Optimization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of homologous and heterologous signal peptides

Christian Degering¹, Thorsten Eggert¹, Michael Puls¹, Johannes Bongaerts³, Stefan Evers³, Karl-Heinz Maurer³, Karl-Erich Jaeger²*

¹evocatal GmbH, Merowingerplatz 1a, D-40225 Düsseldorf, Germany
²Institute of Molecular Enzyme Technology, Heinrich-Heine-University Düsseldorf, Research Centre Jülich, D-52426 Jülich, Germany
³Henkel AG & Co. KGaA, Biotechnology, D-40191 Düsseldorf, Germany

* Corresponding author:

Karl-Erich Jaeger
Phone: +49 2461 613716
Fax: +49 2461 612490
E-mail: karl-erich.jaeger@fz-juelich.de

**Keywords**

*Bacillus subtilis*, *Bacillus licheniformis*, protein secretion, signal peptide, high-throughput screening, Gram-positive
Abstract

*Bacillus subtilis* and *Bacillus licheniformis* are widely used for the large scale industrial production of proteins. These strains can efficiently secrete proteins into the culture medium using the general secretion (Sec-) pathway. A characteristic feature of all secreted proteins is their N-terminal signal peptide which is recognized by the secretion machinery. Here, we have studied the production of an industrially important secreted protease, namely subtilisin BPN’ from *Bacillus amyloliquefaciens*. 173 signal peptides originating from *B. subtilis* and 220 signal peptides from *B. licheniformis* type strain were fused to this secretion target, expressed in *B. subtilis* and the resulting library was analyzed by high throughput screening for extracellular proteolytic activity. We have identified a number of signal peptides originating from both organisms which resulted in significantly increased yield of the secreted protease. Interestingly, we observed that levels of extracellular protease were improved not only in *B. subtilis* which was used as the screening host but also in two different *B. licheniformis* strains. To date, it is impossible to predict which signal peptide will result in a better secretion and thus improved yield of a given extracellular target protein. Our data show that screening of a library consisting of homologous and heterologous signal peptides fused to a target protein can identify more effective signal peptides which result in improved protein export not only in the original screening host but also in different production strains.
Introduction

Gram-positive bacteria of the genus *Bacillus* are industrially well established microorganisms for the production of extracellular proteins. Due to the availability of relatively cheap large scale production systems combined with the ability to secrete up to 20-25 g/l of a target protein into the growth medium, about 60% of commercially available enzymes are presently produced in *Bacillus* species (14, 28).

The closely related strains *B. subtilis* and *B. licheniformis* are widely used as production hosts on an industrial scale and, in contrast to the well known production strain *E. coli*, they are free of endotoxin and possess the GRAS- (generally regarded as safe) status. The complete genome sequences of strains *B. subtilis* 168 (1, 18) and *B. licheniformis* DSM13 (isogenic to ATCC 14580; (25, 32)) are available greatly facilitating the construction of improved production strains.

The Sec-pathway constitutes the main secretion pathway in *B. subtilis* and *B. licheniformis*. Proteins secreted via the Sec-pathway are initially synthesized with an N-terminal hydrophobic signal peptide (SP) consisting of a positively charged N-domain followed by a longer, hydrophobic H-domain and a C-domain consisting of three amino acids which form the signal peptidase recognition site (35). Targeting of a secreted protein to the membrane, the translocation process itself and subsequent processing by a signal peptidase represent the major bottlenecks for efficient translocation and thus production of heterologous proteins (20).

SPs play a crucial role in the efficient translocation of secretory proteins by the Sec machinery. They interact with the SecA protein, the signal recognition particle (SRP), and the signal peptidase (16, 30). The interaction between the SP and the mature protein is known to influence protein export as well (9, 16-17). Therefore, the choice of an efficient signal peptide
for any given target protein is of utmost importance and several approaches were taken to identify efficient SPs for different target proteins (2, 4, 6, 15, 21, 38).

Among the huge number of enzymes produced at a large scale by Bacillus species, proteases are important for diverse industrial applications (25) with subtilisins being used as additives in household detergents (22, 28). We have chosen as a model for secretion optimization the subtilisin “Bacillus Protease Novo type” (BPN’) from B. amyloliquefaciens ATCC 23844, a well-known enzyme belonging to the alkaline serine proteases (5).

We present a novel approach to improve the extracellular production of this protease using different Bacillus host strains. A total number of 393 SPs was fused to the target protein with 173 SPs originating from B. subtilis (termed homologous SPs) and 220 SPs from B. licheniformis DSM13 (termed heterologous SPs). The fusion constructs were cloned and expressed in B. subtilis and the resulting library was screened for extracellular protease activity.

**Materials and methods**

**Bacterial strains, plasmids and cloning**

The bacterial strains and plasmids used in this study are listed in Table 1. B. subtilis TEB1030 was used as an expression host and for cloning as well as for plasmid preparation. B. licheniformis H402 and MW3 were used as expression hosts. All DNA manipulations followed standard procedures (27).
Media, growth conditions and preparation of culture supernatants

Bacterial strains were grown in LB medium (5 g yeast extract l⁻¹; 10 g tryptone l⁻¹; 10 g NaCl l⁻¹; pH 7) at 37 °C supplemented with 50 µg/ml kanamycin. Protease production was monitored on agar plates containing 1 % (v/v) skim milk. For screening, the cultures were grown for 20 hours at 37 °C in deep-well microtiter plates (96 wells, 1 ml LB per well; Greiner Bio-One, Frickenhausen, Germany) using a microplate shaker (600 rpm, TiMix 5, Edmund Bühler GmbH, Hechingen, Germany). Culture supernatants were prepared by centrifugation (20 min, 3200 g, 4 °C) and used immediately to determine proteolytic activity. Each strain was cultured three-fold in parallel in 3 different wells. Cell-free culture supernatants were prepared by micro-filtration (membrane filter, CME, pore-size 0.22 µm) and used for Western blot analysis. A comparison of proteolytic activities in culture supernatants prepared with and without microfiltration did not reveal any differences thus indicating that the proteolytic activities could be assigned to cell-free, i.e. secreted proteases.

High cell density fermentation of B. subtilis

Fed-batch fermentations were performed for 48h with a Labfors fermentor (volume: 7.5 litre, Infors, Germany) using a high cell density cultivation process developed for B. subtilis (24). The dissolved oxygen level was set to 30-40 % and glucose was fed from a stock solution (500 g glucose/l). The pH was adjusted by addition of 10 % NH₃ (w/v) or 4 N phosphoric acid.

Transformation of DNA

B. subtilis was transformed by protoplast transformation essentially as described by Chang and Cohen (7). B. licheniformis was transformed by electroporation (3, 31).
PCR conditions and amplification of *B. licheniformis* DSM13 signal peptides

Amplification of DNA was performed under standard PCR conditions in 50 µl reaction volume with 1 pmol of each primer, 1 U of Phusion High Fidelity polymerase (New England Biolabs, USA) and 100 ng of template DNA. Amplified DNA was purified after gel electrophoresis using the QIAEX II Gel Extraction Kit (QIAGEN, Germany).

Signal peptide encoding DNA sequences for *B. licheniformis* DSM13 were predicted from the genome sequence based on the presence of a SPaseI recognition site (34). 220 SP-encoding fragments were amplified and used for screening in combination with 173 SPs previously amplified from *B. subtilis* (4).

Cloning strategy and construction of the controls

SP libraries were cloned into pBSMul5 as described by Brockmeier et al. (4). The fusion of subtilisin BPN’ with the wild-type SP (wtSP) originating from *B. amyloliquefaciens* was used as benchmark construct which gave an extracellular protease activity in *B. subtilis* of 0.83 +/- 0.1 U/ml. Each tested microtiter plate contained as an internal standard a clone expressing the benchmark construct. The BPN’-encoding gene lacking the DNA encoding the SP, the ribosomal binding site and the spacer sequence served as a control resulting in an extracellular protease activity of < 0.1 U/ml.

Proteolytic activity assay

Protease activity was determined with the substrate succinyl-Ala-Ala-Pro-Phe-ρ-nitroanilide (AAPF; Bachem AG, Weil am Rhein, Germany) dissolved in dimethysulfoxide and finally diluted in 0.1 M Tris-HCl buffer, pH 8.6 containing 1% Brij®35 to a final concentration of 1.1 mM. Assays were performed with the same buffer in microtiter plates (96 wells, 250 µl reaction volume per well; Greiner Bio-One, Frickenhausen, Germany) at 30 °C for 5 minutes. The amount of ρ-nitroanilide released was measured at 405 nm with a molar absorption
coefficient of $8480/M \times cm$ (11). One unit is defined as the activity releasing 1 µmol of $\rho$-nitroanilide per minute. Clones showing high proteolytic activity in the culture supernatants were isolated and independently recultivated in microtiter plates (three-fold replicative determination). The amount of protease protein and proteolytic activity were determined by SDS-PAGE and Western-blotting in culture supernatants of 35 clones with high extracellular proteolytic activity.

**Protein analysis and immunodetection of BPN’**

SDS polyacrylamide gel electrophoresis was performed using a 5 % (w/v) stacking gel and a 15 % separating gel (19). For Western Blot analysis, 10 µl of culture supernatant were loaded on a gel and the separated proteins were subsequently blotted onto a polyvinylidene difluoride membrane (Sequiblot membrane, BioRad, München, Germany) (12). BPN’ protein was detected with a rabbit polyclonal antiserum and horseradish peroxidase-labelled goat anti-rabbit second antibodies (BioRad, München, Germany) and detection using the ECL Western Blot Kit (Thermo Scientific, Rockford, USA).

**Results**

**Design and construction of a signal peptide library consisting of Sec-type signal peptides from B. licheniformis**

For optimization of heterologous protein export in different Bacillus expression strains, a signal peptide library was constructed consisting of 220 B. licheniformis SPs and 173 B.
*subtilis* SPs. Subtilisin protease BPN’ from *B. amyloliquefaciens* ATCC 23844 (K02496.1) served as secretion target and *B. subtilis* was used as expression host strain. DNA fragments encoding SPs were cloned upstream of BPN’ into plasmid pBSMul5 and the resulting library was screened for extracellular protease activity in *B. subtilis* as summarized in Fig. 1.

**Homologous and heterologous signal peptides significantly improve BPN’ export in *B. subtilis***

*B. subtilis* was transformed with the SP library as described above and about 1800 clones (4-fold oversampling) were tested for extracellular protease production on skim milk agar plates. About 900 clones formed a clear halo around the colony, they were cultivated in duplicate or triplicate in different MT plates and proteolytic activities in the culture supernatants were determined allowing to identify eight SPs which efficiently mediated secretion of BPN’ by *B. subtilis* (Fig. 2). SPs dBli00338 and sYbdN increased the extracellular level of BPN’ by about seven-fold as compared to the *B. amyloliquefaciens* wild-type SP (Fig. 2). Characteristics of the best performing SPs are shown in Table 2. Strikingly, we found that not only SPs from the homologous host *B. subtilis* efficiently mediated BPN’ secretion, but also SPs isolated from the heterologous host *B. licheniformis*. In fact, *B. licheniformis* SP dBli00338 was among the most efficient SPs we have identified. *B. subtilis* clones expressing constructs with benchmark SP wtSP and the best performing SP dBli00338 were also cultivated in a laboratory scale fermentor. BPN’ fused to SP dBli00338 was efficiently secreted also under conditions of a high cell density fed-batch cultivation. After 48 hours cultivation, proteolytic activities were 8.9 units/ml for wtSP (at a cell density corresponding to an OD$_{600}$=68.3) and 59.7 units/ml for dBli00338 (OD$_{600}$=65.3) corresponding to a 6-7-fold increase of proteolytic activity in the culture supernatant (Fig. 3 A) with a concomitant increase in extracellular protease protein (Fig. 3, B and C).
Different SPs and expression hosts affect extracellular BPN’ levels

Next, we studied whether the best performing constructs identified in *B. subtilis* would also mediate secretion in two distinct *B. licheniformis* strains of biotechnological relevance. As shown in Fig. 4, we chose three SP-BPN’ fusions with clearly different extracellular protein levels of BPN’ which we transformed into *B. licheniformis* H402 and determined extracellular proteolytic activity and amount of BPN’ protein (Fig. 4).

SP dBli00338 resulted in a nine-fold increase of BPN’ secretion in *B. licheniformis* H402 as compared to the wild-type SP of BPN’ based on extracellular proteolytic activity. For all constructs tested, protein export levels of BPN’ were similar in both *B. subtilis* TEB1030 and *B. licheniformis* H402. These results prompted us to comparatively analyse the previously identified SPs in more detail. We additionally chose as an expression host *B. licheniformis* MW3 which is a variant of strain DSM13 and can easily be transformed. The eight best performing SP-BPN’ fusions previously identified in *B. subtilis* were analyzed together with two fusions which resulted in a significant decrease of extracellular BPN’. Extracellular protease activities were compared to those detected in the screening host *B. subtilis* TEB1030 (Fig. 5A). The fusion sYdjM::BPN’ resulted in an about 3.5-fold increase of protease secretion in each of the three expression hosts while dYdhT::BPN’ showed decreased export relative to the benchmark wtSP::BPN’ in both *B. licheniformis* hosts. The deviation of relative BPN’ export levels in the two *B. licheniformis* hosts to *B. subtilis* were compared for ten different SP-BPN’ fusions (Fig. 5B). Interestingly, the relative protein export levels of the analyzed SP-BPN’ fusions were comparable in both *B. licheniformis* strains to those observed in *B. subtilis* with deviations of less than 20%. BPN’ fused to SP dYdhT from *B. licheniformis* represents a clear exception which showed in both *B. licheniformis* strains a two-fold lower relative extracellular protease level as compared to *B. subtilis* TEB1030 (Fig. 5B).
Discussion

Bacteria belonging to the genus *Bacillus* represent the most important strains for the industrial production of secreted proteins (28). Hence, extensive research into the optimization of protein secretion was performed in *Bacillus* and several patents were filed which address strategies to relieve bottlenecks for protein secretion (14, 20, 23). SPs from extracellular proteins known to be secreted at high levels were identified and modified, e.g. SPs from NprE or AprE (14, 23). Additionally, homologous and heterologous SPs were tested in different hosts (8, 34), however, only very few examples were described for heterologous SPs resulting in a significant increase in secretion efficiency (10).

Screening projects published to date have exclusively been performed with SPs obtained from homologous hosts (4, 21, 38-39). Here, we have used subtilisin BPN’ from *B. amyloliquefaciens* as a model protein and have studied the influence of homologous and heterologous SPs on export efficiency of BPN’ by using three different *Bacillus* strains.

An library consisting of about 400 different SPs fused to subtilisin BPN’ was constructed and tested by high throughput activity screening of *B. subtilis* culture supernatants. Eight SPs were identified with dBli00338 and sYbdN resulting in a seven-fold increase of BPN’ activity in the culture supernatant as compared to wild-type SP. The results obtained by SP screening in microtiter plates were confirmed for the wild-type SP wtSP and SP dBli00338 by high cell density cultivation of *B. subtilis* in a 3 litre labscale fermentor. Interestingly, our screen did not identify SPs from enzymes like AprE and NprE which are known to be secreted at high levels. Instead, we predominantly identified SPs originating from so far uncharacterized so-called “Y”-proteins. An analysis of the extracellular proteomes of *B. subtilis* (29) and *B. licheniformis* (34) for the presence of proteins carrying the SPs shown in Fig. 2 revealed a significant amount of secretion only for protein YbdN. All other proteins were present in only
very low amounts or were not identified at all. It should be noted here that, apart from SPs, additional effects like promoter strengths, gene regulation mechanisms, transcriptional and translational efficiencies and protein folding also affect the amount of secreted proteins. We have also analyzed these secreted proteins with respect to their size, function, and pI, and we were unable to detect any significant similarities between them or to subtilisin BPN'. These results suggest that the general approach of choosing a SP from an efficiently secreted protein to direct secretion of another target protein is clearly not the best strategy.

A detailed analysis of the eight best performing SPs did not reveal any significant similarities with respect to charge of the N-region, hydrophobicity, signal peptidase recognition site or D-score as calculated with the SignalP prediction tool. Thus, high throughput SP screening as described here is the method of choice to quickly identify the most efficient SP for any protein to be secreted.

Another important aspect of this work relates to the fact that industrially relevant production strains are often not amenable to molecular manipulations including DNA transformation (22, 37). Hence, the use of closely related but better accessible strains may be required to accomplish high throughput screening procedures. Therefore, we have determined the levels of extracellular protease of different SP-BPN' fusions which were originally identified in *B. subtilis* in the industrially relevant *B. licheniformis* strain H402 and in *B. licheniformis* type strain DSM13 (MW3). Interestingly, the majority of the SP-BPN' fusions revealed comparable levels of extracellular protease in all three *Bacillus* expression strains. These results suggest performing a high throughput pre-screening in *B. subtilis* and subsequently transferring the best-performing constructs into *B. licheniformis* for further testing. SPs interact with components of the Sec-machinery, but it is also known that the interaction of SPs and the mature part of the secreted proteins influences the secretion efficiency (9, 16-17).

Although *B. subtilis* and *B. licheniformis* are closely related, significant differences were described for components of their Sec-machineries and especially for signal peptidases (32-
33). Most of the constructs with different SPs showed similar relative protease export levels in different *Bacillus* hosts. However, the dYdhT::BPN’ fusion revealed a significantly decreased level of extracellular protease upon transfer into the *B. licheniformis* hosts indicating that the efficiency of SPs can still not be predicted *a priori*.

In summary, we have demonstrated that both homologous and heterologous SPs, when fused to a given target protein, can result in significantly increased amounts of extracellular protein, which can easily be recovered from the culture supernatant. Furthermore, we have demonstrated that a genetically accessible host strain like *B. subtilis* can be used for a preliminary high throughput screening of a SP library and the best performing SP-fusions can subsequently be transferred into a production strain to improve protein export.

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subtilis* by saturation mutagenesis of the N-domain of the AmyE signal peptide. Appl.


Table 1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Description</th>
<th>Source/reference</th>
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<td><strong>I. Strains</strong></td>
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<tr>
<td><em>B. subtilis</em> TEB1030</td>
<td><em>trpC2 his aprE aprE bpf isp1 lipA lipB</em></td>
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<tr>
<td><em>B. licheniformis</em> DSM13</td>
<td>Wild type (isogenic to ATCC 14580)</td>
<td>DSMZ, Germany</td>
</tr>
<tr>
<td><em>B. licheniformis</em> MW3</td>
<td><em>B. licheniformis</em> DSM13 (<em>hur1 hur2</em>)</td>
<td>(35)</td>
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<tr>
<td><em>B. amyloliquefaciens</em> ATCC 23844</td>
<td>Wild type</td>
<td>ATCC, USA</td>
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<td><strong>II. Plasmids</strong></td>
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<td>pBSMul5</td>
<td><em>Bacillus</em> vector, <em>P_{lubA}, repB, Kn</em></td>
<td>This study</td>
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<td>pBSMul5-BPN</td>
<td>pBSMul5 containing 1059 bp <em>EcoRI-BamHI</em> fragment of BPN‘ from <em>B. amyloliquefaciens</em> ATCC 23844 without signal sequence</td>
<td>This study</td>
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Table 2 Characteristics of the SPs identified for BPN* in the SP screening. The constructs below the dashed line represent control constructs.

<table>
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<th>No.</th>
<th>Name</th>
<th>Amino acid sequence</th>
<th>Length (aa)</th>
<th>D-Score*</th>
<th>Charge N-domain</th>
<th>Hydrophobicity (%)</th>
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<td>1</td>
<td>dBlilo0338</td>
<td>MLINKSKFFVFVFVMMLSLSFVMGEVAKA</td>
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<td>2</td>
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<td>30</td>
<td>0.930</td>
<td>3</td>
<td>57</td>
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</table>

*D-score calculated by SignalP 3.0 ([http://www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)).

*The netto charge of the N region was calculated with amino acids D and E defined as –1, R and K defined as +1 and any other amino acid defined as 0.

*The percentage of hydrophobic amino acids in each signal sequence was calculated with the amino acids G, A, V, L, I, M, F, W and P, defined as hydrophobic, and any other amino acid defined as hydrophilic.
Figure Legends

**Fig. 1:** Strategy used for secretion optimization in different *Bacillus* hosts. A. 393 SPs originating from *B. subtilis* and *B. licheniformis* were cloned in front of the gene encoding B. subtilisin BPN’ from *B. amyloliquefaciens* with its propeptide (PP) which was used as a heterologous secretion target protein. Each SP was amplified with an artificial ribosome binding site (RBS) followed by a spacer region and ATG as the standardized start codon. C. *B. subtilis* was used for screening of the signal peptide library and two *B. licheniformis* strains were used to assess protease secretion levels for best performing SP-BPN’-fusions identified previously.

**Fig. 2:** SPs which resulted in the most efficient secretion of BPN’ identified by screening in *B. subtilis* TEB1030. SPs originating from *B. licheniformis* DSM13 are labeled with the prefix "d", SPs from *B. subtilis* 168 with "s". The extracellular enzyme activity obtained with the wild-type SP (wtSP) of BPN’ was defined as the benchmark (100 % corresponding to 0,83 units per ml). Error bars indicate standard deviations between proteolytic activities detected for each construct in at least three independent cultivations.

**Fig. 3:** High cell density cultivation of *B. subtilis* TEB1030 carrying BPN’ fused to SPs wtSP and dBli00338. Cells were grown in fed-batch mode in a fermentor (culture volume: 3 L) and culture supernatants were analyzed for A. extracellular protease activity and amount of extracellular protease protein by B. SDS-PAGE and subsequent staining with Commassie Brilliant Blue, and C. Western blotting using antibodies against subtilisin BPN’. The protein bands correspond to a M<sub>r</sub> of 28 kDa as deduced from positions of molecular weight standards (NEB, broad range, 2-212 kDa, not shown) and thus correspond to the theoretical M<sub>r</sub> = 27,6 kDa of mature BPN’.

**Fig. 4:** A. Extracellular activities and B. amount of protein determined by Western blotting of subtilisin BPN’ in culture supernatants of screening host *B. subtilis* TEB1030 and expression host *B. licheniformis* H402. The protein bands correspond to a M<sub>r</sub> of 28 kDa as deduced from positions of molecular weight standards (NEB, broad range, 2-212 kDa, not shown) corresponding to the theoretical M<sub>r</sub> = 27,6 kDa of mature BPN’. A *B. subtilis* strain with the
vector encoding BPN’ without SP served as a control. Error bars indicate the standard deviation of proteolytic activities detected for each construct in at least three independent cultivations.

Fig. 5: Extracellular enzyme activities of SP-BPN’ fusions identified by screening in *B. subtilis*, and additionally expressed in *B. licheniformis* H402 and *B. licheniformis* MW3. A. Protease activities in culture supernatants of SP-BPN’ constructs expressed in three different *Bacillus* strains were compared to the benchmark construct. B. Secretion efficiencies in *B. licheniformis* H402 and MW3 shown as percent increase or decrease as compared to *B. subtilis*. The bold black line parallel to the X-axis represents the secretion efficiency in *B. subtilis* which is defined as 0 % deviation.