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Insights into Polyether Biosynthesis from Analysis of the Nigericin Biosynthetic Gene Cluster in *Streptomyces* sp. DSM4137

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SUMMARY

Nigericin was among the first polyether ionophores to be discovered, but its biosynthesis remains obscure. The biosynthetic gene cluster for nigericin has been serendipitously cloned from *Streptomyces* sp. DSM4137, and deletion of this gene cluster abolished the production of both nigericin and the closely related metabolite abierixin. Detailed comparison of the nigericin biosynthetic genes with their counterparts in the biosynthetic clusters for other polyketides has prompted a significant revision of the proposed common pathway for polyether biosynthesis. In particular, we present evidence that in nigericin, nanchangmycin, and monensin, an unusual ketosynthase-like protein, KSX, transfers the initially formed linear polyketide chain to a discrete acyl carrier protein, ACPX, for oxidative cyclization. Consistent with this, deletion of either *monACPX* or *monKSX* from the monensin gene cluster effectively abolished monensin A biosynthesis.

INTRODUCTION

Polyether ionophores, such as nigericin (**1**), abierixin (**2**), monensin A (**3**), and nanchangmycin (**4**) (Figure 1), constitute a family of over 120 structurally related natural products with the ability to selectively chelate metal ions and transport them across cell membranes [1]. For example, nigericin, first described in 1951 [2], exerts diverse potent effects on cells through its ability to mediate neutral H^+/K^+ exchange across biological membranes. Polyethers have widespread application in veterinary medicine and animal husbandry and more recently have been identified as agents with activity against drug-resistant strains of malaria [3, 4]. Two polyether biosynthetic gene clusters have so far been characterized, namely, those encoding the biosynthesis of monensin A (**3**) [5, 6] and nanchangmycin (**4**) [7] (dianemycin), respectively. The information obtained from a detailed examination of these biosynthetic clusters has begun to shed light on the mechanism of polyether biosynthesis, and in particular the oxidation of the polyketide chain and its subsequent cyclization [6, 8, 9] to form the characteristic ether rings.

It has been demonstrated that the polyketide skeletons of monensin (**3**) and nanchangmycin (**4**) are generated as for other complex polyketides, by the action of large, modular polyketide synthases (PKSs). Each multifunctional module is able to perform one cycle of chain extension by adding the appropriate extender unit and by carrying out the appropriate level of stereospecific reduction. Variation of both the extender unit and the degree and stereospecificity of the reductive steps at each module enables a huge diversity of complex natural products to be produced by a common mechanism. In the case of monensin A (**3**), the PKS produces a functionalized, branched C_{26} chain containing three double bonds. These double bonds are epoxidized by a flavin-dependent epoxidase, MonCI, to give a putative triepoxide [6, 9]. A homologous epoxidase, NanO, is encoded in the gene cluster for nanchangmycin (**4**) [7]. The novel proteins, MonBI and MonBII (and their proposed counterpart in the nanchangmycin cluster, NanI), have been implicated in ensuring that the cyclization of the multiepoxide intermediate proceeds to give the polyether of the correct stereochemistry [10]. For nanchangmycin (**4**), it has been proposed that the oxidation and cyclization steps take place while the polyketide is anchored to a discrete acyl carrier protein (ACP), NanA10 [7], which also participates in one round of polyketide chain extension. Surprisingly, neither polyether PKS contains the expected intrinsic thioesterase (TE) domain located at the C terminus of the final extension module. Chain release of nanchangmycin (**4**) was originally proposed as being catalyzed by a novel, metal-dependent "chain release domain," located at the end of the final module of the nanchangmycin PKS [7].

We aimed to shed further light on the detailed mechanisms of polyether biosynthesis by characterizing additional clusters of this class. The initial identification of a gene cluster from DSM4137 with very significant homology to that of the published polyether clusters [11] provided the ideal opportunity to do this. It also affords additional genetic material for future biosynthetic engineering to make novel polyethers. The DSM4137 strain was not previously known to produce a polyether, but it was hoped that analysis of the cluster would reveal the likely identity

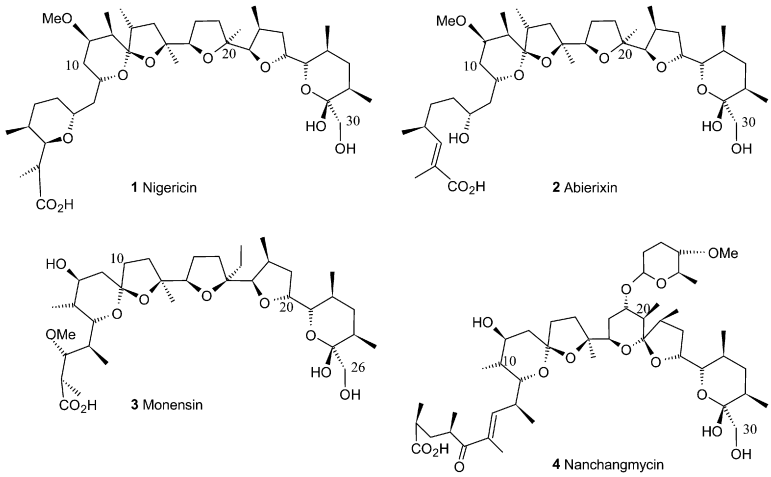

Figure 1. Structures of the Polyethers Nigericin, Abierixin, Monensin, and Nanchangmycin

of the metabolite and enable us to search for it in fermentation broths. This approach, as detailed below, was successful and allowed us to establish that this is an active nigericin biosynthetic cluster. An unexpected discovery from this analysis was the identification of sequence motifs that reinforce the idea [7] that the putative PKS-bound linear intermediate in polyether biosynthesis is transferred by an unusual ketosynthase-like protein to a discrete ACP, on which oxidative cyclization takes place and from which the polyether is finally released by hydrolysis.

RESULTS AND DISCUSSION

Cloning and Sequencing of the Nigericin Biosynthetic Cluster

A genomic library of DSM4137 had previously been screened by hybridization with a 9.5 kbp DNA fragment of *rapA* from the rapamycin PKS of *Streptomyces hygroscopicus* [11]. Preliminary characterization of the hybridizing clones identified multiple loci encoding complex polyketide biosynthesis. This is consistent with previous chemical screening, which has shown that *S. hygroscopicus* and related strains often encode biosynthesis of diverse complex polyketides [12, 13]. DNA sequencing of four overlapping cosmid clones designated AA12, 2G4, 7C2, and NC3 led to the complete sequencing and characterization of one such locus that spanned ~100 kbp and was found to have many features in common with previously sequenced polyether biosynthetic gene clusters. Detailed in silico examination of the predicted modular PKS gene products (see below) was consistent with the cluster encoding the biosynthesis of the polyether nigericin and/or abierixin [14]. The deduced organization of the clustered genes and their putative roles in nigericin biosynthesis are shown in Figure 2.

Evidence that the Biosynthetic Gene Cluster Encodes Biosynthesis of Both Nigericin and Abierixin

The precise margins of the gene cluster can only be conclusively determined by further in vivo genetic analysis. However, in silico analysis allows us a degree of confidence in defining the cluster limits (Table 1). Upstream we have sequenced an additional 15 kbp beyond a gene designated *nigR*, which is homologous to genes found in both the monensin and nanchangmycin gene clusters. Immediately upstream of *nigR* lies a gene with a high degree of sequence similarity to a prolyl-dipeptidyl aminopeptidase of *Streptomyces avermitilis* (BAC69385, 45% identity). It is very unlikely that this gene is involved in polyether biosynthesis. The remaining 15 kbp upstream of *nigR* contains no genes that have previously been identified within complex polyketide gene clusters. The downstream limit of the cluster is identified by the identification of a gene with sequence similarity to an aminopeptidase of *Bacillus thailandensis* (ABC38196, 27% identity). The cluster as sequenced does not contain any recognizable genes for precursor supply, for self-resistance to the antibiotic, or for antibiotic export. *Streptomyces* sp. DSM4137 encodes a very large number of polyketide biosynthetic clusters that have now all been at least partially characterized. Although this cluster is the only plausible candidate, it was nevertheless particularly important to establish that the cluster was active and was correctly identified as governing the synthesis of **1** and/or **2**.

Analysis of the ethyl acetate extracts of the supernatant of *Streptomyces* sp. DSM4137 cultured in medium SM16-1, by using LC-MS, revealed the presence of two natural products, A and B, with the same mass, corresponding to that expected for the sodiated ion of nigericin (**1**), *m/z* 747.5 (Figure 3B). MS/MS analysis of these parent ions revealed two indistinguishable spectra, where the only major fragmentation was that due to water loss, *m/z* 729.4. The MS^3 spectra of the 729.4 fragment, however, revealed clear differences. With the same collision energy, product A was more resistant to the collision-induced dissociation, and the parent ion was still the base peak in the spectrum, while the CO_2-loss fragment (*m/z* 685.4) was only ~10% of the base peak. Product B was more labile to the collision-induced dissociation, and the CO_2-loss fragment (*m/z* 685.4) was the base peak in the spectrum (see Figures S3B and S3C in the Supplemental Data

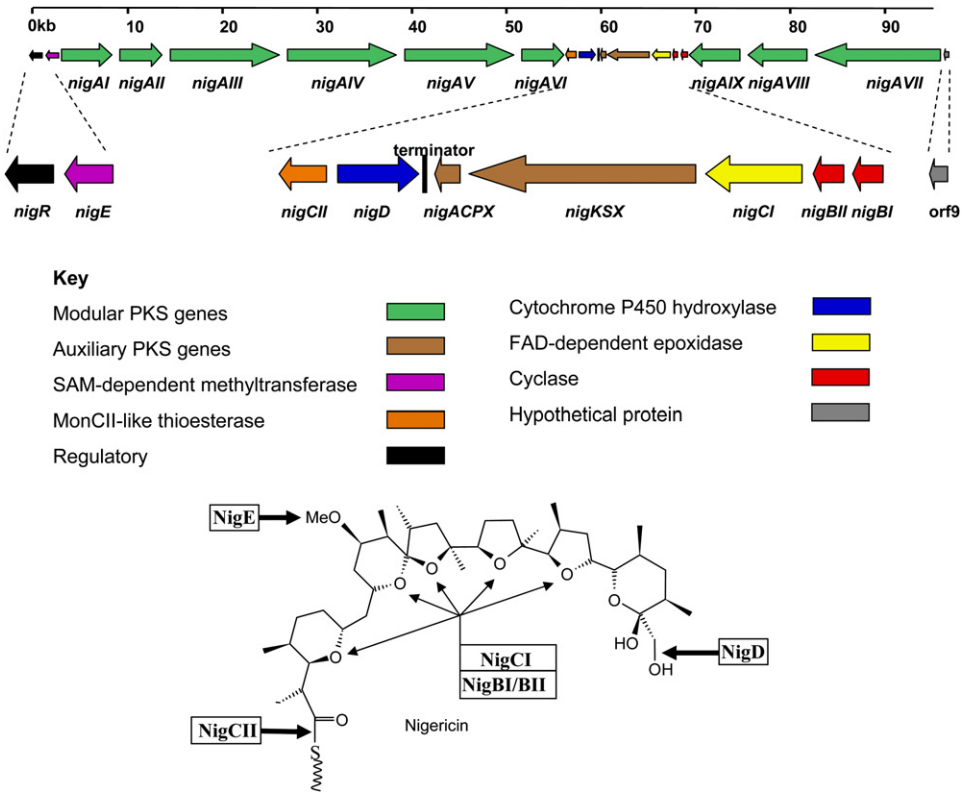

Figure 2. The Organization of the Nigericin Biosynthetic Gene Cluster

The proposed functions of the genes shown are indicated by their color; modular PKS genes are shown in green, auxiliary PKS genes are shown in brown, the SAM-dependent methyltranferase is shown in pink, the thioesterase is shown in orange, the cytochrome P450 hydroxylase is shown in blue, the FAD-dependent epoxidase is shown in yellow, the cyclases are shown in red, and a gene encoding a hypothetical protein is shown in gray.

available with this article online). In order to confirm that the two observed products are nigericin and abierixin, an authentic sample of nigericin was analyzed by LC-MS under the same conditions as for the DSM4137 extracts. The nigericin standard, as anticipated, eluted with the same retention time as that of product B from the DSM4137 extracts (Figure 3A). An MS/MS spectrum of the sodiated parent ion (*m/z* 747.5) and the MS^3 spectrum of the ion after water loss (*m/z* 729.4) were identical to those of product B (see Figure S3A). Therefore, product B from the extracts of DSM4137 is indeed nigericin. No authentic sample of abierixin was available, but the relative retention times of the two compounds as well as their similar mass spectra are consistent with the hypothesis that product A is abierixin. In previous isolations of abierixin (**2**), the supernatant was acidified prior to extraction, whereas, in this case, the supernatant was extracted directly. In the previous studies, only traces of abierixin (**2**) were found compared to ~100 mg L^{-1} nigericin (**1**) [14], whereas, here, approximately equal amounts of nigericin (**1**) and abierixin (**2**) were found. This may be due to strain differences, but perhaps also in part to the acidic conditions previously used, which might affect the relative amounts of nigericin (**1**) and abierixin (**2**). These data taken together provide both proof for the production of the polyether nigericin (**1**) and indications for the production of the polyether abierixin (**2**) by the DSM4137 strain, as predicted from the sequence analysis.

To confirm this, an extensive deletion of the PKS-encoding region of the gene cluster was then undertaken, as described in Experimental Procedures (see also Figure S1). Individual recombinants were checked by Southern hybridization (see also Figure S2), and their products of fermentation were analyzed. Under conditions at which the parent strain produced significant levels of both **1** and **2**, these recombinants produced neither compound, as judged by LC-MS analysis (Figure 3C), indicating that both are products of the same PKS.

Organization of the Nigericin PKS

The biosynthesis of the polyketide backbone of nigericin is encoded by nine large ORFs, *nigAI–nigAIX*, arranged in two convergent groups that encode, respectively, the multimodular PKS multienzymes NigAI–NigAIX. Examination of the amino acid sequence of the individual acyltransferase (AT) domains allows the nature of the extender unit (malonyl versus methylmalonyl) to be deduced [15, 16]. Examination of the reductive loop domains (ketoreductase [KR], dehydratase [DH], and enoyl reductase [ER]) allows the level of reduction to be deduced for

Table 1. Deduced Functions of ORFs in the Nigericin Biosynthetic Gene Cluster

ORF	Number of Amino Acids	Proposed Function in Nigericin/Abierixin Biosynthesis	Closest Protein Homologs, *Organism*, Identity, Similarity (%)	Accession Number
L6	Partial		Sigma factor, *Frankia* sp. CcI3, 55, 68	ABD11893
L5	267		Protein phosphatase, *S. coelicolor*, 41, 56	CAC36845
L4	869		Clp protease, *S. coelicolor*, 68, 78	CAA19619
L3	389		Putative lipoprotein, *S. coelicolor*, 50, 64	CAB63190
L2	207		MerR transcriptional regulator, *M. loti*, 53, 61	BAB48402
L1	628		Prolyl-dipeptidyl aminopeptidase, *S. avermitilis*, 45, 59	BAC69385
nigR	256	Regulator	MonR1, *S. cinnamonensis*, 57, 73	AAO65809
			NanR2, *S. nanchangensis*, 57, 70	AAP42854
nigE	270	*O*-methyltransferase	*O*-methyltranferase, *L. aerocolonigenes*, 41, 55	AAN01212
			O-methyltranferase, *S. avermitilis*, 40, 54	BAA84602
nigAI	2415	Polyketide synthase KSQ ATa ACP	NanAI, *S. nanchangensis*, 56, 63	AF521085
		Polyketide synthase KS ATp DH ACP	MonAI, *S. cinnamonensis*, 55, 63	AAO65796
nigAII	4083	Polyketide synthase KS ATa DH KR ACP	NanAIII, *S. nanchangensis*, 58, 67	AAP42857
		Polyketide synthase KS ATp DH ER KR ACP	MonAIII, *S. cinnamonensis*, 57, 66	AAO65798
nigAIII	4053	Polyketide synthase KS ATp DH KR ACP	MonAV, *S. cinnamonensis*, 50, 59	AAO65800
		Polyketide synthase KS ATa DH ER KR ACP	MonAIV, *S. cinnamonensis*, 50, 59	AAO65799
nigAIV	4055	Polyketide synthase KS ATp DH KR ACP	MonAV, *S. cinnamonensis*, 50, 59	AAO65800
		Polyketide synthase KS ATa DH ER KR ACP	MonAIV, *S. cinnamonensis*, 50, 59	AAO65799
nigAV	4045	Polyketide synthase KS ATp DH KR ACP	MonAV, *S. cinnamonensis*, 51, 59	AAO65800
		Polyketide synthase KS ATp DH ER KR ACP	MonAIV, *S. cinnamonensis*, 48, 57	AAO65799
nigAVI	1701	Polyketide synthase KS ATp KR ACP	NanA7, *S. nanchangensis*, 47, 56	AAP42867
			NanA8, *S. nanchangensis*, 47, 58	AAP42874
nigCII	288	Thioesterase	NanE, *S. nanchangensis*, 42, 54	AAP42868
			MonCII, *S. cinnamonensis*, 40, 53	AAO65791
nigD	419	Cytochrome P450	MonD, *S. cinnamonensis*, 61, 75	AAO65808
			NanP, *S. nanchangensis*, 58, 70	AAP42875
nigAXI	102	Polyketide synthase ACP	ACPX, *S. cinnamonensis*, 61, 75	AAO65789
			NanA10, *S. nanchangensis*, 40, 62	AAP42869
nigAX	1299	Polyketide synthase KS ATa KR	PteA3, *S. avermitilis* MA-4680, 49, 60	BAC68127
			NanA6, *S. nanchangensis*, 49, 58	AAP42860
nigCI	476	Epoxidase	NanO, *S. nanchangensis*, 59, 72	AAP42870
			MonCI, *S. cinnamonensis*, 58, 71	AAO65803
nigBII	155	Cyclase	MonBII, *S. cinnamonensis*, 50, 65	AAO65804
			NanI, *S. nanchangensis*, 45, 57	AAP42871
nigBI	195	Cyclase	MonBI, *S. cinnamonensis*, 52, 70	AAO65805
			NanI, *S. nanchangensis*, 44, 56	AAP42871
nigAIX	1992	Polyketide synthase KS ATp DH KR ACP	MonAIV, *S. cinnamonensis*, 49, 58	AAO65799
			MonAV, *S. cinnamonensis*, 47, 58	AAO65800
nigAVIII	2206	Polyketide synthase KS ATp DH ER KR ACP	MonAIII, *S. cinnamonensis*, 50, 59	AAO65798

Table 1. *Continued*

ORF	Number of Amino Acids	Proposed Function in Nigericin/Abierixin Biosynthesis	Closest Protein Homologs, *Organism*, Identity, Similarity (%)	Accession Number
			FscB, *S.* sp. FR-008, 48, 58	AAQ82565
nigAVII	4787	Polyketide synthase KS ATa KR ACP KS ATa	PteA4, *S. avermitilis*, 47, 57	NP_821591
		Polyketide synthase KR ACP KS ATa KR ACP	PimS2, *S. natalensis*, 44, 56	CAC20921
ORF9	182		nlmOI, unknown function, *S. nanchangensis*, 30, 46	AAS46349
R1	467		Aminopeptidase, *B. thailandensis*, 27, 40	ABC38196
R2	402		IS605 family transposase, *T. fusca*, 75, 86	AAZ54741
R3	185		Unknown function, *C. facinita*, 60, 73	EAS36552
R4	537		Acyl CoA synthetase, *N. facinica*, 53, 63	BAD59000
R5	122		Arsenate reductase, *S. coelicolor*, 85, 94	CAB43945
R6	101		Unknown function, *S. coelicolor*, 69, 79	CAB90852
R7	159		Unknown function, *K. radiotolerans*, 34, 46	EAM72720
R8	199		Unknown function, *S. avermitilis*, 43, 56	BAC72489
R9	301		Unknown function, *M. avium*, 32, 43	AAS03875
R10	122		Transcriptional regulator, *T. fusca*, 51, 65	AAZ54605
R11	176		Tellurium resistance protein, *S. avermitilis*, 63, 74	BAC70171
R12	285		Unknown function, *S. avermitilis*, 47, 55	BAC69717
R13	422		Unknown function, *S. avermitilis*, 67, 79	BAC69716
R14	937		ABC transporter, *S. avermitilis*, 71, 80	BAC73056
R15	291		ABC transporter, *S. avermitilis*, 49, 54	BAC73057
R16	394		Mono-oxygenase, *M. tuberculosis*, 46, 60	AAK45557

Inactive PKS domains are shown in italics. The extender unit specificity of AT domains is denoted by "p" (methylmalonate/propionate) or "a" (malonate/acetate). ORFs L1–L6 lie upstream of the cluster (the left-hand side of Figure 2), ORFs R1–R16 lie downstream of the cluster (the right-hand side of Figure 2). They are not thought to be involved in nigericin biosynthesis.

each such extension. From analysis of the domain complement of the PKS, starting from NigAI incorporating the loading module and reading through to NigAVI, followed by the second group, NigAVII–NigAIX, one would predict the assembly of a polyketide chain entirely consistent with the structure of the presumed polyketide precursor to nigericin (**1**) or the closely related abierixin (**2**). Thus, *nigAI* encodes the loading domain and module 1 of the polyketide chain extension, *nigAII* encodes the module for the 2nd extension module, *nigAIII* encodes modules for extensions 3 and 4, *nigAIV* encodes modules 5 and 6, *nigAV* encodes modules 7 and 8, *nigAVI* encodes the 9th extension module, *nigAVII* encodes extension modules 10–12, *nigAVIII* encodes extension module 13, and *nigAIX* encodes the 14th and final extension module (Table 1).

The arrangement of the first six PKS genes in this cluster shows striking similarities to the corresponding PKS genes in the monensin and nanchangmycin clusters (Figure 4) [5, 7]. The genes encode the same number of monomodular and bimodular proteins, arranged in the same order. The level of reductive processing is also the same at each stage, with the exception of NanA4, which appears to contain inactive or redundant versions of the same domains as MonAIV and NigAIV. In the nanchangmycin structure, these differences result in the C-21 keto group, which is the basis for the second spiroketal center, and the C-19 hydroxyl to which the 4-*O*-methyl-L-rhodinose deoxysugar is attached. The sixth PKS, encoding extension module 9, contains an inactive KR in all three cases. The first extension modules in both the nanchangmycin and monensin clusters contain inactive KR domains and redundant DH domains, while NigAI contains a redundant DH, but no KR, domain. The remainder of NigAI displays high homology to NanA1 and MonAI. This natural KR domain deletion occurs at amino acid 2243 of NigAI and corresponds to a region of ~500 amino acids in MonAI and NanA1. The specificity of the AT domains of extension modules 8 and 9 in NigAV and NigAVI is for methylmalonate, despite high overall sequence similarities to their malonate-specific nanchangmycin and monensin counterparts [16].

The organization of the remaining PKS genes is far more divergent across the three clusters, suggesting multiple mechanisms for the evolution of such biosynthetic

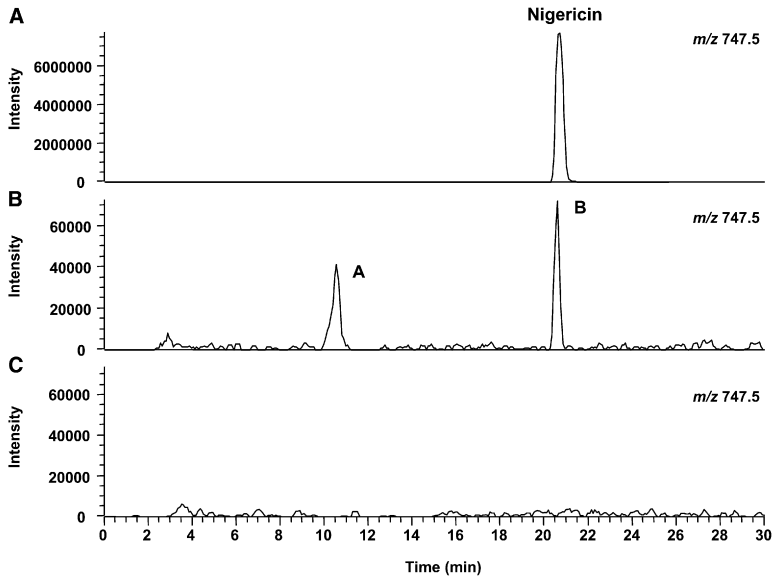

Figure 3. LC-MS Analysis of Nigericin-Related Metabolites Produced by Mutant Strains of *Streptomyces* sp. DSM4137

(A) LC-MS chromatogram of the sodiated ion (*m/z* 747.5) from nigericin standard.

(B) LC-MS chromatogram of the sodiated ion (*m/z* 747.5) from the azalomycin- and meridamycin-deleted strain.

(C) LC-MS chromatogram of the sodiated ion (*m/z* 747.5) from the azalomycin-, meridamycin-, and nigericin-deleted strain.

pathways. It is nevertheless consistent with the unified stereochemical model of polyether biogenesis proposed by Cane and colleagues [17] in which a subgroup of polyethers, named the APPA polyethers, that share the same initial order of subunits (acetate, propionate, propionate, acetate) and the same level of reduction as far as the 12th extension module was identified. These authors also noted the change in the level of reduction at extensions 5 and 6 of nanchangmycin (**4**) (dianemycin) compared to the majority of this class of polyethers.

Organization of Genes Governing Later Steps in Biosynthesis

In addition to the nine large PKS genes required for the synthesis of the polyketide skeleton, the gene cluster was found to encode ten other ORFs, seven of which are located between the two groups of polyether genes. Nine of these ORFs have counterparts in the other known polyether clusters. The immediate product of the action of the PKS is believed, by analogy to the pathway to monensin, to be the full-length linear polyketide (containing four *E* carbon-carbon double bonds) [9], tethered in thioester linkage to the C-terminal ACP domain of the final extension module (module 14). NigCI, the predicted product of *nigCI*, shows high sequence homology to MonCI, the epoxidase responsible for the epoxidation of the polyketide intermediate in monensin biosynthesis. NigCI is therefore predicted to fulfill a similar role in the nigericin pathway.

The products of two small, apparently translationally coupled, genes, *nigBI* (195 amino acids) and *nigBII* (155

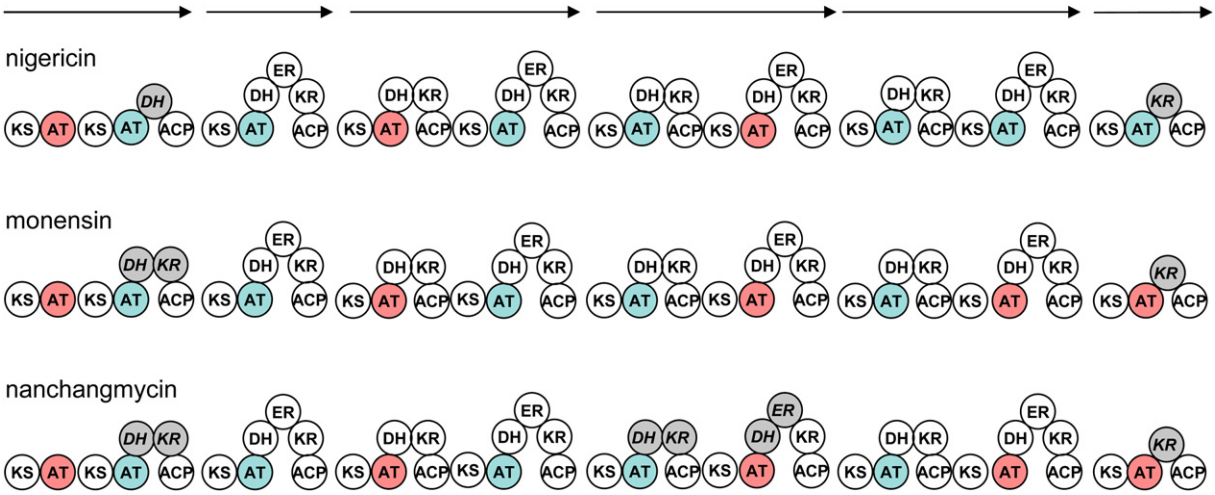

Figure 4. A Comparison of the Organization of the First Nine PKS Modules of Nigericin, Monensin, and Nanchangmycin Biosynthesis

Inactive domains are shaded gray and are named in italics, AT domains specific for malonate extender units are shown in pink, and AT domains specific for methylmalonate extender units are shown in blue.

amino acids), display striking sequence similarity to MonBI and MonBII, respectively. They also display a similar level of sequence similarity to, respectively, the N- and C-terminal domains of NanI (313 amino acids); this arrangement appears to represent a fused protein combining the counterparts of both MonBI and MonBII. NigBI and NigBII also display a significant mutual sequence similarity (31% identity and 27% similarity). Based on a study of the metabolites produced by mutant strains of *Streptomyces cinnamonensis*, the monensin A producer, in which either *monBI* or *monBII* or both *monBI* and *monBII* have been deleted, we have proposed that the two *monB* genes encode novel epoxide hydrolases that help to control the ring opening of the triepoxide intermediate and concomitant polyether ring formation, to produce the correct stereoisomer of monensin (**3**) [10]. NigBI and NigBII are therefore similarly predicted to function as epoxide cyclases. *nigD* encodes a putative cytochrome P450 mono-oxygenase and displays high homology to the enzymes proposed to oxidize the terminal methyl groups in nanchangmycin (C-30) and monensin A (C-26) biosynthesis. The analogous C-30 methyl group is oxidized in the formation of both nigericin (**1**) and abierixin (**2**), and NigD is proposed to carry out this oxidation. This accords with previous work that reported that fermentation of a nigericin-producing strain in the presence of an inhibitor of cytochrome P450 mono-oxygenases led to the accumulation of grisorixin (C-30-dehydroxylnigericin) [18]. This has been proposed to be the last step in the biosynthesis of nigericin [19]. In contrast, abierixin is not bioconverted into nigericin, and it may be a shunt product in which the ring A of nigericin is opened [19] (see also below). *nigCII* lies immediately adjacent to, and is divergently transcribed with respect to, *nigD*. The predicted product shows a high degree of similarity to MonCII of the monensin cluster. It is therefore thought to act as a Te, as recently proposed for MonCII [20] and NanE of the nanchangmycin cluster [21]. Immediately upstream of the first PKS gene, *nigAI*, is *nigE*, encoding a putative *S*-adenosylmethionine (SAM)-dependent *O*-methyltransferase, an obvious candidate for the enzyme catalyzing *O*-methylation of the C-11 hydroxy group. Immediately upstream of *nigE* lies *nigR*. The gene product is presumed to function as a regulator and shows significant homology to both the MonR1 and NanR2 regulators of the monensin and nanchangmycin clusters, respectively. Immediately upstream of *nigAVII* lies a small open reading frame (ORF 9) that is proposed to encode a 182 amino acid protein. The role of this gene product in nigericin biosynthesis is uncertain. There is no homolog in other polyether gene clusters, but a similar gene (AAS46349, 30% identity) has been described in an uncharacterized secondary metabolite cluster in *S. nanchangensis*. The respective timing of the late steps of the biosynthesis of nigericin/abierixin remains to be established.

The Unusual Role of Discrete PKS-like Activities

Until recently [7] it had for many years been tacitly assumed that the oxidative cyclization and other tailoring steps in polyether biosynthesis involve enzyme-free intermediates [17]. However, the discovery that MonCII is a TE that can act on monensyl thioesters [20] suggested the involvement of an enzyme-bound intermediate in these later stages of the pathway of the biosynthesis of the polyether, monensin. MonCII would then act to hydrolyze the monensylthioester from the last ACP of the modular PKS or from another discrete ACP. Similar conclusions have recently been reached for the role of the NanE protein as a TE in nanchangmycin biosynthesis [21].

Such discrete ACPs or closely related peptidyl carrier proteins (PCPs) have previously been uncovered in several biosynthetic pathways to antibiotic natural products: for example, in the provision of the polyketide precursor methoxymalonate [22]; of the pyrrole moiety in pyoluteorin, coumermycin, novobiocin, and clorobiocin biosynthesis [23–25] and nikkomycin biosynthesis [26]; and of intermediates in prodigiosin biosynthesis [23, 27, 28]. Similarly, biosynthesis of the precursor β-hydroxy-tyrosine in the novobiocin [29], balhimycin [30], and teicoplanin [31] pathways takes place on an A-PCP di-domain. The tethering of intermediates to a carrier protein in thioester linkage presumably confers added stability and aids the channeling of such precursors toward formation of the secondary metabolite.

We noted that the nigericin cluster contains an unusual type II (i.e., discrete) ACP, NigACPX, and also an incomplete PKS module, NigKSX, containing an apparently intact KS, an inactive AT domain. These are counterparts of MonACPX and MonKSX of the monensin cluster [6]. The nanchangmycin cluster also contains a KSX, NanA9, and an ACPX, NanA10. It has been previously suggested that the nanchangmycin polyketide chain is transferred to NanA10 and while attached there undergoes the penultimate round of polyketide chain extension involving the KS and AT domains of NanA9. However, the inferred substrate specificity of the AT domain of NanA9 is for malonate, not the methylmalonate unit [7] required by the nanchangmycin structure [15]. Nanchangmycin biosynthesis may involve iteration (obligatory stuttering), a phenomenon in which a single PKS extension module undertakes more than one consecutive round of chain extension [31–35]. Further work will be required to establish if this is the correct explanation.

These shared features of the nigericin, monensin, and nanchangmycin biosynthetic clusters suggested a common mechanism involving the discrete ACP. To obtain further support for the role of the discrete ACP in polyether biosynthesis, we chose to undertake the specific disruption of the *monACPX* gene from the monensin cluster in *S. cinnamonensis* (Experimental Procedures). The use of a hyperproducing strain allowed for particularly convenient and sensitive detection of any polyether-related metabolites accumulated by the mutant strain [9, 10]. The hyperproducing strain produces only monensins A and B. The mutant lacking *monACPX* (BHΔAcpX) was found to produce monensins A and B at ~1000-fold lower levels, accompanied by similar amounts of demethyl-, dehydroxy-, and epi-monensins. These monensin analogs

were previously characterized as fermentation products of mutants specifically disrupted in one or both of the proposed cyclase genes, *monBI* and *monBII* [10]. Complementation of mutant BHΔAcpX with the cloned *monACPX* gene, supplied *in trans* (Experimental Procedures), led to full restoration of monensin production. A second mutant in which the adjacent *monKSX* gene was disrupted (BHΔKSX) was also constructed (Experimental Procedures), and its fermentation gave a similar mixture of metabolites (data not shown). The disruption of the *monKSX* gene could potentially have had a polar effect on the downstream gene, *monACPX*. However, upon complementation of mutant BHΔKSX with *monACPX*, the mixture of metabolites continued to be produced. These results suggest that neither MonACPX nor MonKSX is involved in polyketide chain extension, but that they are important for the correct cyclization of the triepoxide intermediate.

We therefore propose that, for all three of nigericin, monensin, and nanchangmycin, the full-length linear polyketide chain is subsequently transferred from the modular PKS to the type II ACP (NigACPX, MonACPX, or NanA10), catalyzed by the respective KS activity (NigKSX, MonKSX, or NanA9), and then undergoes oxidative cyclization and other tailoring reactions while bound to this carrier protein. There is clear precedent, from previous studies on the biosynthetic pathway to the polyether tetrolide nonactin, for a KS-like enzyme catalyzing acyltransfer rather than C-C bond formation during polyketide biosynthesis [36]. An alternative mechanism is direct, KS-mediated, ACP-to-ACP transfer, as proposed for the observed skipping of an inserted module in an engineered hybrid PKS [37].

Additional support for this mechanistic proposal, of KSX-mediated transfer of the linear polyketide chain to ACPX followed by its oxidative cyclization, came from a close inspection of the amino acid sequences of regions at the C termini of the final extension modules of the PKS (module 14 in the multienzyme NigAIX, module 12 in MonAVIII, and module 14 in NanA11). Such regions, designated as "docking domains" [38], are thought to make specific interactions with partner docking domains at the N termini of downstream subunits, ensuring that the individual proteins within a modular PKS assemble in the correct order. The structure of a docking complex from the erythromycin PKS (DEBS) has recently been characterized by heteronuclear NMR [38]. The structure revealed that the subunits associate through formation of an intermolecular four-α-helix bundle, in which helix 3 of the C-terminal docking domain (Figure 5A) packs against the coiled-coil formed by the N-terminal docking domain; the first two helices of the C-terminal docking domain form another four-α-helix bundle that functions as a dimerization element. As shown by the sequence alignment in Figure 5A, the C termini of NigAIX and MonAVIII show strong sequence homology to the DEBS C-terminal docking domains. MonAVIII has an unusual glycine- and asparagine-rich C-terminal extension [6], but the docking domain model [38] predicts that it would not interfere with docking. NanA11 belongs to a second group of putative docking domains that exhibit overall lower sequence similarity, and for which structural information is not yet available. However, secondary structure prediction with the JPred algorithm [39] supports the presence of a docking α helix in an equivalent position within this group of domains.

Prospective α-helical partners for the C-terminal docking domains were found in the extreme N termini of the unusual KS-like proteins, NanA9, MonKSX, and NigKSX (Figure 5B shows all of the N-terminal partners of the C-terminal docking domains in Figure 5A). Sequence alignment with other N-terminal docking domains shows that, again, the putative docking domains of MonKSX and NigKSX cluster within the DEBS-related sequence group, while that of NanA9 belongs to a second group of N-terminal docking domains. This analysis provides evidence that the KS-like protein docks onto the terminal extension module of the PKS and catalyzes transfer of the full-length polyketide acyl chain from the modular PKS to the cognate ACP (NanA10, MonACPX, or NigACPX). NanA9 appears to have no C-terminal docking domain, and NanA10 (ACP) has no discernible docking domain at either terminus, which further undermines the previous provisional proposal [7]—that these discrete proteins might be involved in polyketide chain extension as an integral part of the modular PKS. Docking domains are also absent from the C-terminal region of NigKSX and MonKSX (NanA9 homologs) and from both the N and C termini of NigACPX and MonACPX (NanA10 homologs).

This revised proposal accounts straightforwardly for the fact that disruption of either *monKSX* or *monACPX* almost abolishes polyether biosynthesis and for the observation that the final extension module of polyether PKSs lacks a conventional pendant C-terminal TE domain for polyketide chain release. Rather, final chain release of the mature polyether is proposed to occur from the discrete ACP after oxidative cyclization [20, 21].

SIGNIFICANCE

Analysis of the nigericin biosynthetic gene cluster and comparison with the pathways to the polyethers monensin and nanchangmycin have highlighted the potential role of unusual PKS-related activities encoded in these clusters and have provided new, to our knowledge, evidence that the KS-like protein catalyzes transfer of the full-length polyketide acyl chain from the modular PKS to a discrete acyl carrier protein (ACP) on which oxidative cyclization to form the characteristic polyether rings takes place. This would be yet another example of the use of such a carrier protein as a mechanistic device for substrate channeling, a familiar feature in the biosynthesis of several other major groups of antibiotics, but one not, to our knowledge, previously found for the late steps of polyketide biosynthesis. These findings also open the way to full deconvolution of the later steps of polyether biosynthesis, and they should accelerate the combinatorial biosynthesis of novel polyethers, as potential leads in the search for new antimalarial drugs.

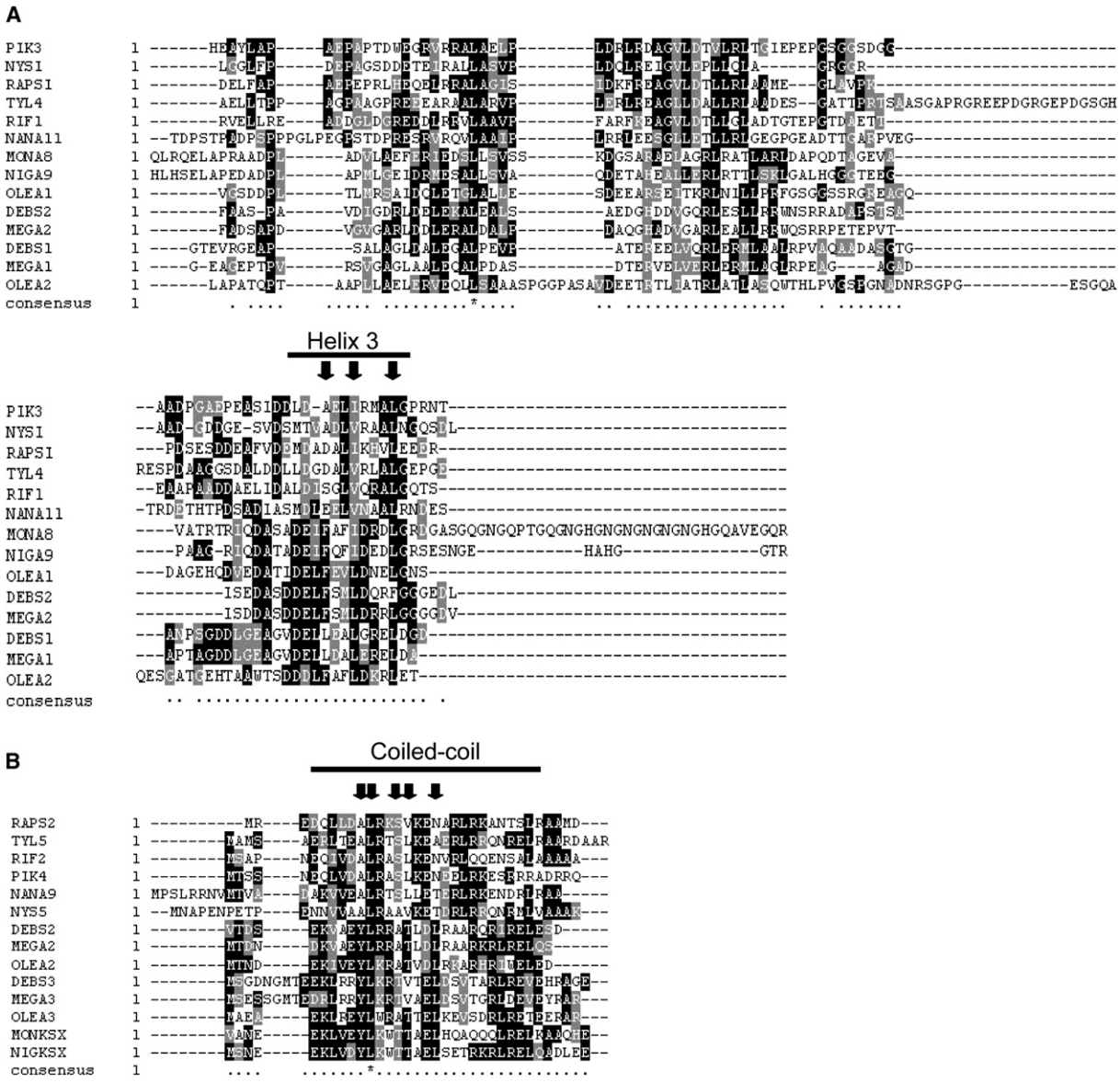

Figure 5. Docking Domains

(A) Multiple alignment of C-terminal docking domains from modular PKSs demonstrates that the docking domains from NigA9 and MonA8 fall into the sequence group with DEBS 2. In contrast, the sequence of the C-terminal docking domain from NanA11 belongs within a putative second group of C-terminal docking domains. The extent of helix 3 (bar) along with the hydrophobic residues present in the docking interface (arrows) are indicated.

(B) Multiple alignment of the N-terminal docking domain partners of the C termini shown in Figure 4A. MonKSX and NigKSX fit into the sequence group with DEBS 3, while NanA9 clusters with the second group of N-terminal docking domains. The extent of the coiled-coil region (bar) as well as the hydrophobic interface residues (arrows) are indicated.

PIK, pikromycin; NYS, nystatin; RAPS, rapamycin; TYL, tylosin; RIF, rifamycin; OLEA; oleandomycin; DEBS, erythromycin.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cloning Vectors

Streptomyces sp. DSM4137 was obtained from the German Microorganism and Cell Culture Collection (DSMZ). A monensin-overproducing strain of *S. cinnamonensis*, A519, was obtained from Prof. I. Agayn (Sofia, Bulgaria) and was used to derive the mutant *S. cinnamonensis* strains described here. The following *Escherichia coli* strains were used: DH10B (GIBCO-BRL, USA) for routine cloning; XL1-Blue MR for cosmid library construction; ET12567 [40] containing the helper plasmid pUZ8002 [41] as a conjugation donor with *Streptomyces*. Routine subcloning was performed in pUC19 [42] or pSG397 [43]. cos2 is a pWE15 [44] (Stratagene, USA)-derived cosmid on which both *monACPX* and *monKSX* are found and was the gift of Dr. M. Oliynyk [6]. The genomic library of *Streptomyces* sp. DSM4137 was created in the SuperCos cosmid vector (Stratagene, USA).

Culture Conditions

E. coli strains were grown on 2xTY medium supplemented with appropriate antibiotics. Liquid *Streptomyces* cultures were routinely grown

in Tryptic Soy Broth (TSB) [45] (Difco Laboratories, USA), and TSBY, SFM [45], and SM16-1 media were used for polyether production. SFM agar and A medium [46] were used for solid cultures. TSBY consists of TSB supplemented with 0.5% (wt/vol) yeast extract. SM16-1 consists of MOPS (20.9 g), L-proline (10.0 g), glucose (20.0 g), NaCl (0.5 g), K_2HPO_4 (2.1 g), EDTA (0.25 g), $MgSO_4.7H_2O$ (0.49 g), $CaCl_2.2H_2O$ (0.029 g), 10× Trace elements No. 1 (1 ml), and Milli-Q water (up to 1 l) and is adjusted to pH 7 with NaOH. $MgCl_2$ (10 mM) is added after autoclaving. 10× Trace elements No. 1 consists of 1 M H_2SO_4 (10 ml), $ZnSO_4.H_2O$ (8.6 g), $MnSO_4.4H_2O$ (2.23 g), H_3BO_3 (0.62 g), $CuSO_4.5H_2O$ (1.25 g), $Na_2MoO_4.2H_2O$ (0.48 g), $CoCl_2.6H_2O$ (0.48 g), $FeSO_4.7H_2O$ (18.0 g), KI (0.83 g), and distilled water (up to 1 l). Ingredients were dissolved in the order given, and the solution was autoclaved prior to use.

Molecular Genetic Procedures

Standard genetic techniques for *E. coli* and in vitro DNA manipulations were followed [47]. Total DNA was isolated from *Streptomyces* by following "procedure B" of Kieser et al. [45]. For the generation of the cosmid library, total DNA was partially digested with Sau3AI, dephosphorylated with shrimp alkaline phosphatase, ligated directly into pSuperCos, and packaged with Gigapack Gold packaging extract (Stratagene, USA) without size fractionation. All procedures were in accordance with the manufacturer's recommendations. Cosmid DNA for complete sequencing was prepared by using Qiagen midi-prep DNA purification kits (Qiagen, Germany) from 50 ml liquid cultures in 2xLB medium with ampicillin and kanamycin selection. The complete sequence of each cosmid was obtained from overlapping Sau3AI fragments obtained from a partial digestion of the parent cosmid. The 2–5 kbp fraction of this digest was eluted from an agarose gel by using the Gene Clean kit (Bio101, BioRad, USA) and was subcloned into pSG397 [43]. The initial colony hybridization of the DSM4137 cosmid library was performed with a digoxigenin-labeled fragment from the rapamycin gene cluster encoding part of the PKS and has been described previously [11].

Deletion of the Entire *nig* Gene Cluster from the Chromosome

Two DNA fragments, a 9 kbp BamHI-KpnI fragment and a 8.6 kbp KpnI-BglII fragment, were cloned from cosmids AA12 and 2G4, respectively, covering each end of the *nig* gene cluster, then inserted into the unique BamHI restriction site in the delivery vector pYH7, a shuttle vector derived from pHZ1358 [48]. A shortened NruI fragment (5714 bp) recovered from the pYH35 insert was moved into the blunted BamHI site of pYH7, creating pYH35s (see Figure S1). DSM4137ΔazlΔmer was used as the parent strain. This is a mutant of DSM4137 in which the meridamycin biosynthetic cluster [49] and a second cluster encoding the biosynthesis of a compound closely related to azalomycin F4 (Y.S. and S.F.H., unpublished data) had previously been deleted. Both deletions were performed by using a similar experimental approach to that described here. The final construct, pYH35s, was introduced into DSM4137ΔazlΔmer by conjugation with donor strain ET12657/pUZ8002 on SFM agar. After incubating at 30°C for 18 hr, exconjugants were selected with 1 ml water containing 100 μg apramycin and 5 mg nalidixic acid. Single colonies from this plate were transferred to an SFM plate with 12.5 μg/ml apramycin for further confirmation of antibiotic resistance. Confirmed colonies were propagated on an SFM plate without antibiotic selection to allow the double crossover to occur. To screen the potential double-crossover mutants, single colonies from nonselective plate were replicated to an SFM plate containing 12.5 μg/ml apramycin. The candidates with the correct phenotype (Apr^S) were chosen for further identification by PCR sequencing and Southern blot (Figure S2), and the resulting mutant was named DSM4137ΔazlΔmerΔnig.

The following primers were used for screening double-crossover mutants: nig-CP1, 5′-CTCGGAATGGAGGAGACTTT-3′; nig-CP2, 5′-GACAACCAGCGGATGAGC-3′. Genomic PCR with the nig-CP1 and nig-CP2 primers was performed in a final volume of 30 μl for 30 cycles of amplification with a programmer (94°C, 5 min; 94°C, 30 s; 55°C, 40 s; 72°C, 50 s; 72°C, 5 min). PCR product (716 bp) was recovered from the gel for sequencing.

Disruption of *monACPX* and *monKSX*

The targeted disruption of *monACPX* and *monKSX* was achieved by using the Redirect PCR targeting system [50] (John Innes Centre, UK) based on Red/ET technology [51]. The manufacturer's instructions were followed to create variants of cos2 [6] in which either *monACPX* (cos2A) or *monKSX* (cos2B) were replaced with a cassette containing *aac(3)IV* to confer apramycin resistance and with *oriT* to allow for conjugation with *Streptomyces* [50]. The oligonucleotides ksx1, 5′-ATGACTGCTCCTGTGTGAGTGAGGTGTTGGCGGGGGTCAATTCCGGGGATCCGTCGACC-3′; ksx2, 5′-GGCTTCACGCGCGAGAACAGGAAGGCTGACCATTCCCGTGTGTAGGCTGGAGCTGCTTC-3′; acpx1, 5′-GACCCCCGCCAACACCTCACTCACACAGGAGCAGTCATGATTCCGGGGATCCGTCGACC-3′; and acpx2, 5′-GGGCGGGGTGGGCGTCGTGCCCGGGTGGTCCAGGGGTCATGTAGGCTGGAGCTGCTTC-3′ were used to create *aac(3)IV-oriT* cassettes with extensions of 36 nt with homology to the regions immediately up and downstream of the targeted genes. The oligonucleotides ksx3, 5′-GAAGTTGCTGTCGTCGCTGAGGGTGGTGTTCAGAAG-3′; ksx4, 5′-GCAAGGCGGGAGTTAGACCCGCTTAAGCAGCCGCG-3′; acpx3, 5′-GACCCACACGCTTCTCCACCTGGGCGCGCTCTCA-3′; and acpx4, 5′-CGCCTCGTCGTCTTCGCCCGGAACCGCTCGCT-3′ were used to verify the presence of the cassettes in the cosmids and later to verify the presence of the disruption in the chromosome. These constructs were transferred to *S. cinnamonensis* by conjugation with donor strain ET12567/pUZ8002 on an A medium plate [45]. After 16 hr of growth at 30°C, the plates were overlaid with apramycin (50 μg/ml) and nalidixic acid (25 μg/ml). Single colonies were patched in parallel onto an A medium plate containing apramycin (50 μg/ml) and an A medium plate containing kanamycin (50 μg/ml). The candidates with the correct phenotype (Apr^R, Kan^S) were chosen for further identification by PCR.

The *monACPX* gene was cloned by using the mutagenic primers acpx8 (5′-GCACATATGACCAGCACCGACCACACCTCCGGCCAGGAC-3′), creating an NdeI site after the start codon, and acpx11 (5′-GGTGCAATTCTCACGCGGT GGTGTGTGCCAGTTCCTGGCCGA-3′), creating an EcoRI site after the stop codon. cos2 was used as template DNA, and Phusion polymerase (New England Biolabs, USA) was used for PCR. After ligation into pUC18, the cloned gene was sequenced and transferred to pIB139 [46] to give pIAXH. The Ω*hyg* gene was isolated as a HindIII fragment blunted with Klenow (New England Biolabs, USA) and was ligated into MscI-cut pIAX to give pIAXH. pIAXH was transferred to both BHΔACPX and BHΔKSX by conjugation as described above.

DNA Sequencing Analysis

Automated DNA sequencing was done on double-stranded DNA templates by using the dideoxynucleotide chain termination method in the University of Cambridge Department of Biochemistry DNA sequencing facility. An Applied Biosystems 800 molecular biology CATALYST robot was used to apply Taq dideoxy terminator sequencing reactions (Big Dye Terminator kit, ABI) to an ABI 373A sequencer according to manufacturer's protocols. Cosmids for initial end sequencing were prepared from overnight growth of 1 ml cultures in 96-well plates in 2xLB medium containing kanamycin and ampicillin. The complete sequence of each cosmid was obtained from overlapping Sau3AI fragments obtained after a partial digestion of the parent cosmid. SeqEd v 1.03 was used for sequence editing. Database searches used the BLAST algorithm [52]. Sequence assembly employed GAP (Genome Assembly Program) version 4.2 [53]. To fill remaining contig gaps and resolve any remaining ambiguities, custom-designed oligonucleotide primers were used to sequence from selected subclones or the parent cosmid. Both strands were completely sequenced with an average 8-fold coverage per base.

Extraction and Analysis of Culture Supernatants

Culture supernatants were extracted twice with an equal volume of ethyl acetate. The combined extracts were dried with anhydrous $MgSO_4$, and the solvent was removed in vacuo. The residue was then redissolved in methanol. Analysis of crude extracts was carried out by ESI LC-MS, ESI LC-MS/MS, and ESI LC-MS^3, by using a Phenomonex Prodigy 5 μm ODS3 100 Å column (250 × 4.6 mm) on a Finnegan MAT LCQ instrument. The column was equilibrated in 20% 20 mM ammonium acetate buffer/80% methanol, and compounds were eluted on a gradient to 100% methanol over 25 min. The mass spectrometer was set to record positive and negative ion scans between 500 and 1600 *m/z*. Nigericin as its sodium salt was purchased from Calbiochem.

Supplemental Data

Supplemental Data include experimental details relating to the deletion and identification of the nigericin gene cluster and are available at http://www.chembiol.com/cgi/content/full/14/6/703/DC1/.

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