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# Improvement of *Aspergillus nidulans* penicillin production by targeting AcvA to peroxisomes



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## ABSTRACT

Aspergillus nidulans is able to synthesize penicillin and serves as a model to study the regulation of its biosynthesis. Only three enzymes are required to form the beta lactam ring tripeptide, which is comprised of L-cysteine, L-valine and L-aminoadipic acid. Whereas two enzymes, AcvA and IpnA localize to the cytoplasm, AatA resides in peroxisomes. Here, we tested a novel strategy to improve penicillin production, namely the change of the residence of the enzymes involved in the biosynthesis. We tested if targeting of AcvA or IpnA (or both) to peroxisomes would increase the penicillin yield. Indeed, AcvA peroxisomal targeting led to a 3.2-fold increase. In contrast, targeting IpnA to peroxisomes caused a complete loss of penicillin production. Overexpression of *acvA*, *ipnA* or *aatA* resulted in 1.4, 2.8 and 3.1-fold more penicillin, respectively in comparison to wildtype. Simultaneous overexpression of all three enzymes resulted even in 6-fold more penicillin. Combination of *acvA* peroxisomal targeting and overexpression of the gene led to 5-fold increase of the penicillin titer. At last, the number of peroxisomes was increased through overexpression of *pexK*. A strain with the double number of peroxisomes produced 2.3 times more penicillin. These results show that penicillin production can be triggered at several levels of regulation, one of which is the subcellular localization of the enzymes.

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#### 1. Introduction

The discovery of the ß-lactam antibiotic Penicillin in 1928 by Alexander Fleming represents a major revolution in medicine and public health and thereby a milestone in human history (Diggins, 1999). Many bacterial infections, which in previous times often caused the death of patients and even resulted in several worldwide epidemics, suddenly could be cured. However, from the first description of the inhibition of bacteria through *Penicillium notatum* cultures to the first human trials or even general application of the antibiotic, many years of research passed. One of the major problems at the time was the yield of penicillin. Even nowadays increasing the yield of penicillin production is still an active research field (Weber et al., 2012).

Penicillin is produced only by a few fungal species, such as *Penicillium chrysogenum* or *Aspergillus nidulans*. Although the molecule can be chemically synthesized since 1957, the worldwide production is still based on the biotechnological process using mainly *P. chrysogenum* (Sheehan and Henery-Logan, 1957). Even if *A. nidulans* strains produce much less penicillin, it is an extremely valuable model system to unravel the molecular

biological basis of the biosynthesis and the enormous complexity of its regulation. *A. nidulans* is amenable to many sophisticated molecular biological methods and in addition offers the great potential of classical genetics (Todd et al., 2007).

The biochemical pathway of penicillin biosynthesis consists of only a few enzymatic steps. First, the non-ribosomal peptide synthetase AcvA activates sequentially the three amino acids  $L-\alpha$ -aminoadipic acid, L-cysteine and L-valine with ATP and forms a tripeptide, which is released from the enzymes by the internal thioesterase activity. This enzyme is encoded by acvA (pcbAB in P. chysogenum). Next, a cyclase converts the tripeptide into a ring structure, isopenicillin N (IPN), the first intermediate with antibiotic activity. The enzyme requires Fe<sup>2+</sup>, molecular oxygen and ascorbate. The enzyme is called IPN synthase and is encoded by ipnA (pcbC). Finally, in A. nidulans and P. chrysogenum isopenicillin N is further converted to penicillin G by a ligase and an acyltransferase, AatA in A. nidulans and PenDE in P. chrysogenum. This reaction requires activated phenylacetic acid as substrate. The activation occurs through coenzyme A (CoA). The AatA enzyme catalyzes two catalytic steps, the removal of the L- $\alpha$ -aminoadipic acid side chain and its replacement with phenylacetic acid. AatA is thus a bifunctional enzyme and consists of two subunits, one of which is posttranslationally processed (García-Estrada et al., 2008). The genes encoding the three main enzymes, AcvA, IpnA, and AatA are clustered in the genome (Aharonowitz and Cohen, 1992; Barredo et al., 1989).

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Many parameters have been described to control the expression of the genes and thereby change the penicillin titers, among which are the carbon source, the nitrogen source, the interaction with other microorganisms, the pH but also light and corresponding light regulators (Atoui et al., 2010; Brakhage, 2013; Kato et al., 2003; Litzka et al., 1999; Nützmann et al., 2013; Nützmann et al., 2011; Ozcengiz and Demain, 2013). Recently, a novel strategy was applied to increase the penicillin yield. The authors achieved the removal of toxic metabolic by-products by overepxression of glyoxalase I and II in *P. chrysogenum*. This increased the yield by 10% (Scheckhuber et al., 2013).

Despite the large body of evidence for transcriptional and epigenetic control of the penicillin biosynthesis gene cluster, much less is known about the effect of compartmentalization of the enzymes of the pathway (Roze et al., 2011; van de Kamp et al., 1999). It was discovered that the last enzyme of the pathway, AatA, localizes to peroxisomes (García-Estrada et al., 2008; Meijer et al., 2010; Müller et al., 1992; Spröte et al., 2009). Thus the biosynthesis involves probably the vacuole as a reservoir for amino acids (the building blocks of penicillin), the cytoplasm for two enzymatic reactions and the peroxisome for the final two steps, the replacement of the L- $\alpha$ -aminoadipic acid by phenylacetic acid. Phenylacetic acid needs to be activated by CoA and hence, both molecules need to be imported into peroxisomes as well, because the activating enzymes also reside in these organelles. The compartmentalization thus requires the shuttle of the substrates: export of amino acids from the vacuole, import of isopenicillin N along with phenylacetic acid and CoA into peroxisomes, export of penicillin G to the cytoplasm and finally the secretion into the medium (Martín et al., 2010; Roze et al., 2011). The substrate flow is only poorly understood, but recently a transporter for phenylacetic acid is identified in the peroxisomal membrane (Fernández-Aguado et al., 2013).

After illustrating the complexity of penicillin biosynthesis it is obvious that improvement of penicillin production can only be a multilayered and continuous process. Overcoming one bottleneck in the biosynthesis normally produces new bottlenecks, which

Table 1

Strains used in this study. Only the relevant genotypes are noted in brackets.

Name	Genotype	Source
SJR2	FGSCA1153 X SKV103#G4; pyroA4, pyrG89, nkuA::bar, veA+	Julio Rodriguez-Romero, KIT
SAH06	SJR2 with pAH05 ( <i>nat</i> ( <i>p</i> ):: <i>ipnA</i> :: <i>PTS</i> 1)	This study
SAH07	SJR2 with pAH04 ( <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS</i> 1)	This study
SAH08	SAH14 with pAH28 (nat(p)::acvA::PTS1 and nat(p)::ipnA::PTS1 and alcA(p)::ipnA)	This study
SAH10	SJR2 with pYZ12 (alcA(p)::mRFP::acuE)	This study
SAH11	SJR2 with pYZ12 and pAH06 (co-transformation); ( <i>alcA</i> ( <i>p</i> ):: <i>pexK</i> and <i>alcA</i> ( <i>p</i> ):: <i>mRFP</i> :: <i>acuE</i> )	This study
SAH12	SJR2 with pAH06 ( <i>alcA</i> ( <i>p</i> ):: <i>pexK</i> )	This study
SAH13	SAH07 and pAH06 ( <i>alcA</i> ( <i>p</i> ):: <i>pexK</i> and <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> )	This study
SAH14	SJR2 with pAH12 ( <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> )	This study
SAH16	SJR2 with pAH14 (alcA(p)::acvA)	This study
SAH19	SAH07 with pAH10 ( <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> and <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> )	This study
SAH21	SAH14 with pAH6 ( <i>alcA</i> ( <i>p</i> ):: <i>pexK</i> and <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> )	This study
SAH23	SJR2 with pAH27 (alcA(p)::CFP::ipnA::PTS1)	This study
SAH24	SJR2 with pAH15 ( <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> )	This study
SAH25	SJR2 with pAH14 and pAH15 (co-transformation); ( <i>alcA</i> ( <i>p</i> ):: <i>acvA</i> and <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> )	This study
SAH28	SAH25 with pAH23 ( <i>alcA</i> ( <i>p</i> ):: <i>acvA</i> and <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> and <i>alcA</i> ( <i>p</i> ):: <i>aatA</i> )	This study
SAH30	SAH14 with pAH23 ( <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> and <i>alcA</i> ( <i>p</i> ):: <i>aatA</i> )	This study
SAH31	SAH14 with pAH10 ( <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> and <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> )	This study
SAH33	SAH23 with pYZ12 (alcA(p)::GFP::ipnA::PTS1 and alcA(p)::mRFP::acuE)	This study
SAH34	SAH14 with pAH21 and pYZ12 (co-transformation); (alcA(p)::GFP::acvA::PTS1 and alcA(p)::mRFP::acuE)	This study
SAH36	SJR2 with pAH23 (alcA(p)::aatA)	This study
SAH37	SAH14 with pAH09 (alcA(p)::acvA::PTS1)	This study
SAH38	SAH6 with pAH12 ( <i>nat</i> ( <i>p</i> ):: <i>acvA</i> :: <i>PTS1</i> and <i>nat</i> ( <i>p</i> ):: <i>ipnA</i> :: <i>PTS1</i> )	This study

Each strain used for penicillin bioassays is made in triplicates by independent transformations.

#### Table 2

Plasmids used in this study.

Name	Genotype	Source
pMCB17apx	pMCB17 version for fusion of GFP to N-termini of proteins of interest	V. P. Efimov, Piscataway, USA
pJET1.2 blunt	Blunt-end PCR fragments cloning vector	Fermentas
pAH04	pJET1.2 blunt with acvA <sup>1 kb C-term</sup> ::PTS1::pyroA::acvA <sup>1 kb 3'UTR</sup>	This study
pAH05	pJET1.2 blunt with <i>ipnA</i> <sup>full</sup> ::PTS1::pyroA::ipnA <sup>1 kb 3'UTR</sup>	This study
pAH06	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>pexK</i> <sup>full</sup> ; <i>pyr4</i> (without <i>GFP</i> )	This study
pAH09	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>acvA</i> <sup>1 kb N-term</sup> ; <i>pyr4</i> (without <i>GFP</i> )	This study
pAH10	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> <sup>full</sup> ; <i>pyr4</i> (without <i>GFP</i> )	This study
pAH11	pMCB17apx with pyroA instead of pyr4; with alcA(p) but without GFP	This study
pAH12	pAH11 with alcA(p)::acvA <sup>1 kb C-term</sup> ::PTS1; pyroA	This study
pAH13	pAH11 with alcA(p)::ipnA <sup>full</sup> ::PTS1; pyroA	This study
pAH14	pAH11 with <i>alcA</i> ( <i>p</i> ):: <i>acvA</i> <sup>1 kb N-term</sup> ; <i>pyroA</i>	This study
pAH15	pAH11 with <i>alcA</i> ( <i>p</i> ):: <i>ipnA</i> <sup>full</sup> ; <i>pyroA</i>	This study
pAH21	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>GFP</i> :: <i>acvA</i> <sup>1 kb N-term; <i>pyr4</i></sup>	This study
pAH23	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>aatA</i> <sup>full</sup> :: <i>PTS1</i> ; <i>pyr4</i> (without <i>GFP</i> )	This study
pAH27	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>GFP</i> :: <i>ipnA</i> <sup>full</sup> :: <i>PTS1</i> ; <i>pyr4</i>	This study
pAH28	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>ipnA<sup>full</sup>::PTS1</i> ; <i>pyr4</i> (without <i>GFP</i> )	This study
pYZ12	pMCB17apx with <i>alcA(p)::mRFP::acuE</i> ; <i>pyroA</i>	Ying Zhang
pYZ14	pMCB17apx with <i>alcA</i> ( <i>p</i> ):: <i>apsB</i> ; <i>pyr4</i> (without <i>GFP</i> )	Ying Zhang

need to be overcome. One obvious bottleneck is the enzymatic activity, and thus it is not surprising that the first high-production strains generated by random mutagenesis carry several copies of the entire penicillin gene cluster (Fierro et al., 1995; Wang et al., 2014). This rational was also followed in early approaches applying molecular biology, namely the overexpression of individual genes (Fernández-Cañon and Peñalva, 1995). Even the combination of increased numbers of the gene cluster in the production strains with overexpression of individual genes led to higher yields, illustrating the high potential of this approach (Weber et al., 2012). However, improvement of the substrate flow could also be achieved by modifications of the compartmentalization.

In this paper we studied the impact of changes of the subcellular residence of the main enzymes of penicillin biosynthesis in *A. nidulans*. Whereas targeting of IpnA to peroxisomes caused a complete loss of the biosynthetic capacity, targeting AcvA to peroxisomes caused a 3.2-fold increase of the penicillin titer.

#### 2. Materials and methods

#### 2.1. Strains, plasmids and culture conditions

Supplemented minimal (MM) media for *A. nidulans* was prepared as described and standard strain construction procedures were used (Hill and Käfer, 2001). Expression of tagged genes under the control of the *alcA* promoter was induced by threonine (Schier et al., 2001; Waring et al., 1989). A list of *A. nidulans* strains used in this study is given in Table 1. *Escherichia coli* Top 10F' was used for cloning (Sambrook and Russel, 1999). Plasmids are listed in Table 2 and oligonucleotides in Table 3.

#### 2.2. Molecular techniques

Standard DNA transformation procedures were used for *A. nidulans* (Yelton et al., 1984) and *E. coli* (Sambrook and Russel, 1999). For PCR experiments, standard protocols were applied using a Biometra Personal Cycler (Biometra, Göttingen, Germany) for the reaction cycles. Genomic DNA was extracted from *A. nidulans* according to Zekert et al. (2010). All plasmids and strains with a peroxisomal targeting signal 1 were sequenced. DNA sequencing was done commercially (MWG Biotech, Ebersberg, Germany).

## 2.3. Tagging of AcvA

In order to generate a construct for *acvA* replacement, a fusion PCR approach was followed (Szewczyk et al., 2006). We first amplified the left and the right borders of the fusion construct using primers P1\_acvA\_PTS, P3\_acvA\_PTS and P4\_acvA\_PTS, P6\_acvA\_PTS. Primers PTS1\_pyroA\_fwd and pyroA\_rev were used to amplify the middle part. Primers P2\_acvA\_PTS and P5\_acvA\_PTS were used to amplify the whole fusion construct out of the three parts with overhangs. This resulting construct was ligated to the vector pJET1.2/blunt resulting in pAH04 and transformed into *A. nidulans* strain SJR2 resulting in SAH07. Homologous integration was confirmed by PCR using three primer pairs (P1\_acvA\_PTS and P5\_acvA\_PTS). The PCR product of the last primer pair was sequenced to confirm the PTS1.

Alternatively, a second plasmid was constructed, in which a PTS1 tagged 1 kb fragment of the 3'-end of *acvA* was under the control of the *alcA*-promoter (pAH12). This vector was transformed into SJR2 resulting in *A. nidulans* SAH14. The PTS1 tagged *acvA* gene was under the control of the natural promoter. Homologous integration of the circular plasmid was confirmed by PCR

Table 3	
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Oligonucleotides used in this study.

Name	Sequence (5'-3')
P1_acvA_PTS	GCTTGCGAGAATGCCTTAG
P2_acvA_PTS	CTGGGCCAGCTGACAAG
P3_acvA_PTS	GAGGAAGCAGTCAAAGCTTGGAATATCTGGCGAGAAATTTCT-
	CGATT
P4_acvA_PTS	GCGACGAATGATGAAGATCTAAAGTGATGCCAACAACGTATG
P5_acvA_PTS	TGGCAAGTAACTAATTAACGGC
P6_acvA_PTS	ACTGTTAGCCACCTTTGCTG
P1_ipnA_PTS	CAGAAACGTCTGTTCTTCGC
P3_ipnA_PTS	GAAGAGCATTGTTTGAGGCGTCAAAGCTTGGAGGTCTGGCC-
	GTTCITGTT
P4_ipnA_PTS	ATCAGTGCCTCCTCTCAGACAGAAGCGAGGGGTGTGGAAC
P5_ipnA_PTS	CCGATCTGGAAGTATAGGC
P6_ipnA_PTS	CGCCTGTGTCTTTTTTGTTTTGC
pyrG_fwd	CGCCTCAAACAATGCTCTTC
pyrG_rev	CTGTCTGAGAGGAGGCAC
PTS1_pyroA_fwd	AAGCTTTGACTGCTTCCTCAAC
pyroA_rev	GATCTTCATCATTCGTCGCTTTC
KpnI_pexK_fwd	CAGGTACCATGGTTGCAGACGCTCTG
PacI_pexK_re	GTTAATTAATTAAGCGGTCTTTCGCCATTG
KpnI_acvA_1000_f	GAGGTACCATGAGCCCTCCCGGGC
acvA_1000_PacI_re	CTTAATTAATTTGTACACGATGGCAAC
KpnI_ipnA_fwd	GAGGTACCATGGGTTCAGTCAGCAAAGC
ipnA_PacI_rev	CTTAATTAATCAGGTCTGGCCGTTCTTG
XhoI_Efimov_fwd	GAAGCTCGAGGTAACAACTAATTGTACTTGGACAAG
AscI_alcA_re	AGGCGCGCCGCTAATTAACTGAGTAGAGAACTGT
AscI_acvA_1000_f	AGGCGCGCCATGAGCCCTCCCGGGC
AscI_ipnA_fwd	AGGCGCGCCATGGGTTCAGTCAGCAAAGC
AscI_aatA_fwd	AGGCGCGCCATGCTTCACGTAACTTGCCAAG
aatA_full_PacI_r	CTTAATTAATCAAATGTTGGCTTGGATCGC
Efimov_seq_re	GTGGAATTGTGAGCGGATAAC
AscI_acvA_1000_	AGGCGCGCCGACAAGCAAGCAATCGATAAC
C-term_f	
acvA_PTS1_PacI_re	GTTAATTAATCAAAGCTTGGAATATCTGGCG
ipnA_PTS1_PacI_re	GTTAATTAATCAAAGCTTGGAGGTCTGG
alcA_seq_fwd	CAAGGTTCTCAGTCTCACCA
pyrG 5′raus	CAATTGCGACTTGGACGAC
pyrG 3′ raus	GAGTATGCGGCAAGTCATG
pyro 5'raus	CGTCAGGAACAGCTGGAAACGCC
pyro 3'raus	GGCCAAGAGAGGATGGTAATTGC
acvA_1350_re	GCTCAAGCAATGCTCTAGAC

using the primers P1\_acvA\_PTS and Efimov\_seq\_re. The PCR product was sequenced to confirm the PTS1.

To get a strain with overexpressed acvA-PTS1, primers KpnI\_acvA\_1000\_f and acvA\_1000\_PacI\_re were used to amplify 1 kb of the 5'-end of acvA. Double digestion with KpnI and PacI of this PCR product together with pMCB17apx and the following ligation led to pAH09, which harbored 1 kb of the 5'-end of acvA under the control of the alcA-promoter. pAH09 was transformed into A. nidulans SAH14 resulting in SAH37. Homologous integration of the circular plasmid was confirmed by PCR using the primers alcA\_seq\_fwd and acvA\_1350\_re. To test the combinations with a strain harboring the PTS1 tagged *acvA* gene under the natural promoter, strains SAH19 and SAH31 were created for the additional overexpression of *ipnA* and strain SAH30 for the additional overexpression of aatA. Primers KpnI\_ipnA\_fwd and ipnA\_PacI\_rev were used to amplify the full ipnA gene. The PCR product and pMCB17apx were double digested with KpnI and PacI and ligated resulting in pAH10. pAH10 was transformed in SAH07 to get SAH19 and in SAH14 to get SAH31. Integration of pAH10 was confirmed by PCR using primers alcA\_seq\_fwd and ipnA\_PacI\_rev. PCR with primers AscI\_aatA\_fwd and aatA\_full\_PacI\_r, double digestion of the PCR product and pMCB17apx with AscI and PacI and the following ligation led to pAH23 which was transformed to SAH14 resulting in SAH30. Integration was confirmed by PCR using primers alcA\_seq\_fwd and aatA\_full\_PacI\_r.

## 2.4. Tagging of IpnA

In order to test whether targeting of IpnA increased penicillin production, a PTS1 targeting signal was added in a fusion PCR approach. Primers P1\_ipnA\_PTS and P3\_ipnA\_PTS were used to amplify the left border, primers P4\_ipnA\_PTS and P6\_ipnA\_PTS for the right border and primers pyrG\_fwd and pyrG\_rev for the middle part. Each of these parts was linked with overhangs in a PCR reaction using the primers P2\_ipnA\_PTS and P5\_ipnA\_PTS. The resulting PCR product was cloned with blunt ends into pJET1.2 (pAH05). pAH05 was transformed into *A. nidulans* strain SJR2 resulting in strain SAH06. Homologous integration was confirmed by PCR using three primer pairs (P1\_ipnA\_PTS and P5\_ipnA\_PTS). The PCR product of the last primer pair was sequenced to confirm the PTS1.

Strain SAH38 was constructed by transformation of pAH12 in SAH06. This strain harbored all three penicillin enzymes (AcvA, IpnA and AatA) in the peroxisomes.

# *2.5. Co-localization of penicillin enzymes with a peroxisomal marker*

Peroxisomal localization of the enzymes was proven with AcuE, a common peroxisomal marker. Double digestion of the vectors pAH13 and pMCB17apx with *AscI* and *PacI* and ligation of the insert of pAH13 and the backbone of pMCB17apx resulted in the plasmid pAH27.

First a strain with GFP tagged IpnA was constructed. pAH27 was transformed into SJR2 and the transformants were screened microscopically resulting in SAH23. The plasmid pYZ12 which harbored mRFP labeled *acuE* under the control of the *alcA*-promoter was transformed into SAH23 resulting in SAH33. For labeling *acvA* with GFP under the control of the *alcA*-promoter and to get a strain with both GFP labeled *acvA* with a PTS1 under the control of the *alcA*-promoter a co-transformation was necessary. A plasmid with a GFP labeled 1 kb part of the *acvA* 5′-end (pAH21) was created by PCR using the primers AscI\_acvA\_1000\_f and acvA\_1000\_PacI\_re, a double digestion of the PCR product and pMCB17apx with *AscI* and *PacI* and ligation of these two parts. pAH21 was transformed together with pYZ12 into SAH14 resulting in SAH34. The transformants were screened microscopically.

## 2.6. Increase of the number of peroxisomes

To test the effect of higher numbers of peroxisomes on penicillin yield *pexK* was overexpressed. Peroxisomes were visualized with AcuE (see above). pYZ12 was transformed into SJR2 to count the number of peroxisomes in wildtype (SAH10). For *pexK* overexpression the full gene was amplified with KpnI\_pexK\_fwd and PacI\_pexK\_re. The resulting PCR product and the plasmid pMCB17apx were double digested with *KpnI* and *PacI* and ligated, resulting in pAH06. pAH06 and pYZ12 were co-transformed into SJR2 resulting in SAH11. SAH10 and SAH11 were screened microscopically. To test the penicillin yield pAH06 was transformed into SJR2 (SAH12).

## 2.7. Overexpression of the penicillin genes

For overexpression of *acvA* primers Ascl\_acvA\_1000\_f and acvA\_1000\_Pacl\_re for PCR and *Ascl* and *Pacl* for double digestion followed by ligation were used to get 1 kb of the *acvA* 5'-end under the control of the *alcA*-promoter (pAH14). pAH14 was transformed into SJR2 resulting in SAH16. For overexpression of *ipnA* primers Ascl\_ipnA\_fwd and ipnA\_Pacl\_rev for PCR and *Ascl* and *Pacl* for

double digestion followed by ligation were used to get the full *ipnA* gene under the control of the *alcA*-promoter (pAH15). pAH15 was transformed into SJR2 resulting in SAH24. For overexpression of *aatA* plasmid pAH23 was transformed into SJR2 (SAH36). For the combination of overexpressions co-transformation of pAH14 (*acvA*) and pAH15 (*ipnA*) into SJR2 were preformed (SAH25). For overexpression of all three enzymes, pAH23 (*aatA*) was transformed into SAH25 resulting in SAH28. Homologous integration of pAH14 was confirmed by PCR using primers alcA\_sec\_fwd and acvA\_1350\_re. Integration of pAH15 was confirmed by PCR using primers alcA\_seq\_fwd and aatA\_full\_PacI\_r.

#### 2.8. Light and fluorescence microscopy

For microscopy cells were grown on cover slips in 0.5 ml minimal medium (MM) containing 2% threonine and 0.2% glucose as carbon source. Medium was supplemented with pyridoxine, uracil, or uridine, depending on the auxotrophy of the strains. Cells were incubated at 28 °C overnight. Fluorescence was observed using standard Zeiss filter combinations no. 09 (fluorescein isothiocyanate, green fluorescent protein [GFP]) and no. 15 (monomeric red fluorescent protein 1 [mRFP]). Images were collected and analyzed with a Zeiss Axiocam and AxioVision software.

#### 2.9. Penicillin bioassay

Penicillin production medium (PPM) was prepared according to the following recipe: corn steep solids, 40 g/l; threonine, 20 g/l; CaCO<sub>3</sub>, 10 g/l; KH<sub>2</sub>PO4, 7 g/l; phenoxyacetic acid, 0.5 g/l; and uridin/uracil 1 g/l each. The medium was filled up to 400 ml of deionized water, and the pH was adjusted to 6.0 with 10 M KOH and autoclaved. Lactose (200 g/l) and MgSO<sub>4</sub>  $\cdot$  7H<sub>2</sub>O (4.5 g/l) were autoclaved separately and lactose (20%, 100 ml), MgSO<sub>4</sub> · 7H<sub>2</sub>O (1 ml), pyro (1000X, 0.1 g/100 ml H<sub>2</sub>O) and biotin (1000X, 0.1 g/ 100 ml  $H_2O$ ) were added.  $4 \times 10^6$  spores of each strain were inoculated in 50 ml medium (100 ml flask) and incubated at 26 °C in a rotary shaker at 200 rpm for 48 h. After incubation mycelium was harvested by filtration with miracloth and dried at room temperature for 48 h. After harvesting supernatants were centrifuged 1.5 min with 2340g and subjected to the penicillin bioassay, with Geobacillus stearothermophilus as indicator organism. G. stearothermophilus was incubated as preculture overnight one day before performing the penicillin assay. Plates were filled with 100 ml LB medium and holes with 0.9 cm diameter were punched into the solid media. 180 µl of the centrifuged supernatant was filled into the holes and the plates were incubated overnight at 55 °C. On the next day diameters of bacterial halos were measured and the penicillin amount was derived from a standard curve. Penicillin was related to the dry mass of the mycelium. All strains for penicillin production exist in triplicate from independent transformations.

For each experiment a penicillinase assay was performed. Supernatants were incubated with 5U penicillinase from *Bacillus cereus* (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions.

#### 3. Results

#### 3.1. Targeting AcvA to peroxisomes increases penicillin production

Three enzymes, AcvA, IpnA, and AatA are required for penicillin biosynthesis, of which AatA, the final enzyme of the pathway, is a



**Fig. 1.** Scheme of penicillin biosynthesis and strategies for peroxisomal targeting of AcvA and IpnA. (A) Scheme of the gene cluster with the three genes *acvA*, *ipnA* and *aatA*. AcvA and IpnA are cytoplasmic proteins whereas AatA is localized in the peroxisomes. LLD-ACV,  $\delta(\iota-\alpha-\text{aminoadipy})$ - $\iota-\text{cysteiny}$ - $\iota-\alpha-\text{AAA}$ ,  $\iota-\alpha-\text{aminoadipic}$  acid. (B) Strategy to target AcvA and IpnA to peroxisomes. (C) Fusion constructs or (D) circular plasmids are used to tag the genes with a PTS1. In both cases the PTS1 tagged genes are under the control of the natural promoter. In case of *ipnA* tagged with a circular plasmid, an additional copy of the gene under the control of the *alcA*-promoter is present.

peroxisomal enzyme (Fig. 1A and B). We hypothesized that changing the residence of one of the other enzymes could have an impact on penicillin biosynthesis. This hypothesis was first tested with the first enzyme of the biosynthetic pathway, AcvA. To target AcvA to peroxisomes a peroxisomal targeting signal 1 (PTS1) was added (plasmid pAH04) and the endogenous copy of acvA in strain SIR2 was replaced by the tagged version (strain SAH07) (Fig. 1C). Strain SJR2 is used, because it harbors the veA wildtype allele, and veA is an important player in the regulation of the production of penicillin and other secondary metabolites (Calvo, 2008; Kato et al., 2003). Homologous integration of the construct was confirmed by PCR followed by sequencing of the PCR product to confirm the presence of the PTS1 sequence. Alternative to gene replacement, a plasmid was constructed, in which a 1 kb fragment of the 3'-end of *acvA* was fused to the PTS1 sequence (pAH12) (Fig. 1D). This vector was transformed into SJR2 resulting in A. nidulans SAH14. Integration of the circular plasmid results in duplication of the 1 kb fragment and expression of the tagged version from the natural promoter and no expression of the truncated version if the strain is grown on glucose medium. If the alcA promoter is induced, only a non-functional protein fragment can be produced. Homologous integration of the circular plasmid and confirmation of the tagging were done by PCR and sequencing of the PCR product. In this paper all strains were done in biological triplicates of independent transformations, but only one strain is listed (Table 1). The two strains generated in the described ways (SAH07 and SAH14) showed no significant difference in penicillin production between each other, but a 3.2-fold higher production compared to wildtype.

In order to test, whether targeting of IpnA also increased penicillin production, a PTS1 targeting signal was added similarly to AcvA tagging. The resulting strain did not produce any penicillin.

The same effect occurred when all penicillin biosynthesis enzymes were targeted to peroxisomes (strain SAH38). Likewise, when additional IpnA was expressed in the cytoplasm (SAH08; transformation of pAH28 in SAH14) a 4-fold higher penicillin titer was obtained compared to that of wildtype (Fig. 2A).

To confirm peroxisomal localization of the enzymes, colocalization studies with acetate malate synthase (AcuE), a peroxisomal protein and common marker, were performed (Sandeman et al., 1991; Zekert et al., 2010). For labeling AcvA with GFP expressed from the *alcA* promoter a 1 kb fragment derived from the 5'-end of acvA was cloned in frame with GFP into pMCB17apx (pAH21). Homologous integration of the circular plasmid results in the duplication of the 1 kb fragment and placement of the fulllength open-reading frame of AcvA-PTS1 fused to GFP and driven by alcA and a truncated 1 kb fragment under the control of the endogenous promoter. pAH21 was transformed together with pYZ12 (AcuE-mRFP) into SAH14 resulting in SAH34. Similarly, IpnA-PTS1 was GFP tagged (pAH27) and transformed into SJR2 (SAH23). This strain was then transformed with pYZ12 (SAH33). Transformants were screened microscopically. Both strains, SAH33 and SAH34, showed nearly 100% co-localization of AcuE with IpnA or AcvA (Fig. 2B).

# 3.2. Increase of the number of peroxisomes results in higher penicillin production

Because targeting of AcvA to peroxisomes increased penicillin production in *A. nidulans*, and because it was previously shown in *P. chrysogenum* that higher peroxisome numbers increase penicillin



**Fig. 2.** Targeting of AcvA to peroxisomes leads to increased penicillin production. (A) Determination of the penicillin titer after 48 h growth in PPM. The mean of three biological replicates is displayed and the standard deviation is indicated. The following strains are compared: WT (strain SJR2), AcvA tagged with PTS1 (strains SAH07 and SAH14), IpnA tagged with PTS1 (strain SAH06) and the combination of the two (strain SAH38). (B) Co-localization of AcvA and IpnA with AcuE. AcvA or IpnA is tagged with GFP and AcuE with mRFP. Cultures are incubated on cover slips at 28 °C overnight and analyzed with the appropriate filter combinations in a fluorescence microscope. All genes are expressed using the *alcA*-promoter.



**Fig. 3.** Increasing numbers of peroxisomes increase penicillin production. (A, B) Visualization of peroxisomes using mRFP tagged AcuE in wildtype (WT) (strain SAH10) and a strain in which *pexK* is overexpressed (strain SAH11). (A) Strains are cultured at 28 °C overnight in microscopy medium with threonine as carbon source to induce the *alcA* promoter. (B) Quantification of peroxisomes per µm hyphal length. The number of peroxisomes is divided by the length of the hypha in µm. The mean of 50 independent hyphae is shown and the standard deviation is indicated. (C) Determination of the penicillin titer after 48 h growth in PPM. The mean of three biological replicates is displayed and the standard deviation is indicated.

production, we aimed at testing the latter effect in *A. nidulans*. An increase of the peroxisome number can be achieved by overexpression of *pexK* (Kiel et al., 2005). First, we transformed pYZ12 into SJR2, which allowed us to quantify peroxisomes in wildtype (SAH10). Next, we constructed a *pexK*-overexpression construct in pMCB17apx (pAH06). pAH06 and pYZ12 were co-transformed into



**Fig. 4.** Effect of overexpression of penicillin genes on penicillin titers. Strains are grown for 48 h in PPM and bioassay is performed as described. For overexpression the *alcA*-promoter is induced by threonine. The following strains are used: WT (strain SJR2), strain SAH16 (*alcA*(*p*)::*acvA*), strain SAH24 (*alcA*(*p*)::*ipnA*), strain SAH36 (*alcA*(*p*)::*acvA* and *alcA*(*p*)::*ipnA*), and strain SAH26 (*alcA*(*p*)::*acvA*) and *alcA*(*p*)::*acvA*).

SJR2 resulting in SAH11. SAH10 and SAH11 were screened microscopically (Fig. 3A) and the number of peroxisomes per  $\mu$ m hyphal length was counted (Fig. 3B). SAH11 showed nearly the double amount of peroxisomes per  $\mu$ m compared to SAH10. Penicillin production was 2.3-fold higher compared to wildtype (Fig. 3C).

# 3.3. Overexpression of the penicillin genes increases the penicillin titer

A first strategy for overproduction of secondary metabolites can either be to increase the number of the corresponding entire gene cluster in the genome of the production strain or to overexpress individual genes of the cluster. Sometimes also combinations of the strategies led tohigher production (Weber et al., 2012). For overexpression of acvA a 1 kb fragment derived from the 5'end of the gene was cloned behind the alcA promoter (pAH14). pAH14 was transformed into SJR2 resulting in SAH16. Homologous integration (required for overexpression of the full-length acvA gene, see above) was confirmed by PCR. The strain showed a 1.4 higher penicillin production compared to that of wildtype. The ipnA gene was constructed in a similar way and resulted in plasmid pAH15. pAH15 was transformed into SJR2 resulting in SAH24. SAH24 showed a 2.8-fold higher penicillin production compared to that of wildtype. Overexpression of aatA (pAH23 transformed into SJR2 (SAH36)) led to a 3.1-fold higher penicillin production. The values for ipnA and aatA are comparable to previous reports (Fernández-Cañon and Peñalva, 1995).

After overexpression of individual genes, we aimed at overexpression of several genes at the same time. To this end, plasmids pAH14 and pAH15 were transformed into SJR2 resulting in SAH25. SAH25 showed a 3.3 fold higher penicillin production compared to wild type. In order to create a strain with all three genes overexpressed, pAH23 was transformed into SAH25. This strain was called SAH28 and had a 6-fold higher penicillin production compared to wildtype (Fig. 4).



**Fig. 5.** Effect of the combination of overexpression and changes of the subcellular localization of the enzymes on penicillin production. The following strains are used: WT (strain SJR2), strain SAH37 (*alcA*(*p*)::*acvA*::*PTS1*), strain SAH19 and SAH31 (*nat*(*p*)::*acvA*::*PTS1* and *alcA*(*p*)::*ipnA*), strain SAH30 (*nat*(*p*)::*acvA*::*PTS1* and *alcA*(*p*)::*acvA*::*PTS1* and *alcA*(*p*)::*acvA*::*PTS1*. The mean of three biological replicates is displayed and the standard deviation is indicated.

3.4. Combination of overexpression and changes of the subcellular localization

Both, overexpression of *acvA* and targeting of AcvA to peroxisomes led to increased penicillin titers. To test if the combination of the two strategies would further increase penicillin production, *acvA* fused to PTS1 was overexpressed using the *alcA* promoter. To achieve this, a 1 kb fragment of the 5'-region of the *acvA* gene was amplified and cloned behind the *alcA* promoter (pAH09). pAH09 was transformed into SAH14, the strain with the PTS1-tagged AcvA. Homologous integration of the circular plasmid into the *acvA* locus led to duplication of the 1 kb fragment and placement of the full open reading frame under the control of the *alcA* promoter. The integration was tested by PCR. The resulting strain (SAH37) displayed a nearly 5-fold higher production compared to wildtype and 3.4-fold compared to simple over-expression of *acvA* or 1.6-fold compared to the strain in which AcvA was targeted to peroxisomes.

Next, we tested the combination of AcvA targeted to peroxisomes and overexpression of *ipnA*. Strains SAH19 and SAH31 showed similar results and produced 5.5-fold more penicillin compared to wildtype. Strain SAH30 combined AcvA peroxisomal localization with overexpression of *aatA* and produced 1.7-fold more penicillin compared to wildtype. The combination of AcvA-PTS1 with increased numbers of peroxisomes (SAH13, SAH21) led to 4.2-fold more penicillin compared to wildtype (Fig. 5).

#### 4. Discussion

In this paper we followed a new strategy to improve penicillin production in *A. nidulans*, targeting of enzymes to peroxisomes and increasing the number of peroxisomes. These approaches were combined with overexpression of certain enzymes.

Overexpression of *pexK* led to a 2-fold increase of the number of peroxisomes and resulted in a 2.3-fold higher penicillin titer. These results are in agreement with a report in *P. chrysogenum*, where overexpression of the gene led to 2-fold higher penicillin titers (Kiel et al., 2005). These and our results indicate that the number of peroxisomes is apparently a rate-limiting step in penicillin biosynthesis under the production schemes used. On the other hand, overproduction of AatA alone already increased the penicillin titers (Fernández-Cañon and Peñalva, 1995). This is essentially also the case in the high-yield production strains, in which several penicillin biosynthesis clusters are present (Weber et al., 2012). It would be interesting to see if the number of peroxisomes in those high-producers is also increased.

Transfer of IpnA to peroxisomes caused its loss of activity. Given that several Cys amino acids are conserved in different IpnA proteins from different fungi, suggesting functional roles of the thiol groups. Site-directed mutagenesis indeed showed the importance for some of them. In case of cysteine 106 the activity dropped about 20-fold (Samson et al., 1985). Because still some activity remained, it was concluded that the thiol groups were not essential but play an important role. Given that peroxisomes harbor catalases, peroxidases and other enzymes involved in e.g. beta-oxidation of fatty acids or production of reactive oxygen species (ROS), it is conceivable to assume that the SH groups in the IpnA protein become oxidized and thus the enzyme is inactivated. The IpnA enzyme contains also Fe<sup>2+</sup> as cofactor, which could also be inactivated by oxidation (Roach et al., 1997). Alternatively or in addition, it could be that the SH-group in the tripeptide produced by ACV became oxidized in peroxisomes. Likewise, it was shown that the bis-ACV disulfide could not be further converted by IpnA (Perry et al., 1988; Roach et al., 1997). This hypothesis is somehow in contrast with the finding that expression of AcvA in peroxisomes led to increased production of penicillin. If bis-ACV indeed is formed in peroxisomes and cannot serve as substrate for IpnA, one has to assume that bis-ACV is exported into the cytoplasm and oxidized in another enzymatic step. This has been shown at least in vitro (Cohen et al., 1994). Another, and yet completely different possibility is that IpnA requires additional factors localized in the cytoplasm. Likewise, it was shown recently that IpnA interacts with proteins of the VeA regulatory complex (Kopke et al., 2013).

In contrast to the data obtained with IpnA, we found that targeting AcvA, the first enzyme in the pathway, to peroxisomes leads to higher penicillin titers. This result shows that AcvA is not only able to function in the oxidative environment of the peroxisomes, but it suggests that the activity can be even stimulated under those conditions. The result also implies that the three building blocks are not rate-limiting and thus can be imported into the peroxisomes in sufficient quantities. In addition the product of the reaction, the tripeptide, needs to be exported into the cytoplasm again. ATP, necessary for the activation of the amino acids, can be even generated within the peroxisomes, because latest results show that portions of several glycolytic enzymes reside in peroxisomes (Ast et al., 2013; Freitag et al., 2012). To date very little is known about such transport processes. But obviously they are not rate-limiting. In summary, we show that localization of AcvA to peroxisomes, increased numbers of peroxisomes and overexpression of the genes lead to increased penicillin titers. Combination of two of the parameters lead to further increases, but combination of all three does not have additional effects on the production. This suggests that other steps become rate-limiting in these strains and thus our results add another puzzle piece to the complex regulation of penicillin biosynthesis and the continuous approach for production improvement. It will be most interesting to analyze the

effect of peroxisomal targeting of AcvA in the high-yield *P. chrysogenum* production strains.

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