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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Production of extracellular heterologous proteins in *Streptomyces rimosus*, producer of the antibiotic oxytetracycline

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Abstract

Among the *Streptomyces* species, *Streptomyces lividans* has often been used for the production of heterologous proteins as it can secrete target proteins directly into the culture medium. *Streptomyces rimosus*, on the other hand, has for long been used at an industrial scale for oxytetracycline production, and it holds 'Generally Recognised As Safe' status. There are a number of properties of *S. rimosus* that make this industrial strain an attractive candidate as a host for heterologous protein production, including (1) rapid growth rate; (2) growth as short fragments, as for *Escherichia coli*; (3) high efficiency of transformation by electroporation; and (4) secretion of proteins into the culture medium. In this study, we specifically focused our efforts on an exploration of the use of the Sec secretory pathway to export heterologous proteins in a *S. rimosus* host. We aimed to develop a genetic tool kit for *S. rimosus* and to evaluate the extracellular production of target heterologous proteins of this industrial host. This study demonstrates that *S. rimosus* can produce the industrially important enzyme phytase AppA extracellularly, and analogous to *E. coli* as a host, application of His-Tag/Ni-affinity chromatography provides a simple and rapid approach to purify active phytase AppA in *S. rimosus*. We thus demonstrate that *S. rimosus* can be used as a potential alternative protein expression system.

Keywords *Streptomyces rimosus* · Heterologous protein production · Gene expression · Gene tools · Reporter system · Phytase

Introduction

Among *Streptomyces* species, *Streptomyces lividans* has often been used for the production of heterologous proteins, which is based on its secretion of target proteins directly into the culture medium. In addition, *S. lividans* has low extracellular proteolytic activity (Binnie et al. 1997) and lacks the restriction systems common to *Streptomyces*, which prevents efficient transformation of *Escherichia coli*-derived DNA, thus avoiding the use of non-methylated DNA (Binnie et al. 1997). *S. lividans* is one of the most versatile *Streptomyces* hosts; however, its slow and filamentous growth and the relatively tedious transformation procedures that it requires have significant disadvantages compared to *E. coli.*

Streptomyces rimosus is the industrial producer of the medically important broad-spectrum antibiotic oxytetracycline. *S. rimosus* has long been used at an industrial scale for oxytetracycline (OTC) production, and it holds 'Generally Recognised As Safe' status. Extensive knowledge of the general microbiology, physiology and molecular biology techniques for the manipulation of *S. rimosus* has accumulated since the 1950s, when the industrial bioprocesses for OTC production were initially developed (Petkovic et al. 2006). There are a number of properties of *S. rimosus* that make this industrial strain attractive as a host for the production of

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heterologous proteins: (1) *S. rimosus* has a rapid growth rate, and as with *E. coli*, it can reach high cell densities in a short period of time; (2) *S. rimosus* does not form complex mycelia, but as for *E. coli*, it grows in the shape of short fragments; (3) *S. rimosus* shows high transformation efficiency using electroporation; and (4) *S. rimosus* can secrete proteins into the culture medium, which facilitates their downstream processing. *S. rimosus* also has potential disadvantages, such as its reported endogenous genetic instability and significant extracellular proteolytic activity, which can cause degradation of any target heterologous protein (Gravius et al. 1993; Renko et al. 1989). As reported by Kieser et al. (1982), *S. rimosus* poorly maintains replicative plasmids, which thus induces plasmid instability, particularly when working with *E. coli*–*Streptomyces* shuttle vectors.

We have demonstrated previously that *S. rimosus* can efficiently express the chalcone synthase *rppA* reporter gene (Magdevska et al. 2010). Champreda and collaborators, on the other hand, reported the expression of the triacyl glycerol lipase gene in *S. rimosus* (Sinsereekul et al. 2010). Thus, this early work by Sinsereekul et al. (2010) and Magdevska et al. (2010) indicated that *S. rimosus* can be used for the expression of heterologous proteins. However, when considering any microbial host as a platform for the production of heterologous proteins, it becomes necessary to develop an efficient and diverse set of genetic tools that can be used to facilitate the production of these heterologous proteins. As mentioned above, the secretion of target proteins out of the cell by *Streptomyces* species is a significant advantage when compared to the *E. coli* host. We have also identified gene homologues of the Sec secretion pathway in the genome of *S. rimosus*. Therefore, this study was specifically focused on an exploration of the utility of the Sec secretion pathway to export heterologous proteins in *S. rimosus*.

Here, we used the catechol 2,3-dioxygenase XylE reporter system from the *Pseudomonas putida* TOL plasmid (Ingram et al. 1989) and commercial phytase AppA from *E. coli* (Golovan et al. 2000) as model systems to evaluate the efficacy of heterologous protein production in *S. rimosus*. The XylE reporter system has been widely used in *Streptomyces* to evaluate the strength of different promoters (Ingram et al. 1989), and the expression of AppA has already been reported in *S. lividans*, *Pichia pastoris*, *Schizosacharomyces pombe* and *Saccharomyces cerevisiae* (Lee et al. 2005; Stahl et al. 2003). Therefore, XylE and AppA appear to be ideal model systems to carry out this comparative study with *S. rimosus* as a potential host for protein production. A selection of potentially useful *Streptomyces* promoters described in the literature and selected signal sequences needed for the export of the target proteins were tested in this study. A stable replicative *E. coli*–*Streptomyces* shuttle vector was also constructed.

In the present study, therefore, the aim was to develop the genetic tool kit for *S. rimosus* and to evaluate the extracellular production of target heterologous proteins in the *S. rimosus* host. We have demonstrated the extracellular production of the industrially important enzyme phytase AppA by *S. rimosus*. Moreover, we have demonstrated the versatility of *S. rimosus* as a host for the production of target heterologous proteins, and analogous to the *E. coli* host, by applying His-Tag/ Ni-affinity chromatography, we have demonstrated a simple and rapid approach to purify active phytase AppA produced by *S. rimosus*.

Materials and methods

Bacterial strains

Plasmid DNA was propagated in *E. coli* DH10β. Non-methylated DNA used to transform *S. rimosus* was prepared in *E. coli* ET12567 (MacNeil et al. 1992). *E. coli* was cultivated in 2TY medium supplemented with 100 μg/mL ampicillin, 75 μg/mL apramycin or 10 μg/mL chloramphenicol when required. The *S. rimosus* M4018 strain (DSM 105900) was used as the host for protein expression (Rhodes et al. 1984). *S. rimosus* was cultivated in tryptic soy broth (TSB) medium to prepare electro-competent cells, as previously described (Pigac and Schrempf 1995), and transformants were selected on agar plates with 30 μg/mL thiostrepton. *S. rimosus* spores were propagated on soya–mannitol agar medium (Kieser et al. 2000) supplemented with 30 μg/mL thiostrepton at 30 °C for 8 to 10 days, and stored in 20% glycerol at − 80 °C. The integrative plasmids pVM and pAB04, and the replicative plasmid pVF, were used to express heterologous proteins in *S. rimosus* (Table S1).

Construction of expression vectors

The plasmids used in this study are listed in Table S1. The pVF vector was constructed using the *Streptomyces* pPZ12 (Chambers and Hunter 1984) and pUC19 (Invitrogen, USA) vectors. Both plasmids were digested with *Pst*I, gel purified, ligated and transformed into *E. coli*, to produce the pVF vector. The plasmid pVF was transformed into *S. rimosus* and isolated from *S. rimosus* transformants. The pVF plasmid from the selected *S. rimosus* transformant was sequenced by primer walking (Fig. S1, Fig. S2A). The pAB04 vector was constructed using pSET152 plasmid linearised with *Msc*I and dephosphorylated. The *tsr* gene for thiostrepton selection was cut out from pUC19 vector containing the *tsr* gene in the MCS using *Nde*I and *Xba*I, filled in with Klenow, and ligated with the previously linearised pSET152 vector. pVM are pTS55 based vectors containing P_{ermE*} promoter, signal sequence and *appA* gene in MCS. Expression–secretion cassettes were

constructed in pBluescript II KS/SK(+) vector using *Eco*RI and *Nde*I restriction sites for cloning of P_{ermE*} promoter, *Nde*I and *Pst*I for signal sequence and *Pst*I and *Xba*I for *appA* gene. The entire cassette was digested with *Hin*dIII and *Bam*HI and inserted in pTS55 expression vector.

The *xylE* gene was modified by removing its native *Nde*I restriction site and was PCR amplified by adding *Nde*I and *Xba*I restriction sites at the 5′ end and the 3′ end, respectively. The resulting *Nde*I-*xylE*-*Xba*I fragment was gel purified and ligated into the pBluescript II KS/SK(+) vector containing the P_{ermE*} promoter, which had previously been treated with *Nde*I/*Xba*I. The cassette containing *xylE* expressed under P_{ermE*} was isolated by digestion with *Eco*RI, end-filled with Klenow, followed by a second digestion with *Xba*I. The DNA fragment containing P_{ermE*}–*xylE* was ligated into the pTS55 vector, which was previously digested with *Hin*dIII, end-filled with Klenow and further cut with *Xba*I, to generate the pVMX3 vector. The P_{ermE*}–*xylE* cassette was digested with *Eco*RI/*Xba*I and ligated into the pAB04 and pVF vectors, which were previously linearised with the same set of enzymes, thus generating pAB04-1 (P_{ermE*}–*xylE*) and pVF1 (P_{ermE*}–*xylE*). The nitrilase (*PnitA*/*NitR*) and tetracycline (*tcp830*) promoters were synthesised based on the information of Herai et al. (2004) and Rodriguez-Garcia et al. (2005), respectively, and delivered into the pEX-based vector (Eurofins, Germany). pEX-*PnitA*/*NitR* and pEX-*tcp830* were digested with *Nde*I/*Xba*I to accommodate the *xylE* gene, which was previously isolated from pAB04-1 using the same set of enzymes, to generate pEX-*PnitA*-1 and pEX-*tcp830*-1. To produce pAB04-2, pEX-*PnitA*-1 was digested with *Hin*dIII and the *PnitA*/*xylE*/*nitR* cassette was end-filled with Klenow, and ligated into pAB04, which was previously digested with *Eco*RI/*Xba*I, end-filled with Klenow and dephosphorylated. Further, pEX-*PnitA*-1 and pVF were both digested with *Hin*dIII, and the fragment *PnitA*/*xylE*/*nitR* was ligated into pVF, which resulted in pVF-2. The transcriptional regulator *tetRiS* (Rodriguez-Garcia et al. 2005) was synthesised under the regulation of the *SF14* promoter and delivered into the pEX-based vector (Eurofins, Germany). The *SF14*-*tetRiS* cassette was digested with *Sac*I/*Bam*HI, treated with Klenow and ligated into pEX-*tcp830*-1, which was previously digested with *Sac*I, end-filled and dephosphorylated, to generate the pEX-*tcp830*–1/*tetRiS* vector. The *tcp830*/*xylE*/*SF14*/*tetRiS* cassette was liberated from pEX-*tcp830*-1/*tetRiS* using *Kpn*I/*Xba*I, end filled with Klenow and ligated into pAB04 and pVF, which were previously digested with *Eco*RI, end-filled with Klenow and dephosphorylated, thus resulting in pAB04-3 and pVF-3.

The signal sequences (Table 1) of the subtilisin inhibitor (*vsi*) (Van Mellaert et al. 1998) and α-amylase (*aml*) from *Streptomyces venezuelae* (Virolle et al. 1988), and the signal sequences of α-amylase (*amy*) (Vigal et al. 1991) and protease B (*sprB*) (Henderson et al. 1987) from *Streptomyces griseus*, were DNA synthesised (Eurofins, Germany). Trypsin-like protease (*srT*) and lipase (*lip*) signal sequences from *S. rimosus* (this study) and the xylanase A signal sequence (*xysA*) from *Streptomyces halstedii* (Diaz et al. 2004) were PCR amplified using genomic DNA of native hosts as the template. The mutated α-amylase signal sequence (*aml**) from *S. venezuelae* (Lammertyn et al. 1998) was PCR amplified using the *aml* native signal sequence that incorporated the mutations in the constructed primers (Table S2). All of the signal sequences were designed with *Nde*I at the 5′ and *Pst*I at the 3′ positions, and ligated into the pBluescript II KS/SK(+) vector that contained the P_{ermE*} promoter, which was previously digested with *Nde*I/*Pst*I. Further, the *xylE* gene was PCR amplified by adding *Pst*I and *Xba*I restriction sites at the 5′ and 3′ positions, respectively, and ligated into each of the eight pBluescript vectors containing P_{ermE*} and selected signal sequences, which were previously digested with *Pst*I and *Xba*I. The DNA fragments containing P_{ermE*}/signal sequence/*xylE* were isolated using *Hin*dIII/*Bam*HI and ligated into the pTS55 vector, which was previously treated with the same enzymes, to yield the pVM series vectors (Table S1).

The phytase (*appA*) gene was synthesised (Eurofins, Germany, GenBank accession number KY951965) without its native signal sequence; codon usage was optimised for the *S. rimosus* strain and delivered in the pBlue-*appAsyn* vector. The DNA fragments that contained the P_{ermE*} promoter and the signal sequences of α-amylase (*aml*) from *S. venezuleae*, trypsin-like protease (*srT*) and lipase (*lip*) from *S. rimosus* were isolated from the pVM8, pVM13 and pVM14 plasmids (Table S1), respectively, cutting with *Eco*RI/*Nde*I and ligated into the pBlue-*appAsyn* vector, which was previously treated with *Eco*RI/*Nde*I. The DNA fragments containing P_{ermE*}–signal sequence–*appAsyn* were digested with *Hin*dIII/*Bam*HI and ligated into the pTS55 vector, which was previously treated with the same set of enzymes, which thus generated the vectors pVM4 (containing the signal peptide from *S. venezuelae* α-amylase), pVM15 (containing the signal peptide from *S. rimosus* trypsin-like protease) and pVM16 (containing the signal peptide from the *S. rimosus* lipase signal sequence) (Table S1). Further, pVM15 was treated with *Eco*RI/*Xba*I, and the fragment P_{ermE*}/*srT*/*appA* was ligated into the pAB04, which was previously linearised with *Eco*RI/*Xba*I, to yield pAB04-4. The fragment *srT*/*appAsyn* was PCR amplified from pAB04-4 using the primers Fw-srT-appA-*Nde*I and Rv-appA-*Xba*I (Table S2), which incorporated an *Nde*I restriction site at the 5′ position, *Xba*I at 3′ position and His-tag nucleotide motif at the C terminus of phytase. The *Nde*I–*Xba*I fragment that contained *srT*/*appAsyn* was ligated into the pEX-PnitA and pEX-tcp830 vectors, and also digested with *Nde*I/*Xba*I, which produced the plasmids pEX-*PnitA*-2 and pEX-*tcp830*-2. Further, pEX-PnitA-2 was treated with *Hin*dIII and the *PnitA*/*srt*/*appAsyn*/*nitR* cassette was ligated in the pVF vector, and also digested

Table 1 Selected signal sequences used in this study, with their secretion efficiencies given as percentages of secreted catechol 2,3-dioxygenase (the C-region splicing site is indicated)

Signal sequence	Number of positively charged AAs in N-region	C-region of signal sequence	Percentage of secreted catechol 2,3-dioxygenase (%)
vsi (subtilisin inhibitor–*S. venezuelae*)	3	GTAQA↓EA	52.3
aml (α-amylase–*S. venezuelae*)	2	GNAPAQA↓VP	45.6
amy (α-amylase–*S. griseus*)	2	PTPAAA↓AP	26.4
sprB (protease B–*S. griseus*)	6	AVPTANA↓ET	26.6
*aml** (mutated α-amylase–*S. venezuelae*)	3	GNAPAQA↓VP	51.7
xysA (xylanase A–*S. halstedii*)	6	AGTAQA↓AG	83.7
srT (trypsin-like protease–*S. rimosus*)	4	QPAAATAAA↓PT	30.4
lip (lipase–*S. rimosus*)	3	SA↓AVSAPRIQ	73.3

with *Hin*dIII and dephosphorylated. For pVF-5, an identical ligation approach was followed using the *Eco*RI restriction enzyme instead of *Hin*dIII in the final cloning step. After sequence confirmation, the plasmids were transformed into *S. rimosus* by electroporation.

Heterologous expression of catechol 2,3-dioxygenase (*xylE*) and phytase (*appA*) genes in *S. rimosus*

The engineered *S. rimosus* strains were cultivated in 5 mL TSB medium (Kieser et al. 2000) and modified NMMP medium (mNMMP) that had the following composition: 2 g $(NH_4)_2SO_4$, 20 g soya peptone, 0.6 g $MgSO_4·7H_2O$, 1 mL minor elements solution (1 g $ZnSO_4·7H_2O$, 1 g $FeSO_4·7H_2O$, 1 g $MnCl_2·4H_2O$, 1 g $CaCl_2$ anhydrous) made up to 800 mL with distilled water, divided into 80 mL aliquots and autoclaved. At the time of use, 15 mL NaH_2PO_4/K_2HPO_4 buffer (0.1 M, pH 6.8) and 2.5 g/L glucose were added. Complex medium (Hranueli et al. 1979) was used to produce AppA.

For expression of the *xylE* gene, 1% (*v*/*v*) *S. rimosus* spore suspension was inoculated into TSB or mNMMP medium, and after 20 to 24 h, 10% (*v*/*v*) inoculum was transferred to fresh TSB or mNMMP medium, and samples were taken for further analysis between 9 and 36 h of cultivation. For production of AppA in the complex medium, 1% (*v*/*v*) spore suspension was inoculated into the seed medium (Hranueli et al. 1979), and after 20 to 24 h, 10% (*v*/*v*) inoculum was transferred into the complex medium, and samples were taken every 24 h during the 7 days of cultivation. The liquid culture media were supplemented with 5 μg/mL thiostrepton when required, and the cultures were cultivated at 30 °C and 220 rpm. The inducers of 0.1% (*w*/*v*) ε-caprolactam or 1 μg/mL tetracycline were added after 4 or 60 h of cultivation in TSB and complex medium, respectively. The cells were harvested at 4 °C and 14,000 rpm for 5 min. Finally, the pellet and supernatant were stored at − 20 °C for further enzymatic analysis.

Enzymatic assays for evaluation of catechol 2,3-dioxygenase and phytase activity produced by *S. rimosus* strains

Catechol 2,3-dioxygenase (XylE) activity was evaluated using the method described by Kieser and collaborators (Kieser et al. 2000). Protein concentrations were determined by the Bradford method (Bradford 1976). Phytase (AppA) activity was determined using the ammonium molybdate method (Lee et al. 2005). The intracellular XylE and AppA activities were analysed by disrupting the cells by sonication, while the extracellular activities were measured directly in the supernatant.

Purification of secreted phytase AppA from *S. rimosus* culture supernatant using His-Tag/Ni-affinity chromatography

Phytase (AppA) production was carried out in 200 mL at a culture shaker scale. The production process started by inoculation of 1% (*v*/*v*) spore suspension of recombinant *S. rimosus* containing the pVF-5 plasmid in seed medium supplemented with thiostrepton. The culture was cultivated at 30 °C and 220 rpm for 20 h. Ten percent (*v*/*v*) of the overnight culture was transferred to complex medium supplemented with 5 μg/mL thiostrepton and cultivated for 4 days. Once the biosynthetic process was completed, the culture was centrifuged at 10,000 rpm and the pellet was discarded. The supernatant was filtered through a 0.22-μm filter paper to remove cell debris and medium components, which can block HisTrap column (5 mL GE HisTrap HP; GE Healthcare). The clear supernatant (around 150 mL) was mixed with 200 mL equilibration buffer (20 mM imidazole, pH 8, 300 mM NaCl, 50 mM Tris, pH 7) and loaded into a 5 mL HisTrap column that was previously equilibrated with five column volumes of washing buffer (25 mM imidazole, pH 8, 500 mM NaCl, 50 mM Tris, pH 7). The column was then washed with 25 column volumes of washing buffer, and the recombinant protein was eluted isocratically with elution buffer (300 mM

NaCl, 250 mM imidazole, pH 8, 50 mM Tris, pH 7). The fractions containing the recombinant phytase were run on SDS-PAGE to confirm the presence of the band at around 45 kDa that represented phytase. The AppA identity was verified by in-gel digestion and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis (Fig. S3).

Statistical analysis

Data was analysed using one-way ANOVA. Tukey test was used to compare mean differences.

Results

Construction of an *E. coli–S. rimosus* replicative shuttle vector

Replicative plasmids are an attractive option considering that they can ensure increased gene copy numbers, which can potentially lead to higher yields of target heterologous proteins. In addition, the transformation efficacy of replicative plasmids is much higher, compared to the integrative type of vectors. *S. rimosus* was initially characterised for its repellency to carry multi-copy vectors (Kieser et al. 1982). However, it was later demonstrated that the pIJ101-derived plasmids pPZ12 (Chambers and Hunter 1984) and pPZ74 (Hunter 1985) can be maintained stably in *S. rimosus*. We therefore constructed a bi-functional multi-copy shuttle vector, pVF, by fusing *E. coli* pUC19 and the pIJ101-based vector pPZ12 (Fig. S1). The pVF plasmid was constructed in *E. coli* and transformed into *S. rimosus*. In the next step, plasmid DNA was isolated from a number of independent *S. rimosus* transformants. One *S. rimosus* transformant was isolated that contained a plasmid with the predicted restriction pattern, therefore establishing the structural stability of the new chimeric vector (data not shown). Importantly, *S. rimosus* transformants that contained the pVF vector did not show any morphological or physiological changes, compared to the parent strain *S. rimosus* M4018. The DNA sequence confirmed that the pUC19 vector sequence remained unaltered. As expected, the pPZ12 backbone contained elements from the pIJ101-based vector (Fig. S1A). Importantly, we have demonstrated the stability of the pVF plasmid in *E. coli* and *S. rimosus*. However, segregation of the pVF vector, which is typical for the pIJ101 type of *Streptomyces* vectors (Kendall and Cohen 1988), was evaluated to establish the rate at which the transformants that contained pVF lose the plasmid in the absence of antibiotic, as described in the Supplementary Information (Fig. S1B). The present data demonstrate that unless thiostrepton is added to the TSB liquid medium, around 30% of the cells can lose the pVF plasmid at every step of sub-cultivation.

Evaluation of selected promoters using the reporter gene *xylE*

Based on published data that demonstrated their functionality in different *Streptomyces* species, a number of promoters were selected for evaluation in *S. rimosus*. We were interested in strong promoters, such as P_{ermE*} (Bibb et al. 1985), and particularly the inducible promoter *PnitA-NitR* (Herai et al. 2004), and the tetracycline inducible promoter *tcp830* (Rodriguez-Garcia et al. 2005). By using the chalcone synthase *rppA* gene as a reporter system, the P_{ermE*}, P_{ermE} and *actII*-ORF4/P*actI* promoters were initially evaluated (Magdevska et al. 2010). The P_{ermE*} promoter showed the highest activity. P_{ermE} showed one-third the activity as that of P_{ermE*}. Interestingly, the *actII*-ORF4/P*actI* promoter/activator system was not active in *S. rimosus*. We also tested the *PxysA* promoter from *S. halstedii* (Ruiz-Arribas et al. 1997) and the *PpstS* promoter from *S. coelicolor* (Sola-Landa et al. 2005); however, these two promoters showed very low activities in *S. rimosus* (data not shown).

Analogous to the *E. coli* expression systems, we particularly aimed at evaluating the inducible promoters *PnitA-NitR* and *tcp830*, and to compare these to the strong constitutive promoter P_{ermE*}. Each of these three promoters was thoroughly evaluated by incorporation of the *xylE* gene into the replicative *E. coli–Streptomyces* shuttle plasmid pVF (Fig. S1A, Table S1) and by the ΦC31-based integrative plasmid pAB04 (Table S1). When the integrative vector pAB04 was used, intracellular XylE activity was measured after 24 h of cultivation in TSB medium. There was relatively low XylE activity when the pAB04-based constructs were integrated into the *S. rimosus* chromosome, with the P_{ermE*} promoter showing the highest XylE activity, of around 0.4 U/mg (Fig. 1a). Addition of the inducers ε-caprolactam and tetracycline to the culture of *PnitA-NitR*- and *tcp830*-containing transformants in *S. rimosus*, respectively, did not induce *xylE* expression, which thus appeared to be non-inducible promoters when the plasmids were integrated into ΦC31 site-specific loci in *S. rimosus*.

Interestingly, when the selected promoters were incorporated into the replicative vector pVF, a significant increase in XylE activity was observed (Fig. 1b). Surprisingly, *S. rimosus* transformants that contained the pVF-based replicative vector pVF-P_{ermE*}/*xylE* showed an abnormal morphology. Compared to the parent strain M4018, these transformants formed soft and bold colonies. Out of 30 independent pVF-P_{ermE*}/*xylE* *S. rimosus* transformants tested, only one transformant was positive for XylE activity, and it reached a specific activity of around 7 U/mg (Fig. 1b). The plasmid DNA from each pVF-P_{ermE*}/*xylE* transformant was isolated to verify the restriction pattern. Not surprisingly, we identified diverse restriction patterns of the isolated plasmids, which thus indicated that there had been in vivo

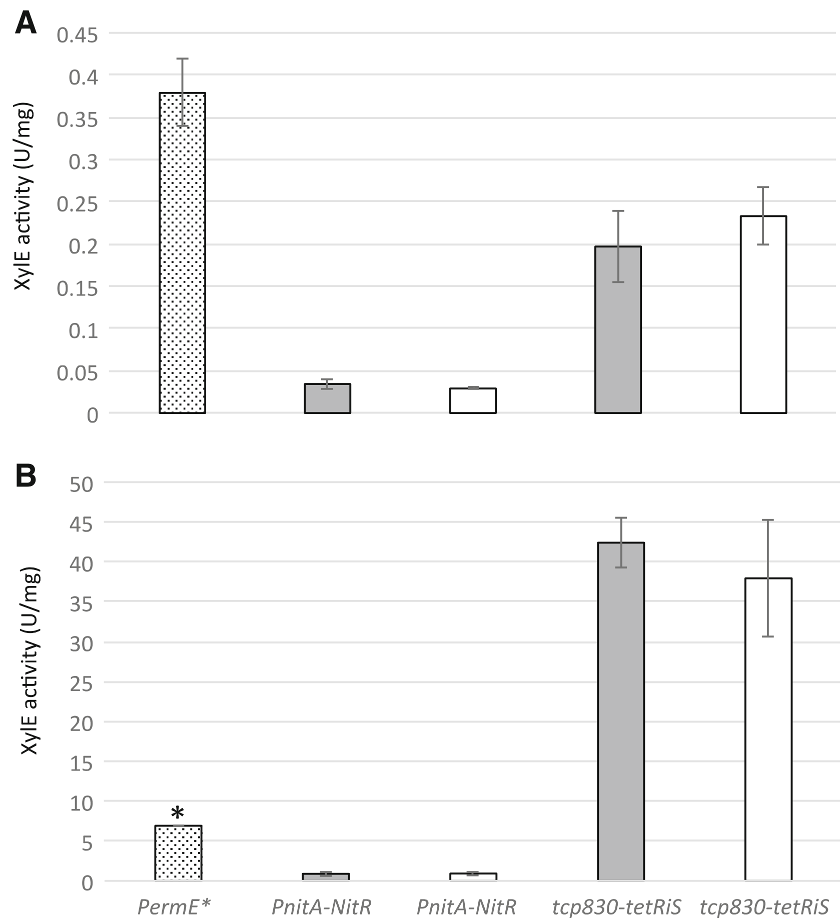

Fig. 1 Intracellular specific XylE activity of *S. rimosus* transformants evaluated after 24 h of cultivation in TSB medium: **a** pAB04 integrative plasmid containing ΦC31 recombinase expression of *xylE*, and **b** pVF replicative *E. coli*–*Streptomyces* shuttle vector containing *xylE*. Grey columns represent non-induced, and white induced, cultures with ε-caprolactam and tetracycline for the *PnitA-NitR* and *tcp830* promoters, respectively. Each construct was analysed with three independent transformants and two replicates, and data are presented as means ± standard error

rearrangements of the pVF vector. However, the single transformant that expressed XylE activity showed the expected restriction pattern.

In contrast, the *S. rimosus* transformants that contained the pVF replicative vector with *xylE* being expressed under the control of the *PnitA-NitR* or *tcp830* promoters showed normal colony morphology. The plasmid isolated from these *S. rimosus* transformants revealed the correct restriction pattern, thus demonstrating the stability of the pVF-based constructs with the *PnitA-NitR* or *tcp830* promoters. When the pVF-based multi-copy vectors containing *xylE* with the *PnitA-NitR* and *tcp830* promoters were tested, the XylE activity was greatly increased. The maximum XylE specific activity with pVF-based vectors reached around 1 U/mg, and 42 U/mg for *PnitA-NitR* and *tcp830*, respectively, compared to 0.034 U/mg and 0.23 U/mg when the same promoters were chromosomally integrated, using ΦC31 recombinase (Fig. 1b). However, the *PnitA-NitR* and *tcp830* promoters unfortunately showed a constitutive nature in the *S. rimosus* background, considering that the addition of ε-caprolactam or tetracycline, respectively, had little impact, if any, on the overall yield of XylE. Considering that only one *S. rimosus* pVF-P_{ermE*}/*xylE* transformant showed XylE activity, statistical analysis could not be performed for this engineered strain of *S. rimosus* (Fig. 1b).

Evaluation of selected signal sequences that target the Sec pathway in *S. rimosus*

In the scope of this study, we aimed to evaluate the potential use of the Sec pathway to achieve the secretion of target heterologous proteins by *S. rimosus*. The *xylE* that encodes catechol 2,3-dioxygenase was shown to be a reliable reporter gene in *S. rimosus*. The pVM vector (Table S1) is a pSAM-based integrative vector that was initially used to evaluate selected signal sequences by fusing the *xylE* gene to the selected *Streptomyces* signal sequences identified in the literature, as described below (Table 1). As demonstrated by Pandza and collaborators (Pandza et al. 1997), pSAM2-based vectors integrate into the *S. rimosus* chromosome at a single location. Therefore, although pSAM-based vectors integrate in the chromosome of *S. rimosus* with lower efficiency compared to ΦC31 recombinase-based vectors, pVM was selected for this initial experiment. Vectors constructed in the scope of the signal sequence evaluation contained the P_{ermE*} promoter, as the highest and most reproducible XylE activity with the pVM integrative vector was achieved under the

regulation of this promoter (Magdevska et al. 2010). Surprisingly, relatively high extracellular activity of XylE was detected in supernatants of *S. rimosus* cultivated in TSB medium, even though the XylE gene reporter did not contain a signal sequence when the testing of the target signal sequences was initiated (data not shown). Considering that XylE without a signal sequence is principally an intracellular protein, this extracellular XylE activity in the *S. rimosus* supernatant was very likely the result of cell lysis during the cultivation. Therefore, to attenuate the leaking of XylE into the culture supernatant, it was necessary to change the cultivation medium. For this purpose, experiments were carried out in the minimal medium NMMP. To ensure growth rate and XylE activity comparable to those observed in TSB medium, the NMMP medium was slightly re-optimised by replacing casamino acids with soya peptone, thus achieving an increase in XylE activity of 1.5-fold. The modified NMMP medium (now designated as mNMMP) that contained 2.5 g/L glucose and 20 g/L soya peptone reduced extracellular XylE activity (i.e. leakage) significantly, while still maintaining similar intracellular XylE activity. Therefore, this NMMP medium re-optimisation improved the testing procedure for the evaluation of the signal sequence efficacy.

With the aim to facilitate the secretion efficacy, the *xylE* and phytase *appA* genes were fused to selected signal sequences (Table 1). XylE is an intracellular enzyme and does not contain a signal peptide. Therefore, only the start codon was removed, and the protein was fused to the short DNA fragments that encoded the selected signal peptides. The entire *E. coli* phytase signal peptide was removed and replaced with selected *Streptomyces* signal peptides. As demonstrated in the literature, export of the target proteins is dependent on the availability of the signal peptidase-cleavage site *Ala-X-Ala*, which is located in the C-region of the enzyme (van Roosmalen et al. 2004). Therefore, in the present study, this motif was maintained unchanged. For this purpose, a *Pst*I restriction enzyme cleavage site was introduced that contained GCA (Ala) in its recognition sequence (Fig. S2). Thus, the *Pst*I restriction site was chosen for the fusion of the signal peptide and the target protein. Eight selected *Streptomyces* signal sequences that belong to the Sec pathway (Table 1) were fused in-frame to the *xylE* reporter gene that was regulated under the P_{ermE*} promoter and then introduced into the *S. rimosus* chromosome using the pVM vector that contained a pSAM2 integrase. Intracellular and extracellular XylE activity was assessed in the cultures of *S. rimosus* transformants after 20 h of incubation in mNMMP.

The signal sequences of xylanase (*xysA*) and lipase (*lip*) from *S. halstedii* and *S. rimosus* showed the highest secretion, with export of 83.7 and 73.3% of the recombinant XylE, respectively (Fig. 2). Approximately half of the XylE activity was detected in the supernatant when using signal sequences from *S. venezuelae*: subtilisin inhibitor *vsi*, α-amylase or a mutated version of the α-amylase signal sequence *aml**. Finally, very low secretion (below 30%) of XylE was observed in the supernatants when the α-amylase *amy* and the protease *sprB* signal sequences from *S. griseus* were used (Fig. 2). However, when considering the highest total activity of XylE in combination with the secretion efficiency, it was the lipase *lip* (pVM14) and the trypsin-like proteinase *srT* (pVM13) signal peptides that performed best (Fig. 2).

Extracellular production of phytase AppA using a *S. rimosus* host

Phytase AppA has been heterologously produced in different hosts, including *S. lividans* and different yeast strains (Lee et al. 2005; Stahl et al. 2003). Phytase (AppA) is an industrially important enzyme that is produced by *E. coli* (Dassa et al. 1990). It is an enzyme with a relatively complex structure, which contains four disulfide bonds; thus, AppA is a very suitable model system to evaluate the production of recombinant proteins in *S. rimosus*. AppA contains a native signal peptide that drives AppA to the periplasm. A synthetic version of the *appA* gene was constructed (designated as *appAsyn*) without its native signal sequence (Fig. S4). Instead, the signal sequences of trypsin-like protease (*srT*) and lipase (*lip*) from *S. rimosus* and α-amylase (*aml*) from *S. venezuelae*, which gave the best results when tested with *xylE*, were fused in-frame to the *appAsyn* gene under the regulation of the constitutive promoter P_{ermE*}. These plasmid constructs, designated as pVM4, pVM15 and pVM16 (Table S1), were integrated into the *S. rimosus* chromosome using a pSAM2 recombinase-based integrative plasmid. Surprisingly, no phytase activity was detected in the TSB or mNMMP media, which is difficult to explain, considering that identical plasmid constructs resulted in relatively high extracellular XylE activity.

In contrast, both extracellular and intracellular AppA activity was detected when the same *S. rimosus* transformants were cultivated in complex medium. Most importantly, all three selected signal peptides evaluated (i.e. *srT*, *lip*, *aml*) facilitated the secretion of AppA. However, different enzyme activities of extracellular AppA were observed (Fig. 3). In agreement with earlier data with the XylE reporter system (Fig. 2), the signal sequences from *S. rimosus*, *srT* and *lip* showed the best secretion, reaching 3.99 and 3.59 U/mL extracellular AppA activity, respectively. Although the α-amylase (*aml*) signal sequence from *S. venezuelae* facilitated the efficient secretion of the phytase, as it reached an activity of 0.77 U/mL, its total AppA activity was much lower compared to *srT* and *lip* (Fig. 3). When mediated by the *aml* and *srT* signal sequences, AppA was produced from the first day of the biosynthetic process, and achieved its highest activity between the third and the fifth days of cultivation. However, when using the

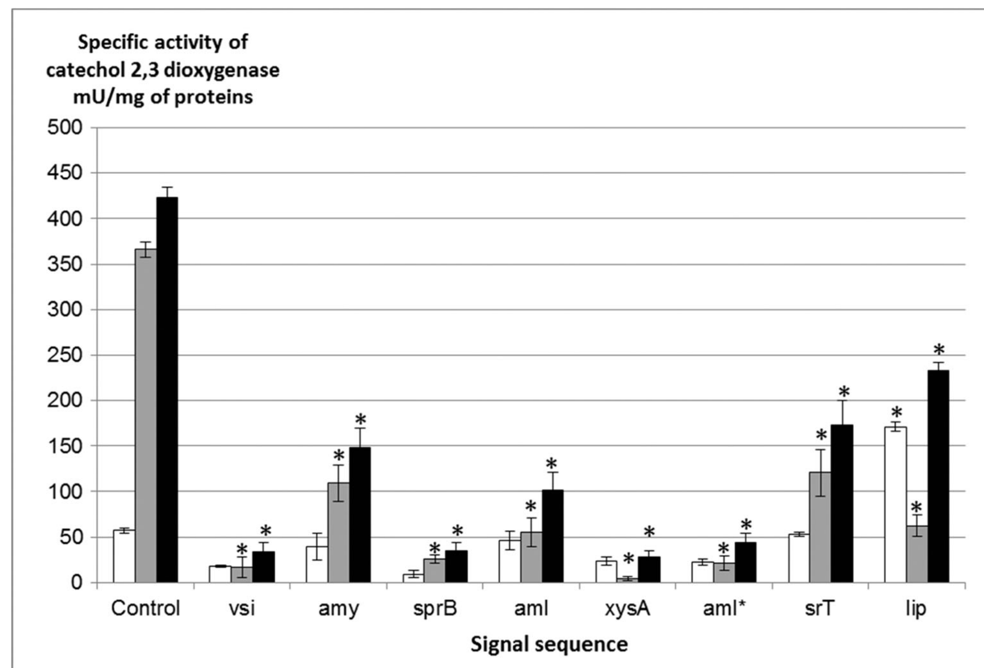

Fig. 2 Specific activities of XylE of different *S. rimosus* transformants that contained an integrative pVM-series vector with different signal sequences. The control corresponds to *S. rimosus* pVMX3 transformants (without signal sequence). White bar: extracellular XylE activity; grey bar: intracellular XylE activity; black bar: total XylE activity. The asterisks indicate that the specific activity was different from that of the control with significance of $P < 0.05$

lip signal sequence, the highest AppA activity was observed after 8 days of cultivation (Fig. 3).

The pSAM2 recombinase-based integrative plasmid pTS55 (Smokvina et al. 1990) that contained the *appA* gene with selected signal sequences resulted in significant extracellular AppA activity (Fig. 3). However, during the initial screening of transformants that contained the *appA* gene, the proportion of AppA-activity-positive colonies never reached over 40% (Table S3). Therefore, additional experiments were carried out that used the integrative vector pAB04 that contained the ΦC31-recombinase and the replicative *E. coli–Streptomyces* shuttle vector pVF (Table S1). Interestingly, practically all of the *S. rimosus* transformants that contained pAB04 derivative vectors showed AppA activity when the P_{ermE*} promoter was evaluated. Independent of the selected promoter, the use of the replicative vector pVF clearly increased AppA activity, as demonstrated in Fig. 4. Therefore, all of the subsequent experiments here were carried out with the pAB04 and pVF vectors.

Construction of the expression plasmids optimal for the production of the phytase AppA in *S. rimosus*

Towards the final stages of this study, the most productive components of the expression system in *S. rimosus* were combined, and they were incorporated into the integrative pAB04 and replicative pVF vectors, to reach the best production of AppA. The phytase *appAsyn* gene was fused in-frame with a trypsin-like proteinase signal sequence (*srT*) that showed the most robust secretion efficiency (Fig. 3b). The *srT-appAsyn* gene was regulated under the P_{ermE*}, *PnitA-NitR* and *tcp830* promoters, which had shown the highest expression profiles in previous studies. Based on the data with the XylE reporter system (Fig. 1), and the signal sequence evaluation (Fig. 3), the P_{ermE*}/*srT-appA* cassette was transferred into pAB04, while the *PnitA-NitR* and *tcp830* promoters that regulated *srT-appA* transcription were ligated into the pVF replicative vector. All of the transformants showed normal morphology, comparable to the parent strain, which confirmed the genetic stability of the pVF vector that carried the *PnitA-NitR* and *tcp830* promoters.

To evaluate the phytase activity, *S. rimosus* transformants were cultivated in complex medium, and samples were collected every 24 h for 7 days. The transformants that contained the pAB04-P_{ermE*}/*srT-appA* integrative vector produced up to 3 U/mL extracellular AppA activity (Fig. 4). Interestingly, the transformants that contained the replicative vector pVF-*PnitA-NitR/srT-appA* produced relatively low extracellular AppA activity, at < 1 U/mL. On the other hand, the replicative vector pVF-*tcp830* showed much higher extracellular AppA activity, to reach up to 5 U/mL. In agreement with earlier studies, the addition of the inducers ε-caprolactam and tetracycline did not significantly influence the final activities of AppA in the supernatants, which thus confirmed the constitutive nature of these promoters in *S. rimosus*. Importantly, the intracellular AppA activity never exceeded 15% of the total phytase activity, which showed the good export capacity of the *S. rimosus* expression system (Fig. 4). A time-course demonstrated that the best time to harvest the recombinant protein was during the fourth day of cultivation. After this time, the AppA

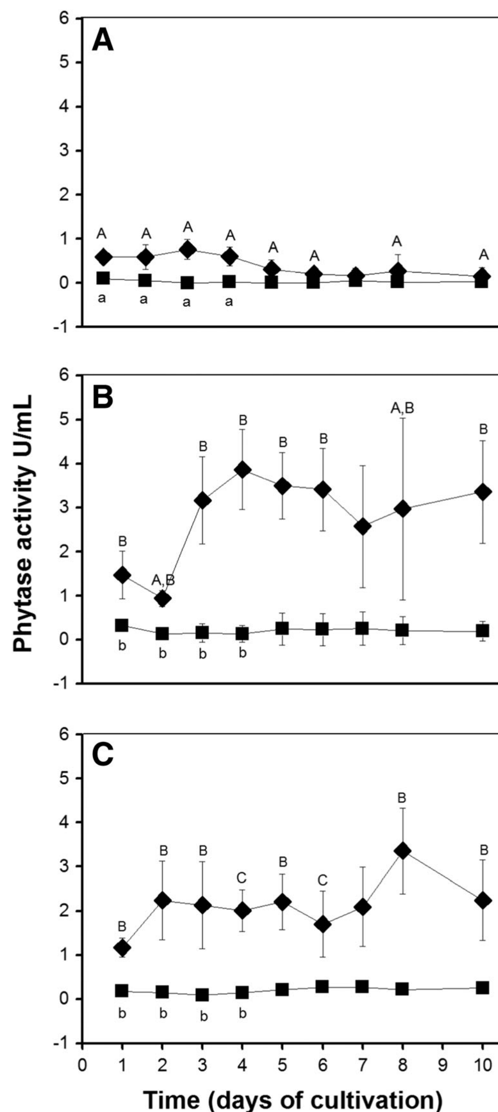

Fig. 3 Production of phytase by *S. rimosus* transformants cultivated in complex medium. Intracellular and secreted AppA activity produced with **a** α-amylase (*aml*), **b** trypsin-like protease (*srT*) and **c** lipase (*lip*) signal sequences. Rectangles, extracellular phytase activity; squares, intracellular phytase activity. Capital letters (A, B, C) indicate significant difference ($P < 0.05$) in the secreted AppA activity between transformants. Small letters (a, b) indicate significant difference ($P < 0.05$) in the intracellular AppA activity between transformants

activity started to decrease (Fig. 4). As demonstrated above, when using the replicative pVF vector in *S. rimosus*, and without the addition of antibiotic, there was significant segregation of this replicative vector. These data demonstrated that over every 24 h around 30% of the cells lost the plasmid (Fig. S1). To reconfirm whether the pVF vector shows a similar segregation phenotype in the complex medium, the AppA production in this medium was analysed without the addition of antibiotic. These data demonstrated that addition of thiostrepton to the complex medium had a profound positive impact on the final yield of AppA. For instance, when medium was supplemented with thiostrepton, after 4 days of cultivation the *S. rimosus* transformants that contained the replicative vector pVF-*tcp830* showed 3-fold higher activity of AppA, to reach an activity of up to 6 U/mL (Fig. 4). On the other hand, the use of the selective marker (thiostrepton) had no effect on the AppA activity when *appA* was integrated into the *S. rimosus* chromosome by ΦC31 recombinase. Thus, independent of the presence of thiostrepton in the complex medium, the AppA activity at the end of the fermentation process reached up to 3 U/mL. Nevertheless, the addition of thiostrepton delayed AppA production, which reached maximum yields after 4 days. In contrast, in the absence of thiostrepton, a similar AppA yield was already reached after 48 h of cultivation (Fig. 4).

Purification and identification of extracellular AppA

In the final stages, we intended to evaluate the applicability of the *S. rimosus* expression platform developed in this study. Analogous to the *E. coli* expression systems where the His-Tag/Ni-affinity chromatography is routinely used for the isolation of target proteins, the isolation of AppA from the culture of recombinant *S. rimosus* was attempted. The *appAsyn* gene was designed to contain a C-terminal polyhistidine-tag, to determine whether production of recombinant AppA was high enough to facilitate a simple procedure for the isolation of extracellular AppA from the complex medium supernatant. The highest-producing *S. rimosus* colony that contained the replicative vector pVF-*tcp830*/*srT*/*appAhis* was cultivated in 200 mL of complex medium. After 4 days of cultivation, the *S. rimosus* biomass was separated by centrifugation, and the supernatant was cleared. The isolation procedure that followed was as described in the 'Materials and methods' section. SDS-PAGE electrophoresis of the eluted protein fractions showed a band of 45 kDa, which was assumed to belong to AppA (Fig. S5A). Further LC-MS/MS analysis of the isolated band from a Coomassie-stained gel confirmed the identity of the purified protein band as phytase AppA (Fig. S3).

Discussion

In addition to the pSAM2 and ΦC31 phage-based integrating vectors used in this study, a replicative *E. coli*–*Streptomyces* shuttle plasmid, pVF, was also constructed. The only disadvantage of this replicative vector was plasmid segregation, which was however typical for pIJ101-based vectors (Kendall and Cohen 1988). At the laboratory scale, this is not really an issue. However, for industrial purposes, this feature represents a drawback. Importantly, loss of AppA activity

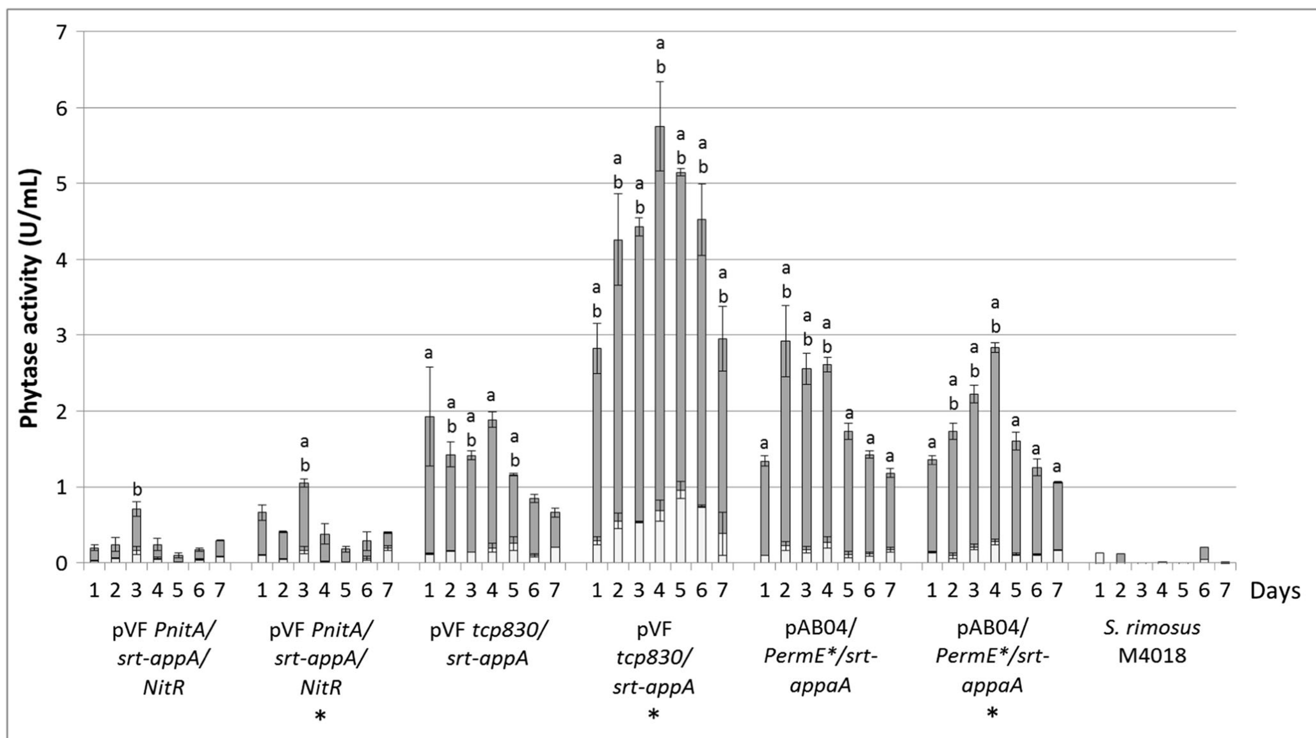

Fig. 4 Phytase activity of *S. rimosus* M4018 transformants containing different plasmid constructs in complex medium. White bars indicate intracellular AppA activity and grey bars represent extracellular AppA activity. Samples were taken for 7 days every 24 h. At least two independent transformants of each construct were analysed in two replicates. Results are presented as the means ± standard error. The recombinant strains cultivated in complex medium supplemented with thiostrepton are marked by asterisk (*). The letters indicate significant difference ($P < 0.05$) in the extracellular (**a**) and intracellular (**b**) AppA activity between the *S. rimosus* M4018 control and *S. rimosus* M4018 transformants

was not observed when integrative vectors were used, which demonstrated the high stability of the *appA* gene that was integrated into the chromosome, which is of industrial importance.

Surprisingly, more than half of the transformants that contained the *appA* gene on the pSAM2-based integrative vector were not active (Table S3), which suggested that the location of the integration site specific for the pSAM2 recombinase was particularly unproductive in *S. rimosus*. Thus, in agreement with literature data, our data confirm that the location of the integration into the chromosome of the host strain can significantly influence the yield of the target product (Bilyk et al. 2017; Mazodier et al. 1990). In contrast, when the vector that contained the ΦC31 *attP* site and the corresponding *int* gene were used, practically all of the transformants showed AppA activity. Therefore, ΦC31-based integrative vectors are clearly the choice when working with a *S. rimosus* host. With the ability to integrate exogenous DNA into the chromosome of *S. rimosus*, we attempted to evaluate the strength and, more importantly, the inducibility of the *PnitA-NitR* and *tcp830* promoters. When integrated into the *S. rimosus* chromosome, the expression of the reporter gene *xylE* demonstrated relatively low activity of the *PnitA-NitR* and *tcp830* promoters, compared to the strong promoter P_{ermE*}. Interestingly, when the replicative plasmid pVF was used, *xylE* expression under the strong constitutive promoter P_{ermE*} induced in vivo deletions of the pVF vector, which was accompanied by morphological instability of these *S. rimosus* transformants; this appears to have occurred due to the extensive burden on the host cell. The genetic instability observed in the transformants that contained pVF-P_{ermE*} was thus avoided by replacing P_{ermE*} with the weaker promoters *PnitA-NitR* and *tcp830*. However, although the pVF replicative constructs that contained *PnitA-NitR* and *tcp830* showed excellent genetic and morphological stability in *S. rimosus*, both of these promoters expressed *xylE* constitutively. In *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, for example, the *PnitA-NitR* promoter was activated with isovaleronitrile instead of ε-caprolactam (Pandey et al. 2009). However, in *S. rimosus*, this analogue did not activate the *PnitA-NitR* promoter (data not shown). Therefore, it appears that the *PnitA-NitR* promoter was never strongly activated in *S. rimosus*.

Similarly, the tetracycline-inducible promoter *tcp830* did not work as an inducible promoter in *S. rimosus*, even though the genome of this strain encodes more than 100 *tetR* homologues. We tested the *tcp830* promoter together with its transcriptional regulator *teTriS* that is regulated by the constitutive promoter *SF14*, which is active in *S. rimosus* (Yin et al. 2015). However, induction of the *tcp830* promoter was never detected. Rodriguez-Garcia et al. demonstrated that for some *Streptomyces* species, the *tcp830* promoter does not have an

'off-state' (Rodriguez-Garcia et al. 2005). Therefore, it can be concluded that *S. rimosus* also belongs to this group. Although they are weaker promoters, *PnitA-NitR* and *tcp830* showed relatively high XylE activity when used with the replicative vector pVF.

Herai and colleagues reported that when the *xylE* gene was under the control of the *PnitA-NitR* promoter in *S. lividans*, and when it was cultivated for 120 h in YEME medium, this reached an intracellular XylE activity of 45 U/mg, when located on the replicative plasmid pSH90 (Herai et al. 2004). In contrast, when expressing the *xylE* gene on the replicative plasmid pVF under the control of *tcp830* in *S. rimosus*, a similar intracellular XylE activity of 42 U/mg was achieved already after 24 h of incubation, thus demonstrating the utility of *S. rimosus* as a heterologous host. These data also indicated that in addition to the strength of a promoter used for the heterologous expression of a target gene, the copy number and timing are of key importance. Therefore, the availability of inducible promoters in *S. rimosus* and the ability to induce the expression of a target gene at the most optimal time would appear to have a positive effect on the final yield of the target protein in *S. rimosus* cultures.

Interestingly, the Tat secretion pathway has been designated as the mayor secretion pathway in *Streptomyces* species such as *Streptomyces coelicolor* (Widdick et al. 2006). However, considering that all of the necessary components of the Sec pathway in *S. rimosus* have been identified (data not shown), we initially decided to evaluate the signal sequences derived from the diverse extracellular proteins associated to the Sec pathway in the literature. The activity of the secreted catechol 2,3-dioxygenase XylE varied significantly, which depended on the signal peptides that were fused to XylE. Interestingly, although a large proportion of the secreted enzyme XylE (83.7%) was observed when using the signal sequence from the xylanase *xysA* of *S. halstedii*, total XylE activity was very low (Fig. 2). There is no advantage of using a signal sequence that leads to high secretion efficacy of the heterologous protein unless the total activity achieved is sufficient. The highest total XylE activity, together with a relatively high secretion efficacy, was observed with a plasmid construct that contained the lipase signal sequence *lip* from *S. rimosus*. A similar secretion efficacy was observed when the *srT* signal sequence of a trypsin-like protease from *S. rimosus* was fused to the *xylE* gene (Table 1). Considering the overall XylE secretion efficacy, both signal sequences that originated from *Streptomyces griseus* showed the worst performance in the *S. rimosus* host, while the native signal sequences that originated from *S. rimosus* were the best. Therefore, based on the present study, we can conclude that the source of the signal sequence appears to have a critical role in the secretion efficacy and the overall yield (i.e. activity) of the target protein. It is reasonable to expect that the choice of signal sequence and the way in which the signal sequences were fused to the *xylE* reporter gene might have an important role in the export efficacy and total yield of the target protein. Therefore, further screening of native signal sequences, their optimisation (Lammertyn and Anne 1998), and optimisation of the signal sequence fusion point should result in significant improvement in the secretion efficacy and the overall yield of the target protein.

It was important to evaluate the secretion of the selected signal peptides in commonly used media. For this purpose, the industrially important *E. coli* enzyme phytase was also selected, which is encoded by the gene *appA* (Dassa et al. 1990). For this study, we evaluated various media: TSB medium (Kieser et al. 2000), mNMMP medium (this study) and complex medium (Hranueli et al. 1979). TSB is common laboratory medium that supports the rapid growth of *S. rimosus*. On the other hand, the complex medium composition resembles the medium used for the production of oxytetracycline, which is not actually designed for protein expression. In contrast to the overnight growth of cultures prepared in TSB medium, the process can take over 7 days in complex medium.

Initially, AppA production was evaluated using the pSAM2-based integrative vector under the regulation of the P_{ermE*} promoter. Surprisingly, no production of AppA could be detected in the TSB and mNMMP media, although identical expression plasmids were used when the XylE activity was assessed. This result is indeed difficult to explain considering that relatively high and reproducible production of AppA was observed when the same *S. rimosus* transformants were cultivated in complex medium (Fig. 3). We can therefore conclude that the choice of cultivation medium does not only have a crucial impact on the productivity of the target protein but also on the overall strategy of the fermentation process.

In agreement with the data initially obtained in the mNMMP medium, the use of the signal sequences *srT* and *lip* facilitated the highest extracellular activity of AppA when cultivated in complex medium. Remarkably, practically all of the AppA was secreted into the supernatant when complex medium was used for the cultivation of the *S. rimosus* transformants. The highest activity of AppA was already observed at the fourth day of cultivation when the *srT* signal sequence was used. Interestingly, the highest activity of XylE was not achieved with *srT*, but *lip* signal sequence, as discussed above. There are many potential reasons why the highest activities of XylE and AppA enzymes are reached with different signal sequences. These two enzymes originate from different hosts and are structurally different. While AppA is enzyme from *E. coli*, which is exported into periplasm, XylE is originally an intracellular protein. This might influence directly transport of the protein by Sec secretion machinery in *S. rimosus*. On the other hand, the addition of *Streptomyces* signal sequence might also affect the mRNA processivity due to formation secondary structures which may also affect protein folding.

Interestingly, AppA activity was reduced and varied after the fourth day of cultivation. Considering that *S. rimosus* produces extracellular proteases when cultivated in complex medium (Renko et al. 1989), the variability observed in AppA activity might have been the result of the proteolytic degradation of AppA, this being one of the potential disadvantages of the *S. rimosus* host. In contrast, the highest activity of AppA detected in the supernatant of *S. rimosus* cultures was observed at the eighth day of cultivation when the *lip* signal sequence was used. Importantly, the performance comparison of the *lip* and *srT* signal sequences was in agreement with the initial testing carried out with the XylE reporter. Therefore, initial evaluation of the selected signal sequences using the catechol 2,3-dioxygenase *xylE* appeared to be a reliable testing procedure in *S. rimosus*.

Next, the most promising gene-tool components were combined, namely the promoter, the signal sequences and the vectors, to further enhance the production of AppA. Two approaches were evaluated in *S. rimosus*: (1) a ΦC31 recombinase-based integrative vector equipped with the P_{ermE*} constitutive promoter and (2) a replicative pVF-based *E. coli–Streptomyces* shuttle vector that contained the *tcp830* promoter. Independent of the vector/promoter combination, the signal sequence *srT* was used to facilitate the secretion of AppA.

The highest production of extracellular AppA reached up to 5 U/mL, and this was achieved when the replicative plasmid pVF that contained the target gene *appA* under the regulation of the *tcp830* promoter was fused to the signal sequence encoded by the trypsin-like proteinase (*srT*). However, when the replicative vector pVF was used in *S. rimosus* without the selective marker thiostrepton, AppA was significantly reduced. On the other hand, the addition of thiostrepton to the medium delayed AppA production. In contrast, there was no need to supplement the medium with any antibiotic when an integrative vector was used. We can therefore conclude that although the activity of AppA was around 40% lower when using an integrative vector, the ΦC31 recombinase-based integrative vector still performed well, thus confirming the industrial value of integrative vectors.

We were able to reproducibly achieve between 3 and 5 U/mL extracellular phytase activity in shaking flasks following 48- and 96-h cultivation times, which depended on what plasmid was used. Although it is not possible to compare directly phytase activity achieved in *S. rimosus* with literature data due to differences in applied enzymatic methods, AppA activity reported in a *S. lividans* host appears to be lower (0.95 U/mL) and significantly longer cultivation times were required (Stahl et al. 2003). The activity of AppA in *P. pastoris* during an 8-day bioprocess was around 50-fold higher, while a 2-day bioprocess with *P. pastoris* reached a yield that was around 20-fold higher (Stahl et al. 2003) than that in the *S. rimosus* expression system. In *S. cerevisiae* and *S. pombe*, on the other hand, the yield of AppA was comparable to that obtained with the *S. rimosus* expression system (Lee et al. 2005). Interestingly, yeast heterologous hosts (Lee et al. 2005) result in the production of glycosylated AppA with good activity but slightly different catalytic properties. Similar to *S. lividans* (Stahl et al. 2003), and as confirmed by LC-MS/MS analysis (Fig. S3), AppA isolated from *S. rimosus* supernatant was of the correct size. We have achieved rapid and efficient purification of the active phytase AppA from *S. rimosus* supernatant by already applying the standard Ni-affinity chromatography methods at the 200 mL flask scale, which is of great utility for laboratory-scale studies (Fig. S5).

The gene tools developed during this study of *S. rimosus* as a host system represent only some initial efforts towards the development of an industrially applicable heterologous protein production platform. In addition to the modest yield of the target protein, at this stage there are a number of shortcomings of the *S. rimosus* protein expression system, such as the lack of inducible promoters and the production of extracellular proteases, which could potentially degrade the target heterologous protein being secreted. The signal peptides used in this study are not likely to be optimal yet. In this study, only the Sec pathway was explored, while the literature from recent years has demonstrated the utility of the Tat pathway in *Streptomyces* species (Gullon et al. 2015; Miyazaki et al. 2013). Thus, there are a number of approaches that can be applied to further improve the *S. rimosus* expression system. We have demonstrated that *S. rimosus* represents a potential alternative protein expression system, particularly when other expression systems fail to produce the target protein in the correctly folded form.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest A.F.C.R. declares that he has no conflict of interest. V.M. declares that she has no conflict of interest. L.K. declares that he has

no conflict of interest. Š.F. is a shareholder of Acies Bio d.o.o.. R.M. declares that he has no conflict of interest, and H.P. declares that he has no conflict of interest.

References

Bibb MJ, Janssen GR, Ward JM (1985) Cloning and analysis of the promoter region of the erythromycin resistance gene (ermE) of *Streptomyces erythraeus*. Gene 38(1-3):215–226. https://doi.org/10.1016/0378-1119(85)90220-3

Bilyk B, Horbal L, Luzhetskyy A (2017) Chromosomal position effect influences the heterologous expression of genes and biosynthetic gene clusters in *Streptomyces albus* J1074. Microb Cell Factories 16(1):5. https://doi.org/10.1186/s12934-016-0619-z

Binnie C, Cossar JD, Stewart DI (1997) Heterologous biopharmaceutical protein expression in *Streptomyces*. Trends Biotechnol 15(8):315–320. https://doi.org/10.1016/S0167-7799(97)01062-7

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72(1-2):248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Chambers A, Hunter I (1984) Construction and use of a bifunctional streptomycete cosmid. Biochem Soc Trans 12(4):644–645. https://doi.org/10.1042/bst0120644

Dassa J, Marck C, Boquet PL (1990) The complete nucleotide sequence of the *Escherichia coli* gene *appA* reveals significant homology between pH 2.5 acid phosphatase and glucose-1-phosphatase. J Bacteriol 172:5497–5500

Diaz M, Adham SA, Ramon D, Gil JA, Santamaria RI (2004) *Streptomyces lividans* and *Brevibacterium lactofermentum* as heterologous hosts for the production of X22 xylanase from *Aspergillus nidulans*. Appl Microbiol Biotechnol 65(4):401–406. https://doi.org/10.1007/s00253-004-1633-3

Golovan S, Wang G, Zhang J, Forsberg CW (2000) Characterization and overproduction of the *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can J Microbiol 46(1):59–71. https://doi.org/10.1139/cjm-46-1-59

Gravius B, Bezmalinovic T, Hranueli D, Cullum J (1993) Genetic instability and strain degeneration in *Streptomyces rimosus*. Appl Environ Microbiol 59(7):2220–2228

Gullon S, Vicente RL, Valverde JR, Marin S, Mellado RP (2015) Exploring the feasibility of the sec route to secrete proteins using the tat route in *Streptomyces lividans*. Mol Biotechnol 57(10):931–938. https://doi.org/10.1007/s12033-015-9883-0

Henderson G, Krygsman P, Liu CJ, Davey CC, Malek LT (1987) Characterization and structure of genes for proteases A and B from *Streptomyces griseus*. J Bacteriol 169(8):3778–3784. https://doi.org/10.1128/jb.169.8.3778-3784.1987

Herai S, Hashimoto Y, Higashibata H, Maseda H, Ikeda H, Omura S, Kobayashi M (2004) Hyper-inducible expression system for streptomycetes. Proc Natl Acad Sci U S A 101(39):14031–14035. https://doi.org/10.1073/pnas.0406058101

Hranueli D, Pigac J, Vesligaj M (1979) Characterization and persistence of actinophage RP2 isolated from *Streptomyces rimosus* ATCC 10970. J Gen Microbiol 114:295–303. https://doi.org/10.1099/00221287-114-2-295

Hunter ISD (1985) Cosmid cloning vectors. Google Patents,

Ingram C, Brawner M, Youngman P, Westpheling J (1989) *xylE* functions as an efficient reporter gene in *Streptomyces* spp.: use for the study of galP1, a catabolite-controlled promoter. J Bacteriol 171(12):6617–6624. https://doi.org/10.1128/jb.171.12.6617-6624.1989

Kendall KJ, Cohen SN (1988) Complete nucleotide sequence of the *Streptomyces lividans* plasmid pIJ101 and correlation of the sequence with genetic properties. J Bacteriol 170(10):4634–4651. https://doi.org/10.1128/jb.170.10.4634-4651.1988

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. The John Innes Foundation Norwich, United Kingdom

Kieser T, Hopwood DA, Wright HM, Thompson CJ (1982) pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol Gen Genet 185(2):223–228. https://doi.org/10.1007/BF00330791

Lammertyn E, Anne J (1998) Modifications of *Streptomyces* signal peptides and their effects on protein production and secretion. FEMS Microbiol Lett 160(1):1–10. https://doi.org/10.1111/j.1574-6968.1998.tb12882.x

Lammertyn E, Desmyter S, Schacht S, Van Mellaert L, Anne J (1998) Influence of charge variation in the *Streptomyces venezuelae* Alpha-amylase signal peptide on heterologous protein production by *Streptomyces lividans*. Appl Microbiol Biotechnol 49:424–430

Lee S, Kim T, Stahl CH, Lei XG (2005) Expression of *Escherichia coli* AppA2 phytase in four yeast systems. Biotechnol Lett 27(5):327–334. https://doi.org/10.1007/s10529-005-0704-6

MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, MacNeil T (1992) Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111(1):61–68. https://doi.org/10.1016/0378-1119(92)90603-M

Magdevska V, Gaber R, Goranovic D, Kuscer E, Boakes S, Duran Alonso MB, Santamaria RI, Raspor P, Leadlay PF, Fujs S, Petkovic H (2010) Robust reporter system based on chalcone synthase *rppA* gene from *Saccharopolyspora erythraea*. J Microbiol Methods 83(2):111–119. https://doi.org/10.1016/j.mimet.2010.08.001

Mazodier P, Thompson C, Boccard F (1990) The chromosomal integration site of the *Streptomyces* element pSAM2 overlaps a putative tRNA gene conserved among actinomycetes. Mol Gen Genet 222:431–434

Miyazaki T, Noda S, Tanaka T, Kondo A (2013) Hyper secretion of *Thermobifida fusca* beta-glucosidase via a tat-dependent signal peptide using *Streptomyces lividans*. Microb Cell Factories 12(1):88. https://doi.org/10.1186/1475-2859-12-88

Pandey AK, Raman S, Proff R, Joshi S, Kang CM, Rubin EJ, Husson RN, Sassetti CM (2009) Nitrile-inducible gene expression in mycobacteria. Tuberculosis (Edinb) 89(1):12–16. https://doi.org/10.1016/j.tube.2008.07.007

Pandza K, Pfalzer G, Cullum J, Hranueli D (1997) Physical mapping shows that the unstable oxytetracycline gene cluster of *Streptomyces rimosus* lies close to one end of the linear chromosome. Microbiology 143(Pt 5):1493–1501. https://doi.org/10.1099/00221287-143-5-1493

Petkovic H, Cullum J, Hranueli D, Hunter IS, Peric-Concha N, Pigac J, Thamchaipenet A, Vujaklija D, Long PF (2006) Genetics of *Streptomyces rimosus*, the oxytetracycline producer. Microbiol Mol Biol Rev 70(3):704–728. https://doi.org/10.1128/mmbr.00004-06

Pigac J, Schrempf H (1995) A simple and rapid method of transformation of *Streptomyces rimosus* R6 and other streptomycetes by electroporation. Appl Environ Microbiol 61(1):352–356

Renko M, Vitale L, Kokalj M, Pokorny M (1989) *Streptomyces rimosus* extracellular proteases. Appl Microbiol Biotechnol 31:38–44 https://doi.org/10.1007/bf00252523, 1

Rhodes PM, Hunter IS, Friend EJ, Warren M (1984) Recombinant DNA methods for the oxytetracycline producer *Streptomyces rimosus*. Biochem Soc Trans 12(4):586–587. https://doi.org/10.1042/bst0120586

Rodriguez-Garcia A, Combes P, Perez-Redondo R, Smith MC, Smith MC (2005) Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria *Streptomyces*. Nucleic Acids Res 33(9):e87. https://doi.org/10.1093/nar/gni086

Ruiz-Arribas A, Sanchez P, Calvete JJ, Raida M, Fernandez-Abalos JM, Santamaria RI (1997) Analysis of xysA, a gene from *Streptomyces halstedii* JM8 that encodes a 45-kilodalton modular xylanase, Xys1. Appl Environ Microbiol 63(8):2983–2988

Sinsereekul N, Wangkam T, Thamchaipenet A, Srikhirin T, Eurwilaichitr L, Champreda V (2010) Recombinant expression of BTA hydrolase in *Streptomyces rimosus* and catalytic analysis on polyesters by surface plasmon resonance. Appl Microbiol Biotechnol 86(6): 1775–1784. https://doi.org/10.1007/s00253-010-2465-y

Smokvina T, Mazodier P, Boccard F, Thompson CJ, Guerineau M (1990) Construction of a series of pSAM2-based integrative vectors for use in actinomycetes. Gene 94(1):53–59. https://doi.org/10.1016/0378-1119(90)90467-6

Sola-Landa A, Rodriguez-Garcia A, Franco-Dominguez E, Martin JF (2005) Binding of PhoP to promoters of phosphate-regulated genes in *Streptomyces coelicolor*: identification of PHO boxes. Mol Microbiol 56(5):1373–1385. https://doi.org/10.1111/j.1365-2958.2005.04631.x

Stahl CH, Wilson DB, Lei XG (2003) Comparison of extracellular *Escherichia coli* AppA phytases expressed in *Streptomyces lividans* and *Pichia pastoris*. Biotechnol Lett 25(10):827–831. https://doi.org/10.1023/A:1023568826461

Van Mellaert L, Lammertyn E, Schacht S, Proost P, Van Damme J, Wroblowski B, Anne J, Scarcez T, Sablon E, Raeymaeckers J, Van Broekhoven A (1998) Molecular characterization of a novel subtilisin inhibitor protein produced by *Streptomyces venezuelae* CBS762.70. DNA Seq 9:19–30

Van Roosmalen ML, Geukens N, Jongbloed JD, Tjalsma H, Dubois JY, Bron S, van Dijl JM, Anne J (2004) Type I signal peptidases of gram-positive bacteria. Biochim Biophys Acta 1694(1-3):279–297. https://doi.org/10.1016/j.bbamcr.2004.05.006

Vigal T, Gil JA, Daza A, Garcia-Gonzalez MD, Martin JF (1991) Cloning, characterization and expression of an alpha-amylase gene from *Streptomyces griseus* IMRU3570. Mol Gen Genet 225(2): 278–288. https://doi.org/10.1007/BF00269860

Virolle MJ, Long CM, Chang S, Bibb MJ (1988) Cloning, characterisation and regulation of an alpha-amylase gene from *Streptomyces venezuelae*. Gene 74(2):321–334. https://doi.org/10.1016/0378-1119(88)90166-7

Widdick DA, Dilks K, Chandra G, Bottrill A, Naldrett M, Pohlschroder M, Palmer T (2006) The twin-arginine translocation pathway is a major route of protein export in *Streptomyces coelicolor*. Proc Natl Acad Sci U S A 103(47):17927–17932. https://doi.org/10.1073/pnas.0607025103

Yin S, Wang W, Wang X, Zhu Y, Jia X, Li S, Yuan F, Zhang Y, Yang K (2015) Identification of a cluster-situated activator of oxytetracycline biosynthesis and manipulation of its expression for improved oxytetracycline production in *Streptomyces rimosus*. Microb Cell Factories 14(1):46. https://doi.org/10.1186/s12934-015-0231-7