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Research Paper

Construction of desosamine containing polyketide libraries using a glycosyltransferase with broad substrate specificity

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Abstract

Background: Combinatorial biosynthesis techniques using polyketide synthases (PKSs) in heterologous host organisms have enabled the production of macrolide aglycone libraries in which many positions of the macrolactone ring have been manipulated. However, the deoxysugar moieties of macrolides, absent in previous libraries, play a critical role in contributing to the antimicrobial properties exhibited by compounds such as erythromycin. Since the glycosidic components of polyketides dramatically alter their molecular binding properties, it would be useful to develop general expression hosts and vectors for synthesis and attachment of deoxysugars to expand the nature and size of such polyketide libraries.

Results: A set of nine deoxysugar biosynthetic and auxiliary genes from the picromycin/methymycin (*pik*) cluster was integrated in the chromosome of *Streptomyces lividans* to create a host which synthesizes TDP-D-desosamine. The *pik* desosaminyl transferase was also included so that when the strain was transformed with a previously constructed library of expression plasmids encoding genetically modified PKSs that produce different macrolactones, the resulting strains produced desosaminylated derivatives. Although conversion of the macrolactones was generally low, bioassays revealed that, unlike their aglycone precursors, these novel macrolides possessed antibiotic activity.

Conclusions: Based on the structural differences among the compounds that were glycosylated it appears that the desosaminyl transferase from the *pik* gene cluster is quite tolerant of changes in the macrolactone substrate. Since others have demonstrated tolerance towards modifications in the sugar substituent, one can imagine employing this approach to alter both polyketide and deoxysugar pathways to produce 'unnatural' natural product libraries.

Keywords: Desosamine; Glycosyltransferase; Macrolactone; Polyketide library; Substrate specificity

1. Introduction

Polyketides are natural products that possess an array of biological activities, finding their way into many useful clinical and agricultural applications. Much of the structural diversity and complexity among this class of compounds can be attributed to the chemistry performed by polyketide synthases (PKSs), large multifunctional enzymes that assemble the 'core' carbon systems of polyketides from small organic acid precursors [1]. In fact, the modular architecture of catalytic domains within PKSs continues to be exploited by different rational and combinatorial engineering approaches to create further polyketide diversity [2–4]. However, some of the structural variability among polyketides does not result from PKSs, but rather is due to post-PKS biosynthetic steps which typically include oxidation and/or glycosylation by unique deoxy and amino sugars. Such modifications are often necessary to impart or enhance the specific biological activity of the molecule. For example, erythromycin A (Fig. 1) contains two deoxysugar moieties, L-cladinose and D-desosamine, that are required for antibacterial activity and the absence of either carbohydrate results in loss of potency. Although some chemical modifications to erythromycin have been discovered which can ameliorate the loss of the cladinose residue [5,6], there has been no substitution found for desosamine. This important deoxyaminosugar is also present in other macrolide antibiotics such as

Abbreviations: 6-dEB, 6-deoxyerythronolide B; DEBS, 6-deoxyerythronolide B synthase; PKS, polyketide synthase

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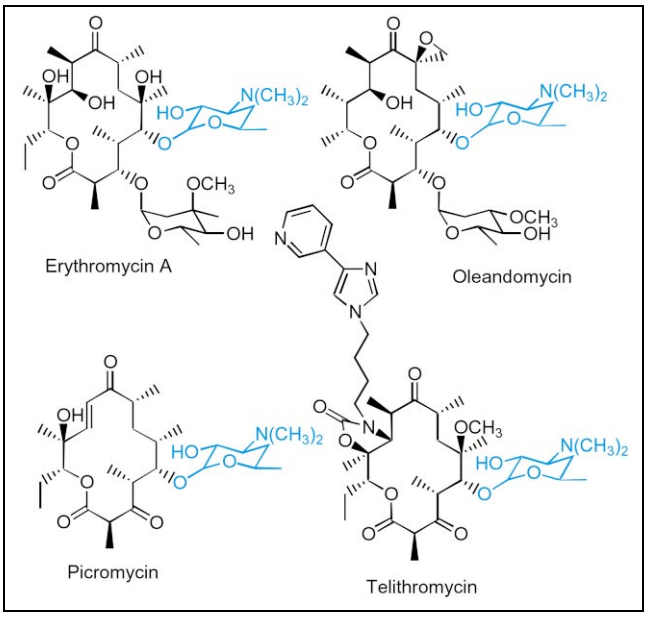

Fig. 1. Desosamine (blue) containing antibiotics. Erythromycin, oleandomycin, and picromycin are naturally occurring polyketide antibiotics. Telithromycin (HMR 3647) is a potent semisynthetic derivative of erythromycin currently in clinical trials.

oleandomycin and megalomicin, and is the only glycoside necessary to confer antibacterial activity to picromycin, methymycin, and the semisynthetic ketolide pharmacophores (Fig. 1).

We and others recently described the production of polyketide libraries generated by genetic modification of macrolide PKSs in which enzymatic domains and entire protein subunits were removed, added, or exchanged in various combinations [3,4,7]. Since these libraries were constructed in heterologous hosts lacking glycosylation pathways, only the corresponding aglycones were produced. We wished to expand the capabilities of the combinatorial biosynthesis strategies described in the previous work to incorporate post-PKS tailoring steps, in particular the addition of deoxysugar components. Some experiments have been performed in which structurally modified macrolactones are subsequently glycosylated in their native hosts [8–12], and also in bioconversion experiments in which a modified aglycone is fed to a PKS-blocked mutant strain [13]. These experiments indicate that glycosyltransferases are able to accept polyketide substrates with some amount of structural alteration. However, neither of these approaches is well-suited for the production and biological screening of large numbers of compounds since most polyketide host organisms are difficult to genetically manipulate and the bioconversion of aglycones requires a tedious initial purification step. A more practical approach is the heterologous expression of deoxysugar biosynthetic pathways in hosts which have been developed for library expression. Although the effort to clone entire deoxysugar biosynthetic pathways in a heterologous organism can be a significant initial investment (most deoxysugars require six or more enzymatic steps whose genes are typically scattered within a polyketide gene cluster), these expression vectors, once made, can be easily combined with those containing PKSs to rapidly engineer glycosylated libraries. Olano et al. recently utilized a two plasmid system to produce L-daunosamine, the deoxyaminosugar of daunorubicin and doxorubicin, in *Streptomyces lividans* [14].

Here we report the development of a single expression vector for the production of desosaminylated macrolides in *Streptomyces*. Desosamine was selected as the sugar constituent because, based on the structures in Fig. 1, it was believed that addition of this single deoxysugar would be sufficient to confer antibacterial activity upon macrolactones to which it was attached. The expression vector was combined with a library of existing PKS expression plasmids to produce several novel glycosylated macrolide compounds in *S. lividans*, providing the first examples in which both polyketide and deoxysugar pathways have been placed in a single heterologous host.

2. Results

2.1. Construction and validation of a desosamine expression system

The picromycin/methymycin (*pik*) gene cluster from *Streptomyces venezuelae* [15] was chosen as the source of desosamine biosynthetic genes rather than other available clusters (i.e. erythromycin, oleandomycin, or megalomicin) for several reasons. First, all of the genes required for biosynthesis of TDP-D-desosamine from glucose-1-phosphate (Fig. 2), a primary metabolite, as well as the desosaminyl transferase are present in the *pik* cluster whereas one or more of the genes are missing or not yet identified in each of the other clusters. Second, the genes from the *pik* cluster are comprised in a single contiguous segment of DNA (the *des* cluster), compared to those in other clusters which are dispersed among other genes, facilitating cloning and plasmid construction. Third, the natural substrates for the desosaminyl transferase from the *pik* gene cluster, narbonolide (**1**) and 10-deoxymethynolide (**2**), are themselves aglycones; in each of the other cases desosamine is attached subsequent to addition of at least one other sugar. Furthermore, the difference in macrolactone ring sizes between narbonolide (**1**) and 10-deoxymethynolide (**2**) (14 and 12 atoms, respectively) suggests that the desosaminyl transferase from this cluster is somewhat forgiving towards its polyketide substrate.

Seven genes in the *des* cluster, *desI, desII, desIII, desIV, desV, desVI*, and *desVIII*, are presumed to be responsible for the biosynthesis of TDP-D-desosamine (Fig. 2) [16]. Also present is the *desVII* gene encoding the glycosyltransferase. In addition to catalyzing the transfer of desosamine to both 12- and 14-membered macrolactones, it has been

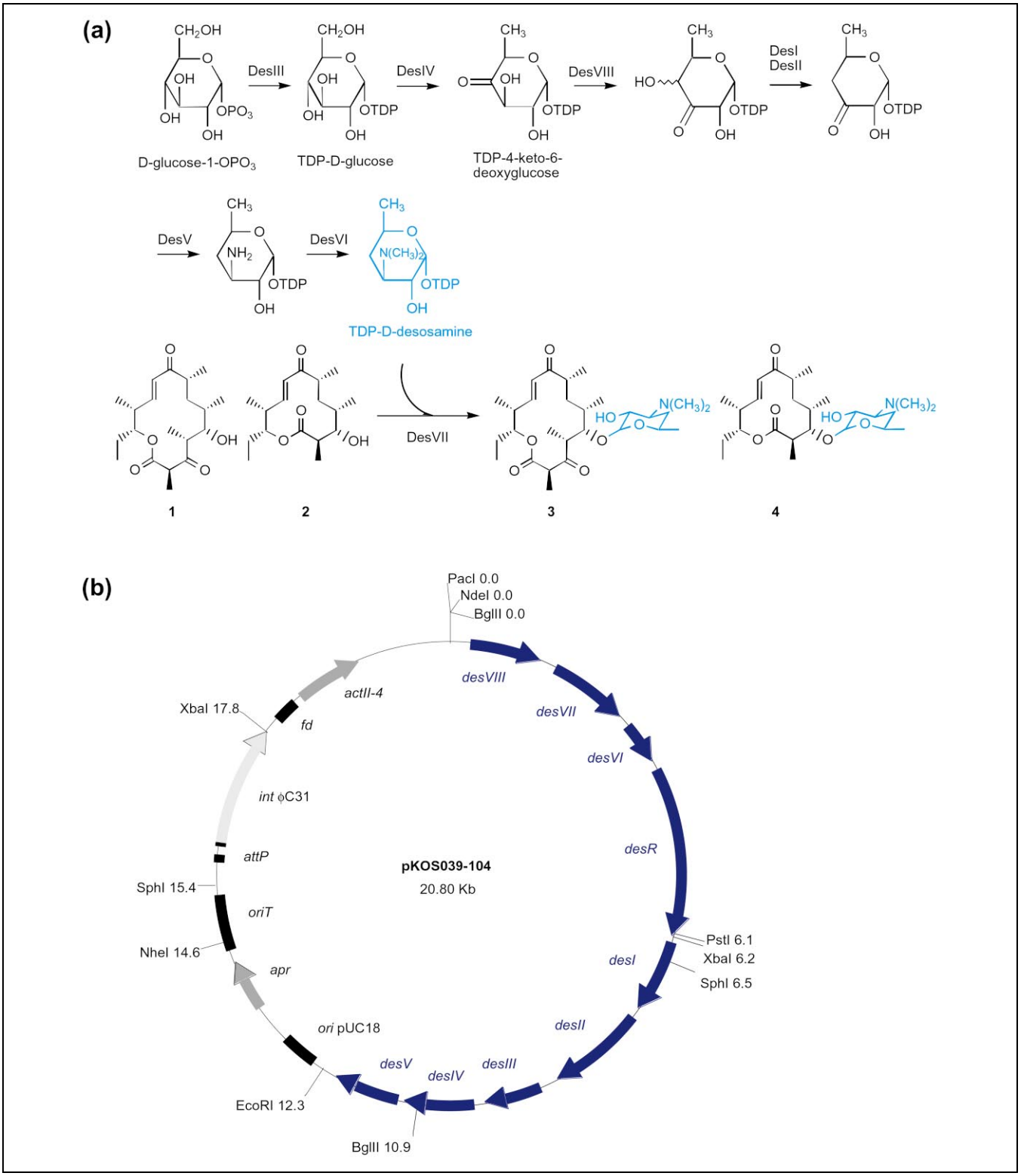

Fig. 2. (a) Enzymes from the *des* cluster of *S. venezuelae* involved in biosynthesis of TDP-D-desosamine and attachment to narbonolide (**1**) and 10-deoxymethynolide (**2**) and (b) construction of an expression vector using the corresponding genes. See text for details.

shown that DesVII is able to incorporate non-natural deoxysugar substrates [16,17]. The *desR* gene encodes a β-glucosidase that removes a glucose residue attached to the C-2′ hydroxyl of desosamine [18]. It is believed that the glucosylation of desosamine containing macrolides like methymycin, picromycin, and oleandomycin, causes inactivation and provides self-resistance to these compounds which are reactivated by a β-glucosidase upon export

[18,19]. *S. lividans* is known to possess at least two such glucosyltransferases which inactivate erythromycin and picromycin by the same mechanism [20] (R. McDaniel, unpublished observations). Therefore, it was important to include this gene for expression in *S. lividans* in order to produce desosaminylated compounds without the glucose modification.

The expression system used here was adopted from the multi-vector system developed for separate expression of erythromycin PKS, or 6-deoxyerythronolide B (6-dEB) synthase (DEBS), subunits in *Streptomyces* [4,21]. Plasmid pKOS39-104 (Fig. 2) contains the *des* genes cloned in a single orientation under control of the *act*I promoter and *act*II-4 activator. Since pKOS39-104 is a derivative of pSET152 [22], it contains the ϕC31-*int-attP* loci for chromosomal integration in *Streptomyces* and can be used in conjunction with our pRM5-based PKS expression plasmid library [3]. *S. lividans* K4-114 was transformed with pKOS39-104 and designated K39-22. Confirmation that this strain produced TDP-D-desosamine was performed by feeding aglycones to the strain and looking for the presence of desosaminylated compounds by LC/MS analysis.

Four aglycones (~10 mg/l each) were fed to liquid fermentations of *S. lividans* K39-22: narbonolide (**1**) and 10-deoxymethynolide (**2**), the natural substrates for DesVII, 3-keto-6-dEB (**5**), and 6-dEB (**6**) (Fig. 3). Fermentation broth from all four aglycone-fed strains displayed antibacterial activity against *Bacillus subtilis* whereas *S. lividans* K39-22 alone produced no detectable activity. LC/MS analysis was consistent with the conversion of each of the aglycones to their corresponding desosaminylated derivatives, narbomycin (**3**), 10-deoxymethymycin (YC17, **4**), 3-keto-5-*O*-desosaminyl-6-dEB (**7**), and 5-*O*-desosaminyl-6-dEB (**8**) (Fig. 3), although a significant amount (~50–90%) of the aglycone remained unconverted in each of the samples. In each case, the parent ion ($M+H^+$) of the expected compound was detected in addition to a characteristic ion at 158 amu which is produced by the desosamine glycoside under the ionization conditions used. In the case of the narbonolide-fed strain, the compound produced was compared to authentic narbomycin obtained from *Streptomyces narbonensis*. For each of the other compounds, the location and/or stereochemistry of desosamine attachment is inferred from the natural substrate specificity of DesVII.

These results established that our *des* expression vector was functional and that the DesVII glycosyltransferase was able to glycosylate non-natural macrolactone substrates. The bioassay results also confirmed our prediction that desosamine is sufficient to confer antibacterial activity to these macrolactones. There were no 2′-*O*-glucosyl derivatives detected which implies the DesR glucosidase included in pKOS39-104 was also operational, although minor glucosylated products were putatively found in subsequent experiments with the strain (see below).

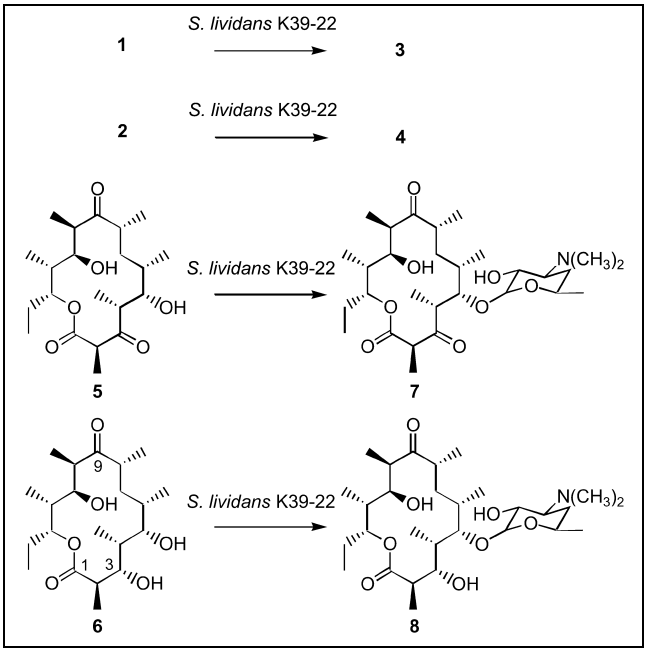

Fig. 3. Bioconversion of aglycones by *S. lividans* K39-22. *S. lividans* K39-22 contains the desosamine expression vector shown in Fig. 2b. Conversion of each aglycone to a desosaminylated derivative was confirmed by LC/MS. Compounds **3** and **4** are the natural products of the desosaminyltransferase, DesVII, expressed in this strain. Aglycones **5** and **6** are novel substrates for DesVII and the assignment of desosamine attachment to C-5 and its stereochemical configuration in compounds **7** and **8** is based on the natural specificity of the enzyme.

2.2. *Co-expression of desosamine and aglycone pathways in S. lividans*

Although expression of both a modular polyketide pathway and a deoxysugar pathway together in a heterologous host has not been reported, the bioconversion results suggested that transformation of *S. lividans* K39-22 with plasmids encoding macrolide PKSs would lead to production of desosaminylated compounds. We introduced plasmids harboring the PKSs which, in *S. lividans*, produce the same four aglycones used in the above bioconversion studies. Plasmid pKOS39-86 contains the picromycin/methymycin PKS and produces both narbonolide (**1**) and 10-deoxymethynolide (**2**) [23]. Plasmid pKAO127 contains DEBS and produces 6-dEB (**6**) [24]. Plasmid pKOS39-18 contains DEBS with a modified terminal module that produces 3-keto-6-dEB (**5**) [23].

Culture broth from each of the transformed strains displayed activity against *B. subtilis* (Fig. 4). LC/MS analysis was performed as above and in each case a new compound with mass corresponding to the predicted desosaminylated polyketide (Fig. 5) was identified. Since these are the same compounds as those produced in the bioconversion experiments, we again infer the regiochemical and stereochemical configurations of desosamine attachment as above. The aglycone precursors produced by the PKS in each

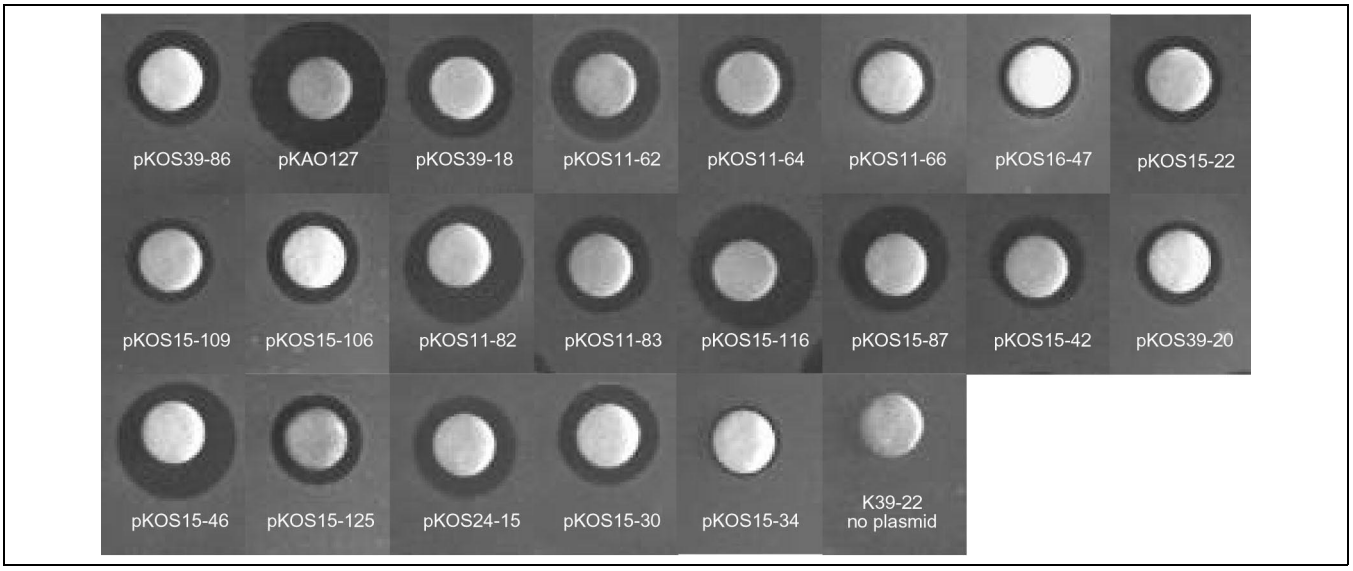

Fig. 4. Biological screening of strains containing engineered desosamine and polyketide biosynthetic pathways. The text below each spot indicates the name of the plasmid used to transform *S. lividans* K39-22. Each plasmid encodes a distinct engineered PKS that produces one or more macrolactones which do not possess antibacterial activity. In the background of *S. lividans* K39-22, inhibition of *B. subtilis* growth can be observed, indicating that a desosaminylated compound(s) is being produced. The inferred corresponding structures are shown in Fig. 5. The size of zone clearing is not indicative of potency since the titers of compound vary for each strain.

strain were also present as well as putative 2′-*O*-glucosyl derivatives of the desosaminylated macrolides. The total yield of narbomycin and 10-deoxymethymycin in *S. lividans* K39-22/pKOS39-86 was approximately 1 mg/l each and represents about a 20% conversion of the total aglycone produced. Thus, although both PKS and deoxysugar pathways function as expected, complete glycosylation of even the natural substrates for DesVII did not occur. *S. lividans* K39-22 contains a copy of the *ermE* macrolide resistance gene and no obvious growth defects were observed with production of the biologically active compounds. These results suggest that a limiting amount of TDP-D-desosamine is being produced by the strain (see Section 3).

2.3. *Production and biological screening of a glycosylated macrolide library*

Over 50 PKS expression plasmids have been constructed and tested in our laboratory using DEBS and other macrolide PKS genes [3,7,23]. These PKSs produce a variety of 14 membered macrolactones in which single or multiple carbon centers have been altered. Each plasmid contains the same pRM5-based vector as above, providing a convenient opportunity to expand and diversify our existing aglycone library by routine transformation of *S. lividans* K39-22. Since a C-5 hydroxyl would be necessary for glycosylation we chose to test a subset of 19 additional plasmids encoding PKSs that produce compounds containing this functional group. Furthermore, the desired polyketides would theoretically possess antibiotic activity and the transformed strains could therefore be readily analyzed in a simple bioassay for production of glycosylated macrolides.

All of the strains transformed and tested displayed antimicrobial activity against *B. subtilis* (Fig. 4). The hypothetical bioactive compounds are shown in Fig. 5. These structures are deduced from the known aglycones produced by the PKSs and the presumed regiospecificity and stereospecificity of the DesVII glycosyltransferase. The presence of desosamine on polyketides from six of these strains was verified by LC/MS analysis. The parent ion of the predicted desosaminylated compound(s) was observed along with the 158 amu desosamine fragment in each of the strains containing plasmids pKOS24-15, pKOS11-62, pKOS15-30, pKOS15-22, pKOS15-106, and pKOS39-20 (Fig. 5). In two of these strains (pKOS11-62 and pKOS15-106), more than one putative desosamine containing compound was detected. In *S. lividans* K39-22/pKOS15-106, compounds with masses corresponding to both 3-hydroxy and 3-keto derivatives were observed (Fig. 5). This is consistent with the production of both aglycones by plasmid pKOS15-106 alone in *S. lividans* (R. McDaniel, unpublished observations). Two compounds were also detected in the strain with pKOS11-62, one of molecular weight consistent with the predicted molecule, 5-*O*-desosaminyl-10-desmethyl-6-dEB, and a putative dehydrated derivative at carbons C-10 and C-11 (Fig. 5). Both aglycones were also identified when the plasmid was originally analyzed in *S. lividans* K4-114 [3], although only the former was reported at that time. As with the first set of plasmids tested, small amounts of 2′-*O*-glucosylated derivatives could also be detected in some of the culture extracts. The yields of the desosamine

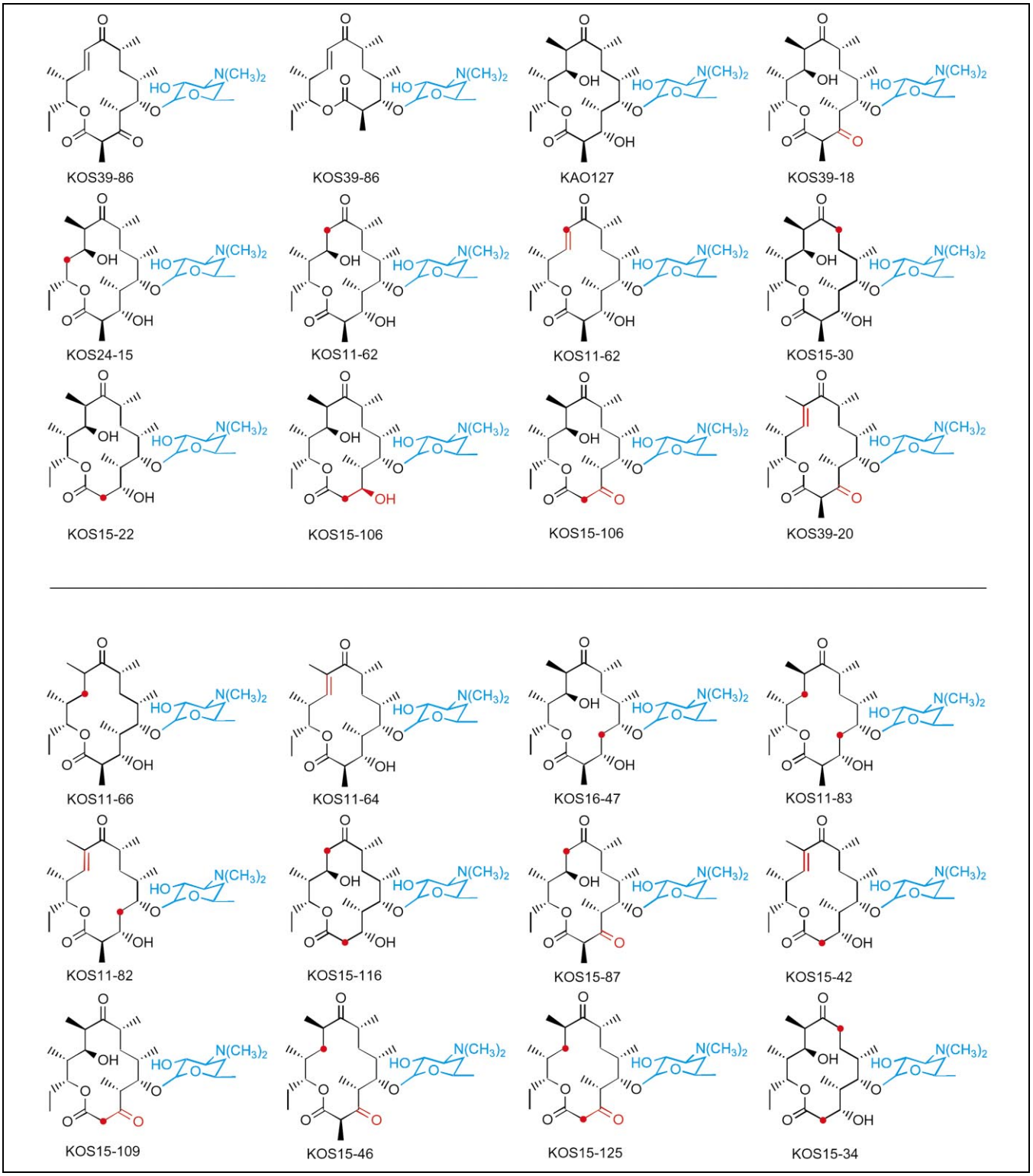

Fig. 5. Putative structures of bioactive compounds in Fig. 4. The PKSs encoded on plasmids pKOS39-86, pKAO127'kan', and pKOS39-18 are described in the text. The remaining plasmids encode DEBS with one or more modifications that produce derivatives of 6-dEB (**6**) (changes in 6-dEB structure are shown in red) [3]. The presence of those compounds above the line are supported by LC/MS analysis while those below the line are hypothetical and assumed to be present based on the antibacterial activity detected and the known structure of the aglycone produced. The regiochemistry and stereochemistry of desosamine attachment is assigned according to the natural specificity of the DesVII enzyme.

containing compounds were too low to determine absolute titers (<1 mg/l) and, therefore, the relative antibacterial activity of the compounds could not be determined from these assays.

3. Discussion

We have demonstrated a minimal set of seven genes (*desI, II, III, IV, V, VI, VIII*) is sufficient for biosynthesis of TDP-desosamine from glucose-1-phosphate in *S. lividans*. The apparent low abundance of TDP-desosamine in the engineered host could be due either to the availability of glucose-1-phosphate in this host or to poor expression of the sugar biosynthesis and/or transferase genes. Alternatively, it is interesting to note that narbonolide and 10-deoxymethynolide are present in the natural picromycin/methymycin producing organism, *S. venezuelae*, and could therefore reflect that one or more of the enzymes from the *des* cluster is relatively inefficient. It may be possible, therefore, to improve the amount of TDP-D-desosamine either by increasing expression levels of these genes and/or by complementing one or more of the enzymes in the pathway with homologs from other clusters such as erythromycin or oleandomycin.

Expression of the minimal desosamine biosynthesis genes together with the DesVII desosaminyltransferase in *S. lividans* has enabled the production of more than 20 glycosylated macrolides with detectable antibacterial activity. The structures of the macrolides that were glycosylated highlight both the remarkable substrate tolerance of the DesVII glycosyltransferase as well as the ability of desosamine to impart biological activity to structurally diverse macrolactones. In addition to their antibacterial properties the desosamine containing compounds presented here may possess additional biological properties that are associated with erythromycin and other macrolides, including motilin receptor antagonism and anti-inflammatory activities. Furthermore, the demonstration by others that DesVII and other glycosyltransferases can also tolerate modifications of the sugar substituent [16,17,25] opens the door to manipulation of both polyketide and deoxysugar pathways for the production of 'unnatural' natural product libraries.

4. Significance

Desosamine is an important deoxyaminosugar present on a number of structurally related macrolide antibiotics such as erythromycin and is the only glycoside present on picromycin, methymycin, and the highly potent semisynthetic ketolides. The desosamine biosynthetic genes from the picromycin/methymycin (*pik*) cluster of *S. venezuelae* were integrated in the chromosome of *S. lividans* to create a host which synthesizes TDP-D-desosamine and can be used in combination with PKS expression plasmids to generate libraries of desosaminylated polyketides. We demonstrate the versatility of the DesVII desosaminyltransferase by creating desosaminylated macrolides from more than 20 different 14-membered lactones. The attachment of desosamine is sufficient to confer antibiotic activity to each of the otherwise inactive aglycones and reinforces the notion that this sugar plays a critical role in the molecular binding properties of erythromycin and related macrolides. This host and others which might be engineered to produce deoxysugar and polyketide tailoring pathways are valuable tools for expanding the size and diversity of polyketides that can be generated by combinatorial biosynthesis.

5. Materials and methods

5.1. Strains, culture conditions, and DNA manipulation

DNA manipulation was performed in *Escherichia coli* XL1-Blue (Stratagene) using standard protocols [26]. *B. subtilis* was grown in LB at 37°C. PCR was performed with *Pfu* polymerase (Stratagene) under conditions recommended by the manufacturer. *S. lividans* K4-114 [24] was used as the host for expression of engineered PKS and desosamine genes. *S. lividans* strains were maintained on R2YE agar plates [27] with appropriate antibiotic selection. *S. lividans* protoplasts were transformed by the standard procedure [27] and transformants were selected using 1 ml of a 1 mg/ml thiostrepton and/or 1 ml of a 2 mg/ml apramycin overlay on R2YE regeneration plates.

5.2. Construction of expression plasmids

Expression plasmid pKOS39-104 was constructed as follows. The 6.0 kb *Bgl*II–*Pst*I fragment containing the picromycin *desVIII, desVII, desVI* and *desR* (partial) genes from cosmid pKOS23-26 [28] was subcloned into the *Bgl*II–*Pst*I sites of pKOS39-98, a pUC19 derivative with a redesigned multiple cloning site. The resulting plasmid, pKOS39-100, contains a *Pac*I site upstream of the *Bgl*II site which is used in a later cloning step. The 6 kb *Sph*I–*Pst*I fragment containing the *desI* (partial), *desII, desIII, desIV* and *desV* genes from pKOS23-26 was subcloned into the *Sph*I–*Pst*I of pUC19 to make pKOS39-102. The remaining 3′-end of the *desR* gene and 5′-end of the *desI* gene were PCR amplified from cosmid pKOS23-26 with the following oligonucleotides (restriction sites shown in italics): *desR* gene, forward 5′-AG*ATGCAT*TTCTGGGATGCCGCCACGGA and reverse 5′-CG*TCTAGA*CGTCACCAGACGTTGACCGTG; *desI* gene, forward 5′-TT*TCTAGA*CGGTGGCCCGGAGGGAACATC and reverse 5′-CG*GAATTC*CGCAGCTGGTCGGCGGCGCA. The two PCR fragments were digested with *Nsi*I–*Xba*I and *Xba*I–*Eco*RI, respectively, and ligated with *Nsi*I–*Eco*RI-digested Litmus 28 (New England Biolabs) to obtain pKOS39-101B. The 6 kb *Sph*I–*Eco*RI fragment of pKOS39-102 was inserted into pKOS39-101B to make pKOS39-103. The 6.4 kb *Nsi*I–*Eco*RI fragment of pKOS39-103 and the 6 kb *Pac*I–*Pst*I fragment of

pKOS39-100 were then ligated together with the 8.5 kb *Pac*I–*Eco*RI fragment of pKOS39-44 [23], yielding the final expression plasmid pKOS39-104 (Fig. 2b).

5.3. Production and analysis of compounds

All strains were grown in 5 ml liquid R2YE medium at 30°C and analyzed following 5 days growth. For bioconversion experiments, aglycones (~10 mg/l) were fed at the start of fermentation. Fermentation broth was analyzed directly by liquid chromatography/mass spectrometry (LC/MS) and evaporative light scattering detection (ELSD) as previously described [23]. An authentic sample of narbomycin prepared from *S. narbonensis* [28] was used to validate production of this compound. For LC/MS analysis of strains containing PKS expression plasmids the cultures were extracted twice with 5 ml of ethyl acetate/triethylamine (99:1), evaporated to dryness and resuspended in 0.5 ml of acetonitrile.

5.4. Antibacterial assays

Extracts prepared from the culture broths as above were assayed for biological activity against *B. subtilis* using an agar plate diffusion method. Samples (5 μl) from each of the extracts were pipetted to sterile filter disks, dried, and placed on an LB plate spread with 20 μl of an overnight culture of *B. subtilis*. The plates were incubated overnight at 37°C to visualize zones of growth inhibition.

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References

[1] D. O'Hagan (1991) The polyketide metabolites, Ellis Horwood, Chichester.
[2] C.R. Hutchinson, Combinatorial Biosynthesis for new drug discovery, Curr. Opin. Microbiol. 1 (1998) 319–329.
[3] R. McDaniel, A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach, M. Betlach, G. Ashley, Multiple genetic modifications of the erythromycin gene cluster to produce a library of novel 'unnatural' natural products, Proc. Natl. Acad. Sci. USA 96 (1999) 1846–1851.
[4] Q. Xue, G. Ashley, C.R. Hutchinson, D.V. Santi, A multi-plasmid approach to preparing large libraries of polyketides, Proc. Natl. Acad. Sci. USA 96 (1999) 11740–11745.
[5] G. Griesgraber, Y.T. Or, D.T.W. Chu, A.M. Nilius, P.M. Johnson, R.K. Flamm, R.F. Henry, J.J. Plattner, 3-Keto-11,12-carbazate derivatives of 6-*O*-methylerythromycin A: Synthesis and in-vitro activity, J. Antibiot. 49 (1996) 465–477.
[6] C. Agouridas, A. Denis, J.-M. Auger, Y. Benedetti, A. Bonnefoy, F. Bretin, J.-F. Chantot, A. Dussarat, C. Fromentin, S.G. D'Ambrierès, S. Lachaud, P. Laurin, O. Le Martret, V. Loyau, N. Tessot, Synthesis and antibacterial activity of ketolides (6-*O*-methyl-3-oxoerythromycin derivatives): A new class of antibacterials highly potent against macrolide-resistant and -susceptible respiratory pathogens, J. Med. Chem. 41 (1998) 4080–4100.
[7] L. Tang, H. Fu, R. McDaniel, Formation of functional heterologous complexes using subunits from the picromycin, erythromycin, and oleandomycin polyketide synthases, Chem. Biol. 7 (2000) 77–84.
[8] S. Donadio, M.J. Staver, J.B. McAlpine, S.J. Swanson, L. Katz, Modular organization of genes required for complex polyketide biosynthesis, Science 252 (1991) 675–679.
[9] S. Donadio, J.B. McAlpine, P.J. Sheldon, M. Jackson, L. Katz, An erythromycin analog produced by reprogramming of polyketide synthesis, Proc. Natl. Acad. Sci. USA 90 (1993) 7119–7123.
[10] X.R. Ruan, A. Pereda, D.L. Stassi, D. Zeidner, R.G. Summers, M. Jackson, A. Shivakumar, S. Kakavas, M.J. Staver, S. Donadio, L. Katz, Acyltransferase domain substitutions in erythromycin polyketide synthase yields novel erythromycin derivatives, J. Bacteriol. 179 (1997) 6416–6425.
[11] D.L. Stassi, S.J. Kakavas, K.A. Reynolds, G. Gunawardana, S. Swanson, D. Zeidner, M. Jackson, H. Liu, A. Buko, L. Katz, Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering, Proc. Natl. Acad. Sci. USA 95 (1998) 7305–7309.
[12] A.F.A. Marsden, B. Wilkinson, J. Cortés, N.J. Dunster, J. Staunton, P.F. Leadlay, Engineering broader specificity into an antibiotic-producing polyketide synthase, Science 279 (1998) 199–202.
[13] J.R. Jacobsen, C.R. Hutchinson, D.E. Cane, C. Khosla, Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase, Science 277 (1997) 367–369.
[14] C. Olano, N. Lomovskaya, L. Fonstein, J.T. Roll, C.R. Hutchinson, A two-plasmid system for the glycosylation of polyketide antibiotics: bioconversion of ε-rhodomycinone to rhodomycin D, Chem. Biol. 6 (1999) 845–855.
[15] Y. Xue, L. Zhao, H.-w. Liu, D.H. Sherman, A gene cluster for the macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity, Proc. Natl. Acad. Sci. USA 95 (1998) 12111–12116.
[16] L. Zhao, D.H. Sherman, H.-w. Liu, Biosynthesis of desosamine: construction of a new methymycin/neomethymycin analogue by deletion of a desosamine biosynthetic gene, J. Am. Chem. Soc. 120 (1998) 10256–10257.
[17] L. Zhao, J. Ahlert, Y. Xue, J.S. Thorson, D.H. Sherman, H.-w. Liu, Engineering a methymycin/pikromycin-calicheamicin hybrid: construction of two new macrolides carrying a designed sugar moiety, J. Am. Chem. Soc. 121 (1999) 9881–9882.
[18] L. Zhao, D.H. Sherman, H.-w. Liu, Biosynthesis of desosamine: molecular evidence suggesting β-glucosylation as a self-resistance mechanism in methymycin/neomethymycin producing strain, *Streptomyces venezuelae*, J. Am. Chem. Soc. 120 (1998) 9374–9375.
[19] L.M. Quiros, I. Aguirrezabalaga, C. Olano, C. Mendez, J.A. Salas, Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*, Mol. Microbiol. 28 (1998) 1177–1185.
[20] G. Jenkins, E. Cundliffe, Cloning and characterization of two genes from *Streptomyces lividans* that confer inducible resistance to lincomycin and macrolide antibiotics, Gene 108 (1991) 55–62.
[21] R. Ziermann, M. Betlach, A two-vector system for the production of recombinant polyketides in *Streptomyces*, J. Ind. Microbiol. Biotechnol. 24 (2000) 46–50.
[22] M. Bierman, R. Logan, K. O'Brien, E.T. Seno, R. Nagaraja, B.E. Schoner, Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp, Gene 116 (1992) 43–49.
[23] L. Tang, H. Fu, M.C. Betlach, R. McDaniel, Elucidating the mechanism of chain termination switching in the picromycin/methymycin polyketide synthase, Chem. Biol. 6 (1999) 553–558.
[24] R. Ziermann, M.C. Betlach, Recombinant polyketide synthesis in *Streptomyces*: Engineering of improved host strains, Biotechniques 26 (1999) 106–110.

[25] S. Gaisser, J. Reather, G. Wirtz, L. Kellenberger, J. Staunton, P.F. Leadlay, A defined system for hybrid macrolide biosynthesis in *Saccharopolyspora erythraea*, Mol. Microbiol. 36 (2000) 391–401.
[26] J. Sambrook, E.F. Fritsch, T. Maniatis (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY.
[27] D.A. Hopwood, M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward, H. Schrempf (1985) Genetic Manipulation of *Streptomyces*: A Laboratory Manual, The John Innes Foundation, Norwich.
[28] M.C. Betlach, J.T. Kealey, M.C. Betlach, G.A. Ashley, R. McDaniel, Characterization of the macrolide P-450 hydroxylase from *Streptomyces venezuelae* which converts narbomycin to picromycin, Biochemistry 37 (1998) 14937–14942.