Effect of protein phosphatase gene *sppA* deletion on hyphal growth in *Streptomyces coelicolor* when combined with different protein kinase gene *afsK* mutations

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Bachelor's degree project 15 credits in Chemistry
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Lund University
2011
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Abstract

Streptomyces species are known for their bacteria-untypical growth and sporulation. Growth by tip extension building up a mycelium consisting of long branching hyphae is a highly polar and regulated process. An essential protein involved in hyphal growth and branching regulation is DivIVA.

In *S. coelicolor* branching and growth is regulated by phosphorylation of DivIVA by the serine/threonine kinase AfsK. The phosphatase SppA dephosphorylates DivIVA and leads to less branching growth, an interaction to be further investigated in this project.

The aim of this project was to combine a *sppA* knockout mutation with different *afsK* modifications, such as deletion and overexpression mutants. A cosmid carrying a new *ΔsppA::vph* allele was constructed using the λ-Red system in *E. coli*. The allele was transferred to *S. coelicolor* and recombinant strains with replacement of the *sppA* gene were selected. Of special interest was the *ΔsppA ΔafsK* double knockout to reveal possible additional DivIVA phosphorylation due to other kinases and to check if the wild type colony size phenotype (previously small colonies were observed in *ΔsppA* strains) would be regained. The obtained strains were examined for growth phenotypes and phosphorylation patterns of DivIVA were visualized by Western blotting. *sppA* knockout mutants were tested for increased sensitivity to overexpression of *afsK*.

The obtained results support the previously raised hypothesis, that SppA and AfsK are direct counteractors on DivIVA phosphorylation. The *sppA afsK* double knockout mutant partly regains the wild type colony size. Sensitivity to overexpression of *afsK* seems to be strongly increased by an *sppA* knockout. As time was limited the results are preliminary and further experiments should be carried out with the generated strains to confirm and extend the results.

1. Introduction

1.1 *Streptomyces coelicolor*

Streptomyces are GC-rich Gram-positive bacteria belonging to the phylum Actinobacteria. They are strict aerobes, mainly inhabiting soil. *Streptomyces* proproduce a wide range of different secondary metabolites, such as geosmin, which is responsible for the typical "wet soil"-smell, and many antibiotics e.g. streptomycin (aminoglycoside from *S. griseus*), erythromycin (macrolide from *S. erythraeus*) and chloramphenicol (*S. venezuelae*).[1] Streptomycetes grow similar to fungi by tip extension, forming a branching vegetative mycelium in which the individual hyphal cells contain multiple copies of the chromosome. Upon nutrient shortage, an aerial mycelium is formed and aerial hyphae develop into long, often spiral-shaped prespore chains, finally dividing and maturing into gray monochromosomal spores[1,2]. The main model organism for genetic and molecular investigations of streptomycetes is *Streptomyces coelicolor*[1].

1.2 Tip extension and its regulation in *S. coelicolor*

Instead of MreB-mediated extension of lateral cell wall, for example as in *Escherichia coli*, *S. coelicolor* grows only at hyphal tips and this is mediated by the essential coiled-coil protein DivIVA. Also generation of new hyphal tips by hyphal branching is directed by assemblies of DivIVA, as it has been shown by *divIVA-egfp* fusion and time-lapse microscopy (but not every DivIVA focus leads to a new branch)[3,4]. Homologs of DivIVA are also involved in directing apical growth of rod-shaped cells in the actinobacterial genera *Corynebacterium* and *Mycobacterium*)[3].

AfsK, one of at least 34 eukaryotic-type Ser/Thr protein kinases found in *S. coelicolor*, is responsible for phosphorylation of DivIVA and affects its localization, leading to modulation of apical growth and more frequent branching[5]. During normal growth, phosphorylation of DivIVA appears at a low level and DivIVA is situated mostly at hyphal tips. However inhibition of cell wall synthesis, e.g. by bacitracin, triggers strongly increased levels of phosphorylated DivIVA and leads to dissolving of the apical foci of DivIVA-EGFP. AfsK was also shown using an *afsK-mCherry* fusion to colocalize with DivIVA at hyphal tips[5]. AfsK activates itself by autophosphorylation of at least two threonine residues and a constitutive mutant AfsK* (threonines T165 and T168 are changed to aspartates, which mimic the phosphorylated active state) can be used to investigate the effects of high AfsK activity effects without blocking cell wall synthesis[5].

These observations have led to the question of how DivIVA is dephosphorylated, and the pSer/pThr protein phosphatase SppA (*Streptomyces* protein phosphatase A, *SCO3941*) was identified as a candidate for dephosphorylating DivIVA[6,7]. *sppA* mutants are known to grow slowly compared to the wild type parent[8]. It was shown that *sppA* mutants, compared to wild type parent, have higher basal levels of DivIVA phosphorylation, higher induced levels after bacitracin treatment, and a slower overall rate of DivIVA dephosphorylation when bacitracin had been washed away[6].

1.3 Aim of this project

In this project the interplay between AfsK and SppA was further investigated. *sppA* knockout mutation was created and combined with different kinds of *afsK* mutations (deletion, overexpression and constitutive overexpression) to identify phenotypic differences between the new strains as well as compared to the wild type. The investigated phenotypes were colony size, phosphorylation pattern after cell wall synthesis inhibition, and sensitivity to AfsK overexpression. Of special interest was the question whether the *sppA-afsK* double knockout would restore the wild type phenotype (compared to the slow growth and small colony phenotype of the *sppA* mutant), as is predicted if assuming that the main role of *sppA* is to dephosphorylate the substrates of AfsK.

Some *sppA* mutants were available before, but this mutants were obtained by transposon insertion. This method still can lead to partly active proteins as the gene sequence is not deleted completely, while the created knockouts can be considered "clean" by complete deletion of the *sppA* gene.

2. Materials and methods

2.1 Strains and growth conditions

Table 1. Strains, cosmids, plasmids

strain	relevant genotype	reference
Escherichia coli		
DY380	DH10B *λcI857 Δ(cro-bio)<>tet*	10,9
ET-12567/ pUZ8002	*dam-12::*Tn9 *dcm-6 hsdM hsdR*$^-$ (pUZ8002 is a defective RK2 plasmid without an *oriT* site, used to drive conjugation)	7
S. coelicolor		
M145	prototrophic, plasmid-free	11
M600	prototrophic, plasmid-free	11
M600 *ΔafsK*	*ΔafsK*	7
M600 pIJ6902	M600 *attB*$_{\Phi C31}$*::*pIJ6902[*tipAp*] empty vector with thiostrepton resistance and thiostrepton inducible promoter (*tipAp)*	5
M600 pKF376	M600 *attB*$_{\Phi C31}$*::*pKF376*[tipAp-afsK]* inducible promoter controlling additional *afsK*	5
M600 pKF275	M600 *attB*$_{\Phi C31}$*::*pKF275*[tipAp-afsK*(T165D, T168D)*]* inducible promoter controlling additional constitutive *afsK**[a]	5
JK II	M600 *ΔsppA::(vph-oriT)*	this work
JK III	M600 *ΔsppA::(vph-oriT) ΔafsK::aac(3)IV*	this work
JK IV	M600 *ΔsppA::(vph-oriT)* pIJ6902	this work
JK V	M600 *ΔsppA::(vph-oriT)* pKF376	this work
JK VI	M600 *ΔsppA::(vph-oriT)* pKF275	this work
cosmids/plasmids		
C08	D78.1.C08_SFD78-1_060 (Kan^r, Amp^r) Cosmid carrying the region around *sppA* from the *S. coelicolor* chromosome, but with *sppA* disrupted by Tn*5062* (carrying apramycin-resistance)	12
pIJ780	contains *oriT* and *vph* (viomycin-resistance) inside priming sites, *amp* (ampicillin-resistance) outside	13
pMS82	vector with hygromycin resistance	6
pKF240	pMS82 with *sppA*	6

[a] usually AfsK activates itself by autophosphorylation, but in AfsK* two mutations have been introduced that mimics autophosphorylation resulting in a constitutively active version of the kinase.

The bacterial strains used in the study are listed in Table 1. *E. coli* strains were cultivated in lysogenic broth (LB) or agar (LA) at 30° or 37°C. *S. coelicolor* strains were cultivated at 30°C on soy flour mannitol medium (SFM)[(11)]. Antibiotic concentrations for *E. coli* were 50 μg/ml for kanamycin, carbenicillin and apramycin (Kana^{50}, Carb^{50}, Apra^{50}). For selection with viomycin (30 μg/ml; Vio^{30}) or hygromycin (100 μg/ml; Hyg^{100}), *E. coli* cells were grown in Difco Nutrient agar (NA) or LA without NaCl. For *S. coelicolor* on SFM medium, antibiotic concentrations were 25 μg/ml for apramycin, 50 μg/ml for kanamycin and 30 μg/ml for viomycin (Apra^{25}, Kana^{50}, Vio^{30}).

Table 2. Primers

designation	sequence (5'-3')	reference
KF893 rev	ACCGCGGGCCGCTGCGACCAGGCAGTCCGAAGCGGTGCAGATGCGGCGCG/TGTAGGCTG GAGCTGCTTC (cosmid 50 bp flank / pIJ780 primer part)	this work[a]
KF894 for	GGGTGCCGCTCTGTACCGTCGGCACCCCGAAAAAGCCGCGCGGTGAACGG/ATTCCGGGG ATCCGTCGACC (cosmid 50 bp flank / pIJ780 primer part)	this work[a]
KF895 for	GATCCGCATTCCTATGATGG	this work[a]
KF896 rev	CAGGGGGTTTCCAGAAATTG	this work[a]
KF581 for	GAGGGGTCGATGACTCAGG	7
KF582 rev	GTGAAGTCGCCGAGGAAC	7
EZR1	ATGCGCTCCATCAAGAAGAG	7
EZL2	TCCAGCTCGACCAGGATG	7

[a] primers obtained from Invitrogen

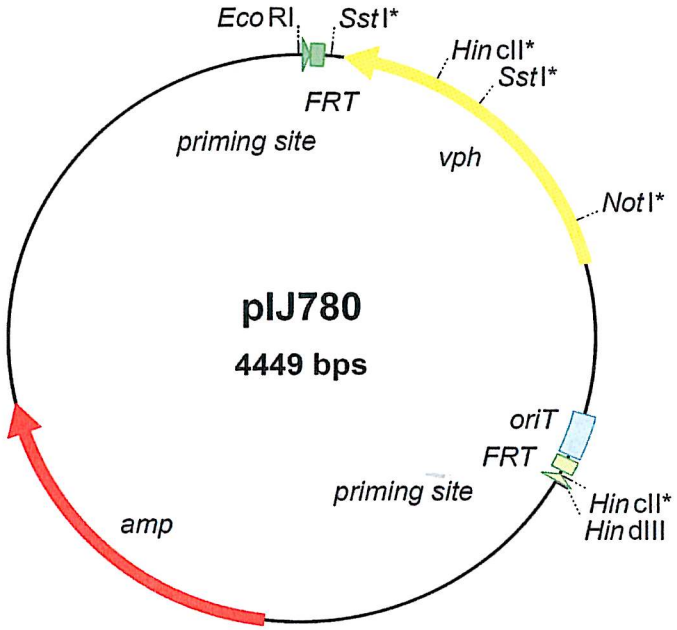

Fig. 1 **pIJ780 carrying *vph-oriT* cassette** used as REDIRECT[13] PCR-template to introduce the cassette into the *sppA* gene on cosmid. The cassette was amplified using PCR primers attached to 50 bp long sequences complementary to sequences flanking *sppA*. Recombination of the amplified *vph-oriT* cassette onto the C08 cosmid resulted in a specific knockout. ***vph***: viomycin-resistance, ***amp***: ampicillin-resistance, ***oriT***: origin of conjugative transfer needed to insert cosmid DNA into the recipient *S. coelicolor* strain, ***FRT***: flippase recognition target (not used in this project).

2.2 PCR targeting

(See App. E for full *sppA*-knockout diagram)

DY380 was used to create clean viomycin-resistant (Vio^R) deletions of the *sppA* gene using λ–Red-mediated recombination. DY380 expresses the *exo*, *bet* and *gam* recombination genes under control of a thermosensitive λ cI repressor. Cosmid C08 was isolated from corresponding *E. coli* strain using modified *DpnI* alkaline lysis minipreparation protocol[14] and introduced into strain DY380 by transformation[15].

Purified pIJ780 (Fig. 1.) was used as template in PCR with primers KF893 and KF894 to amplify the *vph-oriT* cassette flanked by two 50 base pair long sequences corresponding to sequences immediately flanking *sppA*[13]. The PCR product was confirmed in size by 1.2 % agarose gel electrophoresis and purified by JetQuick© spin column Kit (Genomed). Methylated DNA-template was digested by *DpnI* (New England BioLabs© Inc.), the enzyme was heat-inactivated and the PCR product was drop dialysed against water (VSWP membrane filter, 0.025μm (Millipore©)).

Strain DY380/C08 was incubated at 30 °C, shifted to 42°C for 20 min (activating λ-Red high recombination system), electroporated (0 °C, 1.8 kV, 25 μF, 200 Ω, time constant 4.4) with the PCR product and plated on NA $Kana^{50}$ $Carb^{50}$ Vio^{30} at 30 °C selecting for acquired viomycin-resistance (apramycin-resistance, encoded on the transposon Tn*5062* disrupting *sppA* on cosmid C08 should be lost, as was screened on LA $Kana^{50}$, $Carb^{50}$, $Apra^{50}$).

The $Apra^S$ Vio^R colonies were inoculated into low salt LB (0,5 % NaCl) and cosmid DNA was extracted[14]. The knockout of *sppA* was confirmed by Phusion DNA polymerase PCR (Finnzymes, GC-buffer, $T_{annealing}$:60 °C, 10% DMSO, 75" elongation) using KF895 and KF896 primers and *S. coelicolor* M145 chromosomal DNA as control, and by digestion of PCR-product with *NotI* (New England BioLabs© Inc.).

2.3 Conjugation to *S. coelicolor*

(See App. E for full *sppA*-knockout diagram)

A confirmed *ΔsppA::vph* cosmid was introduced by transformation into the methylation-defective conjugation donor strain ET-12567/pUZ8002[15] and plated after phenotypic expression on NA $Kana^{50}$ $Carb^{50}$ Vio^{30}. One donor colony was grown in low salt LB $Kana^{50}$ $Carb^{50}$ Vio^{30} and used for conjugation with *S. coelicolor* strains M600, M600 *ΔafsK*, M600 pIJ6902, M600 pKF376, M600 pKF275, following the previously described procedure[11]. The obtained exconjugants were repeatedly streaked on SFM and screened for loss of resistance to $Kana^{50}$. Recombinants (Vio^R $Kana^S$) were isolated and named JK II, JK III, JK IV, JK V and JK VI, as shown in Table 1. Spores were prepared from each strain and used for strain preservation and inoculation of cultures[11].

2.4 Complementation with pKF240

Different preparations of plasmid pKF240 (constructed by M. Restedt[6]) were checked comparing to the empty pMS82 by restriction with *SacI* (App. D) (one restriction site predicted in pMS82 but two in pKF240, App. C). ET-12567/pUZ8002 was transformed with pKF240[15] and plated on LA (no NaCl) Hyg^{100} to select for the plasmid.

Hyg^R transformants of ET-12567/pUZ8002 were used for conjugation to *S. coelicolor* JK II-VI[11] and plated on SFM plates with Hyg^{25} and other antibiotics as appropriate for each strain.

2.5 Western blotting to detect DivIVA phosphorylation

As bacitracin is known to induce high level phosphorylation of DivIVA, cultures (grown in YEME[11] prepared with 17% sucrose and 5 mM $MgCl_2$ 30 °C) of JK II, JK III, M600 and M600 *ΔafsK* were used to produce cell extracts with and without bacitracin-treatment (30 min, 50 µg/ml). The procedure involved lysis by bead-beating with FASTPREP®-24 and determination of protein concentration by Bio-Rad DC quantification (OD-750 nm, BSA calibrated)[6]. SDS-PAGE and Coomassie Brilliant Blue staining (App. B) confirmed correct quantification of the protein concentration in extracts. Western blotting[5] was performed with DivIVA rabbit-antiserum[16] diluted 5000x as primary antibody and polyclonal swine anti-rabbit immunoglobulin conjugated to horse radish peroxidase (Dako Cytomation) diluted 1000x as secondary antibody. Chemiluminescence detection was achieved using Super Signal West Pico Chemiluminescent Substrate Kit (Pierce). Imaging of Western Blot and Coomassie staining was performed with Kodak image station 440CF with 1D Image Analysis Software.

2.6 Thiostrepton-induced *afsK* overexpression

The strains M600 pIJ6902 (negative control, empty vector), M600 pKF376 (*tipAp-afsK)*, M600 pKF275 (*tipAp-afsK*)* were compared with their *ΔsppA* variants JK IV, JK V, JK VI on SFM plates with different concentrations of thiostrepton. All strains were plated on SFM Apra[25] with thiostrepton concentrations: 0, 0.01, 0.1, 1 and 10 µg/ml. Pictures of plates were taken with RICOH Aficio MP C5000.

3. Results

3.1 Phenotypical interactions of *ΔsppA* and *ΔafsK*

The strains M600 , M600 *ΔafsK*, JK II (M600 *ΔsppA*) and JK III (M600 *ΔsppA ΔafsK*) were plated on SFM at 30 °C to investigate colony size phenotype. It was already known from previous works[6] that *sppA* deletions give a small colony phenotype. One aim of this project was to test whether the slow growth of the *ΔsppA* mutants is caused by AfsK-mediated phosphorylation. To test this, a *sppA* mutation was combined with *ΔafsK*. While JK II confirms a much smaller colony size compared to M600, this effect is less strong in the JK III double mutant compared to the M600 *ΔafsK* (Fig. 2). This suggests that an *afsK* deletion partially suppresses the slow growth phenotype of *sppA* mutants.

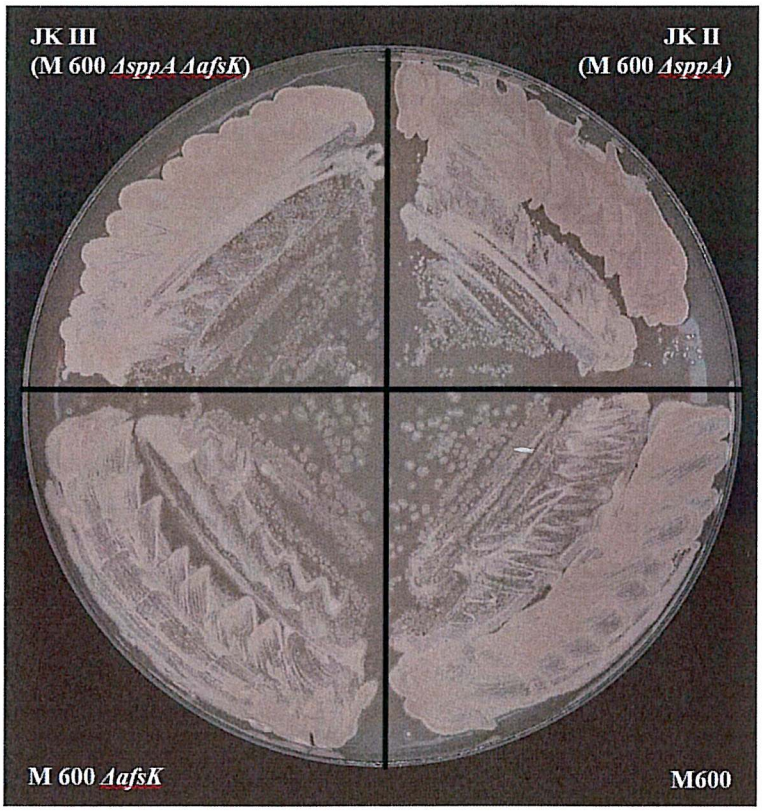

Fig. 2 **Effects of *ΔsppA* and *ΔafsK* on colony size phenotype.** Spores of the strains M600, M600 *ΔafsK*, M600 *ΔsppA* and M600 *ΔsppA ΔafsK* were streaked on antibiotic-free SFM plates. The *ΔsppA* mutant shows the previously reported strong reduction of colony size compared to the wild type. While *ΔafsK* gives no significant altered colony size, it seems to restore partly the colony size in the *ΔsppA ΔafsK* double mutant compared to the *ΔsppA* single mutant. Although the wild type level is not reached, this observation strongly suggests that AfsK and SppA are direct counteractors.

3.2 Sensitivity of *ΔsppA* mutants to *afsK* overexpression

As can be seen in Fig. 3, without thiostrepton-mediated induction of the *tipAp* promoter, the three strains M600 pIJ6902 (negative control, empty vector), M600 pKF376 (*tipAp-afsK)*, M600 pKF275 (*tipAp-afsK**) differ from their *ΔsppA* variants JK IV, JK V, JK VI only by slightly bigger colonies. On the highest level (10µg/ml) of *tipAp*-induction used in the experiment, M600 pIJ6902 and the corresponding *ΔsppA* mutant still show as expected approximately the same colony size phenotypes ratio compared to noninducing conditions (negative control). M600 pKF376 (*tipAp-afsK)* formed smaller colonies compared to without thiostrepton, but significantly larger than the JK V (*tipAp-afsK ΔsppA*) variant, with its tiny colonies and low sporulation (probably due to slow growth). M600 pKF275 (*tipAp-afsK**) differs strongly from JK VI (*tipAp-afsK* ΔsppA*), as the first one shows dense growth of small colonies, while the second produces only few colonies (possibly viable due to additional mutations) on Thio[10] and already significantly decreased growth on Thio[1] (App. A). Although these results are preliminary and further repetitions and documentation are required, they suggest that *sppA* mutants are more sensitive to upregulation of AfsK kinase activity than the *sppA*$^+$ strains.

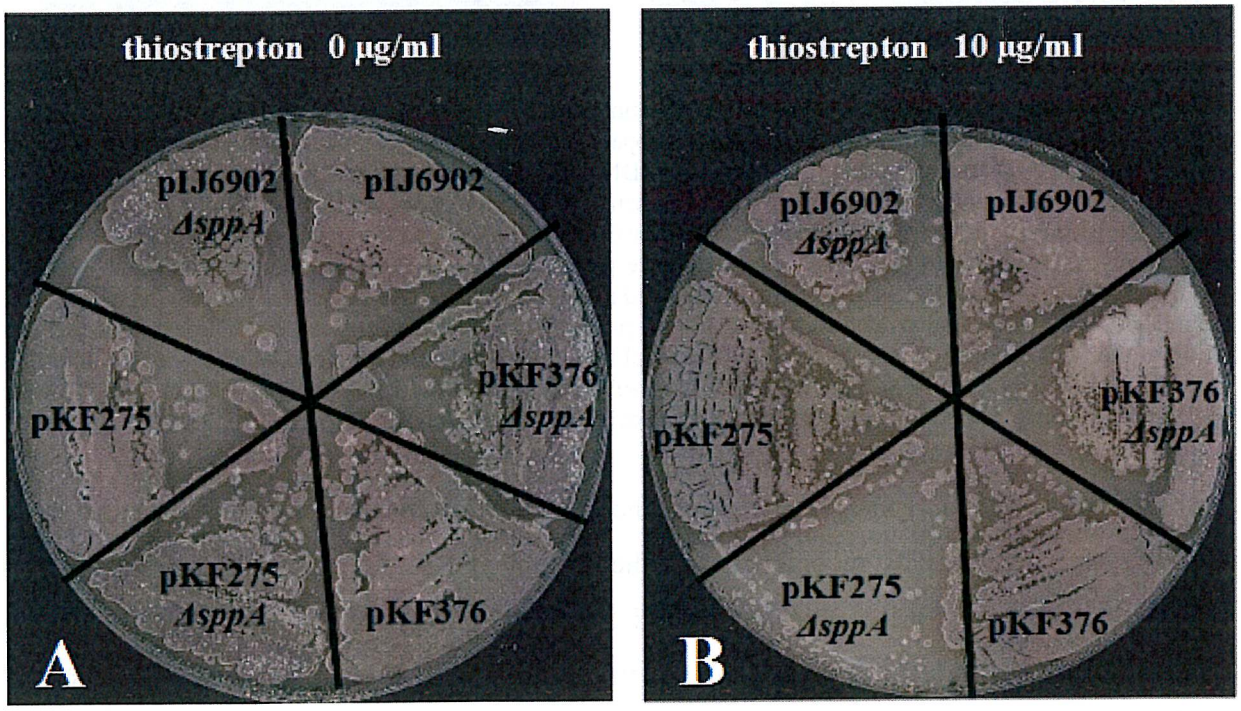

Fig. 3 **Sensitivity of *sppA* mutants to *afsK* overexpression.** To investigate, if *sppA* knockout leads to increased sensitivity to *afsK* overexpression, the strains M600/pKF376 (carries an extra copy of *afsK* under control of the thiostrepton-inducible promoter *tipAp*), M600/pK275 (carries *tipAp-afsK**, AfsK* is a constitutively active form of AfsK) and M600/pIJ6902 (empty *tipAp*-vector as control) are used together with their *ΔsppA* variants. Different levels of *tipAp*-induction were achieved by plating the strains on SFM Apra[25] with different thiostrepton concentrations (0 and 10 µg/ml are shown here, 0.01, 0.1 and 1 µg/ml in App. A) **(A)** on the control plate without thiostrepton all strains show normal growth and sporulation phenotypes with slightly smaller colonies in the *ΔsppA* variants. **(B)** While the empty vector control shows with and without *sppA* only slightly smaller colonies (probably due to the high thiostrepton concentration), *afsK* and *afsK**-overexpressing *sppA*$^+$ strains show only small colonies confirming previous data[(5)]. Overexpression of *afsK* leads in M600/pKF376 *ΔsppA* to extremely small colonies and low sporulation (probably due to slow growth) and overexpression of constitutive *afsK** in M600/pKF275 *ΔsppA* inhibits growth completely, and affects growth already at lower thiostrepton concentrations (App. A).

3.3 Confirmation of *ΔsppA* knockout

Correct knockout of *sppA* phosphatase gene by the *vph-oriT* cassette on the C08 cosmid was confirmed by PCR (Fig. 4A) and restriction (Fig. 4B).

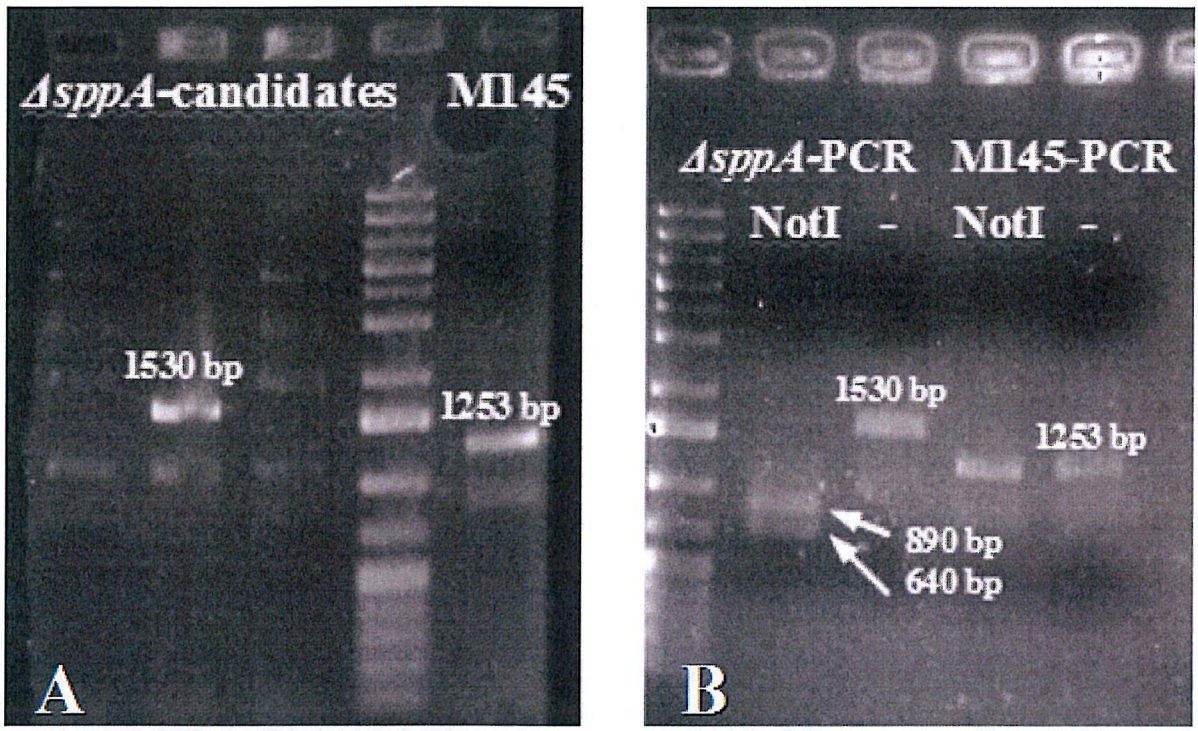

Fig. 4 **Test of *sppA* disruption on cosmid** to confirm insertion of the *vph-oriT* cassette at the correct locus. Given sizes in base pairs are the expected values, consistent with the apparent fragment size, as judged from the used GeneRuler™ 1 kb Plus DNA Ladder (Fermentas). M145 chromosomal DNA as control. **(A)** PCR amplification of the *sppA* locus. Used primers are located outside the *sppA* gene to detect change in distance occurring by insertion (*vph-oriT* cassette is bigger than the wild type *sppA* sequence and much smaller than the the Tn*5062* disrupted *sppA* (>4kbp)). One of the candidate strains showed the correct $Apra^S$ Vio^R resistance pattern producing the expected 1530 bp band while the control (M145 chromosomal DNA used as template) showed a band at 1253 bp due to the undisrupted *sppA* locus. Two other candidates showed several weak bands, probably unspecific PCR products from the Tn*5062* insertion. **(B)** *NotI* restriction of the PCR products shown in panel A. The amplified disruption allele gave two smaller bands of expected size (product additionally cleaved at *NotI* site in the *vph-oriT* cassette) and the 1530 bp band if unrestricted. Control (M145-DNA as template) shows no restriction due to absence of NotI restriction site in *sppA*.

The transfer of this *ΔsppA::vph-oriT* allele onto the *S. coelicolor* chromosome to replace the native $sppA^+$ allele was achieved by recombination. The correct and stable allelic recombination by a double crossover between the conjugated cosmid and the chromosome was confirmed by gain of viomycin-resistance (at least one recombination) and loss of kanamycin-resistance (second recombination and loss of not integrated cosmid parts as it has no *S. coelicolor* origin of replication). PCR tests and Southern Blots to confirm the gene replacement could not be completed within this project due to time limitation.

A complementation test with pKF240 (plasmid carrying the *sppA* gene, App. C) of the new strains JK II-VI was carried out to confirm that the observed phenotypes, e.g. slow growth, are due to the inactivation of *sppA* (Fig 5).

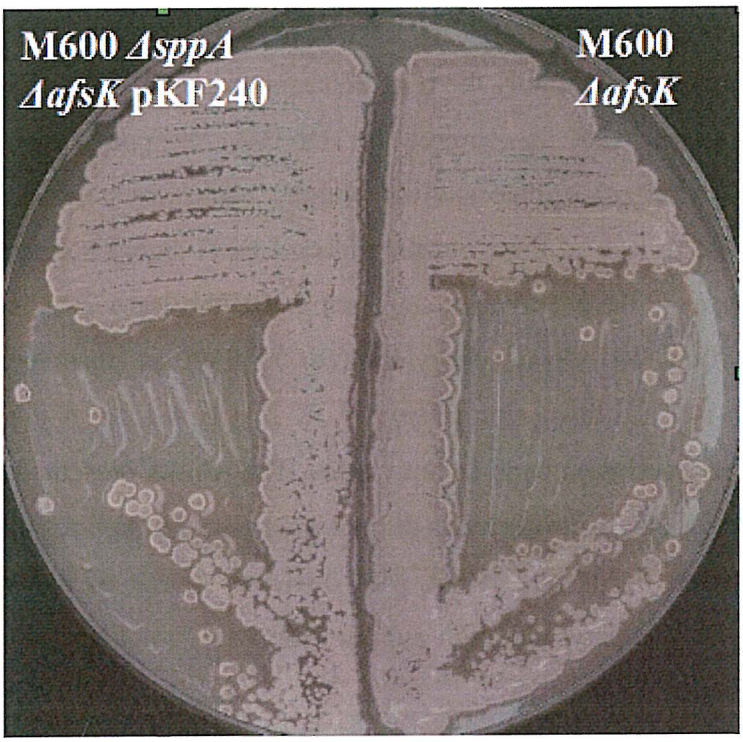

***Fig. 5.* Complementation test of *sppA* knockout with pKF240.** To confirm that the small colony phenotypes of the strains JK II-VI arised only due to the *sppA* knockout, they were complemented with pKF240 (plasmid carrying the sppA gene, App. C). The double knockout strain complementation (M600 ΔafsK ΔsppA pKF240) shows same colony size on pure SFM as M600 ΔafsK. Other strains show comparable results (data not shown).

3.4 DivIVA-phosphorylation

As can be seen in Fig. 6, the phosphorylation pattern of strain M600 supports the data from previous experiments. A fast-migrating band of DivIVA without bacitracin treatment represents nonphosphorylated DivIVA. At least one upshifted band (phosphorylation slows down the DivIVA migration in the SDS-gel)[5] are observed in the bacitracin treated sample. In contrast M600 *ΔafsK* shows also after bacitracin treatment only the band representing unphosphorylated DivIVA, without any clear mobility shift, as AfsK is required for DivIVA phosphorylation in *S. coelicolor*[5]. Unfortunately, the JK II (*ΔsppA*) sample showed without bacitracin treatment no signal and this could not be repeated due to lack of time, but the induced JK II shows a clearly upshifted band of phosphorylated DivIVA, as in the M600 case, suggesting, that the *sppA* deletion was successful and resulted in a similar effect on dephosphorylation of DivIVA as observed for the previously investigated transposon-induced disruption of *sppA*[6]. JK III (*ΔsppA ΔafsK* double mutant) showed only the basal, low phosphorylation band in both cases. The results presented in Fig. 6 have to be treated as preliminary SDS-gel electrophoresis did not give good enough resolution to clearly show discrete and well-separated bands of DivIVA with different degree of phosphorylation, and the signals varied strongly in intensity, although similar amount of total protein was loaded in each lane.

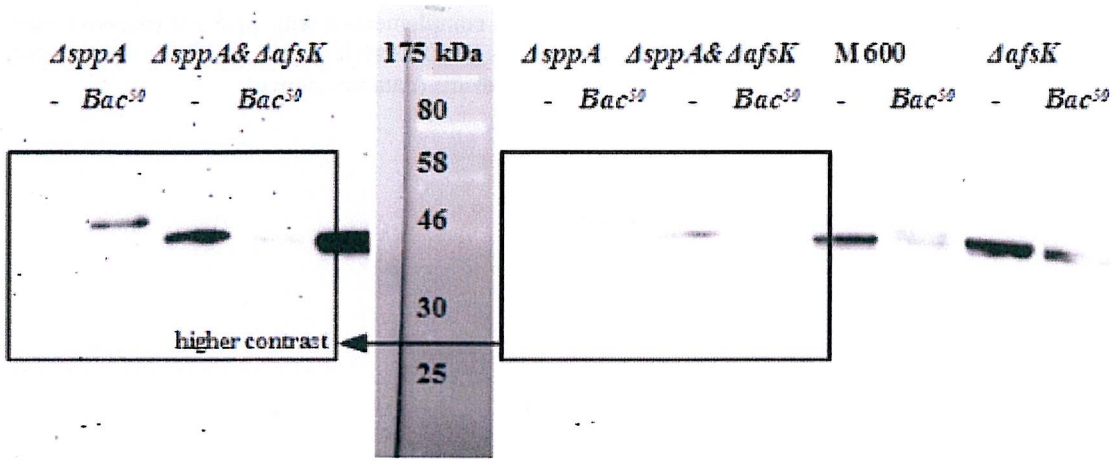

***Fig. 6.* Bacitracin-induced DivIVA phosphorylation in mutants with different combinations of *sppA* and *afsK* alleles** shown by Western blotting. Bacitracin is known to induce phosphorylation of DivIVA that can be detected by Western blotting as phosphorylation leads to significantly slower migration of DivIVA in SDS polyacrylamide gels[5]. Strains with different combinations of *sppA* and *afsK*-knockouts were treated with bacitracin to detect alteration of phosphorylation patterns. The left part of the figure shows an incresed contrast of a part of the blot shown to the right. The fast-migrating band of unphosphoylated DivIVA occurs in all samples not treated with bacitracin and in the sample from treated *ΔafsK* (phosphorylation deficient) mutants JK III and M600 *ΔafsK*, while the bacitracin-treated *ΔsppA* (dephosphorylation deficient) sample shows a more slowly migrating band typical of phosphorylated DivIVA, confirming previous data. For unknown reasons, no signal was detected for the untreated *ΔsppA* strain JK II, although here a mixture of phosphorylated and unphosphorylated DivIVA was expected[6]. In the *ΔsppA ΔafsK* double mutant also the bacitracin-treated sample showed no elevated signal suggesting, that no other kinase is involved in DivIVA phosphorylation.

4. Discussion

The created SppA phosphatase-deficient strains JK II-VI show clean knockouts of the entire *sppA* coding sequence and can be used for further research. The inserted viomycin-resistance gene is not used in other mutants associated with tip extension in the referred research papers, so that the created *sppA* knockout alleles can easily be combined and modified in future experiments.

The λ-Red-mediated recombination appeared to be very efficient. To confirm that no additional mutations, e.g. chromosomal reorganizations, affected the new strains, sequencing or physical mapping methods like Southern blotting could be used.

All five new *ΔsppA* M600 strains show slow growth phenotypes as was shown before using another knockout method and another wild type strain M145, confirming, that the slow growth phenotype arises due to phosphatase-deficiency and is not restricted to the type of mutation or *S. coelicolor* strain.

As colony size phenotypes, Western blotting, *afsK* overexpression experiments, and previous results suggest, SppA phosphatase and AfsK kinase appear to be the main (and only known) proteins affecting DivIVA phosphorylation. Western Blot results are of low resolution and can not be compared quantitatively, but give at least preliminary qualitative results. It shows an upshift of DivIVA phosphorylation for $sppA^{-}afsK^{+}$ and $sppA^{+}afsK^{+}$ after bacitracin-treatment, while the $sppA^{-}$ $afsK^{-}$ double mutant shows no upshift, suggesting that there is no additional DivIVA-phosphorylation pathway that might have been undetectable by fast DivIVA dephosphorylation in previous experiments.

Further it is found that high AfsK activity causes slow growth of an $sppA^{-}$ strain. This is supported by the partial colony size restoration due to *afsK* knockout in M600 *ΔsppA* on the phenotypical level. Lack of SppA leads to highly increased sensitivity to overexpression of *afsK* also suggesting direct counteraction. It should be kept in mind that it is not clear whether the slow growth of $sppA^{-}$ strains is due to phosphorylation of DivIVA or other substrates of the AfsK kinase, maybe even substrates of different kinases.

Unfortunately due to very slow growth and limited project time, many experiments as high resolution Western Blot and contrast phase microscopy could not be carried out or repeated to confirm strains and obtain clear-cut, fully reproducible data.

5. Future Projects

The new *ΔsppA* strains can be used for further studies, but confirmation of their genetic structures around the *sppA* locus should be carried out to confirm correct recombination. As mentioned above, high resolution SDS-PAGE and Western Blot can visualize more detailed phosphorylation patterns to confirm and improve the preliminary data shown in this report.

Next phase contrast microscopy should be done to analyze branching phenotypes of the new strains, best using also fluorescently tagged DivIVA to detect localization depending on the created genotypes. This method could shed more light on the SppA/AfsK interplay and provide further comparisons to previous experiments than only colony size phenotypes.

Another project could be to create an *afsK* complementation of the *sppA*$^{-}$*afsK*$^{-}$-strain JK III to make sure, that the regain of colony size and growth phenotypes in comparison to the *sppA*$^{-}$*afsK*$^{+}$-strain occurs because of the *afsK* knockout.

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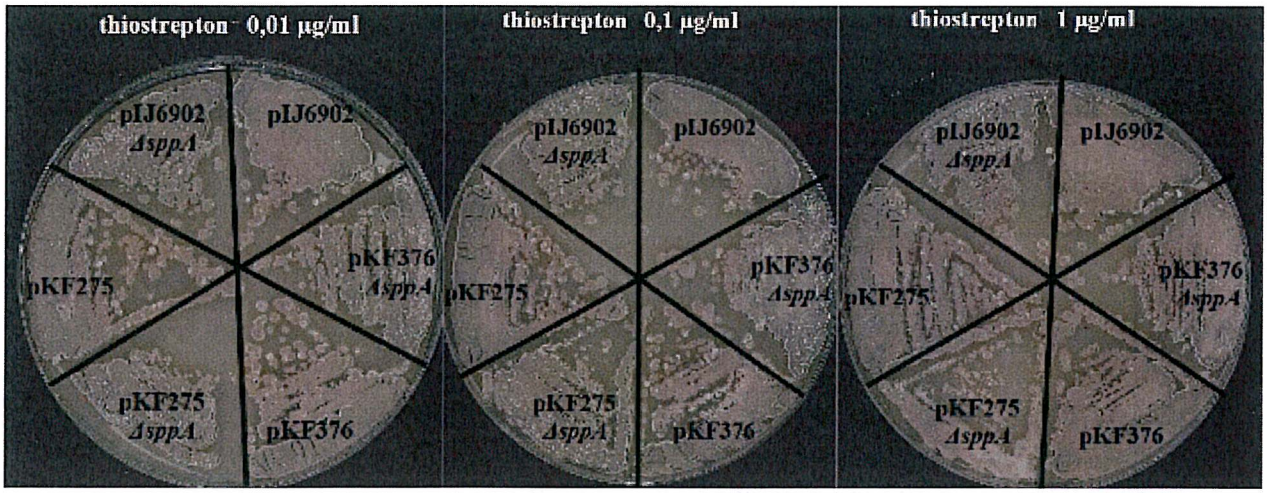
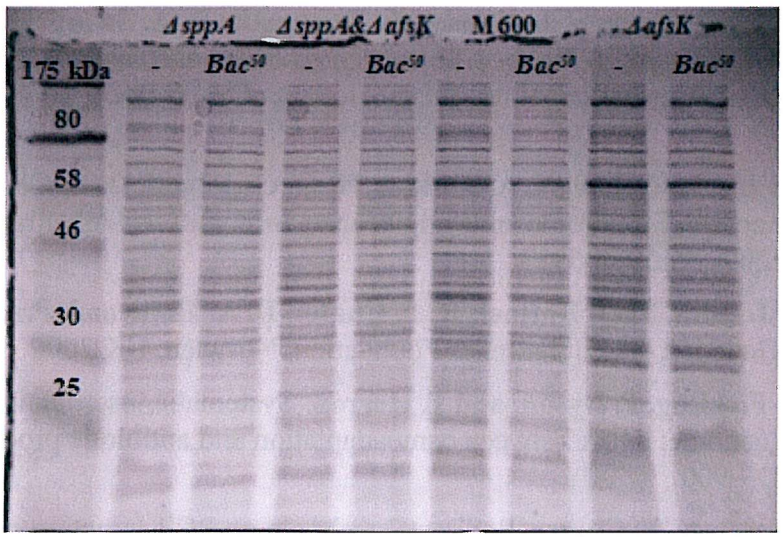
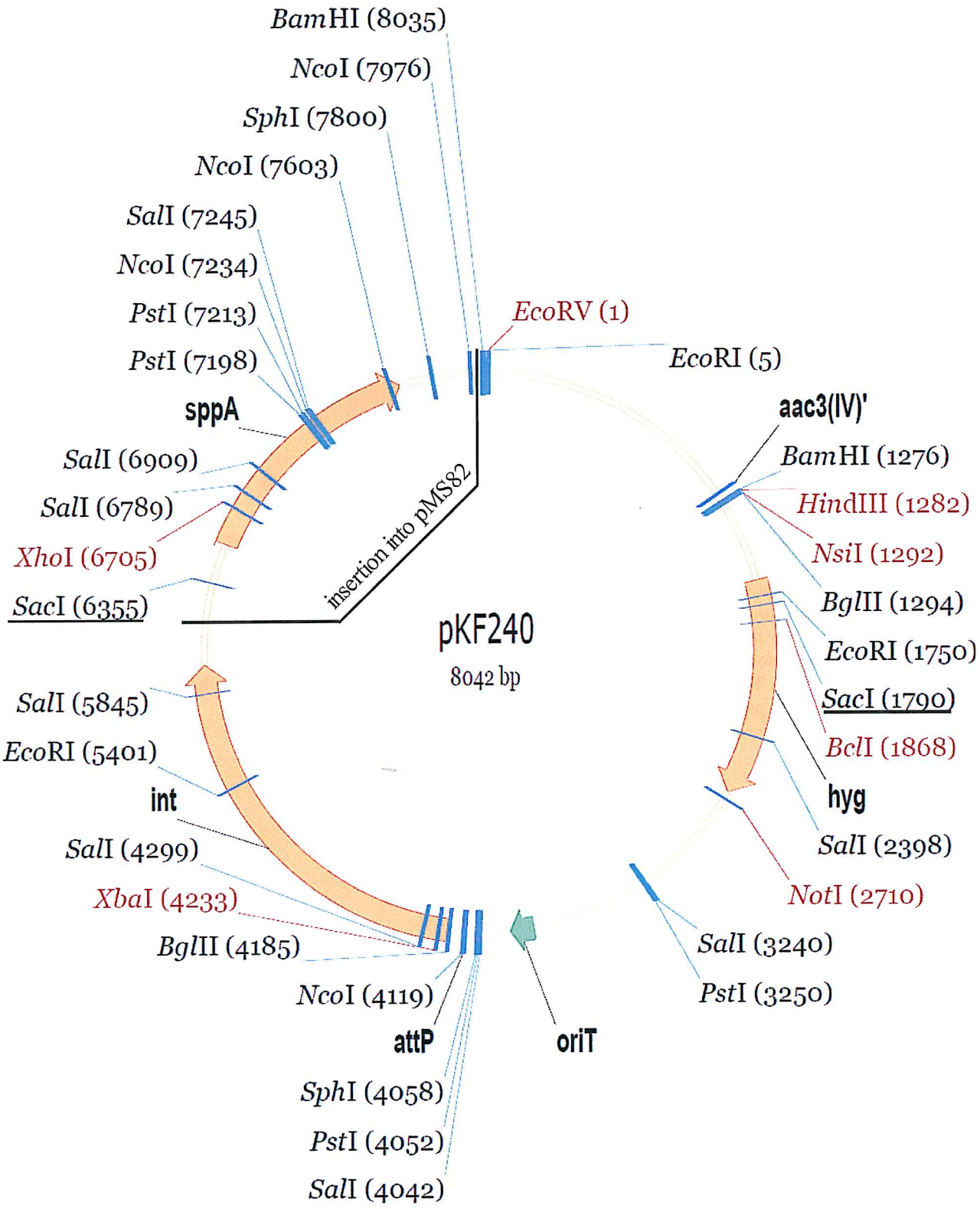
7. Appendix



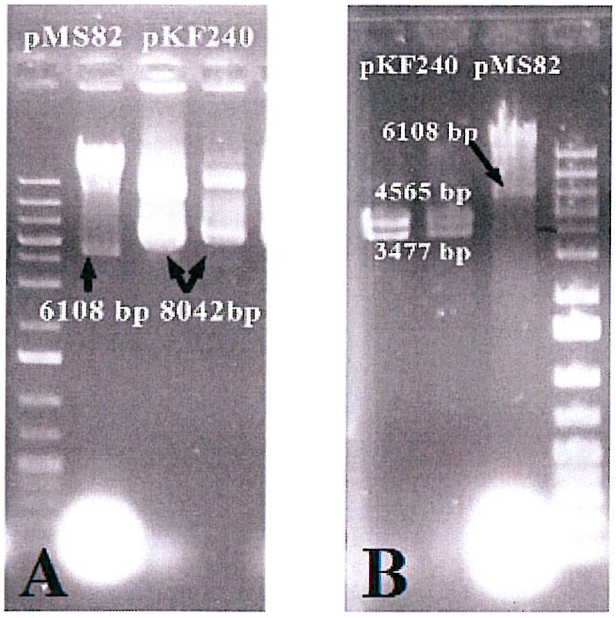
App. A (see 3.2) Colony size phenotypes of *ΔafsK* overexpression strains with intermediate induction. To investigate, if *sppA* knockout leads to increased sensitivity to *afsK* overexpression, the strains M600/pKF376 (carries an extra copy of *afsK* under control of the thiostrepton-inducible promoter *tipAp*), M600/pK275 (carries *tipAp-afsK**, AfsK* is a constitutively active AfsK) and M600/pIJ6902 (empty *tipAp*-vector as control) are used together with their *ΔsppA* variants. Different levels of *tipAp*-induction are achieved by plating the strains on SFM Apra[25] with different thiostrepton concentrations (0 and 10 µg/ml are shown previously, 0.01, 0.1 and 1 µg/ml here). Overexpression of constitutive *afsK** in M600/pKF275 *ΔsppA* inhibits growth completely at 10µg/ml, but already at 1µg/ml a significant decrease of colony size can be observed.



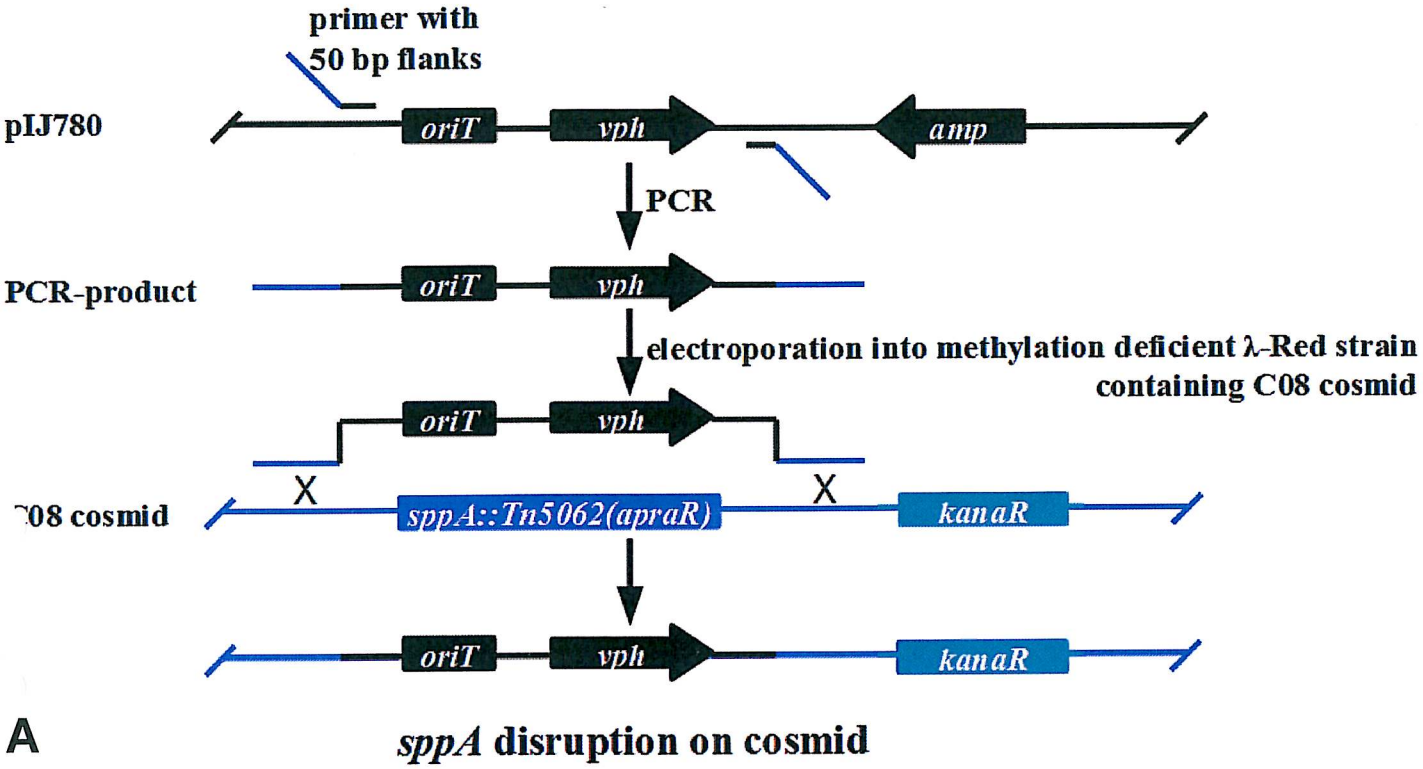
App. B (see 2.5) Coomassie Brilliant Blue staining of protein preparations of bacitracin-treated/untreated cultures (different *ΔsppA ΔafsK* variants). To get quantitatively comparable results in the Western blot of DivIVA-phosphorylation (3.4, Fig.6), the protein content of each sample was quantified and adjusted to approx. 10µg. The shown gel was processed under exactly same conditions as the gel used for Western blotting to check the quantitative comparability. The Bac^{50}/- pairs show comparable protein amounts and the slight trend of increasing protein amount on the gel from left to right cannot explain the complete lack of Western blot-signal for the *ΔsppA* Bac^{0} and low signal for *ΔsppA ΔafsK* Bac^{50} samples. Thus not the SDS-PAGE, but the Western blot method must be modified to get results of higher quality. Marker: Biolabs Prestained Protein Marker broad range 7-175 kDa.

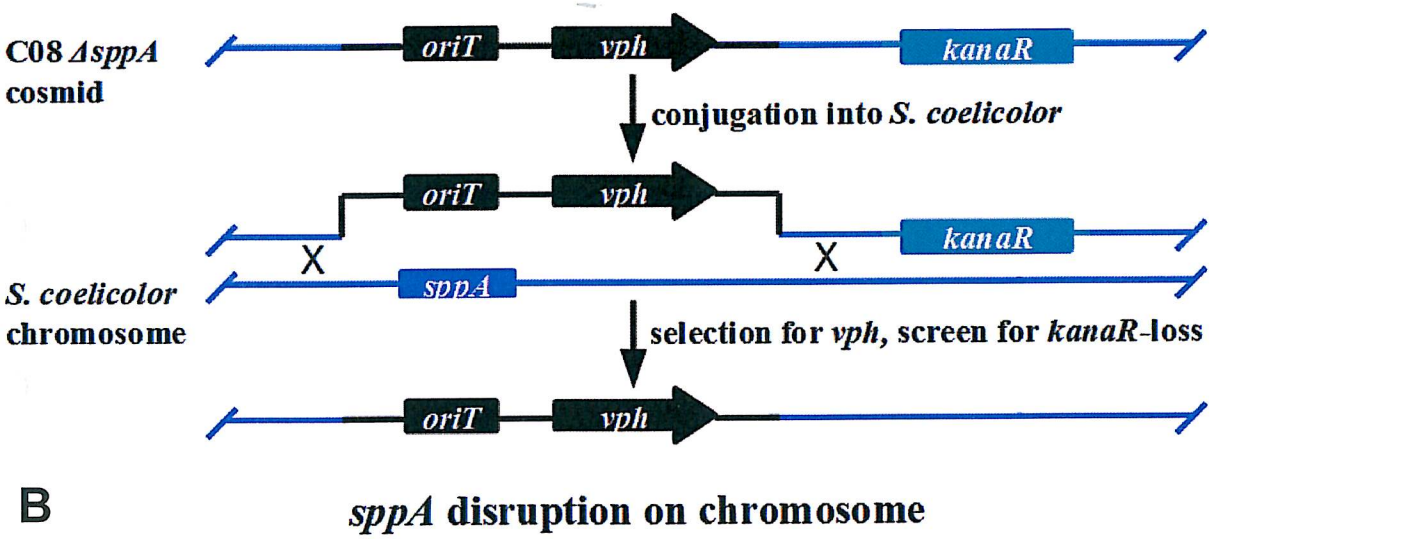


App. C (see 2.4, 3.3) Physical map of pKF240 with common restriction sites and carried genes[6]. pKF240 is the mobilizable pMS82 (6108 bp long) vector carrying the *sppA* gene and was used for complementation of *ΔsppA* strains. It has two *SacI* restriction sites (5′-GAGCT/C-3′) at 6355 and 1790 while pMS82 has only one, used to confirm correct plasmid preparation. ***sppA***: phosphatase gene. ***hyg***: hygromycin-resistance. ***int***: integrase, recombining the vector with the chromosome as it would be lost otherwise because it lacks an origin of replication in *S. coelicolor*. ***attP***: recombination site recognized by the integrase. ***oriT***: origin of transfer in conjugation.



***App. D* (see 2.4) pKF240 confirmation.** pKF240 carries the *sppA* gene cloned in the pMS82 vector and can be used for complementation of *ΔsppA* strains (for detailed plasmid map see App. C). To confirm correct pKF240 preparations, they were digested with *SacI* (New England BioLabs© Inc.) and analyzed on an 1.2% agarose gel. Given sizes in base pairs are the expected values. The size marker is GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) **(A)** Undigested pKF240 preparations and pMS82. The pMS82 (6108 bp) migrated faster since it lacks the *sppA* insert. **(B)** *SacI* digested pKF240 preparations and pMS82 vector. As pMS82 has only one restriction site, the linearized DNA appears as a band at approx. 6 kb. The pKF240 preparations show nearly full restriction by a double band around 4 kb on the DNA-ladder confirming expected structure of pKF240.





App. E* (see 2.2, 2.3) full diagram of sppA knockout generation.** **(A)** creation of *sppA* disruption by the *vph-oriT* cassette on the C08 cosmid. **(B)** transfer of the disruption onto the *S. coelicolor* chromosome. ***sppA: phosphatase gene, ***vph***: viomycin-resistance, ***amp***: ampicillin-resistance, ***oriT***: origin of conjugative transfer needed to insert cosmid DNA into the recipient *S. coelicolor* strain, ***kanaR:*** kanamycin-resistance, ***apraR:*** apramycin-resistance