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Markerless Mutations in the Myxothiazol Biosynthetic Gene Cluster: A Delicate Megasynthetase with a Superfluous Nonribosomal Peptide Synthetase Domain

Stefan Weinig,[1,2] Taifo Mahmud,[3] and Rolf Müller[1,2,*]

[1]Gesellschaft für Biotechnologische Forschung mbH (GBF)
Mascheroder Weg 1
38124 Braunschweig
[2]Institut für Pharmazeutische Biologie
Mendelssohnstr. 1
Technische Universität
38106 Braunschweig
Germany
[3]University of Washington
Department of Chemistry
Seattle, Washington 98195

Summary

Myxobacteria are efficient producers of natural products with various biological activities. Nevertheless, genetic systems for markerless mutagenesis are only available for *Myxococcus xanthus* DK1622, a strain that is not known to produce any secondary metabolites. We report here the development of such a technique for *Stigmatella aurantiaca* DW4/3-1, a multiproducer of natural products. The system is used to further characterize the combined polyketide synthase/peptide synthetase system responsible for myxothiazol biosynthesis. Analysis of a series of point mutations and in-frame deletions shows that the myxothiazol megasynthetase is a rather specialized and delicate system that is hardly capable of processing unnatural intermediates. An in-frame deletion of the *MtaC* oxidation domain results in the production of unchanged bis-thiazole myxothiazol instead of the expected thiazoline-thiazole derivative of the compound. This shows that this domain within a nonribosomal peptide synthetase is not necessary for product formation.

Introduction

Natural products from microorganisms are a major source of bioactive compounds used by the pharmaceutical and the agrochemical industry [1]. Many of these secondary metabolites are formed by large, multifunctional, and modular enzyme complexes termed polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS). These megasynthetases assemble and modify activated short chain carboxylic acids or amino acids, which results in the production of low molecular weight polyketides or peptides [2–4]. This stepwise biochemical process can be correlated to the modular structure of the proteins involved (see accompanying manuscript [47]). Thus, mutagenesis of the corresponding genes aimed at the production of changed or novel structures with improved activities has become a major goal of research related to microbial natural product biosynthesis [5, 6].

As an alternative to the widely used streptomycetes as producing organisms, myxobacteria are a rich source of novel basic structures [7], especially where PKS and NRPS assembly lines are combined [8–14]. Myxothiazol [15, 16] and melithiazol [17, 18] are two effective electron transport inhibitors of the respiratory chain produced by *Stigmatella aurantiaca* DW4/3-1 and *Melittangium lichenicola* Me I46 employing hybrid PKS/NRPS systems (see accompanying manuscript [47] and Figure 1).

Due to the progress in our understanding of the genetics and biochemistry of PKS and NRPS systems, it has become possible to rationally alter chemical structures in a well-defined biosynthetic system, e.g., manipulating the genes encoding the erythromycin PKS [19–23]. Only a few examples of rational design of NRPS are available [24], and many groups have reported (mainly in oral communications) severe difficulties trying to design a variety of biosynthetic systems. This difficulty is mostly attributed to the lack of structural information on the communication between PKS and NRPS modules. Despite the fact that examples of most major domains of the megasynthetases have recently been crystallized [20, 25], there is no information on the structure of a complete PKS and/or NRPS assembly line.

The myxothiazol and melithiazol hybrid PKS/NRPS biosynthetic gene clusters were analyzed recently based on in silico analysis of the corresponding biosynthetic genes (see accompanying manuscript [47] and [8]). However, several novel structural features require further characterization. The development and application of a genetic system for the generation of markerless deletions/insertions or point mutations in the chromosome of *S. aurantiaca* DW4/3-1 that is used to further characterize the myxothiazol genes is reported. These experiments show that the myxothiazol PKS/NRPS is a delicate system in which marginal alterations in the amino acid sequence abolish compound biosynthesis. The complete deletion of the oxidation domain in MtaC shows that it is superfluous for myxothiazol production.

Results and Discussion

Establishing a System for Markerless Mutagenesis in *S. aurantiaca* DW4/3-1

The detailed biochemical analysis of PKS and NRPS biosynthetic pathways is hampered by the complexity of the proteins involved and the lack of in vitro models. Genetic research of myxobacteria is even more difficult due to the lack of plasmids (e.g., for ectopic expression) and the poorly established or completely unavailable genetics for most genera. Most studies on PKS and NRPS pathways have been performed by site-directed and markerless deletions, insertions, or point mutations within the corresponding genes, resulting in modified proteins that produce structurally altered secondary

*Correspondence: rom@gbf.de

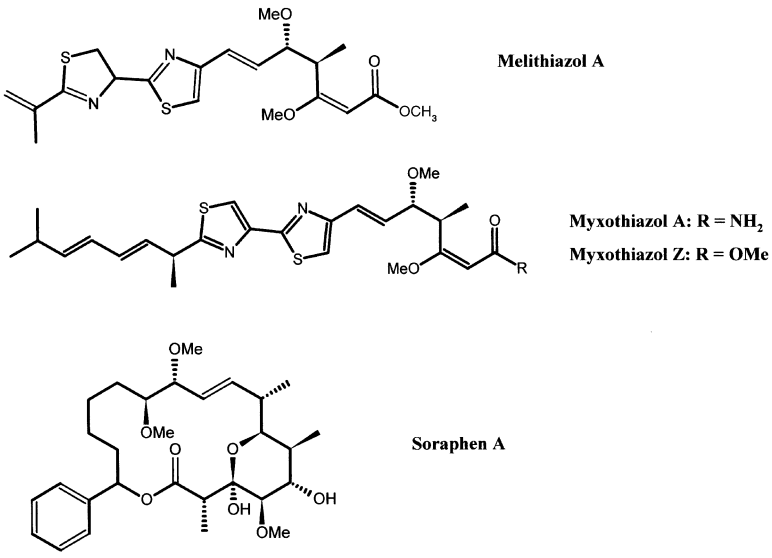

Figure 1. Structures of Myxobacterial Secondary Metabolites

metabolites. Given the severe problems that we encountered while trying to establish a gene inactivation system for the melithiazol producer *M. lichenicola* Me I46 (see accompanying manuscript [47]), and to answer questions arising from the in silico analysis of both the myxothiazol and the melithiazol gene clusters, we developed a genetic system for markerless gene inactivation, deletion, substitution, insertion, and point mutagenesis in *S. aurantiaca* DW4/3-1.

In *M. xanthus*, *galK* is more convenient than *sacB* as a counterselectable marker (e.g., [26]). After a series of experiments employing *galK*, we concluded that this marker is not applicable to *S. aurantiaca* DW4/3-1 (M. Kopp and R.M., unpublished data). On the other hand, the counterselectable *sacB* gene from *Bacillus subtilis* can be used. The procedure is still rather time consuming due to the slow growth of *S. aurantiaca* DW4/3-1. Additionally, only 10%–15% of the sucrose-resistant colonies are kanamycin sensitive, which indicates a relatively high rate of spontaneous resistance in the mutants after counterselection with sucrose. Wu and Kaiser reported an efficiency of 100% in *M. xanthus* [27], which raises the hope that the method can still be optimized in order to assay further functionalities, and not only in the myxothiazol biosynthetic gene cluster.

All of the mutants described below (after first and second crossover events) were confirmed by Southern blot and PCR. Subsequently, the chromosomally mutated DNA fragment was amplified by PCR using a proofreading DNA polymerase, cloned, and resequenced. After verification of the correctness of the mutation, mutants were analyzed for natural product formation. Because the genetic system always gives rise to revertants (Figure 2), it was possible to analyze if the integration and the subsequent excision of the mutagenesis plasmid would cause secondary effects on product formation. This is clearly not the case, because all of the revertants produce myxothiazol at wild-type levels.

Slight Modifications of the Myxothiazol Megasynthetase Lead to the Inactivation of the Biosynthetic Complex, Indicating a Rather Specialized Protein Complex Unable to Process Modified Intermediates

Initially, the potential of the myxothiazol megasynthetase to generate myxothiazol derivatives was investigated. Because similar experiments worked in the erythromycin system [3, 23, 28, 29], we first tried to inactivate the KR of module 1 of MtaB (see accompanying manuscript [47]) by site-directed mutagenesis, which should result in the production of a myxothiazol with a keto-group at C-10 corresponding to C-1 of the isovalerate starter unit (see Figure 1). The conserved NAD(P) binding site was chromosomally mutated from VGGLGGIG to V*II*L*V*GIG (changed amino acids in italics) to prevent the cofactor binding of NAD(P). However, the resulting mutant did not produce any detectable derivative of myxothiazol (S.W. and R.M., unpublished data). In further experiments aimed at the production of desmethyl derivatives of myxothiazol to directly prove the function of previously unknown O-MT domains in PKS, the SAM binding sites of MtaE and MtaF were mutated by site-directed mutagenesis. Here, even single amino acid substitutions led to myxothiazol and desmethyl-myxothiazol negative mutants (S.W., T.M., and R.M., unpublished data). Searching for mass spectra of released and decarboxylated intermediates in the extracts of the culture broth did not result in the detection of any product of the expected mass (T.M. and R.M., unpublished data).

Given the reports on the successful inactivation of domains in some PKS systems, positive results seem to be typical. Nevertheless, a variety of groups in the field do not agree with this statement. Most modification studies seem to result in nonproducers or low producers, and only a few of them appear in the literature. In this study, we demonstrate that relatively simple mutations in the myxothiazol megasynthetase result in non-

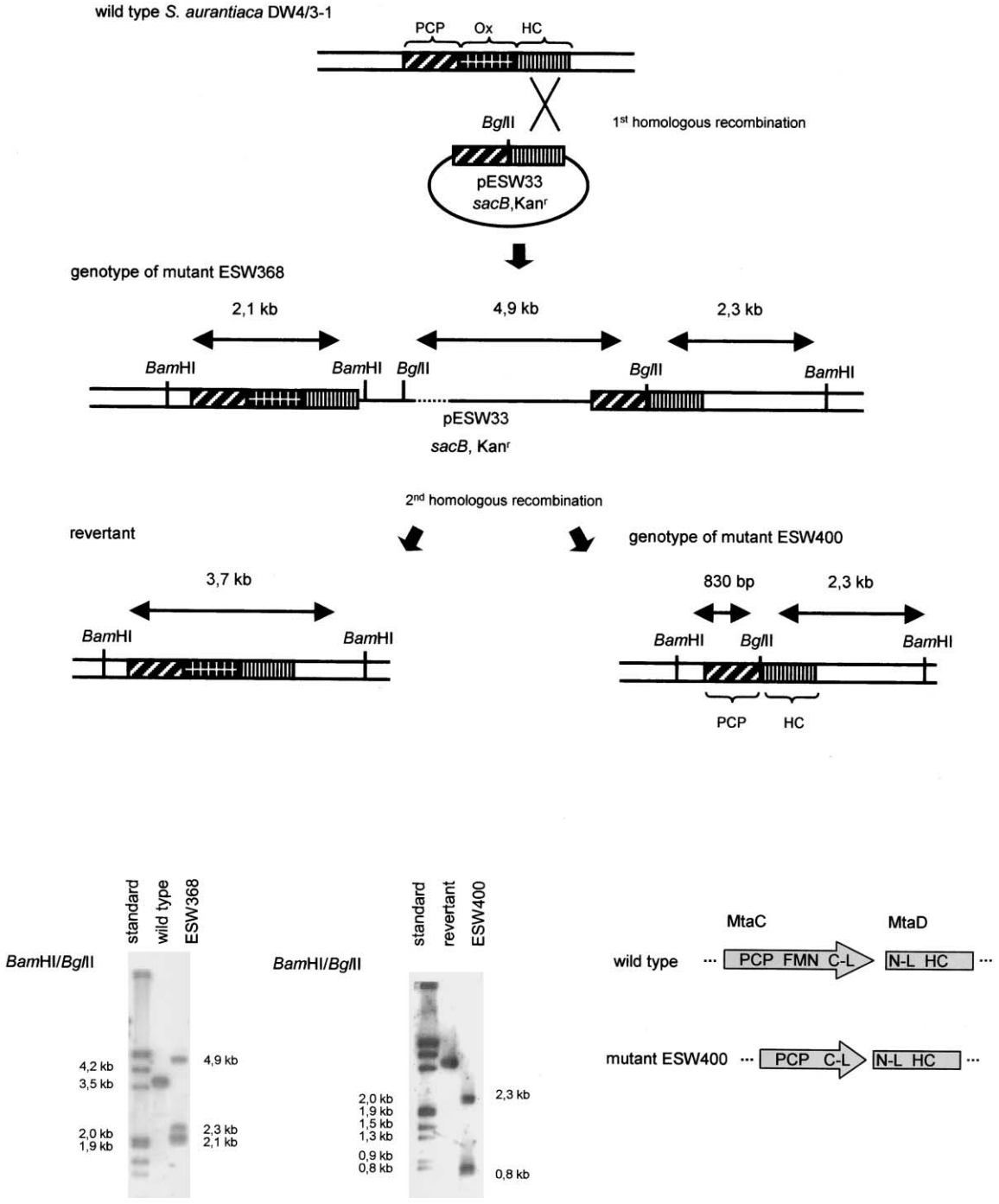

Figure 2. Schematic Diagram Showing the Mutagenesis Strategy for the Generation of Markerless Mutants in *S. aurantiaca* DW4/3-1

The strategy is exemplified by the deletion of the Ox domain of MtaC starting with the integration of pESW33 into the chromosome. The genotype of the merodiploid mutant ESW368 is shown, and the size of the restriction fragments giving signals in the Southern analysis are indicated by arrows. After excision of the plasmid via a second crossover event, two genotypes are possible: the revertant with identical genotype to the wild-type is shown on the left, whereas the deletion mutant ESW400 is shown on the right. In the bottom panel, Southern blot analyses of ESW368, ESW400, revertant, and wild-type in comparison to the DIG-labeled DNA molecular weight marker III (Roche Diagnostics) are shown. Restriction enzymes used are indicated in the picture; the insert of pESW33 was used as a probe. On the lower right, a diagram of the C-terminal part of MtaC and the N-terminal part of MtaD in wild-type and mutant ESW400 is shown. C-L, C-terminal linker region; N-L, N-terminal linker region.

Figure 3. Partial Alignment of the Region of A Domains from MelD and MtaD into which the N-Terminal Parts of the Ox Domains Are Inserted

The highly conserved A domain motif A8, the deletions, and the Ox domain motif Ox-1 are shaded in gray (see text). Asterisks indicate identical amino acids, while colons indicate similar amino acids.

producing mutants instead of producers of the expected derivatives of myxothiazol. In contrast to other biosynthetic systems (e.g., erythromycin, epothilone), the myxothiazol PKS/NRPS hybrid seems to have been optimized evolutionary to produce exactly one compound and not a variety of derivatives. This conclusion is underlined by the fact that almost no biosynthetic derivatives of myxothiazol have been found in *S. aurantiaca* DW4/3-1. In contrast, more than 30 different epothilone derivatives were found in a recent study in the supernatant of *Sorangium cellulosum* So ce90 [30]. Another reason for the absence of myxothiazol analogs might be the lack of an editing type II thioesterase associated with the biosynthetic complex. These enzymes appear to be involved in the release of aberrant products that block the biosynthetic machinery because they are not further processed by the following C or KS domains [31–34]. In the rifamycin system, simple blocking of the PKS release mechanism (lactamization) leads to the production of all possible biosynthetic intermediates in detectable quantities [35, 36]. We have searched the myxothiazol mutants described here using MS-MS techniques for the production of such intermediates and also for the expected altered products without positive results (S.W, T.M., and R.M., unpublished data).

Given the lack of structural information on complete PKS and NRPS megasynthetases, the interactions of the proteins involved are still very difficult to predict, which makes the generation of changed and hybrid natural products predominantly dependent on a biosynthetic system processing a broad range of intermediates. Currently, the researcher's green thumb is very helpful on the way to generate reasonable results. It can be expected that this situation will change when the structural data on complete modules or megasynthetases become available.

Role of the Oxidation Domains in MtaC and MtaD in Myxothiazol Biosynthesis

Due to the presence of Ox domains in MtaC, MtaD, MelC, and MelD (see accompanying manuscript [47] and Figure 1), one would expect melithiazols to be produced as bis-thiazole structures instead of thiazoline-thiazole compounds. Because only thiazoline-thiazoles have been isolated from *M. lichenicola* Me l46, we hypothesized that the Ox domain in MelC may be inactive (which would presumably mean that this is true for MtaC-Ox as well). The lack of activity might be due to the positioning of the domain in the protein, as it is located behind the PCP of MtaC, whereas other Ox domains are inserted between the core motifs of A domains A8 and A9 (in MtaD, MelD, and in the epothilone biosynthetic protein EpoB [37], which is also referred to as EpoP [13]). Another Ox domain is found after the PCP in the bleomycin biosynthetic protein BlmIII [38, 39], but it is not clear whether this domain is active because thiazoline bleomycins are known (phleomycin), and other proteins possibly involved in the oxidation processes can be found in the corresponding gene cluster. In fact, activity as thiazoline oxidase has been clearly demonstrated only for EpoB [37].

To answer the question whether the Ox domain of MtaC is required for myxothiazol biosynthesis, an in-frame deletion of the domain was generated. The domain was deleted from plasmid pESW33 in a way that connected the presumed C-terminal linker of MtaC directly with the PCP of the protein (Figure 2). After verification of the deletion in the chromosome, the resulting mutant ESW400 was analyzed for product formation. Only wild-type levels of myxothiazol and not the thiazoline-derivative were detected. This result clearly demonstrates that the Ox domain of MtaC is superfluous for myxothiazol biosynthesis. This is also true for the MelC-Ox domain in melithiazol formation. Presumably, the Ox domain of MtaD oxidizes both thiazolines during myxothiazol assembly or complements the function of MtaC (which might also be inactive per se). Thus, there must be some differences between the Ox domains of MtaD and MelD, which are 56.4% identical on the amino acid level. In alignments, two gaps occur within the Ox domains (gaps of 1 and 10 amino acids in positions 12 and 21; see Figure 3, numbering according to MtaD-Ox), which indeed might indicate functional differences between the MtaD and MelD Ox domains. Two more experiments were performed dealing with this question: the Ox domain of MtaD was deleted from the wild-type, resulting in mutant strain ESW392, and the A domain including the Ox domain of MtaD was replaced with

Table 1. Plasmids Used in This Study

Name	Description of Construction
pESW26	see accompanying manuscript [47]
pESW33	604 bp PCR product generated using primer NADC1FOR + NADC2REV from cosmid E25, hydrolyzed with NotI/BglII, and 600 bp PCR product generated using primers NADC3FOR + NADC4REV from cosmid E25, hydrolyzed with BglII/SpeI; both fragments ligated into pSWU41 (NotI/SpeI)
pESW36	510 bp PCR product generated using primers NADD1FOR + NADD2REV from cosmid E25, hydrolyzed using NotI/XhoI, and 609 bp PCR product generated with primers NADD3FOR + NADD4REV from cosmid E25, hydrolyzed using XhoI/SpeI; both fragments ligated into pSWU41 (NotI/SpeI)
pESW41	517 bp PCR product generated using primers ENO1FOR + ENO2REV from cosmid E25, hydrolyzed with NotI/SspI, and 508 bp PCR product generated using primers ENO3FOR + ENO4REV from cosmid E25, hydrolyzed with SspI/SpeI; both fragments ligated into pSWU41 (NotI/SpeI)
pESW47	618 bp PCR product generated using primers ISO1 + ISO2 from cosmid E201, cloned into pCR-2.1-TOPO
pESW51	622 bp PCR product generated using primers ISO3 + ISO4 from cosmid E201, cloned into pCR-2.1-TOPO
pESW64	DNA fragment from pESW47 (PvuII/PstI) and DNA fragment from pESW51 (PstI/XbaI); both fragments cloned into pSWU41 (SmaI/XbaI)
pESW204	458 bp PCR product generated using primers MXISO3 + MXISO4 from genomic DNA of *S. aurantiaca* DW4/3-1, cloned into pCR-2.1-TOPO
pESW225	1088 bp PCR-product generated using primers MLD3 + MLD4 from cosmid M2; cloned into pCR-2.1-TOPO
pESW236	631 bp PCR-product generated using primers MYX1FOR + MLD2 from cosmid E25; cloned into pCR-2.1-TOPO
pESW237	620 bp PCR-product generated using primers MLD5 + MYX6REV from cosmid E25; cloned into pCR-2.1-TOPO
pESW241	DNA fragment from pESW237 (HindIII/BamHI) and DNA-fragment from pMSW6 (XbaI/HindIII); both fragments cloned into pSWU41 (XbaI/BamHI)
pESW243	see accompanying manuscript [47]
pMSW5	DNA fragment from pESW236 (XbaI/NotI) and 1.1 kb NotI/EcoRI fragment from cosmid M2 cloned into pBluescript IIKS(−) (XbaI/EcoRI)
pMSW6	DNA fragment from pESW225 (EcoRI/HindIII) cloned into pMSW5 (EcoRI/HindIII)
pMSW12	see accompanying manuscript [47]

the corresponding fragment from MelD (mutant strain ESW627; the substitution took place using highly homologous regions for recombination, see Experimental Procedures). The former experiment should have resulted in a thiazole-thiazoline or a bis-thiazoline myxothiazol, whereas the latter should have given a thiazoline-thiazole myxothiazol. Unfortunately, both strains were blocked in myxothiazol biosynthesis. Thus, the difference between the MtaD and MelD Ox domains remains unclear. It can be concluded that the Ox domain of MtaC is not required for myxothiazol biosynthesis.

The Heptadiene Isomerase Homolog MtaH Is Not Necessary for Myxothiazol Biosynthesis

Downstream of *mtaG/melG* in the myxothiazol and melithiazol gene clusters, a gene encoding a heptadiene isomerase homolog is found (*mtaH/melH*; see accompanying manuscript [47]). Before cloning the melithiazol genes, we thought that this gene was involved in the formation of the unusual triketide starter of myxothiazol, in which the double bonds are located irregularly (Figure 1). Standard PKS biochemistry involves the dehydration of β-keto-intermediates giving rise to Δ2,3 double bonds, whereas in the starter moiety of myxothiazol Δ3,4 double bonds are found. The homology to heptadiene isomerases seemed intriguing because they are involved in double-bond isomerization, and it was thus assumed that inactivation of *mtaH* should result in a myxothiazol with Δ2,3 double bonds. An in-frame deletion of *mtaH* in mutant ESW444 ($mtaH\Delta_{333\text{-}665}$) did not result in any change in myxothiazol formation. The product was purified from the mutant ESW444 and analyzed by H-NMR, as the isomerization would not change the mass of the compound. No difference to wild-type myxothiazol was detected. We assumed that an isogene might be responsible for the complementation of the *mtaH* in ESW444.

The genome sequence of *M. xanthus* DK1622 is currently being determined at the Institute for Genomic Research (TIGR). In the preliminary sequences, a gene with significant similarity to *mtaH* was found and used as a probe to identify a homolog in *S. aurantiaca* DW4/3-1 that represents a putative isogene of *mtaH*. The deduced protein shows 40% identity to MtaH and 89% identity to the protein from *M. xanthus*. It was designated MtaX, and a fragment of the gene was used for the generation of double-mutant ESW463 ($mtaH^{-}/mtaX^{-}$). ESW463 was analyzed similarly to ESW444, but again no modified myxothiazol was detected. Thus, MtaH is not required for myxothiazol formation. The unusual position of the double bonds and the function of *mtaH/melH* in myxothiazol and melithiazol biosynthesis still remain unexplained.

Some other polyketides contain "nonstandard" double bonds as well. Even where the corresponding gene clusters are known, no information about their formation is available (e.g., ansamitocin [40]). Nevertheless, an atypical dehydration step might take place due to intrinsic differences in the DH domain of the PKS. In soraphen A, another "irregular" double bond is located between carbons 9 and 10 (Figure 1). Here, it is evident that the PKS reduces the intermediate completely and an oxidation occurs as a post PKS step [41, 42].

Significance

The molecular analysis of polyketide and nonribosomal peptide biosynthetic pathways from myxobacteria contributed to our knowledge of these systems,

which is mostly from the work on actinomycetes and bacilli. Nevertheless, the essential genetics for detailed study of natural product pathways remain poorly established for this class of microorganisms. A system for markerless in-frame deletions, insertions, and point mutations is described for the secondary metabolite multiproducer *S. aurantiaca* DW4/3-1, which sets the stage for the analysis of the various secondary metabolic pathways in this strain. A series of mutants in the myxothiazol megasynthetase is reported, most of which do not produce novel myxothiazol derivatives. It is assumed that the myxothiazol biosynthetic system does not process the changed biosynthetic intermediates. Similar negative results are usually not published, but are quite often orally communicated. Thus, they contradict the data found in the literature that suggests that almost any site-directed mutagenesis in a PKS and/or NRPS biosynthetic system results in the production of novel structures in good yield. The applicability of the genetic system reported is demonstrated by the fact that all revertants arising from second crossover events regain their ability to produce myxothiazol. Unexpectedly, after chromosomal deletion of the oxidation domain encoded in *mtaC,* it is demonstrated that myxothiazol is still formed as a bisthiazole structure in the resulting mutant.

Experimental Procedures

Bacterial Strains and Culture Conditions

Escherichia coli and *S. aurantiaca* DW4/3-1 and its descendants were cultured as described previously [8, 43]. Batch cultures of 100 ml in 250 ml Erlenmeyer flasks were incubated at 30°C in a gyratory shaker at 160 rpm for 4–5 days.

DNA Manipulations, Analysis, Sequencing, and PCR

Chromosomal DNA from *S. aurantiaca* DW4/3-1 and *M. lichenicola* Me I46 was prepared as described [44]. Southern blot analysis of genomic DNA was performed using the standard protocol for homologous probes of the DIG DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany).

PCR was carried out using *Pfu*-DNA-Polymerase (Stratagene) according to the manufacturer's protocol, with addition of 5% DMSO to the reaction mixture. Conditions for amplification with the Eppendorf Mastercycler gradient (Eppendorf, Germany) were as follows: denaturation for 30 s at 95°C, annealing for 30 s at 60°C, extension for 45 s at 72°C, 30 cycles, and a final extension at 72°C for 10 min.

Primers used for the amplification of internal fragments of *mtaB* are as follows (given in the 5′ to the 3′ direction, introduced restriction sites are underlined): ENO1FOR, 5′-TGC TC**G CGG CCG C**CG AGA ACG CAC AGT CGA T-3′ (introduced NotI restriction site); ENO2REV, 5′-CCG **AAT ATT** ATC ACG ATC AGG TAC GTC GCG TC-3′; ENO3FOR, 5′-GGG **AAT ATT** GGT TGG CAT TGG CCT CGA GCT GG-3′ (introduced SspI restriction site); ENO4REV, 5′-GG**A CTA G**TT GAT GGA CAG CGC CGT CAG GC-3′ (introduced SpeI restriction site); ENO5SEQ, 5′-ACG CTG CCC AGC TTG CCG CG-3′; ERIMTABFOR, 5′-GGC ATC GCC CGG CAG GGT TTG-3′; ENOAB SEQ, 5′-GAC TGG TTG TGG CAG AGG TCT-3′.

Primers used for the amplification of internal fragments of *mtaC* are as follows: NADC1FOR, 5′-ATC **TGC GGC CGC** TTC TTC CTG AAG GAG AGA T-3′ (introduced NotI restriction site); NADC2REV, 5′-TGG **AGA TCT** CTC GAC GTA GTA GGC CGC-3′; NADC3FOR, 5′-ATG **AGA TCT** CAT GGC CTC GTG GGT GGC G-3′ (introduced BglII restriction site); NADC4REV, 5′-GA**A CTA GT**C CGT CGG AAG CAC C-3′ (introduced SpeI restriction site); NADC5SEQ, 5′-AAG CGC CCG TCG ATC GAG C-3′.

Primers used for the amplification of internal fragments of *mtaD* are as follows: NADD1FOR, 5′-CAA G**GC GGC CGC** GCT GGT CAG CCT C-3′ (introduced NotI restriction site); NADD2REV, 5′-CCG G**CT CGA G**AT TCG ACG TCA ATT CCC CAG G-3′ (introduced XhoI restriction site); NADD3FOR, 5′-GCC C**CT CGA G**TT GAG GCG TTA CCT GG-3′ (introduced XhoI restriction site); NADD4REV, 5′-CCG G**AC TAG T**GC CAG ACA TCC CCA CGA T-3′ (introduced SpeI restriction site); NADD5SEQ, 5′-CAA GAT CCA GGG CTA TCG CA-3′; MYX1FOR, 5′-GC**T CTA GA**T CAA CCT GAC CCT CTT CAA C-3′ (introduced XbaI restriction site); MLD2, 5′-TTG A**GC GGC CGC** CTG ACG AAG GAA GAG C-3′ (introduced NotI restriction site); MLD5, 5′-CGG C**AA GCT T**GA TCG AAG CGC-3′ (introduced HindIII restriction site); MYX6REV, 5′-CG**G GAT CC**A TCG ACT GCC GCA AC-3′ (introduced BamHI restriction site).

Primers used for the amplification of internal fragments of *mtaH* are as follows: E3, 5′-TCG GCA GGA AGA AGT CGT C-3′; E4, 5′-CTC GGG ATC CAG CAG GTA G-3′; ISO1FOR, 5′-AC**G AGT CT**G ACG CCT ACC GGG G-3′ (introduced SacI restriction site); ISO2REV, 5′-GG**C TGC AG**G TCA GCG CGG AAG AGG-3′; ISO3FOR, 5′-AC**C TGC AG**G ATG ATC TTC CCG CCC GAG-3′ (introduced PstI restriction site); ISO4REV, 5′-CC**T CTA GA**T GGT TGC CCC CGA TGA CG-3′ (introduced XbaI restriction site); ISO5SEQ, COR 5′-CCC TTC CCC TGA GCC AGG TCG-3′.

Primers used for the amplification of internal fragments of *mtaX* are as follows: MXISO1, 5′-CTG GGC TGG CGC GAA GGA GA-3′; MXISO2, 5′-CAG CAG CGT CAT GAT GTG GGA GAT-3′; MXISO3, 5′-TCC TCG CTG ACG CTG CTG GTG-3′; MXISO4, 5′-ATG TCG GAT GTG CGG CTG TC-3′.

Primers used for the amplification of internal fragments of *melD* are as follows: MLD3, 5′-ATC **GAA TTC** CTC GGC CGC GAG-3′ (naturally occurring EcoRI restriction site); MLD4, 5′-GAT C**AA GCT T**GC CGT TGG CCG TG-3′ (introduced HindIII restriction site); EcoRISEQ, 5′-CGA GCT GAT CCG GGA CGA CAT AGG-3′.

All other DNA manipulations were performed according to standard protocols [45]. Amino acid and DNA alignments were done using the programs of the Lasergene software package (DNAstar Inc.) and ClustalW [46].

Method for the Generation of Markerless In-Frame Mutations in *S. aurantiaca* DW4/3-1

Construction of Mutagenesis Plasmids

Most plasmids used for the generation of double-crossover mutants were created using similar strategies (see Table 1; pESW33, pESW36, pESW41, and pESW64). For example, construction of pESW33 used for the deletion of the MtaC Ox domain was done in the following fashion: a 604 bp PCR product was generated employing *Pfu*-DNA-Polymerase (Stratagene) using oligonucleotides NADC1FOR/NADC2REV and cosmid E25 [8]. The primer combination NADC3FOR/NADC4REV was used to generate a second 600 bp PCRproduct. Both fragments were purified using the Nucleospin Extract Kit (Macherey and Nagel). The 604 bp PCR product was cut using NotI and BglII, and the 600 bp product was hydrolyzed with BglII and SpeI before both fragments were cloned into NotI and SpeI precut pSWU41 [27]. The insert of the resulting plasmid pESW33 was verified via restriction and sequencing analysis.

pESW241 was used for the substitution of the A domain of MtaD with the A domain from MelD. Using oligonucleotides MYX1FOR and MLD2, a 631 bp PCR product was generated employing *Pfu* DNA polymerase (Stratagene) from cosmid E25. This fragment encodes the C-terminal part of the HC domain of MtaD and was cloned into pCR-2.1-TOPO (Invitrogen), resulting in plasmid pESW236. Employing oligonucleotides MLD3 and MLD4, a 1088 bp PCR product from cosmid M2 was generated that encodes the C-terminal part of the A domain of MelD (core motifs A8 to A10, plasmid pESW225). Oligonucleotides MLD5 and MLD6 gave rise to a PCR product 620 bp in size from cosmid E25 that encodes the PCP and part of the KS domain of MtaD (cloned into pCR-2.1-TOPO: pESW237). Plasmid inserts were verified by DNA sequencing. The insert of pESW236 was cut with XbaI and NotI, and cosmid M2 was hydrolyzed with NotI and EcoRI. The latter restriction gave a 1148 bp fragment which was cloned together with the fragment from pESW236 into XbaI/EcoRI precut pBluescript II KS(−) (Stratagene). The resulting plasmid pMSW5 was hydrolyzed with EcoRI/HindIII and cloned into pESW225, precut with the same enzyme combination, which gave plasmid pMSW6. The insert of pMSW6 was cut out of the vector using XbaI/HindIII and cloned together with the HindIII/BamHI insert

from pESW237 into XbaI/BamHI precut pSWU41, which gave plasmid pESW241.

Construction of Mutants

The mutagenesis plasmids were introduced into the chromosome of *S. aurantiaca* DW4/3-1 [11]. Chromosomal DNA of the resulting kanamycin-resistant mutants was isolated and analyzed via Southern experiments to verify site-specific integration. One mutant was grown in tryptone liquid medium for 10–14 days without antibiotic selection in order to induce the second crossover which results in the loss of the integrated plasmid. Every 48 hr, cells were transferred into a new flask (1 ml was used to inoculate 50 ml fresh medium). Next, cells were counted and a dilution series was made: 2.5×10^6 up to 2.5×10^8 cells were plated on tryptone agar supplemented with 5% sucrose. After 12 days, single colonies became visible and were inoculated in 3 ml tryptone liquid medium and grown for 72 hr. In parallel, 70 μl of the culture suspension was transferred as a single drop onto tryptone agar supplemented with either 5% sucrose or 50 μg/ml kanamycin. Growth could be monitored after 72 hr. In a typical experiment, 10%–20% of the mutants showed the kanamycin-sensitive and sucrose-resistant phenotype. These were further analyzed phenotypically and genotypically. In addition, the mutagenized region was reamplified from the chromosome of the mutant using a proofreading polymerase and resequenced to verify the correctness of the mutagenesis. The following mutants were generated: ESW400 (*mtaC*ΔOx$_{3280\text{-}3919}$) using plasmid pESW33, ESW392 (*mtaD*ΔOx$_{3599\text{-}4430}$) using plasmid pESW36, ESW414 (*mtaB* KR$^-$) using plasmid pESW41, ESW444 (*mtaH*$\Delta_{333\text{-}665}$) using plasmid pESW64, and ESW627 (melD$_{swap}$) using plasmid pESW241.

Production and Analysis of Secondary Metabolites in *S. aurantiaca* DW4/3-1 and Its Descendants

The cultivation of the strains, the preparation of the culture extracts, and the conditions for the analysis of the spectrum of secondary metabolites using diode array-coupled HPLC were desribed previously ([8]; see accompanying manuscript [47]).

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