Structure and Biosynthesis of Myxochromides S_{1-3} in *Stigmatella aurantiaca*: Evidence for an Iterative Bacterial Type I Polyketide Synthase and for Module Skipping in Nonribosomal Peptide Biosynthesis**

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The myxobacterium Stigmatella aurantiaca *DW4/3–1 harbours an astonishing variety of secondary metabolic gene clusters, at least two of which were found by gene inactivation experiments to be connected to the biosynthesis of previously unknown metabolites. In this study, we elucidate the structures of myxochromides S_{1-3}, novel cyclic pentapeptide natural products possessing unsaturated polyketide side chains, and identify the corresponding biosynthetic gene locus, made up of six nonribosomal peptide synthetase modules. By analyzing the deduced substrate specificities of the adenylation domains, it is shown that module 4 is most probably skipped during the biosynthetic process. The polyketide synthase MchA harbours only one module and is presumably responsible for the formation of the variable complete polyketide side chains. These data indicate that MchA is responsible for an unusual iterative polyketide chain assembly.*

Introduction

Microorganisms are able to produce a wide variety of natural products with biological activity. Ubiquitous soil bacteria, such as streptomycetes and myxobacteria, are especially rich sources of such compounds. Numerous antifungal, antibacterial and anticancer agents have been isolated from these bacteria and developed by the agrochemical and the pharmaceutical industries. Currently, the potential of bacterial secondary metabolite producers is being evaluated on the genetic level, mainly based on whole-genome sequencing efforts. All of the genome sequences reported to date reveal the presence of many more genomic loci encoding typical secondary metabolite genes (termed SM-biosynthetic gene clusters) than expected. For example, *Streptomyces coelicolor* and *Streptomyces avermitilis* harbour approximately 20 and 30 gene clusters, respectively, for secondary metabolism, most of which encode polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs).[2–4]

NRPSs and PKSs, large multifunctional enzyme systems using acyl-coenzyme A (PKS) or amino acid (NRPS) building blocks, use very similar strategies for the biosynthesis of two distinct classes of natural products (nonribosomally made peptides and polyketides). Although they utilize different classes of substrates, PKSs and NRPSs show striking similarities in the modular architectures of their catalytic domains and assembly line-like mechanisms. The extremely large polyfunctional enzyme systems are organized in modules. Each module is responsible for one discrete chain-elongation step and can be subdivided into domains controlling the choice of the extender unit. Several types of modifications can take place on each intermediate. At a minimum, a typical NRPS module consists of an adenylation (A) domain responsible for amino acid activation, a thiolation (T) domain—also known as peptidyl carrier protein (PCP)—that binds the cofactor 4′-phosphopantetheine (4′PP) to which the activated amino acid is covalently attached and a condensation (C) domain that catalyses peptide-bond formation. Additionally, a variety of optional (e.g., methyltransferase (MT) or epimerization (E)) domains have also been described. These increase the structural diversity of the natural products.[5] Similarly, three domains are necessary as the basic equipment of a PKS elongation module: an acyltransferase (AT) domain for extender unit selection and transfer, an acyl carrier protein (ACP) for extender unit loading and a ketoacyl synthase (KS) domain for decarboxylative condensation of the extender unit (usually malonyl-CoA or methylmalonyl-CoA) with an acyl thioester. The resulting β-keto thioester may subsequently be processed by β-ketoacyl reductase (KR) domains, β-hydroxyacyl

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dehydratase (DH) domains, enoyl reductase (ER) domains and MT domains.[6] Recently, it has been discovered that these modular PKS and NRPS systems can also closely cooperate to form so-called hybrid products.[7–9] Because nature obviously combines all types of enzymatic systems for biosynthetic purposes, especially for the production of secondary metabolites, classifications such as PKS/NRPS hybrids should be handled with care.[10]

Although no complete genome sequence from any myxobacterium has been reported, the ongoing efforts at the Institute for Genomic Research (TIGR) and in the Genomik network at Bielefeld University have already shown that the model myxobacteria *Myxococcus xanthus* DK1622 and *Sorangium cellulosum* So ce56 contain numbers of secondary metabolic gene clusters that equal or exceed those found in the sequenced actinomycete genomes.[11] Nevertheless, only two compounds from *S. cellulosum* So ce56 are known, and no report of any secondary metabolite structure from *M. xanthus* DK1622 is available. Similarly, *S. aurantiaca* has been shown by hybridization experiments and physical mapping to contain a variety of PKS/NRPS gene clusters.[12] Some of these could be correlated to known natural products, whereas the function of others has remained obscure.[8,13–17] Four novel structures were found in *S. aurantiaca* DW4/3–1 after a detailed analysis of metabolite profiles of gene inactivation mutants in each of these clusters and comparison with the wild-type strain.[18] From the genetic analysis, a hybrid PKS/NRPS gene cluster is involved in the formation of three compounds, whereas the fourth compound is biosynthesized by a PKS.

This article reports the production, isolation and structures of the hybrid compounds, cyclic pentapeptides each possessing a polyketide side chain and referred to as myxochromides S_1–S_3. Additionally, the corresponding biosynthetic gene cluster was identified, sequenced and analysed to reveal several novel features. Evidence for "module skipping" in nonribosomal peptide biosynthesis is reported, to the best of our knowledge for the first time. In addition, the PKS involved in myxochromide side-chain biosynthesis is most probably an iteratively acting enzyme with the intrinsic capacity to produce polyketide chains of varying length.

Results

Identification of the myxochromide S biosynthetic gene cluster

During our screening for hybrid PKS/NRPS systems in *Stigmatella aurantiaca* DW4/3–1, the cosmid E196 was identified.[18] Integration of a randomly chosen PKS fragment from this cosmid into the chromosome resulted in a mutant shown to be deficient in the production of at least three substances possessing similar UV spectra. T7 end sequencing of cosmid E196 revealed the presence of a NRPS gene, which indicated that the compounds are biosynthetically derived from a PKS/NRPS hybrid megasynthetase. By using the oligonucleotides 195T3–1 and 195T3–2, a specific NRPS fragment was amplified from cosmid E196. A gene inactivation experiment in *S. aurantiaca* DW4/3–1 with this fragment resulted in the mutant strain EBS66–3, which shows the same myxochromide negative phenotype as the previously described PKS mutant DWE196 (see Figure 1).

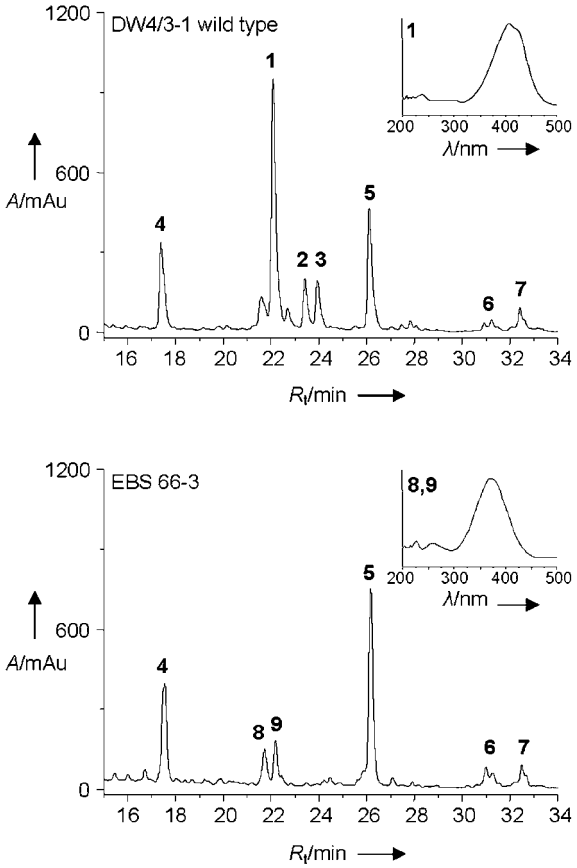

Figure 1. *Phenotypic analysis of S. aurantiaca DW4/3–1 and mutant EBS66–3. HPLC diagrams of the secondary metabolite production spectra of the wild-type strain S. aurantiaca DW4/3–1 and of the mutant EBS66–3. **1**) myxochromide S_1, **2**) myxochromide S_2, **3**) myxochromide S_3, **4**) novel and unknown polyketide, **5**) myxothiazol A, **6** and **7**) dawenols, **8** and **9**) unknown compounds.*

Isolation and structure elucidation of myxochromides S_{1-3} from *S. aurantiaca* DW4/3–1

For the isolation of myxochromides S on large scales, fermentations of strain DW4/3–1 were used.[19] These were performed in bioreactors (150 L) containing Zein liquid medium (85 L) with the adsorber resin Amberlite XAD-16 (1%) as described in detail previously.[19] Fermentation batches of strain DW4/3–1 were harvested 70 h after inoculation, and the myxochromides S were isolated from the culture broth as described in the Experimental Section. The structure determination was carried out with myxochromide S_1, which was obtained in a pure state. The molecular formula $C_{38}H_{54}N_6O_8$ was derived from a HRESI mass spectrum ($[M+Na]^+ = 745.392$) and indicates 15 double bond equivalents. Eight of these are assigned to a heptaenecarboxylic acid moiety, similar to that found in the previously described myxochromide A (**10**, Scheme 1).[20] In addition to the signals from the polyene side chain (14 double bond

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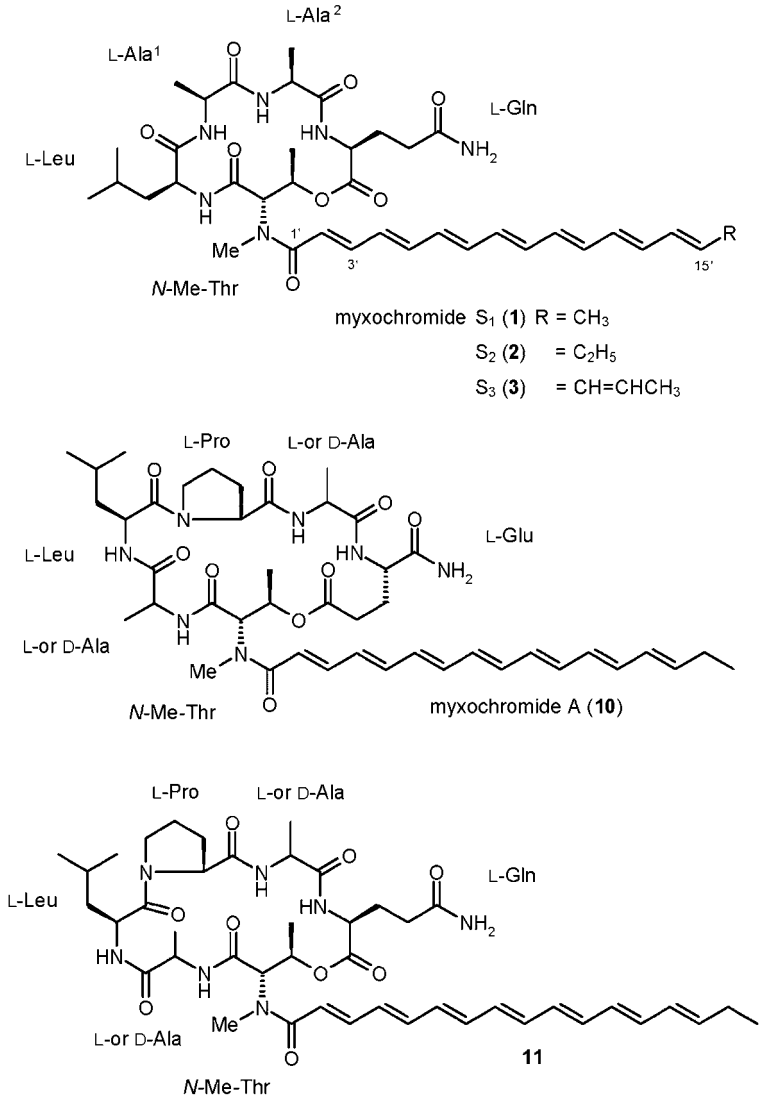


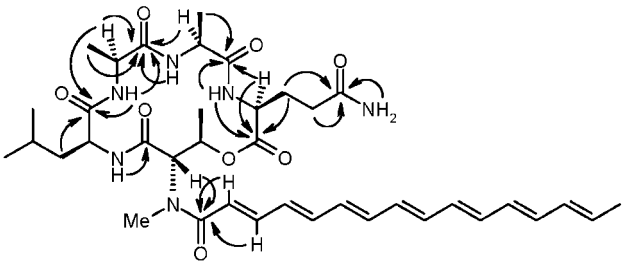
Scheme 1. *Structures of myxochromides. Myxochromides S_{1-3} (**1**–**3**) are produced by* S. aurantiaca *DW4/3–1. Myxochromide A (**10**) was isolated from* M. virescens *Mx v48.[20] Through analogy with the structure of **1** and the mch_{5a} gene cluster we propose a slightly different structure (**11**) for this metabolite (see Discussion).*

To determine the absolute configurations of the amino acids, myxochromide S_1 was hydrolysed, and the resulting mixture was derivatized to afford the corresponding *N*-(trifluoroacetyl)amino acid *n*-propyl esters. Gas chromatographic analysis on a chiral column and comparison with D- and L-configured amino acid standards (data not shown) resulted in the identification of L configurations for all amino acids in the myxochromide S_1 peptide.

HPLC analysis of crude extracts from *S. aurantiaca* DW4/3–1 and side fractions from the isolation indicate the presence of two further myxochromides: myxochromides S_2 and S_3 (**2** and **3**), from their characteristic chromophores ($\lambda_{max} = 406$ nm (**2**) and 424 nm (**3**)) and similar retention times. A HPLC diode array-MS/MS analysis of myxochromides S_{1-3} was performed and indicated that all three compounds have the same peptide core, but differ in the structures of their polyunsaturated side chains (MS/MS data not shown). From the detected molecular masses, myxochromide S_2 (**2**, $[M+H]^+ = 737$) has a propionate starter unit, and myxochromide S_3 (**3**, $[M+H]^+ = 749$) an additional acetate extender unit in the PKS side chain. In a feeding experiment with *S. aurantiaca* DW4/3–1 and D_6-propionic acid, incorporation of D_5-propionate into **2** could be demonstrated in the HPLC-MS analysis of the extract (incorporation rate $> 70\%$; data not shown).

In the agar diffusion test, myxochromide S_1 (40 μg per disc) showed no inhibitory activity against various bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Corynebacterium fascians, Escherichia coli*), yeasts *(Saccharomyces cerevisiae*, *Candida albicans*, *Rhodotorula glutinis*) or filamentous fungi (*Botrytis cinereae*, *Mucor hiemalis*). In cultivated L929 mouse fibroblasts, myxochromide S_1 showed weak cytotoxicity, with an IC_{50} value of 1.3 $\mu g\,mL^{-1}$.

protons between 5.7–7.2 ppm and a CH_3 group at 1.75 ppm), the ^1H NMR spectrum (Table 1) shows signals for five additional fragments. These include one NH_2 and four NH groups (6.5–9.5 ppm), five CH protons (3.5–5.5 ppm), five CH_3 groups, three CH_2 groups and one methine proton in the aliphatic region. The ^{13}C NMR spectrum reveals 38 signals, assigned as seven CH_3, three CH_2, seven sp^3-CH, 14 sp^2-CH and seven CO groups. By proton- and carbon-correlated NMR spectroscopy (COSY, HMQC) the presence of *N*-Me-threonine, leucine, two alanines, glutamine and a heptaenecarboxylic acid moiety attached as a side chain were derived in a straightforward manner. The sequence of the amino acids in the pentapeptide backbone, together with the linkage of the polyunsaturated side chain to the N atom of *N*-Me-threonine, was determined from the HMBC correlations (Scheme 2). The threonine hydroxy group is esterified by the α-carboxyl group of glutamine, resulting in a low-field shift of the Thr-β-CH (5.42 ppm) in the proton NMR.



Scheme 2. *HMBC correlations in myxochromide S_1.*

Sequence analysis of the gene cluster

Sequence analysis of cosmid E196 revealed the presence of a biosynthetic gene cluster of almost 30 kb in size. It comprises three PKS/NRPS genes, which were designated *mchA*, *mchB* and *mchC* (see Figure 2). A putative ribosome binding site

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Table 1. *^{13}C NMR and ^{1}H NMR spectral data of myxochromide S_1 in [D_6]DMSO at 300 MHz.*

Moiety	Functional group	δ_C [ppm]	δ_H [ppm] (multiplicity; $J_{H,H}$ [Hz])
N-Me-L-threonine	–NMe	33.8	3.13 (brs)
	–C=O	170.0	–
	α-CH	57.6	5.42 (brs)
	β-CH	72.4	5.42 (brs)
	β-CH_3	16.1	1.10 (brd; 5.7)
L-leucine	–NH	–	8.37 (m)
	–C=O	174.2	–
	α-CH	52.6	3.88 (m)
	β-CH[a]	39.5	1.34 (m)
	β-CH[b]		1.42 (m)
	χ-CH	24.0	1.64 (m)
	γ-CH_3[a]	22.1	0.88 (d; 6.6)
	γ-CH_3[b]	22.5	0.94 (d; 6.6)
L-alanine[1]	–NH	–	9.38 (d; 6.8)
	–C=O	171.4	–
	α-CH	49.9	3.73 (dd; 6.8, 6.8)
	α-CH_3	14.8	1.25 (d; 6.8)
L-alanine[2]	–NH	-	7.54 (d; 6.6)
	–C=O	170.4 or 170.5	–
	α-CH	51.5	3.58 (dd; 6.8, 6.8)
	α-CH_3	15.1	1.53 (d; 7.3)
L-glutamine	–NH	–	7.66 (d; 9.6)
	–C=O	170.4 or 170.5	–
	α-CH	50.2	4.52 (ddd; 9.6, 9.6, 3.2)
	β-CH_2	26.3	1.64 (m)
			2.06 (m)
	γ-CH_2	30.7	2.06 (m)
	γ-C=O	173.9	–
	δ-NH[a]	–	6.67 (s)
	δ-NH[b]		7.13 (s)
side chain	1′	167.4	–
	2′	120.1	6.60 (d; 15.5)
	3′	142.7	7.17 (dd; 11.3, 14.3)
	5′	–[a]	6.71 (dd; 11.0, 15.0)
	4′,6′-13′	–[a]	6.10–6.65 (m)
	14′	130.6	6.15 (qdd; 1.5; 11.0; 14.5)
	15′	130.8	5.77 (dq; 7.0, 14.7)
	16′	18.2	1.75 (d; 7.0)

[a] Not assigned: δ = 130.8, 132.3, 132.3, 132.5, 134.2, 134.7, 135.4, 136.8, 139.8, 142.7.

of *mchA* and *mchB*. The intergenic region between *mchB* and *mchC* (33 bp) harbours the putative RBS of *mchC* (GAGGA). The codon bias of the reported genes is in accordance with other genes from myxobacteria.[21] The overall G+C content of the *mch*-biosynthetic gene cluster is 68%.

The first open reading frame (ORF) of the myxochromide S biosynthetic operon encodes MchA, a protein predicted to have a size of 232522 Da, and showing striking similarity to polyketide synthases. The following genes encode MchB and MchC, proteins with estimated molecular masses of 332858 and 486881 Da, respectively. These show significant similarity to members of the NRPS family of proteins and display the ordered assembly of conserved C, A and T domains characteristic of such multimodular enzymes.[22] Cosmid E196 does not contain the complete sequence of *mchC*. A plasmid recovery approach starting from chromosomal DNA of the NRPS mutant EBS66–3 and subsequent primer walking on the resulting plasmid pSWMch2 resulted in the identification of the 3-prime end of *mchC* and additional 500 bp located downstream of this gene.

Modular organization of mchABC: MchA represents a PKS that shows the highest homology to other myxobacterial type I PKSs. It contains only one module and a domain organization typical for PKSs. Within the three basic domains (KS, AT and ACP), a complete reductive loop[23] consisting of a KR, a DH and an ER domain is present (see Table 2). These domains could be detected by searching for fingerprint regions described for PKS domains.[24–28] Enoyl reductase domains usually contain the highly conserved sequence LxHxxxGGVG[27] as well as a motif for a putative NADP(H) binding (GxGxxAxxxA[29]). Sequence analysis of the MchA ER domain showed deviations from these highly conserved motifs in relation to ER domains from other modular PKSs and fatty acid synthases (FASs) (see Figure 3). In particular, two alanines that are normally part of the NADP(H) binding site are absent from the MchA ER.

MchB and MchC are bimodular and tetramodular NRPSs, respectively. All six NRPS modules contain C, A and T domains and could be detected by the presence of highly conserved signature sequences imparting each domain type a

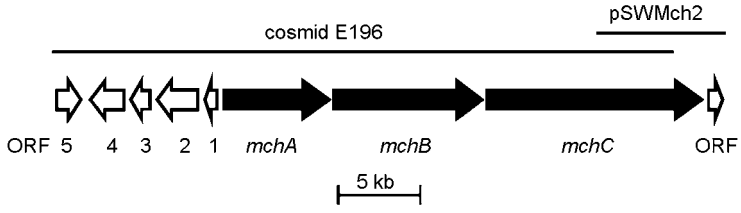

Figure 2. *Map and gene arrangement of the mch biosynthetic gene cluster with flanking regions in S. aurantiaca DW4/3–1. The inserts of cosmid E196 and additional sequences derived from plasmid pSWMch2 are indicated by lines. Genes are shown as arrows, which also indicate the direction of transcription. mchA encodes a PKS, while mchB and mchC encode NRPSs (see Table 2). For the description of ORF1–6, see text.*

Figure 3. *Alignment of the MchA ER domain with the conserved region of other ER domains of PKSs and FASs. Amino acids identical to the consensus sequence (ER core motif:[27] LxHxxxGGVG, putative NADP(H) binding site:[62] GxGxxAxxxA) are boxed in grey. Boldface indicates MchA ER amino acids that differ from the consensus. Rap = ER from rapamycin synthase RapB, Nid = ER from niddamycin synthase NidA, Ery = ER from erythromycin synthase DEBS2, FAS = ER from rat fatty acid synthase, Epo = ER from epothilone synthase EpoA.*

(RBS; AGGAA) is situated in front of *mchA*, while no typical RBS could be found upstream of *mchB*. Overlapping start and stop codons of both genes (ATGA) indicate a translational coupling

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Table 2. *Deduced functions of genes involved in myxochromide S biosynthesis.*

Gene	Size in bp, codon usage (% GC in 1st, 2nd, and 3rd position), size of the deduced protein	Domains (location in the protein sequence)	
mchA	6501 bp (71:51:84) 232.522 Da		KS (16–442)
			AT (561–879)
			DH (889–1090)
			ER (1463–1744)
			KR (1767–2014)
			ACP (2044–2113)
mchB	9174 bp (71:48:85) 332.858 Da	module 1:[a]	C (38–376)
			A′ (531–960)
			MT (961–1403)
			A″ (1404–1437)
			T (1440–1544)
		module 2:	C (1553–1899)
			A (2038–2488)
			T (2490–2594)
			E (2596–2947)
mchC	13407 bp (72:47:86) 486.881 Da	module 3:	C (14–354)
			A (488–946)
			T (949–1054)
		module 4:	C (1057–1391)
			A (1530–1987)
			T (1990–2094)
		module 5:	C (2101–2438)
			A (2578–3033)
			T (3037–3142)
		module 6:	C (3147–3483)
			A (3620–4077)
			T (4081–4186)
			TE (4196–4468)

[a] A N-MT is integrated into the first module of the NRPS A domain. A1′ represents the first part of this A domain containing the core regions A1–A8, while A1″ represents the second part of this domain and contains the core regions A9–A10.

characteristic fingerprint.[22] Sequence alignments of the condensation domains show the presence of the seven described core motifs (C1–C7) (see Figure 4). The A domains of MchBC share the ten known conserved core sequences (A1–A10; see Figure 4), and their thiolation domains contain the core sequence with an invariant serine residue, which represents the 4′PP-attachment site (see Figure 4[22]). Adjacent to or within these essential domains, some modules contain further domains responsible for modifying the biosynthetic intermediate. A methyltransferase (MT) domain is present in the first NRPS module, and is found inserted into the A domain between the core regions A8 and A9. This type of arrangement is unusual but not unprecedented, having also been described in the microcystin[30] and tubulysin[31] biosynthetic proteins McyA and TubB, respectively. The second module contains an epimerization domain located downstream of the T domain. It bears all seven core regions (E1–E7) described for this type of domain.[22] In contrast with modules 1 and 2, the three following modules (modules 3–5) do not contain any additional domains. The last domain of the myxochromide assembly line represents a thioesterase located at the N terminus of module 6.

The binding specificities of the A domains were analysed by comparison of the critical residues responsible for substrate recognition. These were identified and aligned with the known eight amino acid binding pocket residues of the GrsA A domain.[32] Next, they were compared with consensus sequences available in databases (see Table 3,[33,34]). The predicted substrates for the A domains from modules 1 (Thr), 2 (Leu), 3 (Ala), 5 (Ala) and 6 (Gln) correlate well with the amino acid sequence of the myxochromide S peptide core, which consists of a Thr-Leu-Ala-Ala-Gln pentapeptide. The selectivity-conferring residues of module 4 are closest to the consensus sequence of proline, although several variations from normally conserved amino acids in the core regions are conspicuous (see Figure 4). Similar variations could also be found in the T domain core motif of this module. Most notably, the highly conserved serine residue is not in the expected position. In contrast with the A and T domain from module 4, the C domain core motifs do not contain any specific deviations. In general, the C domains of the *mch* biosynthetic gene cluster are clearly less conserved than the A and T domains (see Figure 4).

Table 3. *Comparison of the putative specificity-conferring codes of the A domains in myxochromide S biosynthesis with consensus sequences.[a]*

	residue according to GrsA Phe numbering							
	235	236	239	278	299	301	322	330
Thr consensus	D	F	W	N	I	G	M	V
module 1	**D**	**F**	**W**	**N**	**I**	**G**	**M**	**V**
Leu consensus	D	A	W	L	Y	G	A	V
module 2	**D**	**A**	**W**	**L**	I	**G**	**A**	I
Ala consensus	D	V	W	C	I	A	A	I
module 3	**D**	**V**	M	F	**I**	G	I	V
module 5	**D**	**V**	**W**	V	L	**A**	**A**	**I**
Gln consensus	D	A	W	Q	F	G	L	I
module 6	**D**	**A**	**W**	**Q**	C	**G**	**L**	**I**
Pro consensus	D	V	Q	F	I	A	H	V
module 4	G	A	**Q**	**F**	**I**	**A**	Q	**V**

[a] The numbering of the amino acids corresponds to the GrsA gramicidine synthetase. The GrsA phenylalanine substrate binding pocket has been crystallized,[32] which resulted in the establishment of the nonribosomal code.[60,61] Residues identical with the consensus sequences are shown in bold.

Analysis of the genes flanking the cluster: The 5′ end of the myxochromide S biosynthetic gene cluster is flanked by genes encoding ORF1–5. ORF1 and ORF2 are similar to ABC transporter proteins from *Mycobacterium tuberculosis* (GenBank™ accession number NP_335448.1, 49% identity on the amino acid level) and *Geobacter sulfurreducens* PCA (GenBank™ accession number NP_953236.1, 37% identity on the amino acid level). ORF3 represents a hypothetical protein with 52% identity to a hypothetical protein from *Chloroflexus aurantiacus* (GenBank™ accession number ZP_00018285.1), whereas ORF4 is similar to several K^+/H^+ antiporters, such as the probable K^+/H^+ antiporter from *Gloeobacter violaceus* (GenBank™ accession number NP_923758.1, 44% identity on the amino acid level). ORF5 represents a protein with 56% identity to a permease from *Nostoc punctiforme* (GenBank™ accession number ZP_00106753.1).

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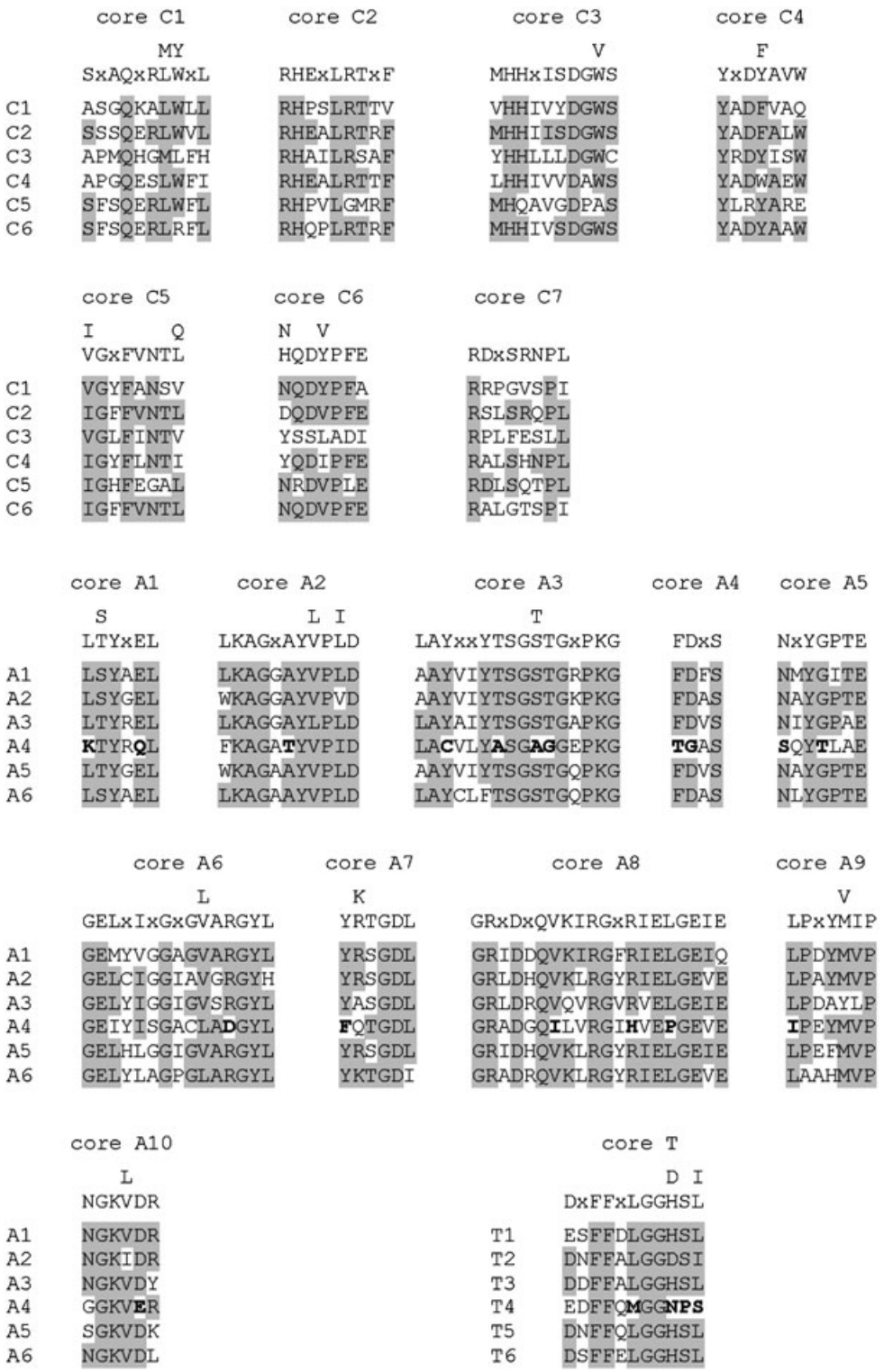

Figure 4. *Alignment of the C, A and T domain core motifs of myxochromide S synthetase from S. aurantiaca DW4/3–1. The consensus sequences of core motifs C1–C7, A1–A10 and of the T domain of NRPSs[22] are indicated in the upper line. Amino acids identical to the consensus sequences are shown in grey. Boldface indicates module 4 amino acids that differ from the consensus.*

At the 3′ end of the cluster ORF6 encodes a polypeptide with homology to a hypothetical protein from *Rhodopseudomonas palustris* (GenBank™ accession number NP_945917.1, 28% identity on the amino acid level).

Discussion

During our screening for hybrid polyketide synthase and nonribosomal peptide synthetase systems in *S. aurantiaca* DW4/3–1

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we identified several such gene loci in the genome of the strain. From gene inactivation experiments and comparison of the wild-type and the mutant metabolite production spectra, three novel natural products corresponding to one of the identified hybrid gene loci and an active copy of the phosphopantetheinyltransferase gene *mtaA* were identified.[18] Consistently with these genetic data, the isolation and structure elucidation of the new metabolites, the myxochromides S_{1-3}, revealed a cyclic peptide core with an attached polyunsaturated polyketide side chain. Sequence analysis of the corresponding gene cluster indicates a three-gene operon encoding the myxochromide S biosynthetic machinery. The first gene product, designated MchA, shows similarities to bacterial type I PKSs. The proteins encoded by the two following genes (*mchB* and *mchC*) show similarities to NRPSs. A detailed analysis of these proteins provides a model for the myxochromide S biosynthetic pathway (see Figure 5), which exhibits several striking deviations from standard PKS and NRPS systems.

Model for myxochromide S biosynthesis: evidence for an iterative bacterial type I PKS lacking chain length control: In modular bacterial type I PKS assembly lines, it appears to be a general biosynthetic rule that each set of domains (termed module) is used only once to catalyse successive cycles of chain extension. This linear, processive mechanism is a paradigm for nonribosomal peptide biosynthesis as well. The number and type of modules within the gene cluster determine the structure of the polyketide and/or peptide product. Sequence analysis of the type I PKS MchA revealed the presence of only one PKS module. No further ORFs with homology to PKSs could be identified in the flanking regions of the myxochromide S biosynthetic gene cluster. In addition, we attempted to identify and inactivate every PKS gene locus in *S. aurantiaca* DW4/3–1, which did not result in the detection of another PKS involved in myxochromide biosynthesis.[18] Therefore, it is most likely that the monomodular PKS MchA catalyses the biosynthesis of the complete unsaturated polyketide side chain in an iterative manner. To date, only one verified example of an iteratively acting bacterial type I PKS has been described: Bechthold and co-workers cloned and heterologously expressed the monomodular protein AviM, which is responsible for the biosynthesis of orsellinic acid in *Streptomyces viridochromogens* Tü57.[35] CalO5 from *Micromonospora echinospora* ssp. *calichensis* has been proposed as another orsellinic acid synthase.[36] SgcE from *S. globisporus* and CalE8 from *M. calichensis* are examples of bacterial type I PKSs also assumed to act iteratively.[36,37] SgcE and CalE8 each consist of four PKS domains (KS, AT, KR and DH) and a fifth domain residing at the C-terminus (TD domain), that seems to be unique to endiyne PKSs. SgcE and CalE8 are believed to catalyse the assembly of linear polyunsaturated polyketides by an iterative process. The resulting polyketide intermediate might subsequently be modified by the enzymes associated with the enediyne PKS into the enediyne cores of C-1027 and calicheamicin. The first example for the iterative use of a type I PKS from a myxobacterium was described for stigmatellin biosynthesis in *Stigmatella aurantiaca* Sg a15.[14] Nevertheless, in stigmatellin biosynthesis only one module of the megasynthetase is used iteratively, whilst all other modules employ standard PKS biochemistry. Further examples of bacterial type I PKSs using single modules iteratively have recently become available.[10,38,39] As the borderlines between different PKS classifications are vanishing, one might also regard biosynthetic machineries involved in unsaturated fatty acid biosynthesis as PKSs. Iterative use of modules has also been described for those.[40–42] The myxochromide S PKS contains the basic equipment of a PKS elongation module plus

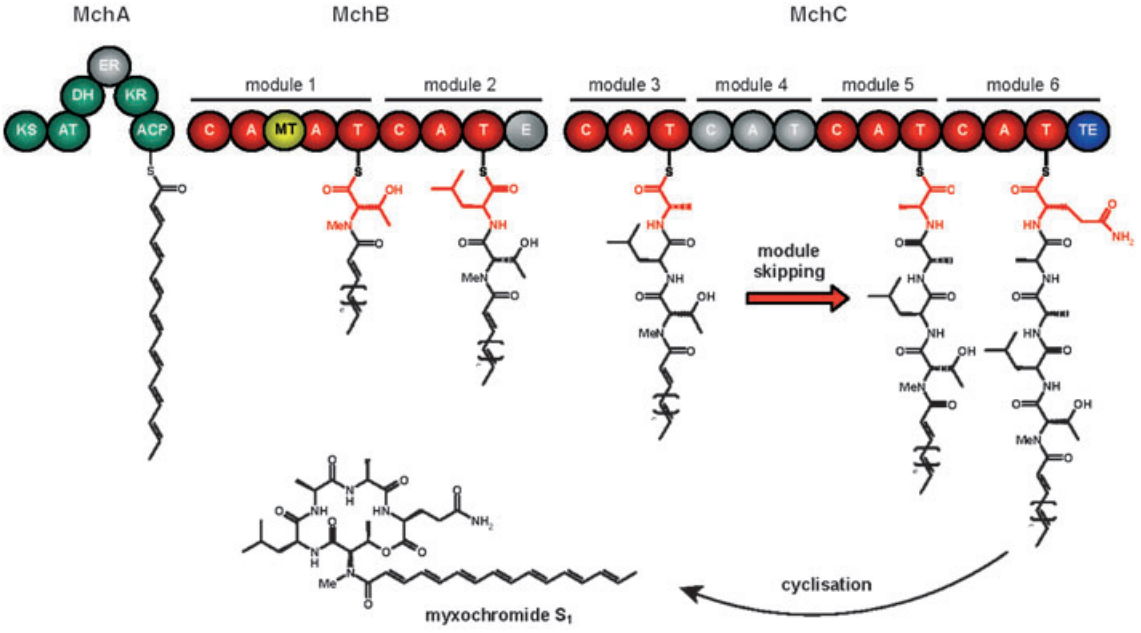

Figure 5. *Model for the biosynthesis of myxochromide S in* S. aurantica *DW4/3–1. PKS domains are shown in green, NRPS domains in red. The methyltransferase (MT) domain is shown in yellow, while the thioesterase (TE) domain is marked in blue. Grey domains are presumably inactive. Transfer of the peptide chain from the T domain of module 3 to the T domain of module 5 is indicated.*

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a complete reductive loop. However, sequence analysis of the MchA ER domain shows variations in the highly conserved fingerprint region (LxHxxxGGVGxxAxxxA) containing the putative NADP(H) binding site (GxGxxAxxxA), which is essential for enzyme function. The MchA ER core region (Lx**L**xxGG**I**Gxx**V**xxx**G**) is significantly altered in parts of the NADP(H) binding site (see Figure 3). This finding correlates well with the polyunsaturated structure of the myxochromide S polyketide side chain, because the ER domain is most probably inactive.

HPLC and HPLC-MS/MS analysis of an extract from *S. aurantiaca* DW4/3–1 shows the production of further myxochromides with similar UV spectra and retention times. In addition to myxochromide S_1 (**1**), in which acetate is used as a starter unit in the C_{16} polyketide chain, the detected mass of one of the other myxochromides indicates the presence of a C_{17} side chain, indicating a propionate starter unit (**2**; see Scheme 1). The MS/MS-based assignment was confirmed by a feeding experiment with D_6-propionic acid, which resulted in a D_5-labelled polyketide chain from compound **2**, verifying the use of propionate as starter unit. Thus, MchA shows flexibility concerning the starter unit. Furthermore, a myxochromide derivative with a C_{18} polyketide group could be detected and verified by MS/MS analyses (**3**; see Scheme 1). This indicates that the programmed iteration during polyketide chain assembly by MchA is not strictly reproducible. Occasionally, additional cycles of chain elongation are performed, resulting in a longer polyketide chain (such as compound **3**). However, the mechanisms underlying the control of chain length in iterative processes in FAS and in type I, type II and type III PKS are still a mystery. The occasional incorporation of additional extender units by MchA could be explained in terms of a phenomenon similar to the "stuttering" process described in modular type I polyketide synthases. It was shown that the hexamodular 6-deoxyerythronolide B synthase (DEBS) from the erythromycin biosynthetic pathway also produces low levels of octaketide products.[43] The structures of these 16-membered macrolactones, when compared with those of the normal 14-membered lactones, indicate that module 4 of DEBS aberrantly operates twice in succession, a process that has been termed "stuttering". A similar process was also assumed to be responsible for the production of epothilones I_1–I_6,[14] containing additional acetate or propionate units in their lactone rings.[44] Even programmed iterative use of single modules in type I PKS for the production of the main compounds of a pathway has been demonstrated.[10] MchA can thus be regarded as a highly unusual iterative type I bacterial PKS lacking perfect chain length control, which could also be referred to as "stuttering."

Model for myxochromide S biosynthesis: first evidence for module skipping in NRPSs: Myxochromide S biosynthesis proceeds with the assembly of the peptide core, which is catalysed by the NRPSs MchB and MchC. These proteins display the ordered assembly of conserved C, A and T domains organized into modules characteristic of such multienzymes.[45] According to the nonribosomal code, the first NRPS module from the myxochromide S biosynthetic gene cluster located on MchB is specific for threonine (see Figure 4). We suggest that the N-terminal C domain of this module catalyses the condensation of the polyunsaturated polyketide chain bound to the MchA ACP with the amino group of threonine. This resembles the assumed roles of the N-terminal C domains of the first modules in the proteins responsible for the biosynthesis of the lipopeptides lichenysin,[46] surfactin,[47] fengycin[48] and mycosubtilin,[49] as well as the putative role of the first C domain in the PKS/NRPS nostopeptolide A synthase.[50] Nevertheless, alignments using these C-domains with standard C-domains catalysing amide bond formation during nonribosomal peptide assembly did not reveal any significant subgrouping in phylogenetic trees (data not shown). A MT domain is found integrated into the A domain of module 1, and most probably catalyses the *N*-methylation of threonine. The biosynthesis continues with the incorporation of leucine by module 2. An E domain, which usually catalyses the epimerization of the incorporated amino acid, could be identified in this module. Surprisingly, L configurations were determined for all amino acids incorporated into myxochromide S. From the sequence analysis, there is no indication that the E domain should be inactive. We speculate that the presence of an E domain in this module is an evolutionary link to the hypothetical biosynthetic gene cluster of myxochromide A (**10**; see Scheme 1) from *M. virescens*. In analogy to myxochromide S biosynthesis, alanine would be the second amino acid expected to be inserted into the myxochromide A peptide core. Because one of the two myxochromide A alanines has the D configuration, the E domain of module 2 could have been optimized for alanine epimerization. After substrate specificity switching by mutation of the A domain from alanine to leucine, the E domain may no longer recognize the new intermediate. We are currently in the process of cloning the myxochromide A biosynthetic gene cluster to evaluate this speculation and to investigate the skipping hypothesis discussed below further. The next three modules of the myxochromide S biosynthetic gene cluster (modules 3–5) contain only the basic equipment of NRPS modules (C, A and T domains). On comparison of the deduced substrate specificities of the A domains, module 3 would be predicted to catalyse the incorporation of alanine (see Table 3). The selectivity-conferring residues of the A domain from module 4 correlate well with the consensus sequence for proline. This is again in good agreement with the hypothetical structure of the myxochromide A synthetase, because **10** contains proline as the fourth amino acid of its peptide core. Surprisingly, proline is not a component of myxochromide S. Additionally, the NRPS part of the myxochromide S gene cluster contains six NRPS modules, although only five amino acids are incorporated into the products. Consequently, it seems likely that module 4 of the myxochromide S NRPS is skipped during the biosynthesis. This module is assumed to have originally activated and incorporated proline in the myxochromide A biosynthesis predecessor protein. Whether this hypothesis is correct remains to be seen through analysis of the myxochromide A biosynthetic genes (see above). Analysis of the A domain from module 4 shows several variations from normally conserved amino acids in the core regions (see Figure 4), which might result in the inactivity of this domain.

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Similar variations can also be observed in the peptide sequence of the T domain from this module. In particular, the highly conserved serine residue, which is necessary for the posttranslational activation of the T domain, is not in the expected position (see Figure 4). From these results, inactivity of module 4 might be the result of mutations in either the A or the T domain, because there is no indication that the C domain is inactive. Thus, to account for the lack of a sixth amino acid in the myxochromide S peptide core, module skipping most probably takes place. To the best of our knowledge, this represents the first example of module skipping in nonribosomal peptide synthetases. One might see the presence of an inactive A domain in the bleomycin megasynthetase as another example. In this case the corresponding T domain is loaded by another A domain acting in trans, which finally results in standard NRPS module assembly logic.[51] Nevertheless, skipping of modules has been observed in PKS systems, especially in non-natural PKS hybrid systems. The Leadlay group investigated this phenomenon with a hybrid tetraketide synthase containing modules from the erythromycin and rapamycin PKSs.[52] Expression of this PKS results in the production of tetraketides (minor products) as well as triketides (major products), which is assumed to be the result of skipping of the "foreign" Rap2 module. However, it was shown by mutagenesis that the ACP of the interpolated Rap2 module is required for chain transfer. As judged by the isolation of a ring-contracted epothilone derivative (epothilone K) from *Sorangium cellulosum*,[44] module skipping most probably also takes place during epothilone biosynthesis. The yield of epothilone K is very low in relation to the other epothilones, which indicates that module skipping in this PKS only takes place very rarely. During myxochromide S biosynthesis, however, module skipping seems to be used exclusively, because all myxochromides isolated so far from *S. aurantiaca* show the same pentapeptide core. A different form of "skipping" had been described earlier by the Sherman group for the pikromycin/methymycin PKS.[53] Here, expression of the full-length PikAIV generates the 14-membered macrolactone, whereas expression of the amino-terminal truncated form of PikAIV leads to some sort of "transcriptional skipping." This happens because the corresponding module is not present in the ORF, so the first condensation cycle in polyketide biosynthesis generates a 12-membered macrolactone. For the unusual skipping process in MchC, two scenarios can be envisioned. Firstly, the whole module might be skipped. In this case, the T domain from the third module would interact with the donor site of the C domain of module 5. Secondly, the peptide chain from module 3 might be transferred to the T domain of module 4 without further extension, because the A domain might not load the T domain with proline. Subsequently, the next condensation would take place. The latter case would require the activation of the T domain by phosphopantetheinylation, presumably of the serine residue next to the proline in the centre of the active site (see Figure 4). Consequently, the incorporation of the fourth amino acid, L-alanine, into the myxochromide S peptide core is catalysed by module 5. Module 6 represents the last module of the biosynthetic gene cluster and contains an additional TE domain. After the incorporation of glutamine into the peptide chain, the TE domain catalyses the cyclization of the peptide molecule, resulting in a macrolactone. The lactonization takes place between the threonine hydroxy group and the glutamine carboxy group. A similar process is assumed for myxochromide A biosynthesis in *M. virescens*. Rather than the proposed structure **10** of myxochromide A (which is based on limited NMR data[20]), we propose a slightly changed structure for this metabolite (**11**; see Scheme 1). This would be the result of the direct incorporation of glutamine rather than glutamic acid into the peptide backbone. If the original structure assignment of myxochromide A **10** were correct, one would have to propose a glutamate-specific module in the corresponding gene cluster of *M. virescens* and a subsequent amidation of the α-carboxy group, as well as an unusual macrocyclization mechanism via the γ-carboxyl group of glutamate.

Experimental Section

Bacterial strains and culture conditions: *Escherischia coli* strains were cultured in Luria Broth at 37 °C. *Stigmatella aurantiaca* DW4/3–1[54] and its descendants were grown in Tryptone medium (1% tryptone, 0.2% $MgSO_4 \cdot 7H_2O$; pH adjusted to 7.2) at 30 °C. Antibiotics (kanamycin sulfate 50 $\mu g\,mL^{-1}$ and streptomycin sulfate 120 $\mu g\,mL^{-1}$) were added where appropriate.

Analysis of secondary metabolites in *S. aurantiaca* DW4/3–1: For secondary metabolite production in shake cultures, strain DW4/3–1 and its descendants were cultivated in Probion liquid medium and the metabolites were extracted and prepared as described previously.[18] To compare the spectrum of secondary metabolites produced by the mutant with that of the wild-type strain, concentrated acetone extracts were analysed on a Hewlett Packard series 1100 HPLC fitted with a diode array detector. Chromatographic conditions were as follows: column ET 125 × 2 mm and precolumn, Nucleosil 120–5-C_{18}; solvents: A) acetonitrile (5%), water (95%), ammonium acetate (5 mM), acetic acid (0.003%), and B) acetonitrile (95%), water (5%), ammonium acetate (5 mM), acetic acid (0.003%); solvent gradient from 10% B at 0 min to 100% B within 30 minutes, followed by 10 minutes with 100% B; flow rate 0.3 $mL\,min^{-1}$ and UV detection at 200–400 nm.

Feeding experiment with D_6-propionic acid: D_6-Propionic acid (1 mM) was added at the beginning of the fermentation to a culture of *S. aurantiaca* DW4/3–1 in Probion liquid medium (50 mL).[18] After 60 h, the adsorber resin XAD-16 (1%) (Rohm & Haas, Germany) was added. The cells were grown at 30 °C for 4 days. Cells and resin were harvested by centrifugation and extracted with methanol. The extract was evaporated and redissolved (in 500 μL of methanol) and the concentrated extract (10 μL) was analysed by HPLC-MS (Agilent 1100 LC system). The chromatographic conditions were the same as used for analytical HPLC. Detection was carried out at 400 nm and for mass detection a Perkin Elmer Sciex API 2000 mass spectrometer fitted with a TurbolonSpray™ source was used.

Fermentation, isolation and structure elucidation of myxochromides S: The strain *S. aurantiaca* DW4/3–1 was cultivated in a 150 L Bioreactor (Bioengineering, Wald, Switzerland) containing Zein liquid medium (85 L, Zein 0.8%, peptone from casein tryptically digested 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, HEPES 10 mM, XAD-16 1%, pH 7.2) as described.[19] The cell mass and the Amberlite XAD 16 adsorber resin from the culture broth were eluted with acetone

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and methanol, respectively. The combined eluates were concentrated in vacuo, resulting in an aqueous layer, which was extracted with ethyl acetate. Evaporation of the solvent yielded a dark oily residue, which was partitioned between methanol and heptane. The concentrated methanol layer was chromatographed with methanol on Sephadex LH-20. The fractions containing myxochromides according to HPLC analysis were combined, concentrated in vacuo and chromatographed on a preparative RP18-HPLC column (Nucleosil C_{18}, solvent: MeOH/H_2O 82:18) to yield pure myxochromide S_1 (140 mg) and mixtures of myxochromides S_2 and S_3 (see Scheme 1).

Myxochromide S_1: yellow solid; TLC (silica gel, ethyl acetate/methanol/water 80:15:10), $R_F = 0.6$; $[\alpha]_D^{22}$ −99.0 (MeOH, $c = 2$ mg mL^{-1}); UV (methanol): 388 sh (4.79), 406 (4.86), and 420 nm sh (4.82); HRESI MS: 745.392 $[M+Na]^+$ (calcd. 745.3901); ^1H and ^{13}C NMR data see Table 1.

Biological assays: The antibiotic activity of myxochromide S_1 against various microorganisms was determined by agar diffusion assay with paper discs. Testing for cytotoxicity against L929 cells (mouse, connective tissue ATCC CCL 1) was determined as reported.[55]

DNA manipulations, analysis, Southern blotting, sequencing, and PCR: Chromosomal DNA from *S. aurantiaca* was prepared as described.[56] Southern hybridizations with homologous probes and chromosomal DNA were performed with the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Molecular Biochemicals) following the manufacturer's recommended protocol. DNA sequencing was performed by use of the Big Dye RR Terminator Cycle Sequencing Kit (PE Biosystems) and the gels were run on ABI-377 sequencers. Sequencing of the cosmid E196[18] was performed by a shotgun approach as described previously.[8] PCR was carried out with use of HotStarTaq Polymerase (Qiagen) according to the manufacturer's protocol. Dimethylsulfoxide was added to a final concentration of 5%. Conditions for amplification with the Eppendorf mastercycler gradient were as follows: 15 min at 95 °C for activation of the polymerase, denaturation for 30 sec at 95 °C, and annealing for 30 sec at 52 °C for degenerate primers and at 60 °C for homologous primers. The extension was run for 45 sec at 72 °C. Thirty cycles were used, and a final extension for 10 min at 72 °C was employed. PCR products were purified by use of the High Pure Product Purification Kit (Roche Molecular Biochemicals) and subsequently ligated into pCR2.1-TOPO by use of the TOPO TA cloning Kit (Invitrogen). All other DNA manipulations were performed by standard protocols.[57] Amino acid and DNA alignments were performed with the programs in the Lasergene software package (DNASTAR Inc.) and Clustal W.[58]

Construction of the *S. aurantiaca* DW4/3–1 mutant strain EBS66–3: After end-sequencing of cosmid E196,[18] two oligonucleotides (195T3–1: 5′-CCCCCAGCTGGAAGAAGTTGTC-3′ and 195T3–2: 5′-CCGGATCGAGCTGGGTGAG-3′) were designed and used to amplify a NRPS fragment from the 3-prime end of the insert. The amplified fragment (407 bp) was cloned into pCR2.1-TOPO (Invitrogen), resulting in plasmid pEBS66, which was introduced into *S. aurantiaca* DW4/3–1 by electroporation as previously described.[18] The introduced plasmid integrated by homologous recombination into the genome, leading to the gene disruption mutant EBS66–3. The integration of the plasmid was verified by genomic Southern blot using the NRPS fragment as a probe (data not shown).

Plasmid recovery from the *S. aurantiaca* DW4/3–1 mutant strain EBS66–3: The vector recovery from chromosomal DNA was performed as described previously.[59] Chromosomal DNA (10 μg) prepared from *S. aurantiaca* DW4/3–1 mutant strain EBS66–3 was digested with *Mlu*I, phenol/chloroform purified and ligated overnight at 16 °C. The ligation mixture (1–3 μL) was electroporated into *E. coli* XL-1 blue cells (electroporation cuvette 0.2 cm; 200 Ω, 25 μF and 2.5 kV cm^{-1}). The vector recovery plasmid pSWMch2 was extracted from resulting kanamycin-resistant colonies.

EMBL database entry: The nucleotide sequence reported here has been submitted to the EMBL database under accession number AJ698723.

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Keywords: bacterial iterative PKS · module skipping · myxobacteria · nonribosomal peptide · polyketides · secondary metabolism

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