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# Genetic engineering of doxorubicin production in *Streptomyces peucetius*: a review*

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**The genetics and biochemistry of daunorubicin and doxorubicin production by *Streptomyces peucetius* is reviewed, with a focus on how such information can be used for the genetic engineering of strains having improved titers of these two antitumor antibiotics.**

**Keywords:** anthracyclines; antibiotic resistance; antitumor drug; biosynthesis; daunorubicin; industrial fermentation; metabolite overproduction; streptomycetes

## Introduction

Daunorubicin (DNR) and doxorubicin (DXR) are clinically important cancer chemotherapeutic agents and, in spite of undesirable acute and long-term toxic effects, DXR remains one of the most widely used antitumor drugs because of its broad spectrum of activity [2,24]. DXR was first isolated in 1969 [1] from *Streptomyces peucetius* subsp *caesius* ATCC 27952, a higher DXR-producing mutant strain derived from the wild-type *S. peucetius* ATCC 29050 strain, and is formed by C-14 hydroxylation of its immediate precursor, DNR (Figure 1). Although a number of organisms (including the 29050 strain) are known to produce DNR [12], *S. peucetius* subsp *caesius* is the only organism reported to produce DXR. The current production of DXR is over 225 kilograms annually due to its wide use and the fact that it is the starting point for the synthesis of numerous analogs and derivatives aimed at improving clinical cancer treatment [2]. Although DXR was discovered as a microbial metabolite, it is produced commercially by semi-synthesis from the more abundant DNR instead of by fermentation. High-DNR producing strains are available world-wide yet apparently lack the ability to make useful amounts of DXR or the DXR produced cannot easily be separated from the DNR that also is present. Consequently, the development of improved strains for DXR production is a beneficial goal since this drug is an expensive product.

## Review

We began our quest in 1987 for basic information about the genetics and biochemistry of DXR production, on the assumption that this work might lead to ways to enhance DXR production considerably. Our studies were carried out with the ATCC 29050 strain, with the intention of extending developments that resulted in enhanced DXR production by the wild-type strain to DNR-producing industrial strains. This was believed to be a sensible approach, although we recognized at the outset that high-producing industrial strains obtained by random mutagenesis can differ in many respects other than just the DNR titer from wild-strains, which might undercut the direct transfer of results from the latter to the former.

The biosynthetic pathway to DNR (**12**) and DXR (**13**) (Figures 1 and 2) had largely been laid out by 1987 through research carried out in several academic and industrial laboratories, most notably at Farmitalia Carlo Erba (now Pharmacia & Upjohn) in Italy and at the former Institute for Microbiology in Jena, Germany. Since this subject has been reviewed recently [15], we have summarized only some recent highlights here. The first stage of the pathway ends with the biosynthesis of $\epsilon$-rhodomycinone (**6**), whose carbon skeleton is made by a type II polyketide synthase (PKS) encoded by the *dpsABCDGEFY* genes (Figure 3) [13,37]. Unlike most other cases, these genes are not grouped in one location within the cluster; *dpsG* and *dpsY* are quite distant from the other *dps* genes yet both of them are required to make 12-deoxyaklanonic acid (**1**) [13,29]. The PKS chooses propionate to initiate assembly of the carbon chain instead of the more commonly used acetate starter unit, and this was initially proposed to involve the *dpsC* and *dpsD* genes [13,29]. We now know that starter unit choice is effected primarily through the *dpsC* gene that encodes a homolog of the FabH $\beta$-ketoacyl:acyl carrier protein synthase III, which is used to initiate fatty acid synthesis in *E. coli.* DpsC, like FabH, is acylated by its substrate and is highly discriminatory, favoring propionyl-CoA over acetyl- or malonyl-CoA (Bao and Hutchinson, unpublished results). Since DpsC, radioactive propionyl-CoA and the *E. coli* AcpP acyl carrier protein charged with malonate (from malonyl-CoA) form an enzyme-bound radioactive product when admixed (Bao and Hutchinson, unpublished results), we believe that DpsC, DpsG encoding an acyl carrier protein and possibly DpsD that produces a malonyl-CoA:acyl carrier protein acyltransferase make $\beta$-ketovalerate bound to DpsG to initiate carbon chain assembly (fatty

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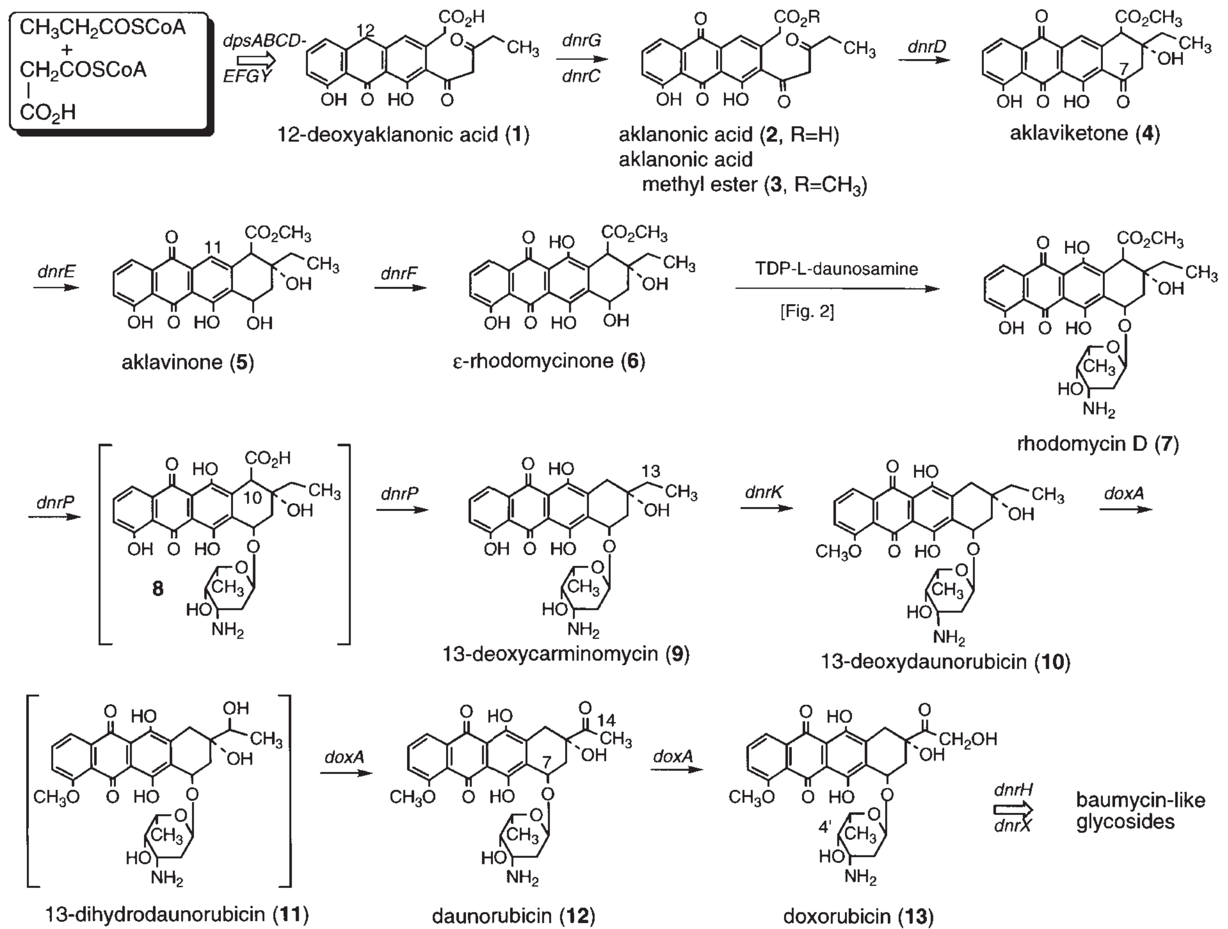


**Figure 1** Abbreviated pathway for biosynthesis of DNR and DXR from propionyl-CoA, malonyl-CoA, and thymidine diphospho (TDP)-l-daunosamine. Open arrows indicate multiple steps between the precursor and product shown. Gene functions are indicated above the steps they govern and defined in the text.

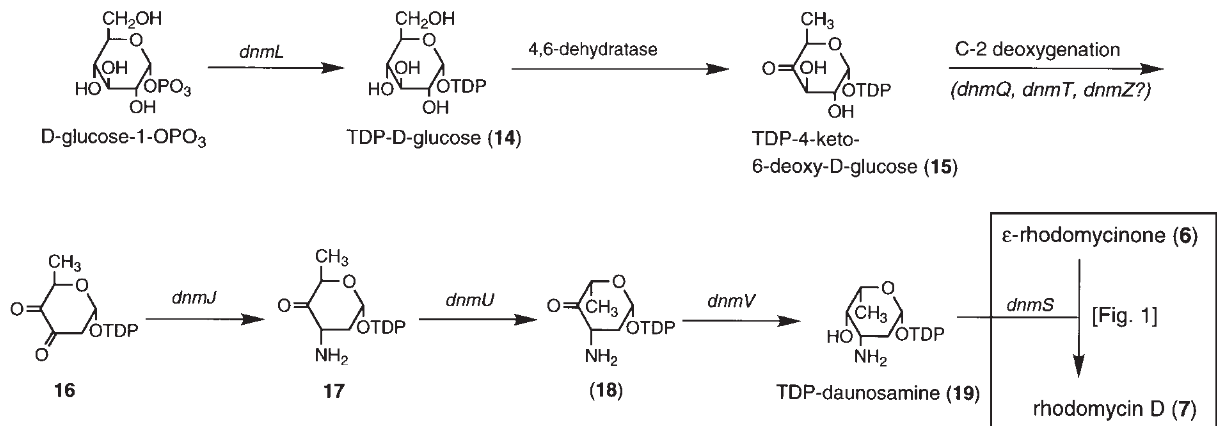


**Figure 2** Hypothesis for the biosynthesis of TDP-l-daunosamine. The genes governing each step are shown above the arrows, but the order and nature of the some of the steps (eg **15–16**) are conjectural.

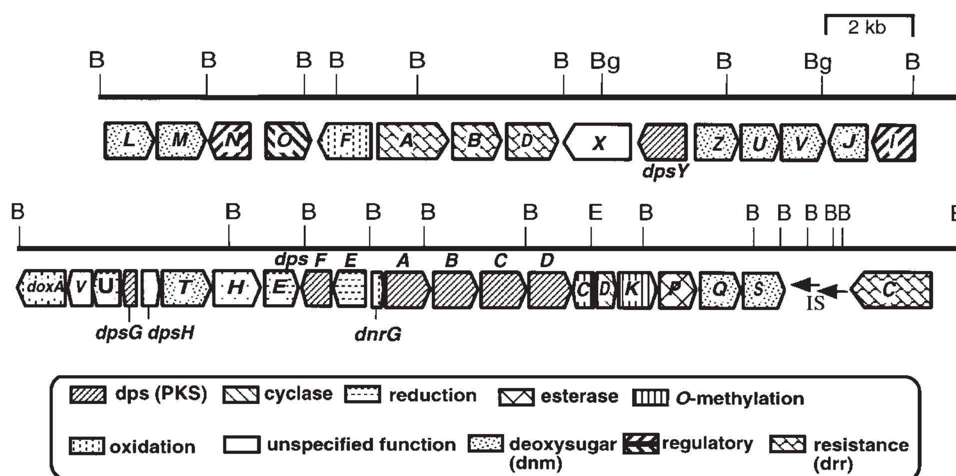


**Figure 3** Physical and functional map of the DNR/DXR gene cluster. The relative sizes of the open reading frames and the direction of gene transcription are designated by pointed boxes, which are shaded according to the types of functions indicated beneath the restriction map of the cluster. Gene functions are defined further in the text and in Figure 1. The solid line indicates the genomic DNA with restriction sites abbreviated as B, *Bam*HI; Bg, *Bgl*II; and E, *Eco*RI.

acid synthesis in *E. coli* is initiated in the same way via $\beta$-ketobutyrate).

After the polyketide stage is completed, $\epsilon$-rhodomycinone (**6**) undergoes glycosylation by TDP-daunosamine (**19**, Figure 2) to rhodomycin D (**7**). It is likely that (**19**) is made by the *S. peucetius dnm* genes as illustrated in Figure 2 by analogy to the biochemistry or deoxysugar biosynthesis in other bacteria [17], and on the basis of recent information about the formation of 2,6-dideoxyhexoses [6] and the 3-amino-3,4,6-trideoxyhexose, desosamine [38]. Rhodomycin D then is converted to 13-deoxycarminomycin (**9**) by the DnrP esterase, most likely via intermediate **8** (Figure 1), followed by *O*-methylation to produce 13-deoxydaunorubicin (**10**) [5]. The latter metabolite undergoes C-13 oxidation in two stages (**11** may be an intermediate), catalyzed by the cytochrome P450 enzyme encoded by the *doxA* gene [20,36]. The product, DNR, is further oxidized to DXR, but this step appears to be an incidental property of the DoxA protein since C-14 hydroxylation is 170-fold less efficient than C-13 oxidation *in vitro* [36].

Our investigations of the biochemistry of DNR and DXR production depended heavily on prior developments that elucidated the genetic basis of DXR production in *S. peucetius* and its close relative, *Streptomyces* sp strain C5 (reviewed in [15] and [31]). The architecture of the gene clusters in both strains is remarkably similar, even at the DNA sequence level, which implies a close evolutionary relationship. Following identification of *S. peucetius* clones bearing type II PKS and DNR/DXR self-resistance genes [26,32], specific genes were characterized by DNA sequence analysis and disruption or replacement as well as by expression, and purification and characterization of enzymes in some cases, to establish the physical and functional relationships of the DXR production genes illustrated in Figure 3. A nomenclature was chosen to differentiate four categories: the eight *dps* genes encode the PKS enzymes, the nine *dnm* genes are for biosynthesis of TDP-daunosamine and its attachment to **6**, the four *drr* genes provide self-resistance to DNR and DXR (some of these genes may facilitate export of these metabolites), and the fourteen *dnr* genes govern all other functions, with *doxA* signifying the novel C-13/C-14 hydroxylase gene. (The roles of the *dpsH* and *dnrV* genes still have not been clarified, although it now seems that *dpsH* is not a true PKS gene [20], contrary to our initial belief [11].) The homologous genes in *Streptomyces sp* strain C5 have been named in the same manner, with '*dau*' used in place of '*dnr*' for the fourth category. We believe that the genes shown in Figure 3 are all that are necessary for DXR production by *S. peucetius*, although this assumption has not been rigorously tested. Their functions were uncovered over a 10-year period, as presented in the following papers: *dpsABCDEF* and *dnrG* [13], *dpsG* [20] *dpsY* [19], and *dpsH* [20]; *dnrC*, *dnrD* and *dnrP* (Lomovaskaya *et al*, unpublished; [22]), *dnrK* [23], *dnrH* and *dnrE* [13,30], *dnrU*, *dnrV* and *doxA* [20], *dnrX* [19], *dnrF* [7,14], *dnrI* [21,33,35], and *dnrN* and *dnrO* [8,25]; *dnmL* and *dnmM* ([10]; Lomosvaskaya *et al*, unpublished), *dnmZ*, *dnmU* and *dnmV* [28], *dnmJ* [21], *dnmT* [30], and *dnmQ* and *dnmS* [27]; *drrA* and *drrB* [14], *drrC* [9,18] and *drrD* (Ali and Hutchinson, unpublished). *S. peucetius* contains at least three additional clusters with type II PKS genes [32], one of which (group I) causes the formation of an insoluble maroon pigment when introduced into *Streptomyces lividans* by transformation [32]; and another locus, *ric2*, specifying DNR/DXR resistance [4], which may be located in the group II PKS cluster identified by Stutzman-Engwall and Hutchinson (Otten and Hutchinson, unpublished; [32]).

Three of the *dnr* genes appear to govern further metabolism of DNR and DXR after their formation. Some of the resulting metabolites are acid-sensitive and can be recognized by the fact that they are isolable from the wild-type strain or ATCC 27952 mutant, but not from the *dnrH* [30] or *dnrX* [19] mutants, in the absence of acid treatment of

the culture before extraction with solvent. Although they have not been isolated and characterized, it is very likely that these compounds are a type of baumycin, acid-sensitive metabolites isolated from various DNR-producing *Streptomyces* sp (see [31]). Baumcyin A1, for example, contains a carbohydrate-derived moiety attached by a glycosidic bond to C-4′ of daunosamine in DNR [34], which is easily hydrolyzed to DNR by aqueous acid. In contrast, the fact that that *dnrU* mutants do not produce significant amounts of (13*S*)-13-dihydro-DNR suggests that DNR is reduced at C-13 after its formation [20]. (It is not known whether the 13-dihydro-DNR (**11**) believed to be an intermediate in the conversion of 13-deoxy-DNR (**10**) to DNR (Figure 1) is identical to the (13*S*)-13-dihydro-DNR that has been isolated from *S. peucetius* and its mutants [20].)

On the basis of the results of the above work, we were able to devise and test three different approaches to increasing DNR and(or) DXR production in the ATCC 29050 strain. The first method involved the regulatory genes, *dnrI* and *dnrN*. Secondary metabolism in actinomycetes is usually developmentally regulated so that antibiotics are produced late in the life cycle, most likely in response to nutrient deprivation, which is believed to trigger the expression of genes that will eventually cause the bacteria to sporulate [3]. In one scenario, antibiotics are believed to protect the bacteria from other bacterial predators while such morphological changes take place and thus their production genes are turned on by the action of transcription factors whose genes often reside in the same cluster as the structural (ie, enzymatic), self-resistance and export genes. For *S. peucetius*, *dnrI* controls the expression of most if not all of the biosynthetic and resistance genes [21,35] through binding of its product to the promoter regions [35], while *dnrN* controls expression of *dnrI* [8,25]. The *dnrO* gene may also be regulatory since *dnrO* mutants do not produce DNR or DXR (Otten and Hutchinson, unpublished), possibly because *dnrN* expression is much reduced in this background (Olano and Hutchinson, unpublished). The products of such positively-acting regulatory genes typically are produced in limiting amounts and, consequently, introduction of extra copies of the regulatory genes into the bacteria often results in noticeably enhanced antibiotic production. This was found for *dnrI* [21,33] and *dnrN* [25,33]; the highest value found for *S. peucetius* 29050 was a 2 to 2.5-fold increase in DNR titer [25,33] but the yield of ϵ-rhodomycinone could be raised nearly 10-fold by *dnrI* alone [33].

Next, during the analysis of the *dnrH* and *dnmT* genes [30], we made two observations that pointed to other ways to increase DNR production. This increased 8-fold in a *dnrH* mutant made by insertional inactivation of the gene with the apramycin resistance gene and the production of ϵ-rhodomycinone fell two-fold. Furthermore, the results of chromatographic analysis of the culture extracts indicated that the amount of acid-sensitive metabolites (see above) had fallen dramatically. Introduction of the *dnmT* mutant into the *dnrH* mutant background led to a further increase in DNR titer (up to nine-fold) and even larger decrease in ϵ-rhodomycinone production (as much as 192-fold). DXR production also was increased (approx. three-fold) in the *dnrH* mutant relative to the wild-strain but did not increase when the *dnmT* gene was introduced into the *dnrH* mutant. Similar changes in the titers of DNR (elevated) and ϵ-rhodomycinone (decreased) were observed when the *dnmT* gene was introduced into the wild-type strain in a high copy number plasmid vector. We concluded from these data: (i) that the DnmT enzyme, which catalyzes the elimination of water from TDP-4-keto-6-deoxyglucose (**15**) as part of the sequence of reactions by which **15** is converted to **16** (Figure 2), is present in limiting amounts in the ATCC 29050 strain; and (ii) that the DnrH enzyme, which resembles bacterial glycosyl transferases acting on NDP-deoxysugars (see [17]), probably catalyzes the addition of carbohydrate-like compounds to DNR or DXR, to produce acid-sensitive metabolites like baumycin A1. (Since 4′-$\alpha$-l-daunosaminyl-DNR is produced by *S. peucetius* [2], *dnrH* may in fact be involved in the formation of this disaccharide.)

Finally, during the analysis of the *dnrU*, *dnrV*, *dnrX* and *doxA* genes [19,20], we found that *dnrX* or *dnrU* mutants individually produced more DXR and less DNR and ϵ-rhodomycinone than their parental strains. We ascribe this outcome to two factors: formation of less acid-sensitive compounds in the *dnrX* background [19] and less (13*S*)-13-dihydro-DNR in the *dnrU* background [20]. This led us to construct *dnrX dnrU* double and *dnrX dnrU dnrH* triple mutants, then ascertain whether the effects of each single mutation were cumulative, with respect to ϵ-rhodomycinone, DNR and DXR production. To our delight, this turned out to be true: DXR production rose approx. two-fold in the *dnrX dnrU* mutant compared with the *dnrX* mutant, or approx. seven-fold compared with the wild-type strain. In both the double and triple mutants, the levels of ϵ-rhodomycinone and DNR were lowered considerably, suggesting that more of these metabolites was converted to DXR. These increases in yield are believed to be due to: (i) blockade of the diversion of DNR and DXR or their precursors to acid-sensitive metabolites, some of which might not be suitable substrates for DoxA [36]; as well as (ii) inhibition of the formation of 13-dihydro-DNR. Further but smaller (36–86%) increases in DXR production were found when the *dnrV* and *doxA* genes were introduced and overexpressed in the *dnrX dnrU* or *dnrX dnrU dnrH* mutants [20]. This outcome probably reflects the fact that since DNR is a poor substrate for the DoxA enzyme (as noted above, DNR is oxidized to DXR 170-fold less efficiently than 13-dihydro-DNR is converted to DNR *in vitro*), increased levels of DoxA are not likely to change the amount of DXR produced easily.

In summary, we have demonstrated that DXR production can be raised considerably in the wild-type strain of *S. peucetius* by genetic engineering, involving enhanced expression of the genes regulating DXR production and at least one of the biosynthetic genes, plus inhibition of steps that convert DNR and (or) DXR to other metabolites, especially those that are thought to be poor substrates for C-14 hydroxylation of DNR. Two of the approaches described above, involving the *dnrH* mutant containing the *dnmT* gene and the *dnrX dnrU dnrH* triple mutant (or their equivalent), were replicated in industrial strains and found to increase DXR yields considerably in such a context [20,30]. Consequently, it is very likely that a high-DXR producing strain of *S. peucetius* could be engineered for

commercial production of this valuable cancer drug by fermentation methods. In the meantime, our work had an ancillary benefit to the current method for DXR production, its semisynthesis from DNR chemically. The overexpression of the *dnrV* and *doxA* genes in a *Streptomyces lividans* strain that also contained the *drrAB* resistance genes, as described by Inventi-Solari *et al* [16], provided the means to bioconvert DNR to DXR efficiently on an industrial scale.

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