***Streptomyces peucetius* daunorubicin biosynthesis gene, *dnrF*: sequence and heterologous expression**

Silvia Filippini,¹ Maria Manuela Solinas,² Umberto Breme,¹ Manuela Birgit Schlüter,² Davide Gabellini,² Giuseppe Biamonti,² Anna Luisa Colombo¹ and Luisa Garofano¹

Author for correspondence: Silvia Filippini. Tel: +39 331 583763. Fax: +39 331 583755.

¹ BioScience Center
PHARMACIA, Via Papa
Giovanni XXIII 23, 20014
Nerviano (Mi), Italy

² Istituto di Genetica
Biochimica ed
Evoluzionistica del CNR,
Via Abbiategrasso 207,
27100 Pavia, Italy

The *dnrF* gene, responsible for conversion of aklavinone to ϵ -rhodomycinone via C-11 hydroxylation, was mapped in the daunorubicin (Dnr) gene cluster of *Streptomyces peucetius* ATCC 29050, close to *drrAB*, one of the anthracycline-resistance genes. The *dnrF* gene was sequenced and should encode a protein of 489 amino acids with a molecular mass of 52 kDa. The deduced DnrF protein shows significant similarities with bacterial FAD- and NADPH-dependent hydroxylases either required to introduce hydroxyl groups into polycyclic aromatic polyketide antibiotics or involved in catabolism of aromatic compounds. Heterologous expression of *dnrF* in *Streptomyces lividans* TK23 and in *Escherichia coli* demonstrated that the gene encodes a NADPH-dependent hydroxylase catalysing the hydroxylation of aklavinone to yield ϵ -rhodomycinone. The enzyme is inactive on anthracyclines glycosylated at position C-7 and its activity decreases to a different extent with other substrate modifications, indicating that DnrF has a significant substrate specificity.

Keywords: aklavinone, hydroxylase, *dnrF* gene, anthracycline, *Streptomyces peucetius*

INTRODUCTION

Daunorubicin (Dnr) and especially its C-14 hydroxylated derivative doxorubicin (Dxr) are important antitumour agents used for the treatment of many different cancers (Arcamone, 1981). Unfortunately, besides their antineoplastic activity, they have long-term undesirable effects such as cardiotoxicity (Myers *et al.*, 1988). For this reason, studies are being done to develop new Dnr derivatives or new anthracyclines showing an increased antitumour spectrum and, at the same time, reduced cardiotoxicity (Cassinelli *et al.*, 1978; Crespi-Perellino *et al.*, 1982; White & Stroshane, 1984).

New possibilities for structural changes, resulting from recent progress in the molecular genetics of *Streptomyces*, have opened a promising line in the development of hybrid antibiotics (Floss & Strohl, 1991; Hopwood *et al.*, 1985b; Hutchinson, 1992; Strohl *et al.*, 1989). This approach requires the understanding of the anthracycline

biosynthetic pathway as well as the function of the single genes and enzymes involved.

Stutzman-Engwall & Hutchinson (1989) and Otten *et al.* (1990) have cloned, in a series of cosmids, the entire Dnr biosynthesis gene cluster of *Streptomyces peucetius* ATCC 29050. Single genes of the cluster have been isolated recently, sequenced and expressed in heterologous systems to enable partial characterization of Dnr biosynthesis enzymes (Madduri *et al.*, 1993).

Previously we reported the isolation of a Dxr-Dnr resistance gene contained in a 5.7 kb fragment (*ric1*) from *S. peucetius* subsp. *caesius* mutant 7600 derived from ATCC 27952 (Colombo *et al.*, 1992). *ric1* belongs to the Dnr cluster described by Stutzman-Engwall & Hutchinson (1989) and the identified resistance gene corresponds to the *drrAB* gene (Guilfoile & Hutchinson, 1991). We also presented preliminary evidence that in the same fragment, close to *drrAB*, is a gene responsible for conversion of aklavinone to ϵ -rhodomycinone via hydroxylation at C-11. This finding is consistent with the observation, made by Guilfoile & Hutchinson (1991), that, in *S. peucetius* ATCC 29050, a spacer of 233 nt separates the *drrAB* gene from an open reading frame (ORF) transcribed in the

Abbreviations: Dnr, daunorubicin; Dxr, doxorubicin; GST, glutathione S-transferase.

The GenBank accession number of the sequence reported in this paper is U18082.

opposite direction. The ORF was only partially sequenced but comparison of the deduced first 79 amino acids revealed a significant similarity with FAD- and NADPH-dependent bacterial hydroxylases (Blanco *et al.*, 1993).

In this paper we report the sequence of the *S. peucetius* ATCC 29050 gene for the aklavinone C-11 hydroxylase (*dnrF*) and its heterologous expression in *Escherichia coli* and *Streptomyces lividans*.

METHODS

Bacterial strains and plasmids. The strains *Streptomyces lividans* TK23 (obtained from D. A. Hopwood, John Innes Institute, Norwich, United Kingdom) and *Streptomyces peucetius* 29050 (Grein *et al.*, 1963), 7600, 7800 and 9700 (Colombo *et al.*, 1992) and the plasmids pWHM3 (Vara *et al.*, 1989), pWHM603 (Guilfoile & Hutchinson, 1991) and *ric1* (Colombo *et al.*, 1992) are described elsewhere. The *ermE** promoter (Gramajo *et al.*, 1991) was isolated from pIJ4070 kindly provided by M. J. Bibb (unpublished results). pUC19, M13mp18 and M13mp19 were purchased from New England Biolabs. pT7-7 was from USB and pGEX-2T was from Pharmacia Biotech. Plasmid pGP1-2 was provided by C. R. Hutchinson. The *Escherichia coli* strains used were JM109 (Yanisch-Perron *et al.*, 1985), DH5 α (BRL) and DH1 (Hanahan, 1983).

Plasmids pFI 15 and pLA-4 contain the *dnrF* gene, obtained by PCR amplification as detailed later, cloned into the *EcoRI* site of pT7-7 and pGEX-2T vectors, respectively.

Plasmid pFI 14 contains a region of 2.2 kb, obtained by partial digestion of pWHM603 with *Bam*HI, cloned into the *Bam*HI site of the pWHM3 vector (see Fig. 1). The 1.7 kb *Xba*I fragment containing the *dnrF* gene from pFI 15 was cloned into the *Xba*I site of pIJ4070 downstream of the *ermE** promoter, obtaining plasmid pFI 16. pFI 16 was double digested with *Sac*I and *Hind*III and the 2 kb fragment, corresponding to the *ermE**-*dnrF* hybrid gene, was inserted into pWHM3 digested with the same enzymes to obtain pFI 20.

Chemicals. Thiostrepton was obtained from Behring Diagnostic. Aklavinone, 10-decarbomethoxy-aklavinone, 11-deoxycarminomycinone, 11-deoxydaunomycinone, 11-deoxy-4-demethyladriamycinone, 11-deoxycarminomycin, 11-deoxydaunomycin and 11-deoxy-4-demethyladriamycin were prepared in the laboratories of Oncology Area Pharmacia Farmitalia-Carlo Erba. Restriction enzymes and other molecular biology materials were purchased from standard commercial sources. All other chemicals and biochemicals, unless specified, were obtained from Sigma.

DNA sequence analysis. The 1.6 kb and 0.6 kb *Bam*HI fragments from pWHM603 (see Fig. 1) containing the *dnrF* gene were subcloned in pUC19, M13mp18 and M13mp19 vectors. Plasmids for sequencing reactions were prepared using Qiagen columns. Single-stranded DNA templates were isolated from *E. coli* JM109 cultures according to the method of Sambrook *et al.* (1989). The templates were sequenced by the chain-termination method with Sequenase 2.0 (USB) using [³⁵S]dCTP α S following the manufacturer's instructions and 7-deaza-dGTP or dITP to reduce the number of sequencing artefacts. Synthetic oligonucleotide primers made by a DNA synthesizer model 391 (Applied Biosystems) were used to obtain the nucleotide sequence of both strands. FASTA and TFASTA searches were performed with the GenBank databank.

Expression of the DnrF protein in *E. coli* and the bioconversion assay. Plasmid pFI 14 was used as template for the amplification of the *dnrF* gene. PCR was carried out with the GeneAmp DNA amplification kit (Perkin Elmer Cetus) ac-

cording to the instructions of the manufacturer except for the addition of glycerol (10%, v/v, final concentration) and formamide (5%, v/v). The primers used in the amplification reaction were: 5'-*dnrF* (5' AGGAATTCAGGTTG GCCTTGACGAAGCCGGATGTC 3') containing the putative GTG translation start site (underlined) and 3'-*dnrF* (5' AGGAATTCGGGACGCTCCGGTCAGCGGGCCAGC 3') 128 nt downstream of the ORF stop codon. Both primers have an *EcoRI* site at their 5' end (bold). Before addition of *Taq* polymerase and dNTPs, the reaction mixture was heated at 95 °C for 7 min, overlaid with mineral oil and then incubated for 3 min at 70 °C. The temperature program was as follows: 25 cycles of 1 min at 94 °C, 1 min at 65 °C and 2 min at 72 °C. The PCR product, with a size of 1.6 kb, was purified from an agarose gel with a QIAquick gel extraction kit (Qiagen), cloned into TA vector (TA cloning kit; Invitrogen) and analysed by restriction enzyme digestion. The 1.6 kb insert, corresponding to the *dnrF* gene, was cut out from the TA vector by *EcoRI* digestion and cloned into the *EcoRI* site of pGEX-2T or pT7-7.

To induce expression of the GST-DnrF fusion protein, mid-log phase DH5 α (pLA-4) cultures (OD₆₀₀ = 0.6) were grown in 2 mM isopropyl- β -D-thiogalactoside (IPTG; Boehringer Mannheim) for an additional 3 h at 37 °C. The fusion protein was extracted and purified using glutathione agarose (G-4510, Sigma) as described by Smith & Johnson (1988).

The expression in *E. coli* of the unfused DnrF protein was achieved by the pT7-7 system. *E. coli* DH1 cells were first transformed with the pGP1-2 plasmid (Tabor & Richardson, 1985) bearing the kanamycin-resistance gene. pGP1-2 enabled the expression of the T7 RNA polymerase under the control of the λ P_L promoter. Kanamycin-resistant cells were transformed with pFI 15 bearing the ampicillin-resistance gene and the entire *dnrF* gene under the control of the ϕ 10 promoter specifically recognized by the T7 RNA polymerase (Tabor & Richardson, 1985). Double transformants were grown at 30 °C. For the induction of *dnrF*, *E. coli* DH1(pGP1-2/pFI 15) cells were grown in 2 $\times$ YT medium (Sambrook *et al.*, 1989) (*made with* 80 μ g ml⁻¹ of both kanamycin and ampicillin). At OD₅₉₀ = 3.0 the temperature was raised to 42 °C for 30 min, then the cells were grown for 120 min further at 30 °C.

For the bioconversion assay anthracyclines dissolved in DMSO at 50 μ g ml⁻¹ and NADPH (5 mM final concentration) were added to a DH1(pGP1-2/pFI 15) induced culture and cells were grown for a further 60 min at 30 °C. The cultures were extracted and analysed by HPLC.

Enzymic assay for the DnrF protein with *E. coli* crude extracts. Induced *E. coli* DH1(pGP1-2/pFI 15) cells were harvested by centrifugation at 3000 g at 4 °C, resuspended in 4 vols cold lysis buffer (25 mM Tris/HCl pH 8.0, 150 mM NaCl, 2 mM EDTA), disrupted by sonication and centrifuged at 15000 g. The assay was performed by shaking the supernatant at 37 °C for 60 min in 100 mM potassium phosphate buffer (pH 7.7) containing NADPH (5 mM) and substrates (at the indicated concentrations) dissolved in DMSO. Further reaction was blocked by adding an equal volume of acetonitrile/methanol (1:1, v/v). After vortexing and centrifugation, the supernatant was filtered and analysed by reverse-phase HPLC.

Enzymic assay for the DnrF protein with *S. lividans* crude extracts. *S. lividans* (pFI 20) was grown in YEME medium (Hopwood *et al.*, 1985a) for 76 h, harvested by centrifugation, washed in PBS, resuspended in 4 vols lysis buffer and disrupted by sonication. After centrifugation at 15000 g the supernatant was used as a crude enzyme preparation.

HPLC analysis. Acetonitrile/methanol (1:1, v/v) was used to extract the metabolites. The extracts were filtered by a Millex-

GV 0.45 µm filter (Millipore) before HPLC analysis. The metabolites were identified by HPLC with a reverse-phase Hypersil ODS-microbore column (Shandon). A linear gradient of 23–67% acetonitrile buffered at pH 2.5 with 12.5 mM sodium monobasic phosphate was applied for 52 min at a flow rate of 0.125 ml min⁻¹. The elution profile was monitored with a Hewlett-Packard diode array detector at 488, 424 or 254 nm.

Streptomyces transformation and bioconversion experiments. Protoplasts were produced from *S. lividans* TK23 grown in YEME medium supplemented with 0.5% glycine at 28 °C as described by Hopwood *et al.* (1985a) and regenerated on R2YE agar. *S. lividans* transformants were selected and maintained on thioestrepton at 50 µg ml⁻¹.

S. lividans TK23(pFI 14) or *S. lividans* TK23(pFI 20) was grown for 48 h in YEME medium at 30 °C. One millilitre of the culture was used to inoculate 25 ml fermentation medium described by McGuire *et al.* (1979). Anthracyclines were added to 68 h cultures and, after 24 h, 650 mg oxalic acid was added. Cultures were incubated at 50 °C for 45 min, then the crude extracts were prepared and analysed by HPLC.

Preparation of antisera to the GST-DnrF fusion protein. Antibodies against the purified recombinant protein were produced in 6 week old Balb/c female mice according to the following protocol: the first intraperitoneal shot contained 50 µg purified recombinant protein in 50 µl sterile PBS plus 50 µl complete Freund adjuvant. Four more injections, at 2 week intervals, were done with the same amount of antigen but without adjuvant. In Western blot analysis the serum was used at a 1:1000 dilution.

Protein analysis. SDS-PAGE and Western blot analysis were performed as previously described (Buvoli *et al.*, 1990). Mouse anti-GST-DnrF antibodies were used at a 1:1000 dilution and revealed by alkaline-phosphatase-conjugated anti-mouse IgG (Promega).

RESULTS

Cloning and sequencing of the aklavinone 11-hydroxylase gene

We previously reported the isolation and characterization of *ric1*, a 5.7 kb DNA fragment from the *S. peucetius* 7600 mutant, which contains the Dxr-Dnr resistance gene

(*drrAB*). Bioconversion experiments with *S. lividans* TK23 (*ric1*) suggested the presence, close to *drrAB*, of the *dnrF* gene, which converts aklavinone (a yellow compound) to ε-rhodomyacinone (red) via 11-hydroxylation (Colombo *et al.*, 1992).

We have now mapped, by means of the same bioconversion assay, the *dnrF* gene within pWHM603 (Guilfoile & Hutchinson, 1991), which contains part of the Dnr cluster from *S. peucetius* ATCC 29050 (Fig. 1). The region conferring the ability to *S. lividans* TK23 to convert the added aklavinone to ε-rhodomyacinone (80% conversion) is located in a 2.2 kb fragment (plasmid pFI 14) (Fig. 1).

The pFI 14 insert was sequenced and, as shown in Fig. 2, the first 118 nt correspond to the 5' end of the *drrAB* gene (non-coding strand) (Guilfoile & Hutchinson, 1991). The ORF for the *dnrF* gene was identified on the basis of previously reported considerations (Bibb *et al.*, 1984) about the distribution of G + C content across codons in *Streptomyces* genes. Thus a region spanning from position 352 to 1822 contains the *dnrF* gene (Fig. 2), beginning with a putative GTG translation start codon and ending with a TGA stop codon. A plausible ribosome-binding site (GGAGG) (Strohl, 1992) is located 6 nt upstream of the GTG codon. The first 237 nt of the ORF were previously sequenced by Guilfoile & Hutchinson (1991) and proposed to be part of the *dnrF* gene.

The *drrAB* and *dnrF* genes are separated by a spacer of 233 nt that contains the *drrAB* promoter (Guilfoile & Hutchinson, 1991) and most likely the divergent *dnrF* promoter. This region is significantly AT-rich than the remaining part of the clone (A + T 46% vs 30%).

Analysis of the deduced *dnrF* gene product

The *dnrF* gene encodes a protein of 489 amino acids with a predicted molecular mass of 52 kDa. Sequence comparison with the GenBank database performed by the FASTA and TFASTA programs showed significant

Fig. 1. Schematic representation of the pWHM603, pFI 14 and pFI 15 inserts. The position of the *drrAB* and the *dnrF* genes is indicated by grey boxes. Black arrowheads indicate the direction of transcription. The AT-rich spacer containing the promoters of the two genes is indicated. The position of the two primers used to amplify the *dnrF* ORF is also shown. Restriction enzyme abbreviations: B, *Bam*HI; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; S, *Sph*I; T, *Sst*I.

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1      G GAT CCC GTA GAC GAG ACC GGC CGG GAC GTT GAG GTC CAG GCC GTC CAC CGC CCT
55    CGT CCC GTT GTA GAC CTT GAC GAG ACC GGA CGT TTC GAT GGC CCG TGT CGG CTG CGT GTT
115   CAC CTAACGCCCCCACTAGTCACATGGAGCGGACAAAGCGTCACTGTAAGTTATTTTCGGTCAATCGGGTTACGTTG
193   AGTACCGTAGCCCCAATGAGGACACTTCATGGCCGCGATGGCTTCGTTCCGCCGAGCCATTTTCGTACGGGGTCCAC
272   CGCGCCTCGTACCCTTTTCAGTGGGGGCGCCGACGCTGGGGTGCGGCCAACAGACGCCCGGAACCACGAGGTTGAG
352   GTG GCC TTG ACG AAG CCG GAT GTC GAT GTC CTC GTG GTG GGC GGC GGT CTC GGG GGG CTG
412   TCC ACC GCC CTG TTC CTC GCC CGC CGG GGG GCG CGG GTC CTG CTG GTG GAG CGG CAT GCC
472   AGC ACC TCG GTC CTG CCC AAG GCG GCA GGC CAG AAC CCG CGC ACC ATG GAA CTG TTC CGC
532   TTC GGC GGC GTG GCC GAC GAG ATC CTG GCC ACG GAC GAC ATC CGC GGC GCC CAG GGC GAC
592   F T I K V V E R V G G R V L H S F A E S
652   TTC GAG GAA CTG GTC GGT GCG ACG GAA CAG TGC ACG CCC ATG CCC TGG GCG CTC GCT CCC
712   CAG GAC CGG GTG GAG CCC GTC CTG GTG GCC CAC GCC GCC AAG CAC GGC GCG GAG ATC CGG
772   Q D R V E P V L V A H A A K H G A E I R
832   GAC CTG GGC ACG GGA GCG GAG AGC ACC GTG AGC GCC CGC TAC CTG GTC GCC GCC GAC GGA
892   CCC CGC AGC GCG ATC CGG GAG AGC CTG GGC ATC ACC CGG CAC GGT CAC GGC ACC CTG GCC
952   CAC TTC ATG GGC GTC ATC TTC GAG GCC GAC CTC ACC GCC GTC GTA CCG CCC GGG TCC ACC
1012  H F M G V I F E A D L T A V V P P G S T
1072  AAC CGG CAC ACC TTC TAC GTC GCT ACG ACC CCT GAA CGC GGC GAG AGG CCG GAG GAC TAC
1132  T P Q R C T E L I R L A V D A P G L V P
1192  GAC ATC CTC GAC ATC CAG GCC TGG GAC ATG GCG GCG TAC ATC GCC GAC CGG TGG CGC GAA
1252  GGC CCG GTG CTG GTG GTC GGC GAT GCC GCC AAG GTC ACC CCG CCC ACC GGG GGC ATG GGC
1312  GGC AAC ACC GCC ATC GGC GAC GGG TTC GAC GTG GCC TGG AAG CTG GCC GCC GTG CTG CGC
1372  GGC GAG GCG GGC GAG CGG CTC CTC GAC AGC TAC GGG GCG GAG CGG TCG CTC GTG TCC CGC
1432  CTC GTC GTC GAC GAG TCA CTC GCC ATC TAC GCC CAG CGC ATG GCT CCC CAC CTG CTC GGC
1492  AGC GTT CCC GAG GAA CGC GGT ACG GCG CAG GTC GTC CTG GGC TTC CGC TAC CGC TCC ACC
1552  GCC GTC GCC GCG GAG GAC GAC CCC GAG CCG ACC GAG GAT CCG CGA CGC CCG TCC GGG
1612  CGC CCC GGC TTC CGC GCA CCC CAC GTC TGG ATC GAA CAG GAC GGC ACA CGG CGT TCC ACC
1672  GTC GAG TTG TTC GGC GAC TGC TGG GTG CTC CTG GCC GCA CCG GAG GGC GGC GCC TGG CCC
1732  GGC CGG CCG CCC GCG CCG CGG ATC TGG GCG TCC GCC TCG ACG TCC ATC TCG TCG GCC
1792  GCG ATG TCG CCG CCC CCT CCG GCG AAC TGA
      A M S P P P P A N *
1822  CGCGGACCTACGGGATCGGGCGGGCGGGGCCAGCTTGGTGCGCCCGGACGGCGTGGTGCCTGGCGTACGGCAGTAGC
1901  GCGGGGAGCGGAGGCCAGGACCACTGAGCACCTGCTCACCCGGCTGCTGGCCCGCTGACCGGAGCGTCCCGCGTCG
1980  GCGGGCCACCAAGGAGCGGTCCGCGCCCGGCGACGCGGCTCGGGCCAGCGCTCCGACAACCTCTGTATGTAATGAAT
2059  CAGTTACAGCGGGCTGTGCACCTCGAACTCGAGCCGAACATCCCGATGGTGAGCGCCAGCCACTCCAGCGAGTGGCG
2138  GCCGAACGACGCCGATGTCCGGTGGTGCAGCCGCTGTGAGGATTCGTCGCGGGCGGATCC

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Fig. 2. Nucleotide sequence of the 2.2 kb insert from plasmid pFI 14 with the deduced amino acid sequence of the *dnrF* ORF in the single-letter code. The putative RBS of the *dnrF* gene is boxed. The first 118 nt are complementary to the 5' end of the *dnrAB* ORF (codon arrangement is represented).

resemblance to two groups of hydroxylase enzymes (Fig. 3): those catalysing the introduction of hydroxyl groups into polycyclic aromatic polyketide antibiotics (Blanco *et al.*, 1993; Decker *et al.*, 1993) and those involved in catabolism of aromatic compounds in different organisms (Blanco *et al.*, 1993; Kalin *et al.*, 1992; Nurk *et al.*, 1991; Perkins *et al.*, 1990; Weijer *et al.*, 1982). No similarity with P-450 cytochromes (Andersen *et al.*, 1993; Stassi *et al.*,

1993; Weber *et al.*, 1991) involved in the hydroxylation of erythromycin, or with *actVA* gene products (Caballero *et al.*, 1991), responsible for hydroxylation of the ring structures in actinorhodin biosynthesis, was found. As shown in Fig. 3, the DnrF enzyme contains two motifs that are common to a number of FAD- and NADPH-dependent enzymes. The first motif, located at the N-terminus of the protein (amino acids 3–47), is the so called

ADP-binding site

consensus		*****VLIVGGGP*GLSAA*FL *R*GVR**L*ER***T***PR																		
DnrF	3	LTKPDVD	V	---	LG	---	T	L	---	A	R	A	VL	V	---	HAS	SVL	K	47	
SchC	10	DTVHRVP	V	---	SLV	---	TSV	---	G	L	---	HM	V	---	HAG	SVH	---	54		
WhiE	16	DRTHRVP	V	---	SLV	---	TSV	---	G	L	---	HT	V	---	HAG	SIH	A	60		
TfdB	5	...IETD	V	---	T	---	A	A	---	GAL	A	Y	---	TM	---	INKYNW	APT	---	46	
PheA	31	THVVETE	T	---	S	---	A	S	---	M	---	STQ	---	ISNIMI	TKYRW	---	ANT	---	75	
TcmG	15	LSTEEVP	---	---	LT	---	---	L	---	---	---	SOH	---	SCR	V	---	KHRG	TVLT	---	59
Pc10	10	GQSADAA	---	---	TT	---	I	---	NE	---	L	R	---	SCRMID	---	LPVAHQTSK	---	54		
PhyA	4	YSESYCD	---	---	A	---	A	---	M	---	RV	---	SKPOLKVR	IIDKRSTK	VYNGQ	---	48			
Hbh	1	...MKT	A	---	A	---	S	---	LLGQL	---	KA	---	IDNVIL	---	QTPDYVLG	---	41			
OtcC	1	...MRYD	V	---	A	---	A	---	T	---	HL	---	CE	---	RLA	A	TLVL	LAERVDFSK	42	

FAD-binding site

consensus		R*GRVFLAGDAAH*HPP*GG*G*NT*IQD*VHL*MKLA*VL*G*A***LLDTY**ER*																																															
DnrF	299	-E	P	L	V	---	K	V	T	-	T	-	M	G	-	A	G	G	F	D	V	A	---	A	-	R	E	G	E	R	---	S	G	A	---	S	356												
PheA	320	Q	K	---	C	C	---	I	K	---	S	H	L	S	-	S	---	S	Y	-	C	---	C	-	K	Q	D	A	P	E	---	E	-	S	T	---	A	377											
TfdB	302	Q	Q	---	C	---	V	R	---	T	N	L	S	-	S	---	S	F	-	A	---	I	M	-	N	T	D	E	S	---	T	I	---	A	359														
SchC	304	-	A	---	---	---	S	---	E	M	S	-	T	A	F	-	S	T	-	G	---	A	H	-	A	---	A	-	G	W	-	G	D	G	---	D	A	---	R	361									
TcmG	327	-	S	---	---	---	V	N	---	A	A	F	-	A	G	G	---	A	H	-	A	---	A	-	K	T	-	G	D	A	---	E	Q	---	L	384													
OtcC	279	-	D	---	L	---	C	I	-	L	-	A	-	Q	-	L	-	L	G	F	-	A	V	-	G	---	G	A	T	I	A	-	T	-	P	P	E	---	E	A	---	R	336						
Pc10	289	-	K	-	N	---	---	C	-	S	-	S	-	S	-	M	-	V	G	M	-	A	F	-	G	---	I	A	M	-	E	R	-	E	-	K	P	D	---	H	T	---	T	346					
PhyA	349	K	D	E	---	I	---	C	-	T	-	S	-	K	A	-	Q	-	M	-	S	M	-	T	Y	-	G	---	G	L	-	T	-	R	-	K	Q	D	I	---	K	-	E	E	---	H	405		
Hbh	277	O	H	---	L	---	---	I	V	---	T	-	A	K	-	L	-	L	A	A	S	-	V	S	T	-	Y	R	L	-	L	I	G	A	T	R	E	-	G	R	G	E	---	R	E	-	S	...	329

Fig. 3. Comparison of the deduced amino acid sequence of the *dnrF* gene with sequences of amino acids encoded by other hydroxylase genes. The alignment shows the similarity among the *dnrF* product and different hydroxylases in the 'ADP-binding site' and in the 'FAD-binding site' motifs, present in many FAD- and NADPH-dependent enzymes. The amino acids in the consensus sequence (on top of each motif) are the ones present in at least half of the compared protein sequences. Asterisks indicate the non-conserved positions. Dashes in the protein sequences represent amino acids identical to the consensus. Grey boxes highlight the more conserved regions. The hydroxylase proteins compared are as follows: SchC (Blanco *et al.*, 1993); WhiE (Blanco *et al.*, 1993); TfdB (Perkins *et al.*, 1990); PheA (Nurk *et al.*, 1991); TcmG (Decker *et al.*, 1993); PcIO (Blanco *et al.*, 1993); PhyA (Kalin *et al.*, 1992); Hbh (Weijer *et al.*, 1982); OtcC (Blanco *et al.*, 1993).

$\beta\alpha\beta$ fold and is involved in binding of the ADP moiety of FAD (Wierenga *et al.*, 1986). The second motif seems to be important in binding the ribityl chain of the flavin moiety of FAD (Eggink *et al.*, 1990; Russel & Model, 1988). These similarities further support the conclusion that the *dnrF* gene encodes for the aklavinone 11-hydroxylase.

Expression of the *dnrF* gene in *Streptomyces* and in *E. coli*

We stated above that, upon transformation with pFI 14, *S. lividans* acquired the ability to bioconvert aklavinone to ϵ -rhodomycinone. Incubations for 1 h gave 11-hydroxylation of 10% of the aklavinone ($50 \mu\text{g ml}^{-1}$) added to

the medium. Complete conversion was seen after 6 h. Since this could be due to low levels of gene expression, we cloned the *dnrF* gene (1605 nt) into the pWHM3 vector under the control of the strong *ermE** promoter (pFI 20 plasmid; see Methods). *S. lividans*(pFI 20) converted 100% of aklavinone ($50 \mu\text{g ml}^{-1}$) to ϵ -rhodomycinone in 1 h incubations. Moreover, this transformant also catalysed the hydroxylation of compounds biochemically derived from aklavinone (see Table 1 and Fig. 4), but it had no activity with molecules other than anthracyclines (Table 1).

For an initial biochemical characterization we expressed the *dnrF* gene in *E. coli* as a fusion with GST. To this aim the *dnrF* gene was PCR-amplified and cloned into the *Eco*RI site of the pGEX-2T vector (see Methods). The

Table 1. Percentage *in vitro* and *in vivo* conversion of different chemical species

Chemical species	Concn ($\mu\text{g ml}^{-1}$)	<i>In vitro</i> conversion		<i>In vivo</i> cell bioconversion	
		<i>E. coli</i> pFI 15	<i>S. lividans</i> pFI 20	<i>E. coli</i> pFI 15	<i>S. lividans</i> pFI 20
Aklavinone	50	100 %	100 %	80 %	100 %
10-Decarbomethoxy-aklavinone	50				90 %
11-Deoxycarminomycinone	50	70 %	100 %	70 %	100 %
11-Deoxydaunomycinone	50		10 %		10 %
11-Deoxy-4-demethyl-adriamycinone	30	10 %	10 %	10 %	10 %
11-Deoxycarminomycin	30	0 %	0 %	0 %	0 %
11-Deoxydaunomycin	30	0 %	0 %	0 %	0 %
11-Deoxy-4-demethyl-daunomycin	30	0 %	0 %	0 %	0 %
1,8-Dihydroxyanthraquinone	50	0 %	0 %	0 %	0 %

GST-DnrF fusion, purified on a glutathione agarose column, was assayed for its ability to convert aklavinone to ϵ -rhodomycinone and used as antigen to raise polyclonal antibodies in a mouse. Since the fusion protein did not show any hydroxylase activity (data not shown), we cloned the *dnrF* gene into the pT7-7 vector that allowed the expression of an unfused DnrF protein. Interestingly, upon transformation with this construct (pFI 15), *E. coli* was able to bioconvert aklavinone to ϵ -rhodomycinone (Table 1). Furthermore, the cell extract prepared from *E. coli*(pFI 15) (see Methods) catalysed the aklavinone 11-hydroxylation in a NADPH-dependent reaction, as determined by HPLC analysis (Fig. 5). Both *E. coli*(pFI 15) cells and crude extracts were active on substrates other than aklavinone, and showed the same specificity observed with *S. lividans*(pFI 20) cells and extracts (Table 1).

To confirm the expression of the DnrF protein in heterologous systems we carried out Western blot experiments using the antibodies raised to the GST-DnrF fusion. As shown in Fig. 6, a protein with an apparent molecular mass of 52 kDa was recognized in extracts from *S. peucetius* ATCC 29050, *E. coli* DH1(pFI 15) and *S. lividans* TK23(pFI 20) while no signal was detected in extracts from *E. coli* DH1(pT7-7) and *S. lividans* TK23. The same protein was present in the *S. peucetius* 7600 strain but absent in the non-producing *S. peucetius* 9700 mutant. Moreover, no protein band was recognized in extracts of the *S. lividans* TK23(pFI 14) strain, which indicates a low level of *dnrF* expression. This result is compatible with the reduced rate of bioconversion observed.

DISCUSSION

In this paper we report the sequencing and expression of the *dnrF* gene from *S. peucetius* ATCC 29050. The gene belongs to the Dxr-Dnr biosynthetic gene cluster and, as previously suggested (Colombo *et al.*, 1992), it is closely associated with the Dxr-Dnr resistance gene *drrAB*. The *dnrF* gene encodes an enzyme of 489 amino acids that

catalyses the conversion of aklavinone to ϵ -rhodomycinone via 11-hydroxylation. Accordingly, the analysis of the deduced DnrF protein sequence revealed the presence of two motifs common to a number of FAD- and NADPH-dependent hydroxylases involved in catabolism of aromatic compounds. As shown in Fig. 3, both regions are formed by alternating divergent and conserved sequences. In particular, the more C-terminal motif seems to be formed by three conserved regions separated by amino acid stretches containing Gly and Pro, two residues very often associated with protein bending. The first conserved box is the only one where Cys or His can be found. It is intriguing to observe that DnrF is the only protein among those shown that lacks these residues. We think that these differences could reflect enzymic specificity and, at the same time, can be used as a measure of the relatedness of different members of this protein family.

For preliminary biochemical characterization we expressed the *dnrF* gene in both *S. lividans* and *E. coli* cells. *In vitro* conversion assays carried out with crude extracts demonstrated that the 11-hydroxylation reaction is NADPH-dependent. DnrF has a significant substrate specificity, since it is completely inactive on molecules other than anthracyclines (see Table 1).

Aklavinone, the natural substrate, is a key intermediate in the biosynthesis of Dxr and Dnr and requires several successive chemical modifications such as hydroxylation, glycosylation, oxidation and methylation. DnrF catalyses the first among these modifications, i.e. 11-hydroxylation of aklavinone (yellow) to ϵ -rhodomycinone (red). The fact that DnrF acts early in the metabolic pathway of Dnr biosynthesis does not mean that 11-hydroxylation is required for all the successive modifications to take place. For instance, the *dnrF* 7800 mutant is able to produce 11-deoxydaunorubicin (yellow) and 11-deoxydoxorubicin (yellow). These results support the alternative hypothesis that the temporal order is simply linked to the substrate specificity of the enzyme because DnrF utilizes the 11-

Fig. 4. Schematic Dnr-Dxr biosynthetic pathway (red compounds) and the structural formula of each compound. The enzymic reaction carried out by the *dnrF* gene product is indicated. Broken arrows indicate yellow compounds derived from the biosynthetic intermediates of the yellow mutant *S. peuceitius* 7800. The box contains the aglycons compounds.

Fig. 5. HPLC analysis of aklavinone bioconversion by *E. coli*(pFI 15) (a) and *S. lividans*(pFI 20) (b) at 254 nm. (c) Aklavinone and ϵ -rhodomyconine relative spectra of the samples from (a). A, Aklavinone; B, ϵ -rhodomyconine.

Fig. 6. Western blot analysis of *E. coli* and *Streptomyces* protein extracts with anti-GST-DnrF mouse polyclonal antibodies. Lanes (a): GST, purified glutathione S-transferase; pLA-4, GST-DnrF fusion protein purified from *E. coli* DH5 α (pLA-4); pT7-7, *E. coli* DH1(pT7-7) total extract; pFI 15, *E. coli* DH1(pFI 15) total extract. Lanes (b): TK23, total extract from *S. lividans* TK23; pFI 14, total extract from *S. lividans* TK23(pFI 14); pFI 20, total extract from *S. lividans* TK23(pFI 20); 29050, total extract from *S. peuceetius* ATCC 29050; 7600 and 9700, total extracts from 7600 and 9700 mutants derived from *S. peuceetius* ATCC 27952. The positions of the molecular mass markers are indicated on the left of each panel.

deoxy compounds, downstream of aklavinone in the biosynthetic pathway, much less efficiently than aklavinone. In particular, the enzyme is completely

inactive on the C-7 glycosylated intermediates (compare the 11-deoxycarminomycin to 11-deoxycarminomycinone conversion in Table 1). Our data also indicate that modifications of the aklavinone molecule can influence the efficiency of the hydroxylation carried out by DnrF. While molecules modified in positions C-10 and C-13 are hydroxylated as efficiently as aklavinone (see 11-deoxycarminomycinone conversion, Table 1 and Fig. 4), molecules 14-hydroxylated or 4-methylated are very poor substrates (see 11-deoxy-4-demethyladriamycinone and 11-deoxydaunomycinone conversion, Table 1 and Fig. 4). The hydroxyl group in position 11 of anthracyclines seems to have a primary biological role, since all the red compounds (11-hydroxylated) are more toxic for *Streptomyces* and more cytotoxic for animals than the yellow 11-deoxy analogues (Oki, 1988).

The *drrAB* gene is one, and probably the most important, of the *Streptomyces* genes conferring resistance to Dxr and Dnr (Colombo *et al.*, 1992; Guilfoile & Hutchinson, 1991). We have previously shown that the *drrAB* mRNA appears in parallel with Dnr production, suggesting that *drrAB* gene expression is regulated at the transcriptional level (Colombo *et al.*, 1992). As suggested previously (Guilfoile & Hutchinson, 1991) and demonstrated in this paper, the *drrAB* and *dnrF* genes are transcribed in opposite directions and are separated by a spacer of 233 nt

where the promoters of both genes are located. It is conceivable that this arrangement can be relevant for a possible coordinate expression of the two genes, as already demonstrated for many prokaryotic and eukaryotic divergent promoters (Beck & Warren, 1988). One indication supporting this idea derives from the *dnrF* 7800 mutant that produces 11-deoxydaunorubicin and 11-deoxydoxorubicin. This mutant fails to express the *drrAB* gene and is sensitive to Dxr and Dnr. It is reasonable to think that the *drrAB* gene is primarily involved in conferring resistance to 11-hydroxylated anthracyclines and therefore is absolutely required after the activation of the *dnrF* gene.

Altogether these considerations point to the *drrAB*–*dnrF* gene system as a possible target for a major control point in the anthracycline biosynthetic pathway, a hypothesis that deserves further analysis.

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