Studies on a Second and Third Ring Cyclization in Anthracycline Biosynthesis

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This paper focuses on study of second and third ring cyclization in anthracycline biosynthesis by a heterologous gene expression. Firstly, anthracycline non-producing *Streptomyces peucetius* mutant, D2 was heterologously complemented to produce daunomycins with plasmids pSgs44 and pSYE66, which contain putative cyclase genes of *S. galilaeus* and *S. nogalater*, respectively. A point mutation in the cyclase gene *dpsY* of D2 has changed glycine to serine resulting inactivation of the enzyme. Secondly, the putative cyclase gene *snoaM* from *S. nogalater*, was expressed in a gene cassette in *S. lividans* TK24 and *S. coelicolor* CH999 to study the influence of the cyclase gene on auramycinone production and the impact of endogenous genes on production profiles. The results obtained confirms that a cyclase closing the second and third ring of a polyketide is essential in anthracycline biosynthesis.

Daunomycin[1] (see Fig. 1 for structure) and especially its 14-hydroxyl derivative, doxorubicin[2], are the most widely used cytotoxic antibiotics in cancer chemotherapy. After their discovery, the biosynthesis of daunomycins and other anthracyclines has been studied intensively[3~10]. All known anthracyclines produced by streptomycetes are generated *via* a similar polyketide pathway. The enzyme complex responsible for biosynthesis of anthracycline polyketide moiety is type II polyketide synthase (PKS II). The diversity of these aromatic polyketide antibiotics arises from structural changes in the aglycone and/or in the sugars attached to the aglycone. The first stable intermediate consists of a 21-carbon aglycone skeleton, and is called aklanonic acid, AA[11]. The earlier biosynthesis intermediates before AA are unstable by their chemical nature or due to the used isolation methods and thus are usually detected as shunt products.

Although the polyketide steps leading to the aglycone are well-studied, there has been speculation whether the second and third ring closures are spontaneous, or whether they require a specific enzyme or enzymes to occur. As the PKS genes are similar in different *Streptomyces* species, it is possible to study gene functions by heterologous gene expression. Here, we report the characterization of D2, a *S. peucetius* var. *caesius* mutant, which produces shunt products derived from unstable biosynthetic intermediates. Originally, D2 was complemented with plasmids expressing putative cyclase genes from *S. galilaeus* and *S. nogalater* leading to restoration of daunomycins production. Later, by introducing a putative cyclase gene isolated from the wild type in D2, the production was restored. Furthermore, the putative cyclase gene of *S. nogalater*, *snoaM*, was expressed in *S. lividans* TK24 and *S. coelicolor* CH999 along with nine other biosynthesis genes to further clarify the role of this enzyme in anthracycline biosynthesis and the impact of endogenous genes on production profiles.
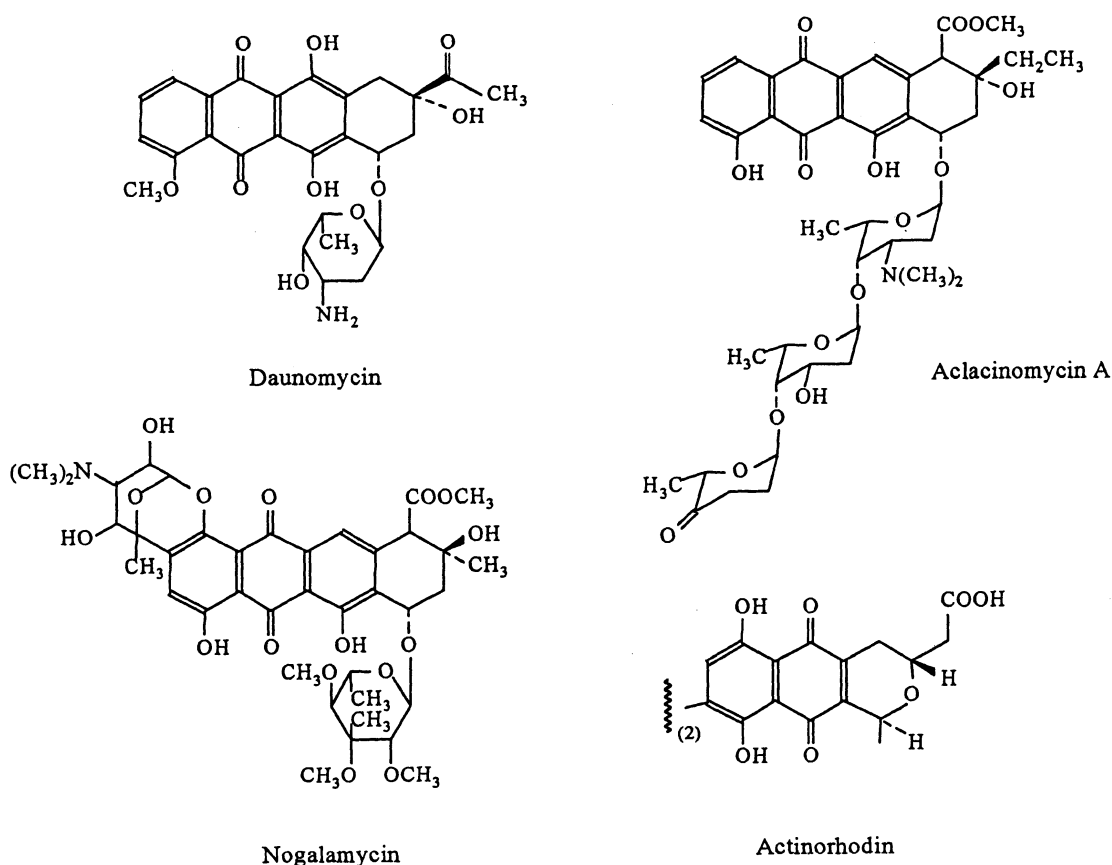
Materials and Methods

Bacterial Strains

Streptomyces peucetius var. *caesius* ATCC 27952 was

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Fig. 1. Structures of nogalamycin, aclacinomycin A, daunomycin and actinorhodin.

Daunomycin

Aclacinomycin A

Nogalamycin

Actinorhodin

used for mutagenesis. As host strains, S. lividans TK24[12] and S. coelicolor CH999[13] were used. The plasmids to be introduced into S. peucetius strains were propagated in TK24 in order to improve the transformation efficiency. Anthracycline biosynthesis genes were cloned from S. nogalater ATCC 27451 and S. galilaeus ATCC 31615. The bacterial strains used are listed in Table 1.

Mutagenesis and Mutant Selection

Cultures grown for NTG mutagenesis were incubated in 250 ml Erlenmeyer flasks containing 50 ml of Tryptone Soya Broth (TSB, Oxoid) and a spring to disperse the mycelium during aeration. All cultivations were performed in an incubator shaker (30°C, 330 rpm), unless otherwise stated. Mycelia for NTG mutagenesis were inoculated from 2-day parental culture broth (1 : 50, by volume) and cultivated for one day. pH of the culture was adjusted to 8.5 with 2% NaOH, and the culture was divided into two parts. One part was used as a control, whereas the other half was treated with 800 μg NTG ml^{-1} for 20 minutes at 37°C in a

shaker. The NTG-treated and control cultures were then centrifuged to remove supernatant, and the cells were resuspended in 50 ml of TSB medium. Cells were grown overnight (30°C, 330 rpm), and serial dilutions (10^{-1}~10^{-6}) of the culture were made in TSB medium. The dilutions were plated on ISP4 plates (Difco) to determine the killing frequency. R2YE plates supplemented with 50 μg/ml spectinomycin[12] were used to detect mutations occurring in treated mycelia. The mutation frequency was estimated from the number of spectinomycin resistant colonies and was 10^{-3}~10^{-4}%, while the killing frequencies were over 90%. The NTG treated culture was diluted and plated on ISP4 agar plates to select colonies differing from the wild type in color or in the ability to form spores. Finally, the selected colonies were picked up, cultured and studied for anthracycline production.

General DNA Manipulations

DNA propagated in E. coli was ligated into pIJ486 derivatives, and introduced into S. lividans TK24.

Table 1.   Bacterial strains and plasmids used.

Strains and plasmids	Characteristics	Reference or source
S. peucetius var. *caesius* ATCC 27952	Daunomycin producer	2
S. lividans TK24	Cloning host	12
S. coelicolor CH999	Cloning host	13
S. nogalater ATCC 27451	Nogalamycin producer	34
S. galilaeus ATCC 31615	Aclacinomycin A producer	27
D2	Anthracyclines non-producing mutant of *S. peucetius*	This work
pIJ486	*Streptomyces* plasmid	35
pIJE486	*ermE* promoter cloned into polylinker of pIJ486	36 8
pSY21	*snoaI-3, snorA* in pIJ486	8
pSY15	*snoaI-3, snorA, snoaB-E* in pIJ486	14
pSY15b	*snoaI-3, snorA, snoaB-E, aknH, dauE* in pIJ486	10
pSY42	*snoaL, gK, gC, gG2, gN, aM, gA, gJ* in pIJ486	16
pSYE66	*snoaM* in pIJE486	This work
pSgs4	*aknS, T, U, V, W, X2, Y* in pIJE486	18
pSgs44	*aknW, X2, Y* in pIJE486	18
pMC9	*snoaI-3, snoaB-E, aknH, dauE* in pIJE486	Unpublished
pMC10aM	*snoaM* added to pMC9	This work
pDpsY	*S. peucetius* var. *caesius* ATCC 27952 *dpsY* in pIJE486	This work

Subsequently, plasmid DNA isolated from TK24 was introduced into *S. peucetius*. All *Streptomyces* strains were transformed by standard methods[12] with minor modifications[14]. DNA isolation and manipulation were carried out by standard procedures[12,15].

Three putative polyketide cyclase genes were amplified by PCR using the following primers: 5'-ATTTCTAGAAG-CCACTGGTAACCACGC-3' and 5'-ATTAAGCTTCGAC-GGGACCTGATCTCC-3' for the *snoaM* gene from *S. nogalater* and 5'-GATTCTAGAGTCACTGGAGCGAAG-GTT-3' and 5'-GATAAGCTTCGGAACGTTCATTCGT-CG-3' for the corresponding cyclase genes from wild type

S. *peucetius* and from the S. *peucetius* mutant D2. PCR was carried out with 25 pmol of each oligonucleotide primer, 1 ng of plasmid template, 0.1 mM of each dNTP, 3% DMSO and 0.8 U of DyNAzyme EXT DNA polymerase (Finnzymes, Finland). The template was initially denaturated by heating at 99°C for 8 minutes followed by 30 cycles of amplification, *i.e.*, denaturation at 96°C for 1 minute, annealing at 59°C for *snoaM* and at 65°C for the cyclase genes from S. *peucetius* wild type and D2 strains and extension at 73°C for 1.5 minutes. The reaction was completed with additional extension for 8.5 minutes. The PCR products obtained were cloned in E. *coli* using a TOPO TA Cloning kit (Invitrogen), according to the manufacturer's instructions and verified by sequencing. The DNA used for sequencing was purified by a Silica Spin Disc Plasmid DNA Miniprep kit (Biometra). DNA sequencing was performed using the automatic ABI DNA sequencer (Perkin-Elmer), according to the manufacturer's instructions. Sequence analysis was carried out using the GCG sequence analysis software package (Version 8, Genetics Computer Group, Madison, Wisconsin, USA).

Expression Constructs

The polyketide cyclase homologue from S. *nogalater*, *snoaM*, amplified by PCR was cloned into pIJE486 downstream of *ermE* promoter, and into pMC9 (Kantola, unpublished) to obtain pSYE66 and pMC10aM, respectively. pSYE66 and pMC10aM were introduced into S. *lividans* TK24. Plasmids pSY42[16], pSY21[8], pSY15[17], and pSYE66 isolated from TK24 were further introduced into D2. Similarly, plasmids pSgs4 and pSgs44 containing the polyketide cyclase homologue from S. *galilaeus*[18] and a plasmid pDpsY containing S. *peucetius* wild type cyclase amplified by PCR were introduced into D2. In addition, pMC9 and pMC10aM were introduced into S. *coelicolor* CH999. The plasmid constructs used are listed in Table 1.

Cultivations

Liquid cultivations for studying anthracycline production were performed in 250-ml Erlenmeyer flasks containing 60 ml of E1 medium consisting of glucose 2%, starch 2%, Pharmamedia 0.5% (Traders protein), yeast extract 0.25%, CaCO$_3$ 0.3%, NaCl 0.3%, MgSO$_4$·7H$_2$O 0.1% and K$_2$HPO$_4$ 0.1% in 1 liter of tap water (pH 7.5)[19]. Fermentation was carried out for 6 to 7 days in 10 liters of E1 medium. Mutagenization and preparation of plasmid DNA were carried out in TSB medium. The plasmid-carrying strains were grown in the presence of 5 μg/ml thiostrepton in liquid medium and 50 μg/ml in solid medium (ISP4 or R2YE). For E. *coli* and *Streptomyces* strains, the general culture conditions were as descriped in SAMBROOK *et al.*[15] and HOPWOOD *et al.*[12].

Detection of Metabolites

A 250-μl sample of E1 culture was adjusted to pH 7.0 by 250 μl of 1 M potassium phosphate buffer, and subsequently extracted with 250 μl of MeOH and 500 μl of CHCl$_3$. The solvent layer was concentrated, and 1~2 μl was spotted on a precoated Kieselgel 60 F$_{254}$ glass plate (E-Merck & Co.), and developed with CHCl$_3$ - MeOH - AcOH, 20 : 5 : 1 (in volume) or toluene - EtOAc - MeOH - HCOOH, 50 : 50 : 15 : 3 (in volume). The production profile of the D2 mutant and the purity of the fractionated compounds were determined by HPLC on a Hewlett Packard 1100 series chromatograph equipped with a LiCHroCART (55×4 mm) RP-18ec column and a diode array detector. The mobile phase consisted of a gradient elution with 0.1% formic acid and MeCN. The flow rate was 1 ml/minute. A mobile phase used for the separation of anthracycline compounds was MeCN-KH$_2$PO$_4$ buffer (60 mM, pH 3.0 adjusted with citric acid). The compounds were separated with a gradient from 65% to 25% KH$_2$PO$_4$ buffer (60 mM, pH 3.0). The flow rate was 1 ml/minute, and detection was done at 254 and 480 nm.

Purification of Metabolites from D2

The fermentation broth (10 liters) was adjusted to pH 3.0 prior to processing. Cells were separated from supernatant with centrifugation, and extracted with 2.5 liters of methanol. Supernatant was treated with 250 g of XAD-7 resin for 1 hour. The products were eluted from the resin with 2 liters of methanol. The combined cell and supernatant extracts were treated with water and subsequently extracted twice with 2 liters of chloroform. The organic layer was evaporated to dryness. HPLC analysis of the residue revealed one major (70% of the integral at 254 nm) and several minor products. The viscous residue was loaded into a (7×10 cm) silica flash column. The column was washed with 1% acetic acid in chloroform, and eluted with a linear methanol gradient up to 30%. Three pooled fractions were further purified in a semi-preparative RP-18 ec column (20×2.5 cm) eluted with a descending gradient of 1% acetic acid and MeCN. Evaporation of MeCN resulted white powdered products, dried under vacuum, yielding 350 mg of product **1**, 22 mg of product **2** and 15 mg of product **3**.

Spectroscopy

NMR spectra were taken on a JEOL JNM-400 spectrometer operating at 400 MHz and 100 MHz for proton and

Fig. 2. Structures of the products obtained from *S. peucetius* D2 and the complementation of the mutant with cyclase containing plasmids which restored the production of daunomycins.
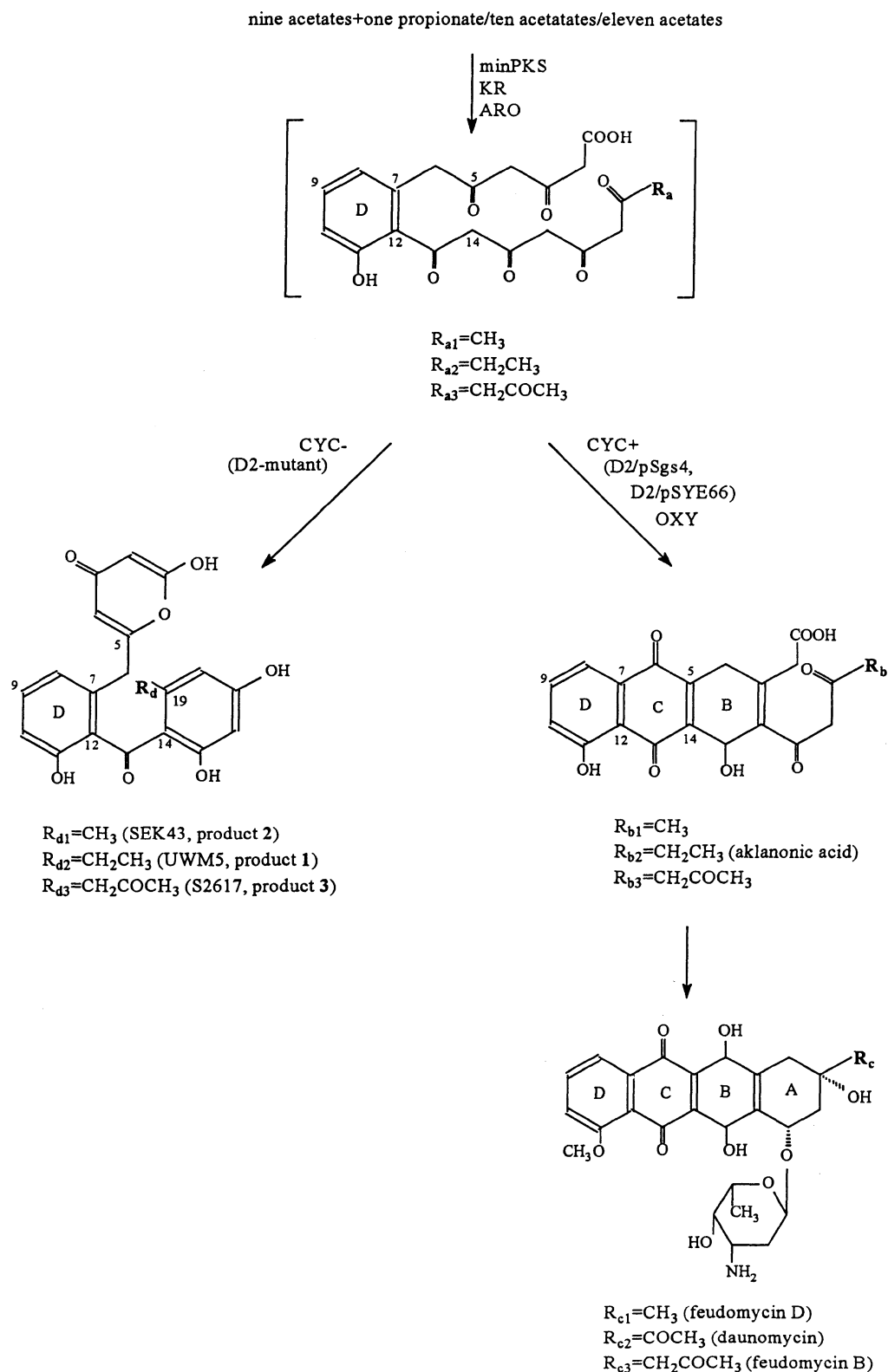
Abbreviations: min PKS=minimal polyketide synthase, KR=polyketide reductase, ARO=aromatase, CYC=second/third ring cyclase, OXY=mono-oxygenase. Only the key intermediates are shown and the arrows are representing multiple steps in biosynthesis. Structure in parenthesis is hypothetical.

carbon respectively, using either a 5-mm normal or an inverse configuration probe. The samples were measured in DMSO-d_6 at 26°C and internally referenced to tetramethylsilane. For HSQC and HMBC measurements, preemptive Bird pulse was employed. EIMS spectra were taken on a VG Analytical Organic mass spectrometry 7070 E. UV spectra were recorded on a Pharmacia biochrom 4060 spectrophotometer in methanol.

Results and Discussion

Characteristics and Products of D2 Mutant

D2 was obtained from the mutagenesis of the wild type *S. peucetius* var. *caesius*. It grows as colorless colonies on ISP4 agar plates, while the wild type has a light orange color. In liquid cultures, D2 did not produce any detectable amounts of anthracyclines, whereas the wild type produces a mixture of baumycins[20,21]. Baumycins are daunomycin-derivatives with additional sugars attached to daunosamine.

The UV spectra of purified products **1**, **2** and **3** (Fig. 2) showed similar chromophores with a substituted aromatic ring. The ¹H NMR spectra indicated two aromatic rings and a 2-hydroxy-4-pyrane ring. Furthermore, the spectra showed four hydroxyl groups, confirmed by saturation transfer upon irradiation of water, which resonated between 9.0 and 14.0 ppm. Two of them were sharp and concentration-independent, indicating an intramolecular hydrogen bonding. In the ¹H NMR spectra **1**, **2** and **3** differed only in the substitution of one aromatic ring. The ¹³C NMR spectra gave 21, 20 and 22 carbons for **1**, **2** and **3**, respectively. The carbons were unambiguously assigned using pHSQC and HMBC measurements. The measured values were in good agreement with the known similar structures UWM5 for product **1**[22] and SEK43 for product **2**[13]. The novel compound **3** had a structure similar to SEK43 except for the extra acetate in the side chain at C-19. The assignments for product **3**, designated as S2617, are given in Table 2. Furthermore, the EIMS gave the correct molecular masses and degradation patterns consistent with the structures.

Of the three products D2 strain produces, UWM5 (**1**) was the main compound, while SEK43 (**2**) and S2617 (**3**) were minor ones. Their structures revealed that a ketoreductase (KR) had reduced the carbonyl group at C-9 of the polyketide skeleton. KR is the first enzyme to act on the nascent polyketide chain, and it induces an aldol condensation between C-7 and C-12[23]. In each identified structure the first ring was also correctly aromatized, indicating that the first ring cyclase/aromatase had acted normally. A typical second ring closure for anthracyclines

Table 2. 1H NMR and 13C NMR spectroscopic data of S2617.

Site	¹H/ppm, mult., J_{HH}/Hz, area	¹³C/ppm (multiplicity)
1	-	170 (s)
1-OH	11.55, brs, 1H	-
2	5.16, d, 2.1, 1H	88.2 (d)
3	-	164.2 (s)
4	5.64, d, 2.0, 1H	99.5 (d)
5	-	165.0 (s)
6	4.38, brs, 2H	38.5 (t)
7	-	137.5 (s)
8	6.76, dd, 7.2, 1.3, 1H	128.2 (d)
9	7.19, dd, 8.1, 7.3, 1H	134.7 (d)
10	6.73, dd, 8.1, 1.3, 1H	118.2 (d)
11	-	156.2 (s)
11-OH	9.78, s, 1H	-
12	-	120.0 (s)
13	-	199.6 (s)
14	-	113.4 (s)
15	-	158.2 (s)
15-OH	12.67, s, 1H	-
16	6.08, d, 1.5, 1H	101.3 (d)
17	-	162.0 (s)
17-OH	10.41, brs, 1H	-
18	6.14, d, 1.6, 1H	118.1 (d)
19	-	140.8 (s)
20	4.11, s, 2H	49.5 (t)
21	-	204.0 (s)
22	2.17, s, 3H	30.2 (q)

was expected between C-5 and C-14 but this reaction had not proceeded in a normal way. Instead, the remaining polyketide tails had folded spontaneously, leading to the shunt products (1, 2, 3) obtained.

The changes in the side chains at C-19 are presumably due to the flexibility of PKS. UWM5, which is a condensation product of nine acetates and a propionate, is a shunt product of daunomycin biosynthesis. SEK43 derived from ten and S2617 from eleven acetates are also shunt products formed from intermediates of S. peucetius products; feudomycin D[24] and feudomycin B[25], respectively. The amounts of the D2 products correlated with the amount of the corresponding anthracycline products in the wild type.

Heterologous Complementation of the D2 Mutant

The plasmids, containing biosynthetic genes for nogalamycin[26] and aclacinomycins[27] (see Fig. 1 for structures), derived from S. nogalater and S. galilaeus respectively, were introduced into D2 by protoplast transformation. Plasmid pSY21 carrying minimal PKS genes for nogalamycin[8] did not complement the D2 mutant. D2/pSY15 also remained non-producing. Although plasmid pSY15 was previously suggested to contain all the genetic information from S. nogalater to produce the first three rings of nogalamycin[17]. D2 was further transformed with pSgs4 which carries genes for aclacinomycin biosynthesis, and with pSY42 which contains nogalamycin biosynthetic genes other than pSY21 and pSY15. As a result, D2 accumulates daunomycins as the wild type and shunt products were no longer detected. Interestingly, sequence analysis revealed that both, pSgs4 and pSY42 contained a gene for putative cyclase, aknW and snoaM, respectively. pSgs4 was further subcloned revealing that pSgs44 expressing aknW alone was able to complement D2. Furthermore, D2 was complemented with plasmid pSYE66 containing snoaM. The complementation results clearly indicate that D2 is a cyclase deficient mutant.

Influence of Endogenous Enzyme Activities of TK24 on Studying Gene Functions

Elucidation of the role of specific enzymes can be problematic due to endogenous activities of the host, which may cause misinterpretation of the results. S. lividans TK24 strain, which is commonly used as a host for expression studies, occasionally produces actinorhodin on ISP4 plates, but the production is suppressed in a liquid medium E1. In contrast to results that the second ring cyclase, Act IV[28]

acting on actinorhodin biosynthetic pathway in S. coelicolor can not act on longer polyketides than octa- and nonaketides in CH999[13], our studies suggest that the corresponding endogenous TK24 cyclase has a role to play in biosynthesis of such polyketide compounds. For example, in our previous studies, S. lividans TK24 carrying pSY15 was able to produce aromatic polyketides with correctly closed first three rings although the construct did not contain snoaM or a related cyclase gene[10,17]. Furthermore, TK24 carrying pMC9 (Kantola, unpublished) or pSY15b[10] was able to produce minor amounts of auramycinone. Construct pMC9 contains nine anthracyclinone biosynthesis genes derived from three different Streptomyces species but no cyclase corresponding to snoaM or aknW, while pSY15b contains the same genes as pMC9 and an activator, snorA. Similarly RAJGARHIA and STROHL[9] were able to produce AA in TK24 transformants and GERLITZ et al.[29] in S. lividans 1326 carrying genes cloned from S. peucetius without genes corresponding to second and third ring cyclases. To get more information whether the cyclizations in TK24 occur spontaneously or by the action of endogenous TK24 enzymes, we expressed pSY15 in a number of unidentified Streptomyces strains that do not produce aromatic polyketides in nature. As expected, similar products as in TK24/pSY15 were not obtained (data not shown). If the cyclization was a spontaneous reaction we should have observed related products as were found in TK24/pSY15.

In addition to correctly folded polyketides, TK24/pSY15 and TK24/pSY15b produced incorrectly folded compounds. In these incorrectly folded compounds[17] (Fig. 3) the first two rings are closed correctly but the remaining tails form a tetrahydropyran ring as in actinorhodin, which is an aromatic polyketide product of S. lividans. This reaction was most probably catalyzed by a product of the locus corresponding to actVI, which acts on the formation of the tetrahydropyran ring found in actinorhodin (Fig. 1) produced by S. coelicolor[30]. This further supports the expression of endogenous genes in TK24. It seems that ActIV and ActVI functions are competitive in TK24/pSY15 and TK24/pSY15b after the second ring closure because both correctly and incorrectly folded products were detected (Fig. 3).

Expression of snoaM in S. lividans TK24 and S. coelicolor CH999

To test the activity of snoaM on auramycinone production, we cloned it into pMC9 to obtain pMC10aM. As expected, the expression of pMC10aM in TK24 resulted

Fig. 3.   Proposed biosynthetic pathways leading to the obtained products in TK24/pSY15[17] and
TK24/pSY15b[10].

Auramycinone
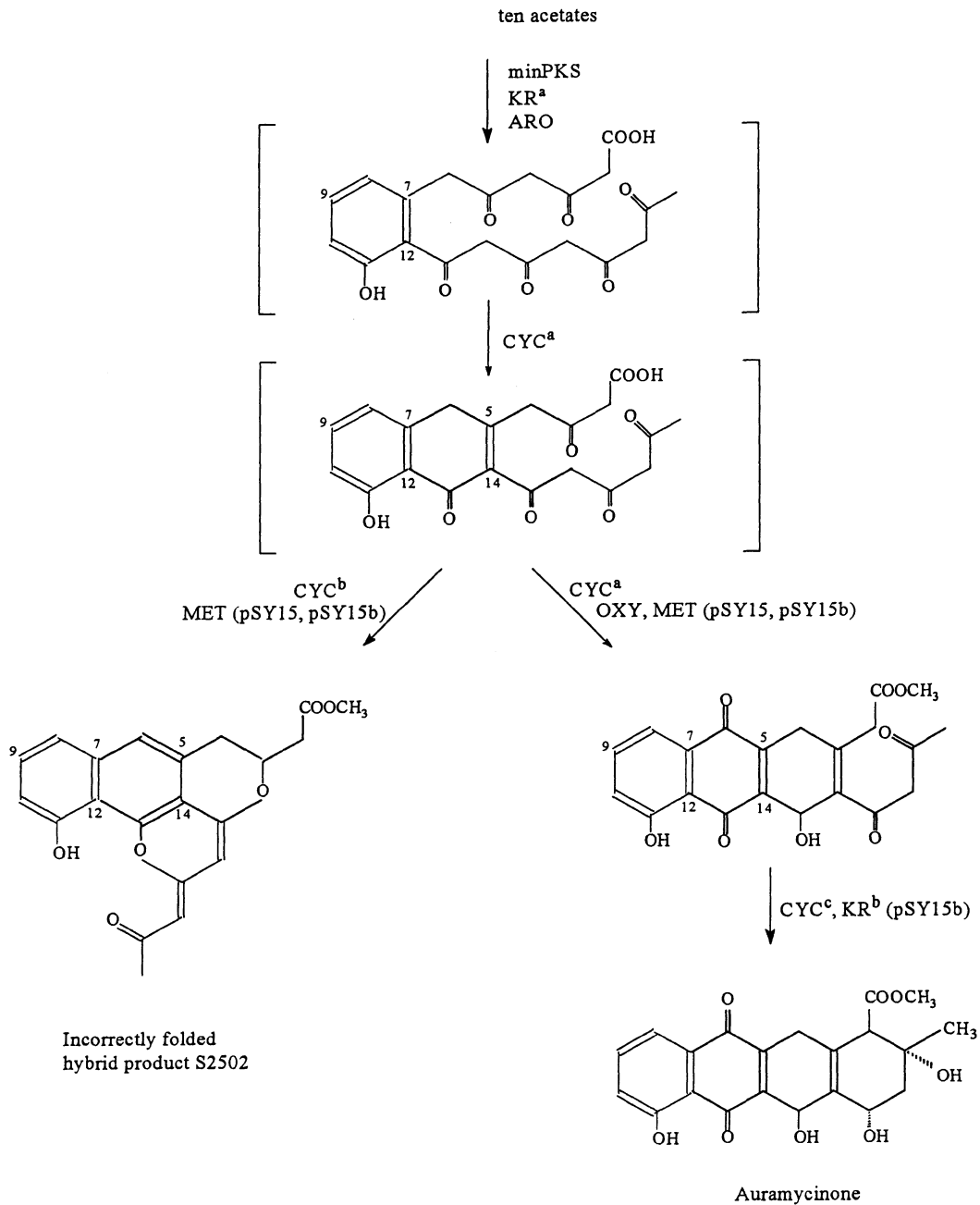
Abbreviations; min PKS=minimal polyketide synthase, KR[a]=polyketide reductase, ARO=aromatase,
CYC[a]=second/third ring cyclase (ActIV), CYC[b]=cyclase involved in the formation of tetrahydropyran ring (ActVI),
MET=methyl transferase, OXY=mono-oxygenase, CYC[c]=fourth ring cyclase, KR[b]=aklaviketone reductase. All
enzyme activities except those of CYC[a] and CYC[b] are derived from plasmids pSY15 and pSY15b. Only the key
intermediates are shown and the arrows are representing multiple steps in biosynthesis. Structures in parenthesis are
hypothetical.

in a ten-fold increase in auramycinone production compared with TK24/pMC9. To further clarify the involvement of the TK24 host strain activities pMC9 and pMC10aM were introduced into S. coelicolor CH999, which is genetically modified strain to lack the genes needed for actinorhodin biosynthesis and thus should not possess endogenous activities influencing on formation of the metabolites. As expected, only CH999/pMC10aM, carrying all the genes for the aglycone formation, was able to produce auramycinone. CH999/pMC9 remained non-producing. These results confirm that the products obtained from the TK24 strain carrying plasmids without a second and third ring cyclase were produced due to the action of TK24 endogenous enzymes.

Sequence Analyses

Comparison of the deduced amino acid sequence encoded by snoaM with database sequences revealed a high degree of similarity to other putative polyketide cyclases found in anthracycline clusters so far. Identities of 73% to AknW from S. galilaeus[18], 71% to ORF1 from S. griseus[31] and 71% to DpsY from S. peucetius[22] were found. Disruption of dpsY in S. peucetius ATCC29050 has led to production of UWM5[22], the major product of D2 also (product 1). BAO et al.[32] further investigated the role of dpsY in the aglycone biosynthesis. They studied constructs with and without dpsY transformed in S. lividans 1326. Their studies revealed that the products obtained were either aberrantly cyclized shunt products or AA depending on whether a construct contained dpsY or not. In addition to cyclases found in anthracycline biosynthetic clusters, SnoaM showed a 65% identity to MtmY[33] implicated in mithramycin biosynthesis. On the contrary, SnoaM did not show any similarity to cyclase ActIV or to those of the other genes involved in actinorhodin biosynthesis. However, this does not exclude the possibility that ActIV has effect on anthracycline biosynthesis.

To further clarify the nature of the mutation in D2 strain a cyclase designated as dpsY[22] was amplified with PCR from wild type and D2 strains. Sequence studies revealed that there is one amino acid difference in the sequences changing glycine at position 191 to serine. All cyclases for the second and third ring closure identified from anthracycline gene clusters sequenced so far (SnoaM, AknW[18], ORF1[31] and DpsY[22]), as well as MtmY[33] from the mithramycin cluster have a glycine at position 191. In addition, there are two other differences in deduced amino acid sequences of wild type and D2 compared to the S. peucetius ATCC 29050 dpsY[22] sequence in the gene bank

(accession number AAC38443): histidine at position 38 is replaced by leucine and aspartic acid at position 126 is replaced by glutamic acid. To confirm the results obtained from heterologous complementation studies we expressed wild type dpsY gene in D2. As expected D2 mutant was complemented with the wild type cyclase.

Conclusion

The results from snoaM expression studies in TK24 and CH999 confirms the necessity of a specific second and third ring cyclase in anthracycline biosynthesis. Furthermore, complementation of D2 with a cyclase gene from two other Streptomyces strains and also with the wild type S. peucetius cyclase are consistent with the suggested function of this enzyme. The point mutation changing glycine to serine at position 191 in D2 dpsY cyclase seems to be crucial to the activity of the enzyme, since all the sequences for the second and third ring cyclases in anthracycline biosynthesis available in the gene bank have a glycine at this position.

It is most likely that the third ring closure proceeds by the action of the same enzyme that acts on the second ring closure, because there are no reports of identified natural intermediates or shunt products from mutants with the first two rings closed correctly. In addition, the results obtained by expressing snoaM in TK24 and CH999 suggest that this enzyme is involved in closures of both the second and third rings. Since anthracycline biosynthesis proceeds similarly in different strains and because the sequence similarities of the PKS regions are high, it can be suggested that the identified S. galilaeus and S. nogalater cyclases have similar functions in their natural context in the biosynthesis of aclacinomycins and nogalamycin, respectively. Finally, though the concomitant action of genes for different antibiotics may cause confusing results as has been discussed in this paper, it provides a powerful tool to generate novel chemical structures for drug discovery.

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