A two-plasmid system for the glycosylation of polyketide antibiotics: bioconversion of ε-rhodomycinone to rhodomycin D

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Background: The biological activity of many microbial products requires the presence of one or more deoxysugar molecules attached to agylcone. This is especially prevalent among polyketides and is an important reason that the antitumor anthracycline antibiotics are avid DNA-binding drugs. The ability to make different deoxyaminosugars and attach them to the same or different aglycones *in vivo* would facilitate the synthesis of new anthracyclines and the quest for antitumor drugs. This is feasible using the numerous bacterial genes for deoxysugar biosynthesis that are now available.

Results: Production of thymidine diphospho (TDP)-L-daunosamine (dnm), the aminodeoxysugar present in the anthracycline antitumor drugs daunorubicin (DNR) and doxorubicin (DXR), and its attachment to ε-rhodomycinone to generate rhodomycin D has been achieved by bioconversion with a strain of *Streptomyces lividans* that bears two plasmids. One contained the *Streptomyces peucetius dnmJVUZTQS* genes plus *dnmW* (previously named *dpsH* and considered to be a polyketide cyclase gene), *dnrH*, which is not required for the formation of rhodomycin D, and *dnrI*, a regulatory gene required for expression of the *dnm* and *drr* genes. The other plasmid had genes encoding glucose-1-phosphate thymidylyltransferase and TDP-glucose-4,6-dehydratase (*dnmL* and *dnmM*, respectively, or *mtmDE*, their homologs from *Streptomyces agrillaceus*) plus the *drrAB* DNR/DXR resistance genes.

Conclusions: The high-yielding glycosylation of the aromatic polyketide ε-rhodomycinone using plasmid-borne deoxysugar biosynthesis genes proves that the minimal information for L-daunosamine biosynthesis and attachment in the heterologous host is encoded by the *dnmLMJVUTS* genes. This is a general approach to making both known and new glycosides of anthracyclines, several of which have medically important antitumor activity.

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Key words: anthracycline antibiotics, combinatorial biosynthesis, daunorubicin, deoxysugars, *Streptomyces*

Received: 14 June 1999
Revisions requested: 19 July 1999
Revisions received: 2 August 1999
Accepted: 11 August 1999

Published: 3 November 1999
Publication was delayed at the authors' request.

Chemistry & Biology December 1999, 6:845–855

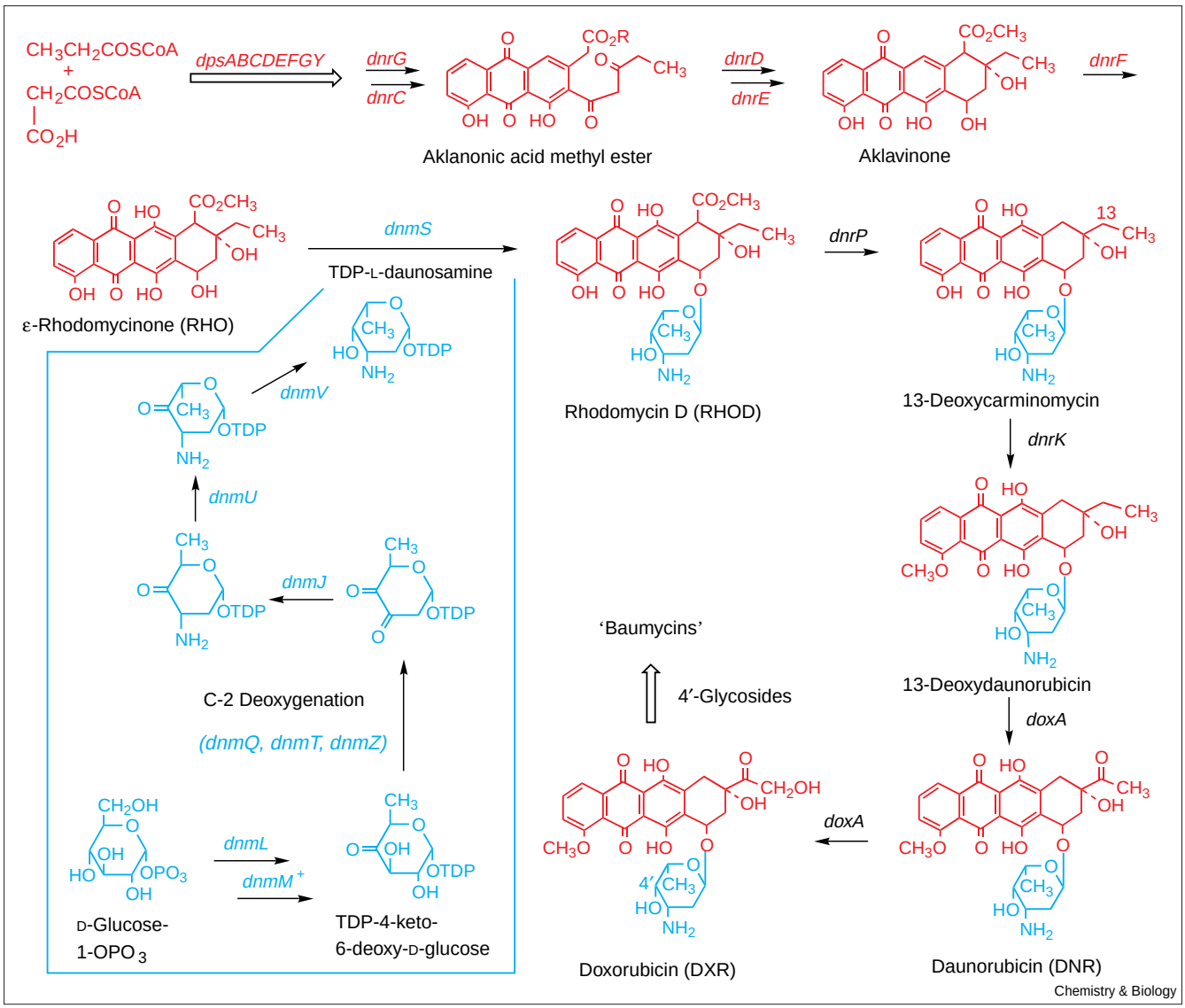
1074-5521/99/$ – see front matter

Introduction

The biosynthesis of daunorubicin (DNR) and doxorubicin (DXR), two anthracyclines produced by *Streptomyces peucetius* ATCC 29050 [1–3] that have potent antitumor activity [4,5], takes place in two stages (Figure 1). A biologically inactive, polyketide-derived aglycone, ε-rhodomycinone (RHO), is formed first, then the 2,3,6-trideoxy-3-aminohexose, L-daunosamine, is attached to give rhodomycin D (RHOD). DNR and DXR result by further metabolism of RHOD and, as with many microbial secondary metabolites, require a deoxyhexose component for their biological activity [6].

The daunosamine biosynthesis genes (Figure 1, inset) have been cloned and sequenced in previous work (reviewed in [7,8]). Nine genes are believed to be involved; *dnmJ*, *dnmL*, *dnmM*, *dnmV*, *dnmU*, *dnmZ*, *dnmT*, *dnmQ*, and *dnmS* (many of these were named '*dnr*' formerly). Their proven or postulated functions are given in Table 1 along with selected homologs from the desosamine, mycarose or oleandrose pathways in other bacteria ([9–16], reviewed in 17–19]). The putative glucose-1-phosphate thymidylyltransferase gene *dnmL*, which presumably governs the first step of daunosamine biosynthesis, is adjacent to *dnmM*, which encodes an apparently nonfunctional thymidine diphospho (TDP)-glucose-4,6-dehydratase resulting from a frameshift mutation in the open reading frame [20]. Interestingly, the gene encoding the functional TDP-glucose-4,6-dehydratase isolated from *S. peucetius* and the closely related *Streptomyces sp.* strain C5 [21] lies somewhere outside the DXR cluster [20]. Although the functions of *dnmT* [22] and *dnmQ* [23] are unknown, recent work on two *dnmT* homologs for granaticin biosynthesis in *Streptomyces violoceoruber Tü22* (*gra-ORF27*) and oleandomycin biosynthesis in *Streptomyces antibioticus Tü99* (*Tü99-ORF10*) has shown that such genes are involved in C2-deoxygenation [24]. C2-deoxygenation might involve two or three proteins [17–19], and in the case of the daunosamine pathway, DnmQ and DnmZ have been proposed to participate in this step along

Figure 1



Proposed pathway for daunosamine biosynthesis (inset) and attachment to RHO in the biosynthesis of DNR and DXR. The sequence of steps in the daunosamine pathway beyond the formation of TDP-4-keto-6-deoxyglucose is unknown.

with DnmT [23,25]. The *dnmS* gene should encode the glycosyltransferase for the DNR pathway [23]. Another putative glycosyltransferase gene, *dnrH* [22] might govern the addition of a second daunosamine [5] or some other carbohydrate-derived moiety to C4′ of DNR. The function of many of these genes has been predicted from the results of gene disruption or replacement experiments that led to the accumulation of RHO, the last intermediate in the biosynthesis of DNR and DXR that lacks a sugar moiety (Figure 1). These postulates also depend upon the roles played by the *dnm* homologs in the biosynthesis of other deoxyhexoses (see Table 1). DnmV and the products of the *avrE* and *eryBIV* genes, however, have been proven to be TDP-4-ketohexulose reductases through the synthesis of 4′-epimeric anthracyclines derived from DNR and DXR [26].

To learn whether the *dnm* genes identified previously are the only ones necessary for glycosylation, we attempted to bioconvert RHO to RHOD by introduction of the *dnm* genes into the heterologous host *Streptomyces lividans*. These genes were cloned together with the *dnrI* regulatory gene [27] for the transcription factor that is required for expression of the DNR and DXR biosynthesis and resistance genes [28–30]. Although *S. lividans* contains a *dnrI* homolog (*actII-ORF4*), this gene appears

Table 1

The TDP-L-daunosamine biosynthesis and glycosyltransferase genes and homologs.

Gene*	Function†	Homolog(s)
dnmL	Glucose-1-phosphate thymidylyltransferase [20]	*mtmD* [32], *avrD* [16], *desIII* [15]
dnmM	TDP-glucose-4,6-dehydratase ([20]; this study)	*mtmE* [32], *avrC* [16], *desIV* [15]
dnmJ	C3-aminotransferase [28]	*eryCI* [9–12], *eryCIV* [9–12], *desV* [15] *oleN2* [14]
dnmZ	Unknown; putative flavoprotein ([25]; this study)	None in deoxysugar pathways
dnmU	Putative TDP-4-keto-6-deoxyglucose-3(5)-epimerase [25]	*eryBVII* [9–12], *avrF* [16]
dnmV	TDP-4-ketohexulose reductase [25]	*eryBIV* [9–12], *avrE* [16]
dnmW	Unknown ([34]; this study)	*actVI-ORFA* [33], *mtmX* [33], *frenX* [33]
dnmT	Putative TDP-4-keto-6-deoxy-2,3-dehydrase [22]	*eryBVI* [9–12], *avrG* [16], *gra-ORF27* [24], *Tü99-ORF10* [24]
dnmQ	Unknown [23]	*eryCII* [9–12], *desVIII* [16], *oleP1* [13]
dnmS	Putative TDP-L-daunosaminyl transferase [23]	*eryCIII* [9–12], *eryBV* [9–12], *desVII* [15], *oleG1* [13,14], *oleG2* [13,14], *avrB* [16]

*The genes are listed in the order that they occur in the DNR/DXR gene cluster. †As proposed in the cited reference(s).

to be poorly expressed and we felt that it might not substitute for *dnrI*. The *drrAB* DNR and DXR resistance genes [31] were also included to give *S. lividans* adequate resistance to RHOD. Finally, to augment the natural activities of glucose-1-phosphate thymidylyltransferase and TDP-glucose-4,6-dehydratase present in *S. lividans*, we used either the *Streptomyces argillaceus mtmD* and *mtmE* genes [32], homologs of *dnmL* and *dnmM* that are involved in the biosynthesis of mithramycin, or *dnmL* and a version of *dnmM* in which the frameshift mutation had been repaired.

The gene cloning and expression experiments were also designed to re-examine the role of the *dpsH* gene in the biosynthesis of TDP-daunosamine and RHOD. This gene was initially thought to encode a type of polyketide cyclase [33] but further investigation has led recently to a different hypothesis, that *dpsH* is involved either in the biosynthesis of TDP-daunosamine or its attachment to RHO [34]. Because of these contradictory results, we prepared an in-frame *dpsH* mutation in the *S. peucetius* chromosome and compared its properties with those of the plasmid-borne daunosamine gene cassettes with and without *dpsH*. As a result we renamed this gene *dnmW*, reflecting its importance in the glycosylation of RHO.

Results

Re-evaluation of the role of the *dpsH* (*dnmW*) gene

In earlier work we made a *S. peucetius dpsH::aphII* mutant, the WMH1666 strain, to assess the importance of *dpsH* for DNR and DXR production [34]. Given that the WMH1666 strain accumulated a considerable amount of RHO but no DNR or DXR, *dpsH* cannot be a polyketide cyclase. The *dpsH::aphII* mutation was complemented only by introducing the *dspH*, *dnmT* and *dnrH* genes together, which indicated that the *dpsH::aphII* mutation must have a polar effect on expression of the *dnmT* and *dnrH* genes [34]. This complicated interpretation of the results and made it difficult to clearly assign a role to the *dpsH* gene. Consequently, to avoid this problem, we constructed a 284 base-pair in-frame deletion between the *Nco*I and *Hinc*II sites in *dpsH* by gene replacement (see Table S1 in the Supplementary material). The resulting 1.7 kb *Kpn*I–*Bal*I fragment containing the in-frame deletion was cloned into the phage-derived KC515 vector as phWHM456 and this phage construct was used to replace the wild-type *dpsH* gene with its in-frame mutant [34]. Three representative mutant strains, WMH1803, WMH1804 and WMH1805, with the correct genotype (data not shown) were grown under standard conditions for DNR and DXR production. Thin layer chromatography (TCL) analysis of the culture extracts showed that all of these strains accumulated considerable amounts of RHO but still produced DNR and DXR. By high-performance liquid chromatography (HPLC) analysis they were found to produce 160–273%, 23–34% and 10–24% of the amounts of RHO, DNR and DXR, respectively, that were produced by the *S. peucetius* 29050 wild-type strain used as a control. These data confirm that *dpsH*, now renamed *dnmW*, has an important but not absolutely essential role in the formation of the anthracycline glycosides RHOD, DNR and DXR. Because glycosylation was not entirely abolished, we speculate that the in-frame *dnmW* mutation was partially suppressed by another locus in *S. peucetius* or that the step in which the DnmW enzyme participates can proceed spontaneously to a small extent. This issue will have to be revisited through studies of purified DnmW in due course.

Construction and evaluation of the repaired *dnmM* gene

So that we could use all of the *dnm* genes in the bioconversion experiments, the frameshift in the wild-type *dnmM*

Table 2

Anthracycline titers of *S. peucetius* 29050, WMH1800, WMH1801 and WMH1802 strains.

	Titers (μg/ml)*	
Strains	DNR	DXR
29050	4.0 ± 0.5	4.6 ± 0.5
WMH1800 *dnmM::aphII*	1.6 ± 0.4	2.4 ± 0.2
WMH1801 *dnmM*$^+$	4.7 ± 0.6	8.4 ± 1.5
WMH1802 *dnmM*$^+$	4.5 ± 0.4	7.2 ± 0.5

*The cultures were grown in APM medium for 120 h and worked up as described in the Materials and methods section. Data are averaged with standard errors obtained from three independent fermentations.

open reading frame was repaired (see the Materials and methods section). The resulting *dnmM*$^+$ gene was introduced by gene replacement into an *S. peucetius dnmM::aphII* mutant (WMH1800) made from the wild-type ATCC 29050 strain (see the Materials and methods section) to give *S. peucetius* WMH1801 and WMH1802 strains containing the *dnmM*$^+$ gene. These two strains, the WMH1800 *dnmM::aphII* strain and the *S. peucetius* 29050 strain were grown under standard conditions for anthracycline production, and the culture extracts were analysed by HPLC. The resulting data showed that the WMH1800 strain produced lower amounts of DNR (40%) and DXR (52%) in comparison with the amounts produced by the 29050 control strain (Table 2). As noted previously, therefore, the *dnmM::aphII* mutation was suppressed by another *S. peucetius* locus that produces functional TDP-glucose-4,6-dehydratase. The WMH1801 and WMH1802 *dnmM*$^+$ strains produced almost the same amounts of DNR (118% and 113%, respectively) as the 29050 control strain, but the amounts of DXR were increased 1.6 to 1.8 times over the control strain (Table 2). The WMH1800 strain produced lower amounts of RHO (57%) in comparison with the 29050 control strain, whereas the WMH1801 and WMH1802 strains produced almost the same amount (95% and 116%, respectively) as the control.

Although these data confirm our earlier suggestion that a better yield of DNR or DXR might be obtained after restoring the frameshift in the wild-type *dnmM* gene, we cannot rationalize the fact that the *dnmM::aphII* mutant produced less RHO, DNR and DXR than the 29050 strain. It is clear, however, from the bioconversion experiments described below that the *dnmM*$^+$ gene functions in *S. lividans* as expected.

Construction of the daunosamine gene cassettes

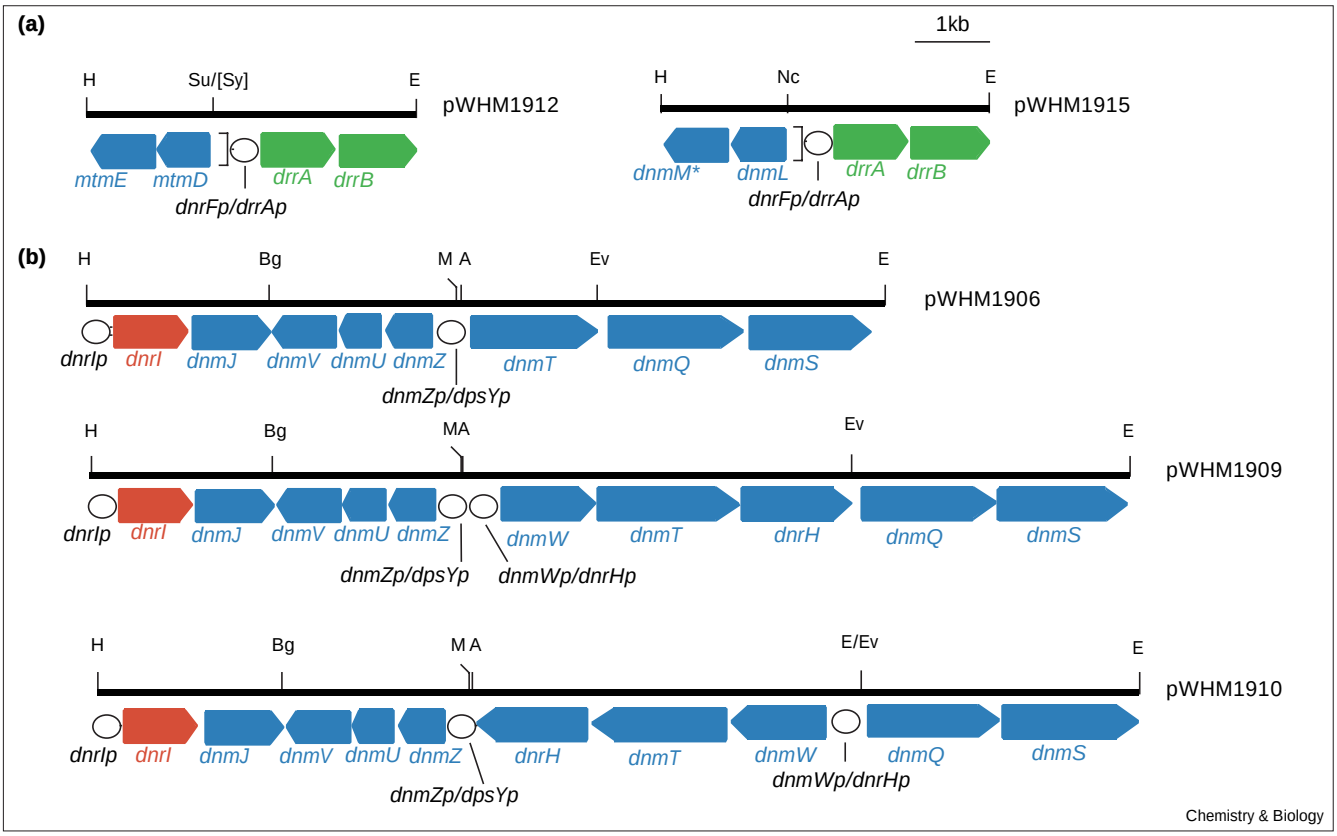
Following completion of the above work, our cloning strategy for the daunosamine genes took two things into consideration. Instead of attempting to clone the *dnm* genes in one orientation downstream of an artificial promoter, which is complicated by the fact that these genes are located in four different regions of the DXR gene cluster [7,8], their natural arrangement and native promoters were used as far as possible. This simplified the cloning work and also allowed enhanced expression of the *dnm* genes by increasing the amount of the positively acting *dnrI* gene. Two plasmid vectors instead of one were used, again for simplification because DnrI can act *in trans* on the *dnm* promoters in both plasmids. The *mtmD* and *mtmE* or *dnmL* and *dnmM*$^+$ genes were cloned on the low copy pWHM601 vector [31] under control of the *dnrF* promoter, as pWHM1912 and pWHM1915, respectively (Figure 2a). The other *dnm* genes were cloned on the high copy pWHM3 vector [35] in three different arrangements (Figure 2b). The *dnrI/dnmJ* and *dnmZ/dnmU/dnmV* genes have the same (native) arrangement in all three plasmids. In pWHM1906 the *dnmT*, *dnmQ* and *dnmS* genes were cloned downstream of the *dpsY* promoter (*dpsY* is a polyketide cyclase gene and is followed by *dnrX* of which the exact function is unknown [36]). In pWHM1909, the *dnmW*, *dnmT*, *dnrH*, *dnmQ* and *dnmS* genes were cloned downstream of the *dnmW* promoter. In pWHM1910, the *dnmW*, *dnmT* and *dnrH* genes were cloned under control of the *dnmW* promoter, and the *dnmQ* and *dnmS* genes under control of the divergently oriented *dpsG* promoter (*dpsG* is a polyketide synthase gene [34]).

Plasmids in which one of the *dnm* genes was deleted were also constructed and evaluated by bioconversion experiments to determine the minimal number of genes required. In each of the following plasmids the *dnm* gene specified in parenthesis was deleted so as not to affect expression of the remaining genes: pWHM1916 (*dnmZ*), pWHM1919 (*dnrH*), pWHM1921 (*dnmW*) and pWHM1924 (*dnmQ*). The experimental details for all of the above constructions are given in the Materials and methods section and in the Supplementary material (Figure S1; Tables S1 and S2).

Complementation experiments to verify functions of *dnm* genes

Before introducing the *dnm* gene cassettes into *S. lividans*, we verified that each *dnm* gene was functional by carrying out complementation experiments with *S. peucetius* mutant strains that accumulate RHO and do not produce DNR. The pWHM1906, pWHM1909 or pWHM1910 (Figure 2; Table S1) was introduced into the *S. peucetius* WMH1425 (*dnrI::aphII*), WMH1524 (*ΔdnmJ*), WMH1621 (*dnmV::aphII*), WMH1628 (*dnmQ::aphII*), WMH1629 (*dnmZ::aphII*), WMH1673 (*dnmU::aphII*), WMH1713 (*dnmT::aphII*), WMH1666 (*dnmW::aphII*) strains (Table S1), and the *S. peucetius var. caesius* H6125 *dnmS* mutant [37,38]). These mutations were complemented when the plasmid was introduced by transformation and the recombinant strains were grown in APM medium [31,34] (Table 3). In most cases, approximately the same amount of DNR was produced by the recombinant strains as by the wild-type control strain. When strains H6125 and

Figure 2



Structure of plasmid-borne daunosamine gene clusters used for biotransformations. (a) pWHM1912 and pWHM1915 cloned in pWHM601. (b) pWHM1906, pWHM1909 and pWHM1910 cloned in pWHM3. Filled wedges oriented in the direction of expression indicate the genes, open brackets indicate partial ORFs, and open black circles indicate the promoter regions of the designated genes. Restriction site abbreviations: A, *Afl*II; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Su, *Stu*I; Sy, *Sty*I; [ ] indicates that the site was blunt-ended.

WMH1713 carried pWHM1906, however, a lower amount of DNR was produced compared with the wild-type strain. No complementation or very low amounts of DNR production were observed when strain WMH1628 carried pWHM1906. We assume that the latter results stem from use of the *dpsY* promoter in pWHM1906 to express the *dnmT, dnmQ and dnmS* genes, or to instability of the mRNA produced from the artificial *dnmTQS* operon. This rationalization is supported by the fact that *dnmT* in its native operon (*dnmW/dnmT/dnrH*) and *dnmQS* were fully functional in pWHM1909 and pWHM1910, neither of which employ the *dpsY* promoter. In addition, the *dnmQS* genes also complemented the *dnmQ::aphII* mutation in the WMH1628 strain when cloned under the control of the *ermE*p* promoter [39] as pWHM1902 (Table 3).

Complementation of the mutations in strains WMH1629 (*dnmZ::aphII*) and WMH1628 (*dnmQ::aphII*) was also achieved (Table 3) by introduction of plasmids pWHM1916 (*ΔdnmZ*) and pWHM1924 (*ΔdnmQ*) (Table 3). In the WMH1629/pWHM1916 recombinant, the levels of RHO, DNR and DXR produced were close to those obtained with the wild-type strain, but in the WMH1628/pWHM1924 strain considerably less DNR and DXR were produced. (The latter result is consistent with the behavior of pWHM1924 in bioconversion experiments described below.) Neither *dnmZ* nor *dnmQ* are strictly essential, therefore, for the biosynthesis of the anthracycline glycosides produced by *S. peucetius*.

Bioconversion of RHO to RHOD in *S. lividans*

Bioconversion experiments using *S. lividans* strains carrying the *dnm* gene constructs cloned in pWHM1906, pWHM1909 or pWHM1910, each together with pWHM1912, were initially performed in four different liquid growth media: R2YE [40], TSB, APM [31,34], or the SMM330 modification of the SMM medium [41]. Seed cultures were prepared by growing a strain for three to four days in R2YE or APM then transferring it to fresh medium containing RHO, after which the cultures were incubated for three additional days before the bioconversion products were extracted by a solvent. Less than 1% bioconversion was

Table 3

***S. peucetius* mutant strains and complementation by different plasmid-borne daunosamine biosynthetic genes.**

Strain	Genotype*	Plasmid	Phenotype
WMH1425	*dnrI::aphII, dnmJ*	–†	–‡
		pWHM1906	DNR/DXR§
WMH1524	*ΔdnmJ*	–	RHO
		pWHM1906	DNR/DXR
WMH1621	*dnmV::aphII*	–	RHO
		pWHM1906	DNR/DXR
WMH1628	*dnmQ::aphII, dnmS*	–	RHO
		pWHM1906	RHO
		pWHM1909	DNR/DXR
		pWHM1910	DNR/DXR
		pWHM1902¶	DNR/DXR
		pWHM1924#	DNR/DXR**
WMH1629	*dnmZ::aphII, dnmU, dnmZ*	–	RHO
		pWHM1906	DNR/DXR
		pWHM1916††	DNR/DXR
WMH1673	*dnmU::aphII*	–	RHO
		pWHM1906	DNR/DXR
WMH1713	*dnmT::aphII*	–	RHO
		pWHM1906	DNR/DXR**
		pWHM1909	DNR/DXR
		pWHM1910	DNR/DXR
WMH1666	*dnmW::aphII, dnmT, dnrH*	–	RHO
		pWHM1909	DNR/DXR
		pWHM1910	DNR/DXR
H6125	*dnmS*	–	RHO
		pWHM1906	DNR/DXR**

*The mutant or nonfunctional genes are indicated. †No plasmid was introduced; all plasmids are shown in Figure 2. ‡No anthracycline metabolites were produced. §Produced at the same level as seen in the wild-type strain. ¶pWHM1902 (Table 2) was modified to express the *dnmQ* and *dnmS* genes under control of the ermE*p promoter. #Same as pWHM1910 (Figure 2) but with in-frame deletion in *dnmQ*. **Produced at a lower level than seen in the wild-type strain. ††Same as pWHM1910 (Figure 2) but with an in-frame deletion in *dnmZ*.

observed using the *S. lividans* WMH1909 (with pWHM1909 and pWHM1912) and WMH1911 (with pWHM1910 and pWHM1912) strains, and no bioconversion with strains WMH1906 (with pWHM1906 and pWHM611), WMH1908 (with pWHM1980 and pWHM611) and WMH1910 (with pWHM1910 and pWHM611) (Table S2). The results from the latter three strains were as expected because the *mtmD* and *mtmE* genes were not included in the plasmids introduced (pWHM611 contains only the *drrAB* resistance genes). When these experiments were repeated after completion of the work with the solid growth medium, however, greater than 85% bioconversion of RHO to RHOD was obtained with the *S. lividans* WMH1911 strain grown in R2YE for seven days but less than 2% when it was grown in APM for the same period.

To check the stability of RHOD under these conditions, 10 μg/ml of it was incubated in the R2YE, APM or SMM330 media for three days, and the culture was worked up under acidic conditions and extracted with chloroform. RHOD was found to be degraded up to 50% because of the oxalic acid treatment, following the procedure of Otten *et al.* [42]; in the absence of the acid treatment, RHOD was stable in R2YE but not in APM (in this case the SMM330 medium was not tested).

We also performed the bioconversion experiments using solid growth media. When strains WMH1910 (without the *mtmD* and *mtmE* genes) and WMH1911 (with pWHM1910 and pWHM1912) were grown in APM solid medium containing 10 μg/ml RHO at 30°C for seven days until well sporulated, we observed ~20% RHO bioconversion with WMH1911 and less than 0.7% bioconversion for the WMH1910 strain. The low bioconversion in the latter case presumably reflects the level of endogenous glucose-1-phosphate thymidylyltransferase and TDP-glucose-4,6-dehydratase activity in *S. lividans*. Using solid TSB, R2YE, SSM330 or APM media, strains WMH1906 (without the *mtmD* and *mtmE* genes) and WMH1907 (with the *dpsY* promoter driving expression of *dnmTQS*) were

unable to bioconvert RHO to RHOD. Successful bioconversion was observed with strains WMH1909 (with pWHM1909 and pWHM1912) grown in R2YE and TSB, and with WMH1911 grown in R2YE, APM, TSB or SMM330. R2YE was the best growth medium (95% bioconversion by WMH1911) followed by APM, TSB and SMM330. No degradation of RHOD was observed under these conditions.

Bioconversion of RHO by strain WMH1912 carrying *dnmL* and the *dnmM*$^+$ gene, instead of *mtmD* and *mtmE* as in strain WHM1911, was analysed in R2YE and showed lower levels of bioconversion than strain WHM1911 (46% of the amount observed with the latter strain). The reason for the apparently lower efficiency of the *dnmL* and *dnmM*$^+$ genes is not known but is less likely to be because of differences in expression than because of differences in the intrinsic activity of the two sets of enzymes.

Bioconversion by *S. lividans* strains carrying plasmids with deletions in specific *dnm* genes — WMH1913/*ΔdnmZ*, WMH1914/*ΔdnrH*, WMH1915/*ΔdnmW* and WMH1916-/*ΔdnmQ* — were performed in R2YE solid medium. In these experiments RHO was bioconverted to RHOD at 90%, 95%, 7% and 29% relative efficiency, respectively, compared with the bioconversion obtained with strain WMH1911 carrying the entire set of *dnm* genes. Similar results were obtained with plasmids bearing combinations of three of these single mutations: *ΔdnmZ*/*ΔdnmQ* (15%); *ΔdnmZ*/*ΔdnrH* (93%); *ΔdnmQ*/*ΔdnrH* (7%) and *ΔdnmZ*/*ΔdnmQ*/*ΔdnrH* (3%). These results confirm our conclusions about the roles of the *dnmZ*, *dnrH*, *dnmW* and *dnmQ* genes stated above; *dnmZ* and *dnrH* are dispensible whereas *dnmW* and *dnmQ* are not absolutely essential in the heterologous host *S. lividans*.

Discussion

Efforts to produce new drugs by combinatorial biosynthesis and related microbiological methods have been described with increasing frequency during the past few years. To date, these have largely involved engineered polyketide synthase (e.g., [43–48]) or nonribosomal peptide synthase ([49,50]) genes, or the introduction of a deoxysugar biosynthetic gene into different hosts (e.g., [51–53]).

A parallel combinatorial approach involving engineered deoxysugar biosynthesis genes is feasible given that considerable information about these pathways is now available [17–19,54]. In fact, a few new macrolides that are closely related to the erythromycin and methymycin/picromycin antibiotics have been produced recently by strains with targeted mutations in such genes [11,12,55,56]. We were not able to make DNR and DXR analogs using this approach because of the small amounts produced by the *S. peucetius dnm* mutants. Moreover, because the construction of strains with chromosomal mutations is inherently time-consuming, a more efficient method for combinatorial biosynthesis with deoxysugar biosynthesis genes is required to fully exploit its potential. McDaniel *et al.* [47] have illustrated clearly the power of plasmid-borne genes in the context of combinatorial biosynthesis and this motivated our studies.

The results of the bioconversion experiments with an anthracycline aglycone confirm the role we had assigned to the *dnm* genes in earlier work, although we still cannot predict the exact function of the DnmW protein. Interestingly, they also demonstrate that the *dnmQ*, *dnmW* and *dnmZ* genes are not absolutely essential in the heterologous host, although the lack of either *dnmQ* or *dnmW* severely reduces the formation of TDP-daunosamine. Whether or not this means that *S. lividans* contains enzymes that function very much like DnmZ, DnmW and DnmQ or that TDP-daunosamine biosynthesis can take place to some extent in their absence awaits studies using the purified enzymes.

Of more interest is our demonstration that artificial operons containing several deoxysugar biosynthesis genes can be constructed on plasmid vectors and can function in alternative regions of the genome as well as in their native arrangement. Two or more vectors, including episomic and integrated forms, could be used as along as the genes are expressed at a similar level. The same approach should be useful with erythromycin where the genes governing mycarose and desosamine biosynthesis also are scattered throughout the erythromycin gene cluster. In fact, we have recently been able to make new daunorubicin, erythromycin and avermectin analogs using the *dnm* and *eryC* genes through the approach illustrated here (K. Kulowski, C. Olano, S.-E. Wohlert and C.R.H., unpublished observations). Consequently, we anticipate that this combinatorial biosynthesis technique will be broadly applicable.

Significance

Expression of the thymidine diphospho (TDP)-daunosamine biosynthesis and glycosyltransferase genes in *Streptomyces lividans* to obtain an efficient conversion of ε-rhodoncycinone (RHO) to rhodomycin D (RHOD) sets the stage for using these as well as other types of deoxysugar biosynthesis genes in combinatorial biosynthesis studies. Structure/function surveys of deoxysugar biosynthesis enzymes can be done much more rapidly with plasmid-borne genes *in vivo* than with their chromosomal counterparts or with the purified enzymes themselves. In this way, the inherent flexibility of the enzymes towards normal and unusual substrates can be assessed expediently, especially for the glycosyltransferases such as DnmS that are the gate-keepers for glycosylation. The approach described should be useful for making glycosides of other types of microbial products

and will facilitate attempts to make new drugs by combinatorial biosynthesis.

Materials and methods

Bacterial strains and plasmids

S. peucetius var. *caesius* H6125 [37,38] was obtained from Ho Coy-Choke (University of Malaya). *S. peucetius* ATCC 29050 mutants used in this work are listed in Table 2. *S. lividans* 1326 [57] was obtained from David Hopwood (John Innes Institute). *E. coli* strain DH5α [58] and plasmids pUC19 [59], pSE380 (Invitrogen), pGEM7Zf(–) (Promega) and Litmus 28 (New England Biolabs) were used for routine subcloning. High-copy-number *Streptomyces* shuttle vector pWHM3 [35] and low-copy-number shuttle vector pWHM601 [31] were from our laboratory. pFLADE [32] used as a source of the *mtmD* and *mtmE* genes was provided by José A. Salas (University of Oviedo). Other *Streptomyces* strains, plasmids and φC31-derived phages used or generated in this study are listed in Table S1.

Media, growth conditions and fermentations

S. lividans strains were maintained on R2YE agar [40] and grown in R2YE liquid medium for preparation of protoplasts and seed inoculum for fermentations. Seed inoculum was also grown in APM media [31,34]. Bioconversion experiments were performed using TSB (BRL Microbiology Systems, Cockeysville, MD), R2YE [40], SMM330 minimal medium containing 330 mM glucose instead of 55 mM as in the original SMM medium [41], and APM media all supplemented with RHO (Pharmacia & Upjohn, Italy) at 10 mg/ml. Analysis of DNR resistance was performed as described in [31] by plating spores of *S. lividans* on TSB agar containing 5, 10 and 15 μg/ml DNR. Cultures for preparation of *S. peucetius* spores were grown on ISP4 solid medium (Difco Laboratories, Detroit, MI). APM seed and production media were used to determine anthracycline production in *S. peucetius*. *Streptomyces* protoplasts were regenerated on R2YE agar. *Streptomyces* plasmids were selected with thiostrepton (obtained from S.L. Lucania, Bristol-Myers-Squibb, Princeton, NJ) at 50 μg/ml in solid medium and 5 μg/ml in liquid medium, or apramycin (Fluka Chemical Co.) at 25 mg/ml. *S. peucetius* mutants containing the resistance gene *aphII* were selected with kanamycin at 20 μg/ml. R2YE agar without sucrose was used for infection of *S. peucetius* with φC31 [60] derivatives. KC515 and KC515-derived phages were propagated as described by Hopwood *et al.* [40]. *E. coli* strains were grown in LB medium [58] supplemented with ampicillin (100 mg/ml), apramycin (25 μg/ml) or tetracycline (15 μg/ml) for the selection of plasmids.

Analysis of anthracycline production or bioconversion

Anthracycline metabolites were extracted from liquid medium with chloroform as described by Otten *et al*. [42] for *S. peucetius* and *S. lividans* bioconversion experiments, and from solid medium using chloroform. TLC analysis were performed as in Grimm *et al.* [61] on precoated silica gel F60/F254 TLC plates (E. Merck) developed in the ascending fashion with chloroform:methanol:acetic acid:water (40:10:0.6:2 v/v) as a mobile phase. HPLC analysis was performed with a Waters (Milford, MA) Nova-Pak® C18 column (3.9 × 150 mm) as in Madduri *et al.* [26] with the elution system: 20% B in A (A, H_2O:trifluoroacetic acid (TFA) 0.1%; B, acetonitrile:TFA 0.078%) to 25% B with a linear gradient for 5 min, followed by 25% B to 40% B with gradient 8 for 10 min, followed by 40% B to 70 % B with a linear gradient for 10 min, and remaining at 70% B for an additional 6 min. RHOD used as a reference both for TLC, HPLC and spectral analysis was provided by William Strohl (Ohio State University). Bioconversion products were purified by TLC and eluted from the silica gel by methanol. Molecular weights were determined by mass spectroscopy using the electrospray ionization mode (ESI–MS) coupled with tandem MS/MS detection of selected ions.

Determination of anthracycline production in *S. peucetius dnm::aphII, dnmM*$^+$ and *dnmW* strains was done as described by Lomovskaya *et al*. [34].

Characterization of RHOD and related products obtained by bioconversion

From the *S. lividans* WMH1911 strain grown in APM solid medium in the presence of RHO (3.5 mg), 210 μg of RHOD were purified by preparative TLC. HPLC analysis showed a retention time identical to that of the RHOD standard, and only a single peak with the expected retention time was observed upon coinjection of the two samples. The RHOD isolated from the bioconversion was characterized initially by low resolution ESI–MS and MS/MS analysis. A parent ion was detected at m/z 558.2 consistent with $[RHOD]H^+$ and the fragmentation pattern exhibited ions corresponding to $[RHOD–H_2O]H^+$ at m/z 540.2, $[RHO]H^+$ at m/z 429.0, $[RHO–H_2O]H^+$ at m/z 411.2, $[daunosamine]H^+$ at m/z 148.0 and $[daunosamine–H_2O]H^+$ at m/z 129.8. The standard and isolated samples of RHOD exhibited identical ^1H NMR spectra. Products isolated from bioconversion with strains WMH1913, WMH1914 and WMH1916 carrying the mutant *dnmZ, dnrH* or *dnmQ* genes, respectively, had the same chromatographic and ESI–MS properties as those of RHOD.

General DNA manipulations

DNA isolation, restriction and ligation were performed according to standard techniques [58]. Qiaex resin (Qiagen, Chatsworth, CA) was used routinely to purify DNA fragments from gel slices. Sequencing reactions using reverse and forward M13 primers were performed with an ABI Prism BigDye terminator cycle sequencing kit at the facilities provided by the University of Wisconsin Biotechnology Center. PCR amplifications were performed in a thermal cycler Perkin–Elmer Cetus model 480. Amplification of *dnrI* with its own promoter and *dnmJ* from pWHM1898 using the primers COA1-COA2 (Table S2), and *dnmT* from pWHM954 (using COA3-COA4, Table S2) were made by denaturing the samples at 95°C for 5 min and subjecting them to three sets of 10 cycles of denaturing at 95°C, 1.5 min, annealing at 50°C first cycle, 60°C second cycle and 65°C third cycle, for 1 min and elongation at 72°C for 5 min followed by 1 cycle of 95°C, 1.5 min, 65°C, 1 min and 72°C for 15 min. For amplification of *dnmQ* and *dnmS* from pWHM1899 (using COA5-COA6, Table S2) the same procedure was used by changing the annealing temperature to 55°C, 60°C and 65°C in the first, second and third cycles, followed by 1 cycle of 95°C, 1.5 min and 72°C, 10 min. PCR mixtures contained 1× PCR buffer [10mM Tris-HCl, pH 8.85 (20°C), 25 mM KCl, 5 mM $(NH_4)_2SO_4$] supplied by Boehringer Mannheim, and $MgSO_4$ 1.5mM for the first two amplifications and 3.5 mM for the last one, which in addition contained DMSO 5%. Pwo DNA polymerase (2.5 U, Boehringer Mannheim) was used in these PCRs. Amplification of a partial *dnmZ* with its own promoter and the *dpsY* promoter was performed with primers COA7B and COA7C (Table S2) and pWHM219 DNA by denaturing the samples at 95°C for 5 min, and then 30 cycles of denaturing (95°C, 30 s), annealing (37°C, 30 s) and elongation (72°C, 3 min) followed by 1 cycle of denaturing (95°C, 30 s) and elongation (72°C, 5 min). Amplification of the operon *dnmLM*$^+$ was performed with primers COA8 and COA9 (Table S2) and pJTR1019 DNA by denaturing the samples at 97°C for 5 min, then 15 cycles of denaturing (97°C, 30 s), annealing (55°C, 45 s) and elongation (72°C, 90 s) followed by 10 cycles of denaturing (97°C, 30 s), annealing (65°C, 45 s) and elongation (72°C, 90 s) and 10 cycles of denaturing (97°C, 30 s) and elongation (72°C, 2 min). *Taq* polymerase (1 U, Promega, Madison, WI) was used in the latter reactions with 1× PCR buffer (50mM KCl, 10 mM Tris–HCl, pH 9.0 (at 25°C), 0.1% Triton X-100), 5mM $MgCl_2$ and DMSO 5%. In all PCR reactions 20 pmol of each primer was used, each dNTP was present at 200 mM and 1 ng of target DNA was used.

Construction of S. peucetius dnmM::aphII *and* dnmM$^+$ *strains*

To prepare the *dnmM::aphII* mutant, a 1.0 kb *Sal*I fragment containing the *aphII* gene from pWHM249 (Table S1) was cloned blunt-ended between the *Xcm*I and *Sac*II sites in a 1.6 kb *Eag*I–*Bam*HI fragment of the wild-type *dnmM* gene in which the frameshift mutation was located. The fragment containing the *dnmM::aphII* disrupted copy was cloned in KC515 as described in Table S1. Protoplasts of *S. lividans* TK24 were transfected with the phage construct and the recombinant phage

was isolated from plaques by a convenient spot-test method [62]. The recombinant phage phWHM461 carrying the *dnmM::aphII* gene was characterized phenotypically by its neomycin and viomycin resistance because the thiostrepton resistance gene in KC515 was replaced by the cloned fragment. The presence of the cloned DNA was confirmed by restriction endonuclease digestion analysis. *S. peucetius* 29050 was infected with phWHM461 (Table S1) and clones resistant to neomycin and viomycin as well as clones resistant only to neomycin were obtained. Clones resistant only to neomycin were expected to have undergone *dnmM* gene replacement through double crossover recombination. PCR analysis of the genomic DNA from six clones was performed to confirm that homologous recombination had taken place between the cloned fragment and genomic DNA in the *dnmM* region. One of the primers for this PCR reaction was located inside the *aphII* gene and another outside of the cloned fragment, 1.2 kb away from the first primer. The WMH1800 strain was chosen as a representative *dnmM::aphII* mutant.

The frameshift in the wild-type *dnmM* gene was repaired using PCR mutagenesis and the structure of the *dnmM*$^+$ gene in the resulting plasmid, pJTR1019, was verified by DNA sequencing. A few other errors in the reported DNA sequence of the last few nucleotides of *dnmL* and the *dnmM* gene were corrected during this work. The recombinant phage containing the *dnmM*$^+$ gene, cloned as a 2.0 kb *Avr*II–*Bam*HI fragment from pJTR1019, was constructed as described in Table S1. The phage was isolated after transfection of *S. lividans* TK24 protoplasts. The phWHM463 containing the *dnmM*$^+$ gene was characterized using a PCR reaction with unique primers located within the cloned fragment and by thiostrepton resistance because the viomycin resistance gene in KC515 was replaced by the cloned fragment.

The *S. peucetius dnmM*$^+$ strain was obtained by replacing the *dnmM::aphII* copy in WMH1800 with the *dnmM*$^+$ fragment. Strain WMH1800 was infected with phWHM463 to obtain clones resistant to neomycin and thiostrepton. From progeny of these clones, two neomycin- and thiostrepton-sensitive clones, WMH1801 and WMH1802, were isolated and expected to be produced as a result of double crossover recombination between chromosomal DNA and cloned *dnmM*$^+$ fragment. PCR analysis with suitable primers revealed the presence of a unique 1.4 kb amplified band in the DNAs from WHM1801 and WMH1802, consistent with replacement of *dnmM::aphII* by the *dnmM*$^+$ gene.

Construction of daunosamine gene clusters

A region of 2,176 bp containing *dnrI* and *dnmJ* under the control of *dnrI* promoter (*dnrIp*) was amplified from pWHM1898 and the resultant product cloned in pUC19 as an *Eco*RI–*Hind*III fragment. An internal 2,064 bp *Bam*HI–*Bgl*II fragment from pWHM1898 was used to replace the most of the PCR product, resulting in pWHM1900 (Figure S1a). In this and all other constructs, any DNA introduced by PCR and not so replaced was verified by sequence analysis. Clone pWHM1900 was digested with *Bgl*II and *Eco*RI and two fragments were cloned in it at these sites; a 1,672 bp *Bgl*II–*Bam*HI fragment from pWHM1898 (containing part of the *dnmJ, dnmV* and *dnmU* genes) and a PCR amplification product (primers COA7B and COA7C, Table S2) from pWHM219 [25] containing a partial *dnmZ* gene and the bifunctional promoter for *dnmZ* and *dpsY* as a *Bam*HI–*Eco*RI fragment. In the resulting pWHM1903 (Figure S1a), an internal 900 bp *Srf*I–*Mlu*I fragment was replaced by the original sequence from pWHM219 to give pWHM1904 (Figure S1a). Construct pWHM1901 (Figure S1b) was obtained by amplifying a 1,590 bp fragment from pWHM954 [22] then cloning this DNA in pUC19 as a *Eco*RI–*Hind*III fragment, which was digested with *Rsr*II–*Bpu*1102I to remove a 1,460 bp fragment and replace it by the original sequence from pWHM954. The *dnm*QS genes were PCR amplified from pWHM1989 as a 2,720 bp fragment that was cloned in pUC19 as an *Eco*RI–*Hind*III fragment. Most of this DNA was replaced by a 3,090 bp *Bss*HII–*Eco*RI fragment from pWHM1989 to give pWHM1902 (Figure S1b). Both pWHM1901 and pWHM1902 were digested with *Eco*RI and *Eco*RV, then the 3,208 bp fragment resulting from pWHM1902 was cloned in pWHM1901 to obtain pWHM1905 (Figure S1b). Finally, pWHM1904 and pWHM1905 were digested with *Hind*III and *Afl*II or *Afl*II and *Eco*RI, respectively, and both fragments were cloned in pWHM3 to give pWHM1906 (Figure 2b).

Two additional plasmids were made to replace *dnmT* in pWHM1905 by the whole operon containing *dnmW, dnmT* and *dnrH*. The source of this operon was pWHM377 containing a 4.0 kb *Sst*I–*Bcl*I fragment from pWHM555 [34]. For the first construct, a 3.9 kb *Eco*RI–*Afl*II fragment was cloned blunt-ended into pWHM1901 previously digested with *Bpu*1102I and the overhanging ends filled in by treatment with the Klenow fragment then digested with *Nae*I to remove *dnmT*. The resulting plasmid, pWHM1901a, was digested with *Eco*RV and *Hind*III and the resulting fragment was cloned into pWHM1902 to give pWHM1907 in which *dnmW, dnmT, dnrH, dnmQ* and *dnmS* are under the control of *dnmW* promoter (Figure S1c). Finally, pWHM1904 and pWHM1907 were digested with *Hind*III and *Afl*II or *Afl*II and *Eco*RI, respectively, and both of these fragments were cloned in pWHM3 to obtain pWHM1909 (Figure 2b). The second construction was made by cloning a 3.9 kb *Eco*RI–*Afl*II fragment into pWHM1901 previously digested with these same restriction enzymes to remove *dnmT*. The resulting plasmid, pWHM1901b, was digested with *Eco*RI, blunt-ended by treatment with the Klenow fragment then digested with *Hind*III and the resulting fragment was cloned into *Eco*RV- and *Hind*III-digested pWHM1902 to give pWHM1908 in which *dnmW*, *dnmT* and *dnrH* are under the control of the *dnmW* promoter and *dnmQ* and *dnmS* under control of the *dpsG* promoter (Figure 2b). Finally, pWHM1904 and pWHM1908 were digested with *Hind*III and *Afl*II or *Afl*II and *Eco*RI, respectively, and both of the resulting fragments were cloned in pWHM3 to obtain pWHM1910 (Figure 2b). In both pWHM1909 and pWHM1910 321 bp from the *dnrE* coding region downstream of *dnrH* were included in the cloning process (*dnrE* encodes a ketoreductase required for RHO biosynthesis [22]).

Construction of daunorubicin resistance plasmids carrying the dnmLM *or* mtmDE *genes*

Genes for the two enzymes common to all the deoxysugar pathways, TDP-glucose-1-phosphate thymidylyltransferase and TDP-glucose-4,6-dehydratase, and the *drrAB* daunorubicin resistance genes were cloned in pWHM601 as follows. The *drrAB* genes were obtained from pWHM611 [31] as a 2.5 kb *Nco*I–*Afl*III fragment and cloned after blunt ending in pUC19 digested with *Sma*I. The correct orientation of *drrAB* was selected and designated pWHM1911, then this plasmid was digested with *Sty*I, blunt ended, and digested with *Hind*III, followed by cloning together with a 2.7 kb *Stu*I–*Hind*III fragment from plasmid pFLADE [32] containing the *mtmDE* genes. From the resulting construction an *Eco*RI–*Hind*III fragment was subcloned in pWHM601 to give pWHM1912 (Figure 2a). In this plasmid the *mtmD* and *mtmE* genes are under the control of the *dnrF* promoter [31]. A second plasmid containing *dnmL* and *dnmM*$^+$ was prepared by PCR amplification of a 2.3 kb fragment from pJTR1019 using primers COA8 and COA9 (Table S2).The *Eco*RI–*Hind*III digested PCR product was cloned in pUC19 and the resultant construction was digested with *Apa*I and *BstE*II. The 400 bp *Apa*I–*Blp*I and 1.9 kb *Blp*I–*BstE*II fragments from pJTR1019 were used to replace most of the PCR DNA in the linearized DNA to give pWHM1913. From pWHM1913 a *Nco*I–*Hind*III fragment was cloned in *Nco*I- and *Hind*III- digested pWHM1911 to obtain pWHM1914. From pWHM1914 the *Eco*RI–*Hind*III fragment was cloned in pWHM601 to give pWHM1915 (Figure 2a, Table S1).

Different strains of *S. lividans* 1326 were obtained by transformation with plasmids containing the *dnm* or *mtm* genes and the *drrAB* resistance genes (Table S1). All strains carrying pWHM1912 tested were resistant to 10 μg/ml DNR (data not shown).

Construction of plasmids with mutant dnm *genes*

Modified versions of pWHM1910 with deletions in some of the genes involved in L-daunosamine biosynthesis were generated as follows.

pWHM1903 that contains a truncated version of *dnmZ* was used to make pWHM1916 by cloning an *Hind*III–*Afl*II fragment from pWHM1903 and an *Afl*II–*Eco*RI fragment from pWHM1908 together in pWHM3. The *ΔdnmZ* gene in pWHM1916 encodes a 60 amino acid protein having only the first 51 amino acids of the deduced DnmZ protein. A plasmid with an in-frame deletion in *dnrH* was constructed by *Bam*HI–*Sgr*AI digestion of pWHM1901b, blunt-ending the resulting DNA followed by religation into a plasmid, then removing a 918 bp fragment to give pWHM1917 from which an *Eco*RI (blunt ended)–*Hind*III fragment was cloned in *Eco*RV- and *Hind*III-digested pWHM1902 to give pWHM1918. From this plasmid an *Afl*II–*Eco*RI fragment was cloned in pWHM3 together with a *Hind*III–*Afl*II fragment from pWHM1904 to give pWHM1919. In the latter plasmid the *ΔdnrH* gene encodes a protein of 137 amino acids without the 306 amino acids between residues 135 and 440 of the DnrH protein. The fragment pWHM1910 with an in-frame deletion in the *dnmW* gene was constructed by digestion of pWHM1908 with *Sph*I (followed by blunt ending) and *Psh*AI, which removes a 393 bp fragment, and religation to give pWHM1920. An *Afl*II–*Eco*RI fragment from pWHM1920 was cloned together with a *Hind*III–*Afl*II fragment from pWHM1904 in pWHM3 to give pWHM1921. In the latter plasmid, the *ΔdnmW* gene encodes a 63 amino acid protein without the 131 amino acids between residue 5 and 135 of the DpsH protein. A frameshift mutation in the *dnmQ* gene was constructed by *Sfr*I digestion of pWHM1905 and religation, followed by removal of a 575 bp fragment to give pWHM1922. An *EcoR*V–*Hin*dIII fragment containing *dnmT* was removed from this plasmid and the remaining DNA was cloned with an *Eco*RI (blunt ended)–*Hind*III fragment from pWHM1901b to give pWHM1923. From this plasmid an *Afl*II–*Eco*RI fragment was cloned in pWHM3 together with a *Hind*III–*Afl*II fragment from pWHM1904 to give pWHM1924. The mutant *dnmQ* gene in the latter plasmid encodes a 251 amino acid protein without the first 220 amino acids of the DnmQ protein.

All of the above plasmids with *dnm* mutations were introduced by tranformation into *S. lividans* 1326 together with pWHM1912 to generate strains WMH1913, WMH1914, WMH1915 and WMH1916 (Table S1).

Supplementary material

Supplementary material with a table of the strains and plasmids used, a table of the oligodeoxynucleotide primers used and a figure showing the structure of the clones used to generate the daunosamine gene clusters is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank William Strohl for an authentic sample of RHOD, Y. Katayama for certain HPLC analyses, J. Kennedy for plasmid pWHM1898, and S.L. Otten for plasmid pWHM1899. This work was supported in part by a grant from the National Institutes of Health (GM31925) and a postdoctoral fellowship to C.O. from the Ministry of Education and Culture in Spain (PF 97 10074407).

References

1. Arcamone, F., *et al.*, & Spalla, C. (1969). Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *Streptomyces peucetius* var. *caesius. Biotechnol. Bioeng.* **11**, 1101-1110.
2. Cassinelli, G. & Orezzi, P. (1963). Daunomycin, a new antibiotic with cytostatic activity. Isolation and properties. *G. Microbiol.* **11**, 167-174.
3. D'bost, M., *et al.*, & Werner, G.H. (1963). Un novel antibiotique a proprietes cytostatiques: la rubidomycine. *C. R. Acad. Sci. Agric. Belg.* **257**, 1813-1815.
4. Arcamone, F. (1981). A novel antibiotic with cytostatic properties: rubidomycin. *Med. Chem. Ser. Monogr.* **17**, 25-31.
5. Arcamone, F.M. (1998). From the pigments of the actinomycetes to third generation antitumor anthracyclines. *Biochimie* **80**, 201-206.
6. Fujiwara, A., & Hoshino, T. (1983). Anthracycline antibiotics. *Crit. Rev. Biotechnol.* **3**, 133-157.
7. Hutchinson, C.R. (1997). Biosynthetic studies of daunorubicin and tetracenomycin C. *Chem. Rev.* **97**, 2525-2535.
8. Hutchinson, C.R. (1999). Genetic engineering of doxorubicin production in *Streptomyces peucetius*. *J. Industr. Microbiol. Biotechnol.* **23**, 647-652.
9. Summers, R.G., Donadio, S., Staver, M., Wendt-Pienkowski, E., Hutchinson, C.R. & Katz, L. (1997). Sequencing and mutagenesis of genes from the erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea* that are involved in L-mycarose and D-desosamine production. *Microbiology* **143**, 3251-3262.
10. Gaisser, S., Böhm, G.A., Cortés, J, & Leadlay, P.F. (1997). Analysis of seven genes from the *eryAI–eryK* region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. *Mol. Gen. Genet.* **256**, 239-251.
11. Gaisser, S., *et al.*, & Leadlay, P.F. (1998). Analysis of *eryBI, eryBIII* and *eryBVII* from the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea. Mol. Gen. Genet.* **258**, 78-88.
12. Salah-Bey, K., *et al.*, & Raynal, M.C. (1998). Targeted gene inactivation for the elucidation of deoxysugar biosynthesis in the erythromycin producer *Saccharopolyspora erythraea*. *Mol. Gen. Genet.* **257**, 542-553.
13. Olano, C., Rodriguez, A.M., Michel, J.M., Mendez, C., Raynal, M.C. & Salas, J.A. (1998). Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltranferases responsible for glycosylation of the macrolactone ring. *Mol. Gen. Genet.* **259**, 299-308.
14. Quiros, L.M., Aguirrezabalaga, I., Olano, C., Mendez, C. & Salas, J.A. (1998). Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antiobioticus. Mol. Microbiol.* **28**, 1177-1185.
15. Xue, Y., Zhao, L., Liu, H.W. & Sherman, D.H. (1998). A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: architecture of metabolic diversity. *Proc. Natl Acad. Sci. USA* **95**, 12111-12116.
16. Occi, J.L., Gerwain, K.M., Ruby, C.L. & MacNeil, D.L. (1991). Abstracts of the annual meeting of the society for industrial microbiology. P49, p.93. Philadelphia, PA.
17. Kirschning, A., Bechthold, A.F.W., & Rhor, J. (1997). Chemical and biochemical aspects of deoxysugars and deoxysugar oligosaccharides. *Top. Curr. Chem.* **188**, 1-84.
18. Liu, H.W., & Thorson, J.S. (1994). Pathways and mechanisms in the biogenesis of novel deoxysugars by bacteria. *Annu. Rev. Microbiol.* **48**, 223-256.
19. Elling, L. (1997). Glycobiotechnology: enzymes for the synthesis of nucleotide sugars. *Advan. Biochem. Eng. Biotechnol.* **58**, 89-44.
20. Gallo, M.A., Ward, J. & Hutchinson, C.R. (1996). The *dnrM* gene in *Streptomyces peucetius* contains a naturally occurring frameshift mutation that is suppressed by another locus outside of the daunorubicin-production gene cluster. *Microbiology* **142**, 269-275.
21. Thompson, M.W., Strohl, W.R. & Floss, H.G. (1992). Purification and characterization of TDP-D-glucose 4,6-dehydratase from anthracycline-producing streptomycetes. *J. Gen. Microbiol.* **138**, 779-786.
22. Scotti, C. & Hutchinson, C.R. (1996). Enhanced antibiotic production by manipulation of the *Streptomyces peucetius dnrH* and *dnmT* genes involved in doxorubicin (adriamycin) biosynthesis. *J. Bacteriol.* **178**, 7316-7321.
23. Otten, S.L., Liu, X., Ferguson, J. & Hutchinson, C.R. (1995). Cloning and characterization of the *Streptomyces peucetius dnrQS* genes encoding a daunosamine biosynthesis enzyme and a glycosyl transferase involved in daunorubicin biosynthesis. *J. Bacteriol.* **177**, 6688-6692.
24. Draeger, G., Park., S.H. & Floss, H.G. (1999). Mechanism of the 2-deoxygenation step in the biosynthesis of the deoxyhexose moieties of the antibiotics granaticin and oleandomycin. *J. Am. Chem. Soc.* **121**, 2611-2612
25. Otten, S.L., Gallo, M.A., Madduri, K., Liu, X. & Hutchinson, C.R. (1997). Cloning and characterization of the *Streptomyces peucetius dnmZUV* genes encoding three enzymes required for biosynthesis of the daunorubicin precursor thymidine diphospho-L-daunosamine. *J. Bacteriol.* **179**, 4446-4450.
26. Madduri, K., *et al.*, & Hutchinson, C.R. (1998). Production of the antitumor drug epirubicin (4′-epidoxorubicin) and its precursor by a genetically engineered strain of *Streptomyces peucetius*. *Nat. Biotechnol.* **16**, 69-74.
27. Stutzman-Engwall, K.J., Otten, S.L. & Hutchinson, C.R. (1992). Regulation of secondary metabolism in *Streptomyces spp.* and overproduction of daunorubicin in *Streptomyces peucetius. J. Bacteriol.* **174**, 144-154.
28. Madduri, K. & Hutchinson, C.R. (1995). Functional characterization and transcriptional analysis of the $dnrR_1$ locus, which controls daunorubicin biosynthesis in *Streptomyces peucetius. J. Bacteriol.* **177**, 1208-1215.

29. Tang, L., Grimm, A., Zhang, Y.X. & Hutchinson, C.R. (1996). Purification and characterization of the DNA-binding protein, Dnrl, a transcriptional factor of daunorubicin biosynthesis in *Streptomyces peucetius. Mol. Microbiol.* 22, 801-813.
30. Furuya, K. & Hutchinson, C.R. (1998). The DrrC protein of *Streptomyces peucetius*, a UvrA-like protein, is a DNA-binding protein whose gene is induced by daunorubicin. *FEMS Microbiol. Lett.* 168, 243-249.
31. Guilfoile, P.G. & Hutchinson, C.R. (1991). A bacterial analog of the *mdr* gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. *Proc. Natl Acad. Sci. USA* 88, 8553-8557.
32. Lombo, F., Siems, K., Braña, A.F., Mendez, C., Bindseil, K. & Salas, J.A. (1997). Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in the earliest steps of biosynthesis of the sugar moieties of the antitumor polyketide mithramycin. *J. Bacteriol.* 179, 3354-3357.
33. Gerlitz, M., Meurer, G., Wendt-Pienkowski, E., Madduri, K. & Hutchinson, C.R. (1997). Effect of the daunorubicin *dpsH* gene on the choice of starter unit and cyclization pattern reveals that type II polyketide synthases can be unfaithful yet intriguing. *J. Am. Chem. Soc.* 119, 7392-7393.
34. Lomovskaya, N., *et al.*, & Hutchinson, C.R. (1999). Doxorubicin overproduction in *Streptomyces peucetius*: cloning and characterization of the *dnrU* ketoreductase and *dnrV* genes and the doxA cytochrome P-450 hydroxylase gene. *J. Bacteriol.* 181, 305-318.
35. Vara, J., Lewandowska-Skarbek, Wang, Y.G., Donadio, S. & Hutchinson, C.R. (1989). Cloning of genes governing the deoxysugar portion of the erythromycin biosynthetic pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). *J. Bacteriol.* 171, 5872-5881.
36. Lomovskaya, N., *et al.*, & Hutchinson, C.R. (1998) The *Streptomyces peucetius dpsY* and *dnrX* genes govern early and late steps of daunorubicin and doxorubicin biosynthesis. *J. Bacteriol.* 180, 2379-2386.
37. Ho, C.C. & Chye, M.L. (1985). Construction of a genetic map of chromosomal auxotrophic markers in *Streptomyces peucetius var. caesius. J. Gen. Appl. Microbiol.* 31, 231-241.
38. Ho, C.C., Wright, M.H. & Hopwood, D.A. (1985). Transformation of protoplasts from *Streptomyces peucetius var. caesius* by plasmid cloning vectors. In *Proceedings of the International Congress on Microbiology in the Eighties* (Lim, G., & Nga, BH., eds.), pp. 275-289, Singapore Society for Microbiology, Singapore.
39. Bibb, M.J., White, J., Ward, J.M., & Janssen, G.R.. (1994). The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. *Mol. Microbiol.* 14, 533-545.
40. Hopwood, D.A., *et al.*, & Schrempf, H. (1985). *Genetic Manipulation of* Streptomyces*: A Laboratory Manual.* The John Innes Foundation, Norwich, United Kingdom.
41. Takano, E., Gramajo, H.C., Strauch, E., Andres, N., White, J. & Bibb, M.J. (1992). Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 6, 2797-2804.
42. Otten, S.L., Stutzman-Engwall, K.J., & Hutchinson, C.R. (1990). Cloning and expression of daunorubicin biosynthesis genes from *Streptomyces peucetius* and *S. peucetius* subsp*. caesius*. *J. Bacteriol.* 172, 3427-3434.
43. Jacobsen, J.R., Hutchinson, C.R., Cane, D.E. & Khosla, C. (1997). Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. *Science* 277, 367-369.
44. Ruan, X., *et al.*, & Katz, L. (1997). Acyltransferase domain substitutions in erythromycin polyketide synthase yield novel erythromycin derivatives. *J. Bacteriol.* 179, 6416-6425.
45. Stassi, D.L., *et al.*, & Katz, L. (1998). Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. *Proc. Natl Acad. Sci. USA* 95, 7305-7309.
46. Marsden, A.F., Wilkinson, B., Cortés, J., Dinster, N.J., Stauton, J. & Leadlay, P.F. (1998). Engineering broader specificity into an antibiotic-producing polyketide synthase. *Science* 279, 199-202.
47. McDaniel, R., Thamchaipenet, A., Gustafsson, C., Fu, H., Betlach, M. & Ashley, G. (1999). Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel 'unnatural' natural products. *Proc. Natl Acad. Sci. USA* 96, 1846-51.
48. Gokhale, R.S., Tsuji, S.Y., Cane, D.E. & Khosla, C. (1999). Dissecting and exploiting intermodular communication in polyketide synthases. *Science* 284, 482-485.
49. Stachelhaus, T., Schneider, A. & Marahiel, M.A. (1995). Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. *Science* 269,69-72.
50. Belshaw, P., Walsh, C.T. & Stachelhaus, T. (1999). Aminoacyl-CoAs as probes of condensation domain selectivity in nonribosomal peptide synthesis. *Science* 284, 486-489.
51. Decker, H., Haag, S., Udvarnoki, G. & Rohr, J. (1995). Novel genetically engineered tetracenomycins. *Angew. Chem. Int. Ed. Engl.* 34, 1107-1110.
52. Solenberg, P.J., Matsushima, P., Stack, D.R., Wilkie, S.C., Thompson, R.C., & Baltz, R.H. (1997). Production of hybrid glycopeptide antibiotics *in vitro* and in *Streptomyces toyocaensis*. *Chem. Biol.* 4, 195-202.
53. Wohlert, S.E., *et al.*, & Rohr, J. (1998). Novel hybrid tetracenomycins through combinatorial biosynthesis using a glycosyltransferase encoded by the *elm* genes in cosmid 16F4 and which shows a broad sugar substrate specificity. *J. Am. Chem. Soc.* 120, 10596-10601.
54. Johnson, D.A. & Liu, H. (1998). Mechanisms and pathways from recent deoxysugar biosynthesis research. *Curr. Opin. Chem. Biol.* 2, 642-649.
55. Zhao, L., Sherman, D.H. & Liu, H.W. (1998). Biosynthesis of desosamine: construction of a new methymycin/neomethymycin analogue by deletion of a desosamine biosynthetic gene. *J. Am. Chem. Soc.* 120, 10256-10257.
56. Zhao, L., Que, N.L.S., Xue, Y., Sherman, D.H. & Liu, H.W. (1998). Mechanistic studies of desosamine biosynthesis: C-4 deoxygenation precedes C-3 transamination. *J. Am. Chem. Soc.* 120, 12159-12160.
57. Hopwood, D.A., Kiesser, T., Wright, H.M. & Bibb, M.J. (1983). Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. *J. Gen. Microbiol.* 129, 2257-2269.
58. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual,* (2nd edn), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. Yanisch-Perron, C., Vieira, J., & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103-119.
60. Lomovskaya, N.D., Chater, K.F. & Mkrtumian, N.M. (1980). Genetics and molecular biology of *Streptomyces* bacteriophage. *Microbiol. Rev.* 44, 206-229.
61. Grimm, A., Madduri, K., Ali, A. & Hutchinson, CR. (1994). Characterization of the S*treptomyces peucetius* ATCC 29050 genes encoding doxorubicin polyketide synthase. *Gene* 151, 1-10.
62. Lomovskaya, N., Fonstein, L., Ruan, X., Stassi, D., Katz, L. & Hutchinson, C.R. (1997). Gene disruption and replacement in the rapamycin-producing *Streptomyces hygroscopicus* strain ATCC 29253. *Microbiology* 143, 875-883.

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