The biosynthetic mta gene cluster responsible for myxothiazol formation from the fruiting body forming myxobacterium *Stigmatella aurantiaca* DW4/3-1 was sequenced and analyzed. Myxothiazol, an inhibitor of the electron transport via the bc$_2$-complex of the respiratory chain, is biosynthesized by a unique combination of several polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), which are activated by the 4'-phosphopantetheinyl transferase MtaA. Genomic replacement of a fragment of mtaB and insertion of a kanamycin resistance gene into mtaA both impaired myxothiazol synthesis. Genes mtaC and mtaD encode the enzymes for bis-thiazoline formation and chain extension on one pure NRPS (MtaC) and on a unique combination of PKS and NRPS (MtaD). The genes mtaE and mtaF encode PKSs including peptide fragments with homology to methyltransferases. These methyltransferase modules are assumed to be necessary for the formation of the proposed methoxy- and $\beta$-methoxy-acrylate intermediates of myxothiazol biosynthesis. The last gene of the cluster, mtaG, again resembles a NRPS and provides insight into the mechanism of the formation of the terminal amide of myxothiazol. The carbon backbone of an amino acid added to the myxothiazol-acid is assumed to be removed via an unprecedented module with homology to monoxygenases within MtaG.

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Little is known about the biochemistry of the formation of myxobacterial compounds and their corresponding gene clusters. A PKS gene (23) and a NRPS gene (24) from myxobacteria were detected by hybridization studies using heterologous gene probes or by Tn5 mutagenesis. Fragments of these genes have been cloned and sequenced. In contrast a great variety of PKS and NRPS genes from Actinomycetes and fungi have been shown to be involved in the biosynthesis of many secondary metabolites (for a review, see Ref. 25). We have established a method for cloning myxobacterial PKS and NRPS genes. Using this approach, both the stigmatellin- and the myxalamid biosynthetic gene clusters of *S. aurantiaca* Sg a15 were identified (22). The frequent occurrence of secondary metabolites in myxobacteria that seem to be synthesized by a combined PKS/NRPS is remarkable (e.g. myxothiazol, epothilones, myxalamids; compare Fig. 1). With probes for PKS and NRPS genes, we detected DNA fragments hybridizing with both gene types in a cosmid library of *S. cellulosum* So ce90 (22) that produces epothilones. In addition one ORF showing homology to PKS and NRPS involved in the biosynthesis of myxovirescin has been reported recently (21).

$^1$ The abbreviations used are: NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; Ppant, 4'-phosphopantetheinyl; MT, methyltransferase; MonoOx, monoxygenase; ACP, acyl carrier protein; PCP, peptidyl carrier protein; KS, $\beta$-ketoacyl-ACP synthase; AT, acyltransferase; ER, enoyl reductase; TE, thioesterase; ORF, open reading frame; bp, base pair(s); kb, kilobase pair(s); HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; MS, mass spectrometry; SAM, S-adenosyl-l-methionine.

New Lessons for Combinatorial Biosynthesis from Myxobacteria
THE MYXOTHIAZOL BIOSYNTHETIC GENE CLUSTER OF *Stigmatella aurantiaca* DW4/3-1*

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Myxothiazol formation from the fruiting body forming myxobacterium *Stigmatella aurantiaca* DW4/3-1 was sequenced and analyzed. Myxothiazol, an inhibitor of the electron transport via the bc$_2$-complex of the respiratory chain, is biosynthesized by a unique combination of several polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), which are activated by the 4'-phosphopantetheinyl transferase MtaA. Genomic replacement of a fragment of mtaB and insertion of a kanamycin resistance gene into mtaA both impaired myxothiazol synthesis. Genes mtaC and mtaD encode the enzymes for bis-thiazoline formation and chain extension on one pure NRPS (MtaC) and on a unique combination of PKS and NRPS (MtaD). The genes mtaE and mtaF encode PKSs including peptide fragments with homology to methyltransferases. These methyltransferase modules are assumed to be necessary for the formation of the proposed methoxy- and $\beta$-methoxy-acrylate intermediates of myxothiazol biosynthesis. The last gene of the cluster, mtaG, again resembles a NRPS and provides insight into the mechanism of the formation of the terminal amide of myxothiazol. The carbon backbone of an amino acid added to the myxothiazol-acid is assumed to be removed via an unprecedented module with homology to monoxygenases within MtaG.

Myxobacteria are Gram-negative soil bacteria that are assigned to the two suborders Cystobacterineae and Sorangineae. Both belong to the $\delta$-group of the Proteobacteria (1). They are distinguished from most other bacteria by their ability to glide in swarms, to feed cooperatively, and to form fruiting bodies upon starvation (2, 3). In addition, they have been shown to produce a wide variety of secondary metabolites with unique structures and biological activities (for reviews, see Refs. 4 and 5). These include the electron transport inhibitors myxothiazol (6), stigmatellin (7), and myxalamids (5, 8) produced by different strains of *Stigmatella aurantiaca* (Cystobacterineae) and the epothilones produced by *Sorangium cellulosum* (Sorangineae) (9) (structures are given in Fig. 1). Due to their antitumor activity, epothilones have attracted great attention (10–12). Myxothiazol as well as epothilones contain a thiazole ring that is formed by the incorporation of cysteine into the polyketide backbone (13). Thiazoline and thiazolidine structures of bacitracin in *Bacillus licheniformis* (14) and the bacterial siderophores yersiniabactin and mycobactin have recently been shown to be biosynthesized by a NRPS$^1$ or a combined PKS/NRPS in *Yersinia pestis* and *Mycobacterium tuberculosis* (15–17). Combinations of PKS and NRPS are known from other microorganisms (18–20) with two examples very recently reported from myxobacteria (*Myxococcus xanthus* (Ref. 21) and *Sorangium cellulosum* (Ref. 22)). No such combinations have been published so far for the formation of a thiazole coupled to a polyketide side chain. In addition to the bis-thiazole moiety, myxothiazol has some unique features: the unusual leucine derived starter unit 3-methyl-butyryl-CoA (13) and the linear polyketide backbone, which includes a $\beta$-methoxy-acrylate and a terminal amide structure.

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**Myxothiazol Biosynthetic Gene Cluster**

**Fig. 1. Examples for myxobacterial secondary metabolites probably biosynthesized by PKS and NRPS.** Epothilone A ($R = H$) and B ($R = CH_3$). Molecule moieties in which amino acids are incorporated are shaded in gray.

This report deals with the isolation and characterization of a gene cluster from *S. aurantiaca* DW4/3-1 that is involved in myxothiazol biosynthesis. It comprises genes encoding PKSs and NRPSs, inactivation of which impairs myxothiazol biosynthesis. In close proximity to this cluster is located *mtaA*, which encodes a putative Ppant transferase. The gene product is revealed that the first primer pair binds to the insert of cosmid E25, and 1% of the adsorber resin XAD-16 (Rohm & Haas). 100-ml batch fermentation contained secondary metabolites after the fermentation, secondarily isolated.

**MATERIALS AND METHODS**

Bacterial Strains and Culture Conditions—Bacterial strains and plasmids are described in Table I. *Escherichia coli* strains and *S. aurantiaca* DW4/3-1 (30) and its descendants were cultured as described previously (29).

Analysis of Secondary Metabolite Production in *S. aurantiaca* DW4/3-1—For the production of secondary metabolites, the strains were cultured as described previously (29). For the production of secondary metabolites, the strains were cultured as described previously (29).

DNA Manipulations, PCR—DNA analyses, sequencing, and PCR—Chromosomal DNA from *S. aurantiaca* was prepared as described (31). Southern analysis of genomic DNA was performed using the standard protocol for homologous probes of the DIG DNA labeling and detection kit (Roche Molecular Biochemicals). PCR was carried out using *Taq* polymerase (Life Technologies, Inc.) according to the manufacturer’s protocol. 5′-MeSO was added to the mixture. Conditions for amplification with the Eppendorf Mastercycler gradient were as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and extension for 45 s at 72 °C; 30 cycles and a final extension at 72 °C for 10 min. Primers used for screening the cosmid library were: SEQBS24–4, 5′-GTACGTTCGGGT-CACCCCTCACG-3′; SEQBS24–23, 5′-GGCGCCGGCGCTTCGACAT-3′; SEQSKAF-4, 5′-GTCCTAGTTGCTCGTGCTG-3′; SEQSKAF-5, 5′-CCACCTCGGCGCCACAGACAACCC-3′.

For double-stranded sequencing of the pBS23 and pBS24 inserts, a dye terminator kit (PE Biosystems) and an ABI Sequencer (Applied Biosystems) were used. pSK4 and pSK9 were sequenced using the T7 polymerase sequencing kit (Amersham Pharmacia Biotech). The inserts of pBS23, pSK4, and pSK9 were sequenced by primer walking. The insert of pBS24 was restricted with *Alu*I, *Hpa*I, and *Sou*3A, respectively. The *Alu*I, *Hpa*I, and *Sou*3A fragments were cloned into the Smal, ClaI, and BamHI sites of pBCSK(+) (-). The resulting subclones were sequenced using universal primers. Sequence gaps were closed by primer walking. The region between E25 and E201 was sequenced using primer walking from plasmid pESW8. Sequencing of cosmids E25 and E201 was performed by a shotgun approach as follows; sheared fragments of the two cosmids were subcloned separately into *pTZ18R*. At least 500 clones were selected from each cosmid library, plasmid DNA prepared (*Qiagen*) and sequenced using Big Dye RR terminator cycle sequencing kit (PE Biosystems) and UPO/RPO-primer (MWG-Biotech). The gels were run on ABI 377 Sequencers, and data were assembled and edited using the XGAP program (32). All other DNA manipulations were performed according to standard protocols (33). Amino acid and DNA alignments were done using the programs of the Lasergene software package (DNASTAR Inc.) and Clustal W (34).

* Colony Hybridization—For colony hybridization of *S. aurantiaca* strains, 5 μl of cell suspension (1 × 10⁸ cells/ml) was spotted onto a nylon membrane (Biodyne B, Pall). The membrane was incubated for 5 min on blotting paper pretreated with 0.5 μl NaOH, 1 μl Tris/HCl, pH 8.0, and 1.5 μl NaCl, 1 μl Tris/HCl, pH 8.0, respectively. After drying for 15 min, the nitrocellulose (Stratagene) was washed with 0.5% SDS, and 50 μl sample that are involved in the production of *S. aurantiaca* DW4/3-1 had a molecular mass of 5000 Da. The PKS/NRPS part of the gene cluster has several unique features. These can be correlated to the 5-methylbutyryl-, bis-thiazole-, β-methoxy-acrylate-, and terminal amide moiety of the compound.
whereas the second pair does not.

Construction of the S. aurantiaca DW4/3-1 Mutant Strains BS47 and BS57—For characterization of mtaA, insertional mutant BS57 was constructed. Plasmid pSKAF that was obtained by inserting an EcoRI fragment which encoded fbfA, fbfB, mtaA, and part of mtaB into pBSKK(−) was restricted by FseI. After removing the extending 3′-ends by T4 DNA polymerase, the kanamycin resistance gene (neo) was inserted, which was obtained from pUC4KIXX (Amersham Pharmacia Biotech) after restriction with SmaI. The plasmid obtained, pBS31, contained the neo gene in the divergent orientation of the mtaA gene. pBS31 was linearized with ScaI and transferred into S. aurantiaca DW4/3-1 wild type by electroporation (22, 34). In the kanamycin-resistant recombinant BS57, the wild type gene was replaced by the mutant mtaA gene, into which the neo gene was inserted (compare Figs. 2 and 3).

The 12-kbp EcoRI fragment of pSKAF was cloned into pBCSKSalA (constructed via restriction of pBCSK(−) with SalI, fill-in using the Klenow fragment of the DNA polymerase, and religation resulting in loss of the SalI restriction site in pBCSK(−)) precut with EcoRI, resulting in plasmid pBS26. After SalI restriction of pBS26, a 2.3-kbp SalI-SalI fragment was replaced by the neo gene from pUC4KIXX, resulting in plasmid pBS27. By electroporation pBS27 was introduced into S. aurantiaca strain DW4/3-1. The resulting electroporants were screened by colony hybridization using pBCSK(−) and the neo gene (derived from pUC4KIXX) as probe, respectively. BS47 (pBCSK(−)−negative and neo-positive) was chosen for further analysis (compare Figs. 2 and 3).

RESULTS

Isolation and Identification of a Ppant Transferase Involved in Myxothiazol Biosynthesis—Sequence analysis of the chromosomal regions adjacent to the S. aurantiaca DW4/3-1 developmental genes fbfA and fbfB (28, 29) led to the detection of a 334-bp open reading frame (ORF) designated mtaA (myxothiazol). The start codon of mtaA is located about 800 bp downstream of the fbfB stop codon (see Figs. 2 and 3). The codon bias is in accordance with other genes from myxobacteria (66.1/50.7/79% G+C in the first, second, and third position, respectively; see Table II) (35). Sequence alignments reveal that mtaA encodes a predicted polypeptide with significant homology to Ppant transferases (26, 27, 36), such as AcpS and EntD from E. coli or Sfp from Bacillus subtilis (Fig. 4). AcpS is the first cloned and characterized Ppant transferase that catalyzes the conversion of the inactive apo form of the fatty acid synthase to the functional form of the enzyme. EntD and Sfp were originally reported to be specific for the activation of the enterobactin- and the surfactin synthetase, respectively. Recently, it was reported that by coexpression Sfp is able to activate fragments of the erythromycin PKS expressed in E. coli (37). Inactivation of mtaA by insertional mutagenesis (compare Fig. 3) resulted in mutant BS57, which is impaired in myxothiazol formation. Mutant BS57 was analyzed for secondary metabolite production by diode-array coupled HPLC and HPLC-MS and did not form any detectable amount of myxothiazol (see Fig. 5; HPLC-MS data not shown). In addition at least one other yet unidentified substance could not be detected in the mutant.

Cloning, Identification, and Sequencing of the mtaA Gene Cluster—About 450 bp downstream of mtaA, a large open reading frame (ORF) was detected and designated mtaB. It encodes a gene product with significant similarity to PKSs. The gene begins with an ATG start codon, which is preceded by a putative ribosome binding site (AGGA), and the codon bias is in accordance with other myxobacterial genes like mtaA (72/52/77). Replacing an internal 2.3-kbp SalI-SalI fragment with the Tn5-derived kanamycin resistance cassette as illustrated in Fig. 3 inactivated the gene and resulted in strain BS47. Mutant BS47 was analyzed for secondary metabolite production by diode-array coupled HPLC and HPLC-MS. BS47 did not form a detectable amount of myxothiazol but still produced the unknown compound missing in BS57 (see Fig. 5; HPLC-MS data not shown).

Since only part of the PKS was encoded by plasmid pSKAF, a cosmide library of S. aurantiaca DW4/3-1 was constructed and screened as described under “Materials and Methods.” Cosmide E25 was mapped and subcloned (see Fig. 2). A 7.3-kilobase pair HindIII/BglII fragment located at the end of E25 was cloned from genomic DNA of S. aurantiaca DW4/3-1 (pESW8) and used as a probe to isolate cosmide E201, which shares with E25 the sequence of the last SauIII restriction site. Cosmids E25 and E201 were shown to be colinear with genomic DNA of S. aurantiaca DW4/3-1 by Southern analysis (data not shown).

Partial sequences of the inserts of plasmids pE25–11, pE25–5.5, and pE25–2 (see Table I) were determined. They showed significant homology to PKSs and NRPSs. Subsequently, cosmide E25 and part of E201 were sequenced. Six large ORFs designated mtaB–mtaG were detected (see Fig. 2). The overall G+C content of the sequenced mta region is 65.9%.

Structural Features of Myxothiazol Biosynthetic Genes—Sequence motifs typical for PKSs (20, 38, 39) and NRPSs (40–42) were detected as shown in Figs. 2 and 4 and Table II. Enzy-
matic activities of the mta PKS and NRPS proteins are colinear with almost all domains and motifs expected for a biosynthetic gene cluster for myxothiazol biosynthesis. One unexpected feature is an enoylreductase (ER) domain present in MtaB (compare Table II, Fig. 4, and “Discussion”). MtaC contains all the necessary domains for adenylation and heterocyclization. MtaD represents a mixed NRPS/PKS with heterocyclization activity (both MtaC and MtaD contain the conserved core motifs for heterocyclization Z1–Z7 (Ref. 41; compare Fig. 4 and Table II). Two direct repeats of about 1.13 kbp were detected in MtaD and MtaF (AT domains). Directly downstream of this repeat in MtaF, a polypeptide fragment homologous to known SAM-dependent methyltransferases (MT) including the putative SAM binding site (41) is found. Following the AT domain of MtaE, a further polypeptide fragment homologous to MTs is located. In Fig. 4 both mta MT domains are aligned with core regions of C-MTs (internal domains) and O-MTs (single proteins) described for different PKS systems. MtaG harbors an unprecedented motif within NRPSs resembling flavin and F420-dependent monoxygenases/hydrogenases (MonoOx) (see Fig. 4). The MonoOx domain is homologous to bacterial luciferases (Prosite signature no. PDOC00397) like LuxA of Xenorhabdus luminescens (Protein Identification Database (PID) code g155414; 21.7% identity and 40% similarity), to the F 420-dependent glucose-6-phosphate dehydrogenase Fgd of Mycobacterium leprae (PID code g3129987; 21.3% and 44%, respectively), to the mitomycin biosynthetic gene MitH from Streptomyces lavandulae (PID code g4731347; 24% and 40%, respectively), and to the methylenetetrahydromethanopterin reductase of Methanobacterium thermoautotrophicum Mer (PID code g2127699; 20.3% and 38%, respectively). Bacterial F420-dependent enzymes have been compared, and areas of
significant similarity have been defined. Fig. 4 shows an alignment of the regions 1 and 2 (43) with the corresponding region of the MtaG MonoOx domain. The domain is inserted in the adenylation domain (between motifs A4 and A5 (Ref. 41)) of the protein. Within the terminal part of MtaG, the core region of thioesterases (44) GXXSXXG followed by a GXX after some 130 amino acids has been detected. The acyl carrier protein (ACP) and peptidyl carrier proteins (PCP) domains of MtaB-MtaG contain the Prosite consensus signature of the putative binding site for the 4′-phosphopantetheine cofactor (Prosite signature no. PS00012, R2082, and L2104). All acyl transferase (AT) domains have been compared for their putative binding specificity for activated acids (compare Fig. 4 and “Discussion”).

**DISCUSSION**

Myxobacteria have been shown to be a valuable source of secondary metabolites with biological activity (4, 5). Nevertheless, little is known about PKS and NRPS systems from this class of microorganisms. We here report the first completely sequenced PKS/NRPS biosynthetic gene cluster from myxobacteria the analysis of which reveals several unique features.

The biosynthesis of the electron transport inhibitor myxothiazol follows a multi-step process. 3-Methylbutyrate, three acetates, two propionates, and two cysteines are condensed giving rise to the carbon framework of the molecule (13). This study demonstrates that the biosynthetic machinery for myxothiazol resembles a typical type I PKS (45) combined with three NRPS modules. Both PKSs and NRPSs need to be transformed from the inactive apo to the active holo form by 4′-phosphopantetheinylation (27). MtaA shows strong sequence similarity to Ppant transferases, and gene inactivation of mtaA leads to a mutant (BS57) not producing myxothiazol. Therefore, MtaA seems to be responsible for the posttranslational modification of MtaB and possibly of more proteins of the myxothiazol biosynthetic machinery in *S. aurantiaca* DW4/3-1. Moreover, the mutant also does not produce at least one additional, still unidentified substance made by the wild type strain that may be synthesized by a PKS and/or NRPS (compare Fig. 5). This indicates MtaA to be responsible for Ppant transfer to proteins catalyzing the biosynthesis of different (secondary) metabolites. A possible polar effect of the insertion of the *neo* gene into *mtaA* in mutant BS47 cannot be excluded. Nevertheless, the distance to *mtaB* suggests that *mtaB* is regulated differently from *mtaA*. In addition, a polar effect would not explain the missing occurrence of the unidentified substance(s) in the mutant.

The modular structure of type I PKSs usually starts with an AT or a CoA-ligase domain responsible for the recognition (and activation in case of the CoA-ligases) of the starter molecule followed by transfer of the activated substrate to the first ACP domain (compare the biosynthetic gene clusters of erythromycin (Ref. 38), rapamycin (Refs. 20 and 46), and rifamycin (Ref. 39)). In the case of *mtaB*, the modular organization looks different; the protein begins with the loading ACP(L), followed by keto synthase (KS) 1, two ATs (AT1 and AT2), dehydratase (DH) 1, ER1, keto reductase (KR) 1, and ACP1 (see Table II). To date, this kind of modular arrangement has only been found in case of the soraphen biosynthetic gene cluster from another myxobacterium (*S. cellulosum*), the sequence of which is available from U.S. patent 5693774. We suppose that MtaB resembles the first multienzyme component of the myxothiazol biosynthetic machinery. In accordance with this suggestion module 1 and the loading domain have presumably been intermixed. One AT would be responsible for 3-methylbutyryl-CoA recognition and for the transfer of the acyl residue to ACP(L), which would resemble the function of a loading domain. The other AT would load the first malonyl group onto ACP1, and KS1 would next condense both activated acyl groups, giving rise to the first diketide intermediate bound to ACP1. Subsequent action of KR and DH domains results in the first double bond of myxothiazol. Soraphen also contains an unusual starter moiety processed by the corresponding PKS (SorA) (benzoate derived from phenylalanine (Ref. 47)) and analogous functions for the first modules of SorA can be assumed. According to the structure of myxothiazol, one would predict the incorporation of a propionate unit by the next module. This would involve an AT specific for methylmalonyl-CoA in contrast to the malonyl-CoA-AT, which is needed for the first
extension step. Both AT types have been compared and characterized for the erythromycin, niddamycin, and rifamycin PKS (39, 48, 49). In Fig. 4, typical malonyl- and methylmalonyl-CoA-ATs from Actinomycetes are compared with ATs of the mta gene cluster. We were unable to define specific regions for substrate binding of malonyl-CoA, methylmalonyl-CoA, or 3-methylbutyryl-CoA.

Derivatives of branched chain α-keto acids are known as the precursors of polyketides of the avermectin type (50), and a cloned from Streptomyces avermitilis (51). An analogous gene locus (esg) was identified in Myxococcus xanthus and could be shown to be necessary for cell signaling during development (52, 53). A homologous gene was detected in S. aurantiaca DW4/3-1.² This suggests S. aurantiaca DW4/3-1 to have a similar activity involved in the synthesis of the myxothiazol starter molecule 3-methylbutyrate. In the first step of avermectin biosynthesis, the α-branched 2-methylbutyrate or isobutyrate is loaded onto the PKS (compare Ref. 54). These metabolites are derived from isoleucine or valine, respectively, whereas the putative starter molecule in myxothiazol biosynthesis is 3-methylbutyrate derived from leucine (13). AT1 of MtaB should be rather specific for 3-methylbutyrate and possibly for other β-branched acids because myxothiazol species that do not contain leucine-derived starters have not been isolated yet.³ It is tempting to speculate that other activated

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² B. Silakowski and R. Müller, unpublished data.
³ G. Höfle, unpublished results.
β-branched acids would be accepted by this AT if fed to the culture or fed to a bkd (esg) negative mutant of *S. aurantiaca* DW4/3-1. The broad substrate specificity of the loading module of the avermectin biosynthetic gene *aveA1* (syn. *avr*) was used to engineer the erythromycin-producing PKS (DEBS1), which resulted in novel erythromycins derived from endogenous branched-chain acid starter units (55), indicating that the 3-methylbutyrate-AT of the *mta* gene cluster could be useful to generate new structures via combinatorial biosynthesis. Unfortunately, the sequence of *aveA1* is not available from common data bases for a comparison to the putative 3-methylbutyrate-AT of the *mta* gene cluster.

After the second chain extension step in myxothiazol biogenesis cysteine adenylation, condensation, cyclization, and bis-thiazole formation takes place, giving rise to an intermediate that finally is extended by the right-hand part of the PKS. A NRPS responsible for thiazoline formation during biogenesis of the polypeptide bacitracin (14) and the combination of PKS and NRPS to form thiazoline and thiazolidine ring structures during the biosynthesis of yersiniabactin and mycobactin have been described recently (15–17). The occurrence of typical motifs of NRPSs responsible for adenylation, condensation, and cyclization in MtaC and MtaD (compare Ref. 41) is in accordance with the predicted biosynthetic machinery for myxothiazol. Interestingly, the bis-thiazole structure in myxothiazol seems to be formed by two independent adenylation and cyclization domains, whereas only one adenylation domain has been reported for three cysteine cyclizations in the yersiniabactin biosynthetic gene cluster (56). Additionally, the NRPS domains for the first ring are encoded on one pure NRPS gene, whereas the second thiazole ring seems to be derived from *mtaD*, which resembles an ORF encoding NRPS and PKS modules. With respect to thiazole ring formation from thiazoline by MtaC and MtaD, no differences to the known systems giving rise to the thiazoline structures bacitracin and yersiniabactin are obvious (*e.g.* an additional NAD binding domain). Possibly intramolecular cross-talk with the ER domain present in module 1 of *mtaB* takes place. This domain could be inactive because the typical motif LXXXXXGGVXXXXXXXA important for functionality of this domain (49) is changed to LXXXXXGLAXXXXLI (compare Fig. 4) and myxothiazol analogs without the corresponding double bond have not been isolated. Because the differences in the core region except for the H to L exchange are not very prominent, one could also speculate that this ER domain is involved in the formation of the thiazole ring(s) from the proposed thiazoline intermediate. Interestingly, it has been speculated that the reduction of one thiazoline to a thiazolidine ring in yersiniabactin may be performed by the ketoreductase (KR) domain of the PKS part of HMWP1 (16). In order to investigate whether this process may be catalyzed by an enzyme encoded downstream of the gene cluster, we have sequenced further downstream of *mtaG*. Within approximately 15 kbp, no gene with homology to oxido-reductases could be detected. Genes with putative function in primary metabolism were found (data not shown), indicating that the end of the gene cluster was reached. Alternatively, it cannot be excluded that this oxidative process may occur non-enzymatically (57).

The PKS genes *mtaE* and *mtaF* contain domains with homology to SAM-dependent methyltransferases. MT domains within PKSs have been reported recently in case of yersiniabactin (15) and lovastatin biosynthesis in *Aspergillus terreus* (58, 59). Whereas these domains are responsible for C-methylation, the MTs of *mtaE* and *mtaF* seem to resemble O-methyltransferases. The latter contain a LDXXGX core sequence, whereas in C-MTs the D seems to be replaced by an E (compare Fig. 4). We suppose that the MT of MtaE catalyzes the O-methylation of the hydroxyl group during myxothiazol biogenesis. Interestingly, there is no KR or DH domain located in *mtaF*. This could well explain how the β-methoxy-acrylate is formed because the enol form of the β-keto intermediate can readily be methylated by the MT of MtaE (compare Fig. 6). The yersiniabactin MT1 in HMWP1 is located behind the AT domain of the protein like the MTs of MtaE and MtaF.

Unexpectedly, we identified another NRPS gene behind *mtaF*. Only two other PKS systems have thus far been characterized with terminal NRPS domains, but in both systems (biosynthesis of lovastatin (Ref. 58) and plithiocerol dimycose-roseate, a *Mycobacterium tuberculosis* cell envelope lipid (Refs. 60 and 61)), the role of these domains remains unclear. None of the derived secondary metabolites has a terminal amide moiety such as that found in myxothiazol. For the polypeptide antibiotic nosiheptide, it has been shown using incorporation studies with 15N-labeled amino acids that the nitrogen of its terminal amide is derived from serine (62). The genetic data presented in this study make it likely that during myxothiazol biosynthesis the myxothiazol acid coupled to the ACP of MtaF is extended with another amino acid (*e.g.* serine). The carbon skeleton of the terminal amino acid would then have to be removed, giving rise to myxothiazol. The myxothiazol precursor is presumably condensed at the PCP of MtaG, and the MonoOx domain within

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**FIG. 6. Proposed action of MtaF and MtaG.** ACP indicates the acyl carrier domain of MtaF, and PCP indicates the peptidyl carrier protein domain of MtaG.
MtaG can act upon this precursor and oxidize the intermediate at the α-position of the amino acid (compare Fig. 6). Dealkylation of the alcohol amide could take place releasing myxothiazol, whereas the carbon skeleton of the terminal amino acid would have to be released from MtaG by subsequent action of the TE domain present at the end of the polypeptide. Terminal amides occur in many bioactive substances including most peptide hormones (63). Their formation in mammals and insects has been studied extensively (64), and it has been shown that they arise from the oxidative cleavage of C-terminal glycine-extended precursors. The enzyme responsible is a bifunctional, copper- and zinc-dependent monooxygenase that first activates the alcohol amide. Similar biochemistry has been described recently for the formation of nicotinamide from nicotinuric acid (65). Despite these similarities, no homology between the described type of enzyme and the MonoOx domain of MtaG could be detected. Because of the sequence similarities with flavin and Fpco-dependent monooxygenases/hydrogenases, it seems likely that the α-position of the extended myxothiazol is hydroxylated by the MonoOx domain, which may also be responsible for the cleavage of the resulting alcohol amide. This could represent a general mechanism for the formation of terminal amides in other PKS and NRPS type structures, e.g. thioestrepton.

The presence of the MonoOx domain in MtaG in the middle of the adenylation domain may indicate that this is a good location to integrate further functionalities into NRPS modules because the MT domain in HMWP2 has been reported at almost the same position (16).

The occurrence of a single protein harboring PKS and NRPS modules (MtaD), of the O-MTs within PKSs (MtaE and MtaF), and of the monooxygcnase within a NRPS (MtaG) provide evidence for combinatorial biosynthesis taking place in S. aurantiaca DW4/3-1. The direct repeat (ATs of MtaD and MtaF) in the gene cluster could indicate that a gene duplication has taken place to integrate further functionalities. This type of strategy in which modules rather than individual enzymatic domains are the building blocks for genetic manipulation has been suggested very recently (37). The data presented in this report show that myxobacteria have an incredible ability to mix and match biosynthetic genes, and the currently broadening knowledge about the PKS/NRPS systems in Gram-negative bacteria will hopefully unravel further genetic and biochemical features.

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