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Review

Bacillus subtilis as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism

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Abstract

Bacillus subtilis is a rod-shaped, Gram-positive soil bacterium that secretes numerous enzymes to degrade a variety of substrates, enabling the bacterium to survive in a continuously changing environment. These enzymes are produced commercially and this production represents about 60% of the industrial-enzyme market. Unfortunately, the secretion of heterologous proteins, originating from Gram-negative bacteria or from eukaryotes, is often severely hampered. Several bottlenecks in the *B. subtilis* secretion pathway, such as poor targeting to the translocase, degradation of the secretory protein, and incorrect folding, have been revealed. Nevertheless, research into the mechanisms and control of the secretion pathways will lead to improved *Bacillus* protein secretion systems and broaden the applications as industrial production host. This review focuses on studies that aimed at optimizing *B. subtilis* as cell factory for commercially interesting heterologous proteins.

Keywords: Cell factory; Chaperone; Heterologous protein; Production; Protease; Secretion

1. Introduction

The continuous discovery of new vaccines and therapeutics asks for the development of efficient systems for the production of pharmaceutical proteins. The choice of an appropriate host and suitable production conditions is crucial for the downstream processing of a pharmaceutical-grade product. *Escherichia coli* and members of the species *Bacillus* are the most frequently used prokaryotes for the industrial production of recombinant proteins. These organisms are above all favored due to the fact that the cultivation of these bacteria in large-scale production systems at high cell densities is easy and usually inexpensive.

For the economical production of recombinant proteins, the existence of stable expression systems is a necessity. At present, about 60% of the commercially available enzymes are produced by *Bacillus* species, mostly being homologous proteins that are naturally secreted in the growth medium, such as alkaline proteases as washing agent or amylases for the starch industry [1–4]. However, *E. coli* is still the most commonly used host for industrial production of pharmaceutical proteins as it is genetically most accessible and therefore first choice in the lead finding phase of a drug development project. Despite the fact that other systems may be superior to *E. coli* both in quality and efficiency, time pressure often has been prohibiting to change from host organism in later stages of development. In this review, we will address the considerations one may have in choosing the best cell factory for pharmaceutical proteins and the efforts that have been employed during the last years to improve *B. subtilis* as protein secretion factory.

In contrast to the well-known Gram-negative bacterium *E. coli*, the Gram-positive bacterium *B. subtilis* is considered as a GRAS organism (*g*enerally *r*ecognized *a*s *s*afe). For that reason, the use of *B. subtilis* for the production of food products is highly favored over the use of *E. coli*. The outer cell membrane of most Gram-negative bacteria, e.g. *E. coli*, contains lipopolysaccharides (LPS), generally referred to as endotoxins, which are pyrogenic in humans and other mammals. These endotoxins complicate product purification, because the end-product should be completely endotoxin-free [5,6]. Furthermore, in comparison to *E. coli*, *B.*

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subtilis is a more attractive host because it has a naturally high secretory capacity and exports proteins directly into the extracellular medium [2]. The secretion of target proteins leads to a natural separation of the product from cell components simplifying downstream processing of the protein. In addition, it may provide better folding conditions compared to the reducing environment in the cytoplasm [7], thereby preventing the formation of inclusion bodies. Despite these clear shortcomings of the *E. coli* system, the use of the highly efficient *Bacillus* secretion hosts has remained limited to bulk industrial enzyme production. Although nearly 80 recombinant protein therapeutics have been approved worldwide (58 approvals in the US) [8], none of these is produced in *Bacillus.* Besides the fact that there is no track record for pharmaceutical registration, the major reservations for using *Bacillus* have been (1) lack of suitable expression vectors; (2) plasmid instability; (3) presence of proteases; (4) occurrence of malfolded proteins. These shortcomings have been studied in detail in recent years and we will exemplify some solutions that have been brought forward by discussing recent attempts to express human pharmaceutical proteins in *Bacillus*. Table 1 lists an overview of the (protein) products that are successfully produced by *B. subtilis*, which should be representative for the range of products that can be produced in *B. subtilis*.

In the past, the optimization of protein production by bacterial strains was performed by empirical approaches, like studying the effects of medium compositions on protein production yields [9,10]. However, the introduction of the recombinant DNA technology has allowed a more directed intervention into the genetics of the production hosts. Overproduction of recombinant proteins has turned out to be a complex process and designing improved production strains requires a comprehensive understanding of the cellular physiology of the cells under overproducing conditions. The choice of a promoter in combination with a DNA vector may work for the overexpression of one protein, but that does not guarantee high-level production of other proteins. To broaden the range of proteins that can be produced in *B. subtilis*, more knowledge on cellular functions and development of better production systems is still needed.

Table 1
Protein products from *B. subtilis*

Product	Yield	Reference
α-amylase (AmyS)	–	[111]
α-amylase (AmyQ)	1–3 g/L	[1]
Proinsulin	1 g/L[a]	[46]
Lipase A	600 mg/L	[112]
Streptavidin	35–50 mg/L	[113]
scFv	10–15 mg/L	[63]
hEGF	7.0 mg/L	[114]
Endoglucanase	8300 U/L	[114]
IFN-alpha 2	0.5–1 mg/L	[115]
Poly(30hydroxybutyrate) depolymerase	1.9 mg purified protein/L	[116]
Endocellulase (Puradax®)	–	[117]
Subtilisin (AprE)	–	[118]

[a] No data on bioactivity.

2. Empirical approaches for production strain optimization

The *Bacillus* secretory pathway can be divided into three functional stages: (1) early stages involving the synthesis of secretory pre-proteins, their interaction (if any) with chaperones and binding to the translocase; (2) translocation across the cell membrane via the Sec (protein *sec*retion of unfolded proteins) or Tat translocase (*t*win-*a*rginine *t*ranslocation of folded proteins); (3) late stages, including removal of the signal peptide, release from the translocase, folding on the trans side of the cell membrane and passage through the cell wall [2]. Thus, several different factors are involved before the produced protein reaches its final destination and conformation and therefore each of these factors can be a bottleneck for high-level production (Fig. 1). For production of heterologous proteins in the medium of *B. subtilis*, it is necessary to use a signal peptide that directs the protein very efficiently to the translocase and that is cleaved efficiently by the signal peptidases (see also the chapter by Van Roosmalen et al. in this special issue). Therefore, modulation of a signal peptide to obtain an efficient signal peptidase cleavage site is sometimes a necessity [11]. Not only on the signal peptides themselves an extensive amount of work has been done, also several fusions have been made with heterologous proteins [12–22]. Furthermore, an increased expression of signal peptidases could enhance the capacity of the secretion machinery, e.g. for AmyQ as shown by Tjalsma et al. [23] and Pummi et al. [24].

2.1. Construction of B. subtilis strains with reduced protease activity

The high quality of the secreted proteins that are produced industrially is, at least in part, due to the presence of cellular ‘‘quality control’’ systems that efficiently remove incompletely synthesized or misfolded proteins. Because the folding of many heterologous proteins is usually inefficient, these quality control systems paradoxically represent major bottlenecks for the production of these heterologous proteins, the greatest problem being proteolytic degradation. A search in the SubtiList database (http://www.genolist.pasteur.fr/SubtiList/) for proteases and peptidases revealed the presence of 24 known and 6 putative proteases and 20 known and 21 putative peptidases. The known proteases and peptidases are partly depicted in Fig. 2. Thus far, most attempts to find solutions were focused on the proteases that are secreted into the growth medium. The deletion of only two major extracellular proteases already declines the proteolytic activity tremendously and improves protein produc-

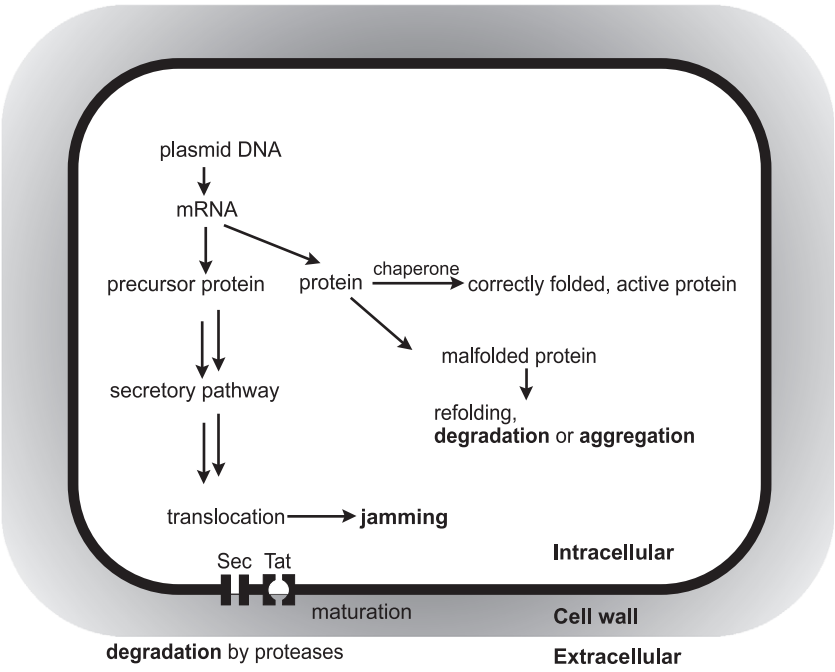

Fig. 1. Schematic presentation of processes that affect the yield of heterologous proteins. Although proteins can be produced intracellularly, this always runs the risk for inclusion body formation when the protein is highly overexpressed. Often extracellular expression is chosen using protease deficient strains to minimize proteolytic degradation.

tion yields [25]. Examples of protease-deficient strains that are mentioned below are listed in Table 2.

B. subtilis strains with deletions in the *aprE* (encoding subtilisin, *a*lkaline *pr*otease *E*) and *nprE* (encoding *n*eutral *pr*otease *A*) genes were the first reported in the same issue of the Journal of Bacteriology in 1984 to produce very low extracellular protease activity [26,27]. The BG2054 strain was tested on protease activity after growth in minimal media supplemented with casein hydrolysate, using azocoll as a substrate in the assay. No protease activity could be detected with this assay. The DB104 and DB105 strains were tested on Schaeffer sporulation agar plates with skim milk, resulting in protease activities of 2.6% and 4.1% compared to the parental strain [27–30]. Also a deletion mutation in the *epr* (encoding *e*xtracellular *pr*otease) gene resulted in a low protease activity in the culture supernatant [31,32]. Since then, strains containing deletion mutations in multiple extracellular proteases have been constructed with extracellular protease activity of less than 0.5% compared to the parental strain [33,34]. These multiple deletion strains allow the production of proteins that are highly sensitive to protein degradation in the parental strain. It has to be noted that when protease activity levels are measured using different substrates under different conditions, it is difficult to compare the outcomes. In a neutral environment, e.g. alkaline proteases are not fully active [35]. Furthermore, it has to be mentioned that inactivation of proteases leads to more cell lysis, which starts already at the point of transition from the exponential to the post-exponential growth phase. Occurrence of intracellular proteases in the medium caused by cellular lysis may result in lower production yields because of degradation of the protein of interest [36].

In a six-extracellular-protease-deficient strain, WB600 (Fig. 2), it was shown that protein degradation is minimized and the production yield of, e.g. β-lactamase [34], streptokinase [37], and the antidigoxin single-chain antibody fragment (5 mg/l in shake flask culture) [38] is improved compared to the production yields in the wild-type strain. With the inactivation of seven known extracellular proteases in the WB700 strain (Table 2), about 0.15% of the wild-type extracellular protease activity could still be detected when these cells were cultivated for 24 h in super-rich medium. Serine protease inhibitors could inhibit this activity. The blood-clot dissolving agent staphylokinase (337 mg/l in a fermentor) [39] and human interleukin-3 (3 mg/l; unpublished observations) are successfully produced in this strain.

Although the presence of an eighth extracellular protease gene in *B. subtilis* could not be excluded at that time (April 1996), it was thought that the residual serine protease activity of WB700 originated from intracellular proteases that were released into the medium through cell lysis. The cytoplasmic Lon protease of *E. coli* is a serine protease, which is known to be involved in the degradation of abnormal or foreign proteins. The Lon homologue in *B. subtilis* (LonA; Fig. 2) was inactivated in WB700 to investigate if LonA was active in the growth medium and thus responsible for the residual protease activity. However, inactivation of LonA did not reduce the extracellular protease activity and is therefore not involved in degradation of secretory proteins [40].

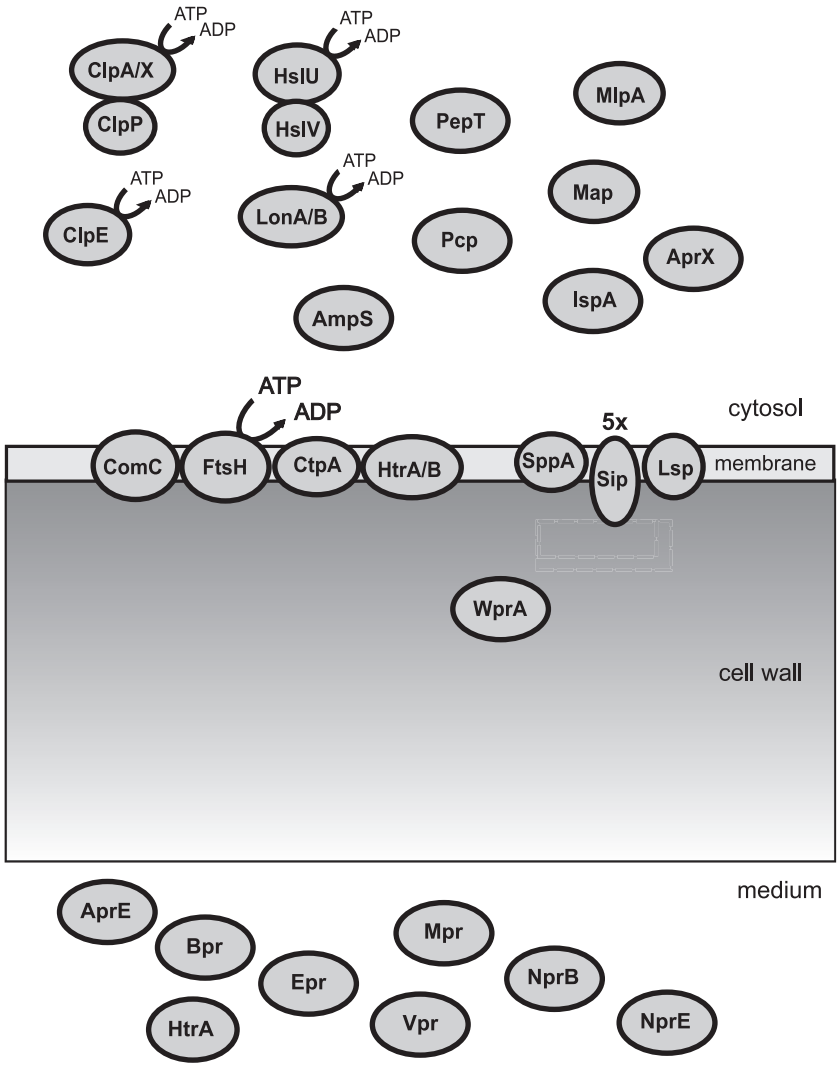

Fig. 2. Proteases and peptidases of *B. subtilis*. The location of the known *B. subtilis* proteases and peptidases in the cellular compartments and the growth medium is indicated. Note that specific proteases and peptidases involved in the sporulation process are not defined in the picture, because they are not expressed during fermentation of protein production strains. Furthermore, peptidases involved in peptidoglycan maturation are not indicated.

In December of 1996 an eighth extracellular protease was reported, named WprA (*w*all *pr*otease *A*) (Fig. 2) [41]. This cell wall-associated protease was shown to be involved in the degradation of secretory proteins prior to release into the culture medium. Unlike other protease genes, which are induced after the transition from the exponential to the post-exponential growth phase, the *wprA* gene is expressed constitutively in exponentially growing cells and up-regulated during the post-exponential phase [42]. When the cell wall protease WprA was inactivated in the WB600 strain, the production of staphylokinase was enhanced compared to the parental strain, although the production levels were lower than those of the WB700 strain, presumably because of different culturing conditions. Stability tests of staphylokinase and streptokinase in spent medium of this strain, LB700, and the strains DB431 (four extracellular proteases inactivated) and WB600 revealed that both enzymes are much more stable in spent medium of LB700, although staphylokinase is more stable than streptokinase [43]. Inactivation of WprA in the WB700 strain led to an even better production system, although in some cases the growth temperature had to be lowered to 30 °C to prevent inclusion body formation. Even after growing the

Table 2
Protease–deficient *B. subtilis* strains mentioned in the review

B. subtilis strain	Protease mutations	Reference
BG2054	Δ*nprE*–522; Δ*apr*–684	[26]
DB104	*nprR2; nprE18;* Δ*aprA3*	[27]
DB105	*nprR2; nprE18;* Δ*aprA3*	[27]
DB431	*nprE*; *aprE*; *epr*; *bpr*; *isp I*; *isp II*	[117]
WB600	*nprE*; *nprB*; *aprE*; *epr*; *mpr*; *bpr*	[34]
WB700	*nprE*; *nprB*; *aprE*; *epr*; *mpr*; *bpr*; *vpr*	[40]
LB700	*nprE*; *nprB*; *aprE*; *epr*; *mpr*; *bpr*; *wprA*	[43]
WB800	*nprE*; *nprB*; *aprE*; *epr*; *mpr*; *bpf*; *vpr*; *wprA*	[63]

cells for 48 h, no degradation of the protein of interest (cellulase) was shown [44]. For production of staphylokinase-hirudin, an artificial heterodimeric protein with thrombolytic and anti-thrombotic activity, the strains WB600, WB700 and WB800 (Table 2) were tested. Both proteins are joined together via a leucine zipper that acts as a heterodimerization domain. For purification purposes, staphylokinase was fused to a lysine-rich domain, whereas hirudin was coupled to a glutamate-rich domain. Production of staphylokinase and hirudin was performed separately and for the first, inactivation of the *wprA* gene in the WB800 strain improved the yield of intact protein dramatically, whereas production of the latter was the same for all strains. In the strains WB600 and WB700 degradation occurs mainly within the lysine-rich domain of the staphylokinase fusion judged from the molecular masses of the degradation products. This suggests that WprA cleaves lysine-rich sequences [45].

To overproduce and secrete human proinsulin (PI), a different approach was used [46]. In the DB431 strain (lacking four extracellular and two intracellular proteases) an *aprE::PI* transcriptional–translational fusion was introduced, in which not only the regulatory region of *aprE* but also the sequence coding for the signal peptide is used to achieve optimal secretion of the proinsulin. After introduction of the *hpr2* (*hpr* encodes a transcriptional repressor of sporulation and extracellular protease genes) and *degU32* (*degU* encodes a two-component response regulator involved in degradative enzyme and competence regulation) mutations, the resulting strain was called BB81.3. These mutations have a positive effect on the transcription of *aprE,* and thus on the *aprE::PI* fusion [46,47]. The supplemented mineral medium used in this expression system induced the *aprE* promoter throughout the exponential phase, which resulted in a production level of 1 g/l of PI in a fermentor.

These studies prove that the gained knowledge on the *Bacillus* proteases leads to still further optimization of production systems for heterologous proteins.

2.2. Coproduction of chaperones

For secretory production of the antidigoxin single-chain antibody (scFv) in *B. subtilis* (see also Section 2.1), the formation of inclusion bodies was found to be a limiting factor [38]. Analysis of the distribution of the protein showed that the secreted fraction represents only 23% of the total scFv fragments produced by the cell. Therefore, strains were constructed producing molecular chaperones to increase the secretory production of the scFv, following the same approach as for *E. coli* [48–50]. Like in *E. coli*, *B. subtilis* has the GroE and the DnaK series of intracellular molecular chaperones. The genes for these chaperones are organized in two operons, the *groE* operon (*groES–groEL*) and the *dnaK* operon (*hrcA-grpE-dnaK-dnaJ-yqeT-yqeU-yqeV*) (http://www.genolist.pasteur.fr/SubtiList/) [51,52]. From studies in *E. coli*, it has been known that these series of molecular chaperones can act either independently or synergistically in a consecutive manner to facilitate the folding and assembly of certain proteins [53–56]. The two operons are both regulated by the repressor HrcA [57–59]. When *hrcA* is inactivated, the intracellular molecular chaperones from the two operons are constitutively produced [57,58]. Overproduction of the antidigoxin scFv in the six-extracellular-protease-deficient *B. subtilis* strain WB600 in which *hrcA* is inactivated results in an increased secretion of the scFv (8 mg/l) compared to the parental strain (5 mg/l). Furthermore, less scFv aggregated in the intracellular insoluble fraction, whereas the total amount of produced scFv was the same. The lipoprotein PrsA is an extracytoplasmic molecular chaperone [60,61] which is bound to the outer surface of the cell membrane and is suggested to mediate protein folding at the late stage of secretion. Overproduction of both the foldase PrsA and the scFv led to an increase in the total amount of scFv. In both the secreted fraction (8.1 mg/l) and the intracellular soluble fraction (14 mg/l, compared to 3.7 mg/l for the parental strain), more scFv was present. Coproduction of both intracellular and extracytoplasmic molecular chaperones also led to a significant decrease of insoluble scFv (1.7 mg/l, compared to 13 mg/l for the parental strain). The total amount of produced scFv was increased 1.3×, but 2.5× more scFv was secreted (12.5 mg/l), and almost 4× more scFv was present in the intracellular soluble fraction (compared to the parental strain), predominantly in the mature form [62]. Inactivation of *hrcA* and overproduction of PrsA in the WB800 strain also result in drastic improvement of the production level of a fibrin-specific scFv (10–15 mg/l) compared to the WB700 strain, which has the same mutations. Looking at the contribution of each mutation in the WB800 strain to the increased production level, it can be concluded that this result was primarily caused by inactivation of *hrcA* and to a less extent by overproduction of PrsA [63].

It has been shown that different chimeric α-amylases, which were built from AmyL (from *Bacillus licheniformis*), AmyQ (from *Bacillus amyloliquefaciens*), and AmyS (from *Bacillus stearothermophilus*), were produced at a lower level than wild-type α-amylase. These mutants had different isoelectric points and were constructed to study the influence of the charge of a secreted protein on passage through the negatively charged cell wall of *B. subtilis.* The chimeric α-amylases were shown to be stable in the growth medium and lower production levels were thought to be due to degradation in a cell-associated location, possibly before or during posttranslocational folding into their native conformations [64]. These observations indicate that when mutagenesis is performed in a protein, this can lead to changed charges in the protein, which can result in lower production levels. Similarly heterologous proteins that exhibit a slower folding in the cell wall microenvironment may be prone to degradation. In those cases the folding of the proteins may need special attention. In a *B. subtilis* mutant named *prsA3*,

the gene encoding the foldase PrsA contains a point mutation. Subtilisin-alkaline phosphatase fusion proteins have been shown to be degraded extensively in this strain. Presumably, this degradation occurs because the limited amounts of PrsA in this strain reduce the rate at which the fusion proteins are folded [60]. When an increased amount of PrsA is introduced by overexpression, the secretion of α-amylases and a protease is increased six- to twofold, respectively, when expressed at high levels [65]. A positive effect on the production of AmyL when the *amyL* gene is expressed at high rates from a multicopy plasmid has been described in more detail by C.L. Jensen in her thesis (1997). The increased AmyL accumulation coincides with a strong reduction in degradation of newly synthesized AmyL in the PrsA-overexpressing strain, indicating that co- or posttranslocational folding is an important factor for efficient secretion. This suggests that the PrsA protein is the rate-limiting component of the secretion machinery, a finding that is of considerable biotechnological interest.

Production of recombinant protective antigen (rPA) from *Bacillus anthracis*, which is used in vaccines against anthrax, was tested in different host organisms. *B. subtilis* turned out to be the best candidate, although even when the WB600 strain was used, the half-life of rPA was only 12 h in spent medium due to residual proteolytic activity. To reduce the proteolytic degradation of rPA, which was thought to occur just after emerging from the translocase, the microenvironment on the trans side of the cytoplasmic membrane was adapted. Because the native structure of rPA contains a Ca^{2+} ion, it is likely that metal ions also are important for the folding of this protein [66]. Metal cations like Ca^{2+}, Fe^{2+}, and Mg^{2+} are concentrated near the cell membrane/wall microenvironment due to D-alanylation of the wall and lipoteichonic acids. These ions serve as folding factors for several secreted proteins. By decreasing the D-alanylation of the cell wall, the density of negative charges in the wall increases, which in turn would increase the rate of protein folding [67]. The D-alanylation of the teichoic acids in the cell wall in *B. subtilis* is regulated by the proteins encoded by the *dlt*-operon (*dltA*–*dltE*). The expression of the operon was placed under control of an inducible promoter, which makes it possible to decrease D-alanylation when the operon is not induced. Indeed, the yield of secreted rPA was significantly increased in this situation [68].

2.3. Thiol-disulfide oxidoreductases

For the activity and stability of many exported heterologous proteins, disulfide bond formation is one of the most important processes. The formation of disulfide bonds in vivo is a fast and effective process, which is catalyzed by thiol-disulfide oxidoreductases [69]. In *E. coli*, disulfide bonds are formed in the periplasm, because the cytoplasm is too reducing for this process. In this organism, six thiol-disulfide oxidoreductases have been found, DsbA–E and DsbG [70–77]. For expression of heterologous proteins in *E. coli*, commercially available systems are designed to circumvent formation of insoluble aggregates or "inclusion bodies" in the cytoplasm by fusing the gene of interest with *dsb*A [78] or with the gene encoding thioredoxin, *trx*A [79]. As fusion proteins with DsbA or TrxA, many troublesome proteins can be made in soluble forms that are biologically active. Hence, the solubility and accumulation level of heterologous proteins synthesized in the *E. coli* cytoplasm can be dramatically increased.

In Bacilli and other Gram-positives, very little is known about disulfide bond formation and isomerization. In these organisms the presence of thiol-disulfide oxidoreductases was questioned for a long time, because their secreted proteins were not found to contain disulfide bridges and the formation of disulfide bonds in secreted heterologous proteins occurred very inefficiently [80]. However, recently the secreted *B. subtilis* sublancin 168 was found to contain two disulfide bonds of which the formation most likely involves the action of thiol-disulfide oxidoreductases ([81]; see also the chapter by Sarvas et al. in this special issue). The first characterized thiol-disulfide oxidoreductase from a Gram-positive eubacterium was the Bdb (*Bacillus d*isulfide *b*ond) protein of *Brevibacillus choshinensis* (formerly known as *Bacillus brevis*) [82]. The orthologue of this Bdb in *B. subtilis* is denoted BdbA and two orthologues of DsbB protein from *E. coli* in *B. subtilis* are denoted BdbB and BdbC [83]. These latter *B. subtilis* proteins functionally correspond to the well-characterized *E. coli* DsbB and DsbA proteins, which catalyze the formation of disulfide bonds in proteins in the periplasmic space [84]. While it is not clear whether BdbA is secreted or retained in the membrane, BdbB and BdbC are membrane proteins with four transmembrane segments, and their catalytic Cys residues are predicted to be exposed on the extracytoplasmic side of the membrane. BdbD has a predicted signal peptide and is the fourth thiol-disulfide oxidoreductase that was identified. Disruption of the *bdbA*, *bdbB*, or *bdbC* genes showed that the absence of BdbB or BdbC, but not BdbA, resulted in the secretion of significantly reduced levels of the two disulfide bonds containing alkaline phosphatase (PhoA) of *E. coli* [83]. Thus, BdbB and BdbC, most likely by catalyzing disulfide bond formation or isomerization, promote extracytoplasmic protein folding.

A recent attempt to produce a staphylokinase fusion with the fibrin-targeting Kringle domain of human plasminogen in *B. subtilis* WB800 failed unfortunately, probably because of malfolded disulfide bridges in the Kringle domain. The fusion of the Kringle domain of plasminogen with staphylokinase will target staphylokinase to fibrin so that bleeding complications are prevented. To achieve production of an active fusion protein in *Pichia pastoris*, mutants of the protein had to be made to prevent *N*-glycosylation [85]. The heterodimeric protein staphylokinase-hirudin was designed to couple an anti-thrombotic protein to a thrombolytic agent (see also Section 2.1). After production of this protein in *B. subtilis* WB800, an in vitro treatment was

necessary to reshuffle the disulfide bonds in hirudin [86]. So it is still a challenge to optimize *Bacillus* host strains in such a way that production of proteins for which correct folding is necessary for activity is possible.

More successful is the production of human epidermal growth factor (hEGF) in *B. choshinensis*. This protein-hyperproducing bacterium was reported to produce up to 20 g/l of endogenous proteins in the medium [87]. After designing a host-vector system for this strain, hEGF as well as bacterial proteins were successfully produced and secreted using this system [88–91]. However, since hEGF contains three disulfide bonds, also incorrectly folded hEGF was produced. Strikingly, it has been reported that incubation of a non-native hEGF multimer with resting *B. choshinensis* cells resulted in the conversion of non-native to native hEGF. This was the first finding suggesting that these cells have a novel thiol-disulfide exchange system. Overexpression of Bdb did not affect this conversion activity [92]. In a recent article, the cloning of the genes *ccdA–catA*, encoding oxidoreductases, and co-expression of these genes with hEGF have been reported. Recently, CcdA of *B. choshinensis* with an associated thiol-disulfide oxidoreductase was proposed to be a homologue of DsbD in *E. coli*, which transfers electrons to DsbC, a periplasmic protein disulfide isomerase (PDI) [93,94]. It is likely that CcdA/CatA functions in the same manner. The action of the CatA protein, which was purified from *B. choshinensis* culturing broth, on the conversion of non-native multimeric hEGF in a resting cell system resulted in a twofold enhancement in production of native hEGF. Coexpression of the CatA protein and hEGF by using two expression vectors promoted the production of native hEGF about 1.3 ×. This slight enhancement was probably caused by a decreased copy number of the plasmid with the gene encoding hEGF due to the coexistence of two plasmids [92].

The same trick has been performed earlier, using a fungal PDI, which was dicistronically expressed with the light chain (LC) of immunoglobulin G on an expression vector or as a fusion protein with the PDI at the N-terminus [95]. Coexpression of LC with PDI did not give an improvement of LC expression (10 mg/l), but expression of the fusion protein led to LC amounts up to 150 mg/l. Even when the active site of PDI, Cys-X-X-Cys, was mutated to Ser-X-X-Ser, the expression level of the fusion protein was this high. This indicates that PDI, besides its function as disulfide bond isomerase, also functions as an important chaperone, thereby preventing protein aggregation.

3. Candidate strategies for further optimization of production strains

Now that the genomes of the important bacterial production hosts *E. coli* and *B. subtilis* have become transparent, we can get a more extensive picture of what is going on inside the cells by means of the 2D-PAGE and DNA-array techniques. Although many empirical approaches led to significantly improved host strains, these new techniques pave the way towards a scientific approach of strain improvements.

3.1. Transcriptional analysis and 2D-gel electrophoresis

It has been shown that *E. coli* responds to the strong overproduction of recombinant proteins by significantly increased mRNA levels of heat shock genes such as *lon* and *dnaK* and increased protein levels of the chaperones GroEL, DnaK, and Tig and decreased levels of ribosomal proteins [96]. Often accumulation of recombinant proteins results in the formation of inclusion bodies, to which host stress proteins like DnaK, GroEL, IbpA, IbpB and OmpT can be associated.

To study the cellular response to overproduction of PorA, an outer membrane protein from *Neisseria meningitidis* that forms cytoplasmic inclusion bodies when produced in *B. subtilis* [97], the transcriptional pattern of the overproducing *B. subtilis* strain was compared with the non-producing strain [98]. Using the DNA macro-array technique, the PorA overproducing strain showed increased mRNA levels from genes coding for the chaperones *dnaK*, *groEL* and *grpE*, the protease *clpP* and the ATPases *clpC* and *clpX*, whereas the mRNA levels of the two potential Lon proteases of *B. subtilis* (*lonA* and *lonB*) were not increased. Genes encoding purine and pyrimidine synthesis enzymes and ribosomal proteins showed the most clearly increased mRNA levels, which is not consistent with data from overexpression experiments in *E. coli.* Here strong overexpression showed a decrease in expression of ribosomal genes. In the paper of Jurgen et al. [98] it is cautiously speculated that *B. subtilis* cells would be able to correct an imbalance in the availability of purines and pyrimidines and ribosomal proteins due to an increase of *porA* expression at the beginning of the stationary phase.

To compare the cytoplasmic protein pattern of PorA overproducing cells, soluble protein fractions from both the control and the overproducing strain were isolated and separated by 2D-PAGE. In the overproducing strain, protein levels of DnaK and GroEL were clearly increased. The protein levels of ClpP and ClpC were moderately increased. These data are consistent with the transcriptome analyses, although the differences are more pronounced. These results were confirmed by Western blot analyses, in which also the presence of PorA in inclusion bodies was shown. Furthermore, the level of the ribosomal proteins RpsB and RplJ was increased in the overproducing strain, whereas the p*I* of other ribosomal proteins was too high to analyze them in these experiments.

Association of ClpP, ClpC and ClpX to the inclusion bodies was shown by electron microscopy using immunogold labelling. These findings demonstrate that overproduction of heterologous proteins in *Bacillus* results in a cellular response that is similar to the heat shock-like response in *E. coli*. The major difference is the important role of the Clp-

machinery of *Bacillus* in the degradation of heterologous proteins [99] and the Lon proteases being offside.

Having these results, one can now go back to the more empirical approach and adjust the production system to obtain better production levels.

3.2. Genomics: minimizing the genome

One of the key questions emerging from the still expanding data set of complete genome sequences is how many genes are essential for life of an organism such as *B. subtilis*. In a recent manuscript, only about 270 genes were shown to be indispensable for growth of *B. subtilis* in a rich medium at 37 °C, which suggests that the majority of the *B. subtilis* genome would be dispensable for growth under defined conditions [100]. For production of heterologous proteins, it may be beneficial to delete these parts of the genome. Any unnecessary gene product that is expressed in a production host represents a potential contaminant that could drive up the cost of product purification, certainly when drugs or vaccines must pass certification. Deletion of the gene coding for that side product is by far the most reliable and effective way to ensure the complete absence of an unwanted component in a biotechnological product [101]. And by deleting parts of the genome, cellular metabolite and energy resources would not be spent to maintain and express the deleted genetic information. Thus, the metabolism would be optimally directed towards the synthesis of both essential and desired gene products. Furthermore, since fewer unwanted proteins are synthesized the metabolic waste might decrease. In the EU-project "*Bacillus* Minimal Genome" [102], a sequential and cumulative approach was explored to delete large dispensable AT-rich regions from *B. subtilis* strain 168, aiming at improvement of the cell factory. Finally, the genome was reduced by 7.7% by deleting two prophages (SPβ and PBSX), three prophage-like elements (prophage 1, prophage 3, and *skin*), and the polyketide synthase (*pks*) operon (Fig. 3).

The results of the genome minimization studies show that this genome engineering did not affect cell viability or the key physiological and developmental processes of *B. subtilis*. Analysis of the metabolic flux ratio (METAFoR) of parental and deletion strains revealed an almost identical intracellular carbon metabolism. Also competence, sporulation, and proteolytic activity were unaffected. Additionally, the secretion capacity of the Δ6 strain for overproduced AmyQ of *B. amyloliquefaciens* was the same for *B. subtilis* Δ6 and *B. subtilis* 168. This suggests that no large energy resources were redirected towards product formation. Furthermore, only few proteins are absent from the extracellular proteome, which indicates that the Δ6 strain is only marginally improved in terms of removal of unwanted byproducts. To what extent the Δ6 strain represents an improved bacterial cell factory remains to be tested. The *B. subtilis* Δ6 strain has one major advantage over conventional *B. subtilis* production strains: it lacks the *Bsu*M restriction-modification system, which was found to be responsible for structural plasmid instability in *B. subtilis*, limiting the application potential of plasmids for high level protein production [103]. Because there is no "serious harm" done to the cells, further reduction still may lead to an improved lean *Bacillus* production machine, especially with regard to reducing unwanted byproducts in the growth medium that are above all inconvenient when a heterologous protein is not expressed and secreted at such high amounts as, e.g., AmyQ.

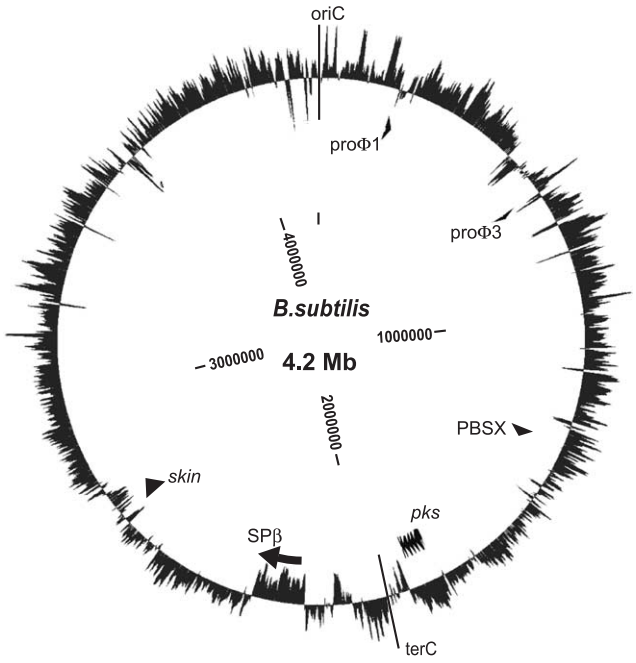

Fig. 3. Relative location of the deleted regions of the *B. subtilis* Δ6 strain on the chromosome of *B. subtilis* 168. The distribution of AT-rich islands on the *B. subtilis* genome (inner side of the circle) is depicted with the Genome Viewer program (http://www.cmbi.kun.nl/genome) in sliding windows of 4000 nucleotides with steps of 200 nucleotides. The outer boundary of the illustration is defined by the window of 4000 nucleotides with the lowest A + T content, whereas the inner boundary is defined by the window with the highest A + T content. The baseline indicates the midpoint between the inner and outer A + T content boundaries. The relative location of the deleted regions from *B. subtilis* Δ6 (SPβ, PBSX, *skin*, prophage 1, prophage 3, and *pks*) is indicated. Note that this plot does not mark PBSX as an AT-rich island, despite the fact that the A + T content of this prophage is higher than the average A + T content of the *B. subtilis* genome [84].

3.3. Engineering the metabolism

The limit of the yield of a biotechnological product is influenced by the energetic household of the host organism. In studies upon the influence of the P-to-O ratio (amount of ATP produced per atom oxygen consumed) in *B. subtilis* on the maximum riboflavin yield, not only the bioenergetic efficiency was estimated, but also homology searches for respiratory chain components from *E. coli* were performed in the genome database of *B. subtilis*. This was done to see whether a logical explanation could be found for a lower P-

to-O ratio in *B. subtilis* than the maximum value that is assumed for *E. coli.* For NADH dehydrogenase I (NDH-I), encoded by *nuoA-N* in *E. coli*, no *B. subtilis* analogue was found, indicating that *B. subtilis* lacks the energy-coupling NDH-I site. It was suggested that one strategy to engineer a *B. subtilis* strain with improved P-to-O ratio might be to functionally express *nuoA-N* from *E. coli* in *B. subtilis*. Overproduction of riboflavin biosynthesis components will not improve the yield, because in bacteria the carbon consumption rate is correlated with the growth rate and thus with biomass formation [104].

For production of two pharmaceutical human proteins, the human leukocyte interferon (IFN-α_1), an important therapeutic protein used as an anti-viral and anti-cancer agent, and erythropoietin (EPO), used for the treatment of anaemia, the metabolic fluxes in the *B. licheniformis* were analyzed theoretically. The metabolic reaction network of *B. licheniformis* contains 105 metabolites and 147 intracellular reactions [105]. The influence of different carbon sources (glucose and citrate) on this intracellular metabolic reaction network during production of IFN-α_1 and EPO was investigated to reveal the potential metabolic bottlenecks in the synthesis of these proteins. Using this strategy, it was possible to pinpoint candidate metabolic engineering sites. When glucose is used as a carbon source, the glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle are active, whereas the gluconeogenesis pathway is inactive, as expected. The fluxes towards the amino acid synthesis pathways are higher with glucose. In the case of citrate, the glycolysis pathway and the reaction from oxaloacetate to citrate are inactive, but the gluconeogenesis pathway is active. The total generation rate of ATP is lower with citrate. According to the model, the highest yield of both IFN-α_1 and EPO would be achieved using glucose as carbon source. Moreover, the EPO synthesis capacity was expected to be 10% lower than that of IFN-α_1. Simply looking at the amino acid distribution in both proteins, it becomes clear that both proteins contain a large number of Leu residues. Therefore, the flux towards Leu would be an ''engineering site''. Because in the synthesis of Leu six enzymes are involved, encoded by the genes *ilvB*, *ilvC*, *ilvD*, *leuA*, *leuCD*, and *leuB*, these genes are candidates for metabolic engineering. Choosing a host strain with a high Leu uptake or synthesis rate may be another possibility. Since it has been reported that the Leu uptake rate is high in *Bacillus pasteurii* [106], this *Bacillus* strain seems to be a potential alternative to produce IFN-α_1 and EPO [107]. Similar results were found in theoretical analyses of *B. licheniformis* producing a serine alkaline protease or α-amylase. In these cases, large amounts of Ala, Ser, Gly and Asn were needed [108].

4. The future of *Bacillus subtilis* as a cell factory

Since approximately two decades, a large amount of information has been gathered about Bacilli. Step by step, these bacteria were optimized to be used as a host strain for production of several proteins, but the success stories were primarily about overproduction of enzymes that were derived from the bacterium itself or a close relative. Nowadays, the complete genome sequence of *B. subtilis* is known and the functions of unknown genes have been studied. With modern techniques, widely used for genomics and proteomics, the road is paved to further optimize the *B. subtilis* production strains that already existed. Still, every recombinant protein is unique and will need some adaptations to the production system, e.g. changing rare codons can optimize translation [109,110]. But with all knowledge about promoters, plasmids, fermentations, signal peptides, secretion machineries, proteases and chaperones, and a large collection of mutant strains, one should be able to rationally choose an optimal production system. Using all advantages of *B. subtilis*, in a few years it should be possible to bring an FDA approved pharmaceutical protein that is produced in *Bacillus* to the market.

References

[1] I. Palva, Molecular cloning of alpha-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*, Gene 19 (1982) 81–87.
[2] M. Simonen, I. Palva, Protein secretion in *Bacillus* species, Microbiol. Rev. 57 (1993) 109–137.
[3] T. Schweder, B. Jurgen, Monitoring of genes that respond to overproduction of insoluble recombinant proteins in *Escherichia coli* and *Bacillus subtilis*, in: O.-W. Merten, D. Mattanovich, C. Lang, G. Larsson, P. Neubauer, D. Porro, P. Postma, J. Teixeira de Mattos, J.A. Cole (Eds.), Recombinant Protein Production with Prokaryotic and Eukaryotic Cells, Kluwer Academic Publishing, Dordrecht, 2001, pp. 359–369.
[4] W.J. Quax, Bacterial enzymes, in: M. Dworkin (Ed.), The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, Springer-Verlag, NewYork, 2003, http://www.link.springer-ny.com/link/service/books/10125/.
[5] D. Petsch, F.B. Anspach, Endotoxin removal from protein solutions, J. Biotechnol. 76 (2000) 97–119.
[6] L. Bredmose, S.M. Madsen, A. Vrang, P. Ravn, M.G. Johnsen, J. Glenting, J. Arnau, H. Israelsen, Development of a heterologous gene expression system for use in *Lactococcus lactis*, in: O.-W. Merten, D. Mattanovich, C. Lang, G. Larsson, P. Neubauer, D. Porro, P. Postma, J. Teixeira de Mattos, J.A. Cole (Eds.), Recombinant Protein Production with Prokaryotic and Eukaryotic Cells, Kluwer Academic Publishing, Dordrecht, 2001, pp. 269–275.
[7] T. Moks, L. Abrahmsen, E. Holmgren, M. Bilich, A. Olsson, M. Uhlen, G. Pohl, C. Sterky, H. Hultberg, S. Josephson, Expression of human insulin-like growth factor I in bacteria: use of optimized gene fusion vectors to facilitate protein purification, Biochemistry 26 (1987) 5239–5244.
[8] J.M. Reichert, C. Paquette, Clinical development of therapeutic recombinant proteins, Biotechniques 35 (2003) 176–185.
[9] F.G. Priest, Products from bacilli, in: C.F. Harwood (Ed.), Handbooks of Biotechnology, Plenum Press, New York, 1989, pp. 293–315.
[10] M.M. Zukowski, Production of commercially valuable products, in: R.H. Doi, M. McGloughlin (Eds.), Biology of Bacilli: Applications to Industry, Butterworth-Heinemann, Boston, 1992, pp. 311–337.
[11] W.J. Quax, A.J. Bonekamp, M.W.E.M. van Tilborg, Correct secretion of heterologous proteins from *Bacillus licheniformis*, in: R.H.

Baltz, G.D. Hegeman, P.L. Skatrud (Eds.), Industrial Microorganisms: Basic and Applied Molecular Genetics, ASM, Washington, 1993, pp. 143–150.
[12] I. Palva, M. Sarvas, P. Lehtovaara, M. Sibakov, L. Kaariainen, Secretion of *Escherichia coli* beta-lactamase from *Bacillus subtilis* by the aid of alpha-amylase signal sequence, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 5582–5586.
[13] K. Ohmura, K. Nakamura, H. Yamazaki, T. Shiroza, K. Yamane, Y. Jigami, H. Tanaka, K. Yoda, M. Yamasaki, G. Tamura, Length and structural effect of signal peptides derived from *Bacillus subtilis* alpha-amylase on secretion of *Escherichia coli* beta-lactamase in *B. subtilis* cells, Nucleic Acids Res. 12 (1984) 5307–5319.
[14] K.Yamane, K. Otozai, K. Ohmura, A. Nakayama, H. Yamazaki, M. Yamazaki, G. Tamura, Secretion vector of *Bacillus subtilis* constructed from the *Bacillus subtilis* alpha-amylase promoter and signal peptide coding region, in: A.T. Ganesan, J.A. Hoch (Eds.), Genetics and Biotechnology of Bacilli, Academic Press, Inc., New York, 1984, pp. 181–191.
[15] N. Vasantha, L.D. Thompson, Secretion of a heterologous protein from *Bacillus subtilis* with the aid of protease signal sequences, J. Bacteriol. 165 (1986) 837–842.
[16] S.R. Fahnestock, C.W. Saunders, M.S. Guyer, S. Lofdahl, B. Guss, M. Uhlen, M. Lindberg, Expression of the Staphylococcal Protein A gene in *Bacillus subtilis* by integration of the intact gene into the *Bacillus subtilis* chromosome, J. Bacteriol. 165 (1986) 1011–1014.
[17] S.R. Fahnestock, K.E. Fisher, Expression of the Staphylococcal Protein A gene in *Bacillus subtilis* by gene fusions utilizing the promoter from a *Bacillus amyloliquefaciens* alpha-amylase gene, J. Bacteriol. 165 (1986) 796–804.
[18] S.L. Wong, R.H. Doi, Determination of the signal peptidase cleavage site in the preprosubtilisin of *Bacillus subtilis*, J. Biol. Chem. 261 (1986) 176–181.
[19] K. Nakamura, Y. Fujita, Y. Itoh, K. Yamane, Modification of length, hydrophobic properties and electric charge of *Bacillus subtilis* alpha-amylase signal peptide and their different effects on the production of secretory proteins in *B. subtilis* and *Escherichia coli* cells, Mol. Gen. Genet. 216 (1989) 1–9.
[20] G. von Heijne, L. Abrahmsen, Species-specific variation in signal peptide design. Implications for protein secretion in foreign hosts, FEBS Lett. 244 (1989) 439–446.
[21] T.V. Borchert, V. Nagarajan, Effect of signal sequence alterations on export of levansucrase in *Bacillus subtilis*, J. Bacteriol. 173 (1991) 276–282.
[22] G. von Heijne, Life and death of a signal peptide, Nature 396 (1998) 111–113.
[23] H. Tjalsma, A. Bolhuis, M.L. van Roosmalen, T. Wiegert, W. Schumann, C.P. Broekhuizen, W.J. Quax, G. Venema, S. Bron, J.M. van Dijl, Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis*: identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases, Genes Dev. 12 (1998) 2318–2331.
[24] T. Pummi, S. Leskela, E. Wahlstrom, U. Gerth, H. Tjalsma, M. Hecker, M. Sarvas, V.P. Kontinen, ClpXP protease regulates the signal peptide cleavage of secretory preproteins in *Bacillus subtilis* with a mechanism distinct from that of the Ecs ABC transporter, J. Bacteriol. 184 (2002) 1010–1018.
[25] E. Ferrari, A.S. Jarnagin, B.F. Schmidt, Commercial production of extracellular enzymes, in: A.L. Sonenshein, J.A. Hoch, R. Losick (Eds.), *Bacillus subtilis* and other Gram-positive bacteria, American Society for Microbiology, Washington, DC, 1993, pp. 917–937.
[26] M.Y. Yang, E. Ferrari, D.J. Henner, Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an in vitro-derived deletion mutation, J. Bacteriol. 160 (1984) 15–21.
[27] F. Kawamura, R.H. Doi, Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases, J. Bacteriol. 160 (1984) 442–444.
[28] M.L. Stahl, E. Ferrari, Replacement of the *Bacillus subtilis* subtilisin structural gene with an in vitro-derived deletion mutation, J. Bacteriol. 158 (1984) 411–418.
[29] G.A. Rufo Jr., B.J. Sullivan, A. Sloma, J. Pero, Isolation and characterization of a novel extracellular metalloprotease from *Bacillus subtilis*, J. Bacteriol. 172 (1990) 1019–1023.
[30] S.R. Fahnestock, K.E. Fisher, Protease-deficient *Bacillus subtilis* host strains for production of Staphylococcal protein A, Appl. Environ. Microbiol. 53 (1987) 379–384.
[31] A. Sloma, A. Ally, D. Ally, J. Pero, Gene encoding a minor extracellular protease in *Bacillus subtilis*, J. Bacteriol. 170 (1988) 5557–5563.
[32] L.F. Wang, R. Bruckner, R.H. Doi, Construction of a *Bacillus subtilis* mutant deficient in three extracellular proteases, J. Gen. Appl. Microbiol. 35 (1989) 487–492.
[33] A. Sloma, G.A. Rufo Jr., K.A. Theriault, M. Dwyer, S.W. Wilson, J. Pero, Cloning and characterization of the gene for an additional extracellular serine protease of *Bacillus subtilis*, J. Bacteriol. 173 (1991) 6889–6895.
[34] X.C. Wu, W. Lee, L. Tran, S.L. Wong, Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases, J. Bacteriol. 173 (1991) 4952–4958.
[35] R. Gupta, Q.K. Beg, S. Khan, B. Chauhan, An overview on fermentation, downstream processing and properties of microbial alkaline proteases, Appl. Microbiol. Biotechnol. 60 (2002) 381–395.
[36] K. Stephenson, S. Bron, C.R. Harwood, Cellular lysis in *Bacillus subtilis*; the affect of multiple extracellular protease deficiencies, Lett. Appl. Microbiol. 29 (1999) 141–145.
[37] S.L. Wong, R. Ye, S. Nathoo, Engineering and production of streptokinase in a *Bacillus subtilis* expression-secretion system, Appl. Environ. Microbiol. 60 (1994) 517–523.
[38] X.C. Wu, S.C. Ng, R.I. Near, S.L. Wong, Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system, Biotechnology (N.Y.) 11 (1993) 71–76.
[39] R. Ye, J.H. Kim, B.G. Kim, S. Szarka, E. Sihota, S.L. Wong, High-level secretory production of intact, biologically active staphylokinase from *Bacillus subtilis*, Biotechnol. Bioeng. 62 (1999) 87–96.
[40] R. Ye, L.P. Yang, S.L. Wong, Construction of protease-deficient *Bacillus subtilis* strains for expression studies: inactivation of seven extracellular protease and the intracellular LonA protease, Proc. International Symposium on Recent Advances in Bioindustry, 1996, pp. 160–169.
[41] P. Margot, D. Karamata, The wprA gene of *Bacillus subtilis* 168, expressed during exponential growth, encodes a cell-wall-associated protease, Microbiology 142 (1996) 3437–3444.
[42] K. Stephenson, C.R. Harwood, Influence of a cell-wall-associated protease on production of alpha-amylase by *Bacillus subtilis*, Appl. Environ. Microbiol. 64 (1998) 2875–2881.
[43] S.J. Lee, D.M. Kim, K.H. Bae, S.M. Byun, J.H. Chung, Enhancement of secretion and extracellular stability of staphylokinase in *Bacillus subtilis* by wprA gene disruption, Appl. Environ. Microbiol. 66 (2000) 476–480.
[44] K. Murashima, C.L. Chen, A. Kosugi, Y. Tamaru, R.H. Doi, S.L. Wong, Heterologous production of *Clostridium cellulovorans* engB, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes, J. Bacteriol. 184 (2002) 76–81.
[45] Q. Lian, S.J. Szarka, K.K. Ng, S.L. Wong, Engineering of a staphylokinase-based fibrinolytic agent with antithrombotic activity and targeting capability toward thrombin-rich fibrin and plasma clots, J. Biol. Chem. 278 (2003) 26677–26686.
[46] J. Olmos-Soto, R. Contreras-Flores, Genetic system constructed to overproduce and secrete proinsulin in *Bacillus subtilis*, Appl. Microbiol. Biotechnol. 62 (2003) 369–373.
[47] A. Martinez, O.T. Ramirez, F. Valle, Improvement of culture conditions to overproduce beta-galactosidase from *Escherichia coli* in *Bacillus subtilis*, Appl. Microbiol. Biotechnol. 47 (1997) 40–45.
[48] G.E. Dale, H.J. Schonfeld, H. Langen, M. Stieger, Increased solu-

bility of trimethoprim-resistant type S1 DHFR from *Staphylococcus aureus* in *Escherichia coli* cells overproducing the chaperonins GroEL and GroES, Protein Eng. 7 (1994) 925–931.
[49] S.C. Lee, P.O. Olins, Effect of overproduction of heat shock chaperones GroESL and DnaK on human procollagenase production in *Escherichia coli*, J. Biol. Chem. 267 (1992) 2849–2852.
[50] J.G. Thomas, F. Baneyx, Protein folding in the cytoplasm of *Escherichia coli*: requirements for the DnaK-DnaJ-GrpE and GroEL-GroES molecular chaperone machines, Mol. Microbiol. 21 (1996) 1185–1196.
[51] G. Homuth, S. Masuda, A. Mogk, Y. Kobayashi, W. Schumann, The dnaK operon of *Bacillus subtilis* is heptacistronic, J. Bacteriol. 179 (1997) 1153–1164.
[52] M. Li, S.L. Wong, Cloning and characterization of the groESL operon from *Bacillus subtilis*, J. Bacteriol. 174 (1992) 3981–3992.
[53] W.A. Fenton, L. Horwich, GroEL-mediated protein folding, Protein Sci. 6 (1997) 743–760.
[54] A. Gragerov, E. Nudler, N. Komissarova, G.A. Gaitanaris, M.E. Gottesman, V. Nikiforov, Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing protein misfolding in *Escherichia coli*, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 10341–10344.
[55] T. Langer, C. Lu, H. Echols, J. Flanagan, M.K. Hayer, F.U. Hartl, Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding, Nature 356 (1992) 683–689.
[56] T. Langer, G. Pfeifer, J. Martin, W. Baumeister, F.U. Hartl, Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity, EMBO J. 11 (1992) 4757–4765.
[57] G. Yuan, S.L. Wong, Isolation and characterization of *Bacillus subtilis* groE regulatory mutants: evidence for orf39 in the dnaK operon as a repressor gene in regulating the expression of both groE and dnaK, J. Bacteriol. 177 (1995) 6462–6468.
[58] G. Yuan, S.L. Wong, Regulation of groE expression in *Bacillus subtilis*: the involvement of the sigma A-like promoter and the roles of the inverted repeat sequence (CIRCE), J. Bacteriol. 177 (1995) 5427–5433.
[59] U. Zuber, W. Schumann, CIRCE, a novel heat shock element involved in regulation of heat shock operon dnaK of *Bacillus subtilis*, J. Bacteriol. 176 (1994) 1359–1363.
[60] M. Jacobs, J.B. Andersen, V. Kontinen, M. Sarvas, *Bacillus subtilis* PrsA is required in vivo as an extracytoplasmic chaperone for secretion of active enzymes synthesized either with or without prosequences, Mol. Microbiol. 8 (1993) 957–966.
[61] V.P. Kontinen, P. Saris, M. Sarvas, A gene (prsA) of *Bacillus subtilis* involved in a novel, late stage of protein export, Mol. Microbiol. 5 (1991) 1273–1283.
[62] S.C. Wu, R. Ye, X.C. Wu, S.C. Ng, S.L. Wong, Enhanced secretory production of a single-chain antibody fragment from *Bacillus subtilis* by coproduction of molecular chaperones, J. Bacteriol. 180 (1998) 2830–2835.
[63] S.C. Wu, J.C. Yeung, Y. Duan, R. Ye, S.J. Szarka, H.R. Habibi, S.L. Wong, Functional production and characterization of a fibrin-specific single- chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production, Appl. Environ. Microbiol. 68 (2002) 3261–3269.
[64] C.L. Jensen, K. Stephenson, S.T. Jorgensen, C. Harwood, Cell-associated degradation affects the yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system, Microbiology 146 (2000) 2583–2594.
[65] V.P. Kontinen, M. Sarvas, The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level secretion, Mol. Microbiol. 8 (1993) 727–737.
[66] C. Petosa, R.J. Collier, K.R. Klimpel, S.H. Leppla, R.C. Liddington, Crystal structure of the anthrax toxin protective antigen, Nature 385 (1997) 833–838.
[67] H.L. Hyyrylainen, M. Vitikainen, J. Thwaite, H. Wu, M. Sarvas, C.R. Harwood, V.P. Kontinen, K. Stephenson, D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*, J. Biol. Chem. 275 (2000) 26696–26703.
[68] J.E. Thwaite, L.W. Baillie, N.M. Carter, K. Stephenson, M. Rees, C.R. Harwood, P.T. Emmerson, Optimization of the cell wall microenvironment allows increased production of recombinant *Bacillus anthracis* protective antigen from *B. subtilis*, Appl. Environ. Microbiol. 68 (2002) 227–234.
[69] J.C. Joly, J.R. Swartz, Protein folding activities of *Escherichia coli* protein disulfide isomerase, Biochemistry 12 (1994) 4231–4236.
[70] J.C. Bardwell, K. McGovern, J. Beckwith, Identification of a protein required for disulfide bond formation in vivo, Cell 67 (1991) 581–589.
[71] S. Kamitani, Y. Akiyama, K. Ito, Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme, EMBO J. 11 (1992) 57–62.
[72] J.C. Bardwell, J.O. Lee, G. Jander, N. Martin, D. Belin, J. Beckwith, A pathway for disulfide bond formation in vivo, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1038–1042.
[73] D. Missiakas, C. Georgopoulos, S. Raina, Identification and characterization of the *Escherichia coli* gene dsbB, whose product is involved in the formation of disulfide bonds in vivo, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7084–7088.
[74] D. Missiakas, C. Georgopoulos, S. Raina, The *Escherichia coli* dsbC (xprA) gene encodes a periplasmic protein involved in disulfide bond formation, EMBO J., (1994) 2013–2020.
[75] D. Missiakas, F. Schwager, S. Raina, Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*, EMBO J. 14 (1995) 3415–3424.
[76] L. Thony-Meyer, F. Fischer, P. Kunzler, D. Ritz, H. Hennecke, *Escherichia coli* genes required for cytochrome *c* maturation, J. Bacteriol. 177 (1995) 4321–4326.
[77] C.L. Andersen, A. Matthey-Dupraz, D. Missiakas, S. Raina, A new *Escherichia coli* gene, dsbG, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins, Mol. Microbiol. 26 (1997) 121–132.
[78] L.A. Collins-Racie, J.M. McColgan, K.L. Grant, E.A. DiBlasio-Smith, J.M. McCoy, E.R. LaVallie, Production of recombinant bovine enterokinase catalytic subunit in *Escherichia coli* using the novel secretory fusion partner DsbA, Biotechnology (N.Y.) 13 (1995) 982–987.
[79] E.R. LaVallie, E.A. DiBlasio, S. Kovacic, K.L. Grant, P.F. Schendel, J.M. McCoy, A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm, Biotechnology (N.Y.) 11 (1993) 187–193.
[80] A. Bolhuis, H. Tjalsma, H.E. Smith, A. de Jong, R. Meima, G. Venema, S. Bron, J.M. van Dijl, Evaluation of bottlenecks in the late stages of protein secretion in *Bacillus subtilis*, Appl. Environ. Microbiol. 65 (1999) 2934–2941.
[81] R. Dorenbos, T. Stein, J. Kabel, C. Bruand, A. Bolhuis, S. Bron, W.J. Quax, J.M. van Dijl, Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168, J. Biol. Chem. 277 (2002) 16682–16688.
[82] T. Ishihara, H. Tomita, Y. Hasegawa, N. Tsukagoshi, H. Yamagata, S. Udaka, Cloning and characterization of the gene for a protein thiol-disulfide oxidoreductase in *Bacillus brevis*, J. Bacteriol. 177 (1995) 745–749.
[83] A. Bolhuis, G. Venema, W.J. Quax, S. Bron, J.M. van Dijl, Functional analysis of paralogous thiol-disulfide oxidoreductases in *Bacillus subtilis*, J. Biol. Chem. 274 (1999) 24531–24538.
[84] L.S. Erlendsson, L. Hederstedt, Mutations in the thiol-disulfide oxidoreductases BdbC and BdbD can suppress cytochrome *c* deficiency of CcdA-defective *Bacillus subtilis* cells, J. Bacteriol. 184 (2002) 1423–1429.
[85] S.C. Wu, F.J. Castellino, S.L. Wong, A fast-acting, modular-structured staphylokinase fusion with Kringle-1 from human plasmino-

gen as the fibrin-targeting domain offers improved clot lysis efficacy, J. Biol. Chem. 278 (2003) 18199–18206.
[86] Q. Lian, S.J. Szarka, K.K. Ng, S.L. Wong, Engineering of a staphylokinase-based fibrinolytic agent with antithrombotic activity and targeting capability toward thrombin-rich fibrin and plasma clots, J. Biol. Chem. 278 (2003) 26677–26686.
[87] S. Udaka, Screening for protein-producing bacteria, Agric. Biol. Chem. 40 (1976) 523–528.
[88] H. Takagi, S. Kagiyama, K. Kadowaki, N. Tsukagoshi, S. Udaka, Genetic-transformation of *Bacillus brevis* with plasmid dna by electroporation, Agric. Biol. Chem. 53 (1989) 3099–3100.
[89] S. Udaka, H. Yamagata, Protein secretion in *Bacillus brevis*, Antonie Van Leeuwenhoek 64 (1993) 137–143.
[90] S. Ebisu, H. Takagi, K. Kadowaki, H. Yamagata, S. Udaka, The efficient production of human epidermal growth factor by *Bacillus brevis*, Ann. N.Y.Acad. Sci. 782 (1996) 115–122.
[91] S. Ebisu, H. Takagi, K. Kadowaki, H. Yamagata, S. Udaka, Production of human epidermal growth factor by *Bacillus brevis* increased with use of a stable plasmid from *B. brevis* 481, Biosci. Biotechnol. Biochem. 56 (1992) 812–813.
[92] A. Miyauchi, M. Ozawa, M. Mizukami, K. Yashiro, S. Ebisu, T. Tojo, T. Fujii, H. Takagi, Structural conversion from non-native to native form of recombinant human epidermal growth factor by *Brevibacillus choshinensis*, Biosci. Biotechnol. Biochem. 63 (1999) 1965–1969.
[93] F. Katzen, M. Deshmukh, F. Daldal, J. Beckwith, Evolutionary domain fusion expanded the substrate specificity of the transmembrane electron transporter DsbD, EMBO J. 21 (2002) 3960–3969.
[94] F. Katzen, J. Beckwith, Disulfide bond formation in periplasm of *Escherichia coli*, Methods Enzymol. 348 (2002) 54–66.
[95] T. Kajino, C. Ohto, M. Muramatsu, S. Obata, S. Udaka, Y. Yamada, H. Takahashi, A protein disulfide isomerase gene fusion expression system that increases the extracellular productivity of *Bacillus brevis*, Appl. Environ. Microbiol. 66 (2000) 638–642.
[96] B. Jurgen, H.Y. Lin, S. Riemschneider, C. Scharf, P. Neubauer, R. Schmid, M. Hecker, T. Schweder, Monitoring of genes that respond to overproduction of an insoluble recombinant protein in *Escherichia coli* glucose-limited fed-batch fermentations, Biotechnol. Bioeng. 70 (2000) 217–224.
[97] M. Nurminen, S. Butcher, I. Idanpaan-Heikkila, E. Wahlstrom, S. Muttilainen, K. Runeberg-Nyman, M. Sarvas, P.H. Makela, The class 1 outer membrane protein of *Neisseria meningitidis* produced in *Bacillus subtilis* can give rise to protective immunity, Mol. Microbiol. 6 (1992) 2499–2506.
[98] B. Jurgen, R. Hanschke, M. Sarvas, M. Hecker, T. Schweder, Proteome and transcriptome based analysis of *Bacillus subtilis* cells overproducing an insoluble heterologous protein, Appl. Microbiol. Biotechnol. 55 (2001) 326–332.
[99] E. Kruger, E. Witt, S. Ohlmeier, R. Hanschke, M. Hecker, The clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins, J. Bacteriol. 182 (2000) 3259–3265.
[100] K. Kobayashi, S.D. Ehrlich, A. Albertini, G. Amati, K.K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S.C. Brignell, S. Bron, K. Bunai, J. Chapuis, L.C. Christiansen, A. Danchin, M. Debarbouille, E. Dervyn, E. Deuerling, K. Devine, S.K. Devine, O. Dreesen, J. Errington, S. Fillinger, S.J. Foster, Y. Fujita, A. Galizzi, R. Gardan, C. Eschevins, T. Fukushima, K. Haga, C.R. Harwood, M. Hecker, D. Hosoya, M.F. Hullo, H. Kakeshita, D. Karamata, Y. Kasahara, F. Kawamura, K. Koga, P. Koski, R. Kuwana, D. Imamura, M. Ishimaru, S. Ishikawa, I. Ishio, D. Le Coq, A. Masson, C. Mauel, R. Meima, R.P. Mellado, A. Moir, S. Moriya, E. Nagakawa, H. Nanamiya, S. Nakai, P. Nygaard, M. Ogura, T. Ohanan, M. O'Reilly, M. O'Rourke, Z. Pragai, H.M. Pooley, G. Rapoport, J.P. Rawlins, L.A. Rivas, C. Rivolta, A. Sadaie, Y. Sadaie, M. Sarvas, T. Sato, H.H. Saxild, E. Scanlan, W. Schumann, J.F. Seegers, J. Sekiguchi, A. Sekowska, S.J. Seror, M. Simon, P. Stragier, R. Studer, H. Takamatsu, T. Tanaka, M. Takeuchi, H.B. Thomaides, V. Vagner, J.M. van Dijl, K. Watabe, A. Wipat, H. Yamamoto, M. Yamamoto, Y. Yamamoto, K. Yamane, K. Yata, K. Yoshida, H. Yoshikawa, U. Zuber, N. Ogasawara, Essential *Bacillus subtilis* genes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 4678–4683.
[101] V. Kolisnychenko , G. Plunkett III, C.D. Herring, T. Feher, J. Posfai, F.R. Blattner, G. Posfai, Engineering a reduced *Escherichia coli* genome, Genome Res. 12 (2002) 640–647.
[102] H. Westers, R. Dorenbos, J.M. van Dijl, J. Kabel, T. Flanagan, K.M. Devine, F. Jude, S.J. Seror, A.C. Beekman, E. Darmon, C. Eschevins, A. de Jong, S. Bron, O.P. Kuipers, A.M. Albertini, H. Antelmann, M. Hecker, N. Zamboni, U. Sauer, C. Bruand, D.S. Ehrlich, J.C. Alonso, M. Salas, W.J. Quax, Genome engineering reveals large dispensable regions in *Bacillus subtilis*, Mol. Biol. Evol. 20 (2003) 2076–2090.
[103] P. Haima, S. Bron, G. Venema, The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg, Mol. Gen. Genet. 209 (1987) 335–342.
[104] U. Sauer, J.E. Bailey, Estimation of P-to-O ratio in *Bacillus subtilis* and its influence on maximum riboflavin yield, Biotechnol. Bioeng. 64 (1999) 750–754.
[105] P. Calik, T.H. Ozdamar, Mass flux balance-based model and metabolic pathway engineering analysis for serine alkaline protease synthesis by *Bacillus licheniformis*, Enzyme Microb. Technol. 24 (1999) 621–635.
[106] L. Beck, T. Jahns, Regulation of leucine transport by intracellular pH in *Bacillus pasteurii*, Arch. Microbiol. 165 (1996) 265–271.
[107] P. Calik, T.H. Ozdamar, Metabolic flux analysis for human therapeutic protein productions and hypothesis for new therapeutical strategies in medicine, Biochem. Eng. J. 11 (2002) 49–68.
[108] P. Calik, T.H. Ozdamar, Carbon sources affect metabolic capacities of *Bacillus* species for the production of industrial enzymes: theoretical analyses for serine and neutral proteases and alpha-amylase, Biochem. Eng. J. 8 (2001) 61–81.
[109] S. Karlin, J. Mrazek, A. Campbell, D. Kaiser, Characterizations of highly expressed genes of four fast-growing bacteria, J. Bacteriol. 183 (2001) 5025–5040.
[110] A.C. McHardy, A. Puhler, J. Kalinowski, F. Meyer, Comparing expression level-dependent features in codon usage with protein abundance: an analysis of 'predictive proteomics', Proteomics 4 (2004) 46–58.
[111] H. Outtrup, B.E. Norman, Properties and application of a thermostable maltogenic amylase produced by a strain of *Bacillus* modified by recombinant-DNA techniques, Starch Stärke 36 (1984) 405–411.
[112] E. Lesuisse, K. Schanck, C. Colson, Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme, Eur. J. Biochem. 216 (1993) 155–160.
[113] S.C. Wu, S.L. Wong, Engineering of a *Bacillus subtilis* strain with adjustable levels of intracellular biotin for secretory production of functional streptavidin, Appl. Environ. Microbiol. 68 (2002) 1102–1108.
[114] K.H. Lam, K.C. Chow, W.K. Wong, Construction of an efficient *Bacillus subtilis* system for extracellular production of heterologous protein, J. Biotechnol. 63 (1998) 167–177.
[115] I. Palva, P. Lehtovaara, L. Kaariainen, M. Sibakov, K. Cantell, C.H. Schein, K. Kashiwagi, C. Weissmann, Secretion of interferon by *Bacillus subtilis*, Gene 22 (1983) 229–235.
[116] R. Braaz, S.L. Wong, D. Jendrossek, Production of PHA depolymerase A (PhaZ5) from *Paucimonas lemoignei* in *Bacillus subtilis*, FEMS Microbiol. Lett. 209 (2002) 237–241.
[117] B. Jones, W.J. Quax, Alzheimer tau test and detergent cellulase: made by genetic engineering (No. 9 in a series of articles to promote a better understanding of the use of genetic engineering), J. Biotechnol. 66 (1998) 229–233.
[118] J.A. Pierce, C.R. Robertson, T.J. Leighton, Physiological and genetic strategies for enhanced subtilisin production by *Bacillus subtilis*, Biotechnol. Prog. 8 (1992) 211–218.