Functional Analysis of the Aureothin Iterative Type I Polyketide Synthase

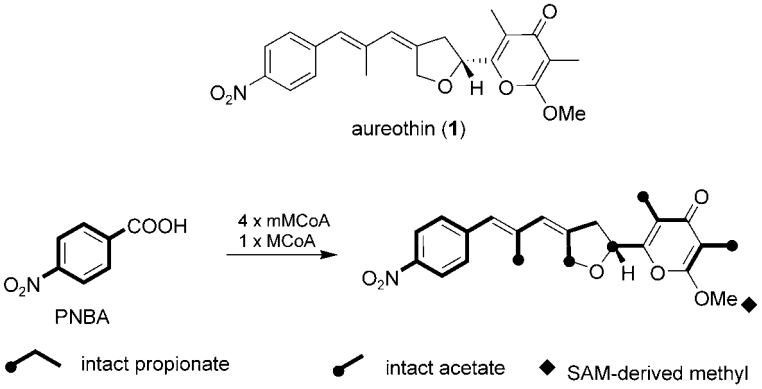
Jing He and Christian Hertweck*[a]

*The modular-type polyketide synthase (PKS) that is involved in aureothin (*aur*) biosynthesis represents one of the first examples in which a single PKS module (AurA) is used in an iterative fashion. Here we report on the heterologous expression of an engineered AurAB fusion protein that unequivocally proves the iterative nature of AurA. In addition, point mutations reveal that* aur *PKS module 4 participates in polyketide biosynthesis despite its aberrant acyltransferase domain.*

Introduction

Bacterial modular type I polyketide synthases (PKS) are giant multifunctional enzymes that play a major role in the biosynthesis of complex polyketides, such as macrolides, polyenes, and polyethers, many of which are endowed with pharmaceutically important properties.[1,2] Since the first discovery of a modular PKS in 1990 (erythromycin PKS),[3] a large number of related bacterial type I PKS gene clusters have been cloned and sequenced. Important examples include the rapamycin,[4] rifamycin,[5] picromycin,[6] avermectin,[7] and candicidin[8] PKSs. Common to these megasynthases is a modular arrangement with sets of ketosynthase (KS) and acyltransferase (AT) domains for chain propagation, and an acyl-carrier protein domain, which serves as an anchor for the acyl intermediate. In addition, depending on to the degree of β-keto processing, full or reduced sets of keto reductase (KR), dehydratase (DH), and enoyl reductase (ER) domains are present in a module. After completion of the polyketide backbone, the chain is released from the thiotemplate by a thioesterase located at the C terminus. In contrast to aromatic, plant, and fungal polyketide synthases, modular PKSs constitute a processing line along which the polyketide backbone is assembled and processed. Usually, the number of modules strictly mirrors the number of elongation and reduction cycles. This one-to-one correspondence of the polyketide metabolite to the modular type I PKS architecture has served as a model for understanding the programming of modular PKSs and has provided the molecular basis for targeted genetic manipulations, which have resulted in natural product derivatives.[2,9,10] Nonetheless, the PKS programming can be error prone. In the fermentation broth of a *Saccharopolyspora erythraea* mutant, Leadlay, Staunton, and co-workers detected trace amounts of ring-enlarged derivatives of the erythromycin aglycone. The incidence of an additional elongation unit in the 16-membered macrolide was rationalized by an aberrant repeated use of a module. This unusual behavior was termed "stuttering",[11] as opposed to "skipping",[12] which involves the omission of a module during chain elongation. A similar observation was made by Höfle and co-workers, who isolated ring-enlarged, as well as ring-contracted, epothilone derivatives as by-products from a large-scale fermentation of *S. cellulosum* So ce90.[13] Although erythromycin and epothilone derivatives are obviously the result of an erratic PKS processing, recent observations provide further evidence that iteration can in fact be a programmed event encoded by the modular PKS. The first genetic indication for an iterative use of a single PKS module was reported by Müller and co-workers, who found that a gene encoding a PKS module is missing in the stigmatellin gene (*sti*) cluster from *Stigmatella aurantia*ca. Since the absent PKS gene could not be detected elsewhere in the genome by hybridization, it was concluded that one PKS module could be compensated for by the repeated use of a functionally equivalent module, either StiH or StiJ.[14]

Recently, we cloned and sequenced the gene cluster that encodes for PKSs responsible for the biosynthesis of the nitroaryl-substituted polyketide aureothin (**1**) in *Streptomyces thioluteus*.[15] Labeling experiments revealed that the aureothin polyketide backbone is assembled from a rare nitrobenzoate starter, one acetate, and four propionate units (Scheme 1).[16,17] Ac-



Scheme 1. *Structure of aureothin and biosynthetic origin of the carbons. PNBA:* p*-nitro benzoate, SAM:* S*-adenosyl methionine, mMCoA: methylmalonyl CoA, MCoA: malonyl CoA.*

[a] *J. He, Dr. C. Hertweck*
Hans-Knöll-Institute for Natural Products Research
Beutenbergstraße 11a, 07745 Jena (Germany)
Fax: (+49) 3641-656705
E-mail: christian.hertweck@hki-jena.de

  DOI: 10.1002/cbic.200400333

cording to the principle of colinearity, five PKS modules would be required for the assembly of the polyketide backbone. However, sequence analyses and heterologous expression of the entire aureothin-biosynthesis gene cluster (*aur*) in a *Streptomyces* host revealed that four modules encoded in the gene cluster are sufficient for catalyzing five rounds of elongation and processing.[15] The domain architecture of the modules suggested that the first module is used twice and gives rise to the diene substructure (Figure 1). Furthermore, the AT of PKS module 4 seemed to be inactive due to a mutated active-site motif. It was assumed that in analogy to the first module, the isosteric module 3 might also operate iteratively in order to accomplish the fifth Claisen condensation.

Almost simultaneously with our findings, the Salas and Leadlay groups jointly communicated that for borrelidin (*bor*) biosynthesis, a single elongation module is used three times.[18] Another unusual PKS, the modules of which seem to be used iteratively, is involved in lankacidin (*lan*) biosynthesis.[19] While classical modular PKSs show a one-to-one correspondence between PKS module architecture and the number of condensation steps, these novel PKS systems clearly breach the generally accepted principle of colinearity. However, sequence comparisons of noniterative and iterative PKSs do not indicate any molecular grounds for iteration, and to date the reason why PKSs act either in an iterative or noniterative fashion remains a mystery. As a prerequisite for gaining a deeper insight into the intriguing code of the *aur* PKS, we had to establish which modules of the *aur* PKS—if any—are operating in a truly iterative fashion.

Results and Discussion

The *aur* gene cluster encodes only four PKS modules for five rounds of chain elongations; this strongly suggests that one module is used twice. However, the possibility that the expression of *aurA* yields two copies of AurA that might assemble head to tail, thus giving a perfectly colinear pentamodular PKS, had to be excluded. In addition, it remained unclear if the fourth module is in fact functional despite its aberrant AT domain.

One attractive option to gain insight into the course of polyketide assembly is the identification of pathway intermediates that are anchored to the thio template during chain elongation and processing. In principle, the hydrolytic activity of a C-terminally fused thioesterase domain can result in a premature release of the polyketide intermediates; this would enable the isolation and elucidation of all pathway intermediates. Impressive examples for repositioning TE domains in the erythromycin[20,21] and rifamycin[22] PKS systems demonstrate the general viability of this approach and encouraged us to perform similar experiments. The *aur* TE domain was placed downstream of each PKS module by using homologous recombination and appropriately fused ACP/TE gene probes. However, all resulting mutants that supposedly express truncated variants of the *aur* PKS were incapable of polyketide production. The failure of the truncated PKS could be explained by a high substrate specificity of the TE or by an incorrect folding of the mutated PKS.

In an alternative approach, to prove the iterative nature of AurA, we aimed at fusing *aurA* and *aurB*. In the case of a covalent attachment of the C terminus of AurA (module 1) with the N terminus of AurB (module 2), the occurrence of a twin AurA protein could be ruled out. A similar strategy has recently proven successful with the iterative *bor* PKS; here genes were fused at the appropriate start/stop codons.[18] Conversely, we aimed to replace parts of ACP1 and KS2 with parts of ACP3 and KS4, including the interdomain region, in order to prevent any incompatibility of the linker region. The high similarity of ACP1 and ACP3, and KS2 and KS4, appeared ideally suited for the targeted substitution of the partial PKS region without affecting the functions of the catalytic domains. Several cloning steps were carried out to yield the desired construct (Figure 2). First, a subclone containing the *aurC* gene was used as template for PCR to amplify the ACP3–KS4 interdomain region and in-

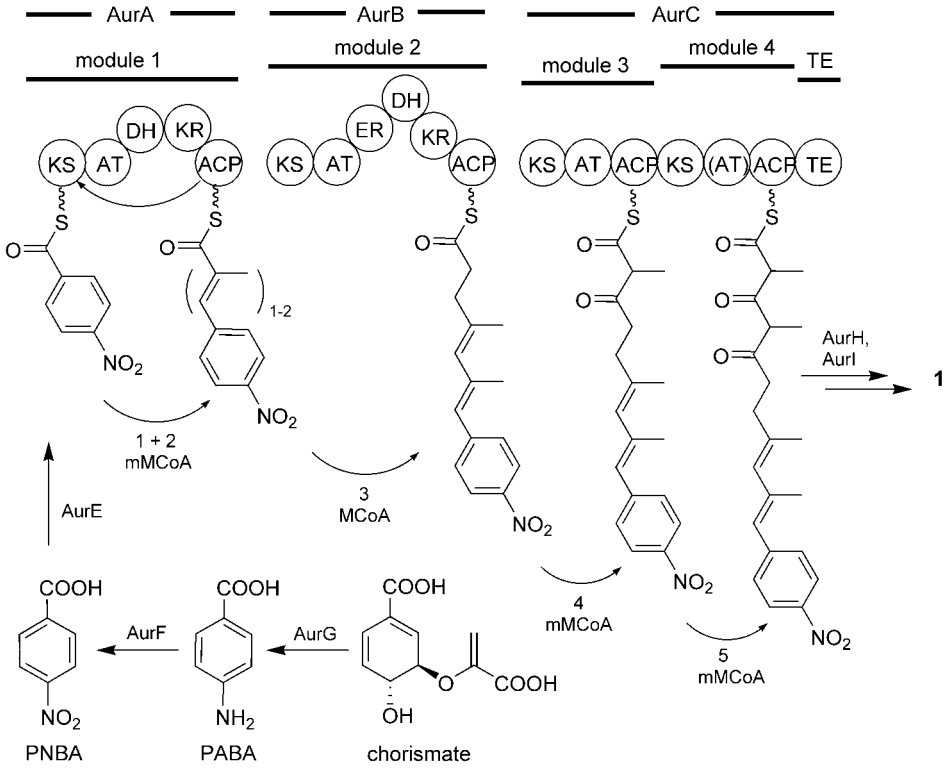


Figure 1. *Model of nonlinear aureothin biosynthesis from chorismate via* p*-aminobenzoate (PABA). For protein functions see Figure 2 caption.*

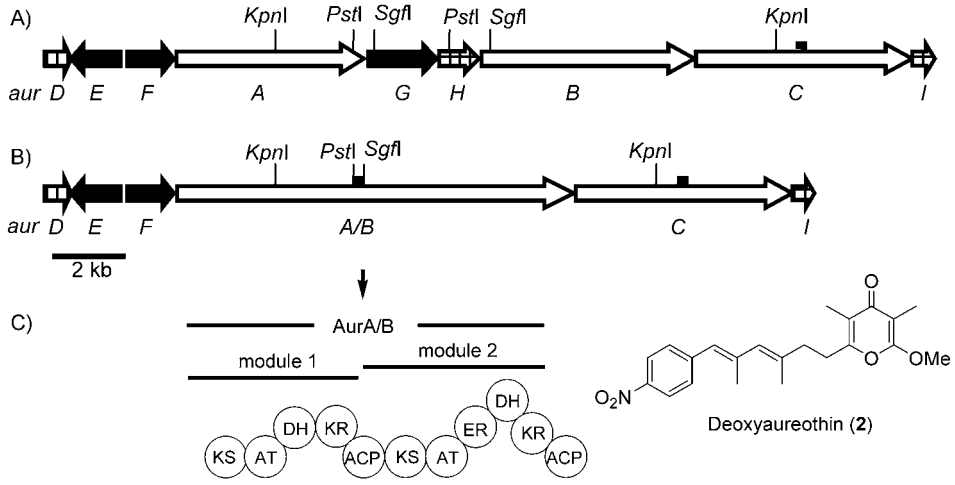


Figure 2. *Organization of A) the* aur *biosynthesis gene cluster, versus B) the engineered construct that was obtained from gene fusion. Derived proteins: AurA (PKS module 1); AurB (PKS module 2); AurC (PKS modules 3 and 4); AurD (transcriptional regulator); AurE (acyl-CoA ligase); AurF (N-oxygenase); AurH (P450 monooxygenase); AurI (methyl transferase). Bars indicate the ACP3–KS4 interdomain area that was amplified by PCR. C) Bimodular A/B fusion protein and deoxyaureothin (**2**) structure.*

troduce two unique restriction sites, *Pst*I and *Sgf*I, that were suitable for executing the subsequent substitution. The PCR product was subcloned and sequenced. Second, from the *E. coli--Streptomyces* shuttle cosmid, pHJ48, which bears the entire *aur* biosynthesis gene cluster, a large (15 kb) *Kpn*I fragment was excised and subcloned. After exchanging the biggest *Pst*I–*Sgf*I fragment with the *Pst*I–*Sgf*I fragment that was obtained by PCR, the altered *Kpn*I fragment was ligated into the *Kpn*I sites of the shuttle cosmid, and the correct direction of the insert was ascertained by restriction digests. In the resulting construct, pHJ86, *aurA* and *aurB* were fused (in-frame) into the same translational unit, while *aurG* and *aurH* were eliminated (Figure 2). After the introduction of pHJ86 into *Streptocmyces albus*, a well-established expression host, the transconjugant *S. albus*::pHJ86, was selected for apramycin resistance and cultured. As the PABA synthase and P450 monooxygenase genes, *aurG* and *aurH*, were eliminated along with the gene fusion, the PABA-derived PKS primer, *p*-nitrobenzoate (PNBA),[16] was added to the culture in order to restore polyketide biosynthesis. TLC analyses of the crude extract from a plate culture revealed the formation of a novel metabolite that carried an orange fluorescent chromophore. The identity of the metabolite with deoxyaureothin (**2**), an aureothin derivative that lacks the tetrahydrofuran ring, was unequivocally proven by HPLC and mass spectrometry (Figure 2). The reference compound **2** was recently generated in our laboratory by targeted inactivation of the multifunctional cytochrome P450 monooxygenase, AurH.[23] Consequently, the polyketide backbone produced by the mutant is identical to aureothin, and four modules clearly catalyze five elongation and reduction cycles. The successful expression of the AurAB fusion protein with concomitant production of deoxyaureothin clearly rules out the possibility that two copies of AurA are forming a twin module (Figure 2). This result—in addition to the domain architecture of AurA—provides the strongest evidence for the iterative use of module 1 during the formation of the diene moiety. The most plausible explanation for the iteration in module 1 is the transfer of the ACP1-bound acyl intermediate back to KS1 until it reaches the defined chain length. The reason and exact mechanism of this unusual iteration are now subject of ongoing studies in our laboratory.

In addition to AurA, we assumed that iteration also occurs in AurC. As sequence analyses revealed, the AT4 domain shows only weak homology to known AT domains and is possibly inactive due to the absent active-site serine. Since module 3 has the same domain arrangement as module 4, it was tempting to speculate that both final chain extensions are catalyzed by module 3 alone. In order to investigate whether module 4 is required for the final chain elongation, site-directed mutagenesis experiments of the KS and ACP active sites were performed.

The targeted regions for mutation were the conserved motifs that accommodate the active-site cystein of KS4 (VDTA**C**SSS) and the phosphopantheinyl-binding serine of ACP4 (GFD**S**L). As previous work on the erythromycin PKS revealed, a cysteine-to-alanine or serine-to-alanine mutation yields inactive PKS domains but does not affect expression or folding of the enzyme.[12] Both manipulations in the *aur* PKS were achieved by exchanging fragments of the gene that encodes the KS4 and ACP4 domains with mutated complements (Figure 3). For inactivation of KS4, a *Pvu*I site was introduced into the active site. The mutated *Kpn*I–*Bsu*36I DNA fragment encoding parts of the mutated KS4 (KS4*) and AT4 was assembled from two different PCR products; a *Kpn*I–*Pvu*II fragment encoding the N-terminal region and a *Pvu*II–*Bsu*36I fragment encoding the C-terminal region. At the junction, the KS4 active-site cysteine codon was replaced by an alanine codon with the introduction of a *Pvu*II site. After exchanging the native DNA fragment with the mutated *Kpn*I–*Bsu*36I fragment in cosmid pHJ48, the resulting plasmid, pHJ146, was introduced into *S. albus* by conjugation.

The ACP4 domain was inactivated in an analogous way. Here, an *Afl*III site was introduced at the phosphopantheinyl attachment site to change the serine codon into an alanine codon. For this purpose, a *Bsu*36I–*Xba*I fragment encoding parts of the AT4, ACP4, and TE domains was assembled from two PCR products, an *Eco*RI–*Afl*III fragment and an *Afl*III–*Xba*I fragment. The mutated *Bsu*36I–*Xba*I fragment (ACP4*) was ligated into the unique *Bsu*36I–*Xba*I sites in cosmid pHJ48, and the resulting plasmid (pHJ144) was introduced into *S. albus*.

Both transconjugants, *S. albus*::pHJ144 and *S. albus*::pHJ146, expressing variants of the *aur* PKS that lacked functional ACP4

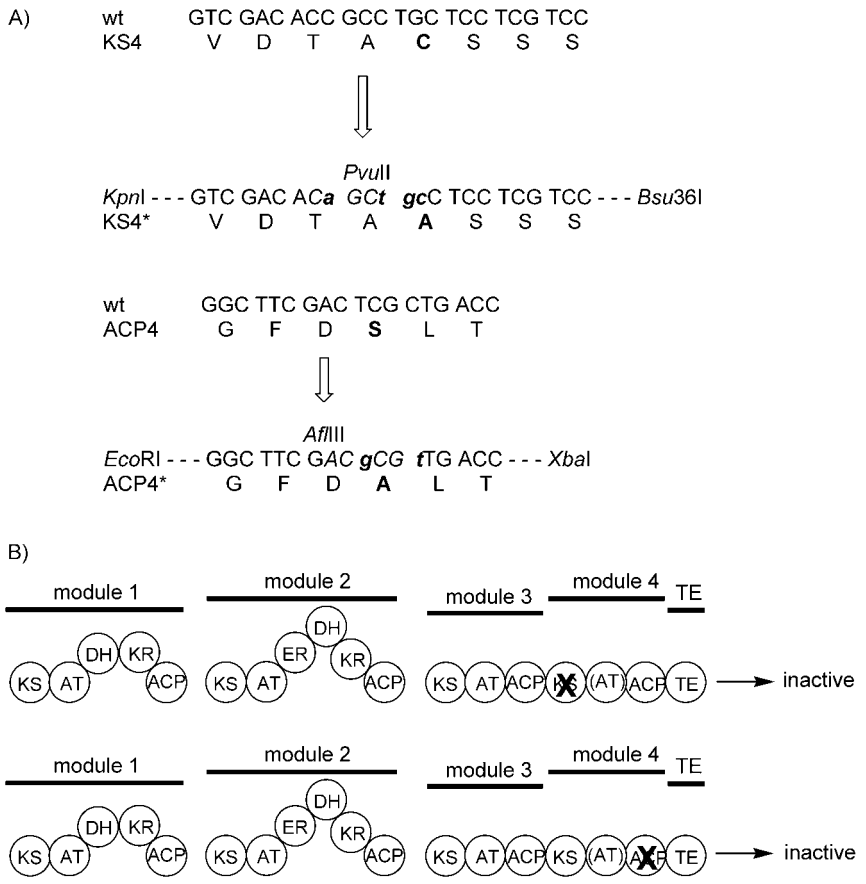


Figure 3. *Inactivation of KS4 and ACP4 to prove the involvement of module 4. A) Nucleotide sequences of wild-type (wt) and mutant, and deduced amino acid sequences; * indicates inactivated domains, letters in bold indicate amino acids exchanged, letters in lower case indicate bases altered. B) Architecture of mutated* aur *PKS variants.*

and KS4 domains, respectively, were fermented under the same conditions as the native aureothin producers. However, thorough TLC, HPLC, and MS analyses revealed that neither aureothin nor a derivative thereof was formed by either of the mutants. In both cases, inactivation the KS or ACP domains of module 4 clearly resulted in a shutdown of polyketide biosynthesis. This strongly suggests that module 4 is required for the biosynthesis of polyketide production, and, despite the apparently inactive AT4 domain, an iterative use of the entirety of module 3 seems not to take place. How can this observation be rationalized? Recently, J. Piel reported the surprising discovery of a type I PKS from a pederus bacterial symbiont in which all PKS modules lack AT domains.[24] This novel PKS system is served by iterative acyltransferases in trans, which are also encoded in the same gene cluster. Similar findings have been made in other more recent cases, such as, the leinamycin[25] and mupirucin PKSs.[26] However, in the *aur* PKS, this mechanism can be excluded.

Interestingly, apparently nonfunctional AT domains have also been identified in other modular type I PKS systems, here AT domains are located within the module (cis-AT), such as the pyoluteorin PKS[27,28] and the neocarzilin PKS, which represents the most recent example of an iterative modular PKS.[29] In these cases, as in the *aur* PKS, how the anomalous AT domains function or how they might be complemented, remains a mystery.

In conclusion, we have unequivocally proven the iterative nature of the first module of aureothin biosynthesis by an engineered gene fusion and heterologous expression of the PKS. The formation of deoxyaureothin by the mutant clearly demonstrates that four PKS modules are sufficient for catalyzing five rounds of Claisen condensations and β-keto processing. In addition, point mutations of the KS4 and ACP4 domains, which result in a shutdown of polyketide biosynthesis, strongly suggest that module 4 is in fact required for the fifth chain elongation despite its aberrant and probably nonfunctional AT domain. These findings set the basis for further studies on the detailed mechanism and evolution of the unusual iterative modular aureothin PKS system.

Experimental Section

Material and general methods: *S. albus*, kindly provided by Prof. Salas, served as host strain for expression experiments. For aureothin production, the wild-type and mutants were cultured in mannitol soya-flour medium for seven days at 28 °C, with shaking. *S. albus* was grown on mannitol soya-flour agar for conjugation. Conjugants were selected with apramycin (30 μg mL^{-1}; Sigma) in both solid and liquid media. *E. coli* strains DH5α and ET12567,[30] which contain the RP4 derivative, pUZ8002, were used for routine subcloning and intergeneric conjugation, respectively. *E. coli* strains were cultured in Luria–Bertani (LB) medium that was supplemented with ampicillin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}), chloromycetin (25 μg mL^{-1}), or apramycin (50 μg mL^{-1}) for plasmid selection. pBluescript II SK(−) was the routine vector for subcloning and the *E. coli–Streptomyces* shuttle vector pSET152 was used for all expression experiments in *Streptomyces*. DNA isolation, plasmid preparation, restriction digest gel electrophoresis, and ligation reactions were carried out according to standard methods.[30]

Isolation and detection of metabolites: For the detection of secondary metabolites, seven-day old-chopped agar plates or liquid culture broths of wild-type and recombinant strains were extracted with ethyl acetate. Metabolic profiles were monitored by TLC, HPLC, and MS by using aureothin and deoxyaureothin as references. HPLC analysis was carried out on a KROMASIL C15 column (Jasco) eluted with methanol:water (75:25) over 20 min at a flow rate of 0.8 mL min^{-1} with UV detection at 345 nm.

In-frame fusion of AurA and AurB: A 15.1 kb *Kpn*I fragment was recovered from cosmid pHJ48 and ligated into pBluescript II SK(−)

to give the plasmid pHJ73. The linker region that was used for the fusion of *aurA* and *aurB* into a single open reading frame was amplified by PCR with two primers, LinkerL (5′-GCGCTGCAGCTGCG-CAACCGGCTCGACG-3′) and LinkerR (5′-GCGATCGCGTCGCGTCCC-GAGGCGACCA-3′) by using an *aurC* subclone as template. The 272 bp PCR product was cloned into the pGEM-T Easy vector (Promega) for sequencing and was then used to substitute a 4.0 kb *Pst*I–*Sgf*I fragment in pHJ73 to give plasmid pHJ83. The 15.1 kb *Kpn*I fragment in cosmid pHJ48 was replaced by the shortened 11.4 kb *Kpn*I fragment from pHJ83 to give the construct, pHJ86. *E. coli* strain ET12567/pUZ8002 was employed as intermediary host to transfer plasmid pHJ86 into *S. albus* by intergeneric conjugation, according to standard procedures.[30]

Inactivation of KS domain of module 4: A 1251 bp *Kpn*I–*Pvu*II fragment encoding the N-terminal region of KS4, up to the mutated active site, was amplified by PCR with two oligonucleotides, KpnIfor (5′-GCCGCCGCCGAGGATGAAGG-3′) and PvuIIrev (5′-GGCAGCTGTGTCGACCGTGATGGCCGGGCCTTC-3′). A 1936 bp *Pvu*II–*Xba*I fragment encoding the C-terminal region of KS4, up to the mutated active site, was amplified by PCR with two oligonucleotides, PvuIIfor (5′-ACAGCTGCCTCCTCGTCCCTCGTCGCCCTCCAC-3′) and XbaIrev (5′-TCTAGATCGGGGAGCGGGGTTTCGTC-3′). Both PCR products were cloned into pGEM-T for sequencing. The *Kpn*I–*Pvu*II and *Pvu*II–*Xba*I fragments and *Kpn*I–*Xba*I-digested pBluescript II SK(−) vector were ligated to give plasmid pHJ145. A 3122 bp *Kpn*I–*Bsu*36I fragment bearing the mutated KS4 gene was recovered from pHJ145 and exchanged with the corresponding fragment of pHJ48. The resulting plasmid, pHJ146, was then transferred into *S. albus* by conjugation.

Inactivation of ACP domain of module 4: A 685 bp *Eco*RI–*Afl*III fragment encoding the N-terminal region of ACP4, up to the mutated active site, was amplified by PCR with two primers, EcoRIfor (5′-GAATTCGTGGCGCTGTACCGGCTGTTGG-3′) and AflIIIrev (5′-GTCAACGCGTCGAAGCCCAGCTCGGGGAGC-3′). A 1046 bp *Afl*III–*Xba*I fragment encoding the C-terminal region of ACP4, up to the mutated active site, was amplified by PCR with the primers, AflIIIfor (5′-TCGACGCGTTGACCGCGGTGGACCTGCGCAAC-3′) and aurATER (5′-GTGTCTAGACGTGCTTGCGCTGGTGGTGGTC-3′). Both PCR products were cloned into pGEM-T for sequencing. The *Eco*RI–*Afl*III and *Afl*III–*Xba*I fragments were ligated into the *Eco*RI–*Xba*I sites of pBluescript II SK(−) to give plasmid, pHJ143. A 1108 bp *Bsu*36I–*Xba*I fragment bearing the mutated ACP4 gene fragment was recovered from pHJ143 and used to substitute a 3743 bp *Bsu*36I–*Xba*I fragment in pHJ48. The resulting plasmid pHJ144 was introduced into *S. albus* by conjugation.

Acknowledgements

Financial support by the DFG priority program SPP1152: "Evolution of metabolic diversity" (HE3469/2) is gratefully acknowledged. We are also grateful to Prof. Jose Salas and Dr. Carmen Mendez, University of Oviedo, Spain, for the kind gift of S. albus.

Keywords: antibiotics · biosynthesis · natural products · polyketides · streptomyces

[1] D. O'Hagan, *The Polyketide Metabolites*, Ellis Horwood, Chichester, **1991**.
[2] J. Staunton, K. J. Weissman, *Nat. Prod. Rep.* **2001**, *18*, 380.
[3] J. Cortés, S. F. Haydcock, G. A. Roberts, D. J. Bevitt, P. F. Leadlay, *Nature* **1990**, *348*, 176.
[4] T. Schwecke, J. F. Aparicio, I. Molnar, A. König, L. E. Khaw, S. F. Haydock, M. Oliynyk, P. Caffrey, J. Cortes, J. B. Lester, G. A. Böhm, J. Staunton, P. F. Leadlay, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7839.
[5] P. R. August, L. Tang, Y. J. Yoon, S. Ning, R. Müller, T.-W. Yu, M. Taylor, D. Hoffman, C.-G. Kim, X. Zhang, C. R. Hutchinson, H. G. Floss, *Chem. Biol.* **1998**, *5*, 69.
[6] Y. Xue, L. Zhao, H. W. Liu, D. H. Sherman, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12111.
[7] H. Ikeda, T. Nonomiya, M. Usami, T. Ohta, S. Omura, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9509.
[8] A. B. Campelo, J. A. Gil, *Microbiology* **2002**, *148*, 51.
[9] C. T. Walsh, *ChemBioChem* **2002**, *3*, 124.
[10] D. E. Cane, C. T. Walsh, C. Khosla, *Science* **1998**, *282*, 63.
[11] B. Wilkinson, G. Foster, B. A. M. Rudd, N. L. Taylor, A. P. Blackaby, P. J. Sidebottom, D. J. Cooper, M. J. Dawson, A. D. Buss, S. Gaisser, I. U. Böhm, C. J. Rowe, J. Cortés, P. F. Leadlay, J. Staunton, *Chem. Biol.* **2000**, *7*, 111.
[12] I. Thomas, C. J. Martin, C. J. Wilkinson, J. Staunton, P. F. Leadlay, *Chem. Biol.* **2002**, *9*, 781.
[13] I. H. Hardt, H. Steinmetz, K. Gerth, F. Sasse, H. Reichenbach, G. Höfle, *J. Nat. Prod.* **2001**, *64*, 847.
[14] N. Gaitatzis, B. Silakowski, B. Kunze, G. Nordsiek, H. Blöker, G. Höfle, R. Müller, *J. Biol. Chem.* **2002**, *277*, 13082.
[15] J. He, C. Hertweck, *Chem. Biol.* **2003**, *10*, 1225.
[16] J. He, C. Hertweck, *J. Am. Chem. Soc.* **2004**, *126*, 3694.
[17] M. Yamazaki, Y. Maebayashi, H. Katoh, J.-I. Ohishi, Y. Koyama, *Chem. Pharm. Bull.* **1975**, *23*, 569.
[18] C. Olano, B. Wilkinson, S. J. Moss, A. F. Brana, C. Mendez, P. F. Leadlay, J. A. Salas, *Chem. Commun.* **2003**, 2780.
[19] S. Mochizuki, K. Hiratsu, M. Suwa, T. Ishii, F. Sugino, K. Yamada, H. Kinashi, *Mol. Microbiol.* **2003**, *48*, 1501.
[20] J. Cortes, E. H. Wiesmann, G. A. Roberts, M. J. B. Brown, J. Staunton, P. F. Leadlay, *Science* **1995**, *268*, 1487.
[21] C. M. Kao, G. Luo, L. Katz, D. E. Cane, C. Khosla, *J. Am. Chem. Soc.* **1995**, *117*, 9105.
[22] Y. Doi-Katayama, Y. J. Yoon, C.-Y. Choi, T.-W. Yu, H. G. Floss, C. R. Hutchinson, *J. Antibiot.* **2000**, *53*, 484.
[23] J. He, M. Müller, C. Hertweck, *J. Am. Chem. Soc.* **2004**, *126*, 16742.
[24] J. Piel, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14002.
[25] Y.-Q. Cheng, G.-L. Tang, B. Shen, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3149.
[26] A. K. El-Sayed, J. Hothersall, S. M. Cooper, E. Stephens, T. J. Simpson, C. M. Thomas, *Chem. Biol.* **2003**, *10*, 419.
[27] B. Nowak-Thompson, N. Chaney, J. S. Wing, S. J. Gould, J. E. Loper, *J. Bacteriol.* **1999**, *181*, 2166.
[28] B. Nowak-Thompson, S. J. Gould, J. E. Loper, *Gene* **1997**, *204*, 17.
[29] M. Otsuka, K. Ichinose, I. Fujii, Y. Ebizuka, *Antimicrob. Agents Chemother.* **2004**, *48*, 3468.
[30] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, U.K., **2000**.

Received: September 15, 2004
Published online on April 5, 2005

