Intramembrane protease RasP boosts protein production in Bacillus

Jolanda Neef†, Cristina Bongiorni‡, Vivianne J. Goosens¹,³, Brian Schmidt² and Jan Maarten van Dijl*¹

Abstract

Background: The microbial cell factory Bacillus subtilis is a popular industrial platform for high-level production of secreted technical enzymes. Nonetheless, the effective secretion of particular heterologous enzymes remains challenging. Over the past decades various studies have tackled this problem, and major improvements were achieved by optimizing signal peptides or removing proteases involved in product degradation. On the other hand, serious bottlenecks in the protein export process per se remained enigmatic, especially for protein secretion at commercially significant levels by cells grown to high density. The aim of our present study was to assess the relevance of the intramembrane protease RasP for high-level protein production in B. subtilis.

Results: Deletion of the rasP gene resulted in reduced precursor processing and extracellular levels of the overproduced α-amylases AmyE from B. subtilis and AmyL from Bacillus licheniformis. Further, secretion of the overproduced serine protease BPN' from Bacillus amyloliquefaciens was severely impaired in the absence of RasP. Importantly, overexpression of rasP resulted in threefold increased production of a serine protease from Bacillus clausii, and 2.5- to 10-fold increased production of an AmyAc α-amylase from Paenibacillus curdlanolyticus, depending on the culture conditions. Of note, growth defects due to overproduction of the two latter enzymes were suppressed by rasP-overexpression.

Conclusion: Here we show that an intramembrane protease, RasP, sets a limit to high-level production of two secreted heterologous enzymes that are difficult to produce in the B. subtilis cell factory. This finding was unexpected and suggests that proteolytic membrane sanitation is key to effective enzyme production in Bacillus.

Keywords: Bacillus, RasP, Site-2 protease, Secretion, Amylase, Protease

Background

Secretory protein production is of critical importance in biotechnology, because this generally delivers high amounts of correctly folded proteins for which downstream processing from the fermentation broth is relatively easy. The Gram-positive bacterium Bacillus subtilis and related bacilli are amongst the best protein producers known today and, therefore, frequently used to produce commercially relevant enzymes. In particular, these organisms are suitable for large-scale high-density fermentation, leading to product yields in the 25 g/l range [1]. Thus, they have a long history in the production of, for example, amylases [2, 3] and proteases [2, 4] used in the food, textile and pharmaceutical industries [5]. Another advantage of B. subtilis and its close relatives is that they lack toxic by-products, such as endotoxin, which makes them suitable for the Qualified Presumption of Safety (QPS) status of the European Food Safety Authority. Accordingly, many Bacillus products have obtained the Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration [6, 7].

Much effort has been made to optimize protein secretion in B. subtilis. Major improvements were achieved by optimizing signal peptides [8, 9] or removing proteases involved in product degradation [10]. For example, the deletion of multiple genes for extracellular proteolytic enzymes allowed efficient production not only of technical enzymes, such as a thermostable β-1,3-1,4-glucanase...
from Clostridium thermocellum [11], but also of pharmaceu-
tical proteins, such as single-chain antibodies [12] or human interleukin-3 [13]. Further improvements in pro-
tein secretion were achieved at lab-scale by overexpres-
sion of the signal peptidase SipS [14], the peptidyl-prolyl
cis/trans isomerase PrsA [15], or the staphylococcal thiol-
disulphide oxidoreductase DsbA [16]. Nonetheless, seri-
bous bottlenecks in the protein export process per se have
remained enigmatic, especially for protein secretion at
commercially significant levels by cells grown to high
density [17].

Notably, it was previously shown that deleting the gene
for the intramembrane protease RasP of B. subtilis led
to elevated levels of the membrane proteins FtsL [18],
HtrA and HtrB, but compromised the production of vari-
ous other membrane proteins [19] and processing of the
α-amylase AmyQ of Bacillus amyloliquefaciens [20, 21].
This focused our attention on a possible role of RasP in
secretory protein production, especially because the bio-
genesis of many membrane proteins relies on the general
secretory pathway [22]. RasP belongs to the family of
site-2 proteases (S2P), more specifically the zink-metallo
proteases, which cleave their substrates within the plane
of the cytoplasmic membrane [23]. These proteases are
conserved in all domains of life where they have roles in
regulated intramembrane proteolysis. For instance, the
Escherichia coli S2P named RseP was shown to cleave sig-
nal peptides upon their signal peptidase-mediated liber-
ation from secretory precursor proteins [24, 25]. B. subtilis
RasP was shown to cleave the anti-sigma factor RsiW
under conditions of oxidative- or temperature stress,
causing induction of the so-called oW regulon, which is
believed to support cell envelope integrity and to mitigate
effects of extracellular stress [20, 21, 26, 27]. Therefore,
the present study was aimed at determining whether RasP
could be a bottleneck for protein production in B.
subtilis. Indeed, our results show that RasP overexpres-
sion can boost protein production in this important cell
factory.

Methods
Bacterial strains and growth conditions
The bacterial strains used in this study are listed in
Table 1. B. subtilis strains were grown at 37 °C, 280 rpm
in Lysogeny Broth (LB; Oxoid Limited), MBU medium
or 5SM12 medium. The MBU medium is similar to the
MBD medium as described by Vogtentanz et al. [28], but
lacks soytone and instead of 7.5% glucose it contains 2.1%
glucose and 3.5% maltodextrin DE13-17. The 5SM12
medium consists of 75 mM K2HPO4, 25 mM NaH2PO4,
12% maltodextrin, 5% Difco Bacto Soytone, 2 mM
sodium citrate, 0.5 mM MgSO4, 0.2 mM MnCl2, 0.03 mM
calcium chloride, and 0.0053% ferric ammonium citrate.

Growth media were supplemented with neomycin 15 µg/
ml or phleomycin 4 µg/ml to select for particular gene
deletions. Chloramphenicol was added to 5 or 25 µg/ml
for, respectively, the selection of chromosomally inte-
grated amylose or protease expression cassettes and their
amplification. In pulse-chase labeling experiments with
cells grown on MBU medium, 2.5 µg/ml chlorampheni-
col was used.

Construction of strains and plasmids
Ex Taq polymerase, dNTPs and buffers used for the con-
struction of mutant strains were purchased from Takara
Bio Inc. Phusion High Fidelity DNA polymerase (New
England Biolabs) was used for the construction of plas-
mids. Primers were obtained from Eurogentec. Construc-
tion of deletion mutants in a B. subtilis Δupp::neoRF
strain was performed using the modified mutation deliv-
ery method described by Fabret et al. [29]. To delete a
particular gene of interest (i.e. rasP or tepA), its 5’ and 3’
flanking regions were amplified using primer pairs desig-
nated P1/P2 and P3/P4 (Table 2). The amplified frag-
ments were fused to a cassette containing a phleomycin
resistance marker, the upp gene and the ci gene [29].
The resulting fusion product was used to transform B. subti-
lis Δupp::neoRF, where competence was induced with 0.3%
xylene due to the presence of a xylose-inducible comK
gene. This resulted in phleomycin resistant and neomy-
cin sensitive strains lacking the target gene. PCR’s using
primer combinations P0/P4 and P0/CI2.rev (Table 2)
were performed to verify the correct deletion. To achieve
overproduction and secretion of AmyE [30], AmyL [31]
or amyE aprE were fused to the 7-codon pro-sequence of
amyE, the amyL gene lacking its signal sequence, or the eighth codon of the BPN
·-Y217L (in short BPN
−
)
]32, 33, 34 and the signal sequence of aprE were fused to the 7-codon pro-sequence of amyE, the amyL gene lacking its signal sequence, or the eighth codon of the BPN signal sequence, respectively. For AmyE, the C-terminal starch-
binding module was removed by the introduction of a
stop codon after the codon for residue 425 in amyE and the complete gene expression cassette was produced syn-
ethetically (GeneOracle, Santa Clara, CA). Transcription of amyE or bpn’ was terminated using the native BPN’
terminator, and the native terminator in case of amyL. To
accomplish the expression and secretion of Properase®
(i.e. the subtilisin variant of Bacillus clausii) or AmyAc
[i.e. an engineered α-amylase from Paenibacillus curd-
lanolyticus that belongs to the AmyAc family (NCBI re-
ference sequence: WP_040711139)], the respective genes
were ordered synthetically (GeneArt, Thermofisher Scien-
tific) and fused to the promoter and signal sequence of
aprE as described above. For transcription termination,
the native BPN’ terminator was used. Genes encoding the
to five afore-mentioned secretory proteins were integrated
Table 1  Bacterial strains used in this study

| Relevant genotypes and phenotypes | Source or reference |
|----------------------------------|---------------------|
| *B. subtilis* strain             |                     |
| Δupp                            | This study          |
| AmyL                            | This study          |
| AmyE                            | This study          |
| BPN’                            | This study          |
| ΔrasP                           | This study          |
| AmyL ΔrasP                       | This study          |
| AmyE ΔrasP                       | This study          |
| BPN’ ΔrasP                       | This study          |
| ΔtepA                           | This study          |
| AmyL ΔtepA                       | This study          |
| BPN’ ΔtepA                       | This study          |
| Proprerase                       | This study          |
| AmyAc                           | This study          |
| AmyAc RasP                       | This study          |
| Plasmids                         | Genencor/dupont     |

| pHTK315-Pspac | Multicopy shuttle vector replicating in E. coli and *B. subtilis* contains IPTG-inducible Pspac Ampβ, Emγ | [44] |
| pHTK315 | pHTK315-Pspac derivative, Emγ is replaced by Kmδ, Ampζ, Kmθ | This study |
| pHTK315K-ΔrasP | pHTK315K-Pspac derivative, contains IPTG-inducible rasP, Ampβ, Kmθ | This study |

Tbpn’ terminator structure of *B. amylobacter* bpn’; Emγ erythromycin resistant; Phleoα phleomycin resistant; Neoβ neomycin resistant; Neoγ neomycin sensitive; Kmδ chloramphenicol resistant; Ampζ ampicillin resistant; Kmθ kanamycin resistant

into the aprE locus by single cross-over recombination using a vector based on plasmid pH101 [35]. Lastly, gene amplification was achieved by growing transformants at increasing chloramphenicol concentrations up to 25 µg/ml.

To complement the ΔrasP mutation, plasmid pHTK315, a derivative of pHT315-Pspac [36] containing the *kan* gene for kanamycin resistance was constructed using the CPEC strategy described by Quan et al. [37]. Plasmid pHT315-Pspac was amplified by PCR using the primers pHT315-Pspac fw and pHT315-Pspac rev. In parallel, the *kan* gene was amplified from the vector pGDL48 [38], using the primers kanaCPEC fw and kanaCPEC rev both containing approximately 30 bp overlap with the pHT315-Pspac vector. The two resulting PCR fragments were fused and amplified by PCR, and the resulting amplicon was used to transform competent cells of *E. coli* strain TG1. The plasmid thus obtained was named pHTK315. Next, plasmid pHTK315-rasP was constructed following the same strategy. For amplification of pHTK315, the primer combination pHT315rasP fw/ pHT315rasP rev was used, and rasP was amplified using the primer combinations rasP fw/rasP rev. Both fragments were fused by PCR based on the 30 bp overlaps. The resulting amplicon was used to transform competent cells of *E. coli* TG1, and the plasmid thus obtained was named pHTK315-rasP. The correctness of pHTK315 and pHTK315-rasP was verified by sequencing and, subsequently, these plasmids were introduced into *B. subtilis* using xylose-induced competence, as described above. After the transformation, mutant strains containing amplified *amyE*, *amyL* or bpn’ expression cassettes were selected by repeated transfers to fresh LB plates containing 25 µg/ml chloramphenicol.

Analysis of secreted protein production by LDS-PAGE
Cultures were inoculated from LB plates with 25 µg/ml chloramphenicol and grown for approximately 8 h in LB broth with 25 µg/ml chloramphenicol. These cultures were diluted 1000-fold in MBU medium with 2.5 µg/ml
chloramphenicol in Ultra Yield Flasks™ (Thomson Instrument Company) and incubated for approximately 16 h at 37 °C, 280 rpm in a Multitron orbital shaker (Infors) at high humidity. After measuring and correcting for the optical density at 600 nm (OD_{600}), equal amounts of cells were separated from the culture medium by centrifugation. For the analysis of extracellular proteins, proteins in the culture medium were precipitated with trichloroacetic acid (TCA; 10% w/v final concentration), dissolved in LDS buffer (Life Technologies) and heated for 10 min at 95 °C. Next, proteins were separated by LDS-PAGE using 10% NuPage TCA-precipitated culture medium samples. Labeled proteins were separated from the culture medium by centrifugation.

### Pulse-chase protein labeling experiments

Pulse-chase labeling of *B. subtilis* proteins was performed using Easy tag [35S]-methionine (PerkinElmer Inc.). Immunoprecipitation and LDS-PAGE were performed as described previously [39] using the following adaptations. Cells were grown for 16 h in MBU with 2.5 µg/ml chloramphenicol as described before and diluted 1 h prior to the actual labeling to OD_{600} ~0.7 in fresh MBU with 2.5 µg/ml chloramphenicol. Labeling was performed with 25 µCi [35S]-methionine for 30 s before adding an excess amount of unlabeled methionine (chase; 0.6 mg/ml final concentration). Samples were collected at several time points, followed by direct precipitation of the proteins with 10% TCA (w/v) on ice. Precipitates were re-suspended in lysis buffer (10 mM Tris pH 8, 25 mM MgCl₂, 200 mM NaCl and 5 mg/ml lysozyme). After 10–15 min incubation at 37 °C, lysis was achieved by adding 1% (w/v) SDS and heating for 10 min at 100 °C. Specific polyclonal antibodies against AmyE or AmyL were used for immunoprecipitation of the respective labeled proteins in STD-Tris buffer (10 mM Tris pH 8.2, 0.9% (w/v) NaCl, 1.0% (w/v) triton X-100, 0.5% (w/v) sodium deoxycholate) with the help of Protein A affinity medium (Mabselect Sule, GE Healthcare Life Sciences).

Because of the high proteolytic activity of BPN’, which also degrades antibodies, the immunoprecipitation of BPN’ was performed in the presence of a specific serine protease inhibitor (4 mM, Pefablock SC, Roche). Due to a specific binding of the BPN’ antibodies to unidentified cellular proteins of *B. subtilis*, the immunoprecipitation of BPN’ was only performed to assay secreted BPN’ in TCA-precipitated culture medium samples. Labeled proteins were separated by LDS-PAGE using 10% NuPage gels (Life Technologies) and visualized using a Cyclone Plus Phosphor Imager (Perkin Elmer). Quantification of the obtained data was achieved by making use of the ImageJ software.

For pulse-chase labeling studies on the complementation of the ΔrasP mutation, cells containing pHTK315-rasP or control cells containing the empty vector pHTK315 were pre-cultured for 20 h in MBU with 2.5 µg/ml chloramphenicol, because these cells grow slightly slower than cells without such plasmids. One hour prior to labeling with [35S]-methionine, the cells were diluted to an OD_{600} of ~0.7 in fresh MBU with 2.5 µg/ml chloramphenicol containing 25 µM isopropyl β-d-1-thiogalactopyranoside (IPTG) necessary for the induction of the P_{spac} promoter. The pulse-chase protein labeling was performed as described above.

### Assays for Properase or AmyAc production

To analyze Properase secretion by cells overexpressing rasP or wt control cells, the respective strains were pre-cultured for 5 h in LB at 37 °C. From these pre-cultures 1.5 OD units were used to inoculate 25 ml of MBU medium in Ultra Yield Flasks™, and culturing was continued at 37 °C (250 rpm, 70% humidity). Samples were withdrawn from the cultures at 18, 25, 41, 48 and 65 h of growth for OD_{600} readings and protease activity.
measurements. OD600 was determined using a SpectraMax spectrophotometer (Molecular Devices, Downingtown, PA, USA). Protease activity in the samples was determined by incubating sample aliquots with the synthetic substrate N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Sigma Chemical Co) and absorbance readings at 405 nm using a SpectraMax spectrophotometer, as described previously in WO 2010/144283.

To test the effect of rasP overexpression on the production of AmyAc, four colonies from the rasP overexpressing strain or the wt control strain were used to inoculate LB with 25 µg/ml chloramphenicol and the resulting pre-cultures were grown for 4 h at 37 °C. Next, 0.075 OD units from a pre-culture were used to inoculate 2 ml of medium (5SM12 or MBU) in 24 deep well microtiter plates and cultures were grown for 48 h at 37 °C under vigorous shaking. Samples were withdrawn at 18, 25, 41 and 48 h of culturing for OD600 readings and amylase activity measurements. Amylase activity in whole-broth samples was assayed with the Ceralpha HR kit (Mega-zyme, Wicklow, Ireland) and absorbance readings at 400 nm according to the manufacturer’s instructions.

**Results and discussion**

As a first approach to assess the possible function of RasP in protein secretion under fermentation-mimicking conditions, the rasP gene was deleted from the B. subtilis genome and the secretion of three representative model proteins was assessed in the resulting ΔrasP strain. Specifically, the secreted model proteins were the α-amylase AmyE from B. subtilis, the α-amylase AmyL from Bacillus licheniformis and the serine protease BN’ from B. amyloliquefaciens. The respective genes were expressed to high levels using the aprE promoter, which is a preferred promoter for enzyme production at industrial scale [40]. As shown in Fig. 1, ΔrasP cells grown to stationary phase in about 16–20 h of culturing secreted less AmyE, AmyL or and BN’ than wild-type cells. This was clearly not the case for control cells lacking the tepA gene, which encodes an unrelated cytoplasmic protease [41]. Furthermore, the rates of AmyE and AmyL precursor processing as determined by pulse-chase labeling with [35S]-methionine after 16 h of growth were substantially reduced in ΔrasP cells compared to wild-type cells (Fig. 2a). In case of BN’ we were unable to detect cell-associated precursor forms of this protein but, nonetheless, we showed that the rate of appearance of the mature [35S]-methionine-labeled BN’ in the growth medium was strongly slowed down in cells lacking rasP (Fig. 2b). Together, these findings clearly demonstrate that RasP is needed for efficient processing and secretion of mature AmyE, AmyL and BN’. As illustrated with AmyE, the wild-type rate of precursor processing and secretion of the mature protein were restored when rasP was expressed from a plasmid in the ΔrasP cells (Figs. 3, 4). This shows that the ΔrasP mutation can be complemented with rasP expressed in trans. Of note, the wild-type cells grew to higher optical densities at 600 nm (OD600 ~ 25) than the ΔrasP cells (OD600 ~ 15), but this effect was corrected for in the loading of gels shown in Figs. 1 and 3, and in the pulse-chase labelling experiments in Figs. 2 and 4 comparable amounts of cells were used.

Because the removal of RasP had significant influence on the processing and secretion of AmyL, AmyE and BN’, we wanted to know whether rasP overexpression could be beneficial for protein secretion in B. subtilis. Notably, in ‘wild-type’ B. subtilis the secretion of AmyL, AmyE and BN’ is already very efficient. Moreover, these enzymes are produced really well in other industrial Bacillus species, which gives the optimization of their production in B. subtilis a lower commercial impact. Therefore, we focused our attention on two other enzymes, namely a protease (‘Properase’) from B. clausii and AmyAc from P. curdlanolyticus, which are both commercially valuable but hard to produce. Especially the large-scale production of enzymes of the AmyAc family is very challenging in Bacillus species. To overexpress rasP, this gene was placed under control of the very strong spoVG promoter (PsapoVG). Importantly, the PsapoVG
promoter is a constitutive promoter and its strength is comparable to that of the promoter of aprE, which was used to express the secreted proteins employed in

Fig. 2 Reduced rates of AmyE, AmyL and BPN' secretion in rasP mutant cells. a Processing of the precursor proteins of AmyE or AmyL by signal peptidase was analyzed by pulse-chase protein labeling with [35S]-methionine, immunoprecipitation of AmyE or AmyL from culture samples with specific antibodies, LDS-PAGE and phosphorimaging as described in "Methods." The positions of precursor (p) and mature (m) forms of AmyE and AmyL are indicated. Data from three independent experiments were analyzed with ImageJ to assess the kinetics of precursor processing, and the results are plotted below the autoradiographs. The plot shows the relative amounts (%) of the precursor forms of AmyE (black symbols) or AmyL (white symbols) in the ΔrasP (triangle) or wt (square) strains at different time points after the chase with non-radioactive methionine (t = 0). Error bars show the standard deviation. b Secretion of BPN' was analyzed by pulse-chase labeling with [35S]-methionine, immunoprecipitation from growth medium fractions devoid of cells with specific antibodies, LDS-PAGE and phosphorimaging as described in "Methods." The position of mature BPN' is indicated (m). Data from three independent experiments were analyzed with ImageJ to determine the kinetics of BPN' appearance in the growth medium, and the results are plotted below the autoradiographs. The plot shows the average of the calculated ratio of secreted BPN' in the ΔrasP (triangle) or wt (square) strains relative to the amount of BPN' secreted immediately after the chase with non-radioactive methionine (t = 0) in the wt strain. Error bars indicate the standard deviation.

Fig. 3 IPTG-dependent complementation of the ΔrasP mutation in AmyE-producing cells containing pHTK315-rasP. ΔrasP mutant bacteria overproducing AmyE and carrying either pHTK315-rasP or the empty vector pHTK315 were grown for 16 h in MBU with 2.5 µg/ml chloramphenicol. Next rasP expression in cells containing pHTK315-rasP, where rasP is transcribed from the IPTG-dependent Pspac promoter, was induced for 4 h by the addition of IPTG to different end concentrations as indicated. ΔrasP mutant bacteria carrying the empty vector pHTK315 were also treated with IPTG as a negative control. Proteins in the growth medium were precipitated with TCA and separated by LDS-PAGE. Protein bands were visualized with the SimplyBlue SafeStain. The AmyE band is indicated with an arrow, and the molecular weights of marker proteins are indicated on the left of the gel (in kDa).

Fig. 4 Complementation of pre-AmyE processing in ΔrasP mutant cells. Processing of pre-AmyE (p) to mature AmyE (m) was analyzed by pulse-chase protein labeling with [35S]-methionine in IPTG-induced ΔrasP mutant or wt cells containing either pHTK315-rasP or the empty vector pHTK315. Pre-AmyE and mature AmyE were immunoprecipitated with specific antibodies, separated by LDS-PAGE, and visualized by a phosphorimaging. Data from two independent experiments were analyzed with ImageJ to assess the kinetics of pre-AmyE processing to the mature form, and the results are plotted below the autoradiographs. Specifically, the plot shows the relative amounts (%) of the precursor form of AmyE in ΔrasP mutant (triangle) or wt (square) cells carrying pHTK315-rasP (white symbols) or pHTK315 (black symbols) at different time points after the chase with non-radioactive methionine (t = 0).
this study (data not shown). Next, the capability of the resulting strain to secrete the highly active Properase, which is even capable of degrading prions, was tested. A potential problem caused by high-level Properase production is a negative effect on the viability of *B. subtilis*. As shown in Fig. 5a, PspoVG-driven expression of *rasP* enhanced both cell viability and the secretion of Properase in the production phase when cells were grown in MBU medium. Of note, high-level *rasP* expression led to an increase in Properase production of about threefold. To further obtain proof-of-principle that PspoVG-driven *rasP* expression may be beneficial for secretory protein

![Fig. 5](image.png)

**Fig. 5** Improved production of Properase and AmyAc upon overexpression of *rasP*. 

(a) Growth (left panel) and extracellular Properase activity (right panel) of cells cultured in MBU medium. Measurements on cells that overexpress *rasP* from the strong PspoVG promoter are indicated with black lines and measurements on wt cells are indicated with grey lines. 

(b) Growth (left panel) and extracellular AmyAc activity (right panel) of cells that overexpress *rasP* from the PspoVG promoter (black lines) or wt cells (grey lines) cultured in MBU medium. 

(c) Growth (left panel) and extracellular AmyAc activity (right panel) of cells that overexpress *rasP* from the PspoVG promoter (black lines) or wt cells (grey lines) cultured in 5SM12 medium. All plots in (a–c) show average values from three independent experiments, and the error bars represent the standard deviations of the respective measurements.
production, we investigated the effect on production of a bacterial α-amylase belonging to the AmyAc family. Similar to Properase, the expression of the AmyAc enzyme had a negative impact on growth in MBU medium and, in this case, both growth and amylase production remained relatively low unless rasP was overexpressed (Fig. 5b). In fact, AmyAc production by cells grown in MBU was up to tenfold increased upon rasP overexpression. Interestingly, expression of the AmyAc enzyme does not impact on growth in 5SM12 medium, which allowed us to distinguish between growth effects and effects of rasP overexpression on production of the secreted AmyAc enzyme. As shown in Fig. 5c, the yield of this enzyme in the 5SM12 growth medium was about 2.5-fold increased, which implies that the improved productivity was mostly directly related to rasP overexpression rather than an enhanced cell density of the culture. The main difference between the 5SM12 and MBU media is that the 5SM12 medium contains soytone and 3.4-fold more maltodextrin. This suggests that AmyAc production may have a negative impact on nutrient acquisition by cells grown in MBU, which can be bypassed either by rasP overexpression or the provision of soytone and/or additional maltodextrin. Altogether, our results imply that rasP overexpression gives significant benefits for producing secretory proteins commercially.

**Conclusions**

In conclusion, our present study shows that the S2P intramembrane protease RasP sets the limit to efficient extracellular production of two proteins in *B. subtilis*, namely Properase and an AmyAc type amylase. These proteins are difficult to produce, which is partly due to effects on cell growth and/or viability in late stages of the fermentation process. Enhanced expression of rasP can overcome these negative effects, and seems even capable of boosting the secretion of the AmyAc type amylase up to tenfold. Our present findings are unprecedented, giving the first proof-of-principle that overexpression of a protease that cleaves within the plane of the cytoplasmic membrane of a bacterium can lead to improved protein production. The precise mechanism by which RasP exerts this effect in *Bacillus* is not yet known but, based on knowledge from other studies on S2P proteases, we envisage at least three possible scenarios. Firstly, RasP may facilitate the removal of cleaved signal peptides from the membrane [25], secondly, RasP may clear the membrane of mislocalized secretory precursor proteins that may interfere with essential membrane processes [20], or thirdly, overproduced RasP may modulate expression of αW-dependent genes that somehow influence productivity [26]. In these three scenarios, rasP overexpression could increase the fitness of the producing cells through the prevention of membrane and cell envelope perturbations. This would then lead to enhanced growth and productivity. A fourth possible explanation would be that RasP activity precludes potentially inhibitory effects of accumulating signal peptides on the SecA preprotein translocation motor [42] and/or on signal peptidases that convert translocated precursors of secretory proteins to the mature form [43]. Of course, combinations of these four scenarios are also conceivable. Irrespective of the precise mechanisms, we conclude that RasP can be applied to boost protein secretion in *Bacillus*, and that the overexpression of this and other S2P proteases represents a promising avenue for future cell factory engineering.
