Characterization of the Alnumycin Gene Cluster Reveals Unusual Gene Products for Pyran Ring Formation and Dioxan Biosynthesis

Terhi Oja,[1] Kaisa Palmu,[1] Hanna Lehmussola,[1] Outi Leppäranta,[1] Kati Hännikäinen,[1] Jarmo Niemi,[1] Pekka Mäntsälä,[1] and Mikko Metsä-Ketelä[1,2,*]
[1]Department of Biochemistry and Food Chemistry, University of Turku, FIN-20014 Turku, Finland
[2]Division of Molecular Structural Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Tomtebodavägen 6, 4tr, S-171 77, Stockholm, Sweden
*Correspondence: mikko.mk@gmail.com
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SUMMARY

Alnumycin is closely related to the benzoisochroma-nequinone (BIQ) polyketides such as actinorhodin. Exceptional structural features include differences in aglycone tailoring that result in the unique alnumycin chromophore and the existence of an unusual 4-hy-droxymethyl-5-hydroxy-1,3-dioxan moiety. Cloning and sequencing of the alnumycin gene cluster from *Streptomyces* sp. CM020 revealed expected biosynthesis genes for polyketide assembly, but several genes encoding subsequent tailoring enzymes were highly atypical. Heterologous expression studies confirmed that all of the genes required for alnumycin biosynthesis resided within the sequenced clone. Inactivation of genes *aln4* and *aln5* showed that the mechanism of pyran ring formation differs from acti-norhodin and granaticin pathways. Further inactivation studies identified two genes, *alnA* and *alnB*, involved in the synthesis and attachment of the dioxan moiety, and resulted in the production of the polyketide prealnumycin.

INTRODUCTION

Alnumycin (**1**, Figure 1) is an aromatic polyketide antibiotic first isolated in 1998 from *Streptomyces* sp. DSM 11575 (Bieber et al., 1998) and *Streptomyces griseorubiginosus* strain Mer-K1115, under the name K1115 B$_1$ (Naruse et al., 1998). It was shown to have several biological activities, including antibiotic, cytostatic, gyrase inhibitory, and topoisomerase inhibitory (Björn and Jörg, 1998). Structurally, alnumycin is an isochromanequi-none compound, closely related to the benzoisochromanequi-none (BIQ) polyketides, such as actinorhodin (**2**), medermycin (**3**), granaticin (**4**), and frenolicin (**5**), whose biosynthetic pathways have been characterized in detail over the years (Caballero et al., 1991; Bibb et al., 1994; Fernandez-Moreno et al., 1991, 1992, 1994; Hallam et al., 1988; Ichinose et al., 1998, 2003). There are, however, several unusual features in the structure of alnumycin, which makes it an intriguing research target. First, the chiral carbon at position 2 (carbon 3 in polyketide number-ing), which is characteristically found in all BIQ metabolites, is

missing as a result of a double bond between carbons 2 and 3 (Figure 1). Second, unlike in BIQ pathways, the first carbon of the polyketide chain is missing as a result of decarboxylation during biosynthesis. Third and foremost, alnumycin contains a sugar-like 4-hydroxymethyl-5-hydroxy-1,3-dioxan moiety, which is highly uncommon in natural products, and there is no previous knowledge about its biosynthesis.

Studies on the biosynthetic pathways of BIQ antibiotics have produced a remarkable amount of information on formation of polyketide aglycones, (Hopwood, 1997; Hertweck et al., 2007) and structural studies on the actinorhodin ketoacyl synthase heterodimer (Keatinge-Clay et al., 2004), acyl carrier protein (Crump et al., 1997), ketoreductase (Hadfield et al., 2004; Kor-man et al., 2004), and monooxygenase (Sciara et al., 2003) proteins have clarified in great detail many issues concerning polyketide biosynthesis. Furthermore, stereochemical control of third ring cyclization has also been under thorough investi-gation in the actinorhodin, granaticin, and medermycin path-ways (Taguchi et al., 2001, 2004; Li et al., 2005), and use of longer starter units has been shown in the R1128 biosynthetic pathway (Pan et al., 2002; Marti et al., 2000). However, despite this vast amount of work, there remain unanswered questions regarding the function of several common "unknown" genes found from BIQ pathways. These include, for instance, the possible role of ActVI-OrfA in either cyclization (Fernandez-Moreno et al., 1994) or transcription regulation (Taguchi et al., 2007) and the exact roles of *act*VA gene products in tailoring reactions in actinorhodin biosynthesis (Caballero et al., 1991).

The combination of a biosynthetically familiar polyketide skeleton coupled to an unusual tailoring pattern prompted us to investigate the underlying alnumycin biosynthetic pathway to shed light into formation of the pyran ring and synthesis of the dioxan moiety. Here, we report the cloning and sequencing of a gene cluster responsible for alnumycin biosynthesis from *Streptomyces* sp. CM020 and heterologous expression of the gene cluster in *Streptomyces albus* (Chater and Wilde, 1980). In addition, structural elucidation of metabolites from four blocked pathway mutants yields insights into third ring cycliza-tion and biosynthesis of the dioxan moiety. Knowledge about the gene products involved in dioxan synthesis and attachment sets the ground for interesting combinatorial biosynthesis possi-bilities regarding the use of this building block in generating BIQ compound analogs.
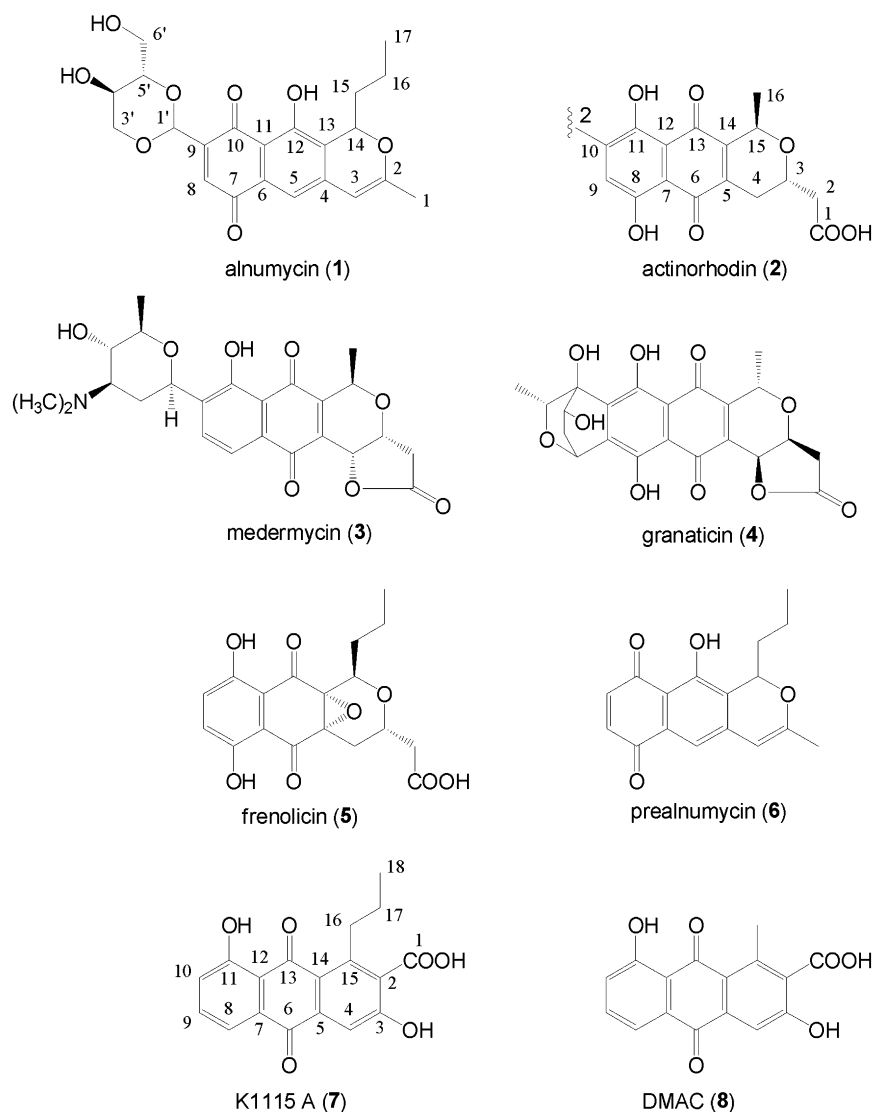
cosmid by homologous recombination in *E. coli* to allow intergenic conjugation of the resulting expression cosmid pAlnuori into *Streptomyces albus* (Chater and Wilde, 1980). Cultivation of transformants in E1soy medium supplemented with XAD-7 resin resulted in the production of alnumycin, which was confirmed by HPLC-UV/Vis and LC-MS monitoring (Figure 2) by comparative analysis against the metabolic profile of the producing strain *Streptomyces* sp. CM020.

Organization of the Genomic Region Involved in Alnumycin Biosynthesis

Analysis of a ~31 kb DNA sequence with the program Artemis (Rutherford et al., 2000) revealed the presence of 32 open reading frames (ORFs) that could be involved in alnumycin biosynthesis. Two of these (Figure 3, left) were omitted from the expression cosmid pAlnuori, indicating that *alnR1* and *aln1* are not absolutely required for alnumycin synthesis, but their involvement in, for instance, regulation of biosynthesis in the native host cannot be ruled out. Based on sequence comparisons with database proteins, 22 probable structural genes and 10 genes possibly involved in regulation and transport were identified. Putative functions for the gene products, which are summarized in Table 1, were assigned by BLAST searches (Altschul et al., 1990) and gene inactivation experiments (see text herein).

Genes Involved in Polyketide Synthesis

The carbon skeletons of aromatic type II polyketides are synthesized by the iterative action of minimal PKS complexes, which consist of a β-ketoacyl synthase heterodimer (KS_α and KS_β/CLF) that catalyzes the condensation of malonyl-CoA substrates and an acyl carrier protein (ACP) onto which the growing polyketide is tethered during synthesis (Hopwood, 1997; Hertweck et al., 2007). In systems that employ starter units other than acetyl-CoA, a combination of similar gene products (a β-ketoacyl synthase III, an acyl transferase and another ACP) is used to synthesize a starter unit of specific length (Marti et al., 2000; Meadows and Khosla, 2001; Tang et al., 2004). The structure of alnumycin suggested that it is synthesized from a four-carbon butyryl-CoA starter unit, and in accordance a full set of starter

RESULTS

Cloning, Sequencing, and Heterologous Expression of the Alnumycin Biosynthetic Gene Cluster

A homologous hybridization probe was generated by PCR using a set of degenerate primers designed to amplify KS_α gene fragments from actinomycetes (Metsä-Ketelä et al., 2002). Phylogenetic analysis revealed that the sequence amplified from *Streptomyces* sp. CM020 clustered with KS_α genes from known BIQ antibiotic pathways (data not shown). Such similarity was expected for alnumycin biosynthetic genes, and therefore the PCR product was used to screen a genomic cosmid library constructed in vector pFD666 as described in Experimental Procedures.

Sequencing of a 31.6 kb region from cosmid p020P1 revealed unique *Nhe*I-*Spe*I restriction sites that were used to repackage a 29.9 kb fragment from the cluster into the shuttle cosmid vector pKC505 (Richardson et al., 1987) for expression studies. The *oriT* origin of replication was sequentially cloned into the shuttle
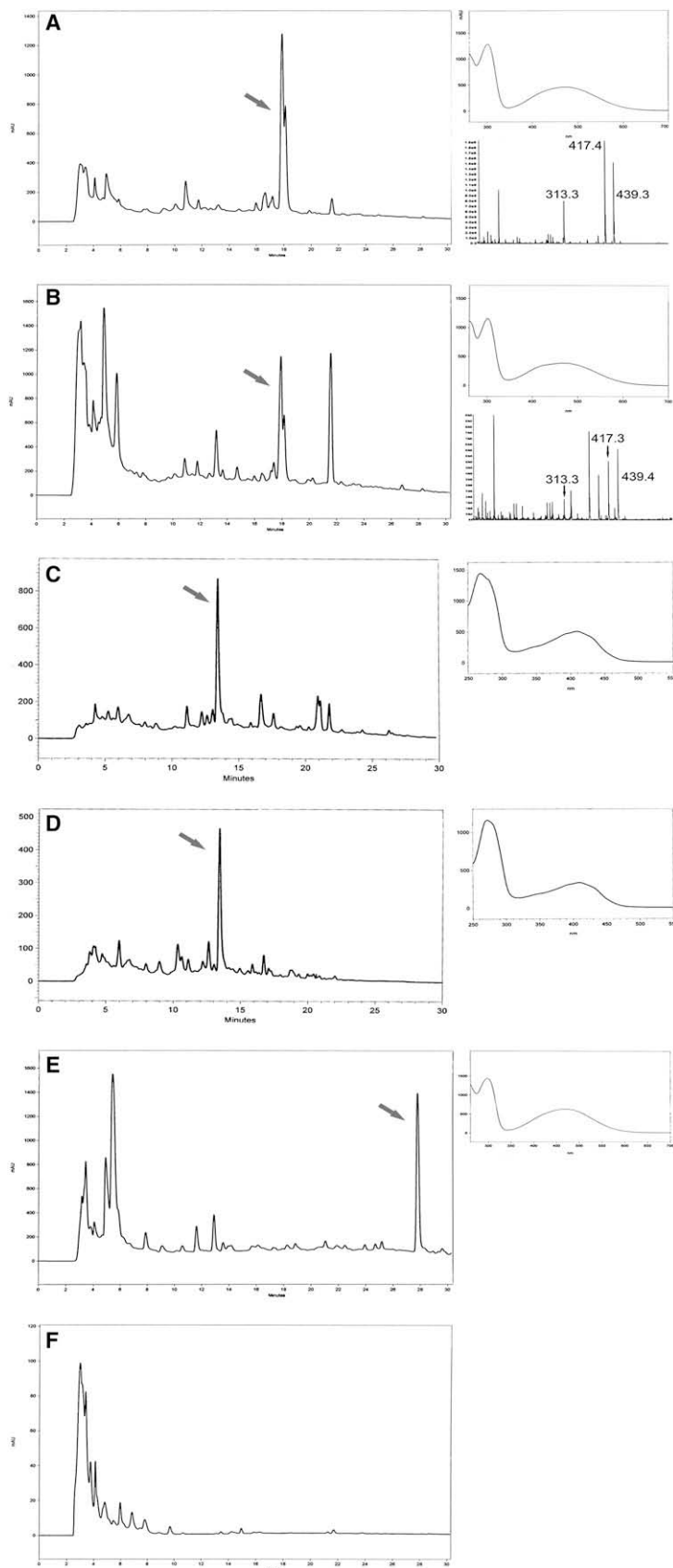
Figure 2. Monitoring of Metabolites Produced by Wild-Type and Recombinant Strains

HPLC-UV-Vis profiles at 298 nm of crude extracts from (A) wild-type *Streptomyces* sp. CM020, (B) *S. albus*/pAlnuori, (C) *S. albus*/pAoriΔaln4, (D) *S. albus*/pAoriΔaln5, (E) *S. albus*/pAoriΔind, and (F) *S. albus*. Results from LC-MS analysis in positive mode are also shown for (A) and (B).
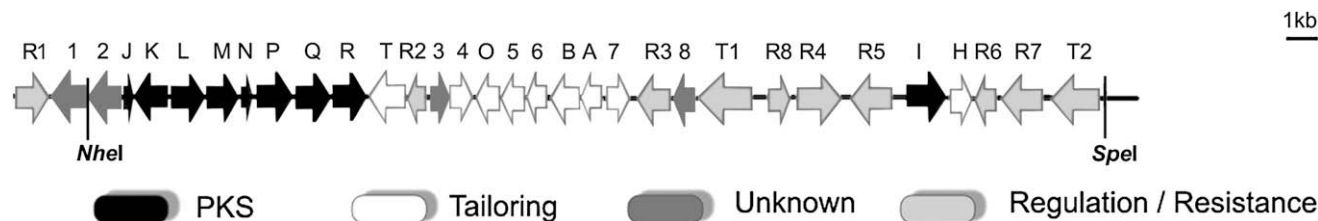
Figure 3. Organization of the *aln* Biosynthetic Gene Cluster
PKS genes responsible for polyketide carbon skeleton synthesis are marked in black; genes responsible for post-PKS tailoring reactions are white; genes of unknown function are dark gray; and genes involved in regulation/resistance are light gray.

unit genes were discovered from the cluster. The starter unit ketosynthase *alnI* was found to reside apart from the rest of the PKS genes separated by ∼22 kb from the starter unit acyl transferase *alnK* (Figure 3). The starter unit ACP *alnJ* resided next to *alnK*. In general, genes encoding starter and minimal PKS (minPKS, *aln*LMN) subunits were most similar to those found from the frenolicin pathway reflecting the same chain length of these compounds (Bibb et al., 1994).

After the formation of the polyketide chain, the biosynthesis continues by the action of a ketoreductase (KR, *alnP*) at C-9, followed by aromatization of the first ring by an aromatase (ARO, *alnQ*) and formation of the second ring in a controlled manner between C-5 and C-14 by a specific cyclase (CYC, *alnR*). The gene products involved in these reactions are typically highly conserved, and the *aln* gene products were in complete agreement with findings from the BIQ pathways. The product of the second ring cyclization is a bicyclic intermediate that can be said to define the end of the PKS part of the pathway (Ichinose et al., 2003).

Identification of Genes Involved in Pyran Ring Formation

Though genes encoding PKS steps were highly conserved in the alnumycin cluster, genes putatively responsible for post-PKS tailoring reactions held, in general, little resemblance to the genes found in the other BIQ clusters. The next biosynthetic step on BIQ pathways is the stereospecific reduction at C-3, which ultimately controls the stereochemistry of the two chiral centers, leading to (3S, 15R) configuration in actinorhodin and (3R, 15S) in granaticin (Figure 1). In the actinorhodin and granaticin pathways, proteins belonging to different enzyme families have been shown to catalyze the ketoreduction step (Taguchi et al., 2001, 2004), but remarkably, no gene products homologous to either of these were found from the alnumycin cluster. The most likely candidate for the ketoreduction step was *aln4*, which is similar in amino acid sequence to aldo-keto reductases (Table 1). Considering that the actinorhodin C-3 ketoreductase ActVI-ORF1 has been shown to use NADPH as a cofactor (Itoh et al., 2007), it is noteworthy that the aldo-keto reductase superfamily contains several NADPH-dependent members.

On the actinorhodin pathway, it has been proposed that hemiketal formation after C-3 ketoreduction might be spontaneous, but enzymatic catalysis of both the ring closure and the subsequent dehydration would be necessary for efficient pyran ring formation in vivo (Ichinose et al., 1999). However, no obvious gene products were discovered from the alnumycin cluster that could catalyze the cyclization and dehydration steps. For the cyclization step, a possible candidate was the gene product

of *aln5*, which is similar (46%) in sequence to the C-terminal domain of a modular polyketide synthase enzyme StiJ from *Stigmatella aurantiaca*. The domain in StiJ is, however, unusual, and it is not related to thioesterase (TE) domains, which normally are responsible for cyclization reactions in modular type I PKS pathways.

To obtain experimental evidence for the involvement of *aln4* and *aln5* in alnumycin biosynthesis, both genes were individually inactivated from the shuttle cosmid pAlnuori by homologous recombination in *E. coli* (Datsenko and Wanner, 2000). The resulting expression constructs were transformed into the heterologous host *S. albus* by conjugation to obtain the mutant strains *S. albus*/pAori∆*aln4* and *S. albus*/pAori∆*aln5*. The production profiles of the two mutant strains were found to be identical by HPLC-UV/Vis monitoring (Figure 2), which showed a single main metabolite peak that was clearly distinct from alnumycin. For structural elucidation of the metabolite, *S. albus*/pAori∆*aln5* was cultivated in a large scale, and the compound was purified by chloroform extraction, column chromatography, and preparative HPLC. The molecular formula $C_{18}H_{14}O_6$ was verified by EI-HR-MS (*m/z* observed 326.078200, calculated 326.079038), and the ^{1}H and ^{13}C NMR spectra were found to be similar to those of K1115 A (7, Figure 1; Naruse et al., 1998). The structure was confirmed using ^{1}H^1-H COSY, HSQC, and HMBC techniques (data not shown). In essence, the evidence presented here suggests that K1115 A is a shunt product from the alnumycin pathway analogous to 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC) from the actinorhodin pathway (McDaniel et al., 1993).

Probable Post-PKS Genes

In the actinorhodin pathway, quinone formation is the next biosynthetic step, which has been shown to proceed through the action of anthrone oxidase ActVA-6 (Sciara et al., 2003), followed by the action of the two-component FMN monooxygenase system of ActVA-ActVB that is responsible for hydroxylation at C-8 (Valton et al., 2006). In addition, early mutagenesis studies suggest that the *actVA-3* gene product has some role during the oxidative tailoring of actinorhodin intermediates (Caballero et al., 1991). In the alnumycin cluster, no genes homologous to the actinorhodin monooxygenase could be found. Gene products that could be assigned to this part of the biosynthetic pathway with confidence were the AlnT and AlnH pair that resembled the actinorhodin ActVA-ActVB system, respectively, and Aln6 and Aln7, which showed similarity to each other and to ActVA-3 from the actinorhodin pathway. In conclusion, the set of tailoring genes

Table 1. Proposed Functions of *aln* Gene Products

Protein	Size (aa)	Category	Function	Closest Sequence Similarity		
				Protein, Origin	Sim/Id (%)	Accession No.
AlnR1	168	Regulation	MarR family regulator	TroR, *Streptomyces avermitilis* MA-4680	69/53	NP_828826
Aln1	231	Unknown	Unknown	SCO5557A, *Streptomyces coelicolor* A3(2)	55/45	NP_629692
Aln2	172	Unknown	Unknown	Francci3_4104, *Frankia* sp. CcI3	54/40	YP_483181
AlnJ	84	PKS	Starter unit acyl carrier protein	FrnJ, *Streptomyces roseofulvus*	72/56	AAC18105
AlnK	414	PKS	Starter unit acyl transferase	HedF, *Streptomyces griseoruber*	54/42	AAP85359
AlnL	421	PKS	Ketoacyl synthase α	FrnL, *Streptomyces roseofulvus*	86/76	AAC18107
AlnM	428	PKS	Ketoacyl synthase β	ZhuA, *Streptomyces* sp. R1128	76/66	AAG30188
AlnN	84	PKS	Acyl carrier protein	FrnN, *Streptomyces roseofulvus*	71/59	AAC18109
AlnP	263	PKS	Ketoreductase	ORF5, *Streptomyces antibioticus*	79/68	CAC05675
AlnQ	331	PKS	Cyclase/aromatase	FrnQ, *Streptomyces roseofulvus*	60/51	AAC18112
AlnR	311	PKS	Cyclase	FrnR, *Streptomyces roseofulvus*	78/65	AAC18113
AlnT	413	Tailoring	Hydroxylase	LndZ5, *Streptomyces globisporus*	65/52	AAR16420
AlnR2	198	Regulation	TetR family regulator	AcrR, *Rhodococcus* sp. RHA1	56/40	YP_706368
Aln3	155	Unknown	Unknown	Franean1DRAFT_4417, *Frankia* sp. EAN1pec	66/53	ABW11619
Aln4	354	Tailoring	Ketoreductase	CflavDRAFT_0972, Bacterium Ellin514	68/50	ZP_02965856
AlnO	152	Tailoring	Oxidoreductase, pyridoxine 5′-phosphate oxidase	SCO1156, *Streptomyces coelicolor* A3(2)	73/51	NP_625448
Aln5	176	Tailoring	Involved in pyran ring formation	Bcenmc03DRAFT_4709, *Burkholderia cenocepacia* MC0-3	50/37, 75 length	ZP_01564599
Aln6	359	Tailoring	Oxidase	SwitDRAFT_4883, *Sphingomonas wittichii* RW1	56/43	ZP_01606335
AlnB	227	Dioxan synthesis	Phosphatase, phosphoglycolate phosphatase	IdgB, *Pectobacterium chrysanthemi*	54/40	AAF74780
AlnA	306	Dioxan synthesis	Involved in dioxan synthesis/attachment	IdgA, *Pectobacterium chrysanthemi*	67/50	AAF74779
Aln7	352	Tailoring	Oxidase	SwitDRAFT_4883, *Sphingomonas wittichii* RW1	56/43	ZP_01606335
AlnR3	271	Regulation	SARP family regulator	Gra-orf9, *Streptomyces violaceoruber* Tu22	71/59	CAA09630
Aln8	186	Unknown	Unknown	SAV2891, *Streptomyces avermitilis* MA-4680	58/39	NP_824067
AlnT1	513	Export	Transporter	SCO3199, *Streptomyces coelicolor* A3(2)	63/42	NP_627413
AlnR8	166	Regulation	MarR family regulator	SAP1p77, *Streptomyces avermitilis* MA-4680	67/52	NP_828826
AlnR4	430	Regulation	Two-component sensory kinase	Gra-orf11, *Streptomyces violaceoruber* Tu22	54/40	CAA09632

Table 1. Continued

Protein	Size (aa)	Category	Function	Closest Sequence Similarity		
				Protein, Origin	Sim/Id (%)	Accession No.
AlnR5	201	Regulation	Two-component response regulator	NcnR, *Streptomyces arenae*	75/60	AAD20272
AlnI	332	PKS	Starter unit ketoacyl synthase	FrnI, *Streptomyces roseofulvus*	75/68	AAC18104
AlnH	180	Tailoring	Flavin reductase	FrnH, *Streptomyces roseofulvus*	66/56	AAC18103
AlnR6	218	Regulation	Two-component response regulator	CitB, *Streptomyces* sp. WA46	84/73	BAC78370
AlnR7	381	Regulation	Two-component sensory kinase	ORF20, *Streptomyces* sp. Strain WA46	61/46	BAC78369
AlnT2	741	Export	Transporter	SCO3166, *Streptomyces coelicolor* A3(2)	74/58	NP_627382

typical to BIQ pathways that were present in the cluster were identical to those found from the granaticin pathway.

Genes of Unknown Function

Several deduced gene products were discovered from the cluster for which no function could be assigned based on sequence information, but which might hold a role in post-PKS reactions. Two of these, Aln3 and Aln8, had no sequence similarity to any proteins of known function. Aln3 was found to be similar to a protein AclJ of unknown function from the aclacinomycin biosynthetic gene cluster, whereas Aln8 was most similar to hypothetical protein SAV2891 from *S. avermitilis*.

The deduced gene product of *aln2* was most similar (54%) to a β-subunit of a proposed aromatic ring hydroxylating dioxygenase from a *Frankia* species. Aln2 also held sequence similarity (50%) to ActVI-ORFA from *S. coelicolor* A3(2) and to several ActVI-ORFA homologs from *Streptomyces* species. ActVI-ORFA has recently been shown to affect transcription of the *actVI* genetic loci (Taguchi et al., 2007), and because of the sequence similarity it is possible that Aln2 also has a regulatory role.

Inactivation of Genes Involved in Dioxan Biosynthesis

The gene cluster contained two apparently translationally coupled genes *alnA* and *alnB*—which encoded proteins homologous to IndA and IndB, respectively—that have been implicated to be involved in the biosynthesis of the blue pigment indigoidine in *Erwinia chrysanthemi* (Reverchon et al., 2002). However, recently it has been shown that indigoidine is synthesized solely by an unusual nonribosomal peptide synthetase (NRPS) in *Streptomyces lavendulae* (Takahashi et al., 2007). According to sequence analysis, AlnB belongs to the haloacid dehalogenase superfamily (HAD) (Kuznetsova et al., 2006), but no function has been described for proteins homologous to AlnA. The crystal structure of an IndA-like protein TM1464 from *Thermotoga maritima* has recently been determined, which revealed an unidentified ligand slightly larger than glycerol-3-phosphate in the putative active site (Levin et al., 2005). In alnumycin biosynthesis, erythritol phosphate would be an excellent building block for the dioxan moiety and would be in agreement with the description of the unknown ligand from TM1464. These observations prompted us to delete *alnA* and *alnB* from the expression cosmid pAlnuori by homologous recombination.

Transformation of *S. albus* with the mutated cosmid pAoriΔind resulted in the production of a novel metabolite as shown by HPLC-UV/Vis analysis, in which the compound had the characteristic spectrum of alnumycin, but a markedly different retention time. Hence the compound was named prealnumycin (**6**, Figure 1). For full structural elucidation, the strain was cultivated in larger scale and the compound was isolated by column chromatography and preparative HPLC. The molecular formula of prealnumycin ($C_{17}H_{16}O_4$) was readily inferred by EI-HR-MS (*m/z* observed 284.105500, calculated 284.104859). Literature searches revealed that the ^{1}H and ^{13}C NMR spectra of **6** were exceedingly similar to those of **1** (Table 2) except for the dioxan part of the molecule for which the signals were missing. In agreement, two aromatic proton signals at 6.83 ppm and 6.87 ppm, which showed AB coupling because of their highly similar chemical environment, confirmed that in place of the dioxan moiety there was a single hydrogen at position 9 in **6**.

To test the effect of individual *alnA* and *alnB* mutations, a two-plasmid system was used to complement the double-knockout mutant strain. First, the genes *alnA* and *alnB* were cloned together into the high copy-number vector pIJ487 (Hopwood et al., 1985) in *Streptomyces lividans* TK24, resulting in plasmid pIJa7AB. Transformation of the plasmid to *S. albus*/pAoriΔind by protoplast transformation reestablished production of **1** (Figure 1) in levels comparable to *S. albus*/pAlnuori (data not shown). Complementation with single genes in the vector pIJE486 (Ylihonko et al., 1996) did not have any effect on the production profile, and **6** remained the main metabolite detected, confirming that both gene products are required for biosynthesis and attachment of the dioxan moiety.

Regulatory and Resistance Genes

A high number of putative regulatory genes were discovered to reside in the gene cluster. In total, gene products from eight ORFs held sequence similarity to proteins from known regulatory families. These included two pairs, putatively AlnR4/AlnR5 and AlnR7/AlnR6, of two-component sensory kinase/response regulators; two proteins, AlnR1 and AlnR8, belonging to the MarR family of regulators; and AlnR2, which was classified as a member of the TetR family of DNA binding proteins (Table 1). Finally, AlnR3 had sequence similarity with the SARP family of pathway-specific antibiotic biosynthesis activators (Wietzorrek and Bibb, 1997).

Table 2. Observed NMR Signals from Prealnumycin (6) in Chloroform-d_1 and K1115 A (7) in Chloroform-d_1:DMSO-d_6 (5:1)

	Prealnumycin (6)		K1115 A (7)	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	1.96 (s)	20.4		169.0
2		158.4		129.5
3	5.60 (s)	100.0		159.1
4		139.5	7.76 (s)	112.7
5	7.12 (s)	114.4		136.7
6		131.2		182.1
7		184.5		132.1
8	6.83 (d)	138.8	7.71 (dd)	117.9
9	6.87 (d)	139.0	7.60 (t)	134.9
10		188.9	7.27 (dd)	124.1
11		112.9	13.13 (s, OH)	161.8
12	12.16 (OH)	156.8		116.7
13		122.3		188.9
14	5.65 (dd)	73.0		121.9
15	1.51 (m)	34.9		146.6
	2.00 (m)			
16	1.47 (m)	18.1	3.21 (m)	34.3
	1.55 (m)		3.24 (m)	
17	0.96 (t)	13.8	1.67 (m)	23.6
			1.71 (m)	
18			1.08 (t)	14.3

Carbon numbering is the same as that used in Figure 1.

The number of gene products involved in resistance was more conservative as sequence analysis revealed two proteins, AlnT1 and AlnT2, that were homologous to transmembrane proteins.

DISCUSSION

Aromatic polyketides isolated from natural sources offer only a few core polyketide aglycon structures, which are formed by controlling starter unit selection, chain length formation, possible ketoreduction, and regiochemistry of cyclization events (Hopwood, 1997; Hertweck et al., 2007). The enormous variety seen in the structures of the metabolites isolated originates mostly from numerous tailoring or post-PKS reactions (Rix et al., 2002). Commonly these are subtle changes in functional groups such as oxygenations, reductions, and methylations; glycosylation remains the most widely used means to generate major structural changes to these compounds (Hertweck et al., 2007). Other more drastic changes include rearrangements of C-C bonds in the polyketide aglycones (Xu et al., 2005; Kharel et al., 2007) and in some cases incorporation of more unusual building blocks from other pathways (Trefzer et al., 2002). Cloning of the genes responsible for alnumycin synthesis gives insight both into the pyran ring formation and the biosynthesis of the highly unusual dioxan moiety, which provides new interesting possibilities for utilization of this building block in combinatorial biosynthesis approaches (Rix et al., 2002; Reeves, 2003). Particularly, given the structural similarities, an obvious starting point would be the replacement of the deoxysugar residue of medermycin (Figure 1) with the alnumycin dioxan moiety. Medermycin has been shown to have a number of biological activities, including cytotoxity against adriamycin-, aclarubicin- and bleomycin-resistant cells (Brimble et al., 1999; Bililign et al., 2005); it would therefore be interesting to see the effect of the dioxan moiety on the activity.

Early Biosynthetic Steps Are Related to the BIQ Pathways

Analysis of the alnumycin gene cluster reveals that the gene products discovered are in agreement with the established model for polyketide assembly (Figure 4). Especially, sequence similarity with BIQ enzymes catalyzing early biosynthetic steps was significant. From the widely accepted polyketide biosynthesis model it can be deduced that first a four-carbon starter unit is synthesized by AlnIJK in a manner analogous to R1128 biosynthesis (Meadows and Khosla, 2001; Tang et al., 2004). No gene products for reduction of the starter unit diketide could be found and therefore it is probable that these activities are borrowed from primary fatty acid metabolism. Following polyketide chain synthesis by AlnLMN, the nascent carbon skeleton is most likely reduced, aromatized, and cyclized by AlnP, AlnQ, and AlnR, respectively, to generate the common bicyclic intermediate found in the BIQ pathways (Figure 4).

A Novel Type of Ketoreductase Is Responsible for the Pyran Ring Formation

After formation of the two-ring intermediate, the alnumycin biosynthetic pathway deviates from the traditional BIQ biosynthetic routes. The gene inactivation experiments revealed two genes, *aln4* and *aln5*, involved in the next step, as disruption of either of these genes completely abolished alnumycin formation and caused the production of the shunt product K1115 A as the sole main product (Figure 2). The result can be interpreted as another remarkable example of the convergent evolution of antibiotic biosynthesis pathways; the alnumycin system presents a third alternative solution for pyran ring formation in addition to the two mechanisms identified previously on the actinorhodin and granaticin pathways (Figure 5).

A likely difference in these three systems is that the Aln4 aldo-keto reductase probably acts on the C-15 keto group instead of the C-3 keto group that is reduced in BIQ biosynthesis (Figure 5; Taguchi et al., 2004). The benefit of this model is the direct generation of a double bond between carbons 2 and 3 (carbons 3 and 4 in polyketide numbering in Figure 5), which is seen in the structures of **1** and **6** (Figure 1) after subsequent cyclization and dehydration steps. On the granaticin pathway, Gra-ORF5 and Gra-ORF6 are proposed to catalyze the ketoreduction at C-3 in a cooperative manner, the former alone being a ketoreductase at C-9 (Ichinose et al., 2001). In an analogous fashion, the relatively small-sized Aln5 might function as an accessory protein for the reductase Aln4 on the alnumycin pathway.

Alnumycin Tailoring Reactions Proceed Through Unusual Enzymatic Reactions

Following third-ring cyclization, the biosynthesis is likely to continue by formation of the quinone moiety through hydroxylation at carbon 7 (carbon 8 in polyketide numbering in Figure 4) by AlnT/AlnH and subsequent oxidation. The Aln6 and Aln7 gene
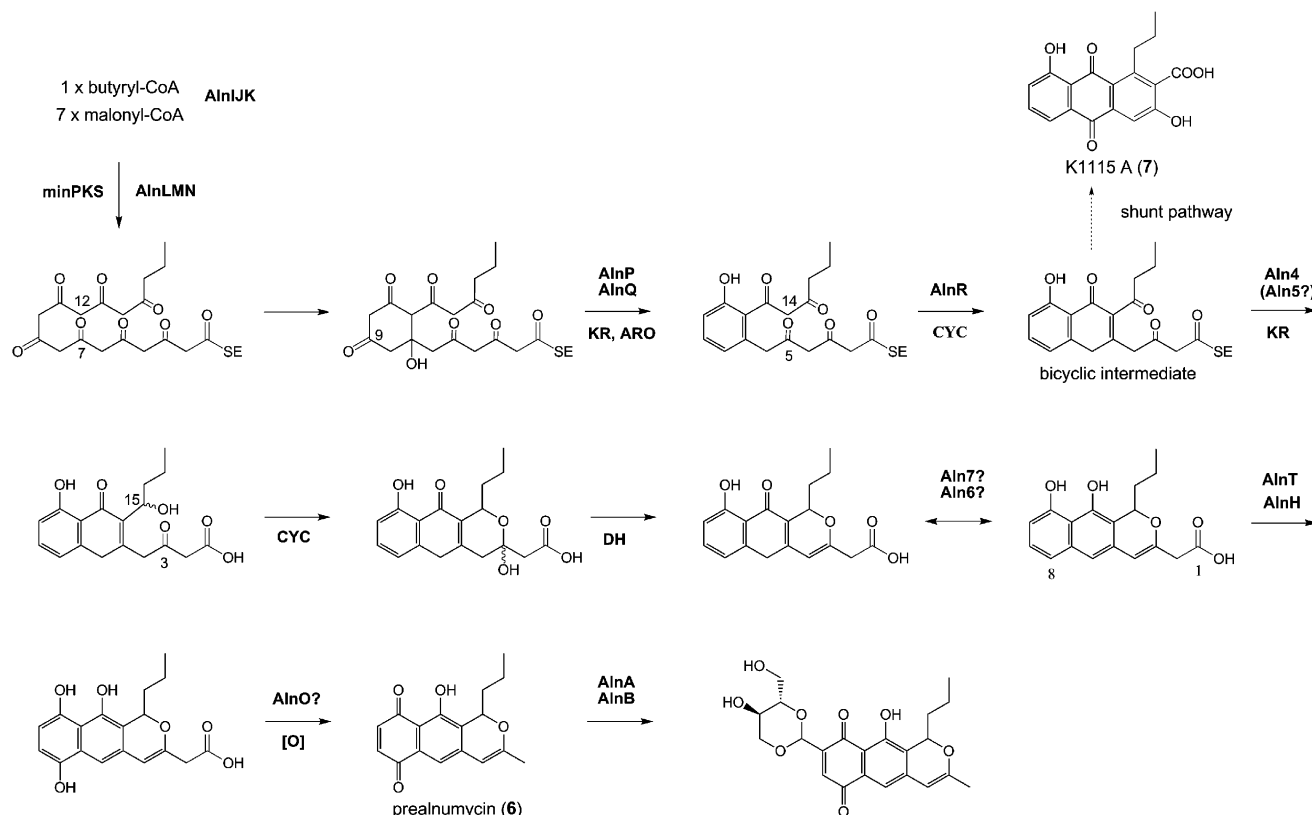
Figure 4. Model for Alnumycin Biosynthesis
The carbon numbering shown is calculated beginning from the enzyme-bound carbon of the formed polyketide chain and differs from the carbon numbering of the end product alnumycin.

products might be involved in stabilization of the reactive intermediates at this step, as has been proposed to occur in actinorhodin biosynthesis (Caballero et al., 1991). It is interesting to note, given the opposite order of the quinone and aromatic rings, how an identical set of gene products to the granaticin pathway is most likely responsible for the generation of the distinct alnumycin chromophore. Formation of the quinone moiety requires oxidation, but whether this occurs nonenzymatically or by AlnO, for instance, remains to be established. Likewise, the timing and mechanism of decarboxylation of the nascent polyketide cannot be deduced *in silico* (Figure 4). In many cases, like in the pathways for angucyclic compounds (Kulowski et al., 1999; Metsä-Ketelä et al., 2003) and the anthracycline steffimycin (Gullon et al., 2006), decarboxylation occurs during last-ring cyclization, whereas in the case of alnumycin this is unlikely as a methylene group separates the carboxyl moiety from the nascent ring. The alnumycin cluster has enough unassigned ORFs (e.g., *aln3* and *aln8*) to provide enzymes that could catalyze reactions at this part of the biosynthetic pathway.

The gene inactivation studies indicated that the final biosynthetic step is synthesis and attachment of the dioxan moiety by *alnA* and *alnB*. Isolation of **6** from a blocked pathway mutant suggests that the whole 4-hydroxymethyl-5-hydroxy-1,3-dioxan moiety is synthesized prior to attachment and not constructed directly on the alnumycin aglycone in a stepwise manner. Logically, formation of the dioxan moiety should proceed via acetal

formation between erythritol and formaldehyde in a manner analogous to the synthesis of 5-hydroxy-1,3-dioxan (García et al., 2000). The substrates for these reactions are most likely phosphorylated as the involvement of AlnB, which is homologous to phosphatases, in the reactions implies. This is also supported by the observation of the unknown phosphorylated ligand in the crystal structure of TM1464 (Levin et al., 2005), which has 30% sequence identity with AlnA. It is possible that one of the gene products catalyzes acetal formation while the other is responsible for attachment of a complete dioxan moiety to the alnumycin aglycone. No other potential candidates for dioxan attachment could be found by sequence analysis, and, especially, no gene products homologous to glycosyl transferases, which have been shown to catalyze C-aryl transfers (Bililign et al., 2005), were identified within the gene cluster. The exact nature of the substrates and the specific reactions catalyzed by AlnA and AlnB are currently under in vitro investigations in our laboratory.

SIGNIFICANCE

Alnumycin is an aromatic polyketide antibiotic related in structure to the much-studied benzoisochromanoquinones (BIQ) such as actinorhodin. Several atypical features in the structure of alnumycin suggested that numerous biosynthetic steps might proceed in an unusual manner, which
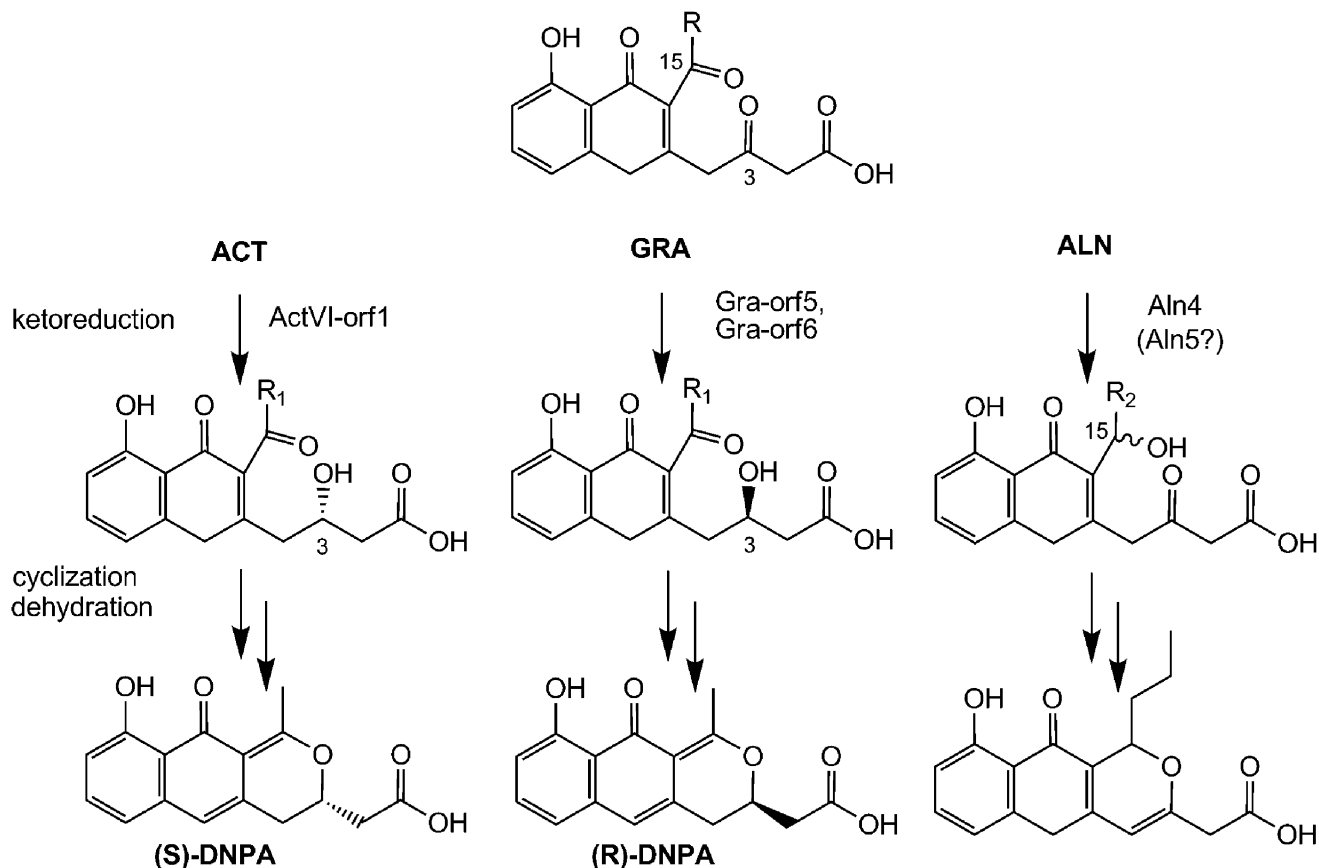
Figure 5. Comparison of Reactions Involved in Pyran Ring Formation in Actinorhodin, Granaticin, and Alnumycin Biosynthesis
$R_1 = -CH_3$ and $R_2 = -CH_2CH_2CH_3$. Multiple arrows indicate several biosynthetic steps.

prompted us to investigate the underlying biosynthesis pathway by molecular genetics.

Cloning, sequencing, and heterologous expression of the alnumycin gene cluster isolated from *Streptomyces* sp. CM020 confirmed the presence of a complete set of biosynthetic genes sufficient for alnumycin biosynthesis. Sequence analysis indicated that early biosynthetic steps were in agreement with the established model for polyketide assembly, but several genes encoding subsequent tailoring enzymes were highly atypical. No genes homologous to those encoding ketoreductases involved in pyran ring formation in either actinorhodin or granaticin pathways were present. Inactivation studies confirmed that on the alnumycin pathway the ketoreduction step is catalyzed by a gene product homologous to aldo-keto reductases. This surprising finding confirms that on related antibiotic biosynthesis pathways, proteins belonging to three different enzyme families catalyze a highly similar biosynthetic step.

The most remarkable feature of alnumycin is the sugar-like 4-hydroxymethyl-5-hydroxy-1,3-dioxan moiety attached to the aglycone. The structure is highly unusual in natural products, and no previous information exists about the underlying biosynthetic pathway. Gene inactivation experiments identified two genes, *alnA* and *alnB,* involved in its biosynthesis. The finding suggests that the entire dioxan

moiety is synthesized prior to attachment and not constructed in a stepwise manner directly to the aglycone.

The work presented here sets the ground for more detailed mechanistic studies on the unusual biosynthetic enzymes and opens up interesting combinatorial possibilities for use of the dioxan moiety in the generation of natural product diversity via pathway engineering.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions

The alnumycin producing *Streptomyces* sp. CM020 was obtained from Galilaeus Oy (Kaarina, Finland). *Streptomyces albus* (Chater and Wilde, 1980) and *Streptomyces lividans* TK24 (Hopwood et al., 1985) were used as heterologous hosts. For production of metabolites, *Streptomyces* species were cultivated in modified E1 medium (Ylihonko et al., 1994), where soy flour (grocery store grade) was used instead of Pharmamedia, for 4–5 days at approximately 300 rpm at 28°C. Amberlite XAD-7 (1 g/50 ml) was added for adsorption of metabolites from cultivation media. For generation of *S. albus* protoplasts, cultures growing for 1–3 days in TSB media (Oxoid; Cambridge, UK), including 5 mM MgCl₂ and 0.5% glycine, were combined, whereas *S. lividans* TK24 was grown in YEME media (Hopwood et al., 1985) supplemented with 0.5% glycine for 3 days. Protoplasts were regenerated on R2YE plates. MS (Kieser et al., 2000) and ISP4 plates (Difco; Detroit, MI) were used for storage on solid medium. Apramycin (Fluka; Buchs, Switzerland) at 50 μg/ml and thiostrepton (Calbiochem; San Diego) at 50 μg/ml and 40 μg/ml were used for selection in solid and liquid media, respectively.

Escherichia coli strains XL1-Blue MRF′ (Stratagene; La Jolla, CA) and TOP10 (Invitrogen; Carlsbad, CA) were used as cloning hosts. Transformation of *S. albus* by intergenic conjugation was conducted using *E. coli* ET12567/pUZ8002 (Kieser et al., 2000). *E. coli* strains were cultivated in LB medium supplemented with appropriate antibiotics for selection of plasmids and cosmids.

General DNA Techniques

Isolation of plasmid and cosmid DNA from *Streptomyces* species was performed using conventional techniques. Restriction enzyme-digested fragments were recovered from agarose gels by QIAquick Gel Extraction Kit (QIAGEN; Venlo, The Netherlands), and plasmid DNA from *E. coli* was isolated using QIAprep® Spin Miniprep Kit (QIAGEN). Phusion™ DNA Polymerase (Finnzymes; Espoo, Finland) was used in PCR amplification reactions according to manufacturer's instructions. Southern hybridization was performed using a DIG-labeled (Roche; Switzerland) probe amplified by PCR with degenerated primers designed to amplify part of the KS_{α} gene (Metsä-Ketelä et al., 2002).

Cloning and Sequencing of the Alnumycin Gene Cluster

The genomic DNA of *Streptomyces* sp. CM020 was isolated by standard procedures (Kieser et al., 2000). For construction of a genomic library, DNA was partially digested with *Sau*3AI, ligated into cosmid vector pFD666 (ATCC) digested with BamHI and packaged using Gigapack III XL (Stratagene). Thirteen positive colonies were chosen from colony hybridization for further screening by restriction analysis, and the cosmid p020P1 was selected for sequencing. Both strands of an area of 31.6 kb were completely sequenced using a transposon-based Template Generation System (Finnzymes) and an automatic ABI 310 DNA sequencer (Applied Biosystems; Foster City, CA). ORFs were detected with the program Artemis (Rutherford et al., 2000), and Vector NTI Suite version 9.0 (Invitrogen) was used for sequence analysis. All restriction enzymes were purchased from Fermentas (Vilnius, Lithuania).

Construction of Expression Cosmids

Most of the alnumycin gene cluster was cloned into an *E. coli-Streptomyces* shuttle vector pKC505 (Kieser et al., 2000) for heterologous expression in *Streptomycetes*. The cosmid p020P1 was digested with *Nhe*I and *Spe*I and the 29.9 kb Klenow treated (Fermentas) digestion product was ligated into pKC505 digested with HpaI and BamHI; Klenow treated and dephosphorylated; and repackaged using Gigapack III XL (Stratagene). The resulting cosmid pAlnu lacked the *oriT* origin of replication required for transformation by conjugation from *E. coli* to *Streptomyces*. For this purpose, the *aac(3)IV* region of pKC505 in pAlnu was substituted by an *aac(3)IV oriT* fragment, which was generated by PCR using pSET152 (Kieser et al., 2000) as a template. The λ Red recombinase system (Datsenko and Wanner, 2000) using homologous recombination was used for the substitution, which was performed in two steps. First, 70 nt and 69 nt primers were designed to amplify the gene coding for chloramphenicol acetyl transferase, using the plasmid pKD3 (Datsenko and Wanner, 2000) as a template: pkccmfor (5′)GTCACGCTGAAA ATGCCGGCCTTTGAATGGGTTCATGTGCAGCTCCATCAGATCGGCACGTA AGAGGTTC and pkccmrev (5′)CAGCGCGACCTTGCCCCTCCAACGTCATC TCGTTCTCCGCTCATGAGAGAATAGGAACTTCGGAATAGG. The 5′ ends of the primers consisted of 50 nt segments homologous to *aac(3)IV* region. The 0.9 kb-long PCR product was used for homologous recombination in *E. coli* TOP10 transformed with pAlnu and the helper plasmid pKD46 (Datsenko and Wanner, 2000) to substitute the *aac(3)IV* area by the chloramphenicol resistance cassette generating pAlnucm^R. Second, the chloramphenicol resistance cassette was substituted in a similar manner by a fragment containing both *aac(3)IV* and *oriT*. The fragment was generated by PCR using the primers aprorifor (5′)GATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAA GATCACTCATCAGCAAAAGGGGATGAT and aprorirev (5′)GCCCTGCCACTC ATCGCAGTACTGTTGTATTCATTAAGCATCTGCCGACAGCCAAAGGGTTCG TGTAGAC, and pSET152 as a template. *E. coli* TOP10, including pAlnucm^R and pKD46, was transformed with the 0.75 kb-long PCR product resulting in pAlnuori.

Inactivation of *aln4*, *aln5*, *alnA*, and *alnB* and Construction of the Complementation Plasmids

The ORFs *aln4*, *aln5*, *alnA*, and *alnB* were deleted from the cosmid pAlnuori again using the λ Red recombinase system (Datsenko and Wanner, 2000).

The primers consisted of 50 nt homology region followed by 20 nt priming sequence for amplifying of the cm^R area with flanking FRT (FLP recognition target) sites: aln4fcamfor

(5′)GTGCGCTACACGCTTCTCGGCAGGACCGGGGTCCGGATATCCCGG CTGGCGTGTAGGCTGGAGCTGCTTC (start codon underlined) and aln4f-camrev

(5′)GTTCAGGCACCGCACTCCCGGAGGAGGAGCGCTCAGGCGGCCCC GTACCGATGGGAATTAGCCATGGTCC. The resistance gene was subsequently eliminated using the helper plasmid pFLP2 (Hoang et al., 1998) expressing FLP recombinase, leaving behind a fragment consisting of 50 bp from the 5′ end of *aln4*, a 103 bp scar sequence, and 18 bp from the 3′ end in place of *aln4* in pAori∆*aln4*.

The inactivation of ORF *aln5* was performed similarly, using the primers aln5fcamfor

(5′)CCGGAGGAGGTCGTCGGAGTGTGGCGGCTGGCCTCGTACACGGAG GTGGGGTGTAGGCTGGAGCTGCTTC and aln5fcamrev

(5′)GTGCGTCTCCTTCTCCTGCTGTTCCGCCGTCTCGTCGGACGCCGTG CTGTATGGGAATTAGCCATGGTCC. Elimination of the resistance gene resulted in a fragment with 56 bp from the 5′ end of *aln5*, the 103 bp scar sequence, and 53 bp from the 3′ end in place of *aln5* in pAori∆*aln5*.

The overlapping genes *alnA* and *alnB* in the cosmid pAlnuori were inactivated in a similar manner as *aln4* and *aln5*, using the primers indfor (5′)CATGG AACGACAGCCCGACCAGCTGCTAGAGGTCAGCGACGAGATCGCCAGTGT AGGCTGGAGCTGCTTC (start-codon underlined) and indrev (5′)GTCGTCG CCCGCGATGACCGTCAGCCGGGTGTCCAGACCGGTGAGTTCGG

ATGGGAATTAGCCATGGTCC. FLP recombinase-mediated elimination of the resistance gene left behind 49 bp from the 5′ end of *alnA*, the 103 bp scar sequence, and 302 bp from the 3′ end of *alnB* in place of *alnA* and *alnB*. The resulting cosmids pAori∆*aln4*, pAori∆*aln5* and pAori∆ind were introduced into *S. albus* by intergenic conjugation. All *S. albus* mutant strains were verified by restriction analysis of cosmid DNA, which was first introduced back into *E. coli*.

For complementation of the pAori∆ind double mutant with individual genes, plasmids pIJEHaA and pIJEHaB were constructed. Insert DNA was derived from plasmids pB∆HaA and pB∆HaB that were designed for recombinant protein production in *E. coli*, and each contains a PCR-amplified and sequenced gene cloned into a modified pBADHisB (Invitrogen) vector (Kallio et al., 2006). Plasmid pB∆HaA was digested with BamHI and *Hind*III, and the 1.04 kb insert fragment was cloned into similarly digested pIJE486 to obtain pIJEHaA in *S. albus*/pAori∆ind. Plasmid pB∆HaB was also digested with BamHI and *Hind*III, resulting in a 0.6 kb BamHI fragment and a 0.2 kb BamHI-*Hind*III fragment, which were cloned in a three-way ligation into pIJE486 in *S. albus*/pAori∆ind. Plasmid pIJEHaB was constructed via transformation of *S. lividans* TK24, and finally, *S. albus*/pAori∆ind was transformed with plasmid isolated from *S. lividans* TK24/pIJEHaB.

An 11.7 kb BglII fragment from the cosmid p020P1 was cloned into pBluescript (Fermentas) digested with BamHI, resulting in plasmid pB020P1B1.2, and a blunt ended 4.6 kb PagI fragment from pB020P1B1.2 was cloned into pBluescript digested with *Eco*RV. The latter plasmid was digested with *Xba*I and *Hind*III, and the 3.9 kb insert fragment, including *alnA*, *alnB*, and *aln7*, was cloned into the *Streptomyces* vector pIJ487 (Hopwood et al., 1985) digested with *Xba*I and *Hind*III in *S. albus*/pAori∆ind, resulting in plasmid pIJa7AB.

Analysis of Metabolites

From cultivations with XAD-7, the resin was separated from the culture broth by repeated decanting, and the compounds were extracted with acetonitrile for further analysis. Samples from *S. albus*/pAori∆*aln4* and *S. albus*/pAori∆*aln5* cultivations were extracted under acidic conditions. HPLC samples were analyzed with a Shimadzu SCL-10Avp (Shimadzu; Kyoto, Japan) with a Merck LiChroCART 250-4 RP-18 (5 mm) column using a 18 min gradient from 50% to 99% acetonitrile in 0.1% formic acid.

Purification and Structural Determination of K1115 A and Prealnumycin

For large-scale production of metabolites, *S. albus*/pAori∆*aln5* was cultivated for 7 days in 250 ml, 500 ml, and 1 l flasks (total volume 6 l), where XAD was added 1 day prior to harvesting. XAD and whole cells were collected by

centrifuging, and the pellets were stored at −20°C until extraction with acetone. K1115 A was purified by repeated chloroform extraction in both neutral and acidic conditions, followed by silica gel chromatography in chloroform using a methanol gradient from 5% to 30%. As a final step, K1115 A was purified by preparative HPLC with a Merck Hitachi L-6200A using a LiChrospher 100 RP-18 (10 μm) column and a 45 min acetonitrile gradient from 50% to 100% in 0.1% formic acid.

For production of metabolites, *S. albus*/pAoriΔind was grown in 250 ml and 500 ml flasks (total volume 6 l). The compounds were extracted from XAD using a water:isopropanol gradient. Prealnumycin was subsequently subjected to repeated silica gel chromatography using a chloroform:methanol gradient and LH-20 size exclusion chromatography using dichloromethane:tetrahydrofuran (20:5) as the eluent. As a final step, prealnumycin was purified by preparative HPLC with a Merck Hitachi L-6200A using a Phenomenex Phenyl-Hexyl 250-10 (10 μm) column and a 30 min acetonitrile gradient from 15% to 100%.

LC-MS and NMR Measurements

Analytical samples were analyzed by LC-ESI-MS (Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer) using the same column and conditions as in other HPLC measurements. High-resolution EI-HR-MS data was collected with Micromass ZAB-oa-TOF mass spectrometer. NMR spectra were obtained with a Bruker Avance 400 MHz NMR spectrometer. ^{1}H and ^{13}C NMR chemical shifts are referenced to tetramethylsilane.

ACCESSION NUMBERS

The nucleotide sequence has been deposited into the GenBank database under accession number EU852062.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Bibb, M.J., Sherman, D.H., Omura, S., and Hopwood, D.A. (1994). Cloning, sequencing and deduced functions of a cluster of *Streptomyces* genes probably encoding biosynthesis of the polyketide antibiotic frenolicin. Gene *142*, 31–39.

Bieber, B., Nuske, J., Ritzau, M., and Grafe, U. (1998). Alnumycin a new naphthoquinone antibiotic produced by an endophytic *Streptomyces* sp. J. Antibiot. (Tokyo) *51*, 381–382.

Bililign, T., Griffith, B.R., and Thorson, J.S. (2005). Structure, activity, synthesis and biosynthesis of aryl-C-glycosides. Nat. Prod. Rep. *22*, 742–760.

Björn, B., and Jörg, N. April 1998. Alnumycin useful as antibiotic. German patent DE19745914.

Brimble, M.A., Duncalf, L.J., and Nairn, M.R. (1999). Pyranonaphthoquinone antibiotics—isolation, structure and biological activity. Nat. Prod. Rep. *16*, 267–281.

Caballero, J.L., Martinez, E., Malpartida, F., and Hopwood, D.A. (1991). Organisation and functions of the *actVA* region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. *230*, 401–412.

Chater, K.F., and Wilde, L.C. (1980). *Streptomyces albus* G mutants defective in the *Sal*GI restriction-modification system. J. Gen. Microbiol. *116*, 323–334.

Crump, M.P., Crosby, J., Dempsey, C.E., Parkinson, J.A., Murray, M., Hopwood, D.A., and Simpson, T.J. (1997). Solution structure of the actinorhodin

polyketide synthase acyl carrier protein from *Streptomyces coelicolor* A3(2). Biochemistry *36*, 6000–6008.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. *97*, 6640–6645.

Fernandez-Moreno, M.A., Caballero, J.L., Hopwood, D.A., and Malpartida, F. (1991). The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell *66*, 769–780.

Fernandez-Moreno, M.A., Martinez, E., Boto, L., Hopwood, D.A., and Malpartida, F. (1992). Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. J. Biol. Chem. *267*, 19278–19290.

Fernandez-Moreno, M.A., Martinez, E., Caballero, J.L., Ichinose, K., Hopwood, D.A., and Malpartida, F. (1994). DNA sequence and functions of the *act*VI region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). J. Biol. Chem. *269*, 24854–24863.

García, N., Compañ, V., Díaz-Calleja, R., Guzmán, J., and Riande, E. (2000). Comparative study of the relaxation behaviour of acrylic polymers with flexible cyclic groups in their structure. Polymer *41*, 6603–6611.

Gullon, S., Olano, C., Abdelfattah, M.S., Brana, A.F., Rohr, J., Mendez, C., and Salas, J.A. (2006). Isolation, characterization, and heterologous expression of the biosynthesis gene cluster for the antitumor anthracycline steffimycin. Appl. Environ. Microbiol. *72*, 4172–4183.

Hadfield, A.T., Limpkin, C., Teartasin, W., Simpson, T.J., Crosby, J., and Crump, M.P. (2004). The crystal structure of the actIII actinorhodin polyketide reductase: proposed mechanism for ACP and polyketide binding. Structure *12*, 1865–1875.

Hallam, S.E., Malpartida, F., and Hopwood, D.A. (1988). Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene *74*, 305–320.

Hertweck, C., Luzhetskyy, A., Rebets, Y., and Bechthold, A. (2007). Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. Nat. Prod. Rep. *24*, 162–190.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J., and Schweizer, H.P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene *212*, 77–86.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M., and Schrempf, H. (1985). Genetic manipulation of *Streptomyces*: a laboratory manual (Norwich, UK: The John Innes Foundation).

Hopwood, D.A. (1997). Genetic contributions to understanding polyketide synthases. Chem. Rev. *97*, 2465–2497.

Ichinose, K., Bedford, D.J., Tornus, D., Bechthold, A., Bibb, M.J., Revill, W.P., Floss, H.G., and Hopwood, D.A. (1998). The granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tu22: sequence analysis and expression in a heterologous host. Chem. Biol. *5*, 647–659.

Ichinose, K., Surti, C., Taguchi, T., Malpartida, F., Booker-Milburn, K., Stephenson, G., Ebizuka, Y., and Hopwood, D. (1999). Proof that the *act*VI genetic region of *Streptomyces coelicolor* A3(2) is involved in stereospecific pyran ring formation in the biosynthesis of actinorhodin. Bioorg. Med. Chem. Lett. *9*, 395–400.

Ichinose, K., Taguchi, T., Bedford, D.J., Ebizuka, Y., and Hopwood, D.A. (2001). Functional complementation of pyran ring formation in actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2) by ketoreductase genes for granaticin biosynthesis. J. Bacteriol. *183*, 3247–3250.

Ichinose, K., Ozawa, M., Itou, K., Kunieda, K., and Ebizuka, Y. (2003). Cloning, sequencing and heterologous expression of the medermycin biosynthetic gene cluster of *Streptomyces* sp. AM-7161: towards comparative analysis of the benzoisochromanequinone gene clusters. Microbiology *149*, 1633–1645.

Itoh, T., Taguchi, T., Kimberley, M.R., Booker-Milburn, K.I., Stephenson, G.R., Ebizuka, Y., and Ichinose, K. (2007). Actinorhodin biosynthesis: structural

requirements for post-PKS tailoring intermediates revealed by functional analysis of ActVI-ORF1 reductase. Biochemistry 46, 8181–8188.

Kallio, P., Sultana, A., Niemi, J., Mäntsälä, P., and Schneider, G. (2006). Crystal structure of the polyketide cyclase AknH with bound substrate and product analogue: implications for catalytic mechanism and product stereoselectivity. J. Mol. Biol. 357, 210–220.

Keatinge-Clay, A.T., Maltby, D.A., Medzihradszky, K.F., Khosla, C., and Stroud, R.M. (2004). An antibiotic factory caught in action. Nat. Struct. Mol. Biol. 11, 888–893.

Kharel, M.K., Zhu, L., Liu, T., and Rohr, J. (2007). Multi-oxygenase complexes of the gilvocarcin and jadomycin biosyntheses. J. Am. Chem. Soc. 129, 3780–3781.

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).

Korman, T.P., Hill, J.A., Vu, T.N., and Tsai, S.C. (2004). Structural analysis of actinorhodin polyketide ketoreductase: cofactor binding and substrate specificity. Biochemistry 43, 14529–14538.

Kulowski, K., Wendt-Pienkowski, E., Han, L., Yang, K., Vining, L.C., and Hutchinson, C.R. (1999). Functional characterization of the jadI gene as a cyclase forming angucyclinones. J. Am. Chem. Soc. 121, 1786–1794.

Kuznetsova, E., Proudfoot, M., Gonzalez, C.F., Brown, G., Omelchenko, M.V., Borozan, I., Carmel, L., Wolf, Y.I., Mori, H., Savchenko, A.V., et al. (2006). Genome-wide analysis of substrate specificities of the Escherichia coli haloacid dehalogenase-like phosphatase family. J. Biol. Chem. 281, 36149–36161.

Levin, I., Miller, M.D., Schwarzenbacher, R., McMullan, D., Abdubek, P., Ambing, E., Biorac, T., Cambell, J., Canaves, J.M., Chiu, H.J., et al. (2005). Crystal structure of an indigoidine synthase A (IndA)-like protein (TM1464) from Thermotoga maritima at 1.90 A resolution reveals a new fold. Proteins 59, 864–868.

Li, A., Itoh, T., Taguchi, T., Xiang, T., Ebizuka, Y., and Ichinose, K. (2005). Functional studies on a ketoreductase gene from Streptomyces sp. AM-7161 to control the stereochemistry in medermycin biosynthesis. Bioorg. Med. Chem. 13, 6856–6863.

Marti, T., Hu, Z., Pohl, N.L., Shah, A.N., and Khosla, C. (2000). Cloning, nucleotide sequence, and heterologous expression of the biosynthetic gene cluster for R1128, a non-steroidal estrogen receptor antagonist. Insights into an unusual priming mechanism. J. Biol. Chem. 275, 33443–33448.

McDaniel, R., Ebert-Khosla, S., Hopwood, D., and Khosla, C. (1993). Engineered biosynthesis of novel polyketides. Science 262, 1546–1550.

Meadows, E.S., and Khosla, C. (2001). In vitro reconstitution and analysis of the chain initiating enzymes of the R1128 polyketide synthase. Biochemistry 40, 14855–14861.

Metsä-Ketelä, M., Halo, L., Munukka, E., Hakala, J., Mäntsälä, P., and Ylihonko, K. (2002). Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal DNA genes from various Streptomyces species. Appl. Environ. Microbiol. 68, 4472–4479.

Metsä-Ketelä, M., Palmu, K., Kunnari, T., Ylihonko, K., and Mäntsälä, P. (2003). Engineering anthracycline biosynthesis toward angucyclines. Antimicrob. Agents Chemother. 47, 1291–1296.

Naruse, N., Goto, M., Watanabe, Y., Terasawa, T., and Dobashi, K. (1998). K1115 A, a new anthraquinone that inhibits the binding of activator protein-1 (AP-1) to its recognition sites. II. Taxonomy, fermentation, isolation, physicochemical properties and structure determination. J. Antibiot. (Tokyo) 51, 545–552.

Pan, H., Tsai, S., Meadows, E.S., Miercke, L.J., Keatinge-Clay, A.T., O'Connell, J., Khosla, C., and Stroud, R.M. (2002). Crystal structure of the priming beta-ketosynthase from the R1128 polyketide biosynthetic pathway. Structure 10, 1559–1568.

Reeves, C.D. (2003). The enzymology of combinatorial biosynthesis. Crit. Rev. Biotechnol. 23, 95–147.

Reverchon, S., Rouanet, C., Expert, D., and Nasser, W. (2002). Characterization of indigoidine biosynthetic genes in Erwinia chrysanthemi and role of this blue pigment in pathogenicity. J. Bacteriol. 184, 654–665.

Richardson, M.A., Kuhstoss, S., Solenberg, P., Schaus, N.A., and Rao, R.N. (1987). A new shuttle cosmid vector, pKC505, for streptomycetes: its use in the cloning of three different spiramycin-resistance genes from a Streptomyces ambofaciens library. Gene 61, 231–241.

Rix, U., Fischer, C., Remsing, L.L., and Rohr, J. (2002). Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat. Prod. Rep. 19, 542–580.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.A., and Barrell, B. (2000). Artemis: sequence visualization and annotation. Bioinformatics 16, 944–945.

Sciara, G., Kendrew, S.G., Miele, A.E., Marsh, N.G., Federici, L., Malatesta, F., Schimperna, G., Savino, C., and Vallone, B. (2003). The structure of ActVA-Orf6, a novel type of monooxygenase involved in actinorhodin biosynthesis. EMBO J. 22, 205–215.

Taguchi, T., Ebizuka, Y., Hopwood, D.A., and Ichinose, K. (2001). A new mode of stereochemical control revealed by analysis of the biosynthesis of dihydrogranaticin in Streptomyces violaceoruber Tu22. J. Am. Chem. Soc. 123, 11376–11380.

Taguchi, T., Kunieda, K., Takeda-Shitaka, M., Takaya, D., Kawano, N., Kimberley, M.R., Booker-Milburn, K.I., Stephenson, G.R., Umeyama, H., Ebizuka, Y., and Ichinose, K. (2004). Remarkably different structures and reaction mechanisms of ketoreductases for the opposite stereochemical control in the biosynthesis of BIQ antibiotics. Bioorg. Med. Chem. 12, 5917–5927.

Taguchi, T., Okamoto, S., Lezhava, A., Li, A., Ochi, K., Ebizuka, Y., and Ichinose, K. (2007). Possible involvement of ActVI-ORFA in transcriptional regulation of actVI tailoring-step genes for actinorhodin biosynthesis. FEMS Microbiol. Lett. 269, 234–239.

Takahashi, H., Kumagai, T., Kitani, K., Mori, M., Matoba, Y., and Sugiyama, M. (2007). Cloning and characterization of a Streptomyces single module type non-ribosomal peptide synthetase catalyzing a blue pigment synthesis. J. Biol. Chem. 282, 9073–9081.

Tang, Y., Koppisch, A.T., and Khosla, C. (2004). The acyltransferase homologue from the initiation module of the R1128 polyketide synthase is an acyl-ACP thioesterase that edits acetyl primer units. Biochemistry 43, 9546–9555.

Trefzer, A., Pelzer, S., Schimana, J., Stockert, S., Bihlmaier, C., Fiedler, H.P., Welzel, K., Vente, A., and Bechthold, A. (2002). Biosynthetic gene cluster of simocyclinone, a natural multihybrid antibiotic. Antimicrob. Agents Chemother. 46, 1174–1182.

Valton, J., Fontecave, M., Douki, T., Kendrew, S.G., and Niviere, V. (2006). An aromatic hydroxylation reaction catalyzed by a two-component FMN-dependent Monooxygenase. The ActVA-ActVB system from Streptomyces coelicolor. J. Biol. Chem. 281, 27–35.

Wietzorrek, A., and Bibb, M. (1997). A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold. Mol. Microbiol. 25, 1181–1184.

Xu, Z., Jakobi, K., Welzel, K., and Hertweck, C. (2005). Biosynthesis of the antitumor agent chartreusin involves the oxidative rearrangement of an anthracyclic polyketide. Chem. Biol. 12, 579–588.

Ylihonko, K., Hakala, J., Niemi, J., Lundell, J., and Mäntsälä, P. (1994). Isolation and characterization of aclacinomycin A-non-producing Streptomyces galilaeus (ATCC 31615) mutants. Microbiology 140, 1359–1365.

Ylihonko, K., Tuikkanen, J., Jussila, S., Cong, L., and Mäntsälä, P. (1996). A gene cluster involved in nogalamycin biosynthesis from Streptomyces nogalater: sequence analysis and complementation of early-block mutations in the anthracycline pathway. Mol. Gen. Genet. 251, 113–120.