In vitro reconstitution of the myxochelin biosynthetic machinery of *Stigmatella aurantiaca* Sg a15: Biochemical characterization of a reductive release mechanism from nonribosomal peptide synthetases

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Microorganisms produce iron-chelating compounds to sequester the iron essential for growth from the environment. Many of these compounds are biosynthesized by nonribosomal peptide synthetases, some in cooperation with polyketide synthases. Myxochelins are produced by the myxobacterium *Stigmatella aurantiaca* Sg a15, and the corresponding gene cluster was cloned recently. We have undertaken to express heterologously the myxochelin biosynthetic machinery in *Escherichia coli*. To activate the involved proteins posttranslationally, they were coexpressed with the phosphopantetheinyltransferase MtaA from the myxothiazol biosynthetic gene cluster. Phosphopantetheinylation of the carrier proteins could be verified by protein mass analysis. Six active domains in proteins MxcE, MxcF, and MxcG are capable of assembling myxochelin from ATP, NAD(P)H, lysine, and 2,3-dihydroxybenzoic acid *in vitro*. This fact demonstrates that the condensation domain of MxcG performs two condensation reactions, creating the aryl-capped α-amide and the aryl-capped γ-amide of the molecule. A previously unknown type of reductive release is performed by the reduction domain of MxcG, which alternatively uses NADPH and NADH to set free the peptidyl-carrier protein-bound thioester as an aldehyde and further reduces it to the alcohol structure that can be found in myxochelin A. This type of reductive release seems to be a general mechanism in polyketide and nonribosomal peptide biosynthesis, because several systems with C-terminal similarity to the reductase domain of MxcG can be found in the databases. Alternatively, the aldehyde can be transaminated, giving rise to a terminal amine.

Iron is one of the most important micronutrients used by bacteria, because it is needed as a cofactor for a large number of enzymes (1). To acquire iron from the extracellular medium, almost all aerobic bacteria produce and secrete low-molecular-weight iron-chelating compounds termed siderophores. The Fe^{3+}-chelator complex is recovered subsequently by the cell through specific uptake systems (2). Nonribosomal peptide synthetases (NRPSs) and/or polyketide synthases (PKSs) have been shown to be responsible for the biosynthesis of several of these small iron-chelating molecules [e.g., enterobactin in *Escherichia coli* (3), yersiniabactin in *Yersinia pestis* (4), and mycobactin in *Mycobacterium tuberculosis* (5)]. These enzymatic systems also direct the formation of an immense variety of secondary metabolites with biological activity which are currently used or developed as pharmaceuticals and agrochemicals [e.g., the erythromycins (6), rapamycins (6), epothilons (7, 8), myxothiazols (9), and bacitracin (10)]. Hybrid-gene clusters encoding PKS and NRPS modules on single ORFs have been reported recently (9, 11, 12) and provide ideal candidates to study PKS–NRPS interaction, which will enlarge the possibilities for combinatorial biosynthesis. Further knowledge can be gained from the study of systems with heretofore unique features, as is the case for the biogenesis of several iron chelators and some additional systems such as myxothiazol or mycosubtilin. One further example is the myxochelin-type siderophore (compare Fig. 1), the biosynthetic gene cluster that we recently cloned from *Stigmatella aurantiaca* Sg a15 and analyzed (13).

S. aurantiaca belongs to the myxobacteria, which are assigned to the class *Proteobacteria* (14). In comparison to most other bacteria, they show some unique features: their abilities to glide in swarms, to feed cooperatively, to form fruiting bodies upon starvation (15, 16), and their capability to produce a wide variety of secondary metabolites with different biological activities (17).

It has been suggested that the NRPS involved in myxochelin biosynthesis (MxcG) could perform two rounds of condensation using one condensation domain. In addition, MxcG harbors an C-terminal domain with homology to NAD(P)H-binding motifs, which can be found in several other PKS and NRPS systems (13). Nevertheless, the function of these domains remains unclear. The modular logic of the myxochelin biosynthetic operon led us to assume that this terminal domain encodes the machinery for a new type of reductive release of the carrier protein-bound thioester-intermediate from NRPS and PKS systems. By using heterologous expression and *in vitro* reconstitution of the myxochelin megasynthetase, we analyzed the biochemistry performed by MxcG in close cooperation with MxcE and MxcF.

Materials and Methods

Construction of Plasmids Containing *mxcE*, *mxcF*, *mxcG*, and the Coding Region of the Thiolation Domain of *mxcG* (*pcpG*). The expression plasmids used in this study were generated after amplification of the target genes by PCR. Primer sequences were derived from GenBank accession no. AF299336. A Mastercycler Gradient (Eppendorf) was used to amplify the genes *mxcE*, *mxcF*, and *mxcG* from chromosomal DNA of *S. aurantiaca* Sg a15 in 30 cycles of the following cycling profile: denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 90 s at 72°C (an extension for 4 min at 72°C to amplify *mxcG* was applied). A final extension at 72°C was carried out for 10 min. The PCR was carried out with *Pfu* DNA polymerase (Strat-

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Abbreviations: NRPS, nonribosomal peptide synthetases; PKS, polyketide synthases; DHBA, dihydroxybenzoic acid; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight.

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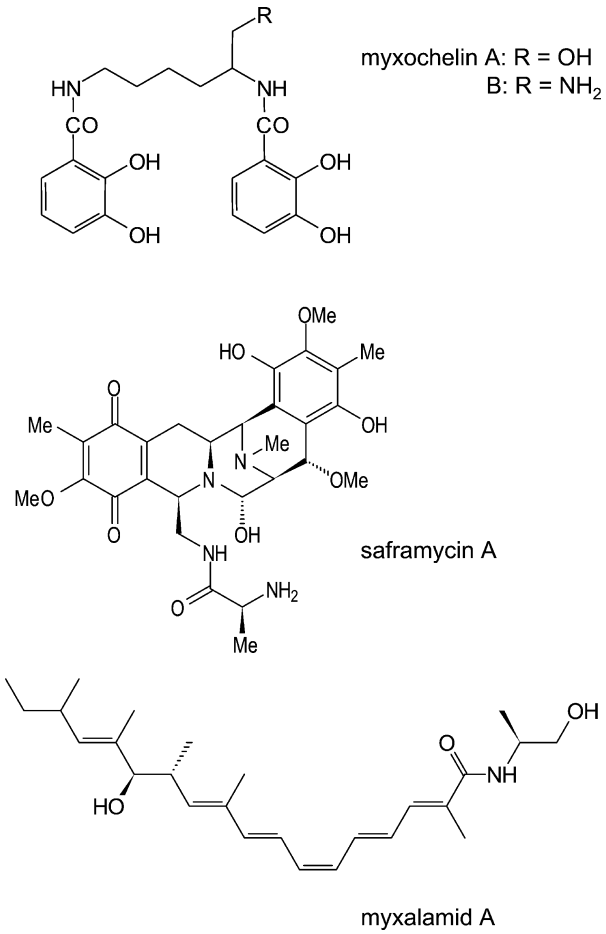

Fig. 1. Structures of myxochelin A and B, saframycin A, and myxalamid A.

agene) according to the manufacturer's protocol with the following primer pairs: (*i*) forward: 5′-GAGGTGCA*CATATG*ACCTCGCCCAGCCCTCA-3′ and reverse: 5′-TGGGAAAA*CTCGAG*TCGGTGAGCGAGTGTTTC-3′; (*ii*) forward: 5′-AGAGTCCA*CATATG*GCACTTCCCGCCATCG-3′ and reverse: 5′-TCGA*GAATTC*CGCGGTGGGGCGGACGCC-3′; (*iii*) forward: 5′-AGTACTGA*CATATG*CGGGATTCACAGGAAAC-3′ and reverse: 5′-TG*GAATTC*CCGGTGTTCCGGAGGCCTC-3′. The coding region of the peptidyl-carrier protein (PCP) from MxcG was determined as described (18). The amplification of *pcpG* (*iv*) was carried out under the conditions described above, except for a 20-s extension phase of the PCR. The primers used for *iv* were forward: 5′-AGCTGGAT*CATATG*GCACTGCGCCTCGTG and reverse: 5′-AGCATGGC*CTCGAG*GATGCCTTCGCCTTC-3′. The forward primers introduced an *Nde*I restriction site (shown in italics), whereas the reverse primer introduced *Xho*I (*i*, *iv*) and *Eco*RI (*ii*, *iii*) restriction sites, respectively (shown in italics). The *Nde*I/*Xho*I- (*i*, *iv*) and *Nde*I/*Eco*RI- (*ii*, *iii*) hydrolyzed PCR products were ligated with pCYB2 (New England Biolabs), creating the plasmids pMXCE, pMXCF, pMXCG, and pCPG. The cloning strategy of the four PCR fragments into pCYB2 led to modifications in the C terminus of the expressed proteins: cloning of *mxcE* and *pcpG* into the *Xho*I restriction site of the vector led to an extension of the resulting proteins by the amino acids LEPG, whereas the *Eco*RI ligation of *mxcG* and *mxcF* into pCYB2 resulted in an expression product extended by the amino acids EFLEPG. In all three plasmids, a translational fusion of the target proteins with an intein-chitin-binding domain encoded by the vector was generated to facilitate the subsequent purification of the target proteins.

The accuracy of the gene insertion into pCYB2 was checked by DNA sequencing, which was performed by means of Big Dye RR terminator cycle sequencing kit (Perkin–Elmer), p*Tac* forward-intein reverse primer (New England Biolabs), and an ABI Prism-System 377 Sequencer (Applied Biosystems).

Overproduction and Purification of MxcE, MxcF, and MxcG. A culture of *E. coli* XL1 Blue carrying pMXCE was grown in LB medium (1 liter) supplemented with 100 μg of ampicillin at 37°C to reach an OD_{600} of 0.4, and, subsequently, the expression was induced with 0.25 mM isopropyl β-D-thiogalactoside (IPTG). At the same time, the incubation temperature was shifted down to 30°C. The cells were harvested by centrifugation after an additional 6-hr incubation and washed with a sodium–Tris–EDTA buffer (20 mM Tris·HCl/50 mM NaCl/0.1 mM EDTA, pH 8.0). Cells were resuspended in 1 ml of lysis buffer (20 mM Tris·HCl/500 mM NaCl/0.1 mM EDTA/0.1% Triton X-100, pH 8.0) and lysed by sonification with a French press (Sonopuls, Bandelin, Berlin). The purification of the target protein was performed as described (18), according to the manufacturer's protocol (Impact I One-Step Protein Purification System, New England Biolabs), with 12 ml of chitin beads for the affinity chromatography. After overnight cleavage of the target protein from the intein-chitin-binding domain with 20 mM DTT, MxcE was eluted with 35 ml of elution buffer (20 mM Tris·HCl/50 mM NaCl/0.1 mM EDTA, pH 8.0). Finally, the protein of interest was concentrated 35-fold through filtration by using Centriplus RC YM-10 spin columns (Millipore). After purification of 1 liter of starting culture, the yield of MxcE was 2 mg.

For the expression and purification of apo-MxcF and apo-MxcG, the above described procedures were used.

Complete posttranslational phosphopantetheinylation of apo-MxcF and apo-MxcG was achieved by coexpression of MtaA (9) with one of the proteins MxcF and MxcG, either after transformation of *E. coli* XL1 Blue/pSUMtaA (18) by pMXCF or pMXCG. The purification of the target holoproteins led to yields of 1.8 mg of MxcF and 3 mg of MxcG when starting cultures of 1-liter volume were used.

The detection of the isolated proteins was achieved after separation with 10% gels prepared as described by Laemmli (19) and after staining with colloidal Brilliant blue G (Sigma). Eluted proteins were sequenced by N-terminal Edman degradation.

Overproduction and Purification of the Thiolation Domain of MxcG (PCPG). Expression and purification of the thiolation domain of MxcG was carried out as described (18).

Determination of the Posttranslational Activation of MxcF and MxcG. Mass spectral analysis of the purified proteins using a positive-ion matrix-assisted laser desorption ionization (MALDI) analysis was performed. Molecular masses of the isolated proteins were determined by MALDI with a Reflex time-of-flight (TOF) instrument (Bruker, Billerica, MA). The eluted apo-forms and holo-forms of PCPG and MxcF were desalted by multiple dilutions and reconcentrations with deionized water (pH 8.0) using Microcon YM-30 spin columns (Millipore). The analysis was carried out as described (18).

***In Vitro* Assay for Myxochelin Formation by MxcE, MxcF, and MxcG.** Incubations of 1 ml each contained 25 mM Tris·HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 3 mM 2,3-dihydroxybenzoic acid (DHBA), 1.5 mM lysine, 3 mM ATP, 1.5 mM NADPH (alternatively 1.5 mM NADH), 500 nM MxcE, 500 nM MxcG, and 5 μM MxcF and were incubated at 30°C after starting the reaction with NADPH or NADH. The incubations were frozen at −20°C after 0–65 hr. The myxochelins were extracted three

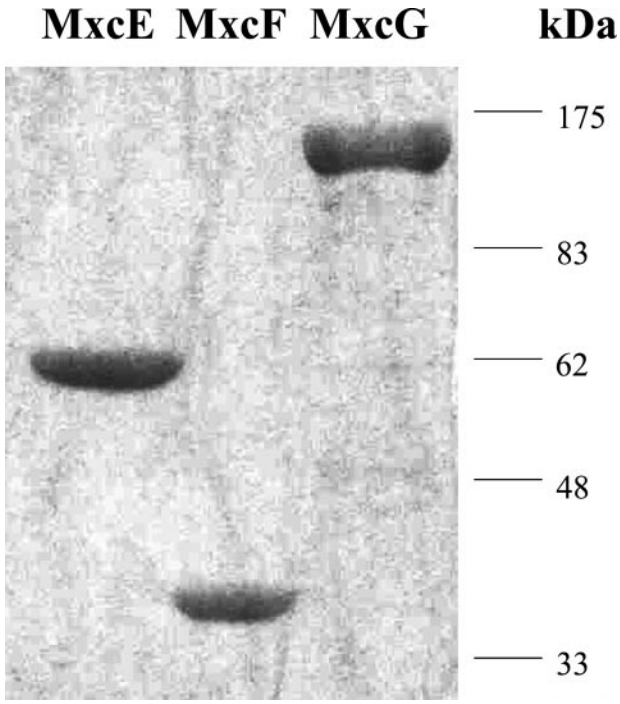

Fig. 2. Purified MxcE, MxcF, and MxcG after expression in *E. coli*. In a 10% polyacrylamide gel, sizes were estimated by using a prestained broad-range protein marker (New England Biolabs). Calculated masses of the recombinant proteins are as follows: MxcE, 59,940 Da; holo-MxcF, 35,089 Da; holo-MxcG, 157,936 Da. N-terminal protein sequencing revealed that the first amino acid is cleaved off from MxcE and MxcF after expression in *E. coli*.

times with equal volumes of ethylacetate, dried, and redissolved in 120 μl of methanol. Aliquots of 5 μl each were analyzed by HPLC as described (13). The identity of myxochelin A was verified by using HPLC-MS analysis (13). The amount of myxochelin formed was estimated by using integrated peak areas in comparison to those of a myxochelin standard curve.

Results

The analysis of the myxalamid (compare Fig. 1) and the myxochelin biosynthetic megasynthetase-systems from *S. aurantiaca* Sg a15 revealed the presence of terminal reductase (R-domains; refs. 13 and 20). Neither of the corresponding gene clusters encodes a thioesterase, which is usually needed for chain release of the biosynthetic intermediate bound as a thioester to the last carrier protein of the biogenetic complex (see *Discussion*). Because the chemical structure of both myxochelin A and myxalamid A seems to correlate well with a chain-termination process by means of the reduction of the last PCP-bound thioester-intermediate to the corresponding alcohol, we analyzed this hypothesis biochemically.

Heterologous Expression and Posttranslational Activation of the Myxochelin Biosynthetic Proteins. MxcE, MxcF, and MxcG were expressed in *E. coli* as intein-chitin-fusion proteins that enabled their efficient one-step enrichment (Fig. 2). MxcF and MxcG were coexpressed with MtaA to posttranslationally activate the aryl-carrier protein (ArCP) domain in MxcF and the PCP domain in MxcG. The broad substrate specificity of MtaA could be shown recently for an acyl-carrier protein (ACP) domain of the *mta* gene cluster (9), a PCP of unknown function from *Sorangium cellulosum* So ce90, and the ArCP of the enterobactin synthetase EntF (18). MxcF and the PCP domain of MxcG, which was expressed separately as intein-chitin fusion, were analyzed for posttranslational activation after coexpression in comparison to expression without MtaA. After coexpression, purification, and MALDI-TOF analysis of the protein masses, both proteins showed mass increases of ≈340 daltons, which corresponds to the mass of the attached phosphopantetheinyl group (see Table 1). Thus, both carrier proteins could be activated in *E. coli* by using MtaA, proving the protein to be a valuable tool for the expression of PKS and NRPS proteins in active form. Without coexpression of MtaA, no (MxcF) or only very little (PCPG) activation was observed, indicating that the phosphopantetheinyltransferases present in *E. coli* are not able to perform this reaction sufficiently. Interestingly, the phosphopantetheinyltransferase EntD of *E. coli* cannot activate MxcF, although MxcF shows 42% identity and 62% similarity with EntB, the cognate substrate of EntD.

Table 1. Determination of the *in vivo* phosphopantetheinylation of the isolated proteins MxcF and MxcG by coexpression with MtaA using MALDI-TOF analysis

Protein	Calculated mass, Da	Determined mass, Da
Apo-MxcF	34,749	34,714
Holo-MxcF	35,089	35,046
Apo-PCPG	11,123	11,126
Holo-PCPG	11,463	11,466

MxcE, MxcF, and MxcG Are Sufficient for the Assembly of the Myxochelins. Enzymatic reactions were set up, starting the incubations with the three enzymes, ATP, NADPH (alternatively NADH), lysine, and DHBA. Incubations were between 0 and 65 hr, and the products were extracted, concentrated, and analyzed by HPLC (Fig. 3). Myxochelin A was identified on the basis of retention time, cochromatography with an authentic sample, UV-spectrum, and mass spectrum. The amount of myxochelin A in the extracts was calculated on the basis of the peak areas of a dilution series of an authentic sample. The amount of myxochelin A found in the incubations is given in Fig. 3 *Inset*. Control reactions omitting the peptide synthetase, ATP, or NADPH or after denaturation of the enzymes did not result in any detectable amount of the product. The system is able to reduce the thioester-bound myxochelin acid with both cosubstrates, NADPH and NADH, albeit more slowly for the latter compound.

Domains with Similarity to the Terminal Reductase Domain of MxcG Can Be Found in Other PKS and NRPS Systems. There are several examples from the database in which C-terminal R domains can be found (see Fig. 4), such as a PKS (HetM) involved in heterocyst formation in *Anabaena* sp. (22) and a hybrid PKS/NRPS (MxaA) system from *S. aurantiaca* Sg a15 responsible for the formation of the myxalamids (20). In addition, there is the R domain of the saframycin synthetase SafA (23) from *Myxococcus xanthus* (compare Fig. 1). Further examples of terminal R domains of unknown function in PKS and NRPS systems can be found when searching the genomes of completely sequenced microorganisms. In the genome of *Streptomyces coelicolor*, a putative PKS (GenBank accession no. AL512902) has been located showing 36% identity and 49% similarity to MxcG over the last 400 aa of the protein. A putative NRPS found in the genome of *Pseudomonas aeruginosa* (National Center for Biotechnology Information protein list no. E83137) shows 28% identity and 47% similarity. Another NRPS system is involved in the glycopeptidolipid biosynthesis of *Mycobacterium smegmatis* (24), showing 31% identity and 46% similarity to the R domain of MxcG.

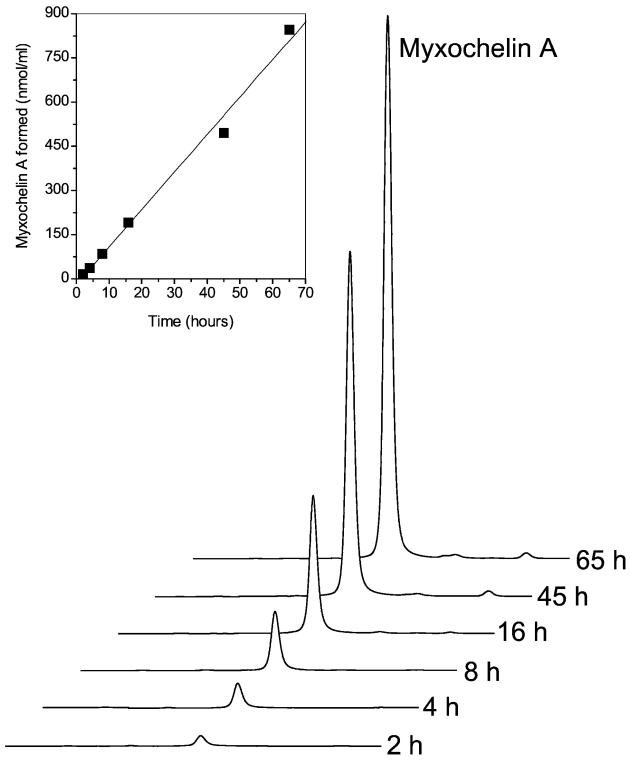

Fig. 3. *In vitro* myxochelin A production. The amount of myxochelin A formed in incubations and the corresponding HPLC diagrams are shown from one representative of three time-course experiments.

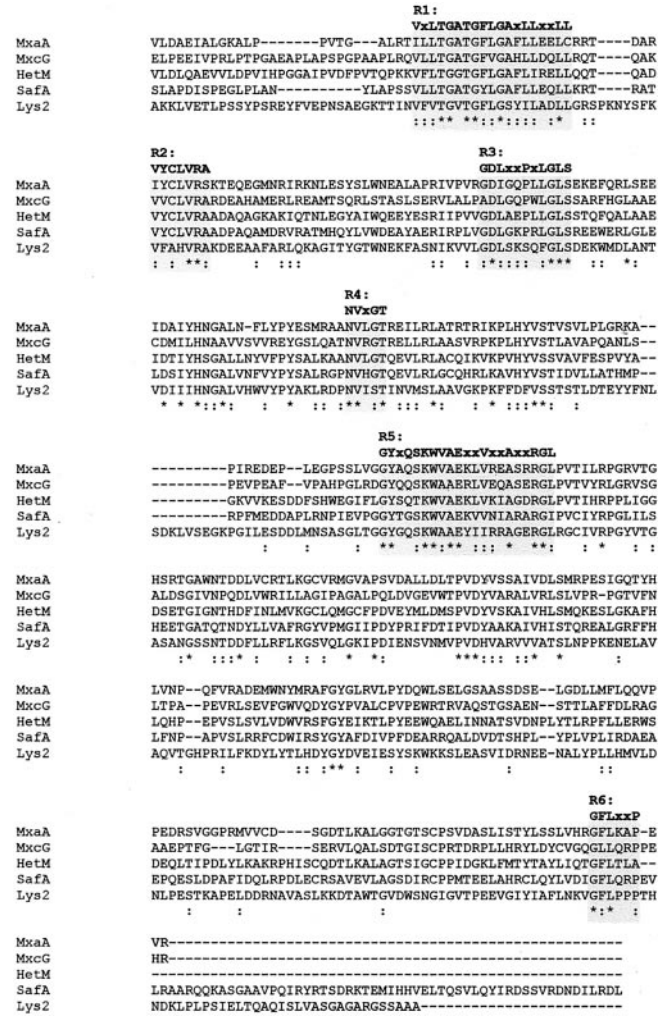

Fig. 4. Alignment of reductase domains. Alignment of R domains of several PKS and NRPS systems, in which the last 386 aa of MxaA (*S. aurantiaca*), the last 393 aa of MxcG (*S. aurantiaca*), the last 395 aa of HetM (*Anabaena* sp.), the last 448 aa of SafA (*M. xanthus*), and the last 409 aa of Lys2 (*Saccharomyces cerevisiae*) are aligned [see text; performed with CLUSTAL W (21)]. Consensus sequences of the six redefined core motifs R1–R6 (shown shaded in gray) are shown in bold. These core regions are based on ref. 26. ''x'' represents any amino acid. The amino acids shown in bold occur in at least 80% of the sequences given. *, identical amino acids; :, similar amino acids.

Discussion

The biosynthesis of the myxochelin-type siderophore reveals new insights into nonribosomal peptide and polyketide biosynthesis. Usually, complex modular NRPS systems comprise adenylation (A) domains, which activate the amino acid that is to be incorporated next, and condensation (C) domains, which form the amide linkage with the growing peptide chain. These events happen while the intermediates are tethered to the way-stations of NRPS, the PCPs. The PCPs need to be activated with a phosphopantetheinyl group serving as a swinging arm to pass the intermediates on from module to module. Additional modules may be incorporated in these systems to perform additional reactions, such as N-methylations, heterocyclizations, and oxidations (9, 25, 26). Finally, the compound is set free from the last carrier protein by hydrolysis which is usually catalyzed by a thioesterase (TE) domain that can set free the acid or perform an intramolecular ester formation, resulting in macrolactone structures. Very similar biochemistry is used in PKS systems for chain termination, also involving TE domains.

Alternative chain terminations have been reported; an amide synthase is involved in the formation of the macrolactam structure of the rifamycins (27, 28), and an unusual NRPS module with a monooxygenase domain is used for the formation of the terminal amide in the myxothiazols (9).

Here, we describe the myxochelin megasynthetase, which reveals some additional features of NRPS systems. The reductase domain that can be found at the C terminus of MxcG performs a previously unknown type of reductive release in that it sets free, after two rounds of reduction, the alcohol that corresponds to the PCP-bound thioester of the bis-aryl-capped lysine (myxochelin A; compare Fig. 5). Most likely, similar R domains (Fig. 4) perform reductive chain-termination biochemistry in both PKS and NRPS systems. The terminal NRPS of the myxalamid (see Fig. 1) megasynthetase, MxaA, also contains an R domain (see Fig. 4). It has been speculated that MxaA extends the intermediate polyketide with an alanine that finally gets reduced to an 2-aminopropanol moiety by the R domain, perfectly matching the chain release by means of a two-step reduction described here (20). This two-step reduction involves an aldehyde intermediate that can be subjected to a reductive aminotransferase reaction, representing yet another possibility for the chain-termination reaction. The aminotransferase reaction gives rise to terminal amino groups, as in the case of myxochelin B biosynthesis (Fig. 5). Already, a putative aldehyde aminotransferase (MxcL), presumably performing this reaction, has been located in the myxochelin biosynthetic operon (13), thus providing evidence that the two alternative chain-termination processes are performed in parallel in *S. aurantiaca*. Further, there is the reductase domain of the saframycin (see Fig. 1) NRPS from *Myxococcus xanthus* (23, 29); it has been speculated that its R-domain forms an aldehyde and, subsequently, intramolecular Schiff bases are formed from a putative Ala-Gly-Tyr-Tyr-S-PCP intermediate. Finally, there is a close

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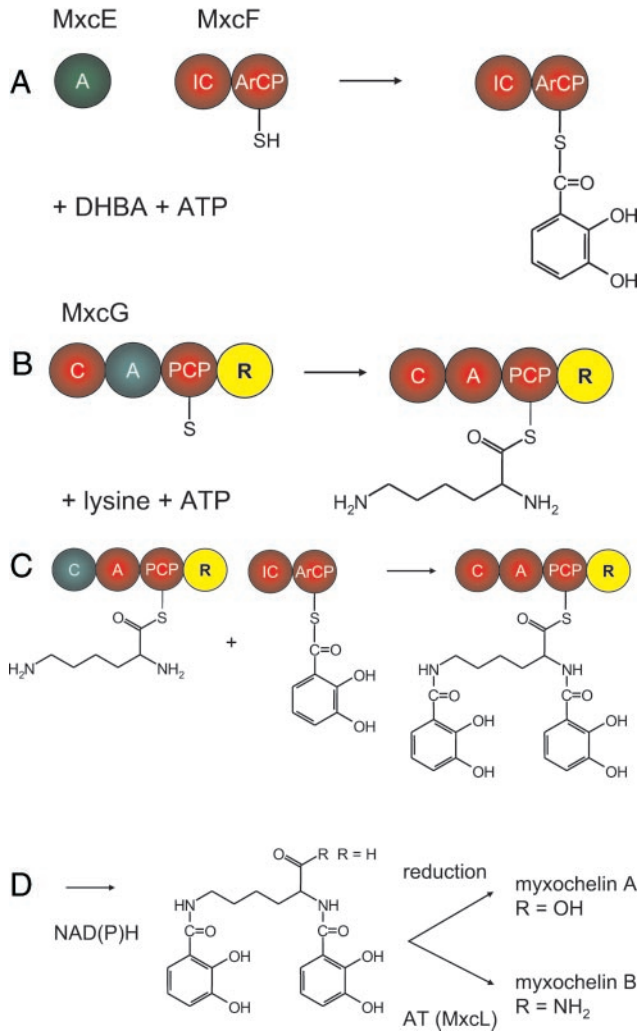

Fig. 5. Scheme for myxochelin A and B biosynthesis. (*A*) The A domain of MxcE adenylates DHBA and attaches it to the ArCP of MxcF. MxcF is a bifunctional protein with an isochorismatase (IC) module, which is relevant for DHBA biosynthesis from chorismate. (*B*) The A domain of MxcG adenylates lysine and tethers it to the PCP of the protein. Active domains in each step are shown in green. (*C*) The C domain of MxcG creates the amide linkage between DHBA and the two amino groups of lysine. It is not known which amide is formed first. Usually, C domains perform only one round of condensation during nonribosomal peptide biosynthesis (see text). (*D*) The R domain of MxcG reduces the PCP-bound thioester to the aldehyde intermediate. Subsequently, either the R domain of MxcG further reduces the aldehyde to the alcohol, or the aldehyde is reductively transaminated by an aminotransferase (AT), presumably MxcL.

connection between these secondary metabolic reactions to primary metabolism in yeast; the Lys2 protein has been shown to reduce the PCP-bound thioester of α-aminoadipate to α-aminoadipate semialdehyde, which is reductively transaminated to lysine subsequently (30). It has been speculated already that the R domains of SafA and Lys2 perform an alternative strategy for peptide-chain termination by releasing a linear aldehyde (31, 32). Based on this possibility, Konz and Marahiel (26) defined core regions of putative reductase domains. In Fig. 4, these core regions are redefined based on the sequences aligned. In contrast to earlier assumptions (13, 32), no further oxidoreductase protein is needed to reduce the aldehyde to the alcohol. Here, we provide direct biochemical evidence that the MxcEFG complex is capable of reducing the PCP-bound thioester to the aldehyde and, further, to the alcohol structure.

Additional examples of terminal-reduction domains of unknown function in PKS and NRPS systems can be found when searching the databases (see *Results*). It is likely that most of these domains are involved in chain-termination reactions similar to those described herein. Thus, terminal R domains seem to be responsible for a frequently used type of reductive release mechanism in PKS and NRPS systems. The corresponding biosynthetic modules will presumably benefit combinatorial biosynthesis in the future.

The second unusual part of MxcG is its C domain. MxcE activates DHBA as adenylate and tethers it to the ArCP of MxcF (Fig. 5). The ArCP-bound DHBA-thioester is delivered next to the megasynthetase MxcG, the A domain of which activates lysine and attaches it to the PCP. The C domain of MxcG must be capable of performing two different condensation reactions: it creates the aryl capped α-amide of lysine and links DHBA to the γ-amide of the molecule. This finding implies that MxcF delivers activated DHBA to the complex twice. It is not clear whether one molecule of MxcF is attached to the biosynthetic complex and reloaded with DHBA after the first condensation reaction, resulting in the monoacylated intermediate. Alternatively, two different charged MxcF proteins may be used for two acylation reactions. To our knowledge, besides the vibriobactin system, in which VibF is responsible for the bisacylation of norspermidine (33), this domain is the only C domain in NRPS systems that has dual activities during chain assembly.

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