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A novel screening system for secretion of heterologous proteins in *Bacillus subtilis*

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Summary

High-level production of secretory proteins in *Bacillus subtilis* leads to a stress response involving the two-component system CssRS and its target genes *htrA* and *htrB*. Here, we used this sensing system in a reporter strain in which *gfp* is under control of P_{htrA}, the secretion stress responsive promoter of *htrA*. Overexpression of heterologous secretory proteins in this strain results in green fluorescent cells, which can be separated from non-secreting, low fluorescent cells using a fluorescence-activated cell sorter (FACS). Using this principle, genomic libraries of uncharacterized prokaryotic organisms, expressed in the reporter strain, can be screened for genes encoding secretory proteins.

Introduction

Prokaryotic enzymes, which are used in food- and other industries on large scales, can efficiently be produced using the Gram-positive soil bacterium *Bacillus subtilis* and related species (Harwood and Cranenburgh, 2008). Discovery of new enzymes with improved properties is of commercial interest. As these enzymes may be present in less commonly used, exotic prokaryotes with unknown genome sequence, an easy high-throughput system (HTS) to screen these organisms can overcome time-consuming and costly species-specific analyses.

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Enzymes produced on an industrial scale using *B. subtilis* or other bacilli are generally secreted proteins, since they can easily be isolated and purified from the growth medium. *Bacillus subtilis* has the capacity of secreting proteins to high levels, which makes it, together with its GRAS status, a favourable host for enzyme production (Ferrari *et al*., 1993).

In *B. subtilis*, the overproduction of proteins (either endogenous or heterologous) that are secreted across the cytoplasmic membrane commonly causes a stress at the membrane-cell wall interface by the accumulation of misfolded protein or by export jams. This problem is counteracted by the cells by increasing the expression of two related quality-control proteins, i.e. HtrA and HtrB, which display proteolytic activity at the membrane–cell wall interface. The *htrA* and *htrB* genes are regulated by the two-component system CssRS. CssS, the membrane-located sensor, senses accumulation of unfolded or misfolded protein, probably in the pseudo periplasm and transfers the signal to CssR, the response regulator, which in turn activates the expression of *htrA* and *htrB*, as well as the *cssRS* genes (Noone *et al*., 2000; Darmon *et al*., 2002; Westers *et al*., 2006).

Here, we describe the use of the CssRS-mediated secretion stress response as a reporter system for the (over)production of secretory proteins, by fusing the promoter of *htrA* to the gene encoding green fluorescent protein (*gfp*). Secretion stress, provoked by the overproduction of a secretory protein and sensed by CssS, will lead to induction of the *htrA* promoter and expression of *gfp*. This enables the screening of large genomic libraries for genes encoding secretory proteins in a reporter strain of *B. subtilis* that will be green fluorescent when a secretory protein is expressed (Fig. 1). The screening can conveniently take place by use of a fluorescence-activated cell sorter (FACS), which enables the fast recovery of living fluorescent (protein secreting) cells.

Results

*GFP is expressed from P*htrA *upon AmyQ overexpression in a P*htrA–gfp *reporter strain*

To test the feasibility of using the *htrA* promoter (P*htrA*) fused to *gfp* as a reporter for secretion stress in *B. subtilis*, a P*htrA–gfp* fusion was integrated into the chromosome at the *htrA* locus in strain 168, and in the *amyE* locus in the

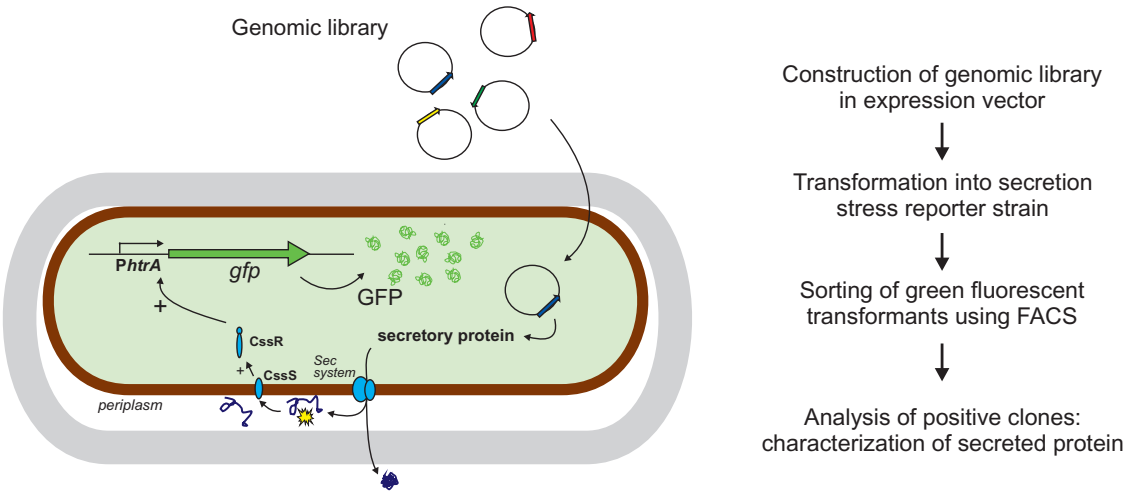

Fig. 1. Principle of secreted enzyme screening system. A *Bacillus subtilis* reporter strain carrying a secretion stress responsive P*htrA–gfp* fusion in the chromosome is transformed with a genomic expression library of a prokaryotic organism. Clones that express a secreted protein will become green fluorescent (GFP) by the CssRS-mediated stress response and activation of P*htrA*. These positive clones can be separated from negative clones using a fluorescence-activated cell sorter (FACS). The secreted protein can be further characterized and tested for possible application.

Δ*htrA* strain BV2003 (Hyyrylainen *et al*., 2001). The resulting strains were transformed with either pKTH10 (Palva, 1982), which strongly expresses *amyQ*, encoding the secreted α-amylase of *Bacillus amyloliquefaciens* and known to induce CssRS-mediated *htrA* expression (Hyyrylainen *et al*., 2001), or with the empty vector pUB110. Cells were grown to stationary phase and inspected by fluorescence microscopy (Fig. 2A–D). Clear induction of *gfp* expression was observed in cells harbouring pKTH10 compared with cells with the empty vector. Second, the level of GFP was significantly higher in the Δ*htrA* strain than in the wild-type strain. This is in agreement with the compensatory overexpression from the *htrA* promoter due to reduced proteolytic activity (or negative autoregulation by HtrA activity) as described by Noone and colleagues (2000). An increase in background level of GFP was observed in the Δ*htrA* strain (Fig. 2D). A more precise study of the GFP response in the wild-type and Δ*htrA* background was carried out using plasmid pDGamyQ, carrying *amyQ* under control of the IPTG inducible promoter P*spac*. This plasmid was transformed into wild-type background strain HT-A and the Δ*htrA* strain BV-A, both containing the P*htrA–gfp* fusion in the *amyE* locus. Cells were induced with a range of IPTG concentrations and GFP fluorescence was measured using flow-cytometry (Fig. 2E and F). The level of AmyQ secreted into the medium was analysed using SDS-PAGE (Fig. 2G and H). The GFP response was dependent on the level of induction and AmyQ secretion in both the wild-type and Δ*htrA* background. This dose-dependency in the wild-type background is in agreement with previous observations (Westers *et al*., 2004). The separation between uninduced and fully induced cells appeared better in the wild-type background, with fluorescence peaks at 20 and 240 arbitrary units respectively. However, in absolute levels, the difference in average fluorescence between uninduced and fully induced cells was bigger in the Δ*htrA* background, with peaks at 220 and 700 A.U. respectively. In addition, in the wild-type background two populations of cells with low (background) and higher fluorescence were observed at intermediate expression level. The nature of this effect, which was not observed with the Δ*htrA* reporter strain, was unclear. The *htrA* deletion was used in the reporter strain for proof of principle experiments.

The CssRS-mediated secretion stress response is specific for extracellular protein accumulation

For a selective screening system, the stress sensor of the reporter strain, i.e. CssS, should discriminate between proteins that are secreted into the medium and proteins destined for other locations such as the cytoplasm and cytoplasmic membrane. To test this, the P*htrA–gfp* fusion was integrated at the P*htrA* locus of the SURE system expression strain NZ8900 (Bongers *et al*., 2005), allowing for high-level expression. A range of different his-tagged proteins of different origin and localization were overproduced using the SURE expression vector pNZ8902: AmyQ, the secreted α-amylase of *Bacillus amyloliquefaciens*; Usp45, a highly secreted protein of unknown function of *Lactococcus lactis*; XynA, a secreted xylanase of *B. subtilis*; MntA, a manganese binding lipoprotein of *B. subtilis*; LmrA, a membrane located ABC-transporter of *Lactococcus lactis*; YwbN, a TAT system secreted protein of *B. subtilis*; LasB, or elastase, a secreted protease of *Pseudomonas aeruginosa*, TEM-1 β-lactamase,

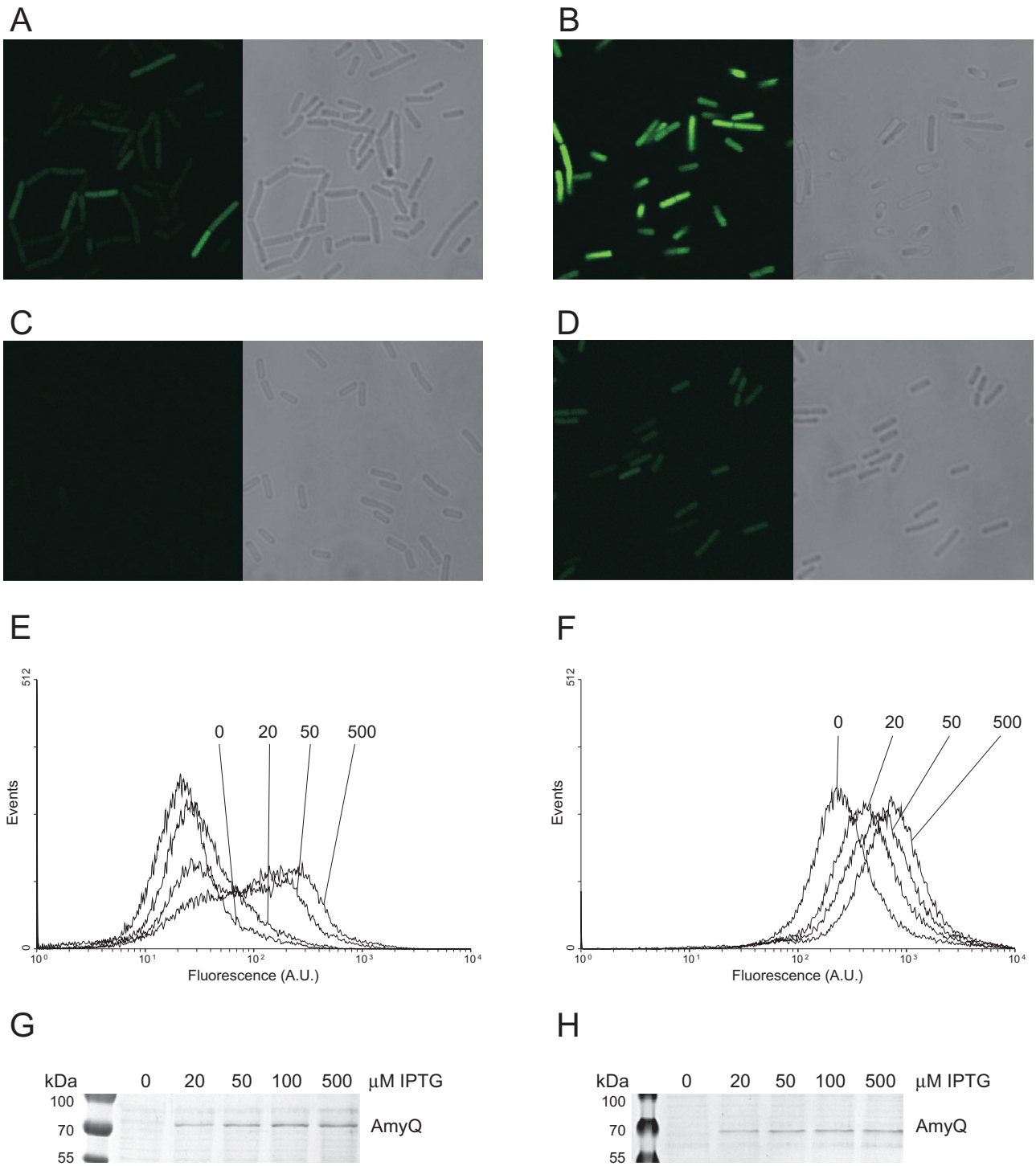

Fig. 2. P*htrA*-driven GFP fluorescence as a reporter of secretion stress. (A–D) Fluorescence (left) and light (right) microscopic images of reporter strains 168-A (A and C) and BV-A (Δ*htrA*, B and D) expressing α-amylase from pKTH10 (A and B) or harbouring the empty vector pUB110 (C and D). (E and F) Flow-cytometry analysis of GFP response of HT-A (wild-type background) and BV-A harbouring pDGamyQ after 1 h of IPTG induction. IPTG concentrations are indicated in µM. Note that the scale of the *x*-axis is logarithmic. (G and H) SDS-PAGE showing AmyQ levels in the supernatants of the cultures used in (E) and (F).

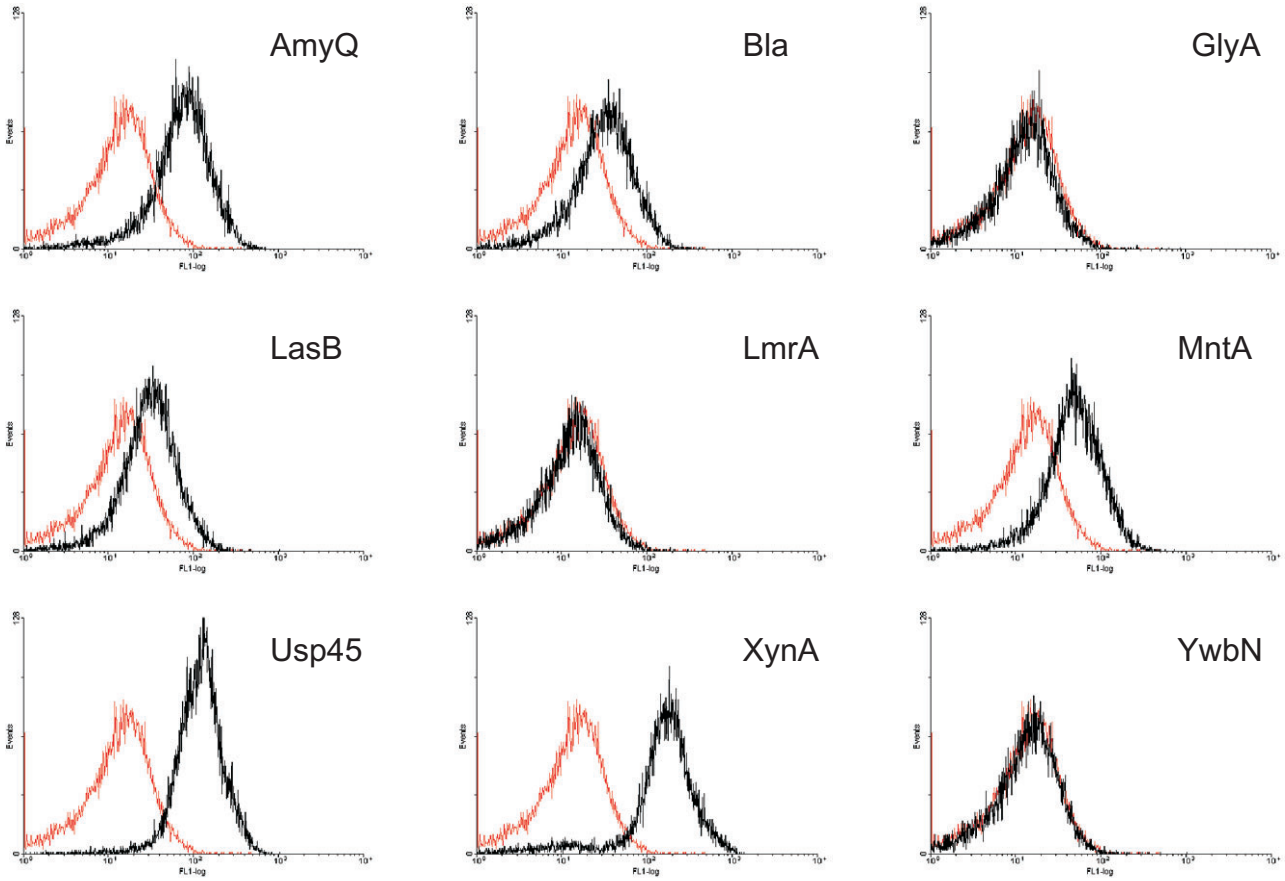

Fig. 3. Secretion stress response to overexpression of different proteins, measured by flow cytometric analysis of GFP fluorescence in reporter strain HT100A. Red lines: HT100A cells harbouring the empty vector pNZ8902; black lines: HT100A cells harbouring pNZ8902-based constructs for expression of the indicated proteins. Expression was induced with subtilin for 1 h.

a secreted and cell wall-associated β-lactamase of *Escherichia coli*; and GlyA, a cytoplasmic serine hydroxymethyltransferase of *B. subtilis*. Protein production was confirmed and protein levels and localization were tested by cell fractionation, SDS-PAGE and Western blotting using his-tag antibody (data not shown). To test the P*htrA*-driven *gfp* expression in response to overproduction of the proteins, flow-cytometry was performed with cells in which expression was induced for 1 h with subtilin (Fig. 3). All proteins destined to the medium gave a clear GFP response, except for the TAT secreted YwbN. However, more than 90% of YwbN was found in the cytoplasmic fraction after cell fractionation, and only a small amount in the medium, probably due to a lower secretion capacity or lower expression of the TatAyCy translocation system (Jongbloed *et al*., 2004). A rough relation between the amount of protein secreted into the medium and GFP response, as described above for AmyQ, also became clear from the other secretory proteins (data not shown). Overproduction of the cytosolic protein GlyA and the membrane protein LmrA did not yield increased GFP signals in the reporter strain, suggesting that CssS senses accumulation of misfolded protein on the pseudo-periplasmic side of the membrane rather than in the membrane. In other studies of *lmrA* overexpression, specific stress signals were observed that indicated that membrane protein overproduction induces different responses (B.C. Marciniak, H. Trip, P.J. van der Veek and O.P. Kuipers, unpubl. data). Taken together, the P*htrA*-driven GFP response is specific for proteins accumulating on the extracytoplasmic side of the cell and is dose-dependent.

Construction of a reporter strain for high-throughput screening

To screen prokaryotic genomic libraries for genes encoding unknown secretory proteins with possible industrial application, a reporter strain was constructed that carries the P*htrA*–*gfp* fusion at the *amyE* locus and had three other features: First, as a mother strain, the *nprE aprE* mutant strain DB104 was used (Kawamura and Doi, 1984). NprE (neutral protease) and AprE (alkaline protease) are

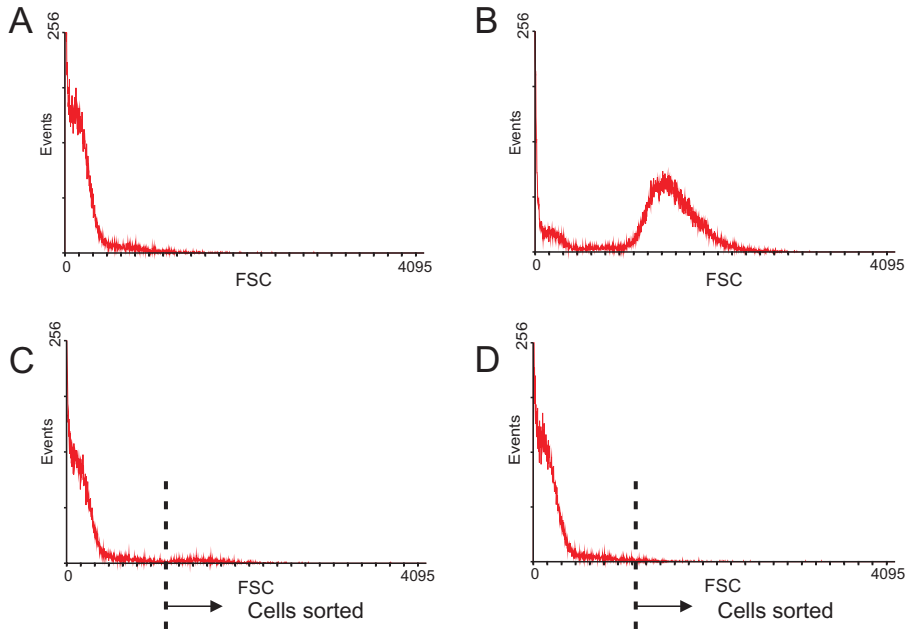

Fig. 4. FACS sorting of α-amylase secreting cells from a large population. (A and B) Fluorescence analysis of cells of reporter strain VT210A harbouring the empty vector pUB110 or the *amyQ* expression vector pKTH10 respectively. Based on (A) and (B), a threshold was set (dashed line in C and D) for sorting fluorescent cells from a mixed population of pUB110 (95%) and pKTH10 (5%) harbouring cells (C), or from a population of cells obtained after transformation with a mixture of 5% pKTH10 and 95% pUB110 plasmid DNA and overnight growth (D).

extracellular proteases that account for more than 90% of the extracellular protease activity of *B. subtilis*, and their absence increases the stability of secreted proteins of heterologous origin in particular (Nakamura *et al*., 1991; Wu *et al*., 1991). This is an advantage for downstream analysis of GFP-positive clones from which the secreted protein has to be characterized. Second, the *htrA* gene was disrupted, thereby removing the negative autoregulation of P*htrA* by HtrA (see Fig. 2). Although the background GFP level is also increased by this mutation, the difference in absolute fluorescence between cells with and without secretion stress was higher than in the wild-type background. Third, the BsuM DNA modification and restriction system (Ohshima *et al*., 2002) was eliminated by deletion of *ydiS*, which encodes the restriction nuclease. Disruption of *ydiS* increases transformation efficiency by 10-fold for plasmids of 6 kb and more than 100-fold for plasmids of 15 kb (Haima *et al*., 1987). Higher transformation efficiency will greatly facilitate the transformation of genomic libraries into the reporter strain when a full coverage of the genome is desired. The final reporter strain was named VT210A and has the following genotype: *nprE aprE*, *amyE*::P*htrA–gfpmut-1*, Cm^r, *htrA*::pMutin2,Em^r, *ydiS*::Sp^r.

Proof of principle: sorting of secretory protein producing cells using a FACS

The reporter strain VT210A was transformed with a mixture of plasmid DNA of pUB110 (empty vector) and pKTH10 (strongly expressing the secreted α-amylase gene *amyQ*) in a ratio of 20:1. After transformation, a small part of the cells was spread on TY agar plates containing kanamycin for plasmid selection, and starch for indication of α-amylase secretion. The rest of the cells was transferred to liquid TY medium containing kanamycin and incubated overnight with shaking. The separate plasmids were transformed to VT210A as well, performing the same operations as for the co-transformation. The next day, cultures were analysed for GFP fluorescence using a MoFlo FACS. First, fluorescence signals of cells of VT210A harbouring the empty vector pUB110 and of VT210A harbouring the AmyQ overproducing plasmid pKTH10 were analysed separately. On basis of these results (Fig. 4A and B), a cell sorting limit was manually set (dashed line in Fig. 4C and D), such that cells giving a GFP fluorescence higher than this limit were sorted. Subsequently, the co-transformation culture was applied to the FACS and sorted cells were collected in a tube. To verify that the sorted fluorescent population had been enriched for α-amylase secreting cells, different dilutions of the collected cells were spread on TY agar containing starch. The same was done with the input overnight co-transformation culture. After growth, the colonies were covered by Lugol's solution to visualize halo formation caused by α-amylase activity. Over 90% of the sorted cells indeed secreted α-amylase, whereas in the input culture only 0.5–0.8% of the cells secreted α-amylase (Table 1). This result clearly shows that it is possible to

Table 1. Enrichment of AmyQ secreting cells using fluorescence-activated cell sorting.

	Dilution[a]	Number of colonies	Number of AmyQ$^+$ colonies	Percentage AmyQ$^+$
Before sorting	NA[b]	> 2000	15	< 0.75%
After sorting	10^0	~ 1000	> 900	> 90%
	10^{-1}	111	104	94%
	10^{-2}	9	8	89%

a. Dilutions of cells collected in TY medium after fluorescence-activated cell sorting.
b. Cells from an overnight culture obtained after transformation of a mixture of pUB110 and pKTH10 were plated from different dilutions to obtain an appropriate number of AmyQ secreting colonies.

select secreting cells by discrimination in fluorescent signal between normal and stressed cells.

A genomic expression bank of the plant root-colonizing *B. amyloliquefaciens* was constructed by cloning partially digested chromosomal DNA downstream the *amyQ* promoter of a pKTH10 derived *E. coli*/*B. subtilis* shuttle vector. This partial library was transformed into reporter strain VT210A. Following the procedure described above, green fluorescent cells were sorted by a FACS and propagated individually in TY medium in microtitre plates. Supernatants of cultures were analysed using a high-throughput mass spectrometry procedure, resulting in the identification of *B. amyloliquefaciens* proteins in 11 cultures. From these, four supernatants contained proteins with a typical N-terminal signal peptide: β-mannanase (encoded by *gmuG*), a putative proteoglycan hydrolase (encoded by *ganA*), which was identified in 2 of the 11 supernatants, and the oligo binding protein AppA. The positive clones from which proteins without a clear signal peptide were identified do not necessarily represent false positives: in the study of Antelmann and colleagues (2001), 50% of the proteins identified in the extracellular proteome of *B. subtilis* were predicted to be cell associated because of the absence of a signal peptide or the presence of specific cell retention signals in addition to an export signal. Upon overproduction, similar proteins of *B. amyloliquefaciens* may induce the CssRS-mediated stress response in *B. subtilis*. The result shows a significant enrichment in secretory proteins from a partial genomic library of a heterologous organism and thereby the applicability of the screening system.

Discussion

In this study we describe the development of a screening system for heterologous prokaryotic secretory proteins. The CssRS-mediated secretion stress response of *B. subtilis*, translated into green fluorescence by the use of a fusion of the *htrA* promoter and *gfp*, proves to be a suitable reporter to select for cells that are secreting overproduced homologous and heterologous proteins. The response is selective for proteins that reach the extracellular space, while overproduced cytosolic and membrane proteins, or secretory proteins that are somehow poorly secreted, do not give a GFP response in the reporter strain. Using a FACS, this fluorescence response enabled the separation of cells that are overproducing a secretory protein (α-amylase) from a large population of non-secreting cells. Similarly, a significant enrichment of secretory proteins of *B. amyloliquefaciens* was obtained after expressing a partial genomic library and subsequent FACS screening. The proof-of-principle experiments demonstrated the potential of this screening system to screen uncharacterized prokaryotic organisms for novel secretory proteins of possible industrial interest. Advantages of this system are: (i) The high-throughput manner of screening. A fluorescent cell sorter can measure and sort tens of thousands of cells within minutes, whereas cloning of genomic libraries and subsequent transformation to the reporter strain can be highly efficient using modern cloning techniques such as the Gateway cloning system (Invitrogen). (ii) Selected clones are easily recovered, allowing rapid identification of the expressed gene by DNA sequencing and analysis of the secreted protein by MASS spectroscopy. (iii) The secretion stress response is generally dose-dependent and therefore the system is to a certain extent selective for high-level producing clones. (iv) The high recovery rate of cells after FACS sorting allows multiple rounds of analysis and selection, increasing the enrichment in positive clones. Additionally, the reporter strain can be provided with a second reporter construct, using a different fluorescent protein. The use of a second reporter construct and possibly a third would thereby make a more defined selection of clones possible. Fine tuning or enhancement of the signal may be desirable, which can be achieved by mutating the reporter strain as was done here by deletion of *htrA*. This had an increasing effect on GFP levels, but also resulted in a higher background GFP level in cells without secretion stress. Analysis of the GFP response with different levels of AmyQ production in the wild-type and the Δ*htrA* background showed that in both cases the GFP response was dose-dependent. However, the relative increase in GFP fluorescence upon AmyQ production was higher in the wild-type background, whereas in absolute GFP fluorescence levels the effect was stronger in the Δ*htrA* strain (Fig. 2). Although the *htrA* deletion was used here in the proof of principle experiments, good separation between secreting and non-secreting cells may also be obtained using the wild-type background, possibly with less false positives.

This secretion stress reporter system may be used in other applications as well, for example in monitoring secretion levels during (industrial) fermentations. An

unstable variant of GFP with reduced half-life may be used to allow for real-time monitoring of secretion stress (Andersen *et al.*, 1998).

Experimental procedures

Bacterial strains and media

Bacillus subtilis NZ8900 (Bongers *et al.*, 2005) was used for overexpression of secretory proteins and testing of the secretion stress response. *Bacillus subtilis* DB104 (Kawamura and Doi, 1984) was used for construction of the final reporter strain. *Escherichia coli* DH5α and *Lactococcus lactis* MG1363 were used for cloning purposes. TY (Sambrook *et al.*, 2009) was used as growth medium for *B. subtilis* and *E. coli* and M17 (Difco) based medium supplied with 0.5% glucose was used for *L. lactis*. Antibiotics were added when appropriate, at 5 μg ml^{-1} for chloramphenicol and kanamycin, 100 μg ml^{-1} for spectinomycin and 2 μg ml^{-1} for erythromycin. Strains and plasmids used are listed in Table 2.

Recombinant DNA techniques

Procedures for cloning and transformation of *E. coli* were carried out as described before (Sambrook *et al.*, 2009). *Lactococcus lactis* was transformed by electroporation. *Bacillus subtilis* was transformed using natural competence as described before (Kunst and Rapoport, 1995).

Construction of secretory protein overexpression vectors

Except for *lmrA*, all genes for inducible overexpression were amplified from chromosomal DNA of the originating host, using primers listed in Table S1. Sequences coding for C-terminal His6-tags were incorporated in the reverse primers. Suitable restriction sites were incorporated in both forward and reverse primers. Genes to be expressed with the SURE system were ligated into pNZ8902 and subcloned in *L. lactis* MG1363 for propagation. *lmrA*, containing a sequence coding for an N-terminal His6-tag, was cloned from the existing vector pNHLmrA (Margolles *et al.*, 1999) into pNZ8902 using NcoI and XbaI restriction sites. For IPTG inducible expression, *amyQ* was amplified using primers amyQ-pDG-f and amyQ-pDG-r and ligated into pDG148, yielding pDGamyQ. *Escherichia coli* was used as a cloning host. The sequence of all constructs was confirmed by sequencing (ServiceXS, the Netherlands).

Construction of the reporter strains 168-A, BV-A, HT-A and HT100A

To test whether a transcriptional fusion of the *htrA* promoter and *gfp* could generate a detectable GFP level upon secretion stress, a first reporter strain was constructed with the P_{htrA}–*gfp* fusion integrated at the *htrA* locus. For this, a 530 bp promoter region of *htrA* (P_{htrA}) was amplified by PCR from chromosomal DNA of *B. subtilis* 168 with primers PhtrA-fw and PhtrA-rv, introducing KpnI and HindIII restriction sites. The digested product was ligated into KpnI- and HindIII-digested pSG1151 (Lewis and Marston, 1999), a *B. subtilis* non-replicating integration vector, upstream of the *gfp-mut1* gene (Cormack *et al.*, 1996). The resulting plasmid was transformed into *B. subtilis* 168 and chloramphenicol-resistant clones were tested by PCR for site-specific integration of the plasmid into the chromosome by single-cross-over

Table 2. Strains and plasmids used in this study.

Strains	Genotype or properties	Reference
B. subtilis		
168	*trpC2*	Kunst *et al.* (1997)
DB104	*his nprE aprE*	Kawamura and Doi (1984)
168-A	168 P*htrA*::P*htrA–gfpmut-1*, Cm^r	This work
BV2003	168 *htrA*::pMutin2, Em^r	Hyyrylainen *et al.* (2001)
BV-A	168 *amyE*::P_{htrA}–*gfpmut1*, Cm^r, *htrA*::pMutin2, Em^r	This work
HT-A	168 *amyE*::P_{htrA}–*gfpmut1*, Cm^r	This work
NZ8900	168 *amyE*::*spaRK*, Km^r	Bongers *et al.* (2005)
HT100A	168 P*htrA*::P*htrA–gfpmut-1*, Cm^r, *amyE*::*spaRK*, Km^r	This work
VT210A	DB104 *amyE*::P_{htrA}–*gfpmut1*, Cm^r, *ydiS*::Sp^r *htrA*::pMutin2, Em^r	This work
L. lactis		
NZ9000	*pepN::nisRK*	De Ruyter *et al.* (1996)
Plasmids		
pUB110	High copy number *S. aureus* plasmid, Km^r	Palva *et al.* (1981)
pKTH10	pUB110 harbouring the *amyQ* gene of *B. amyloliquefaciens*; Kmr	Palva (1982)
pKTHM10	*E. coli–B. subtilis* shuttle vector harbouring the *amyQ* gene of *B. amyloliquefaciens*; Apr, Kmr	Zanen *et al.* (2005)
pDG148	*E. coli–B. subtilis* shuttle vector, containing P*spac*	Stragier *et al.* (1988)
pDGamyQ	pDG148 harbouring *amyQ* of *B. amyloliquefaciens*	This work
pSG1151	*gfpmut1*, Ap^r Cm^r	Lewis and Marston (1999)
pNZ8902	SURE expression vector carrying the inducible $P_{spaSmut}$; Em^r	Bongers *et al.* (2005)
pNZ-amyQ	pNZ8902 harbouring *amyQ* of *B. amyloliquefaciens*	This work
pNZ-usp45	pNZ8902 harbouring *usp45* of *L. lactis* MG1363	This work
pNZ-mntA	pNZ8902 harbouring *mntA* of *B. subtilis*	This work
pNZ-lmrA	pNZ8902 harbouring *lmrA* of *L. lactis* MG1363	This work
pNZ-lasB	pNZ8902 harbouring *lasB* of *P. aeruginosa* PAO1	This work
pNZ-xynA	pNZ8902 harbouring *xynA* of *B. subtilis*	This work
pNZ-ywbN	pNZ8902 harbouring *ywbN* of *B. subtilis*	This work
pNZ-glyA	pNZ8902 harbouring *glyA* of *B. subtilis*	This work

at the P_{htrA} locus. The reporter strain, containing the P_{htrA}–*gfpmut-1* fusion as well as P_{htrA} upstream of *htrA*, was named 168-A.

To test the effect of disruption of *htrA* on the level of P_{htrA}-induced *gfp* expression, strain BV-A was constructed as follows: *gfpmut-1* was amplified together with an upstream multiple cloning site from pSG1151 with the primers gfpmut-fw and gfpmut-rv, introducing a Bpu1101I restriction site downstream of *gfpmut-1*. The PCR product was ligated in KpnI and Bpu1101I digested pDL (Yuan and Wong, 1995), containing homologous flanking regions of the *amyE* locus for site specific recombination, resulting in pDL–gfp. The *htrA* promoter (P_{htrA}) was amplified by PCR from chromosomal DNA of *B. subtilis* 168 with the primers PhtrA-fw and Phtr-rv, introducing KpnI and HindIII sites and ligated into KpnI- and HindIII-digested pDL–gfp, upstream of the *gfp* gene. The resulting plasmid pDL-PhtrA–gfp was transformed into *B. subtilis* BV2003 (*htrA*::pMutin2) and *amyE* replacement by the P_{htrA}–*gfpmut-1* fusion and the chloramphenicol resistance gene was confirmed by PCR on chromosomal DNA. Strain HT-A was constructed similarly, by transformation of *B. subtilis* 168 with pDL-PhtrA–gfp. BV-A and 168-A were transformed with pKTH10, from which the α-amylase gene of *Bacillus amyloliquefaciens*, *amyQ*, is constitutively expressed (Palva, 1982), and with the empty vector pUB110 (Palva *et al*., 1981). HT-A and BV-A were transformed with pDGamyQ. Strain HT100A, constructed for testing the overproduction of a range of different secretory proteins, was obtained by transforming strain 168-A with chromosomal DNA of strain NZ8900 (*amyE*::*spaRK*, Km^r). Transformants were selected on TY plates containing 5 μg ml^{-1} kanamycin and 1% (w/v) starch, and screened for starch halo formation to confirm integration of the *spaRK* and kanamycin resistance genes in the *amyE* locus.

Construction of reporter strain VT210A

Bacillus subtilis VT210A was constructed in three steps. First, the P*htrA–gfpmut-1* fusion was integrated in the *amyE* locus of *B. subtilis* strain DB104 (*nprE aprE*), which has less than 5% of the wild-type extracellular protease activity due to disruption of the genes encoding neutral protease (NprE) and alkaline protease (AprE) (Kawamura and Doi, 1984), by transformation with pDL-PhtrA–gfp (see above). Second, the *htrA* gene was disrupted by transformation of chromosomal DNA of *B. subtilis* BV2003, which has a disrupted *htrA* gene by integration of pMutin2 (Hyyrylainen *et al*., 2001) and selection on plates containing chloramphenicol. The obtained strain was named VT200A. Third, the BsuM DNA restriction system was disrupted by replacement of *ydiS* with a spectinomycin resistance gene. For this, a *ydiS* deletion cassette was constructed: a *ydiS* upstream flanking region (flr1) was amplified from chromosomal DNA using the primers flrbsu1-f and flr1bsu1-r. A spectinomycin resistance cassette (Sp^r) was amplified from pDG1726 (Guerout-Fleury *et al*., 1995) with the primers spbsu-f and spbsu-r. The *ydiS* upstream flanking region and the spectinomycin resistance gene were fused in a PCR in which both fragments served as one template upon recombination of the homologous ends introduced by primers flrbsu1-r and spbsu-f. The fused fragments were cloned in pCR-XL-TOPO (Invitrogen), resulting in pCR-XL-TOPO-flr1-sp^r. A second *ydiS* downstream flanking region, amplified using primers flrbsu2-f and flrbsu2-r, was cloned in pCR-XL-TOPO, resulting in pCR-XL-TOPO-flr2. The flr1-$spec^r$ fragment was restricted from pCR-XL-TOPO-flr1-sp^r using XhoI and NsiI, and ligated into pCR-XL-TOPO-flr2, digested with XhoI and PstI (compatible with NsiI). This resulted in the deletion construct pTOPO-flr1-$spec^r$-flr2, which was transformed into strain VT200A. Spectinomycin-resistant transformants were checked for the *ydiS* deletion by PCR on chromosomal DNA. The final reporter strain obtained was designated VT210A.

GFP analysis by fluorescence microscopy and flow cytometry

Strain 168-A and BV-A containing either pKTH10 or pUB110 were grown overnight in TY with kanamycin. Cells were diluted 20-fold, grown to early stationary phase and then prepared for analysis by fluorescence microscopy. Flow cytometry was performed with a FACSCanto flow cytometer (BD Biosciences) (Fig. 2), or with an EPICS-XL flow cytometer (Beckmann Coulter) (Fig. 3). Strain HT-A and BV-A containing pDGamyQ were grown to mid exponential phase and then induced for 1 h with IPTG at concentrations indicated in Fig. 2. HT100A cells harbouring pNZ8901 or a pNZ8901 derived expression construct were grown similarly, but induced for 1 h with 0.1% of subtilin containing medium from a *B. subtilis* ATCC5533 overnight culture (Bongers *et al*., 2005). Cells were diluted in 50 mM KPi, pH 7.0, to an appropriate density for flow cytometry.

Construction of partial genomic expression library of B. amyloliquefaciens

A *B. subtilis* expression vector was constructed based on the *E. coli*/*B. subtilis* shuttle vector pKTHM10 (Zanen *et al*., 2005), which contains the *amyQ* gene and its promoter region from pKTH10. The *amyQ* gene was removed leaving a BamHI restriction site for cloning of fragments downstream of the *amyQ* promoter. Chromosomal DNA of *B. amyloliquefaciens* was partially digested with Sau3AI and subjected to agarose gel electrophoresis. DNA fragments ranging from 2 to 4 kb were isolated from the gel and ligated into the BamHI digested pKTHM10 derivative. After transformation of *E. coli* DH5α and spreading on TY plates containing 50 μg ml^{-1} ampicillin, a subset of clones was analysed for presence of inserts in the plasmids. Approximately, 90% of the clones contained an insert with an average length of 2.1 kb. Plasmid DNA of all obtained clones was isolated by harvesting colony material from plates, followed by a standard plasmid DNA isolation procedure. This DNA was used to transform the reporter strain VT210A.

Fluorescence-activated cell sorting and analysis of positive clones

Cells of strain VT210A, transformed with a mixture of pKTH10 and pUB100 or the partial genomic library of *B. amyloliquefaciens*, were grown overnight in TY medium containing 5 μg ml^{-1} kanamycin and subjected to flow cytometry

and cell sorting using a MoFlo FACS (Dako-cytomation). A blue laser of 80 mW and 488 nm was used. A typical FACS experiment comprised analysis of 20.000 events (cells). Fluorescence cut-off values for sorting positive cells were determined on basis of empty vector control cells, or by setting a certain percentage of highest fluorescent cells. Positive cells transformed with pKTH10 or pUB110 were collected in one tube containing TY and tested for α-amylase secretion by plating on TY medium containing 1% (w/v) starch and 5 μg ml^{-1} kanamycin. After overnight growth, halo formation by α-amylase activity was visualized using Lugol's solution. Positive cells from the *B. amyloliquefaciens* library were collected individually in 96-well microtitre plates containing TY with 5 μg ml^{-1} kanamycin. After overnight growth, supernatants were collected and subjected to standard trypsin digestion and mass spectrometry analysis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers sequences.

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