Chapter 7
Engineering *Streptomyces peucetius* for Doxorubicin and Daunorubicin Biosynthesis

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Contents

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Abstract Doxorubicin and daunorubicin are notable members of the type II polyketide synthase family and clinically important cancer chemotherapeutic agents and are produced by a mutant strain *S. peucetius* ATCC 27952. They belong to the anthracycline-type antitumor drugs. Doxorubicin remains one of the most widely used antitumor drugs for the treatment of various cancers because of its broad spectrum of activity. As a result, numerous works have been carried to unravel the biosynthetic pathway and the underlying regulatory mechanisms to gain insight into the mechanisms of the genes involved. Consenquently, there is a need to develop an overproducing strain at the industrial scale, to produce doxorubicin as an anticancer drug. Therefore a significant amount of progress has been made in unraveling the bottlenecks in the pathway, manipulating the biosynthesis, improving production, and generating novel derivatives by engineering *S. peucetius* strain.

Here we review in depth, various pathway engineering approaches and strategies that have been applied during these courses of time, since the discovery of these compounds, for the efficient production of daunorubicin and doxorubicin. The major pathway engineering approaches discussed in this chapter are divided into three parts: the first part includes the engineering of the thymidine diphosphate-L-daunosamine biosynthesis pathway genes which is important for the enhanced production of the glycone which in turn is used for the glycosylation reaction. Similarly the second part includes the engineering of the polyketide genes responsible for the production of the aglycone moiety that undergoes several modifications to generate the important compounds doxorubicin and daunorubicin. Lastly, we discuss the engineering of the several regulatory genes involved either directly or indirectly in regulation and control of the production of daunorubicin and doxorubicin.

7.1 Introduction

Streptomyces belonging to the group of actinomycetes are filamentous gram-positive bacteria that undergo morphological and physiological differences to produce a wide range of secondary natural products. The soil-dwelling *Streptomyces* are the key producers of numerous molecules including antibiotics, antivirals, anticancer, and other bioactive molecules, owing to their secondary metabolism. These products may include useful therapeutic agents, such as antibiotics, antifungals, and antitumor, and thus a better understanding of the biosynthetic pathway and the regulatory mechanism of genes at the molecular level would provide useful insights into the fundamental issue of secondary metabolism in *Streptomyces* spp., ultimately helping to engineer strains for overproduction of these useful metabolites (Hao and Hutchinson 2006). Polyketide secondary metabolites in microbes are mainly produced via various polyketide synthases (type I, type II, type III) and non-ribosomal polyketide synthases (NRPKS) enzymes. Among these, type II polyketide synthases consist of a significant and chemically diverse group of bacterial secondary metabolites, such as tetracyclines and actinorhodin produced by *S. coelicolor*; rhodomycinone, doxorubicin, and daunorubicin produced by *S. peucetius*; jadomycin A

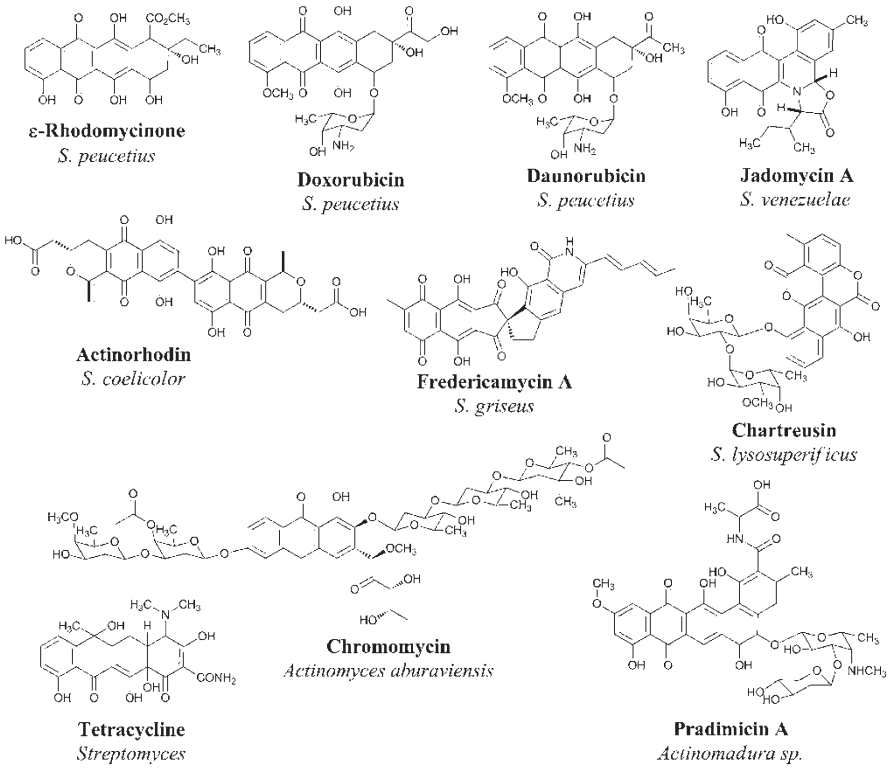

Fig. 7.1 Structures of different type II polyketides synthases (PKSs) produced by various actinomycetes including *S. peucetius* major products ε-rhodomycinone, daunorubicin, and doxorubicin

produced by *S. venezuelae*; pradimicins produced by *Actinomadura* sp.; charteusin produced by *S. lysosuperificus*; chromomycin produced by *Actinomyces aburaviensis var. verrucosus*; and many more (Fig. 7.1).

Doxorubicin and daunorubicin are notable members of the type II polyketide synthase family, and clinically important cancer chemotherapeutic agents. These molecules are produced by *Streptomyces peucetius* ATCC 27952 which is a mutant strain of *S. peucetius* 29050 (Arcamone et al. 1969). Daunorubicin was discovered in 1962, when it was first isolated from *Streptomyces caeroleorubidus* in France; however, it was also isolated from *S. peucetius* in Italy and the Soviet Union with different names (Aubel-Sadron and Londos-Gagliardi 1984), such as rubidomycin, daunomycin, and rubomycin. Daunorubicin is reported to be produced from a number of organisms; however, doxorubicin was exclusively produced by *S. peucetius subsp. caesius* (Grein 1987). Doxorubicin is a chemotherapy medication belonging to the anthracycline and antitumor drug family and is also known by its trade name Adriamycin. It is routinely used in the treatment of numerous human cancers, including breast, ovarian, liver, lung, bladder, gastric, and thyroid cancers, multiple myeloma, non-Hodgkin's and Hodgkin's lymphoma, Kaposi's sarcoma, neuroblastoma, soft tissue sarcoma, and pediatric cancers (Cortes-Funes and Coronado 2007;

Thorn et al. 2011). Because of its broad spectrum of activity, doxorubicin remains one of the most widely used antitumor drugs for the treatment of various cancers (Allwood et al. 2002).

The biosynthesis of both daunorubicin and doxorubicin is initiated by a type II polyketide synthase starting from one propionyl-CoA starter unit and extended by nine malonyl-CoA units, to produce a decaketide that is converted to aklanonic acid that leads to the formation of an aglycone ε-rhodomycinone (Hutchinson 1997). The aglycone of daunorubicin, ε-rhodomycinone, is a tetracyclic ring consisting of quinone-hydroquinone groups lying adjacent to each other, along with a methoxy group, a short carbonyl side chain. The sugar is attached to aglycone by a glycosidic bond and is known as L-daunosamine, which consists of 3-amino-2, 3, 6-trideoxy-L-fucosyl moiety, and is synthesized from D-glucose 1-phosphate using a variety of genes. Finally, series of post-modifications, like methylation, decarboxylation, and hydroxylation, leads to the formation of daunorubicin and ultimately doxorubicin. Doxorubicin is the C-14 hydroxylated form of its immediate precursor, daunorubicin. The only difference between these two molecules is the side chain of doxorubicin, which terminates with a primary alcohol, whereas daunorubicin terminates with a methyl group (Fig. 7.1) (Minotti et al. 2004).

S. peucetius has a self-resistance system that helps it to overcome the toxicity of the antibiotic daunorubicin and doxorubicin inside the cell. The four genes, namely, *drrA*, *drrB*, *drrC*, and *drrD*, present in the doxorubicin biosynthetic gene cluster of *S. peucetius* mediate the self-resistance. The first two genes *drrA* and *drrB* belong to ABC transporter type I and together form an ATP-dependent efflux pump to remove daunorubicin out of the cell (Brown et al. 2017; Guilfoile and Hutchinson 1991; Kaur and Russell 1998), whereas the third gene, *drrC*, imparts resistance through excisional repair by binding to DNA at regions intercalated by daunorubicin and then removing it (Prija and Prasad 2017). DrrD is a flavin-binding protein involved in the self-resistance mechanism, and DrrD devoid mutant exhibits partial loss of self-resistance to daunorubicin (Karuppasamy et al. 2015).

7.1.1 Objective

Doxorubicin and daunorubicin have been of interest since their discovery in 1962 and their use as a potent anticancer drug in various forms of cancer. There has been significant work involving the enhanced production of these important metabolites from *S. peucetius* using various approaches. The drive to develop a recombinant strain of industrial importance for mass production of this anticancer drug has been addressed in this chapter. Using the fermentation process combined with pathway engineering strategies and engineering the regulatory genes with modifications at the molecular level have been discussed and explained in this chapter thus providing an overview of the doxorubicin and daunorubicin biosynthesis in *S. peucetius.*

7.1.2 Mode of Action of Doxorubicin

Doxorubicin is one of the most potent US Food and Drug Administration-approved anthracycline classes of anticancer agents. It exerts its antiproliferative activity on tumor cells via three proposed mechanisms: (1) DNA binding by intercalation between DNA double helix and disrupt DNA replication and transcription process, (2) disruption of topoisomerase-II-dependent DNA repair, and (3) production of free radicals ultimately damaging cell components such as cell membranes, nucleic acids, and proteins (Fig. 7.2). Collectively, these modes of actions result in DNA disruption and loss of DNA mismatch repair function that ultimately leads to cell death (Gewirtz 1999; Thorn et al. 2011). However, cardiotoxicity is the major factor limiting its medicinal use as it alters iron and calcium regulations in mitochondria (Swain et al. 2003; Carvalho et al. 2009).

Doxorubicin and most of the anthracycline class of compounds intercalate between deoxyribonucleic acid (DNA) base pairs and bind with DNA associated

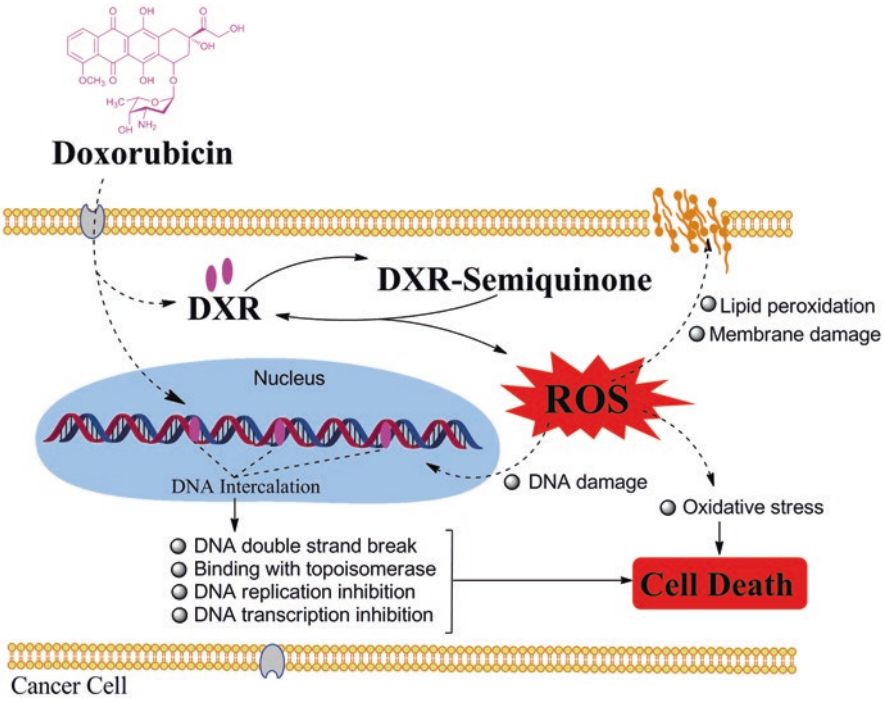

Fig. 7.2 Mode of action of doxorubicin (DXR) inside the cancer cell. Doxorubicin interacts with the DNA by intercalation, disruption of topoisomerase-II-dependent DNA repair, and inhibition of the replication and transcription process. Doxorubicin is oxidized to doxorubicin semiquinone which is unstable intermediate and converted back to DXR-releasing reactive oxygen species (ROS) that causes oxidative stress and induces damage to cell membrane, lipid peroxidation, and DNA damage leading to cell death

enzymes (topoisomerase II), inhibiting DNA replication and ribonucleic acid (RNA) transcription. The aglycone molecule intercalates in between DNA strands, whereas the sugar unit binds to the minor groove in the DNA by displacing water molecules and ions. The amino sugar (L-daunosamine) attached at the seventh hydroxyl position of the ring sits in the minor groove and intercalates the flanking base pairs immediately adjacent to the flanking site, which stabilizes the binding complex of doxorubicin and the DNA molecule. The amino group and hydroxyl group of the sugar facing outside the minor groove interact with the polymerase enzymes, thus inhibiting their function in DNA replication (Pigram et al. 1972; Frederick et al. 1990). Alternatively inside the cell, doxorubicin undergoes one electron reduction to form a DOX-semiquinone radical, an unstable metabolite. It is reoxidized back to doxorubicin and leads to the formation of reactive oxygen species and hydrogen peroxide. These reactive radicals cause oxidative stress to the cell that destructs multiple cell components such as cell membrane, nucleic acids, and ultimately trigger apoptotic pathways of cell death (Fig. 7.2) (Doroshow 1986; Thorn et al. 2011).

7.2 Biosynthesis of Daunorubicin and Doxorubicin

The gene cluster of *S. peucetius* has been studied extensively, because of its ability to produce doxorubicin and daunorubicin, which have broad clinical applications. *S. peucetius* 29050 complete genetic map was first published in 1999 by Lomovskaya et al. The *S. peucetius* ATCC 27952 strain was sequenced, and its genome was analyzed in 2004 (Parajuli et al. 2004). The 8.7-Mb genomic sequence analysis of *S. peucetius* ATCC 27952 identified a 40 kb daunorubicin and doxorubicin biosynthetic gene cluster, which is 5.3 % of the total genome consisting of 37 open reading frames (Fig. 7.3). Biosynthesis of doxorubicin is completed in three stages: (A) formation of the aglycone, ε-rhodomycinone through condensation of one propionyl-coenzyme A and nine malonyl coenzyme A precursor units; (B) formation of activated amino sugar moiety, thymidine diphosphate (TDP)-L-daunosamine from D-glucose-1-phosphate; and finally (C) glycosylation of ε-rhodomycinone by glycosyltransferase, followed by different post-modifications like decarboxylation, methylation, and hydroxylation to produce the final compound (Hutchinson and Colombo 1999). In recent years, detail study of the daunorubicin and doxorubicin biosynthetic gene cluster that comprises catalytic enzymes, transcription factors,

Fig. 7.3 Physical and functional map of the daunorubicin and doxorubicin gene cluster. The relative sizes of the open reading frames and the direction of gene transcription are designated by pointed boxes, which are colored according to the types of functions that are indicated below

and resistance gene has improved the understanding of the biosynthesis machinery and regulatory mechanisms that control the doxorubicin biosynthesis.

7.2.1 Biosynthesis of ε-Rhodomycinone

Doxorubicin biosynthesis starts with the formation of an important intermediate ε-rhodomycinone (Dickens et al. 1995), whose entire carbon backbone is synthesized by a type II polyketide synthase (PKS) enzyme that is encoded by the *dpsABCDGEFY* genes (Fig. 7.4). A 21-carbon decaketide is initially formed by serial condensation of 9 malonyl-CoA units to 1 propionyl-CoA starter unit, and this multistep reaction involves enzymes from the polyketide synthase family, like 3-oxoacyl ACP synthase (*dpsA*) (Meurer and Hutchinson 1995), ketosynthases (*dpsB* and *dpsC*) (Grimm et al. 1994; Bao et al. 1999), acyltransferase (*dpsD*), and an acyl carrier protein (*dpsG*) (Lomovskaya et al. 1999). The ketoreductase (*dpsE*) carries out reduction of the decaketide, followed by aldol condensation, and then three steps of ring cyclization catalyzed by DpsF and DpsY, to form 12-deoxyalkanoic acid (Lomovskaya et al. 1998). A keto group is introduced into this intermediate by monooxygenase (*dnrG*) to form alkalonic acid, which is subsequently converted to aklaviketone by alkalonic acid-S-adenosyl-L-methionine methyl ester transferase, encoded by a homodimeric protein *dnrC* (Madduri and Hutchinson 1995). Further, alkalonic acid methyl ester cyclase, encoded by *dnrD*, carries out the cyclation reaction. Finally, the 7-oxo moiety of aklaviketone is reduced to a hydroxyl group, to form ε-rhodomycinone in two sequential steps executed by the enzymes aklaviketone reductase, encoded by *dnrH*, and a hydroxylase, encoded by *dnrF* (Filippini et al. 1995).

7.2.2 Biosynthesis of Thymidine Diphosphate-L-Daunosamine

The biosynthesis of thymidine diphosphate-L-daunosamine involves a seven gene cluster, namely, *dnmL*, *dnmM*, *dnmU*, *dnmT*, *dnmJ*, and *dnmV*. The biosynthesis begins from D-glucose-1-phosphate. The sequential action of two enzymes glucose-1-phosphate thymidylyl transferase and thymidine diphosphate-D-glucose 4, 6-dehydratase, encoded by *dnmL* and *dnmM*, respectively, catalyzes the first two enzymatic reactions to generate the intermediate thymidine diphosphate-4-keto-6-deoxy-D-glucose (TKDG) (Gallo et al. 1996). DnmU, an epimerase, carries out the epimerization of thymidine diphosphate-4-keto-6-deoxy-D-glucose to thymidine diphosphate-4-keto-6-deoxy-L-glucose, to which a keto group and an amino group is added at the C-3 position, followed by the enzyme hydratase and aminotransferase, which are encoded by *dnmT* and *dnmJ*, respectively. Finally, *dnmV*, a ketoreductase, reduces the C-4 ketone to a hydroxyl group, to generate thymidine diphosphate-D-daunosamine (Fig. 7.4) (Otten et al. 1997).

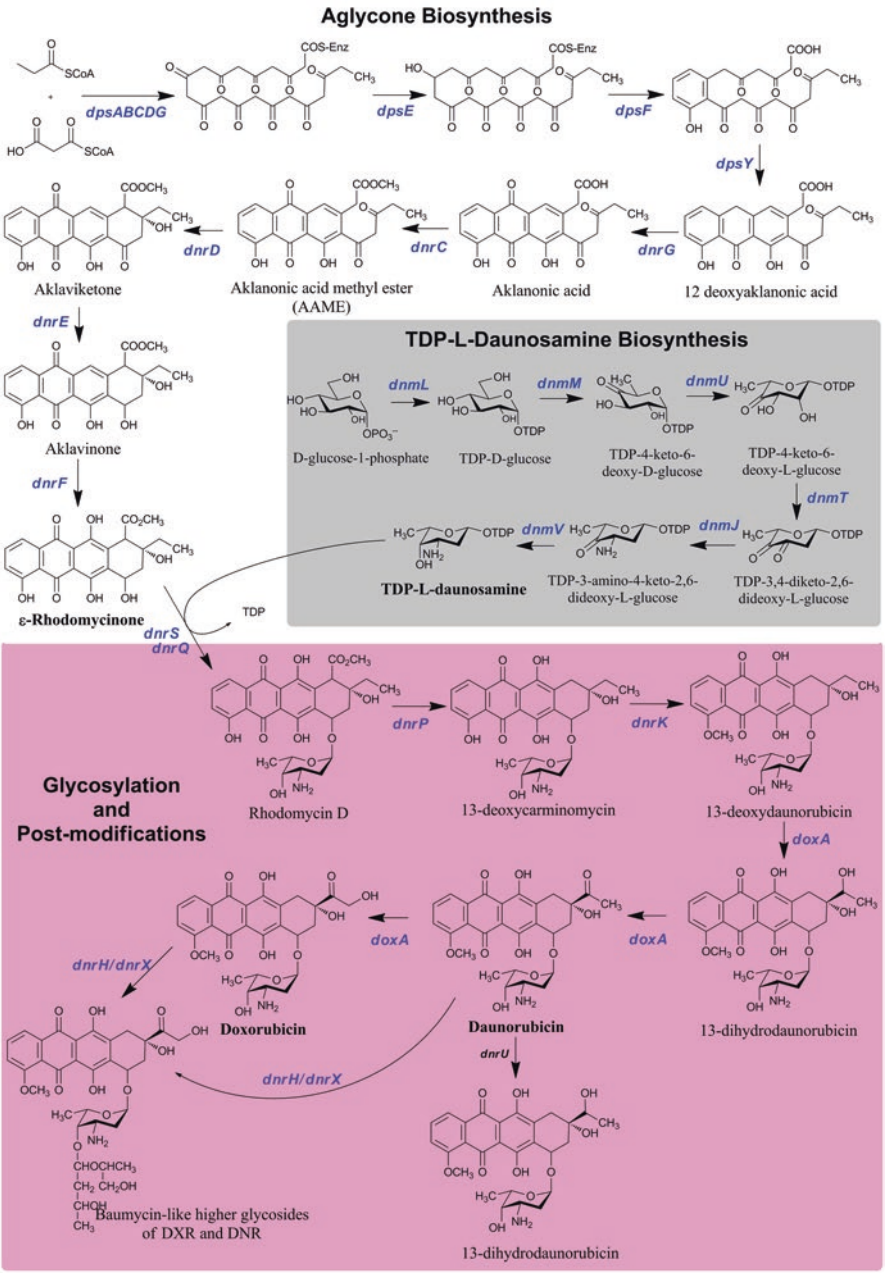

Fig. 7.4 Biosynthetic pathways of doxorubicin (DXR), daunorubicin (DNR), and ε-rhodomycinone from propionyl-CoA and malonyl-CoA along with biosynthetic pathway for thymidine diphosphate-L-daunosamine starting from D-glucose-1-phosphate. Also shown in the figure is the pathway for the glycosylation and post-modification of the final compound daunorubicin and doxorubicin into 13-dihydrodaunorubicin and baumycin-like higher glycosides

7.2.3 Glycosylation and Post-modifications

After the completion of the polyketide stage, ε-rhodomycinone is converted to rhodomycin D, a daunosamine conjugated derivative by the enzyme *DnrS*. Rhodomycin D is then converted to 13-deoxycarminomycin (Furuya and Hutchinson 1998) by the *DnrP* esterase, and this is followed by *O*-methylation by the methyltransferase encoded by *DnrK*, to produce 13-deoxydaunorubicin (Dickens et al. 1997). The latter metabolite undergoes C-13 oxidation by the cytochrome P450 enzyme in two stages, first forming an intermediate 13-dihydrodaunorubicin, and then daunorubicin. *DoxA* is responsible for both steps (Walczak et al. 1999). Daunorubicin is eventually hydroxylated by the same *DoxA* enzyme at the C-14 position, to generate doxorubicin (Fig. 7.4).

7.2.4 Regulation of Daunorubicin and Doxorubicin Biosynthesis

S. peucetius has various types of regulatory genes that control the production of daunorubicin/doxorubicin, which include transcription factors *dnrO*, *dnrN*, and *dnrI*, transcriptional repressor *drrD*/*dnrW*, transcriptional control by a coherent feed forward loop, self-resistance, and feedback regulation (Jiang and Hutchinson 2006). The *dnrO* is the major transcriptional regulator located adjacent to the *dnrN* gene. It encodes a protein that has a helix-turn-helix DNA binding domain close to its *N*-terminal region and belongs to a member of the TetR family of transcriptional regulators. The inactivation of *dnrO* leads to the complete loss of anthracycline antibiotics biosynthesis in *S. peucetius.* DnrO is essential for the expression of the pathway-specific *dnrN* transcriptional activator, and this in turn activates *dnrI* (Otten et al. 2000). DnrI, being the master regulator, binds to the several regions of polyketide synthases and activate the efflux regulatory genes (Madduri and Hutchinson 1995; Tang et al. 1996). In contrast, DnrO negatively regulates biosynthesis pathway genes due to self-repression phenomena (Lei and Parekh 2005). The self-repression of *dnrO* is an important event, as it is the key factor for the feedback regulation of daunorubicin biosynthesis, and this activates the transcription of *dnrN* and *dnrI*, which in turn leads to the activation of daunorubicin biosynthesis in a sequential manner (Ajithkumar and Prasad 2010). Thus, the existence of tightly regulated antibiotic biosynthesis machinery has been explained and extensively studied by making use of *dnrO*, *dnrN*, and *dnrI* transcription regulator mutants, leading to the better understanding of the doxorubicin biosynthesis in *S. peucetius* (Vasanthakumar et al. 2013).

7.3 Pathway Engineering and Production of Daunorubicin and Doxorubicin

The production of daunorubicin and doxorubicin from *S. peucetius* is hindered by several factors such as (1) the low availability of thymidine diphosphate-L-daunosamine sugar, (2) low efficiency of glycosylation reaction, (3) cytotoxicity, and (4) regulatory mechanisms. This could be overcome by generating a robust *S. peucetius* strain capable of producing practical amount of target molecules using recent biotechnological tools. Till date, several studies have been performed to enhance the production of daunorubicin and doxorubicin from this strain. The basic approaches used to enhance the production of daunorubicin and doxorubicin are summarized in Fig. 7.5.

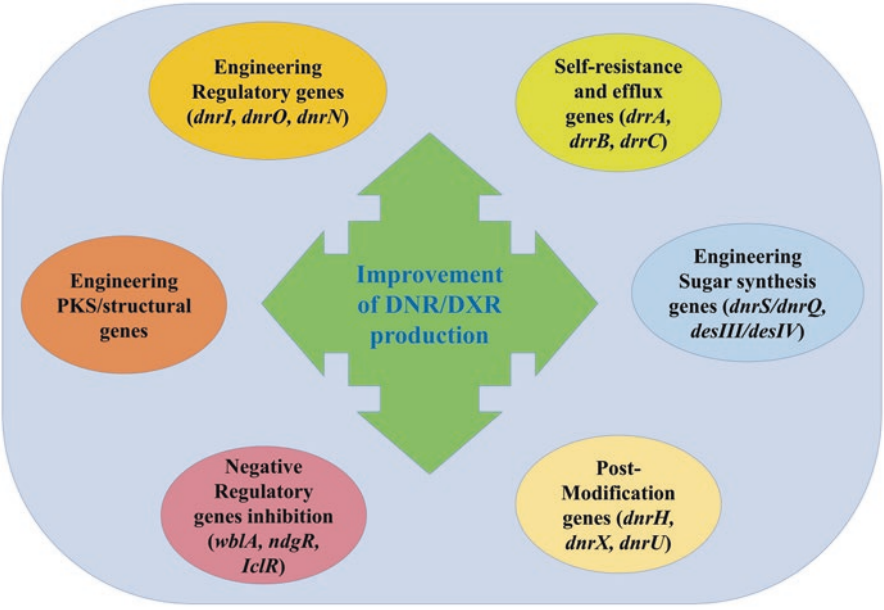

Fig. 7.5 Overall scheme used for enhanced production of daunorubicin and doxorubicin (DNR/DXR) from *S. peucetius*. The scheme shows the pathway engineering strategy and modification of regulatory genes; structural genes; sugar synthesis genes, overexpressing the positive regulators; and the resistance genes followed by inactivation of the post-modification genes thereby improving the yield of both daunorubicin and doxorubicin

7.3.1 Engineering of Thymidine Diphosphate-L-Daunosamine Biosynthesis Pathway Genes

The deoxysugar moieties constitute a very important role in the production of daunorubicin and doxorubicin. Thymidine diphosphate-L-daunosamine sugar formation and its glycosylation by the enzyme DnrS along with DnrQ are considered the rate-limiting step in the biosynthesis of doxorubicin (Dekleva et al. 1985). During daunorubicin and doxorubicin biosynthesis, there is a limited production of glycosylated intermediates because of the low glycosylation efficiency of DnrS/DnrQ glycosyltransferases. Therefore, Malla et al. (2009) explored the overall effects of glycosyltransferase expression for the efficient glycosylation of ε-rhodomycinone and expression of sugar genes to increase the thymidine diphosphate-L-daunosamine pool. Homologous TDP-sugar biosynthesis genes from *S. venezuelae* ATCC 15439, *desIII* (glucose-1-phosphate thymidylyltransferase), and *desIV* (thymidine diphosphate-D-glucose 4, 6-dehydratase) were cloned and overexpressed. Additionally, *dnrS*, along with *dnrQ*, which codes for the activator protein DnrQ, were also overexpressed, and their effects were analyzed in *S. peucetius* ATCC 27952. Introduction of multicopies of *dnrS*/*dnrQ* produced noticeable 2.8-fold enhancement over the parental strain. Furthermore, co-overexpression of *dnrS*/*dnrQ* along with *desIII*/*desIV* increased the doxorubicin production by a 5.6-fold more than the *S. peucetius* parental strain.

DnrH encodes a glycosyl transferase involved in the post-modification stages of the daunorubicin and doxorubicin biosynthesis. Studies involving the *dnrH* mutant produced by inactivation of this gene led to an eightfold increase in daunorubicin production and twofold decrease in ε-rhodomycinone accumulation. Introduction of *dnmT* mutant into the *dnrH* mutant, daunorubicin production increased ninefold compared to the wild-type *S. peucetius*. Doxorubicin production was also improved approximately threefold in the *dnrH* mutant in comparison to the wild-type strain (Scotti and Hutchinson 1996). This is due to the fact that daunorubicin and doxorubicin are further modified into baumycin, like higher glycoside, by these post-modification enzymes, and thus their deletions lead to high production titers of both daunorubicin and doxorubicin. It has also been reported that the DnmT enzyme is present in limiting amounts in the *S. peucetius* (Dickens et al. 1996).

7.3.2 Engineering of the Polyketide Synthase Genes

Earlier studies carried out by Ye et al. (1994), Gerlitz et al. (1997), Bao et al. (1999), Lomovskaya et al. (1999), and Strohl et al. (1998) have shown the importance of the early polyketide synthase genes, like dpsABCDFGY, to the doxorubicin pathway, as their inactivation leads to complete or partial loss in daunorubicin and doxorubicin production. Thus there is a strong possibility that the overexpression of these genes may lead to a significant increase in the production of these anthracyclines in

S. peucetius. Studies carried out by the disruption of the late modifying genes of the doxorubicin biosynthetic pathway, such as *dnrU*, *dnrV*, and *dnrX*, found that individual *dnrX* or *dnrU* mutants produced more doxorubicin than their parental strains, whereas the production of daunorubicin and ε-rhodomycinone decreased (Lomovskaya et al. 1998; Lomovskaya et al. 1999). Doxorubicin production increased approximately twofold in the double *dnrX* and *dnrU* mutant when compared with only *dnrX* mutant, which was an approximate sevenfold increase, when compared with the wild-type strain.

This increase in production is accredited to daunorubicin not being able to be converted to 13-dihydrodaunorubicin, and neither daunorubicin and doxorubicin being able to be further modified to acid-sensitive metabolites, due to the deletion of these modifying enzymes (Walczak et al. 1999). Additionally, when the *dnrV* and *doxA* genes were introduced and overexpressed in the above *dnrX*, *dnrU*, and *dnrH* mutants, a smaller increase in doxorubicin production was observed (Lomovskaya et al. 1999), possibly due to the fact that the oxidation rate of daunorubicin to doxorubicin is 170-fold less efficient than the conversion rate of 13-dihydrodaunorubicin to daunorubicin. Hence, the increased levels of DoxA are less likely to change the extent of doxorubicin production in *S. peucetius*.

As mentioned earlier, *S. peucetius* ATCC 27952 self-resistance system imparts resistance against the toxicity of the antibiotic daunorubicin and doxorubicin inside the cell, and helping in this endeavor are the four resistance genes *drrA*, *drrB*, *drrC*, and *drrD*. Owing to this fact, when three of these resistance genes *dnrABC* were cloned under strong *ermE** promoter into the pIBR25 expression vector, the recombinant expression strains, pDrrAB25, pDrrC25, and pDrrABC25, produced more doxorubicin than the parental strain, with a 2.2-fold increase in pDrrAB25, a 5.1-fold increase in pDrrC25, and a 2.4-fold increase in pDrrABC25. Thus, doxorubicin production is positively affected when the resistance genes are introduced in multiple copies (Malla et al. 2010a).

7.3.3 Engineering of the Regulatory Genes

Secondary metabolite production in *Streptomyces* spp. is regulated by two different classes of regulatory genes: cluster-situated regulators and global regulators or pleiotropic regulatory genes. Most of these cluster-situated regulators control the biosynthesis of a particular antibiotic and are also known as pathway-specific regulators. On the other hand, the global regulatory genes may not always be present in biosynthetic gene cluster but regulate morphological and physiological differentiation and secondary metabolite biosynthesis in *Streptomyces* (Umeyama et al. 2002). The study of these regulatory genes provides a theoretical basis for antibiotic biosynthesis in *Streptomyces* and also helps to increase the yield of antibiotics by the use of pathway engineering and manipulation of these regulatory genes at molecular level.

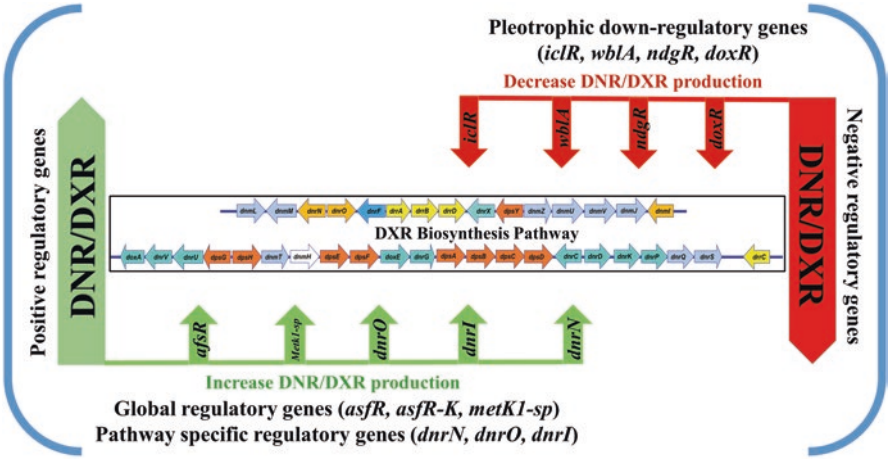

Fig. 7.6 Schematic representation of genes involved in regulation of daunorubicin and doxorubicin (DNR/DXR) production in *S. peucetius*. Global regulatory genes like *asfR* and *Metk1*-sp and pathway-specific regulatory genes like *dnrO*, *dnrI*, and *dnrN* act as positive regulatory genes and thus have positive effect in daunorubicin and doxorubicin production, whereas the pleiotropic downregulatory genes such as *iclR*, *wblA*, *ndgR*, and *doxR* decrease the daunorubicin and doxorubicin production and hence the negative regulatory genes. Overexpression or inhibition of the regulatory genes has a profound effect in the production of the final compound doxorubicin

Similarly in *S. peucetius*, as discussed earlier, DnrI, DnrN, and DnrO act as transcriptional regulator and control production of daunorubicin and doxorubicin (Fig. 7.6). The DnrO is the major transcription regulator, and its inactivation leads to complete loss of antibiotic production. In *S. peucetius*, DnrI is required for the transcription of biosynthetic and resistance genes of the daunorubicin and doxorubicin gene cluster and thus controls the expression of almost all of the biosynthetic and resistance genes (Madduri and Hutchinson 1995), while DnrN controls the expression of DnrI (Otten et al. 1995). Consequently, the introduction of positively acting regulatory genes like DnrI and DnrN has profound effects on the production of antibiotics like daunorubicin. In *S. peucetius*, the production of daunorubicin was increased 2.5-fold, whereas the ε-rhodomycinone yield was raised to nearly 10-fold (Otten et al. 1995; Stutzman-Engwall et al. 1992).

Introduction of regulatory genes such as *dnrN*, *dnrI*, *afsR*, and *metK1*-sp under strong *ermE** promoter increased doxorubicin production by 1.2-fold in recombinant strains NI (with dnrN-dnrI), 1.4-fold in NIS (with *dnrN-dnrI-metK1*-sp), and 4.3-fold in NIR (with *dnrN-dnrI-afsR*) (Malla et al. 2010b). AfsR is a global regulator which constitutes the AfsK-AfsR system. The expression of *afsR* from both *S. peucetius* ATCC 27952 and *S. venezuelae* in *S. peucetius* enhanced production of doxorubicin by fourfold and eightfold, respectively (Parajuli et al. 2005). Furthermore the overexpression of this pleiotropic activator *afsR* enhanced other antibiotics such as actinorhodin in *S. lividans*, clavulanic acid in *S. clavuligerus*, and streptomycin in *S. griseus* (Maharjan et al. 2009).

S. peucetius does not contain a functional copy of bldA-tRNA. Although *bldA* is non-essential for the survival of the *Streptomyces* species, it plays an important role in secondary metabolism. When the regulatory gene *dnrO* codon was thoroughly examined, a TTA codon was found which is hardly encoded by bldA-tRNA. Multiple engineered strains of *S. peucetius* were generated by heterologously expressing *bldA* and *dnrO* individually and a combination of both *bldA* and *dnrO*. Overexpression of these pathway-specific negative regulators enhanced the production of daunorubicin 1.25-fold, as compared to the parental strain (Pokhrel et al. 2016). Likewise these genes, engineering of genes, like dephosphocoenzyme A (*coaE*), which catalyzes the last step in the biosynthesis of the cofactor coenzyme A, has been shown to have positive increase in doxorubicin production. When these two genes *coaA* and *coaE* were overexpressed independently in the doxorubicin-producing wild-type strain, there was 1.4- and 1.5-fold increase in doxorubicin production, respectively. Both genes in combination exhibited 2.1-fold enhancement in doxorubicin production (Lee et al. 2014).

Besides overexpressing positive regulators to enhance production titer, as mentioned above, there are a few negative regulators present in *S. peucetius*, whose overexpression or inactivation may have a negative or positive effect on daunorubicin and doxorubicin production (Fig. 7.6). One such negative regulator is *wblA*, which controls antibiotic production and morphological differentiation in actinomyces, and when this *wblA* regulator from *S. coelicolor* was introduced into the doxorubicin-overproducing strain, it led to significant decrease in the production of doxorubicin (Kang et al. 2007).

The *dox R* regulator belonging to the *IclR* type family of transcription regulator was found in the genome of *S. peucetius*, and when overexpressed in *S. peucetius* strain, it strongly repressed the production of antibiotics. Furthermore, it exerted an adverse consequence on the regulatory system of doxorubicin, wherein the binding of DoxR inhibited the *dnrI* expression, leading to the blockade of doxorubicin production (Chaudhary et al. 2014). Another regulatory gene *ndgR* which is a regulator for nitrogen source-dependent growth and antibiotic production, similar to an *IclR*-like regulator from *S. coelicolor*, can bind to the promoters in the doxorubicin biosynthetic gene cluster in *S. peucetius* (Yang et al. 2009), and its inactivation in *S. coelicolor* leads to increased actinorhodin production. Thus, the deletion of the *doxR* and the *ndgR* regulatory genes may also have a positive effect on daunorubicin and doxorubicin production.

7.4 Conclusion

Because of low production yield of doxorubicin and high market demand, engineering of *S. peucetius* strain is a beneficial goal. Until the late 1990s, the annual production of doxorubicin was over 225 kg, and it was the most widely used anticancer drug. Moreover, doxorubicin is also considered as lead molecule to generate other value-added derivatives by enzymatic and chemical modifications with improved

pharmacological properties for clinical cancer treatment (Arcamone et al. 1997; Allwood et al. 2002). Although doxorubicin can be produced semi-synthetically from its precursor daunorubicin, the process is tiresome, and the yield is quite low. Thus, sustainable fermentation technology combined with pathway engineering approaches is currently needed to enhance the production of these drugs (Hutchinson and Colombo 1999; Malla et al. 2010c).

In summary, we conclude that by identification of the key steps in *S. peucetius* that hinder daunorubicin and doxorubicin production, like the low availability of thymidine diphosphate-L-daunosamine sugar and the low efficiency of glycosylation, cytotoxicity, and the regulatory mechanisms, daunorubicin and doxorubicin production can be raised significantly in the wild-type strain of *S. peucetius* by genetic engineering. This would involve overexpression of the genes regulating doxorubicin production and also the genes in the biosynthetic pathway, along with the deletion of negative regulators and inhibiting the post-modification steps of daunorubicin and doxorubicin into other metabolites (Table 7.1). Additionally, high-daunorubicin-and doxorubicin-producing strains can be generated by overexpression of the genes in the sugar pathway of thymidine diphosphate-L-daunosamine, a very

Table 7.1 Increase in daunorubicin and doxorubicin production by the engineering of the PKS pathway, sugar pathway, and regulatory pathway genes in *S. peucetius*

Gene overexpression/ inactivated	Effect in production	References
dnrI + dnrN overexpression	2.5-fold increase in daunorubicin	Otten et al. (1995) and Stutzman-Engwall et al. (1992)
dnrH inactivation	Eightfold increase in daunorubicin and threefold increase in doxorubicin	Scotti and Hutchinson (1996)
dnmT + *dnrH* inactivation	Ninefold increase in daunorubicin	Scotti and Hutchinson (1996)
dnrS/*dnrQ* overexpression	2.8-fold increase in doxorubicin	Malla et al. (2009)
dnrS/*dnrQ* + *desIII*/*desIV* overexpression	5.6-fold increase in doxorubicin	Malla et al. (2009)
dnrX + *dnrU* inactivation	Sevenfold increase in doxorubicin	Lomovskaya et al. (1998) and Lomovskaya et al. (1999)
drab overexpression	2.2-fold increase in doxorubicin	Malla et al. (2010a)
drrC overexpression	5.1-fold increase in doxorubicin	Malla et al. (2010a)
*drab C*overexpression	2.4-fold increase in doxorubicin	Malla et al. (2010a)
dnrN-dnrI overexpression	1.2-fold increase in doxorubicin	Malla et al. (2010b)
dnrN-dnrI-metK1-sp overexpression	1.4-fold increase in doxorubicin	Malla et al. (2010b)
dnrN-dnrI-afsR overexpression	4.3-fold increase in doxorubicin	Malla et al. (2010b)
afsR overexpression	Fourfold increase in doxorubicin	Parajuli et al. (2005)
bldA-dnrO overexpression	1.25-fold increase in doxorubicin	Pokhrel et al. (2016)
coaA overexpression	1.4-fold increase in doxorubicin	Lee et al. (2014)
coaE overexpression	1.5-fold increase in doxorubicin	Lee et al. (2014)

important step in the production of daunorubicin and doxorubicin, along with the engineering of the polyketide synthase genes. Engineering of wild-type strain using combined effect of regulatory genes and other biosynthesis genes along with self-resistance and cofactors limiting genes using state-of-the-art systems/synthetic biology and metabolic engineering tools could certainly generate a high-doxorubicin-producing strain for commercial production of these valuable anticancer drugs.

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