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Activity-independent screening of secreted proteins using split GFP

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ABSTRACT

The large-scale industrial production of proteins requires efficient secretion, as provided, for instance, by the Sec system of Gram-positive bacteria. Protein engineering approaches to optimize secretion often involve the screening of large libraries, e.g. comprising a target protein fused to many different signal peptides. Respective high-throughput screening methods are usually based on photometric or fluorimetric assays enabling fast and simple determination of enzymatic activities. Here, we report on an alternative method for quantification of secreted proteins based on the split GFP assay. We analyzed the secretion by *Bacillus subtilis* of a homologous lipase and a heterologous cutinase by determination of GFP fluorescence and enzyme activity assays. Furthermore, we identified from a signal peptide library a variant of the biotechnologically relevant *B. subtilis* protein swollenin EXLX1 with up to 5-fold increased secretion. Our results demonstrate that the split GFP assay can be used to monitor secretion of enzymatic and non-enzymatic proteins in *B. subtilis* in a high-throughput manner.

1. Introduction

Microbial proteins including enzymes are widely used for a variety of industrial applications, e.g. for the production of chemicals and use as pharmaceuticals (Adrio and Demain, 2014). Protein production is often carried out using recombinant microorganisms like *Escherichia coli*, *Bacillus subtilis* or *Saccharomyces cerevisiae* where different optimization strategies are applied to increase product yields and thus economic benefit (Nijland and Kuipers, 2008; Rosano and Ceccarelli, 2014; Borodina and Nielsen, 2014). In addition to enzymes, many nonenzymatic proteins are of special interest for biotechnological applications, for example collagen as a biomaterial for tissue regeneration (Khan and Khan, 2013; An et al., 2014), pharmaceutical recombinant antibodies (Lee and Jeong, 2015) or cytokines like interleukin-3 (Nielsen, 2013; Westers et al., 2006).

For many enzymes, photometric and fluorimetric activity assays exist, for example for lipases (Jaeger and Kovacic, 2014), proteases (Kasana et al., 2011) and different biomass-degrading enzymes (Kracun et al., 2015), which allow high-throughput screenings aiming at improved production yields or cultivation conditions. In several other cases, such simple assays do not exist. For instance, determination of

cellulase, β-glucanase or xylanase activities (Gusakov et al., 2011) requires 3,5-dinitrosalicylic acid (DNS) or Nelson-Somogyi (NS) reagent to quantify the amount of reducing sugars released from polysaccharides. These assays are difficult to carry out since they include – among other steps – incubation in boiling water for several minutes. For proteins lacking enzymatic activity as for example pharmaceutical antibodies or hormones, more sophisticated immunological detection methods like Western-blotting or enzyme-linked immunosorbent assays (ELISA) must be established. To overcome such problems, target proteins are fused to reporter proteins like the green fluorescent protein (GFP) for detection, quantification and more detailed analyses (Snapp, 2005). For example, a GFP fusion with a non-hydrolytic cellulose-binding module was used to study its interaction with cellulose (Hong et al., 2007). Also, the secretion of a glucoamylase by *Aspergillus niger* was monitored *in vivo* using a GFP fusion (Gordon et al., 2000). These fusions can be easily detected also at higher throughput in microtiter plates by using respective fluorescence readers or by flow cytometry (Delvigne et al., 2015). However, GFP is a relatively large protein (26.9 kDa) which as a fusion partner can affect production yields, solubility and bioactivity of the target protein (Waldo et al., 1999). These problems are minimized by using the split GFP assay. Here, a small

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peptide containing the C-terminal, eleventh β-sheet of GFP (GFP11) is fused to a protein of interest, while the non-fluorescing detector protein containing the β-sheets 1–10 (GFP1-10) is produced separately (Cabantous et al., 2005; Cabantous, 2006). Only after combining the GFP11 tagged protein with the detector either *in vivo* or *in vitro* in a screening procedure, a fluorescent GFP is self-assembled; hence, production of the target protein labeled with a small tag can be monitored simply by fluorescence determination. Several applications of the split GFP method have been reported, including high-throughput *in vivo* solubility assays (Santos-Aberturas et al., 2015), facilitation of protein crystallization (Nguyen et al., 2013) and functional fluorescent labeling of antibodies (Ferrara et al., 2011). Also, protein–protein interactions (Blakeley et al., 2012), membrane fusions (Ishikawa et al., 2012) and membrane protein stability (Errasti-Murugarren et al., 2017) were analyzed. Furthermore, split GFP and derivatives with different fluorescence spectra were applied to monitor the secretion of recombinant proteins into the endoplasmic reticulum of HeLa cells (Kamiyama et al., 2016). We therefore reasoned that the split GFP detection system should be adaptable to monitor protein secretion in *B. subtilis*.

The Gram-positive bacterial protein production host *B. subtilis* possesses the GRAS (generally recognized as safe) status and secretes many proteins co-translationally *via* the Sec pathway (Nijland and Kuipers, 2008; Kang et al., 2014; Degering et al., 2010). Sec-dependent secretion requires a signal peptide (SP) located at the N-terminus of the secreted protein (reviewed by H. Tjalsma et al. (Tjalsma et al., 2000)). Considerable effort was put into understanding and improving secretion because downstream processing of secreted proteins is easy and cost-efficient. Optimization strategies include, among others, the screening of SP libraries (Degering et al., 2010; Brockmeier et al., 2006a) which harbor a set of known SPs and allow identifying the optimal combination of a SP and a target protein resulting in significantly improved yields of secreted protein.

Here, we have tested a signal peptide library combined with the activity-independent split GFP detection system to monitor and optimize protein secretion in *B. subtilis*. The well characterized homologous lipase LipA secreted by *B. subtilis* (Eggert et al., 2001; Dartois et al., 1992; Fulton et al., 2015) was used as proof of concept demonstrating that split GFP fluorescence can be used for quantification of lipase in *B. subtilis* supernatants without affecting enzymatic activity. With a signal peptide screening for the heterologous enzyme cutinase (Cut) from the fungus *Fusarium solani pisi* (Martinez et al., 1992), we could demonstrate that fluorescence determined with the split GFP assay correlated with enzymatic activity and the amount of secreted protein. Furthermore, we used the split GFP assay to identify a SP-fusion variant of *B. subtilis* swollenin EXLX1 (Kerff et al., 2008; Kim et al., 2009; Georgelis et al., 2011; Silveira and Skaf, 2016) which showed a 5-fold increased secretion yield. EXLX1 is a homologue of plant expansins (Sampedro and Cosgrove, 2005) and fungal swollenins (Brotman et al., 2008) and acts by cell wall loosening thereby improving enzymatic degradation by disaggregating cellulosic structures. This group of proteins has biotechnological applications with respect to improving enzymatic breakdown of cellulosic biomass (Jager et al., 2011; Kim et al., 2013; Georgelis et al., 2015).

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Initial proof-of-concept experiments were performed with lipase-negative *B. subtilis* 168 derived strain TEB1030 (Eggert et al., 2003) lacking genes *lipA* and *lipB*. Signal peptide screenings were carried out with the protease-deficient strain *B. subtilis* DB431 derived from *Bacillus* Genetic Stock Center (BGSCID 1A1097DB430) for Cut screening or DB430 for EXLX1 screening (Doi et al., 1991; Doi et al., 1986). *B. subtilis* 168 derivatives, *Escherichia coli* DH5α (Grant et al., 1990) and *E. coli* BL21(DE3) (Studier and Moffatt, 1986) were cultivated in LB medium

(10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.5) at 37 °C supplemented with 50 μg/ml kanamycin (*B. subtilis*) or 100 μg/ml ampicillin (*E. coli*) for plasmid stabilization. Transformation was carried out using chemical competent *E. coli* cells (Sambrook and Russell, 2001) as well as by protoplast formation (Chang and Cohen, 1979) or natural competence (Anagnostopoulos and Spizizen, 1961) for *B. subtilis*. SP screenings were carried out in 96-well microtiter plates with 150 μl LB medium (for EXLX1 screening) or deep-well plates with 1 ml LB medium (for Cut screening), re-cultivation for verification and all other cultivations were performed in shake flasks with 10 ml LB medium. Single clones were transferred to pre-cultures and incubated with agitation overnight. Freshly inoculated cultures were subsequently cultivated with agitation for 6 h and supernatants were isolated by centrifugation in 1.5 ml reaction tubes (21,000 × g, 1 min) or in plates (4000 × g, 20 min).

2.2. Recombinant DNA techniques and gene cloning

Gene cloning was performed using standard methods (Sambrook and Russell, 2001). Kits for PCR and plasmid purification were purchased from Analytic Jena (Jena, Germany), enzymes from Thermo Fisher Scientific (St. Leon-Roth, Germany).

2.3. Construction of signal peptide library plasmid pBSMul1lipA_SPBox

The *B. subtilis lipA* gene was amplified from the pET22lipA (Fulton et al., 2015) using the primer pair *Eco*RI_fw: 5 CGCGGAATTCGCTGA-ACAC 3 and *Hin*dIII_rev: 5 AGTGCGGCCGCAAGCTTGTCGACGTAATG-TTCATTAATTCGTATT 3. The *Eco*RI/*Hin*dIII *lipA* fragment was subsequently ligated into the *E. coli* − *B. subtilis* shuttle vector pBSMul1 (Brockmeier et al., 2006b) resulting the pBSMul1lipAsslipA. Here, the *lipA* gene is fused to its native signal sequence *sslipA* separated by an *Eco*RI restriction site. The *Hin*dIII restriction site downstream of the *lipA* gene in pBSMul1lipAsslipA was deleted by QuikChange PCR® (Edelheit et al., 2009) and re-inserted upstream of the *sslipA* gene using the primer pairs ΔHindIII_Cterm_fw (5′ CCGTCGACGCGGCCGCG 3′)/ ΔHindIII_Cterm_rev (5′ CGCGGCCGCGTCGACGG 3′) and HindIII_N-term_fw (5′ GCGATTTACATAATAAGCTTAAGGAGGACATATG 3′)/Hin-dIII_Nterm_rev (5′ CATATGTCCTCCTTAAGCTTATTATGTAAATCGC 3′). The resulting vector pBSMul1lipA_SP was hydrolyzed with *Hin*dIII/ *Eco*RI removing *sslipA* which was subsequently replaced by the signal peptide toolbox library (Brockmeier et al., 2006a) consisting of equimolar amounts of *Hin*dIII/*Eco*RI signal sequence fragments coding for 173 Sec-specific signal peptides of proteins secreted by *B. subtilis*. This signal peptide toolbox was ligated into pBSMul1lipA_SP resulting the pBSMul1lipA_SPbox with 173 signal sequence-encoding DNA fragments fused to the 5′-end of *lipA*.

2.4. Construction of GFP11 fusions and of signal peptide libraries

Fusions of target proteins with a GFP11 tag and an additional linker region were constructed by a 2-step PCR approach as described in (Santos-Aberturas et al., 2015) using template plasmids pBSMul1lipAsslipA for *lipA-11* and pBSMul1cut, which harbors an *Eco*RI-*Bam*HI *cutinase* fragment from pBSMul3cut (Brockmeier et al., 2006a), for *cut-11*. In the first PCR, the target gene was amplified using a plasmid-specific primer binding upstream of the gene of interest (pBSMul_for 5′ GGAGCGATTTACATAATAAGGAGGACATATG 3′) and a second gene-specific primer that omits the stop codon but inserts the first part of the GFP11 tag (lipA-rev-fu1 5′ TGATCACGAGATGTAGAGCCGCCGCCA GAGCCGCCATCAGAGCCGATAAGATTCGTATTCTGGCCCCCGCCGTTC 3′; cut-rev-fu1 5′ TGATCACGAGATGTAGAGCCGCCGCCAGAGCCGCC-ATCAGAGCCGATAAGAGCAGAACCACGGACAGCCCGAACCTTC   3′). The resulting PCR product was used in a second PCR with the same forward primer and a second primer that binds specifically to the first part of GFP11 tag and includes the residual sequence of the GFP11 tag,

a stop codon and a *Xba*I cut site (rev-fu2 5′ TATATCTAGATTATGTGATGCCAGCAGCGTTAACGTATTCATGAAGAACCATGTGATCACGAGAT GTAGAGCCGCCGCC 3′). Therefore, the second PCR product contains the target gene sequence fused to the complete GFP11 tag. It was hydrolyzed with *Eco*RI and *Xba*I and ligated into a likewise hydrolyzed vector pBSMul1 (Brockmeier et al., 2006b). The resulting vectors pBSMul1-lipA-11 and pBSMul1-cut-11 contain the N-terminal signal sequence of *lipA* followed by an *Eco*RI restriction site and the target gene fusion under control of a strong constitutive promoter for Gram-positive bacteria (P_{HpaII}). Fragment EXLX1-11 was synthesized by Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific) and similarly cloned into pBSMul1 based on the *yoaJ* gene sequence (accession ID: BSU18630) with an additional *Nde*I site flanking the start codon, an *Eco*RI site behind the signal peptide coding sequence (codon 26) and an *Xba*I site downstream of the stop codon. Naturally occurring restriction sites were avoided using synonymous codons and an additional alanine codon was introduced upstream of the *Eco*RI site to restore the signal peptidase recognition motif.

The plasmids pBSMul1-cut-11 and pBSMul1-EXLX1-11 were used to construct the respective signal peptide libraries. The artificially inserted *Eco*RI site between the signal peptide sequence and the sequence coding for the mature protein was used to extract the mature protein sequence fused with GFP11 by hydrolysis with *Eco*RI and *Xba*I. These fragments were ligated into the signal peptide library plasmid pBSMul1lipA_SPBox. After transformation, about 1000 single *E. coli* DH5α colonies were washed off from agar plates and plasmids were isolated to generate a library containing different signal peptides fused to the target gene and the GFP11 tag. *B. subtilis* was transformed with these libraries and cultures grown out of single clones were analyzed regarding extracellular target enzyme activity and formation of split GFP fluorescence.

2.5. Cloning, production and purification of GFP1-10 detector

For the production of the GFP1-10 fragment (detector) in *E. coli* BL21(DE3), vector pET22b-sfGFP1-10 was constructed. Therefore, a DNA fragment coding for a superfolder GFP variant with additional mutations (Cabantous et al., 2005) was synthetized by Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific) and GFP1-10 fragment was amplified by PCR using primer sfgfp_fw (5′ ATATCATATGAGCAAAGGAGAAGAAC 3′) and sfgfp_trunc_rev (5′ ATATAAGCTTTCACTTTTCGTTGGGATCTTTCG 3′). PCR product and vector pET22b(+) (Novagen) were hydrolyzed with *Nde*I and *Hin*dIII and ligated resulting in pET22b-sfGFP1-10 which encodes the detector fragment under control of a T7 promoter.

GFP1-10 detector was produced in *E. coli* BL21(DE3) and purified from the inclusion body fraction as previously described (Santos-Aberturas et al., 2015). Briefly, *E. coli* BL21(DE3) harboring pET22b-sfGFP1-10 was inoculated to an O.D._{580nm} = 0.05, supplemented with 1 mM ITPG after 2 h of cultivation and harvested by centrifugation (3000 × g, 20 min) after additional 5 h. The cell pellet was suspended in TNG buffer (100 mM Tris-HCl pH 7.4, 100 mM NaCl, 10% (v/v) glycerol), sonicated and again centrifuged to sediment cell debris and inclusion bodies containing the detector molecule. This procedure was repeated twice. The resulting inclusion body pellet was dissolved in 9 M urea to reconstitute soluble GFP1-10. After another centrifugation step (14,000 × g, 20 min), supernatant was mixed with TNG buffer (differing from the original protocol) containing 10 mM EDTA to obtain ready-to-use detector solution.

2.6. Fluorescence-based screening

Formation of GFP11 fusion proteins was monitored by mixing 20 μl of sample (culture supernatant) with 180 μl of detector solution in a microtiter plate and incubation with gentle agitation at room temperature to support formation of fluorescent GFP protein. Fluorescence was measured using a Tecan Infinite M1000 Pro (Tecan) with λ_{Ex} = 485 nm (bandwidth 10 nm) and an emission spectrum was recorded from 505 to 550 nm in 5 nm steps (bandwidth 5 nm, gain 120). GFṔs specific emission maxima at λ_{Em} = 510–515 nm was used for calculation.

2.7. Determination of enzymatic activity

Lipolytic activity of lipase and cutinase was measured using the chromogenic substrate *p*-nitrophenyl-palmitate (*p*NPP) as described by U. K. Winkler and M. Stuckmann (Winkler and Stuckmann, 1979). Prior to use, 6 mg *p*NPP was dissolved in 2 ml isopropanol and subsequently mixed with Sørensen buffer pH 8 containing 20 mg gum arabic and 41.4 mg sodium deoxycholic acid. If necessary, samples were diluted with LB medium and subsequently 20 μl of sample were mixed with 180 μl of this substrate solution and incubated at 37 °C. Absorption was measured at λ_{Abs} = 410 nm over 15 min in a SpectraMax 250 plate reader (Molecular Devices) and was used to calculate volume activities (U/ml) with a molar absorption coefficient of 15,000 $M^{-1}\,cm^{-1}$.

2.8. SDS-PAGE und Western-blot analysis

Samples of supernatant were mixed with 2× SDS sample buffer and separated by SDS-PAGE as described by U. K. Laemmli (Laemmli, 1970). Cutinase was detected by Western-blotting on a PVDF membrane (BioRad) with cutinase-specific antibodies (Brockmeier et al., 2006a), goat-anti-rabbit IgG (H + L)-HRP conjugate (BioRad) and an ECL Western-Blotting Detection Reagent (Amersham Pharmacia, Great Britain).
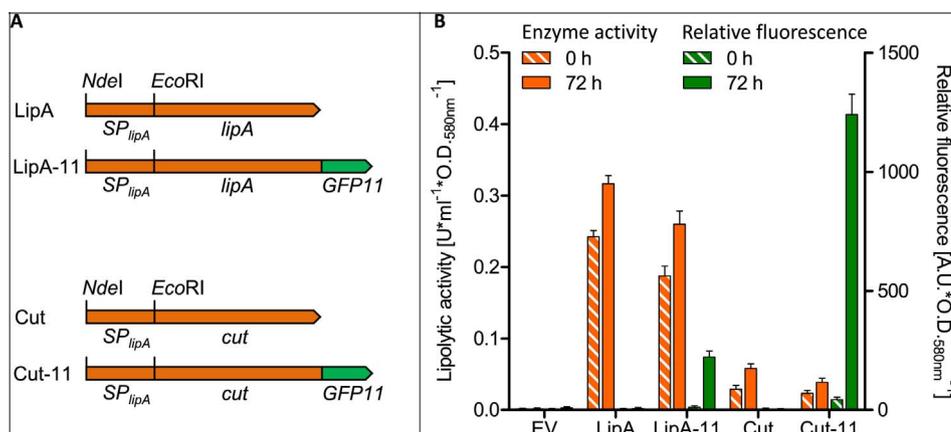

Fig. 1. Lipase and cutinase fusions with GFP11 tag are secreted as active enzymes by *B. subtilis*.
(A) Orientation of fusion constructs consisting of signal peptide (SP_{lipA}), native protein and GFP11 tag. (B) *B. subtilis* was cultivated for 6 h carrying an empty vector (EV) or producing either wildtype lipase (LipA), cutinase (Cut) or GFP11 tagged variants (LipA-11, Cut-11). 20 μl of each supernatant were mixed with 180 μl split GFP detector solution and lipolytic activity (with *p*NPP as the substrate) and relative fluorescence (caused by split GFP) were determined immediately (0 h) and after 72 h incubation as suggested previously (Santos-Aberturas et al., 2015).
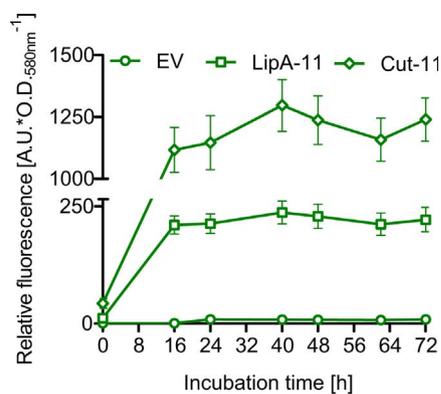
Fig. 2. Development of split GFP fluorescence depends on incubation time. Culture supernatants of *B. subtilis* harboring the empty vector control pBSMul1 (EV) or producing either GFP11-tagged lipase (LipA-11) or cutinase (Cut-11) were incubated with split GFP detector solution as described above and fluorescence was determined at the indicated time points.

3. Results and discussion

3.1. Enzyme secretion by B. subtilis can be monitored with split GFP assay

Enzyme production by microorganisms is usually monitored and quantified by appropriate activity assays as described e.g. for lipolytic enzymes (Jaeger and Kovacic, 2014). However, detection of proteins without enzymatic activity or of enzymes without knowledge of their substrate specificity is difficult. Here, antibody-based detection methods are often used, for example Western-blotting or ELISA assays, but they are usually not suitable for high-throughput screenings, mainly due to laborious protocols and high costs. An additional non-enzymatic detection method is provided by split GFP which allows labeling a target protein with a small GFP-derived peptide tag which can combine with a truncated GFP thus forming mature GFP resulting in an easily detectable fluorescence signal (Cabantous et al., 2005; Santos-Aberturas et al., 2015). We have adapted this method to monitor protein secretion by the industrially important bacterium *Bacillus subtilis* 168 (Nijland and Kuipers, 2008; Schallmey et al., 2004).

This wildtype strain is known to secrete a number of proteases including subtilisin into the culture supernatant (Westers et al., 2004); we therefore used multiple protease-deficient *B. subtilis* strains DB 431 and 430 lacking several intra- and extracellular proteases to avoid premature degradation of target and detector proteins. The homologous lipase LipA and the heterologous fungal cutinase Cut from *Fusarium solani pisi* were used as model enzymes because both are known to be secreted into the culture medium by *B. subtilis via* the Sec pathway (Brockmeier et al., 2006a; Dartois et al., 1994). Fusion of the LipA SP to the N-terminus of Cut (Fig. 1A) ensured secretion as shown previously (Brockmeier et al., 2006a). Furthermore, both enzymes were C-terminally fused to a GFP11 tag with an additional linker region as proposed in (Santos-Aberturas et al., 2015) resulting in constructs LipA-11 and Cut-11. Plasmid-encoded variants with and without GFP11 tag were produced in *B. subtilis* and the presence of LipA or Cut, respectively, was determined in supernatants by measuring lipolytic activity and by the split GFP assay (Fig. 1B). The C-terminal fusion of a GFP11 tag only slightly influenced the lipolytic activity of both enzymes. As expected, no fluorescence was detected immediately after addition of split GFP detector solution, but significant fluorescence was detectable after 72 h for GFP11 tagged samples (Fig. 1B).

These results lead to the conclusion that the split GFP assay is not only suitable for detection of intracellular soluble proteins in *E. coli* as shown previously (Santos-Aberturas et al., 2015), but can also be used to detect and quantify proteins secreted by *B. subtilis*.
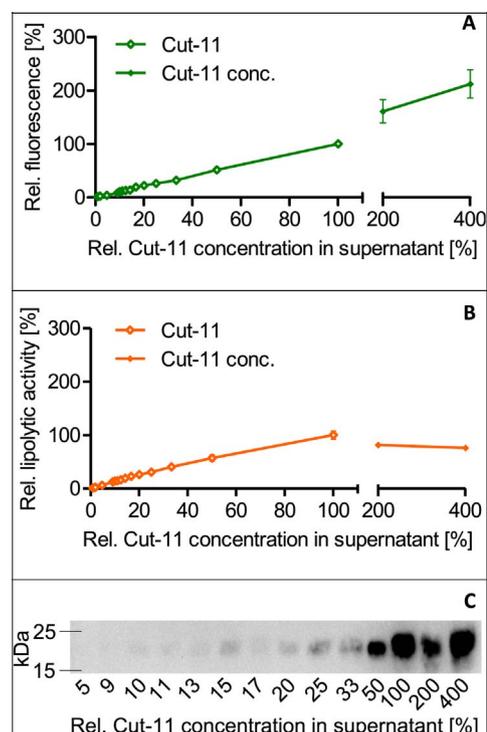

Fig. 3. Determination of split GFP detection range. *B. subtilis* supernatant containing Cut-11 (100%) was combined with supernatants of *B. subtilis* harboring an empty vector (% < 100) or 2- or 4-fold concentrated by vacuum centrifugation (200%, 400%). (A) Relative fluorescence caused by split GFP; (B) lipolytic activity determined with *p*NPP as the substrate; and (C) immunodetection by Western-blotting using a cutinase-specific antibody.

3.2. Split GFP detection range in culture supernatants

After having shown that split GFP can monitor enzyme secretion, we tested whether this assay is also suitable for screening of larger samples, e.g. derived from SP libraries (Degering et al., 2010; Brockmeier et al., 2006a). For this, we determined absolute split GFP fluorescence for LipA-11 and Cut-11 at different time points (16, 24, 40, 48, 62 h) in addition to the previously used incubation time of 72 h (Fig. 2).

To assess the sensitivity of the assay, supernatants of *B. subtilis* secreting Cut-11 were sequentially diluted with supernatants obtained from the empty vector control sample or 2- to 4-fold concentrated by vacuum centrifugation. As shown in Fig. 3A, the observed fluorescence intensity correlates with Cut-11 concentration (linear regression $y = 0.99x + 0.45$ with $R^2 = 0.99$ for diluted samples). The sensitivity of the split GFP assay was comparable to that of the enzymatic assay (Fig. 3B) and superior to the immuno-detection using cutinase-specific antibodies (Fig. 3C). The observed loss of lipolytic activity in the concentrated samples (Fig. 3B) which apparently did not affect fluorescence yield (Fig. 3A) may indicate a partial enzyme denaturation during the concentration procedure.

3.3. Screening of a SP-cutinase library with split GFP assay

Next, we addressed the putative applicability of the split GFP assay for high-throughput screening of secreted enzymes. To this end, Cut-11 was fused to a signal peptide library (Brockmeier et al., 2006a) and supernatants of about 500 *B. subtilis* DB430 clones each harboring a different SP-Cut-11 fusion were tested for lipolytic activity and protein amount using the split GFP assay; Cut-11 fused to the LipA signal peptide served as a reference. The fluorescence and enzymatic activity in the supernatant were determined for each clone (Fig. 4 A, displayed in descending order) and 152 clones with increased fluorescence were identified. Interestingly, we also observed several variants which
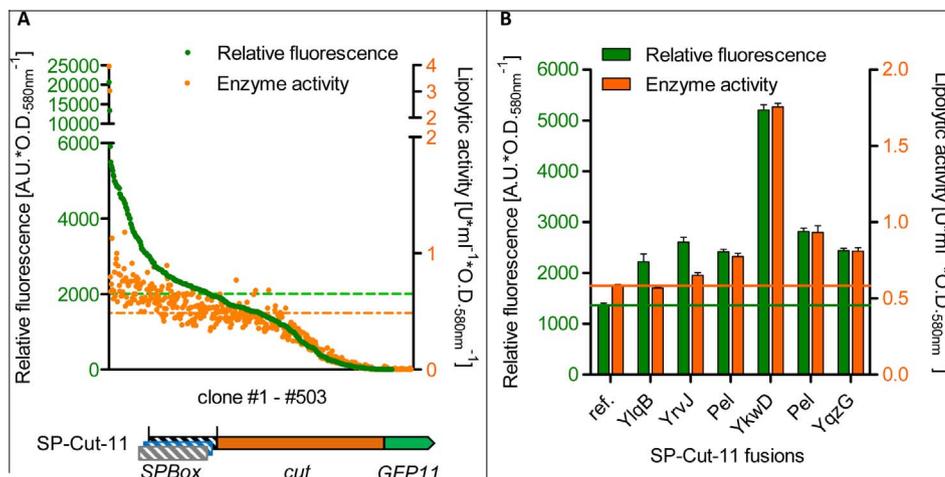
Fig. 4. Screening of a SP-cutinase library using the split GFP assay.

(A) The gene *cut-11* was fused to a *B. subtilis* signal peptide library (SPBox) and about 500 clones were screened for improved secretion as determined by fluorescence of split GFP in culture supernatants. (B) Selected variants were re-cultivated, re-analyzed, and the corresponding signal peptides were identified by sequencing. Horizontal lines indicate fluorescence (green) and enzymatic activity (orange) of the reference construct Cut11 fused to LipA SP.
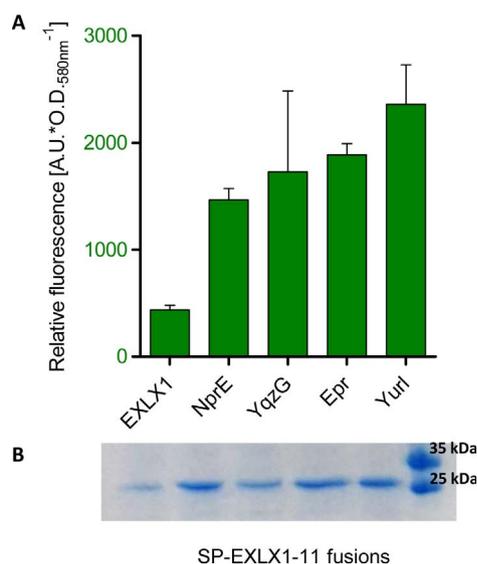

Fig. 5. Screening of a SP-swollenin EXLX1 library using the split GFP assay. EXLX1-11 gene was fused to a *B. subtilis* signal peptide library and fusion protein amount was monitored in the supernatant of over 500 clones (data not shown). Best variants with increased protein amount were re-cultivated in shaking flasks, re-analyzed by split GFP assay (A) and SDS-PAGE (B), and the corresponding signal peptide was identified by sequencing.

showed higher fluorescence indicating higher amounts of secreted protein but lower enzymatic activity as compared to the reference and *vice versa*. The reasons for this observation are unknown, but the respective SP may influence both folding and secretion efficiencies positively or negatively.

To confirm the results obtained by screening of the SP library, we chose to cultivate five clones which had shown improved cutinase secretion as biological triplicates in shaking flasks to determine fluorescence, lipolytic activity and identify the respective signal peptides by sequencing (Fig. 4B). Except for variant SP_{YlqB}-Cut-11, all variants indeed showed an increased amount of protein and lipolytic activity in the supernatants. In a previous screening campaign, these signal peptides were also identified to improving the secretion of a cutinase by *B. subtilis* (Brockmeier et al., 2006a). In this study, Brockmeier et al. had identified the signal peptide of the minor extracellular serine protease (SP_{Epr}) as the most effective for cutinase secretion. For so far unknown reasons, this SP was not identified in our screening.

3.4. Screening of a SP-swollenin library with split GFP assay

Secretion of recombinant non-enzymatic proteins has been analyzed indirectly in *B. subtilis* by monitoring the secretion stress response (Trip et al., 2011). The split GFP assay should be particularly useful to directly monitor secretion of such proteins. Therefore, we decided to analyze and optimize the secretion of swollenin EXLX1 (also named YoaJ), an accessory protein of important biotechnological relevance which is produced by *B. subtilis* and increases the accessibility of densely packed polysaccharides thereby facilitating their enzymatic depolymerization (Kerff et al., 2008; Kim et al., 2009; Georgelis et al., 2011; Kim et al., 2013; Georgelis et al., 2015; Lin et al., 2013).

EXLX1 of *B. subtilis* 168 tagged with GFP11 was fused to the SP library, and the variants were expressed in *B. subtilis* as described above for Cut-11. Culture supernatants of 552 clones were analyzed with the split GFP assay (data not shown). Subsequently, plasmids of the best performing clones were isolated and re-transferred to *B. subtilis*. The cells were grown in shaking flasks and the amount of secreted EXLX1 protein was determined *via* split GFP fluorescence and SDS-PAGE (Fig. 5). SPs giving highest yields of secreted proteins were identified by sequencing as belonging to the extracellular proteases NprE and Epr, the extracellular ribonuclease YurI as well as to YqzG, a protein of unknown function probably contributing to spore formation (Wang et al., 2006).

4. Conclusions

The bacterium *B. subtilis* is well known for its capacity to produce and secrete a wealth of biotechnologically relevant proteins and enzymes (Nijland and Kuipers, 2008). Consequently, considerable efforts were directed to optimizing secretion (Kang et al., 2014), however, screening for improved secretion can be difficult if high-throughput assays are not available as for enzymes which form difficult-to-determine products or proteins which lack enzymatic activity. We have demonstrated here that the split GFP assay can be used to determine the amount of proteins secreted by *B. subtilis* into the culture supernatant. The target protein is fused to a 30 amino acid tag comprising the C-terminal β-sheet of GFP and apparently does not negatively affect secretion. After being secreted, this fusion construct can complement a truncated GFP (GFP 1-10) yielding a fluorescence signal that can easily be read out also at high throughput. Using homologous and heterologous enzymes, we could show that split GFP signals correlate well with enzymatic activities and amount of secreted proteins. Furthermore, a SP library was constructed with the non-enzymatic swollenin protein EXLX1 which has been suggested for potential use in assisting enzymatic degradation of cellulosic biomass (Kerff et al., 2008; Georgelis et al., 2015). The split GFP assay enabled us to identify SP

variants which led to up to 5-fold more secreted swollenin protein than the wildtype SP. In summary, our data demonstrate that the split GFP assay can be used, also at high-throughput, to detect and quantify enzymes and non-enzymatic proteins secreted by *B. subtilis*. It is reasonable to assume that this assay is transferable to other target proteins and secretion host strains as well.

Acknowledgements

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