Chemistry & Biology, Vol. 9, 1017–1026, September, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00223-5

A Gene Cluster from a Marine *Streptomyces* Encoding the Biosynthesis of the Aromatic Spiroketal Polyketide Griseorhodin A

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Summary

The telomerase inhibitor griseorhodin A is probably the most heavily oxidized bacterial polyketide known and features a unique epoxyspiroketal moiety crucial for its activity. To gain insight into which tailoring enzymes generate this pharmacophore, we have cloned and fully sequenced the griseorhodin biosynthesis gene cluster. Among other unusual features, this aromatic polyketide synthase (PKS) system encodes an unprecedented number of functionally diverse oxidoreductases, which are involved in the oxidative modification of a polyaromatic tridecaketide precursor by cleavage of three carbon-carbon bonds. The cluster was highly unstable on a variety of shuttle plasmids but could finally be functionally expressed in its entirety in *Streptomyces lividans* using a novel integrative cosmid vector. The availability of the tailoring system now opens up the possibility of engineering nonnatural biosynthetic pathways yielding novel pharmacologically active analogs with a similar pharmacophore.

Introduction

Many clinically important natural products belong to the aromatic polyketide group. In bacteria, these metabolites are usually synthesized by type II polyketide synthases (PKSs) [1,2] in a two-stage process. A first sequence of biosynthetic steps leads to the generation of the aromatic core structure and is, with one known exception [3], highly similar in all type II PKSs. Core synthesis commences with the iterative condensation of an acyl-coenzyme A (CoA) starter and a defined number of malonyl-CoA extender units. This elongation cycle is catalyzed by the minimal PKS, an association of the three proteins ketosynthase α (KS_α), ketosynthase β (KS_β), and acyl carrier protein (ACP). The resulting poly-β-oxoacyl-CoA chain is then, after an optional C9 ketoreduction, regiospecifically cyclized by cyclases (CYC) to yield the polyaromatic core skeleton. The structural diversity produced at this point is surprisingly small, and the intermediates generally exhibit no or low biological activity. In contrast, a striking plethora of structural features and pharmacological properties can be introduced during the tailoring stage. A large variety of tailoring enzymes introducing a wide range of structural features is known from type II systems. Since these steps in most cases also lead to the generation of the pharmacophore moieties, tailoring genes are of high chemical and pharmacological importance.

In combinatorial biosynthesis, biosynthetic genes from diverse origin are exploited to construct and heterologously express hybrid pathways [2, 4]. This technique can yield polyketides not encountered in nature and is therefore a powerful complementation to traditional microbial drug development programs, which require an ever-increasing effort for the identification of structurally novel drug candidates. Most hybrid compounds have so far been obtained through manipulation of core PKS enzymes, but a number of groundbreaking studies focusing on tailoring enzymes such as glycosyl transferases already highlight their relevance for drug discovery [5]. In view of the impact of post-PKS proteins on bioactivity and structural diversity, we have to thoroughly understand their combinatorial potential and to enlarge the toolbox of available enzymes. We are interested in oxygenases to generate hybrid polyketides and have therefore chosen to clone genes responsible for the biosynthesis of griseorhodin A [6], a member of the rubromycin family [7] and probably the most heavily oxidized bacterial polyketide known. Most rubromycins (Figure 1) are strong inhibitors of human telomerase and retroviral reverse transcriptase [8, 9] and possess an aplanar, axially chiral structure due to the presence of a highly unusual spiroketal moiety [10]. The system plays a crucial role in telomerase inhibition, since compounds with opened ketal function such as α-rubromycin are less active by two orders of magnitude [8]. Genes responsible for the formation of the spiroketal pharmacophore are therefore potentially useful tools to generate rubromycin analogs with improved pharmacological profiles. Here we report the cloning, sequencing, and heterologous expression of the griseorhodin A biosynthesis gene cluster, a system encoding an exceptionally large number of diverse oxidoreductases.

Results

Cloning of the Griseorhodin Cluster

In the course of a study on bacterial symbionts of marine invertebrates, we detected a large community of Actinomycetes associated with the marine tunicate *Aplidium lenticulum* (J. P., R. Quinn, B.S. Moore, unpublished data). One of the isolated strains, *Streptomyces* sp. JP95, produces a number of rubromycin-type compounds with griseorhodin A as the major component. This strain was selected for further cloning work. Labeling studies by Zeeck and coworkers suggest that the rubromycins are assembled either from two independent polyketide chains or from only one that is oxidatively cleaved at the tailoring stage [7]. Since similar methoxynaphthoquinone moieties as in rubromycins are found in the pradimicins and in fredericamycin A, we speculated that these compounds are biosynthetically related. Consequently, griseorhodin biosynthesis could

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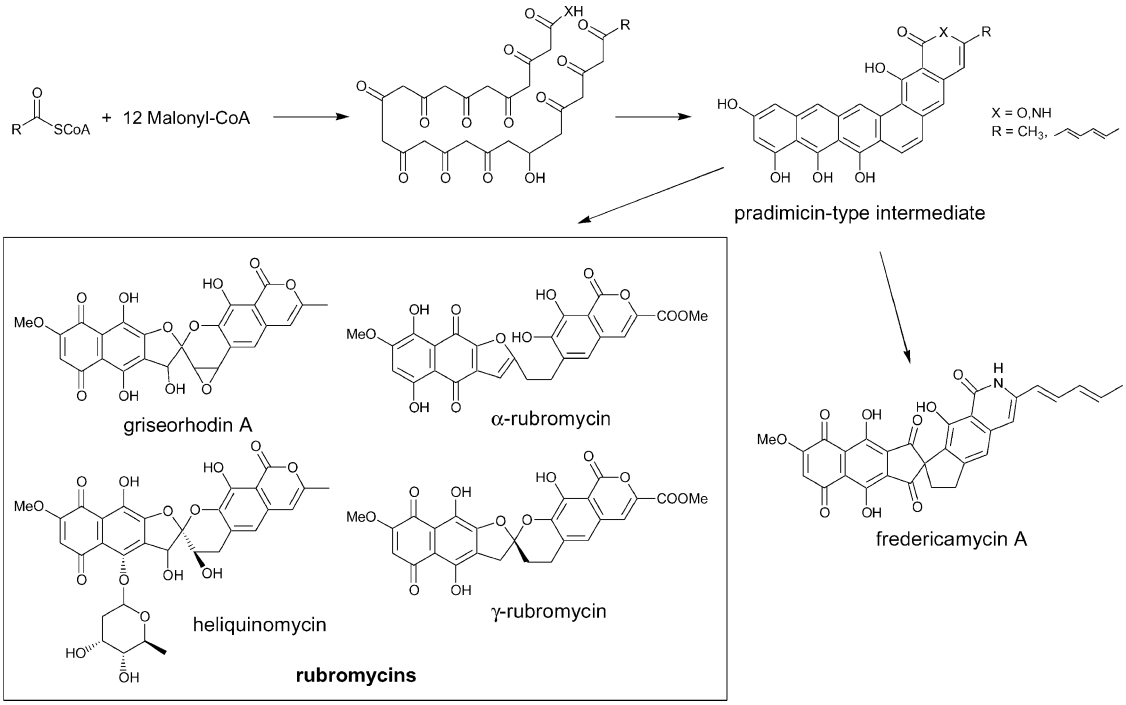

Figure 1. Proposed Biosynthesis of the Rubromycins and Related Compounds

proceed via a pradimicin-type precursor that loses two carbon atoms during an oxidative degradation sequence (Figure 1). We therefore aligned KS_α sequences from the two known pradimicin clusters [11, 12] and the fredericamycin cluster (C.R. Hutchinson, personal communication) in order to identify motifs only conserved in enzymes from pradimicin-type pathways. Several PCR primer pairs designed from such motifs indeed gave rise to products of the expected size and with a sequence most similar to the pradimicin and fredericamycin KS_α. During the later course of our cloning work, Minas and coworkers deposited 6 kb of sequence from the *S. collinus* rubromycin cluster at GenBank, whose KS_α gene sequence was 85% identical to ours (accession number AF293355). This extremely high similarity increased our confidence that we had amplified a gene from the correct cluster. A cosmid library prepared from *Streptomyces* sp. JP95 DNA was screened by Southern hybridization with a probe derived from our KS_α fragment, which yielded nine positive cosmids mapping to the same region. As sequencing of the insert ends of cosmid pGR6C2 revealed only putative non-PKS genes, this cosmid likely contained the entire griseorhodin cluster and was selected for further analysis.

Figure 2 shows a map of the completely sequenced cluster. It spans a 34.2 kb region and contains 33 ORFs designated as *grhA* to *grhV*. According to database homologies, 9 ORFs are core PKS genes, 11 are tailoring genes, 4 are involved in regulation and resistance, and 9 are of unassigned or unknown function (Table 1). The most remarkable feature is the presence of 11 ORFs with similarity to genes involved in redox processes. This number is unprecedented among type II PKS systems but in agreement with the highly oxidized structure of griseorhodin. In the following sections, the putative functions of *grh* genes are discussed in more detail.

Polyketide Core Genes

The minimal PKS is encoded by *grhA* (KS_α), *grhB* (KS_β), and *grhC* (ACP), which all resemble their counterparts in other type II PKS systems. GrhA contains an acyltransferase GHSxG motif around Ser349 and a conserved Cys170 putatively involved in acyl binding during condensation [13]. This cysteine is replaced by a glutamine in the otherwise very similar GrhB, which is a general characteristic of KS_β proteins. As expected for an enzyme with a strong influence on polyketide chain length [14], the closest homologs to GrhB are found in PKSs generating the largest known polyketides, such as rubromycin, fredericamycin, and pradimicin. The ACP gene *grhC* is located 26 kb upstream from the two KS ORFs, representing a rare case of a disconnected minimal PKS. The only other examples among the around 30 described type II systems are found on the daunorubicin [15] and the R1128 [16] biosynthetic gene clusters. Surprisingly, the rubromycin sequence contains a grouped minimal PKS, although the part of the cluster deposited at GenBank is otherwise virtually identical with the *grhT* through *grhU* region.

Two putative PKS accessory genes were identified that are often recruited from other pathways but sometimes found as parts of type II PKS clusters. GrhF resembles phosphopantetheinyl transferases [17]. Such proteins attach the phosphopantetheinyl anchor group to ACPs and peptidyl carrier proteins of fatty acid synthases, PKSs, and nonribosomal peptide synthetases. The *grhG* product is similar to the transcarboxylase component of acyl-CoA carboxylases catalyzing carboxyl

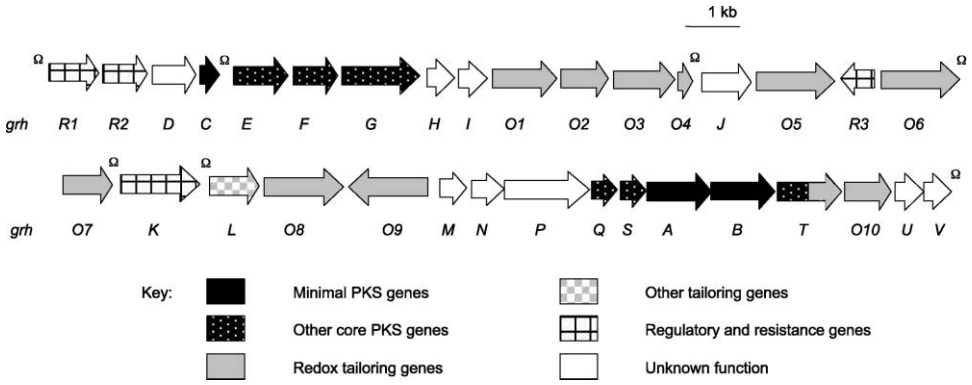

Figure 2. Organization of the Griseorhodin Biosynthesis Gene Cluster in *Streptomyces* sp. JP95
Each arrow represents the direction of transcription of an ORF.

transfer reactions between biotin and acyl-CoA derivatives [18]. The best studied members of this family are from fatty acid biosynthesis pathways, where they synthesize malonyl-CoA. Another acyl-CoA carboxylase component, JadJ, has recently been shown to positively influence jadomycin production rates [19]. GrhG probably has a similar function as supplier of biosynthetic building blocks.

Cyclization of aromatic open chain precursors is generally performed by at least two cyclases. Downstream of the ACP gene *grhC* lies *grhE* with high homology at the protein level to bidomain CYCs. These enzymes, often termed aromatases, are known to perform a cyclization reaction between carbon atoms 7 and 12 and a subsequent elimination of water to yield the first aromatic ring [2]. Two additional CYCs were identified that contain only one catalytic domain. The ORF *grhQ* is strikingly similar to the rubromycin CYC *rubE*. Intriguingly, the next closest homologs of GrhQ are TcmI [20], catalyzing a kinked fourth ring cyclization, and angucycline cyclases of the JadI group, responsible for the introduction of a kink at the third ring [21]. GrhQ could therefore generate a structurally related cyclized moiety. The deduced protein sequence of GrhT consists of two halves being homologous to CYCs and oxidoreductases, respectively. The same architecture is found on the closest homolog, RubG. The 150 aa N-terminal sequence of GrhT exhibits strongest similarity to a second group of aromatases catalyzing a cyclization of the second ring between C7 and C17 and directing first-ring closure between C9 and C14, such as TcmN [22]. Interestingly, TcmN is besides GrhT and RubG the only other known bifunctional CYC, but carries an O-methyltransferase instead of an oxidoreductase domain on its C-terminal half. The best matching database entries to GrhS for which functional studies exist are WhiE-ORFII [23] and TcmJ [24]. The role of these proteins is not perfectly understood, but they seem to enhance yields of cyclized products through stabilization of the minimal PKS-cyclase protein assembly. The closest related protein to GrhS is RubD, annotated as putative cyclase in the database. GrhQ, GrhS, GrhA, and GrhB seem to be translationally coupled, since their genes overlap each other by 4 nt.

Tailoring Steps

The griseorhodin structure features highly oxygenated epoxyspiroketal and naphthoquinone moieties. In line with a biosynthesis consisting of a large number of oxidative steps, a remarkable variety of putative oxidoreductases are encoded on the *grh* PKS. According to similarity searches, only one of the twelve *grh* tailoring enzymes does not catalyze an electron transfer reaction. GrhO1 aligns well with a small group of oxidoreductases containing covalently bound flavin-adenine dinucleotide (FAD) [25]. Members of this family are involved in diverse types of oxidative modifications and include the enterocin EncM participating in a Favorskii-type carbon-carbon rearrangement [26] and three enzymes involved in mitomycin biosynthesis and resistance [27]. The putative conserved FAD binding histidine [25] can be found at position 75. GrhO3 is most similar to oxygenases of the cytochrome P450 family, such as RifS [28]. The cysteine residue presumably serving as heme attachment site [29] is located at aa 365, the threonine most likely involved in oxygene binding at aa 259. P450-catalyzed reactions in bacteria are continuously fed with electrons by NADPH-ferredoxin reductases via ferredoxins to keep the P450 in a reduced state [30]. A ferredoxin as component of the GrhO3 relay chain is putatively encoded by *grhO4*, whose translated product is highly similar to many ferredoxins from PKS clusters. Three of the four possible iron binding cystein residues (at positions 10, 16, and 54) could be identified. We did not find a candidate gene for the NADPH-ferredoxin reductase on the cluster, indicating that this enzyme is shared with other pathways. GrhO5, GhrO8, and GrhO9 exhibit significant homology to FAD-dependent monooxygenases involved in hydroxylations of aromatic systems. All sequences contain a monooxygenase (Pfam: PF01360) and an FAD binding domain pattern (PF01494). Closest characterized enzymes in the database to all three proteins are 4-methyl-5-nitrocatechol oxidase from a *Burkholderia* sp. [31] and the tetracenomycin A2 triple hydroxylase TcmG [32]. The same patterns and a strong overall resemblance to monoxygenases identified GrhO6 as being functionally related, but, notably, the closest homolog identified by database searches is MtmOIV. This protein performs a rare Bayer-Villiger-type

Table 1. Deduced Functions of the Open Reading Frames Shown in Figure 2.

Protein	Amino Acids	Proposed Function	Sequence Similarity (Protein, Origin)	Similarity/Identity	Protein Accession Number	Reference
GrhR1	273	Transcriptional activator	TylS, *Streptomyces fradiae*	77%/65%	AAD40804	[41]
GrhR2	273	Transcriptional activator	SAR, *S. ambofaciens*	85%/74%	CAB72140	[41]
GrhD	271	Thioesterase	ORF5, *S. rochei*	68%/58%	BAA87906	[45]
GhrC	86	ACP	ORF3, *S. rochei*	63%/52%	BAA87909	[45]
GrhE	323	CYC	SimA5, *S. antibioticus*	51%/35%	AF324838	[46]
GrhF	267	Phosphopantetheinyl tranferase	Gra-ORF32, *S. violaceoruber*	48%/39%	CAA09659	[59]
GrhG	521	Acetyl-CoA carboxylase	PgaI, *S.* sp. Pga64	88%/81%	AAK57534	
GrhH	113	Unknown				
GrhI	122	Unknown				
GrhO1	475	FAD-dependent oxygenase	MitR, *S. lavendulae*	57%/44%	AAD28454	[27]
GrhO2	257	2-Oxoacyl-ACP reductase	MtmTII, *S. rimosus*	61%/50%	CAA07756	[38]
GrhO3	416	Cytochrome P450	PteD, *S. avermitilis*	70%/55%	BAB69310	
GrhO4	67	Ferredoxin	PteE, *S. avermitilis*	73%/60%	BAB69311	
GrhJ	295	Unknown	Dra0019, *Deinococcus radiodurans*	41%/31%	AAF12362	
GrhO5	537	FAD-dependent monooxygenase	DntB, *Burkholderia* sp. RASC	56%/44%	AAC44479	[31]
GrhR3	172 or 175	MarR family regulatory protein	2SCD64.11, *S. coelicolor*	57%/44%	AL391406	
GrhO6	520	FAD-dependent monooxygenase	MtmOIV, *S. argillaceus*	56%/45%	AAC64929	[33]
GrhO7	325	NADPH:quinone oxidoreductase	ActVI ORF2	59%/43%	Q53927	[37]
GrhK	529	Efflux protein	UrdJ, *S. fradiae*	58%/40%	AAF00219	[43]
GrhL	343	Methyltransferase	TcmO, *S. argillaceus*	60%/47%	P39896	[39]
GrhO8	534	FAD-dependent monooxygenase	DntB, *Burkholderia* sp. RASC	55%/40%	AAC44479	[31]
GrhO9	529	FAD-dependent monooxygenase	DntB, *Burkholderia* sp. RASC	57%/44%	AAC44479	[31]
GrhM	149 or 150	Unknown	ORF5, *Actinomadura hibisca*	47%/36%	JC5854	[11]
GrhN	147	Unknown	Rv0580c, *Mycobacterium tuberculosis*	48%/30%	CAA17451	
GrhP	625	Asparagine synthase	TcsG, *S. aureofaciens*	58%/46%	BAB12569	
GrhQ	123	CYC	RubE, *S. collinus*	89%/78%	AAG03065	
GrhS	144	Unknown	RubD, *S. collinus*	81%/75%	AAG03066	
GrhA	426	KS_{α}	RubA, *S. collinus*	91%/85%	AAG03067	
GrhB	420	KS_{β}	RubB, *S. collinus*	83%/77%	AAG03068	
GrhT	402	Bifunctional CYC–3-oxoacyl-ACP reductase	RubF, *S. collinus*	77%/69%	AAG03070	
GrhO10	249	3-oxoacyl-ACP reductase	RubG, *S. collinus*	87%/79%	AAG03071	
GrhU	107	Unknown	RubH, *S. collinus*	83%/72%	AAG03072	
GrhV	102	Unknown	ORF9, *A. hibisca*	54%/38%	BAA23152	[11]

ketone-to-ester conversion to cleave a carbon-carbon bond during mithramycin biosynthesis [33]. Since three carbon-carbon bond cleavages are necessary for spiroketal formation, GrhO6 could thus play an important role in the generation of this moiety. GrhO7 aligns well with enzymes of the NADPH:quinone oxidoreductase/ζ-crystallin (QOR) group. [34]. QORs usually generate semiquinone radicals from quinones in a one electron transfer reaction [35] and seem to play a role in cell detoxification [36]. The closest homolog, ActVI-ORF2, participates together with the functionally related ActVI-ORF4 in the 1,4-reduction of an enone moiety [37]. GrhO2, GhrO10, and the C-terminal half of GrhT show good end-to-end similarity to enzymes of the short chain dehydrogenase/reductase (SDR) family and contain the expected characteristic sequence patterns (PF00106 and PF00678). The closest homolog of GrhO2 is MtmTII with unknown function [38], while GrhO10 and the GrhT domain match best to the rubromycin proteins RubG and RubF and the pradimicin ORF7 product [11], which are all uncharacterized. Other closely related proteins are nearly exclusively FabG enzymes, i.e., 3-oxoacyl-ACP reductases from fatty acid pathways.

The only identified tailoring gene most likely not in-

volved in redox processes is the putative methyltransferase gene *grhL*. The deduced protein is most similar to the tetracenomycin 8-O-methyltransferase TcmO [39]. Since griseorhodin contains a methoxy group, the presence of a methyltransferase was expected.

Regulation and Resistance

Database searches revealed three genes with probable regulatory function on the cluster. GrhR1 and GrhR2 are similar to transcriptional activators of the *Streptomyces* antibiotic regulatory protein (SARP) family [40]. Although these proteins are part of many PKS systems, only the type I tylosin cluster was so far known to contain more than one copy of SARP genes [41]. There the SARPs TylS and TylT together with additional proteins are part of a complex, multitiered signaling network [41]. While the close GrhR1 homolog TylS functions as a master regulator controlling the expression of yet another regulatory gene, TylT had no obvious effect on tylosin biosynthesis. SARPs bind to promoter regions to activate the transcription of downstream genes and often contain a TTA codon involved in regulation [40]. No TTA codons, however, are present in *grhR1* or *grhR2*. GrhR3 is highly homologous to transcriptional repressors of the MarR family. Many of these proteins regulate multiple antibiotic resistance by binding to specific operators [42]. Transcription of downstream genes is induced upon recognition of a broad variety of small molecule substrates by the regulator. During griseorhodin biosynthesis, GrhR3 could have a similar function of resistance control and bind upstream of *grhK*. The protein product of this ORF resembles transporters encoded on many antiobiotic gene clusters and imparting self-resistance to the producer, such as UrdJ [43].

Genes with Unassigned or Unknown Functions

GrhD has high end-to-end similarity with thioesterases (TEs) and features the active site Ser81 within a highly conserved GHSxG motif [44]. To our knowledge, the only other examples of a TE gene as part of a type II PKS system are ORF5 from a cluster with unknown function on the *S. rochei* linear plasmid pSA2 liter [45], to which it is most homologous, and the simocyclinone SimC3 [46]. In contrast, TE proteins and domains are integral components of most type I PKSs, where they are responsible for the proper assembly and release of the complex polyketide chain [47, 48]. How chain release is controlled in type II PKS systems is still a mystery. Nevertheless, release mechanisms could be different for the assembly of very large aromatic polyketides such as griseorhodin and could require an additional TE. Alternatively, GrhD could act as esterase and may hydrolize ester intermediates generated by Baeyer-Villiger oxidations. The *grhP* protein strongly resembles class II glutamine amidotransferases that generate carboxamides, for example, PhzH, involved in phenazine-1-carboxamide synthesis [49]. Sequence alignment of GrhP with related proteins revealed that the N-terminal Cys crucial for acyl binding [50] is exchanged against Ser. GrhP could therefore be inactive. The three ORFs *grhM*, *grhU*, and *grhV* are strikingly similar on an amino acid level to uncharacterized genes from the *Actinomadura hibisca* pradimicin cluster [11] and the unpublished *rub* cluster. No information about their possible function could be gained by available pattern and profile analysis tools. Similarity searches with GrhJ and GhrN yielded only proteins with unknown function, while the *grhI* and *grhJ* products do not resemble any proteins in the database.

Heterologous Expression of the Entire Griseorhodin Cluster

Our initial trials to confirm that the *grh* cluster is involved in griseorhodin biosynthesis were met with unexpected difficulties. The griseorhodin producer proved to be highly refractory against the introduction of foreign DNA by a variety of methods, thus precluding knockout studies. Since the cluster is slightly smaller than the average cosmid insert, our alternative strategy was the heterolous expression of the complete pathway in *S. lividans* on a suitable shuttle cosmid. Expressions of large gene clusters from plasmids are often challenged by genetic instability resulting in plasmid rearrangements. To be able to quickly test expressions from a variety of plasmids, we developed a flexible cloning strategy. pWEB, containing restriction sites rare in GC-rich Actinomycetes on both sides of the insert, was used for cosmid library construction. The intact insert of pGR6C2 containing the complete *grh* cluster could therefore be excised with SspI and EcoRV and ligated directly to the blunt ends of the desired linearized expression vector. To facilitate an efficient introduction into *E. coli*, vectors with a *cos* site were chosen. However, expressions from the replicative vector pOJ446 and the integrative vector pOJ436 [51] suffered from extensive plasmid rearrangements in *E. coli*. This stability problem was overcome by using the shuttle vector pHZ132 [52], but no detectable amounts of griseorhodin were produced in *Streptomyces lividans*. As pHZ132 carries the pSG5 replicon, we suspected the relatively high copy number to be the cause for these expression problems. We therefore constructed the new vector pAY1 based on the stable integrative plasmid pSET152, containing a *cos* site, the *oriT* region for conjugative transfer, and the ϕC31 attachment site. After inserting the *grh* region into this vector, we observed only 20% rearrangement in *E. coli*. The resulting plasmid pMP31 was transformed into *S. lividans* ZX1 and gave rise to colonies that developed the purple color characteristic for griseorhodin. Figure 3 shows the HPLC-MS trace of the extract of one of the purple strains, *S. lividans* ZX1(pMP31a). According to UV and APCI-MS data, the extract of *S*. sp. JP95 contains griseorhodin A and at least three other related compounds. The same four metabolites were also produced by the recombinant strain. The sequenced cosmid insert therefore contains all the genetic information necessary to synthesize the complete set of fully elaborated griseorhodin molecules.

Discussion

The paper presented here reports the cloning and sequence analysis of a gene cluster encoding the biosynthesis of the extensively tailored aromatic polyketide

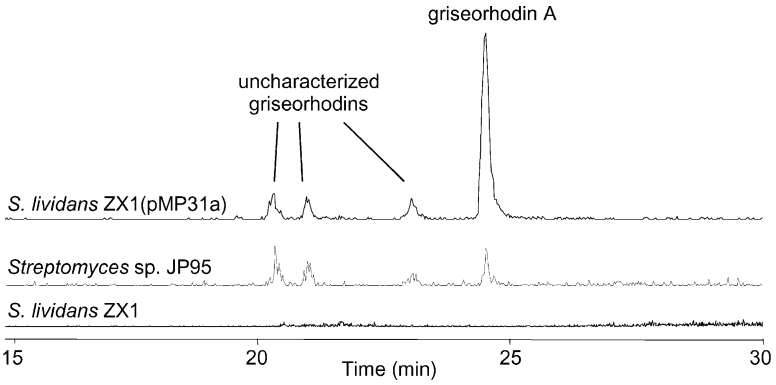

Figure 3. LC-ESI-MS Trace of Extracts from the Wild-Type Griseorhodin Producer *S*. sp. JP95, *S. lividans* ZX1, and *S. lividans* ZX1(pMP31a) Containing the Entire *grh* Cluster

Masses were recorded in the range between m/z = 490 and 494.

griseorhodin A. The griseorhodin producer *Streptomyces* sp. JP95 is an isolate from the marine ascidian *Aplidium lenticulum* that harbors a rich and diverse flora of Actinomycetes. At this point, we do not know whether these bacteria are accidental, temporary guests of *A. lenticulum* or perhaps involved in a symbiotic relationship. However, that at least some producing Actinomycetes could indeed be true symbionts is suggested by the presence of 5-deoxyenterocin in the same animal (R. Quinn, personal communication). This compound is assembled by a type II PKS [3], an enzyme family exclusively known from Actinomycetes.

Expression of the entire griseorhodin cluster in *S. lividans* was accomplished by a flexible cloning strategy that allowed for the rapid testing of various vectors. Among all plasmids, the cluster was only stably maintained and functionally expressed using the novel integrative cosmid vector pAY1 and yielded the same set of griseorhodins as the wild-type strain. The cloned region is therefore solely responsible for the biosynthesis of these metabolites in *S. lividans*. Sequence analysis indicates that the *grh* operon spans 34.2 kb and consists of 33 ORFs. The upstream end of the cluster seems to be represented by the activatory gene *grhR1*. Encoded further upstream are strong homologs to the E1 and E2 units of the 2-oxoglutarate dehydrogenase complex, which belongs to primary metabolism. The downstream *grh* boundary is most likely *grhV*. It is followed by an inverted repeat putatively serving as transcriptional terminator and, further downstream, ORFs similar to tripeptide transporters and several unknown proteins never found as PKS components.

How is griseorhodin biosynthesized? Important insight into the assembly of rubromycin-type compounds is available from studies conducted by Minas, Bailey, and coworkers. Heterologous expression of a fragment of the rubromycin cluster in *S. coelicolor* resulted in the production of the novel antibiotic collinone (Figure 4) [53]. This metabolite features a hexacyclic, pradimicin-like structure with two carbon atoms more than rubromycin and very likely represents a shunt product of the early tailoring stage. Unfortunately, no information about the genes present on the expressed cluster fragment has been published. Given the obvious relatedness between the griseorhodin and rubromycin pathway, a similar tridecaketide is also a plausible griseorhodin intermediate. This compound would consequently lose two carbon atoms during post-PKS tailoring. In accordance with the biosynthesis of a pradimicin-related intermediate, there are large similarities between the pradimicin, *grh*, and *rub* clusters. Close counterparts to all genes from the pradimicin system [11], with the exception of the ACP (see below), are present on the *grh* cluster. This includes the products of the pradimicin ORFs 5, 8, and 9, to which no known related proteins exist apart from the *grh* and the *rub* system. The notable difference between pradimicin and griseorhodin/rubromycin biosynthesis lies in the ketide chain lengths: while pradimicin is a dodecaketide, the *grh* and *rub* clusters produce tridecaketides. Even more striking is the similarity between the *rub* fragment available at GenBank and the corresponding *grh* region. The nucleotide sequences are 80% identical over the whole 6.3 kb region and show the same order of ORFs. A curious divergence is the remarkably clean deletion of a 200 nt region that should encode the *grh* ACP, which is instead located more than 20 kb upstream. A phylogenetic analysis (data not shown) revealed that while the *rub* and pradimicin ACPs are closely related, the *grh* ACP is more similar to the mithramycin *mtmS* [54]. The isolated ACP gene is therefore presumably not the result of a DNA rearrangement within the cluster but rather of a gene acquisition from a different pathway.

Another surprising feature of the *grh* cluster is the coexistence of *grhE* (an *actVII* homolog) [13] with *grhV*, which is related to *tcmN* [22]. ActVII and TcmN represent two types of aromatases that both perform the first cyclization of open polyketide precursors [2]. Since the ActVII type usually directs a ring closure of C9-reduced substrates between C7 and C12, but the TcmN type catalyzes a C9-C14 cyclization, these enzymes stand at a switchpoint of aromatic polyketide biosynthesis and channel intermediates toward different structural fates. The presence of both types of enzymes in the same pathway is unprecedented and should theoretically result in a mixture of differently cyclized products. The fact that we were unable to detect ActVII-type cyclization products in the extracts, that no ActVII homolog is known from clusters with otherwise similar sets of CYCs, and that the unreduced griseorhodin precursor should represent a bad substrate for ActVII CYCs indicates that GrhE either performs chemistry different to ActVII or is inactive. We suspect that *grhE* has been integrated into the cluster together with the directly adjacent ACP gene *grhC* but is not needed for griseorhodin biosynthesis.

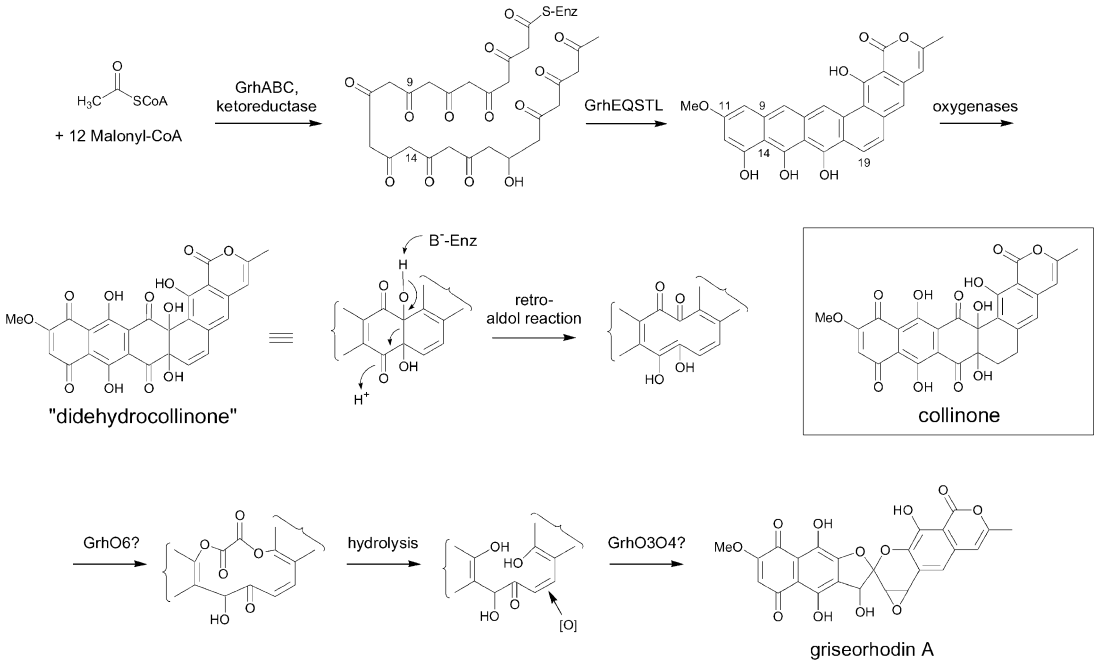

Figure 4. Possible Biosynthetic Pathway Leading to the Formation of Griseorhodin A via the Hypothetical Intermediate Didehydrocollinone
The engineered metabolite collinone from a partial expression of the rubromycin cluster is shown for comparison.

Collinone and all rubromycin members except the griseorhodins feature a rare fully reduced ethylene unit at C19/20. The epoxide function of griseorhodin A could be generated from the same moiety by a rare hydroxylation–dehydrogenation sequence similar to hyoscyamine biosynthesis [55]. However, since no homolog of the rather unusual hyoscyamine epoxidase, but rather a putative P450/ferredoxin pair encoded by *grhO3* and *grhO4* was found, it seems likely that the epoxide is instead formed by a more conventional P450-catalyzed double bond epoxidation. The uncleaved hexacyclic intermediate could therefore resemble the hypothetical didehydrocollinone (Figure 4). This compound could be produced by C19 ketoreduction at the precyclization stage, presumably catalyzed by GrhO2, GrhO10, or GrhT, subsequent post-PKS O-methylation by GrhL, and five ring hydroxylations by GrhO5, GrhO8, and/or GrhO9. Interestingly, the closest homolog to Grh5 and GrhO9 from a polyketide pathway is the tetracenomycin A2 triple hydroxylase TcmG that produces an angular diol moiety reminiscent of the C/D ring portion of collinone [56]. A similar multiple hydroxylation performed by a single enzyme perhaps also occurs during griseorhodin biosynthesis.

The final biosynthetic steps between the putative didehydrocollinone intermediate and griseorhodin A apparently feature rather exotic chemistry. To generate the spiroketal pharmacophore, three carbon-carbon bonds have to be cleaved to extrude a C2 unit. Although the exact mode of cleavage is still unknown, the striking similarity of GrhO6 to MtmOIV suggests that this enzyme plays an important role in final tailoring. MtmOIV is a "Baeyer-Villigerase" responsible for the aromatic ring cleavage during mithramycin biosynthesis via oxygen insertion and lactone ring opening [33]. Such a reaction can also be envisaged to account for the spiroketal system. One possible pathway that could generate this moiety is the hypothetical retro-aldol/Baeyer-Villiger sequence shown in Figure 4, but alternative routes, perhaps involving radicals, are also conceivable.

Through cloning of the griseorhodin biosynthesis gene cluster, many diverse and unsusual oxidoreductases are now available for studies in combinatorial biosynthesis. It remains to be established if these enzymes that could potentially generate a pharmacophore moiety are catalytically flexible enough to convert substrates from related pathways. In contrast to the often freely interchangeable core PKS proteins, the much higher substrate specificity of tailoring enzymes still represents a major challenge in hybrid metabolite production. However, the growing number of studies successful in producing tailoring hybrids illustrates that novel biologically active compounds can indeed be obtained by searching for new tailoring tools [5] and by modifying available tailoring enzymes to increase substrate flexibility [57].

Significance

With the griseorhodin biosynthesis cluster from the marine isolate *Streptomyces* sp. JP95, a tridecaketide synthase system has been completely cloned, sequenced, and heterologously expressed for the first time. In contrast to all other known aromatic PKSs, the cluster encodes two regulators of the SARP family, an ACP gene separated from the other minimal PKS genes by 26 kb, and two types of aromatase genes. Most striking, however, is the presence of 11 ORFs encoding various oxidoreductases. This unprecedented number of redox enzymes in a single bacterial PKS pathway is responsible for a remarkable post-

PKS modification of a hexacyclic pradimicin-type precursor, during which three carbon-carbon bonds are cleaved to generate a unique epoxyspiroketal moiety. This unit is also the pharmacophore responsible for the telomerase-inhibiting activity of griseorhodin A. With the complete tailoring system available, alteration of its components or its expression in other polyketide pathways could now provide access to novel drug candidates with similar spiroketal pharmacophores.

Experimental Procedures

Bacterial Strains, Plasmids, Culture Conditions, and DNA Manipulations

Streptomyces sp. JP95 was isolated on B1 agar (2.5 g peptone, 1.5 g yeast extract, 1.5 ml glycerol, 28.8 g instant ocean, 17 g agar per l) from the marine ascidian *Aplidium lenticulum* at Heron Island, Queensland, Australia. For further culturing in liquid and solid medium, 2CM was used (1 l contains 10 g soluble potato starch, 1 g NaCl, 2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 2 g $CaCO_3$, 2 g $MgSO_4 \cdot 7H_2O$, 2 g tryptone, and 1 ml inorganic salt solution consisting of 0.1% each of $FeSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, and $ZnSO_4 \cdot 7H_2O$). *S. lividans* ZX1 (a kind gift of Dr. Xiufen Zhou, Shanghai Jiaotong University) was grown on R5 for protoplast transformation and on 2CM for all other experiments. Streptomycetes were routinely cultured at 30°C. Shake-flask cultures were grown in Erlenmeyer flasks equipped with steel springs at 200 rpm. *E. coli* XLI-Blue was used for standard subcloning techniques and grown on LB plates or in liquid LB medium. *E. coli* EPI305 was used for cosmid library preparation and grown in liquid LB supplemented with 10 mM $MgSO_4$ or on LB plates. pBX531 containing a part of the fredericamycin cluster was kindly provided by Dr. C. Richard Hutchinson (KOSAN Biosciences, Inc.). pBluescript II SK(-) (Stratagene) was used as vector for cloning in *E. coli* and for sequencing.

Purification of Griseorhodin A

1 liter of a *Streptomyces*. sp. JP95 liquid culture was acidified to pH 3.5 with HCl and extracted repeatedly with EtOAc until the organic layer was almost colorless. The organic solution was dried over Na_2SO_4, and the solvent was removed in vacuo. The residue (684 mg) was chromatographed on Sephadex LH-20 using methanol, and the major red fraction containing griseorhodin A was subjected to further chromatography in a $CHCl_3$:methanol 95:5 to 85:15 gradient on KH_2PO_4-impregnated silica. Fifty-seven milligrams of pure griseorhodin A were obtained, whose identity was confirmed [6] by nuclear magnetic resonance and mass spectrometric methods.

Cosmid Library Construction and Screening

A library of *Streptomyces*. sp. JP95 DNA in *E. coli* EPI305 was constructed in the cosmid vector pWEB (Epicentre). Conserved motifs of KS_α proteins involved in the biosynthesis of pradimicin-type compounds were identified by sequence alignment and used for the design of PCR primers. The following primer pairs successfully amplified the desired products: KS2F (5′-YGSYGCSARTGC GACTTCG-3′) – KS2R (5′-CCGAYCATSGACTTGATGGAGC-3′), KS4F (5′-GAGGCSVTCGMSGACAGCGG-3′) – KS1R (5′-AGTCSAGGTCR CAYTCSGGGTC-3′), and KS4F – KS2R. ^{32}P-labeled hybridization probes prepared from these PCR products served to identify positive cosmids.

DNA Sequencing

pGR6C2 was sonicated, end-repaired by BAL-31 and Klenow fragment, and size-fractionated by gel electrophoresis to obtain fragments of 1–2 kb lengths. These were ligated into the EcoRV site of pBluescript II SK(-) and end sequenced with the BigDye Terminator Ready Mix (Applied Biosystems) and an ABI3700 sequencer (Applied Biosystems). Remaining gaps were filled by targeted subcloning and the use of specific primers.

Construction of the *grh* Expression Plasmids

pAY1 was constructed by inserting the 3.4 kb EcoRV–BglII fragment of pWEB containing the *cos* site into pSET152 that had been cut with EcoRV and BamHI. The various expression cosmids carrying the *grh* cluster were constructed by cutting cosmid pGR6C2 with SspI and EcoRV. The insert was isolated from a low-melting point agarose gel by treatment with GELase (Epicentre) and ligated into the EcoRV site of pAY1 or pOJ446, the PvuII site of pOJ436, or the blunted BamHI site of pHZ132. Cosmids were packaged and used for transfection into *E. coli* according to the pWEB manufacturer's direction. Ten clones were analyzed for each construction to assess plasmid stability.

Heterologous Expression of the Griseorhodin Cluster

Protoplasts were prepared from *Streptomyces* ZX1 and transformed with the *grh* plasmids according to standard procedures [58]. After protoplast regeneration became visible, plates were overlaid with the appropriate antibiotic. Resistant colonies were grown on fresh plates and checked for the development of purple color. Production of griseorhodin was confirmed by extracting a small amount of mycelium with 1 ml of 95:5:1 EtOAc/MeOH/HOAc. The organic solvent was removed in vacuo, redissolved in MeOH, and analyzed. An Agilent HP1100 series high performance liquid chromatography (HPLC) system was linked to a Finnigan MAT LCQ mass spectrometer, using APCI ionization and operating in the positive ion mode. A Grom ODS-3 C18 column was used at a flow rate of 0.2 ml/min with a linear gradient of 0.5% trifluoroacetic acid (TFA) in water to 0.5% TFA in acetonitrile over a period of 27 min. Griseorhodin A was identified by comparison of the retention time, UV spectrum, and mass spectrum with an authentic sample.

Acknowledgments

We thank I. Höfer (Fachhochschule Jena) and D. Schnabelrauch (MPI for Chemical Ecology) for technical assistance, Prof. C.R. Hutchinson (KOSAN Biosciences, Inc.) for the plasmid pBX531 and information about the fredericamycin cluster, and Profs. W. Boland, B.S. Moore and R. Quinn for support and valuable discussion. Prof. John Hooper (Queensland Museum) and Dr. M. Butler (Astra Pharmaceuticals, Inc.) are gratefully acknowledged for helping with the collection and identification of *A. lenticulum*. A.L. was a recipient of a postdoctoral fellowship from the DAAD.

Received: July 10, 2002
Revised: August 8, 2002
Accepted: August 8, 2002

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Accession Numbers

The GenBank accession number for the *grh* cluster is AF509565.