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Optimization of Protease Secretion in *Bacillus subtilis* and *Bacillus licheniformis* by Screening of Homologous and Heterologous Signal Peptides▽

Christian Degering,[1,2] Thorsten Eggert,[2] Michael Puls,[2] Johannes Bongaerts,[3] Stefan Evers,[3] Karl-Heinz Maurer,[3] and Karl-Erich Jaeger[2]*

Institute of Molecular Enzyme Technology, Heinrich-Heine-Universität Düsseldorf, Research Center Jülich, D-52426 Jülich, Germany[1]; evocatal GmbH, Merowingerplatz 1a, D-40225 Düsseldorf, Germany[2]; and Henkel AG & Co. KGaA, Biotechnology, D-40191 Düsseldorf, Germany[3]

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***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 peptides, which are recognized by the secretion machinery. Here, we have studied the production of an industrially important secreted protease, namely, subtilisin BPN′ from *Bacillus amyloliquefaciens*. One hundred seventy-three signal peptides originating from *B. subtilis* and 220 signal peptides from the *B. licheniformis* type strain were fused to this secretion target and 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 produced 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 better secretion and thus an improved yield of a given extracellular target protein. Our data show that screening a library consisting of homologous and heterologous signal peptides fused to a target protein can identify more-effective signal peptides, resulting in improved protein export not only in the original screening host but also in different production strains.**

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 of bacteria to secrete up to 20 to 25 g/liter 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 species *Bacillus subtilis* and *Bacillus licheniformis* are widely used as production hosts on an industrial scale, and, in contrast to the well-known production species *Escherichia coli*, they are free of endotoxin and have GRAS (generally *r*egarded *a*s *s*afe) status. The complete genome sequences of strains *B. subtilis* 168 (1, 18) and *B. licheniformis* DSM13 (isogenic to ATCC 14580) (26, 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 to identify efficient SPs for different target proteins were taken (2, 4, 6, 15, 21, 38).

Among the huge number of enzymes produced on 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 *Bacillus 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 were 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.

* Corresponding author. Mailing address: Institute of Molecular Enzyme Technology, Heinrich-Heine-Universität Düsseldorf, Research Center Jülich, D-52426 Jülich, Germany. Phone: 49 2461 613716. Fax: 49 2461 612490. E-mail: karl-erich.jaeger@fz-juelich.de.
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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
B. subtilis TEB1030	*trpC2 his nprE aprE bpf ispI lipA lipB*	13
B. licheniformis DSM13	Wild type (isogenic to ATCC 14580)	DSMZ, Germany
B. licheniformis MW3	*B. licheniformis* DSM13 (*hsdR1 hsdR2*)	37
B. licheniformis H402		Henkel AG & Co. KGaA, Germany
B. amyloliquefaciens ATCC 23844	Wild type	ATCC
Plasmids		
pBSMul5	*Bacillus* vector P_{HpaII}; *repB* Km^r	This study
pBSMul5-BPN	pBSMul5 containing 1,059-bp EcoRI-BamHI fragment of the BPN′ coding sequence from *B. amyloliquefaciens* ATCC 23844 without signal sequence	This study

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 and 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 $liter^{-1}$, 10 g tryptone $liter^{-1}$, 10 g NaCl $liter^{-1}$, pH 7) at 37°C supplemented with 50 μg/ml kanamycin. Protease production was monitored on agar plates containing 1% (vol/vol) skim milk. For screening, the cultures were grown for 20 h at 37°C in deep-well microtiter plates (96 wells, 1 ml LB medium 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, 3,200 × *g*, 4°C) and used immediately to determine proteolytic activity. Each strain was cultured 3-fold in parallel in 3 different wells. Cell-free culture supernatants were prepared by microfiltration (membrane filter, cellulose acetate/nitrate, 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 48 h with a Labfors fermentor (volume: 7.5 liter; Infors, Germany) using a high-cell-density cultivation process developed for *B. subtilis* (24). The dissolved oxygen level was set to 30 to 40%, and glucose was fed from a stock solution (500 g glucose/liter). The pH was adjusted by addition of 10% NH_3 (wt/vol) 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 a 50-μl reaction volume with 1 pmol of each primer, 1 U of Phusion high-fidelity polymerase (New England Biolabs), 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 signal peptidase type I (SPase I) recognition site (34). Two hundred twenty 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 the 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; it produced an extracellular protease activity of <0.1 U/ml.

Proteolytic activity assay. Protease activity was determined with the substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF; Bachem AG, Weil am Rhein, Germany) dissolved in dimethyl sulfoxide and finally diluted in 0.1 M Tris-HCl buffer, pH 8.6, containing 1% Brij35 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 min. The amount of *p*-nitroanilide released was measured at 405 nm using a molar absorption coefficient of 8,480/M × cm (11). One unit is defined as the activity releasing 1 μmol of *p*-nitroanilide per minute. Clones showing high proteolytic activity in the culture supernatants were isolated and independently recultivated in microtiter plates (3-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% (wt/vol) stacking gel and a 15% separating gel (19). For Western blot analysis, 10 μl of culture supernatant was loaded on a gel and the separated proteins were subsequently blotted onto a polyvinylidene difluoride membrane (Sequiblot membrane; Bio-Rad, Munich, Germany) (12). BPN′ protein was detected with a rabbit polyclonal antiserum and horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (Bio-Rad, Munich, Germany) using the ECL Western blot kit (Thermo Scientific).

RESULTS

Design and construction of a signal peptide library consisting of Sec type signal peptides. For optimization of heterologous protein export in different *Bacillus* expression strains, a signal peptide library consisting of 220 *B. licheniformis* SPs and 173 *B. subtilis* SPs was constructed. Subtilisin protease BPN′ from *B. amyloliquefaciens* ATCC 23844 (NCBI accession no. K02496.1) served as the secretion target, and *B. subtilis* was used as the 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 1,800 clones (4-fold oversampling) were tested for extracellular protease production on agar plates containing skim milk. About 900 clones formed a clear halo around the colony; they were cultivated in duplicate or triplicate in different microtiter plates, and proteolytic activities in the culture supernatants were determined, allowing identification of 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 7-fold compared to the *B. amyloliquefaciens* wild-type SP (Fig. 2). Characteristics of the best-performing SPs are

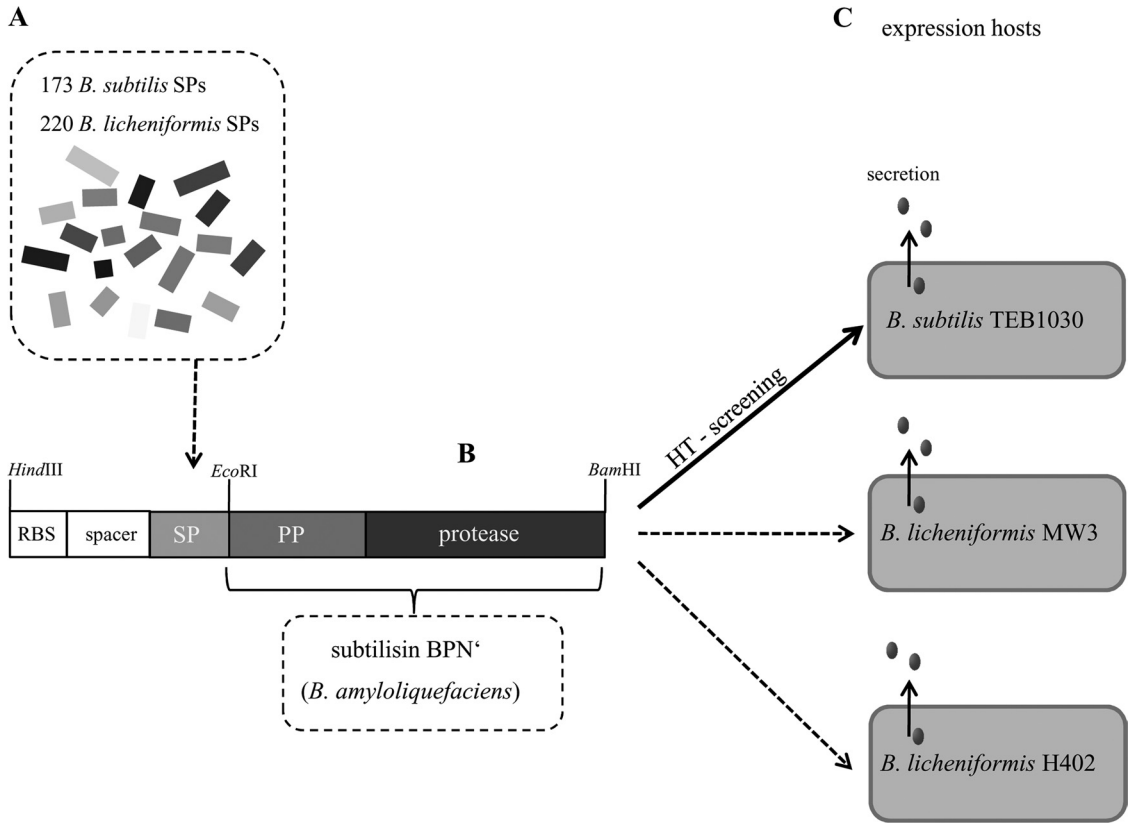

FIG. 1. Strategy used for secretion optimization in different *Bacillus* hosts. Three hundred ninety-three SPs originating from *B. subtilis* and *B. licheniformis* (A) were cloned in front of the gene encoding subtilisin BPN′ from *B. amyloliquefaciens* with its propeptide (PP) (B), which was used as 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 the best-performing SP-BPN′ fusions identified previously. HT, high throughput.

shown in Table 2. Strikingly, we found that not only SPs from the homologous host *B. subtilis* but also SPs isolated from the heterologous host *B. licheniformis* efficiently mediated BPN′ secretion. In fact, *B. licheniformis* SP dBli00338 was among the most efficient SPs we have identified. *B. subtilis* clones expressing constructs with the benchmark SP, wtSP, and the best-performing SP, dBli00338, were also cultivated in a laboratory scale fermentor. BPN′ fused to SP dBli00338 was also efficiently secreted under the conditions of high-cell-density fed-batch cultivation. After 48 h of cultivation, proteolytic activities were 8.9 units/ml for wtSP (at a cell density corresponding to an optical density at 600 nm [OD_{600}] of 68.3) and 59.7 units/ml for dBli00338 (OD_{600} = 65.3), corresponding to a 6- to 7-fold increase of proteolytic activity in the culture supernatant (Fig. 3A), with a concomitant increase in extracellular protease protein (Fig. 3B 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′. We transformed these into *B. licheniformis* H402 and determined extracellular proteolytic activity and amount of BPN′ protein (Fig. 4).

SP dBli00338 produced a 9-fold increase of BPN′ secretion in *B. licheniformis* H402 compared to the wild-type SP of BPN′ based on extracellular proteolytic activity. For all constructs tested, levels of protein export of BPN′ were similar in both *B. subtilis* TEB1030 and *B. licheniformis* H402. These results prompted us to comparatively analyze 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 produced a significant decrease in extracellular BPN′. Extracellular protease activities were compared to those detected in the screening host *B. subtilis* TEB1030 (Fig. 5A). The fusion sYdjM::BPN′ produced an approximately 3.5-fold increase of protease secretion in each of the three expression hosts, while dYdhT::BPN′ produced decreased export relative to the benchmark, wtSP::BPN′, in both *B. licheniformis* hosts. The deviations of relative BPN′ export levels in the two *B. licheniformis* hosts with respect to *B. subtilis* levels were compared for 10 different SP-BPN′ fusions (Fig. 5B). Interestingly, the relative protein export levels of the analyzed SP-BPN′ fusions for both *B. licheniformis* strains were comparable to those observed in *B. subtilis*, with deviations of less than 20%. BPN′ fused to SP dYdhT from *B. licheniformis* represents a clear exception, showing in both *B. licheniformis*

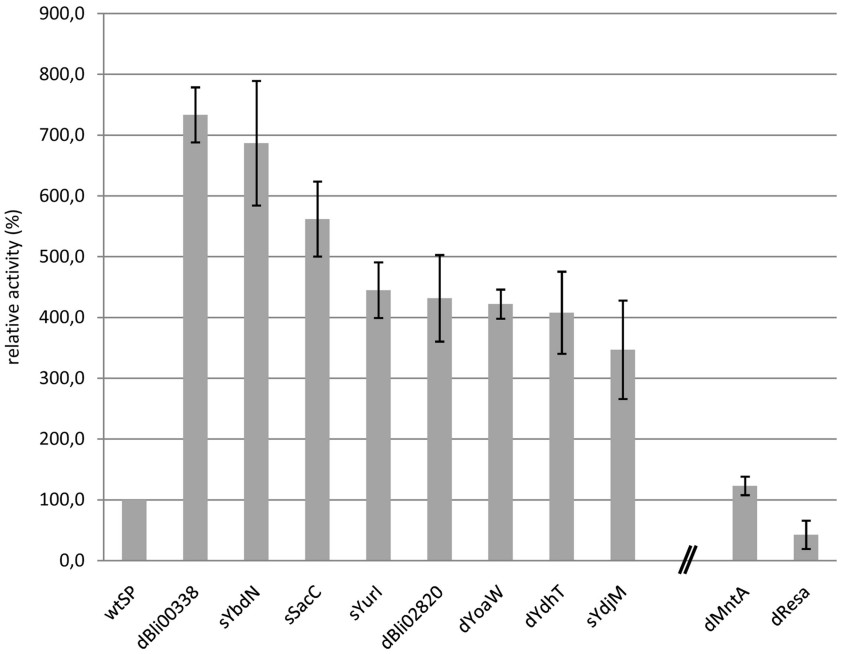

FIG. 2. SPs which produced the most efficient secretion of BPN′, identified by screening in *B. subtilis* TEB1030. SPs originating from *B. licheniformis* DSM13 are labeled with the prefix "d," and SPs from *B. subtilis* 168 are labeled with "s." The extracellular enzyme activity obtained with the wild-type 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.

strains an extracellular protease level 2-fold lower than that for *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 which address strategies to relieve bottlenecks for protein secretion were filed (14, 20, 23). SPs from extracellular proteins known to be secreted at high levels were identified and modified, e.g., SPs from NprE and AprE (14, 23). Additionally, homologous and heterologous SPs were tested in different hosts (8, 36); however, only very few examples of heterologous SPs producing significant increases in secretion efficiency have been described (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.

A library consisting of about 400 different SPs fused to

TABLE 2. Characteristics of the SPs identified for BPN′ in the SP screening

SP	Amino acid sequence	Length (aa[e])	D score[a]	Net charge of N domain[b]	Hydrophobicity (%)[c]
dBli00338	MLINKSKKFFVFSFIFVMMLSLSFVNGEVAKA	32	0.897	3	53
sYbdN	MVKKWLIQFAVMLSVLSTFTYSASA	25	0.930	2	48
sSacC	MKKRLIQVMIMFTLLLTMAFSADA	24	0.927	3	58
sYurI	MTKKAWFLPLVCVLLISGWLAPAASASA	28	0.915	2	68
dBli02820	MRMKRLRMRKHLLIAVCTLALLLSSPIVSDA	31	0.936	6	52
dYoaW	MKKIVCLMVFSIMTAFGIHIQPAEA	25	0.864	2	56
dYdhT	MKKNIVCSIFALLLAFAVSQPSYA	24	0.870	2	58
sYdjM	MLKKVILAAFILVGSTLGAFSFSSDASA	28	0.915	2	57
dMntA[d]	MKWKQTLAIAAALILILAAGCSSKSSS	27	0.783	2	59
dResA[d]	MKKKRFYIRTGILLVLLAALGYTLYSAVFQNTESV	35	0.617	5	51
wtSP[d]	MRGKKVWISLLFALALIFTMAFGSTSSAQA	30	0.930	3	57

[a] D score calculated by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).
[b] Calculated with amino acids D and E defined as −1, R and K defined as +1, and any other amino acid as 0.
[c] Percentage of hydrophobic amino acids in each signal sequence, calculated with amino acids G, A, V, L, I, M, F, W, and P defined as hydrophobic and any other amino acid defined as hydrophilic.
[d] Control construct.
[e] aa, amino acids.

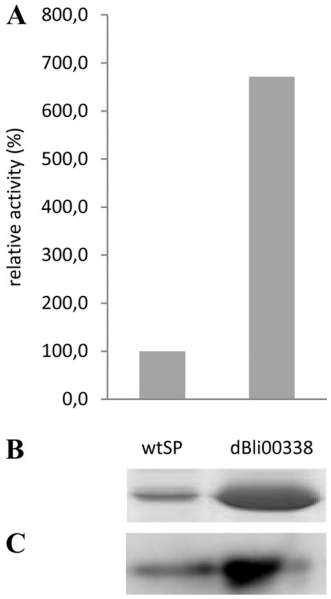

FIG. 3. High-cell-density cultivation of *B. subtilis* TEB1030 carrying BPN′ fused to SPs wtSP and dBli00338. Cells were grown in fed-batch mode in a fermentor (culture volume: 3 liters), and culture supernatants were analyzed for extracellular protease activity (A) and amount of extracellular protease protein (B and C) by SDS-PAGE and subsequent staining with Coomassie brilliant blue (B) and Western blotting using antibodies against subtilisin BPN′ (C). The protein bands correspond to a molecular mass of 28 kDa, as deduced from positions of molecular weight standards (New England Biolabs [NEB]; broad range, 2 to 212 kDa, not shown) and thus correspond to the theoretical molecular mass of 27.6 kDa of mature BPN′.

subtilisin BPN′ was constructed and tested by high-throughput activity screening of *B. subtilis* culture supernatants. Eight SPs were identified, with dBli00338 and sYbdN producing a 7-fold increase of BPN′ activity in the culture supernatant compared to wild-type SP. The results obtained by SP screening in microtiter plates were confirmed for wtSP and SP dBli00338 by high-cell-density cultivation of *B. subtilis* in a 3-liter laboratory scale 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 small amounts or were not identified at all. It should be noted 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 between them and subtilisin BPN′. These results suggest that the general approach of choosing an 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

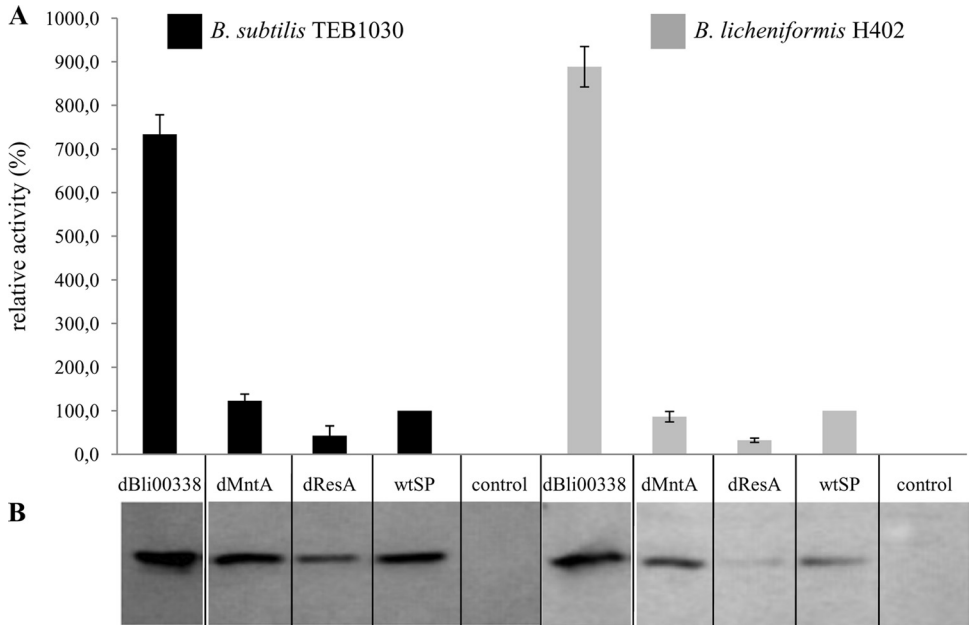

FIG. 4. Extracellular activities (A) and amount of protein (B) determined by Western blotting of subtilisin BPN′ in culture supernatants of screening host *B. subtilis* TEB1030 and expression host *B. licheniformis* H402. The protein bands correspond to a molecular mass of 28 kDa, as deduced from positions of molecular weight standards (NEB; broad range, 2 to 212 kDa, not shown) corresponding to the theoretical molecular mass of 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 deviations of proteolytic activities detected for each construct in at least three independent cultivations.

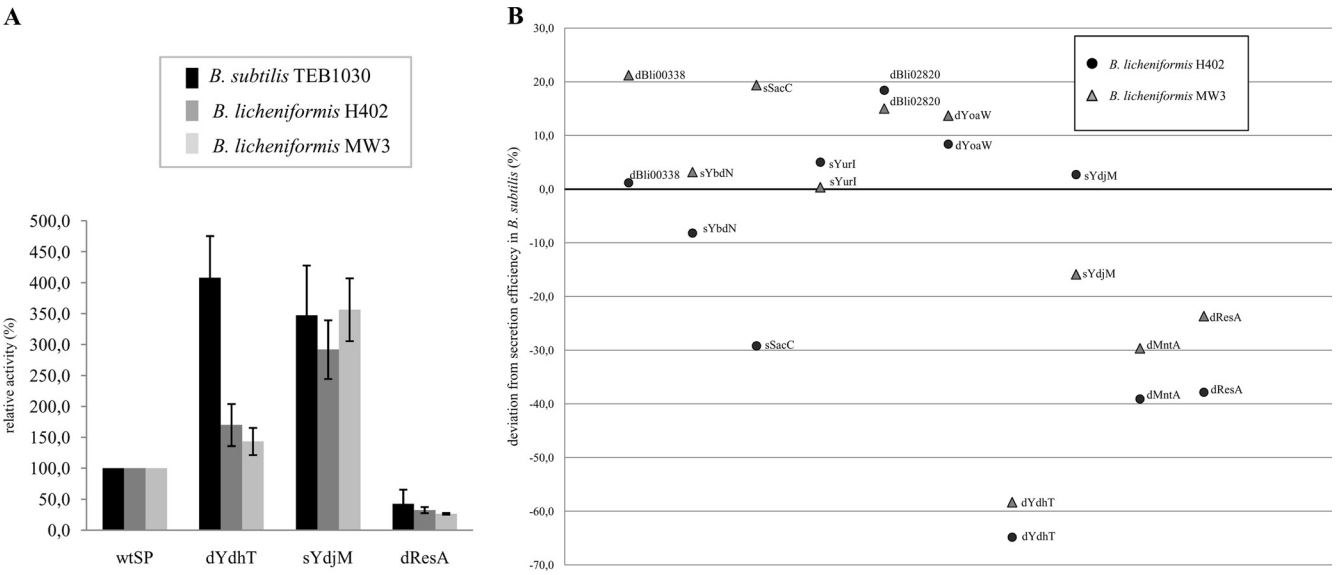

FIG. 5. Extracellular enzyme activities of SP-BPN′ fusions identified by screening in *B. subtilis* and additionally expressed in *B. licheniformis* H402 and *B. licheniformis* MW3. (A) Protease activities in culture supernatants of SP-BPN′ constructs expressed in three different *Bacillus* strains were compared to the benchmark construct. (B) Secretion efficiencies in *B. licheniformis* H402 and MW3 shown as percent increases or decreases compared to that in *B. subtilis*. The boldface line represents the secretion efficiency in *B. subtilis*, which is defined as 0% deviation.

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 proteases 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 that high-throughput prescreening in *B. subtilis* and subsequent transfer of the best-performing constructs into *B. licheniformis* for further testing should be performed. 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 in components of their Sec machineries and especially their signal peptidases have been described (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 produce 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 an SP library and that the best-performing SP fusions can subsequently be transferred into a production strain to improve protein export.

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