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Expression of *Streptomyces peucetius* Genes for Doxorubicin Resistance and Aklavinone 11-Hydroxylase in *Streptomyces galilaeus* ATCC 31133 and Production of a Hybrid Aclacinomycin

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The aklavinone 11-hydroxylase gene and two doxorubicin resistance genes cloned from *Streptomyces peucetius* subsp. *caesius* ATCC 27952 were introduced into doxorubicin-sensitive *Streptomyces galilaeus* ATCC 31133, an aclacinomycin producer. The doxorubicin resistance genes *drrA* and *drrB* endowed *S. galilaeus* with high-level resistance to doxorubicin, indicating that the resistance mechanism for doxorubicin might be different from that for aclacinomycin A. Transformation of *S. galilaeus* ATCC 31133 with plasmid pMC213 containing the aklavinone 11-hydroxylase gene (*dnrF*) resulted in the production of many red pigments. A new metabolite was purified, and the position of the newly introduced hydroxyl group was determined. This result indicated that the aklavinone 11-hydroxylase gene was stably expressed in *S. galilaeus* ATCC 31133 and that it gave rise to a hybrid aclacinomycin A which showed highly specific in vitro cytotoxicity against leukemia and melanoma cell lines.

The anthracyclines are an important class of antibiotics and include such compounds as doxorubicin (DXR), aclacinomycin, and tetracenomycin, which exhibit extraordinary cytotoxicity toward tumor cells. The genetics and biochemistry of the formation of anthracyclines, particularly DXR (16) and tetracenomycin (11), have been well characterized, and the entire sets of biosynthetic genes for these drugs have been cloned. The biosynthesis of aclacinomycins has also been studied extensively by Umezawa and his coworkers (7, 15). However, there is no report concerning any aclacinomycin resistance gene, the self-resistance mechanism of the aclacinomycin producer, or the biosynthetic genes, except for that concerning the part of the gene cluster encoding the polyketide synthase (18).

There has been a growing need to find new anthracyclines with fewer side effects and greater therapeutic efficacy. Because of the diminishing returns of screening for new metabolites from nature, the hybrid antibiotic approach of using well-characterized antibiotic-producing strains as well as targeted gene disruption represents a potent alternative for the development of new derivatives (6). *Streptomyces* spp. can produce a variety of antibiotics in environmental culture conditions, and different strains are found to produce structurally related compounds. More interestingly, they share a part of the biosynthetic pathway leading to common intermediates, which opens up the possibility of creating hybrid antibiotics.

Previous biosynthetic studies revealed that the pathways leading to daunorubicin (DNR) and aclacinomycin share a common intermediate, aklavinone (15). After the formation of aklavinone, the biosynthetic pathways diverge to a hydroxylation reaction for the production of DXR and a glycosylation reaction for the production of aclacinomycin A (ACM-A) (Fig. 1). Recently, we cloned and characterized the DXR resistance genes and *dnrF* encoding the aklavinone 11-hydroxylase from *Streptomyces peucetius* subsp. *caesius* ATCC 27952 (3, 4). *Streptomyces galilaeus* should be a good host to study whether or not the genes are heterologously expressed and able to confer DXR resistance or to introduce a hydroxyl group into the aclacinomycin skeleton.

In this report, we describe the expression of DXR resistance and aklavinone 11-hydroxylase genes in *S. galilaeus*, the production of a hybrid anthracycline, 11-hydroxyaclacinomycin A (Fig. 1), and its in vitro cytotoxicity against human tumor cell panels.

Distribution of the *dnrF* gene among *Streptomyces* spp. We previously reported the cloning of DXR resistance genes (*drrA* and *drrB*) (4) and an aklavinone 11-hydroxylase gene (*dnrF*) (3) from *S. peucetius* subsp. *caesius* ATCC 27952, the DXR producer. Restriction maps constructed on the basis of the results of single and double restriction enzyme digestions of *dnrF*, *drrA*, and *drrB* loci are shown in Fig. 2. We performed Southern hybridization analyses to investigate the distribution among *Streptomyces* spp., including several anthracycline producers, of DNA that is homologous to *dnrF* and *drrA*. A digoxigenin-labeled 2.1-kb *Pst*I fragment which covers 1.0 kb of *dnrF* as well as 1.1 kb of *drrA* was used as a probe. Total chromosomal DNAs from *Streptomyces lividans* 1326, aclacinomycin-producing *S. galilaeus* ATCC 31133, baumycin-producing *Streptomyces* sp. strain C5 (9), and DXR-producing *S. peucetius* ATCC 27952 were digested with *Pst*I and used for Southern blots. Hybridizing bands with sizes of 2.1 kb and 4.0 kb were detected in the genomic DNAs of *S. peucetius* ATCC 27952 and *Streptomyces* sp. strain C5, respectively, suggesting that strain C5 is a close relative of *S. peucetius*. However, genomic DNA of *S. galilaeus* strains did not give any hybridization signal (Fig. 3). This result is not surprising, because the hydroxylation

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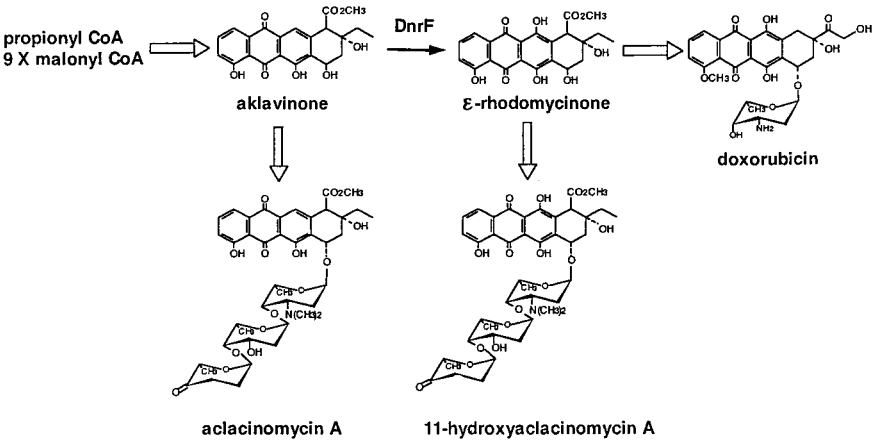

FIG. 1. Biosynthetic pathways of ACM-A, 11-hydroxyaclacinomycin, and DXR. DnrF, which catalyzes the conversion of aklavinone to ε-rhodomycinone, is indicated by a thin solid arrow, and open arrows indicate the multiple enzymatic steps. CoA, coenzyme A.

reaction of aklavinone at the C-11 position is necessary for the biosynthesis of DXR but not for that of ACM-A. However, *S. galilaeus* ATCC 31133 is known to produce cinerubin (1-hydroxyaclacinomycin A) (14). We therefore concluded that the gene encoding the aklavinone 1-hydroxylase would be different from that encoding the aklavinone 11-hydroxylase.

Expression of the *drrA* and *drrB* genes in *S. galilaeus* ATCC 31133. Expression of the *drrA* and *drrB* genes was studied by the transformation of ATCC 31133 with pIJ702, pMC1, and pMC4. The results of the experiments, in terms of the resistance levels of strains grown on R2YE plates to DNR, DXR, and ACM-A, are summarized in Table 1. *S. lividans* 1326, its pIJ702, pMC1, and pMC4 transformants, and *S. peucetius* ATCC 27952 were also tested. Drug resistance was initially tested with gradient plates, and further testing was done by inoculating approximately 10^5 spores of each strain onto plates containing 5, 10, 20, 30, 40, 60, 80, 100, 200, 300, and 400 μg of DXR per ml and 5, 10, 20, 30, 40, 60, 80, and 100 μg of ACM-A and DNR (each) per ml. *S. galilaeus* cells transformed with pMC1 and pMC4 exhibited higher levels of resistance to DXR and DNR than did untransformed *S. galilaeus* cells. As can be seen from Table 1, the level of resistance to DXR for the transformants was 15-fold greater than that obtained with the pIJ702 vector as a control. Some partial resistance was observed in the host cells transformed with the vector alone, but the reasons for this partial resistance are unknown (2). Although *S. galilaeus* itself is not markedly more sensitive to DNR than to DXR, in the transformants, *drrA* and *drrB* apparently confer significantly higher levels of resistance to DXR by comparison with those for DNR.

In a similar experiment, *S. galilaeus* transformed with *drrA* and *drrB* was tested for its level of resistance to ACM-A. As can be seen from Table 1, there were no significant differences in the levels of aclacinomycin resistance for *S. galilaeus* transformed with pIJ702 and the pMC1 and pMC4 transformants. *S. peucetius* ATCC 27952, however, was sensitive to ACM-A.

The deduced products of *drrA* and *drrB* have been shown to be similar to the multidrug resistance protein gp170, which mediates the outward transport of such anticancer drugs as DXR in drug-resistant cancer cells (1). This ATP-dependent efflux of cytotoxic drugs would be accomplished by a mechanism analogous to the mechanism of self-resistance conferred by DrrA and DrrB in the DXR producer (2). However, ACM-A was equally active against both DXR-resistant and -sensitive p388 cell lines, indicating that ACM-A is not likely to be a substrate of the multidrug resistance protein gp170 in cancer cells (19). The lack of an increase in the resistance to ACM-A of the transformants with *drrA* and *drrB* indicated that ACM-A is also not a substrate of DrrA and DrrB. This result, together with the absence of any detectable sequence similarity for strain ATCC 31133 and *drrA*, suggests that the ATP-dependent efflux system of *S. galilaeus* is different from that of *S. peucetius.*

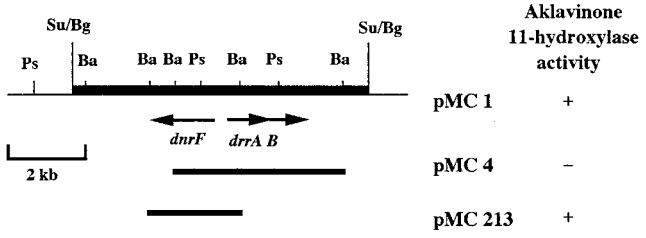

FIG. 2. Restriction maps of *dnrF* and *drrAB* loci. The restriction maps were constructed on the basis of the results of single and double restriction enzyme digestions. The thick solid line indicates a series of clones from the region of the DXR biosynthetic gene cluster containing the *drrAB* and *dnrF* loci. The thinner lines indicate a part of pIJ702, and the arrows indicate the open reading frames and directions of transcription. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Ps, *Pst*I; Su, *Sau*3AI; +, positive activity of aklavinone 11-hydroxylase; −, negative activity.

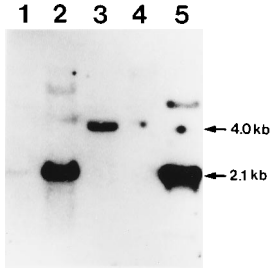

FIG. 3. Distribution of DNA sequences homologous to that of the *dnrF* gene in *Streptomyces* spp. A digoxigenin-labeled, 2.1-kb *Pst*I fragment of pMC4 was used as a probe. DNA-DNA hybridization was performed by the method of Southern (17). The genomic DNAs from various *Streptomyces* strains were digested with *Pst*I and used. Lane 1, *S. galilaeus* ATCC 31133; lane 2, *S. peucetius* ATCC 27952; lane 3, *Streptomyces* sp. strain C5; lane 4, *S. lividans* 1326; lane 5, *Pst*I-digested plasmid pMC1.

TABLE 1. Resistance of *Streptomyces* strains and their transformants to DXR, DNR, and ACM-A

Strain	Plasmid	MIC (μg/ml)		
		DXR	DNR	ACM-A
S. lividans 1326	None	<5	<5	<5
	pIJ702	20	10	5
	pMC1	400	100	5
	pMC4	400	80	5
S. peucetius ATCC 27952	None	40	10	<5
S. galilaeus ATCC 31133	None	<5	<5	20
	pIJ702	20	10	30
	pMC1	300	40	30
	pMC4	300	60	20

Expression of the *dnrF* gene in *S. galilaeus* ATCC 31133 to produce a hybrid metabolite. Our previous work on the cloning of the aklavinone 11-hydroxylase gene (3) enabled us to construct several subclones to locate the hydroxylase gene from a plasmid (pMC1) containing a 7.5-kb DNA fragment carrying the DXR resistance gene locus (4) (Fig. 2).

We transformed *S. galilaeus* ATCC 31133 with plasmid pMC213, which contained the aklavinone 11-hydroxylase gene (*dnrF*). The transformants, which were grown on R2YE plates (5), produced reddish pigments, which indicated that the gene was expressed in the host strain. One of the transformants cultivated in SGM medium (13) containing 10 μg of thiostrepton (Sigma) per ml yielded a new metabolite, which was purified by the following procedure. The culture broth (5 liters) was separated into the mycelial cake and the supernatant by centrifugation. The mycelial cake was extracted with acetone, and the extract was concentrated in vacuo to a small volume. The concentrate was extracted twice with chloroform and concentrated in vacuo again. The residue was loaded onto a cartridge column packed with a silica gel (Xpertek; P. J. Cobert Associates, Inc.) and eluted successively with chloroform and chloroform-methanol (1:1 [vol/vol]). The fractions with red spots, showing R_f values similar to those for ACM-A by thin-layer chromatography (Kieselgel 60 F_{254}; Merck Co.) developed with chloroform-methanol (15:1 [vol/vol]), were collected. The crude material was purified by high-performance liquid chromatography (HPLC) with a normal-phase column (3.9 by 300 mm) (μ-Porasil; Waters Co.) eluted with a solvent system of chloroform-methanol-acetic acid-water (68:20:10:2 [vol/vol/vol/vol]) supplemented with triethylamine to a final concentration of 0.01%. The elution profile was monitored with a Spectrafocus UV detector (Spectra-Physics) set at 289 nm.

The hybrid antibiotic 11-hydroxyaclacinomycin A was a reddish powder with a melting point of 160 to 162°C and was easily soluble in methanol and chloroform. The fast atom bombardment-mass spectrometric analysis showed a molecular ion peak at an *m/z* of 828 $(M + H)^+$ for this hybrid, with a 16-mass-unit difference from that of ACM-A (13) implying the addition of one oxygen atom to the molecule. The UV-visible spectrum showed maximum absorption at 235, 292, and 492 nm, indicating the presence of another hydroxyl group in the molecule which is causing a bathochromic shift (data not shown). Hydrolysis of the purified compound in 0.1 N HCl solution gave ε-rhodomycinone, according to thin-layer chromatography and HPLC analyses. The exact position of hydroxylation was further confirmed by ^{1}H nuclear magnetic resonance spectral analysis (Fig. 4). The proton signals of 11-hydroxyaclacinomycin A were very similar to those of ACM-A, except for the absence of a singlet at 7.58 ppm corresponding to the H-11 peak of ACM-A. This result was supported by the characteristic ^{13}C nuclear magnetic resonance signal shift resulting from the hydroxylation of aromatic carbon (120.8→156.9 ppm, data not shown). This result clearly showed that the aklavinone 11-hydroxylase gene has been expressed in *S. galilaeus* ATCC 31133 and is producing a hybrid antibiotic, 11-hydroxyaclacinomycin A, by introducing an OH group into the expected position (Fig. 1).

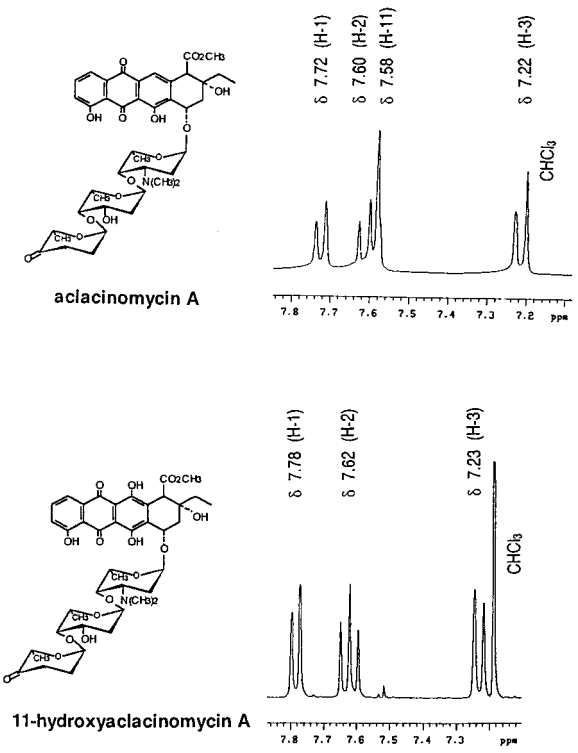

FIG. 4. ^{1}H nuclear magnetic resonance spectra of the aromatic regions of ACM-A and 11-hydroxyaclacinomycin A (300 MHz in $CDCl_3$).

11-Hydroxyaclacinomycin A was previously produced by microbial glycosylation of ε-rhodomycinone with a mutant that does not produce aclacinomycin (8, 15) and that is different from the one reported on here. The significance of this report is that endowment of the strain with aklavinone 11-hydroxylase activity by molecular genetic means resulted in the production of the hybrid antibiotic. Recently, Niemi et al. (12) reported the production of ε-rhodomycinone in *S. galilaeus* transformed with a cloned DNA fragment from *Streptomyces purpurascens* ATCC 25489, which indicated that the aklavinone 11-hydroxylase gene is present in the cloned DNA fragment.

In vitro cytotoxicity of 11-hydroxyaclacinomycin A. The in vitro cytotoxicity of 11-hydroxyaclacinomycin A for various types of human tumor cell lines was tested by the Developmental Therapeutic Program of the National Cancer Institute (10) in order to evaluate the drug's selectivity for particular tumor types.

As can be seen from Table 2, the compound showed selectivity for leukemia and melanoma, with average 50% lethal concentrations (LC_{50}) of $10^{-6.31}$ M and $10^{-6.4}$ M, respectively. It was most active against HL-60 leukemia cells, HCC-2998 colon cancer cells, and SK-MEL-2 and UACC-62 melanoma cells, with LC_{50} of less than 10^{-8} M.

The cytotoxicity results reported here indicate that 11-hy-

TABLE 2. In vitro test results for 11-hydroxyaclacinomycin and ACM-A activity against human cancer cell lines

Cancer cell panel	Cell line	Log LC_{50} (M)	
		11-Hydroxyaclacinomycin	ACM-A
Leukemia	CCRF-CEM		>−5.00
	HL-60(TB)	<−8.00	>−5.00
	K-562	−5.75	>−5.00
	MOLT-4	−6.04	>−5.00
	RPMI-8226	−5.78	>−5.00
	SR	−5.98	>−5.00
Non-small-cell lung cancer	A549/ATCC	−4.61	>−5.00
	EKVX	−6.59	>−5.00
	HOP-62	−4.64	>−5.00
	HOP-92	−4.26	−5.64
	NCI-H226	−5.01	>−5.00
	NCI-H23	−5.23	−6.13
	NCI-H322M	−4.24	>−5.00
	NCI-H460	−4.40	−5.24
	NIH-H522		−6.71
Colon cancer	COLO205		−6.37
	HCC-2998	<−8.00	−5.40
	HCT-116	−4.34	
	HCT-15	−4.38	>−5.00
	HT-29	−4.89	>−5.00
	KM12	>−4.00	−5.69
	SW-620	−4.75	−5.34
Central nervous system cancer	SF-268	−4.10	−5.04
	SF-295	−4.09	−5.19
	SF-539	−4.93	−5.61
	SNB-19		>−5.00
	SNB-75		−6.25
	U251	−4.25	>−5.00
Melanoma	LOXIMVI	−4.30	−5.23
	MALME-3M	−7.17	−6.25
	M14	−6.63	−6.39
	SK-MEL-2	<−8.00	−6.07
	SK-MEL-28	−5.55	−6.43
	SK-MEL-5	−7.53	−6.52
	UACC-257	>−4.00	−6.34
	UACC-62	<−8.00	−7.06
Ovarian cancer	IGROV1	−4.47	>−5.00
	OVCAR-3	−4.14	>−5.00
	OVCAR-4	−4.93	−5.90
	OVCAR-5	−4.41	>−5.00
	OVCAR-8	−4.37	>−5.00
	SK-OV-3	−4.41	>−5.00
Renal cancer	786-0	−5.08	−5.40
	ACHN	−4.49	>−5.00
	CAKI-1	−5.32	
	SN12C	−4.63	−6.68
	TK-10	−4.41	>−5.00
	UO-31	−4.72	>−5.00
Prostate cancer	PC-3	−4.84	−6.32
	DU-145	−4.61	>−5.00
Breast cancer	MCF7	−4.60	>−5.00
	MCF7/ADR-RES	>−4.00	−5.29
	MDA-MB-231/ATCC	>−4.00	>−5.00
	HS 578T	−4.23	−6.09
	MDA-MB-435	−6.94	
	MDA-N	−6.74	−6.39
	T-47D	>−4.00	>−5.00
Average log LC_{50}		−5.17	−5.48
Delta		2.83	1.58
Range		4.00	2.06

droxyaclacinomycin A is more toxic than ACM-A by 1 to several orders of magnitude. These results suggest that a hydroxyl group at C-11 of aglycon may play an important role in biological activities. *S. galilaeus* transformed with pMC213 is likely to produce a new series of 11-hydroxylated aclacinomycins corresponding to the aclacinomycin analogs produced by strain ATCC 31133. The effects of hydroxylation on the other aclacinomycin analogs should be evaluated by examining their antitumor activities.

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