Mutation in *afsR* leads to A-factor deficiency in *Streptomyces griseus* B2682

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Abstract

Background/Aims: A-factor, a γ-butyrolactone autoregulator, in *Streptomyces griseus* is involved in the regulation of differentiation and antibiotic production. Here we studied the *Streptomyces griseus* B2682-AFN (A-factor negative) bald mutant that harbors a nonsense mutation in the *afsR* gene encoding a pleiotropic regulator. Our aim was to prove that this mutation is the cause of the A-factor deficiency in AFN. We also studied if AfsR regulates A-factor production by AfsA, which is supposed to be the only specific key enzyme in A-factor biosynthesis.

Methods: Wild *afsR* was cloned to the pHJL401 shuttle vector and was transformed to the *S. griseus* AFN and B2682 strains. During phenotypic characterization sporulation, antibiotic, protease, A-factor and AfsA protein production were studied.

Results: Transformation of AFN by a wild *afsR* restored its phenotype including sporulation, antibiotic, extracellular protease and A-factor production. Introduction of *afsR* to the B2682 wild type strain resulted in antibiotic and extracellular protease overproduction that was accompanied with elevated A-factor level. AfsA was detected both in AFN and B2682.

Conclusions: AfsR has an effect on the regulation of A-factor production in *S. griseus*. The presence of AfsA is not sufficient for normal A-factor production. AfsR regulates A-factor biosynthesis independently of AfsA.

Introduction

Streptomyces comprises Gram-positive, soil dwelling bacteria that show complex morphological differentiation terminated in sporulation [Flärdh and Buttner, 2009]. On solid medium the formation of substrate hyphae from a germinating spore is followed by the emergence of aerial hyphae, which segments into chains of spores [Flärdh and Buttner, 2009]. They are also able to produce a wide variety of secondary metabolites that makes streptomycetes one of the most important industrial microbial genus since many of the known antibiotics derive from their cultures [Chaudhary et al., 2013; Horinouchi, 2007]. The production of immunosuppressants, enzyme inhibitors, and industrial enzymes also contributes to their biotechnological significance [Chaudhary et al., 2013]. The spread of multidrug resistant bacterial pathogens represents great concern for future medicine and encourages the search for new antibiotics. According to genome mining studies streptomycetes are still promising candidates for antibiotic research [Chaudhary et al., 2013; Zhu et al., 2014]. Understanding the temporal and conditional cues that activate antibiotic production in streptomycetes would facilitate the improvement of screening efforts [Chaudhary et al,. 2013; Zhu et al., 2014].

Morphological development and secondary metabolism are simultaneously affected by various nutritional environments and controlled by extracellular signaling molecules such as γ-butyrolactones [Horinouchi, 2007]. In *S. griseus* A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) is produced in a growth-dependent manner and diffuses freely across the cell membrane. When its extracellular concentration reaches a critical level it binds to the A-factor receptor protein (ArpA) leading to the transcription and translation of *adpA* [Horinouchi, 2002]. AdpA then activates a variety of genes that are required for secondary metabolism, morphological development and extracellular protease production forming an AdpA regulon [Ohnishi et al., 2005]. The biosynthesis of A-factor is a multistep

pathway, in which the role of AfsA proved to be essential by catalyzing a ß-ketoacyl transfer from the 8-methyl-3-oxononanoyl-acyl carrier protein to the hydroxyl group of dihydroxyacetone phosphate, thus producing an 8-methyl-3-oxononanoyl-dihydroxyacetone phosphate ester [Kato et al., 2007]. The key role of AfsA in A-factor biosynthesis is supported by the observations that i) mutants having *afsA* deficiency are not able to produce A-factor, ii) introduction of *afsA* to A-factor nonproducing strains resulted in overproduction of A-factor, iii) introduction of *afsA* alone to *E. coli* caused the host to produce a substance having A-factor activity [Ando et al., 1997; Hara et al., 1983; Horinouchi et al., 1984; Kato et al., 2007].

Secondary metabolism is also under the regulation of pleitropic regulators in streptomycetes, e.g. by AfsR. In *S. coelicolor* AfsR is activated by phosphorylation in response to unknown environmental stimuli and acts by inducing the transcription of *afsS* [Lee et al., 2002]. AfsS then is proved to be important in the induction of secondary metabolite production and also modulates nutritional stress response in *S. coelicolor* [Lee et al., 2002; Lian et al., 2008]. AfsR and/or AfsS-like regulators proved to be important in the regulation of secondary metabolite production in other streptomycetes as well including *S. lividans* [Kim et al., 2001], *S. clavuligerus* [Parajuli et al., 2005], *S. venezuelae* [Maharjan et al., 2009], *S. pristinaespiralis* [Jin et al., 2012], *S. avermitilis* [Lee et al., 2000], *S.peucetius* [Parajuli et al., 2005] and *S. scabies* [Seipke et al., 2011]. But only a little, controversial information is available about AfsR in *S. griseus*. Earlier studies suggest that AfsR may be conditionally involved in the regulation of morphological differentiation but not directly needed for secondary metabolite production [Umeyama et al., 1999]. No information is available about AfsS in *S. griseus*.

In this work we studied the *S. griseus* B2682-AFN strain that is not able to produce sufficient amount of A-factor to support its differentiation [Birkó et al., 2007]. The A-factor deficiency of this strain is accompanied with the block of differentiation and secondary metabolite

production that is restored by the addition of A-factor [Birkó et al., 2007]. Previous studies showed that *afsA* is functional and transcribed in AFN [Birkó et al., 2007]. However, a nonsense mutation was detected in the *afsR* gene [Zhang, 2015] that might be the cause of the A-factor deficiency of this strain. To prove this hypothesis we transformed AFN with a functional *afsR.* The transformation restored its phenotype that confirms the role of AfsR in the regulation of sporulation, antibiotic, protease and A-factor production in *S. griseus*. We also present that this regulation is independent of the A-factor biosynthetic AfsA enzyme.

Results

Transformation of AFN by afsR

The A-factor deficient *S. griseus* AFN strain is a spontaneous bald mutant of the *S. griseus* B2682 wild type strain [Birkó et al., 2007]. To shed light on the genetic cause of the non-differentiating phenotype of AFN its genome was sequenced and a nonsense mutation was identified in the *afsR* (SGR3012) gene [Zhang, 2015]. In *S.griseus* the *afsR* gene encodes an 974 amino acid protein [Ohnishi et al., 2008]. Its promoter region contains the consensus sequence of sigma70 (-10 region: AGGCAGACT, -35region: CTGACG, predicted by the BPROM program) [Solovyev and Salamov 2011]. This suggests its expression during the growth phase that was confirmed by Umeyama et al. [1999]. In AFN a G → A transition occurred in the 2642. position that resulted in the change of Trp codon to Stop codon [Zhang, 2015]. This causes the production of a 94 amino acid shorter protein product that could lead to the non-differentiating phenotype of AFN. In order to confirm this hypothesis we transformed the AFN strain with a functional copy of *afsR*.

A 3.1 kb DNA fragment containing the *afsR* gene and its promoter sequence was cloned to the pHJL401 low copy number shuttle vector resulting in pHJL401-*afsR*.This construct was transformed to AFN that led to the *S. griseus* AFNpHR strain. In order to study the effect of

extra *afsR* copy to the phenotype of a wild type strain the pHJL401-*afsR* construct was also introduced to B2682 resulting in *S. griseus* B2682pHR. The pHJL401 empty construct was also transformed to AFN and B2682 that led to the *S. griseus* AFNpH and *S. griseus* B2682pH strains that were used as controls in the phenotypic studies.

Phenotypic characterization of the S. griseus B2682, AFN, B2682pHR and AFNpHR strains

In the phenotypic studies the intensity of growth, production of spores, antibiotics, and extracellular protease were investigated on rich (R5) and on minimal medium (MM). A-factor production was also detected.

In liquid R5 intensive growth was observed and the strains reached stacionary phase approximately 20 hours after inoculation (Fig 1/A). No significant difference was observed in the growth intensity of the strains and the specific growth rates were also comparable (0.99 ± 0.001). When growth was tested in liquid MM the strains were precultured in R5 before transfer to MM. This culturing condition is also suitable for the induction of differentiation in the liquid cultures of *S. griseus* [Daza et al., 1989; Birkó et al., 2007]. Nutritional shift-down decreased the length of exponential growth phase and all the strains entered stationary phase approximately 4 hours after shift (Fig 1/B). No significant difference was observed in the growth rates in MM either between the strains (0.98 ± 0.002).

Aeral mycelium and spore production were studied on solid R5 and MM medium. *S. griseus* B2682 strain produced aerial mycelium on both of the media but in case of the AFN strain it was medium dependent. No aerial hyphae could be discerned with the naked eye on rich R5 medium but spores could be seen by light microscopy inside the colonies (Fig 2/A). These spores were formed likely from the substrate mycelium and their shape and size were less regular than those formed from the aerial mycelium in case of the B2682 strain (Fig 2/A). Aerial mycelium formation and sporulation were detected in AFN growing on MM although

the amount of spores was reduced compared to the B2682 strain (Fig 2/B). Aerial hyphae formation was restored in the AFNpHR strain on R5 (Fig 2/A). Transformation of B2682 strain with pHJL401-*afsR* did not alter its sporulation (Fig 2/A and 2/B).

Antibiotic production was detected by a growth inhibition assay, as follows. The strains were grown for 7 days on R5 or MM then antibiotic testing medium mixed with *B. subtilis* spores was overlaid on the *S. griseus* colonies. Clear zones – with 39 ± 1 mm on R5 and 30 ± 1 mm on MM in diameter - indicating antibiotic production could be seen around the colonies of the B2682 strain (Fig 2/A and 2/B). But no antibiotic production was detected in the case of the AFN strain neither on R5 or MM (Fig 2/A and 2/B). Transformation of AFN with *afsR* restored its antibiotic production on both R5 and MM (Fig 2/A and 2/B). Moreover, it resulted in antibiotic overproduction especially on R5medium (Fig 2/A; 52 ± 2 mm inhibitory zone diameter) compared to the B2682 strain. The B2682pHR strain was also capable of antibiotic overproduction on R5 (Fig 2/A; 47 ± 2 mm inhibitory zone diameter). All the strains produced less antibiotics on MM than on R5 medium (Fig 2/A and 2/B).

Extracellular protease production was studied in liquid cultures. Extracellular protease activity increased continuously in the cultures of the B2682 strain on both R5 and MM (Table 1). In the case of the AFN strain only negligible extracellular protease production was observed (Table 1). Transformation of the AFN strain with pHJL401-*afsR* markedly increased its extracellular protease production compared to the AFN strain and this activity proved to be higher than the protease activity measured in the B2682 strain in both R5 and MM (Table 1). The extracellular protease formation of the B2682pHR strain was also elevated compared to the B2682 strain (Table 1). We observed that all the strains produced more extracellular protease enzymes in MM than in R5 (Table 1).

It is generally accepted that A-factor is produced during the growth phase [Horinouchi, 2002]. Since much more intensive growth was observed in liquid R5 than in MM A-factor production was studied in rich medium only. A-factor production of the strains was determined by Mass Spectrometry. The B2682 wild type strain produced 27.3 ng ml^{-1} A-factor (Table 2) that is in good agreement with the previous observation that A-factor reaches 25 to 30 ng ml^{-1} concentration in late exponential cultures [Horinouchi ,2002]. The AFN strain produced lower amount of A-factor than the B2682 strain as expected (Table 2). In the AFNpHR strain elevated A-factor production was observed that was comparable with the A-factor level produced by the B2682 strain (Table 2). It is also important to mention that introduction of *afsR* to B2682 resulted in increased A-factor production compared to the B2682 strain (Table 2).

Transformation of the empty pHJL401 plasmid to the AFN or B2682 strains did not influence their sporulation, antibiotic, extracellular protease or A-factor production (data not shown).

Detection of AfsA protein production in S. griseus AFN and B2682 strains

In our further studies we investigated whether AfsR affects A-factor biosynthesis by regulating AfsA that is considered to be the only specific key enzyme in A-factor biosynthesis [Ando et al., 1997]. The *afsA* gene encodes a 314 amino acid protein [Ohnishi et al., 2008]. The promoter region of *afsA* contains the consensus sequence for sigma70 (-10 region: CTGCAAGAT, -35region: TTCCCG [Solovyev and Salamov 2011] that suggests its expression during the growth phase. It is in good accordance with the fact that A-factor is produced during growth [Horinouchi, 2002].

In order to study the presence of AfsA protein an anti-AfsA antibody was required. For this purpose we produced the AfsA protein as an AfsA-GST fusion protein in *E. coli* (Supplementary text; Supplementary Figure). The purified AfsA-GST and AfsA proteins were

used for the immunization of rabbits. The AfsA protein was identified by Western blotting. To ascertain translation of *afsA* in the *S. griseus* AFN and B2682 strains submerged cultures were studied according to previous transcription studies [Birkó et al., 2007]. The same culturing conditions were used as described earlier: strains were pregrown in R5 and transferred to MM. Samples were taken before (0 h sample, cultured in R5) and 2 hs after the transfer (2 h sample, cultured in MM). This condition made AfsA detection possible during intensive growth – when A-factor is produced – and after the induction of differentiation by nutritional shift-down. AfsA protein was detectable in both of the conditions (Fig 3). The presence of AfsA was detected not only in the B2682 but also in the AFN strain at both of the media (Fig 3). Although this is not a quantitative measurement our result suggests that AfsA production is decreased after the induction of differentiation in B2682 that did not occur in the AFN mutant (Fig 3). Similar results were obtained for the synthesis of *afsA* mRNA [Birkó et al., 2007].

Discussion

The γ-butyrolactone molecules serve as key factors in the control of secondary metabolism in streptomycetes. However, little is known about the regulation of their synthesis. Shedding light on the regulatory processes in details has a great significance in the development of antibiotic overproducing strains since these molecules can enhance the production of antibiotics at very low concentration. Elevated γ-butyrolactone production may also support the induction of cryptic antibiotic gene clusters and industrially relevant extracellular proteases. Our results raise the possible role of AfsR in the regulation of morphological and physiological differentiation by influencing A-factor biosynthesis in *S. griseus*.

Here we studied the A-factor deficient *S. griseus* B2682-AFN strain that harbors a nonsense mutation in the *afsR* gene [Birkó et al., 2007; Zhang, 2016]. The non-differentiating

phenotype of AFN could be restored by exogenously added A-factor or by introducing the *facC* gene to AFN - that is responsible for the synthesis of factor C, which is an autoregulatory protein essential for the induction of differentiation in *S. albidoflavus* (formerly known as *S. griseus* 45H) [Birkó et al., 2007; 2009; Kiss et al., 2008]. These observations suggest that all the genes that function in the mode of action or in the biosynthesis of A-factor – including AfsA - are functional in AFN [Birkó et al., 2007; 2009]. The fact that AFN harbors a mutation in the *afsR* gene suggests that this mutation might be responsible for the bald phenotype of AFN and raises the possible role of AfsR in the regulation of A-factor biosynthesis. To prove this hypothesis we introduced a wild *afsR* copy to the AFN strain. Due to the transformation the phenotype of AFN was restored including sporulation, extracellular protease and antibiotic production. Moreover the A-factor production of this transformant was also comparable with the A-factor level of the B2682 wild type strain. Increasing the copy number of *afsR* in B2682 resulted in elevated A-factor, extracellular protease and antibiotic production. Elevated production of secondary metabolites by the enhanced expression of AfsS and/or AfsR-like regulators was also experienced in other streptomycetes including *S. coelicolor* [Floriano and Bibb, 1996; Matsumoto et al., 1995], *S. lividans* [Vogtli et al., 1994], *S. avermitilis* [Lee et al., 2000], *S. noursei* [Sekurova et al., 1999], *S. peucetius* [Parajuli et al., 2005] and *S. venezuelae* [Maharjan et al., 2009]. It is also important to mention that introduction of *afsR* from *S. peucetius* – sharing 61% sequence identity with *afsR* of *S. griseus* - to *S. griseus* also resulted in streptomycin overproduction [Parajuli et al., 2005]. However, to the best of our knowledge the effect of AfsR to γ-butyrolactone biosynthesis has not been studied yet. According to our results – both the mutation in *afsR* or increasing its copy number influenced A-factor production – AfsR might have a role in the regulation of A-factor biosynthesis in *S. griseus*.

Our results show that medium composition has a great influence on differentiation. Nutrient limitation is able to induce aerial mycelium and spore formation even in an A-factor deficient strain. However, A-factor seems to be essential in the induction of antibiotics and extracellular protease production regardless of medium composition. Rich medium supports the production of more antibiotics that is in good agreement with the fact that secondary metabolites require nitrogen compound precursors [Romero-Rodríguez et al., 2018]. On the contrary, elevated extracellular protease production was observed during nutrient limitation. This observation confirms that these enzymes are responsible for the degradation of substrate mycelium in order to provide nutrients and energy for spore formation [Kato et al., 2002; Kim et al., 2008].

We also studied if AfsR affects the biosynthesis of A-factor by influencing the A-factor biosynthetic AfsA enzyme that is supposed to be the only specific key enzyme of A-factor biosynthesis [Ando et al., 1997]. In this case the decreased level of A-factor would have been accompanied with the decreased amount/absence of AfsA protein in the AFN strain. However, Western blot results confirmed the presence of AfsA protein in the AFN strain that was comparable to the level observed in the B2682 strain in R5. Our results suggest that during intensive growth the i) the role of AfsR is independent of AfsA in the regulation of A-factor biosynthesis, ii) the presence of AfsA alone is not enough for the sufficient production of A-factor, iii) there is no correlation between the A-factor and AfsA level in *S. griseus*. We hypothesize that A-factor biosynthesis during growth is controlled by other mechanisms, e.g. by the availability of substrates for A-factor biosynthesis as it was also suggested earlier [Horinouchi, 2007]. The concentration of dihydroxyacetone phosphate (derives from glycolysis) and 8-methyl-3-oxononanoyl-acyl carrier protein (derives from the condensation of isobutyryl-CoA and acetate units leaked from the fatty acid biosynthesis) highly depends on the physiological state of the cell. We suggest that AfsR might affect the metabolic flux

that fuels A-factor biosynthesis in the cell. It is also important that *afsA* expression was decreased after the induction of differentiation by nutritional shift-down in the B2682 strain that did not occur in AFN. This is in good agreement with the observation that A-factor production is repressed after the induction of differentiation that might be mediated by AdpA in a two-step regulatory feedback loop [Kato et al., 2004]. Our future aim is to shed light on this regulation in details that could lead to the better understanding of the regulation of A-factor biosynthesis.

Experimental procedures

Streptomyces strains and culture conditions

The strains used in this study were *S. griseus* NRRL B2682 (wild type parental strain, in short B2682) and its spontaneous A-factor negative bald mutant *S. griseus* NRRL B2682-AFN (in short AFN) [Birkó et al., 2007; 2009]. The following strains were created in this study: *S. griseus* NRRL B2682 pHJL401 (in short B2682pH), *S. griseus* NRRL B2682 pHJL401-*afsR* (in short B2682pHR), *S. griseus* NRRL B2682-AFN pHJL401 (in short AFNpH), *S. griseus* B2682-AFN pHJL401-*afsR* (in short AFNpHR). All the strains were maintained on solid R5 medium [Kieser et al., 2000] at 30 °C for 7 days. In the case of the *S. griseus* AFNpH, AFNpHR, B2682pH and B2682pHR strains the medium was supplemented with 25 μg ml^{-1} thiostrepton.

To investigate areal mycelium and spore formation *Streptomyces* strains were cultivated on solid R5 or MM at 30 °C for 7 days. To assay growth kinetics, the production of AfsA protein and extracellular proteases submerged cultures were used. In these experiments two different culturing conditions were used. During the phenotypic studies in R5 *Streptomyces* strains were cultivated in 50 ml R5 medium for 96 h (30 °C; 250 rpm) and samples were taken 48, 72 and 96 hours after inoculation (for extracellular protease production). When the strains

were tested on MM, the strains were precultured in R5 medium until A_{550} = 0.7. Then mycelia were separated and transferred into 50 ml minimal medium (MM) [Kieser et al., 2000] and further cultivated for 72 h. Samples were taken at the time of shift (0 h) and 2 (for Western Blot) or 24, 48 and 72 hours (for extracellular protease detection) after transfer. Growth intensity was studied in the liquid cultures photometrically (A_{550}). Note that samples with absorbance higher than 1 were diluted with the appropriate medium before measurement. In the case of the *S. griseus* AFNpH, AFNpHR, B2682pH and B2682pHR strains the medium was supplemented with 2.5 μg ml^{-1} thiostrepton.

Transformation of Streptomyces strains

The pHJL401 plasmid (low copy number,~10 copies/cell [Kieser et al., 2000]), and its derivative -pHJL401-*afsR*- were used for transformation. The pHJL401-*afsR* plasmid was constructed as follows. The *afsR* sequence and the 192 bp upstream sequence containing the promoter region of *afsR* was amplified by PCR with B2682 genomic DNA as a template using the following primers: *afsR*-F 5'-GCG*TCTAGA*CTTGTGCAGAGCGTCACCCACT-3' (with an XbaI site shown by italics) and *afsR*-R 5'-GCC*GAATTC*AACGATTGATGAACGCCTCCGC-3' (with an EcoRI site shown by italics). Promoter position was predicted by the BPROM program [Solovyev and Salamov 2011]. PCR fragment was cloned to the pHJL401 plasmid between the XbaI and EcoRI sites. The pHJL401-*afsR* plasmid purified from the *E. coli* ET12567 methylation deficient strain was used for transformation [MacNeil et al., 1992].

During the transformation of *S. griseus* B2682 and AFN strains the PEG-assisted transformation of *Streptomyces* protoplasts method was used [Kieser et al., 2000]. *S. griseus* strains were cultivated in liquid GYM medium. Protoplasts were prepared by incubating the culture in lysozyme solution (1 mg ml^{-1}) as described elsewhere [Kieser et al., 2000]. The

number of the protoplasts was determined using a haemocytometer. During transformation 5 µg plasmid DNA (pHJL401 or pHJL401-*afsR*) was added to 4 x 10^9 protoplasts in the presence of P-buffer that was supplemented with 25 % PEG [Kieser et al., 2000]. For regeneration protoplasts were spread on the surface of R5 agar plates and were incubated at 30 °C for 24 h. Selection of transformants was carried out by flooding 1.5 ml thiostrepton containing solution (0.66 mg ml^{-1}) on the surface of the agar plates [Kieser et al., 2000]. Negative controls were used in every transformation experiment in order to check transformation efficiency.

Detection of antibiotic production

Antibiotic production of *S. griseus* colonies were assayed on surface grown cultures. *Streptomyces* strains were grown on solid R5 for 7 days at 30 °C. Antibiotic medium (5 g l^{-1} meat extract, 1 g l^{-1} Na_2HPO_4, 0.1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} Bacto peptone) mixed with *Bacillus subtilis* ATCC 6633 spores was overlaid on the *Streptomyces* colonies. The plates were further incubated at 37 °C for 18 h and the formation of growth inhibition zone around the *Streptomyces* colonies was detected [Horinouchi et al., 1984].

Determination of protease activity

0.1 ml fermentation broth samples were incubated (60 min, 37 °C) in 0.2 mol l^{-1} Na_2HPO_4/0.1 mol l^{-1} citric acid buffer (pH 8) with azocasein substrate (12.5 mg ml^{-1} final concentration) as described elsewhere [Szilágyi et al., 2011]. One unite (U) of protease activity was defined as an amount of enzyme resulting in 1 OD increase in the absorbance at 440 nm. Protease activities are presented in U ml^{-1}.

Detection of A-factor production

A-factor was extracted from liquid grown cultures after 48 h of growth with equal amount of chloroform as described earlier [Birkó et al., 2007]. A-factor concentrations are presented in ng ml^{-1} culture. A-factor concentration of the extracts was determined by Mass Spectrometry (MS). These measurements were obtained on a Waters QTOF Premiermass spectrometer (Milford, USA) equipped with an electrospray (ESI) source. The instrument was operated in negative ion mode using enhanced sensitivity scan focused to 241 m/z and fragment ion scan mode (MSMS) of 241 m/z. Fragment ion scans and the retention time data was used to confirm the presence of the molecule in the sample. The quantitative determination of A-factor based on the mass chromatogram of the 241 ion. Ion intensities were compared to the intensities of the standard solution. Matrix effect was excluded using standard spiked biological samples. Samples were subjected to gradient HPLC separation before MS analysis. (Mobile phases were A: water and formic 99.95/0.05 v/v, B: acetonitrile, formic acid 100:0.05 v/v HPLC apparatus: Applied Biosystems 140C; flow rate: 250 µl min-1; gradient: 40-100 15 min; column: Kinetex 5u EVO C18 100A C18, 150x2.1 mm).

Antibodies

The primary antibody used to detect the presence of AfsA protein in this study was rabbit polyclonal antibody. AfsA protein was produced as AfsA-GST fusion protein by *E. coli* BL21 cells harboring the pGEX-4T1-*afsA* plasmid (Supplementary text; Supplementary Figure). Purified AfsA-GST and AfsA proteins were used for the immunization of New Zealand white rabbits. The antibodies were purified on protein A column. Rabbit immunization and antibody purification were performed by Covalab (www.covalab.com). The secondary antibody in Western Blot studies was horseradish peroxidase-coupled anti-rabbit antibody (Covalab).

SDS-PAGE and Western Blotting

Mycelia of *S. griseus* AFN and B2682 strains derived from submerged cultures were centrifuged, washed and resuspended in PBS. Cells were disrupted by sonication. Proteins were separated by electrophoresis in a 12% polyacrylamide gel. Western blot was prepared using a Trans Blot Semi Dry Transfer Cell (Bio-Rad) and PVDF membrane in Towbin buffer according to the manufacturer's instructions. After transfer the membrane was incubated in blocking buffer (50 g l^{-1} non fat milk powder in PBS) for 1 h, and washed in PBS. For immunodetection the blot was incubated with the primary polyclonal antibody (1:1000 dilution, 4 °C, overnight). Before the incubation with secondary antibody (1:2000 dilution) the blot was washed in PBS. For chromogenic detection 3.3-diaminobenzidine (0.5 g l^{-1} in PBS supplemented with 0.3% H_2O_2) was used as substrate.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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Table 1. Extracellular protease activity in the submerged cultures of *Streptomyces griseus* AFN, B2682, AFNpHR and B2682pHR strains.

Strains	**Specific protease activity U ml $^{-1}$**					
	48 h		72 h		96 h	
	R5	MM	R5	MM	R5	MM
AFN	0 ± 0.01	0.1 ± 0.1	0.01 ± 0.01	0.3 ± 0.1$^{\#}$	0.01 ± 0.01	0.2 ± 0.1$^{\#}$
B2682	1.43 ± 0.1	1.4 ± 0.2	2.1 ± 0.2	3 ± 0.3$^{\#}$	3 ± 0.2	3.6 ± 0.3$^{\#}$
AFNpHR	2.54 ± 0.2*	2.8 ± 0.3*	4 ± 0.3*	4.2 ± 0.5*	5.1 ± 0.3*	5.9 ± 0.5*$^{\#}$
B2682pHR	1.44 ± 0.2	1.7 ± 0.2	2.2 ± 0.2	3.7 ± 0.3*$^{\#}$	3.5 ± 0.2*	4.3 ± 0.4*$^{\#}$

The strains were inoculated to R5 and cultivated for 96 hours. When protease production was studied in MM the strains were pregrown in R5 than transferred to MM and cultivated further for 72 hours . Samples were taken 48, 72 and 96 hours after inoculation. All data represent mean ± SD values calculated from 4 independent experiments. * stands for significant ($p < 0.05$) differences from the B2682 strain calculated by the Student's *t*-test. $^{\#}$ stands for significant ($p < 0.05$) differences between the activities detected in R5 and MM in each of the strains calculated by the Student's *t*-test.

485 **Table 2.** A-factor production of the cultures of *Streptomyces griseus* AFN, B2682, AFNpHR
486 and B2682pHR strains.

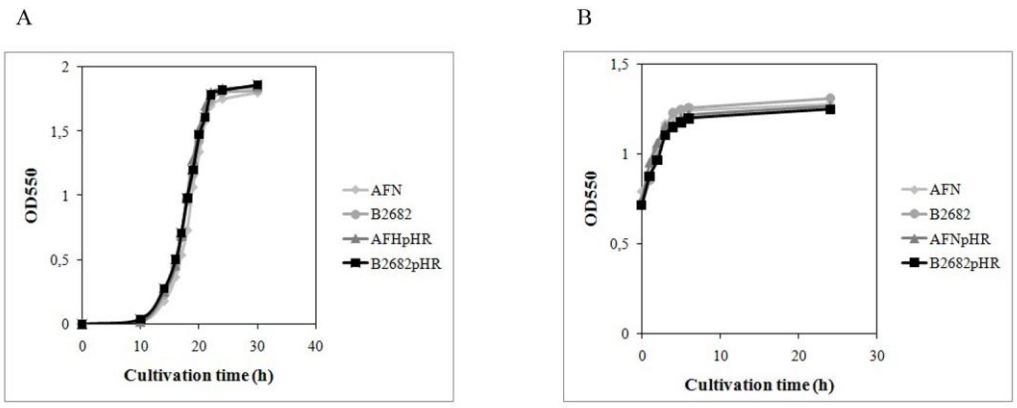
Strains	**A-factor concentration ng ml $^{-1}$ culture**
AFN	13.6 ± 1.5
B2682	27.3 ± 2.5
AFNpHR	25 ± 2.7*
B2682pHR	38.8 ± 3.9**

487

488 All data represent mean ± SD values calculated from 4 independent experiments. Symbols *
489 or ** stand for significant differences from the AFN or B2682 strains calculated by the
490 Student's *t*-test ($p < 0.01$).

491

Figure 1



Growth curves of the liquid cultures of *Streptomyces griseus* AFN, B2682, AFNpHR and B2682pHR strains in R5 (A) and in MM (B). Strains were inoculated to R5 and cultivated for 30 hours (A). When growth was tested in MM the strains were pregrown in R5 to A_{550}=0.7 and transferred to MM (B).

506 **Figure 2/A**

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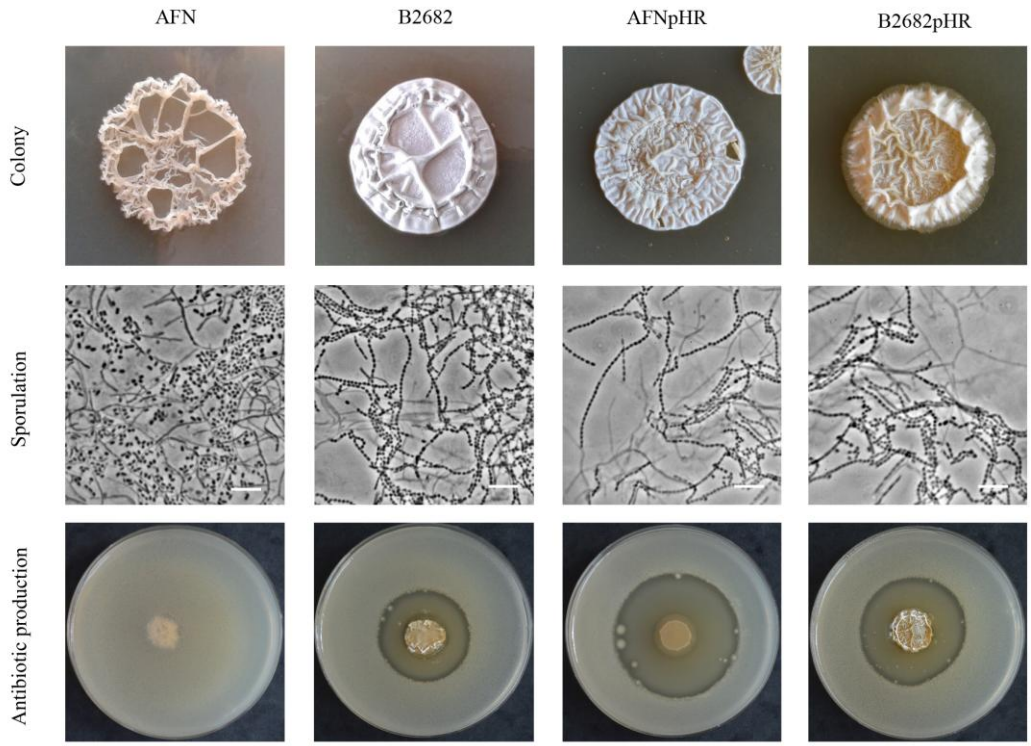

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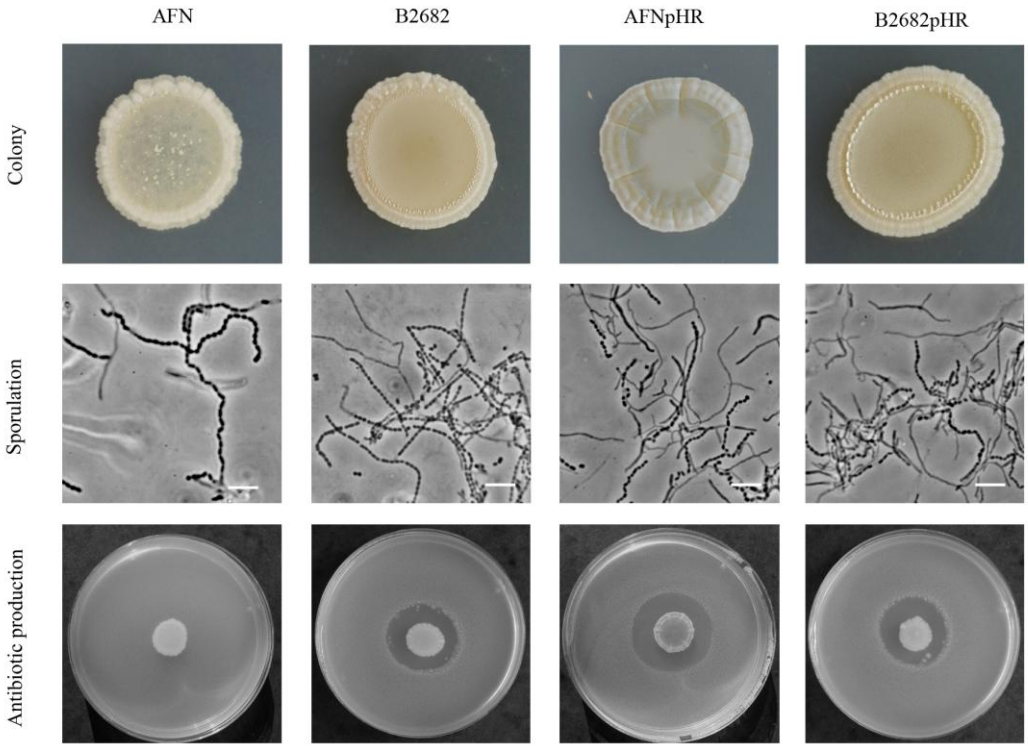
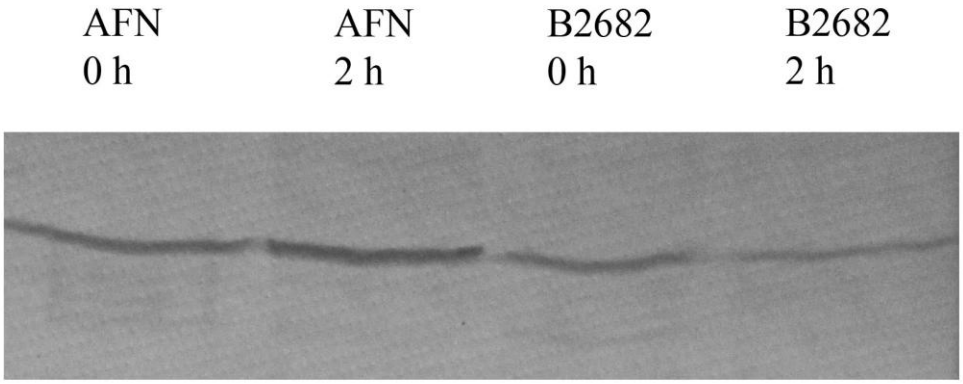
Figure 2/B



Sporulation and antibiotic production of *Streptomyces griseus* AFN, B2682, AFNpHR and B2682pHR strains on R5 (A) and on MM (B). *Streptomyces* colonies were grown on solid R5. The photos and microscopic images (Bar = 10 µm) of the cultures were taken 7 days after inoculation. To detect antbiotic production agar medium mixed with *Bacillus subtilis* spores was overlaid on the *Streptomyces* colonies after 7 days of incubation. The formation of growth inhibition zone of *Bacillus subtilis* around the *Streptomyces* colonies showed the production of antibiotics.

Figure 3



Detection of AfsA protein in the submerged cultures of *Streptomyces griseus* AFN and B2682 strains by Western blot. Differentiation was induced by nutritional shift-down. Samples were taken at the time of shift (0 h sample, cultured in R5) and 2 h after nutritional shift down (2 h sample, cultured in MM).

Supplementary material

Expression and purification of recombinant AfsA-GST

To produce AfsA-GST fusion protein *afsA* was cloned to the pGEX-4T1 plasmid (GE Healthcare) as follows. The *afsA* sequence was amplified by PCR with B2682 genomic DNA as a template using the following primers: *afsA*-F 5'-*GAATTC*ATGCCCGAAGCAGCAGTCTTGATCG-3' (with an EcoRI site shown by italics) and *afsA*-R 5'-*GCGGCCGC*TCAGCCGTGGGCCCGGGGGCCGGAC-3' (with a NotI site shown by italics). The blunt-end PCR product was cloned to the pJET1.2 plasmid (Thermo Fisher Scientific). The EcoRI-NotI fragment was excised from pJET1.2-*afsA* and cloned between the EcoRI and NotI sites of pGEX-4T1, resulting in pGEX-4T1-*afsA*, which was transformed to *E. coli* BL21.

During the production of AfsA-GST this *E. coli* strain was incubated in Luria Broth (LB) medium supplemented with 100 μg ml^{-1} ampicillin (30 °C; 250 rpm). Fusion protein expression was induced by 0.1 mM IPTG. Cells harvested by centrifugation were washed and suspended in PBS buffer. Cells were disrupted by sonication. Expression yielded low amount of fusion protein in the soluble fraction due to the formation of insoluble inclusion bodies (Supplemetaly Figure). In order to dissolve the inclusion bodies the pellet obtained from sonication was solubilized with 5M urea [Harper and Speicher, 2011]. To refold the denatured AfsA-GST urea was removed by successive dialysis [Harper and Speicher, 2011]. To purify the recombinant AfsA-GST protein Gluthatione GST-bind resin (Millipore) was used by following the manufactural instructions. GST-fusion protein bound to the resin was either eluted directly by 10 mM reduced gluthatione containing elution buffer or was cleaved on the resin by thrombin. Each of the steps were monitored by SDS-PAGE (Supplemetary Figure).

Reference

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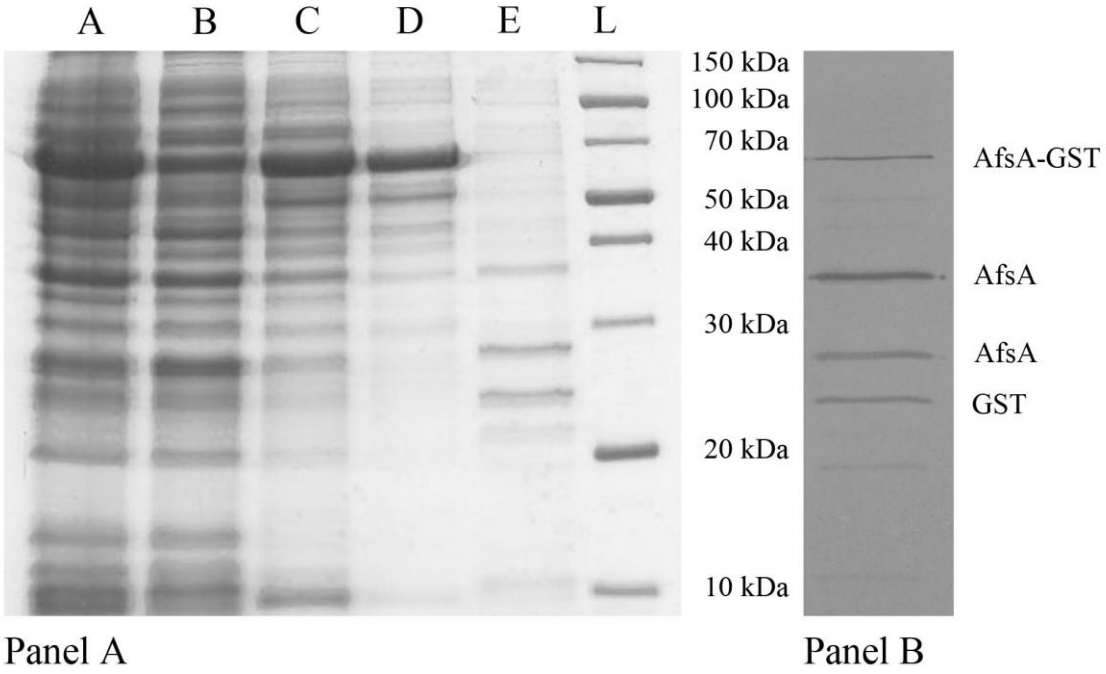
Supplementary Figure



Purification of recombinant AfsA (Panel A) and its detection by antibody (Panel B). Panel A: The AfsA-GST fusion protein was purified from the culture of *E. coli* BL21 harboring pGEX-4T1-*afsA* with Gluthatione GST-bind resin and was cleaved by thrombin. Symbols: A: sediment after sonication, B: supernatant after sonication, C: sediment after urea treatment, D: purified AfsA-GST fusion protein, E: AfsA and GST proteins after the cleavage of the fusion protein by thrombin, L: protein molecular weight ladder. Purified AfsA-GST and AfsA proteins were used for the immunization of rabbits. Panel B: The purified rabbit polyclonal antibodies proved to be active against the recombinant AfsA-GST and AfsA proteins by Western blot. Note that the AfsA protein (36 kDa) contains a thrombin cleavage site that results the smaller fragment (28 kDa).