Isolation and characterization of stable mutants of *Streptomyces peucetius* defective in daunorubicin biosynthesis

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Abstract

Daunorubicin and its derivative doxorubicin are antitumour anthracycline antibiotics produced by *Streptomyces peucetius*. In this study we report isolation of stable mutants of *S. peucetius* blocked in different steps of the daunorubicin biosynthesis pathway. Mutants were screened on the basis of colony colour since producer strains are distinctively coloured on agar plates. Different mutants showed accumulation of aklaviketone, *e*-rhodomycinone, maggiemycin or 13-dihydrocarminomycin in their culture filtrates. These results indicate that the mutations in these isolates affect steps catalysed by *dnrE* (mutants SPAK and SPMAG), *dnrS* (SPFS and SPRHO) and *doxA* (SPDHC) gene products.

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Introduction

Streptomyces peucetius produces the antitumour anthracycline antibiotic daunorubicin. The strain used in our laboratory does not sporulate under laboratory conditions. However, the *S. peucetius* strain ATCC 29050 does sporulate, albeit poorly. On the basis of studies with blocked mutants of *Streptomyces* sp. strain C5, Bartel *et al*. (1990) have proposed a pathway for the biosynthesis of daunorubicin. This pathway starts with the synthesis of a linear polyketide by the successive condensation of a propionyl CoA starter unit with nine malonyl CoA extender units catalysed by polyketide synthase (PKS). This linear polyketide is regiospecifically folded and cyclized to give the first stable yellow coloured intermediate aklanonic acid, which undergoes several side-group modifications to yield aklavinone. Aklavinone is the precursor common to anthracyclines of the daunorubicin–aclacinomycin–rhodomycin family. Aklavinone is hydroxylated at C-11 to yield a red compound, *e*-rhodomycinone. Mutants defective in aklaviketone C-7 reductase, which retain C-11 hydroxylase activity, accumulate maggiemycin. In *S. peucetius* wild type, maggiemycin does not accumulate since aklaviketone is completely converted into aklavinone by the *dnrE*-encoded enzyme, aklaviketone C-7 reductase. *e*-Rhodomycinone is glycosylated with daunosamine sugar which is synthesized through a separate pathway starting from UDP-glucose. The glycosylated rhodomycinone (rhodomycin) undergoes side-group modifications to give the penultimate product daunorubicin. In some cases daunorubicin is hydroxylated at the C-14 position to give the final product doxorubicin (also called adriamycin). Doxorubicin is more potent than daunorubicin in antitumour activity.

Although several blocked mutants of *S. peucetius* have already been isolated, those that are available from the American Type Culture Collection are very leaky (Strohl *et al*. 1989). In this report we describe isolation of stable mutants defective in three steps of the antibiotic biosynthetic pathway, and as far as we know this is the first report of isolation of stable mutants in *S. peucetius*. NTG was used as the mutagen since this compound is known to induce multiple, clustered lesions and has been shown to

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be ideal for isolating mutants with variations in antibiotic yields in *S. fradiae* (Baltz 1986) and *S. coelicolor* (Rudd and Hopwood 1979). In addition the daunorubicin biosynthetic pathway proposed in the related strain *Streptomyces* sp. strain C5 was based on studies with blocked mutants derived by NTG mutagenesis. Isolation of these mutants reinforces the view that the daunorubicin biosynthetic pathway could be similar in different *Streptomyces*.

Materials and methods

Biochemicals and chemicals: *e*-Rhodomycinone was obtained from W. R. Strohl, Merck Research Laboratories, USA. Aklanonic acid, aklavinone and aklaviketone were obtained from K. Eckardt, Academy of Sciences, Jena, Germany. All other chemicals and biochemicals were purchased from standard commercial sources.

Bacterial strains, media and culture conditions: *Streptomyces peucetius* ATCC 29050 was obtained from W. R. Strohl, Merck Research Laboratories, USA. *Bacillus subtilis* was obtained from the American Type Culture Collection (ATCC; Rockville, USA). SMA agar medium containing 2% each of soybean and mannitol (pH 7.2) was used for routine propagation of *Streptomyces* cultures. Production medium agar (PMA) (Johdo *et al*. 1991) and nitrate defined medium with 0.5% w/v yeast extract (NDYE) (Dekleva *et al*. 1985) used in this study have already been described. For anthracycline production, *Streptomyces* cultures were grown in NDYE for 48 h, and the mycelia were spread-plated on PMA.

Mutagenesis: *Streptomyces peucetius* mycelia were cultivated to mid-logarithmic phase in NDYE and were mutagenized as described previously (Vetrivel and Dharmalingam 2000). Mutagenized mycelia were plated onto PMA agar plates and incubated at 30°C for 7 days.

Analysis of anthracycline production: *Streptomyces* cultures were grown in PMA for 7 days and anthracyclines were extracted as described previously (Bartel *et al*. 1990). Total anthracycline was resuspended in 600 µl of chloroform, loaded onto TLC plates (silica gel 60, F_{254}; Merck, Germany). The solvent systems chloroform : heptane : methanol (10 : 10 : 3; SS1), benzene : acetone : methanol (100 : 10 : 1; SS2) and chloroform : methanol : water : acetic acid (80 : 20 : 0.2 : 2; SS3) (Bartel *et al*. 1990) were used for separation of aglycones and glycones respectively. Authentic daunorubicin (DNR) and *e*-rhodomycinone (RHO) were used as standards. Spots were visualized under ultraviolet light and photographed. An aliquot of total anthracycline was taken, air-dried, resuspended in methanol, and subjected to HPLC (Shimadzu, Japan) using 65% v/v methanol, 35% v/v phosphorylated water (pH 2) as solvent system (Bartel *et al*. 1990). The flow rate was maintained at 1 ml per min and the samples were read at 254 nm. Authentic standards were used for comparison.

Bioassay: Agar plugs were taken from five-day-old *Streptomyces* cultures grown on PMA and placed over *B. subtilis* lawn on LB agar. Alternatively, total extracted anthracycline was resuspended in 600 µl of chloroform, aliquots of 10 µl were loaded on Whatman filter paper discs, and the discs were kept over a lawn of *B. subtilis*. Authentic daunorubicin spotted on Whatman filter paper discs were used as control.

Results

Isolation of NTG-induced mutants of S. peucetius

The *Streptomyces peucetius* strain used, described in our earlier reports (Vetrivel and Dharmalingam 2000; Vetrivel *et al*. 2001), is different compared to the *S. peucetius* ATCC 29050 strain, particularly in its sporulation property. The ATCC strain sporulates poorly but the strain used in our lab does not sporulate. In addition, this strain is red coloured on PMA because of the accumulation of daunorubicin and its pathway intermediates inside the mycelium. Therefore we screened for colonies that are colourless or varied in colour on agar media after mutagenesis. Surviving colonies showed wide variety of colouration ranging from near-colourless to all shades of orange, red, yellow, violet and blue on SMA plates. First, the mutants were screened for production of bioactive compounds. Agar plugs of uniform size were cut out from lawns of mycelia grown for 5 to 6 days on SMA and placed over a lawn of *B. subtilis* (data not shown). Eight mutants showing variation in bioactivity were purified, cultured, and used for further analysis. A photograph of the colonies of these mutants is shown in figure 1. All eight mutants were examined for their stability by repeated restreaking on SMA plates for several generations. All the mutants grew to normal-size colonies on plates, and seven were of nonsporulating phenotype similar to the parent. SPFS is the only mutant that sporulated on SMA. The mutants that failed to show any bioactivity were grouped into three classes on the basis of intermediates accumulated by them. To identify the defective step in the daunorubicin biosynthetic pathway of these mutants, cultures were grown on solid medium (SMA) and the antibiotic/intermediates were analysed. Total anthracycline extracts from these four mutants did not show any bioactivity against *B. subtilis* (figure 2). This indicated that these mutants failed to produce the antibiotic and the intermediates produced are also inactive against *B. subtilis*. TLC separation (figure 3) of total anthracycline from these mutants using solvent system SS3 showed that these mutants do not accumulate daunorubicin. A summary of the relevant portion of the pathway and the step affected in the different mutants are shown in figure 4.

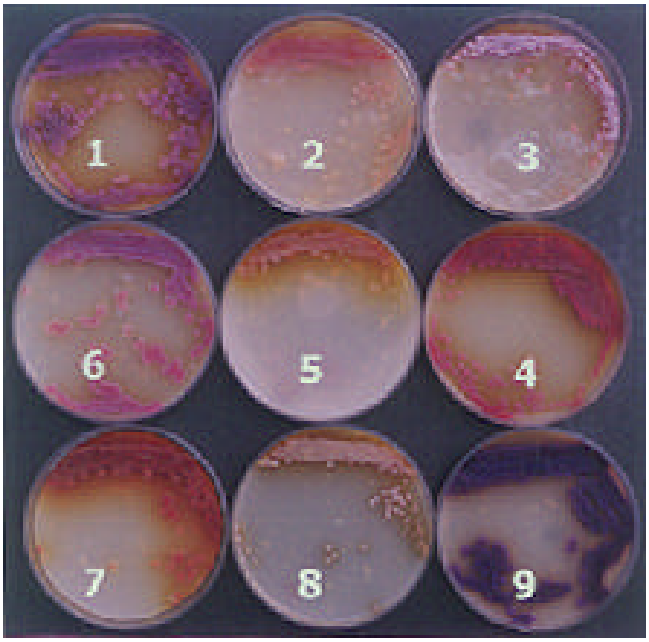

Figure 1. Colony morphology and pigment colouration of NTG-induced mutants of *S. peucetius*. Mutants isolated as described in Materials and methods were plated on SMA. 1, *S. peucetius*; 2, SP1C; 3, SPFS; 4, SPRHO; 5, SPDHC; 6, SPAK; 7, SP3D; 8, SP3A; 9, SPMAG.

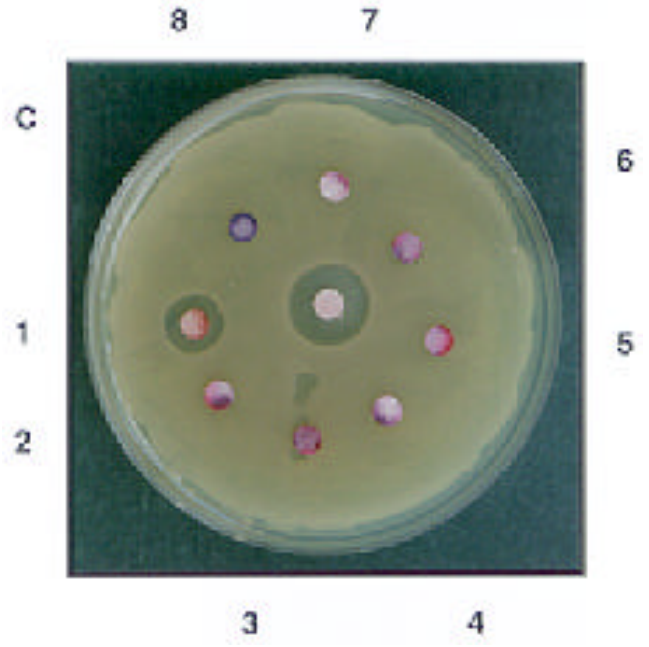

Figure 2. Bioassay of total anthracycline extracted from NTG-induced mutants of *S. peucetius* on *B. subtilis*. Anthracyclines were extracted from the cultures as described in Materials and methods, aliquots were loaded on Whatman 3 mm discs, and the discs were kept over *B. subtilis* lawn. Centre spot, daunorubicin standard (5 µg); 1, *S. peucetius*; 2, SPFS; 3, SPRHO; 4, SPDHC; 5, SPAK; 6, SP3D; 7, SP3A; 8, SPMAG.

Characterization of class 1a mutant

The class 1a comprises mutant SPMAG. Colonies of this mutant are bluish-red on both PMA and SMA (figure 1). Such blue-coloured mutants have been isolated earlier from *Streptomyces* sp. strain C5 and found to accumulate maggiemycin (Connors *et al*. 1990). SPMAG also accumulates maggiemycin as revealed by TLC separation with both SS1 and SS2 solvent systems (figure 5,a&b, lane 10). HPLC separation also confirmed the accumulation of maggiemycin (42% of total anthracycline) by this mutant (figure 6). There was also a blue compound in addition to maggiemycin in the total anthracycline from this mutant that did not migrate in the silica gel column developed with SS1 or SS2 solvent system, indicating glycosylated nature of this compound. This compound could be eluted from the silica column when methanol was used as mobile phase following SS1 solvent system. HPLC separation of the blue compound eluted from this column gave a single peak with a retention time of 4.2 that did not correspond to any known intermediates of daunorubicin biosynthetic pathway (data not shown). The identity of this compound is yet to be determined.

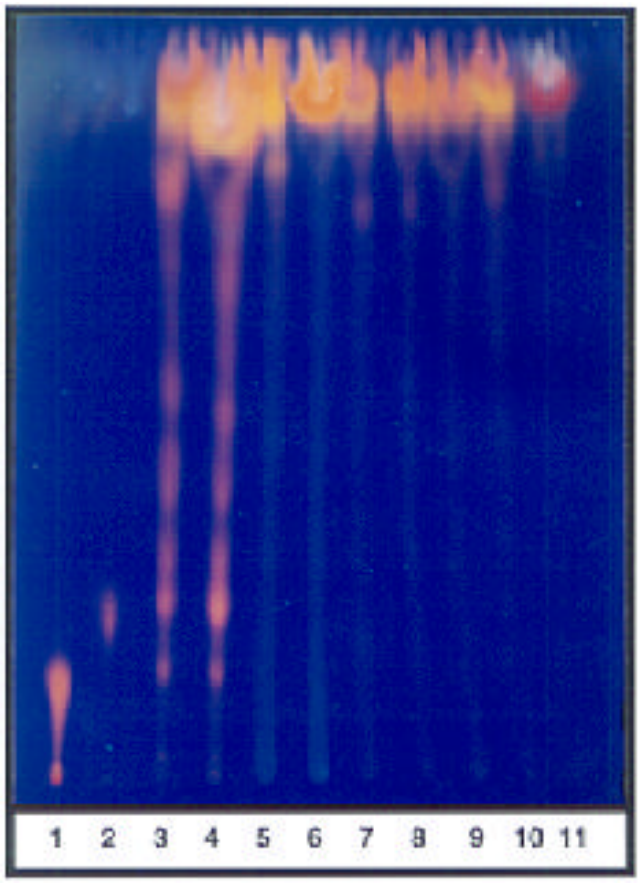

Figure 3. TLC separation of total anthracycline extracted from NTG-induced mutants using SS3 solvent system. Lanes: 1, doxorubicin standard; 2, daunorubicin standard; 3, *S. peucetius*; 4, SP1C; 5, SPFS; 6, SPRHO; 7, SPDHC; 8, SPAK; 9, SP3D; 10, SP3A; 11, SPMAG.

Characterization of class 1b mutant

The class 1b comprises mutant SPAK, which accumulated aklaviketone. SPAK is violet-red on SMA (figure 1). *e*-Rhodomycinone is the major intermediate along with trace amounts of aklaviketone (TLC using SS1 and SS2 solvent systems; figure 5,a&b, lane 7). Even though maggiemycin and aklaviketone were not separable on TLC with either solvent system, identification of trace amounts of aklaviketone as the accumulated product was done by HPLC separation (figure 6). The HPLC data also show accumulation of *e*-rhodomycinone in the extracts. These results indicate that SPAK is a leaky mutant accumulating aklaviketone in addition to *e*-rhodomycinone.

Characterization of class 2a mutant

The class 2a comprises mutant SPRHO, which accumulated *e*-rhodomycinone. Colonies of SPRHO are violet-red on PMA plates and deep red on SMA plates (figure 1). Total anthracycline from this mutant contains predominantly *e*-rhodomycinone (TLC using SS1 or SS2 solvent system; figure 5,a&b, lane 5). HPLC separation showed that 73% of the total anthracycline from this mutant was *e*-rhodomycinone and there was no detectable level of daunorubicin (figure 6), corroborating the TLC analysis. To confirm this result, the major fraction in HPLC was collected, extracted with chloroform, and analysed on TLC using SS1 solvent system. The extracted compound comigrated with authentic *e*-rhodomycinone (figure 7). These results confirm that SPRHO accumulates *e*-rhodomycinone. Earlier studies also showed that this intermediate accumulated in most of the daunorubicin-producing strains (Scotti and Hutchinson 1996). This indicates that even in the wild-type strain conversion of *e*-rhodomycinone to daunorubicin is incomplete and a small amount of *e*-rhodomycinone remains unconverted.

Characterization of class 2b mutant

The class 2b comprises mutant SPFS, which also accumulated *e*-rhodomycinone. SPFS was found to sporulate well on solid media. This mutant is red on SMA and sporulation starts at 72 h of growth on SMA (unpublished results). Further, TLC separation of total anthracycline from this mutant using SS1 and SS2 solvent systems (figure 5,a&b, lane 4) showed accumulation of *e*-rhodomycinone and compounds with R_f values of 0.24 (figure 5a, lane 4; table.1) and 0.14 (figure 5b, lane 4). The latter intermediates could not be identified. HPLC separation

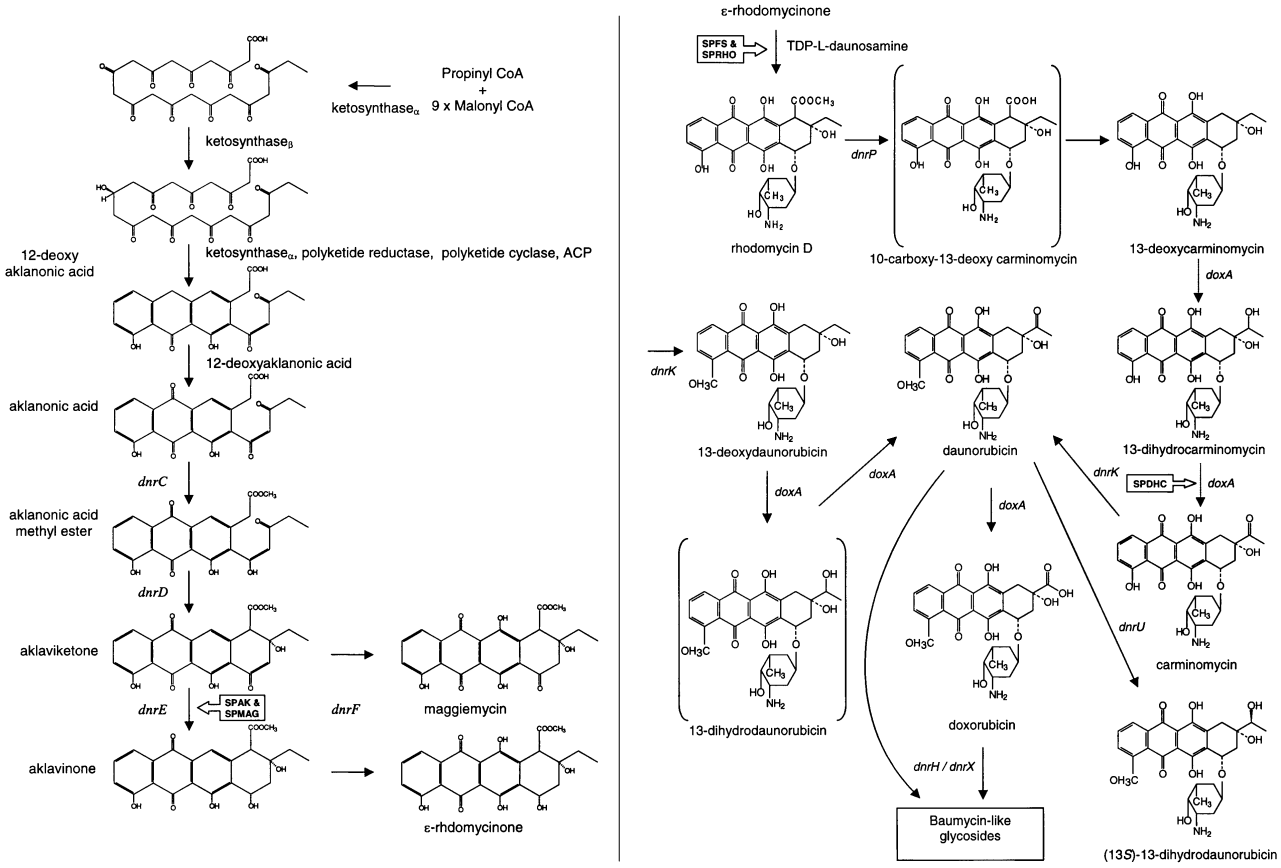

Figure 4. Daunorubicin biosynthetic pathway showing the defective steps in the NTG-induced mutants of *S. peucetius* isolated in this study.

also showed accumulation of *e*-rhodomycinone (36% of total anthracycline) (figure 6), confirming TLC data. This mutant is particularly useful for genetic characterization of the antibiotic biosynthetic pathway because of its proficiency in sporulation.

Characterization of class 3 mutant

The class 3 comprises mutant SPDHC, which is orange-red on SMA (figure 1) and accumulates 13-dihydrocarminomycin (42% of total anthracycline extract) as revealed by HPLC separation (figure 6). This compound could not be identified by TLC as it does not migrate in any of the three solvent systems. But in HPLC a major fraction with a retention time of 3.737 was identified which corresponds to authentic 13-dihydrocarminomycin. This mutant also showed accumulation of trace amount of *e*-rhodomycinone (figure 5,a&b, lane 6).

Discussion

Mutants of *S. peucetius* obtained by UV irradiation or by NTG or EMS chemical treatment have been reported to revert to wild-type phenotype at a very high frequency (Connors *et al*. 1990). Several blocked mutants of *S. peucetius* have been isolated earlier; however, those that are available from ATCC were leaky since they were shown to produce the final product (Strohl *et al*. 1989). In this study we have isolated mutants of *S. peucetius* by NTG mutagenesis. These mutants are stable and affect distinct steps in the biosynthetic pathway. Elucidation of the daunorubicin biosynthetic pathway was based on blocked mutants of *Streptomyces* sp. strain C5 and on leukaemomycin-producing *S. griseus* strains IMET JA 3933 (Eckardt and Wagner 1988), IMET JA 5142 (Wagner *et al*. 1984) and IMET JA 5570 (Wagner *et al*. 1981). Many of the results obtained for *Streptomyces* sp. strain C5 were also reported to be similar to those obtained with *S. griseus* mutants, indicating that the same pathway might be operating in different strains producing daunorubicin. The physical organization of biosynthetic genes in the daunorubicin clusters of *Streptomyces* sp. strain C5 and that in *S. peucetius* are nearly identical. In addition, nucleotide sequence data also show that the biosynthetic pathways are very similar in *Streptomyces* sp. strain C5 and *S. peucetius* (Strohl *et al*. 1997).

One point to consider is whether there are additional mutations affecting steps subsequent to the one that is blocked in the identified mutants. One way to confirm the absence of additional mutations is to feed the mutants with the appropriate precursor following the blocked step. SPMAG appears to be defective in aklaviketone C-7

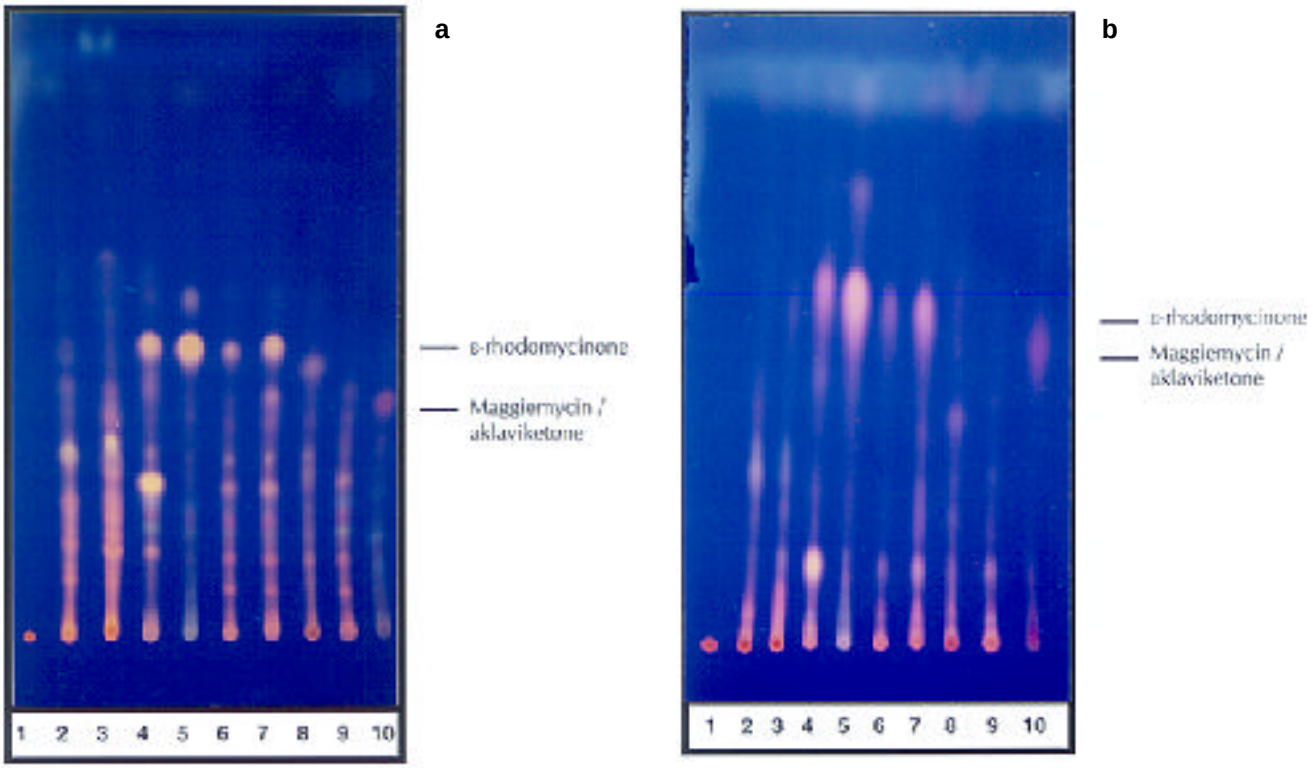

Figure 5. TLC separation of total anthracycline extracted from NTG-induced mutants using SS1 and SS2 solvent systems. (a) SS1 solvent system. Lanes: 1, daunorubicin standard; 2, *S. peucetius*; 3, SP1C; 4, SPFS; 5, SPRHO; 6, SPDHC; 7, SPAK; 8, SP3D; 9, SP3A; 10, SPMAG. (b) SS2 solvent system. Lanes: 1, daunorubicin standard; 2, *S. peucetius*; 3, SP1C; 4, SPFS; 5, SPRHO; 6, SPDHC; 7, SPAK; 8, SP3D; 9, SP3A; 10, SPMAG.

reductase. However, the precursor aklaviketone (figure 4) does not accumulate since this product is converted into maggiemycin. Interestingly, aklaviketone-accumulating mutants of *Streptomyces* sp. strain C5 were isolated and were shown to be defective in aklaviketone C-7 reductase (Connors *et al*. 1990). These mutants carry intact aklavinone C-11 hydroxylase activity since maggiemycin accumulated in these mutants. It is likely that in SPMAG also aklaviketone C-7 hydroxylase is still functional. In addition to maggiemycin, a blue-coloured compound also could be identified. The glycosylated nature of the blue compound (based on TLC analysis) accumulated by this mutant indicates that the DnrS enzyme, the presumed glycosyl transferase, is able to attach daunosamine sugar to this unusual blue-pigmented aglycone. Structure determination of this compound would reveal the type of anthracycline chromophore responsible for the blue pigmentation of the mutant and determination of the pathway responsible for its production could reveal novel aspects of the biosynthetic pathway. SPAK appears to be a leaky mutant accumulating aklaviketone (6%) in addition to *e*-rhodomycinone (44%). The accumulation of aklaviketone in this mutant could be due to reduced activity or lower level of aklaviketone C-7 reductase. The absence of maggiemycin, a C-11 hydroxylated aklaviketone, in this mutant indicates that the amount of aklaviketone (6%) may not be sufficient for the aklavinone C-11 hydroxylase to produce measurable quantity of maggiemycin. The accumulation of *e*-rhodomycinone could be due to defect in addition of sugar to the aglycone since subsequent modification could occur only when ε-rhodomycinone is converted to rhodomycin by the transfer of daunosamine. Therefore the defect in SPRHO could be in biosynthesis of daunosamine sugar, or in addition of this sugar to *e*-rhodomycinone, or both. The *dnrS*-encoded product has been reported to be TDP-daunosamine glycosyl transferase, which catalyses the addition of daunosamine sugar to *e*-rhodomycinone (Otten *et al*. 1995). Introduction of *dnrS* either by transformation or conjugation into this mutant would show whether SPRHO has a defective glycosyl transferase. Determination of the quantity of daunosamine sugar in total anthracycline of this mutant would indicate status of

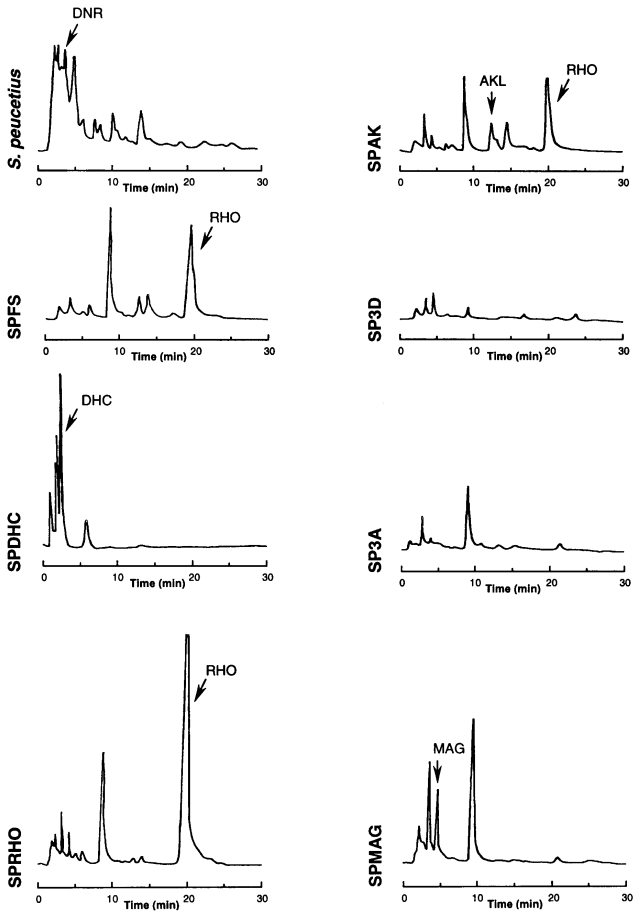

Figure 6. HPLC separation of total anthracycline from NTG-induced mutants of *S. peucetius*. DNR, daunorubicin; AKL, aklaviketone; DHC, 13-dihydrocarminomycin; RHO, *e*-rhodomycinone; MAG, maggiemycin.

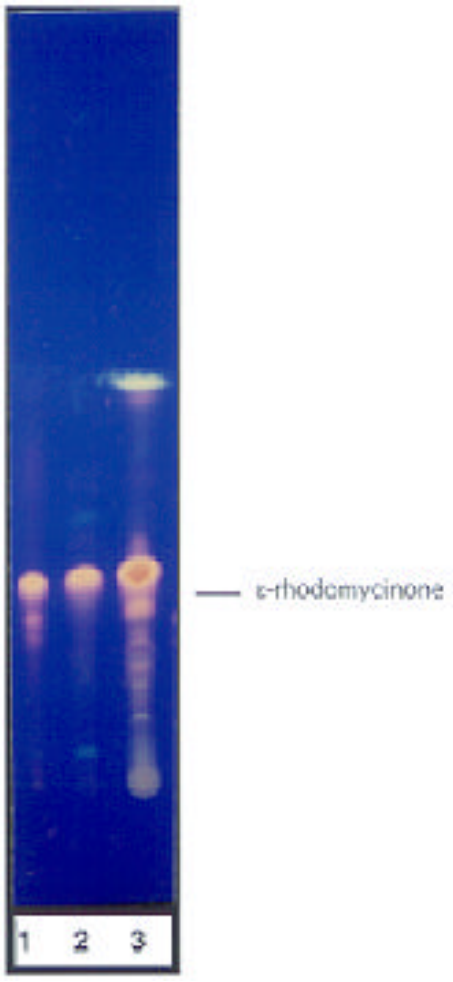

Figure 7. TLC separation of HPLC-eluted *e*-rhodomycinone fraction of SPRHO mutant using SS1 solvent system. Lanes: 1, *e*-rhodomycinone standard; 2, *e*-rhodomycinone HPLC-eluted fraction; 3, SPRHO total anthracycline.

the biosynthesis of the sugar. Mutations with phenotypes resembling SPRHO were described in *S. galilaeus* and *Streptomyces* sp. strain C5. SPFS is a sporulating mutant and the step affected in SPFS also could be very similar to what is described above for SPRHO. The enhanced sporulation and its correlation with defect in antibiotic biosynthesis is not clear. Restoration of antibiotic production in this mutant would give us a good strain for genetic studies.

Dickens *et al*. (1997) have reported that a single DoxA enzyme catalyses the following three reactions. (i) C-13 hydroxylation of 13-deoxycarminomycin to 13-dihydrocarminomycin, (ii) C-13 oxidation of 13-dihydrocarminomycin to carminomycin, and (iii) C-14 hydroxylation of daunorubicin to doxorubicin. Therefore we presume that the block in the SPDHC mutant is probably due to a defective C-13 oxidation activity of DoxA. Since this mutant accumulates 13-dihydrocarminomycin, the C-13 hydroxylation activity of DoxA is probably not affected.

These mutants show that the pathway of biosynthesis of doxorubicin in *S. peucetius* could be very similar to the one described in *Streptomyces* sp. strain C5. Further, these mutants could be of considerable use in identifying the function or functions affected in these strains and one

Table 1. TLC analysis of anthracyclines accumulated by NTG-induced mutants of *S. peucetius.*

Mutant	Colony colour	Fluorescence[a]	R_f value[b]	Identified products
SPFS	Red	Orange	*0.46	*e*-Rhodomycinone
		Orange	0.28	ND
		Orange	*0.24	ND
		Blue	0.22	ND
		Blue	0.18	ND
		Orange	0.14	ND
SPRHO	Deep red	Orange	0.53	ND
		Orange	*0.46	*e*-Rhodomycinone
SPDHC	Orange-red	Orange	0.46	*e*-Rhodomycinone
		Orange	0.28	ND
		Orange	0.24	ND
		Orange	0.14	ND
		Red	0.07	ND
		Red	0.00	13-Dihydrocarminomycin
SPAK	Violet-red	Red	*0.46	*e*-Rhodomycinone
		Orange	0.38	Aklaviketone
		Orange	0.28	ND
		Orange	0.24	ND
		Red	0.14	ND
		Red	0.07	ND
SP3D	Deep red	Orange	*0.42	ND
		Orange	0.28	ND
		Red	0.14	ND
SP3A	Yellowish-red	Red	0.40	ND
		Orange	*0.24	ND
		Blue	0.22	ND
		Blue	*0.18	ND
		Red	*0.14	ND
		Red	*0.07	ND
SPMAG	Bluish-red	Red	*0.38	Maggiemycin
		Blue	0.22	ND
		Blue	0.18	ND
		Blue	0.14	ND
		Blue	0.00	ND

*Major compounds.
[a]Fluorescence under UV (365) light.
[b]Separated using solvent system SS1.
ND, Not determined.

could also identify the defects in the genes by cross-feeding or genetic complementation using cloned genes. Both these approaches are being explored now.

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