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MINI-REVIEW

Genetic engineering of macrolide biosynthesis: past advances, current state, and future prospects

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Abstract Polyketides comprise one of the major families of natural products. They are found in a wide variety of bacteria, fungi, and plants and include a large number of medically important compounds. Polyketides are biosynthesized by polyketide synthases (PKSs). One of the major groups of polyketides are the macrolides, the activities of which are derived from the presence of a macrolactone ring to which one or more 6-deoxysugars are attached. The core macrocyclic ring is biosynthesized from acyl-CoA precursors by PKS. Genetic manipulation of PKS-encoding genes can result in predictable changes in the structure of the macrolactone component, many of which are not easily achieved through standard chemical derivatization or total synthesis. Furthermore, many of the changes, including post-PKS modifications such as glycosylation and oxidation, can be combined for further structural diversification. This review highlights the current state of novel macrolide production with a focus on the genetic engineering of PKS and post-PKS tailoring genes. Such engineering of the metabolic pathways for macrolide biosynthesis provides attractive alternatives for the production of diverse non-natural compounds. Other issues of importance, including the engineering of precursor pathways and heterologous expression of macrolide biosynthetic genes, are also considered.

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Introduction

Macrolides (Fig. 1) are a highly diverse group of polyketides that are used in therapeutics, mainly as antibiotics (erythromycin, oleandomycin, pikromycin, tylosin), immunosuppressants (FK506, rapamycin), antiparasitics (avermectin), antifungal agents (amphotericin B), and antitumor agents (epothilone) (O'Hagan 1991; Fischbach and Walsh 2006). Macrolide activities stem from the presence of a macrolactone ring (aglycone) with one or more deoxysugars and aminosugars attached (Weymouth-Wilson 1997). Macrolides are classified into different groups according to the number of atoms in the lactone ring, e.g., 12-, 14-, 15-, or 16-membered ring, etc., and are synthesized by polyketide synthases (PKSs). Three classes of bacterial PKSs have been identified so far (Shen 2003). First, type I PKSs are multifunctional enzymes whose domains are organized into modules, each of which controls the incorporation of a precursor into a polyketide backbone during chain elongation, generating complex polyketides such as macrolides. Since the discovery of erythromycin PKS (6-deoxyerythronolide B synthase [DEBS]) (Cortes et al. 1990; Donadio et al. 1991) and rapamycin (Rap) PKS (Schwecke et al. 1995), many type I modular PKSs were identified (Fischbach and Walsh 2006). In contrast, type II PKSs are multienzyme complexes carrying a single set of enzymatic activities for the iterative biosynthesis of multiaromatic products (Hertweck et al. 2007). Lastly, type III PKSs, also known as chalcone synthase-like PKSs, are homodimeric enzymes that catalyze iterative condensation reactions (Austin and Noel 2003).

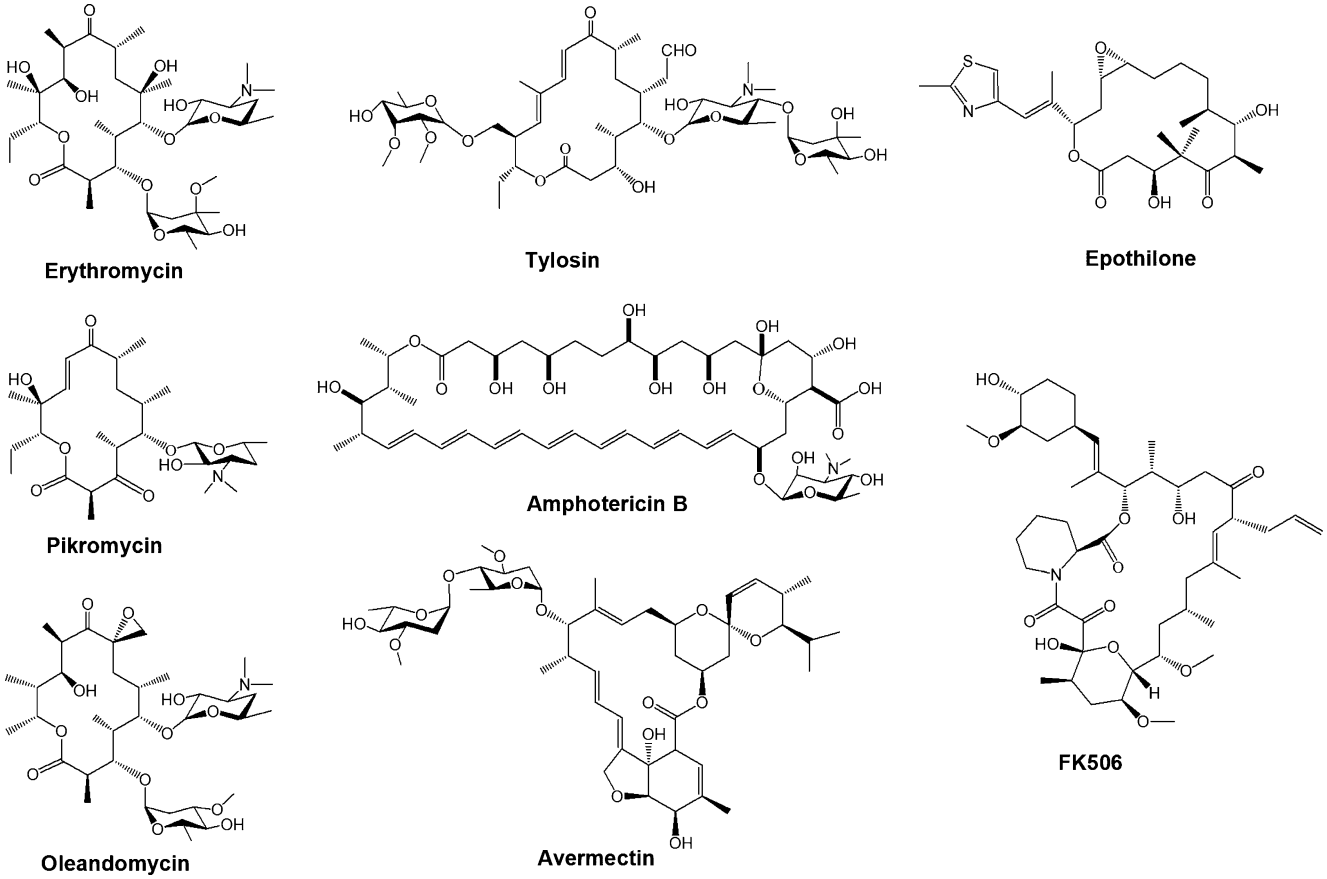

Fig. 1 Examples of macrolide compounds. Macrolides have macrocyclic lactone chemical structures to which one or more deoxysugars can be attached. They are classified into different groups according to the number of atoms on the macrolactone ring: 14-membered (e.g., erythromycin, pikromycin, oleandomycin), 16-membered (tylosin, epothilone, avermectin), 23-membered (FK506), and 36-membered (amphotericin B)

Type I modular PKS assemblies are formed from giant multifunctional enzymes consisting of one to many modules that catalyze successive condensations of activated coenzyme A (CoA) thioesters (usually acetyl-, propionyl-, malonyl-, or methylmalonyl-CoA). Each module contains a set of domains for β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP), which is required for polyketide elongation, as well as optional β-keto processing domains, ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). Figure 2 shows the schematic organization of a modular PKS, as exemplified by the pikromycin (Pik) PKS of *Streptomyces venezuelae*. The Pik PKS locus encodes a system of six modules comprised of four proteins, designated PikAI, PikAII, PikAIII, and PikAIV. PikAI is fronted by a loading domain (KS^Q) that catalyzes the decarboxylation of the starter unit methylmalonyl-CoA to generate propionate, while it has been proposed that PikAIII and PikAIV terminate the acyl chain elongation with a thioesterase (TE) domain contained in PikAIV. Once the products of the Pik PKS are released from the multienzyme, they are further elaborated by post-PKS "tailoring" enzymes (PikC monooxygenase and DesVII glycosyltransferase), which introduce hydroxyl functional groups and decorate the carbon skeleton with a D-desosamine sugar moiety (Xue and Sherman 2001).

The recent widespread dissemination of antibiotic-resistant pathogenic bacteria as well as the need for bioactive molecules with new or improved pharmacological properties has fostered increased interest in the discovery of novel macrolide analogs (Demain and Sanchez 2009). There are several strategies for the development of novel antimicrobial agents or other biologically active molecules. First, clinically useful macrolides have been traditionally discovered through the screening of natural sources, such as soil microorganisms, for the presence of the desired activities. Erythromycin and other early antibiotics discovered by this approach are still widely used. In addition to classical screening, chemical derivatization of natural products by semi-synthesis is also able to provide novel macrolides. Large numbers of natural macrolide compounds have been chemically modified to yield semi-synthetic antibiotics such as clarithromycin and ketolides (Zhanel et al. 2001). A third strategy for the generation of novel bioactive macrolides is

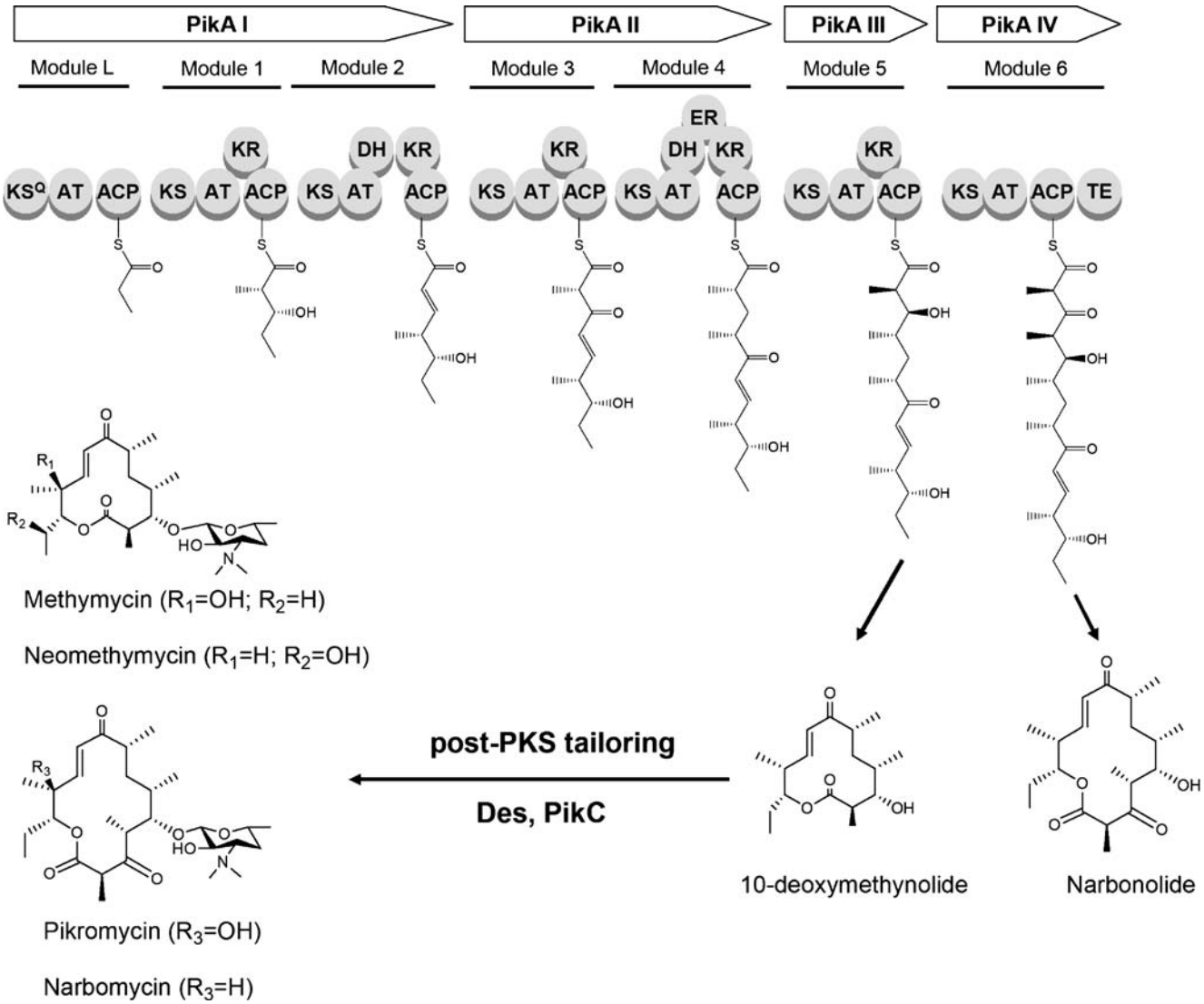

Fig. 2 Schematic representation of type I pikromycin polyketide synthase. The pikromycin polyketide synthase (Pik PKS) consists of a loading module, six elongation modules, and a thioesterase. The domain organization of PikAI–PikAIV and PikAI–PikAIII is consistent with the PKS for the 14-membered ring, narbonolide, and the 12-membered ring, 10-deoxymethynolide, respectively. In the process of post-PKS modification, both products are converted to methymycin and pikromycin. The Des cluster consists of eight enzymes (DesI-VIII) in desosamine biosynthesis and transfer and PikC (P450 hydroxylase) accept two positions in the macrolactone system for hydroxylation steps

total organic synthesis of an intact molecule. Although chemical derivatization and total synthesis have been and will continue to be important tools for the development of new molecules with enhanced pharmacological activity, many sites on the macrolactone rings and/or the sugar moiety are not susceptible to chemical modification. In contrast to the aforementioned approaches, genetic engineering or combinatorial biosynthesis can be used to generate new macrolide analogs through manipulation of the genes involved in their biosynthesis (McDaniel et al. 2005; Oh et al. 2007).

In this review, we describe past and current attempts in the genetic engineering of macrolide biosynthesis, including engineering of macrocyclic aglycone biosynthesis and post-PKS modification steps such as glycosylation and oxidation (Fig. 3). We also present approaches for the engineering of precursors to improve production of useful macrolides as well as recent developments in the heterologous expression of macrolide biosynthetic pathways. Finally, we discuss the future prospects of generating novel macrolides and further improving the efficiency of their production.

Engineering of macrocyclic aglycone biosynthesis

The colinearity between the catalytic domains present and the structure of their products makes modular PKSs

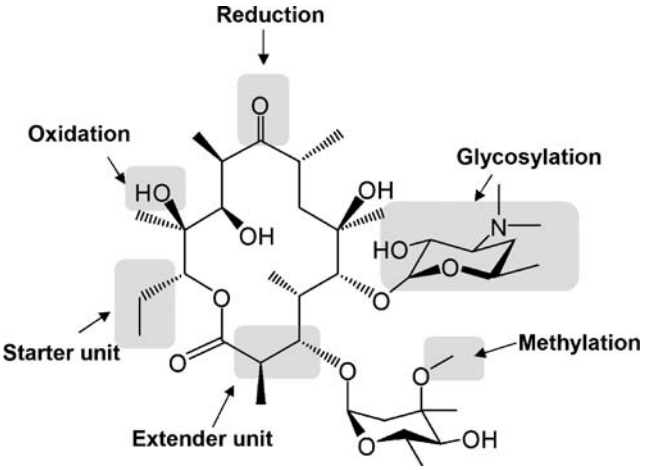

Fig. 3 Several biosynthetic targets for genetic engineering to generate novel macrolides. A wide variety of structural changes can be introduced into macrolactone ring biosynthesis by genetic engineering as follows: alteration of starter and extender units required for PKS and alteration of the extent of β-carbon processing and chain length. Modification of glycosylation and oxidation patterns by flexible GTs and monooxygenases, respectively, can lead to the generation of structurally-altered non-natural macrolides

attractive systems for the genetic engineering of novel polyketides (Khosla 1997). As the number and type of modules control macrolide chain length, unnatural chain lengths can be generated through insertion and deletion of PKS modules, thereby contributing to macrolide structural diversity. It has been possible to change the chain length by module deletion and addition at the genetic level. In a previous report, unnatural triketide and hexaketide lactones were biosynthesized by a deletion mutant comprising DEBS modules fused to the TE that can release chains of diverse lengths (C6–C15; Kao et al. 1996). Also, the ability to extend PKS assembly lines by the insertion of modules demonstrated that the individual modules of natural PKSs can be combined combinatorially to generate novel macrolide libraries. For example, unnatural extended PKSs were created by the engineered insertion of the Rap PKS module 2 or 5 into alternative sites within DEBS1-TE, generating two novel tetraketides. Moreover, four different novel octaketides containing either an acetate or propionate starter unit were biosynthesized by the addition of the Rap PKS module into the DEBS modules (Rowe et al. 2001).

The incorporation of unnatural starter units provides another way of modifying the structure of a macrolide chain (Fig. 3). The widely used starter units such as propionyl-CoA and acetyl-CoA are determined by the specificity of the AT domain of the loading module (Moore and Hertweck 2002). Thus, swapping of the loading module can lead to changes in the side chain originating from the starter unit. When the DEBS loading module was replaced with the loading module from the avermectin PKS of *Streptomyces avermitilis*, several novel analogs of erythromycin containing branched starter units were obtained (Marsden et al. 1998). In addition, when the oleandomycin or tylosin (Tyl) PKS loading module was used to replace the DEBS loading module, each hybrid produced a triketide lactone exclusively derived from an acetate or propionate starter unit, respectively (Long et al. 2002).

Since the structure of the side chain of a macrolide is determined by the extender unit incorporated into the macrolide backbone by the AT domain, exchange of one extender unit with another will result in alteration of the side chain of the final polyketide (Fig. 3). Strategies for altering extender unit specificity though replacement with other AT domains or site-specific mutagenesis of the active site of the AT domain have led to the production of novel macrolides. For example, by replacing methylmalonyl-specific DEBS AT domains with heterologous AT domains that have specificity for ethylmalonyl, methoxylmalonyl, or malonyl units, the corresponding extender units have been incorporated in the chain elongation step to generate numerous erythromycin analogs (Stassi et al. 1998; Kato et al. 2002; Hans et al. 2003). Similarly, when the methoxymalonyl-ACP-specific AT domain of the FK520 PKS of *Streptomyces hygroscopicus* was exchanged with malonyl-, methylmalonyl-, or ethylmalonyl-specific AT domains, the strains produced the expected FK520 analogs. The engineered 13- and 15-desmethoxy analogs of FK520 showed decreased immunosuppressive properties but enhanced neurite outgrowth in the cell cultures and accelerated nerve regeneration in the rat model (Revill et al. 2002). Moreover, when the active site of the AT domain was mutated from the canonical methylmalonyl-specific motif to the canonical malonyl-specific motif by site-specific mutagenesis, the desired novel analog of 6-deoxyerythronolide B, 6-desmethyl-6-deoxyerythronolide B, was obtained (Reeves et al. 2001).

Manipulation of the extent of β-carbon processing (ketoreduction, dehydration, and enoyl reduction) leads to structural diversity (Fig. 3). When DEBSs were engineered through replacement of sets of β-carbon processing domains with other domains from Rap PKS modules, a library of more than fifty macrolides was generated that would have been difficult to obtain using chemical synthesis (McDaniel et al. 1999). In addition, inactivation of the KR domain in DEBS module 6 through site-specific mutagenesis not only achieved production of a novel keto derivative of 6-deoxyerythronolide but also identified key residues in the active site of KR domains (Reid et al. 2003). Power et al. (2008) have demonstrated that replacement of the C-7 hydroxyl group on the amphotericin B with a keto group by inactivation of the KR domains of amphotericin PKS leads to generation of 7-oxo- and 15-deoxy-15-oxo-amphotericin analogs. Among these analogs, 7-oxo-amphotericin B showed decreased hemolytic activity but

retained antifungal activity (Power et al. 2008). Likely, Brautaset et al. (2008) have recently obtained several nystatin analogs with reduced hemolytic activity but high antifungal activity by inactivation of DH or KR domain of the nystatin PKS (Brautaset et al. 2008).

Furthermore, combining complete subunits from heterologous PKS clusters can create functional hybrid PKSs. It has been shown that replacement of the PKS subunit of Pik PKS in *S. venezuelae* by the corresponding subunits from heterologous modular PKSs (Tyl PKS and DEBS) resulted in recombinant strains producing both 12- and 14-membered ring macrolactones (Yoon et al. 2002). Recently, a genetic approach to the design of synthetic PKS has been developed to facilitate the generation of "unnatural" natural products by the interchange of modules and domains in PKS. The 14 modules from eight PKS (erythromycin, soraphen, epothilone, geldanamycin, leptomycin, rifamycin, rapamycin, and pikromycin PKS) were synthesized and then associated in 154 bimodular combinations, leading to the production of 72 triketides (Kodumal et al. 2004; Menzella et al. 2005). A major advantage of this strategy is that a large number of PKS genes can be rapidly examined for the production of novel macrolactones.

Engineering of post-PKS modification steps

A common structural feature of a large portion of bioactive macrolides is the glycosylation of core macrocyclic ring molecules (Fig. 3). The sugar moieties (Fig. 4) attached to such macrolides by glycosyltransferases (GTs) are usually essential for biological activity (Weymouth-Wilson 1997). In recent years, it has been shown that many GTs display subtrate flexibility toward their acceptor or sugar donor and sometimes, to both. Thus, these enzymes have been and will become more and more important tools to generate novel glycosylated macrolides (Rix et al. 2002).

DesVII from *S. venenzuelae* attaches a D-desosamine moiety to 10-deoxymethynolide or narbonolide during the biosynthesis of methymycin and pikromycin (Fig. 2). This GT displays flexibility regarding the sugar donor, and it has been shown to transfer various sugars including the intermediate deoxysugars of D-desosamine that are produced after inactivation or replacement of genes involved in D-desosamine biosynthesis (Rix et al. 2002; Hong et al. 2004; Borisova et al. 2006; Melançon and Liu 2007). Besides its natural acceptor substrates 10-deoxymethynolide (12-membered ring macrolactone) and narbonolide (14-membered ring), DesVII is able to attach natural or unnatural sugars to the linear precursor of 10-deoxymethynolide as well as various sizes of macrolactone rings such as the 16-membered tylactone (Tang and McDaniel 2001; Yoon et al. 2002; Kao et al. 2005; Jung et al. 2007; Borisova et al. 2008).

EryCIII from the erythromycin producer *Saccharopolyspora erythraea* transfers a D-desosamine moiety to 3-α-L-mycarosyl erythronolide B and is capable of transferring an alternative sugar-donor substrate such as D-mycaminose and D-angolosamine (Yuan et al. 2005; Schell et al. 2008). Gaisser and coworkers demonstrated that EryCIII could accept the altered macrolide substrates 3-*O*-(2'-*O*-methyl-L-rhamnosyl)-erythronolide B and 3-*O*-(2', 3'-bis-*O*-methylrhamnosyl) erythronolide B (Gaisser et al. 2001). EryBV, which catalyzes the transfer of L-mycarose to erythronolide B in *S. erythraea*, is also a flexible GT that can glycosylate erythronolide B with various sugar donors including D-glucose and L-rhamnose (Zhang et al. 2007).

OleG1 is the D-desosaminyl transferase involved in oleandomycin biosynthesis in *Streptomyces antibioticus*. The natural sugar substrate (D-desosamine) can be linked to the unnatural substrate 3-*O*-L-mycarosyl erythronolide B by OleG1 (Doumith et al. 1999). Another GT from *S. antibioticus*, OleG2, is responsible for transfer of L-olivosyl to oleandolide. This GT can attach the non-cognate deoxysugars L-mycarose and L-rhamnose to the alternative aglycones erythronolide B and 6-deoxyerythnolide B (Doumith et al. 1999).

TylMII from *Streptomyces fradiae* transfers a D-mycaminose moiety to the 16-membered tylactone (Fish and Cundliffe 1997). This sugar transferase is able to attach various sugar donors to its native aglycone (tylactone) or non-native aglycone (narbonolide; Gaisser et al. 2000; Melançon et al. 2005; Jung et al. 2007). Furthermore, the novel tylosin derivatives 4'-deoxy-5-*O*-D-mycarminosyl-tylonolide and 4'-deoxy-20-dihydro-demycarosyltylosin were produced in *S. fradiae* through the heterologous expression of *nbmK* and *nbmJ*, which are involved in C4-deoxygenation during D-desosamine biosynthesis in *Streptomyces narbonesis* and transfer of the corresponding sugars by TylMII (Butler et al. 2002).

AveBI, from the avermectin biosynthetic gene cluster in *S. avermitilis*, is the GT responsible for the transfer of L-oleandrosyl to avermectin aglycones. AveBI can also catalyze the glycosylation of several aglycones related to avermectin with various sugar donors, such as 6-deoxy-D-glucose, D-olivose, L-olivose, and L-digitoxose (Wohlert et al. 2001; Zhang et al. 2006).

Although the flexibility of several wild-type GTs described here has provided new opportunities for efficient glycorandomization, the stringency of other GTs limits enzymatic glycodiversification and demonstrates the need for the development of platform technologies for GT engineering or evolution. Recently, an engineered oleandomycin GT (OleD) library was constructed by error-prone PCR using the wild-type OleD gene as a template and was screened through a fluorescence-based assay. Several engineered OleDs selected during screening showed altered catalytic

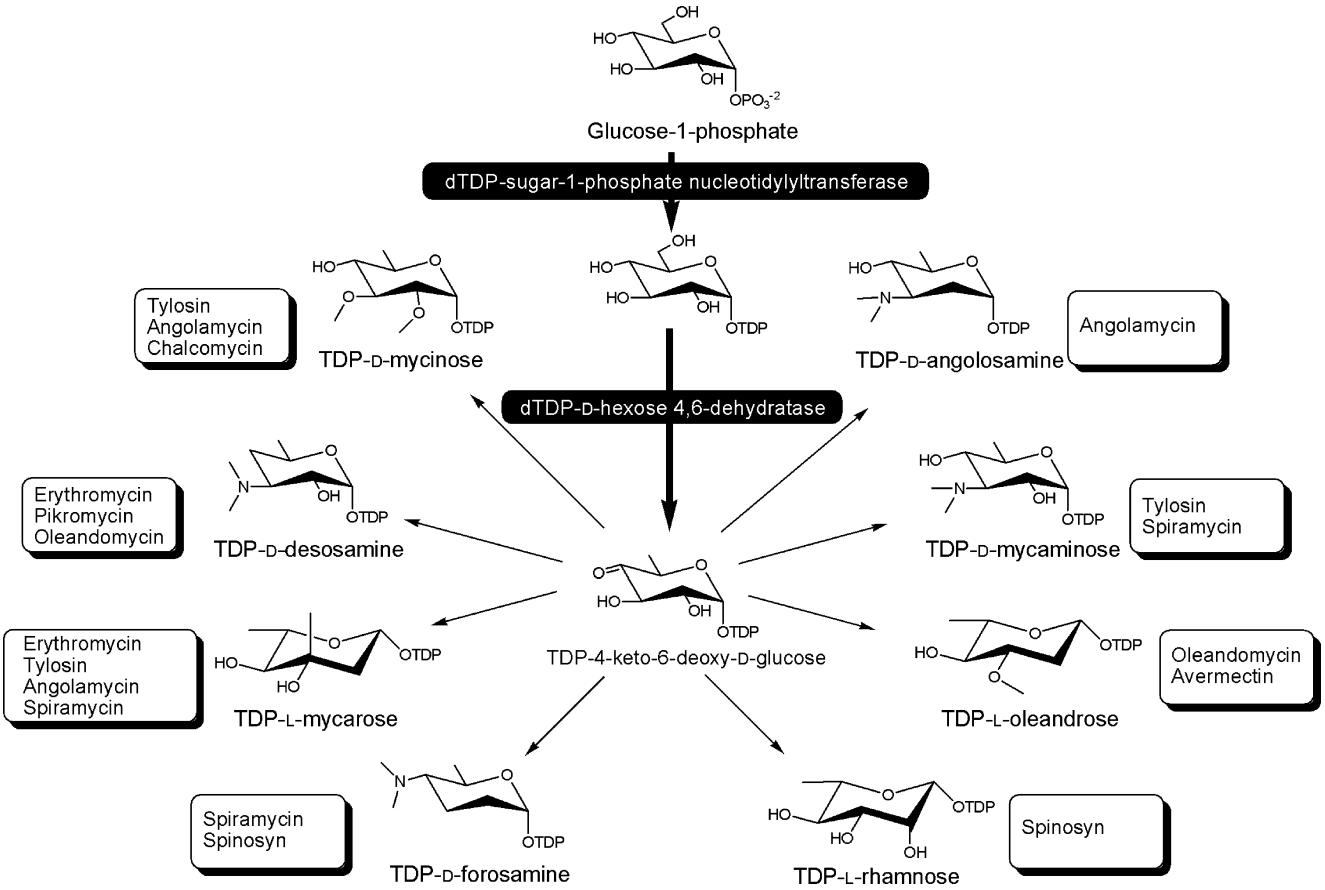

Fig. 4 Schematic representation of the pathways for 6-deoxysugar nucleotide biosynthesis. Two early enzymatic steps in the biosynthesis of the common intermediate dTDP-4-keto-6-deoxy-D-glucose are indicated. Structural diversity in the sugar family arises from further modifications of the 4-keto-6-deoxy intermediate through specific enzymatic reactions that affect different carbons of the hexose chain

efficiency and/or substrate flexibility (Williams et al. 2007; Gantt et al. 2008). Therefore, genetic engineering provides the potential to create broadly promiscuous GTs, presenting opportunities for in vivo and in vitro glycodiversification.

It has been reported that, in some cases, the GT/auxiliary protein pair is important for the efficient transfer of the deoxysugar in the biosynthesis of macrolide compounds. TylMII/TylMIII from *S. fradiae* and MycB/MydC from the mycinamicin biosynthetic gene cluster in *Micromonospora griseorubida* have been identified as GT/auxiliary protein pairs involved in glycosylation (Melançon et al. 2004). It has also been shown that EryCIII can be activated by the cognate auxiliary protein EryCII from *S. erythraea* or the non-cognate auxiliary protein AknT from *Streptomyces galilaeus* (Yuan et al. 2005). Likewise, DesVII requires its companion protein, DesVIII, for optimal activity. Furthermore, Hong and coworkers demonstrated that homologues of DesVIII, including EryCII, OleP1 from *S. antibioticus*, and DnrQ from *Streptomyces peucetius*, can functionally replace DesVIII (Hong et al. 2007).

Oxidation, such as hydroxylation and epoxidation catalyzed by cytochrome P450 monooxygenases, is often one of the key post-PKS tailoring steps that results in structural and biological diversity of macrolide compounds (Figs. 3 and 5). Therefore, engineering of monooxygenases toward a range of alternative substrates would be helpful in generating novel products with potentially greater or altered biological activities (Rix et al. 2002). The cytochrome P450 monooxygenase from *S. venezuelae*, known as PikC (Fig. 2), accepts YC-17, narbomycin, methymycin, and pikromycin as natural substrates (Zhang and Sherman 2001; Lee et al. 2006b) and can also act on diverse unnatural macrolactones (Yoon et al. 2002; Lee et al. 2005). In a recent report, 5-*O*-desosaminyl erythronolide A, a potent precursor of ketolides, the latest generation of antimicrobials derived from erythromycin A, was produced by the expression of PikC in an EryBV GT deletion mutant of *S. erythraea* (Basnet et al. 2008). PikC, having a similar function to EryK (erythromycin C-12 hydroxylase) but with more relaxed substrate specificity, can introduce a C-12 hydroxyl group into the unnatural substrate 5-*O*-desosaminyl erythronolide B. X-ray structure analysis has provided new information regarding the diverse hydroxylation patterns of PikC, and site-specific mutagenesis based on crystal struc-

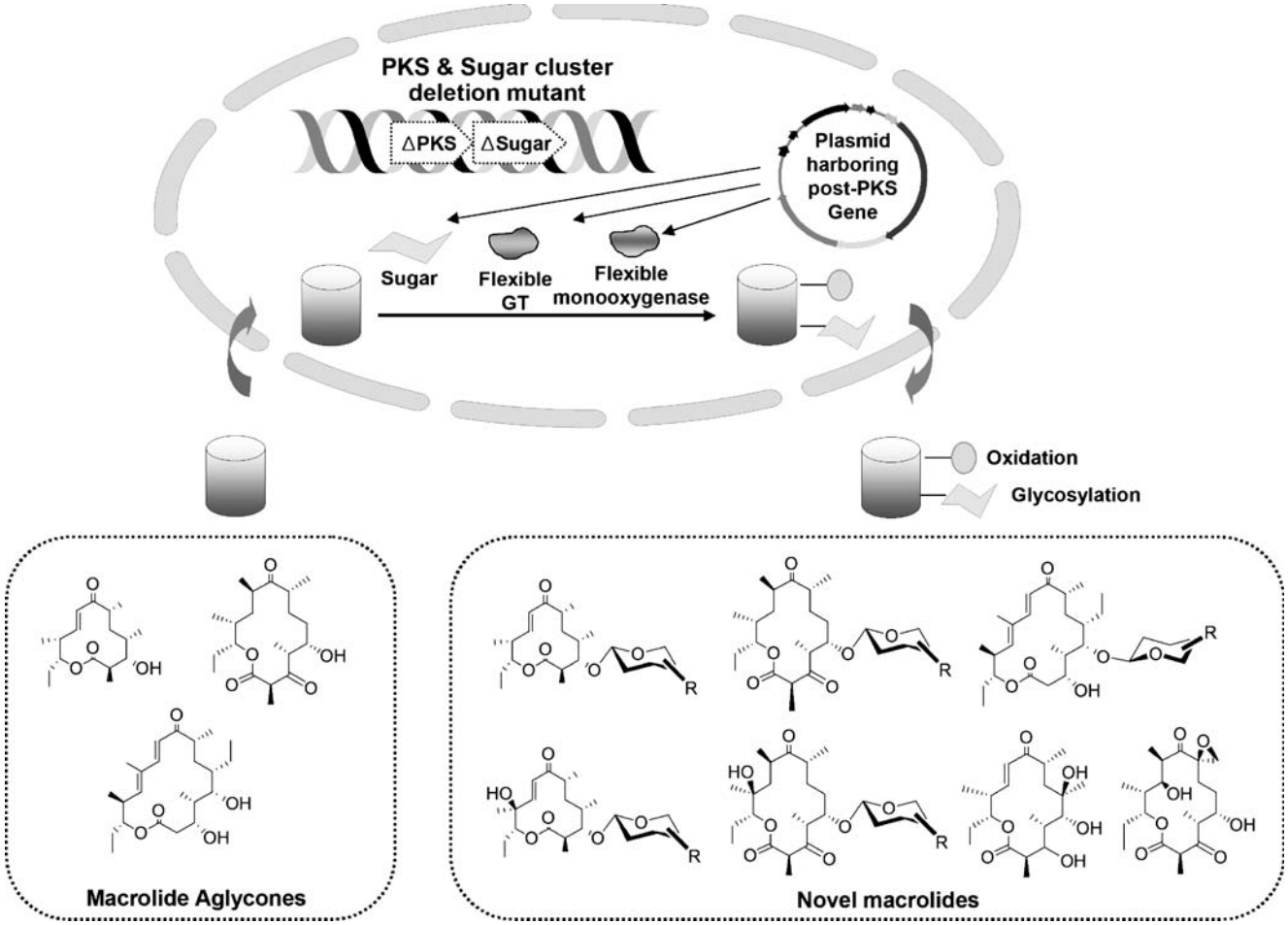

Fig. 5 Concept of combinatorial biosynthesis of post-PKS modification steps. Combinatorial biosynthesis is the application of genetic engineering to the modification of natural product biosynthetic pathways in order to produce unnatural or hybrid natural products. Generating glycosylated and/or oxygenated metabolites via combinatorial biosynthesis requires a flexible GT and/or monooxygenase active toward the sugar acceptor (aglycone) and sugar donor substrates

tures has revealed several PikC residues that are essential for the hydroxylation of 12- and 14-membered ring macrolides, as well as D-desosamine binding (Sherman et al. 2006). In addition, in another study, site-directed mutagenesis of PikC led to enhanced hydroxylation activity toward indole, an unnatural substrate with a completely different structure from native PikC substrates (Lee et al. 2006a).

EryF from *S. erythraea* is responsible for the hydroxylation of the erythromycin precursor 6-deoxyerythronolide B (Shafiee and Hutchinson 1988). EryF possesses flexible specificity for several macrolide substrates related to 6-deoxyerythronolide B, producing hydroxylated 6-deoxyerythronolide B derivatives (Andersen et al. 1993). X-ray crystallographic studies revealed the EryF residues that are critical for 6-deoxyerythronolide B hydroxylase activity (Cupp-Vickery et al. 1994). Moreover, an A245T mutant of EryF enabled the oxidation of the alternative substrate testosterone (Xiang et al. 2000). EryK, known as the P450 monooxygenase responsible for the conversion of erythromycin D to erythromycin C (Lambalot et al. 1995), shows a certain degree of substrate flexibility and can hydroxlateYC-17 and narbomycin, natural substrates of PikC from *S. venezuelae* (Lee et al. 2004). It has also been shown that overexpression of *eryK* in *S. erythraea* results in the improved bioconversion of 15-fluoro-6-deoxyerythronolide B to 15-fluoro-erythromycin (Desai et al. 2004). The cytochrome P450 monooxygenase OleP from the oleandomycin biosynthetic gene cluster of *S. antibioticus*, which mediates epoxidation of the oleandomycin macrolactone (Rodriguez et al. 1995), can accept six different unnatural erythronolide B-related substrates by the heterologous expression of *oleP* (in *S. erythraea*) and feeding with various unnatural aglycones (Gaisser et al. 2002).

Combining the use of these substrate-flexible post-PKS tailoring enzymes with combinatorial biosynthesis may provide a valuable tool for producing unnatural macrolides, as exemplified in Fig. 5. The aglycone can be glycosylated

by flexible GTs using sugar substrates that are combinatorially biosynthesized and hydroxylated by flexible monooxygenases. Moreover, increasing knowledge of the broad substrate spectrum and X-ray crystal structure of GTs and P450 monooxygenases involved in the biosynthesis of macrolides will provide new biotechnological approaches for the generation of novel glycosylated or hydroxylated derivatives.

Engineering of precursor pathways

Macrolide aglycones are assembled from the appropriate extender units by PKS. Knowledge of the metabolic pathways involved in the supply of PKS precursors required for biosynthesis may enable control of the carbon flux and lead to improved production of useful bioactive macrolides (Olano et al. 2008). Extender units for macrolide biosyntheis can be divided into two types, CoA-linked and ACP-linked. CoA-linked extender units are the classical precursors of PKS and include malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, and chloroethylmalonyl-CoA, whereas ACP-linked extender units include methoxylmalonyl-ACP, hydroxymalonyl-ACP, and aminomalonyl-ACP (Chan et al. 2009; Eustáquio et al. 2009).

Malonyl-CoA and methylmalonyl-CoA are the most commonly used extender units for macrolide biosynthesis. The incorporation of malonyl-CoA and methylmalonyl-CoA into the growing macrolides proceed via a condensation between a neighboring ACP-linked acyl thioester and ACP-linked malonate and methylmalonate derivative (acetate and propionate, respectively) generated by the decarboxylation. Malonyl-CoA is usually derived from the carboxylation of acetyl-CoA by an acetyl-CoA carboxylase (Bianchi et al. 1990). In contrast, methylmalonyl-CoA can be produced via three generally accepted pathways (Fig. 6): (1) isomerization of succinyl-CoA, catalyzed by methylmalonyl-CoA mutase (MCM); (2) carboxylation of propionyl-CoA by propionyl-CoA carboxylase (PCC) or methylmalonyl-CoA transcarboxylase; and (3) catabolism of valine (Chan et al. 2009). Engineering of the methylmalonyl-CoA metabolite node of the erythromycin producers *Aeromicrobium erythreum* and *S. erythraea* has led to enhanced production of erythromycin, depending on the fermentation medium used. Erythromycin production was enhanced by the knockout of the MCM gene *mutB* plus cultivation of *A. erythreum* or *S. erythraea* in a carbohydrate-based medium, while in an oil-based medium, duplication of the MCM operon (*meaA*, *mutB*, *meaB*, *mutR*) led to increased production of erythromycin in *S. erythraea* (Reeves et al. 2007). Recently, the expression of *mutB* from *S. erythraea* in the FK506 producer *Streptomyces clavuligerus* CKD1119 led to 3-fold and 1.5-fold increases in the methylmalonyl-CoA pool and FK506 production, respectively (Mo et al. 2009). Overproduction of 6-deoxyerythrolide B was also achieved by heterologous expression of the PCC genes *pccB* and *accA1* from *Streptomyces coelicolor* in an engineered *Escherichia coli* strain expressing DEBS PKS (Murli et al. 2003). Valine catabolism can be a source of methlymalonyl-CoA precursors in macrolide biosynthesis (Fig. 6). Isotopic labeling experiments have shown that isobutyryl-CoA-formed valine catabolism is subsequently converted to methylmalonyl-CoA for the biosynthesis of several macrolide antibiotics including tylosin and leukomycin (Chan et al. 2009). In addition, inactivation of the valine dehydrogenase gene (*vdh*), which encodes the first enzyme of the valine catabolism pathway, reduced the production of tylosin and spiramycin in *S. fradiae* and *Streptomyces ambofaciens*, respectively (Tang et al. 1994). These results suggest that valine may serve as a precursor for the biosynthesis of macrolide aglycones. However, these are not the only pathways that can be engineered to supply methylmalonyl-CoA for macrolide biosynthesis. Epothilone B production was improved approximately 2.5-fold compared with that of wild-type *Sorangium cellulosum* by the heterologous expression of propionyl-CoA synthetase, which converts propionate into propionyl-CoA, the precursor of methylmalonyl-CoA (Han et al. 2008). Furthermore, heterologous expression of methylmalonyl-CoA ligase and feeding with methylmalonate in *E. coli* led to the accumulation of a high level of methylmalonyl-CoA (Murli et al. 2003).

Ethylmalonyl-CoA (Fig. 6), which can also be used as an extender unit in the biosynthesis of a variety of macrolide compounds, such as tylosin, concanamycin A, and spiramycin, appears to be derived from the carboxylation of butyryl-CoA (Chan et al. 2009). At least two routes lead to the production of butyryl-CoA. One route involves the isomerization of the valine catabolite, isobutyryl-CoA, by isobutyryl-CoA mutase (ICM). The second route involves the condensation of two acetate units and terminates with the reduction of crotonyl-CoA to butyryl-CoA by crotonyl-CoA reductase (CCR) (Liu and Reynolds 2001). CCR catalyzes not only this reduction but also the reductive carboxylation of crotonyl-CoA to ethylmalonyl-CoA with NADPH and CO_2 (Erb et al. 2007). Unlike the extender units discussed above, chloroethylmalonyl-CoA has been required solely by the salinosporamide PKS system (Chan et al. 2009).

ACP-linked extender units such as methoxymalonyl-ACP, hydroxymalonyl-ACP, and aminomalonyl-ACP can be considered to be macrolide precursors. These precursors are unique to polyketide biosynthesis, while CoA-linked extender units can be used for both fatty acid and polyketide biosynthesis. The first reported ACP-linked extender unit, methoxylmalonyl-ACP, has been proposed as a precursor of

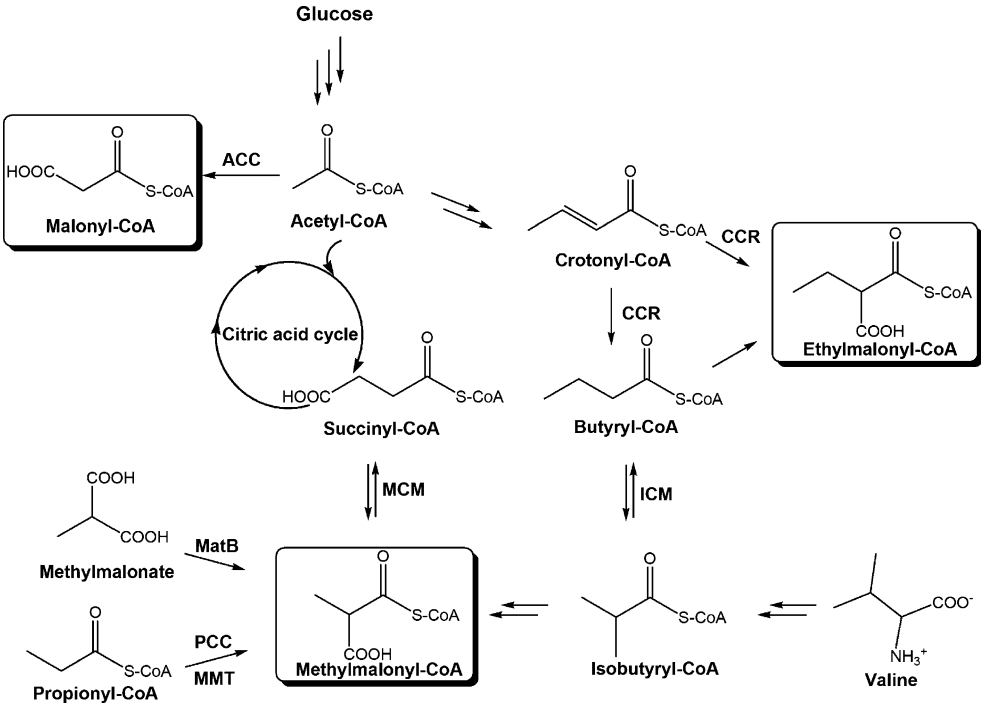

Fig. 6 Proposed pathways for precursor supply engineering. In macrolide biosynthesis, a CoA-linked extender unit plays a role as a carrier of carboxylic acid precursors. Abreviations: *ACC* acetyl-CoA carboxylase, *MCM* methylmalonyl-CoA mutase, *MatB* methylmalonyl CoA ligase, *MMT* methylmalonyl-CoA transcarboxylase, *PCC* propionyl-CoA carboxylase, *CCR* crotonyl-CoA carboxylase/reductase, *ICM* isobutyryl-CoA mutase

the macrolides concanamycin, midecamycin, and FK520. The ACP-linked units, hydroxymalonyl-ACP and aminomalonyl-ACP, have also been found in the biosynthesis of several polyketide compounds including zwittermicin A and aflastatin A (Chan et al. 2009). The overproducing ability of an *S. fradiae* strain was extended by introducing the biosynthetic pathway for methoxylmalonyl-ACP from the FK520 producer *Streptomyces hygroscopicous*. The resulting strain was able to produce more than 1 g/L of midecamycin, which requires a methoxymalonyl extender unit when midecamycin PKS is coexpressed (Rodriguez et al. 2004). Although the engineering of hydroxymalonyl-ACP and aminomalonyl-ACP in macrolide biosynthesis has not been reported to date, study of these extender units may become more prevalent and they are likely to be exploited for the expansion of macrolide diversity.

Heterologous expression of macrolide biosynthetic genes

Newly developed technologies and increased genetic knowledge have enabled the manipulation of macrolide biosynthetic pathways to improve yield and produce novel compounds as mentioned above. However, such an approach is often limited by the poor growth and decreased opportunity for genetic manipulation associated with the original hosts. This drawback has created the need to develop heterologous hosts for the production of high levels of the desired natural products or to generate novel bioactive compounds by genetic engineering. Table 1 presents numerous examples of macrolide products generated by heterologous expression systems.

Streptomyces or related actinomycetes, which are Gram-positive bacteria with a high GC content, produce a wide range of pharmacologically relevant natural products as secondary metabolites. Therefore, these bacteria have come to be known as robust heterologous hosts for macrolide production. The most widely used bacteria for such purposes are *S. coelicolor* and *Streptomyces lividans*. *S. coelicolor* has been used for the biosynthesis of macrolides including erythromycin (Kao et al. 1994), oleandomycin, pikromycin (Tang et al. 2000a), epothilone (Tang et al. 2000b), and megalomycin (Hu et al. 2003). *S. lividans* has been tested as a heterologous host for the production of oleandomycin (Shah et al. 2000) and soraphen A (Zirkle et al. 2004). Similary, *S. fradiae* (Rodriguez et al. 2004; Ward et al. 2004) and *S. erythraea* (Volchegursky et al. 2000) have also been successfully used to express several PKS gene clusters.

Among well-developed heterologous actinomycete strains, the pikromycin-producing *S. venezuelae* has recently been selected as a favorable host for the production of macrocyclic natural products due to its rapid growth, relative ease of genetic manipulation, and abundant supply of substrates, such as malonyl-CoA (Yoon et al. 2002; Jung et al. 2006; Park et al. 2007, 2008a, b). In a previous study, the entire Tyl PKS gene cluster (approximately 40 kb) of *S. fradiae* and the epothilone PKS gene cluster (approximately 60 kb) of *S. cellulosum* were expressed in *S. venezuelae* mutant DHS2001 bearing a deletion of the

Table 1 Examples of macrolides produced in heterologous hosts

Compound	Host	Reference
Chalcomycin	*S. fradiae*	Ward et al. 2004
Epothilone	*S. coelicolor*, *M. xanthus*, *E. coli*, *S. venezuelae*	Tang et al. 2000b; Julien and Shah 2002; Mutka et al. 2006; Park et al. 2008b
Eerythromycin	*S. coelicolor*, *E. coli*	Kao et al. 1994; Pfeifer et al. 2001
Megalomycin	*S. coelicolor*, *S. erythraea*	Hu et al. 2003; Volchegursky et al. 2000
Midecamycin	*S. fradiae*	Rodriguez et al. 2004
Oleandomycin	*S. coelicolor*, *S. lividans*	Tang et al. 2000a; Shah et al. 2000
Pikromycin	*S. coelicolor*	Tang et al. 2000a
Soraphen A	*S. lividans*	Zirkle et al. 2004
Tylosin	*S. venezuelae*	Jung et al. 2006

native Pik PKS gene cluster, resulting in production of tylactone and epothilone, respectively (Jung et al. 2006; Park et al. 2008b).

Besides the actinomycetes, which are the most prolific producers of macrolides, *E. coli* can also serve as a host for heterologous expression due to its rapid growth rate and the existence of well-developed molecular biology tools. The first reported heterologous macrolide to be produced by *E. coli* was 6-deoxyerythronolide B, the precursor of erythromycin (Pfeifer et al. 2001). Heterologous production of epothilones C and D in *E. coli* has also been achieved (Mutka et al. 2006). In addition, *Myxococcus xanthus*, the best-characterized myxobacterium, is amenable to engineering and has a relatively shorter doubling time than the original epothilone producer *S. cellulosum*; as a result, it has been engineered for the biosynthesis of epothilone (Julien and Shah 2002). Although several hosts have been used successfully for the heterologous production of many macrolides, there are still some limitations that need to be overcome. One of these is associated with the relatively low levels of heterologously produced macrolides. Recently, Jung et al. reported that improved production of heterologous tylactone and desosaminyl tylactone can be achieved by the overexpression of the regulatory gene *pikD* in *S. venezuelae* (Jung et al. 2008). Another recent study also showed that overexpression of the PikD regulator in *S. veneuzuelae* resulted in enhanced heterologous production of epothilones (Park et al. 2008b). These results suggest one approach to overcoming the problem of low expression levels encountered during heterologous macrolide production.

Prospects

Biosynthesis of "unnatural" natural macrolides in soil microbes through genetic engineering approaches has been extensively investigated. Recently, research efforts regarding the generation or discovery of more diverse macrolides have shifted to the exploration of new sources. First, natural products derived from marine microorganisms or invertebrates have attracted great attention since their metabolic capacities offer the potential to produce compounds with several interesting pharmacological activities (Blunt et al. 2006). Next, multiorganism-derived pools of genomic DNA (metagenomes) can also provide rich sources of new natural products. It is widely accepted that about 99% of microbes are non-cultivable under standard laboratory conditions. However, the use of metagenomics may circumvent the need for cultivation of these organisms through the isolation of bacterial DNAs and the construction and screening of DNA clone libraries (Daniel 2005). Thus, if metagenomics is coupled with the genetic engineering tools and heterologous expression systems described herein, an untapped pool of resources could be opened up in which new bioactive molecules could be discovered.

One of the key challenges of the genetic engineering approach to macrolide biosynthesis is the relatively reduced levels of production mentioned above. To achieve an efficient production platform, it may be preferable to express genetically engineered macrolide PKSs in hosts that have undergone industrial strain improvement (Rodriguez et al. 2003). Moreover, the availability of the genome sequences of polyketide-producing strains such as *S. coelicolor* (Bentley et al. 2002) and *S. avermitilis* (Ikeda et al. 2003) in combination with the use of advanced techniques for genomic and/or proteomic analysis will provide valuable information for the improved production of macrolides generated by genetic engineering.

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