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Starter Unit Choice Determines the Production of Two Tetraene Macrolides, Rimocidin and CE-108, in *Streptomyces diastaticus* var. 108

Elena M. Seco, Francisco J. Pérez-Zúñiga, Miriam S. Rolón, and Francisco Malpartida*
Centro Nacional de Biotecnología
Campus de la UAM
28049 Cantoblanco
Madrid
Spain

Summary

***Streptomyces diastaticus* var. 108, a newly isolated strain [1], produces two closely related tetraene macrolides (rimocidin and CE-108) as well as oxytetracycline. A region of 19,065 base pairs of DNA from the *S. diastaticus* var. 108 genome was isolated, sequenced, and characterized. Ten complete genes and one truncated ORF were located. Disruption of these genes proved that this genomic region is part of the biosynthetic cluster for the two tetraenes. The choice of starter units by the loading module and the in vivo availability of the starter metabolites are crucial for the final ratio of the two macrolides. A second type I PKS, unrelated to tetraene biosynthesis, was also identified; disruption of these genes suggests that they would code for enzymes involved in the biosynthesis of a polyketide that might compete metabolically with rimocidin production.**

Introduction

Species of the genus *Streptomyces* produce a large variety of secondary metabolites. Most strains make several metabolites, not always structurally related. *S. coelicolor* is a typical example; it produces several secondary metabolites, including actinorhodin, prodigiosin, calcium-dependent antibiotic, spore-associated pigment, and methylenomycin. Moreover, many other secondary metabolites were suggested when the genomes of this and another species, *S. avermitilis*, were fully sequenced [2, 3]. Although the chemical diversity of these metabolites is very important as a source of natural products, it may sometimes lead to an undesirable bottleneck for the industrial production of compounds of interest due to metabolic competition between them [4].

The polyketides are a large group of secondary metabolites. Their importance arises from the facts that they include different and potentially useful biological activities (antibacterial, antifungal, immunosuppressant, antiparasitic, antitumor, etc) and that the peculiarities of their biosynthetic pathways have facilitated a pioneering activity in the field of secondary metabolism aimed at the generation of novel molecules through combinatorial biosynthesis [5]. All polyketides share a similar biosynthetic mechanism derived from a polyketide synthase activity (PKS) in which condensation of different elongation units (typically malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA) gives rise to a partially oxidized polyketide chain. The sequence of steps (condensations, keto reductions, dehydrations, enoyl reductions, etc.) is programmed in two different ways among PKS enzymes: type I PKSs are complexes of multifunctional polypeptides [6–8], whereas in type II PKS each biochemical function is performed by a separate polypeptide, forming a multienzymate complex. While the first group is the choice for biosynthesis of complex macrolides, the latter is used for biosynthesis of aromatic polyketides [9]. Irrespective of the organization of the PKSs, the primary carbon skeleton of the resulting polyketide is subjected to further modifications such as methylation, oxidation, glycosylation, etc., by so-called "tailoring" steps.

The polyenes are a large group of polyketides, most of them with antifungal activity. They contain a large macrolactone ring with several conjugated double bonds forming a chromophore responsible for their characteristic physical and chemical properties (strong light absorption, photolability, and poor solubility in water). Most contain a sugar moiety (usually mycosamine) attached to the macrolactone ring. According to the number of their conjugated double bonds, they are classified as trienes, tetraenes, pentaenes, hexaenes, and heptaenes; the number of double bonds determines their characteristic ultraviolet/visible light spectrum [10, 11]. The main target of these compounds is the fungal cell membrane; they interact with ergosterol-forming channels, ultimately causing loss of ions, destruction of electrochemical gradients, and finally cell death [12]. New biological properties have recently been reported for some polyenes: antibacterial, antiviral, insecticide, and immunostimulating activities [10, 13].

Biosynthetic gene clusters for the polyenes pimaricin, amphotericin, nystatin, and candicidin have recently been described [14]; all of these PKSs are type I modular enzymes. The loading modules of nystatin, amphotericin, and pimaricin, unlike those of other modular type I PKS, such as that for erythromycin biosynthesis [6, 7], are located on a separate protein, while the extension modules are distributed over several PKS components organized as bi-, tri-, tetra-, or hexa-modular proteins. The candicidin cluster seems to be structurally intermediate, with the loading domain fused to the first elongation module. It is noteworthy that these hexamodular PKS are the biggest multifunctional proteins so far reported in the literature.

Streptomyces diastaticus var. 108, a newly isolated strain, produces two closely related tetraenes, rimocidin and CE-108. Rimocidin has been known since 1951 as an antifungal compound [15], and its structure and stereochemistry were previously elucidated [16, 17]. CE-108 is an antifungal tetraene structurally related to rimocidin; its structure has recently been determined [1]. The two tetraenes are recovered from the fermentation broths of *Streptomyces diastaticus* var. 108 in a ratio

*Correspondence: fmalpart@cnb.uam.es

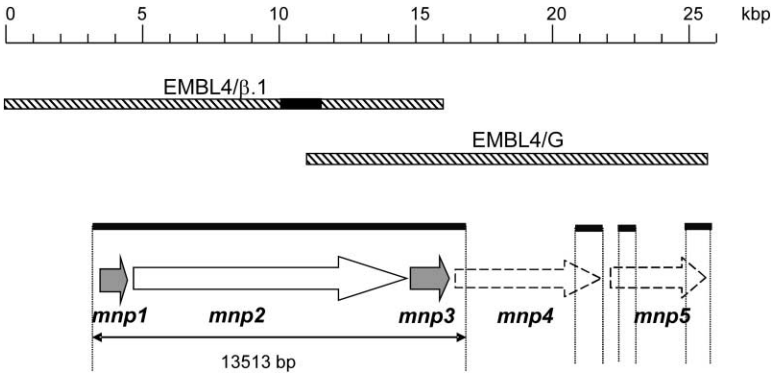

Figure 1. Partial Organization of a Biosynthetic Gene Cluster (*mnp*) for an Unidentified Polyketide Compound

The EMBL4/β1 and EMBL4/G recombinant phages overlapping the cloned region are shown. The 1.5 kbp ApaI fragment used for insertional inactivation is indicated by a black bar within the EMBL4/β1 phage. Arrows with dotted lines are the partially sequenced regions; the fully sequenced region is also indicated.

that is medium dependent; while in some media the main polyene compound is rimocidin (up to 75% of total polyenes), by changing the composition this ratio can even be inverted. The two tetraenes differ in the aglycone moiety, with a methyl side chain in CE-108 instead of the propyl group in rimocidin (see Figure 6). Assuming that both compounds are derived from the same biosynthetic pathway, this difference presumably reflects differential loading of the PKS and suggests a flexibility of the PKS in recognizing different metabolites as starter units. We undertook the molecular cloning and characterization of the biosynthetic genes for these polyenes in order to gain insight into the biochemical and/or genetic controls leading to in vivo discrimination between the two molecular species.

Because the producing strain can be easily genetically manipulated, we thought that *S. diastaticus* var. 108 and the biosynthetic cluster for the two tetraenes would serve as useful tools for modifying polyene biosynthetic genes in attempts to generate new bioactive compounds.

Results

Preliminary Genetic Characterization of the Producer Strain

In order to assess the amenability of *S. diastaticus* var. 108 to genetic manipulation, we tested several vectors for their suitability for introducing DNA fragments and further genome manipulation. High and low copy number replicative plasmids, such as those based on the pIJ101 or SCP2 [18] and pSG5 [19] replicons, as well as integrative plasmid vectors based on ϕC31 site-specific integration [20] were introduced successfully by standard transformation as described previously [18]; those carrying oriT can also be introduced by interspecies conjugal transfer [20]. Both *att*$^+$ and *att*$^-$ ϕC31 phage derivatives were also used successfully.

Cloning Antibiotic Biosynthetic Genes

In order to isolate the genes of the putative rimocidin and CE-108 gene cluster, a *S. diastaticus* var. 108 genomic library was constructed in the *E. coli* EMBL4 phage vector (see Experimental Procedures). For screening the library, two different probes were used.

Heterologous Probe

A DNA fragment of the *ery* cluster carrying the *eryAII* gene [8] in an *E. coli* vector (kindly provided by P. Leadlay) was used for probing a genomic library of 10,000 recombinant phages. Two overlapping lambda recombinant phages (EMBL4/β1 and EMBL4/G) were isolated, covering a total of 26 kbp. The DNA was partially sequenced; five open reading frames were identified, of which three (*mnp1*, *mnp2*, and *mnp3*) were fully characterized and two (*mnp4* and *mnp5*) were deduced from

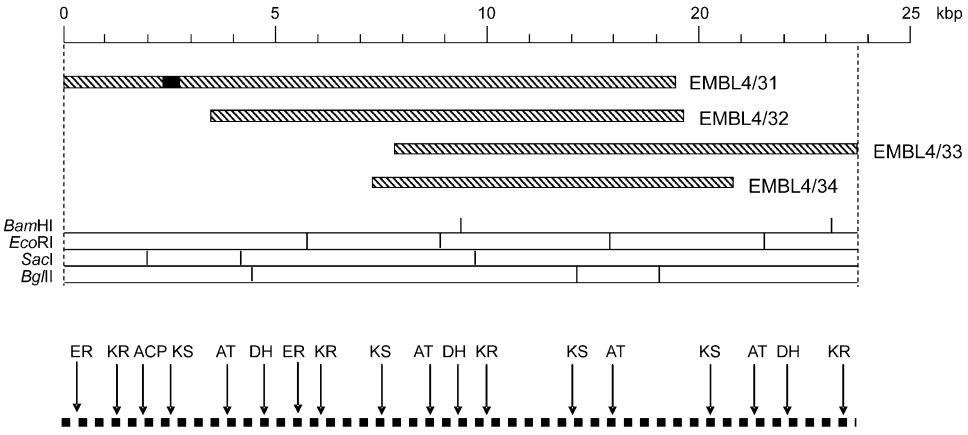

Figure 2. Partial Characterization of a Genomic Region Carrying a Modular PKS for Rimocidin and CE-108 Biosynthesis

EMBL4/31, EMBL4/32, EMBL4/33, and EMBL4/35 are recombinant phages covering 24 kbp of the *rim* cluster. The catalytic domains shown above the thick dotted line were deduced from random DNA sequencing along the recombinant phages. The black rectangle within EMBL4/31 represents the region used for disruption of the PKS gene corresponding to the KS108.1 fragment.

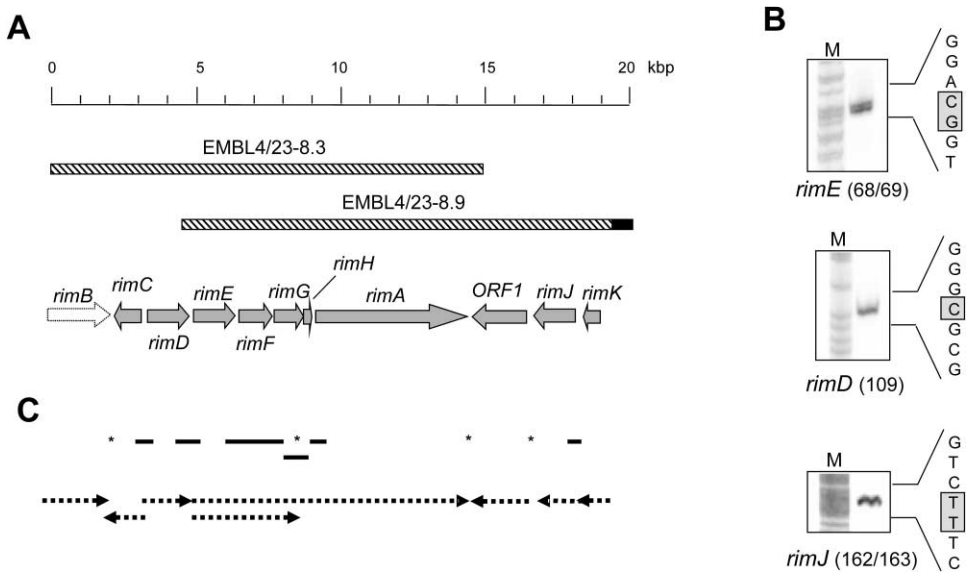

Figure 3. Organization of a Genomic Region Involved in Several Steps of Rimocidin and CE-108 Biosynthesis

(A) EMBL4/23.8-3 and EMBL4/23.8-9 are recombinant phages isolated with the homologous probe generated by a PCR fragment. The C terminus of the RimB PKS is indicated by a dotted arrow.

(B) Mapping the transcription start sites. The S1 protected fragments are shown for *rimD* and *rimJ* as monocistronic genes and *rimE, rimF, rimG, rimH*, and *rimA* as a polycistronic transcript. M, Maxam & Gilbert reactions (G + A ladder). The corresponding transcription start is shown in gray boxes. The numbers in parentheses indicate the position upstream of the putative translation start codon.

(C) Transcriptional organization of the *rim* genes located in this chromosomal region. The fragments used for S1 endonuclease protection assays are indicated with continuous lines. Asterisks show the positions where inverted repeated sequences were located. The deduced transcripts are represented by dotted arrows.

their partial DNA sequence (see Figure 1). The deduced products of *mnp2*, *mnp4*, and *mnp5* resembled known modular PKSs. The deduced protein Mnp2 is a polypeptide of 3180 amino acids carrying conserved domains putatively involved in two condensation cycles for a polyketide chain. The domains are arranged in the order KS-AT-DH-KR-ACP—KS-AT-KR-ACP; the first and second AT domains contain the typical signature for methylmalonyl-CoA and malonyl-CoA, respectively. The deduced products of *mnp1* and *mnp3* resemble P450 monooxygenases and probably would be involved in post-PKS modifications, as in many other polyketide biosynthetic pathways. The products of the partially identified *mnp4* and *mnp5* code for PKS components each involved in a single condensation step; Mnp5 contains a thiosterase domain at the C terminus, suggesting that it would be involved in the last condensation step and release of the final polyketide chain.

To determine if the cloned genes are involved in rimocidin and/or CE-108 biosynthesis, we disrupted *mnp2* using the *att*$^-$ phage PM1. A 1.5 kb ApaI fragment internal to the coding region of *mnp2* was first cloned in the polylinker of the *E. coli* vector pIJ2925; the resulting fragment was recovered by digesting with BamHI/SacI and cloned into BamHI/SacI-digested PM1. The resulting PM1 recombinant (PM1-114) was used to generate lysogens (*S. diastaticus* var. 108/PM1-114). The expected insertion within the target gene was confirmed by Southern blotting. No phenotypic differences between these lysogens and the wild-type could be observed at this stage. Several lysogens were cultivated in SYM2, and the fermentation broths were extracted with methanol and analyzed by HPLC. All of the lysogens still produced rimocidin and CE-108, indicating that the cloned region is not involved in biosynthesis of these polyenes. Moreover, production of rimocidin (but not of CE-108) in the disruptants was found to be increased by 25% to 30% compared to the wild-type. This finding suggests that the cloned genes are likely being expressed, and they are probably involved in the production of a different polyketide; this pathway might well use the same building blocks as those used for rimocidin biosynthesis. Attempts to identify antimicrobial activity associated with these genes (HPLC and TLC fractionations) were unsuccessful.

Homologous Probe

Two oligonucleotide primers were designed matching the consensus region of the KS domains of known modular PKSs (see Experimental Procedures). The polymerase chain reaction (PCR) was used to amplify a DNA region from the *S. diastaticus* var. 108 genome with these primers. Different PCR fragments of 500 bp were isolated and cloned into the polylinker of the *E. coli* vector pIJ2925. The resulting clones were analyzed by restriction pattern and DNA sequencing. Four different DNA fragments (KS108.1, KS108.2, KS108.3, and KS108.4) were identified; the translated products of all of them showed good similarities with the KS domains of modular PKSs. The KS108.1 fragment was cloned in the PM1 phage, and the resulting recombinant phage (PM1-108.1) used for infecting wild-type *S. diastaticus* var. 108. Lysogens were selected with thiostrepton and found to no longer produce either rimocidin or CE-108. Southern blot analysis, using the KS108.1 fragment as

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain or Plasmid	Properties	Reference
E. coli XL-1 blue MRA	host for maintaining the gene library	Stratagene
E. coli JM101	general cloning host	[42]
P. chrysogenum *ATCC10003*	antifungal activity assays	ATCC
S. lividans TK21	general cloning host	[43]
S. diastaticus var. 108	wild-type (WT) CE-108 and rimocidin producer	[1]
S. diastaticus var. 108/KC859	WT derivative by integration of KC859 (WT, control)	
S. diastaticus var.108/PM1-114	WT derivative with *mnp2* disrupted by integration of PM1-114	this work
S. diastaticus var. 108/PM1-108.1	WT derivative by integration of PM1-108.1, CE-108, and rimocidin nonproducer	this work
S. diastaticus var. 108/PM1-500	WT derivative with *rimA* disrupted by integration of PM1-500, CE-108 and rimocidin nonproducer	this work
S. diastaticus var. 108/PM1-754	WT derivative with *rimB* disrupted by integration of PM1-754, CE-108, and rimocidin nonproducer	this work
S. diastaticus var. 108/PM1-752	WT derivative with *rimC* disrupted by integration of PM1-752	this work
S. diastaticus var. 108/PM1-715	WT derivative with *rimD* disrupted by integration of PM1-715	this work
S. diastaticus var. 108/PM1-751	WT derivative with *rimE* disrupted by integration of PM1-751	this work
S. diastaticus var. 108/PM1-757	WT derivative with *ORF1* disrupted by integration of PM1-757	this work
S. diastaticus var. 108/PM1-709	WT derivative with *rimJ* disrupted by integration of PM1-709	this work
pER-1	EcoRI-HindIII fragment from pIJ4090 carrying the $ermE_p$* [18] cloned in pIJ2925	this work
EMBL4	vector used for the gene library	[40]
PM1	att^- ϕC31 derivative, *hyg, tsr*	[38]
KC859	att^+ ϕC31 derivative, *xylE, tsr*	[39]
pCNB5006	0.3 kb KpnI-BamHI fragment carrying the erythromycin promoter from *ermE* cloned into the KpnI/BamHI sites of pIJ2925	this work
pSM114	1.5 kb ApaI fragment of *mnp2* cloned into the HincII site of pIJ2925	this work
PM1-114	1.5 kb BamHI-SacI fragment from pSM114 cloned into the BamHI/SacI sites of PM1	this work
pKS108.1	0.5 kb PCR product amplified from the *S. diastaticus* 108 genome cloned into the HincII site of pIJ2925	this work
PM1-108.1	0.5 kb BamHI-PstI fragment from pKS108.1 cloned into the BamHI/PstI sites of PM1	this work
pSM500	1.3 kb *Sal*I-*Sac*I fragment of *rimA* cloned into the *Sal*I/*Sac*I sites of pIJ2925	this work
PM1-500	1.3 kb BamHI-PstI fragment from pSM500 cloned into the BamHI/PstI sites of PM1	this work
pSM754	1.2 kb SmaI-SalI fragment of *rimB* cloned into the SmaI/SalI sites of pIJ2925	this work
PM1-754	1.2 kb BglII-PstI fragment from pSM754 cloned into the BamHI/PstI sites of PM1	this work
pSM752	0.5 kb EheI fragment of *rimC* cloned into the HincII site of pIJ2925	this work
PM1-752	0.5 kb BamHI-PstI fragment from pSM752 cloned into the BamHI/PstI sites of PM1	this work
pSM715	0.6 kb EheI-HincII fragment of *rimD* cloned into the HincII site of pIJ2925	this work
PM1-715	0.6 kb *BamHI-PstI* fragment from pSM715 cloned into the BamHI/PstI sites of PM1	this work
pSM751	0.8 kb SalI fragment of *rimE* cloned into the SalI site of pCNB5006	this work
PM1-751	1.2 kb BglII-PstI fragment from pSM751 cloned into the BamHI/PstI sites of PM1	this work
pSM757	0.5 kb ApaI-XmaI of *ORF1* cloned into the HincII site of pIJ2925	this work
PM1-757	0.5 kb BamHI-PstI fragment from pSM757 cloned into the BamHI/PstI sites of PM1	this work
pER1-709	0.8 kb BamHI-SacI fragment of *rimJ* cloned into the BamHI/SacI sites of pER1	this work
PM1-709	1.1 kb SacI-XbaI fragment from pEL1-709 cloned into the SacI/BamHI sites of PM1	this work

probe, showed that the insertion took place within a 3 kbp SacI fragment, and consequently this fragment must be part of the biosynthetic cluster for the two tetraenes. In order to isolate this SacI fragment, we generated in pIJ2925 a mini-library enriched in SacI fragments; 200 clones were tested for amplification with the designed oligonucleotides. With this strategy, we identified two clones carrying different SacI fragments of nearly 3 kbp; the DNA sequence of the fragment used for disruption (KS108.1) was found in one of them. This clone was therefore used for screening the *S. diastaticus* var. 108 library, allowing us to isolate 24 kbp of the chromosomal region overlapping the disrupted 3 kbp SacI fragment where the insertional inactivation occurred. The region was partially sequenced, revealing several catalytic domains belonging to one or more type I PKSs responsible for at least five elongation steps for rimocidin and CE-108 assembly (Figure 2).

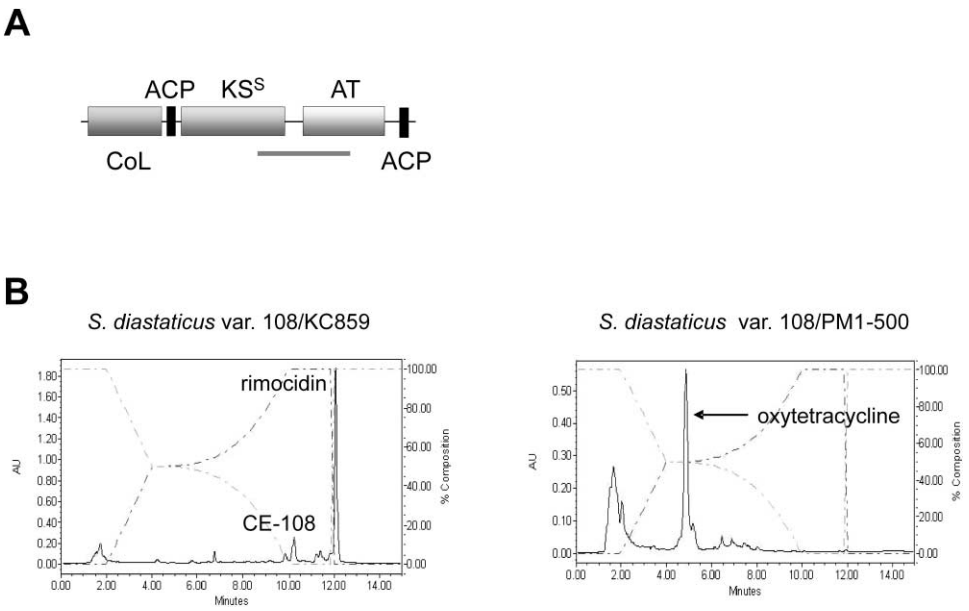

Figure 4. Functional Organization of RimA

(A) Schematic organization of RimA. CoL, carboxylic acid-CoA ligase; ACP, acyl carrier protein; KS^S, ketosynthase with the Cys→Ser substitution in the condensing active site; AT, acyltransferase. The fragment used for insertional inactivating is indicated by the black line below.

(B) HPLC analysis of the fermentation broth of *S. diastaticus* var. 108/KC859 (wild-type, control), left panel, and *S. diastaticus* var. 108/PM1-500 (*rimA* disruptant), right panel. The strains were grown on SYM2 medium without glucose, and the production was measured from total fermentation broths extracted with methanol and expressed as optical density at 304 nm.

Similarly, a second 3 kbp SacI DNA fragment isolated from the mini-library was completely sequenced, revealing an internal segment of a type I PKS. To determine if the cloned fragment is part of the tetraene biosynthetic cluster, a 1359 bp SacI/SalI fragment was used for insertional inactivation. The resulting *S. diastaticus* var. 108/PM1-500 lysogens failed to produce rimocidin and CE-108, confirming that the cloned fragment is indeed part of the rimocidin biosynthetic cluster. Using this fragment as probe, an additional 19 kbp overlapping this fragment was isolated. Its DNA sequence revealed 12 ORFs, one of which is truncated at the 3′ end (see Figure 3A); the genes were named *rimB*, *rimC*, *rimD*, *rimE*, *rimF*, *rimG*, *rimH*, *rimA*, *ORF1*, *rimJ*, and *rimK*.

Molecular Analysis of the Deduced Genes

The product of the *rimA* gene is a polypeptide of 1737 amino acids with a deduced molecular mass for the monomer of 181 kDa. RimA resembles type I PKSs, showing 70% identity with PimS0, the putative loading module of pimaricin [21]. It has several well-defined catalytic domains: CoL, ACP, KS^S, AT, and a second ACP at the C terminus (see Figure 4A). This arrangement, along with the presence of the KS^S, found in the presumed loading modules of pimaricin, nystatin, and amphotericin [21–23], suggests that RimA is the loading module for rimocidin and CE-108 biosynthesis. Bearing in mind the structural differences between the two polyenes, it can be hypothesized that RimA might play a pivotal role in the final ratio between rimocidin and CE-108 production. The complete DNA sequence of this region confirmed that the SacI fragment used for this disruption was in fact part of the *rim*A gene. It is noteworthy that the tetraene-blocked mutants overproduce oxytetracycline (see Figure 4B), suggesting pathway competition, as suggested above for the *mnp* genes.

The truncated *rimB* gene encodes 632 amino acids of the C terminus of a protein with high similarity to type I PKSs. It has KR and ACP domains, suggesting that this gene is also an integral part of a modular PKS for tetraene production. Disruption of *rimB* generated polyene nonproducer mutants (*S. diastaticus* var. 108/PM1-754), confirming that RimB is also involved in elongation steps for rimocidin and CE-108 biosynthesis.

Convergently to *rim*B lies *rim*C; its product is a deduced protein of 375 amino acids with similarity to tyrosine phosphatases. The role of this protein in the tetraene pathway remains to be determined. Interestingly, this peptide shows similarities with a small region near the C terminus of the PimS0 protein (between amino acids 1480 and 1654) [21]. This region is missing in RimA, which is shorter than the PimS0 protein, explaining the discontinuity found before the last ACP domain when RimA and PimS0 are aligned. This finding suggests a common ancestor for the two proteins and that RimC probably diverged from that ancestor during evolution of the biosynthetic pathway. Disruption of *rimC* (*S. diastaticus* var. 108/PM1-752 lysogens) had a slight effect on the ratio between rimocidin and CE-108 in favor of rimocidin (data not shown); this could be attributable to changes in in vivo availability of starter units.

The *rimD* gene is divergently transcribed from *rimC*. Its product shows the highest similarity (85%) to the PimE [21] protein of the pimaricin gene cluster. Like PimE, RimD resembles cholesterol oxidases, which occur in other polyene biosynthetic clusters [24], but with

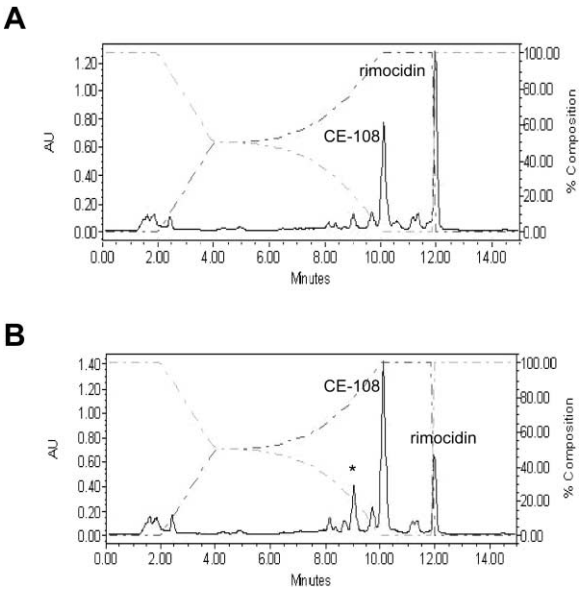

Figure 5. HPLC Analysis of the Fermentation Broth of *S. diastaticus* var. 108 Recombinants

(A), *S. diastaticus* var. 108/KC859 (wild-type, control); (B), *S. diastaticus* var. 108/PM1-709 (*rimJ* disruptant). The strains were grown on SYM2 medium. The overproduced tetraene is indicated with an asterisk on top of the peak. Polyenesa were extracted and measured as in Figure 4B.

no biosynthetic role assigned. A *rimD* disruption in *S. diastaticus* var. 108/PM1-715 lysogens showed no significant change in rimocidin and CE-108 biosynthesis. Nevertheless, by S1 mapping we detected its putative transcription start point (Figure 3C) and concluded that *rimD* is actively transcribed as a monocistronic transcript.

Two hundred and thirty base pairs downstream from *rimD* lies *rimE*, encoding a protein highly similar to glycosyl transferases, with as much as 69% identity with the PimK protein. Based on these similarities, the predicted function would be attachment of the sugar mycosamine to the aglycone moiety of rimocidin and CE-108. Insertional inactivation of *rimE* using the recombinant phage PM1-751 carrying an internal fragment of the gene gave rise to rimocidin nonproducer strains. No polar effect downstream of the disrupted gene is expected because the disruption was carried out using the *ermE* promoter (see Table 1). HPLC analysis of methanol extracts of the cultured *S. diastaticus* var. 108/PM1-751 lysogens failed to show any putative aglycone moiety. Because this can be due to a possible chemical instability of the aglycone compounds, we cultivated the *rimE* disruptant in the presence of 1% Amberlite XAD 16 resin in order to exclude the compounds from the fermentation broth and prevent expected degradation. The compounds bonded to the resin were finally extracted with methanol for further analysis; in this way, both rimocidin and CE-108 aglycones were successfully detected by HPLC-MS, indicating that the RimE protein is indeed a glycosyl transferase and that it can transglycosylate both aglycones. No antifungal activity could be detected for any of the aglycone moieties, confirming the crucial role of the amino sugar for biological activity.

The deduced product of *rimF*, located 53 bp downstream from *rimE*, resembles aminotransferases involved in polyketide biosynthesis with a maximum identity of 85% to PimC from *S. natalensis* [25]. This protein is likely to be involved in the biosynthesis of mycosamine.

The *rimG* gene lies 28 bp downstream from *rimF*. Its product strongly resembles cytochrome P450 monooxygenases; the maximum identity (82%) is to PimG from *S. natalensis*. Eighty-six base pairs downstream of the stop codon of *rimG* lies *rimH*, whose product is a small peptide (6.3 kDa) encoding a ferredoxin. On the basis of its homology, RimG and RimH could participate in the specific oxidation required for generating the carboxylic acid group of rimocidin and CE-108, and RimH would serve as electron donor, as for many monooxygenase reactions [26].

Seventy-eight base pairs downstream of *rimA*, and convergently transcribed to this gene, lies the stop codon of ORF1. Based on similarities with other known proteins, ORF1 would encode a transposase. The protein is unlikely to be involved in biosynthesis of the polyenes; a probable transposable element was also described in the amphotericin gene cluster [23]. This gene might be a vestige of the evolution of this cluster, suggesting a possible horizontal transfer. As expected, disruption of *ORF1* in *S. diastaticus* var. 108/PM1-757 had no effect on polyene productions.

Next to ORF1 lies *rimJ*; its stop codon is located 130 bp upstream from the putative translation start codon of ORF1. The RimJ product resembles crotonyl-CoA reductases. The maximum identity (87%) is to a crotonyl-CoA reductase from *S. coelicolor* A3(2) (EMBL AL93127) and, to a lesser extent, to that from *Streptomyces collinus* [27]. Crotonyl-CoA reductases play an important role in the formation of butyryl-CoA through condensation of two acetyl-CoA units [27]. Since acetyl-CoA and butyryl-CoA seem to be the starter units of the CE-108 and rimocidin tetraenes, respectively, it is likely that RimJ provides the butyryl-CoA needed for biosynthesis of rimocidin. Disruption of *rimJ* did not abolish rimocidin production as expected; instead a marked decrease in rimocidin production occurred, while CE-108 was significantly increased. An additional tetraene compound with less retention time was overproduced by the *rimJ* disruptant (see Figure 5).

Finally, 306 bp upstream of the putative translation start codon of *rimJ* lies the stop codon of *rimK*. RimK showed similarities to known acetyltransferases. The maximum similarity was to *ORF*H from *Streptomyces kasugaensis* M338-M1 [28]; ORFH is an acetyltransferase for kasugamycin, thus generating an inactive kasugamycin-derived compound. *Streptomyces diastaticus* var. 108 does not seem to be sensitive to any of the produced tetraenes, and so it is unlikely that RimK plays a self-resistance role; nevertheless, there are some reports of N-acetylated derivatives of tetraenes [29]. The N-acetylated products are almost devoid of antifungal activity compared with the nonacetylated compounds. The possibility that *rimK* is not part of the rimocidin biosynthetic cluster cannot be ruled out.

Transcriptional Analysis of the Cloned Genes

In order to gain a general picture of the transcription pattern of the cloned genes, S1 mappings were carried

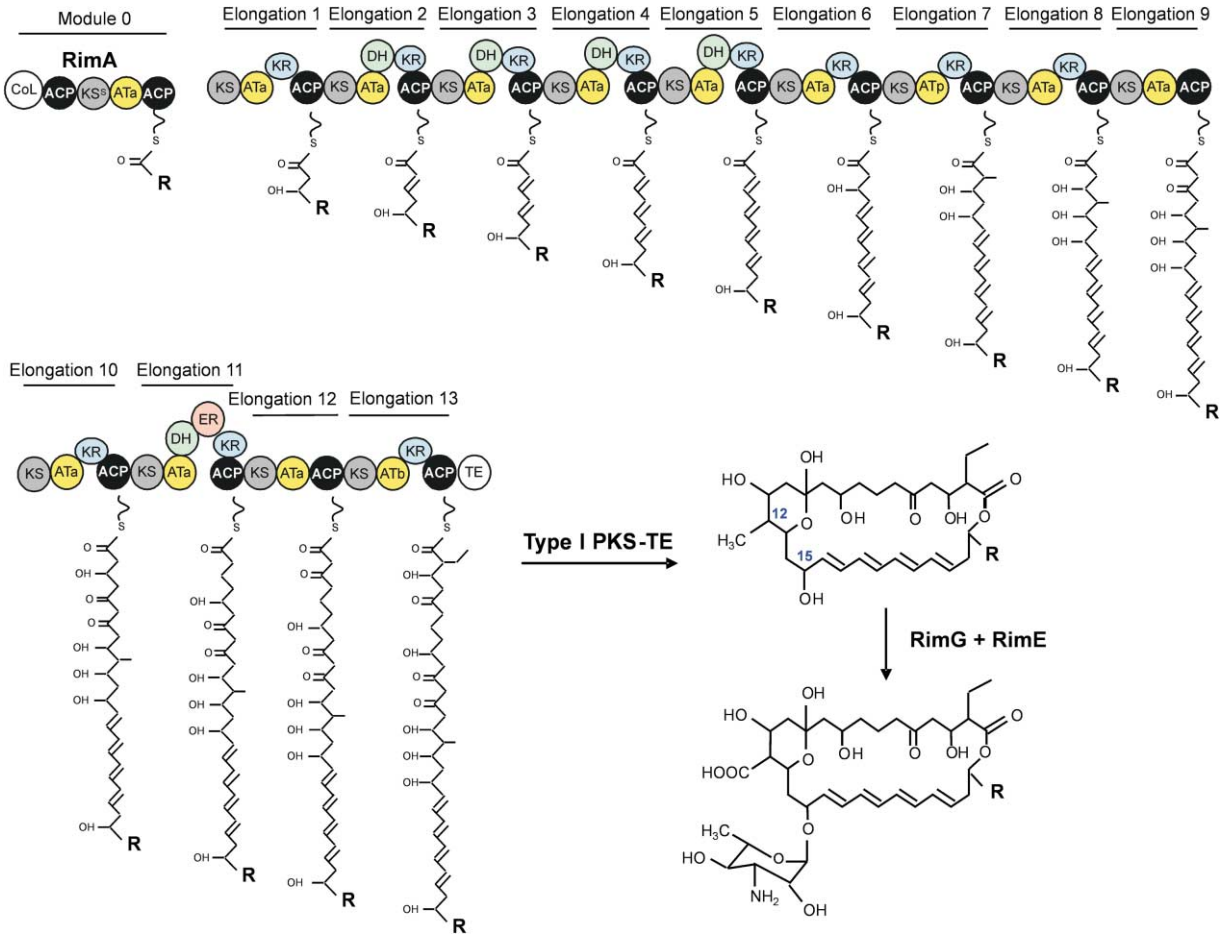

Figure 6. Proposed Model for Rimocidin and CE-108 Biosynthesis

The proposed scheme shows the expected elongation steps and the domains needed for rimocidin and CE-108 biosynthesis. **R**, $-CH_3$ (CE-108); **R**, $-CH_2$-CH_2-CH_3 (rimocidin); ATa, ATp, and ATb, AT domains specific for acetate, propionate, and butyrate, respectively.

out (see Figure 3). By using different overlapping DNA fragments, we deduced that *rimE, rimF, rimG, rimH*, and *rimA* are encoded in a polycistronic transcript longer than 9 kb. The transcription start point lies 68–69 bp upstream of the putative translation start codon (ATG) of *rimE* (see Figure 3B). Between the end of this transcript and that of *ORF1*, an inverted repeat sequence was identified; this is likely to be a transcriptional terminator. In addition to this polycistronic transcript, a shorter transcript can also be detected; its transcriptional end point was located at the end of the *rimG*. The *rimD* gene seems to be transcribed as a monocistronic messenger; its transcription start point was located 109 bp upstream of its putative GTG start codon. The *rimJ* transcript starts 162–163 bp upstream of its putative translation start codon (GTG) (Figure 3B). This result and the presence of the inverted repeats at the end of the other genes suggest the transcriptional pattern of these *rim* genes shown in Figure 3C.

Proposed Model for the Rimocidin and CE-108 Biosynthetic Pathway

The two tetraenes differ only in the side chain (methyl or propyl) (Figure 6), suggesting that the choice between acetyl-CoA or butyryl-CoA as the starter unit by the loading module (RimA) determines production of CE-108 or rimocidin and thus the final ratio between the two products. The tentative proposed biosynthetic scheme is given in Figure 6, based on the chemical structure of the polyenes. Thirteen elongation steps would generate the macrolactone rimocidinone or its biosynthetic variant CE-108; all of them (except elongation steps 7 and 13) would incorporate acetate by decarboxylative condensation of malonyl-CoA units (ATa), while elongations 7 and 13 would incorporate propionate (ATp) and butyrate (ATb) by decarboxylative condensation of methylmalonyl-CoA and ethylmalonyl-CoA units, respectively [30]. The DNA sequence of the whole biosynthetic cluster will give complete information about the general organization of the elongation modules within the corresponding PKS.

Once the polyketide chain is released and the macrolactone ring formed, it is expected that two post-PKS modifications would yield the final structures of both tetraenes. One of them is based on a specific oxidation of the methyl group at C-12 to generate a carboxylic group that is highly conserved in most polyenes. This modification is probably carried out by a cytochrome

P-450 monooxygenase. The deduced sequences of the cytochrome P-450 monooxygenases involved in specific modifications required in the biosynthesis of polyenes can be divided into two phylogenetic groups, depending on the target site: the exocyclic methyl branch and the polyol region [14]. Since only one cytochrome P-450 monooxygenase is required for rimocidin and CE-108 biosynthesis, and PimG shows the best homology with cytochrome P-450 monooxygenases involved in oxidation of the exocyclic methyl branch of other polyenes, we propose that RimG is responsible for this modification at C-12 in both tetraenes. RimH would serve for electron transport to RimG.

The second expected post-PKS modification is glycosylation of the aglycone structures. Based on phenotypic characterization of the *rimE* disruptant, this seems to be carried out by RimE, which would be responsible for incorporation of mycosamine at the C-15 position of the rimocidin and CE-108 aglycones. RimF (a putative aminotransferase) is likely to be involved in the synthesis of mycosamine. It is noteworthy that in the *rimE* disruptant, in addition to the expected aglycones, two additional compounds were detected. Based on LC-MS analysis they might well be rimocidin and CE-108 aglycones with an exocyclic methyl branch at C-12 instead of the carboxyl group. No GDP-mannose dehydratase gene was found on the cloned fragments, although presumably it could be linked to the remainder of the biosynthetic cluster, unlike the remaining mycosamine biosynthetic genes, which could be recruited from other sources such as primary metabolism, as suggested for other polyene clusters [14].

Discussion

Streptomyces diastaticus var. 108 is a typical actinomycete in the sense that it produces several secondary metabolites. In addition to the aromatic polyketide oxytetracycline, two macrolide polyenes (rimocidin and CE-108) are made by this strain [1]. The catalog of secondary metabolites produced by this strain is increased with the accidental isolation of another modular PKS gene cluster (*mnp*) as described in this paper. The pathways for all three secondary metabolites are probably expressed at the same time, because there are differences in the final balance of such metabolites in the fermentation broths of the blocked mutants. While, in *mnp* disruptants, only rimocidin but not CE-108 is increased (suggesting a competition for butyryl-CoA units between pathways), *rimA* disruption generates oxytetracycline overproducer strains. These results might reflect an in vivo competition between these pathways for the same metabolite as the building blocks for generating the backbone of their corresponding final products. Apart from these secondary metabolic pathways, some primary metabolic pathways such as fatty acid biosynthesis also use the same building units, and so this competition might constitute a bottleneck for production of secondary metabolites, as seems to happen in *Streptomyces cinnamonensis* [31] and *Streptomyces avermitilis* [32]. Nevertheless, in *S. diastaticus* var. 108, this competition may be partially bypassed because of the crotonyl-CoA reductase (RimJ) encoded in the rimocidin/CE-108 biosynthetic gene cluster. Moreover, disruption of *rimJ* did not abolish rimocidin production, and this is consistent with the existence of another Ccr activity probably related to primary metabolism, as described for the biosynthesis of monensin [31]. Alternatively, butyryl-CoA units could also be provided by catabolism of valine [27]. Ccr activity also plays a role in the formation of ethylmalonyl-CoA units (via butyryl-CoA carboxylase), which can account for the ethyl side chain of rimocidin and CE-108 being introduced by the last extender module (see Figure 6). The reduction of the availability of etylmalonyl-CoA and butyryl-CoA units in the *rimJ* mutant might have facilitated incorporation of an alternative unit by the loading module and/or the last extender module eliciting overproduction of a new tetraene with lower retention time, as shown in Figure 5.

In the proposed model for rimocidin and CE-108 biosynthesis (Figure 6), the PKS loading module seems to play a crucial role in determining the balance of the two tetraenes in the fermentation broth. The protein can probably recognize both acetyl-CoA and butyryl-CoA as starter units, giving rise to the methyl or propyl side chains of the resulting polyenes. As in other polyene biosynthetic pathways [21–23], the loading module for rimocidin/CE-108 biosynthesis is on a polypeptide independent of those carrying the condensation steps; in this loading module the cysteine residue of the conserved active site is replaced by a serine (KS^S). This change seems to generate a PKS with much lower condensing activity while still retaining some decarboxylative activity [14, 33]. As for NysA [34], RimA alternatively could also recognize dicarboxylic acyl-CoA esters as starter units and then decarboxylate either malonyl-CoA or ethylmalonyl-CoA to provide the starter units for CE-108 and rimocidin biosynthesis, respectively. The organization of RimA and PimS0 [21] as loading modules is highly conserved. Despite the head-to-tail similarities that comprise the domains CoL-ACP-KS^S-AT-ACP on both PKSs, with no other structural differences, RimA has a broad specificity in choosing the starter units, which has also been reported for the loading module of AVRS1 [35]. RimA is shorter than its homolog PimS0, but the missing amino acids at the carboxyl end are located in a separate polypeptide (RimC), which is similar to a fragment of the carboxyl end of PimS0. We have no explanation for the putative role of this small polypeptide, but it might reflect a remnant of a common ancestor. Because disruption of *rimC* produced differences in the ratio of rimocidin and CE-108, RimC might choose the starter units for RimA or, alternatively, modify the balance of carboxylic acids inside the cell. The presence of a gene coding for a crotonyl-CoA reductase (Ccr) within the *rim* cluster argues in favor of the importance of such availability in the choice of one or the other unit. Interestingly, linked to *rimJ* is ORF1, which encodes a putative transposase; the presence of this gene within the *rim* cluster points to the possibility that *rimJ* could have been recruited from another chromosomal location.

Significance

The loading module for rimocidin and CE-108 biosynthesis seems to have a relaxed specificity in recogniz-

ing starter units. This ability would make this strain an attractive tool for generating new polyene compounds by engineering other related clusters. Unlike other polyene producers, *S. diastaticus* var. 108 is amenable to genetic manipulation and thus can be used as recipient of foreign DNA in combinatorial biosynthesis. This, and the broad specificity of the loading module, makes the producer strain a good candidate for hosting recombinant genes in attempts to generate novel compounds by combinatorial biosynthesis.

Experimental Procedures

Bacterial Strains, Cloning Vectors, and Growth Conditions

Bacterial strains and plasmids are described in Table 1. *Streptomyces diastaticus* var. 108 and its derivatives were routinely cultured in R5 [18] and SYM2 [36] media. *Streptomyces lividans* TK21 was used for propagation of PM1 and KC859 phages. *E. coli* strains were grown in Luria-Bertani (LB) agar or in LB broth [37]. *Penicillium chrysogenum* ATCC 10003 was used for testing antifungal activity and was grown in MPDA medium (2% malt extract, 2% glucose, 0.1% Bactopeptone). For gene disruption experiments, the phage PM1 [38] was used for insertional inactivation and KC859 [39] as control; the corresponding constructions are indicated below.

Genetic Procedures

The *E. coli* strains were grown and transformed as described elsewhere [37, 42]. *Streptomyces* strains were manipulated as described previously [18, 43]. RNAs were extracted from cultures grown on solid medium (SYM1, [36]). Other methods were as in Kieser et al. [18].

Construction of a Gene Library

For the construction of the gene library, genomic DNA of *S. diastaticus* var. 108 was partially digested with Sau3AI and size fractionated by sucrose gradient centrifugation. Fractions with an average size of 20 kbp were pooled and ligated into the λ EMBL4 phage [40] previously digested with BamHI and SalI. The ligation mixture was in vitro packaged using the Promega λ DNA Packaging System (cat. # K3154) following the manufacturer's recommendations and used to transfect *E. coli* XL1-blue MRA.

Construction of a Homologous DNA Probe for Screening *S. diastaticus* var. 108 Genomic Library

Two primers (PimD and PimR) were designed matching the consensus sequences generated by multiple alignments between several known type I PKSs (from the pimaricin and erythromycin gene clusters) overlapping several KS domains. The primers were as follows: PimD, 5′-CGC GCC GTC CTG GTT GAC CGC CG-3′; PimR, 5′-ATG GAC CCG CAG CAG CGG CTG CT-3′. The PCR mixture (50 μl) contained 0.1 μg *S. diastaticus* var. 108 total DNA, 10 pm each PimD and PimR primers, 200 μM of each dNTP, Tris HCl (pH 8), KCl 0.1 M, and 2 U of the *Taq* DNA polymerase. PCR was performed with 1 cycle of denaturation (5 min to 95°C), 5 cycles of 2 min to 97°C, 1 min to 55°C and 1 min to 72°C, and 20 cycles of 2 min to 95°C, 1 min to 55°C and 1 min to 72°C, and 1 cycle of final extension of 7 min to 72°C.

S1 Endonuclease Assays

High-resolution S1 mapping was performed as described previously [41]. The probes were labeled in a single strand by PCR amplification; the corresponding oligonucleotide was previously 5′ labeled with γ-^{32}P-ATP using polynucleotide kinase. For DNA/RNA hybridization, 50 μg of total RNA was mixed with 5000 cpm of ^{32}P-labeled probe.

Construction of Disruptant Strains

The disruptant strains were constructed by inserting an internal fragment of the target gene into the PM1 phage, previously cloned in an intermediate *E. coli* plasmid (see Table 1). To prevent polar effects during disruption, the cloned fragment was inserted downstream of the erythromycin promoter before generating the final construction in PM1 (PM1-751). The recombinant phages were transfected into *S. lividans* TK21, [18] and those carrying the desired DNA were selected. *S. diastaticus* var. 108 was infected with the selected phages and allowed to sporulate before selecting the corresponding lysogens, as previously described [18].

Assay of CE-108 and Rimocidin Production and Biological Activity

0.2 ml of total culture was extracted with 0.8 ml of methanol. The extracts were filtered and the quantitative determination of polyenes was performed with a Waters 600S Controller HPLC equipped with a Waters 996 diode array, as described elsewhere [1].

HPLC-MS Assays

The mass spectra were determined in an 1100MSD HPLC connected to a quadrupole Agilent Technology Detector using electrospray as source and a positive ionization mode. The chromatographic conditions were the same as described above.

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References

1. Pérez-Zúñiga, F.J., Seco, E.M., Cuesta, T., Degenhardt, F., Rohr, J., Vallín, C., Iznaga, Y., Pérez, M.E., González, L., and Malpartida, F. (2004). CE-108, a new macrolide tetraene antibiotic. J. Antibiot. (Tokyo), in press.
2. Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., et al. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature *417*, 141–147.
3. Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M., and Omura, S. (2003). Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. Nat. Biotechnol. *21*, 526–531.
4. Cropp, A., Chen, S., Liu, H., Zhang, W., and Reynolds, K.A. (2001). Genetic approaches for controlling ratios of related polyketide products in fermentation processes. J. Ind. Microbiol. Biotechnol. *27*, 368–377.
5. Hopwood, D.A. (1999). Forty years of genetics with Streptomyces: from in vivo through in vitro to in silico. Microbiol. *145*, 2183–2202.
6. Cortés, J., Haydock, S.F., Roberts, G.A., Bevitt, D.J., and Leadlay, P.F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. Nature *348*, 176–178.
7. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J., and Katz, L. (1991). Modular organisation of genes required for complex polyketide biosynthesis. Science *252*, 675–679.
8. Bevitt, D.J., Cortes, J., Haydock, S.F., and Leadlay, P.F. (1992). 6-Deoxyerythronolide-B synthase 2 from *Saccharopolyspora erythraea*. Cloning of the structural gene, sequence analysis and inferred domain structure of the multifunctional enzyme. Eur. J. Biochem. *204*, 39–49.
9. Hopwood, D.A. (1997). Genetic contributions to understanding polyketide synthases. Chem. Rev. *97*, 2465–2497.
10. Gil, J.A., and Martín, J.F. (1997). Polyene antibiotics. In Biotechnology of Antibiotics, W.R. Strohl, ed. (New York: Marcel Dekker), pp. 551–575.
11. Hamilton-Miller, J.M.T. (1973). Chemistry and biology of the polyene macrolide antibiotics. Bacteriol. Rev. *37*, 166–196.
12. Bolard, J. (1986). How do the polyene macrolide antibiotics

affect the cellular membrane properties? Biochim. Biophys. Acta *864*, 257–304.
13. Nair, M.G., Putnam, A.R., Mishra, S.K., Mulks, M.H., Taft, W.H., Keller, J.E., Miller, J.R., Zhu, P.P., Meinhart, J.D., and Lynn, D.G. (1989). Faerifungin: A new broad-spectrum antibiotic from *Streptomyces griseus* var. *autotrophicus*. J. Nat. Prod. *52*, 797–809.
14. Aparicio, J.F., Caffrey, P., Gil, J.A., and Zotchev, S.B. (2003). Polyene antibiotic biosynthesis gene cluster. Appl. Microbiol. Biotechnol. *61*, 179–188.
15. Davisson, J.W., Tanner, F.W., and Finlay, A.C. (1951). Rimocidin, a new antibiotic. Antibiot. Chemother. *1*, 289–290.
16. Cope, A.C., Axen, U., and Burrows, E.P. (1966). Rimocidin II. Oxygenation pattern of the aglycone. J. Am. Chem. Soc. *88*, 4221–4227.
17. Sowinski, P., Pawlak, J., and Borowski, E. (2003). Stereostructure of Rimocidin. J. Antibiot. (Tokyo) *48*, 1288–1291.
18. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical Streptomyces Genetics (Norwich, UK: The John Innes Foundation Norwich).
19. Muth, G., Nubbaumer, B., Wohlleben, W., and Puhler, A. (1989). A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. Mol. Gen. Genet. *219*, 341–348.
20. Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene *116*, 43–49.
21. Aparicio, J.F., Colina, A.J., Ceballos, E., and Martín, J.F. (1999). The biosynthetic gene cluster for the 26-membered ring polyene macrolide pimaricin: a new polyketide synthase organization encoded by two subclusters separated by functionalization genes. J. Biol. Chem. *274*, 10133–10139.
22. Brautaset, T., Sekurova, O.N., Sletta, H., Ellingsen, T.E., Strom, A.R., Valla, S., and Zotchev, S.B. (2000). Biosynthesis of the polyene antifungal antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway. Chem. Biol. *7*, 395–403.
23. Caffrey, P., Lynch, S., Flood, E., Finnan, S., and Oliynyk, M. (2001). Amphotericin biosynthesis in *Streptomyces nodosus*: deductions from analysis of polyketide synthase and late genes. Chem. Biol. *8*, 713–723.
24. Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., et al. (2001). Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Proc. Natl. Acad. Sci. USA *98*, 12215–12220.
25. Aparicio, J.F., Fouces, R., Mendes, M.V., Olivera, N., and Martín, J.F. (2000). A complex multienzyme system encoded by five polyketide synthase genes is involved in the biosynthesis of the 26-membered polyene macrolide pimaricin in *Streptomyces natalensis*. Chem. Biol. *7*, 895–905.
26. O'Keefe, D.P., and Harder, P.A. (1991). Occurrence and biological function of cytochrome-P450 monooxygenases in the actinomycetes. Mol. Microbiol. *5*, 2099–2105.
27. Wallace, K.K., Bao, Z.Y., Dai, H., Digate, R., Schuler, G., Speedie, M.K., and Reynolds, K.A. (1995). Purification of crotonyl-CoA reductase from *Streptomyces collinus* and cloning, sequencing and expression of the corresponding gene in *Escherichia coli*. Eur. J. Biochem. *233*, 954–962.
28. Ikeno, S., Tsuji, T., Higashide, K., Kinoshita, N., Hamada, M., and Hori, M. (1998). A 7,6 kb DNA region from *Streptomyces kasugaensis* M338–M1 includes some genes responsible for kasugamycin biosynthesis. J. Antibiot. (Tokyo) *51*, 341–352.
29. Cañedo, L.M., Costa, L., Criado, L.M., Fernandez Puentes, J.L., and Moreno, M.A. (2000). AB-400, a new tetraene macrolide isolated from *Streptomyces costae*. J. Antibiot (Tokyo) *53*, 623–626.
30. Haydock, S., Aparicio, J.F., Molnar, I., Schwecke, T., Konig, A., Marsden, A.F.A., Galloway, I.S., Staunton, J., and Leadlay, P.F. (1995). Divergent sequence motifs correlated with the substrate specificity of (methyl)malonyl-CoA:acyl carrier protein transacylase domains in modular polyketide synthases. FEBS Lett. *374*, 246–248.
31. Liu, H., and Reynolds, K.A. (1999). Role of crotonyl coenzyme A reductase in determining the ratio of polyketides monensin A and monensin B produced by *Streptomyces cinnamonensis*. J. Bacteriol. *181*, 6806–6813.
32. Denoya, C.D., Fedechko, R.W., Hafner, E.W., McArthur, H.A., Morgenstern, M.R., Skinner, D.D., Stutzman-Engwall, K., Wax, R.G., and Wernau, W.C. (1995). A second branched-chain alpha-keto acid dehydrogenase gene cluster (*bkdFGH*) from *Streptomyces avermitilis*: its relationship to avermectin biosynthesis and the construction of a *bkdF* mutant suitable for the production of novel antiparasitic avermectins. J. Bacteriol. *177*, 3504–3511.
33. Witkowski, A., Joshi, A.K., Lindqvist, Y., and Smith, S. (1999). Conversion of a β-ketoacyl synthase to a malonyl decarboxylase by replacement of the active-site cysteine with glutamine. Biochemistry *38*, 11643–11650.
34. Brautaset, T., Borgos, S.E.F., Sletta, H., Ellingsen, T.E., and Zotchev, S.B. (2003). Site-specific mutagenesis and domain substitutions in the loading module of the nystatin polyketide synthase, and their effects on nystatin biosynthesis in *Streptomyces noursei*. J. Biol. Chem. *278*, 14913–14919.
35. Marsden, A.F., Wilkinson, B., Cortes, J., Dunster, N.J., Staunton, J., and Leadlay, P.F. (1998). Engineering broader specificity into an antibiotic-producing polyketide synthase. Science *279*, 199–202.
36. Atlas, R.M. (1993). Microbiological Media (Boca Raton, FL: CRC Press).
37. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press).
38. Malpartida, F., and Hopwood, D.A. (1986). Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. *205*, 66–73.
39. Angell, S., Schwarz, E., and Bibb, M.J. (1992). The glucose kinase gene of *Streptomyces coelicolor* A3(2): its nucleotide sequence, transcriptional analysis and role in glucose repression. Mol. Microbiol. *6*, 2833–2844.
40. Frischauf, A.M., Lehrach, H., Poustka, A., and Murray, N. (1992). Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. *170*, 827–842.
41. Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, Third Edition (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press).
42. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene *33*, 103–119.
43. Davis, N.K., and Chater, K.F. (1990). Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. Mol. Microbiol. *4*, 1679–1691.

Accession Numbers

The DNA sequence for an unidentified polyketide gene cluster carrying *mnp1*, *mnp2*, and *mnp3* and the 5′ end of *mnp4* was deposited in GenBank under accession number AY423269. The DNA sequence of 19055 bp including the CE-108 and rimocidin gene cluster was deposited under accession number AY442225.