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Engineering Anthracycline Biosynthesis toward Angucyclines

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The biosynthesis pathways of two anthracyclines, nogalamycin and aclacinomycin, were directed toward angucyclines by using an angucycline-specific cyclase, *pgaF*, isolated from a silent antibiotic biosynthesis gene cluster. Addition of *pgaF* to a gene cassette that harbored the early biosynthesis genes of nogalamycin resulted in the production of two known angucyclinone metabolites, rabelomycin and its precursor, UWM6. Substrate flexibility of *pgaF* was demonstrated by replacement of the nogalamycin minimal polyketide synthase genes in the gene cassette with the equivalent aclacinomycin genes together with *aknE2* and *aknF*, which specify the unusual propionate starter unit in aclacinomycin biosynthesis. This modification led to the production of a novel angucyclinone, MM2002, in which the expected ethyl side chain was incorporated into the fourth ring.

Aromatic polyketides consist of a large group of structurally diverse natural products that exhibit a broad range of biological activities. Despite the structural diversity found within different aromatic polyketides, they all arise from a fundamentally similar biosynthesis pathway which has been elucidated for many representatives of this class of natural products (for a review, see reference 4). Biosynthesis begins with the condensation of a specific number of small carboxylic acids by the iterative action of the minimal polyketide synthase (minPKS) complex, which consists of two ketosynthases (KS_α and KS_β) and an acyl carrier protein (ACP) onto which the growing polyketide chain is attached via a flexible phosphopantetheinyl arm. The polyketide chain is subsequently folded into a range of different aromatic compounds by various ketoreductases (KR), cyclases (CYC), and aromatases (ARO). Finally, tailoring reactions catalyzed by diverse oxygenases (OXY), methylases, and reductases, as well as possible noncatalyzed reactions, often further modify the aglycone formed. In this work the latter turned out to be important because of the reactive nature of the isolated intermediates (see below).

The convergent biosynthesis pathways have prompted research into combinatorial biosynthesis, a method that uses genetic engineering of metabolic pathways to provide modified natural products for drug discovery (6, 8). Early research led to the identification of a set of design rules (12) which could be used to engineer novel aromatic polyketides in a predictable manner. However, later research has shown that in many instances different combinations of antibiotic biosynthesis genes have not resulted in the production of the expected compounds. In particular, the *jadI* cyclase gene from the biosynthesis pathway of the angucycline antibiotic jadomycin has been reported to be incompatible with tetracenomycin (11) and daunorubicin (20) biosynthesis genes. Similarly, the tetracenomycin (*tcmI*) and daunorubicin (*dpsY*) cyclase genes were nonfunctional with jadomycin biosynthesis genes (11, 20).

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We have recently isolated a silent antibiotic biosynthesis gene cluster that exhibits strong sequence homology to the known angucycline gene clusters (M. Metsä-Ketelä, unpublished results) from soil isolate *Streptomyces* sp. strain PGA64. The cluster contains a cyclase gene designated *pgaF*, according to the other homologous angucycline cyclases *lanF* and *urdF* from the landomycin (19) and urdamycin (3) biosynthesis pathways, respectively. All of these cyclases also display sequence homology to the *jadI* cyclase, which has been proven to be responsible for the cyclization of the fourth, angular ring in jadomycin biosynthesis (11).

Our previous investigations on the biosynthesis pathways of the anthracycline antibiotics nogalamycin (17, 24) and aclacinomycins (14) (Fig. 1) combined with the genes from the silent angucycline gene cluster presented us an opportunity to study the interface of these two classes of aromatic polyketides and to assess the extent to which these pathways are compatible. In particular, the nogalamycin biosynthesis genes offered a good starting point for this study, since nogalamycin biosynthesis proceeds identically, up to a point, to the proposed biosynthesis pathways of angucyclines (Fig. 2); in both pathways the polyketide chain synthesized is composed of 10 acetates and the C-9 ketone is reduced to hydroxyl, followed by cyclization between C-7 and C-12 and aromatization of the first ring. The second ring is also closed in a similar manner, and differences start to appear only in the third cyclization event, in which opposite carbons (C-16 in nogalamycin and C-4 in angucyclines) carry out the nucleophilic attack.

The biosynthesis of aklavinone, the aglycone moiety of aclacinomycin, progresses highly similarly to that of nogalamycin, with the exception of the use of a propionate starter unit, which results in an ethyl side chain in the fourth ring (14), and the stereochemistry in the cyclization of the fourth ring, which is opposite in the nogalamycin and aclacinomycin biosynthesis pathways (16). In this study, the differences in anthracycline starter unit utilization could be used to investigate the substrate flexibility of PgaF, as priming of the minPKS complex with propionate results in an unnatural substrate for PgaF that is composed of a longer carbon chain (Fig. 2). Here we report

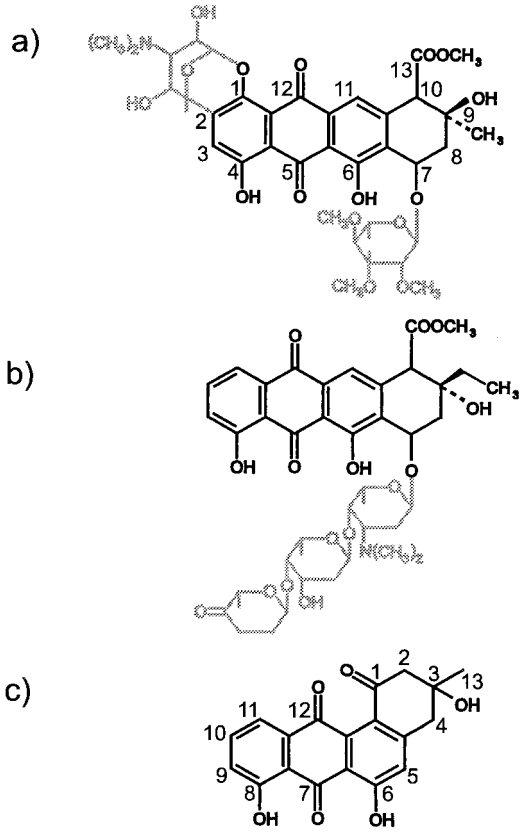

FIG. 1. Structures of anthracycline antibiotics nogalamycin (a), produced by *S. nogalater*, and aclacinomycin A, produced by *S. galilaeus* (b), and an example of angucycline antibiotic rabelomycin (c). Black and gray lines indicate atoms derived from PKS and deoxysugar biosynthesis pathways, respectively.

on the use of the *pgaF* cyclase to divert the biosynthetic pathways of two anthracyclines, nogalamycin and aclacinomycin, toward angucyclines.

MATERIALS AND METHODS

Bacterial strains and cultivation of bacteria. The bacterial strain designated PGA64 was obtained from a soil sample collected in Barcelona, Spain. One gram of the dried soil sample was suspended in 5 ml of H_2O and centrifuged to remove solid substances. The supernatant containing bacterial strains was spread as 100-μl aliquots on International *Streptomyces* Project (ISP) medium 4-related (ISP4) agar plates in which fructose replaced starch as a carbon source and that were supplemented with heximide (25 μg/ml). Soil isolate PGA64 was purified on agar plates by repeated plating. Cultures were maintained on ISP4 agar (Difco, Detroit, Mich.), which is a medium commonly used to isolate *Streptomyces* and closely related genera from soil. Manipulations of *Streptomyces* DNA were carried out with *Escherichia coli* XL2-Blue (Stratagene, La Jolla, Calif.). The *Streptomyces* strains used were *Streptomyces nogalater* ATCC 27451 (2), *Streptomyces galilaeus* ATCC 31615 (13), and *Streptomyces lividans* TK24 (9). *E. coli* XL2-Blue and *Streptomyces* strains were grown at 37 and 30°C in Luria-Bertani medium (15) and E1 medium (23), respectively.

PCR amplifications and cloning procedures. Amplification of DNA fragments by PCR was performed with 100 pmol of each oligonucleotide primer, 0.1 to 100 ng of plasmid or chromosomal DNA template, 5 mmol of each deoxynucleoside triphosphate, and 2 U of DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland). The template was initially denaturated by heating at 99°C for 5 min, followed by 30 cycles of amplification, i.e., denaturation at 96°C for 1 min, annealing at 55 to 62°C (depending on the primers) for 2 min, and extension at 73°C for 1 min 30 s. The primers that were used are shown in Table 1. The PCR products were cloned with the TOPO-TA cloning system (Invitrogen, Carlsbad, Calif.) and sequenced with an automatic ABI310 DNA sequencer (Applied Biosystems, Foster City, Calif.). DNA isolations and manipulations were carried out by standard procedures (9, 15). DNA propagated in *E. coli* was introduced by standard protoplast transformation into *S. lividans* TK24 (9).

Expression constructs. The nogalamycin biosynthesis genes were previously amplified (J. Kantola, unpublished results) by PCR with oligonucleotide primers that contain unique restriction sites at their ends (Table 1) to facilitate the construction of the desired expression vectors. The genes *snoa1-a3*, *snoaD*, and *snoaE* were combined in pUC19 (22) one at the time by using standard molecular biology methods (15) to give plasmid pMC5. The angucycline cyclase *pgaF* was inserted as a Klenow fragment-treated *Eco*RI fragment to a similarly blunt-ended *Nhe*I site of the pMC5 cassette, resulting in plasmid pMC6B (Fig. 3). To investigate the effect of the aclacinomycin minPKS genes, the *sno* minPKS genes were first removed from plasmid pMC6BD by *Xba*I-*Nhe*I digestion, and the *akn* genes were inserted as an *Xba*I-*Nhe*I fragment to give plasmid pSJ10 (Fig. 3). Plasmid pMC6BD was used in this case, because the *Nhe*I restriction site in pMC6B was no longer intact, as it was used for insertion of the *pgaF* cyclase gene. Plasmid pMC6BD, which causes the production of the same compounds as pMC6B in *S. lividans* TK24, differs from pMC6B in that it contains an aromatase (the *pgaL* gene from the silent angucycline gene cluster) different from the homologous nogalamycin *snoaE* aromatase. Both constructs were ligated as *Xba*I-*Hin*dIII fragments to similarly digested vector *Streptomyces*(pIJE486) and introduced into *S. lividans* TK24. In this system, all of the genes are expressed from the constitutive *ermE* promoter from *Saccharopolyspora erythraea* (9), which has been cloned into the polylinker of *Streptomyces* cloning vector pIJ486 (18).

Isolation and purification of products. For structure elucidation the strains were grown in 250-ml flasks (a total of 2 liters of E1 medium supplemented with thiostrepton [50 μg/ml] and XAD-7 absorbent [20 g/liter] at 30°C and 330 rpm) for 7 days. Compounds were extracted with acidic methanol-chloroform (1:3) and evaporated to dryness. The crude extract was subjected to silica gel liquid chromatography and was further separated by reversed-phase high-pressure liquid chromatography on a Merck Hitachi instrument (L-6200A/L-4250).

Instrumental analysis. Structural elucidation of the compounds by nuclear magnetic resonance (NMR) was performed with a JEOL JNM-GX 400 spectrometer in dimethyl sulfoxide ($DMSO_{d6}$). The spectra were run at ambient temperatures in the solvent, and both the ^1H and ^{13}C NMR spectra were referenced internally to tetramethylsilane, which was assigned a value of 0 ppm.

Nucleotide sequence accession number. The 16.5-kb DNA fragment sequenced from *Streptomyces* sp. strain PGA64 has been submitted to GenBank and given accession number AY034378.

RESULTS AND DISCUSSION

Deduced function of *pgaF* gene product. The gene cluster designated *pga* was cloned from a rubromycin-producing soil isolate, *Streptomyces* sp. strain PGA64. Analysis of the 16.5-kb DNA fragment sequenced revealed that the region was most similar to gene clusters involved in the biosynthesis of angucycline metabolites (Metsä-Ketelä, unpublished). In particular, the cluster harbors a gene designated *pgaF*, which is homologous to various cyclases found in angucycline biosynthesis gene clusters. These cyclases are distinct from other cyclases found in databases, and although they do show a lower degree of similarity to other cyclases involved in secondary metabolite production, they are indicative of angucycline biosynthesis. *pgaF* encodes a short 109-amino-acid peptide which is most related to the *lanF* gene product from the landomycin biosynthesis (19) pathway (82% identity, 92% similarity), followed by the *jadI* gene product (79% identity, 86% similarity), which has been shown to cyclize the last angular ring in jadomycin biosynthesis (11).

Conversion of nogalamycin biosynthetic pathway toward angucyclines. We have recently elucidated the biosynthesis pathway of auramycinone by stepwise cloning of genes and isolation of the compounds produced (7; Kantola, unpublished). From these studies we acquired plasmid pMC5, which contains

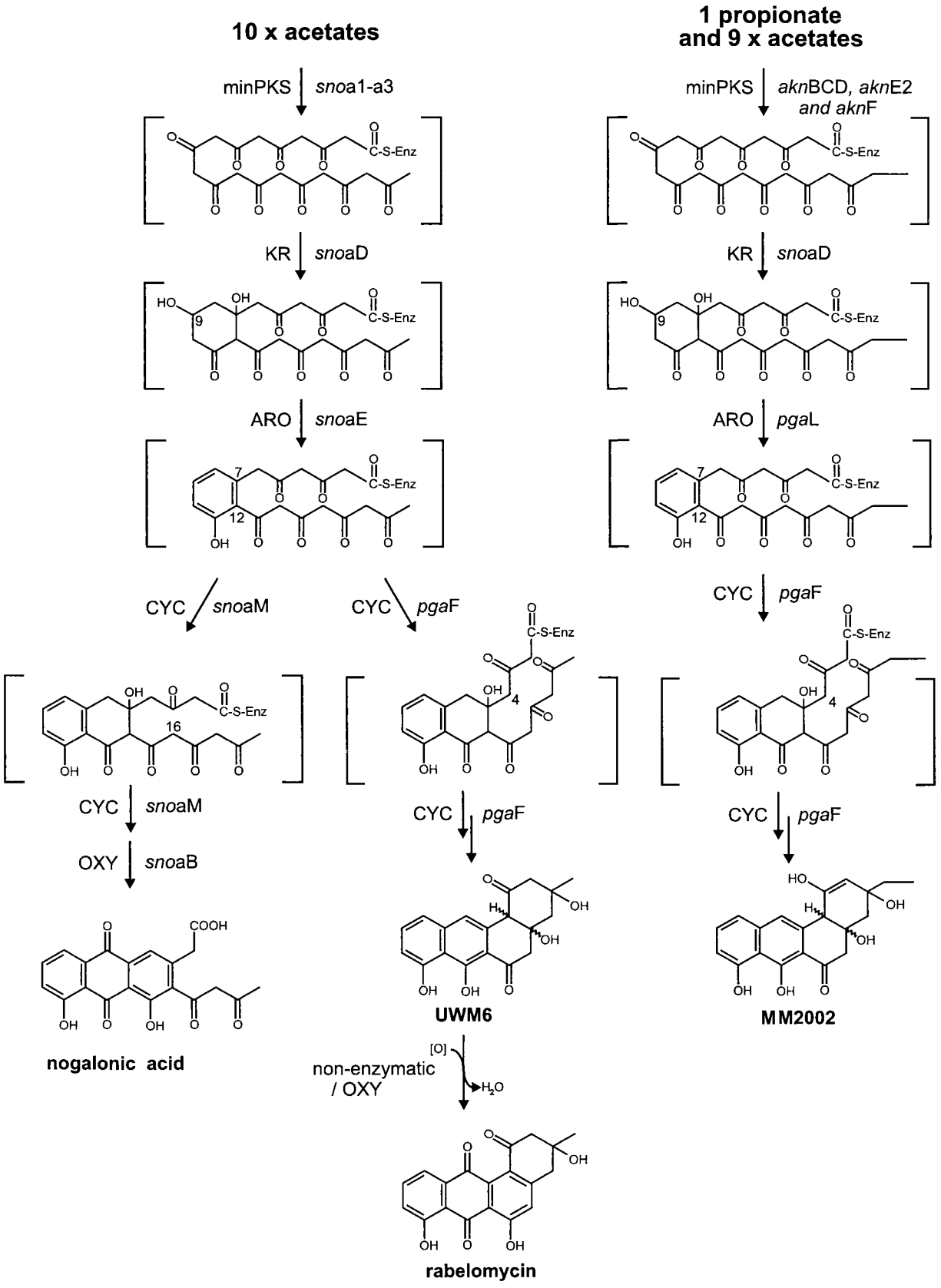

FIG. 2. Structures and hypothetical biosynthesis pathways of the compounds produced in this study. The pathway resulting in the production of nogalonic acid is described elsewhere in detail (14). Structures within brackets indicate hypothetical intermediates.

TABLE 1. Sequences of oligonucleotide primers used in amplification of the genes used in this study[a]

Gene	Forward and reverse primer sequences	Restriction site	Reference
snoa1 and *snoa2*	GATTCTAGAACTTCAAGACGATCAAAGC	*Xba*I	Kantola, unpublished
	GATAAGCTTTTAATTAACTTACCGCGGCTCCGG	*Hin*dIII and *Pac*I	Kantola, unpublished
snoa3	GATTTAATTAACGGTAAGAGGCCACGGAA	*Pac*I	Kantola, unpublished
	GATAAGCTTGCTAGCAGTTCCGTGCCGTACACA	*Hin*dIII and *Nhe*I	Kantola, unpublished
snoaD	GATGCTAGCTTGTATTCCGCCGTGTC	*Nhe*I	Kantola, unpublished
	GATAAGCTTGTTAACTGTCATGGTCCTCCGTCC	*Hin*dIII and *Hpa*I	Kantola, unpublished
snoaE	GATGTTAACGGCCTGGGAAACTACTGA	*Hpa*I	Kantola, unpublished
	GATAAGCTTATGCATGAACCGTCCTCCGCTGT	*Hin*dIII and *Nsi*I	Kantola, unpublished
pgaF	CTTAAGCCCGCTCACCCGAAGAAGAG	*Afl*II	This work
	TACGTACTTCACTGTGTGGCCGTCCA	*Sna*BI	This work
pgaL	GTTAACGACCTGAAACCGACCCCTGA	*Hpa*I	This work
	ATGCATGTGTGGTCCTCGCTCATGTC	*Nsi*I	This work
aknBCDE2F	GATTCTAGAGCCCGAACTGTATCTGGTA	*Xba*I	12
	GATAAGCTTGCTAGCAGGACAAACGTCAGCAGCT	*Nhe*I	12

[a] The *Hin*dIII sites at the ends of the *sno* reverse primers were used as insertion sites for the indicated gene.

the genes that are responsible for the putatively mutual part of auramycinone and angucyclinone biosynthesis, namely, *snoa1-a3* (minPKS), *snoaD* (KR), and *snoaE* (ARO). The putative angucycline cyclase *pgaF* was amplified from *Streptomyces* sp. strain PGA64 by PCR; inserted into the pMC5 cassette, resulting in plasmid pMC6B (Fig. 3); and introduced into *S. lividans* TK24 by standard protoplast transformation methods (9).

Fermentation of *S. lividans* TK24/pMC6B resulted in the production of two previously undetected compounds that appeared in different ratios in separate flask culture fermentations after 7 days of cultivation. The two compounds were isolated from the culture broth, and structural elucidation of the compounds by NMR (Table 2) revealed that the compounds were UWM6 and rabelomycin, which have previously been described as intermediates in the jadomycin biosynthesis pathway (11). Additionally, during the NMR work the instability of UWM6 under the conditions used was observed, as the sample slowly turned red; the maximum of the visible spectrum changed from 440 to 510 nm. This indicates additional conjugation within the molecule, which could result from the elimination of two water molecules and the formation of a fully aromatized derivative of UWM6 that is aromatized similarly to 6-hydroxytetrangulol (21). Furthermore, this transformation

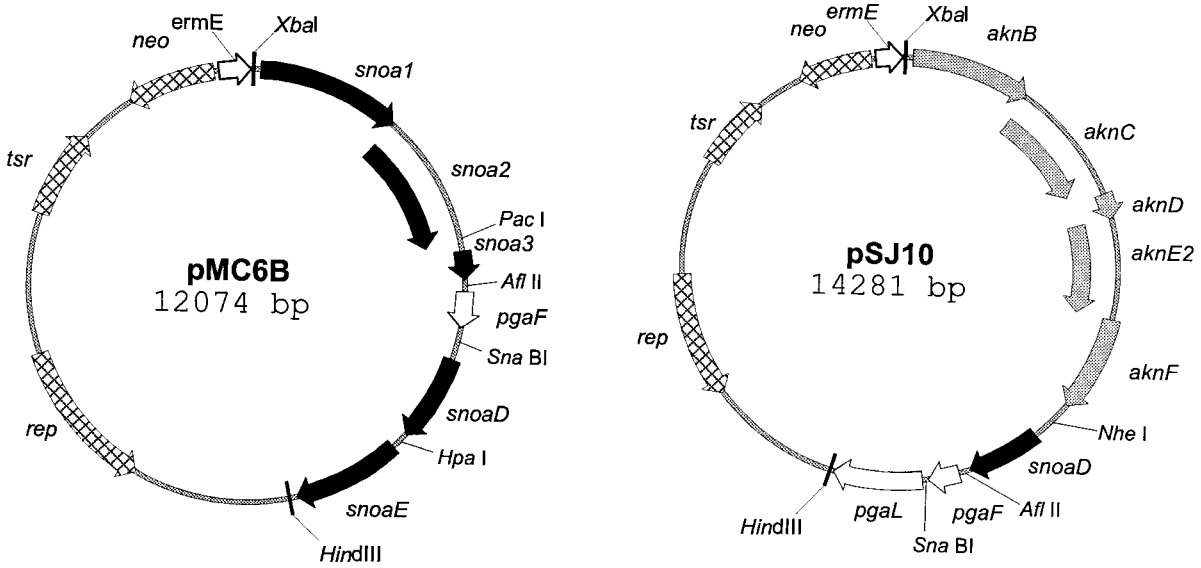

FIG. 3. Diagrams of expression constructs used in this work. The genes have been obtained from *S. nogalater* (*sno*; black), *S. galilaeus* (*akn*; gray), and *Streptomyces* PGA64 (*pga*; white); and they encode the following proteins: KS_{α} (*snoa1*, *aknB*), KS_{β} (*snoa2*, *aknC*), ACP (*snoa3*, *aknD*), starter unit selection (*aknE2*, *aknF*), KR (*snoaD*), ARO (*snoaE*, *pgaL*), and CYC (*pgaF*). The antibiotic biosynthesis genes were cloned in the pIJ486 vector under the constitutive *ermE* promoter (8).

TABLE 2. ^{1}H and ^{13}C NMR spectral data for compounds MM2002 and UWM6

Site	MM2002		UWM6	
	^{1}H NMR spectrum (400 MHz)	^{13}C NMR spectrum (100 MHz)	^{1}H NMR spectrum (400 MHz)	^{13}C NMR spectrum (100 MHz)
1		166.1 (s)		203.9 (s)
1-OH	brd[a], exchangeable			
2A	5.95, 1H, s	123.5 (d)	3.00, 1H, d, 18.0	57.5 (t)
2B			2.58, 1H, d, 18.0	
3		72.4 (s)		72.3 (s)
3-OH	brd, exchangeable		brd, exchangeable	
4A	2.72, 1H, d, 17.6	42.6 (t)	2.90, 1H, d, 17.8	46.9 (t)
4B	2.50, 1H, d, 17.6		2.56, 1H, d, 17.8	
4a		71.1 (s)		70.0 (s)
4a-OH	brd, exchangeable		brd, exchangeable	
5A	2.92, 1H, d, 17.8	48.2 (t)	3.18, 1H, d, 17.9	53.1 (t)
5B	2.78, 1H, d, 17.8		2.87, 1H, d, 17.9	
6		202.0 (s)		202.7 (s)
6a		113.0 (s)		110.0 (s)
7		163.4 (s)		164.6 (s)
7a		108.9 (s)		108.7 (s)
7-OH	16.0, 1H, s		15.9, 1H, brs	
8		157.5 (s)		157.2 (s)
8-OH	9.66, 1H, s		9,67, 1H, s	
9	6.84, 1H, d, 7.8	110.7 (d)	6.78, 1H, dd, 7.8, 0.9	110.8 (d)
10	7.47, 1H, dd, 8.2, 7.8	132.2 (d)	7.45, 1H, dd, 8.1, 7.8	132.2 (d)
11	7.17, 1H, d, 8.2	118.2 (d)	7.18 1H, dd, 8.0, 0.9	118.0 (d)
11a		139.1 (s)		138.6 (s)
12	7.01, 1H, s	121.1 (d)	7.14, 1H, s	124.8 (d)
12a		133.2 (s)		132.9 (s)
12b	3.87, 1H, s	58.2 (s)	3.80, 1H, s	62.0 (d)
13	2.26, 2H, m	29.9 (t)	1.31, 3H, s	23.6 (q)
14	1.11, 3H, t, 7.2	10.5 (q)		

[a] brd, broadened.

was accelerated in acidic conditions, which are favorable to the elimination reaction.

Of the two compounds identified, UWM6 has been suggested to be the compound that results from the direct action of the corresponding jadomycin biosynthesis genes and rabelomycin has been suggested to emerge from oxidation of UWM6 in flask cultures or from the concomitant action of endogenous genes of the host strain, *S. lividans* (11). These suggestions were based on the fact that the principal product was UWM6 on R2YENG agar plates. Our results coincide with the nonenzymatic model for UWM6 oxidation, since addition of an absorbent, XAD-7, to liquid cultures shifted the production greatly toward UWM6 and only minor amounts of rabelomycin were produced. This can be interpreted to mean that the UWM6 produced by the strain binds to XAD-7 after export to the culture medium and is subsequently protected from oxidation.

Interestingly, studies on the total synthesis of angucyclinones present an attractive analogy and a possible explanation for the nonenzymatic oxidation of the C-12 carbon; Krohn et al. (10) have reported a method for the total synthesis of a rabelomycin derivative in which the C-1 ketone is synthesized by photoinduced oxygenation upon exposure to daylight. They suggested that the reaction could proceed through a diradical intermediate derived from photoinduced hydrogen abstraction at C-1 by the neighboring C-12 carbonyl group. In the biosynthetic route presented here, a similar diradical could conceivably be formed after dehydration of UWM6 and photoinduced hydrogen abstraction at C-12 by the C-1 carbonyl group. The hydrogen abstraction can then be followed by reaction with oxygen and subsequent ketone formation.

Conversion of aclacinomycin biosynthetic pathway toward angucyclines. To further test the ability of *pgaF* to act in conjunction with unnatural biosynthesis genes, we replaced the *sno* minPKS genes with the corresponding genes *aknB*, *aknC*, and *aknD* as well as genes *aknE2* and *aknF* from the aclacinomycin pathway. The *aknE2* and *aknF* genes, which are homologous to the *dpsC* and *dpsD* genes from the daunorubicin biosynthesis pathway (1), respectively, are responsible for the initiation of biosynthesis from a longer propionate starter unit (14). This expression construct, which was designated pSJ10 (Fig. 3) and which was transformed into *S. lividans* TK24, was cultivated in E1 medium with XAD-7. The compounds produced were purified as described above for *S. lividans* TK24/pMC6B. According to the NMR results (Table 2), the strain produced the expected UWM6 derivative, MM2002, which, however, appeared in the NMR solvent used as an enol tautomer (Fig. 2). The accumulation of MM2002 in the culture medium demonstrated that the *pgaF* cyclase is capable of processing a C_{21} polyketide chain and cyclizing the last ring correctly.

The *jadI* jadomycin cyclase, unlike the *pgaF* cyclase described here, has been reported to exhibit strict context-dependent behavior and nonfunctionality with the *tcm* and *dps* biosynthesis genes. Tetracenomycin biosynthesis proceeds through an unreduced intermediate of the first ring, which subsequently leads to cyclization between C-9 and C-14 that is different from the folding pattern of reduced polyketides like anthracyclines and angucyclines. The differences in the early

part of the biosynthesis of unreduced and reduced polyketides are the most likely reasons why these two pathways were incompatible, as investigators acknowledged previously (11).

On the contrary, the biosynthesis of daunorubicin proceeds in a manner highly similar to the biosynthesis of aclacinomycins, as described above, and no clear explanation for the incompatibility of the *jad* and *dps* biosynthesis cyclases could be presented (20). However, it is interesting that the expression constructs used in the *jad* and *dps* biosynthesis experiments also included the *tcmJ* gene from the biosynthesis pathway of tetracenomycin. The exact function of *tcmJ* has not been elucidated, but it has been reported to improve production of tetracenomycin in its native cluster (5). Nonetheless, in light of the fact that the *pgaF* cyclase is functional with two different sets of anthracycline biosynthesis genes, it would be interesting to see if the inclusion of *tcmJ* in the expression constructs has an effect on the nonfunctionality of the *jad* and *dnr* genes.

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