Novel Insights into Siderophore Formation in Myxobacteria

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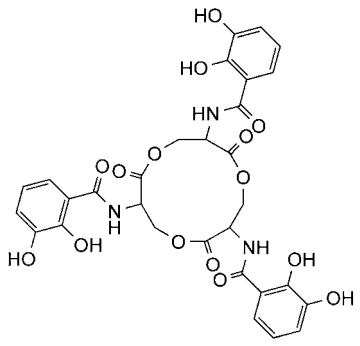
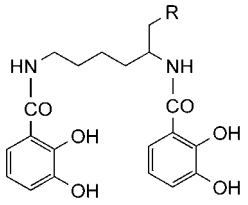
The myxochelins are catecholate-type siderophores produced by a number of myxobacterial strains, and their corresponding biosynthetic gene clusters have been identified in Stigmatella aurantiaca *Sg a15,[1] and* Sorangium cellulosum *So ce56; the latter being presented in this work. Biochemical and genetic studies described here further clarify myxochelin biosynthesis. In addition to the myxochelin A biosynthetic complex, the aminotransferase MxcL is required in order to form myxochelin B, starting from 2,3-dihydroxy benzoic acid and* L*-lysine. Additionally, the substrate specificity of the myxochelin A biosynthetic complex was analyzed in vitro; this led to the formation of novel myxochelin derivatives. Furthermore, MxcD was over-expressed and its function as an active isochorismic acid synthase in* Escherichia coli *was verified by complementation studies, as was activity in vitro. The organization of the myxochelin gene cluster of* S. cellulosum *So ce56 was compared to that of the Sg a15 gene cluster. The comparison revealed that although the organization of the biosynthetic genes is completely different, the biosynthesis is most probably extremely similar.*

Introduction

Iron plays an essential role in microbial metabolism since it is an integral part of many enzymes and enzymatic complexes. Studies with mycobacteria have revealed that up to 60 μg of iron per gram cell mass are needed to ensure growth.[2] However, under aerobic and physiological conditions, iron forms insoluble $Fe(OH)_3$. To supply this iron, microorganisms are forced to develop highly specific iron-uptake mechanisms. These mechanisms are based on the production, secretion, and recovery of low-molecular-weight compounds, also called siderophores, with high affinity for Fe^{3+}. The best known example of a bacterial iron-uptake and -recovery system is represented by the catecholate enterobactin (Scheme 1) produced by *Escherichia coli*.[3,4] Efficient recognition of the iron–siderophore complex and transport into the transmembrane space are performed by porine-like transmembrane proteins, which form extracellular loops that have high affinity for the iron–siderophore complex,[5] followed by active transport into the cytoplasm. Once the complex has reached the cytosol, iron is released from the siderophore by hydrolysis, a process that is catalyzed by an esterase.[4] In comparison to this and other similar microbial systems,[6,7] little is known about iron acquisition in myxobacteria.



enterobactin



myxochelin

Scheme 1. *Structures of myxochelins A (R = OH) and B (R = NH_2) and enterobactin produced by* E. coli.

Myxobacteria comprise Gram-negative soil bacteria, which belong to the δ-group of proteobacteria and show some outstanding and unique features. They exhibit a complex life cycle, including the formation of fruiting bodies upon starvation.[8–10] Over the past two decades, myxobacteria have been intensively studied because of their ability to produce a wide variety of secondary metabolites with important biological function.[11–13] Several myxobacterial gene clusters responsible for the biosynthesis of secondary metabolites have been cloned and characterized, thus providing extraordinary new insights into secondary-metabolite formation.[14–26]

These facts and the knowledge that myxobacteria harbor the largest known bacterial chromosomes (12.6 Mb in *S. cellulosum* So ce56[27–29]) have triggered independent sequencing projects with two different model strains. *Myxococcus xanthus*

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DK1622, belonging to the suborder *Cystobacterineae*, is being sequenced at the Institute for Genomic Research and *S. cellulosum* So ce56 (*Sorangineae*) by a consortium at the Gesellschaft für Biotechnologische Forschung and Bielefeld and Saarland Universities.

Two types of siderophores are known to be produced by myxobacteria. The hydroxamate-type nannochelins were isolated from *Nannocystis exedens*,[30] and the catecholate-type myxochelins A and B (Scheme 1) were identified in different strains of *S. aurantiaca*,[19] *Myxococcus xanthus* (unpublished data), and *Angiococcus disciformis*.[31]

Recently, the myxochelin biosynthetic gene cluster from *S. aurantiaca* Sg a15 was cloned and analyzed (Figure 1 A.[19]) It was found to represent the only known myxobacterial gene cluster responsible for the acquisition of iron. Myxochelin biosynthesis was reconstituted in vitro after heterologous expression of the biosynthetic proteins.[15] Myxochelin A biosynthesis occurs on MxcG, a nonribosomal peptide synthetase (NRPS) consisting of four active domains: a condensation domain (C), an adenylation domain (A), a peptidyl carrier protein (PCP), and a terminal reductase domain (R; Figure 1 C). Two further enzymes (MxcE and MxcF) are involved in the formation of myxochelin A, starting from 2,3-dihydroxy benzoic acid (2,3-DHBA) and L-lysine. 2,3-DHBA is activated by the adenylating enzyme MxcE and is transferred to the phosphopantetheinylated aryl carrier protein (ArCP) domain of MxcF. The NRPS, MxcG, is responsible for the activation and the transfer of L-lysine to its activated PCP domain. 2,3-DHBA is then condensed with each of the free amino residues of the activated L-lysine-PCP-thioester, a process catalyzed by the C domain. The terminal reductase domain of MxcG then catalyzes the release of the PCP-bound thioester intermediate from the enzyme by reduction to the alcohol (Figure 1 C);[15] this leads to myxochelin A, the minor myxochelin product of *S. aurantiaca* Sg a15. The major product of this strain, however, is myxochelin B, in which the alcohol group is replaced by an amino function (Scheme 1). The biosynthesis of myxochelin B has until now not been elucidated in detail.

This study deals with the verification of the myxochelin biosynthetic pathway in *S. aurantiaca* Sg a15 starting with chorismic acid. Because MxcL contains a putative PLP-binding site typical for aminotransferases, it was speculated that it is responsible for the biogenesis of myxochelin B.[15] To show that *mxcL* is involved in myxochelin B formation, gene-inactivation experiments were carried out. These experiments revealed that four enzymes, MxcE, F, G, and L are necessary to form myxochelins A and B starting from 2,3-DHBA and L-lysine. We elucidated the biosynthesis of DHBA in *S. aurantiaca* Sg a15 by biochemical characterizing of MxcD. Furthermore, novel myxochelin derivatives were generated in vitro by using the enzymes MxcE, F, and G and alternative substrates.

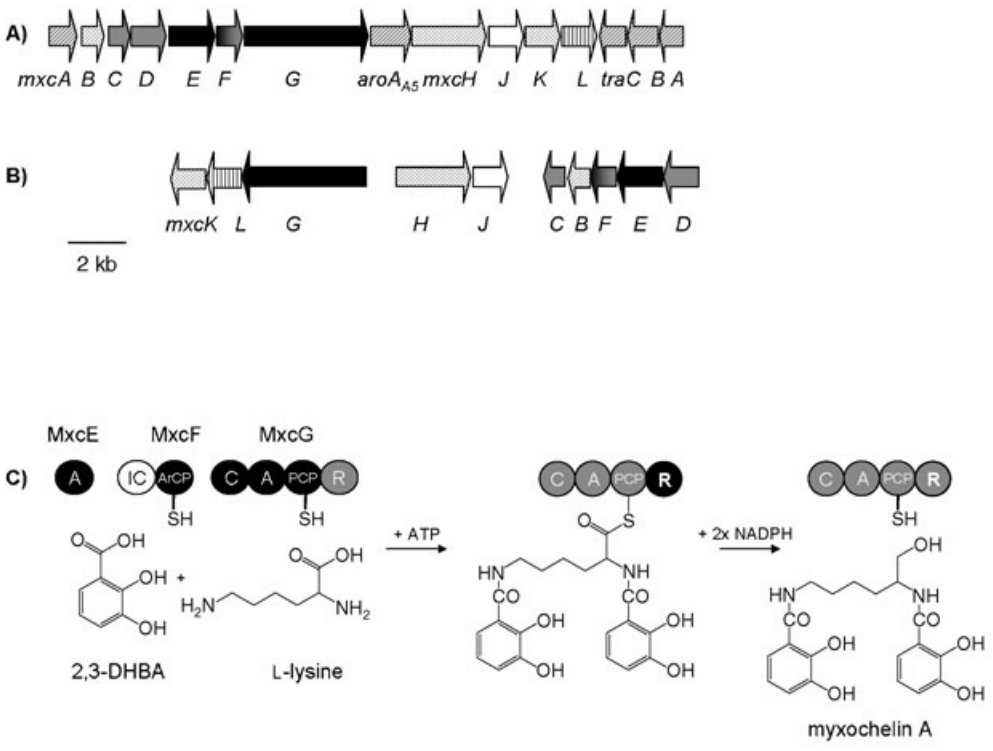


Figure 1. *Comparison of gene arrangements in the myxochelin biosynthetic gene cluster isolated from A)* S. aurantiaca *Sg a15 and B)* S. cellulosum *So ce56. Arrows indicate the transcriptional orientation of the genes. Genes that are not present in each cluster are indicated by diagonal lines. Genes responsible for myxochelin A biosynthesis are shown in black;[15]* mxcL*, which codes for an aminotransferase, is shown by vertical lines, and genes believed to be responsible for DHBA biosynthesis are represented by grey arrows; genes possibly responsible for utilization and transport of iron are depicted in dots. Genes with unknown function are shown in white. C) Scheme of myxochelin A biosynthesis. MxcE, F, and G are responsible for the activation and condensation of 2,3-DHBA with* L*-lysine resulting in the PCP-bound thioester. As previously described,[15] the thioester is then reduced to myxochelin A alcohol. Active domains during the respective biosynthetic steps are shown in black. The isochorismate lyase domain of MxcF (IC) is not needed for myxochelin A biosynthesis and is shown in white.*

Myxochelin was found to be produced by *S. cellulosum* So ce56, and the corresponding gene cluster was analyzed and compared to *S. aurantiaca* Sg a15.

Results

Myxochelin formation in *S. aurantiaca* Sg a15: The aminotransferase MxcL is required for myxochelin B formation

We recently demonstrated that myxochelin A formation is based on the activity of MxcE, F, and G, which are responsible for the condensation of two molecules of 2,3-DHBA with the amino groups of L-lysine, and the reduction of the corresponding intermediate by the R domain of MxcG (see Figure 1 and Introduction). Nonetheless, *S. aurantiaca* Sg a15 produces two myxochelins: A and B. The latter compound represents the major product of this strain (Figure 2), and the steps for myxochelin B formation in *S. aurantiaca* Sg a15 remain to be elucidated.

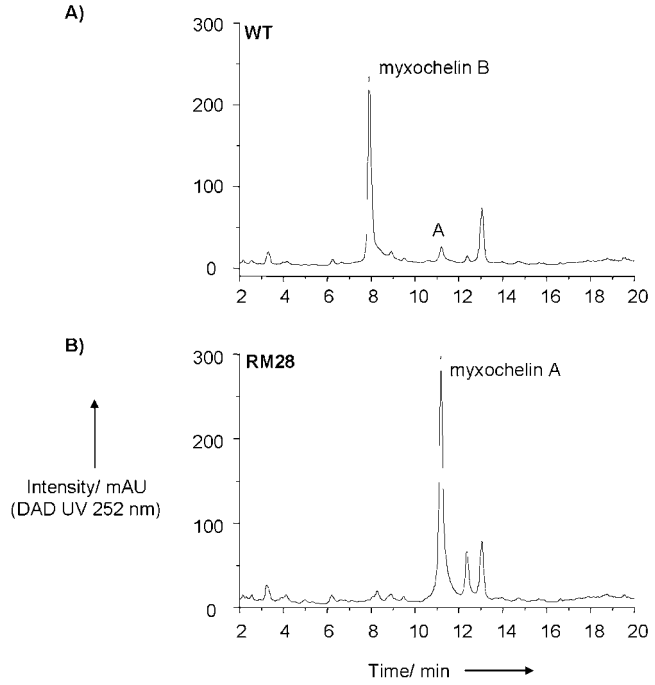


Figure 2. *HPLC analysis of mutant RM28 in comparison to wild-type (WT)* S. aurantiaca *Sg a15.*

MxcL is similar to several amino transferases whose sequences are available from various databases.[19] Therefore, it was suggested that MxcL could be involved in myxochelin B biosynthesis.[15] To determine whether MxcL is responsible for the formation of Myxochelin B, gene inactivation of *mxcL* was performed by insertional mutagenesis with the plasmid pRM28. The insertion occurred by homologous recombination over the internal 502 bp fragment of *mxcL*, which was cloned into pCR2.1TOPO. This resulted in mutant RM28. The specific insertion of pRM28 into the chromosome was verified by Southern blot analysis (data not shown), and the phenotypic analysis of the resulting mutant was performed by DAD-HPLC analysis (Figure 2). In contrast to *S. aurantiaca* Sg a15 wild type, the mutant RM28 was not able to produce myxochelin B. Instead, myxochelin A was detected at levels comparable to the amount of myxochelin B produced by *S. aurantiaca* Sg a15.

These results demonstrate the specific aminotransferase activity of MxcL during myxochelin B biosynthesis.

MxcA has no effect on the biosynthesis of myxochelins

MxcA shares sequence similarity with several proteins that exhibit oxidoreductase activity. Although *mxcA* is located upstream of a DNA region defined as a ferric-uptake regulator (FUR) binding site, which seems to regulate myxochelin biosynthesis,[19] it was speculated that the deduced protein is part of the myxochelin biosynthetic machinery. Previously we demonstrated that MxcA had no effect on the biosynthesis of myxochelin A.[15] However, an influence on myxochelin B production could not be excluded. Therefore, *mxcA* was inactivated by insertional mutagenesis as described for *mxcL*, but by using the palsmid pNGM34. Upon phenotypic analysis by DAD-HPLC, both mutant NGM34 and *S. aurantiaca* Sg a15 exhibited the same metabolite production pattern (data not shown). For this reason any influence of MxcA on myxochelin B formation was excluded.

Substrate recognition by the myxochelin biosynthetic complex

Recently, myxochelin A biosynthesis was reconstituted in vitro.[15] To analyze the substrate specificity of the enzymatic complex, structural analogues of 2,3-DHBA were tested for their incorporation into the myxochelin backbone. We utilized the in vitro assay for myxochelin A production as described in the Experimental Section. The calculated reaction velocity of myxochelin production was 4.2 nmol mL^{-1} min^{-1}, and 405 μg of myxochelin A were obtained after incubation for 90 min. Native substrates were then substituted by their structural analogues in the reaction mixture, for example, 3,4-DHBA, singly hydroxylated benzoic acid derivatives and amino derivatives of benzoic acid. The last compounds represent important intermediates of primary and secondary metabolism, for example, anthranilic, acid is used for aurachin biosynthesis in *S. aurantiaca* Sg a15[1] and 2,3-dihydro-2,3-dihydroxybenzoic acid is an intermediate of the DHBA biosynthetic pathway.[32] L-Ornithine was used to replace L-lysine. From all of these assays, only two novel compounds (**1** and **2**) were identified by HPLC as shown in Figure 3. The assay revealing compound **1** was performed with 2-hydroxy benzoic acid (2-HBA), and compound **2** was isolated from the assay including 3-HBA in place of 2,3-DHBA. Quantification of the products revealed total yields of 37 μg mL^{-1} for compound **1** and 11 μg mL^{-1} for compound **2**. The structures of both **1** and **2** are further supported by mass spectrometric analysis (data not shown).

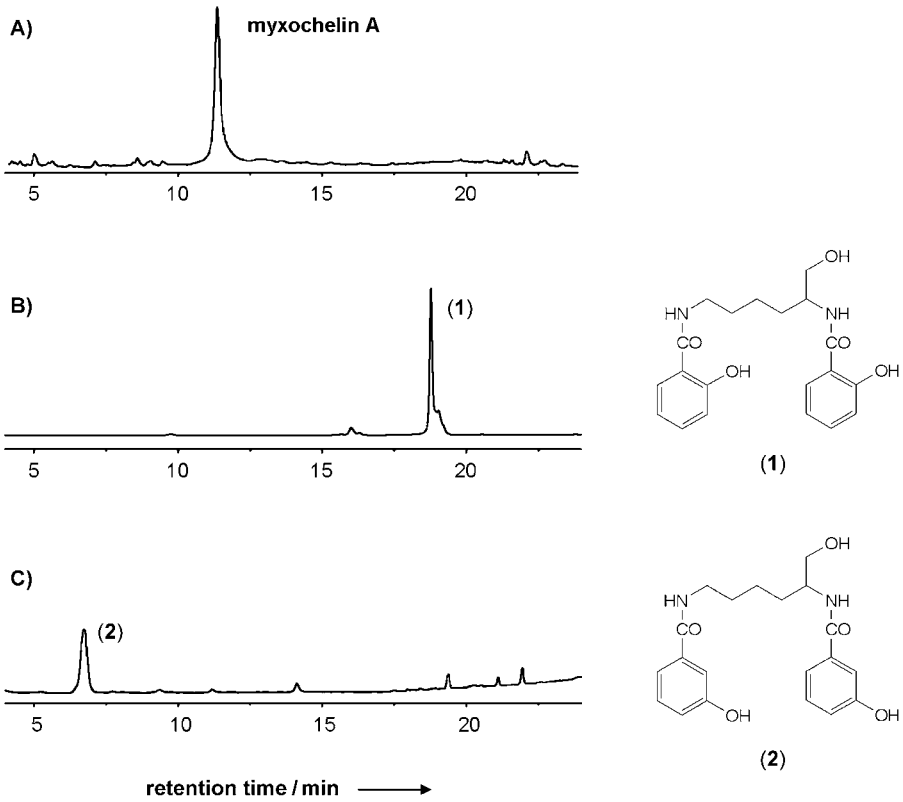


Figure 3. *HPLC analysis of the in vitro experiments which determine the specificity of the myxochelin biosynthetic complex. A) In vitro reconstitution of myxochelin A biosynthesis. Assays in which 2,3-DHBA is substituted with B) 2-HBA and C) 3-HBA.*

Biosynthesis of 2,3-DHBA in *S. aurantiaca* Sg a15

Biosynthesis of 2,3-DHBA appears to be unique to microbes, and appears to involve a three-step process starting from chorismic acid. MxcC, MxcD, and MxcF have homologies to proteins involved in 2,3-DHBA biosynthesis in *E. coli*. Gene-inactivation experiments revealed that MxcD is essential for myxochelin biosynthesis, although polar effects of mutagenesis could not be excluded.[19]

To verify the role of MxcD in this biosynthetic pathway, we set out to heterologously express *mxcD* in *E. coli* strain PBB8. This *E. coli* mutant harbors a deletion in both isochorismate synthase-encoding genes, *entC* and *menF*, and therefore enterobactin biosynthesis in *E. coli* PBB8 is abolished completely.[33]

E. coli PBB8 that carried the pMxcD or p422 plasmids (p422 expresses endogenous isochorismate synthase EntC from *E. coli*,[33] data not shown for *E. coli* PBB8 harboring p422) were grown on minimal media plates containing chromazurol S. The colonies showed a yellow "halo" due to the restored production of enterobactin (Figure 4). *E. coli* PBB8 cultured under the same conditions do not produce a "halo". The same effect is visible around *E. coli* PBB8 carrying the empty control plasmid pCYB2 (Figure 4). Thus expression of MxcD in *E. coli* PBB8 complements the "missing" isochorismate synthase activity so that enterobactin production is restored.

In addition, the specific activity of the expressed recombinant enzyme was determined in vitro. This revealed a specific isochorismate synthase activity of 945 pmol mg^{-1} protein per minute. The control reaction, which was performed in parallel, did not reveal any conversion of chorismate to isochorismate.

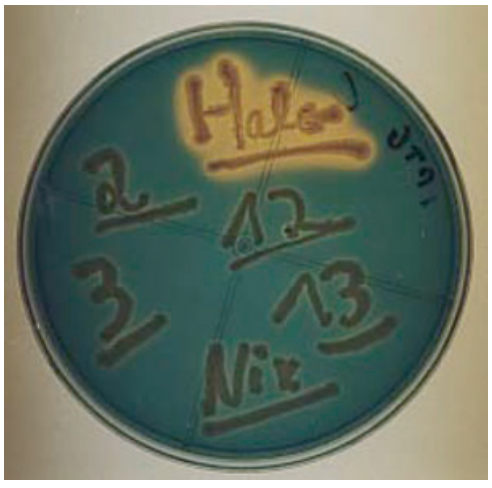


Figure 4. *Complementation of the isochorismic acid synthase-negative strain* E. coli *PBB8 by MxcD. After being grown on CAS-agar media,* E. coli *PBB8 transformed with pMxcD (Halo) are detected by a characteristic yellow zone around colonies. The yellow zone is absent around* E. coli *PBB8 (Nix) and* E. coli *PBB8 transformed with pCYB2 or pCR2.1 (2, 3 and 12, 13, respectively).*

Genes downstream of the *mxc* locus in *S. aurantiaca* Sg a15

The DNA sequence downstream of the *mxc* biosynthetic gene cluster was analyzed by using the frame-plot software offered by the National Institute of Infectious Disease in Japan. This software enables prediction of protein-coding regions in bacterial DNA which have a high G and C content.[34] The prediction is based on the fact that myxobacterial DNA has an extremely high G and C distribution at the third letter of each codon. The putative open reading frames (ORFs) were then analyzed by using the blast software from the German Research Centre of Biotechnology in Braunschweig. A cluster of three ORFs, named *traA, traB,* and *traC*, that seemed to form an operon was identified (Figure 1 A). *traB* seems to start immediately downstream of *traA* with an ATG that overlaps with the TGA of

Table 1. *Sequence comparison of genes and their encoded proteins from the myxochelin biosynthetic gene cluster in* S. aurantiaca *Sg a15 and* S. cellulosum *So ce56. MegAlign from the DNASTAR package was used.*

Gene	Identity [%]	Protein	Identity [%]
mxcB/*mxcB*$_{so}$	51.4	MxcB/MxcB$_{so}$	41.1
mxcC/*mxcC*$_{so}$	69.9	MxcC/MxcC$_{so}$	67.8
mxcD/*mxcD*$_{so}$	61.3	MxcD/MxcD$_{so}$	52.3
mxcE/*mxcE*$_{so}$	68.5	MxcE/MxcE$_{so}$	68.0
mxcF/*mxcF*$_{so}$	64.7	MxcF/MxcF$_{so}$	50.6
mxcG/*mxcG*$_{so}$	68.0	MxcG/MxcG$_{so}$	63.0
mxcH/*mxcH*$_{so}$	59.1	MxcH/MxcH$_{so}$	46.1
mxcJ/*mxcJ*$_{so}$	54.6	MxcJ/MxcJ$_{so}$	41.7
mxcK/*mxcK*$_{so}$	48.0	MxcK/MxcK$_{so}$	34.9
mxcL/*mxcL*$_{so}$	68.6	MxcL/MxcL$_{so}$	61.3

traA. *traC* also begins with an ATG codon that appears to overlap with the TGA of *traB*.

The presumed translational products of these genes show homologies to hemin ABC transporters (Table 2). *traA* seems to encode a protein that has the highest similarity to a putative periplasmic binding protein identified in *Deinococcus radiodurans* (protein ID gi|10957442, 46% identity), *Streptomyces coelicolor* (protein ID gi|21220741, 37% identity), or *Vibrio parahaemolyticus* (protein ID gi|28900278, 35% identity). The deduced product of *traB* shows homologies to several cytoplasmic proteins from *D. radiodurans* (protein ID gi|10957443, 65% identity), *Gloeobacter violaceus* (protein ID gi|37520148, 55% identity), and *Pseudomonas putida* (protein ID gi|26991372, 49% identities). Ten putative transmembrane regions were identified from the deduced protein sequence. *traC* appears to code for a protein that has high identity to several ATP-binding proteins identified in *D. radiodurans* (protein ID gi|10957444, 56% identity), *G. violaceus* (protein ID gi|37520147, 44% identitiy), or *P. aeruginosa* (protein ID gi|15599900, 46% identity). Conserved ATP-binding motifs (p loop) usually start with a serine residue, which is then followed by a glycine-rich sequence and typically terminates with a conserved leucine.[35] A similar motif was identified in the deduced protein of *traC* (SGGEQQRVHFARVL).

Table 2. *Proteins encoded by genes located downstream of the myxochelin biosynthetic operon.*

gene	size [bp]	Codon usage [%][a]	M_W of the resulting protein [Da]	postulated function	identity [%] to protein with PID
traA	810	82.1	29416	hemin ABC transporter, periplasmic binding protein	46% to NP_051557, *D. radiodurans*
traB	1053	84.9	36732	hemin ABC transporter, permease protein	48% to NP_051558, *D. radiodurans*
traC	906	74.8	31934	hemin ABC transporter, ATP-binding protein	42% to NP_051559, *D. radiodurans*

[a] GC content in 3rd position.

The myxochelin biosynthetic gene cluster in *S. cellulosum* So ce56

The DNA sequence obtained from the *S. cellulosum* So ce56 genome project was checked for genes with homology to *mxcG* from *S. aurantiaca* Sg a15, and an ORF that has 68% identity to that of *mxcG* was identified. Close to *mxcG*, nine further genes were identified that had identities of up to 70% to analogous genes from the myxochelin biosynthetic gene cluster from *S. aurantiaca* Sg a15 (Table 1). Sequence analysis of the *mxc* locus in *S. cellulosum* So ce56 revealed the organization of the genes as shown in Figure 1B. In contrast to the *mxc* locus of *S. aurantiaca* Sg a15 (Figure 1A), a different organization of the genes was observed. The identified *mxc* genes in the genome of *S. cellulosum* So ce56 appear to form three transcriptional units. The first putative operon consists of *mxcD*, *E*, *F*, *B,* and *C*. A 974 bp DNA region separates *mxcC* and the following gene, *mxcJ*, but no further ORFs were identified in this DNA region. *mxcJ* is part of the second transcriptional unit formed by the genes *mxcH* and *J*. The third putative transcriptional unit is formed by *mxcG*, *mxcL*, and *mxcK*. The ATG of *mxcG* is located 1148 bp upstream of the *mxcH* ATG. By using the promoter-prediction software provided by the fruit fly genome project, a bidirectional promoter was identified. This software was originally optimized to predict promoter regions in the fruit fly genome, whereas the modified version of the software can also be used for promoter predictions in prokaryotic DNA. In contrast to the *mxc* biosynthetic gene cluster of *S. aurantiaca* Sg a15, no putative FUR-binding site could be identified in the *mxc* locus of *S. cellulosum* So ce56.

The *aroA*$_{A5}$ gene, which is present in the myxochelin biosynthetic operon of *S. aurantiaca* Sg a15 and has been shown to be involved in aurachin biosynthesis (probably required for the biosynthesis of chorismic acid),[1] is absent in the *mxc* locus of *S. cellulosum* So ce56. The *traA–C* genes located downstream of *mxcL* in *S. aurantiaca* Sg a15, believed to be involved in Fe-myxochelin transport, are also missing in the *mxc* locus of *S. cellulosum* So ce56. Therefore, all genes required for myxochelin A and B formation are present in *S. cellulosum* So ce56.

Discussion

Myxochelin B formation

Previously, it was proposed that the putative aminotransferase encoded by *mxcL* (Figure 1) is responsible for myxochelin B formation in *S. aurantiaca* Sg a15.[15] In this work, we present

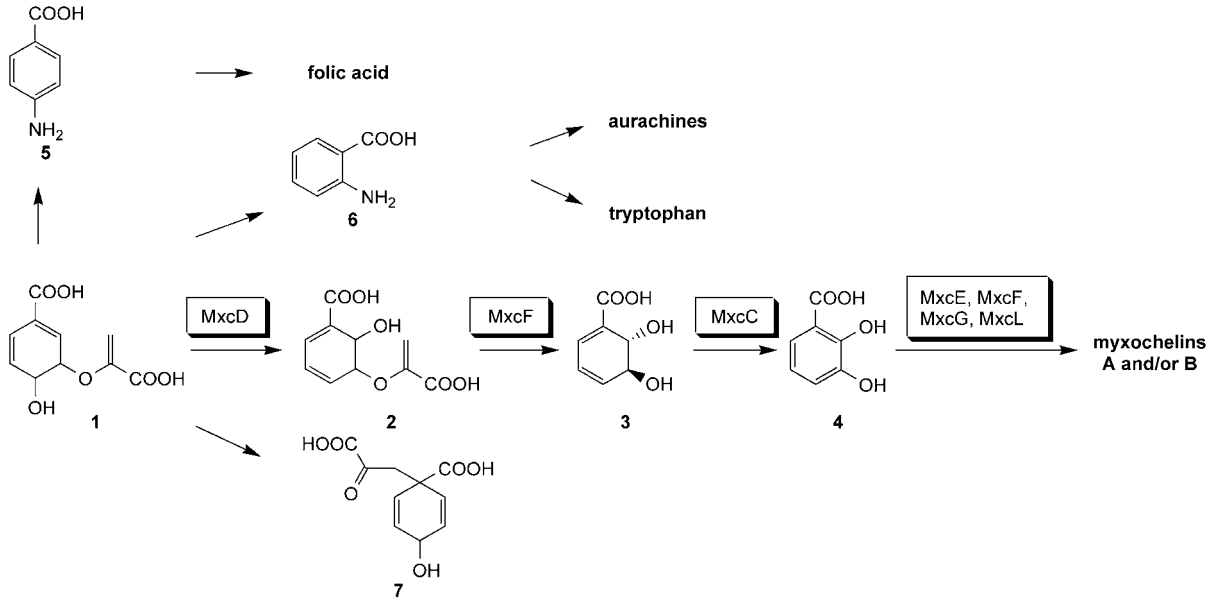
direct evidence that MxcL is essential for myxochelin B formation in *S. aurantiaca* Sg a15. This conclusion was obtained by performing gene inactivation of *mxcL*. The *mxcL* knock-out mutant RM28 was unable to produce myxochelin B, whereas myxochelin A was produced at increased titer. These data suggest that the *mxcL*$^-$ mutant is unable to transaminate a myxochelin aldehyde intermediate, which is obtained after a first round of reduction of the thioester intermediate catalyzed by the terminal R domain of MxcG (Scheme 2). It is believed that a second round of reduction results in myxochelin A formation.

It is very likely that for myxochelin B biosynthesis, starting from L-lysine and 2,3-DHBA, four enzymes, MxcE, F, G, and L, are necessary (compare Figures 1 and 3). However, the amino donor required for the transamination reaction (Scheme 2) remains unknown. To gain further insight into this question, we have expressed and purified MxcL from *E. coli* (data not shown). Despite serious efforts, reconstitution of myxochelin B formation in vitro has not been successful.

The inactivation of *mxcA* did not show any influence on myxochelin formation; this showed that the corresponding enzyme is not part of the biosynthetic complex.

A number of myxobacterial strains have been tested for myxochelin production. Myxochelin A production has been reported for *Archangium gephyra* 3947,[19] while *Angiococcus disciformis*[31] and *M. xanthus* Mx x48 produce both myxochelins A and B (unpublished data; all strains belong to the *Cystobacterineae* suborder). These data have been extended here, and we show that *S. cellulosum* So ce56, which belongs to the suborder *Sorangineae*, produces both myxochelin A and B (see below). In analogy to mutant RM28, *A. gephyra* 3947 appears to lack an active aminotransferase analogous to MxcL. This effect could be due to point mutations or gene deletions. *mxcL*-equivalent genes were identified in the chromosomes of *S. aurantiaca* Sg a15,[19] *S. cellulosum* So ce56 (this work), and *M. xanthus* DK1622 (TIGR, unpublished data).

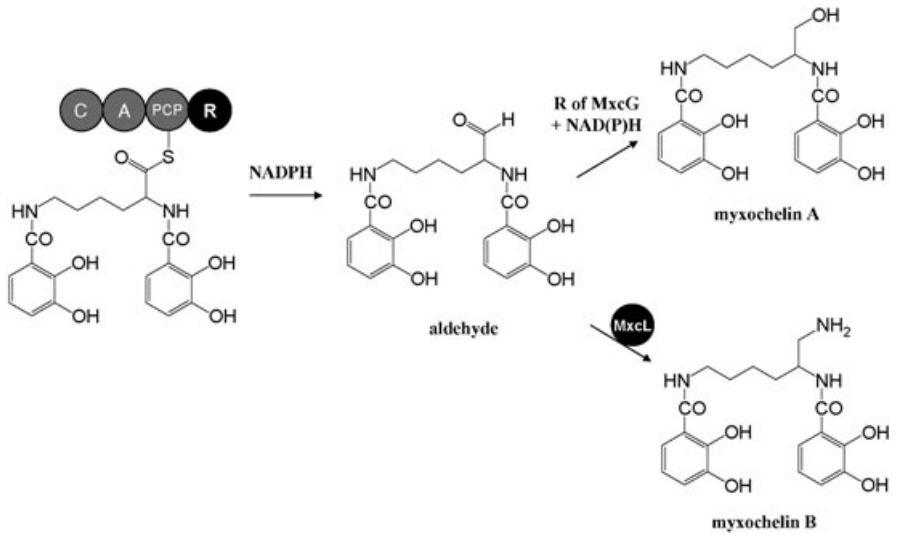
Chain-release mechanisms during nonribosomal peptide biosynthesis involving reductive domains and/or aminotransferases have been reported in primary and secondary metabolism. In analogy to myxochelin A formation, it is speculated that during myxalamide biosynthesis, a single R domain is responsible for reduction of the thioester intermediate to the alcohol.[18] In *Cryptosporidium parvum*, a gene encoding a multimodular polyketide synthase (PKS) was identified, which also terminates with a reductive domain. The resulting product has not been identified, but it is very likely that the PKS is responsible for the formation of a polyketide alcohol.[36] CmlP, an NRPS involved in chloramphenicol biosynthesis, terminates with an R domain.[37] It was speculated that two enzymes are involved in the two-step reduction of the corresponding thioester intermediate bound at the PCP of CmlP to give *N*-dichloroacetyl-*p*-aminophenylserinol, the precursor of chloramphenicol. Considering the elucidation of myxochelin A biosynthesis, it is likely that both rounds of reduction are performed by the terminal R domain of CmlP. Reductive transaminations during nonribosomal peptide formation have also been reported, for example, during lysine formation, the terminal R domain of Lys2 reduces the PCP-bound thioester, resulting in α-aminoadipate semialdehyde, which is then transaminated to give saccharopine.[38,39] Here we report that both reductive chain-release mechanisms can coexist during nonribosomal peptide biosynthesis, that is, the thioester intermediate located at the PCP of the NRPS is initially reduced to an aldehyde. The following steps depend upon the presence of an aminotransferase. The aldehyde intermediate can be transaminated if an aminotransferase is present. If the aminotransferase is lacking (Scheme 2) the aldehyde is reduced to the alcohol.



Scheme 2. *Isochorismic acid as an important intermediate of primary and secondary metabolism of* S. aurantiaca *Sg a15. The myxochelin biosynthetic pathway is highlighted with boxes. (**1**) chorismic acid; (**2**) isochorismic acid; (**3**) 2,3-dihydro-2,3-DHBA; (**4**) 2,3-DHBA; (**5**)* p*-amino-benzoic acid; (**6**) anthranilic acid; (**7**) prephenic acid.*

2,3-DHBA biosynthesis

Myxochelins A and B belong to the catecholic-type siderophores (see above) and contain DHBA. Biosynthesis of DHBA has been analyzed extensively in the past decade.[40–44] The biosynthesis is based on the activity of three enzymes that convert chorismic acid via isochorismic acid and 2,3-dihydro-2,3-DHBA to DHBA (Scheme 3). Proteins with homology to these enzymes (MxcC, MxcD, and MxcF) are encoded within the myxochelin biosynthetic operon of *S. aurantiaca* Sg a15.[19]



Scheme 3. *Scheme showing an alternative reductive release mechanism in nonribosomal peptide biosynthesis. The myxochelin thioester intermediate that is formed by MxcE, MxcF, and MxcG can be reduced by two alternative mechanisms. After two rounds of reduction catalyzed by the R domain of MxcG, the alcohol is produced (compare with Figure 1). In the presence of the aminotransferase MxcL, the aldehyde that results after the first reductive cycle can then be transaminated to form myxochelin B.*

After elucidating the biosynthesis of myxochelin in *S. aurantiaca* Sg a15, starting with 2,3-DHBA and L-lysine, verification of DHBA biosynthesis was attempted. MxcD functions as an isochorismic acid synthase and catalyzes the rearrangement of chorismic acid to isochroismic acid. By using in vivo complementation experiments in an ischorismic acid synthase-negative *E. coli* mutant PBB8,[33] the isochorismic acid synthase function of MxcD was verified. *E. coli* PBB8 is unable to produce enterobactin since DHBA is absent. Heterologous expression of MxcD in the mutant restored enterobactin biosynthesis. Additionally, the isochorismic acid synthase activity of MxcD was determined in vitro by using whole-cell extracts of *E. coli* PBB8 *entC*$^-$/*menD*$^-$ that expressed MxcD.

Although inactivation of *mxcD* in *S. aurantiaca* Sg a15 indicated its involvement in myxochelin biosynthesis, a polar effect could not be excluded.[19] In this study, we supply additional evidence that MxcD performs the conversion of chorismic acid to isochorismic acid, which is then directed into myxochelin biosynthesis (Scheme 3). Analysis of MxcD and earlier biochemical characterization of MxcF,[15] in combination with the in silico characterization of *mxcC*,[19] led us to conclude that biosynthesis of 2,3-dihydro-2,3-DHBA in *S. aurantiaca* Sg a15 is very similar to other microorganisms. Two further steps are needed to give DHBA. Isochorismic acid must be converted to 2,3-DHBA, which is most probably catalyzed by MxcF, a bifunctional enzyme with a C-terminal ArCP domain responsible for DHBA binding.[15] It is likely that the latter compound is then oxidized by MxcC to give DHBA (Scheme 3), which serves as substrate for MxcE, MxcF, and MxcG.[15] Biochemical verification of these reactions seems redundant. The responsible proteins, MxcF and MxcC, show high identity to the already well characterized analogous enzymes of the DHBA biosynthetic pathway in *E. coli* (up to 63% similarity).

Generation of novel myxochelins

The substrate specificity of the myxochelin-forming enzyme complex was examined in vitro. By substituting the natural substrates with structural analogues, novel myxochelin derivatives were generated (Scheme 2). In total, seven DHBA analogues and L-ornithine were supplied to substitute 2,3-DHBA or L-lysine, respectively, in the reaction mixture. Of these, only 2- and 3-hydroxybenzoic acids were utilized by the myxochelin biosynthetic complex to give compounds **1** and **2**. This indicates a rather high substrate specificity of the enzyme complex. In NRPSs, two domains are responsible for specificity. The adenylation domain defines specificity during selection and activation of the substrate. Marahiel and co-workers have deduced the so called "nonribosomal code", which consists of ten amino acids that seem to be responsible for the recognition and activation of the substrate.[45–47] The second factor defining substrate specificity during nonribosomal peptide synthesis is the condensation domain.[48] In the experiments described above, the condensation domain should catalyze the acylation of the amino residues of L-lysine with activated DHBA analogues. Studies of the crystal structure of the C domains have revealed that its acceptor site is highly specific for its substrate, whereas the substrate specificity at the donor site is usually more relaxed.[49] Following the rules of nonribosomal peptide biosynthesis, the DHBA analogues should be bound at the relaxed site of the C domain, irrespective of whether its highly specific acting acceptor site is being loaded with its native substrate, L-lysine. Because the specificity-defining part of the C domain in MxcG is loaded with its native substrate, L-lysine, there is the possibility that all tested DHBA derivatives could be condensed to the amino residue L-lysine. Therefore, it is more likely that sub-

strate specificity is defined by the A domains of the complex. Using pyrophosphate exchange assays, Marahiel and co-workers demonstrated that the DHBA-activating enzyme of the Bacillibactin synthetase strictly binds DHBA and salicylic acid (= 2-HBA). In this work, we demonstrate that the DHBA-activating enzyme MxcE can additionally adenylate 2-HBA and 3-HBA.

Transport of the iron–myxochelin complex in Gram-negative *S. aurantiaca* Sg a15

Previous sequence analysis of cosmids CA5 and CA5a revealed three genes (*mxc*B, *mxcH*, and *mxcK*) that were located in the myxochelin biosynthetic regulon, and which might be responsible for myxochelin transport.[1] However, studies of siderophore transport in Gram-negative bacteria have shown that other proteins are actually required for this purpose.[7] Further sequence analysis of cosmid CA5a revealed additional genes (*traA–traC*) with homology to hemin ABC transporters, which were located downstream of *mxcL* (Figure 1 A and Table 1). In combination with *mxcB* and *mxcH*, *traA–C* could form the myxochelin-uptake system in *S. aurantiaca* Sg a15. A porin-forming enzyme like MxcH[19] with outer cellular receptors would be required for the recognition and the transport of myxochelins into the periplasmic space. The remaining steps of siderophore transport into the cytoplasmic space are highly conserved among bacteria.[4] TraA is homologous to periplasmic binding proteins that are usually responsible for the translocation of siderophores through the periplasmic space. Myxochelins could be directed to transporters that are located at the cytoplasmic membrane. TraB shows homologies to several cytoplasmic transmembrane proteins, like the well-characterized proteins FepD and FepG (30% identity), which are responsible for the enterobactin transport into the cytoplasm in *E. coli*.[4,50] As in the case of enterobactin transport in *E. coli*, two proteins are usually necessary to form a channel through the cytoplasmic membrane. Besides *traB*, no further genes encoding a transmembrane protein were identified in the *mxc* locus. Active transport through the membrane could be enabled by TraC, which belongs to the family of ATP-hydrolyzing enzymes. Finally, the iron could be released from the myxochelin siderophore by MxcB, which shows homology to iron utilizing proteins.[19]

The *mxc* locus in *S. cellulosum* So ce56

The myxochelin biosynthetic gene cluster from *S. cellulosum* So ce56 was identified. Although the strain has been screened extensively for the production of secondary metabolites, no siderophores have been detected. After the myxochelin biosynthetic gene cluster was identified in the genome of *S. cellulosum* So ce56, the secondary-metabolite production pattern of the strain was reanalyzed for the presence of myxochelins A and B, and both myxochelins were indeed found to be produced by *S. cellulosum* So ce56.

It is surprising that the gene organization in both *mxc* clusters is quite different. This finding might reflect the fact that *S. aurantiaca* Sga 15 and *S. cellulosum* So ce56 are representatives of the two known myxobacterial suborders, *Cystobacterineae* and *Sorangineae*, respectively. The identities of the encoded proteins were found to be between 35 and 70% (see Table 1). As the corresponding genes are also quite similar in sequence (48–70% identities), one is tempted to speculate that the myxochelin pathway has not evolved independently and convergently in the two organisms.

It is known that the myxochelin iron-uptake system is used by several myxobacterial strains belonging to the suborder *Cystobacterineae* (refs. [31,19] and unpublished data). The identification of the *mxc* locus in *S. cellulosum* So ce56 indicates that the myxochelin siderophore system is widespread among myxobacteria.

Experimental Section

Bacterial strains and culture conditions: *E. coli* strains and *S. aurantiaca* Sg a15 and its descendants were cultured as described previously.[15,19]

Modified in vitro assay for myxochelin A production and biosynthesis of novel myxochelin derivatives: Heterologous expression of myxobacterial proteins in *E. coli* XL1Blue was carried out as described elsewhere.[15]

The assay for myxochelin A formation was performed at 30 °C in a total volume of 200 μL containing Tris-HCl (50 mmol L^{-1}, pH 8.0), NaCl (50 mmol L^{-1}), $MgCl_2$ (10 mmol L^{-1}), 2,3-DHBA (3 mmol L^{-1}), L-lysine (1.5 mmol L^{-1}), ATP (3 mmol L^{-1}), NADPH (1.5 mmol L^{-1}), MxcE (500 nmol L^{-1}), MxcG (500 nmol L^{-1}), and MxcF (5 μmol L^{-1}). Reactions were frozen in liquid nitrogen after 0–90 min and were stored at −80 °C before extraction.

The assay described above was used to determine the substrate specificity of the MxcEFG enzymatic complex. In these experiments, 2,3-DHBA or L-lysine was substituted with structural analogues. In total, seven DHBA analogues (2-hydroxybenzoic acid; 3-hydroxybenzoic acid; 4-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid; 2,3-dihydroxy-2,3-dihydro-benzoic acid, 2-amino-benzoic acid and 4-amino-benzoic acid) and L-ornithine were examined. The reactions were incubated for 16 h at 30 °C and stored at −80 °C before extraction.

Each of the reactions (200 μL) was made up to 1 mL with Tris-HCl (50 mmol L^{-1}, pH 8.0), which contained NaCl (50 mmol L^{-1}). The samples were extracted three times with equal volumes of ethylacetate. The combined ethylacetate extracts were dried and the residues were then dissolved in of methanol (120 μL). The amount of myxochelins formed was estimated by using integrated peak areas in comparison to those of a myxochelin standard curve. HPLC analysis was performed as published previously.[19]

Construction and analysis of *S. aurantiaca* Sg a15 mutants: An internal fragment of *mxcL* was PCR amplified by using cosmid CA5a[19] as template and oligonucletides RM214: 5′-TCGTGGAGAGCATTCGCAA-3′ and RM215: 5′-GTCGAAGAATTCCTGCGCGC-3′ as primers. Amplification was carried out with *Taq*-DNA Polymerase (Gibco). The resulting 509 bp DNA fragment was cloned into pCR2.1TOPO (Invitrogen) by using the TOPO TA Cloning Kit (Invitrogen). This gave rise to plasmid pRM28. pRM28 was electroporated into *S. aurantiaca* Sg a15[51] to give mutant RM28. Mutant NGM34 (*mxcA*$^-$) was made by a similar approach, and the 500 bp DNA fragment was amplified by using oligonucleotides NGM3: 5′-

GGGTTCCAAATGGGGCTACAC-3′ and 5′-CGGGGCGGTCTTCTGCTC-3′ as primers and cosmid CA5 as template.

Culture and extraction from the wild-type and mutant strains were performed as previously described by using Amberlite XAD-16 absorber resin.[19] Phenotypic analysis was carried out by diode-array detection HPLC (DAD-HPLC) analysis as described elsewhere.[19]

Construction of a plasmid harboring *mxcD* for heterologous expression in *E. coli*: *mxcD* was PCR amplified by using *Pfu*-DNA polymerase (Stratagene). Cosmid CA5 was used as template and the oligonucleotides MXCDF 5′-GAGTGCCT*CATATG*ACCGAACGTCCC-3′ and MXCDR 5′-GGCG*GAATTC*CGACTGCACCTCTGCGTTCT-3′ as primers. PCR reactions had following cycle profile: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. Thirty cycles were carried out on a gradient cycler from Eppendorf. Restriction sites introduced into the oligonucleotides are shown in italics. The resulting 1245 bp DNA fragment was digested with *Nde*I and *Eco*RI and ligated into the *Nde*I/*Eco*RI sites of pCYB2 (NEB); this resulted in plasmid pMxcD. The gene insertion into pCYB2 was verified by DNA sequencing. DNA sequencing was performed by using the Big Dye RR terminator cycle sequencing kit (PE Biosystems), p*Tac* forward/intein reverse-primer (New England Biolabs) and an ABI Prism-System 377 Sequencer (Applied Biosystems).

In vivo determination of isochorismate sythase activity of MxcD: Chromazurol S (CAS) agar plates[52] were used to determine the in vivo activity of MxcD. *E. coli* PBB8,[33] which were transformed with pMxcD, were grown overnight on LB-Agar plates. A single colony was transferred onto CAS-agar plates[52] and was incubated for 20 h at 30 °C.

Additionally, *E. coli* PBB8 carrying pCYB2 as a negative control and *E. coli* PBB8 harboring p422[53] as a positive control and expressing EntC from *E. coli* were grown under the same conditions. Finally, the CAS-agar plates were transferred to 6 °C and were incubated for an additional hour. The color of the CAS-medium is based on the presence of Fe^{3+}. In the absence of iron, the dye is yellow. In presence of Fe^{3+}, Chromazurol S forms a blue Fe^{3+} complex.

In vitro determination of isochorismate sythase activity of MxcD: *E. coli* PBB8[33] harboring pMxcD were grown in LB medium at 37 °C until the cell density reached OD_{600} 2.0. For protein extraction cells were harvested and treated as previously described for *entC* and *menF* characterization.[53] The isochorismate synthase activity assay was performed in a 100 μL reaction containing 80 μg total protein of the crude cell-free extract (5 mM chorismate, 1 mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, and 200 mM Tris-HCl, pH 7.5). The reaction mixture was incubated for 30 min at 37 °C, quenched with methanol (75 μL), and analyzed with DAD-HPLC. Isochorismic acid was determined by UV spectroscopy cochromatography with an authentic sample as described elsewhere.[29]

DNA-analysis: DNA sequences from cosmid CA5a and from the genome of *S. cellulosum* So ce56 were obtained as previously described.[19,13]

Isolation of the myxochelins from *S. cellulosum* So ce56: *S. cellulosum* So ce56 was grown in a synthetic medium (SM; 100 mL, in distilled water: 5 g L^{-1} asparagine, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.008 g L^{-1} ethylenediamine-tetraacetic acid, 0.008 g L^{-1} iron(III) sodium salt, 11.5 g L^{-1} HEPES, pH 7.2). The medium was autoclaved, and the following separately sterilized compounds were added from stock solutions: $CaCl_2 \cdot 2H_2O$ (0.5 g L^{-1}), $K_2HPO_4 \cdot 3H_2O$ (0.06 g L^{-1}), maltose (2 g L^{-1}). After incubation for 4 days at 32 °C, 10 mL of the culture was centrifuged at 6000 rpm and the pellet was washed once with sterile HEPES buffer solution (10 mL, 100 mM, pH 7.2). This cell suspension was used to inoculate Fe-EDTA-free SM medium (100 mL). After incubation for one week on a rotary shaker at 30 °C, 10 mL of the culture was used to inoculate a second passage in the Fe-salt-free medium. Because of poor growth, the third passage was inoculated with preculture (20 mL). This liquid culture contained 2% of the absorber resin XAD-16 (Rohm and Haas, Frankfurt) and was incubated for two weeks. The resin was harvested by sieving and eluted with methanol. Myxochelins A and B were detected in a 100:1 concentrated eluate by HPLC-MS and compared with authentic samples of myxochelin A ($m/z = 405$ [$M+H^+$]) and myxochelin B ($m/z = 404$ [$M+H^+$]).

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[1] B. Silakowski, B. Kunze, R. Müller, *Arch. Microbiol.* **2000**, *173*, 403.
[2] J. J. De Voss, K. Rutter, B. G. Schroeder, C. E. Barry, 3rd, *J. Bacteriol.* **1999**, *181*, 4443.
[3] V. Braun, *Front. Biosci.* **2003**, *8*, s1409.
[4] K. N. Raymond, E. A. Dertz, S. S. Kim, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3584.
[5] S. K. Buchanan, B. S. Smith, L. Venkatramani, D. Xia, L. Esser, M. Palnitkar, R. Chakraborty, D. van der Helm, J. Deisenhofer, *Nat. Struct. Biol.* **1999**, *6*, 56.
[6] L. E. Quadri, *Mol. Microbiol.* **2000**, *37*, 1.
[7] J. D. Faraldo-Gomez, M. S. Sansom, *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 105.
[8] M. Dworkin, *Microbiol. Rev.* **1996**, *60*, 70.
[9] *Myxobacteria II*, 2nd ed. (Eds.: M. Dworkin, D. Kaiser) American Society for Microbiology, Washington DC, **1993**.
[10] A. Koch, D. White, *BioEssays* **1998**, *20*, 1030.
[11] H. Reichenbach, G. Höfle in *Drug Discovery from Nature* (Eds.: S. Grabley, R. Thieriecke) Springer, Berlin, **1999**, pp. 149–179.
[12] H. Reichenbach, G. Höfle in *Myxobacteria II*, 2nd ed. (Eds.: M. Dworkin, D. Kaiser) American Society for Microbiology, Washington DC **1993**, pp. 347.
[13] K. Gerth, S. Pradella, O. Perlova, S. Beyer, R. Müller, *J Biotechnol* **2003**, *106*, 233.
[14] N. Gaitatzis, A. Hans, R. Müller, S. Beyer, *J. Biochem.* **2001**, *129*, 119.
[15] N. Gaitatzis, B. Kunze, R. Müller, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11136.
[16] N. Gaitatzis, B. Silakowski, B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle, R. Müller, *J. Biol. Chem.* **2002**, *277*, 13082.
[17] B. Silakowski, H. U. Schairer, H. Ehret, B. Kunze, S. Weinig, G. Nordsiek, P. Brandt, H. Blöcker, G. Höfle, S. Beyer, R. Müller, *J. Biol. Chem.* **1999**, *274*, 37391.
[18] B. Silakowski, G. Nordsiek, B. Kunze, H. Blöcker, R. Müller, *Chem. Biol.* **2001**, *8*, 59.
[19] B. Silakowski, B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle, R. Müller, *Eur. J. Biochem.* **2000**, *267*, 6476.
[20] B. Silakowski, B. Kunze, R. Müller, *Gene* **2001**, *275*, 233.

[21] S. Weinig, T. Mahmud, R. Müller, *Chem. Biol.* **2003**, *10*, 953.
[22] S. Weinig, H. J. Hecht, T. Mahmud, R. Müller, *Chem. Biol.* **2003**, *10*, 939.
[23] J. Ligon, S. Hill, J. Beck, R. Zirkle, I. Monar, J. Zawodny, S. Money, T. Schupp, *Gene* **2002**, *285*, 257.
[24] I. Molnar, T. Schupp, M. Ono, R. Zirkle, M. Milnamow, B. Nowak-Thompson, N. Engel, C. Toupet, A. Stratmann, D. D. Cyr, J. Gorlach, J. M. Mayo, A. Hu, S. Goff, J. Schmid, J. M. Ligon, *Chem. Biol.* **2000**, *7*, 97.
[25] L. Tang, S. Shah, L. Chung, J. Carney, L. Katz, C. Khosla, B. Julien, *Science* **2000**, *287*, 640.
[26] B. Julien, S. Shah, *Antimicrob. Agents Chemother.* **2002**, *46*, 2772.
[27] H. Chen, I. M. Keseler, L. J. Shimkets, *J. Bacteriol.* **1990**, *172*, 4206.
[28] B. Neumann, A. Pospiech, H. U. Schairer, *J. Bacteriol.* **1992**, *174*, 6307.
[29] S. Pradella, A. Hans, C. Sproer, H. Reichenbach, K. Gerth, S. Beyer, *Arch. Microbiol.* **2002**, *178*, 484.
[30] B. Kunze, W. Trowitzsch-Kienast, G. Höfle, H. Reichenbach, *J. Antibiot.* **1992**, *45*, 147.
[31] B. Kunze, N. Bedorf, W. Kohl, G. Höfle, H. Reichenbach, *J. Antibiot.* **1989**, *42*, 14.
[32] D. Franke, V. Lorbach, S. Esser, C. Dose, G. A. Sprenger, M. Halfar, J. Thommes, R. Müller, R. Takors, M. Müller, *Chem. Eur. J.* **2003**, *9*, 4188.
[33] C. Dahm, R. Müller, G. Schulte, K. Schmidt, E. Leistner, *Biochim. Biophys. Acta* **1998**, *1425*, 377.
[34] M. J. Bibb, P. R. Findlay, M. W. Johnson, *Gene* **1984**, *30*, 157.
[35] M. Saraste, P. R. Sibbald, A. Wittinghofer, *Trends Biochem. Sci.* **1990**, *15*, 430.
[36] G. Zhu, M. J. LaGier, F. Stejskal, J. J. Millership, X. Cai, J. S. Keithly, *Gene* **2002**, *298*, 79.
[37] J. He, N. Magarvey, M. Piraee, L. C. Vining, *Microbiology* **2001**, *147*, 2817.
[38] D. E. Ehmann, A. M. Gehring, C. T. Walsh, *Biochemistry* **1999**, *38*, 6171.
[39] J. Casqueiro, S. Gutierrez, O. Banuelos, F. Fierro, J. Velasco, J. F. Martin, *Mol. Gen. Genet.* **1998**, *259*, 549.
[40] C. Earhart in *Iron Transport in Microbes, Plants, and Animals*, (Eds.: G. Winkelmann, D. van der Helm, J. Neilands) VCH, Weinheim, **1987**, pp. 67 – 84.
[41] M. S. Nahlik, T. P. Fleming, M. A. McIntosh, *J. Bacteriol.* **1987**, *169*, 4163.
[42] B. A. Ozenberger, T. J. Brickman, M. A. McIntosh, *J. Bacteriol.* **1989**, *171*, 775.
[43] C. T. Walsh, J. Liu, F. Rusnak, M. Sakaitani, *Chem. Rev.* **1990**, *90*, 1105.
[44] J. Liu, N. Quinn, G. A. Berchtold, C. T. Walsh, *Biochemistry* **1990**, *29*, 1417.
[45] T. Stachelhaus, H. D. Mootz, M. A. Marahiel, *Chem. Biol.* **1999**, *6*, 493.
[46] G. L. Challis, J. Ravel, C. A. Townsend, *Chem. Biol.* **2000**, *7*, 211.
[47] J. J. May, N. Kessler, M. A. Marahiel, M. T. Stubbs, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12120.
[48] P. Belshaw, C. Walsh, T. Stachelhaus, *Science* **1999**, *284*, 486.
[49] T. A. Keating, C. G. Marshall, C. T. Walsh, A. E. Keating, *Nat. Struct. Biol.* **2002**, *9*, 522.
[50] S. S. Chenault, C. F. Earhart, *J. Gen. Microbiol.* **1992**, *138*(10), 2167.
[51] S. Beyer, B. Kunze, B. Silakowski, R. Müller, *Biochim. Biophys. Acta* **1999**, *1445*, 185.
[52] B. Schwyn, J. B. Neilands, *Anal. Biochem.* **1987**, *160*, 47.
[53] K. Buss, R. Müller, C. Dahm, N. Gaitatzis, E. Skrzypczak-Pietraszek, S. Lohmann, M. Gassen, E. Leistner, *Biochim. Biophys. Acta* **2001**, *1522*, 151.

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