Production of hybrid anthracycline antibiotics by heterologous expression of *Streptomyces nogalater* nogalamycin biosynthesis genes

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A cluster of anthracycline biosynthetic genes isolated from *Streptomyces nogalater* was expressed in *Streptomyces lividans* and in *Streptomyces galilaeus*. A 12 kb DNA fragment cloned from this cluster in pIJ486 caused the production of a novel compound when introduced into *S. lividans*. The compound is derived from nogalonic acid methyl ester, an early intermediate in nogalamycin biosynthesis. Complementation with the cloned 12 kb fragment of *S. galilaeus* mutants blocked in aclacinomycin biosynthesis caused the production of hybrid anthracyclines. Cloning of the nogalamycin gene cluster should make possible a detailed study of the biosynthesis of this interesting antibiotic, as well as the production of novel anthracyclines of potential value as cytostatic drugs.

Keywords: *Streptomyces nogalater*, anthracycline, nogalamycin, gene cloning

INTRODUCTION

Anthracyclines, produced mainly by *Streptomyces* spp., are aromatic polyketide antibiotics widely used in cancer chemotherapy. Nogalamycin is a member of an anthracycline group produced by *Streptomyces nogalater* (Bhuyan & Dietz, 1965; Wiley *et al.*, 1977; Wiley *et al.*, 1968). Several derivatives of nogalamycin have been prepared and their pharmacological properties characterized (Li & Krueger, 1991); one of them, menogaril, is undergoing clinical trials for use in cancer chemotherapy. So far, studies of nogalamycin have been mainly concerned with the biological and chemical properties of the compound. No biosynthetic studies have been described since Wiley *et al.* (1978) showed that acetate is the starting unit in the pathway.

We have previously reported a study on the biosynthetic pathway for aclacinomycins (Ylihonko *et al.*, 1994). Aklavinone, the aglycone moiety of aclacinomycins, is a precursor of several natural anthracyclines (Oki *et al.*, 1975). The aglycone of nogalamycin differs from aklavinone in having the opposite stereochemistry at position 9 (Fig. 1), and in the side chain, where a methyl group replaces an ethyl group. The C-9 side chains each derive from the starter unit for polyketide synthesis, which is an acetate in nogalamycin and a propionate in aklavinone

biosynthesis. Therefore, aklavinone is not a precursor of nogalamycin, but the steps in the biosynthetic pathway (Fig. 1) are probably analogous.

In this paper, we describe the cloning and expression in *Streptomyces lividans* of a gene cluster from *S. nogalater*, which caused the production of a novel compound derived from nogalonic acid methyl ester, a proposed intermediate in the biosynthesis of nogalamycin. The gene cluster was implicated in nogalamycin biosynthesis by gene disruption and was further characterized by the complementation of *Streptomyces galilaeus* mutants blocked in the pathway for biosynthesis of aclacinomycins, the anthracyclines of wild-type *S. galilaeus*. Besides characterizing the gene cluster, this offered a way to produce hybrid antibiotics by the technique of Hopwood *et al.* (1985b). The hybrid anthracyclines produced have auramycinone, with a methyl side chain at position 9 as in nogalamycin, as the aglycone moiety. The C-9 side chain of anthracyclines is derived from a biosynthetic starter unit selected by the polyketide synthase (PKS). Modification and glycosylation of the aglycone are, however, dependent on the host strains used. With this system it may be possible to produce a series of novel anthracyclines that can be screened for cytostatic activity.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. E1 medium [2% (w/v) glucose, 2% (w/v) soluble

Abbreviations: MPLC, medium pressure liquid chromatography; PKS, polyketide synthase; tsr, thiostrepton.

Fig. 1. (a) The proposed biosynthetic pathway for the auramycinone glycosides produced by *S. galilaeus* recombinant strain H028(pSY15). The stereochemistry at C-9 in nogalamycin is opposite to that in auramycinone. In aklavinone there is an ethyl group instead of a methyl group at C-9. (b) Structures of nogalamycin and aclacinomycin A.

starch, 0.5% Farmamedia (Trader's Mill Protein Co.), 0.25% yeast extract, 0.1% K_2HPO_4 , 0.1% $MgSO_4 \cdot 7H_2O$, 0.3% NaCl, 0.3% $CaCO_3$] was used to culture cells for anthracycline production (Ylihonko *et al.*, 1994). For other cultures, ISP4 agar (Difco) or TSB medium (Tryptone Soya Broth, Oxoid) were used. Culture conditions for *Streptomyces* strains were as described by Hopwood *et al.* (1985a). Standard culture conditions were used for *Escherichia coli* strains (Sambrook *et al.*, 1989).

DNA isolation and cloning. *E. coli* plasmid isolation, transformation and DNA manipulations were carried out by standard procedures (Sambrook *et al.*, 1989). For isolation of total DNA, *S. nogalater* was grown in TSB medium supplemented with 0.5% glycine. DNA was isolated by the method of Hopwood *et al.* (1985a) and partially digested with *Sau3A*I. Fragments of DNA about 20 kb in size were purified on a 0.3% agarose gel and ligated to λ EMBL4 *Bam*HI arms. The ligation mixture was packaged into phage particles and plated with *E. coli* GM2163.

Hybridization. The *actI* probe was the 0.8 kb *Bgl*II fragment from pIJ2345 comprising parts of *actI* ORF1 and ORF2 (Fernandez-Moreno *et al.*, 1992). The *acm* probe was the 3 kb *actI*-hybridizing *Bam*HI fragment isolated from *S. galilaeus* (Niemi *et al.*, 1994). The probes were labelled with 50 μ Ci (1.85 MBq) [α - 32 P]CTP by nick-translation (Sambrook *et al.*, 1989). Membranes were hybridized in a Techne hybridization oven and washed with $2 \times$ SSC ($1 \times$ SSC is 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0)/1% (w/v) SDS, or with $0.2 \times$ SSC/0.1% SDS at 65 °C. Plaques giving hybridization signals

were isolated and purified as described elsewhere (Niemi *et al.*, 1994).

Gene disruption. The 0.5 kb and 1.5 kb *Eco*RI-*Bam*HI fragments from the *actI*-hybridizing region (Fig. 2) were cloned in the integration vector pGM160, and digested with *Eco*RI and *Bam*HI. The resulting plasmids pGSn1 and pGSn2, respectively, were introduced by transformation into *S. lividans* TK24 and recombinant clones were selected with thiostrepton (tsr). Plasmids were extracted from TK24 and introduced into *S. nogalater*. The transformants were transferred to plates of ISP4 agar containing tsr (50 μ g ml $^{-1}$) for sporulation. The spores were used to inoculate TSB medium and cultures were grown at 39 °C, without selection pressure, to eliminate the autonomously replicating plasmids. Four-day-old TSB cultures were plated on ISP4 agar supplemented with tsr and resistant clones were assayed for nogalamycin production. This method allows selection of single crossover products only.

Subcloning in *Streptomyces*. Small DNA fragments from λ clones were first cloned in *E. coli* XL1-Blue using pUC19. pIJ486 was used for all subclones in *Streptomyces* strains. Recombinant plasmids were introduced by transformation into *S. lividans* as described by Hopwood *et al.* (1985a), and after isolation from *S. lividans* were transferred to *S. galilaeus*. For transformations in *S. galilaeus*, the procedure for *S. lividans* was modified by using SGYEME instead of YEME (110 g instead of 340 g sucrose l $^{-1}$) (Hopwood *et al.*, 1985a), supplemented with 0.8% glycine. Thiostrepton (200 μ g per transformation plate), was added after 40–48 h.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic	Source or reference
Strains		
<i>S. nogalater</i>		
ATCC 27451		Bhuyan & Dietz (1965)
Sno-366	Nogalamycin non-producing	K. Ylihonko and others (unpublished)
<i>S. lividans</i> TK24		Hopwood <i>et al.</i> (1985a)
<i>S. galilaensis</i>		
ATCC 31615		Fujiwara <i>et al.</i> (1980)
H028	Aclacinomycin non-producing	Ylihonko <i>et al.</i> (1994)
H071	Aclacinomycin non-producing	Ylihonko <i>et al.</i> (1994)
H054	Glycosylation-deficient mutant	Ylihonko <i>et al.</i> (1994)
<i>E. coli</i>		
GM2163		Raleigh <i>et al.</i> (1988)
XL1-Blue		Stratagene
Plasmids		
pUC19		Pharmacia
pIJ486		Ward <i>et al.</i> (1986)
pIJ2345	<i>actI</i> (2.2 kb) probe in pBR329	Malpartida <i>et al.</i> (1987)
pACM5	<i>acm</i> probe in pBR322	Niemi <i>et al.</i> (1994)
pGM160	<i>E. coli-Streptomyces</i> vector, temperature-sensitive <i>Streptomyces</i> replicon	Muth <i>et al.</i> (1989)
pGSn1	pGM160-derived integration construction	This work
pGSn2	pGM160-derived integration construction	This work
pSY15	pIJ486 derivative, expression construction	This work
pSY6	pIJ486 derivative, expression construction	This work
pSY1/pSn1	pIJ486 derivative, expression construction/pUC19 derivative for sequencing	This work

Fig. 2. Restriction map of *S. nogalater* genomic DNA carrying part of the nogalamycin biosynthetic cluster. The arrows represent the sizes of *S. nogalater* DNA fragments inserted into pIJ486 (shown by dashed lines). The segment hybridizing with the *actI* probe is shown by a filled bar. The *acm*-hybridizing region (Sno1 and Sno3) is to the left of the 2 kb *actI*-hybridizing *EcoRI* fragment.

Detection of anthracyclines and their intermediates. *S. lividans* and *S. galilaensis* strains were grown in E1 medium (Ylihonko *et al.*, 1994), and 5 µg tsr ml⁻¹ was added to cultures of plasmid-containing strains. After 7 d, samples (0.5 ml) were extracted with chloroform or with toluene/methanol (1:1, v/v) under acidic and neutral conditions. Portions (10–20 µl) of the toluene or chloroform phase were applied to a TLC plate (Kieselgel 60 F₂₅₄, Merck) and developed with chloroform/methanol (10:1, v/v) or with toluene/ethyl acetate/methanol/formic acid (50:50:15:3, by vol.).

Two HPLC systems were used to characterize the products and to determine the purity of MPLC (medium pressure liquid chromatography) fractions. Both systems used an octadecyl reverse phase column (RP-18). The solvent systems were acetonitrile/buffer (7:3, v/v) (S-1) and acetonitrile/buffer/methanol (5:3:2, by vol.) (S-2), the buffer being 60 mM potassium dihydrogen phosphate adjusted with citric acid to pH 3. The compounds were detected with a UV monitor at 254 nm.

Cosynthesis. Portions (1 ml) from each of two 48-h-old E1 cultures (Ylihonko *et al.*, 1994) were used to inoculate 50 ml E1 medium in a 250 ml Erlenmeyer flask. The flasks were shaken (330 r.p.m.) at 28 °C. After incubation for 2–3 d the co-culture was assayed for anthracyclines by TLC.

Production of anthracycline metabolites. The compounds were produced in 7 l fermenters containing 5.5 l E1 medium supplemented with tsr (5 µg ml⁻¹). The fermentation was carried out at 28 °C with aeration at 6 l min⁻¹ and with stirring at 280 r.p.m. After 4–6 d, the broth was separated by centrifugation. Anthracyclines and their intermediates were extracted from the pellet with methanol. The methanol extract was added to the broth supernatant and the solution was extracted with chloroform. The chloroform extracts were evaporated, then resolubilized in a small amount of chloroform. The solution was chromatographed on a silica gel column with chloroform/acetic acid (200:1, v/v) (neutral intermediates and aglycones) and with gradients of chloroform/methanol/acetic acid (200:0:1 to 200:20:1, by vol.) (glycosides) using a MPLC system. Fractions containing pure compounds were combined and evaporated to dryness.

Spectroscopy. ¹HNMR spectra were recorded with a Jeol JNM-GX400 system. The spectra were assigned by comparing them to those of aklavinone and aklavinone glycosides.

RESULTS

Isolation of *actI*- and *acm*-hybridizing DNA from *S. nogalater*

actI DNA, encoding the actinorhodin PKS (Fernandez-Moreno *et al.*, 1992), has been successfully used to identify the biosynthetic genes of different polyketide antibiotics (for example, Bibb *et al.*, 1994; Sherman *et al.*, 1989; Yu *et al.*, 1994). Southern blotting was used to select a convenient probe for localizing the PKS gene cluster from *S. nogalater*. Genomic DNA from *S. nogalater* digested with *EcoRI* showed two non-contiguous hybridizing fragments (2 kb and about 15 kb) when the 0.8 kb *BglII* fragment of *actI* was used as a probe. With the *acm* probe, only one signal, over 15 kb in size, was obtained but was estimated to be too weak for plaque screening.

A *Sau3A* library of *S. nogalater* genomic DNA in λEMBL4 was screened using the *actI* probe. Five hybridizing clones

(Sno1, Sno2, Sno3, Sno6, Sno10) were identified. Three of them, Sno1, Sno2 and Sno3, covered over 20 kb of contiguous DNA (Fig. 2), while two clones (Sno6 and Sno10) were not located in this region. The region covered by Sno1, Sno2 and Sno3 also hybridized with the *acm* fragment, and was therefore selected for subcloning.

The *actI* probe identified two separate DNA regions, but was used in plaque screening because of its strong signals, whilst the *acm* probe was used to select one of the two DNA regions for further subcloning. Two *actI*-hybridizing regions have also been found in *Streptomyces coelicolor*, where they represent genes for actinorhodin and spore pigment formation (Malpartida *et al.*, 1987; Davis & Chater, 1990). The *acm* probe seems to be very useful in identifying anthracycline biosynthetic genes because, in addition to the gene cluster from *S. nogalater*, we have recently used it to identify two anthracycline clusters, a rhodomycin cluster from *Streptomyces purpurascens* (Niemi *et al.*, 1994) and an anthracycline cluster from *Streptomyces peucetius* (unpublished results).

Disruption of a putative nogalamycin gene cluster

To implicate the cloned Sno cluster in nogalamycin biosynthesis, pGSn1 and pGSn2 were constructed (Fig. 2). pGSn2 consisted of almost the whole of ORF1 and 300 bp of the upstream sequence, whilst the 0.5 kb insert of pGSn1 contained 150 bp of the 3' end of ORF1 and 350 bp of the 5' end of ORF2 (Fig. 2). (The ORFs for the minimal PKS are numbered according to those for granaticin, Sherman *et al.*, 1989.) Because of the very low transformation frequency of *S. nogalater*, pGSn1 and pGSn2 were first constructed in *S. lividans* and then transferred into *S. nogalater*. The *S. nogalater* transformants were plated on ISP4 agar for sporulation. After plasmid curing, five tsr-resistant clones were obtained from both the pGSn1 and pGSn2 transformants.

Three of five pGSn1 clones retained tsr resistance during cultivation and were analysed for nogalamycin production. Two were non-producing and the third produced a low yield of nogalamycin-related metabolites. Evidence for plasmid integration in the two non-producing clones was supported by Southern hybridization. Three of the five putative pGSn2 integrants produced nogalamycin at wild-type levels, and based on Southern hybridization, they still carried free plasmids; the other two putative integrants produced diminished amounts of nogalamycin. Overall, the results implicated the cloned Sno cluster in nogalamycin biosynthesis.

Subcloning of λ clones in *S. lividans*

Subcloning of the 6 kb *EcoRI* fragment from λ clone Sno3, and of the 12 kb *BglII* fragment from Sno1, into pIJ486 gave pSY6 and pSY15, respectively (Fig. 2). pSY6 and pSY15 were then introduced by transformation into *S. lividans* TK24. Subcloning in pUC19 of the 2 kb fragment excised from Sno1 with *EcoRI* gave pSn1. This plasmid was extracted from *E. coli*, and the insert was ligated into pIJ486 and introduced into TK24 to give

Table 2. Properties of the compounds produced by *S. lividans* TK24 and *S. galilaeus* mutant H028 transformed with pSY15

Compound	Producer	Colour (vis/UV)	R _F value*	R _t (min)†	Identity‡
1	TK24(pSY15)	Yellow/brown	0.76 (a)	5.80 (S-1)	ND
2	TK24(pSY15)	Yellow/orange	0.85 (a)	5.60 (S-1)	ND
3	TK24(pSY15)	Yellow/orange	0.87 (a)	4.16 (S-1)	I
4	H028(pSY15)	Yellow/orange	0.22 (b)	2.92 (S-1) 3.52 (S-2)	IV
5	H028(pSY15)	Yellow/orange	0.75 (b)	2.92 (S-1) 4.02 (S-2)	V
6	H028(pSY15)	Yellow/yellow	0.87 (b)	3.67 (S-1) 4.50 (S-2)	III

ND, Not determined.

* Mobile phases for TLC were (a) toluene/ethyl acetate/methanol/formic acid (50:50:15:3, by vol.) and (b) chloroform/methanol (10:1, v/v).

† R_t, HPLC retention time. HPLC conditions are described in Methods.

‡ The structures of compounds I, III, IV and V are shown in Fig. 1.

pSY1. A restriction map of the λ clones with the fragments used for subcloning is shown in Fig. 2.

S. lividans does not produce anthracyclines and is typically colourless or blue on ISP4 agar, but *S. lividans* TK24 carrying pSY15 differed from the parent strain in giving a brown colour on ISP4 agar. When cultured in the anthracycline production medium E1, TK24 did not produce any coloured compounds, but yellow and red compounds were detected in a sample from the TK24(pSY15) culture. The properties of the main products of TK24(pSY15) are shown in Table 2. One of the products (I, Fig. 1) was determined by NMR spectroscopy (Table 3) to be a novel compound by comparing the spectrum with those of aklavinone and auramycinone. The aromatic portion of this compound is the same as the aromatic portion of the two aglycones. The fourth ring is not condensed, since the integral of the singlet corresponding to C-10 in the aglycone gives two protons instead of one. The position corresponding to C-9 carries a proton because the methyl at position 13 gives a doublet instead of a singlet. Compound I is suggested to be a shunt product derived from nogalonic acid methyl ester, a hypothetical intermediate of nogalamycin biosynthesis similar to the aclacinomycin precursor, aklanonic acid methyl ester (Ylihonko *et al.*, 1994).

pSY6, which lacks the *actI*-hybridizing region, did not cause production of anthracyclines or their intermediates in *S. lividans*. Also, pSY1, containing the *actI*-hybridizing part of pSY15, failed to cause production of anthracycline metabolites in E1 medium.

Cosynthesis

Structural analysis of compounds produced by *S. lividans* TK24(pSY15) suggested that the 12 kb *Bgl*II fragment from *S. nogalater* encodes several enzymes for anthra-

cycline biosynthesis. To test this, TK24(pSY15) was co-cultured with *S. nogalater* Sno366 (L. Cong, K. Ylihonko & P. Mäntsälä, unpublished results). The latter strain failed to produce any coloured compound in E1 medium, and was characterized as a mutant blocked early in the nogalamycin pathway. The co-cultures produced nogalamycin, thus suggesting that the compounds are probably derived from the nogalamycin pathway because they were converted to this product by Sno366.

Complementation of *S. galilaeus* mutant H028 and production of hybrid anthracyclines

S. galilaeus ATCC 31615 produces aclacinomycins, which consist of aklavinone and a sugar moiety (rhodosamine-2-deoxyfucose-cinerulose) attached to C-7 (Fujiwara *et al.*, 1980). *S. galilaeus* mutant H028 (Ylihonko *et al.*, 1994), which does not produce any anthracycline metabolites, was transformed with pSY15. The transformants were cultured in E1 medium and their products were first characterized by TLC and HPLC. The properties of the products from H028(pSY15) are shown in Table 2. The structures of the aglycone and two glycosides were then determined by ¹HNMR spectroscopy (Table 3). The main compound of H028(pSY15) was aglycone III (auramycinone) which was also the aglycone moiety in the glycoside fraction. Auramycin-like hybrid glycosides were determined to be compounds IV (auramycinone-rhodosamine-deoxyfucose) and V (auramycinone-rhodosamine-deoxyfucose) (Fig. 1) by comparing their NMR spectra with that of aclacinomycin S (aklavinone diglycoside). The spectrum of IV was nearly identical to this compound, except that the side chain at C-9 gave a singlet for a methyl group instead of the ethyl group of aklavinone. Compound V lacked, additionally, the dimethylamine group and gave two double triplets of rhodose 2',3'. Compound IV has earlier been reported as a

Table 3. ¹HNMR data for compounds I, III, IV and V

Site	I	III	IV	V	AcmS*
1	7.85 dd, 7.5, 1.2, 1H	7.84 dd, 7.5, 1.2, 1H	7.84 dd, 7.6, 1.4, 1H	7.86 d, 7.6, 1H	7.84 d, 7.7, 1H
2	7.73 dd, 8.5, 7.5, 1H	7.71 dd, 8.5, 7.5, 1H	7.70 t, 8.3, 7.6, 1H	7.70 t, 8.4, 7.6, 1H	7.68 t, 8.4, 7.7, 1H
3	7.35 dd, 8.5, 1.2, 1H	7.32 dd, 8.5, 1.2, 1H	7.32 dd, 8.3, 1.4, 1H	7.32 d, 8.4, 1H	7.31 d, 8.3, 1.2, 1H
4-OH	11.91 s, 1H	11.97 s, 1H	12.03 s, 1H	12.03 s, 1H	12.05 s, 1H
6-OH	12.59 s, 1H	12.74 s, 1H	12.70 brs, 1H	12.73 s, 1H	12.69 brs, 1H
7		5.39 brs, 1H	5.28 brs, 1H	5.26 brs, 1H	5.27 brs, 1H
8A	3.27 dd, 17.8, 2.6, 1H	2.63 dd, 15.0, 5.1, 1H	2.61 dd, 14.9, 4.1, 1H	2.62 dd, 14.5, 4.2, 1H	2.52 dd, 14.9, 4.2, 1H
8B	3.12 dd, 17.8, 9.1, 1H	2.24 ddd, 15.0, 2.1, 1.0, 1H	2.20, 14.9, 1H	2.20 d, 14.5, 1H	2.29 d, 14.9, 1H
9-OH		4.06 brs, 1H	4.78 brs, 1H	4.38 s, 1H	4.56 brs, 1H
10	4.42 m, 2H	4.06 d, 1.0, 1H	4.11 s, 1H	4.11 s, 1H	4.12 s, 1H
11	7.72 s, 1H	7.70 s, 1H	7.67 s, 1H	7.68 s, 1H	7.70 s, 1H
13	1.29 d, 6.5, 3H	1.42 s, 3H	1.39 s, 3H	1.39 s, 3H	
13A					1.75 m, 1H
13B					1.51 m, 1H
14					1.09 t, 7.4, 3H
15	3.72 s, 3H	3.72 s, 3H	3.71 s, 3H	3.72 s, 3H	3.70 s, 3H
1'			5.54 brs, 1H	5.50 brs, 1H	5.52 brs, 1H
2'			1.85 m, 2H	1.95 dt, 9.0, 3.9, 2H	1.87 dd, 6.8, 2.2, 2H
3'			3.63 brs, 1H	1.77 dt, 9.0, 3.0, 2H	3.64 brs, 1H
3'-NMe ₂			2.18 brs, 6H		2.18 brs, 6H
4'			3.74 brs, 1H	3.75 brs, 1H	3.74 brs, 1H
5'			4.01 q, 7.1, 1H	4.17 q, 1H	4.00 q, 6.8, 1H
6'			1.21 d, 7.1, 3H	1.27 d, 3H	1.28 d, 6.8, 3H
1''			5.01 brs, 1H	4.97 brs, 1H	5.01 brs, 1H
2''			2.08 dd, 11.7, 4.6, 2H	2.07 dd, 12.0, 4.9, 2H	2.08 m, 1H
3''			4.13 m, 1H	4.17 m, 1H	4.13 m, 1H
4''			3.62 brs, 1H	3.59 brs, 1H	3.75 brs, 1H
5''			4.53 q, 7.1, 1H	4.17 q, 1H	4.54 q, 6.1, 1H
6''			1.29 d, 7.1, 3H	1.27 d, 3H	1.20 d, 6.1, 3H

* AcmS, aklavinone-rhodosamine-deoxyfucose.

mutant product (Hoshino *et al.*, 1982). Compound V, auramycinone with neutral glycosides, is a novel compound similar to an aklavinone glycoside isolated earlier from H054, a mutant of *S. galilaensis* defective in glycosylation (Ylihonko *et al.*, 1994).

DISCUSSION

To establish that we had cloned the biosynthetic genes for nogalamycin we determined their function by gene disruption. Putative enzymes for biosynthesis of anthra-

cyclines were deduced from the structure of a compound produced by heterologous expression. In addition, we demonstrated that it is possible to use the genes as a cluster when constructing strains to produce hybrid anthracyclines.

Because of the restriction barriers in *S. galilaensis*, *S. lividans* TK24 was used as a primary cloning host. The *S. nogalater* genes in pSY15 were expressed in TK24, and surprisingly a shunt product (I), derived from the anthracycline intermediate nogalonic acid methyl ester, was formed. The polyketide backbone formed was not derived from

genes for actinorhodin, although it is possible that they also take part in the biosynthesis of tricyclic compounds. Bartel *et al.* (1990) have complemented a PKS-deficient *S. galilaeus* mutant with a gene for actinorhodin but this gene product acts in the early stage of biosynthesis using the polyketide backbone as a substrate. However, an aromataase and a cyclase for actinorhodin do not accept carbon chains longer than 16 as substrates (McDaniel *et al.*, 1995), suggesting that the gene products encoded by pSY15 are responsible for cyclizations of aromatic rings. On the basis of the ability of pSY15 to complement a *S. galilaeus* mutant accumulating 2-OH anthracycline metabolites, we have shown that it encodes a ketoreductase, which eliminates a hydroxyl group at C-2 of such metabolites (Ylihonko *et al.*, 1996). These findings suggest that the activities needed to form the anthracycline chromophore are encoded in the 12 kb *Bgl*II fragment from *S. nogalater* genomic DNA.

The best-documented gene cluster for anthracyclines is that of daunomycin (Grimm *et al.*, 1994; Ye *et al.*, 1994). However, biosynthetic intermediates were obtained by heterologous expression only when large fragments were cloned since the genes for acyl carrier protein (ACP) and a transcriptional activator (*dnrI*) are not closely linked to other minimal PKS components (Grimm *et al.*, 1994). Usually the three ORFs for minimal PKS in aromatic polyketides (the β -ketosynthase, the chain length factor (CLF) and the acyl carrier protein; McDaniel *et al.*, 1994a, b) are closely linked. The unusual arrangement of three ORFs for minimal PKS of daunomycin was not found in the *Sno* cluster, allowing expression of a tricyclic intermediate by a single pSY15 clone. [The sequence (EMBL accession number Z48262) of the *actI*-hybridizing region in pSY15 contains three ORFs for the 'minimal PKS'].

The most significant result was the successful production of 'hybrid' anthracyclines by direct complementation of the *S. galilaeus* anthracycline-non-producing mutant with the plasmid pSY15. The fact that the anthracyclines produced contained a methyl side chain at C-9 suggests that the genes from *S. nogalater* determined the starter unit used in biosynthesis of the hybrid anthracyclines. *S. lividans* TK24(pSY15) did not produce auramycinone (Fig. 1, III), although it was able to produce a tricyclic intermediate of it. Therefore, it seems that the cluster in pSY15 lacks a cyclase needed to complete formation of the intact aglycone. In the *S. galilaeus* mutant, the corresponding enzyme is derived from the host. The stereochemistry of the aglycone formed is characteristic of the products of *S. galilaeus* rather than *S. nogalater* metabolites, whereas the side chain at C-9 is acetate-derived, as in nogalamycin (Fig. 1). On the basis of the structure of the aglycone, the hybrid compounds are related to auramycin anthracyclines.

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