The Biosynthesis of the Aromatic Myxobacterial Electron Transport Inhibitor Stigmatellin Is Directed by a Novel Type of Modular Polyketide Synthase*

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Deductions from the molecular analysis of the 65,000-bp stigmatellin biosynthetic gene cluster are reported. The biosynthetic genes (stiA–J) encode an unusual bacterial modular type I polyketide synthase (PKS) responsible for the formation of this aromatic electron transport inhibitor produced by the myxobacterium Stigmatella aurantiaca. Involvement of the PKS gene cluster in stigmatellin biosynthesis is shown using site-directed mutagenesis. One module of the PKS is assumed to be used iteratively during the biosynthetic process, which seems to involve an unusual transacylation of the biosynthetic intermediate from an acyl carrier protein domain back to the preceding ketosynthase domain. Finally, the polyketide chain which is presumably catalyzed by a novel C-terminal domain in StiJ that does not resemble thioesterases, is cyclized and aromatized. The presented results of feeding experiments are in good agreement with the proposed biosynthetic scheme. In contrast to all other PKS type I systems reported to date, each module of StiA–J is encoded on a separate gene. The gene cluster contains a “stand alone” O-methyltransferase and two unusual O-methyltransferase domains embedded in the PKS. In addition, inactivation of a cytochrome P450 monoxygenase-encoding gene involved in post-PKS hydroxylation of the aromatic ring leads to the formation of two novel stigmatellin derivatives.

The antifungal natural product stigmatellin A (Fig. 1) inhibits the electron flow in the respiratory chain of beef heart submitochondrial particles within the cytochrome bc1 complex (5) and has been shown to be a powerful inhibitor of the photosynthetic electron transport (6). The compound has been used widely in studies dealing with the characterization of electron transport processes (e.g. Ref. 7). Several types of stigmatellins are produced by different myxobacterial species, including Stigmatella aurantiaca (8, 9). This bacterium was developed as a model organism to study secondary metabolism in myxobacteria (10–15), because this class of microorganisms has been established as potent producers of natural products with biological activity over the last decades (16, 17). Nevertheless, there still is only limited knowledge of the basis of secondary metabolism in these fascinating Gram-negative bacteria, because most studies on PKS systems have focused on actinomycetes or fungi (1, 3). Given the fact that myxobacteria have the largest of all known bacterial chromosomes (18) and a very complex life cycle (19), one can imagine secondary metabolites having diverse functions during morphological and physiological differentiation. A multitude of secondary metabolic gene sets has been located in the chromosome of S. aurantiaca, and structure-genome relationships have been established using gene inactivation studies (20). The focus of these studies has thus far been to elucidate novel mechanisms of secondary metabolite formation and to find new natural products.

We report here a set of genes responsible for stigmatellin formation in S. aurantiaca. Surprisingly, a bacterial type I PKS facilitates the formation of two novel stigmatellin derivatives.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ421825. This article is dedicated to Prof. Dr. T. Hartmann on the occasion of his 65th birthday.

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1 The abbreviations used are: PKS, polyketide synthase; ACP, acyl carrier protein; AT, acyltransferase; bp, base pairs; Cy, cyclization; Da, Dalton; DH, β-hydroxy-acyl-thioester dehydratase; KR, β-ketoacyl-ACP reductase; KS, β-ketoacyl-ACP synthase; O-MT, O-methyltransferase; ORF, open reading frame; PK, polyketide; S, spacer region; HPLC, high pressure liquid chromatography.
For production of secondary metabolites, the mutant was cultivated in 5 liters of Zein liquid medium containing 0.8% zein, 0.1% peptone (from casein tryptically digested), 0.1% MgSO₄, 7H₂O, 50 mM Hepes buffer, pH 7.2, and 1% of the adsorbent resin XAD16 (Rohn & Haas). Incubation was done at 30 °C on a gyratory shaker at 160 rpm for about 3 days. Cell mass and adsorbent resin were collected and extracted twice with acetonitrile. The extract was concentrated to the water phase and extracted with ethyl acetate. The organic extract was dried with Na₂SO₄ and evaporated to give 2.7 g of a dark oil. This was dissolved in dichloromethane and applied to a silica gel column (35 g). Elution with dichloromethane/methanol, 96:4, resulted in crude stigmatellin X (0.21 g), and elution with dichloromethane/methanol, 96:4, resulted in crude stigmatellin Y (0.21 g). Repeated RP 18 chromatography (acetonitrile, 50 mM ammonium acetate buffer, pH 5.5, 75:25) afforded pure stigmattelin X (18 mg) and stigmatellin Y (12 mg).

Stigmatellin X—DC (silica gel, dichloromethane/methanol, 95.5:4.5, Rf = 0.5; UV (methanol), 258, 268, 279, and 320 nm; ¹H NMR (CD₃OD), 6.30 (d, J = 2.2 Hz, 6H); 6.28 (d, J = 2.2 Hz, 8H); 12.98 (s, 5OH); electrospray ionization-mass spectrometry (negative ions), m/z 469 (M–H)⁻.

Stigmatellin Y—DC Rf = 0.36; UV (methanol), 238, 256, 269, 279, and 305 nm; ¹H NMR (CD₃OD), 6.36 (d, J = 2.0 Hz, 6H, 6.37 (d, J = 2.0 Hz, 6H, 3.90 (s, 5-OCH₃); ¹³C NMR (CD₃OD), 163.6 (C-1a), 107.4 (C-4a), 161.9 (C-5), 96.8 (C-6), 160.5 (C-7), 95.7 (C-8); nuclear Overhauser effect, irradiation of 5-OCH₃ at δ 3.90 enhanced only 6-H at δ 6.37; electrospray ionization-mass spectrometry (negative ions), m/z 483 (M–H)⁻.

Biological Activity of Stigmatellins

The antibiotic activity of stigmatellins against Saccharomyces cerevisiae cultured in glucose-free N3 medium (glycerol 2%, casein peptone 1%, yeast extract 1%, phosphate 50 mM, pH 6.3) was determined using the agar diffusion assay with paper discs (8). The assay used to determine the inhibition of NADH oxidation in beef heart submitochondrial particles was described previously (5). In this study, a UNICAM UV-visible spectrometer UV2 was employed to record the data.

DNA Manipulations, Analyses, Sequencing, and PCR

Chromosomal DNA from S. aurantiaca was prepared as described (21). Southern analysis of genomic DNA was performed using the standard protocol for homologous probes of the Digoxigenin DNA labeling and detection kit (Roche Molecular Biochemicals). PCR was carried out using Taq polymerase (Invitrogen) according to the manufacturer’s protocol. 5% MeSO₄ was added to the mixture. Conditions for amplification with the Eppendorf mastercycler were as follows: denaturation 30 s at 95 °C, annealing 30 s at 60 °C, and extension 45 s at 72 °C, 30 cycles and a final extension at 72 °C for 10 min.

Screening of the cosmids CS4 and CS4a was performed by a shotgun approach as follows. Sheared fragments of the two cosmids were subcloned separately into pTZ18R (Amersham Biosciences). At least 500 clones were selected from each cosmid library, and plasmid DNA was prepared (Qiagen) and sequenced using Big Dye RR Terminator Cycle Sequencing Kit (PE Biosystems) and UPO/RPO primer (MWG Biotec). The gels were run on ABI 377 sequencers, and data were assembled and edited using the XGAP program (50). All other DNA manipulations were performed according to standard protocols (22). Amino acid and DNA alignments were done using the programs of the Lasergene software package (DNASTAR Inc.) and ClustalW (23).

Screening of the Cosmid Library

The preparation of the cosmid library from S. aurantiaca Sg a15 has been described (10). Approximately 2200 cosmids harboring single colonies were picked into 96-well microtiter plates, grown in LB medium overnight, and replicated. To one copy of the library, 25% glycerol was added, and the plates were frozen at −80 °C. The second copy was transferred onto nylon membranes and used for colony hybridizations. Cosmid CS4 harboring a KS fragment encoding part of the stigmatellin biosynthetic gene cluster has been reported (10). After sequencing of this cosmid, a probe homologous to the end of CS4 could be generated using primers C22 (5’-CAGCGAGCGGCTGGCCCGAGATTG-3’) and C23 (5’-CTTGGGAGGAGAGCAAAAACA-3’). This probe was employed in a colony hybridization against the complete library. One of the resulting cosmids (CS4a) was chosen for sequencing of the rest of the gene cluster.
Construction of the S. aurantiaca Sg a15 Mutant Strains NGS12 and NGS910

For characterization of stiJ and stiL insertion mutants, S. aurantiaca Sg a15 NGS12 and NGS910 were constructed. Therefore, internal fragments of stiJ and stiL were amplified as follows: using primers NGS1 (5'–GGCCGCCGCTGCCCCCTCC–3') and NGS2 (5'–GCTGCGTGATCTCGGTCGCTCA–3') a 505-bp DNA fragment encoding part of stiJ was amplified and subsequently inserted into pcR2.1TOPO (according to the manufacturer's protocol, Invitrogen) resulting in plasmid pNGS10. To amplify a 470-bp DNA fragment of stiL, the primers NGS9 (5'–GAAGCCGCGCTCCGGAGATGAT–3') and NGS10 (5'–GCGGGGAGTATGACCA–3') were employed in a PCR. Plasmid pNGS910 was obtained and harbors the 470-bp DNA fragment in pcR2.1TOPO.

The plasmids were transferred into S. aurantiaca Sg a15 wild type by electroporation (10). Verification of the mutant strains was performed in Southern experiments (data not shown).

Gene Inactivation Resulting in Mutants Unaffected in Stigmatellin Biosynthesis

Several genes located upstream of the stigmatellin biosynthetic gene cluster were inactivated via insertional mutagenesis, because they seemed to encode proteins that might be involved in stigmatellin biosynthesis (see below). Therefore, 500 bp of ORF8 (cytochrome P450 monooxygenase-like protein) (a), 580 bp of ORF7 (serine/threonine kinase-like protein) (b), 430 bp of a ORF6 (methyltransferase-like protein) (c), and 403 bp of ORF2 (similar to a protein with unknown function) (d) were amplified via PCR as described above using the following primers: (a) C5, 5'–GACCGGTCTGCCGGCAACTT–3' and C6, 5'–GACTTGCGCGCCCGCACTCC–3'; (b) C7, 5'–GGAGAGGCCGGCCATCCTATGT–3' and C8, 5'–GGCAATTCTCCGGCGCTTCTCT–3'; (c) C9, 5'–GCTGGCAGCCTCTCTCTTACAA–3' and C10, 5'–GGCGGGGATGATGAT–3'; (d) C11, 5'–GGGCCGGGCCCTCCCTC–3' and C32, 5'–GGAGGCCGGGCCCTCCCTC–3'. The amplification products (a–d) were inserted into pcR2.1TOPO creating the plasmids pCBS18 (a), pCBS19 (b), pCBS23 (c), and pCBS24 (d). These constructs were electroporated into S. aurantiaca Sg a15 as described and resulting mutants (CBS18 (a), CBS19 (b), CBS23 (c), and CBS24 (d)) were verified using Southern experiments (data not shown). All of these mutants were analyzed for stigmatellin production and found to be stigmatellin-positive. No differences in the amount of stigmatellin produced in comparison to wild type were obvious in these experiments.

RESULTS

Cloning and Identification of the Stigmatellin Biosynthetic Gene Cluster via Gene Disruption—A cosmids library of S. aurantiaca Sg a15 was prepared. Subsequently, a variety of cosmids hybridizing to PKS probes were isolated and used to amplify specific KS fragments of the PKS genes encoded on the respective DNA locus. One of these fragments, amplified from cosmids CS4, was used for a gene inactivation experiment in S. aurantiaca Sg a15 and resulted in a stigmatellin-negative phenotype (10). During the course of this study, the sequence of cosmid CS4 was determined, revealing the presence of several open reading frames (ORFs) with similarity to bacterial type I PKSs, which were designated stia–stiE (see Fig. 2). To verify the expected involvement of the PKS genes in stigmatellin biogenesis, the gene disruption mutant of the previous study was identified as a stic insertion. Subsequently, an overlap-
backbone and the C-methyl groups of stigmatellin are solely derived from acetate (after conversion to malonate) and propionate (after conversion to methylmalonate) (Table II and see Fig. 5). The C-2 of acetate is incorporated into propionate units and 32, for example). This affords a free rotation of the acyl
phloroglucinol intermediate, which in the biosynthetic sequence is first cyclized to a chromone and later hydroxylated at the 8-position (compare Fig. 5).

**Analysis of Genes Flanking the PKS Gene Cluster**—Upstream of **stiA** a gene encoding a putative cellulase (ORF1 which shows 31% identity and 47% homology to cellulase P23548 from *Paenibacillus polymyxa*) can be found. Further upstream, genes encoding ORF2–8 were detected. ORF2 is similar to a hypothetical 43.2-kDa protein from *S. coelicolor* (GenBank™ accession number AL049707, 37.9% identity). ORF3 represents a putative ribosome binding factor with 30% identity to the ribosome binding factor A of *S. aurantiaca* (GenBank™ accession number AJ000999, 41% identity). ORF4 is similar to several eucaryotic protein kinases, e.g. -chains, *Rattus norvegicus* from *Rattus norvegicus* (GenBank™ accession number X96560, 53% identity). ORF6 and ORF7 represent proteins with homology to methyltransferases and protein kinases, respectively. ORF8 is most similar to the probable methyltransferase MLP3908 from *Rhizobium loti* (GenBank™ accession number MLP3908, 64.9% identity), whereas ORF7 shows highest similarity to a protein kinase-like protein from *S. coelicolor* (GenBank™ accession number Q53839, 32.9% identity). ORF8 is similar to a variety of cytochrome P450-dependent enzymes,
e.g. GenBank™ accession number AL049754 from Streptomyces coelicolor (37.5% identity).

ORFs 2 and 7 were inactivated, because they were initially thought to be involved in the regulation of stigmatellin biosynthesis (mutants CBS24 and BS19, respectively). Analysis of stigmatellin formation revealed that the amount of product formed in the mutants did not differ from the wild type. ORFs 6 and 8 were also subjected to gene inactivation experiments (mutants CBS23 and CBS18, respectively), because they seemed to be involved in the post-PKS hydroxylation and methylation (see Fig. 5) expected to be necessary to build up stigmatellins. Product spectra of these mutants did not reveal any difference to wild type spectra.

In the ~8 kbp sequenced further upstream in cosmid CS4, no ORF with homology to PKSs could be detected. Downstream of stiJ, two genes encoding proteins with presumed function in stigmatellin biosynthesis were detected and designated stiK and stiL. StiK represents a protein with homology to several methyltransferases, e.g. with 33% identity and 54% homology to UbiG of Pasteurella multocida (GenBank™ accession number NP 245777). StiL shows homology to a variety of P450-dependent monoxygenases and is 32% identical and 48% similar to a propionate hydroxylase from E. coli (GenBank™ accession number NP 286090).

Downstream of stiL, a gene encoding a protein (ORF9) with homology to polynucleotide adenyltransferases can be found. ORF9 shows 33% identity and 46% similarity to PcnA from Treponema pallidum (GenBank™ accession number NP 218711) and 73% identity to a polynucleotide adenyltransferase that was identified downstream of the myxothiazol biosynthetic gene cluster (11) of S. aurantiaca DW4/3-1.2 Downstream of ORF9, additional genes similar to and organized in the same way as those located after the polynucleotide adenyltransferase behind the myxothiazol gene cluster of S. aurantiaca DW4/3-1 were found. No genes encoding proteins with homology to PKSs were detected.

Verification of the Role of StiL as Aromatic Hydroxylase; Inactivation of the Gene Results in the Formation of Novel Stigmatellins—Inactivation of stiL was performed as described under “Materials and Methods.” Due to the structure of the gene cluster and its comparison to the natural product, we assumed that the encoded monoxygenase would be responsible for the hydroxylation of the aromatic ring found in stigmatellin after PKS assembly. Surprisingly, two novel compounds were found when mutant strain NG8910 was analyzed

Fig. 4. Model for stigmatellin polyketide chain biosynthesis. PKS domains are shown in green. The O-MT domains are shown in yellow, and the Cy domain is shown in red. * indicates a presumably inactive domain. S, spacer region. For clarification, StiG is shown in the upper and in the lower row of the figure. Back transfer of the PK chain from either the ACP of StiH or StiJ to the preceding KS domain is indicated. Incorporation of intact acetate and propionate units is shown using blue and red bonds, respectively.

2 S. Weinig, B. Silakowski, and R. Müller, unpublished data.
OH group presumed to be incorporated via the action of StiL. Surprisingly, stigmatellin Y lacks one of the methyl groups and stigmatellin X lacks both methyl groups of the methoxy moieties found attached to the aromatic moiety. Both compounds do still carry the methoxy groups attached to the PK side chain.

**Biological Activity of Stigmatellins X and Y**—The biological activities of stigmatellins A, X, and Y were compared. The diameter of the inhibition zone determined in the agar diffusion test with *S. cerevisiae* grown on glucose-free medium was 32 mm for stigmatellin A, 0 mm for stigmatellin X, and 13 mm for stigmatellin Y (5 μg on a 6-mm paper disc). NADH oxidation in submitochondrial particles of beef heart was inhibited by 50% at a concentration of 7.7 ng/ml (15 nM) stigmatellin A and 10.2 ng/ml (21 nM) stigmatellin Y. Stigmatellin X was shown to be four times less active (IC50 31 ng/ml; 66 nM) *in vitro*. Stigmatellin X is not active *in vivo*, which may be due to the phenolic OH group forming a strong H-bond to the 4-carbonyl.

**DISCUSSION**

Since the cloning of the first sets of aromatic PKSs from streptomycetes, numerous aromatic PKS genes have been identified. The studies revealed that aromatic polyketides in bacteria are made by type I PKSs that consist of several discrete proteins carrying a set of iteratively used enzyme activities. To date, the following two exceptions have been noticed: the iterative type I PKS involved in the biosynthesis of orsellinic acid in *Streptomyces viridochromogenes* (33) and a non-iterative type I PKS involved in the biosynthesis of the aromatic moiety of pyoluteorin in *Pseudomonas fluorescens* (34). In the latter case, PtbA and PtbC proteins carry three modules without loading and thioesterase domains. These are thought to catalyze the
formation of the resorcinol ring within pyoluteorin. AviM from *S. viridochromogenes* has been shown to be the orsellinic acid synthase by heterologous expression and strongly resembles fungal PKSs. The protein consists of a KS, AT, and ACP domain and does not harbor a thioesterase domain either.

We report here the cloning and analysis of the stigmatellin biosynthetic gene cluster, a novel bacterial type I PKS that catalyzes the formation of an aromatic chromone ring with a polyketide side chain.

*Model for the stigmatellin Polyketide Chain Assembly.—The presumed biosynthesis of stigmatellin starts with StiA, the first PKS gene of the cluster. Its modular organization (see Fig. 4) is different from most other PKSs but similar to several myxobacterial initiating double modules (responsible for loading with the first activated short chain carboxylic acid and also for the first chain extension step). As is the case with MxaF, the first biosynthetic protein of the myxalamid biosynthetic machinery of *S. aurantiaca* Sg a15 (14), MtaB, the first biosynthetic protein of the myxothiazol biosynthetic complex of *S. aurantiaca* DW4/3-1 (11), and the first biogenesis protein of soraphen biosynthesis in *Sorangium cellulosum* (49), the modular organization appears as follows with an ACP, followed by KS, two ATs (AT1 and AT2), DH, S, and KR followed by the second ACP. Most other initiating proteins of PK biosynthesis follow the pattern AT1-ACP1-KS-AT-DH-S-KR-ACP. We speculated that this unusual organization is of consequence for the specificity of the loading domains for the activated chain initiating carboxylic acids, which are of unusual nature in myxalamid (isobutyryl-CoA or 2-methyl-butyryl-CoA), soraphen (benzoyl-CoA (35)), and myxothiazol (3-methylbutyryl-CoA) biogenesis. In light of the findings in StiA, this assumption does not hold, because here we find the same arrangement of ATs, but only the commonly employed acetyl-CoA is used as a starter for stigmatellin biosynthesis (see below). It seems reasonable to suppose that in all of these systems the first AT (AT1) is responsible for the recognition of the different starter units, whereas the second AT (AT2) loads the extender unit (methylmalonate for stigmatellin) (compare Fig. 4). How this arrangement of the ATs fits into the theory of the three-dimensional structure of PKSs (recently reviewed in Ref. 36) and the closely related fatty-acid synthases (37) remains obscure. Further experiments are in progress to clarify their function.

StiB would next extend the PK chain with malonyl-CoA. Surprisingly, it lacks the DH domain that would be required for the formation of the second double bond. A DH domain that on first sight seems superfluous for the system is located in StiG and might be able to perform the dehydration step. Interestingly, this parallels myxalamid biogenesis in the same producing strain; within the biosynthetic gene cluster there is also a DH domain missing (14), which would actually be required for myxalamid biosynthesis. If the dehydration giving rise to the second double bond does not occur, one would expect the compound not to have the typical stigmatellin UV spectrum, because it has been shown that the triene moiety is the major chromophore of the compound (9). StiC harbors all the domains expected for extension with malonyl-CoA and further modification.

StiD and StiE are unusual PKS modules, because they contain the core sequence motifs for SAM binding ((D/E)XGX) domains (38). Interestingly, this motif carries an Asp in all of the *O*-methyltransferases (O-MT) identified, whereas C-MTs have the core sequence EXGX (compare with Ref. 11). Such O-MT domains have only been reported once in a PKS (in the proteins of the myxothiazol biosynthetic complex MtaE and MtaF). C-MT domains within PKSs have been found recently, e.g. in the yersiniabactin biosynthetic complex of *Yersinia pestis* (27) and in lovastatin biosynthetic proteins of *Aspergillus terreus* (28, 40). We supposed 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, which was assumed to explain how the β-methoxyacrylate of myxothiazol is formed, because the enol form of the β-keto intermediate can readily be methylated by the MT of MtaF (11) (compare Fig. 1). This would also explain why the homologies between the O-MT domains of StiD, StiE, and MtaE are significantly higher than those of these three domains to the MtaF O-MT (see “Results”).

StiF harbors all domains necessary for a chain extension following the complete cycle of reduction including an enoyl reductase domain (Fig. 4).

Next, StiG incorporates methylmalonyl-CoA. No reduction of the keto group takes place as there is no KR domain present. Nevertheless, StiG contains a DH domain, which might complement the missing DH domain of StiB (see above). After the action of StiG, the two modules encoded by StiH and StiJ must add three more molecules of malonyl-CoA to the growing PK chain. StiH harbors a KR domain that seems to be inactive, because the typical NADPH-binding site is lost (data not shown). The addition of three malonyl-CoA units by two modules must involve an unusual transacylation from the ACP of StiH or StiJ back to the preceding KS domain. Similar biochemistry has been described as “stuttering” during erythromycin biosynthesis, where module 4 has been shown to be able to incorporate two methylmalonate extender units in an iterative fashion resulting in the formation of octaketides instead of the usual heptaketides (41). Comparable results have been obtained when analyzing the culture broth of the epothilone producer *S. cellulosum* So ce90, in which ring-expanded 18-membered macrolides could be found as minor components indicating that stuttering also takes place during epothilone biosynthesis (42). Nevertheless, during erythromycin and epothilone biogenesis this process only leads to the formation of side products. In contrast, only stigmatellin A is known from *S. aurantiaca* Sg a15. Because fungal type I PKS systems are known to iteratively use their modules giving rise to the final product, one could describe the stigmatellin biosynthetic system as a mixture of bacterial and fungal type I PKS. We believe that these classifications are outdated. The data presented here, the stuttering phenomenon in the erythromycin model and the systems governing pyoluteorin and orsellinic acid biosynthesis, strongly indicate that all types of biosynthetic mixtures and hybrids are possible and are also employed in evolutionary optimized biocombinatorial approaches by microorganisms. Similar observations can be made regarding PKS and non-ribosomal peptide synthetases. These had been regarded as two unrelated biosynthetic systems, until recently several combinations of both protein types have been reported (e.g. Refs. 11 and 25). It can be expected that more novel biosynthetic systems will be analyzed in the future, and more new combinations of machineries for biogenesis will be found (e.g. mixtures of type III PKS with type I and type II PKS, additional combinations with non-ribosomal peptide synthetase, or prenylating enzymes).

At this time we cannot exclude the possibility that the “missing domain” of the stigmatellin PKS is encoded somewhere else in the chromosome of *S. aurantiaca* Sg a15. This would be very unusual, because genes responsible for the formation of secondary metabolites have been shown to be clustered in actinomycetes and myxobacteria. We have tried to identify representative cosmid of all PKS regions in the chromosome of *S. aurantiaca* DW4/3-1 (20) and *S. aurantiaca* Sg a15 (10), and we
inactivated those that were found. All stigmatellin negative mutants could be related to the gene cluster reported in this communication.

Additionally, one could argue that either two molecules of StiH or StiJ are assembled in each biosynthetic complex giving rise to the stigmatellin. This would bring up the need to produce twice as much of the respective protein in comparison to the other proteins of the complex. This assumption is contrasted by the organization of the sti operon, which does not provide any indication for stiH or stiJ regulation being different from the other PKS genes. Also, the latter speculation would not fit into the linker theory, which says that docking regions are encoded at the N- and C-terminal ends of the proteins of the biosynthetic complex that need to interact, enabling specific binding between modules encoded on different proteins (43, 44). If one protein were to be used twice, the docking regions could impossibily be specific for one partner protein alone.

**Model for the Formation of the Chromone Ring**—The biosynthetic scheme presented above would result after PK chain assembly in the thioester shown in Fig. 4 bound to the ACP of StiJ. Most PKS systems harbor C-terminal thioesterase domains that catalyze the hydrolytic release of the terminal thioester using either water (resulting in the release of the free acid) or a free OH group of the same molecule for the release, the latter resulting in lactone structures (2). Some further release mechanisms are as follows: the amide syntheses used in the biogenesis of macrolactams (45, 46), reductive release catalyzed by reduction domains resulting in aldehydes that can either be reductively transaminated or further reduced to the alcohol (15, 47), formation of terminal amides employing a derivative of peptide synthetase biochemistry (11), and the terminal Caisen cyclase that acts during fungal naphthopyrone synthesis (48). None of the domains responsible for these chain termination mechanisms shows homology to the terminal domain found in StiJ. Neither does this designated Cys (cyclization) domain show homology to any protein from the data bases. Although this does not prove any catalytic function of the domain in stigmatellin biogenesis, we speculate that this domain catalyzes the formation of the chromone ring of stigmatellin (see Fig. 5), because spontaneous cyclization of the last ACP-bound intermediate seems rather unlikely. It can be deduced from the feeding experiments presented in Fig. 5 that first the aromatic ring and next the chromone structure is formed, because two types of acetate incorporations into the aromatic ring can be detected. This finding can only be explained if a symmetrical intermediate exists, in which the aromatic ring can freely rotate around the C–C bond to the PK chain. Further experiments will be needed to biochemically characterize the role of the presumed Cys domain.

**Post-PKS Reactions**—After the chromone ring is formed, the two phenolic OH groups must be methylated, and the third phenolic OH group must be introduced into the structure. We initially identified a putative methyltransferase gene (orf6) and an ORF with similarity to cytochrome P450-dependent monooxygenases (orf8) upstream of the gene cluster. These genes were inactivated, but the resulting mutants did not show any differences in stigmatellin formation when compared with wild type. After cosmid CS4a was sequenced, stiK and stiL were found. These genes are located directly behind the PKS genes, transcribed in the same direction, and there is no indication for them being regulated differently from the PKS genes. To prove the presumed function of the methyltransferase homologue StiK and the monooxygenase homologue StiL in the presumed post-PKS processes, we performed a gene inactivation of stiL and analyzed the product spectrum of the resulting mutant. As expected, the mutant was unable to form stigmatellin A. Surprisingly, two new peaks were detected in the HPLC with the typical stigmatellin chromophore (Figs. 1 and 3). The structures of these compounds were solved, and it was shown that both do not carry the phenolic OH group that is expected to be inserted by StiL. However, both compounds do not exactly resemble stigmatellin A, because either one or both of the methoxy groups on the aromatic ring are replaced by OH groups. This indicates that hydroxylation by StiL takes place before the two methylation steps are performed (presumably by StiK), because the changed structure of the substrate of StiK seems to inhibit methylation efficiency. It could even be possible that StiK does not methylate the non-hydroxylated substrate at all, and only nonspecific methylation occurs.

**Genes Located Adjacent to the Stigmatellin Biosynthetic Gene Cluster**—It seems to be a common feature for myxobacteria that they employ proteins that have previously been thought to be used predominantly or solely by eukaryotes (12, 39). Two more examples of these are represented by ORFs 4 and 5, which are similar to eukaryotic potassium channels and to acyl-CoA-binding proteins. Because the putative methyltransferase ORF6 shows similarity to several S-adenosylmethionine-dependent enzymes involved in biotin biosynthesis, one could speculate that the acyl carrier protein ORF5 might be specific for this pathway in *S. aurantiaca* Sg a15.

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