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Using the IPTG-Inducible P*grac*212 Promoter for Overexpression of Human Rhinovirus 3C Protease Fusions in the Cytoplasm of *Bacillus subtilis* Cells

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Abstract

Expression and secretion of recombinant proteins in the endotoxin-free bacterium, *Bacillus subtilis*, has been thoroughly studied, but overexpression in the cytoplasm has been limited to only a few proteins. Here, we used the robust IPTG-inducible promoter, P*grac*212, to overexpress human rhinovirus 3C protease (HRV3C) in the cytoplasm of *B. subtilis* cells. A novel solubility tag, the N-terminal domain of the *lysS* gene of *B. subtilis* coding for a lysyl-tRNA synthetase was placed at the N terminus with a cleavage site for the endoprotease HRV3C, followed by His-HRV3C or His-GST-HRV3C. The recombinant protease was purified by using a Ni–NTA column. In this study, the His-HRV3C and His-GST-HRV3C proteases were overexpressed in the cytoplasm of *B. subtilis* at 11% and 16% of the total cellular proteins, respectively. The specific protease activities were 8065 U/mg for His-HRV3C and 3623 U/mg for His-GST-HRV3C. The purified enzymes were used to cleave two different substrates followed by purification of the two different protein targets, the green fluorescent protein and the beta-galactosidase. In conclusion, the combination of an inducible promoter P*grac*212 and a solubility tag allowed the overexpression of the HRV3C protease in the cytoplasm of *B. subtilis*. The resulting fusion protein was purified using a nickel column and was active in cleaving target proteins to remove the fusion tags. This study offers an effective method for producing recombinant proteins in the cytoplasm of endotoxin-free bacteria.

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Introduction

Bacillus subtilis is currently the best-studied Gram-positive bacterium in the scientific world. *B. subtilis* strain 168 and its derivatives are nonpathogenic and free of exo- and endotoxins. Its ease of genetic manipulation and genetically well-characterized expression systems enable this species to be used as an ideal workhorse for industrial and pharmaceutical purposes including the production of recombinant microbial enzymes and chemicals [1, 2]. Owing to its FDA status as GRAS (generally regarded as safe), its capacity for protein secretion [3–5], the absence of significant codon bias [6], the amenability for genetic engineering and abundant toolboxes [7], and extensive information on its fermentation processes [8, 9], *B. subtilis* has been chosen as an alternative host for overexpression and secretion of heterologous proteins [9–11].

In contrast to *B. subtilis*, the widely used Gram-negative bacterium *Escherichia coli* produces recombinant proteins mainly in the cytoplasm. Since *E. coli* contains the potent immunostimulatory endotoxin lipopolysaccharide, scientists sought to use an endotoxin-free platform for the synthesis of desired products [12]. Similar to other expression hosts, in certain cases, *E. coli* produces little or no recombinant protein, often because of inhibitory effects caused by the heterologous proteins [13]. Different states of recombinant enzymes can also confer low or high activities of the same protein expressed in each cell [14]. Therefore, an alternative intracellular expression system such as that offered by the endotoxin-free and GRAS *B. subtilis* is needed to produce proteins in the cytoplasm. Only a few proteins have been expressed at levels of more than 10% of the total cellular protein: GFP [15–18], β-galactosidase [17, 19–21], GUS [15], *B. subtilis* HtpG [22], *B. subtilis* PbpB4 [22], the B subunit of *E. coli* heat-labile toxin (LTB) [23], listeriolysin O [24], HIV P24 antigen [25], 2,3-dioxygenase (C23O) (about 25% of total cellular protein) [26], CAT protein from *Staphylococcus aureus* [27], and two membrane proteins from *E. coli*, OmpA and OmpF [28]. Recent studies indicate that pMTBs72 backbones [29] such as the pHCMC and pHT01 plasmids use the theta replicating mechanism conferring structural stability of the plasmids. In contrast, most of the *B. subtilis* overexpression studies published before used the rolling-circle mechanism resulting in structural instability [30, 31]. In this study, a structurally stable plasmid carrying the P*grac*212 promoter was used for overexpression of recombinant proteins in *B. subtilis*. P*grac*212 is a strong promoter belonging to the P*grac* family [32], but differing from P*grac*01. It contains the mRNA-controllable stabilizing element (CoSE) conferring an extremely stable mRNA [33] that allows beta-galactosidase (BgaB) expression equal to that reported for P*grac*100 [34].

Solubility enhancement tags and affinity purification sequences are essential tools that keep recombinant proteins soluble and simplify purification [35, 36]. However, some tags can alter the structural or functional integrity of a recombinant protein and these have to be removed after purification by specific endoproteases such as thrombin, enterokinase, the tobacco etch virus (TEV) protease or the human rhinovirus 3C protease (HRV3C) [37–39]. HRV3C cleaves between glutamine and glycine residues of the canonical site LEVLFQ/GF [35, 38, 40]. The HRV3C endoprotease has up to 10×higher activity at 4 °C than 37 °C [41] and does not cleave at undesired cryptic sites as reported for other proteases [36].

Overexpression of useful proteins in the cytoplasm of the endotoxin-free bacterium *B. subtilis* using structurally stable plasmids in combination with the robust IPTG-inducible promoter P*grac*212 has not been reported so far. In this work, we overexpressed the reporter, HRV3C in *B. subtilis*, purified the protease, and tested its ability to cleave target proteins with subsequent purification.

Materials and Methods

Materials

The strains, plasmids, proteins, and primers used in this study are listed in Table 1. The *E. coli* strain OmniMAX (Invitrogen) was used for all cloning experiments, and *B. subtilis* strain 1012 (MoBiTec) was used to express the two proteases fused with six histidines, and in one case with 6×His plus glutathione S-transferase: His-HRV3C and His-GST-HRV3C. Cells were routinely grown in Luria broth (LB) at 37 °C under aeration and shaking at 200 rpm, with additional conditions as indicated. *E. coli* was grown in the presence of 100 µg/mL ampicillin and *B. subtilis* with 10 µg/mL chloramphenicol.

Construction of Recombinant Plasmids

The *bgaB* gene of pHT212 was replaced by the N-terminal part of the *B. subtilis lysS* gene (*lysSN*), fused to the HRV3C cleavage site (CS), a 6×His tag and a multicloning site (LysSN-HRV3C/CS-His-MCS). Therefore, plasmid pHT399 contained a DNA sequence encoding LysSN-HRV3C/CS-His-MCS with a Strep-tag under control of the P*grac*212 promoter. The BsLysSN enhances the solubility and folding of target proteins [42]. The protein sequence of LysSN-HRV3C/CS-His-MCS with the Strep-tag is shown in Supplement 1. DNA sequences coding for HRV3C (631 bp) and GST-HRV3C (1322 bp) were

Table 1 Bacterial strains, plasmids, proteins, and oligonucleotides used in this study

Bacterial strains	Genotype	Source/references
E. coli OmniMAX	F′ {*proAB lacI*^{q} *lacZ*ΔM15 *Tn*10(Tet^R) Δ(*ccdAB*)} *mcr***A** Δ(*mrr hsdRMS-mcrBC*) Φ 80(*lacZ*)ΔM15 Δ(*lacZYA-argF*)U169 *endA*1 *recA*1 *supE*44 *thi*-1 *gyrA*96 *relA*1 *tonA pan D*; used for cloning	Invitrogen
B. subtilis 1012	*leuA8 metB5 trpC2 hsrM1*	[54]; could be obtained at MoBiTec
Plasmids	**Description**	**Source/reference**
pGEX4T-HRV3C	Source for the fusion gene GST-HRV3C endoprotease	Lab collection synthesized by Phu Sa company
pHT01	P*grac*01, inducible	[31]
pHT212	Pgrac212-*bgaB*	[33]
pHT399	P*grac*212-BslysSN-HRV3C/CS-His-MCS	This work
pHT399B	pHT399 + HRV3C (BslysSN-HRV3C/CS-His-HRV3C), for expression His-HRV3C	This work
pHT399C	pHT399 + GST-HRV3C (BslysSN-HRV3C/CS-His-GST-HRV3C-Strep-tag), for expression of His-GST-HRV3C	This work
Proteins	**Description**	
BsLysSN-GFP	BsLysSN-HisTag-HRV3C/CS-GFP, used as a substrate for HRV3C	From the lab, produced in *B. subtilis* (unpublished)
BsLysSN-BgaB	BsLysSN-HisTag-HRV3C/CS-BgaB, used as a substrate for HRV3C	From the lab, produced in *B. subtilis* (unpublished)
Oligonucleotide	**Sequence 5′→3’**	**Used for**
ON607	TCACCATCACGGATCCGGCGGTGGCCTGGTTCC	Using to amplify the HRV3C gene to construct pHT399B
ON607B	TCACCATCACGGATCCATGTCCCCTATACTAGGTTATTGGAA	
ON608	GGATGGCTCCAAGCGAGGCAGATCGTCAGTCAGTCAC	Using to amplify the HRV3C gene to construct pHT399B
ON608B	GGATGGCTCCAAGCGGATTGTTTCTCTACAAAATATTGTTTTTTAAG	

P*grac*01 (another name is P*grac*) and P*grac*212 are the names of the two different promoters

obtained by PCR reactions with appropriate primer pairs (Table 1) using pGEX 4T-HRV3C as a template (Genbank ID, MN103550). These amplicons were inserted into pHT399 (derived from pHT212 [33]) by the InFusion method to generate pHT399B (His-HRV3C) and pHT399C (His-GST-HRV3C), respectively, and both plasmids were transformed into *B. subtilis* 1012.

The resulting plasmids were confirmed by restriction enzyme analysis and DNA sequencing as containing the fusion genes encoding BsLysSN-HRV3C/CS-His-HRV3C (pHT399B, for His-HRV3C) and BsLysSN-HRV3C/CS-His-GST-HRV3C-Strep-tag (pHT399C, for His-GST-HRV3C). The protein sequence of BsLysSN-HRV3C/CS-His-HRV3C and His-HRV3C are shown in Supplement 2, while BsLysSN-HRV3C/CS-His-GST-HRV3C-Strep-tag and His-GST-HRV3C with the Strep-tag are shown in Supplement 3. Map of plasmid pHT399B and expression cassette of pHT399B and pHT399C are shown in Supplement 4.

Expression of the Recombinant Proteins

The plasmids obtained from *E. coli* OmniMAX were inserted into *B. subtilis* 1012 by natural transformation [20]. To identify an appropriate temperature for the overexpression of the recombinant proteins, *B. subtilis* 1012 cells harboring pHT399B or pHT399C were grown at 23 °C, 30 °C, and 37 °C till the optical density at 600 nm (OD_{600nm}) reached 0.8. Then, the synthesis of proteins was induced by the addition of 0.5 mM IPTG. Cells were harvested by centrifugation at 6000×*g* for 10 min. The number of *B. subtilis* cells was equivalent to those present in 1 ml of culture with an OD_{600nm} of 6. The cells were suspended in 500 µl of lysis buffer (20 mM Tris–HCl, pH 7.2 and 15% (w/v) sucrose) containing 200 µg/ml lysozyme and incubated at 37 °C for 10 min. Then, the samples were sonicated and centrifuged at 2000×*g* for 5 min. The supernatants representing total proteins were centrifuged at 13,000×*g* for 5 min, and these supernatants were collected as soluble proteins. The pellets

were resuspended at the original volume and represented the insoluble proteins. Each protein sample was analyzed by SDS-PAGE to determine the temperature at which the amount of soluble recombinant protein was highest. The optimal IPTG concentration (0, 0.01, 0.05, 0.1, 0.5, 1 mM) and induction time (0, 2, 4, 6, 8, 10, and 12 h) were also determined.

Purification of Recombinant Proteins by His-Trap (Ni–NTA) Column

B. subtilis 1012 cells carrying either pHT399B or pHT399C were cultured in one liter of LB medium at 37 °C and when the OD_{600} reached 0.8, IPTG was added to 1 mM, the temperature was reduced to 23 °C, and the two cultures were further incubated with shaking for 12 h to allow expression of the HRV3C fusion proteins. Cells were harvested by centrifugation at 6000×*g* for 10 min. Next, the cells were resuspended in binding buffer (30 mM Tris–HCl, pH 8.0; 500 mM NaCl; 5% glycerol) containing lysozyme (20 µg/ml) and incubated at 37 °C for 10 min. DNase I was added to a final concentration of 20 µg/ml and PMSF at 0.5 mM before sonication, and soluble proteins were obtained in the supernatants after centrifugation at 19,000×*g* at 4 °C for 30 min. The protein solutions were clarified by membrane filtration (pore size 0.22 µm). The clarified samples were pumped through 5 ml His-trap columns at a flow rate of 2 ml/min and recombinant proteins fused to the His-tag bound to the Ni–NTA in the columns. All other proteins without a His-tag were removed from the columns by washing with 30 ml of binding buffer containing 5 mM imidazole. The recombinant proteins were eluted from the columns with buffer containing 10, 20, 40, 60, 80, 100, 120, 160, 250, or 500 mM imidazole. 2-ml fractions were collected from each elution and the fourth fraction containing the eluted proteins was run on SDS-PAGE.

Determination of HRV3C Protease Activity and Purification of Cleaved Proteins

The activity of the eluted proteases (initial concentration, 14 µM) was tested on the substrates, BsLysSN-His-HRV3C/CS-GFP and BsLysSN-His-HRV3C/CS-BgaB. These substrate proteins were first dialyzed against cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0 at 25 °C) and then 100 µg of each substrate was incubated with 100 µl of protease at dilutions of 5- to 1000-fold for 16 h at 4 °C. The SDS-PAGE and AlphaEaseFC software were used to determine the percentage of intact vs. cleaved substrates, and the activity of HRV3C proteases was calculated. After that, the samples were applied to a His-trap column to confirm the loss of the His-tag.

SDS-PAGE

Bacillus subtilis 1012 carrying either the plasmid pHT399B or pHT399C was grown in LB in the presence of chloramphenicol (Fig. 1). Cells were sedimented by centrifugation and resuspended in lysis buffer. After lysis by sonication and clarification by centrifugation at 2000×*g* for 5 min, supernatants were sampled for measuring total protein(T) and the remainder was subjected to a second centrifugation step at 13,000 g for 10 min. Further protein samples were taken from the supernatant (S) and from the pellets (P) after resuspension. Aliquots of 100 µl of each protein samples were mixed with 25 µl of 5× SDS-PAGE sample buffer, heated at 95 °C for 5 min, and then centrifuged at 13,000×*g* for 5 min. The supernatants were loaded onto SDS-PAGE gels, and electrophoresis was carried out at 25 mA.

Results

Construction of pHT399B (His-HRV3C) and pHT399C (His-GST-HRV3C)

Two different expression vectors constructed contain the HRV3C endoprotease gene and additional sequences. Both vectors were based on pHT212, an *E. coli–B. subtilis* shuttle vector containing the beta-galactosidase (*bgaB*) gene fused to the IPTG-inducible P*grac*212 promoter [33] derived from the P*grac* promoter [22]. The only difference between these two promoters is that P*grac*212 carries the mRNA stabilizing element CoSE, which results in higher recombinant protein production. It has been shown that RNA molecules can also function as chaperones and are extremely effective in helping the folding of a variety of proteins [42, 43]. In a recent publication, a small tRNA-binding N-terminal domain of lysyl-tRNA synthetase of *E. coli* has been shown to assist the de novo folding and trimeric assembly of the RID-HA1 hemagglutinin [44]. In the present publication, we evaluated the expression of the recombinant proteins His-HRV3C and His-GST-HRV3C fused to the N-terminal domain of the *lysS* gene of *B. subtilis* coding for a lysyl-tRNA synthetase (BsLysSN) to facilitate protein expression.

Expression Levels of His-HRV3C and His-GST-HRV3C Under Different Growth and Induction Conditions

Bacillus subtilis 1012 carrying pHT399B or pTH399C was incubated at three different temperatures, and the levels of recombinant proteins produced under these conditions were quantified by SDS-PAGE. In the case of pHT399B (His-HRV3C), all the recombinant protein was present in the soluble fraction if growth was done at 23 °C or 30 °C, while at 37 °C, about 50% of the protein appeared in the

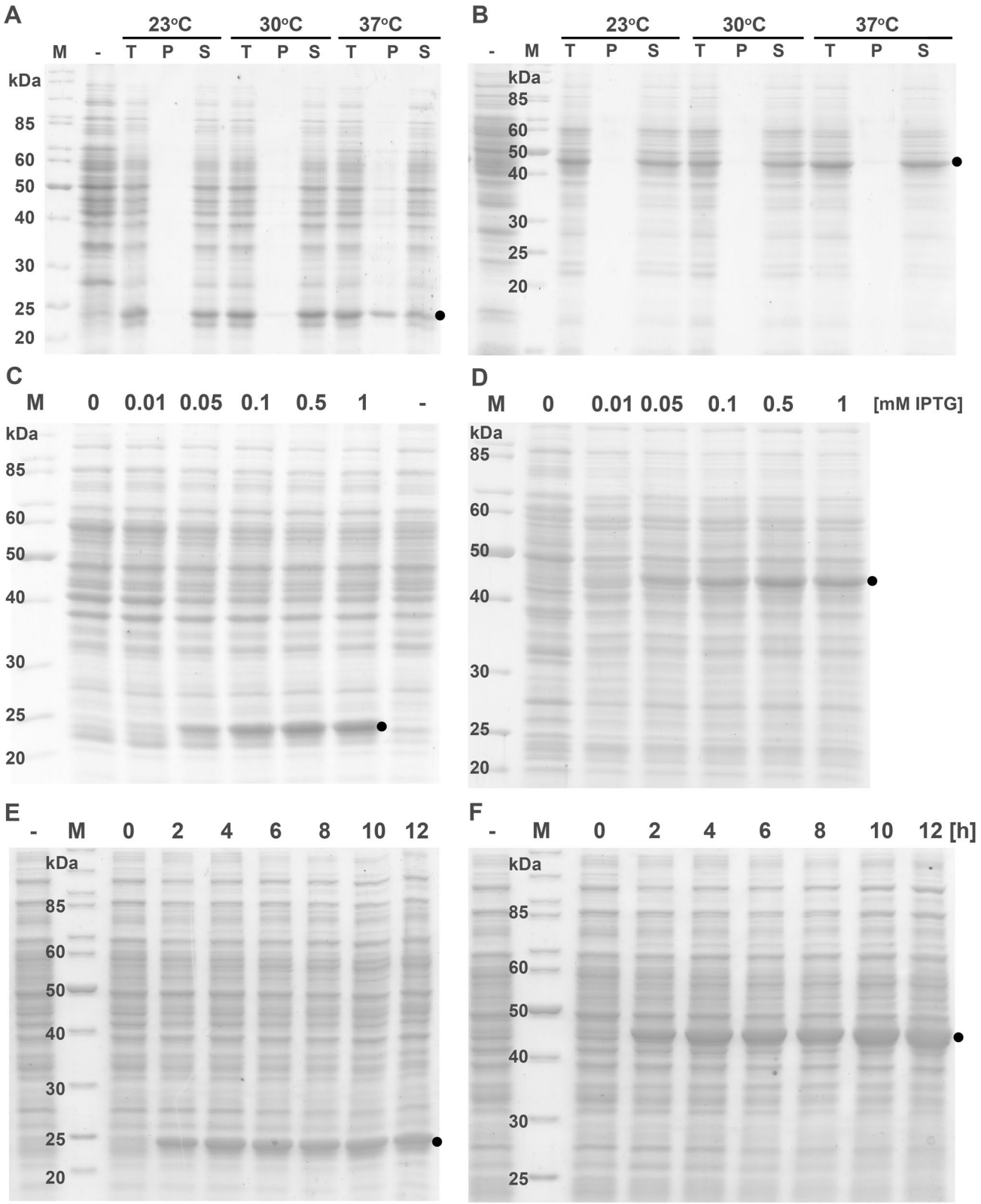

Fig. 1 Influence of temperature and IPTG concentration on the production of His-HRV3C and His-GST-HRV3C. *B. subtilis* containing the plasmids pHT399B (His-HRV3C) or pHT399C (His-GST-HRV3C) were incubated in LB medium at different temperatures (**a**, **b**), or induced with different IPTG concentrations (**c**, **d**) for up to 12 h (**e**, **f**). –, empty vector; *T*, the total amount of proteins; *P*, proteins present in the pellets and *S*, the supernatant after centrifugation. The locations of the recombinant proteins are indicated by the black dot

insoluble fraction (Fig. 1a). In the case of pHT399C (His-GST-HRV3C), the recombinant protein remained soluble at all three temperatures (Fig. 1b). To determine the optimal IPTG concentration for inducing protein expression, bacteria carrying pHT399B were grown at 30 °C and those containing pHT399C at 37 °C in the presence of increasing concentrations of IPTG (Fig. 1c, d). A concentration of 1 mM IPTG resulted in the highest expression levels of the HRV3C proteases, and this concentration was chosen for inducing expression in subsequent experiments. The *B. subtilis* strain carrying pHT399B was grown at 30 °C and that containing pHT399C at 37 °C with 1 mM IPTG and samples were taken every two hours up to 12 h. The amount of His-HRV3C protein expressed from pHT399B was almost identical between 4 and 12 h (11% of total protein), while the His-GST-HRV3C encoded by pHT399C was highest at 12 h (16% of total protein) (Fig. 1e, f).

Purification of His-HRV3C and His-GST-HRV3C

The soluble isolates of the recombinant proteins were purified on a nickel column which allows binding of proteins with a histidine tag. Protein isolates of His-HRV3C showed a band at 22 kDa before loading on the column (*BC*) which became even more prominent after binding and elution from the column (*AC)* (Fig. 2a). The His-HRV3C protein was recovered at an imidazole concentration of 80–160 mM. When the same experiment was carried out with bacteria producing the His-GST-HRV3C protein, this protein also bound to the Ni–NTA column and was eluted after addition of 60–160 mM imidazole (Fig. 2b).

The Protease Activity of His-HRV3C and His-GST-HRV3C

The activity of the purified proteases was determined with two substrates, BsLysSN-His-HRV3C/CS-GFP and BsLysSN-His-HRV3C/CS-BgaB. When BsLysSN-His-HRV3C/CS-GFP was used for cleavage, the lowest concentrations of His-HRV3C and His-GST-HRV3C corresponding to 1 unit of HRV3C protease able to cleave > 95% of 100 µg of target protein after 16 h at 4 °C were similar at a 250-fold dilution (Fig. 3a, b). This means that the fusion tag (6×His-GST) did not alter the HRV3C protease activity compared to 6×His alone. The specific protease activity of His-HRV3C was measured at 8065 U/mg and that of His-GST-HRV3C at 3623 U/mg. When the BsLysSN-His-HRV3C/CS-BgaB substrate was used for cleavage, the two proteases cleaved 95% at fivefold dilution (Fig. 3c, d). With the substrate protein BsLysSN-His-HRV3C/CS-BgaB, the specific protease activity of His-HRV3C was 161 U/mg and of His-GST-HRV3C was 72 U/mg. The reason for this difference could be that the folding of this substrate led to partial sequestering of the HRV3C cleavage site from the protease.

Application of the HRV3C Proteases for the Purification of GFP and BgaB

The two purified HRV3C endoproteases, His-HRV3C and His-GST-HRV3C, were used to cleave the substrate proteins BsLysSN-GFP (BsLysSN-HisTag-HRV3C/CS-GFP) and BsLysSN-BgaB (BsLysSN-HisTag-HRV3C/CS-BgaB). The BsLysSN-His6-HRV3C/CS-GFP (Fig. 4a, b) target was cleaved to more than 95% by both proteases, while the

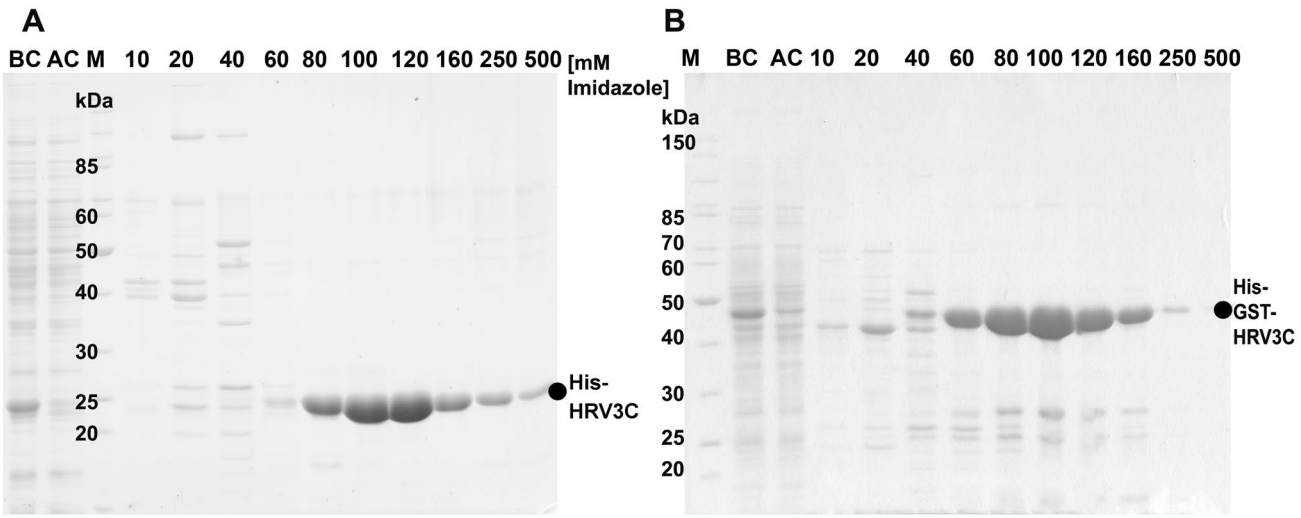

Fig. 2 Purification of HRV3C using Ni–NTA columns. *BC*, protein isolates before loading on the Ni–NTA column; *AC*, flow-through samples collected after protease binding to the Ni–NTA column. The fusion proteins binding to the Ni–NTA column were eluted using a buffer with the indicated concentrations of imidazole (10 to 500 mM). The protein samples were analyzed by SDS-PAGE

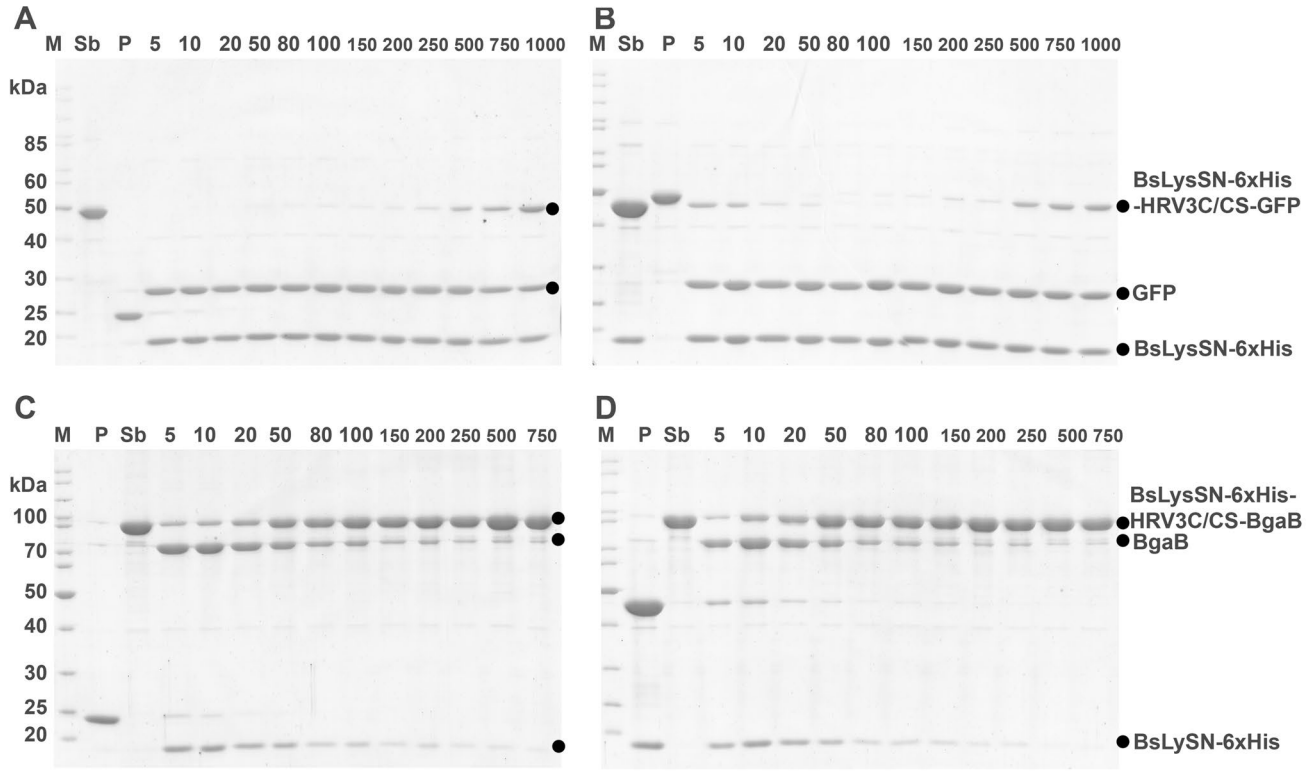

Fig. 3 Analysis of protease activities of His-HRV3C and His-GST-HRV3C using different substrates. Proteases (*P*) His-HRV3C (**a**, **c**) and His-GST-HRV3C (**b**, **d**) were tested by digesting 100 µg aliquots of the two substrates (*Sb*): BsLysSN-His6-HRV3C/CS-GFP (**a**, **b**) and BsLysSN-His-HRV3C/CS-BgaB (**c**, **d**). The stocks of protease at an initial concentration of 14 µM diluted from 5 to 1000 times

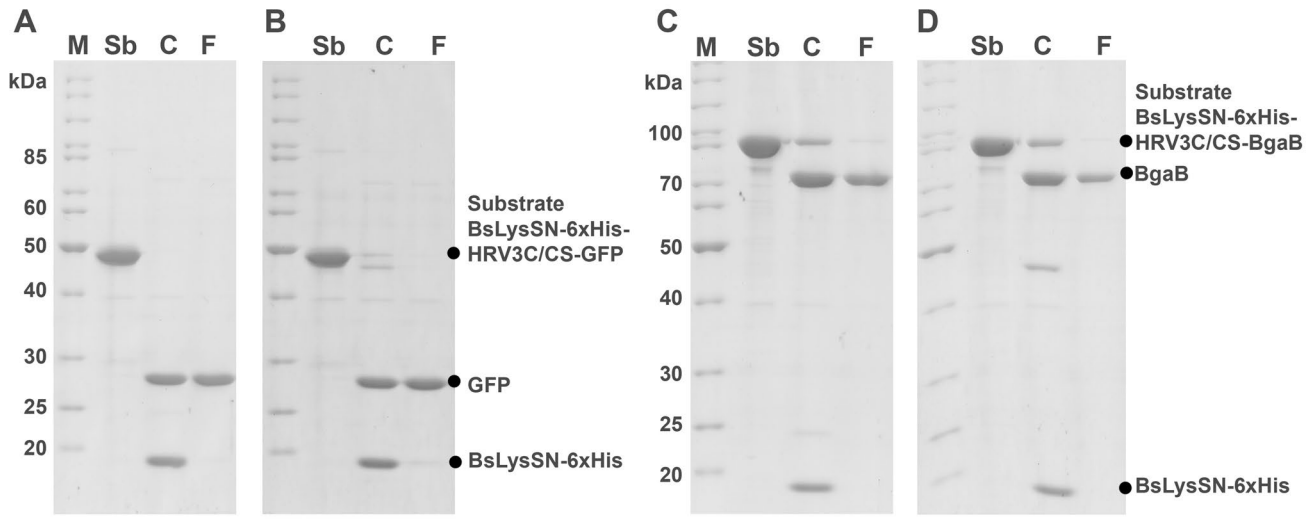

Fig. 4 Testing of the proteases for purification of GFP and BgaB. The substrates (*Sb*) BsLysSN-6×His-HRV3C/CS-GFP (**a**, **b**) and BsLysSN-6×His-HRV3C/CS-BgaB (**c**, **d**) were cleaved by His-HRV3C (**a**, **c**) or His-GST-HRV3C (**b**, **d**) for 16 h at 4 °C. The mixture after cleavage (*C*) was loaded on a Ni–NTA column. The flow-through (*F*) contained the target proteins GFP or BgaB. The dots indicate the location of the isolated proteins

BsLysSN-His6-HRV3C/CS-BgaB (Fig. 4c, d) was cleaved only to about 95%. The recombinant proteins GFP and BgaB in the cleaved reactions were isolated from the flow-through after loading the mixtures consisting of the purification tag and the recombinant proteins on the His-trap column. The target proteins which had been cut by the HRV3C protease lost their His-tag so they could not bind to the Ni–NTA column and were eluted in the flow-through solution. All the proteins having the 6×His affinity tag were retained on the Ni–NTA column. Finally, pure GFP (Fig. 4a, b, lands F) and BgaB (Fig. 4c, d, lands F) could be collected in the flow-through. These results proved that the purified HRV3C proteases could be applied to release GFP and BgaB from fusion forms containing the HRV3C cleavage site.

Discussion

This study demonstrates the use of a structurally stable plasmid carrying the robust IPTG-inducible promoter, P*grac*212 [33], to produce recombinant proteins in the cytoplasm of *B. subtilis*. The human rhinovirus 3C protease (HRV3C) [39] was either fused to a His-tag or a GST-tag, and both the recombinant proteases were overexpressed at 11% and 16%, of the total cellular proteins, respectively, and purified using a nickel column. The specific protease activities were measured to be 8065 U/mg for His-HRV3C and 3623 U/mg for His-GST-HRV3C.

In recent years, HRV3C has been used to develop new purification methods which are more simplified and faster than the traditional approach. For example, in 2014, a new system was developed to produce native proteins in *E. coli*, allowing for the removal of the fusion tag via intracellular self-cleavage by this protease [45]. In 2019, the simplified method to remove fusion tags named Cell Lysate Purification system based on HRV3C protease (CLP3C) was developed, in which strains expressing HRV3C protease and the other expressing substrates were mixed before (co-fermentation method) or after (post-fermentation method) inducing with IPTG, followed by cell disruption and incubation at 4 °C, overnight for cleavage [46]. However, these methods were only based on naturally lipopolysaccharide (LPS) producing host *E. coli*, which need the additional steps to remove LPS. In contrast, the HRV3C expressed in the endotoxin-free bacteria *B. subtilis* could be easily applied for those new purification methods to produce other recombinant proteins in *B. subtilis* using a similar approach as described for *E. coli*.

Intracellular expression of recombinant proteins in *B. subtilis* could be used to produce antigens as an oral vaccine delivery vector. It has been demonstrated that the heat-labile enterotoxin, B subunit (LTB), expressed in the cytoplasm of *B. subtilis* at deficient levels that could not be seen on SDS-PAGE. The purified protein was able to induce the immune response and confer partial protection to mice using lethal challenges with purified LTB [47]. The high expression system for *B. subtilis* as described here could be useful to develop *B. subtilis* as vaccine vehicles.

A large number of strong inducible and constitutive promoters have been identified and used for recombinant protein production, and there are still many attempts to discover novel promoters [48–50]. To improve the stability of the recombinant protein and allow simple purification, stabilizing peptides and purification tags can be added [9]. We used the IPTG-inducible P*grac*212 promoter and added three different peptide domains: (i) The small tRNA-binding N-terminal domain of the *E. coli* lysyl-tRNA synthetase to assist the de novo folding of the recombinant protein [44], (ii) a His-tag or His- and GST-tag to allow a one-step purification of the recombinant proteins, and (iii) the HRV3C sequence recognized by HRV3C protease [35] thereby allowing to cleave between these three tags to release the recombinant protein. As recombinant protein, we used first the HRV3C protease and analyzed the influence of the growth temperature and the IPTG concentration on the production of the protease. Based on these data, we fused the coding sequences of GFP and BgaB to the fusion tags in our vectors to further verify the application of this approach in *B. subtilis*. We could express GFP and BgaB fusions in *B. subtilis* (unpublished data) and used the purified HRV3C enzymes to cleave two substrate proteins followed by isolation of the two protein targets, GFP and BgaB. In summary, our data nicely prove the successful application of our vectors for the intracellular production of recombinant proteins in *B. subtilis*.

As the next step, our vector systems carrying different recombinant genes could be analyzed in the genome-reduced *B.* subtilis 168 cells. Several genomes reduced *B. subtilis* strains have been constructed so far. The first strain constructed lost 332 nonessential genes [51]; another group deleted 0.99 Mbp [52], and the smallest chromosome constructed so far lost 1.51 Mbp [53, 54]. All these strains allowed an increase production of recombinant proteins as compared with the wild-type strain 168.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

Research Involving Human and Animal Rights This study did not use animals or samples from human for experiments.

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