisse et al., 1993). After 24 h, the amount of LipA markedly decreased in the 168 strain (Fig. 7). In contrast, degradation of LipA in the Dpr9 was effectively inhibited, compared with the 168 strain. However, after 36 h, the production of LipA in Dpr9 was reduced by approximately 10% (Fig. 7). These results showed that LipA was also degraded in the Dpr9 strain. Overproduction of both homologous (LipA) and heterologous (AmyQ and hIL-3) proteins induces the expression of *htrA* and *htrB* by the CssRS system (Darmon et al., 2002; H. Westers et al., 2006). From the currently available data, it seems most likely that limitation of both proteases of HtrA and HtrB improved the yield of heterologous proteins (Vitikainen, M., H. L. et al., 2005). To confirm the effect of HtrA and HtrB on the degradation of secreted proteins, we examined the production of LipA of *B. subtilis* in the *htrA* and/or *htrB* deficient *B. subtilis* strains. We constructed Dpr9ΔhtrA,
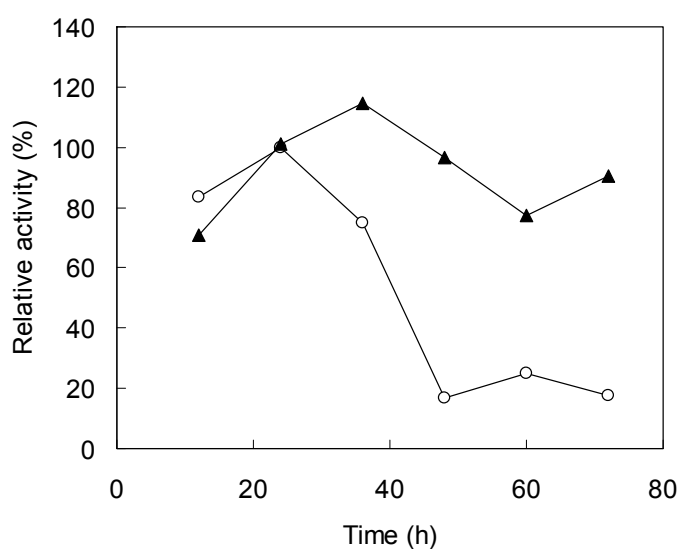

Fig. 7. Time course of LipA activity in the Dpr9 mutant. Cells were cultured in modified 2xL broth at 30°C. The accumulation of LipA in the culture medium was measured at various incubation times. Open circles, wild type strain; closed triangles, Dpr9 mutant.

Dpr9ΔhtrB, and Dpr9ΔhtrA/B (with eleven inactivated proteases), and evaluated each strain for the production of LipA. No effect on LipA production was observed in Dpr9ΔhtrA and Dpr9ΔhtrB. However, the production of LipA by the Dpr9ΔhtrA/B strain was at 1100 mg/L, which is 1.2-times higher than that of the Dpr9 strain (Fig. 8). These results suggest that inactivation of both *htrA* and *htrB*, as well as the nine proteases, has improved the productivity of *B. subtilis* for the production of LipA.
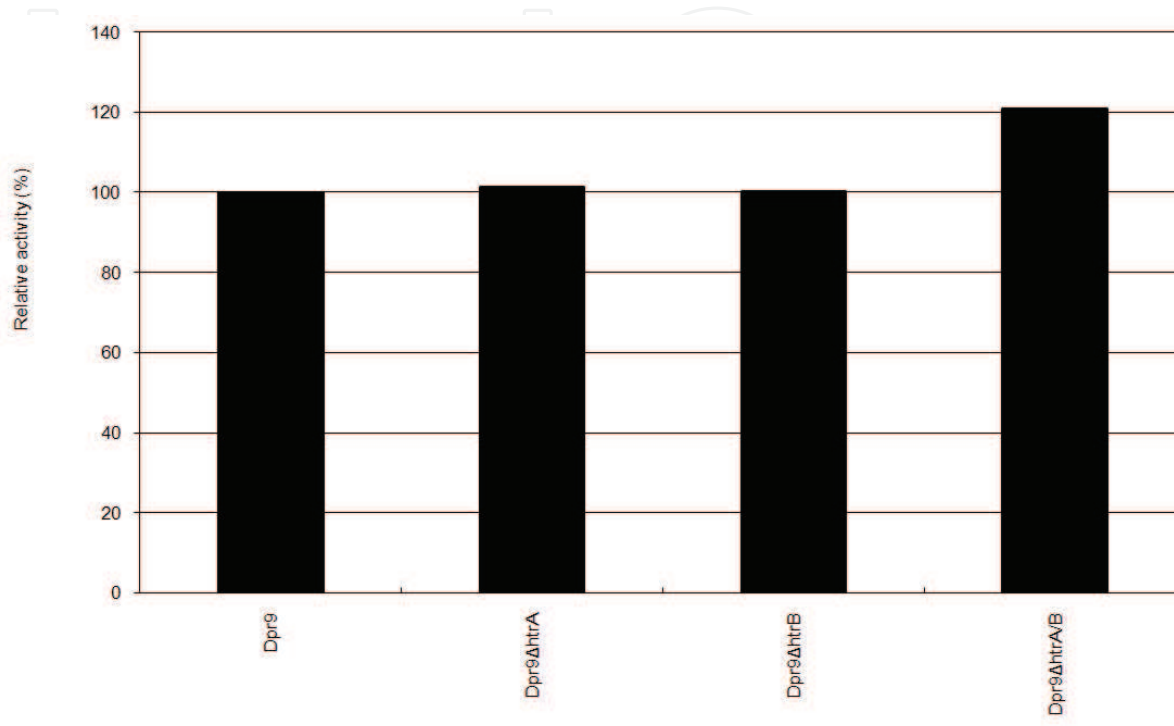

Fig. 8. Enhanced productivity of LipA in the absence of both *htrA* and *htrB*. Cells were cultured in modified 2xL broth at 30°C. The accumulation of LipA in the culture medium was measured at 48 h. The relative activities of LipA are shown (the amount of Dpr9 was set to 100%).

2.2.3 Transcriptional regulation of *htrB* and *htrA* by reciprocal cross regulation

We predicted that there was no difference between the productivities of LipA in the Dpr9ΔhtrA and Dpr9ΔhtrB strains, because the inactivation of either *htrA* or *htrB* results in a compensating overexpression of the other gene (Noone et al., 2001). To confirm that the overexpressions of *htrA* and *htrB* are caused by the inactivation of the other gene, we examined the level of expression of the *htrB-lacZ* fusion for the Dpr9ΔhtrA mutant, as well as the similar expression of the *htrA-lacZ* fusion for the Dpr9ΔhtrB mutant. Cells carrying pHY300PLK (control) and pHLApm (LipA overexpression) were cultured in modified 2xL broth for 48 h. As shown in Table 1, Dpr9ΔhtrA cells harboring pHLApm transcribed *htrB-lacZ* at a 4-fold increased level, compared with Dpr9 harbouring pHLApm (from 0.51 to 2.30 U). Similarly, a 10-fold increase in the *htrA-lacZ* expression level was observed in the Dpr9ΔhtrB mutant (from 0.41 to 4.26 U). The expressions of *htrB-lacZ* and *htrA-lacZ* also demonstrated reciprocal cross regulation in cells carrying pHY300PLK. These observations suggest that the overexpression of *htrB* in Dpr9ΔhtrA and of *htrA* in Dpr9ΔhtrB might affect LipA production. The expression level of *htrB-lacZ* in LipA-producing Dpr9 was 2.4-times higher than that of non-LipA-producing Dpr9 (Table 1). There was almost no change in the

expression level of *htrA-lacZ*, between Dpr9 cells harboring pHLApm and Dpr9 cells harbouring pHY300PLK. The expression of the *htrB-lacZ* reporter gene fusion has previously been shown to be more sensitive to secretion stress than the *htrA-lacZ* reporter gene fusion (Hyyryläinen et al., 2001). These results suggest that Dpr9 produced LipA in weak response to secretion stress.

Expressed gene	Strain	Plasmid	Expression [a]
htrB-lacZ	Dpr9	pHY300PLK	0.22±0.01
	Dpr9ΔhtrA	pHY300PLK	1.46± 0.11
	Dpr9	pHYLApm	0.51± 0.11
	Dpr9ΔhtrA	pHYLApm	2.30± 0.02
htrA-lacZ	Dpr9	pHY300PLK	0.38± 0.03
	Dpr9ΔhtrB	pHY300PLK	1.29± 0.01
	Dpr9	pHYLApm	0.41± 0.03
	Dpr9ΔhtrB	pHYLApm	4.26± 0.02

[a] One activity unit is defined as 1 nmol of *O*-nitrophenyl-ß-D-galactopyranoside hydrolysed per min per μg of OD_{600}. The results presented are the average of three individual experiments. Plus/minus values represent standard deviations.

Table 1. Expression of transcriptional fusions between the *htrA* and *htrB* promoters and *lacZ* reporter gene in various genetic backgrounds.

3. Conclusion

This chapter focused on biotechnological approaches to optimization of heterologous protein and enzyme production by multiple protease-deficient mutations in *B. subtilis*. Section 2.2 described the identification of AprX protease using gelatin zymography and the effects of AprX on heterologous protein production. The nine-protease-deficient KA8AX strain (lacking nine genes encoding eight extracellular proteases and AprX) effectively prevented proteolysis of α-amylase-A522-PreS2 [PreS2 antigen of human hepatitis B virus (HBV) fused with the N-terminal 522 amino acids of *B. subtilis* α-amylase] in the late stationary growth phase and improved the yield of the fusion protein. In addition, AprX was detected in the culture medium due to leakage on cell lysis during the late stationary growth phase. Section 2.3 described that the inactivation of nine-proteases and both *htrA* and *htrB* (resulting the Dpr9ΔhtrA/B mutant) improved the productivity of LipA in *B. subtilis*. In particular, the productivity of the LipA in the Dpr9ΔhtrA/B strain was 1100 mg/L, an optimal yield which is 1.8-times higher than that of previously reported. There was no difference in the productivities of LipA in the Dpr9ΔhtrA and Dpr9ΔhtrB strains, compared with that of Dpr9. Because the transcriptions of *htrA* and *htrB* are controlled by reciprocal cross regulation, overexpression of *htrB* in the Dpr9ΔhtrA strain and of *htrA* in the Dpr9ΔhtrB strain might affect LipA production. The previous approach for effective protein production was to generate a strain which has the inactivation of eight extracellular proteases in *B. subtilis* as the host. We reported that AprX leaked outside of cells, and HtrA/HtrB membrane-bond proteases of *B. subtilis* were also key proteases involved in the degradation of natural and heterologous proteins. In addition, nine- or eleven-protease-deficient strains of *B. subtilis* were helpful in improving protein productivity. Our findings, described in this chapter should contribute to the generation of hosts to be further optimized for protein production.

4. Acknowledgment

5. References

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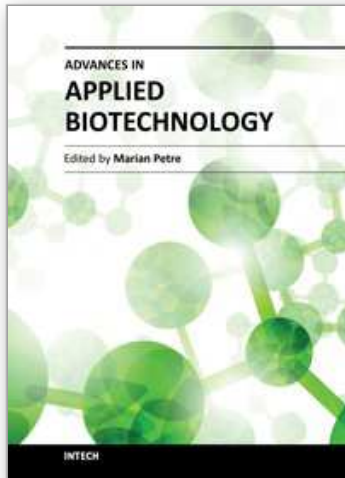
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