Hybrid anthracycline antibiotics: production of new anthracyclines by cloned genes from *Streptomyces purpurascens* in *Streptomyces galilaeus*

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A DNA segment cloned from *Streptomyces purpurascens* ATCC 25489 close to a region that hybridized to a probe containing part of the actinorhodin polyketide synthase caused *S. galilaeus* ATCC 31615 to produce new anthracyclines. When transformed with certain sub-clones of this segment, the host produced glycosides of ϵ -rhodomycinone, β -rhodomycinone, 10-demethoxycarbonylklavinone and 11-deoxy- β -rhodomycinone in addition to those of aklavinone, the natural anthracyclines of *S. galilaeus*. The first two compounds are *S. purpurascens* products and the other two are novel compounds that conceptually are structural hybrids between *S. galilaeus* and *S. purpurascens* products. Three glycosides of one of the novel aglycones, 11-deoxy- β -rhodomycinone, were purified and found to possess cytotoxic activity against L1210 mouse leukaemia cells. Separate regions of the cloned *S. purpurascens* DNA are responsible for modification of the *S. galilaeus* host product at the 10- and 11-positions.

Keywords: *Streptomyces purpurascens*, anthracycline antibiotics, hybrid antibiotics, cloning

INTRODUCTION

Anthracyclines are an important group of antibiotics used in cancer chemotherapy. Most of them are either actinomycete metabolites or their semi-synthetic derivatives. The complexity of their structures has made syntheses difficult, and has restricted the development and testing of new members of this group. Natural anthracycline aglycones are produced by the polyketide pathway (Eckardt & Wagner, 1988). We sought to apply the principle of hybrid antibiotics (Hopwood, 1981; Hopwood *et al.*, 1985b) to the search for new anthracycline molecules.

Aklavinone is a precursor of the aglycones of most

anthracyclines (Eckardt & Wagner, 1988). Producers of other anthracyclines should contain the enzymes that modify aklavinone to these anthracyclines. The aklavinone glycoside producer, *Streptomyces galilaeus* (Oki *et al.*, 1975; Fujiwara *et al.*, 1980), should therefore be an ideal host for the production of hybrid anthracycline antibiotics. The strategy used was, firstly, to identify modifying genes by cloning biosynthetic clusters from other anthracycline producers by cross-hybridization; the probe used was a conserved segment of the actinorhodin biosynthetic genes (Malpartida *et al.*, 1987). Portions of the clusters were then transferred into *S. galilaeus* to identify genes causing the production of novel compounds in combination with the aklavinone biosynthetic genes of the host.

METHODS

Bacteria and plasmids. The organisms and vectors are listed in Table 1.

General recombinant DNA methods. Standard methods (Maniatis *et al.*, 1982; Hopwood *et al.*, 1985a) were used, with the exceptions described below. Plasmids were isolated by the

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Abbreviation: tsr, thiostrepton.

Table 1. Bacteria and vectors used

Designation		Reference	Source	Notes
<i>Streptomyces galilaeus</i>	ATCC 31615	Fujiwara <i>et al.</i> (1980)	ATCC, Rockville, MD, USA	
<i>Streptomyces galilaeus</i>	H067*		K. Ylihonko (unpublished)	
<i>Streptomyces purpurascens</i>	ATCC 25489	Shirling & Gottlieb (1969)	ATCC, Rockville, MD, USA	
<i>Streptomyces lividans</i>	TK24	Hopwood <i>et al.</i> (1985a)	D. A. Hopwood, John Innes Institute, Norwich	
<i>Escherichia coli</i>	GM2163	Raleigh <i>et al.</i> (1988)	<i>E. coli</i> Genetic Stock Center†	
<i>Escherichia coli</i>	NM538	Frischauf <i>et al.</i> (1983)	Promega	
Bacteriophages	λEMBL3	Frischauf <i>et al.</i> (1983)	Promega	
	λEMBL4	Frischauf <i>et al.</i> (1983)	Amersham	
Plasmids	pIJ486	Ward <i>et al.</i> (1986)	D. A. Hopwood, John Innes Institute, Norwich	<i>actI</i> probe <i>acm</i> probe: in pBR322 (Bolivar <i>et al.</i> , 1977)
	pIJ487	Ward <i>et al.</i> (1986)		
	pIJ2345	Malpartida <i>et al.</i> (1987)		
	pACM5	This work		
	pRDM6	This work		

* H067 was produced from ATCC 31615 by NTG mutagenesis using a modification of the method of Baltz (1986), and selected for lysozyme sensitivity.

† *E. coli* Genetic Stock Center, Department of Biology 255 OML, Yale University, New Haven, USA.

alkaline lysis method (Kieser, 1984) unless otherwise stated. Restriction fragments were isolated from agarose gels using the GeneClean system (Bio 101).

Preparation of hybridization probes and hybridization conditions. The *actI* probes were the 2.2 kb *Bam*HI fragment or the 0.8 kb *Bgl*II fragment (designated *actI0.8*) from pIJ2345. The *acm* probe was the 3 kb *Bam*HI fragment from pACM5. The plasmids were isolated by neutral lysis, followed by caesium chloride/ethidium bromide gradient centrifugation, isopropanol extraction and ethanol precipitation. The probe fragment was isolated by preparative agarose gel electrophoresis after digestion with the appropriate restriction enzyme; about 100 ng was labelled with 50 µCi [α^{32} -P]ATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹)] by random priming (Feinberg & Vogelstein, 1983, 1984), and the labelled probe was separated from unincorporated label by chromatography on Sephadex G-25. Membranes were prehybridized for at least 6 h at 65 °C in 1% (w/v) SDS, 1 M NaCl, 5 × Denhardt's solution and 100 µg calf thymus DNA ml⁻¹ before addition of the denatured probe and a further 100 µg carrier DNA ml⁻¹. Hybridization was performed for at least 16 h. The membranes were washed twice with 100 ml 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) plus 1% SDS at room temperature for 5 min, and twice with 300 ml of the same solution at 65 °C for 30 min. They were subsequently autoradiographed.

Isolation of total DNA from *S. galilaeus* and *S. purpurascens*. Total DNA was isolated by a modification of the method of Hopwood *et al.* (1985a) from cultures grown in 50 ml SGYEME medium. To prepare SGYEME medium, a solution containing Difco yeast extract (3 g l⁻¹), Difco Bacto-peptone (5 g l⁻¹), Oxoid malt extract (3 g l⁻¹), glucose (10 g l⁻¹) and sucrose (100 g l⁻¹) was autoclaved and supplemented with 2 ml sterile 2 M MgCl₂ l⁻¹ and 80 ml sterile 10% (w/v) glycine solution l⁻¹. Cultures were incubated in 250 ml Erlenmeyer flasks, each with a steel spring baffle, at 30 °C for 50 h. In phenol extractions, samples were mixed by careful inversion instead of vortexing;

solutions containing DNA were transferred with a wide-bore pipette. DNA was spooled on a glass rod after layering the aqueous solution with 1 vol. isopropanol. The precipitate was dipped in 70% (v/v) ethanol, dried *in vacuo* for 5–10 min, and dissolved in 1–2 ml TE buffer (Maniatis *et al.*, 1982). The concentration and molecular size of the DNA were determined by electrophoresis in 0.3–0.6% agarose gels.

Preparation of λ gene banks from *S. galilaeus* and *S. purpurascens* genomic DNA. Genomic DNA (> 30 kb) prepared as above was digested partially with *Sau*3AI and fractionated by sucrose gradient centrifugation. Fractions of about 20 kb were ethanol precipitated and ligated with λ vector arms prepared with *Bam*HI cohesive ends (λEMBL3 with *S. galilaeus* DNA, λEMBL4 with *S. purpurascens* DNA.) Ligations were monitored by agarose gel electrophoresis in 0.3% agarose, and packaged into λ particles (Rosenberg *et al.*, 1985; Rosenberg, 1987).

Isolation and characterization of hybridizing clones. The prepared λ gene banks were plated using *Escherichia coli* GM2163 as host on 150 mm diameter Petri dishes at a density of 4000–10000 plaques per plate, and two replicate filters were lifted from each plate using nylon membranes (Colony/Plaque Screen, DuPont NEN) according to the manufacturer's instructions. Plaques giving matching hybridization signals on duplicate filters were picked, purified by plating the eluted phage at low plaque density and repeating the hybridization screening, and used to prepare phage stocks by confluent plate lysis. Phage DNA was prepared as described by Kaslow (1986), except that it was precipitated with isopropanol.

The λ clone inserts were restriction mapped by single and double digestion; assignment of fragments was confirmed by Southern hybridization of the digested DNA.

Subcloning in *Streptomyces*. Fragments were isolated from λ clones by preparative gel electrophoresis followed by GeneClean treatment. pIJ486 was linearized with suitable restriction

enzyme(s), treated with calf intestinal alkaline phosphatase where the cohesive ends were rejoinable, and ligated with the isolated fragments using T4 DNA ligase. *S. lividans* was transformed as described by Hopwood *et al.* (1985a). Mycelium of *S. galilaeus* used for transformation was grown in SGYEME instead of YEME, and regeneration plates were overlaid with thiostrepton (tsr) solution after 40–48 h. Plasmid DNA from *S. lividans* transformed *S. galilaeus* at a frequency of about $1 \mu\text{g}^{-1}$, and from *S. galilaeus* at about $10^4 \mu\text{g}^{-1}$.

Detection of anthracycline antibiotics produced by *S. galilaeus*. *S. galilaeus* strains were grown at 28.5°C in 250 ml Erlenmeyer flasks containing 60 ml E1 medium [g l^{-1} : glucose, 20; soluble starch, 20; Pharmamedia (Traders' Mill Protein Co., Fort Worth, Texas), 5; yeast extract, 2.5; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1; NaCl, 3; CaCO_3 , 3; in tap water, pH adjusted to 7.5)] using high aeration (300 r.p.m.). With plasmid-containing strains, thiostrepton (tsr) was added at $5 \mu\text{g ml}^{-1}$.

For screening novel products, *S. galilaeus* was grown for 7 d, after which a 1 ml sample was centrifuged; the cell pellet was extracted with 500 μl toluene/methanol (1:1, v/v) and 250 μl 0.1 M sodium phosphate buffer, pH 7.0, was mixed with the extract. After centrifugation a sample of the toluene phase was applied to a TLC plate (Kieselgel 60 F₂₅₄, Merck) and developed with toluene/ethyl acetate/methanol/formic acid (50:50:15:10, by vol.).

To detect aglycones of the glycosides produced, the toluene phase obtained above was extracted with 0.1 M HCl, and the aqueous phase hydrolysed for 30 min in a boiling water bath. The aglycones were extracted into toluene, and analysed by TLC on oxalic-acid-treated silica (see below).

Production of hybrid glycosides. Hybrid *S. galilaeus* strains were maintained on ISP4 agar (Difco) containing $5 \mu\text{g tsr ml}^{-1}$. To produce a seed culture, 60 ml medium E1 containing tsr in a 250 ml Erlenmeyer flask was inoculated with a colony and grown for 4 d at 28.5°C in a rotary shaker at 330 r.p.m. The culture was transferred to 5.5 l medium E1 sterilized in a 7 l fermenter and supplemented with tsr (to $5 \mu\text{g ml}^{-1}$) and antifoam (5 ml polypropylene glycol P 2000, Fluka). The production culture was grown for 5 d at 30°C , 350 r.p.m., and with aeration at 61 min^{-1} . The fermenter contents were transferred to a 10 l vessel and stirred for 45 min with 400 g Celatom (Johns-Manville), 47 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 24.4 g citric acid, 500 ml water and 3 l methyl ethyl ketone. The extract was vacuum-filtered, and the phases were allowed to separate overnight after addition of 500 g NaCl. The organic phase was dried with 400 g Na_2SO_4 .

Isolation and hydrolysis of the glycoside fraction. The organic phase was evaporated *in vacuo*, redissolved in 150 ml toluene, and mixed with 150 ml isopropanol, 150 ml 0.1 M HCl and 75 ml hexane. The lower phase was removed and extracted with 40 ml dichloromethane. The dichloromethane phase was further extracted with 30 ml 1 M sodium phosphate buffer, pH 7.0. The upper phase of the first extraction was mixed with 75 ml isopropanol and 150 ml 0.1 M HCl; the lower phase was then treated in the same way as the lower phase from the first extraction. The combined dichloromethane extracts were evaporated *in vacuo* and the residue, redissolved in toluene, was extracted with 1 vol. 0.1 M HCl. The aqueous phase was heated at 85°C for 2 h, then extracted three times with 0.5 vol. toluene. The combined toluene extracts were washed with an equal volume of 0.1 M sodium phosphate buffer, pH 7.0, and evaporated to dryness. The aglycone products so obtained were redissolved in a small volume of toluene.

Fractionation of the aglycone mixture. The aglycones in the

toluene solution were purified by chromatography on a $4 \times 20 \text{ cm}$ column of oxalic-acid-treated silica (Brockmann *et al.*, 1965). Fractions eluted with 10% (v/v) acetone in toluene were analysed by TLC as described below. Those containing two components were fractionated again on a $1.5 \times 50 \text{ cm}$ column with 5% acetone in methylene chloride. The aglycones obtained were crystallized by dissolving in boiling methanol, concentrating the solution under a stream of nitrogen until crystals began to form, and allowing the solution to cool.

Large-scale fermentation of the hybrid strain and purification of aglycone IV. *S. galilaeus* ATCC 31615(pRDM6) was grown for 7 d in 550 l E1 medium containing tsr in a 750 l pilot fermenter; the culture was hydrolysed in the fermenter by adjusting the medium to pH 2 and heating to 85°C for 2 h. The hydrolysate was extracted with methyl ethyl ketone, and the extract was concentrated by vacuum distillation. A crude extract obtained was dissolved in 3 l toluene.

To 500 ml of the crude extract, representing the product from about 100 l culture from the fermenter, 100 g of oxalic-acid-treated silica was added. After stirring, the silica was recovered on a sintered glass filter, and washed twice with 500 ml toluene. Crude aglycone IV was eluted from the silica with acetone. The eluate was concentrated *in vacuo*, and dissolved in 100 ml methanol. The insoluble material was removed, and the solution was evaporated *in vacuo*. The residue was dissolved in 100 ml toluene; 100 ml hexane was added, and the clarified solution was again evaporated *in vacuo*. Aglycone IV in the residue was dissolved in toluene and chromatographed on oxalic-acid-treated silica on a $4 \times 20 \text{ cm}$ column. Fractions eluted with 10% acetone in toluene were analysed by TLC on oxalic-acid-treated silica plates; those containing mostly aglycone IV were extracted with 1 vol. 0.1 M sodium phosphate buffer, pH 7.0, to remove oxalic acid, dried over Na_2SO_4 , evaporated to dryness, and crystallized from methanol. The yield of pure aglycone IV was 10.1 mg.

Purification of aglycone IV glycosides. A portion (2 ml) of the glycoside solution obtained above was applied as a band to a TLC plate and developed with chloroform/methanol/acetic acid (20:5:1, by vol.). Yellow products at R_F values of 0.40 (IV A) and 0.55 (IV B) were recovered.

About 200 ml glycosidic extract was extracted with 0.05 M HCl, and the aqueous phase was incubated for 30 min at 55°C . The solution was neutralized with 20 ml 1 M sodium phosphate buffer, pH 7.0, and extracted three times with chloroform. The combined chloroform extracts were evaporated and the residue, dissolved in 20 ml dichloromethane, was chromatographed on a Kieselgel 60 (Merck) column with dichloromethane/methanol/acetic acid (100:20:1, by vol.). Fractions containing a yellow product with an R_F value of 0.21 (IV T) by TLC were pooled. Acetic acid was removed by extracting with water to which NaOH was added until the aqueous phase was neutral. The dichloromethane phase was evaporated and the residue, dissolved in 2 ml methanol and 20 ml toluene, was rechromatographed on a silical gel column with toluene/methanol (1:1, v/v) as eluent.

Cytotoxicity testing. The cytotoxicity of anthracycline glycosides was tested *in vitro* against L1210 mouse leukaemia cells using the method of Matsuzawa *et al.* (1981) modified for microtitre plates, and with cell numbers determined using a Coulter cell counter.

Analytical methods. Anthracycline glycosides were analysed by TLC on Kieselgel 60 F₂₅₄ using chloroform/methanol/acetic acid (30:4:1, by vol., first dimension) and toluene/ethyl acetate/methanol/formic acid (50:50:15:3, by vol., second

Fig. 1. Restriction maps of λ clones and the corresponding portion of the *S. purpurascens* chromosome. The segment to which the *acm* probe cross-hybridized is indicated by hatching. B, *Bam*HI; E, *Eco*RI; N, *Not*I; P, *Pvu*II; X, *Xho*I.

dimension). Aglycones were analysed by TLC on oxalic-acid-treated silica as follows: Kieselgel 60 F₂₅₄ plates were dipped in 0.25 M oxalic acid, dried in the oven at 110 °C and developed with chloroform/acetone (10:1, v/v). NaHCO₃-treated plates were similarly impregnated with a 0.5 M solution. NMR spectra were recorded with a 200 MHz Bruker AM-200 instrument, and mass spectra with a VG Analytical 7070E Organic Mass Spectrometer using chemical ionization with methane or isobutane.

Construction of the deletion series. Unidirectional deletions of cloned *S. purpurascens* DNA were made by the method of Henikoff (1984, 1987) using plasmid DNA isolated from *S. galilaeus*. After deletion of DNA, the religated plasmid mixture was introduced by transformation into *S. galilaeus*.

RESULTS

Cloning a fragment from *S. galilaeus* homologous to *actI* of *S. coelicolor*

As has been observed for other anthracycline producers (Stutzman-Engwall & Hutchinson, 1989), probes from the *actI* region of the actinorhodin polyketide synthase gene cluster hybridized to more than one *Bam*HI fragment of *S. galilaeus* DNA. Signals were detected for 3 kb and 9 kb fragments. A λ gene bank was constructed from *S. galilaeus* total DNA. For efficient cloning of *S. galilaeus* DNA in *E. coli*, it was necessary to use a *mcrB*⁻ host, GM 2163 (Raleigh *et al.*, 1988). The clones were then propagated in NM538. Plaques hybridizing with the *actI*0.8 probe were purified, and a 3 kb *Bam*HI hybridizing DNA fragment from one of them was subcloned into the *Bam*HI site of pBR322, producing pACM5. The insert in this plasmid, designated *acm*, hybridized with unique 3, 8 and 3 kb *Bam*HI fragments from *S. galilaeus*, *S. purpurascens* and *S. peucedinus*, respectively.

Hybrid anthracycline production by cloned *S. purpurascens* DNA

A λ gene bank from *S. purpurascens* was prepared in the same manner as that from *S. galilaeus*; six hybridizing clones were isolated using the *acm* probe. Restriction

mapping showed that these clones covered 23 kb of contiguous DNA (Fig. 1).

Various restriction fragments from the clones were transferred into *S. galilaeus* by cloning into the polylinker region of the high copy number plasmid pIJ486. Because *S. galilaeus* appears to have a relatively strong restriction barrier, the constructions were first made in *S. lividans* and then used to transform *S. galilaeus*. *S. galilaeus* H067, which is more easily transformed than the ATCC 31615 strain, but is a poor producer of anthracyclines, was used as intermediate host in most cases.

pRDM6, which contains the 12.6 kb insert of λ rdm6 subcloned using the flanking vector *Eco*RI sites, caused the production of anthracyclines that clearly differed from those of the host. On hydrolysis, they yielded several aglycones in addition to aklavinone, the aglycone produced by the host. Cultures of *S. galilaeus* containing pRDM6 in media without *tsr* selection rapidly lost their ability to produce aglycones other than aklavinone. When pRDM6 isolated from the original transformant was used to transform plasmid-free *S. galilaeus*, the new transformants produced the same additional aglycones, implicating pRDM6 in their production.

Purification and identification of aglycones produced by the hybrid strain

Cultures of the hybrid strain contained a complex spectrum of products: two-dimensional TLC of the crude glycoside fraction showed at least 28 components apparently containing an anthracyclinone chromophore. The mixture was hydrolysed and the aglycones were fractionated by column chromatography on oxalic-acid-treated silica. Two different solvent systems were used because pairs not resolved with dichloromethane/acetone (Brockmann *et al.*, 1965, but with dichloromethane replacing chloroform) could be resolved with toluene/acetone. The main fractions were designated I–V in the order they eluted; pairs resolved with dichloromethane/acetone were identified with letters. Thus, seven aglycones

Fig. 2. Observed anthracycline aglycones produced by *S. galilaeus* carrying pRDM6, and enzyme activities expected to be required for their production. The untransformed host produces glycosides of IA (aklavinone), and the gene donor *S. purpurascens* produces glycosides of IB and IIB (ϵ - and β -rhodomyconine).

(IA, IB, IIA, IIB, III, IV and V) were resolved (Fig. 2, except III and V). Comparison with authentic aglycones by TLC, mass spectroscopy and proton NMR identified IA, IB and IIB as aklavinone, ϵ -rhodomyconine and β -rhodomyconine, respectively.

Mass spectroscopy of IIA using direct chemical ionization (DCI) gave a molecular mass of 354 Da. Interpretation of the proton NMR spectrum led to the identification of IIA as 10-demethoxycarbonylklavinone (Fig. 2). Tanaka *et al.* (1980) previously prepared this aglycone chemically; their proton NMR data are very similar to ours. Authentic 10-demethoxycarbonylklavinone (a kind gift of Dr H. Tanaka, Mercian Corp., Japan) was indistinguishable from IIA by TLC on oxalic-acid-treated silica and on NaHCO_3 -impregnated silica with chloroform/acetone (10:1, v/v).

The molecular mass of IV was 370 Da, and the proton NMR spectrum was interpreted as that of 11-deoxy- β -rhodomyconine. Because reference data for this molecule were unobtainable, we compared the ^{13}C -NMR spectrum of IV with those of aklavinone and β -rhodomyconine

Table 2. Chemical shifts and suggested assignment of ^{13}C -NMR chemical shifts of aglycone IV, aklavinone and the aglycone portion of β -rhodomyconin A

Reference data are from Oki (1984). Chemical shift data are given in p.p.m. relative to $[^{13}\text{C}]$ tetramethylsilane.

Carbon no.	Aklavinone	β -Rhodomyconin A	Aglycone IV
14	6.7	6.6	6.52
13	32.5	32.9	29.03
8	34.9	30.5	35.38
16	52.6		
10	56.7	66.7	72.10
7	62.5	70.4	61.12
9	71.9	71.8	73.01
5a	114.7	112.1	114.06
4a	115.8	116.0	115.99
1	120.4	119.7	119.31
11	121.5	157.3	120.87
3	124.8	124.9	124.33
11a	132.7	111.5	131.85
6a	133.1	135.2	132.85
12a	133.6	133.3	133.41
2	137.7	137.1	137.36
10a	142.9	138.7	148.25
6	161.4	156.9	160.57
4	162.8	162.7	161.30
15	171.5		
12	181.3	186.2	181.16
5	192.8	190.7	191.93

(Table 2). The chemical shift data support the proposed structure for IV.

The proton NMR spectra of IIA and IV, as well as the ^{13}C -NMR spectrum of IV, have been deposited with the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, UK, as Supplementary Publication no. SUP 28027 (4 pages).

On the basis of proton NMR spectra, and the observation that in milder hydrolysis conditions their amounts are much reduced, III and V were identified as 7-epimers of IIA and IV, respectively, and concluded to be artefacts of hydrolysis.

Restriction fragments of the insert in λ rdm6 were subcloned in pIJ486. The smallest tested restriction fragment to cause the production of all the hybrids was the 6.2 kb *Bam*HI-*Sau*3AI (–*Eco*RI) fragment designated EB3 in Fig. 3.

To locate more accurately the genes responsible for the various modifications, a deletion series was constructed from the clone containing EB3 in pIJ486. Fig. 3 shows the aglycones produced by *S. galilaeus* transformed with various members of the deletion series. The largest fragment causing the production of all hybrids is 1.3 p1 (5.0 kb). Further shortening of the fragments eliminated

Fig. 3. Deletion series of the 6.2 kb *Bam*HI-*Sau*3AI (-*Eco*RI) fragment of λ rdm6 and aglycones produced by *S. galilaeus* carrying the remaining fragments in pIJ486.

production of both aglycones with an 11-hydroxyl group. With fragments shorter than 3.6 kb (2-24A) no hybrid production was detected by TLC.

The EB3 fragment was also subcloned into pIJ487, which has the opposite orientation of the polylinker region from pIJ486. *S. galilaeus* transformed with this construct produced only glycosides of aklavinone (IA) and ϵ -rhodomycinone (IB). The same products were observed when the 8.1 kb *Bam*HI fragment (6.5–14.6 kb, Fig. 1) was subcloned in pIJ486.

Purification and biological activity of some glycosides of aglycone IV

Three glycosidic components, IV A, IV B and IV T, were purified from the glycosidic mixture. On hydrolysis, all of them yielded aglycone IV; on mild hydrolysis IV A and IV B yielded IV T. The mass spectrum of IV T shows a molecular ion peak at 528, corresponding to aglycone IV + rhodosamine. When IV A and IV B were added to cultures of *S. galilaeus* ATCC 31615 under conditions where biotransformation of aclacinomycin B to aclacinomycin A takes place (Hoshino *et al.*, 1983), IV B was transformed into IV A, and the latter remained unchanged.

The concentrations of the glycosides were estimated assuming molar extinction values similar to aclacinomycin A, and their *in vitro* cytotoxicity against mouse leukaemia

cell line L1210 was tested. The potencies of all three (ED_{50} 75, 24 and 22 nmol l⁻¹ for IV A, IV B and IV T, respectively) were within an order of magnitude, although less, than that of aclacinomycin A (10 nmol l⁻¹).

DISCUSSION

Cross-hybridization with *actI* (Malpartida *et al.*, 1987) again proved successful in the isolation of genes participating in the biosynthesis of a polyketide. The anthracycline modifying genes described in this study were isolated from *S. purpurascens* using a homologous fragment from *S. galilaeus* that had been isolated using *actI*. Although it has not been shown that this fragment is part of the aklavinone biosynthetic cluster, it also recognizes the 3 kb *Bam*HI fragment in *S. peucetius* which is part of the biosynthetic cluster of ϵ -rhodomycinone (Stutzman-Engwall & Hutchinson, 1989; K. Ylihonko, unpublished data).

pRDM6 caused *S. galilaeus* to synthesize new anthracycline products. The aglycones identified can be arranged in a plausible biosynthetic pattern (Fig. 2) and could all be produced by enzyme activities expected in *S. purpurascens*, which makes glycosides of ϵ - and β -rhodomycinone. The overall amount of anthracycline glycosides produced by the hybrid strains was similar to that of the host (about 100 mg l⁻¹). However, this is divided over a large number of products, so it was not possible to produce enough glycosides for definitive structural determination. By mild

Fig. 4. Proposed structures of the three 11-deoxy- β -rhodomyacinone glycosides purified and tested for biological activity in this work.

hydrolysis, higher anthracycline glycosides yielded predominantly monoglycosylated products. Rhodosamine glycosides could be distinguished from daunosamine glycosides by their partitioning to the toluene phase at pH 7.0, and further by the mass spectrum of IV T. We propose that the three glycosides tested for biological activity have the structures shown in Fig. 4. The cytotoxic activity of these compounds in a simple *in vitro* test is comparable to that of anthracyclines presently in clinical use (Matsuzawa *et al.*, 1981), but only further tests can show whether these new glycosides could be clinically useful.

Otake *et al.* (1985) described glycosides of 11-deoxy- β -rhodomyacinone having daunosamine glycosylation; unfortunately further data are not available to us. As far as we know, the 11-deoxy- β -rhodomyacinone glycosides with rhodosamine glycosylation described in this work are new.

The cloned segments of *S. purpurascens* DNA did not cause the large increase in anthracycline production observed when cloned *dnrR*₁ and *dnrR*₂ segments of *S. peuceitius* DNA were introduced into a producing host (Stutzman-Engwall *et al.*, 1992).

Whether production of the new compounds is due to activation of host genes (so-called silent genes: Jones & Hopwood, 1984) by the cloned DNA cannot be excluded by present data. During strain development of the host in our laboratory, mutants producing 11-hydroxylated anthracyclines have never been seen (these would be clearly observable due to their colour) but mutants producing anthracyclines modified at the 10 position could conceivably have been missed. Preliminary bio-transformation experiments using *S. lividans* transformed with pRDM6 and its subclones have not been successful. The fact that the DNA regions causing modifications at

the 10- and 11- positions can be separated also speaks against the silent gene hypothesis.

It is remarkable that only the 11-modification was expressed, when the EB3 fragment was inserted in the opposite orientation, or about 1.5 kb more DNA was included in the insert. We believe, that in the active subclones the 10-modifications are artefactually expressed from a vector promoter or some other *cis*-acting genetic element. As the insert in pIJ486 and pIJ487 is flanked on one side by an efficient transcription terminator (Ward *et al.*, 1986), the most probable explanation seems to be, that the promoter of the *tsr* resistance gene in the vector causes transcription as indicated in Fig. 3.

We expected that the regulation of anthracycline biosynthesis would be sufficiently conserved to allow the biosynthesis genes to be expressed in the producer of a related antibiotic. This appeared to be true for the 11-modification, but our results indicate that the 10-modifications are not naturally expressed in *S. galilaeus*.

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